

Charged aerosol detection - factors affecting uniform analyte response

Authors

Michael Menz¹, Benjamin Eggart¹,
Katherine Lovejoy¹, Ian Acworth²,
Paul Gamache², Frank Steiner¹

¹Thermo Fisher Scientific,
Germering, Germany

²Thermo Fisher Scientific,
Chelmsford, MA, USA

Keywords

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Goal

To discuss why charged aerosol detection produces uniform analyte response for non-volatiles, factors that affect this uniform response, and ways to improve response for semi-volatiles and volatiles.

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Introduction

The charged aerosol detector (CAD) is a universal detector used in conjunction with high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) to measure all non-volatile and many semi-volatile analytes in a sample. It is commonly used for the analysis of species that cannot be detected using traditional UV/Vis approaches due to their lack of a chromophore.

Since the CAD is different than commonly used optical detectors, the general technology will be briefly described. CAD uses pneumatic nebulization of the mobile phase from the analytical column to form an aerosol. The size distribution of the droplets within the aerosol entering the drying tube is well controlled with a maximum size of several micrometers after removal of large droplets within a spray chamber. In the evaporation tube the solvent is evaporated from the droplets and dried particles, or stable liquid particles (lipids), remain. Their size distribution typically ranges from a few nanometers to several hundred nanometers depending on the analyte concentration and density. In the next step, the dried particles are positively charged by diffusion charging, which involves collision of particles with gas ions created

via a corona discharge. After removing excess gas ions using an ion trap, the aggregate charge of all particles is measured via an ultra-sensitive electrometer. The charge on the dried particle is proportional to the particle diameter and thus the mass concentration of the analyte.¹

The CAD shows outstanding uniform response

The CAD is a mass-flow sensitive detector (response is proportional to mass of analyte reaching the detector per unit time) with outstanding uniformity of response (i.e., response being independent of an analyte's chemical structure or physicochemical properties).¹ As an example of the response uniformity of this detector, Figure 1 shows the CAD response for a diverse range of substances related to the pharmaceutical, industrial, molecular biology, and food markets. Note that the variability of response was < 6%. This analysis was performed without a column. Flow Injection Analysis (FIA) was used to exclude chromatographic effects (e.g., analyte degradation or loss on column). See Appendix A for details of the experiment, performed on a Thermo Scientific™ Dionex™ Corona™ Veo™ CAD/Thermo Scientific™ Vanquish™ Flex CAD.

Due to its response uniformity, and unlike UV detectors, the CAD can quantify unknown substances for which it is

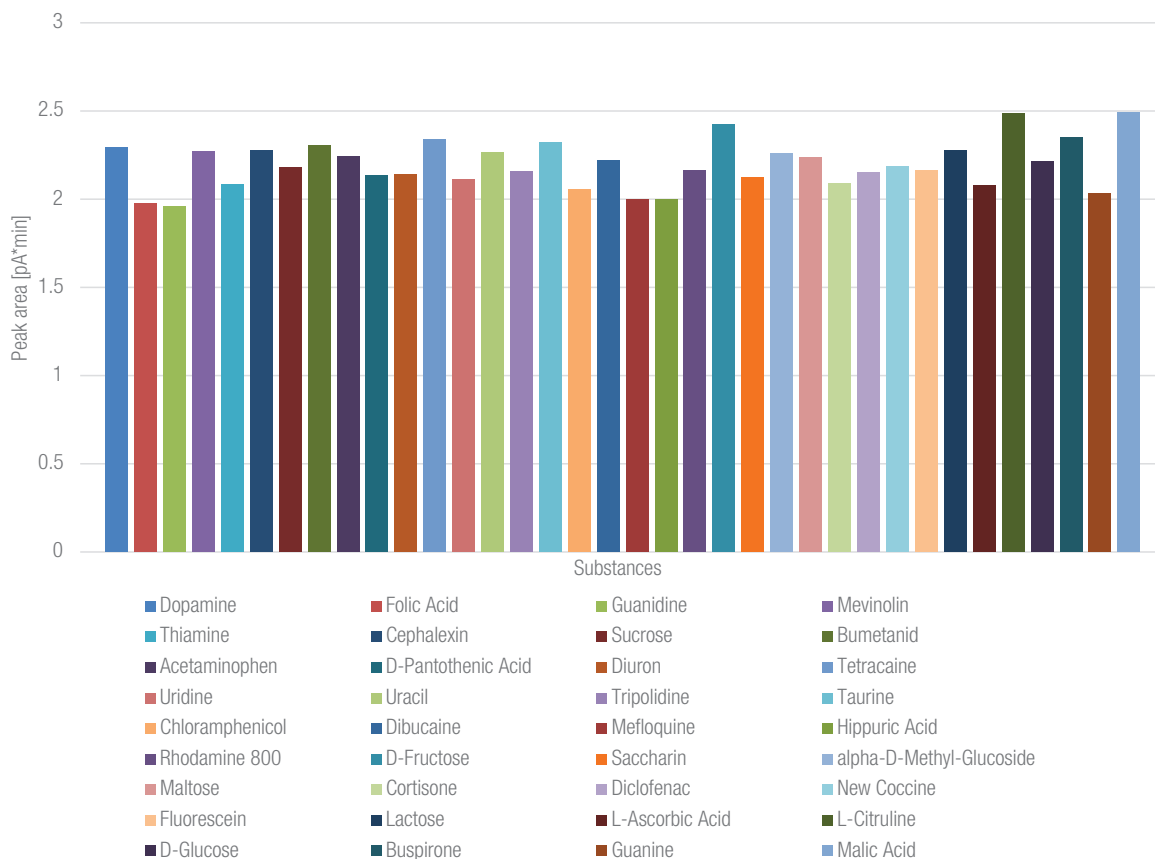


Figure 1. Response of the CAD to 36 compounds (0.5 µg each) by flow injection

impossible to use a standard, using the calibration curve of a substance for which a standard exists (called a universal calibrant). Figure 2 shows the analysis of contaminants extracted from biopharmaceutical cell culture bags for an extractables and leachables study (see Appendix B for further details). UV response varied dramatically according to the analyte's extinction coefficient. Conversely, the CAD response was sufficiently uniform to allow accurate quantification of unknown substances in the extracts. The uniform response of the CAD positions this detector as a powerful tool for pharmaceutical laboratories throughout all stages of drug development.²

Facilitating uniform response by optimal CAD operation

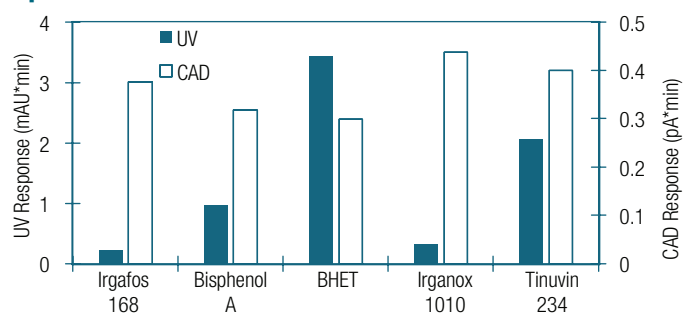


Figure 2. Comparison of UV detector and CAD response to analytes in an extractables application

The CAD response can be influenced by four factors affecting the up-stream spray drying process:

- **Mobile phase composition:** Changes in organic content of the mobile phase during gradient elution can impact detector response. Obviously, this is not a concern when using isocratic conditions or the Thermo Scientific™ Vanquish™ Duo Inverse Gradient Workflow available with Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) version 7.2.8.²
- **Analyte volatility:** Loss of analyte response is due to its evaporation during nebulization and drying processes (evaporation temperature effect).
- **Salt formation:** The interaction between ionizable analyte and mobile phase additives (i.e., pH modifiers, pH buffers, and ion pairing agents). Salt formation can be used practically to convert analytes that behave as semi-volatiles and volatiles into those that behave more like non-volatiles.
- **Analyte density:** This is only a minor influence on analyte response.

The impact of these factors will be addressed more fully below, including practical and theoretical tips.

Effects of mobile phase solvent composition

As the mobile phase composition changes during an analytical solvent gradient, the response of the CAD varies as a function of the volatility, surface tension, and viscosity of the blended mobile phase. In a reversed-phase gradient separation, the organic composition increases over time. As the organic content increases, the efficiency of nebulization increases, thus increasing the percentage of analyte reaching the detector. This results in higher response for the later eluting peaks. Conversely, when organic content is low and aqueous content is high, such as at the beginning of the gradient, nebulization is less efficient with less analyte mass detected.

To achieve uniform response with a CAD, a constant composition of mobile phase must reach the detector inlet. This constant composition is accomplished by a technique that is often referred to as a “make-up” or “inverse” gradient. A second gradient pump generates the inverse of the analytical gradient. The combination of the streams from both pumps prior to the detector yields a constant composition of the mobile phase mixture entering the detector at any point in time.

Implementation of the inverse gradient requires some calculations, including void volume of the column and gradient delay volume of the two flow paths. An inverse gradient wizard is incorporated into Chromeleon CDS 7.2.8 to facilitate these calculations for a binary or even a ternary gradient. For analytical gradient methods that have a relatively small (e.g., <50%) change in solvent composition, the wizard also provides the calculations to minimize total flow rate or to maximize the percentage of a given solvent. In both cases, the eluent flowing into the CAD is kept at a constant solvent composition in order to provide uniform response. Minimizing the total flow rate reduces solvent consumption while the option to maximize the percentage of a given solvent can be used to optimize performance. For example, use of a higher percentage of a solvent (A, B, or C) that has lower viscosity and surface tension and also has a low level of nonvolatile and semivolatile impurities can provide better detection limits. The use of the inverse gradient wizard is described in Appendix C.

The following two impurity analyses for the chemotherapy agent paclitaxel and for the antiretroviral agent tenofovir disoproxil fumarate show the use of the inverse gradient for real applications.

Paclitaxel example of inverse gradient

A stressed paclitaxel sample (See Appendix D for sample preparation and experimental details) was analyzed using the Vanquish Duo Inverse Gradient Workflow with the Vanquish Flex Dual Pump and compared to the same system configuration without gradient compensation (Figure 3). Both setups were able to detect the same number of peaks but there was a significant difference in analyte response between the two approaches.

For the thermally and oxidatively stressed sample shown in Figure 3, the combined peak areas for all impurities were found with the CAD to be 63.9% of the paclitaxel, or active pharmaceutical ingredient (API), peak area when not using inverse gradient compensation (blue trace) and 53.8% of the API when using inverse gradient compensation (black trace). This large (~10%) difference in determined impurity content is attributed to the influence of solvent composition on the CAD response. As expected in reversed-phase gradients, response factors for later eluting peaks are higher when not using inverse gradient compensation (see Figure 3 inset). These data highlight the capabilities of the Vanquish Duo Inverse Gradient Workflow to achieve uniform response with a CAD and thus minimize quantitation errors.

Tenofovir example of inverse gradient

The antiretroviral drug combination of tenofovir disoproxil fumarate and emtricitabine is used to treat HIV/AIDS patients. Impurity profiling by reversed-phase liquid chromatography is difficult because both early-eluting polar as well as late-eluting hydrophobic impurities are present. The two active ingredients have different extinction coefficients, so quantitation by UV requires standards for the active ingredients and all impurities. However, due to the uniform response of the CAD, all components in the sample can be quantified using a **single** calibration curve. See Appendix E for chromatographic details and system parameters. Figure 4 shows the calibration curves with analytical gradient only and with inverse gradient applied (see Figure 5 for chromatograms). The inverse gradient serves to normalize the peak height and area relative to the analytical gradient and to reduce baseline drift. An example of this is shown in Table 1. Here, analysis of 30 ng of all substances quantified using the tenofovir disoproxil calibration curve, led to a marked underestimation (6–25 fold) when using the analytical gradient only, but a slight overestimation when using the inverse gradient. The accuracy of quantitation of API and impurities is thus greatly improved upon implementation of the inverse gradient.

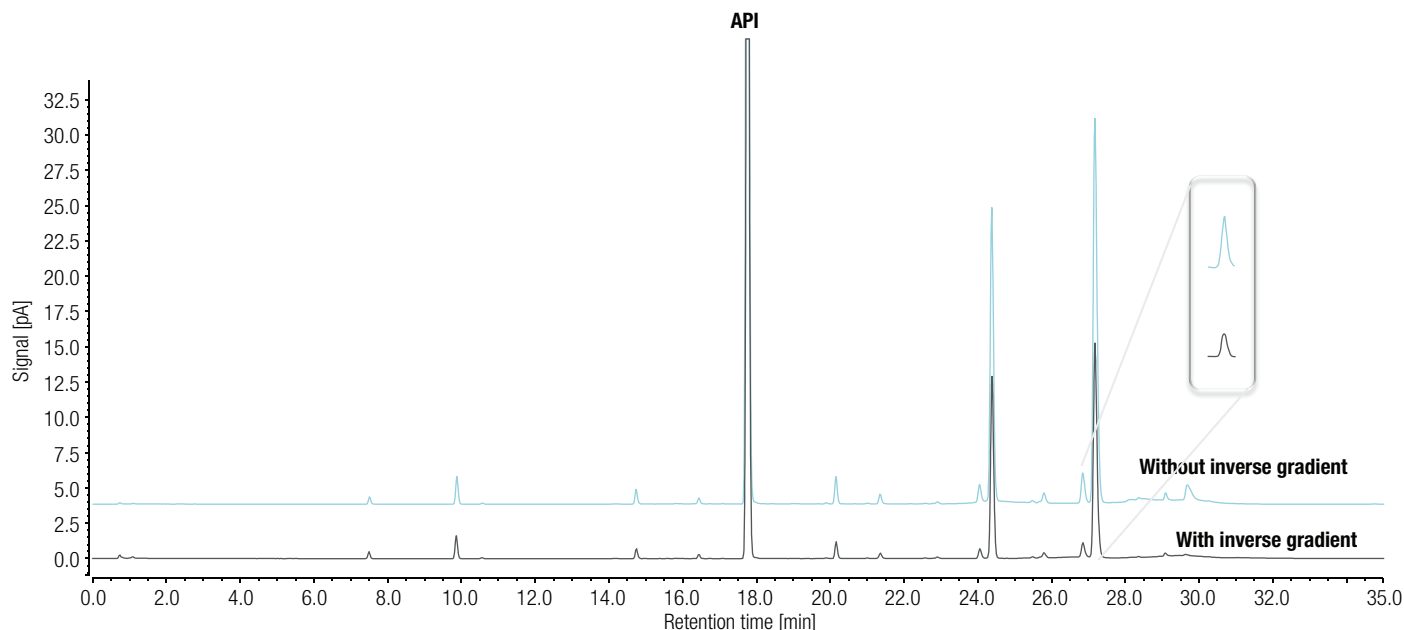


Figure 3. Comparison of CAD response for a sample of paclitaxel and impurities with (gray trace) and without (blue trace) applying inverse gradient compensation using the Vanquish Flex Duo system. The active pharmaceutical ingredient (labeled API in the figure) is paclitaxel.

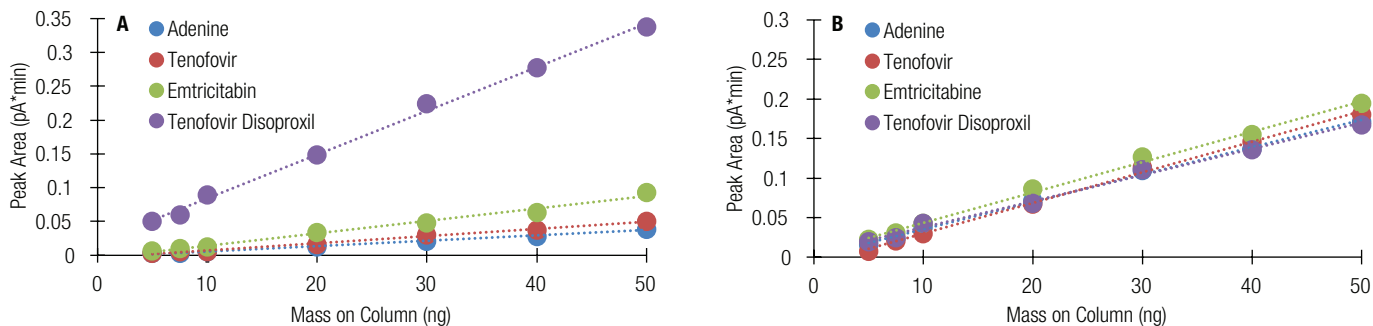


Figure 4. Calibration curves for tenofovir disoproxil and impurities either (A) with analytical gradient only and (B) with inverse gradient

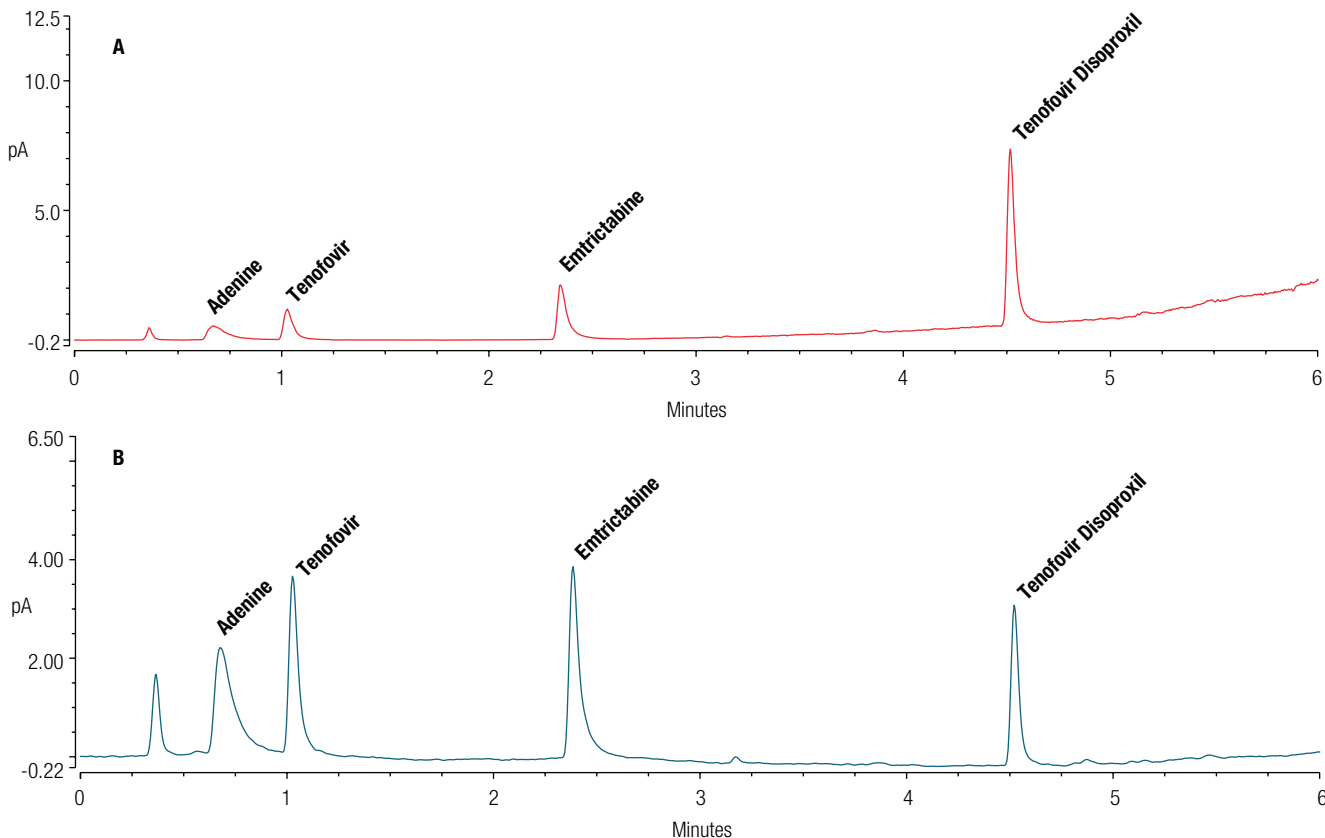


Figure 5. Comparison of chromatograms for 20 ng of each substance either (A) with analytical gradient only or (B) with the inverse gradient

Table 1. Single calibrant quantification results for tenofovir disoproxil fumarate and impurities with and without the inverse gradient

Analyte	R ² , Inverse Gradient Curves	Reinjection, 30 ng, with Inverse Gradient (ng)	Reinjection, 30 ng, without Inverse Gradient (ng)
Adenine	0.997	29.6	1.2
Tenofovir	0.998	32.5	1.7
Emtricitabine	0.996	36.2	4.8
Tenofovir disoproxil	0.994	32.0	31.6

Effects of analyte volatility

Besides ensuring that the CAD always sees a constant gradient composition, the user should also consider an analyte's volatility and propensity to form salts with other components in the mobile phase or sample. Analytes fall into three different categories (Figure 6): the majority show uniform response and are non-volatile; the second group (Group A in Figure 6: dopamine-HCl, guanidine-HCl, and diclofenac disodium salt) show a greater response than expected due to salt formation; the third group (Group B in Figure 6: oxalic acid) include semi-volatiles and show a lesser response than expected. Additionally, some analytes are too volatile to show a signal by the CAD. This section will address semi-volatiles. The following section will focus on salt formation and its impact on volatility.

The ability to predict whether the CAD can measure a particular analyte is of considerable interest. Several studies have described approximate cut-offs beyond which all analytes behave as non-volatiles.¹ For example, one study of a large and diverse compound library showed that any substance with a boiling point above 400 °C was found to behave as a non-volatile. Another similar study showed that any analyte with both an

enthalpy of vaporization above 65 kJ/mol and a molecular weight above 350 g/mol behaved as a non-volatile. Similar cut-offs have also been described in relation to vapor pressure. While these volatility limits are useful as rough guidelines, it should be noted that they depend on instrument design and conditions, especially evaporation temperature. The specific process of spray-drying is also not fully explained by these parameters alone. Thus, for analytes with values beyond a given volatility limit (e.g., boiling point < 400 °C) there may be unexpected outliers and differences in sensitivity between analytes.¹ Some of these differences might be due to formation of ionic salts within droplets as they dry, a topic that will be further discussed in the following section. Future research into spray drying and gas-to-particle partitioning will help improve predictions of LC-CAD response.

The evaporation temperature (T_e) can be used to alter the "selectivity" of the CAD. Reducing T_e is generally expected to produce more uniform response and a broader detection scope since selectivity is effectively reduced. However, higher background current and noise may result due to more sensitive detection of semi-volatile impurities that are likely to be present. Using higher T_e may reduce background currents and noise,

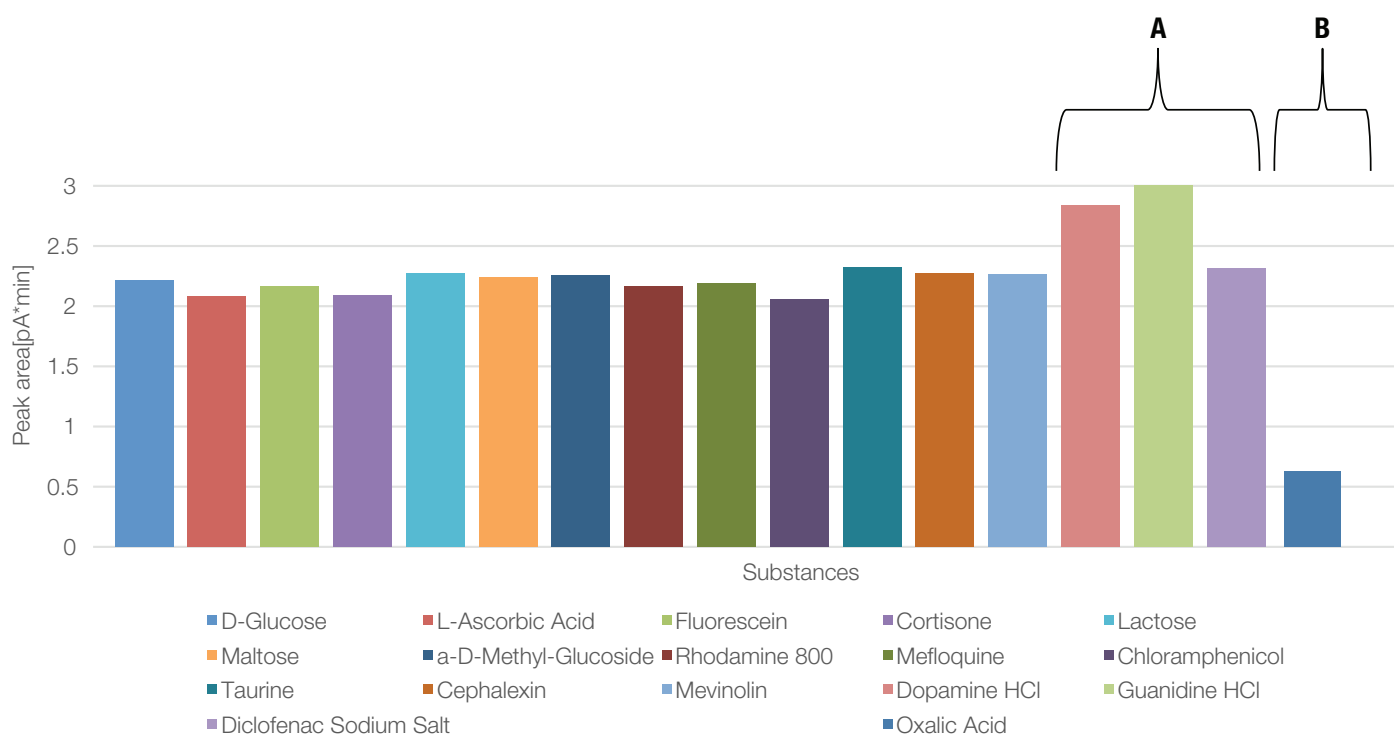


Figure 6. Influences that can affect uniform response in charged aerosol detection. (A = salt formation; B = semi-volatiles). See Appendix A for conditions for flow injection analysis.

but more analytes will behave as semi-volatiles resulting in loss of signal, especially at low levels. In general, it is best to use the lowest T_g that consistently produces the required sensitivity limits. This should also provide the best reproducibility and most uniform response between analytes. It is also important when selecting a T_g to examine method robustness and stability of each analyte based on sample concentrations that are near the limit of detection.

Effects of salt formation

As shown in Figure 1, the majority of compounds give similar responses, thus behaving as nonvolatiles. However, salt formation in aerosol droplets between the ionized analyte and oppositely charged ions can be a key factor influencing response. A simple example of salt formation affecting response using FIA is shown in Group A of Figure 6 (see Appendix A for experimental). Since there is no chromatographic separation, the oppositely charged ions involved in salt formation are counterions present in the starting material or powder (chloride in the case of dopamine). For chromatographic separations, mobile phase additives are the more common source of counterions. If an ionogenic substance inherently (i.e., in the absence of mobile phase additives) behaves as a non-volatile, detector response increases proportionally according to the additional, relative molar mass of the counterion upon salt formation. If a substance inherently behaves as a volatile or semi-volatile but forms a non-volatile salt with a mobile phase additive, detector response can improve by much more than the relative molar mass of the counterion. These two cases are discussed in detail with examples below.

Salt formation with non-volatile ionizable analytes

As mentioned above, the potential for non-volatile ionizable compounds to form salts can lead to decreased

response uniformity among analytes. To minimize this effect, it is essential to choose mobile phase additives with low molar mass. For this purpose, formic acid and ammonium formate are the typical additives of choice. Higher molecular weight additives, including ion pairing agents such as trifluoroacetic acid, increase analyte mass more than ions of lower molecular weight and are more detrimental to the CAD's uniform response. Regardless of the additive used, if the extent of analyte ionization is known (from functional group pK_a and mobile phase pH) then it is possible to "normalize" the response as shown below for the example of dopamine-HCl:

$$\text{Corrected Response} = \text{Response} * \frac{M_w(\text{dopamine})}{M_w(\text{dopamine} + \text{HCl})}$$

Figure 6 shows that both dopamine and guanidine, which are associated with HCl, give increased responses when compared to the average value of 2.19 $\text{pA} \cdot \text{min}$ for the 13 non-volatile compounds. By correcting the response, the peak areas for dopamine and guanidine decreased from 2.83 and 3.16 $\text{pA} \cdot \text{min}$ to 2.29 and 1.95 $\text{pA} \cdot \text{min}$, respectively. Thus, when salt formation is taken into account, the response of dopamine and guanidine are similar to the other non-volatile analytes measured.

Use of this correction in a chromatographic method can be shown for the previously discussed tenofovir disoproxil fumarate example (see Appendix E for the experimental parameters). The four analytes in the standard sample, adenine, tenofovir, emtricitabine, and tenofovir disoproxil, are all singly positively charged in an aqueous solution of 0.1% formic acid. The calibration curves with and without the application of an inverse gradient are shown in Figure 7. After the correction for salt formation is made, the curves show great similarity. The quantitative results for evaluating using a single

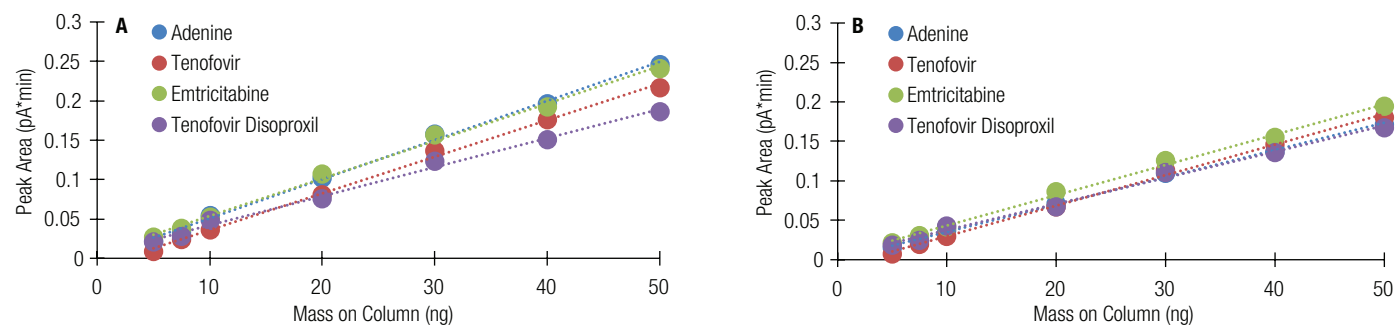


Figure 7. Calibration curves for analytes in the tenofovir application are shown (A) before correction for one acetate per analyte molecule and (B) after correction. All of the analytes are expected to exist as singly charged cations (+1) in the eluent.

calibrant sample containing 30 ng of each substance are shown in Table 2. When not corrected for salt formation the response was overestimated by up to 33%. However, when taking salt formation into account the response for all but one analyte was just 1.3% of the expected value. Without the column and using flow injection analysis and no mobile phase additive, the peak areas of these four analytes have an RSD of only 6.2% after correction, showing that the use of salt correction restores the expected uniform response.

Table 2. Comparison of single calibrant data for tenofovir disoproxil before and after salt correction. The salt correction calculation assumed one acetate per molecule.

Analyte	Reinjection, 30 ng, No Salt Correction (ng)	Reinjection, 30 ng, Salt Correction (ng)
Adenine	38.3	29.6
Tenofovir	34.9	32.5
Emtricitabine	40.1	36.2
Tenofovir disoproxil	31.8	32.0

Influence of mobile phase additive on the CAD response can be minimized by using a low molar mass additive and maximized with larger molar mass additives like TFA, and HFBA. The effect is more pronounced for lower molar mass analytes. If formic acid had been used in this application instead of the higher molecular weight acetic acid, the response increase due to salt formation would have been smaller. In general, additives with molecular weights that are as low as possible should be used to minimize the disruption of the CAD's uniform response.

It should be noted that the widely used conditions of low pH (e.g., 0.1% formic acid) mobile phase with reversed-phase chromatography are in part chosen to ensure that most acidic analytes are fully neutral while basic analytes are fully protonated. This avoids inter-conversion between ionic-neutral forms during the separation, which can lead to peak tailing and poor retention reproducibility. Under these conditions, the CAD response of only basic analytes should be affected by salt formation.

Using the simple calculation outlined in this discussion, response uniformity amongst ionizable analytes can be maintained when the use of buffers or pH modifiers are necessary.

Salt formation with volatile ionizable analytes

Several studies have shown that the intentional formation of salts (e.g., from mobile phase additives, such as volatile acids or bases), can broaden the range of compounds that can be measured by the CAD.¹ In this case, salt formation can be advantageous by enabling better detection of volatile analytes (e.g., volatile basic analyte + volatile acidic modifier = non-volatile signal-producing salt). The potential disadvantage of this approach is that it can lead to increased background and noise (e.g., volatile basic impurity + volatile acidic modifier = non-volatile noise-producing salt). Judicious choice of pH and additive can address these disadvantages.

An example of the stabilization of semi-volatiles using intentional salt formation was illustrated using oxalic acid as an example (Group B of Figure 6). These semi-volatile substances give a lower signal intensity compared to the typical non-volatile compounds. To overcome effects of their volatility, triethylamine (TEA) can be used to form a non-volatile salt complex when analyzing acidic compounds (Figure 8). Oxalic acid was diluted and analyzed with a basic mobile phase (80/20/0.01 ACN/H₂O/TEA, pH ~10.5; see Appendix A for more details).

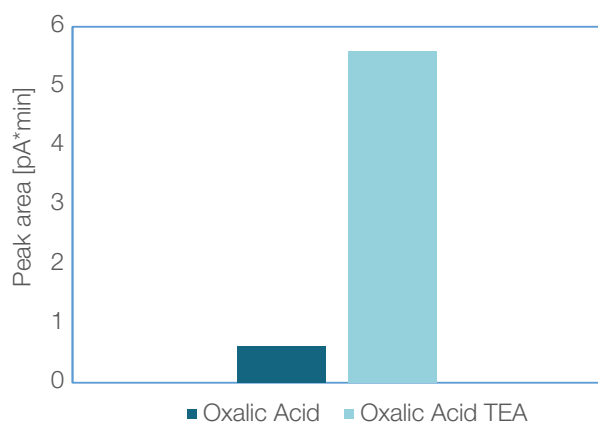


Figure 8. Utilization of salt formation to enhance signal intensity for the semi-volatile compound oxalic acid (0.5 µg each)

By reducing evaporation during the drying process, TEA salt formation leads to an enhanced detector response of approximately ten-fold for oxalic acid. The following relationship can be used to calculate the response of the pure compounds and corrected for the weight of the associated TEA.

$$\text{Response} * \frac{\text{Molar Mass Oxalic Acid}}{\text{Molar Mass Oxalic Acid} + \text{Molar Mass TEA}}$$

These data are summarized in Table 3.

Intentional salt formation can overcome sensitivity issues for semi-volatile and volatile substances. Using this approach, the signal intensity of semi-volatile analytes is similar to those of non-volatile compounds (Figure 1).

Analyte density and other considerations

Dried particle diameter varies by the cube root of solute density. Solute density therefore has only a minor influence on sensitivity. For this reason and because experimental density values are scarce in the literature, a density correction is not frequently applied. If the solutes in an application are known to have disparate densities, a simple compensation would be to multiply the CAD response for each analyte by the cube root of its density. If the analyte is likely to have formed a salt in the dried particle, the density of the predicted salt should be used.¹

There are a number of additional considerations, unrelated to the CAD detection, which may influence the observed response uniformity. These include the purity of the material that was used to prepare the sample (e.g., 10 mg of a powder that is 95% pure contains only 9.5 mg of the main component), changes during storage and preparation (e.g., adsorption of water by hygroscopic powders), weighing and dilution errors, analyte degradation, analyte loss on the column, and injection reproducibility.

Conclusion

Inherent universal response of non-volatile and most semi-volatile compounds is a superior feature of the CAD compared to classic detection options like UV-Vis. We have presented simple considerations and techniques to further increase analyte response uniformity.

- The Vanquish Duo Inverse Gradient Workflow can compensate the analytical gradient with a second low-pressure gradient pump to avoid bias occurring from different solvent compositions. This approach gives a more uniform response and more reliable quantitation.
- Salt formation between ionizable analytes can influence response uniformity. This effect can be minimized by choosing low molar mass mobile phase additives and response can be normalized using the described calculations.
- Volatility is a crucial consideration in CAD response. It is best to use the lowest evaporation temperature that consistently produces the required sensitivity limits. This should provide the most uniform response between analytes. The formation of salts can markedly improve the response for difficult to detect volatile compounds, which have ionisable functional groups.

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Table 3. Peak areas for for a volatile ionizable analyte with and without salt formation and salt correction

Compounds	Peak Area for 0.5 µg without TEA (pA*min)	Peak Area for 0.5 µg with TEA (pA*min)	Peak Area for 0.5 µg after Salt Correction (pA*min)
Oxalic Acid	0.626	5.583	2.629

Appendix A: Experimental details for flow injection analysis

Instrumentation

Thermo Scientific™ Vanquish™ Flex Duo UHPLC system for Inverse Gradient consisting of:

- System Base Vanquish Flex (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Dual Pump F (P/N VF-P32-A-01)
- Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VF-A10-A-02) with a 25 µL sample loop
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
- Corona Veo / Vanquish Flex CAD (P/N VF-D20-A)

Flow injection conditions

Capillary from

Autosampler to CAD: 0.1 × 550 mm Thermo Scientific™ Viper™ Capillary

Mobile Phase:

For flow injection without TEA:

A: Water (20%)

B: Acetonitrile (80%)

For flow injection with TEA:

A: Water with 0.01% TEA, pH ~10.5 (20%)

B: Acetonitrile with 0.01% TEA (80%)

Flow Rate: 0.4 mL/min

Injection Volume: 1 µL

CAD Settings: 35 °C evaporation temperature, 5 Hz data collection rate, 0.5 s filter

Data Processing: Chromeleon CDS 7.2.8

Recommended lab consumables and equipment

- Fisher Scientific™ LC/MS grade Acetonitrile (P/N A955-212)
- Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136171)
- Fisher Scientific™ Triethylamine (P/N O4885-1)

Sample preparation

Samples were prepared in mobile phase A. Specifically, when flow injection was performed without TEA, samples were dissolved in water. When flow injection was performed with TEA, samples were prepared in 0.01% TEA.

Appendix B: Extractables example for comparison of UV detector and CAD response

Instrumentation for analysis of extractables

Vanquish Flex Duo UHPLC system for Inverse Gradient consisting of:

- Thermo Scientific™ Vanquish™ Flex Dual Pump (P/N VF-P32-A-01)
- Thermo Scientific™ Vanquish™ Flex Split Sampler (P/N VF-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
- Thermo Scientific™ Vanquish™ Flex Diode Array Detector (2.5 µL titanium flow cell) (P/N VF-D11-A)
- Vanquish Flex / Corona Veo CAD (P/N VF-D20-A)

Chromatographic conditions for analysis of extractables

Column: Thermo Scientific™ Accucore™ C18, 100 × 2.1 mm, 2.6 μm (P/N 17126-102130)

Mobile Phase: A: 4 mM Formic Acid in water
B: Isopropanol

Analytical Gradient:	<i>Time (min)</i>	<i>%B</i>
	0	5
	10.5	100
	12	100
	12.1	5
	16	5

Inverse Gradient:	<i>Time (min)</i>	<i>%B</i>
	0	100
	0.728	100
	11.228	5
	12.728	5
	12.9	100
	16	100

Flow Rate: 0.4 mL/min

Column Temp.: 45 °C forced air mode,
45 °C active pre-heater

Autosampler Temp.: 4 °C

Injection Volume: 2 μL

UV Detector Settings: 10 Hz data collection rate,
0.5 s response time,
4 nm bandwidth, 210, 220,
254, 280, 300, 320 nm and
190-345 nm (3D field)

CAD Settings: 35 °C evaporation temperature,
10 Hz data collection rate,
3.6 s filter, 1.0 power function
value

Data Processing: Chromeleon CDS 7.2.8

Recommended lab consumables and equipment

- Fisher Chemical™ Optima™ LC/MS grade isopropanol (P/N A461)
- Barnstead GenPur xCAD Plus Ultrapure Water Purification System (P/N 50136171)
- Fisher Chemical™ Optima™ LC/MS grade formic acid (P/N A117)

Sample preparation

Eighteen reference compounds were selected based on literature reports of extractables present in cell culture bags and were purchased from Sigma-Aldrich, Steinheim, Germany.³ Dilutions were prepared in methanol from 1 mg/mL standards (in suitable solvents: hexane, methanol, or acetone) at 1, 2, 5, 10, 20, and 50 μg/mL, except for butylparaben, eicosane, and tetracosane, which were prepared at 10, 20, 50, 100, 200, and 500 μg/mL. Four different types of single-use cell culture bags, the inner layer of which was made of ethylenevinyl acetate and different density grades of polyethylene, were investigated. Extracts were prepared by flushing the inside of the bags with 50/50 isopropanol/water, then removing and evaporating the flushing solution to dryness.

Eluent preparation

Eluent A was prepared by adding 150 μL 99.5% formic acid to 1000 mL water. Eluent A had a final pH of 3.1.

Appendix C: Use of the Inverse Gradient Wizard

Starting with version 7.2.8, Chromeleon CDS features an inverse gradient wizard that facilitates design and implementation of inverse gradient methods. The wizard encompasses two stages. The first stage allows the user to define the fluidic confirmation based on the inverse gradient capillary kit for the Vanquish Dual Gradient Pump. The second stage is initiated when the user programs an instrument method. The two stages are detailed below.

The first stage of the wizard requires the user to confirm the fluidic configuration. Only after confirming the fluidic configuration can the user program an instrument method. The user must set the fluidic configuration by clicking the valve icon above the ePanels (Figure 9).

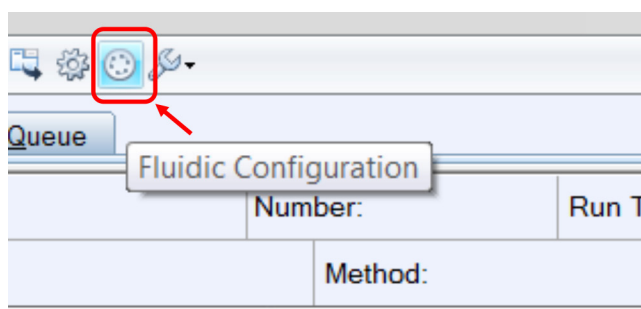


Figure 9. The valve icon above opens the fluidic configuration dialog.

The “Select Fluidic Configuration” step assumes a certain fluidic configuration. For the calculation using an assumed fluidic configuration, the wizard offers the user a drop-down list (Figure 10) of workflow kits that might be installed with the modules in the configured system. If the user selects “Vanquish Inverse Gradient,” the wizard automatically incorporates modules from the instrument configuration manager and capillaries from the inverse gradient capillary kit (P/N 6036.2010) into the calculation.

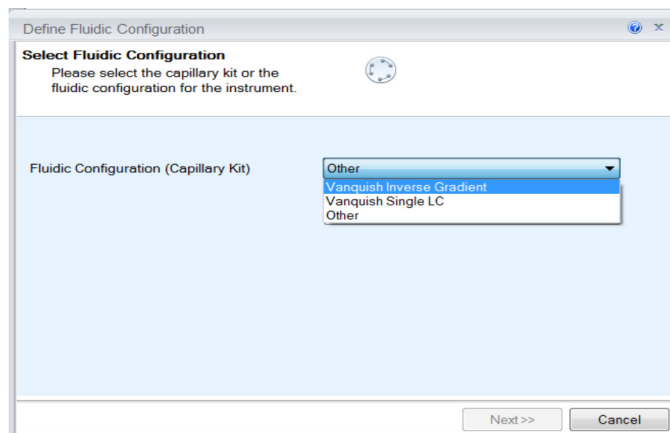


Figure 10. The fluidic wizard allows the user to select relevant workflows based on the system configuration and capillary kit.

After selection of the capillary kit, the next step of the wizard, “Assign Modules,” asks the user to assign the right and left pump heads to the inverse and analytical gradients (Figure 11). This step also asks the user to choose a column if more than one column with a tag is present. Additionally, if no active pre-heater is connected to the column compartment, Chromeleon CDS adds a passive pre-heater to the fluidic description in this step.

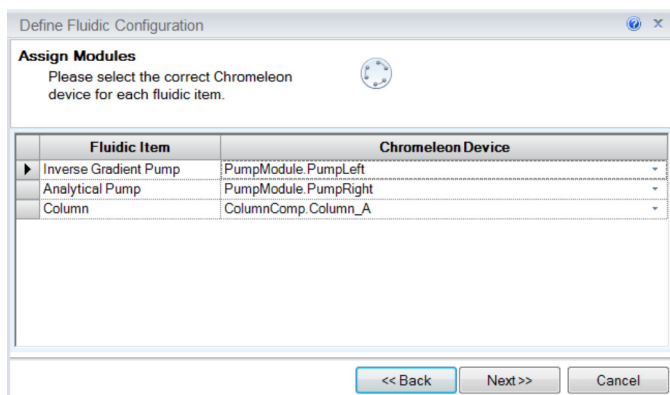


Figure 11. In the “Assign Modules” step, the user must assign the pump heads to the analytical or inverse gradients.

The next step, “Define Column Volume,” calculates the column volume using the equation:

$$\text{Column Volume } (\mu\text{L}) = \frac{\pi}{4} * d^2 * L * 63\%$$

Where d is the column’s inner diameter in mm, L is the column’s length in mm, and 63% is the void volume factor. The wizard either automatically calculates the effective void volume based on the column tag (Figure 12) or it requires the user to either enter the values for column length and inner diameter or to enter the column void volume (Figure 13).

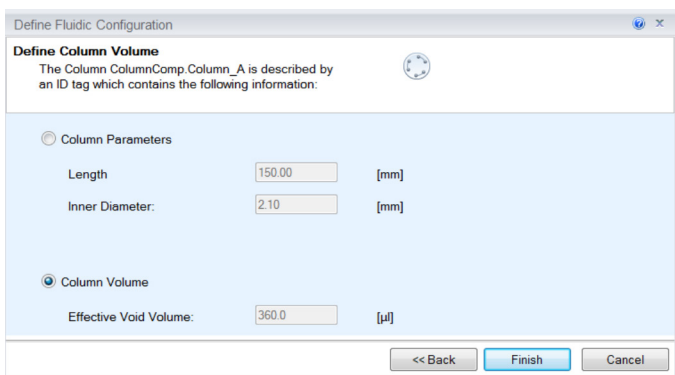


Figure 12. The “Define Column Volume” step when a column tag is present

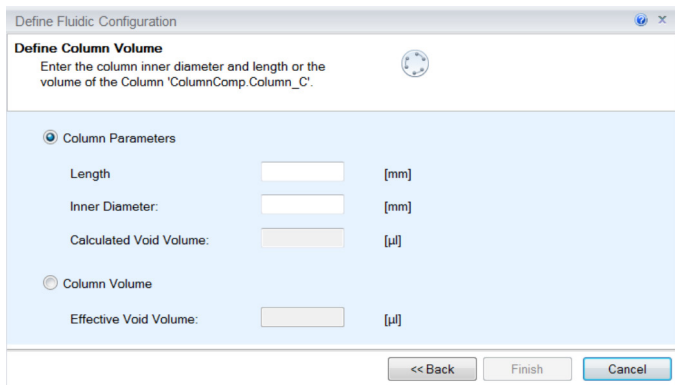


Figure 13. The “Define Column Volume” step when a column tag is not present

Clicking “Finish” after the “Define Column Volume” step saves the fluidic configuration and ends the first stage of the wizard.

The second stage of the wizard automatically programs the inverse gradient when the user initializes an instrument method. Upon creating a method, the user must make an “Instrument Method Workflow Selection” (Figure 14).

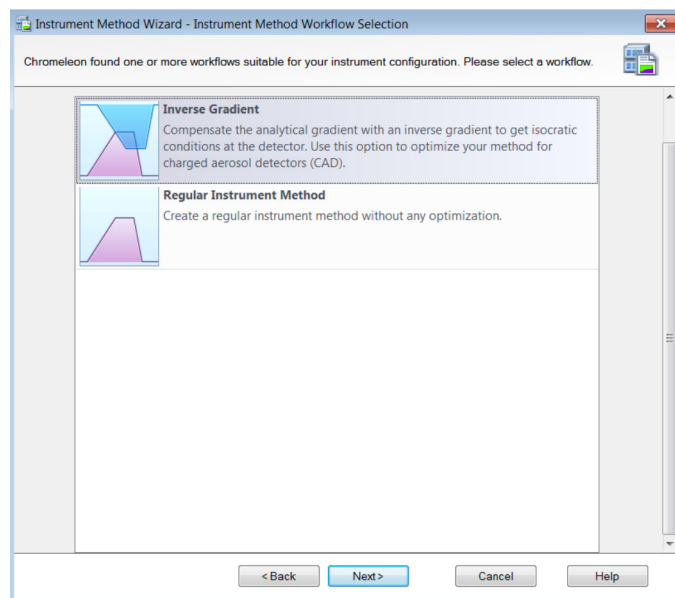


Figure 14. Upon creating an instrument method, the user chooses a workflow.

After entering method parameters such as run time and pressure limits for the analytical and inverse gradient pumps, the “Inverse Gradient: Options” step allows a choice of modes for calculating the inverse gradient. Although the wizard defaults to a “keep solvent composition” mode (Figure 15), it also offers a “minimize flow” mode, for the case that the user wants to limit the flow at the detector to reduce baseline noise from eluent impurities, and “maximize %X” flows, for the case that the user wants to improve sensitivity by maximizing the amount of the solvent that is most volatile, least viscous or has the lowest surface tension.

After selecting the calculation mode, the user must type in the desired analytical gradient. The inverse gradient is updated as the user types and can be viewed on a second tab (Figure 16). The inverse gradient incorporates an isocratic hold step equivalent to the difference in gradient delay volume between the analytical and inverse flow paths.

After the “Flow Gradients” step, the instrument method editor continues through settings for the autosampler, column oven, and other system modules, as usual.

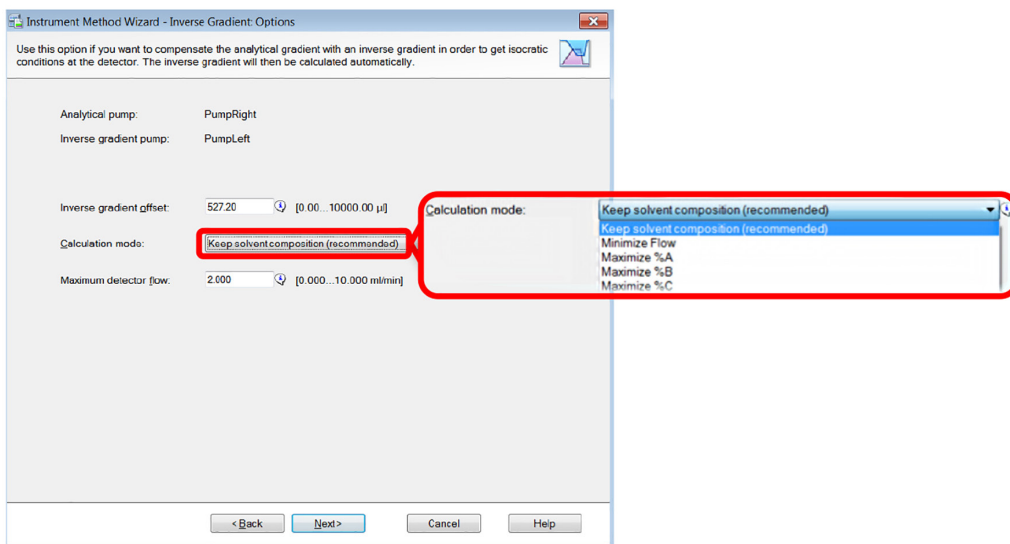


Figure 15. Drop-down box with modes to minimize flow or to maximize the level of a certain eluent

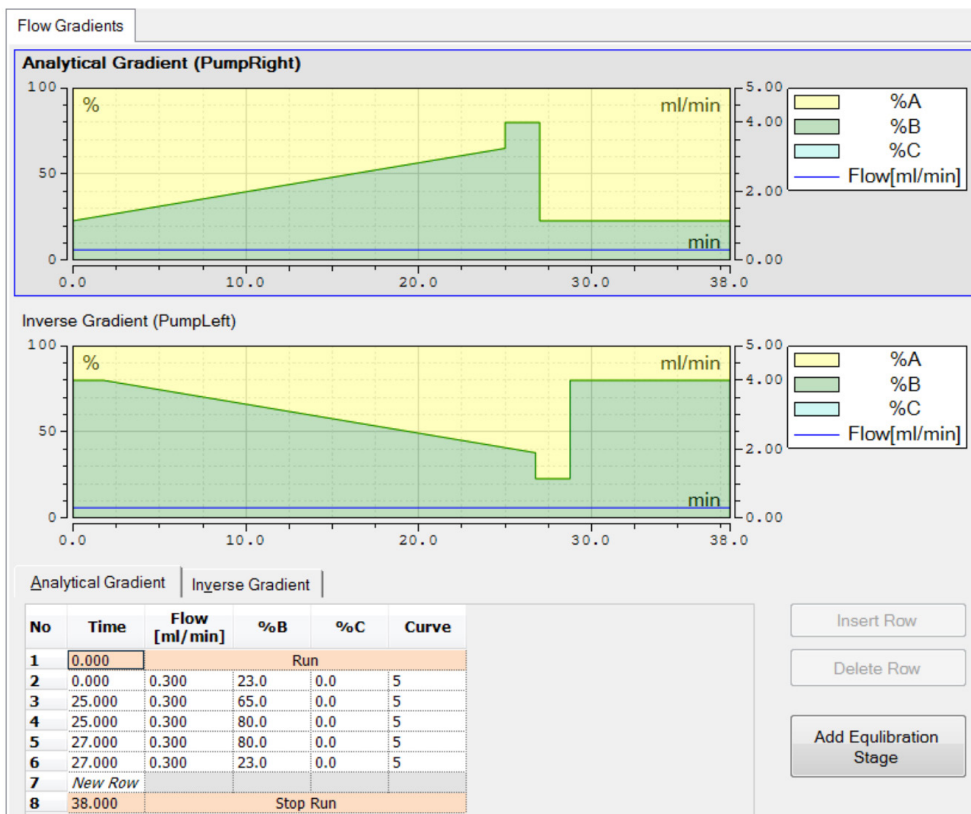


Figure 16. Editing the analytical gradient and automatic calculation and updating of the inverse gradient

Appendix D: Inverse gradient example, paclitaxel

This application was published as Thermo Fisher Scientific Application Note 72594.² Additional details, analyses, and conclusions are available in the full application note.

Instrumentation

Vanquish Flex Duo UHPLC system for Inverse Gradient consisting of:

- System Base Vanquish Flex (P/N VF-S01-A-02)
- Dual Pump F (P/N VF-P32-A-01)
- Split Sampler FT (P/N VF-A10-A-02) with a 25 μ L sample loop
- Column Compartment H (P/N VH-C10-A-02)
- Vanquish Flex CAD (P/N VF-D20-A)
- Inverse Gradient Kit for Vanquish Duo (P/N 6036.2010)

Materials

- Thermo Scientific™ Accucore™ Pentafluorophenyl (PFP) column, 150 \times 2.1 mm, 2.6 μ m, L43 (P/N 17426-152130)
- Fisher Scientific LC/MS grade Acetonitrile (P/N A955-212)
- Barnstead GenPure xCAD Plus Ultrapure Water Purification System (P/N 50136171)
- Thermo Scientific™ Digital Heating Shaking Drybath (P/N 88880028)

Sample preparation for the stressed paclitaxel (active pharmaceutical ingredient)

For the thermal degradation study, a 100 μ L volume of a 1 mg/mL paclitaxel solution (purchased from European Pharmacopeia, Strasbourg, France) was diluted with 350 μ L methanol and 50 μ L dimethyl sulfoxide (DMSO) in a 1.5 mL microcentrifuge tube. The solution was heated at 65 °C for 2 hours using a digital heating shaking drybath. The degraded sample was then analyzed immediately without further sample preparation.

Experimental conditions

Column:	Accucore PFP 2.1 x 150 mm, 2.6 μ m
Mobile Phase:	A: Water, Ultra-pure (18.2 M Ω -cm at 25 °C) B: Acetonitrile
Flow Rate:	0.3 mL/min analytical gradient, 0.3 mL/min inverse gradient
Analytical Gradient:	23–60% B in 25 min
Inverse Gradient:	23–60% A in 25 min
Column Temperature:	35 °C forced air mode, 35 °C active pre-heater
Injection Volume:	1 μ L
UV Detector Settings:	227 nm, 5 Hz data collection rate, 1 s response time
CAD Settings:	50 °C evaporation temperature, 5 Hz data collection rate, 3.6 s filter, 1.0 PFV
Data Processing:	Chromeleon CDS 7.2.8

Appendix E: Inverse gradient and salt formation example, tenofovir

Instrumentation

Vanquish Flex UHPLC system for Inverse Gradient consisting of:

- System Base Vanquish Flex (P/N VF-S01-A-02)
- Dual Pump F (P/N VF-P32-A-01)
- Split Sampler FT (P/N VF-A10-A-02)
- Column Compartment H (P/N VH-C10-A-02)
- Corona Veo / Vanquish Flex CAD (P/N VF-D20-A)

Chromatographic conditions for the impurity analysis of tenofovir disoproxil fumarate

Column: Thermo Scientific™ Accucore™ aQ, 2.6 μm, 2.1 x 100 mm (P/N 17326-102130)

Mobile Phase: A – Water with 0.1% Acetic Acid
B – Methanol
C – Acetonitrile

Gradient: 0–4 min: 0–70% B, 0–15% C
4–4.5 min: 70% B, 15% C
4.5–5 min 70–25% B, 15–70% C
5–6 min: 25% B, 70% C
6–6.1 min: 25–0% B, 70–0% C
6.1–15 min: 0% B, 0% C

Flow Rate: 0.6 mL/min

Temperature: 40 °C still-air mode, active pre-heater at 40 °C

Injection Volume: 1 μL. 5 μL for flow injection

CAD Settings: Vanquish Flex CAD (Equivalent to Corona Veo)
Evaporator temp.: 35 °C
Filter: 3.6
Data collection rate: 20 Hz
Power function value: 1.00

Data processing: Chromeleon CDS 7.2

Eluent preparation

Mass spectrometric grade methanol and acetonitrile were used as eluent B and C. Solvents were refreshed weekly to reduce background noise. The pH of eluent A, 1000 mL water with 1 mL acetic acid, was 3.5.

Sample preparation

USP standards were used for tenofovir, emtricitabine, and tenofovir disoproxil fumarate. Samples of 1 mg/mL were prepared in water. Adenine was prepared at 0.1 mg/mL in 0.1% acetic acid for solubility reasons. Tenofovir disoproxil calibration standards were prepared at 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 7.5, and 5 μg/mL in water. All other analytes were prepared at concentrations of 50, 40, 30, 20, 10, 7.5, and 5 μg/mL in water. Samples were measured in quintuplet.

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