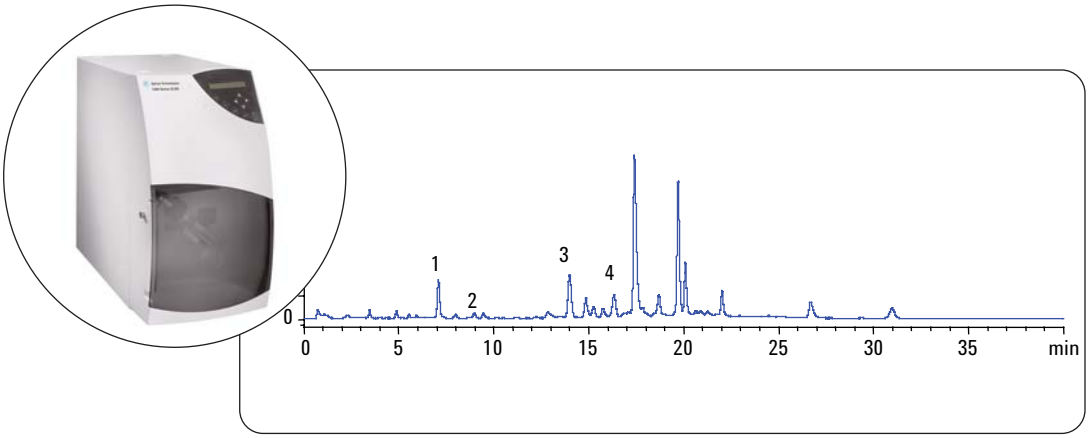
Analysis of traditional Chinese medicines with the Agilent 1200 Series evaporative light scattering detector

Application Note

Zhixiu Xu



Abstract

Traditional Chinese medicines often contain components that must be detected by HPLC, but that lack a chromophore and so do not produce signals with an ultraviolet (UV) detector. The Agilent 1200 Series evaporative light scattering detector (ELSD) is an excellent alternative because it detects all solutes that are less volatile than the mobile phase. In the *Chinese Pharmacopoeia 2005*, several TCMs require the ELSD as the detection method, and this Application Note illustrates two examples – the flavonoids in *Ginkgo biloba* L. and astragaloside in *Astragali*.

Agilent Equipment

- 1200 Series Rapid Resolution LC system
- 1200 Series evaporative light scattering detector

Application Area

- Traditional Chinese medicine (TCM)

Agilent Technologies

Introduction

The evaporative light scattering detector is increasingly being used as a quasi-universal detector for non-UV-absorbing analytes in HPLC systems. Especially in herbal medicines, an increasing number of non-UV-absorbing compounds need to be detected without derivatization. The Agilent 1200 Series HPLC family now has a new member – the evaporative light scattering detector – to help with such applications.

The Agilent 1200 Series ELSD can detect all solutes that are less volatile than the mobile phase. It produces a signal only for the nonvolatile particles that are generated from the sample. If the compounds have no chromophores and are less volatile than the LC solvents, the ELSD is a good detector that can provide both ease of use and good sensitivity. Gradient mobile phases do not interfere with detection. When a mobile phase produces strong absorption under a specific UV wavelength, the ELSD is an excellent alternative that maintains a stable baseline and produces strong signals.

The ELSD principle of operation consists of three main successive processes:

a) Nebulization of the chromatographic eluent using nitrogen or air,
b) Evaporation of mobile phase at relatively low temperature, and
c) Light scattering by the residual particles, which ideally consist of analyte molecules.

Figure 1 shows a schematic of the various stages of detection.

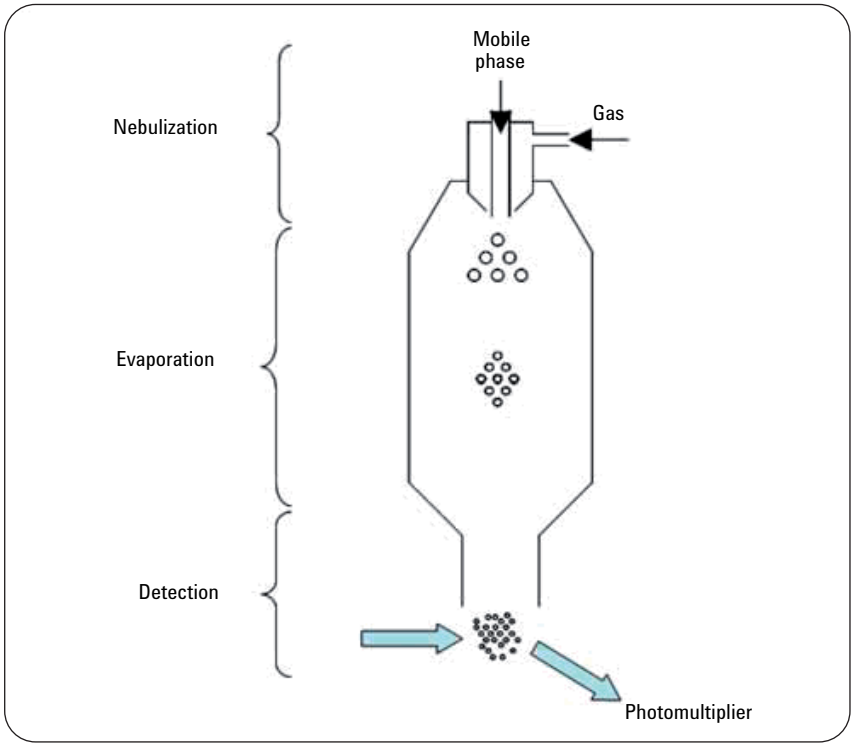


Figure 1
Cross-sectional view of the Agilent 1200 Series evaporative light scattering detector.

Some components of TCMs have no chromophores, so they need a universal detector for detection. Compared with other general detectors, the Agilent 1200 Series ELSD provides significant benefits. Mass spectrometry (MS) is more expensive and requires well-trained, knowledgeable operators. MS is not normally used for the routine analytical work that is done in quality control departments. The refractive index detector provides universal detection, but can be used only for isocratic analyses, so is not fit for separation of complex mixtures. A system with a UV detector that is connected in series with an ELSD can be used for simultaneous determination of multiple components with various structures, with or without chromophores.

In the *Chinese Pharmacopoeia 2005*, there are several TCMs that require ELSD as the detection method for certain components. Examples include the flavonoids in *Ginkgo* and astragaloside in *Astragali*.

Ginkgo biloba L. and its extracts are not only very famous in China, but also are used worldwide to treat cardiovascular and cerebrovascular diseases. The therapeutic effects are due to the ginkgolides and bilobalide that are present together with the flavonoids. Quality control methods must determine the amount of the flavonoids in *Ginkgo* and its products.

Radix Astragali, in Chinese Huangqi, is one of the most widely used TCMs in China.

Pharmaceutical studies and clinical practice have demonstrated that *Radix Astragali* possesses many biological functions; therefore, it is used for the treatment of nephritis, diabetes, hypertension, and other diseases. One of the effective components, astragaloside, has no chromophores and needs a universal detector to determine the amount of the compound.

In this Application Note, the separation and the quantitative analysis of the *Ginkgo* flavonoids and astragaloside are studied. The note also provides some general advice on use of the Agilent 1200 Series ELSD.

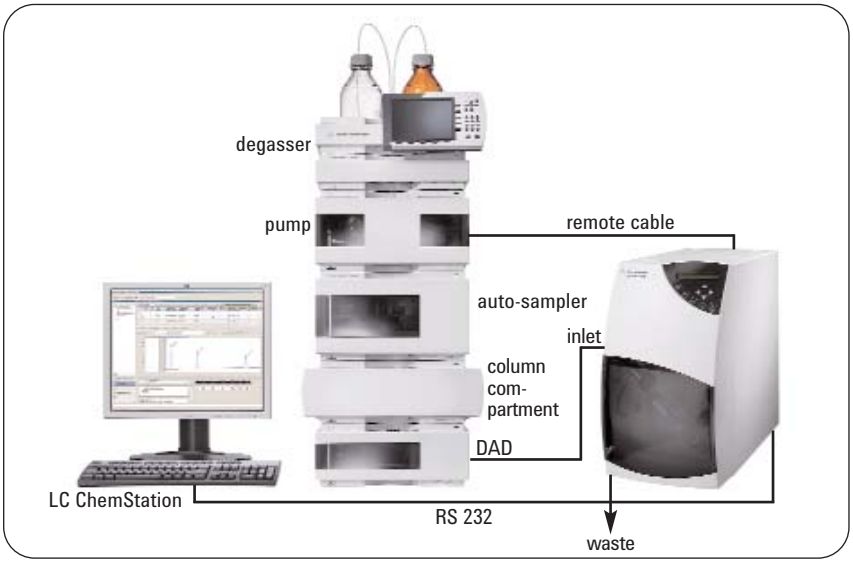


Figure 2
Agilent 1200 Series RRLC system with Agilent 1200 Series evaporative light scattering detector.

Experimental

Equipment

For development of the Rapid Resolution LC (RRLC) method, an Agilent 1200 Series RRLC system with the following modules was used:

- Agilent 1200 Series binary pump SL with vacuum degasser
- Agilent 1200 Series high-performance autosampler SL
- Agilent 1200 Series thermostatted column compartment SL
- Agilent 1200 Series diode array detector SL with micro flow cell (2 µL volume, 3 mm path length)
- Agilent 1200 Series evaporative light scattering detector with standard nebulizer
- Agilent ChemStation B.03.02 for data acquisition and evaluation
- Agilent ZORBAX XDB-C18 Rapid Resolution High Throughput (RRHT) column, 3.0 x 50 mm, 1.8 µm particle size

System setup

The HPLC modules are plumbed in the usual ways, as shown in figure 2. The Agilent 1200 Series ELSD is connected with the computer through an RS-232 cable. The remote cable connects the Agilent 1200 Series ELSD with any module of the HPLC.

System checkout

Before starting an experiment, a test run should be done to make sure that the Agilent 1200 Series ELSD is in good condition and has sufficient performance.

Test run conditions:

- Sample: caffeine at 250 µg/mL (or similar concentration)
- Solvent: isocratic, 80 % water, 20 % acetonitrile
- Flow rate: 1 mL/min
- Injection volume: 20 µL
- Column: Agilent ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, 5 µm (or other column with comparable size)
- Thermostatted column compartment (TCC) temperature: 40 °C
- ELSD temperature: 40 °C
- ELSD pressure: 3.5 bar (51 psi)
- ELSD gain: 7
- ELSD filter: 1 second
- Typical system: Agilent 1200 Series standard LC system (Please note that the Agilent 1200 Series RRLC system can be used as the standard system when conventional HPLC is needed.)

After the method has been run, the response of the detector should be recorded when the instrument is in good condition after the installation. Then one should use the same method every time the performance check is needed.

Nonvolatile buffers cannot be used with the ELSD. If nonvolatile salt goes into the ELSD, the detector cannot distinguish whether the nonvolatile particles came from

the sample or from the buffer. Therefore, the baseline increases dramatically and the signal-to-noise is reduced for the sample.

Because ELSD can detect only compounds that are less volatile than the mobile phase, when new methods are developed, another detector should be used to make sure all the compounds can be eluted from the column.

Standards and sample preparation

The following standards, samples, and solvents were used for these experiments:

- Standards were purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products).
- Solvents (methanol, acetonitrile, tetrahydrofuran) were purchased from Thermo Fisher Scientific.
- Water was obtained from a Millipore pure water system.
- The *Astragali* sample was purchased from a TCM drug store.
- *Ginkgo* extracts were kindly provided by a customer.

Samples were prepared by weighing 1 gram sample, dissolving it in 5 mL methanol, mixing in an ultrasonic bath for 30 minutes, then filtering and saving the clear liquid for injection.

Method for *Ginkgo* analysis

- Mobile phase: A = water,
  B = tetrahydrofuran/methanol with a volume ratio of 10/25
- Flow rate: 0.7 mL/min
- Gradient: 0 min, 12 %B;
  10 min, 16 %B;
  15 min, 22 %B;
  20 min, 30 %B
- ELSD: temperature = 40 °C,
  pressure = 50 psi,
  gain = 7,
  filter = 3 seconds

Method for *Astragali* analysis

- Mobile phase: A = water,
  B = acetonitrile
- Flow rate: 0.7 mL/min
- Gradient: 0 min, 20 %B;
  1 min, 30 %B;
  5 min, 35 %B,
  30 min, 100 %B
- ELSD: temperature = 40 °C,
  pressure = 50 psi,
  gain = 5,
  filter = 3 seconds

Results and Discussion

Analysis of *Ginkgo*

Ginkgo and its products have a very complex composition and require a good separation by HPLC. Isocratic methods were used frequently in the past because of limitations in the instrumentation. The disadvantages of isocratic methods include long analysis time and lower resolution. A gradient method was used in this application to get better separation in a shorter time.

Figure 3 shows chromatograms of standards of *Ginkgo* flavonoids, and compares the signals on ELSD

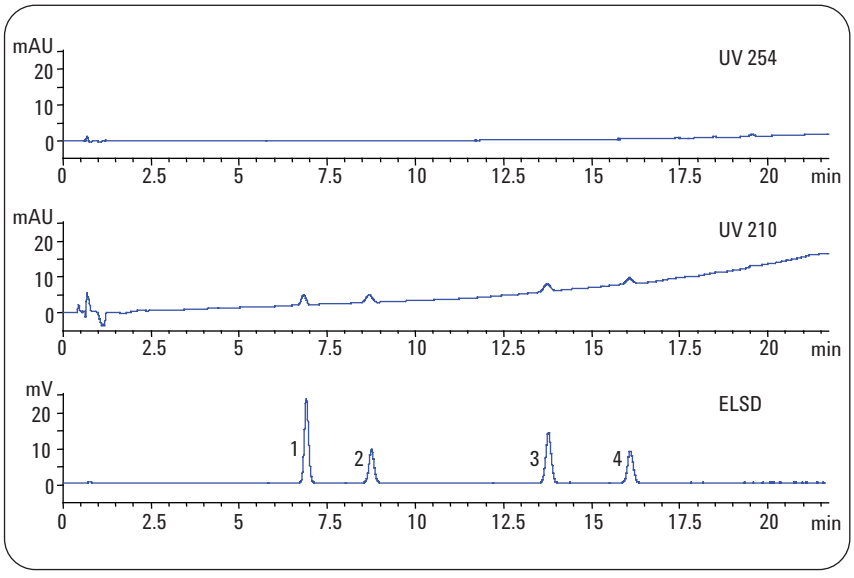


Figure 3
Comparison of chromatograms of *Gingko* flavonoids between ELSD and different channels of UV.

and two channels of UV. The flavonoids have very low response at a wavelength of 210 nm and no response at 254 nm. Comparison of the sensitivity between UV and ELSD shows why it is necessary to use ELSD to determine the flavonoids in *Ginkgo*. By checking complementary UV signals, this method can also be used to tell if the peaks are pure flavonoids.

The chromatogram in figure 4 shows that there are many peaks in the *Ginkgo* sample that can be detected by ELSD. The sample composition is very complex and the ELSD information can help to find more components, including those that lack chromophores. The four flavonoid peaks are well-separated from each other and from the other components.

When running ELSD methods, even when there are no more peaks in the ELSD signal, one still needs to monitor the UV channels to make sure all the components are eluted out of the system. This avoids contamination of the column and system.

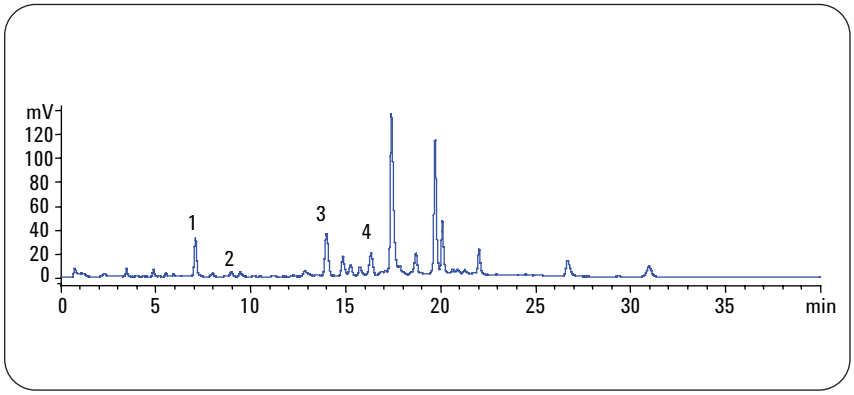


Figure 4
ELSD chromatogram of *Ginkgo* sample, with the flavonoid peaks numbered.

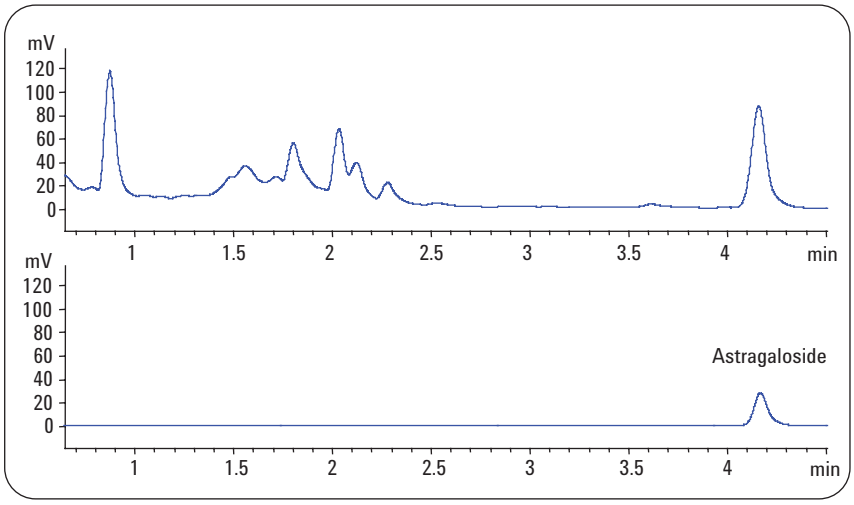


Figure 5
ELSD chromatogram of *Astragali* sample (top) and astragaloside standard (bottom).

Analysis of *Astragali*

When developing the HPLC conditions for analysis of astragaloside in *Astragali*, one must separate all the standards and the other components in the real TCM samples. To reduce the separation time, a gradient analysis can be used. Isocratic analysis can be used when the separation time is not too long and peak capacity is sufficient.

The chromatogram at the top of figure 5 shows a gradient analysis of an *Astragali* sample, while the one at the bottom shows the astragaloside standard. The top chromatogram shows good separation of the astragaloside from the other sample components.

Quantitative analysis

The ELSD is quite special when it is used for quantitative analyses. which is determined by the ELSD instrumentation and the way the analytes are detected.
The ELSD usually considered to be a non-linear detector with a response according to $A=am^b$

where A is peak area, “a” is the response factor, “b” is the slope of response line and “m” is the mass injected on the column.

“a” and “b” depend on many factors, for example, sample concentration, solvent properties, as well as the parameter of the instrument. “b” is related to the ratio of the particle size and wavelength of the light source because the different particle sizes have a different dominant theory to scatter the light and therefore have different response equations[3]. Normally “b” is between 0.7-2.0.

Researchers found some interesting results which are published in different scientific journals [4-7]. So far no single theory can solve all problems and research is still ongoing. However, this does not have an impact on using the ELSD as a good detector to detect compounds without chomophores.

Scientists discovered that different light scattering processes depend on particle size. The ELSD sensitivity and response linearity are related to the size distribution and number of particles formed during the nebulization and evaporation processes. The amount of light scattered per unit mass of material depends on the average particle diameter and the particle size distribution. Therefore, different instrument and different chromatogram conditions can greatly influence the ELSD response. During a gradient chromatogram the response of a given amount of analytes can vary as much as 10 times in magnitude as a result of the changing mobile phase compositions [3-5].

Compound name	Function	Linear range (µg)	Regression factor
Ginkgolide A (peak 3)	y = 562.24805 x -128.48353	0.2-4	0.99889
Ginkgolide B (peak 4)	y = 461.26026 x -134.18949	0.33-6.6	0.99976
Ginkgolide C (peak 2)	y =1413.64682 x - 399.10437	0.2-4	0.99843
Bilobalide (peak 1)	y=1085.84028 x - 289.42956	0.2-4	0.99828
Astragaloside	y = 1985.90624 x - 282.78609	0.1-2	0.99882

Table 1
Quantitative results.

Compound name	Detection limit (ng)
Ginkgolide A	12.5
Ginkgolide B	19.5
Ginkgolide C	30
Bilobalide	15
Astragaloside	22

Table 2
Detection limits of standards.

Although the light scattering detector is considered to be non-linear it has been shown that linearity can be obtained in a limited concentration range [4]. There are also other approaches with the regression equation when scientists use the different methods for analysis [5]

In this application, we found the linear response within the given range of standards injected to the column with HPLC-ELSD conditions developed in our lab. Some papers were published in Chinese that describe similar phenomena when the quantitative analysis was done on TCM standards [8]. There are also methods using the log(Area) and log(mass) to regress the equation for quantitative analysis. The different results may be due to the fact that different systems and parametere were used. We strongly recommend ELSD users to develop the specific quantitative equation based on the HPLC- ELSD system they are using. The gas and the solvent used for the analysis need to be “clean” which means they should contain no unvolatile particles because particles can raise the baseline and destroy the reproducibility and sensitivity. The quantitative results are shown in table 1. The peak number corresponds to the number shown in figure 3.

The detection limit is highly related to the conditions developed on the ELSD. For example, the ELSD parameters for gain and filter influence the detection limit. The limits listed in table 2 were obtained using methods that were similar to those used for the sample analyses. The numbers shown in table 2 give the basic idea of the amount of each standard that can be detected with the current methods.

Conclusion

Two methods that used the Agilent 1200 Series RRLC and the Agilent 1200 Series ELSD were developed in this application. The methods achieved good separation of the standards and TCM components. The detection limits of the TCM standards also showed that the system is capable of detecting low levels of components. The quantitative analyses done here give the holistic idea of how to meet the requirements of the *Chinese Pharmacopoeia*. The methods can be used for the quality control of TCMs that contain related components.

References

1.
Pharmacopoeia of the People's Republic of China, **2005.**

2.
L.-W. Qi et al., "Quality evaluation of *Radix Astragali* through a simultaneous determination of six major active isoflavonoids and four main saponins by high-performance liquid chromatography coupled with diode array and evaporative light scattering detectors", *J. Chromatogr. A*, *1134*, *162-169*, **2006.**

3.
Wang Qiao'e et al. "Progresses on Evaporative Light-scattering Detection" *J. of Instrumental analysis (Chinese)*, Vol. 25, No.6 ,126-132, **2006.**

4.
Roger Trones et. al "Modified laser light-scattering detector for use in high temperature micro liquid chromatography" *J. of Chrom A*, 814, 55-61, **1998**.

5.
B.T. Mathews et al. "Improving Quantitative measurements for the Evaporative Light Scattering Detector " *Chromatographia*, 60 ,625-633, **2004.**

6.
E.Oppenheimer et. al "Examination of the concentration response Evaporative Light Scattering Mass Detector" *J. Chrom* 323, 297-304, **1985**.

7.
M, Dreux et al. "The Evaporative Light Scattering Detector- a Universal Instrument for Non-Volatile Solutes in LC and SFC" *LC-GC Int*, March **1996**.

8.
Su Hong et al. "Determination of Jujubaside A in Ziziphi Spinosae Mixture by HPLC-ELSD" *J. of Modern Food and Pharmaceuticals (Chinese)*, Vol. 17 No 6, 35-37, **2007**.

Zhixiu Xu is an Application Chemist at Agilent Technologies, Shanghai, China.

www.agilent.com/chem/1120

Published November 1, 2008
Publication Number 5989-8922EN

Agilent Technologies