

Ensuring High Sensitivity and Consistent Response in UHPLC-MS Analyses



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INTRODUCTION

High sensitivity for low limits of detection always requires a careful optimization of detection parameters. Unlike most optical detectors, sensitivity in mass spectrometry is highly dependent on the composition of the liquid phase that introduces the eluting samples from an UHPLC column to the MS detector. As the MS ion source conditions are typically optimized for one solvent composition, an isocratic LC separation with this eluent would be best suited for constantly high sensitivity in LC-MS hyphenated methods. However, the analysis of complex, unknown mixtures typically requires gradient elution, thus severely compromising efficient ion formation and sensitivity, especially at gradient compositions strongly deviating from the optimized average. A very powerful solution to ensure constant eluent compositions and thus ionization conditions, both in electrospray (ESI) and atmospheric pressure chemical ionization (APCI), is the Inverse Gradient Compensation concept. In a dedicated LC setup, one LC pump delivers the analytical elution gradient while a second one uses a mirrored setup for the delivery of an inverted gradient profile. Both streams are merged after the HPLC column and before the MS ion source so that the total solvent proportion in the ion source adds up to a constant ratio, ensuring best ion formation throughout the whole separation.

This principle has already been applied successfully to the near-universal-response Corona[®] Charged Aerosol Detector (CAD[®]). However, one major drawback remains: the compensation flow stream leads to a dilution of the column effluent by a factor depending on the flow rate ratio between the analytical and the compensation gradient. Using the simplest approach, in which the compensation gradient just mirrors the analytical gradient, the column effluent will be diluted by a factor of two, affecting sensitivity in concentration-dependent detection approaches using most spectroscopic detectors. This study will investigate whether the consistent response in APCI provides an increased sensitivity, surpassing potential drawbacks caused by sample dilution when performing a typical pharmaceutical application such as the UHPLC separation of different diuretics.

SYSTEM SETUP FOR CONVENTIONAL GRADIENT ELUTION AND COMPENSATION BY INVERSE GRADIENTS

The Inverse Gradient concept has been intensively tested for use with the Corona CAD detector series. This detector, similar to an APCI mass spectrometer, relies on the mobile phase removal by a nebulization process, leading to the formation of small particles from the residual analyte molecules. These particles become charged when they collide with a charged reactant gas, and the moving charged particles can be detected by an appropriate counter. As the signal intensity relies primarily on the particle-building efficiency, it strongly depends on the solvent composition. Compensating the mobile phase composition change during a gradient separation by merging the column effluent with the appropriate inverse amount of organic modifier will lead to constant nebulizing conditions, thus leading to a nearly uniform response.

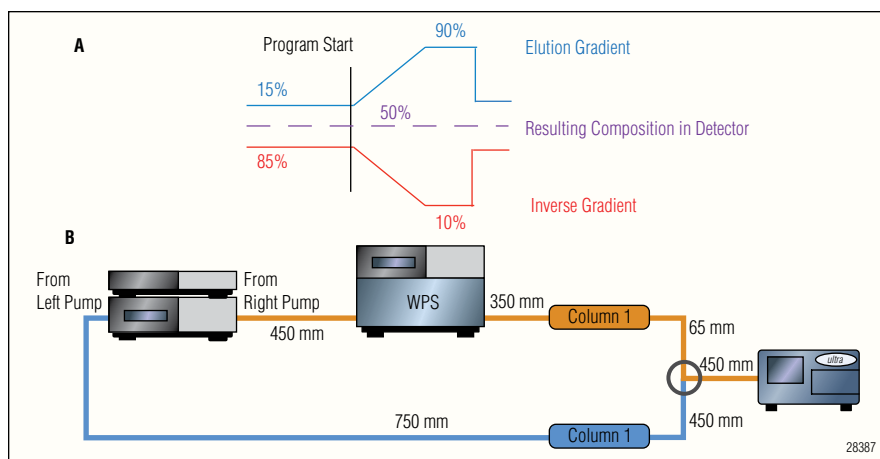


Figure 1. A) Inverse Gradient Compensation, and B) instrument setup for a Corona ultra detector.

Figure 1 illustrates the solvent gradient compensation method and the flow scheme for inverse gradients based on the UltiMate[®] 3000 x2 Dual System with a Corona CAD detector. The special design, which includes a DGP-3600RS pump, integrates two ternary low-pressure gradient pumps in one housing. This allows for the easy delivery of the required compensation gradient using the second pump stream. In the conventional, uncompensated mode, only the right gradient pump is used. When compensating the analytical eluent composition with an inverse gradient, the left pump delivers the inverted solvent composition over a second column of the same type as the analytical column, providing an additional gradient delay volume that helps to synchronize the two eluent streams. In the compensation setup, analytical and compensation flow stream are merged by a mixing tee prior to the Corona *ultra*[™] detector.

As the Corona CAD is a mass-sensitive, not concentration-dependent detector, sample dilution by a factor of two (due to the merging flow after the column) will not affect detection sensitivity. Figure 2 illustrates this effect in a separation of six different diuretics (Figure 3) and detection with a Corona *ultra* CAD detector.

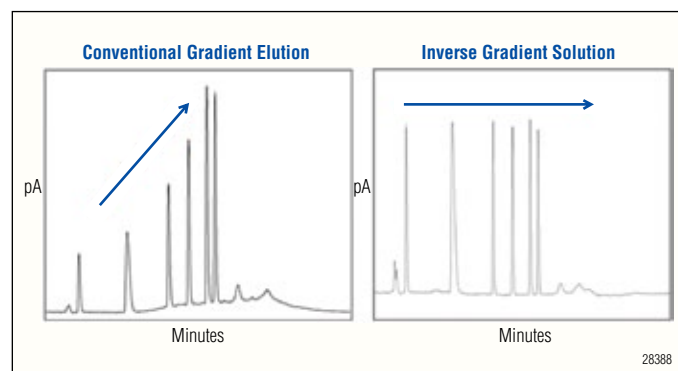


Figure 2. Illustration of the inverse gradient effect on the response of a mass-sensitive detector (Corona *ultra* CAD detector) for a separation of six diuretics (see Figure 3).

It is expected that ESI- and APCI-based LC-MS methods will respond in a more complex way. Both ESI and APCI are described as being predominantly concentration-sensitive detection methods. This study compared the MS sensitivity by monitoring the response factors and the signal-to-noise ratios of the same six diuretics—amiloride, triamterene, chlorthalidone, furosemide, bumetanide, and ethacrynic acid—after separating them using an Acclaim[®] RSLC 120 C-18, 2.2 μ m, 2.1 \times 100 mm column (see Table 1 for chromatography conditions). The compound structures as well as the theoretical m/z values of the neutral compounds are depicted in Figure 3. Sample concentrations varied from 0.5–12.5 μ g/mL.

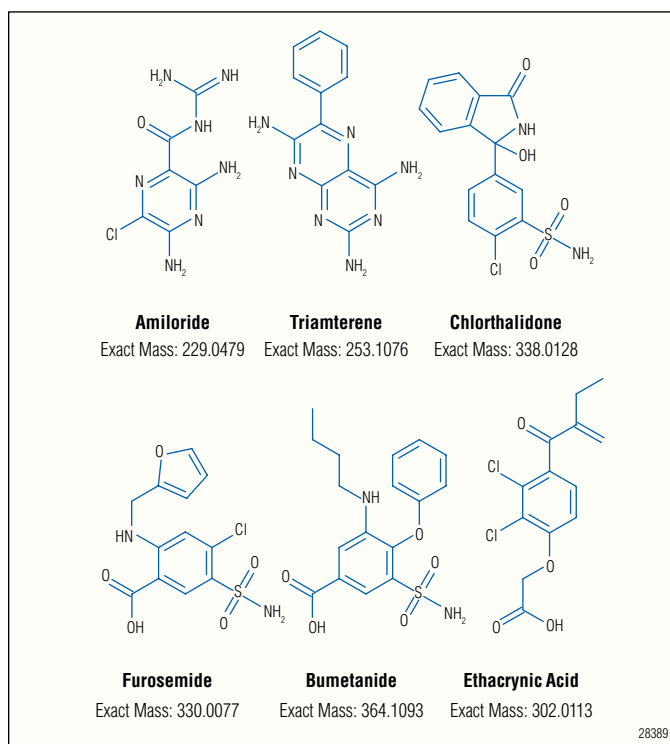


Figure 3. Chemical structures and m/z of diuretics in sample test mix.

2 Ensuring High Sensitivity and Consistent Response in UHPLC-MS Analyses

The Inverse Gradient setup with the mass spectrometer generally followed the scheme in Figure 1. The two solvent streams were combined in a t-piece prior to the UV detector, which was in line with the APCI-TOF-MS. The inverse compensation gradient was delayed artificially by an additional 0.27 min isocratic prestep to account for the larger gradient delay volume of the analysis flow path, due to longer connection capillaries and the autosampler contribution. APCI was selected as the ionization method because it provides better sensitivity than ESI and can more easily handle flow rates of more than 1 mL/min without the need for flow splitting. For details of the instrument setup, please refer to Figure 4. Separation conditions are listed in Table 1.

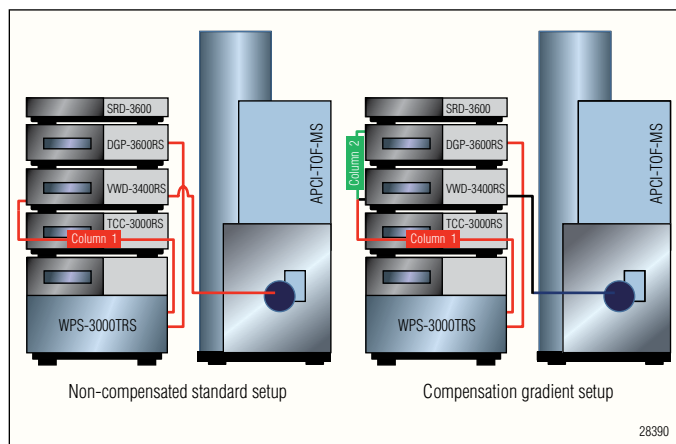


Figure 4. Instrument setup.

Table 1. Gradient Elution and Separation Conditions of the Diuretics Sample Test Mix			
Analytical Gradient		Compensation Gradient with Column	
Time (min)	%B	Time (min)	%B
0.00	15	0.00	85
		0.27	85
1.80	90	2.07	10
2.40	90	2.67	10
2.40	15	2.67	85
7.00	15	7.00	85

System

UltiMate 3000 x2 Dual Rapid Separation LC-MS System consisting of the following modules:

- SRD-3600 Solvent Rack
 - DGP-3600RS Dual Ternary RS Pump
 - WPS-3000TRS Well Plate Sampler
 - TCC-3000RS Thermostatted Column Compartment
 - VWD-3400RS Four-Channel Variable Wavelength Detector with semi-microflow cell (2.5 µL)
 - Bruker microTOF-Q II with APCI source
- All modules were connected with 0.005 in. (0.13 mm) i.d. Viper™ fingertight fittings.

LC and MS Conditions

- Columns: Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm
- Mobile Phases: A: Water + 0.1% formic acid
B: Acetonitrile + 0.1% formic acid
- Gradient: See Table 1
- Flow Rate: 0.65 mL/min on analytical and inverse gradient, as appropriate
- Inj. Volume: 2 µL
- Column Temperature: 35 °C
- Detection: UV: Wavelength, 214 nm; Data Collection Rate, 80 Hz
APCI Parameters: Nebulizer Pressure, 0.25 MPa; Dry Gas Flow, 4.0 L/min; Dry Temp., 300 °C; Vaporizer Temp., 450 °C; Capillary Voltage, (+/-)3500 V; End Plate Offset, (-/+)100 V; Polarity switch (+/-) after 1.5 min

CONVENTIONAL GRADIENT DETECTION VS INVERSE GRADIENT DETECTION USING APCI-MS

In preliminary experiments, ESI and APCI were tested for analyte detection and signal intensity, leading to the following results:

- APCI was superior to ESI in terms of sensitivity (data not shown).
- However, as can be deduced from the chemical properties of the diuretics, it was not possible to detect all analytes with one constant ion source polarity. With ESI in both polarities, not more than three compounds could be detected, while APCI(+) revealed four, and APCI(-) five of six compounds.

Thus, the MS detection featured a polarity switch starting with APCI(+) and turning into APCI(-) mode after 1.3 min. Ion source tuning was optimized to the parameters listed above by direct infusion of a concentrated diuretics stock solution (F = 0.026 mL/min) into the UHPLC pump stream (F = 1.274 mL/min) using a syringe pump, ending up with a total source flow of 1.30 mL/min at a composition A:B of 50:50 v:v which equals the total flow rate in compensation mode.

The relative gradient delay difference between the analytical and the compensation flow path was determined to be 0.27 min by step-gradient change monitoring. Figure 5 shows UV chromatograms of a conventional gradient elution (red) with a total flow of 0.65 mL/min, and the Inverse Gradient concept application of merging the eluent streams prior to the UV detector (blue), for the two diuretics. As both the elution and the inverse gradient start with a small baseline increase, the UV signal doesn't end in an entirely flat baseline; however, the extensive baseline rise seen in the uncompensated separation can be reduced significantly. Furthermore, UV detection clearly illustrates the strong sample dilution by the compensation eluent; the peak heights and areas are half the size (blue) of the conventional run (red).

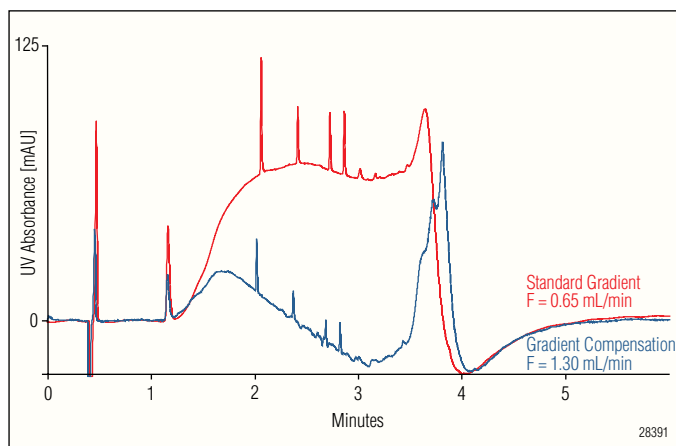


Figure 5. Illustration of gradient compensation effect in UV detection.

With this setup, a calibration series was run for the six diuretics in the range from 0.5–12.5 µg/mL. In addition to the standard gradient and the compensation approach, in a third set of experiments the separation column effluent was merged with a constant mobile phase of 85% B as a make-up flow instead of the inverse gradient. This provides the same flow rate into the APCI source, but without balancing the eluent composition. A signal change in the compensation approach could thus be more easily related to a solvent composition or a dilution effect. Figure 6 contrasts the outcomes of this experiment.

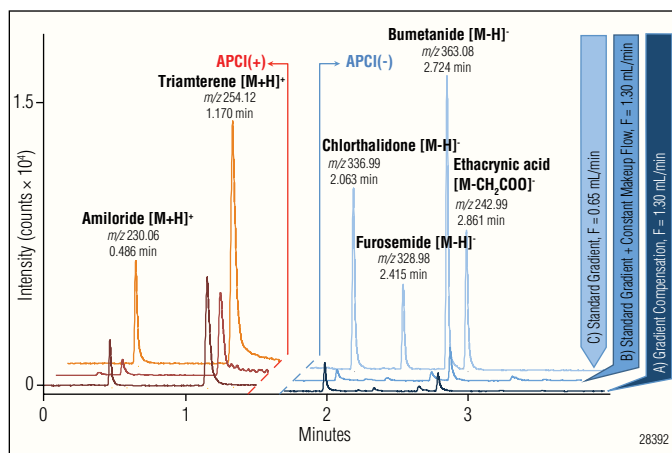


Figure 6. Base peak chromatograms of the separation of 7.5 µg/mL diuretics in APCI-TOF-MS (mode switched after 1.3 min). A) Sample with, and C) without compensation, as well as B) with noncompensating make-up flow.

Chromatogram C shows the UHPLC-APCI-TOF-MS base peak signal of the uncompensated (red) separation shown in Figure 5, offering a very good signal intensity. APCI typically performs best at higher organic solvent contents, leading to more sensitive detection along the gradient profile.

When superimposing this separation with a make-up flow of 85% B, leading to a still changing overall solvent composition in the ion source, a significant reduction in the signal intensity can be observed (chromatogram B). This holds especially true for the more hydrophobic analytes in APCI(-) mode. It demonstrates the concentration sensitivity of APCI, as the higher sample dilution and the reduced ionization efficiency under these conditions negatively impact signal intensity.

However, if the compensation gradient flow is merged (chromatogram A) resulting in an eluent mixing ratio in the APCI source of 50% B, the signal intensity loss due to the doubled flow rate is much less pronounced. This is especially true for those analytes that elute at gradient compositions much different from the 50% B elution composition in the timeframe around 2.2 min, when this gradient proportion reaches the detector. More polar analytes, i.e., amiloride and triamterene, profit from the higher organic percentage related to the normal mode. Hydrophobic analytes i.e., bumetanide or ethacrynic acid, are less sensitively detected than with the pure make-up flow because they are eluted in a less organic mobile phase. These observations are reflected by the calibration results.

Figure 7 compares the response factors (A) and the signal-to-noise ratios at 12.5 µg/mL (B) of the different analytes. For better clarity, all response factors were normalized to the respective triamterene signal, it being the most sensitive analyte in standard mode. Leveling the detection composition to 50% B has different effects on each individual analyte, depending on its hydrophobicity and elution gradient composition, as illustrated by the higher sensitivity of the highly polar amiloride and the nonpolar ethacrynic acid in comparison to the remaining diuretics. It should be noted that between triamterene and chlorthalidone, the APCI source switches polarity to negative mode, which implies different ion formation conditions for the four remaining analytes. Moreover, the Inverse Gradient approach ensures better peak area precision and slightly improved linearities (data not shown). In addition, as the differences in the signal-to-noise ratios reveal, the noise level is significantly reduced with the compensation approach, leading to higher signal quality.

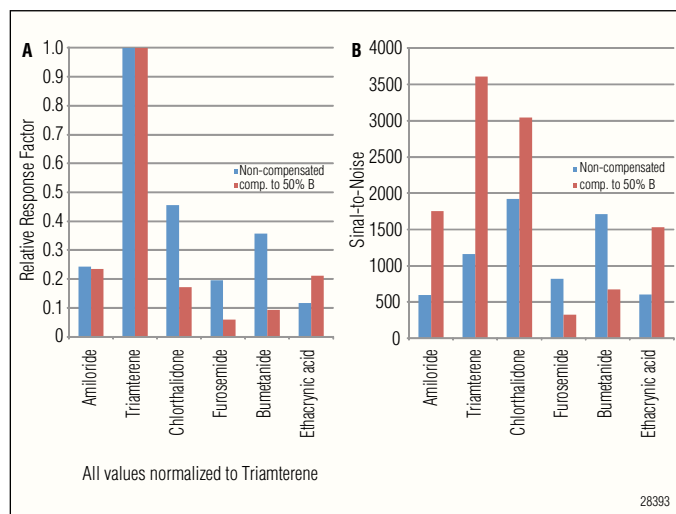


Figure 7. Normalized response factors and signal-to-noise ratios in compensated and non-compensated mode. Compounds are listed in order of elution in the chromatogram.

CONCLUSION

While the concept of Inverse Gradients opens up an amazing potential for universal calibration with a charged aerosol detector, it has ambivalent effects on the signal quality in concentration-dependent ionization modes for LC-MS.

- The overall response factors of the analytes of interest are reduced due to the higher sample dilution caused by the higher volume flows in the ion source.
- While the higher flow rates merely dilute the sample, balancing the eluent composition prior to its passing into the detector actually changes the relative response factors of the different analytes along the gradient. However, analyte response with MS can never be as uniform as with a charged aerosol detector due to the very different analyte ionization properties. At best, an Inverse Gradient approach with MS reduces the widespread variations in the analyte signal and ensures at least a more homogeneous response, as was achieved here, with some concessions.
- The overall signal-to-noise ratios can benefit from the constant mobile phase composition in the MS source due to improved noise levels.

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