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Development and Validation of Fingerprints of *Turnera diffusa* Extracts Obtained by Use of High-Performance Liquid Chromatography with Diode Array Detection and Chemometric Methods[a]

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Summary. A high-performance liquid chromatographic method with diode-array detection (HPLC–DAD) has been developed and validated to establish the fingerprint of *Turnera diffusa*. Hydroalcoholic extracts were obtained from 19 raw herbal samples collected in different regions of México. Separation was performed on a 150 mm × 3.9 mm (4-µm particle) C_{18} column, using a gradient of methanol and 0.1% aqueous trifluoroacetic acid as the mobile phase. Chromatograms were recorded at 254 nm. To identify each peak, both retention time and peak spectrum were used. Intraday and interday relative standard deviations were <3% for retention time and <12% for relative areas. Extracts were stable in solution for up to 60 days. Results from a robustness study showed that the amount of ethanol in the mobile phase had a substantial effect on retention time. The relative areas of 12 peaks common to the chromatograms obtained from 19 authenticated *T. diffusa* samples were chosen to construct a principal-components analysis (PCA) model. The soft independent modeling of class analogy (SIMCA) method based on the PCA model was used to evaluate the quality of eleven commercial products.

Key Words: HPLC fingerprints, chemometrics, *T. diffusa,* herbal products, quality control

Introduction

Chromatographic fingerprinting is a useful tool for evaluation of the quality of herbal medicines and their related products. This technique has been accepted by the World Health Organization (WHO), the US Food and Drug Administration (FDA), and the European Medicines Agency as a strategy for assessment of the quality of herbal medicines [1–3]. It has been suggested that herbal medicines can be accurately identified and authenticated by use of chromatographic fingerprints, even if the amounts and/or con-

centrations of the constituents are not exactly the same in different samples of a herbal medicine [4, 5].

Thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), and electrophoretic methods are the main techniques used for fingerprint analysis [4–7]. HPLC is one of the most widely used methods because of the high precision, sensitivity, and reproducibility of the fingerprints [1, 3–5, 8–10]. With the additional UV spectral information that can be acquired by use of a photodiode-array detector (DAD), qualitative analysis of complex samples of herbal medicines becomes much easier [5, 11].

Turnera diffusa (damiana) is a small shrub that grows to a height of 1–2 m and bears aromatic, serrated leaves 10–25 cm long. Small yellow flowers bloom in early to late summer, and are followed by small fruit with a sweet smell and a fig-like flavor. The medicinal part of the plant is its leaves, which are harvested during the flowering season. Damiana, which is found throughout Mexico, Central America and the Caribbean islands, and parts of South America [12, 13], is recorded as having been used as an aphrodisiac in the ancient Mayan civilization. It has been used to treat conditions such as depression, anxiety, sexual inadequacy, debilitation, menstrual irregularities, gastric ulcers, and constipation [13]. In Mexico, the plant is also used to treat asthma, bronchitis, neurosis, diabetes, dysentery, dyspepsia, headaches, paralysis, nephritis, spermatorrhea, stomachache, and syphilis [14]. No method of quality control is available for this plant, and markers are unknown.

We recently described use of a TLC method for evaluation of the quality of several commercial products containing several plants, including *T. diffusa* [15]. The technique has several advantages when used for routine monitoring—many samples can be analyzed in parallel, it requires minimum sample cleanup, and it is simple and economical. Nevertheless, when multicomponent samples, for example herbal extracts, are under consideration, more sensitive and appropriate methods are needed, for example HPTLC and HPLC.

A chromatographic fingerprint of a herbal medicine is a multivariate system because it represents many of the constituents of the herbal product, so the information content of a chromatogram containing many peaks must be determined by use of a variety of approaches [5, 16, 17]. Principal-components analysis (PCA) is a powerful tool for analysis of multivariate data. PCA compresses the original data into a new set of variables called the "principal components" (PCs). PCA analysis groups samples and variables according to similarities or correlation between them. PCA can construct a theoretical model of the fingerprints using a data set obtained from samples

of the same class (calibration samples) [18, 19]. Unknown samples can be compared with the PCA model previously constructed, and recognized or rejected as a member of a class using the soft independent modeling of class analogy (SIMCA) [18, 19].

In this study, we used HPLC–DAD to develop a simple, rapid, and validated method for the qualitative analysis of *T. diffusa*. PCA was used to establish a chromatographic fingerprint model, and by use of this model a series of commercial products was analyzed using the SIMCA method.

Experimental

Reagents

HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained from Laboratorios Monterrey (Monterrey, NL, Mexico). Before use, these mobile phase components were filtered through 0.45-µm Nylon filters (Waters Corporation, Milford, MA, USA). Samples were filtered through Nylon acrodiscs (Waters Corporation). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), and reagent-grade ethanol were from Fermont (Monterrey, NL, Mexico).

Plant Materials

Turnera diffusa samples were collected from different regions of Mexico. Their origins are listed in *Table I*. The plants were authenticated at the Institutional Herbarium of the Biological Sciences School of the Universidad Autónoma de Nuevo León.

Table I. Turnera diffusa samples collected from different regions of México

Sample	Region of México	Date of collection
1	Cadereyta Jiménez, N.L.	December 2004
2	Zuazua, N.L.	December 2005
3	Ciudad Victoria , Tamaulipas	October 2006
4	La Cruz de Elote, Sinaloa	October 2006
5	Rayones, N.L.	November 2006
6	Jaumave, Tamaulipas	December 2006
7	Marín, N.L.	January 2007
8	San Luis Potosí, S.L.P.	January 2007
9	Cañada del Lobo, S.L.P.	January 2007

Table I. (continued)

Sample	Region of México	Date of collection
10	Zuazua, N.L.	February 2007
11	Nuevo Padilla, Tamaulipas	July 2007
12	Marín, N.L.	October 2007
13	Dr González, N.L.	October 2007
14	Linares, N.L.	October 2007
15	Cadereyta Jiménez, N.L.	October 2007
16	Pesquería, N.L.	October 2007
17	Zuazua, N.L.	October 2007
18	Rayones, N.L.	October 2007
19	Villaldama, N.L.	October 2007

S.L.P., San Luis Potosí; N.L., Nuevo León

Commercial Products

Commercial products containing *Turnera diffusa* as unique component were purchased from several drug stores, markets, and herbal stores in the states of Nuevo Leon, Tamaulipas, San Luis Potosi, Baja California, and Veracruz, Mexico. They consisted of the whole aerial parts from the plant, or their powder.

Sample Preparation

The collected plants were dried at room temperature (27°C). The aerial parts (leaves and stems) were ground, and passed through a 40-mesh sieve. A portion (~2 g) was accurately weighed and extracted three times with 5 mL 9:1 (v/v) ethanol–water at 27°C by vortex mixing for 3 min. The extracts were filtered through a Whatman No. 40 filter paper and evaporated to dryness under reduced pressure by rotary evaporation at 37 ± 2°C. All the extracts were stored at 4°C. The same extraction process was used for the commercial products.

Before analysis, the sample extracts (15 mg) were dissolved in DMSO (1 mL). All samples were then filtered through 0.45-µm Nylon filters before injection for chromatographic analysis.

Equipment and Chromatographic Conditions

Liquid chromatography was performed with a Waters 2690 Alliance system with a 996 photodiode-array detector. Compounds were separated on a 150 mm × 3.9 mm, 4-µm particle, AccQ Tag C_{18} column (Waters Corporation) with a gradient prepared from 0.1% TFA (component A) and methanol (component B) as mobile phase. Before use, the mobile phase was degassed and filtered through a 0.45-µm filter. The gradient program started with 70% A and 30% B; this was changed to 30% and 70% B in 25 min then maintained constant for 5 min. The mobile phase flow rate was 0.4 mL min^{-1}. Before the first injection, the column was preconditioned for an hour with the initial phase, and each run was followed by an equilibration period of 10 min under the initial conditions.

The column temperature was maintained at 30°C and the injection volume was 10 µL. The DAD was set at 254 nm to acquire the chromatograms. UV spectra were acquired in the range 200–400 nm (2-nm resolution).

Method Validation

Precision

Precision was evaluated by measurement of the relative standard deviation (RSD, %) of retention times, relative peak heights, and relative peak areas for the most intense signals. Intraday RSDs were calculated for five injections of an extract of *T. diffusa* (raw herbal sample) on the same day. Interday precision was calculated by injecting the same solution on five different days. The RSD (%) of the extraction procedure was determined by extraction of the same sample on four different days.

Selectivity

Retention times and UV spectra were used to identify the chromatographic peaks common to all 19 samples. The chromatographic fingerprint and UV spectra of the peaks obtained from *Turnera diffusa* were also compared with those obtained, under the same conditions, from an extract from *Chrysactinia mexicana*, a plant popularly known as "damianita" [12].

Robustness

Robustness was tested by assessing the effects of slight variation of the temperature, the initial composition of the mobile phase, and the amount of TFA in the mobile phase. Values slightly higher and lower than the optimum values were used. The variables that were modified, and their values, are presented in *Table II*.

Table II. The conditions used for evaluation of the robustness of the method

Variable	Optimized condition	Modified condition	
		High value	Low value
Column temperature (°C)	30	32	28
Concentration of TFA (%)	0.1	0.15	0.05
Initial proportion (%) of MeOH	30	32	28

Three replicates were analyzed for each change of conditions. In all cases robustness was assessed by measurement of retention times (t_R). The results were evaluated by use of eq. (1) [20].

$$\text{If } \left|Vt_R - vt_R\right| > \sqrt{s(0.95)} \tag{1}$$

the evaluated variable was regarded as affecting the chromatographic process significantly. Vt_R and vt_R are the mean retention times obtained for the high and low values defined for each variable, respectively, and s is the standard deviation of t_R obtained from analysis of precision.

Stability

To determine the stability of the components in the extracts, portions of an extract were stored at 4°C for 120 days and analyzed, by use of the HPLC method, at 30-day intervals. The results were evaluated using control graphs constructed using the results obtained from measurement of precision.

Data Analysis

Data were analyzed by use of a Pentium 4 processor (Windows XP). Relative peak areas and peak heights were calculated and standardized by use

of Microsoft Office Excel software. PCA and SIMCA analyses were performed by use of Unscramble v.9.2 program (Umetrics).

Results and Discussion

Optimization of the Extraction Conditions

To select the optimum conditions for extraction, two different sets of conditions reported in the literature, which differed in the proportion of solvents (ethanol–water), and time and type of agitation (vortex mixing and ultrasonic bath) were evaluated [6, 21]. Reproducibility was assessed, and the numbers of peaks in the chromatograms were determined to evaluate the extraction efficiency. Under the conditions selected, and described in the Experimental section, 42 peaks were observed.

Although a similar number of signals was obtained by use of an ultrasonic bath, this type of agitation had some disadvantages. It required more time (30 min), its reproducibility was not sufficient, the peaks were less intense, and the bath temperature increased with time. This last factor could cause the decomposition of some extract components.

Optimization of the HPLC Conditions

To develop a fingerprint for *T. diffusa*, we assayed three columns (a 125 mm × 4 mm, 5 µm particle, MetaChem Hypersil BDS C_8, a 100 mm × 4.6 mm Chromolith C_{18}, and a 150 mm × 3.9 mm, 4-µm particle, AccQ Tag C_{18}), two organic mobile phases components (methanol and acetonitrile), addition of TFA to the mobile phase, and several modifications of the gradient. The reproducibility, analysis time, and number of peaks in the chromatograms were the criteria used in optimization of the separation conditions.

The greatest number of peaks (46) and the best reproducibility were obtained by using the AccQ Tag C_{18} column under the conditions described in the Experimental section.

Although acetonitrile has been recommended for improving the selectivity of these separations, we obtained better results when we used methanol (i.e., more peaks were observed). The importance of controlling the pH of the mobile phase when analyzing ionizable compounds by RP–HPLC is well recognized and easily understood. TFA can be used both to control the pH of the mobile phase and to improve the selectivity of the separation [20]. Addition of 0.1% TFA to the mobile phase improved both the resolution

and the peak symmetry of the chromatograms. Most of the major peaks were separated to baseline

To obtain a large number of detectable peaks on the HPLC chromatograms, spectra were recorded between 200 and 400 nm for all peaks. The best results (i.e., the largest number of peaks detected) were obtained at 254 nm.

Validation of the Method

Precision

Precision was evaluated as the RSD (%) of retention times, relative peak heights, and relative peak areas of the signals with the greatest intensity (16 peaks). *Table III* shows the results obtained for intraday and interday precision. For 16 of the 17 peaks, intraday RSD for both relative peak areas and relative peak heights were less than 8%. Interday RSD for the same 16 signals were less than 10%. The peak at t_R = 2.87 min had RSD values of 12% and 16% for intraday and interday precision, respectively. That peak did not separate well from Peak 1 (t_R = 2.64).

Table III. Results from determination of the intraday and interday precision of the chromatographic system

Characteristic	Intraday RSD (%)	Interday RSD (%)
t_R	0–1.42*	0.10–2.82*
Relative area	0.84–8.02*	2.35–12.05*
Relative height	0.87–7.95*	2.71–16.01*
n	5	15

*Evaluation for 16 peaks

RSD for the extraction procedure was determined by extraction of the same sample on four different days. *Table IV* shows the results obtained from determination of the precision of the method, including the extraction step.

Table IV. Results from evaluation of the extraction process used for *Turnera diffusa* plants

Characteristic	RSD (%)
t_R	0.40–3.33*
% Area	7.93–14.91*
% Height	5.07–19.54*
n	4

*Evaluation for 16 peaks

Selectivity

Table V shows the retention times and characteristic spectra of each of the 14 common signals obtained from the 19 *Turnera diffusa* samples analyzed. Identification criteria were retention times and the spectra obtained at the apex of each signal, after baseline subtraction. None of the signals selected by us to construct the fingerprint model of *Turnera diffusa* were found in the chromatogram obtained from an extract of *Chrisantinia mexicana.*

Table V. Mean retention times, spectra, and relative areas of the peaks common to the 19 samples of *Turnera diffusa*

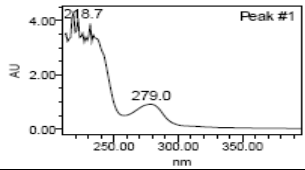
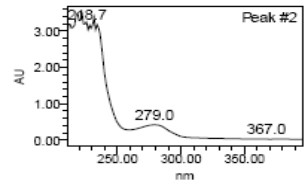
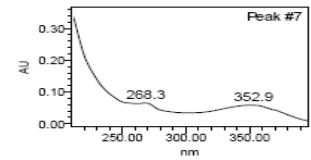
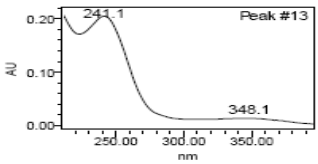
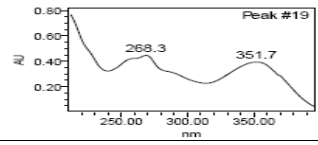
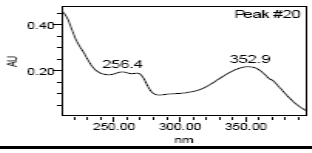
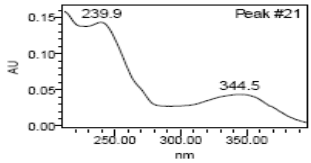
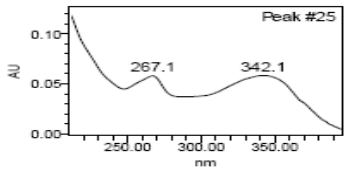
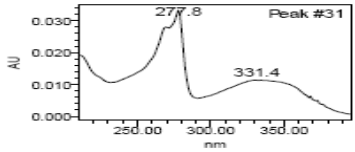
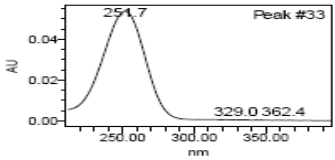
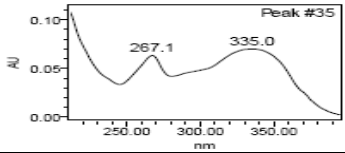
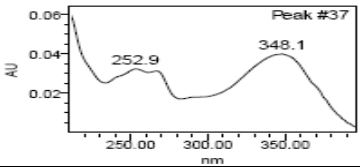
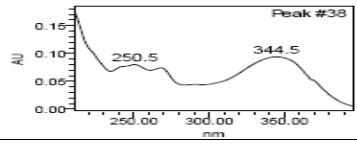
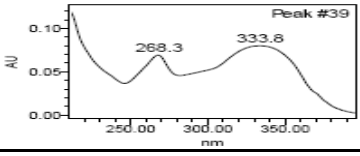
Peak	t_R	UV–visible spectrum	Relative area (%)	
			Mean	Range
1	2.64		26.01	7.03–59.59
2	3.30		20.16	7.20–34.82
3	7.19		5.02	1.02–10.73
4	11.48		6.55	0.36–14.30
5	15.53		9.44	2.69–17.38
6	16.22		4.18	0.58–11.71

Table V. (continued)

Peak	t_R	UV–visible spectrum	Relative area (%)	
			Peak	Range
7	17.20		2.32	0.30–6.50
8	20.40		2.75	1.26–4.31
9	23.85		1.97	0.15–6.68
10	25.07		2.83	0.46–6.86
11	26.26		3.58	0.98–11.23
12	27.02		3.17	0.27–8.19
13	29.60		5.36	2.78–11.86
14	32.70		6.54	1.24–15.47

Robustness

Robustness was assessed by modification of several experimental conditions to values higher and lower than the optimum values then measurement of t_R. The method was affected by changes in temperature, solvent composition, and amount of TFA in component B. During the chromatographic assays we perceived slight changes in the t_R values of some of the peaks, which is why it was very important to confirm the signals by use of the UV spectra. Variations in t_R can originate over time from uncontrollable fluctuations in temperature and pressure, and from matrix effects and the degradation of the stationary phase [22].

Stability

Stability was evaluated as a function of the total number of peaks and of the relative heights of the most intense peaks. The results were evaluated by use of control graphs constructed using the results obtained from the precision experiment, with a limit of ±2 s. The extract was analyzed 30, 60, 90, and 120 days after preparation. The results for the stability as a function of the relative heights of the 17 peaks showed the extract was stable until day 60 after its preparation when stored at 4°C. On day 90, four of the components of the extract of *T. diffusa* had lost their stability, and on day 120, more components were unstable. The total number of peaks did not change. On the basis of on these results, all extracts were analyzed within 30 days of their preparation.

Standardization of the Chromatographic Fingerprints of *Turnera diffusa*

Comparison of chromatographic fingerprints can be used to demonstrate both the "sameness" of samples and the "differences" between them. In this work we are using similarities between chromatograms obtained from different samples to establish a chemometric model to be used for quality control of products prepared from damiana. The peaks present in all 19 raw herbal samples were designated "common peaks" for *T. diffusa* (*Fig. 1*). Fourteen common peaks were found in the chromatograms of all the raw herbal samples, on the basis of both their retention times and their spectra (*Table V*). UV spectra were recorded at the apex of each of the signals and were visually compared, taking into consideration their wavelength of maximum absorbance.

The areas of the peaks common to all the samples were analyzed by using Excel 2003 to calculate the relative peak areas of the 14 common peaks in the chromatograms:

$$\text{Relative area (\%)} = (\text{peak area} \times 100)/\Sigma(\text{areas of common peaks}) \quad (2)$$

Before chemometric analysis by PCA the relative areas were standardized by use of eq. (3):

$$Astd = (Ao - Aavg)/s \quad (3)$$

where *Ao* is the relative percentage area of a peak, and *Aavg* and *s* are the average and the standard deviation of the relative areas of this peak in all the samples.

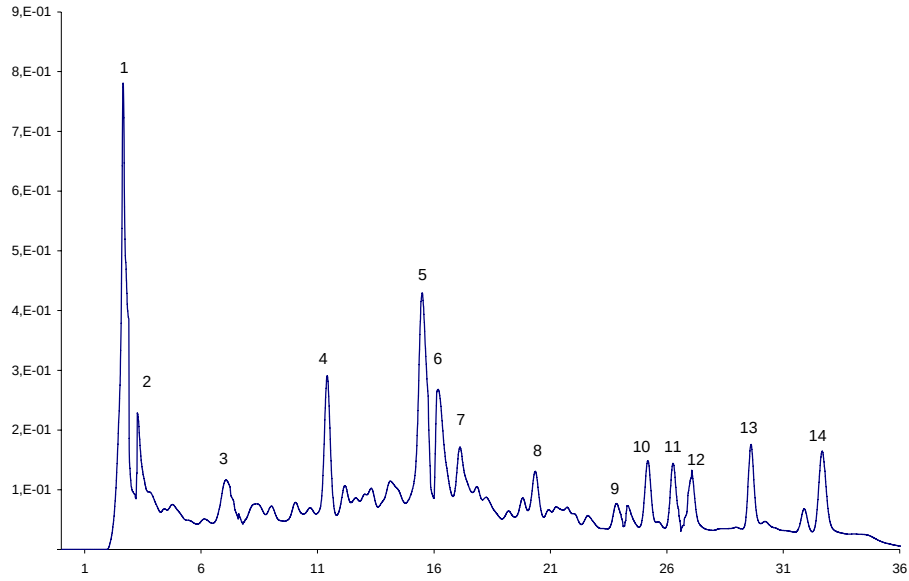


Fig. 1. Chromatogram obtained from an extract of *Turnera diffusa* with the 14 peaks common to all the raw herbal samples

Principal-Components Analysis

The objective of this study was to establish a chemometric (PCA) model using the chromatographic fingerprint of *T. diffusa* for quality control of commercial herbal products containing this plant.

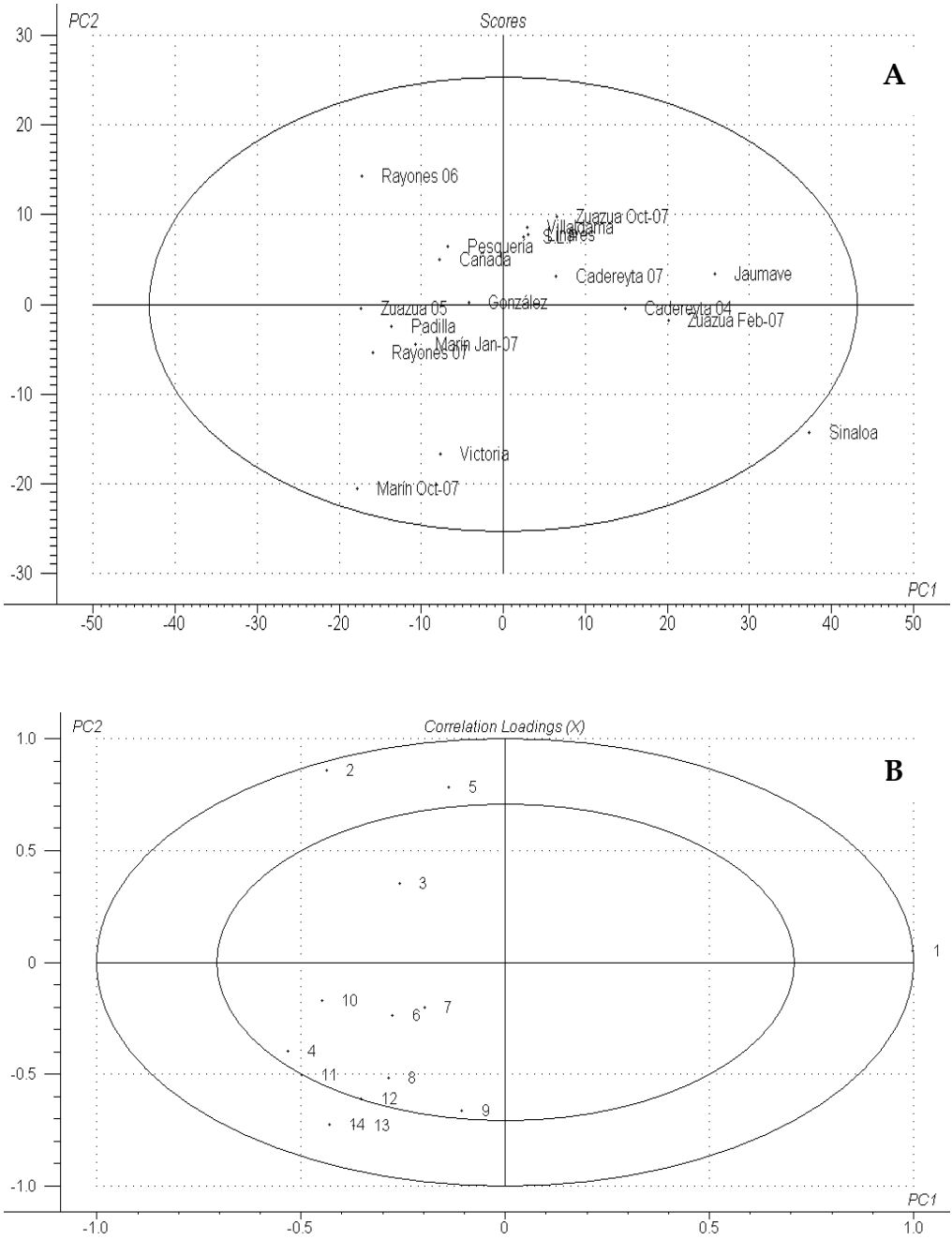


Fig. 2. (A) The PCA score (samples) and (B) the loading (variables) plots for 19 *T. diffusa* samples. Numbers 1–14 correspond to the relative areas of peaks 1–14, respectively

PCA is a popular method that can be used to summarize multivariate data. The simplest approach in applying it to chromatographic data is to use the raw chromatogram data set for each sample. The variability in t_R and in detector response makes this approach difficult. Some mathematical models have been used to resolve this problem [5, 22–24], but they have limited applicability.

Some authors have described the use of data extracted from the peaks of interest in the chromatogram. For example, Chen et al. [8] described a liquid chromatographic (LC)–UV method for characterization of authentic *Ginkgo biloba* materials and commercial products. They used the normalized areas of 21 characteristic chromatographic peaks in the PCA analysis, with good results. Similarly, in fingerprint analysis of *Eucommia* bark, Ni et al. constructed a data matrix containing the absolute peak areas of the seven principal peaks from the plant; using this model they could discriminate between herbs from different regions of China [9]. Therefore, to construct our PCA model, we used the characteristic peaks present in all the raw herbal samples. Fourteen peaks were common to the 19 plants analyzed. The relative areas of these peaks were calculated in an Excel database and exported to Unscramble v. 9.2 software for PCA analysis. All the data were standardized before chemometric analysis by PCA, as recommended by Miller and Miller [19]. If the variables were not standardized, the variable with the greatest variance might dominate the first main component.

Initially, a data matrix of 19 samples (raw herbal samples) × 14 variables (relative areas of peaks 1–14) was submitted for PCA. The samples (scores) and variables (loadings) plots are presented in *Fig. 2*. Using this model, *T. diffusa* from Sinaloa had the highest positive score on PC1. From the loading plot, we observed that Peak 1 had a very high positive value in PCA1. Therefore, this peak contributed strongly to the variance of the data. This peak in the sample from Sinaloa was the largest chromatographic peak in the sample, and its relative area was significantly different from those of the other samples. With this observation in mind, and considering that Peaks 1 and 2 could not be resolved using our conditions, we decided to exclude it from our model.

The new model consisted of a data matrix of 19 samples × 12 variables. The samples (scores) and variables (loadings) plots are presented in *Fig. 3*. In this new model, all the variables make similar contributions to the variance of the data. Some clusters observed in the score plot explain some of the similarities between the samples. For example, Cañada and SLP are samples collected in the same region (San Luis Potosi). These samples had the highest positive score on PC1 and a similar negative score on PC2, and we associated with variable 3 (loading plot). In the same way, two of the three samples from Tamaulipas (Victoria and Padilla) had the highest nega-

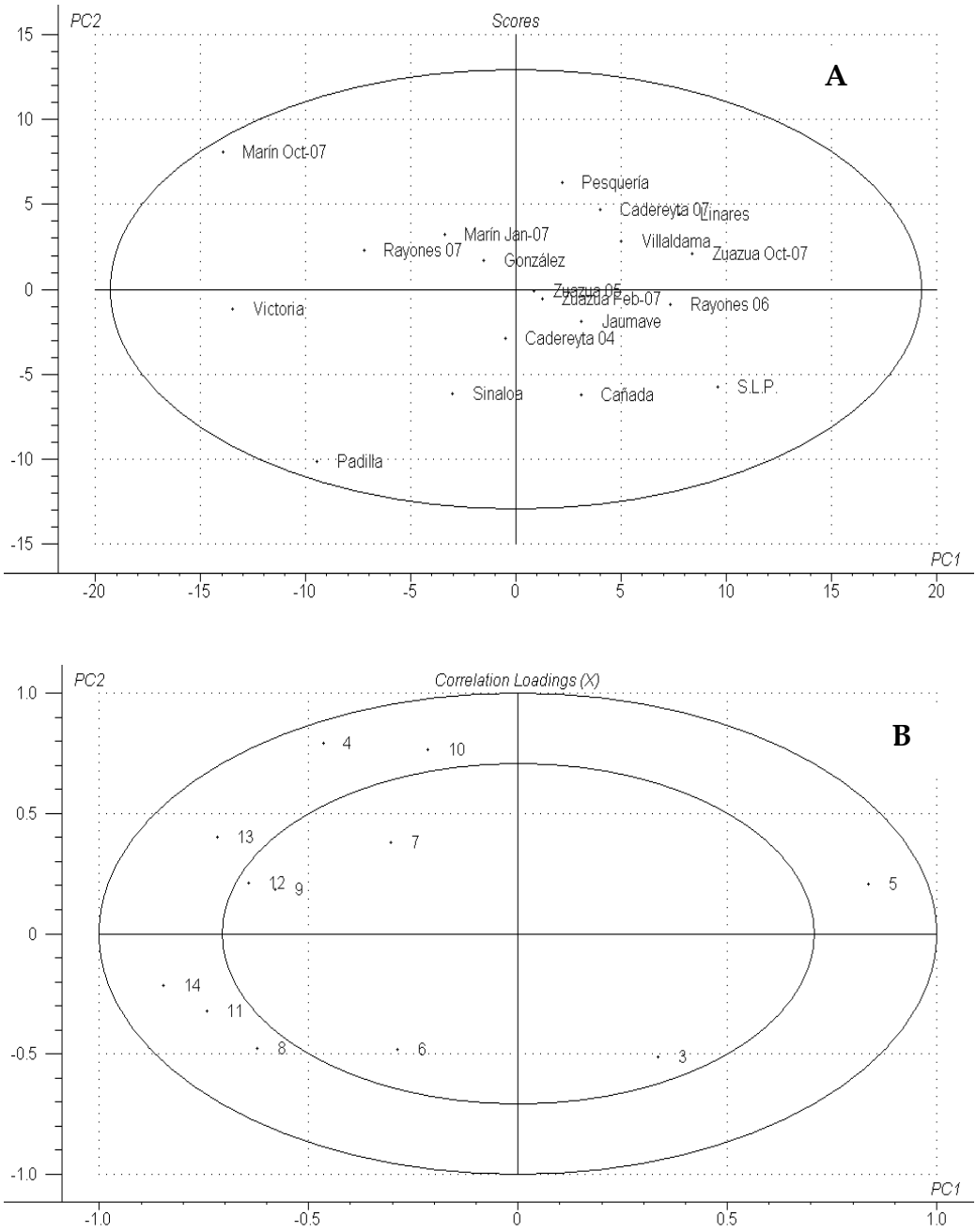


Fig. 3. (A) The PCA score (samples) and (B) the loading (relatives areas of peaks 3–14) plots for the 19 *T. diffusa* samples without variables (peaks) 1 and 2

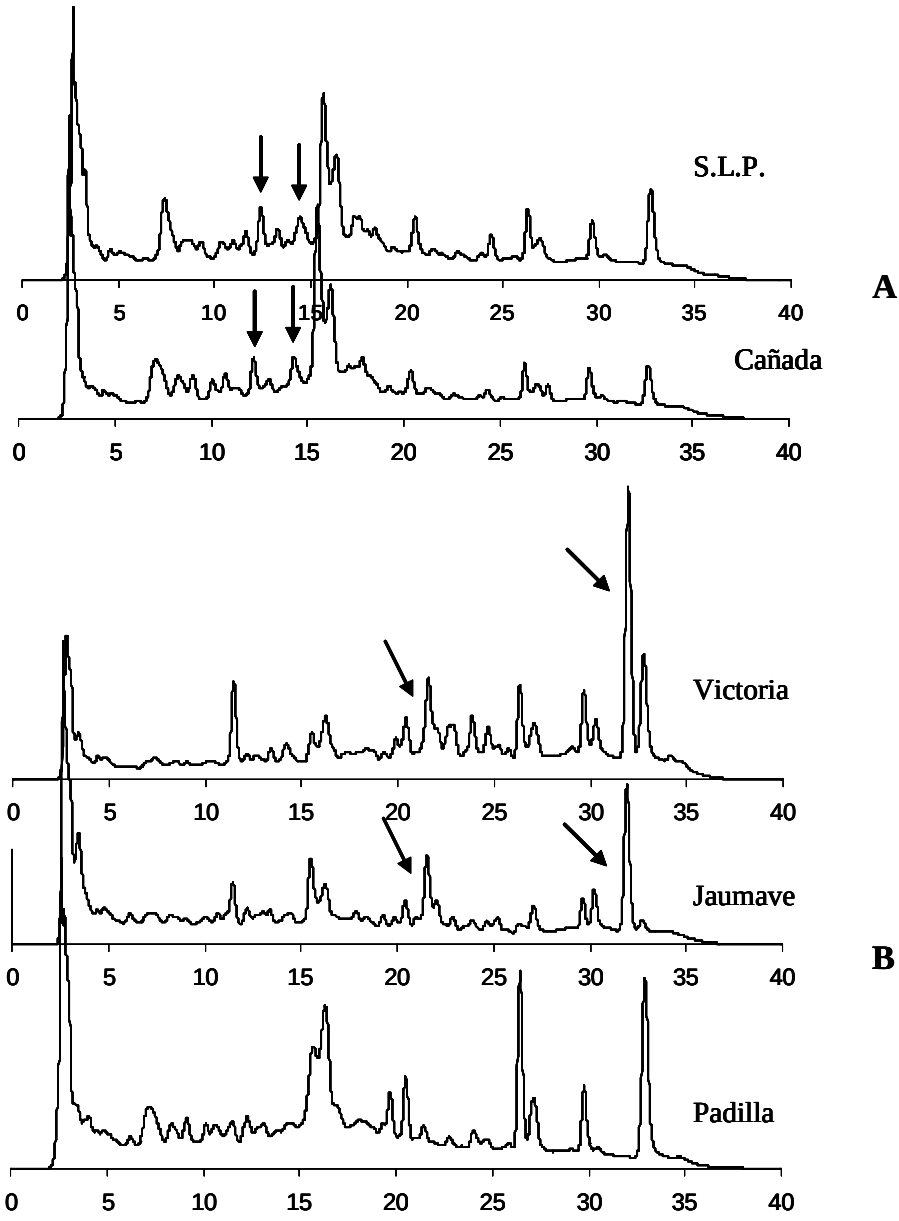


Fig. 4. Chromatograms of *Turnera diffusa* extracts. (A) Obtained from plants collected in two different regions of San Luis Potosi. (B) Obtained from plants collected in three different regions of Tamaulipas

tive scores on PC1. A sample collected in Jaumave (Tamaulipas) clustered with four of the samples collected in the Nuevo Leon region (negative scores on PC2). All the samples with positive scores on PC2 were collected in Nuevo Leon in 2007.

In the chromatograms for two plants collected in San Luis Potosi (Cañada and SLP), two peaks with t_R values of 12 and 14 min were observed which did not appear in the chromatograms of the other samples (*Fig. 4*). These peaks were not taken into consideration in constructing the PCA model, because they were not common to all samples. However, in future, these peaks could be used to distinguish plants from this region. Although a sample collected in Jaumave was not similar to the other plants collected in Tamaulipas in the PCA analysis, its chromatogram had two peaks with t_R values of 21 and 31 min, which were also present only in the Cd. Victoria sample (*Fig. 4*). These peaks were not present in the chromatogram of the sample from Nvo. Padilla.

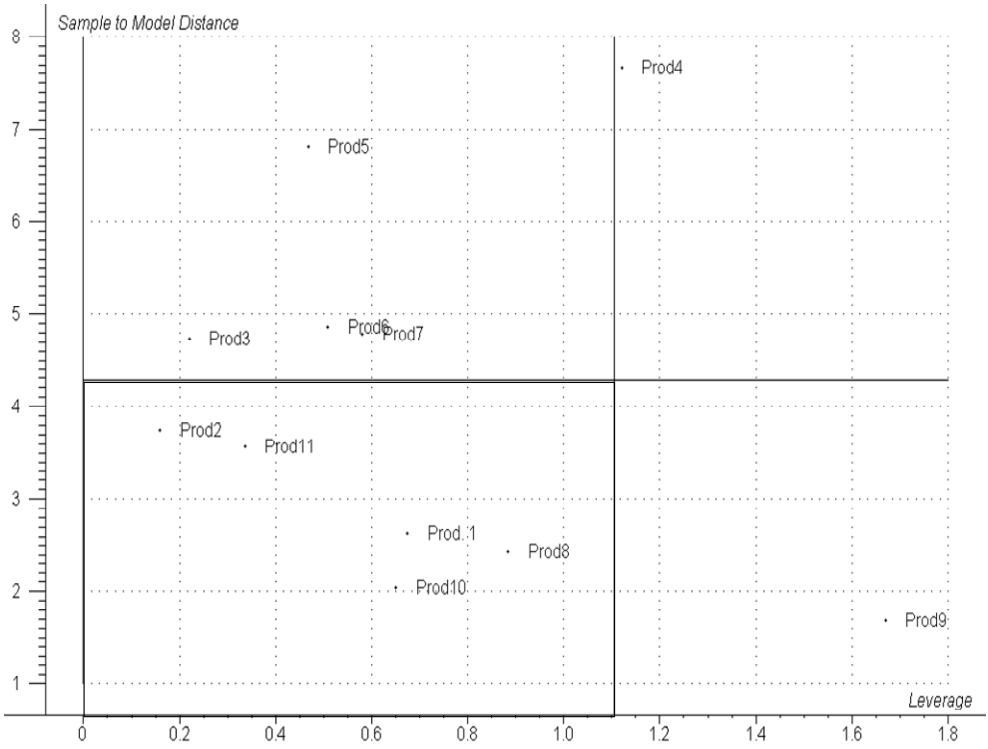


Fig. 5. A plot of sample-to-model distance (Si) against leverage (Hi) was used to evaluate eleven commercial products of *T. diffusa*

Applicability

SIMCA classification based on the predefined PCA model described above was used to evaluate the quality of eleven commercial products obtained from several drug stores, markets, and herbal stores in different regions of Mexico.

A graph of sample-to-model distance (Si) vs sample leverage (Hi) was used to identify the samples that were accepted or rejected by our model (*Fig. 5*). Products 1, 2, 8, 10, and 11, with values below 4.3 for Si and 1.1 for Hi, were accepted by the model at the 1% significance level. The other products were rejected. Product 9 was rejected because its leverage was high (1.7), even though its distance to the model value was low (1.8).

Conclusions

In this study, 19 samples of native *T. diffusa* were collected from different regions of Mexico. An HPLC–DAD analytical method for obtaining the fingerprint of the plant was developed and validated. Fourteen common peaks were found in the 19 samples. The retention time and UV–visible spectrum were used to identify each peak. A PCA model with 19 samples and 12 variables was constructed and used with a SIMCA classification method to evaluate eleven commercial products of *T. diffusa*. Only five of the eleven products evaluated were recognized by the model as *T. diffusa*.

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