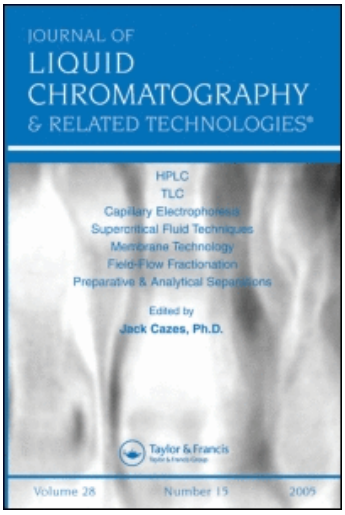

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High-Performance Liquid Chromatographic Determination of Triptonide, Triptriolide, and Triptophenolide in Ethyl Acetate Extract of Tripterygium Wilfordii Hook F

Yanping Mao[a]; John J. Cai[a]; Xuelian Tao[a]; Li[a]; Peter E. Lipsky[a]
[a] Southwestern Medical Center at Dallas, University of Texas, Dallas, TX, USA

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRIPTONIDE, TRIPTRIOLIDE, AND TRIPTOPHENOLIDE IN ETHYL ACETATE EXTRACT OF TRIPTERYGIUM WILFORDII HOOK F

Yanping Mao, John J. Cai, Xuelian Tao,
Li Ma, Peter E. Lipsky

University of Texas
Southwestern Medical Center at Dallas
5323 Harry Hines Blvd.
Dallas,TX 75235-8884, USA

ABSTRACT

To monitor the composition of extracts of the Chinese herbal remedy Tripterygium wilfordii Hook F (TwHF), a rapid, selective, and sensitive reverse phase HPLC method was developed for the quantitative determination of some of the major or active diterpenoid components, triptonide (**1**), triptophenolide (**2**), and triptriolide (**3**). Ethyl acetate extracts of TwHF were extracted with chloroform, filtered, and then purified by passage through mixed columns of silica gel and alumina B for **1** and **2**, and an alumina B cartridge for **3**. The columns were eluted with ethyl acetate : chloroform, 1 : 9, for **1** and, 3 : 7, for **2**. The alumina B cartridge was washed with ethyl acetate : chloroform, 1 : 9 and then **3** was eluted with ethyl acetate : methanol, 8 : 2.

The elutions were chromatographed on a silica-based reverse phase HPLC C_{18} column with the mobile phase of water : acetonitrile, 67 : 33, 54 : 46, and 86: 14 for **1**, **2**, and **3**, respectively, with UV detection performed at 218 nm. Linear detection responses were observed for the calibration curves in the concentration ranges of 0.64-20.48 mg/L for **1**, 4.04-60.60 mg/L for **2**, and 0.31-19.84 mg/L for **3**. The average recoveries were 96.88%, 102.54%, and 100.09%, and the limits of quantitation were 0.174 mg/L, 0.049 mg/L, and 0.112 mg/L for **1**, **2**, and **3**, respectively.

INTRODUCTION

The ethyl acetate (EA) extract of Tripterygium wilfordii Hook F (TwHF) has been used to treat a variety of autoimmune and inflammatory diseases, including rheumatoid arthritis, for many years in China.[1,2] It has been noted to cause marked clinical improvement in a number of autoimmune inflammatory diseases.[3-4] Moreover, the EA extract of TwHF has been shown to inhibit immune and inflammatory activities in vitro.[5]

Kupchan et al. first isolated and characterized some of the active compounds of TwHF, including triptolide, tripdiolide, and triptonide in 1972.[6] These components were isolated from the ethyl acetate layer of an ethyl acetate - water partition of TwHF. Since then, more than 70 components, including alkaloids, diterpenoids, triterpenoids, sesquiterpenoids, carbohydrates, and glycosides have been isolated and structurally characterized.[7] Among the components, the diterpenoids of TwHF that contain an α, β-unsaturated lactone, have been confirmed to be the major active components of TwHF.[8]

Previous quantitative evaluations of components of TwHF have focused on triptolide, one of the major active diterpenoids of TwHF. Analytical methods, including gas chromatography[9] and thin-layer chromatography scanning densitometry[10,11] have been developed to detect and quantitate triptolide. In 1994, we reported a reverse phase HPLC method for the determination of triptolide and another active diterpenoid, tripdiolide.[12] No previous reports have focused on the quantitative determination of other diterpenoids in TwHF.

Although triptolide and tripdiolide have been recognized as potent components of TwHF, the use of these purified components as therapeutics was limited by toxicity. Compared with triptolide, the EA extract of TwHF was found to have comparable immunosuppressive and anti-inflammatory activity but lower toxicity.[13] This has suggested that the activity of the EA extract may involve cooperative interactions of many different active components. Moreover, previous observations have shown that the active ingredients of

TwHF are unstable and may change in concentration and composition during preparation and storage.[14] Many of the diterpenoids of TwHF degrade and some of them, such as the triptolide and triptonide, can interconvert into one another under certain condition.[15] Furthermore, different methods of preparation of the EA extract may contain different amounts of diterpenoids. Therefore, the immunosuppressive and anti-inflammatory activity of various batches of the EA extract of TwHF may vary. To insure uniformity in manufacture and storage, a method to assess the concentrations of the active diterpenoids is essential. In this communication, we report the development and validation of a reverse phase high performance liquid chromatography method for the quantitative determinations of triptonide (**1**), an anti-inflammatory and immunosuppressive diterpenoid in TwHF; triptriolide (**3**), a highly active anti-inflammatory diterpenoid with less toxicity than other active diterpenoids;[16] and triptophenolide (**2**), an inactive diterpenoid that is found at high concentration in EA extracts.

EXPERIMENTAL

Plant Material

TwHF was collected from the south of Fujian province, P. R. of China in 1994. The plant was peeled, dried, and stored at 4°C and chopped to a coarse powder before extraction.

Chemicals and Reagents

Authentic standards of **1**, **2**, and **3** were isolated from TwHF, their composition verified by IR, UV, MS, and NMR, and the results compared with published data.[6,16,17] Acetonitrile was HPLC grade (Aldrich Chemical Company); methanol, chloroform, and ethyl acetate were reagent grade (E. M. Science Chemicals); and water was Millipore pure prepared by a Millipore Milli-Q Plus device. Alumina (WB-2: basic, activity grade 1) and silica gel (28-200 mesh, average pore diameter: 22 A) were obtained from Sigma Chemical Co., and screened with a #120 mesh before use.

Instrument Conditions

The Waters HPLC instruments was used to quantitate **1**, **2**, and **3** in the EA extract of TwHF. It was outfitted with an U6K injector, a 486 detector set at 218 nm, and two model 510 pumps for mobile phase delivery. Millennium software version 2.00 (Waters Assoc.) was used for data processing. A 3.9 x

300mm stainless steel column prepackaged with Nova-Pak $C^{18,}$ with a particle size of 4 μm, was purchased from Waters and used to separate samples. An HPLC precolumn with an insert packed with Nova-Pak C_{18} (Waters Assoc.) was used to extend the life of the column.

The model ULTRAsonik 2QT / H ultrasonic water bath was purchased from NEY Barkmeyer division (Yucaipa, CA) and used in both the solvent degassing and sample preparation.

Instrument Calibration

Authentic standards of **1**, **2,** and **3** were weighted accurately and dissolved in methanol in an ultrasonic bath for 15 minutes before analysis. For determination of linearity and the determination of the concentration range, calibration standards were prepared of **1** of 0.64, 1.28, 2.56, 5.12, 10.24, 20.48 mg / L; of **2** of 4.04, 8.08, 12.12, 16.16, 20.20, 40.40, 60.60 mg / L; and of **3** of 0.31, 0.62, 1.24, 2.48, 4.96, 9.92, 19.84 mg / L. The injection volume was 5 μL for each sample.

Repetitive calibrations of standard solutions of **1, 2, 3** yielded correlation coefficients of absorbance versus concentration of analytes of > 0.999.

EA Preparation

Three batches of EA were prepared by the method of Chen et al.[18] from the woody portion of the roots of TwHF.

Preparation of Quality Control (QC) Samples

Extraction

About 0.45 g of the EA extract powder of TwHF extracted from ~25 g of plant material, was weighed accurately and extracted with 10 mL chloroform two times, each for 10 minutes in an ultrasonicator at room temperature. The extract solution was filtered in a vacuum and the residue was washed with chloroform two times.

The chloroform solutions were combined and the volume was adjusted by evaporating or adding chloroform to give a final solution equivalent to 1g plant material / mL, calculated according to the yield of EA extract.

Clean up

Triptonide and Triptophenolide: A 25mL column containing 1.6 g silica gel and 1.6 g alumina B was prepared, conditioned with chloroform : ethyl acetate 7 : 3, followed by 20 mL chloroform. One mL of sample solution was added to the column and **1** eluted with 100 mL of chloroform : ethyl acetate (9 : 1) at a flow rate of 2 mL / min.

Using the same procedure, except that only 0.5 mL of the EA extract solution was loaded, compound **2** was eluted with 120 mL of chloroform : ethyl acetate (7 : 3).

Triptriolide: An alumina B cartridge was conditioned by passage of 10 mL of ethyl acetate : methanol (9 : 1), followed by 15 mL of chloroform. One mL of the sample solution of **3** was loaded, and the cartridge was washed with 10 mL of chloroform : ethyl acetate (9 : 1) and then eluted with 20 mL of ethyl acetate : methanol (8 : 2).

The analytical fractions were evaporated to dryness gently and frozen (-15°C). The samples were dissolved in 1.0 mL of methanol for **1**, 4.0 mL of methanol for **2,** and 2.0 mL of methanol for **3** before analysis by HPLC.

Method Accuracy

The method accuracy (recoveries) was determined by computing the ratio of the amount of the extracted compounds from the EA extract spiked with known amounts of diterpenoid authentic standards (0.8, 1.2, 1.6, 2.0 mg /L for **1** and **3**, and 1.6. 2.4, 3.2, 4.0 mg/L for **2**) to the amount of the corresponding diterpenoid in the same preparation of the EA extract of TwHF without added components. Each sample was assessed in triplicate.

Precision

For inter-experiment reproducibility, the aliquots of QC samples were evaporated to dryness and stored at -15°C. The assay procedure was repeated on different days using the same QC samples spiked with **1**, **2,** and **3** at high, moderate, and low concentrations by performing replicate analyses and comparing the results with calibration curves of **1**, **2,** and **3**.

Same-day reproducibility was assessed by performing replicate analyses of samples spiked with high, moderate, and low concentrations of **1**, **2**, and **3** on the same day.

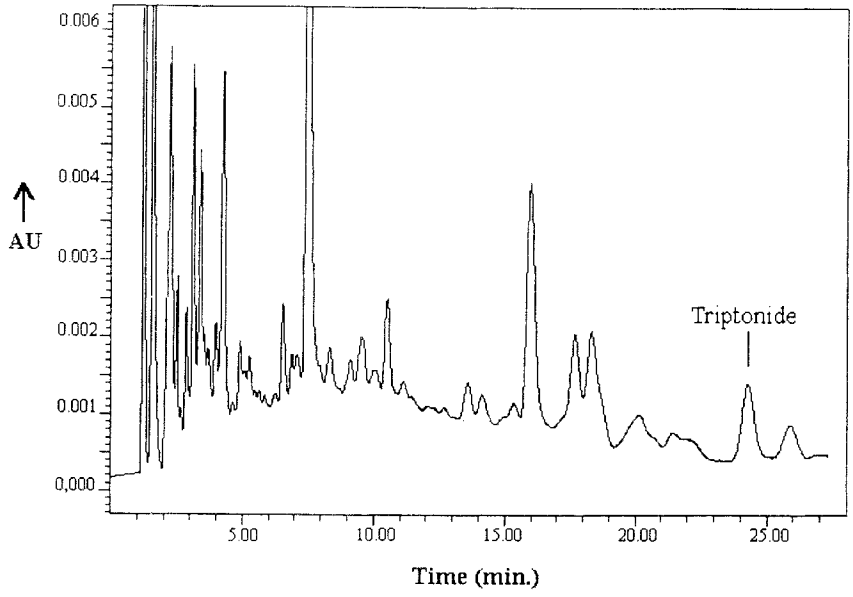

Figure 1. The HPLC Chromatogram of Triptonide in the EA Extract of TwHF. Mobile Phase: Acetonitrile : Water 33 : 67, Flow Rate: 1 mL / min.

Stability

The stability of **3** in water has been studied in detail and has exhibited a k_{25} (reaction speed constant at 25°C) of $1.8x10^{-4}$. The methanol solution of compound **1** has exhibited lability after storing for 48 hours at room temperature.[15] Therefore, methanol solutions were spiked with concentration of **1**, **2**, and **3** (2.078 ug/mL for **1**, 10.100 μg/mL for **2**, and 2.074 μg/mL for **3**) and assessed twice in hourly intervals within the first 6 hours and then at the 20th hour at room temperature.

Detection Limits and Quantitation Limits

The detection limits of **1**, **2**, and **3** were defined as the concentrations at which the signal to noise radio (S/N) equaled 3. The quantitation limits were defined as the lowest concentration at which the relative standard deviation (RSD) for six replicate injections was less than 5%.

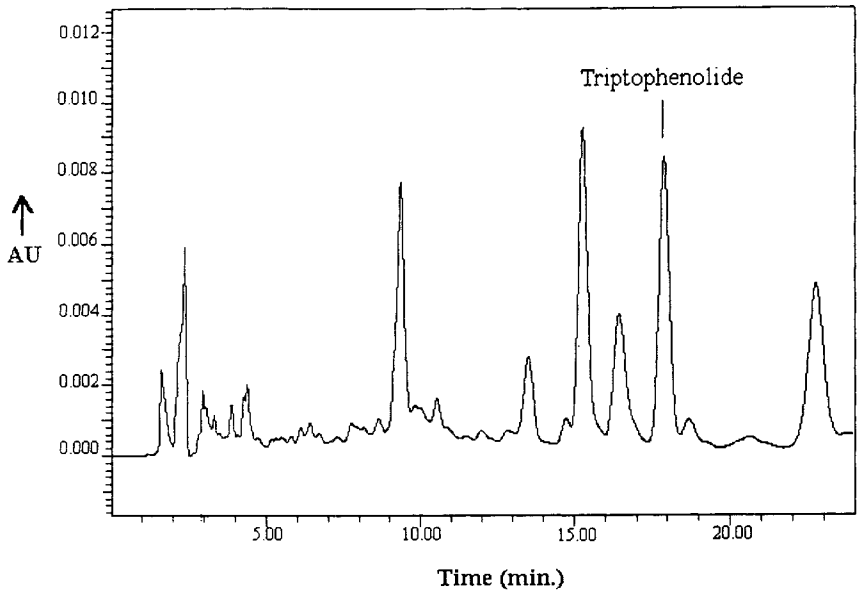

Figure 2. The HPLC Chromatogram of Triptophenolide in the EA Extract of TwHF. Mobile Phase: Acetonitrile : Water 47 : 53, Flow Rate: 1mL / min.

HPLC Analysis of QC Sample Preparations

Eight μL of sample preparations of **1** and **3** and 10 μL of **2** were injected into the chromatograph with a 10 μL syringe. Each sample was analyzed in triplicate. The mobile phase and flow rate are shown under the corresponding chromatograms (Figure 1-3). The peaks of **1**, **2,** and **3** in the chromatograms of the QC sample solutions were identified by comparing the retention times with that of reference standards.

The contents of **1**, **2** and **3** were calculated by comparing the peak area of the sample preparations with the external standard curves of authentic standards.

RESULTS AND DISCUSSION

The chromatograms of the QC samples of compounds **1**, **2,** and **3** are shown in Figure 1-3. Analysis of the EA extracts indicated that **1**, **2,** and **3** can be separated effectively using the established conditions and exhibited

Table 1

The Quantitative Determination of Triptonide, Triptophenolide and Triptriolide in Three Different Batches of the EA Extract of TwHF

Batch	Mean ± SD (μg/g EA)	RSD %	n
	Triptonide		
1	112.44 ± 7.04	6.26	8
2	102.74 ± 6.44	6.27	8
3	93.76 ± 16.85	7.97	9
	Triptophenolide		
1	2609.46 ± 111.82	4.28	4
2	2177.74 ± 82.73	3.80	4
3	1250.26 ± 108.64	8.69	4
	Triptriolide		
1	63.82 ± 3.26	5.10	5
2	69.86 ± 2.14	3.17	4
3	52.91 ± 2.22	4.20	4

Table 2

Linearity and Ranges of Detection of Triptonide, Triptophenolide and Triptriolide

Sample Name	Range (mg/L)	Line's Equation	Correlation Coefficient
Triptonide	0.64 - 20.48	$y = -0.6305 + 5.2585 \times 10^{-4}x$	1.0000
Triptophenolide	4.04 - 60.60	$y = -3.7844 + 2.7780 \times 10^{-4}x$	0.9999
Triptriolide	0.31 - 19.84	$y = 0.1067 + 5.7211 \times 10^{-4}x$	1.0000

resolution values of 1.7, 1.1, and 1.3, with the closest peaks, respectively. The retention times of **1**, **2**, and **3** were about 24.3, 17.9, and 11.0 minutes. 49 EA extract samples of 3 separately prepared batches have been analyzed. The results are shown in Table 1.

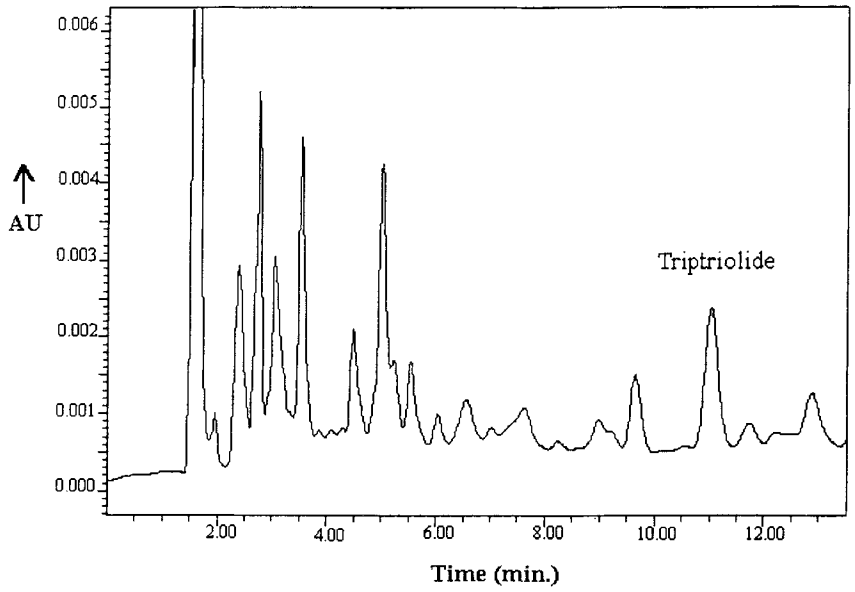

Figure 3. The HPLC Chromatogram of Triptriolide in the EA Extract of TwHF. Mobile Phase: Acetonitrile : Water 14 : 86, Flow Rate: 1mL / min.

Table 3

Percent Recoveries of Triptonide, Triptophenolide, and Triptriolide in the EA Extract

Triptonide		Triptophenoide		Triptriolide	
Theoretical Concn. (mg/L)	**Recovery (%)**	**Theoretical Concn. (mg/L)**	**Recovery (%)**	**Theoretical Concn. (mg/L)**	**Recovery (%)**
2.869	97.36	13.463	104.75	2.766	97.84
3.283	98.29	14.291	105.94	3.182	96.61
3.698	96.66	15.099	96.01	3.599	105.04
4.113	95.17	15.907	103.46	4.003	100.84
Mean	96.88%	Mean	102.54%	Mean	100.09%
SD	1.31%	SD	4.47%	SD	3.74%
RSD	1.35%	RSD	4.36%	RSD	3.74%

Table 4

Reproducibility of the Determinations of Triptonide, Triptophenolide, and Triptriolide

Sample	Theoretical Conc. (mg/L)	Experimental Conc. (mg/L)	RSD (%)
	Same-day Reproducibility		
Triptonide	0.643	0.670 + 0.0230	3.44
	5.140	5.015 ± 0.0162	0.32
	20.56	19.55 ± 0.1623	0.83
Triptophenolide	2.02	2.054 ± 0.026	1.27
	16.16	16.023 ± 0.158	0.975
	32.32	32.755 ± 0.254	0.761
Triptriolide	1.237	1283 ± 0.00698	0.544
	9.896	9.643 ± 0.0925	0.959
	19.79	18.980 ± 0.146	0.766
	Inter-Experimental Reproducibility		
Triptonide	2.869	2.784 ± 0.0551	1.98
	3.283	3.398 ± 0.0712	2.10
	4.113	4.053 ± 0.158	3.90
Triptophenolide	13.463	13.653 ± 0.566	4.15
	14.291	14.517 ± 0.410	2.82
	15.907	15.664 ± 0.335	2.14
Triptriolide	2.766	2.879 ± 0.101	3.51
	3.182	3.095 ± 0.088	2.84
	4.013	4.046 ± 0.127	3.14

Linearity and Range

The examined linear ranges were 0.64-20.48 mg/L for **1**, 4.04-60.6 mg/L for **2**, and 0.31-19.84 mg/L for **3**. The correlation coefficients for the compounds showed very good linear dependence of concentration with peak area within the determination ranges (Table 2).

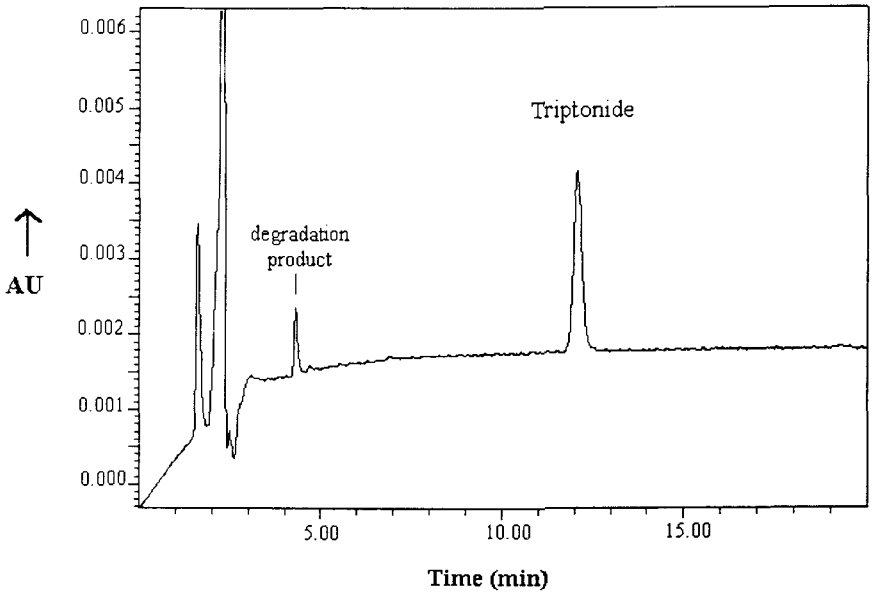

Figure 4. Triptonide stored in methanol at room temperature for 20 hours. Mobile phase: Acetonitrile : Water 44 : 56, Flow Rate: 1mL / min.

Table 5

Stability of Triptonide, Triptophenolide, and Triptriolide, in Methanol Solution

Time (h)	Peak Area Triptonide	Triptophenolide	Triptriolide
0	20780	191900	18148
1	20858	189380	18421
2	21405	195729	18822
3	20359	188785	18809
4	20712	190072	17274
5	21474	191826	18176
6	20526	185541	18450
	X = 20873 SD = 421 RSD = 2.02%	X = 190462 SD = 3180 RSD = 1.67%	X = 18300 SD = 526 RSD = 2.87%

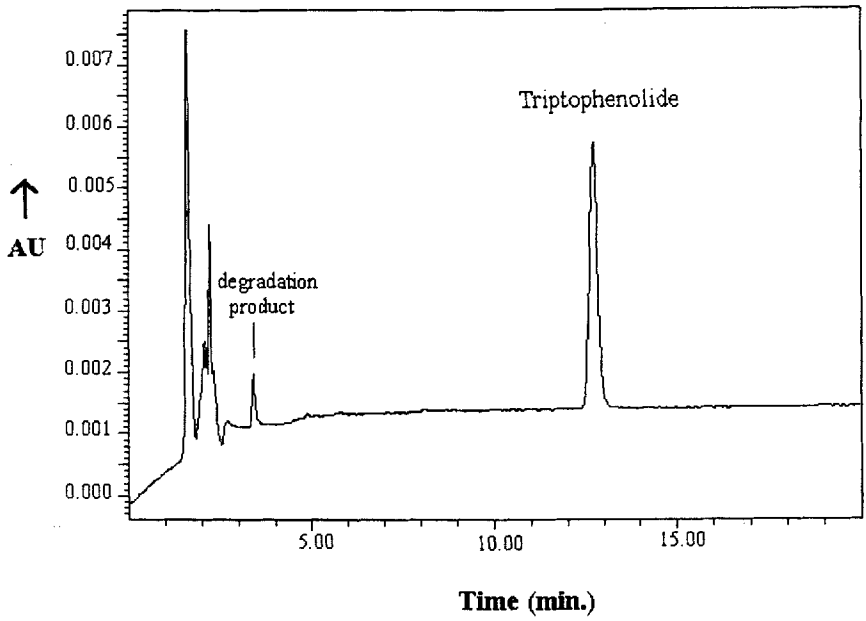

Figure 5. Triptophenolide stored in methanol at room temperature for 20 hours. Mobile phase: Acetonitrile : Water 50 : 50, Flow Rate: 1 mL / min.

Recovery

Compounds **1**, **2**, and **3**, showed quantitative recoveries from QC samples. The mean percentage recoveries of **1**, **2**, and **3** were 96.88 ± 1.31, 102.54 ± 4.47, and 100.09 ± 3.74, respectively. See Table 3.

Precision and Stability

Same-day and inter-experiment reproducibility were tested by replicate injections of various levels of **1**, **2**, and **3** and QC samples. The RSD of the same-day precision and the interexperiment variability for all three compounds were less than 1.56% and 4.20%, respectively. The results are presented in Table 4.

Table 4 also indicates that compounds **1**, **2**, and **3** are relatively stable in dry conditions at -15°C. Their stability results in methanol indicate that degradation products appeared in the chromatograms after the 20th hour for **1**, **2**, and **3** (Figure 4-6), but no obvious degradation occurred within 6 hours at room

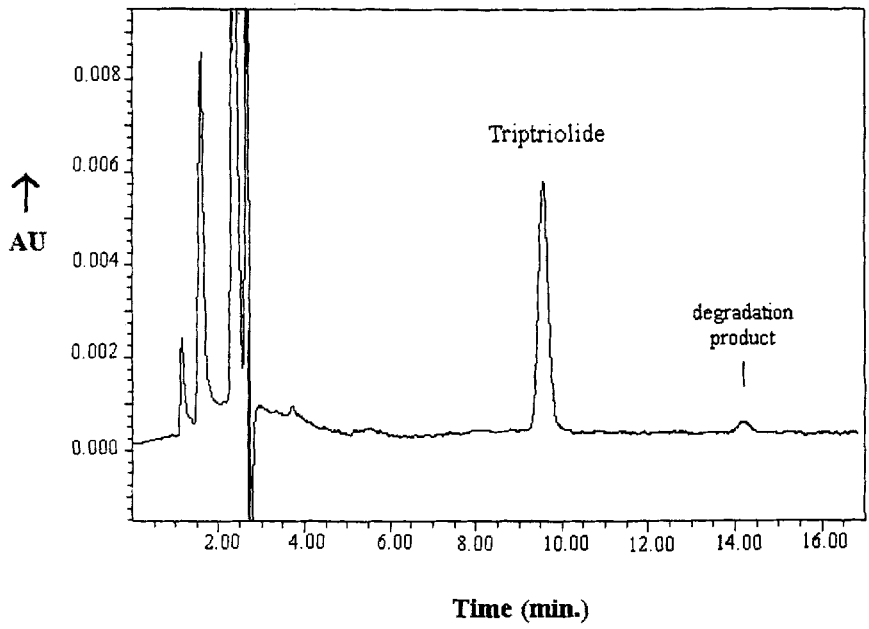

Figure 6. Triptriolide stored in methanol at room temperature for 20 hours. Mobile phase: Acetonitrile : Water 15 : 85, Flow Rate: 1mL / min.

temperature (Table 5). Therefore, for the reliable analysis, the QC samples or standards can be kept frozen in a dry state for several days, but accurate quantitation requires dissolution in methanol immediately before the HPLC analysis.

Detection limits and quantitation limits

Large injection amounts could pollute the injector loop and HPLC column, and can interfere with accurate determinations, whereas small injection amounts might influence the accuracy of the determination because of the minimal amount of analyte contained in the large and complex sample mixture. A limit test, therefore, was carried out to determine the optimal injection amount. The detection limits assessed by concentration of analyte in the test samples were approximately 0.0414 mg/L, 0.0178 mg/L, and 0.0344 mg/L for **1**, **2**, and **3**, respectively; and the quantitation limits were 0.174 mg/L, 0.112 mg/L, and 0.049 mg/L. The injection volumes were 5μL for all the limit tests. The limits of quantitation data are shown in Table 6.

Table 6

Determination of the Limits of Quantitation of Triptonide, Triptophenolide and Triptriolide

Sample	Sample No.	Peak Area (uV sec^2)	Mean, SD, & RSD
Triptonide	1	1794	
	2	1663	
	3	1723	
	4	1704	Mean=1741 (uV sec^2)
	5	1708	SD = 70.57 (uV sec^2)
	6	1856	RSD = 4.06%
Triptophenolide	1	997	
	2	1059	
	3	958	
	4	991	Mean=986 (uV sec^2)
	5	923	SD = 41.37 (uV sec^2)
	6	992	RSD = 4.19%
Triptiolide	1	1223	
	2	1248	
	3	1302	
	4	1285	Mean=1276 (uV sec^2)
	5	1249	SD = 46.27 (uV sec^2)
	6	1351	RSD = 3.63%

General Considerations

For the determination of **1** in the EA extract, several kinds of columns and cartridges of basic alumina, neutral alumina and silica gel have been used, but no satisfactory isolation was found. The mixed columns were used to eliminate most of the impurities and concentrate **1** and **3**. It appeared to provide the best method to remove interference and obtain a symmetric peak for **1** and also permit a good recovery of **3**. A number of solution systems were tested for analyte elution. Each of these was less effective than the one employed, yielding either broad peaks or poor recoveries.

To achieve an accurate analytical result and good separation by chromatography, the HPLC column required washing with acetonitrile : water (80 : 20) after each injection of the QC sample.

Sixty mL of elution solution usually was sufficient for the elution of more than 96% of **1** and **3**. However, additional elution solution was necessary to ensure the complete elution of **1** and **3** and to avoid potentially confounding factors such as the quality of packing and the use of different cartridges.

The quantitation of these compounds in the EA extract was performed without internal standards. As was found for the chromatographic properties of **1**, **2**, and **3**, the components of the EA extract eluted very near to each other. Therefore, it was very difficult to insert an internal standard. Since the recoveries of **1**, **2**, and **3** were previously noted to be > 96.88%, the use of an internal standard was not essential.

In summary, the described HPLC method permitted a rapid determination of **1**, **2**, and **3** in the EA extract of TwHF. The technique was found to exhibit good accuracy, precision, and reproducibility. The sensitivity of the assay is sufficient to monitor the composition of the EA extract accurately.

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