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The effect of analyte acidity on signal suppression and the implications to peak purity determinations using atmospheric pressure ionization mass spectrometry

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Abstract

The effect of a co-eluting halogenated phenol, spiked at 1% of the main analyte level, has been examined for a series of halogenated phenols using LC–MS techniques. Similarly, the effect of co-eluting anilines has been investigated. The purpose of the work presented here was to evaluate the degree of signal suppression for structurally similar halogenated phenols and for similar anilines utilizing atmospheric pressure chemical ionization (APCI) in the negative mode and electrospray (ESI) in positive mode, respectively. A correlation between the effects of analyte ionization efficiency resulting from co-eluting compounds (signal suppression) and pK_a has been made for these compounds. It was found that minimal signal suppression occurs when the spiked impurity has a similar (ΔpK_a < 1.5) acidity when compared to the main peak it is co-eluting with. The degree of signal suppression sharply increases when the difference in pK_a's between the main peak and the spiked impurity was greater than 1.5 units. Thus, when the main peak is much less acidic (more than 1.5 pK_a difference) than the co-eluting impurity, signal suppression of the latter would not occur in negative mode APCI. Similarly, when the main peak is much less basic than the co-eluting peak, signal suppression of the impurity will also not be found for aniline compounds in positive mode ESI. Furthermore, the degree of signal suppression decreases as a function of sample load such that injections of 3 μg or less show no discernible impact on the spiked impurity peak. Ultimately, these results indicate that the use of mass spectrometry (MS) in peak purity determinations requires numerous considerations prior to assessing main peak purity. The optimization of sample load during an impurities assay will maximize co-eluting impurity signal as purity determinations by mass spectrometry made at sample loads above the 3 μg (sample load) threshold increase the risk for false negative assessment of impurities.
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Keywords: Peak purity; Signal suppression; Co-elution; LC–MS; Impurities

1. Introduction

An important aspect of analytical method validation in the pharmaceutical industry includes ensuring that the method is stability indicating and that the method is selective for the analyte of interest. The common reporting threshold for impurities is defined by the International Conference on Harmonization (ICH) as 0.05% in the active pharmaceutical ingredient, and 0.1% in the final drug product. The ICH Harmonised Tripartite Guideline on the Validation of Analytical Procedures: Text and Methodology Q2 [1] goes on to define peak purity specificity "as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present and that peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry)" [1]. When separated from the main peak these threshold-reporting limits are routine; however, detecting an impurity at this level if it coelutes with the main peak is challenging. Therefore, the ability to confirm with high level of certainty the absence of a co-eluting impurity is highly sought after.

There are several different approaches utilized to address the issue of peak purity. John Dolan gives a systematic approach to confirm peak homogeneity [2]. In the case study presented there, the peak shape (i.e., tailing) gave the chromatographer an indication that the main peak may not be pure. The recommendation

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that followed included fraction collection in order to reduce the ratio of the main peak to the impurity, thus allowing for a better separation on a subsequent injection. Assessing peak purity is more complicated if an indicator such as fronting or tailing does not present itself. Assuming that reversed phase HPLC is being utilized for the impurity assay; this activity could utilize different column chemistry. There are several guidelines published on column selectivity that can be used in the selection process. Other approaches might include using an orthogonal separation technique, such as capillary electrophoresis, or normal phase chromatography. Regardless of the outcome of these orthogonal experiments, there would still be an impetus to demonstrate unequivocally that there are not multiple peaks under the parent peak.

Historically, there has been an emphasis on the use of diode array detection (DAD) for peak purity assessments; however, this type of measurement is not without its limitations. It is generally recognized that the impurity must either have a significantly different absorption profile or be sufficiently resolved from the parent peak in order to maximize the effectiveness in peak purity determination. There have been many improvements in the processing algorithms from ratioing to chemometric analysis [3,4]. However, the limits still appear to be somewhere in the 0.2–2% range under known conditions where the peaks do not completely coincide and there are spectral dissimilarities [5,6]. An evaluation of different techniques for peak purity assessment on a diode array detector had been reported by Fabre et al. [7]. There in the best case scenario, the investigators were able to detect 0.5% of a co-eluting impurity with a resolution of only 0.14. Using alternative data analysis techniques, the same impurity could not be detected using the same chromatographic conditions at levels equivalent to 10% of the main peak. A ranking of analysis techniques with their limitations was also presented.

Recently, on the recommendation of the ICH, there has been a surge in the use of mass spectrometry as a powerful tool for peak purity assessment [8–11]. Antonovich and Keller reported that for 24 drug impurity permutations, 75% of the peaks were detected at less than 1.0% level, with 33% of the peaks detected at the 0.1% level [10]. Mulholland et al. utilized LC–MS/MS in multiple reaction-monitoring mode for the detection of as little as 0.001% of prednisone in hydrocortisone [8]. Bryant et al. demonstrated detection of 0.02% of a co-eluting impurity [11]. As with DAD peak purity assessments, mass spectrometry is not a technique without limitations. When considering the limitations of mass spectrometry for peak purity assessment, one must first consider the parameters that influence the ionization mechanism. It has been demonstrated that ionization efficiency is a function of many parameters including solvation energy [12], polarity [13], pK_a of the analyte [14], and surface activity [15]. Cech and Enke have done several studies on the question of acidity as related to negative ion response in electrospray [15–18]. The work by Henriksen et al. concluded that pK_a is an important parameter in choosing positive or negative polarity but is not useful in predicting response factors, and that in some cases, the $\log P$ of a molecule may be more indicative of its ionization efficiency [13]. In a conflicting study by Banks et al. on the response factors for a simple set of nucleosides (uridine, guanosine, adenosine, cytidine), a near-linear relationship between pK_a and signal response was observed [14]. The above studies were conducted on pure analytes; the understanding of ionization efficiency in a binary system becomes even more complicated with regards to signal suppression. In addition, signal suppression effects have been suggested to be related to multiple factors that can ultimately affect the uncertainty in peak purity determination. Tang et al. did an investigation of signal suppression observed in multi-component systems at various molar concentrations using electrospray ionization in positive ion mode [19]. It was found that charge competition is prevalent in the ESI analysis for highly concentrated analyte mixtures. Furthermore, charge competition was concluded to be one of the main factors contributing to signal suppression but this effect becomes negligible when very dilute samples are used. The ratio used to calculate the electrospray ionization charge capacity for a solution can be used to determine the sample concentration limit to minimize charge competition effects [19].

The variability in the results reported above (0.001–10% detection of impurities) indicates that there could be a great deal of uncertainty when making the claim of peak homogeneity. In DAD analysis, the compounds must have different absorption characteristics and the co-eluting impurity must be partially resolved. These criteria are often not satisfied due to the fact that the co-eluting analytes are often structurally similar and have similar UV profiles. In peak purity assessment by mass spectrometry, enhanced specificity is achieved because the impurity will have a unique mass-to-charge ratio (unless the impurity is a structural isomer). However, unknown response factors caused by solvation energy, pK_a, surface activity and polarity of the analyte, solution chemistry, and signal suppression severely impact the chromatographer's ability to interpret the data. Thus, the use of either of the techniques discussed above requires some assumptions to be made.

This paper presents the impact of signal suppression on peak purity assessments on very simple systems of similar molecules in the positive pneumatically assisted ESI and negative APCI modes under the conditions usually used for LC–MS assay. Only the pK_a values of the molecules are correlated to the degree of signal suppression in order to illustrate the complexity in the measurement of peak homogeneity. Practical guidelines for maximum sample load and instrument parameters are presented for peak purity assessment regarding the levels typically injected for a peak versus total (% area) impurity assay.

2. Experimental

2.1. Reagents

All reagents, with the exception of 2,4,6-trichloroaniline, were obtained from Sigma–Aldrich (Oakville, ON). 2,4,6-Trichloroaniline was obtained from Acros (NJ, USA). Methanol and acetonitrile were HPLC grade (EMD, NJ, USA). Water was purified using the Gradient A10 system (Millipore, Cambridge, ON).

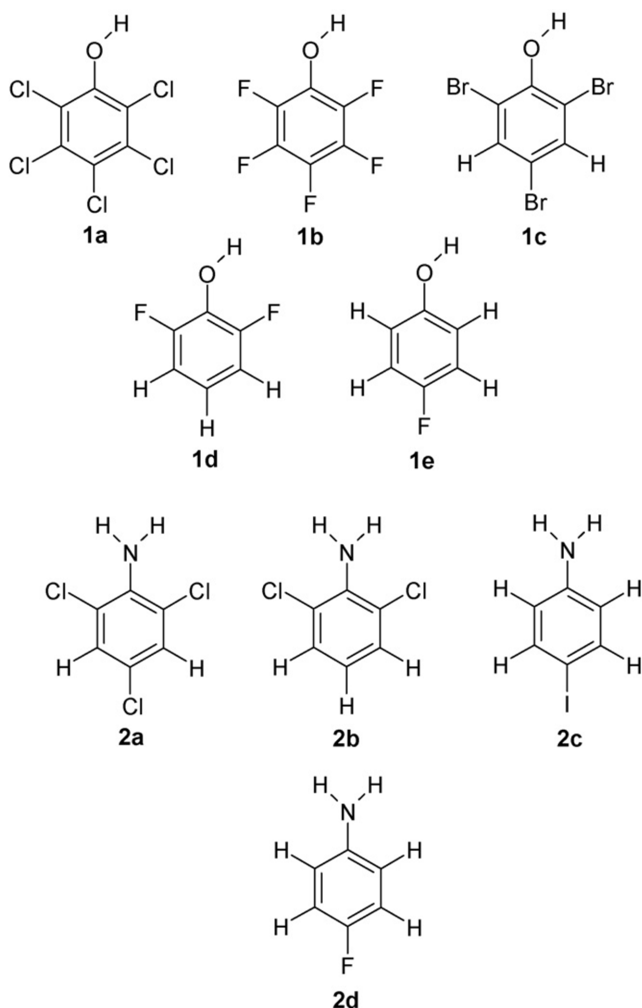

Fig. 1. Structures of halogenated phenols and halogenated anilines used as main peak components.

2.2. Sample preparation

To determine the response of halogenated phenols in the absence and presence of a highly concentrated main component peak, compounds **1a**–**1e** (Fig. 1) were dissolved individually in acetonitrile:water (50:50, v/v) to make 10 mg/mL solutions. Separately, a solution of each of the other halogenated phenols were dissolved in acetonitrile:water (50:50, v/v) to make 0.1 mg/mL solutions. A complete list of the halogenated phenols evaluated is presented in Table 1. The final mixtures were prepared by pipetting 1.0 mL of each of the 10 mg/mL solutions and 1.0 mL of each of the 0.1 mg/mL impurity solutions directly into separate 2 mL HPLC vials (final concentrations of 5 mg/mL and 0.05 mg/mL for main peak and impurity, respectively). The response controls were made with 1:1 dilutions of the 10 mg/mL main peak and 0.1 mg/mL impurity stock solutions in acetonitrile:water (50:50, v/v) in 2 mL HPLC vials. All samples were analyzed with negative mode APCI ionization using the Agilent 1100 single quadrupole and the Thermo Finnigan LCQ Deca ion trap mass spectrometer. To determine the response of halogenated anilines in the absence and presence of a highly concentrated main component peaks **2a**–**2d** (Fig. 1), these compounds were prepared similarly as described above and analyzed with positive mode ES ionization. A complete list of halogenated anilines evaluated is presented in Table 2.

For sample load analysis, the 10 mg/mL stock solutions of compounds **1a**–**1c** and the 0.1 mg/mL impurity stock solutions were diluted according to the dilution scheme illustrated in Table 3. In all cases, spiked impurity compounds were at 1% of the main peak compound concentration. As control, sample solutions of main peak compound (with no impurity) and impurity solutions (with no main peak) were also analyzed. Furthermore, a second experiment was performed with a decreasing sample load ratio for main peak:impurity (from 100:1 to 1:1) using the dilution scheme illustrated in Table 4.

Table 1
pK_a of halogenated phenols

Compound	pK_a	Monitoring *m*/*z*
Pentachlorophenol (**1a**)	4.74[a]	264.8
Pentafluorophenol (**1b**)	5.53[b]	183.0
2,4,6-Tribromophenol (**1c**)	6.08[c]	328.7
2,4,6-Trichlorophenol	6.23[d]	194.9
2,6-Dibromophenol	6.67[b]	250.8
2,4,6-Trifluorophenol	6.94[e]	147.0
2,6-Difluorophenol (**1d**)	7.51[b]	129.0
3,5-Difluorophenol	8.66[e]	129.0
4-Iodophenol	9.33[f]	218.9
4-Bromophenol	9.37[f]	170.9
4-Chlorophenol	9.41[f]	127.0
4-Fluorophenol (**1e**)	9.89[f]	111.0

[a] Ref. [20].
[b] Ref. [21].
[c] Ref. [22].
[d] Ref. [23].
[e] Theoretical value only. Ref. [24].
[f] Ref. [25].

2.3. Instrumentation

A 1100 series HPLC (Agilent, Mississauga, Ont.) was used for the analysis of halogenated phenols at a flow rate of 0.5 mL/min using 45:55 water:methanol mobile phases. No HPLC column was used to ensure full co-elution, and the injection volume was set at 2.5 μL. A single quadrupole mass spectrometer (Agilent, Mississauga, Ont.) was used with an APCI source. The mass spectra were acquired using selected

Table 2
pK_a of halogenated anilines

Compound	pK_a	Monitoring *m*/*z*
2,4,6-Trichloroaniline (**2a**)	−0.03[a]	195.9
2,6-Dichloroaniline (**2b**)	0.42[a]	162.0
3,5-Dichloroaniline	2.51[a]	162.0
4-Iodoaniline (**2c**)	3.81[b]	220.0
4-Bromoaniline	3.89[b]	172.0
4-Chloroaniline	3.98[b]	128.0
4-Fluoroaniline (**2d**)	4.65[b]	112.1

[a] Ref. [26].
[b] Ref. [25].

Table 3
Dilution scheme for sample load analysis with a constant sample load ratio of 100:1 for main peak:impurity

Sample	Dilution scheme (% of 5 mg/mL main peak)	Volume of main peak stock solution (μL)	Sample load (μg)	Volume of impurity stock solution (μL)	Sample load (μg)	Volume of sample solvent (μL)
1	100	1000	12.500	1000	0.12500	0
2	75	750	9.375	750	0.09375	500
3	50	500	6.250	500	0.06250	1000
4	25	250	3.125	250	0.03125	1500
5	10	100	1.250	100	0.01250	1800
6	1	10	0.125	10	0.00125	1980

The impurities used in this experiment are as listed: pentafluorophenol, 2,4,6-trifluorophenol, 2,6-difluorophenol, 4-iodophenol and 4-fluorophenol.

ion monitoring (SIM). The gas temperature was 350 °C, vaporizer temperature was 400 °C, drying gas flow rate was set at 5.0 L/min, and nebulizer pressure was 35 psig. For the APCI source, the capillary voltage was set at 3000 V, and the corona current was set to 40 μA.

In the cases where the ion trap mass spectrometer was used, an 1100 series HPLC (Agilent, Mississauga, Ont.) was used to introduce the mobile phase and the sample. The HPLC conditions are identical to those listed above, with the exception of the injection volume, which was increased to 5.0 μL. An ion trap mass spectrometer (Thermo Electron, ME, USA) was used with an APCI source. The vaporizer temperature was set at 400 °C, the capillary temperature was 350 °C, the sheath gas flow rate was set at 100, the auxiliary gas flow rate was set to 60, the capillary voltage was set to −39.00 V, the tube lens offset was set to 15 V, and the corona discharge current was set to 4.5 μA.

A 1100 series HPLC (Agilent, Mississauga, Ont.) was used for the analysis of halogenated anilines at a flow rate of 0.5 mL/min with acetonitrile:water:formic acid (50:50:0.1, v/v/v) with mobile phase. Again, a HPLC column was not used. The injection volume was set at 5 μL for the pK_a and signal suppression experiment and 2.5 μL for the sample load experiments. A single quadrupole mass spectrometer (Agilent, Mississauga, Ont.) was used with an ESI source. The gas temperature was set to 350 °C, the rate of the drying gas was set to 12.0 L/min, and the nebulizer pressure was set at 35 psig. For the ESI source, the capillary voltage was set at 3000 V.

2.4. Data analysis

The analyte peaks obtained in SIM mode were manually integrated in off-line data analyses. In the mass spectra obtained from the single quadrupole MS, the peaks were integrated from 0 min to 1 min (Agilent ChemStation software) for consistency. In the mass spectra obtained from the ion trap mass spectrometer, the peaks were integrated from 0 min to 0.5 min (Thermo Xcalibur software) for consistency.

2.5. "Degree of signal suppression" calculation

The degree of signal suppression was calculated by determining the ratio of the peak area of a 1% spiked level of "impurity" in both the presence and absence of the 5 mg/mL co-eluting main peak.

3. Results and discussion

3.1. Evaluation of halogenated phenols

Solutions containing 5 mg/mL of main peak compounds **1a–1e** with a 1% spiked level of "impurity" halogenated phenols of varying pK_a's were dissolved in acetonitrile:water (50:50, v/v) and analyzed using negative mode APCI. SIM data for the $[M-\mathrm{H}]^-$ ion of the impurity in the presence and in the absence of the main component peak were collected. Solutions containing the halogenated phenols at the 1% level in the absence of the 5 mg/mL main component were used as controls. The degree of signal suppression was plotted against the pK_a values of impurities, as shown in Fig. 2A. This figure illustrates the degree of signal suppression afforded when the impurity at the 1% level were analyzed under co-eluting conditions with a main peak. The results are presented in order of increasing pK_a values of the impurities. The pentafluorophenol impurity peak in the presence of, for example, pentachlorophe-

Table 4
Dilution scheme for sample load analysis with variable sample load ratios for main peak:impurity

Sample	Ratio of main peak to impurity	Volume of main peak stock solution (μL)	Sample load (μg)	Volume of impurity stock solution (μL)	Sample load (μg)	Volume of sample solvent (μL)
1	100:1	1000	12.500	1000	0.125	0
2	75:1	750	9.375	1000	0.125	250
3	50:1	500	6.250	1000	0.125	500
4	25:1	250	3.125	1000	0.125	750
5	10:1	100	1.250	1000	0.125	900
6	1:1	10	0.125	1000	0.125	990

The impurities used in this experiment are as listed: pentafluorophenol, 2,4,6-trifluorophenol, 2,6-difluorophenol, 4-iodophenol and 4-fluorophenol.

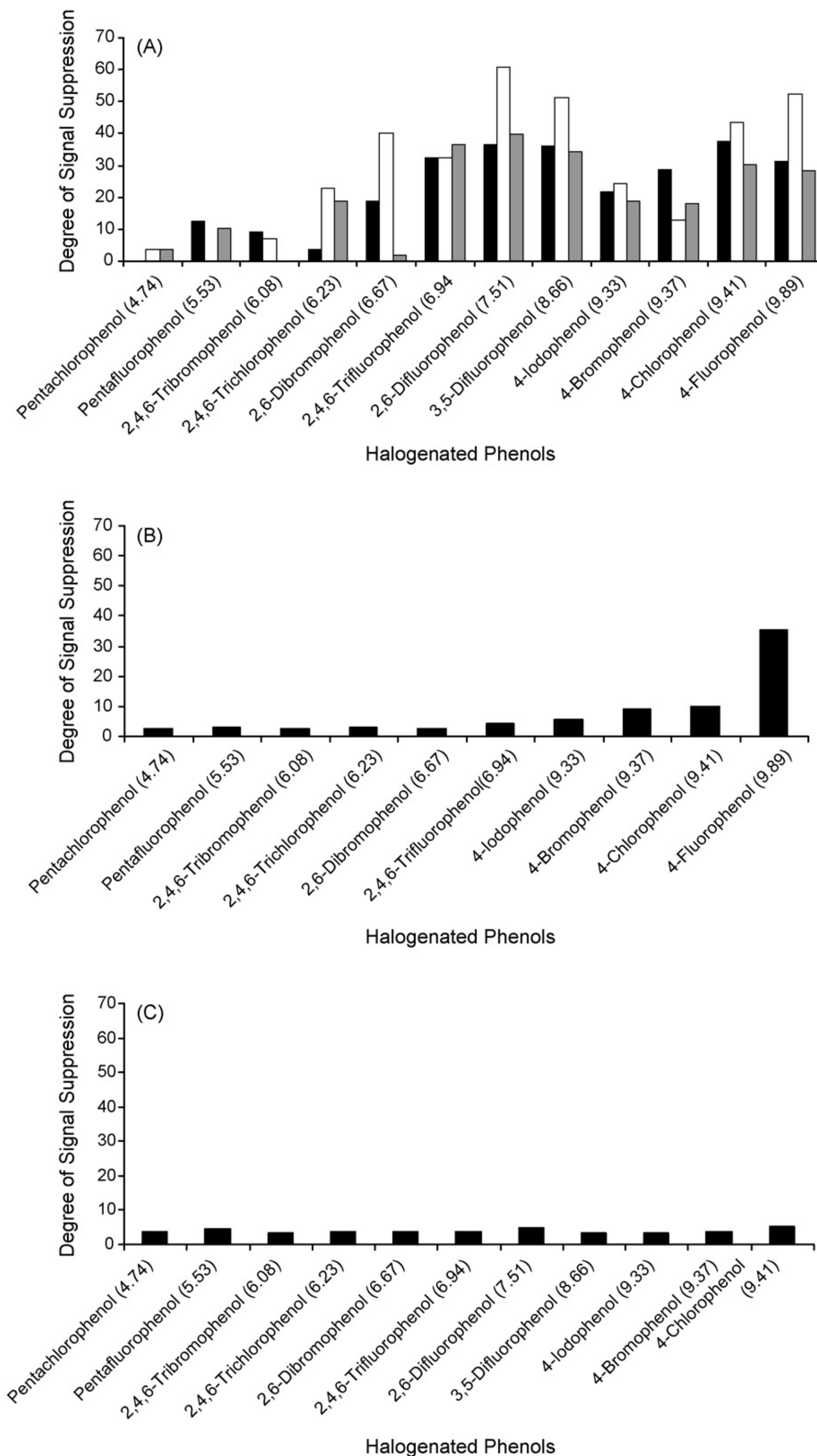

Fig. 2. The plots in this figure describe the relationship between the degree of signal suppression and pK_a for the series of halogenated phenols. (A) For this plot, three acidic compounds: pentachlorophenol (black), pentafluorophenol (white) and 2,4,6-tribromophenol (grey) are treated as main peaks while all others are impurities. (B) The relatively less acidic compound 2,6-difluorophenol is taken as the main peak compound in this plot. (C) 4-Fluorophenol, the least acidic halogenated phenol, is the main peak compound. pK_a values for each of the compounds are indicated in parentheses.

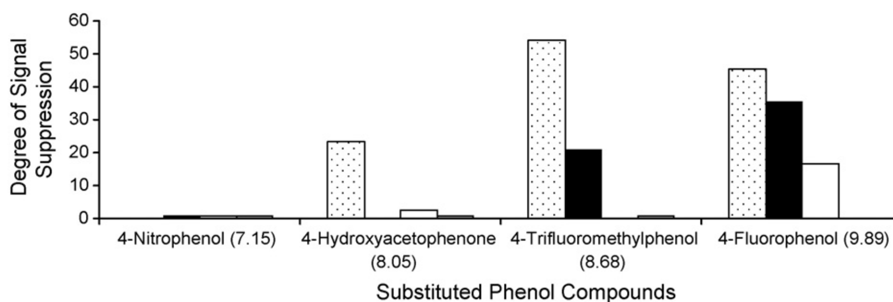

Fig. 3. This figure describes the degree of signal suppression as a function of the pK_a of a series of substituted phenols. The scheme of this figure is similar to Fig. 2 where each of the phenols is in turn treated as the main peak and impurity. The legend of the figure is as follows: 4-nitrophenol (dotted), 4-hydroxyacetophenone (black), 4-trifluoromethylphenol (white) and 4-fluorophenol (grey). pK_a value for 4-trifluoromethylphenol obtained from Ref. [20]. All other pK_a values obtained from Ref. [17].

nol, experienced an approximate 12-fold decrease in its MS peak area.

When acidic halogenated phenols were treated as the main peak component, the degree of signal suppression afforded a highly variable trend as a function of pK_a. While signal suppression is clearly a function of multiple aspects and interactions, it appears that in this simple case study, the degree of signal suppression and hence peak purity determination thresholds are impacted by greater than 10-fold depending simply on the difference in pK_a between the main peak and the co-eluting impurity.

In the presence of acidic halogenated phenols (**1a**–**1c**), impurities that have pK_a's that are 0–1.5 pK_a units higher than the pK_a of the main peak afford slightly variable but relatively low degrees of signal suppression. Since there is a similar affinity for deprotonation for both the main peak and impurity from strictly an acid–base chemical viewpoint, it is believed that the mechanism of signal suppression amplified by acid–base chemistry and proton transfer is not the predominant cause of signal intensity changes. Impurities that have pK_a's 1.5 units greater than the main peak show greatly amplified degrees of signal suppression. This is because the less acidic nature of the impurity causes it to be protonated as a result of the high concentration of the acidic main peak (and H^+ ions) present in both solution and gas phase. It is known that the acidity of halogenated phenols increase in the gas phase and therefore the phenomenon of gas phase proton transfer may be included as a plausible explanation for a portion of the signal suppression [15,16,27,28].

In Fig. 2B, a less acidic phenol was used as the main peak component and the results afford two different trends. As observed in Fig. 2A, impurities that have a greater pK_a than the main peak show some level of signal suppression. Conversely, all impurity peaks that have pK_a's that were lower than that of the main peak show no discernible signal suppression. This was further shown in Fig. 2C were the least acidic main peak component was used. In the presence of 4-fluorophenol, none of the halogenated phenols showed any signal suppression when present at 1% levels of the main peak. In these cases, acid–base chemistry does not cause any significant changes in the ionization of the impurities present and therefore has no effect on their signal intensity.

An experiment was carried out with a different series of substituted phenol compounds (4-nitrophenol, 4-hydroxyacetophenone, 4-trifluoromethylphenol and 4-fluorophenol) to investigate a relationship between other substituted phenols in the presence of acidic main peaks. The intent of the experiment was to determine if signal suppression would occur for phenols where the halogen atom is not directly bonded to the phenyl ring (with the exception of 4-fluorophenol). From Fig. 3, similar trends were observed in terms of signal suppression where the acidic phenols induce more signal suppression than the less acidic ones.

3.2. Sample load analysis

The effect of sample load analysis sample load as it pertains to signal suppression has already been reported [13]. In an attempt to better understand the effect sample load has on this system, solutions described in Table 3 were injected with varying sample loads of the main peak from 0.125 μg to 12.5 μg with a 1% spiked impurity. As expected, the degree of signal suppression decreases as the sample load of the main peak is decreased. In Fig. 4, the degree of signal suppression is plotted against the varied amount of sample load of the main peak compound for pentachlorophenol, pentafluorophenol and 2,4,6-tribromophenol.

In all cases, signal suppression decreases and becomes negligible at sample load of less than 3 μg. When the sample load is above 3 μg, the degree of signal suppression can range anywhere from 5- to 40-fold. Plots shown in Fig. 4 also confirm that the maximum attainable degree of signal suppression is a function of pK_a of the impurity. As mentioned previously, there is more than one factor that may be responsible for the decrease in signal suppression observed with decreasing sample load. One of the apparent factors is deprotonation competition between the main peak component and the impurity. A decrease in concentration of a more acidic main peak results in a decrease of H^+ ions and an increase in the deprotonation of the less acidic impurity peak. This would increase the $[M-H]^-$ impurity ion and thus enhance its signal response in the mass spectrometer. Charge competition is another factor that plays a role when the injected sample loads for both main peak and impurity are decreased. Since there is a smaller concentration of analyte and impurity ions in solution and in the gas phase, there is a decrease in charge competition, resulting in a decrease in the suppression

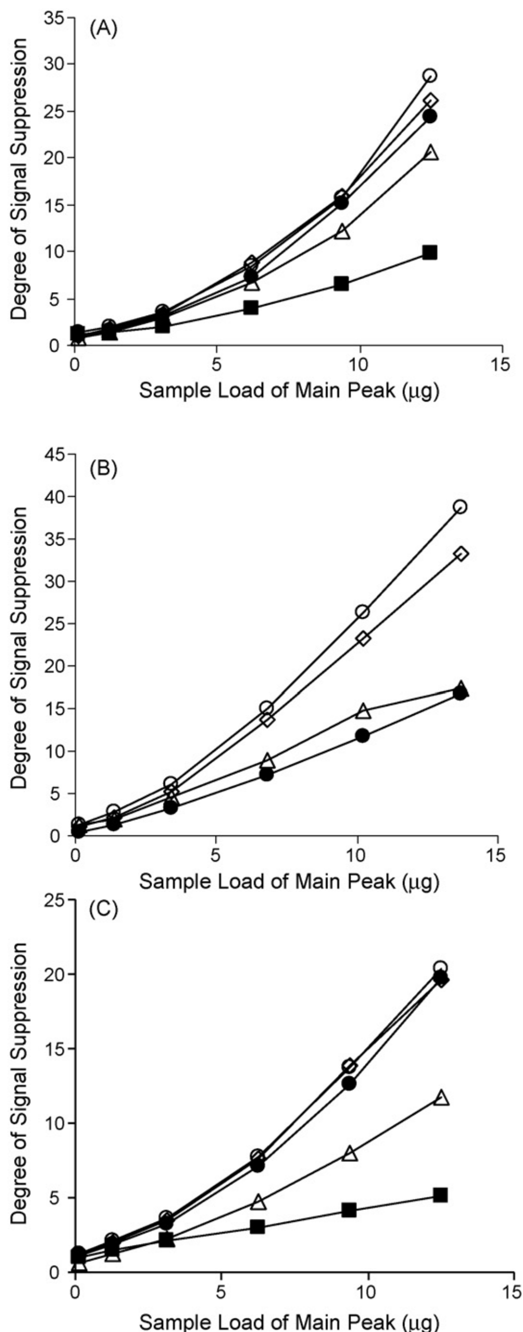

Fig. 4. Degree of signal suppression as a function of sample load for selected halogenated phenols. The three acidic phenols investigated are (A) pentachlorophenol (4.7), (B) pentafluorophenol (5.5) and (C) 2,4,6-tribromophenol (6.04). Symbols: (■) pentafluorophenol (5.5), (●) 2,4,6-trifluorophenol (6.94), (◊) 2,6-difluorophenol (6.98), (△) 4-iodophenol (9.33), and (◯) 4-fluorophenol (9.89).

of the impurity signal. Lastly, source saturation should be taken into consideration. Although it is believed that this phenomenon does not play a major role in this experiment because of the correlation between signal response and sample load, it is possible that it could contribute to some degree of impurity signal suppression. It can be concluded that source saturation is a minor impact factor in this experiment and does not affect the observed results and conclusions.

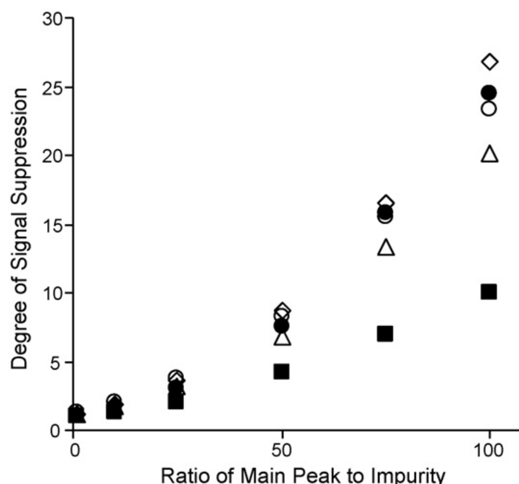

Fig. 5. This is a plot for the degree of signal suppression as a function of decreasing sample load ratio of main peak to impurity from 100:1 to 1:1. Pentachlorophenol is the main peak. Symbols: (■) pentafluorophenol (5.5), (●) 2,4,6-trifluorophenol (6.94), (◊) 2,6-difluorophenol (6.98), (△) 4-iodophenol (9.33), and (◯) 4-fluorophenol (9.89).

A sample load experiment was carried out for pentachlorophenol to observe the degree of signal suppression as a function of decreasing sample load for the main peak, while the impurity sample load remain constant. As seen from Fig. 5, this experimental setup also afforded similar trends to the previous sample load analyses.

The analogous experiments (with the exception of the experiment described in Table 4) were carried out for the halogenated phenols using an ion trap mass spectrometer. The results obtained from these experiments show a similar trend between pK_a and the degree of signal suppression for impurities and a decrease in signal suppression of the impurity as a function of decreasing main peak sample load.

3.3. Evaluation of halogenated anilines

The 5 mg/mL stock solutions of halogenated anilines **2a** and **2b** with a 1% spiked level of impurity (halogenated anilines listed in Table 2) were dissolved in 50:50 water:acetonitrile and analyzed using positive mode ES mass spectrometric detection. Solutions containing the halogenated anilines at the 1% level without the main component were used as a control. The data presented in Fig. 6 exhibit similar trends to the data presented in Fig. 2 for halogenated phenols. The abnormally large signal suppression ratio for 4-bromoaniline (Fig. 6A) was most likely due difficulties in signal integration because of its extremely low level.

The protonated forms of 2,4,6-trichloroaniline and 2,6-dichloroaniline (pK_a −0.03 and 0.42, respectively) do not induce signal suppression of the impurities regardless of their pK_a value. This trending is similar to that observed for the least acidic phenol, 4-fluorophenol (Figs. 2C and 6B). All three compounds did not undergo any significant proton transfer reactions, and therefore its presence, even at 5 mg/mL, did not affect the protonation/deprotonation of the impurities. Con-

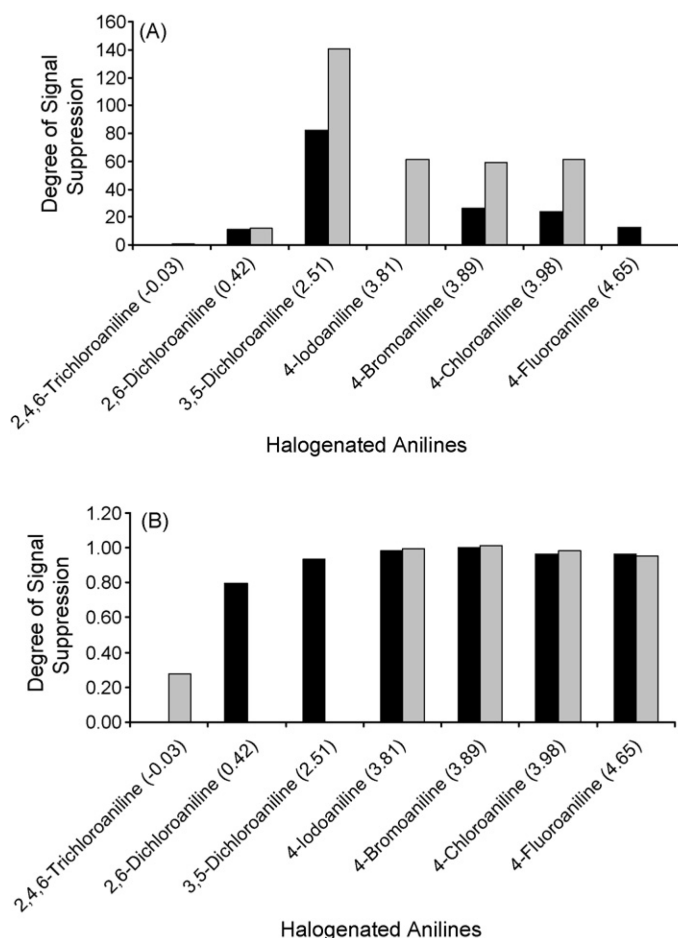

Fig. 6. Plots for the degree of signal suppression as a function of impurity pK_a for the series of halogenated anilines. (A) 4-Iodoaniline (black) and 4-fluoroaniline (grey) are the main peak compounds while the others are impurities. (B) The two compounds with the lowest pK_a's, 2,4,6-trichloroaniline (black) and 2,6-dichloroaniline (grey) are the main peaks. pK_a values for the compounds are indicated in parentheses.

versely, the more basic anilines, 4-iodo- and 4-fluoroaniline, showed behavior similar to that of the acidic halogenated phenols (Figs. 2A and 6A). In these cases, the anilines remain protonated and are able to participate in the gas phase proton transfer that creates conditions conducive for signal suppression. It should be noted that there is the assumption here that the difference in surface activities of any two co-eluting anilines has a negligible effect on responses.

Although the number of anilines used for the experiment was limited, it was expected that they would show a similar but reversed relationship in regards to the degree of signal suppression as a function of the impurity pK_a. However, 2,4,6-trichloroaniline and 2,6-dichloroaniline do not have good response at this impurity concentration, therefore accurate signal suppression data could not be obtained.

3.4. Determination of peak purity effectiveness

Peak purity analyses have been reported using both UV and mass spectrometric techniques. It is widely accepted that peak

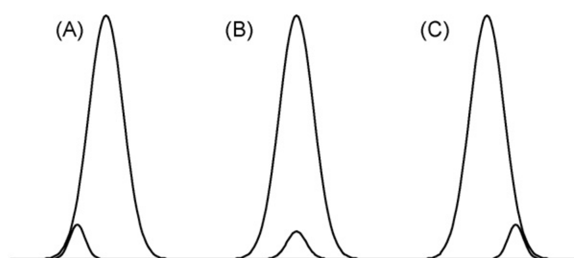

Fig. 7. Main peak with a co-eluting impurity at the (A) upslope, (B) apex, and (C) downslope. The impurity peak at the apex position will experience the greatest degree of signal suppression.

purity measured by mass spectrometry is more sensitive allowing for the detection of even perfectly co-eluting peaks down to the 0.1% level. Although more sensitive, peak purity by mass spectrometry also has a number of factors that must be addressed in order to maximize the potential of success in the purity determination. It is widely published in the literature that signal suppression can be due to a variety of factors such as pK_a, charge competition, mobile phase composition, and LC–MS parameters. In order to attain maximum effectiveness in a peak purity analysis by mass spectrometry, the following experimental factors must be considered and optimized.

3.5. Sample load threshold

It would be intuitive to think that increasing the sample load in a LC–MS peak purity analysis would increase the chances of success of detecting a low level impurity that may be co-eluting under the main peak. However, based on the effects of sample load on signal suppression, it appears that it is more beneficial to target a sample load of less than 3 μg (for the main analyte); since in almost all cases, the identity, and hence the pK_a, of any possible co-eluting entity is unknown. By targeting a sample load of between 1 μg and 3 μg, signal response of the impurity is maximized while the suppression effect is minimized.

3.6. Relative position of the co-eluting peaks

The maximum signal suppression for a perfectly co-eluting impurity will occur at the apex of the main chromatographic peak. As a co-eluting impurity shifts away from the apex of the main peak, the concentration of analytes will decrease, and the impact of charge competition and acid–base chemistry will be diminished (Fig. 7).

3.7. pK_a of potential co-eluting impurities with the main peak

As this work has shown, under the worst-case scenario of perfectly co-eluting peaks, signal suppression is impacted by the relative difference in pK_a value between the impurity peak and the main peak. If the pK_a of the co-eluting peak is 1.5 units greater than the main peak, in an unbuffered environment, the potential exists for there to be a substantial degree of signal suppression. This, in turn, will decrease the effectiveness of the peak purity analysis.

3.8. LC–MS parameters

There are two major factors in LC–MS instrumentation that will affect peak purity determination: flow rate, and the mobile phase used in the LC run. It is expected that with a decrease in the flow rate, there will be a decrease in charge competition and increase in ionization efficiency [15]. A decrease in flow rate will also increase the linear dynamic range of the ionization source and the sensitivity of the mass detector. Moreover, different mobile phases, including pH, additives (such as buffers) and organic content will impact the sensitivity of the signal response, not only for binary systems investigated in this experiment, but for analytes in general.

Although its effects on signal suppression are not investigated in this paper, another factor that should be considered is the sample solvent. The choice of a protic or an aprotic sample solvent can enhance or minimize signal suppression, depending on the compounds being investigated and the ionization mode of the mass spectrometer.

4. Conclusions

It is clear that single quadrupole MS coupled with either an ESI or an APCI source is a useful tool for the detection of co-eluting impurities in HPLC analysis during research and development. Mass spectrometric peak purity determination has been shown to be a versatile, sensitive, and rapid technique for the determination of LC peak purity. However, the technique cannot be expected to reliably detect all impurities at the 0.1% limit of detection level. This work shows that, although signal suppression is the culmination of a number of interrelated interactions, the pK_a of a co-eluting impurity as well as the sample load being injected can greatly impact peak purity determination. Knowledge of solution chemistry and spectral interpretation is critical to the successful application of this technique for peak purity measurements. Used appropriately, co-eluting impurities can be rapidly determined down to low levels. In addition, both the molecular mass and the retention time of the impurity can be determined; and unlike UV methods, compounds with identical or similar UV spectra can be distinguished. However, since the identity of a co-eluting impurity is usually unknown, performing analyses at low sample load will afford a more reliable measurement.

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