

# Chromatographic Fingerprinting of Flos Chrysanthema Indici Using HPLC

## INTRODUCTION

The flower of the Flos chrysanthemi indicum, *Dendranthema indicum* L. (*D. indicum*), is a common medicinal plant known in China as wild chrysanthemum. The Chinese Pharmacopeia Edition 2005 (CP 2005) regulates its use as an herbal medicine.<sup>1</sup> It is used with the belief that it improves eyesight and cures fever, swelling, erysipelas (a bacterial infection of the skin), sore throat, and headache.<sup>2</sup> *Dendranthema lavandulaefolium* (Fish) Mak is another type of flowering plant in the same genus as *D. indicum* that grows in the same types of environments, appears similar to *F. chrysanthemi*, and is often mislabeled as such when the plant is harvested.

Only the flower of *D. indicum* plant can be used as the herbal medicine described in CP 2005. That method regulates *F. chrysanthemi* using an HPLC method for determination of linarin, which exists in both *D. indicum* and *D. lavandulaefolium*.<sup>2</sup> For this reason, the quality control for *F. chrysanthemi* using this method is both inadequate and inaccurate. A previously developed method for evaluating the quality of this species used

HPLC to determine the content of chlorogenic acid and linarin in samples.<sup>3</sup> However, the ability to determine other components unique to *F. chrysanthemi* is critical for more accurate quality evaluation.

Traditional Chinese medicine (TCM) considers chromatography as an effective means to control TCM quality<sup>4-7</sup> with several published reports on chromatographic fingerprinting of *F. chrysanthemi*.<sup>8-10</sup> Flavonoids and homo-chlorogenic acids are two types of compound classes in this species that can be used as markers for identification. Tan *et al.* obtained an HPLC chromatographic fingerprint of chlorogenic acid, caffeic acid, and linarin, among other compounds, in samples of *F. chrysanthemi*.<sup>10</sup>

Here, the authors establish an HPLC chromatographic fingerprint of *F. chrysanthemi* based on discriminating the characteristic peaks of chlorogenic acid and flavonoids (luteolin-7-*o*-glucoside, linarin, luteolin and apigenin; structures shown in Figure 1). The authors also compare an ultrasonic extraction technique to Accelerated Solvent Extraction (ASE®)<sup>6</sup> for isolation of the target components from the samples.

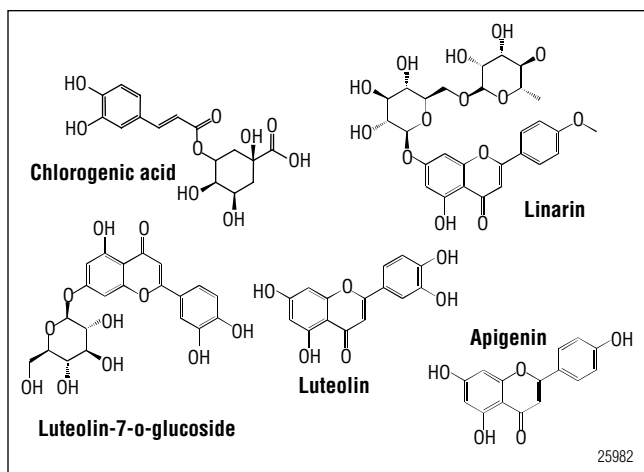


Figure 1. Structures of chlorogenic acid, luteolin-7-o-glucoside, linarin, luteolin, and apigenin.

## EQUIPMENT

Summit® HPLC system\*

DPG 680 Pump with SRD-100 Solvent Rack  
with degasser

TCC-100 Thermostatted Column Compartment

ASI-100T Autosampler

PDA-100 UV/Vis detector

UltiMate® 3000 HPLC system\*

HPG 3400A Pump with SRD 3400 Solvent Rack  
with degasser

TCC-3000 Thermostatted Column Compartment

WPS-3000TSL Autosampler

VWD-3400 UV/Vis detector

Chromeleon® 6.80 SP3b Chromatography Data System

Multi-Variate Statistical Package (MVSP)™ 3.1

Kudos SK3200LH Ultrasonic Generator, Kudos

Ultrasonic Instrumental Co., Shanghai, China

ASE 200 Accelerated Solvent Extractor

\* The data in this application note was collected using both systems. A photodiode array detector (PDA-100 or PDA3000) is required for spectral scans such as those in Figure 2.

## REAGENTS AND STANDARDS

Water; Milli-Q® Gradient A10

Acetonitrile (CH<sub>3</sub>CN), HPLC grade, Fisher Scientific

Methanol (MeOH), HPLC grade, Fisher Scientific

Phosphoric acid (H<sub>3</sub>PO<sub>4</sub>), analytical grade, SCRC, China

Acetic acid (HAc), analytical grade, SCRC, China

Ethyl acetate, analytical grade, SCRC, China

Chlorogenic acid, purity ≥ 98%, Huyun Pharmaceutical Co., Shanghai, China

Linarin, purity ≥ 98%, Research Center of Standardization of CTM, Shanghai, China

Luteolin-7-o-glucoside, purity ≥ 98%, lab-prepared (extract from *F. chrysanthemi*)

Luteolin, purity ≥ 98%, lab-prepared (extract from *F. chrysanthemi*)

Apigenin, purity ≥ 98%, lab-prepared (extract from *F. chrysanthemi*)

## SAMPLES

Twelve samples were purchased from commercial retailers and eight samples were collected from different regions in China and used for this study. Sample details are shown in Table 1. Flower samples of both *D. indicum* and *D. lavandulaefolium* were collected and separated by Professor Wu Lihong (Shanghai Univ. of TCM) and Professor Liu Shoujin (Anhui Univ. of TCM). These certified collected samples were stored in the Shanghai Research Center of TCM Standardization.

**Table 1. Twenty Flos Chrysanthemi Samples**

Purchased Sample #	Location of Purchase	Collected Sample #	Location of Collection	Plant Type
1	Henan 1	13	Shanghai Univ. of TCM	<i>D. indicum</i>
2	Henan 2	14	Weiyuan Sichuan	<i>D. lavandulaefolium</i>
3	Dalian, Liaoning	15	Huangwan Sichuan	<i>D. indicum</i>
4	Tongrentang, Beijing	16	Qingyang 1 Anhui	<i>D. lavandulaefolium</i>
5	Zhangjiang, Shanghai	17	Qingyang 2 Anhui	<i>D. indicum</i>
6	Yunnan	18	Tianmashan Shanghai	<i>D. indicum</i>
7	Suzhou, Jiangsu	19	Luanyang Henan	<i>D. lavandulaefolium</i>
8	Huayu, Shanghai	20	Henan	<i>D. indicum</i>
9	Guangdong 1			
10	Guangdong 2			
11	Xiamen, Fujian			
12	Hangzhou, Zhejiang			

**CONDITIONS****ASE Conditions**

Solvent:	Methanol/Ethyl acetate (1:1, v/v)
Temperature:	100 °C
Pressure:	1500 psi
Static Time:	5 min
Static Cycles:	3
Flush:	60 %
Purge:	100 s

**Chromatographic Conditions**

Column:	Acclaim® 120 C18, 5 µm, 4.6 × 250 mm (P/N: 059149)
Column Temp.:	30 °C
Mobile Phase:	Gradient (Table 2) A. CH <sub>3</sub> CN B. HAc/water (0.1%, v/v)
Flow Rate:	1.0 mL/min
Injection Vol.:	10 µL
UV detection:	Absorbance @ 326 nm

**PREPARATION OF STANDARDS AND SAMPLES****Standard Solutions**

Weigh approximately 4 mg chlorogenic acid, luteolin-7-*o*-glucoside, linarin, luteolin, and apigenin and bring to volume in 10 mL volumetric flasks. The concentration of each analyte is approximately 0.4 mg/mL.

**Sample Preparation**

Weigh approximately 0.5 g of each dried and powdered sample and pass each through a 250 µm sifter. Purchased samples were already dried; collected samples were air dried.

Ultrasonic method: Add the weighed sample to a 25 mL volumetric flask, and bring to volume with a solution of methanol/ethyl acetate (1:1, v/v). After 30 min of ultrasonic extraction, bring to volume with the methanol/ethyl acetate solution to replace the liquid lost by evaporation.

ASE method: Add the weighed sample to a 5 mL stainless steel extraction cell equipped with two cellulose filters on the bottom. Samples should nearly fill the cell. Program the ASE system to perform the extraction using the conditions above and transfer the extracts into separate 25 mL volumetric flasks. Bring flasks to volume with methanol /ethyl acetate(1:1).

Prior to injection, the dilution should be filtered through a 0.45 µm filter (Millex-HN® Syringe Filter Unit).

## RESULTS AND DISCUSSION

### Optimization of Chromatographic Conditions

Figure 2 shows the UV absorption spectra of chlorogenic acid, luteolin-7-*o*-glucoside, linarin, luteolin, and apigenin. Two absorption bands are found between 210 and 400 nm in the UV absorption spectrum of luteolin-7-*o*-glucoside, linarin, luteolin, and apigenin, which are characteristic of flavonoids.<sup>11</sup> 326 nm was selected for the analysis, as chlorogenic acid and flavonoids both demonstrated good absorbance at that wavelength.

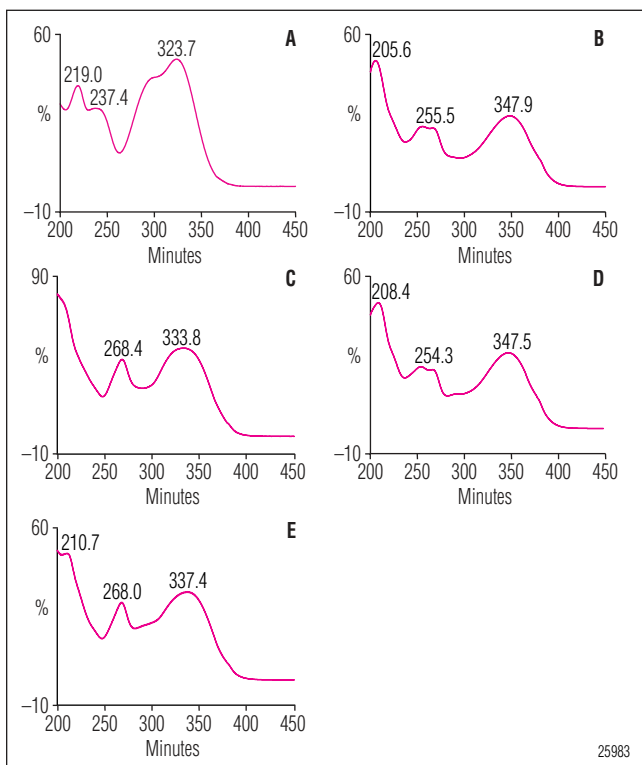


Figure 2. UV-vis absorption spectra obtained using the PDA-100 absorbance detector. (A) chlorogenic acid, (B) luteolin-7-*o*-glucoside, (C) linarin, (D) luteolin and (E) apigenin

To optimize separation of the component peaks of homo-chlorogenic acids and flavonoids in *F. chrysanthemi*, several mobile phases were investigated, including methanol/water, acetonitrile/water, and acetonitrile/acidic aqueous solution (HAc and H<sub>3</sub>PO<sub>4</sub>). Empirical data demonstrate that a chromatogram with good resolution of over 20 peaks is obtained using the acetonitrile/0.1% HAc solution described in Table 2 (Figure 3).

**Table 2. Separation Gradient (A- CH<sub>3</sub>CN, B- 0.1% HAc)**

Time (min)	A (%)	B (%)
0.0	10	90
15	22	78
20	22	78
40	55	45
45	90	10
45.1	10	90
55	10	90

### Chromatographic Performance

Linarin (peak 11, Figure 3), is an active component in *F. chrysanthemi* with good UV absorption, and was selected as the reference peak to calculate the retention times and relative peak areas of the other peaks. These values were used to estimate instrument and method reproducibilities.

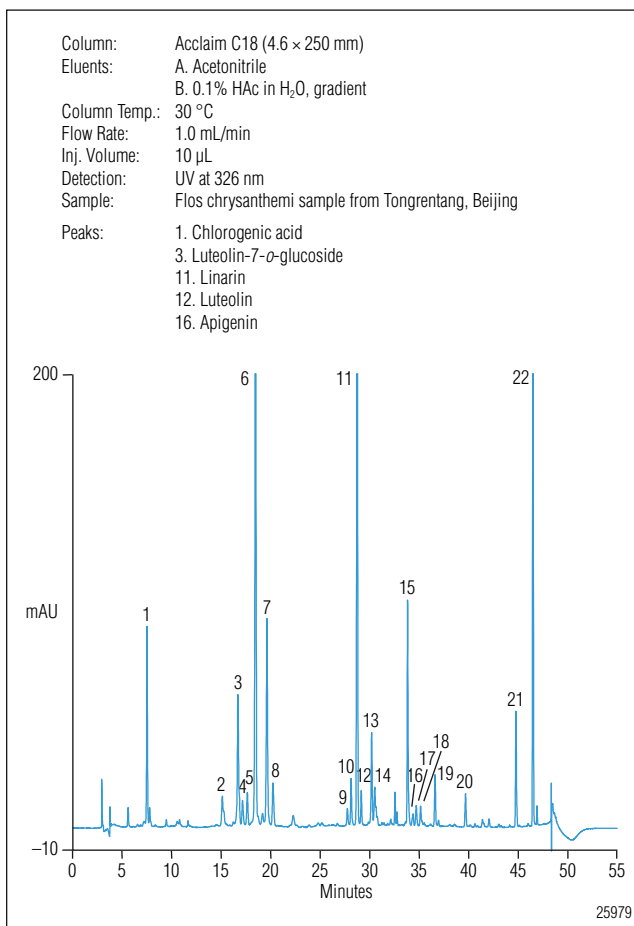


Figure 3. Chromatogram of *F. chrysanthemi* sample 4, purchased from a commercial retailer in Beijing.

Table 3. Results of Instrument and Method Reproducibility Tests				
Peak	Instrument Reproducibility		Method Reproducibility	
	Retention Time RSD	Peak Area RSD	Retention Time RSD	Peak Area RSD
1	0.23	0.66	0.20	1.91
3	0.09	0.79	0.11	0.78
6	0.10	0.44	0.08	0.93
7	0.10	0.46	0.06	0.52
10	0.03	0.50	0.02	0.75
11	0.00	0.00	0.00	0.00
16	0.03	0.66	0.13	0.55

Instrument reproducibility was estimated by making six consecutive replicate injections of a sample solution; and the method reproducibility was estimated by preparing six replicate sample powders as specified in the sample preparation section, and making one injection for each sample solution. The RSDs for retention time and peak area are shown in Table 3. These data demonstrate that this method is appropriate for establishing a chromatographic fingerprint of *F. chrysanthema*.

#### Identification of Homo-chlorogenic Acids and Flavonoids in the HPLC Fingerprint of *F. Chrysanthema*

Overlays of the chromatograms of all samples are shown in Figure 4. Chlorogenic acid (peak 1) and four flavonoids (luteolin-7-*o*-glucoside—peak 3; linarin—peak 11; luteolin—peak 12; and apigenin—peak 16) were identified by their retention times and UV absorption spectra.

Twenty-two peaks were observed in each sample. The UV absorption spectra of peaks 2 and 4–7 are similar to that of chlorogenic acid (peak 1) with maximum absorption at approximately 323 nm; they can therefore be regarded as homo-chlorogenic acids. Peaks 8, 10, 13 and 17–20 have two absorption zones between 210 and 400 nm, the same zones found in the UV absorption spectra of luteolin-7-*o*-glucoside (peak 3), linarin (peak 11), luteolin (peak 12), and apigenin (peak 16). This strongly suggests that these peaks are flavonoids.

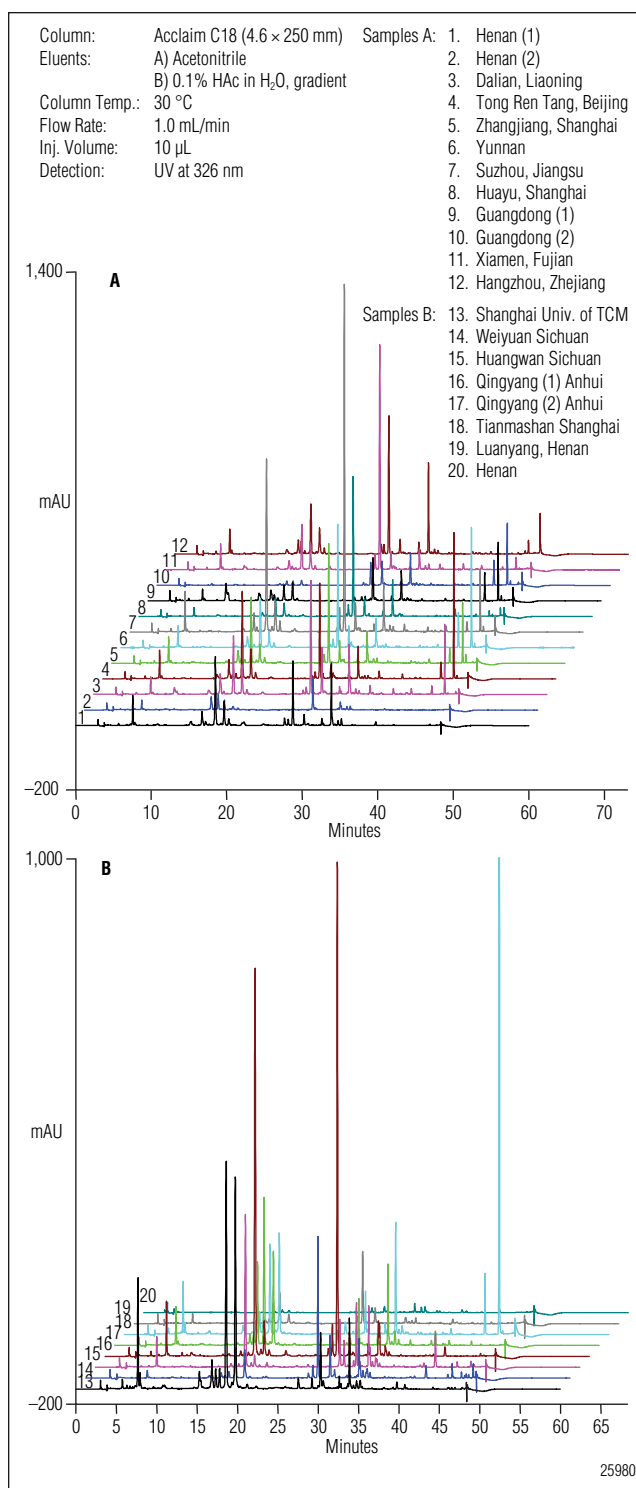


Figure 4. Overlay of chromatograms of (A) twelve purchased samples and (B) eight collected samples. Sample numbers are identified in Table 1, and chromatographic conditions are described in Figure 3.

## Principal Component Analysis of Flavonoids in *F. Chrysanthema*

Principal component analysis (PCA) of flavonoids in samples can be determined by calculating the related chromatographic data using Multi-Variate Statistical Package 3.1 (MVSP 3.1) software. Analysis results are summarized in Figure 5. The eight collected samples are found in two regions; *D. indicum* samples 13, 15, 17, 18, and 20 are found in Region 1, and *D. lavandulaefolium* samples 14, 16, and 19 are found in Region 2. The twelve purchased samples (1-12) are all in Region 3, which is located between Region 1 and Region 2, demonstrating that they are a mixture of *D. indicum* and *D. lavandulaefolium*. This suggests that the workers who harvest *F. chrysanthemi* cannot discriminate between *D. indicum* and *D. lavandulaefolium*.

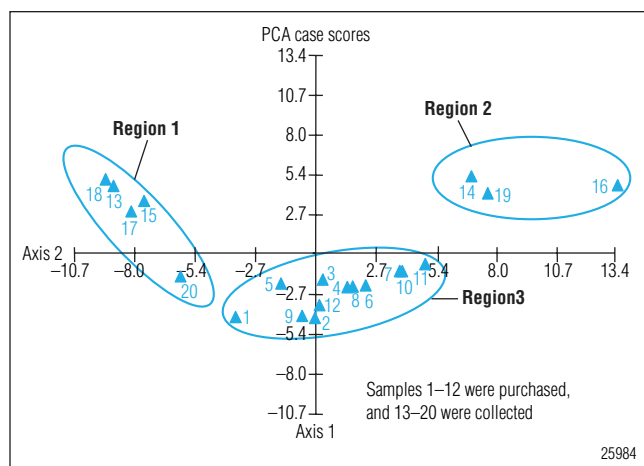


Figure 5. Principal component analysis of flavonoids in samples of *flos chrysanthemi*. Samples 1–12 were purchased from commercial retailers, and samples 13–20 were collected in the field.

## Using ASE for Sample Preparation and the UltiMate 3000 HPLC System for Separation

Ultrasonic extraction and ASE are efficient means for extracting homo-chlorogenic acids and flavonoids from *F. chrysanthemi* samples. Separations of the extract can also be performed on the UltiMate 3000 HPLC system; Figure 6 shows the chromatograms from the UltiMate 3000 HPLC of a sample of *F. chrysanthemi* extracted using ultrasonic and ASE extractions. Although both extraction methods extract the same major compounds, ASE demonstrates higher extraction efficiency for the compounds eluting before 25 min.

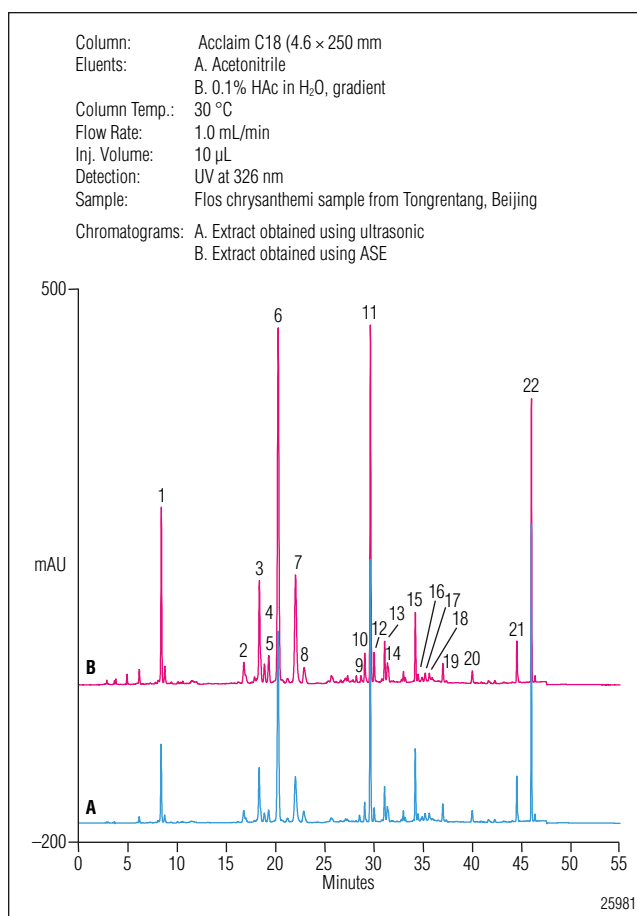


Figure 6. Overlay of chromatograms of the extracts obtained using (A) ultrasonic extraction and (B) accelerated solvent extraction on the UltiMate-3000 HPLC system. *F. chrysanthemi* sample purchased from retailer in Beijing. Peaks: 1-chlorogenic acid, 3-luteolin-7-*o*-glucoside, 11-linarin, 12-luteolin and 16-apigenin. Chromatographic conditions are described in Figure 3.

## CONCLUSION

This HPLC method was developed to separate homo-chlorogenic acids and flavonoids with good resolution. Based on this separation, an efficient chromatographic fingerprint of *F. chrysanthemi* was established. Quality control of this species can be achieved with excellent results using the chromatographic fingerprint and data processing software established in this application note. This HPLC method can distinguish between *D. indicum* and *D. lavandulaefolium*, an identification that can be difficult using only visual inspection. The advantages provided by the ASE system are short extraction time, low solvent consumption, and high extraction efficiency. While both extraction techniques yielded the same compounds, the ASE system extracted higher

amounts of the compounds which eluted in the first half of the chromatogram, and therefore demonstrated greater accuracy than ultrasonic extraction.

### ACKNOWLEDGEMENT

The authors would like to thank Ms. Liu Tingna (Shanghai University of Traditional Chinese Medicine) for her assistance in the completion of this project.

### REFERENCES

1. Chinese Pharmacopoeia Vol. 1, **2005**, p 219.
2. Chinese Material Medica, Vol. 7, Shanghai Science and Technology Publishers, **1999**, p 801.
3. National Chinese Patent Medicine Specifications, Medical Lung System (1<sup>st</sup>), State of Food and Drug Administration, **2002**, p 428.
4. Xie, P. X., Chen, S. B., Liang, Y. Z., Wang, X. H., Tian, R.T., Upton, R., Chromatographic fingerprint analysis—a rational approach for quality assessment of traditional Chinese herbal medicine, *J. Chromatogr. A*, 1112, **2006**, 171–180.
5. Chen, C., Zhang, H., Xiao, W., Yong, Z. P. Bai, N., High-performance liquid chromatographic fingerprint analysis for different origins of sea buckthorn berries, *J. Chromatogr. A*, 1154, **2007**, 250–259.
6. Ji, Y. B., Xu, Q. S., Hu Y. Z., Heyden, Y. V., Development, optimization, and validation of a fingerprint of Ginkgo Biloba extracts by high-performance liquid chromatography, *J. Chromatogr. A*, 1066, **2005**, 97–104.
7. Shen S., Sha Y. F., Deng C. H., Zhang X. M., Fu, D. X., Chen J. K., Quality assessment of flos chrysanthemi indicis from different growing areas in China by solid-phase microextraction-gas chromatography-mass spectrometry, *J. Chromatogr. A*, 1047, **2004**, 281–287.
8. Chen K. L., Li P., Ye C. J., HPLC fingerprints of Flos Chrysanthemi from Futianhe region and other cultivation breeds, *Chin. Tradit. Herb Drugs*, 35, **2004**, 1050–1053.
9. Zhao Y. C., Liu G. J., Ren B. Z. Fingerprint of Flos Chrysanthemi by HPLC, *Chin. Tradit. Herb Drugs*, 36, **2005**, 1052–1054.
10. Tan X. J., Jia Y., Qi D. D., Chen X. H., Bi K. S., Establishment of chromatographic fingerprint and quality assessment of flos chrysanthemi indicis by RP-HPLC, *Chin. J. Pharm. Anal.*, 27, **2007**, 29–32.
11. Chinese Medica Chemistry, Shanghai Science and Technology Publishers, **1996**, p 291.

Summit, UltiMate, Chromeleon, ASE, and Acclaim are registered trademarks of Dionex Corporation.  
Milli Q and Millex are registered trademark of Millipore Corporation.  
Multi-Variate Statistical Package is a trademark of Microsoft Corporation.

Passion. Power. Productivity.



#### Dionex Corporation

1228 Titan Way  
P.O. Box 3603  
Sunnyvale, CA  
94088-3603  
(408) 737-0700

#### North America

U.S./Canada (847) 295-7500

#### South America

Brazil (55) 11 3731 5140

#### Europe

Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294  
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0  
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380  
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

#### Asia Pacific

Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735  
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190  
Taiwan (886) 2 8751 6655

[www.dionex.com](http://www.dionex.com)



LPN 2092 PDF 4/09  
©2009 Dionex Corporation