DIETARY SUPPLEMENTS

Chemical Fingerprinting of *Valeriana* Species: Simultaneous Determination of Valerenic Acids, Flavonoids, and Phenylpropanoids Using Liquid Chromatography with Ultraviolet Detection

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The roots and rhizomes of various *valeriana* species are currently used as a sleeping aid or mild sedative. A liquid chromatography method has been developed that permits the analysis of chlorogenic acid, lignans, flavonoids, valerenic acids, and valpotrates in various valerian samples. The best results were obtained with a Phenomenex Luna C18(2) column using gradient elution with a mobile phase consisting of water and 0.05% phosphoric acid and 2–100% acetonitrile–methanol (1 + 1) with 0.05% phosphoric acid. The flow rate was 0.8 mL/min and ultraviolet detection was at 207, 225, 254, 280, and 325 nm. Different valerian species and commercial products showed remarkable quantitative variations. Chlorogenic acid (0.2–1.2%), 3 lignans, linarin (0.002–0.24%), and valepotriates were detected in all the *valeriana* species analyzed. Highest amounts of valerenic acids were detected in *V. officinalis* L., trace amounts in *V. sitchensis,* and none in the other species analyzed.

Valerian is the common name for over 200 worldwide plant species of the genera *valeriana* (valerianaceae; 1, 2). The roots and rhizomes of several species of this genus are currently used as a sleep aid or mild sedative and as a gastrointestinal spasmolytic agent (3, 4). Animal and clinical studies have demonstrated the central nervous system depressant effect for several *valeriana* species (4–7). The most important commercial species in herbal medicine are *V. officinalis* L. (European valerian); *V. jatamansi* Jhones (synonym *V. wallichii* DC, Indian or Pakistan valerian); *V. fauriei* Briq. (Japanese valerian); and *V. procera* Kunth [synonym *V. edulis* subsp. *procera* (Kunth) Meyer, Mexican valerian] (8). These species show large differences with regard to chemical constituents, as well as in their morphology. Consequently, the phytomedicines prepared from these species are characterized by different chemical composition (9). More than 150 compounds have been identified; none appears to be solely responsible for the biological activities attributable to valerian, suggesting that many compounds may act synergistically (4). The terpenoid esters named valepotriates (notably valtrate and dihydrovaltrate); their decomposition products, the baldrinals; and various components of the essential oil, in particular, the valerenic acid derivatives, are considered to be the most important compounds responsible for the biological activities of valerian. However, some experimental facts cast doubts upon the real involvement of these substances from different valerians with similar activities (2, 4, 10). Recently, lignans and flavonoids (hesperidin, linarin, and 6-methylapigenin) have been found to be active principles in valerian species, showing activity in the central nervous system (11–14). Chlorogenic acid was isolated from *V. officinalis* L. (4) and found to have antioxidant properties (15).

Analysis of *valeriana* species was initially focused on valepotriates; these compounds were exclusively found in the family valerianaceae (16, 17). In the 1980s, the valerenic acids started to attract attention, and analytical procedures for these compounds were developed for *V. officinalis* L. (18, 19). The United States Pharmacopoeia 28 NF 23 described a liquid chromatographic (LC) method to quantitate valerenic acid in plant material, powder, and tablets prepared from *V. officinalis* L. An LC method has also been developed to quantitate valerenic acids and lignans in *V. officinalis* L. (20). Analytical methods previously reported for the analysis of valerian have several shortcomings. The main shortcomings are as follows: (*1*) valepotriates cannot be used as reference standards due to

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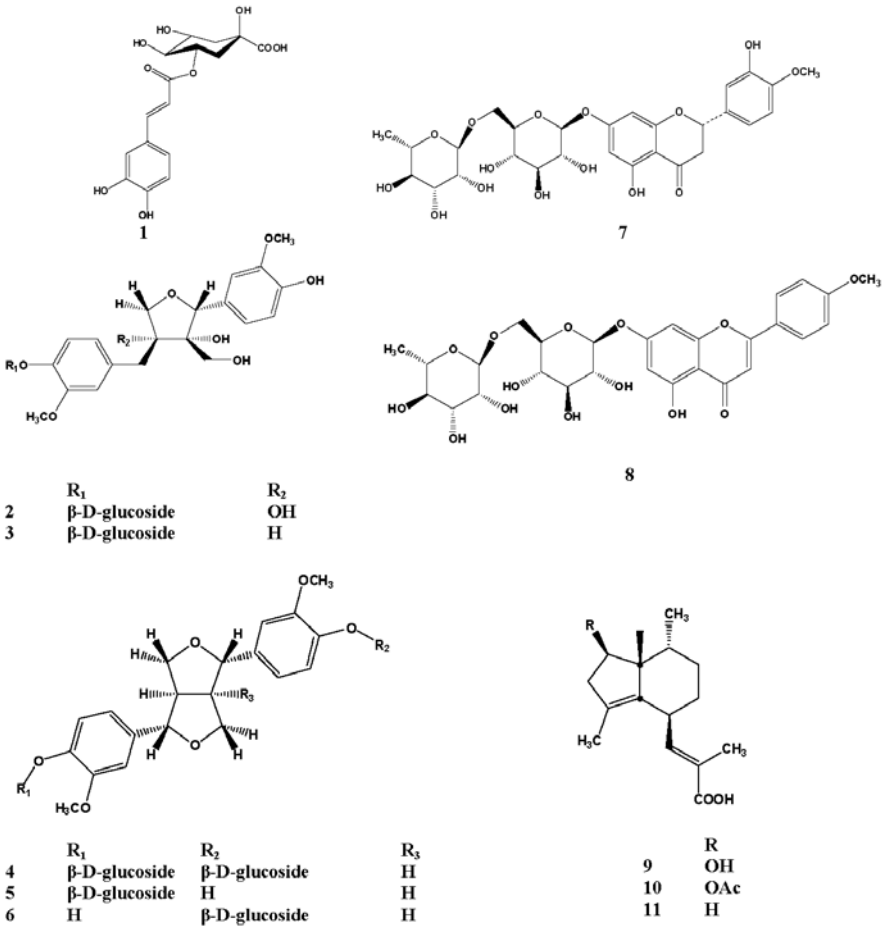

Figure 1. Structures of chlorogenic acid, lignans, flavonoids, and valerenic acids. Numbered compounds are identified in text.

their rapid decomposition; (*2*) analytical methods developed for valerenic acid apply only for *V. officinalis* L.; and (*3*) no methods of analysis exist for various commercially important valerian species containing chemical constituents other than valerenic acids.

In the present study, special attention was given to the Mexican valerian (*V. procera* Kunth or *V. edulis* subsp. *procera*) because of the following reasons: (*1*) it is a commercially important species; (*2*) the use of this species as a sleeping aid is supported by pharmacological and clinical studies (6, 7, 10); and (*3*) no methods were developed for quality control of this species. Therefore, herein is reported a simple and precise LC method for the qualitative and quantitative analysis of chlorogenic acid (**1**), 5 lignans [massoniresinol-4′-*O*-β-D-glucoside (**2**), berchemol-4′-*O*-β-D-glucoside (**3**), pinoresinol-4,4′-di-*O*-β-D-glucoside (**4**), 8-hydroxypinoresinol-4′-*O*-β-D-glucoside (**5**), pinoresinol-4-*O*-β-D-glucoside (**6**); 2 flavonoids [hesperidin (**7**) and linarin (**8**)]; and 3 sesquiterpenes [hydroxyvalerenic acid (**9**), acetoxyvalerenic acid (**10**), and valerenic acid (**11**)] (Figure 1) in *V. procera* Kunth. A comparative study between different valerian species was performed as well. The numbering of analytes was provided in the order of elution. In the present study, wild Mexican valerian and the cultivated Mexican valerian were also compared.

Experimental

Chemicals

The standard analyte hesperidin was purchased from Sigma (St. Louis, MO); linarin was purchased from Chromadex (Santa Ana, CA); and hydroxy valerenic acid, acetoxy valerenic acid, and valerenic acid were purchased from Apin Chemicals (Abingdon, Oxon, UK). Lignans (**2**–**6**) were isolated at the National Center for Natural Products Research (NCNPR), and their identity and purity were confirmed by LC and thin-layer chromatography methods and comparison with published infrared, nuclear magnetic resonance, and mass spectrometry data (11, 21).

HPLC grade acetonitrile, methanol, and phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Water for the LC mobile phase was purified in a Milli-Q system (Milli-Q Academic, Millipore, Bedford, MA). Products of *V. officinalis* L. (Capsules VOP7–VOP9) and *V. procera* Kunth (Drops VPP1, Solid Extracts VPP2–VPP3, and Fluid Extracts VPP4–VPP6) were obtained through online and personal contacts.

Table 1. Calibration data [regression equation and correlation coefficient (R^2)] and limit of detection (LOD) for analytes 1–8 and 11

Analyte	Regression equation (1/x weighting)	r^2	LOD, μg/mL	Wavelength, nm
1	$y = 3.83 \times 10^4x + 1.33e + 002$	0.9998	0.3	325
2	$y = 1.84 \times 10^4x - 3.28e + 002$	0.9999	0.5	225
3	$y = 1.15 \times 10^4x + 9.74e + 002$	0.9999	0.3	225
4	$y = 1.20 \times 10^4x + 6.29e + 002$	0.9999	0.5	225
5	$y = 2.54 \times 10^4x + 3.95e + 002$	0.9999	0.5	225
6	$y = 1.48 \times 10^4x + 1.32e + 003$	0.9999	0.3	225
7	$y = 2.00 \times 10^4x + 1.76e + 003$	0.9994	0.1	280
8	$y = 1.79 \times 10^4x - 1.05e + 002$	0.9999	0.3	280
11	$y = 4.65 \times 10^4x + 2.54e + 004$	0.9999	0.1	225

Plant Material

Various species of *V. officinalis* L. (Frontier Natural Products Co-op, Norway, IA); *V. edulis* Nutt. ex Torr. & A. Gray. (Switzerland trail, near Gold Hill, CO); *V. jatamansi* Jhones (India); and *V. sitchensis* Bong. (Sauk Mountains, North-Cascade, WA) were studied. Different populations of same species *V. procera* Kunth collected from different locations in Mexico [Estado de Mexico (VP-1 and VP-2), Distrito Federal (VP-3 and VP-4), and Puebla (VP-5)] were also studied. VP-1 to VP-4 were micropropagated according to a procedure previously reported (22) and harvested in November 2004, except VP-3 that was harvested in November 2002. VP-5 was wildly grown and harvested in November 2004. VP-1 was a 6-month-old plant, and VP-2 to VP-4 were 12-month-old plants. Mexican valerian plant samples were identified by botanists of the Herbario del Centro Medico Nacional Siglo XXI. Voucher specimens of all *valeriana* samples are deposited at the NCNPR.

Standard Solutions for Accuracy and Precision Determination

Individual stock solutions of standard analytes were prepared at a concentration of 0.5 mg/mL in methanol. The quantitation was performed using 7 levels of external standards. The ranges obtained were 0.6–6.0 to 60–80 μg/mL depending on the concentration of each stock solution. Table 1 shows the calibration data and calculated limit of detection (LOD). LOD and limit of quantitation (LOQ) were defined, respectively, as signal-to-noise ratio equal to 3 and 10; LOD ranged from 0.1 to 0.5 μg/mL.

Sample Preparation

Finely powdered dried plant material (0.2 g) of *valeriana* or an adequate amount of powdered solid extract or capsule were sonicated in 2.5 mL methanol for 15 min followed by centrifugation for 10 min at 3300 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated 3 times, and respective supernatants were combined. The final volume was adjusted to 10 mL with methanol. The aliquots of the liquid formulations (5.0 mL) were diluted with equal volumes (5.0 mL) of methanol and mixed thoroughly. Prior to use, all samples were filtered through a 0.45 μm nylon membrane filter.

Instrumentation and Chromatographic Conditions

The HPLC system consisted of Waters Corp. (Milford, MA) Model 6000A pumps, Model U6K injector, Model 680 automated gradient controller, Model 996 photodiode array detector (PDA), and a computerized data station equipped with Millennium software. Separation was achieved on a Luna C18(2) column, 150 × 3.0 mm id, 5 μm particle size (Phenomenex Inc., Torrance, CA) operated at 30°C. The column was equipped with a 2 cm LC-18 guard column (Supelco, Bellefonte, PA). The mobile phase consisted of water (A) and acetonitrile–methanol (1 + 1) (B), both containing 0.05% phosphoric acid, which were applied in the gradient elution sequence shown in Table 2.

Each run was followed by a 5 min wash with 100% acetonitrile and an equilibration period of 15 min. The flow rate was adjusted to 0.8 mL/min, and the injection volume was 10 μL. Chlorogenic acid was detected at 325 nm, lignans

Table 2. Gradient elution conditions

Gradient	Time, min	Flow rate, mL/min	A, %	B, %	Curve
1	0.00	0.80	98.0	2.0	6
2	10.00	0.80	83.0	17.0	6
3	25.00	0.80	50.0	50.0	6
4	40.00	0.80	0.0	100.0	6

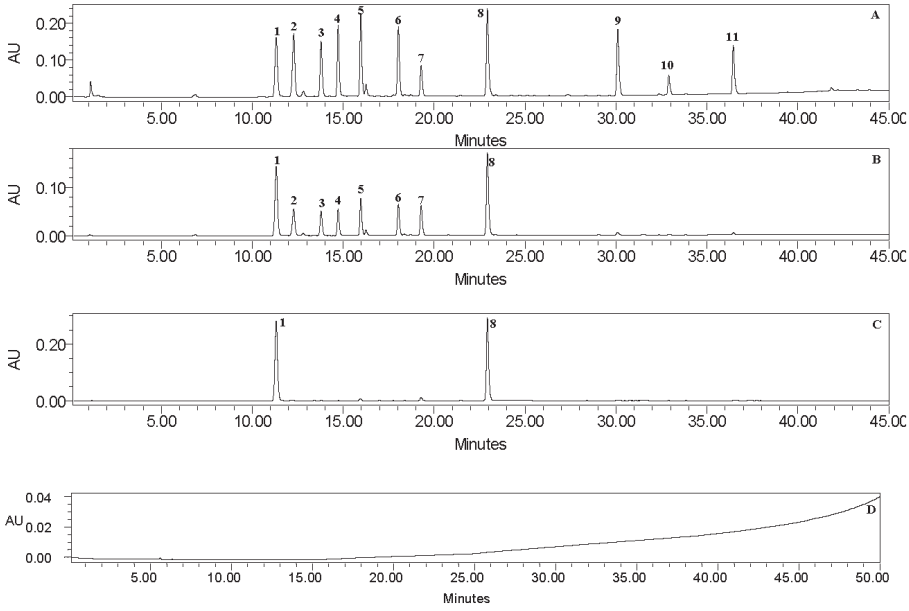

Figure 2. Typical liquid chromatograms of chlorogenic acid, ligans, flavonoids, and sesquiterpenoidal standards (compounds 1–11) at 225 nm (A), 280 nm (B), 325 nm (C), and chromatogram of blank solution (D) from 200 to 400 nm.

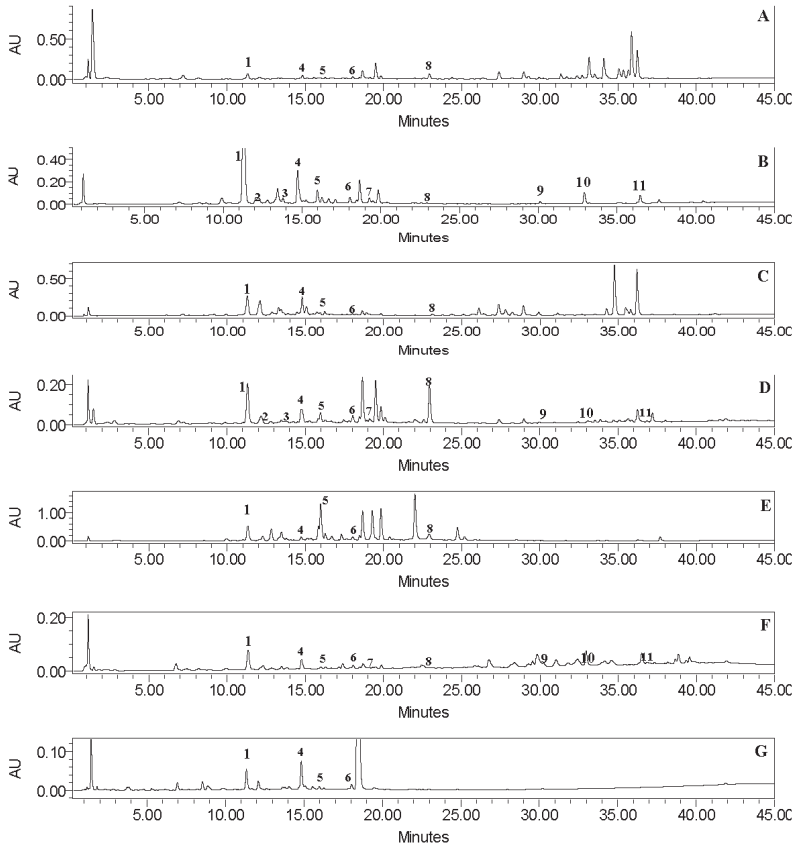

Figure 3. Comparison of chromatograms at 225 nm of *V. procera* Kunth (A), *V. officinalis* L. (B), *V. edulis* Nutt. Ex Torr. & A. Gray. (C), *V. sitchensis* Bong. (D), *V. jatamansi* Jhones (E), an extract of dietary supplement containing *V. officinalis* L. (F), and an extract of dietary supplement containing *V. procera* Kunth (G).

and valerenic acids at 225 nm, flavonoids at 280 nm, and valepotriates at 207 and 254 nm.

Chromatographic Conditions

The different columns tried were Luna C18(2), Synergi Max-RP 80 A, Aqua, Lichrospher, and Synergi Polar-RP (Phenomenex Inc.). Composition of the mobile phase was crucial as well, and an acidic system improved the peak symmetry of all analytes significantly. The best separations were observed with the Luna C18(2) column using water and methanol–acetonitrile (1 + 1) both containing 0.05% phosphoric acid as the mobile phase. Variation of the column temperature between 25–40°C did not cause significant change in the resolution, but changes in retention time were observed. Thus, the column was used at 30°C at a flow rate of 0.8 mL/min. The method allowed for the separation of all 11 analytes in 37 min (Figure 2).

Accuracy, Precision, and Linearity

The calibration graph showed a linear correlation between sample concentration and peak area. Intraday and interday variations were determined with standards. Analyses were performed in triplicate on 3 different days, and each concentration point was injected in triplicate. Purity of the standards was confirmed by the PDA data of all peaks of interest. In order to determine the accuracy of the method, one sample was spiked with a known amount of the standard analytes. Recovery rates were found to be between 97.3 and 103.2%. An indicator for precision is the relative standard deviation (RSD). All samples were injected in triplicate, and the RSD of standard analytes was below 3.0%. Calibration

Table 3. Analytes detected or quantitated in various species, populations, and products of valerian

		Analyte, %												
Study	Name	1	2	3	4	5	6	7	8	9	10	11	12	13
Species	*V. jatamansi*	0.19	–[a]	DUL[b]	0.13	0.02	0.03	–	0.24	–	–	–	+[c]	+
	V. procera	0.65	–	–	DUL	0.002	0.02	–	0.07	–	–	–	+	+
	V. edulis	0.24	–	–	0.27	0.005	0.01	–	DUL	–	–	–	+	+
	V. sitchensis	0.47	0.12	0.05	0.17	0.50	0.12	0.57	0.03	DUL	DUL	0.003	+	+
	V. officinalis	1.16	0.01	0.04	0.41	0.06	0.05	0.02	0.002	+	+	0.89	+	+
Population	VP-1	0.09	–	–	DUL	DUL	0.01	–	0.03	–	–	–	+	+
	VP-2	0.17	–	–	DUL	DUL	0.01	–	0.03	–	–	–	+	+
	VP-3	0.06	–	–	DUL	.0006	0.01	–	0.02	–	–	–	+	+
	VP-4	0.15	–	–	DUL	0.005	0.01	–	0.02	–	–	–	+	+
	VP-5	0.0003	–	–	DUL	DUL	DUL	–	DUL	–	–	–	+	+
Products	VPP1[d]	20.24	–	–	DUL	1.67	5.33	–	0.50	–	–	–	+	+
	VPP2	0.10	–	–	DUL	0.02	0.01	–	0.04	–	–	–	+	+
	VPP3	0.05	DUL	–	DUL	0.01	0.01	–	0.04	–	–	–	+	+
	VPP4[d]	55.80	–	DUL	DUL	20.11	9.14	–	16.25	–	–	–	+	+
	VPP5[d]	254.2	–	–	DUL	12.58	50.26	–	46.25	–	–	–	+	+
	VPP6[d]	40.0	–	–	DUL	5.61	13.12	–	19.13	–	–	–	+	+
	VOP7	–	–	–	–	–	–	–	–	–	–	–	–	–
	VOP8	0.08	0.02	0.01	0.06	0.004	0.01	DUL	0.001	+	+	0.02	–	–
	VOP9	0.16	0.03	0.02	0.12	0.01	0.04	0.07	0.005	+	+	0.08	+	+
Stability	$VO_{4°C}$[d]	195.7	17.45	137.9	580.4	63.24	82.03	2.22	1.03	+	+	452.5	+	+
	$VO_{22°C}$[d]	185.3	18.75	141.5	576.4	67.71	88.71	2.54	1.15	+	+	449.2	+	+
	$VO_{50°C}$[d]	184.4	19.90	134.1	580.3	63.29	88.86	2.23	1.01	+	+	451.6	–	–
	$VP_{4°C}$[d]	34.74	–	–	DUL	1.33	27.89	–	13.53	–	–	–	+	+
	$VP_{22°C}$[d]	33.23	–	–	DUL	1.14	25.30	–	13.84	–	–	–	+	+
	$VP_{50°C}$[d]	30.58	–	–	DUL	1.84	26.07	–	14.00	–	–	–	DUL	DUL

[a] – = Not detected.

[b] DUL = Detected under the limit of quantitation.

[c] + = Detected.

[d] Liquid samples; the content of the analytes is given in μg/mL.

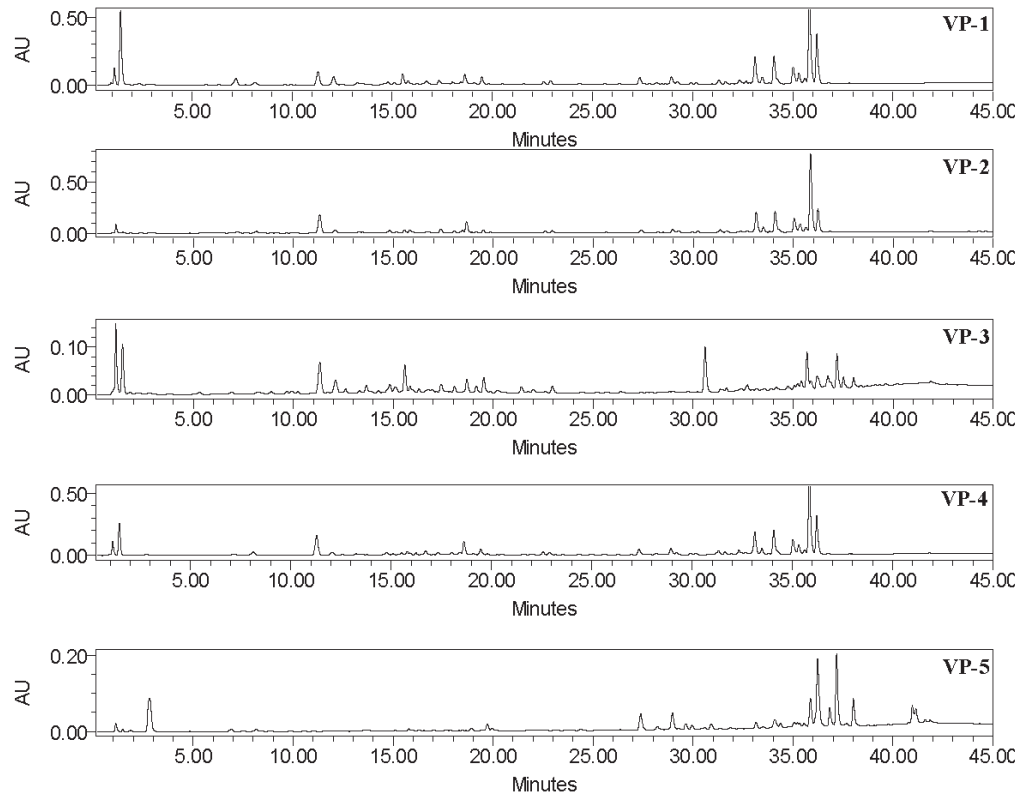

Figure 4. Liquid chromatograms of 5 *V. procera* Kunth populations at 225 nm.

data (Table 1) indicated the linearity of the detector response for all standard analytes from 0.60–80.0 μg/mL. The LOD was found to be in the range from 0.1 to 0.5 μg/mL for the standard analytes.

Plant Analysis

Figure 3 shows the variations of chlorogenic acid, lignans, flavonoids, and valerenic acids in 5 different *valeriana* species. Because each *valeriana* species had a distinctly different chromatogram, it was possible to develop a chemical profile in order to distinguish among *valeriana* species. Analytes **1**, **4**–**6**, and **8** were detected in all the species analyzed (Table 3). *V. officinalis* L. showed the highest concentration (1.157%) of chlorogenic acid (**1**), whereas *V. jatamansi* Jhones showed the lowest concentration (0.193%) for this analyte. Analyte **2** was detected in

Table 4. Characteristics of the commercial products analyzed containing *V. procera* or *V. officinalis*

Sample	Type of product	Species	Origin	Amount of valerian extract[a]
VPP1	Drops	*V. procera*	Mexico	5%
VPP2	Solid extract	*V. procera*	Mexico	—
VPP3	Solid extract	*V. procera*	Mexico	—
VPP4	Fluid extract	*V. procera*	Mexico	—
VPP5	Fluid extract	*V. procera*	Mexico	—
VPP6	Fluid extract	*V. procera*	Mexico	—
VOP7	Soft gelatin capsule	*V. officinalis*	Mexico	540 mg
VOP8	Capsule	*V. officinalis*	Egypt	100 mg
VOP9	Capsule	*V. officinalis*	United States	450 mg

[a] — = Not specified.

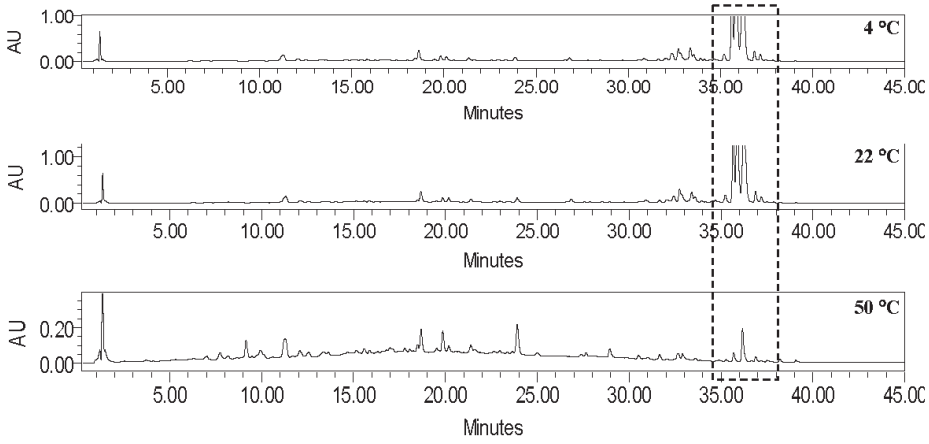

Figure 5. Chromatograms from heat-stability studies of an extract of *V. procera* Kunth treated at 4, 22, and 50°C showing decrease of valepotrate content at 50°C; detection was at 254 nm.

V. sitchensis Bong. (0.12%) and *V. officinalis* L. (0.013%). Analyte **3** was detected in *V. sitchensis* Bong. (0.053%) and *V. officinalis* L. (0.043%), and there were trace amounts in *V. jatamansi* Jhones. Content of **4** was highest in *V. officinalis* L. and trace amounts in *V. procera* Kunth. The ranges for Analytes **5** and **6** were between 0.002–0.5% and 0.012–0.122%, respectively, in the different species. Both analytes were in high concentrations in *V. sitchensis* Bong. and in low concentrations in *V. procera* Kunth and *V. edulis* Nutt. ex Torr. & A. Gray. Hesperidin (**7**) was detected in *V. sitchensis* Bong. (0.56%) and in *V. officinalis* L. (0.02%). The detection range of linarin (**8**) for the various *valeriana* species was between 0.002–0.23%. Highest concentration was present in *V. jatamansi* Jhones (0.23%), and trace amounts were in *V. edulis* Nutt. ex Torr. & A. Gray. species. Hydroxyvalerenic acid (**9**), acetoxyvalerenic acid (**10**), and valerenic acid (**11**) were not detected in various *valeriana* species, except in *V. officinalis* L. and *V. sitchensis* Bong. In *V. officinalis* L., 3 valerenic acids were detected. The content of valerenic acid detected in *V. officinalis* was 0.88%, and trace amounts appeared in *V. sitchensis* Bong. Dihydrovaltrates (**12**) were detected at 207 nm and valtrates (**13**) at 254 nm; these compounds were present in all of the species analyzed. *V. sitchensis* Bong. showed the least amount of these valepotriates, and *V. edulis* Nutt. ex Torr. & A. Gray. and *V. procera* Kunth showed the highest amounts.

As shown in Figure 4, the fingerprint method was validated by testing different populations of *V. procera* Kunth. Various populations of *V. procera* Kunth showed the same profile. Analytes **1**, **4–6**, and **8** were present, and a high content in valepotriates was observed in *V. procera* Kunth species. The valerenic acids (**9–11**) were absent in *V. procera* Kunth. However, the changes in the chromatographic profile of VP-3 (Figure 4) from VP-1, VP-2, and VP-4 may have been due to the difference in location, harvest time, and storage conditions (3). VP-5 grown wildly showed a different profile (Table 3).

The availability of unique fingerprints, with qualitative and quantitative data for each species provided a number of benefits, including authentication of samples, determination of chemotaxonomic markers, and identification of constituent patterns related to specific geographic location. Thereafter, the 11 analytes tested could be analyzed in *V. officinalis* L. and *V. sitchensis* Bong. *V. procera* Kunth and *V. edulis* Nutt. ex Torr. & A. Gray did not contain analytes **2**, **3**, **7**, or **9–11**. Analytes **1** and **8** were in higher amounts in *V. procera* Kunth than in *V. edulis* Nutt. ex Torr. & A. Gray. *V. jatamansi* Jhones did not show the presence of **2**, **7**, **9–11** and analyte **8** was present in higher concentration.

Product Analysis

Nine products were analyzed (Table 4): 6 products (VPP1-VPP6) labeled to contain *V. procera* Kunth and 3 products (VOP7–VOP9) labeled to contain *V. officinalis* L. The products had the same profile of the corresponding plant material with exception of VOP7, which did not show the presence of any of the analytes analyzed. VOP8 and VOP9 showed the presence of valerenic acids, lignans, hesperidin, and trace amounts of linarin (Table 3). Valepotriates were not detected in VOP8. The range of linarin detected in products VPP1–VPP6 was from 0.50 μg/mL (VPP1) to 46.3 μg/mL (VPP5). More variation in linarin content was observed in liquid products (0.5 to 46.3 μg/mL) than in solid products (0.04%). Lignans and valepotriates were detected in VPP2–VPP6, but valerenic acids and hesperidin were not detected in these products.

Heat-Stability Studies

Heat stability studies for valepotriates and valerenic acids had been reported previously (9, 20), however, no studies to date had been reported for the presence of other components in valerian species. Samples of freshly prepared tinctures from *V. officinalis* L. and *V. procera* Kunth were stored at 4, 22, and 50°C and subsequently analyzed. After storage for 2 weeks, the content of lignans, linarin, hesperidin, or valerenic acids

did not change much, whereas the valepotriates content was decreased significantly in tinctures of both species (Table 3). The tinctures prepared with *V. officinalis* L. and *V. procera* (Figure 5) were more sensitive at 50°C, and the content of valepotriates in the samples decreased significantly.

Conclusions

The method described determines presence or absence of valerenic acids in various *valeriana* species. The method was based on the analysis of chlorgenic acid, 5 lignans, 2 flavonoids, and 3 valerenic acids. The quantitation of chlorogenic acid, lignans, and flavonoids may present an important option to perform quality control analysis of plant material and final products prepared with valerian species that do not contain valerenic acids. Finally, the fingerprinting for *V. edulis*, *V. jatamansi*, *V. officinalis*, *V. procera,* and *V. sitchensis* was described. The LC profile of various valerian species was different with respect to the content of different analytes.

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