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Mechanistic Investigation of Ionization Suppression in Electrospray Ionization

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We show results from experiments designed to determine the relative importance of gas phase processes and solution phase processes into ionization suppression observed in biological sample extracts. The data indicate that gas phase reactions leading to the loss of net charge on the analyte is not likely to be the most important process involved in ionization suppression. The results point to changes in the droplet solution properties caused by the presence of nonvolatile solutes as the main cause of ionization suppression in electrospray ionization of biological extracts. (J Am Soc Mass Spectrom 2000, 11, 942–950) © 2000 American Society for Mass Spectrometry

One of the most common analyses in the pharmaceutical industry is the analysis of biological fluids for a target analyte and related species. The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has now become the standard technique throughout the pharmaceutical industry for quantitative analysis of drug compounds and related materials. The general applicability, with the inherent selectivity and sensitivity, has made LC-MS/MS the most important modern quantitative analytical technique in the industry. Routine analysis of samples from studies aimed at determining safety, efficacy, and pharmacokinetic and pharmacodynamic properties of new drug entities is now accomplished primarily by the use of atmospheric pressure ionization (API) LC-MS/MS sample analysis.

Even with the wide-spread use and remarkable success of API LC-MS/MS in quantitative analysis, there are still problems with the technique that can invalidate quantitative results [1, 2].

A number of researchers have shown that the response observed in electrospray can be affected by factors other than analyte concentration (Table 1) [3–12]. Most of these factors can be controlled to be reproducible from sample to sample, in which case they may affect overall sensitivity, but not the quantitative abilities of the technique. The solution pH, electrolyte concentration, and solution properties of the electrospray droplets will depend on the exact composition of the droplet which may vary from sample to sample. One of the often-observed consequences of unexpected changes in these factors is the variability in target compound response typically referred to as ionization suppression.

Sample molecules start out in the solution phase in the LC mobile phase. The liquid is formed into charged drops by the electrospray source. While in the solution phase in the drops, the analyte has several possible pathways. As the solvent evaporates, the analyte may precipitate from solution either as solid compound or as a coprecipitate with other nonvolatile sample components. Alternatively, the analyte may remain in the unevaporated portion of the liquid stream and consequently collect on the interface plate of the mass spectrometer. Analyte might also be transferred to the gas phase as an ion, a neutral gas phase molecule, or as part of a charged solvent cluster. Once in the gas phase as an ion or charged solvent cluster, the charge might be lost through neutralization reactions, charge stripping, or charge transfer to another gas phase species. Only in the case of an isolated ion or charged solvent cluster reaching the declustering region of the API source is there any possibility of observing the analyte ion in the mass spectrometer. Any mechanism that might decrease the production rate of small droplets, and ultimately gas phase analyte ions, could participate in ionization suppression [13–18].

The objective of the experiments described below was to determine the relative importance of gas phase and solution phase processes to the ionization suppression observed in typical study sample extracts. The information obtained about the causes of ionization suppression may lead to practical ways to eliminate the ionization suppression problems observed during analysis of study sample extracts.

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Table 1. Factors affecting electrospray ion formation

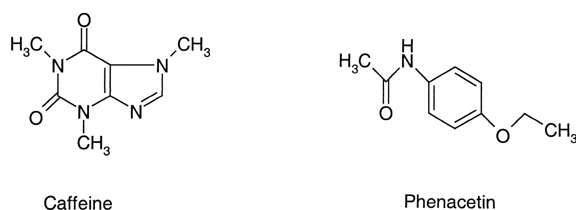
System variables	Compound variables	Method variables
Electric field	Surface activity	Flow rate
ES–capillary diameter	Proton affinity	Electrolyte concentration
ES–capillary voltage	pKa	pH
Distance to counter electrode	Solvation energy	Solvent properties (boiling point, surface tension, etc.)
Heat capacity of ambient gas		
Solvent saturation level of ambient gas		

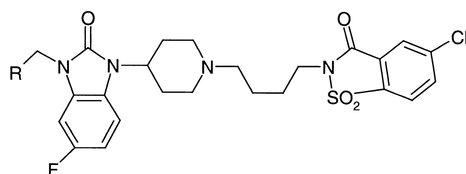
Experimental

The experiments described below were carried out with a variety of drug molecules. Data for experiments conducted with Urapidil, Phenacetin, and two Merck structural analogs are presented (Figure 1). Each experiment was repeated a minimum of three times to provide an assessment of the reproducibility of the reported results. Representative data are shown.

Materials

Phenacetin, caffeine, and urapidil were purchased from Sigma (St. Louis, MO) and used without further purification. The Merck compound and structural analog were synthesized by Merck (West Point, PA). All solvents used were high-performance LC (HPLC) grade or better.





Merck Compound A, R= CH_2F
Merck Compound B, R= CF_3

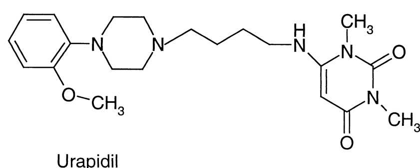


Figure 1. Structures of the analytes used to generate the reported results.

Extractions

Three common sample preparation techniques were used to generate the blank extracts: methyl *t*-butyl ether (MTBE) liquid–liquid extraction, protein precipitation, and solid-phase extraction. The liquid–liquid extractions were performed by adding 3 mL of MTBE to 0.5 mL of control dog plasma buffered to pH 7.4 with 0.1 M K_2HPO_4 in 13 × 100 mm glass test tubes with caps. After agitating in a shaker for 10 min, these samples were spun for 10 min at 3000 rpm in a Beckman (San Ramon, CA) GS-6 centrifuge. The top liquid layer was transferred into clean 12 × 75-mm glass test tubes. The tubes were then evaporated to dryness at 50 °C under dry nitrogen in a Turbo Vap (Zymark, Hopkinton, MA).

Solid-phase extractions were performed using Oasis HLB (30 mg) cartridges from Waters (Milford, MA) and Empore C8 disks from 3M (St. Paul, MN). Oasis cartridges were preconditioned with 1.0 mL of 100% methanol followed by 1.0 mL of distilled water. Blank plasma samples (0.5 mL) were then transferred from 10 × 75-mm glass test tubes to the cartridges and loaded on the cartridges at a flow rate of approximately 2 mL/min. The cartridges were then rinsed with 0.250 mL of distilled water and 1.0 mL of 5% methanol. The samples were eluted with 1 mL of 100% methanol. All samples extracted with SPE cartridges were evaporated to dryness in a Turbo Vap at 50 °C under dry nitrogen.

Protein precipitation samples were prepared by adding 1 mL of acetonitrile containing 0.1% TFA to 0.5 mL of control dog plasma in 2-mL plastic microcentrifuge vials. The samples were mixed on a vortex mixer for 1 min and centrifuged for 20 min at 11,000 rpm. The supernatant liquid was decanted into 12 × 75-mm glass test tubes and evaporated to dryness under nitrogen in a Turbo Vap at 50 °C. Dried samples from all three preparations methods were each reconstituted in 150 μL of 50/50 acetonitrile/water containing 0.1% formic acid, sonicated in an ultrasonic bath for 10 min, mixed on a vortex mixer for 1 min, and transferred to microinjection vials.

Infusion Chromatograms

Postcolumn infusions. The postcolumn infusion system is schematically represented in Figure 2. The chromatographic portion system consisted of a Hewlett-Packard model 1050 HPLC and autosampler (Palo Alto, CA).

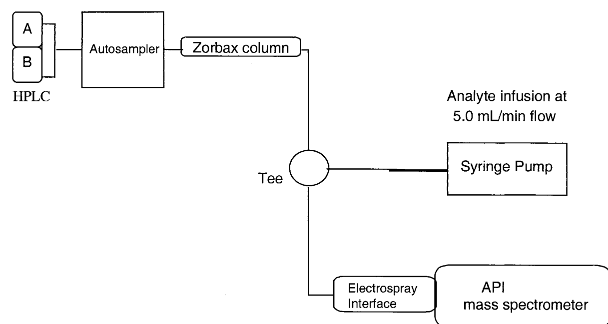


Figure 2. Schematic diagram of the postcolumn infusion system used to generate infusion chromatograms of sample extracts.

Chromatographic separation for the infusion experiments with both APCI and electrospray ionization (ESI) was performed using a Zorbax SB-C18 (2.1 × 50 mm, 5 μm) column supplied by MacMod (Chadds Ford, PA) at a flow of 250 μL/min for ESI and 500 μL/min for APCI. Isocratic chromatographic conditions were used with a mobile phase composed of 50% solvent A (90/10 acetonitrile/water containing 0.1% formic acid) and 50% solvent B (10/90 methanol/water containing 0.1% formic acid). Blank plasma samples (10–20 μL) were injected onto the Zorbax column. 5×10^{-6} M analyte dissolved in 50% acetonitrile; 50% water with 0.1% formic acid was infused, postcolumn, through a zero dead volume tee using a Harvard Apparatus Model 2400 (South Natick, MA) syringe pump at a rate calculated to deliver approximately equal concentrations of analyte to each interface. Effluent from the HPLC column combined with the infused analytes and entered a PE Sciex API III+ (PE Sciex, Concord, Ontario, Canada) mass spectrometer through an ESI (Turbo Ionspray from PE Sciex) LC/MS interface or an APCI (Heated Nebulizer from PE Sciex) LC/MS interface.

Mass spectrometer settings were optimized using 10 μL injections of 1×10^{-6} M test compounds. Argon collision gas used for fragmentation in SRM mode was set at 250×10^{13} atoms cm^2. The ion spray voltage was +4300 V.

Combined ESI/APCI Source Experiments

For the combined source experiments, the IonSpray source of the Sciex API3+ was modified by the addition of a discharge needle. A discharge needle from the APCI source was attached by a metal conductor to the Valco tee of the ESI sprayer. The needle current and the electrospray current of the combined ESI/APCI source were supplied by the same HV power supply used with the original API3+ sources. The discharge needle on the modified source was located just above the spray approximately halfway between the ESI sprayer and the interface plate. This is similar to the needle positioning for the APCI source. The spray is positioned to spray across the mass spectrometer inlet and it contacts the interface plate on the side opposite the sprayer (Figure 3). No change in mass spectrometer operating conditions was required to operate the combined source. All adjustable mass spectrometer parameters were unchanged from the ESI settings.

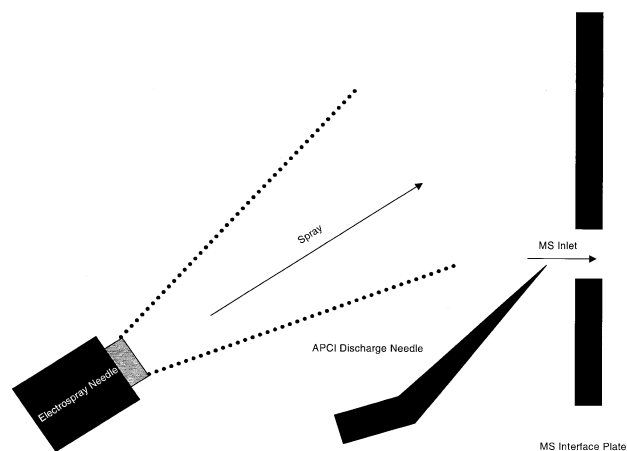


Figure 3. Schematic diagram of the ESI–APCI combined source showing the positioning of the ESI sprayer, the APCI discharge needle, and the mass spectrometer inlet.

Dual ESI Sprayer Experiments

For the dual sprayer experiments, the IonSpray ion source from the API3+ was modified by replacing the single needle sprayer valco tee with a custom built four needle sprayer head that accommodates the same tubing arrangement and needle size as the original API3+ IonSpray. The sprayers themselves were constructed using PE Sciex replacement parts for the IonSpray source. This assures that the spray characteristics are as similar as possible between the dual sprayer and the single sprayer arrangements. Nebulizing gas is supplied to all four sprayers from one source. The entire spray head is fed pressurized nitrogen from the gas source normally used for the single sprayer. The pressure and flow were adjusted to create a stable spray. Voltage was also supplied to the common spray head, rather than to individual sprayers. The common point for gas and current ensures that the settings for each of the spray needles are identical.

Infusion experiments were conducted with only two of the four sprayers operating. The experiments were performed using the conditions described above for infusion experiments.

Spray Collection Experiments

Comparison of plasma extract and mobile phase. Protein precipitation with acetonitrile as described above was used to generate the extracts sprayed. Analyte was spiked into either the reconstituted precipitation samples or an equal volume of mobile phase at a final concentration of 500 ng/mL. These samples were infused into the ESI interface and the analyte response

was monitored in selection reaction monitoring (SRM) mode.

Collection of material remaining in the solution was accomplished by taping a piece of aluminum foil (approximately 1 in. × 2 in.) to the interface plate of the API3+ where the spray hits the plate. A 10 min infusion at 10 μL/min was used. Upon completion of the infusion, the foil was carefully removed, cut into strips, and placed in a 20 mL scintillation vial. Three infusions were collected on foil for the 500 ng/mL solutions prepared in extract and in mobile phase. The foil from each test was treated separately. To each scintillation vial, 1 mL methanol was added and the vial was vortex mixed for 1 min. 1 mL of acetonitrile was then added and the sample was again vortex mixed for 1 min. This solution was transferred to a 12 × 75 mm glass tube and evaporated to dryness at 50 °C under nitrogen (Turbo Vap). The residue was reconstituted with 150 μL of 20% acetonitrile + 0.1% formic acid. A 20 μL aliquot of each sample was injected onto the analytical column. Drug response was measured in SRM by integrated peak area.

Comparison of acetate and sulfate. The same sample preparation procedure used above for the protein precipitation samples was used to prepare samples for the comparison of acetate and sulfate containing samples. The test analyte solution was prepared by diluting an aqueous stock 1 mg/mL analyte standard to a final concentration of 2 μg/mL in either (50% 10 mM ammonium acetate/50% acetonitrile) or (50% 10 mM ammonium sulfate/50% acetonitrile).

LC-MS/MS analysis. The LC-MS system described above for postcolumn infusions was used for analysis of the collection samples. Chromatographic separation was performed using a Discovery C18 (2.1 × 50 mm, 5 μm) column from Supelco (Belefont, PA) at a flow of 1.5 mL/min. Gradient elution conditions were used with a mobile phase composition starting at 0% acetonitrile containing 0.1% formic acid:100% (water, 0.1% formic acid) for 1.0 min, followed by a 1.5 min linear gradient to 95% (acetonitrile, 0.1% formic acid):5% (water, 0.1% formic acid). This mobile phase composition was held for 0.5 min and then dropped to the initial conditions in a single step. The column was reequilibrated for 1.5 min at the initial conditions before the start of the next injection sequence. The flow to the ESI interface was split to deliver 100 μL/min to the interface and 1.4 mL/min to waste. Analyte SRM transitions were monitored. Peak areas were determined by integration using the Sciex quantitation software, MacQuan 1.6 (PE Sciex, Concord, Ontario, Canada).

Results and Discussion

Gas Phase Processes

ESI–APCI comparison. In both electrospray ionization and atmospheric pressure chemical ionization, analyte in a liquid stream must be converted into gas phase ions that can be sampled by the mass spectrometer. There are important differences in the way each technique produces the charged analyte. In electrospray, analyte is ionized in the liquid phase inside the electrically charged droplets. The analyte ions in solution are then liberated from the liquid phase into the gas phase [5]. In APCI, the neutral analyte is transferred into the gas phase by vaporizing the liquid in a heated gas stream. Ionization occurs in a separate step by chemical ionization of the gas phase analyte [18]. Comparing the results of ionization suppression experiments performed using ESI and APCI provides a means of studying the relative importance of gas phase and solution phase processes in ionization suppression.

One possible explanation for ionization suppression in electrospray is that gas phase reactions involving analyte ions and other sample components result in the loss of charge from the analyte ion [8]. If processes involving the reaction of gas phase analyte and other gas phase species are the major causes of ionization suppression, then the suppression effects should be similar with APCI and ESI.

The data in Figure 4 show the results from an infusion experiment using urapidil as the analyte and injection of a plasma sample prepared by acetonitrile protein precipitation. The suppression is much more severe with ESI than APCI. Because gas phase species capable of reacting with gas phase analyte ions should be present in both APCI and ESI at similar levels, the intense suppression observed with ESI indicates that gas phase processes are not likely to be dominant in ionization suppression for these samples.

Dual ESI sprayer. Although the results from the previous experiment seem to indicate that gas phase processes are not the controlling factors in ionization suppression in ESI, we cannot rule out the possibility that the addition of the heat in APCI changes the system energetics enough to make the comparison invalid. Therefore, we constructed a dual-spray electrospray source for the Sciex API3+. If gas phase processes, disrupted by the heated gas in the APCI source, are responsible for ionization suppression, then the dual ESI source should show this interaction. The new sprayer is mounted in place of the original spray needle on the IonSpray source of the API3+. The arrangement of the sprays is such that the spray plumes do not intersect at any point. One sprayer is aimed at the interface plate on one side of the orifice and the second on the opposite side (Figure 5). The only possible interactions between the sprays should be in the gas phase as the ions are extracted into the high vacuum region of the mass spectrometer. By infusing analyte into one sprayer and material known to cause ionization suppression into the other, any important interaction occurring in the gas phase should be observed.

Figure 6 shows data collected from each of the sprayers. Both sides were shown to ionize analyte and

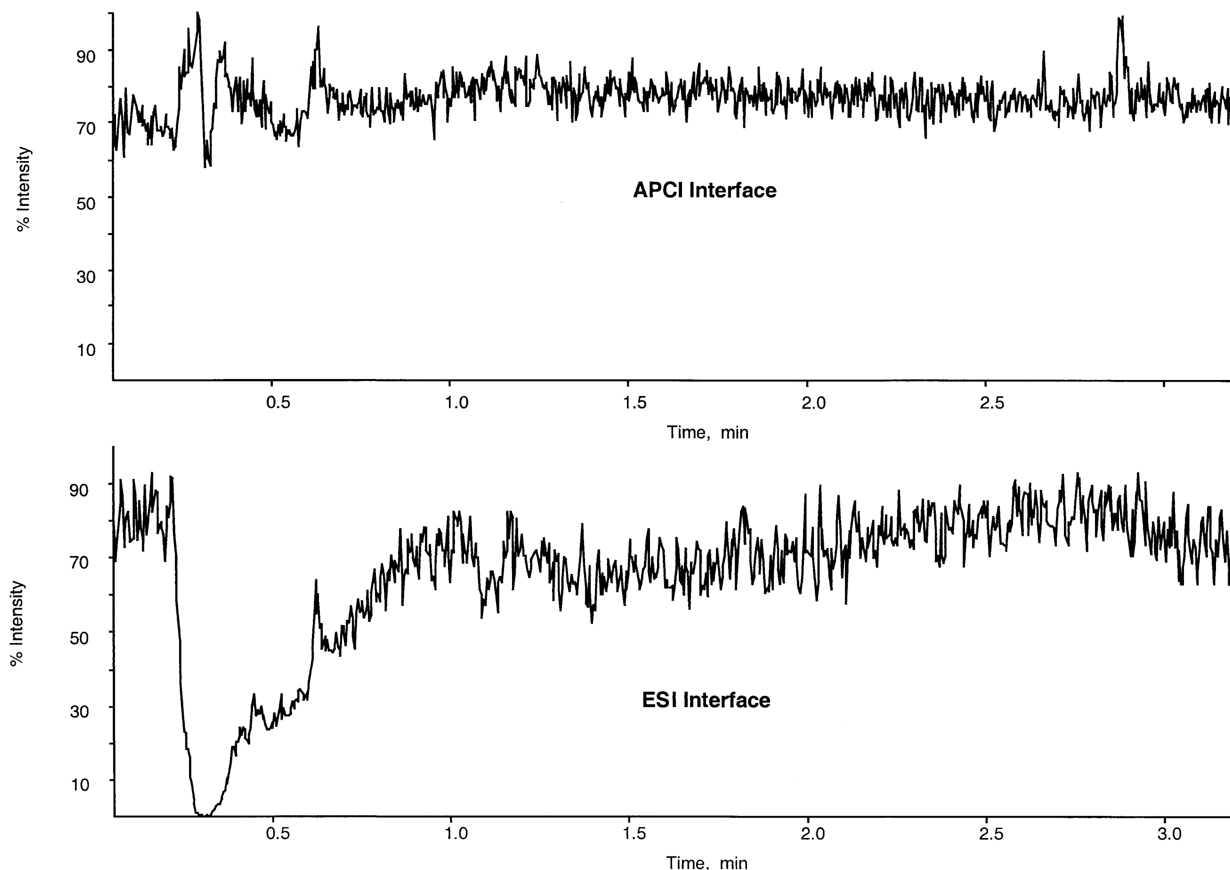


Figure 4. Comparison of infusion chromatograms generated using APCI and electrospray ionization. The chromatograms show the effect of study sample components eluting from the analytical column on the response of a postcolumn infusion of 10 μM urapidil. Direct comparison of the loss of response observed with APCI (top panel) and electrospray (bottom panel) shows the loss to be much greater with electrospray for the same injected protein precipitation sample.

to be subject to ionization suppression by performing infusion experiments on each sprayer independently. Figure 6 shows infusion chromatograms from the sprayers running simultaneously with two different structural analogs being infused through each sprayer. An aliquot of a plasma extract was injected through sprayer one with the postcolumn infusion of analyte A, whereas only the infused analyte B was introduced through sprayer two. The figure shows no effect on the response of sprayer two. The reverse experiment, injection of plasma extract through sprayer two, provides the same results for sprayer one. Because no interaction is observed between the two sprayers, it is not likely that gas phase reactions are important to the suppression of ionization observed with study sample extracts.

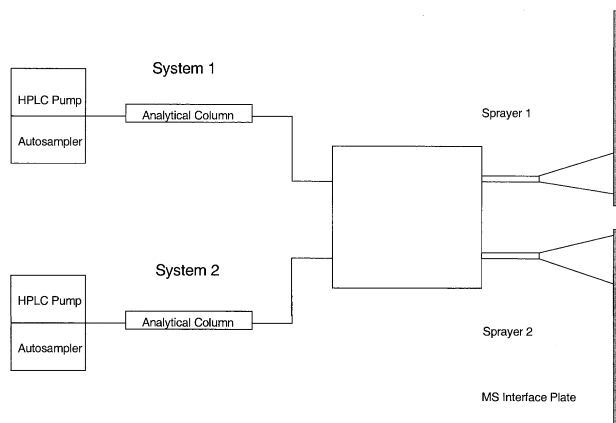


Figure 5. Schematic diagram of the two-sprayer experimental layout showing the relative position of the two independent sprays and the inlet to the mass spectrometer.

Combined APCI/ESI source. Another possible explanation for the difference in ionization suppression observed between APCI and ESI is that although analyte is being transferred to the gas phase by electrospray under suppression conditions, the species transferred into the gas phase is not charged. The formation of a neutral analyte species rather than a charged species could be due to depletion of available charge from the drop as interfering sample components are ionized [19].

To test this hypothesis, a combined ESI–APCI source was designed and built. An APCI discharge needle was

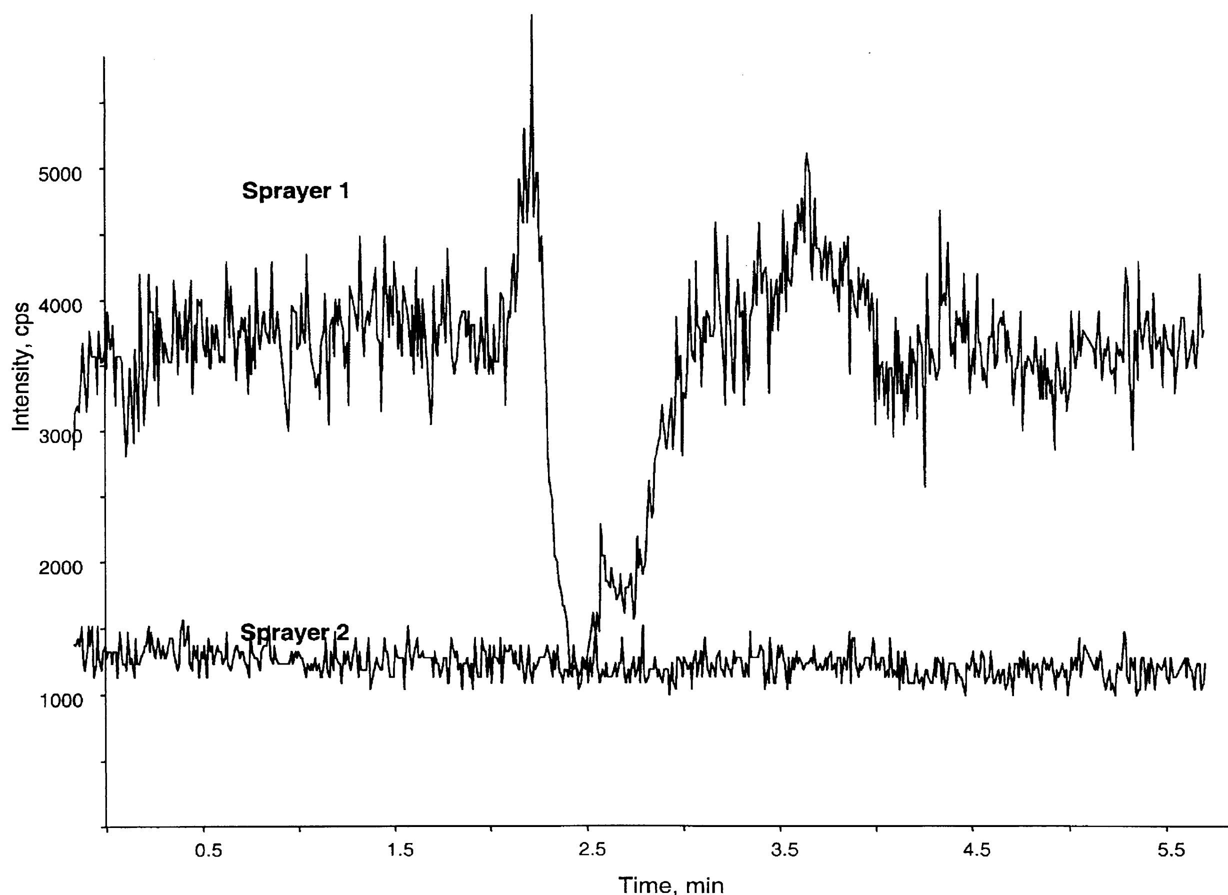


Figure 6. Infusion chromatograms acquired with the two sprayers running simultaneously. The traces are SRM chromatograms for two structural analogs infused independently, one into each sprayer. The negative peak in the sprayer 1 trace is the result of ionization suppression from a plasma protein precipitation sample injected through sprayer 1 only. No effect is observed for the response of the analog being introduced through sprayer 2.

added to the ESI source to provide charge in excess of what is available from the electrospray (Figure 3). If the analyte is being evaporated as a neutral species, then the APCI should ionize the neutral analyte and reduce the degree of ionization suppression observed with ESI alone. Figure 7 shows the results of the infusion experiment with Merck compound A and a plasma protein precipitation sample. Virtually no change in the suppression is observed with the discharge needle in place indicating that the release of gas phase neutral analyte from the solution under suppression conditions is not likely to be the cause of ionization suppression.

The results of the three experiments designed to test the importance of gas phase mechanisms, the APCI–ESI comparison, the ESI–APCI combined source, and the dual ESI source indicate that the controlling factors are not likely to be related to gas phase processes. Consequently, solution phase and solid-phase processes are left as possible controlling factors in ionization suppression of plasma extracts.

Solution and Solid-Phase Processes

Interface plate collection experiments. In the case of both solution phase and solid-phase ionization suppression processes, the analyte is not transferred from the solution into the gas phase. To determine if the analyte is remaining in the solution phase and not being transferred to the gas phase, a series of experiments was undertaken to measure the amount of analyte accumulating where the spray contacts the interface plate. Data were collected under conditions of no ionization suppression and severe ionization suppression. Analyte in mobile phase and analyte in plasma extract were each infused at a rate of 5 μL/min through the Sciex API3+ IonSpray interface. A piece of aluminum foil was taped to the interface plate where the spray contacted the plate. 100 μL of each sample was passed through the sprayer while SRM data was acquired for the analyte. The material collected on the aluminum foil was redissolved in the mobile phase and analyzed on an analyt-

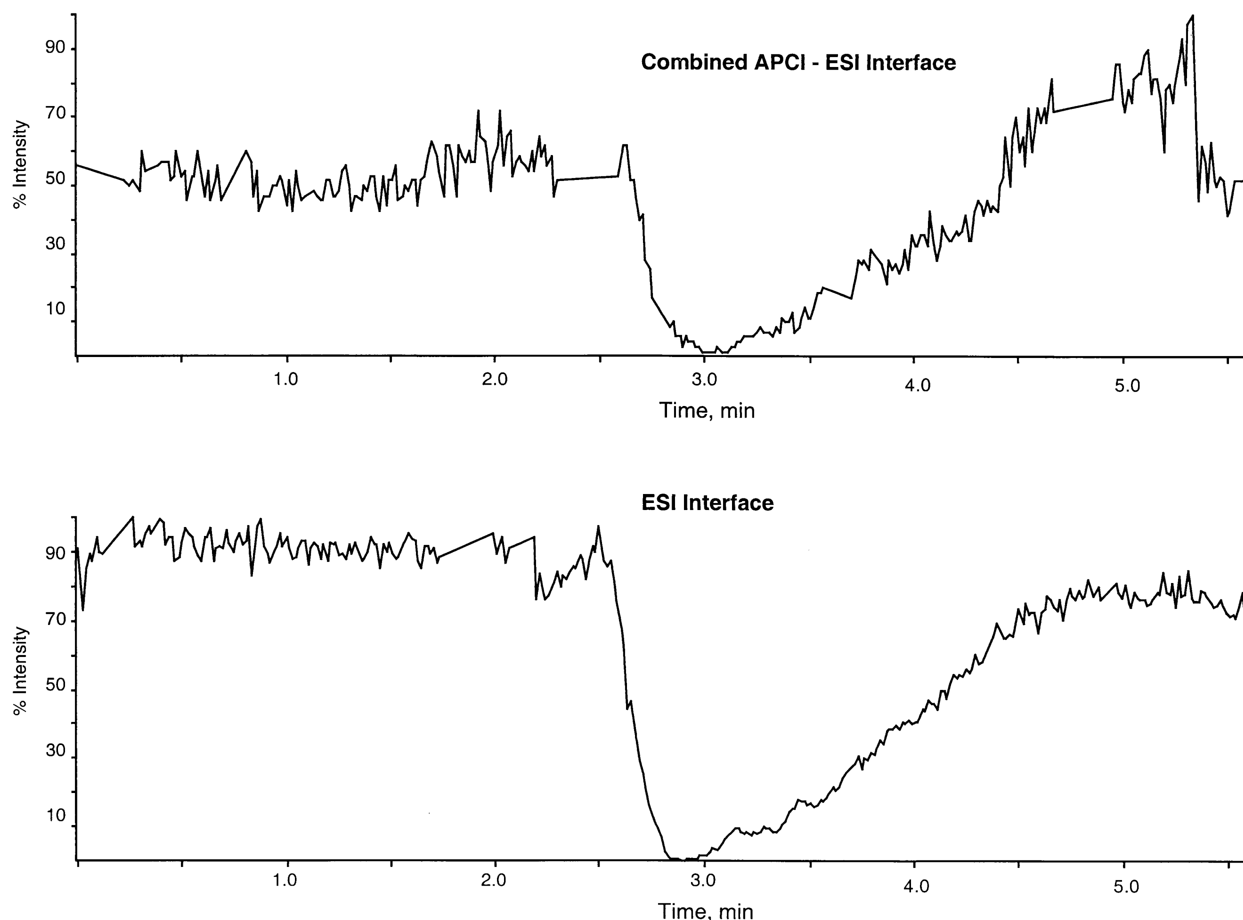


Figure 7. Comparison of infusion chromatograms from the ESI–APCI combined source and the ESI source. No change in the degree of ionization suppression is observed for the ESI–APCI combined source when compared to the ESI source.

ical HPLC column with a gradient capable of separating the interfering extract components from the analyte. The integrated peak areas for the analyte collected from the aluminum foil for each experiment were compared to assess the relative amount of analyte remaining in solution. If the ionization suppression is caused by the analyte not being transferred from the solution phase into the gas phase, then there should be more analyte detected in the redissolved material from the infusion of analyte in plasma extract than the infusion of analyte in mobile phase. The results are shown in Table 2. The larger peak areas measured for the analyte in the plasma infusion collected from the interface plate shows that the analyte is staying in solution and not reaching the gas phase.

Table 2. Peak areas of phenacetin and caffeine measured from interface plate collection of infused extract and mobile phase

Trial	Mobile phase infusion	Extract infusion
Phenacetin		
1	26,222	112,837
2	34,412	103,276
3	20,612	94,854
Caffeine		
1	1028	4654
2	1126	4267
3	589	3814

Precipitation in ESI and APCI. Under conditions that yield severe ESI ionization suppression, a small reduction in APCI response is typically observed. Because there is very little chance for analyte to pass through the APCI vaporization region and remain in solution, it seems likely that the mechanism for suppression in APCI is solid formation. The precipitate may form as the concentration of analyte and other nonvolatile sample components increases with solvent evaporation, as either the pure analyte or as a solid coprecipitate with other nonvolatile sample components.

Solid formation may also affect the ESI response but, based on the minor response loss observed with APCI, it is not likely to account for the difference observed between APCI and ESI. The difference is more likely to be a result of a change in colligative solution properties perhaps caused by the presence of nonvolatile materials in the extracts. The same materials that will not evapo-

Table 3. Peak areas of phenacetin and caffeine measured from interface plate collection of infused acetate and sulfate solutions

Trial	Sulfate infusion	Acetate infusion
Phenacetin		
1	53,959	20,231
2	56,784	23,571
3	1,155,098	35,356
Caffeine		
1	3137	930
2	2792	1010
3	3365	1619

rate and lead to solid formation in APCI may cause a change in colligative solution properties in electrospray that inhibits the formation of offspring droplets and ultimately, analyte ions. The effect on the colligative solution properties before the electrosprayed solution reaches saturation, would make ESI more sensitive to the presence of nonvolatile components and ionization suppression than APCI.

Nonvolatile solute. The hypothesis that ionization suppression is caused by the presence of nonvolatile solute in solution with the analyte was tested using ammonium acetate, a common LC-MS mobile phase additive that does not cause significant ionization suppression, as a model volatile solute and ammonium sulfate, a salt that does cause significant ionization suppression, and as a model nonvolatile solute. The amount of analyte collected on the interface plate when ammonium sulfate and ammonium acetate solutions containing analyte were electrosprayed was measured. If the presence of the nonvolatile solute in solution with the analyte is preventing efficient ion formation by ESI, then the ammonium sulfate collection should contain more analyte than the same experiment done with ammonium acetate. The integrated peak areas for both model systems shown in Table 3 support the idea that the nonvolatile solute might be responsible for ionization suppression in electrospray.

Previous results comparing the degree of ionization suppression observed with different extraction procedures show that the cleanest, most selective extractions, produce samples with the least ionization suppression [20]. The more general extraction procedures produce samples with the most severe ionization suppression. This observation can be correlated with the amount of nonvolatile material produced by each extraction method. The amount of nonvolatile material left by each extraction method measured as the weight of dry residue remaining after solvent evaporation was measured for three common sample preparation methods, MTBE liquid–liquid extraction, Oasis solid-phase extraction, and acetonitrile protein precipitation. The average of three measurements shows the samples prepared by the protein precipitation method to have the most nonvolatile material, 3.35 mg, whereas the samples prepared by solid-phase and liquid–liquid extraction have significantly less (0.3 and 0.2 mg, respectively). The same trend is observed for the severity of ionization suppression in study samples. The protein precipitation was shown to have the most severe ionization suppression and the liquid–liquid and solid-phase procedures to have the least [20]. These observations support the hypothesis that nonvolatile sample components are mainly responsible for ionization suppression in study sample extracts.

The effect of high concentrations of nonvolatile material on the ESI response has been documented [5]. The underlying physical chemical reason for the suppression of ionization however has not been investigated thoroughly. Several possibilities exist. The nonvolatile material may precipitate as the solvent is evaporated and bring analyte into the precipitate as an impurity. Data from APCI–ESI comparisons indicates that, although precipitation may occur in both systems and cause some ionization suppression, it is not the dominant cause of ionization suppression in electrospray. Another possible effect of high concentrations of nonvolatile solutes is elevation of the solution boiling point. An increase in the solution boiling point might cause less efficient solvent evaporation and decreased offspring droplet formation rates. The surface tension of the liquid might also be increased as a result of concentrating nonvolatile solute, thus, leading to less efficient offspring droplet formation and eventually reduced analyte ion production. A combination of these processes may be occurring under normal plasma sample analysis and cause the loss of ESI response associated with ionization suppression.

Conclusions

Ionization suppression typically observed in sample extracts from biological samples is not likely to be caused by reactions occurring in the gas phase. It is most likely that ionization suppression is the result of high concentrations of nonvolatile materials present in the spray with the analyte. The nonvolatile solute causing ionization suppression can be any chemical structure. Salts such as sulfates and phosphates, are well known to cause ionization suppression. However, our results suggest that the effect is more generally applicable to any nonvolatile solute, including analyte. The exact mechanism by which the nonvolatile materials inhibit release of analyte into the gas phase has not been clearly demonstrated, although a likely list of effects relating to the attractive force holding the drop together and keeping smaller droplets from forming should account for a large portion of the ionization suppression observed with ESI. The results from APCI experiments indicate that analyte precipitation may also be responsible for a small portion of electrospray ionization suppression.

The results indicate that nonvolatile materials must be removed from the sample in order to avoid the ionization suppression typically observed with plasma

extracts. Once these interferences have been removed, however, there is no guarantee that suppression of ionization will no longer be a problem. Other mechanisms with reduced effects may still change the analyte response. Ion pairing agents that behave like trifluoroacetic acid, for example, may play a role in ionization suppression. The effect of ion pairing agents was not directly tested by any of the experiments described above. The importance of surface activities of the analyte and interfering compounds may also play an important role in ionization suppression. More work is needed in these areas to obtain a thorough understanding of the causes of ionization suppression.

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