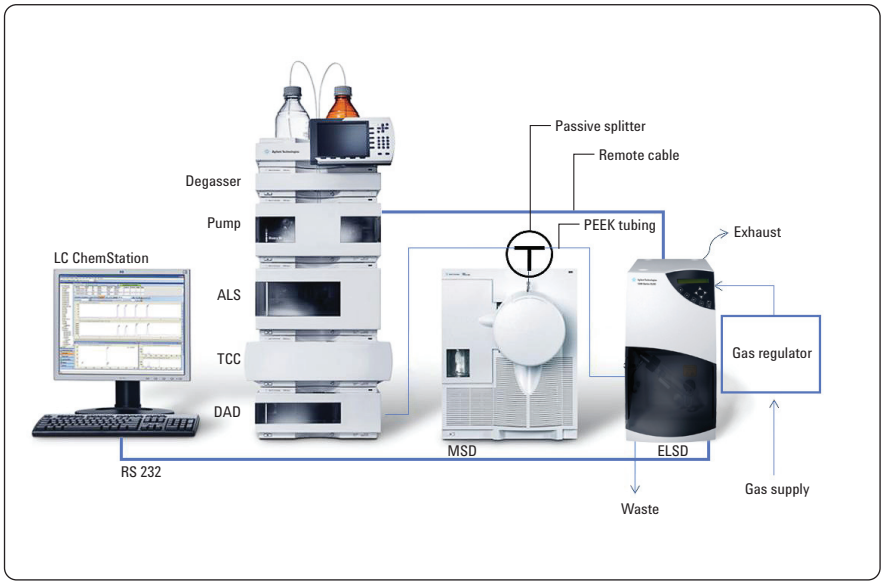
RRLC impurity profiling to detect non-UV absorbing compounds using diode array detection, single quadrupole MS and evaporative light scattering detection

Technical Note



Introduction

In drug development, the risk of missing an impurity can have drastic consequences. Impurities may lack UV absorbance or ionize poorly. This Technical Note describes an arrangement consisting of an Agilent 1200 Series Diode Array Detector (DAD), an Agilent 6140 Single Quadrupole LC/MS system and an Agilent 1200 Series Evaporative Light Scattering Detector (ELSD) which allows identification of such impurities. Mass spectrometry (MS) and ELSD complement each other because volatile compounds are detected by MS while poorly ionizing compounds can be detected by ELSD. Therefore, this combination provides the greatest coverage for identifing impurities.

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Equipment setup

To connect the Single Quadrupole LC/MS system (referred to as mass selective detector, MSD) and the ELSD we use a passive flow splitter with appropriate tubing, while maintaining additional UV/DAD detection in series. The Agilent ChemStation software controls the DAD, the ELSD and the MSD from a single point, making operation simple. This note shows the effect on flow split ratio when using combinations of various PEEK tubings and provides step-by-step instructions on setting up the system. In addition, we show how to tune the system so that the output flow is in line with the specified operating flow rates of the respective nebulizers.

The figure on the front page shows a configuration where the ELSD is on the right of the MSD to ensure that the distances between nebulizers are minimal. The passive splitter allows the instruments to run in parallel. This figure also depicts the additional cable connections needed to set up the ELSD.

Detection of compounds with varying UV activity using ELSD and MSD in parallel

Glutamine (non UV active), sulfamethoxazole, sulfadimethoxine and topiramate (non UV active) were analyzed using the ELSD and the MSD system in parallel, while using the DAD in series with the MSD.

Figure 1 shows that non UV active compounds such as glutamine and topiramate can easily be detected with ELSD and MSD in parallel. Additionally, UV analysis in series provides detection of impurities that are UV active in the same run. The small peak at 0.3 min in the UV/Vis trace is due to injection.

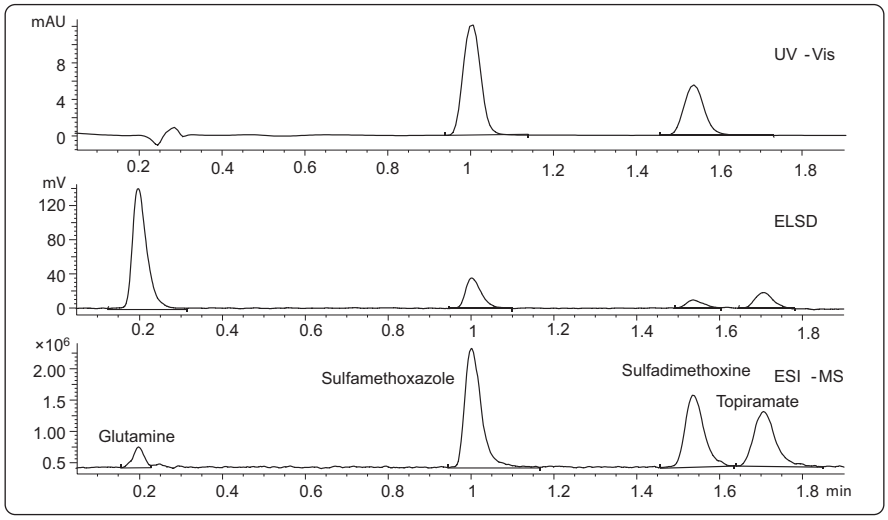

Figure 1
Analysis of glutamine, sulfamethoxazole, sulfadimethoxine and topiramate (10 µg/mL each) using UV/Vis (254 nm), ELSD and ESI-MS detection (time aligned data).

Experimental parameters

Details

Column	50×3.0 mm, 1.8 µm ZORBAX Eclipse XDB C18; operated at room temperature	
Mobile phase	Buffer A: 0.1% formic acid in Milli Q water Buffer B: 0.1% formic acid in acetonitrile	
Isocratic run	Run Time (min) 2.0	Mobile phase composition 25% B
Flow	1 mL/min	
Sample preparation	All standards were of concentration 10 µg/mL in 10% buffer B and 90% water. 5 µL of the sample was injected	
DAD	Peak width = >0.1min 254 nm and 210 nm	
UV Flow cell	2 µL, 3mm, 120 bar	
ALS	Injector with needle wash for 5 sec using 50% buffer A and 50% buffer B	
T connector	30 cm/red in arm A, 20 cm/yellow in Arm B and 74 cm/red in Arm C. (see Table 1 for a description of the arms)	
ELSD parameters:		
Standard nebulizer	Temperature Pressure Gain Filter Peakwidth	55 °C 3.5 bar 12 1 100 – 10 Hz
AP-ES MSD parameters:		
ESI source	Scan range 100-500 amu, peak width 0.02 min, step size – 0.1, positive polarity and one signal is monitored – cycle time = 0.18 sec/cycle	
Drying gas	13 L/min	
Nebulizer pressure	40 psig	
Dry gas temperature	300 °C	
Capillary voltage	3000 V	
Fragmentor	120 V	

Preliminary considerations for a DAD/ELSD/MSD setup

Minimizing the distance between the detectors keeps the tubing length short, which decreases peak broadening. ELSD response is more sensitive to peak widths of eluted compounds. Shorter tubing length maintains resolution and thus ensures that impurities eluting relatively close to each other do not decrease MS signal due to ion suppression. Larger tubing ID or lengthy tubing increases the system's dead volume thereby causing peak dispersion, that is, loss in resolution.

The passive splitter divides the flow and the absolute amount of the compounds in the same ratio, however the concentration of the compounds in the solvents is not affected by the split. Concentration sensitive detectors such as the electrospray ionization (ESI) MSD show the same response with or without a splitter; however, mass-based detectors such as an atmospheric pressure chemical ionization (APCI) MSD will show a reduced response because of the split. Recent studies have shown that ELSD behaves like a concentration sensitive detector in some cases.[1] With concentration sensitive detectors at the either end of the splitter, it is important to consider the detection limit and saturation limit of the respective detectors. The detection limit of MSD is in the subpicogram range while ELSD is at the low nanogram range on-column.

The saturation limit of MSD is evident when the count approaches the value of 9×10^6. As seen in Figure 1, a sample concentration of 10 µg/mL produced an MSD signal intensity of 2×10^6, well below the saturation limit while the ELSD signal is enhanced by working at an increased gain value of 12.

Obtaining appropriate flow split ratios using correct tubing lengths, tubing ID's and nebulizers

A passive splitter divides the solvent flow from the DAD to the ELSD and LC/MS. Knowing the flow from the passive splitter is mandatory in order to divert a larger portion of the flow into the ELSD and a smaller portion into the MS. This is because a low flow rate increases the sensitivity of the LC/MS. At the same time, this information is required to perform the experiment within the allowed flow rates of the respective nebulizers. The choice of tubing length, ID and back pressure from the nebulizer affect the split ratio and the final output flow as seen in Table 1.

For clarity, the passive splitter arms are labeled as "A", "B" and "C" (Figure 2) in this note.

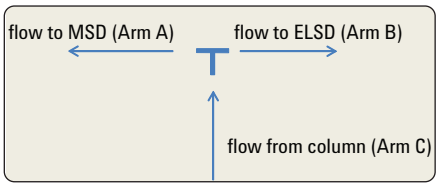

Figure 2
The passive splitter arms are labeled as A, B and C to distinguish the influx and destination of the flow.

Here, various combinations of PEEK tubing ID's and lengths are used in Arms A and B. Due to the dilution effects seen with larger ID tubing (blue 0.254 mm) it is best to use the smaller ID red (0.13 mm) or yellow (0.18 mm) tubings to connect the two detectors.

The major factor affecting the split is the back pressure contributed by nebulizers. The nebulizers from the MSD source (the ESI or multimode source have the same back pressure effect) show less effect on split as compared to ELSD nebulizers. The back pressure contributed by the tubing length changes the flow split ratio (compare rows D and E, as shown in Table 1).

Working within the specifications of nebulizer and UV flow cell:

After splitting, the resulting flow rates in both Arms A & B are reduced. Table 1 shows the split ratios under various conditions. As an example, row B demonstrates that using an initial flow rate of 1 mL/min in Arm C, allows the flow to split to 430 µL/min (Arm A) and 570 µL/min (Arm B).

Care is to be taken to ensure that the new flow rates are within the specifications of the nebulizers. Additionally, the back pressure from the nebulizers and the choice of tubing should not exceed the pressure limit of the UV flow cell.

	Length [cm] tubing color		Flow split ratio A:B				
A	Arm A (MSD)	Arm B (ELSD)	A → waste B → waste	A → waste B → ELSD standard nebulizer	A → waste B → ELSD RRLC nebulizer	A → ESI MSD B → waste	A → ESI MSD B → ELSD standard nebulizer (theoretical flow split)
B	10 cm/ Yellow	40 cm/ Blue	43:57	84:16	94:6	15:85	55:45
C	10 cm/ Red	40 cm/ Yellow	58:41	75:25	88:12	36:64	54:45
D	20 cm/ Red	40 cm/ Yellow	42:58	60:40	79:21	28:72	45:55
E	40 cm/ Red	40 cm/ Yellow	27:73	43:57	64:36	18:82	31:69
F	30 cm/ Red	30 cm/ Yellow	27:73	48:52	70:30	20:80	38:62
G	30 cm/ Red	20 cm/ Yellow	20:80	42:58	70:30	14:86	32:68
H	40 cm/ Red	20 cm/ Red	33:67	48:52	68:32	27:73	41:59

Table 1
Flow split ratios with PEEK tubing of various diameters and lengths in Arms A and B of the passive splitter. Theoretical flow split was calculated based on flow ratios of nebulizers to waste (column 3 and 4) and normalizing the waste ratios (column 2). Results in Table 1 were obtained using 30% acetonitrile and a flow rate of 1 mL/min (Arm C). PEEK tubings were used as supplied and were not calibrated.

Table 2 shows the optimal working flow rate of various nebulizers, and the pressure limit for the DAD SL flow cells. In Table 1, Row G, the combination of tubing used in the experiment above (Cover page figure) results in 32:68 split where the back pressure contribution from ESI (MSD) is less compared to the standard nebulizer (ELSD). Since the operating flow rate is 1 mL/min, the split ratio obtained is well within the optimal operation flow rate of the nebulizers as shown in Table 2. For 30% acetonitrile, the ESI/multimode source back pressure is less than 1 bar, while ELSD standard nebulizer is less than 3 bar, while the ELSD rapid resolution LC (RRLC) nebulizer is within 10 bar. Therefore, the back pressure effect onto the standard UV flow cell is minimal.

While split ratios are independent of the mobile phase flow rates, the viscosity of the liquid can affect the split ratios. Viscosity changes during the mixing of solvents in gradient runs can potentially lead to different flow ratios than those shown above. For example, methanol and water mixtures in gradient runs show larger viscosity changes than acetonitrile and water mixtures.

Factors influencing peak shape

In UV, ELSD and MSD detection, improper data acquisition rates (Hz) can lead to apparent peak broadening. In LC/MS, at a data rate of >6 spectra/sec (or cycles/sec) peak widths begin approaching those of the UV data.[2] The number of active signals acquired, scan or SIM mode of acquisition, mass range in scan mode, step size and peak width settings all affect cycle times. ChemStation will automatically calculate cycle time based on the settings. Decreasing cycle times and monitoring peak shape can be achieved by optimizing each of the above parameters. The step-by-step procedure is provided below.

Besides improper MS signal settings, peak broadening is also caused by dispersion within the UV flow cell, large tubing length and ID, and a higher swept volume T connector. Previously, dispersion in UV flow cells was shown to contribute a 30% increase in MS peak width.[3] To minimize dispersion, we recommend a 2-µL flow cell and a least swept volume T connector. Another factor for peak broadening is the sample

(A)

Nebulizers	Specified flow rate
MSD ESI	0.1-1 mL/min
MSD Multimode/APCI	0.1-1.5 mL/min
MSD Capillary	0.001-0.1 mL/min
ELSD Standard nebulizer	0.2-2.5 mL/min
ELSD RRLC nebulizer	0.2-1.4 mL/min
ELSD Large flow nebulizer	0.8-5 mL/min
ELSD Semimicro nebulizer	0.02-1.2 mL/min
ELSD Micro nebulizer	0.002-0.08 mL/min

(B)

UV flow cell for DAD SL	UV flow cell pressure limit
Standard - 13 µL, 10 mm	120 bar
Semimicro - 5 µL, 6 mm	120 bar
Micro – 2 µL, 3 mm	120 bar

Table 2
Specified flow rates for the various nebulizers (A) and pressure limits for UV flow cells (B). The experimental flow rate is the major factor in determining the appropriate nebulizer .

concentration. The MS peak width will broaden as the detector approaches saturation. To maintain an optimal MS peak width performance, the MS spectral response can be maintained close to 2 $\times 10^6$. Above 2 $\times$ 10^6 to ~9 $\times$ 10^6 detector response becomes nonlinear. A concentration of around 10 µg/mL is a good starting point where most compounds are detected in ELSD and below the saturation limit of MS.

Step-by-step instructions to set up ELSD and MSD in parallel using passive splitter

1. Bypass the UV detector and the column. Using your experimental flow rate and a midgradient percentage mobile phase, measure the flow rate when both the Arms (A and B) are connected to waste and when either of them is connected to the ELSD or MSD (Table 1). Calculate the theoretical flow to the ELSD and MSD using Table 1 as reference.

2. Check if the output flow matches the specifications of the chosen nebulizers (Table 2).

3. With the flow on, measure the pressure difference with and without the ELSD/MS connected. The pressure should be within the specification of the UV flow cell back pressure limit. Using a starting concentration of around 10 µg/mL, optimize the concentration based on the signal of ELSD and saturation limit of the MS signal (~9×10^6).

4. Optimize the data acquisition signal settings of the ELSD, UV and MS by selecting the appropriate data rate and obtaining the best peak shape.

Conclusion

For profiling of impurities and compounds which have varying UV activity or ionization efficiencies, ELSD and LC/MS in parallel provide an effective solution for detection. In this Technical Note we show an easy setup using the Agilent 1200 Series ELSD and the Agilent 6140 LC/MS system for detection in parallel (Table 3). The use of a passive flow splitter can be applied to other detector combinations as well.

Product	Part No
Agilent LC/MS Single Quad ES High Throughput Bundle	G6140AA
Agilent 1200 Evaporative Light Scattering Detector	G4218A
PEEK T connector (0.57 µL swept vol)	5022-2144
PEEK tubing, Blue ID 0.254 mm	5042-6463
PEEK tubing, Yellow ID 0.18 mm	5042-6462
PEEK tubing, Red ID 0.13 mm	5042-6461

Table 3
Equipment used.

References

1.
A.D Villiers, T. G´orecki, F. Lynen, R. Szucsb, P. Sandra, "Improving the Universal Response of Evaporative Detection by Mobile Phase Compensation," J. Chromatography A, 1161:183-191, 2007.

2.
D. McIntyre, " An LC/MS System Designed for Rapid High Throughput Analysis of Pharmaceutical Compounds," Agilent Application Note, Publication number 5989-5917EN

3.
U. Huber, "Fast LC analysis with quadrupole MS detection – without loss of spectra quality. Combining the Agilent 6140 quadrupole MS with the Agilent 1200 Series Rapid Resolution LC system," Agilent Application Note, Publication number 5989-7591EN.

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