Journal of Chromatography A, 1229 (2012) 172–179

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of volatile bases by high performance liquid chromatography with aerosol-based detection

Ryan D. Cohen*, Yong Liu**, Xiaoyi Gong

Merck Research Laboratories, Merck & Co., Inc., Rahway, NJ 07065, USA

ARTICLE INFO

Article history:
Received 30 November 2011
Received in revised form 11 January 2012
Accepted 12 January 2012
Available online 20 January 2012

Keywords:
Nano quantity analyte detector
Charged aerosol detector
NQAD
CAD
Volatile bases
HPLC

ABSTRACT

The development and performance of two methods are described whereby low levels of volatile bases are quantified by HPLC using either a charged aerosol detector (CAD) or a nano-quantity analyte detector (NQAD). A test set of 12 volatile bases (ammonia, hydrazine, methylamine, ethylamine, diethylamine, triethylamine, isobutylamine, N,N-diisopropylethylamine, morpholine, piperazine, ethylenediamine, and 1,4-diazabicyclo[2.2.2]octane) were analyzed. The studied compounds all exhibit poor UV chromophores and are typically undetectable by aerosol-based detection when using conventional volatile mobile phases. The ability to detect these analytes by CAD or NQAD depended on their propensity towards formation of a low volatility salt between the target analyte and mobile phase modifier. Trifluoroacetic acid (TFA) was found to significantly improve detection of most volatile bases. A low concentration (0.2 mM) of hydrochloric acid was additionally needed to enable detection of ammonia. The compounds were separated under hydrophilic interaction liquid chromatography (HILIC) conditions on a zic-*p*HILIC column. For all analytes, limits of detection (LOD) were measured in the range of 1–27 ng on column, which is comparable to previously reported detection limits for non-volatile analytes.

1. Introduction

Aerosol-based detectors, such as the evaporative light scattering detector (ELSD), charged aerosol detector (CAD), and nano-quantity analyte detector (NQAD), are commonly used for HPLC detection of analytes with poor UV chromophores. These detectors have often been referred to as "universal", in the sense that they provide a near uniform response dependent on the mass of analyte and irrespective of molecular structure so long as mobile phase composition remains constant [1–3]. However, a considerable shortcoming has been that the analyte must be of sufficiently low volatility for detection to occur. Moreover, semi-volatile molecules, such as ibuprofen and caffeine, exhibit diminished response relative to lower volatility molecules, and this bias increases at lower concentrations [3,4].

There have been several publications on detection by either ELSD or CAD for inorganic ions (e.g., Na^+, Cl^-, PO_4^{3-}, Ca^{2+}) and low molecular weight organic ions (e.g., tartrate, maleate, piperazine) [5–11]. In most reports, a mobile phase containing a volatile buffer, such as ammonium acetate, was employed. It is noteworthy that for Cl^- ion, the conjugate acid is HCl, which is a gas at ambient temperature and pressure, and presumably too volatile for aerosol-based detection. Lantz et al. [5] first postulated that the ammonium chloride salt, formed between the Cl^- analyte and NH_4^+ ions from the mobile phase buffer is the aerosol species actually observed in the ELSD.

Generally, there is a decrease in volatility of ionizable molecules upon salt formation. By pairing ammonia with trifluoroacetic acid (TFA), the vapor pressure of the ammonium trifluoroacetate salt is markedly reduced relative to the free acid/base [12]. If this decrease is large enough, then the salt species formed should be detectable. This behavior allows for analysis of both ammonia and TFA by HPLC with aerosol-based detection (*viz.*, as a single, conjugated peak).

In the current study, we investigate the use of mobile phase modifiers that can enable the detection of volatile bases. Two different types of aerosol-based detectors, CAD and NQAD, were studied to demonstrate that the phenomenon is generally applicable to this class of HPLC detectors. The CAD was commercialized by ESA in 2004, and there have been more than 50 publications documenting its performance for non-volatile analytes such as lipids [13,14], pharmaceuticals [15–17], polymers [18,19], parabens [20], vitamins [21], amino acids [8], and counterions [9,10]. This detector operates by first nebulizing the HPLC eluent into an aerosol, followed by drying, then mixing with a stream of positively charged N_2 gas, and lastly detection by an electrometer [22,23]. For non-volatile analytes with high-purity volatile mobile phases, limits of detection (LOD) have typically been measured to 0.3–2.5 μg/mL [8,13,14,17,20,21]. Response is inherently non-linear and is best fit by a power function of the form $f(x) = ax^b$; although, over short

* Corresponding author. Tel.: +1 732 594 1605; fax: +1 732 594 3887.
** Corresponding author. Tel.: +1 732 594 9040; fax: +1 732 594 3887.
E-mail addresses: ryan.cohen@merck.com (R.D. Cohen), yong.liu2@merck.com (Y. Liu).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2012.01.036

ranges of up to 2 orders of magnitude, a linear fit is reasonable [8].

The NQAD is a commercially available condensation nucleation light scattering detector (CNLSD) that was introduced by Quant Technologies in 2009. To date, there have been several literature reports describing the use of this instrument for analysis of surfactants [24], antibiotics [25], polymers [26], and amino acids [27]. NQAD uses water condensation to increase the size of the aerosol particles prior to light scattering detection [28,29]. This greatly enhances limits of detection relative to ELSD with 10–100 fold improvements reported [26,30]. In addition, several investigators have demonstrated a linear response up to 2 orders of magnitude while ELSD produces a non-linear response for even small ranges [24,25]. Hutchinson et al. [31] recently evaluated the performance of this detector versus ELSD and CAD for a test set of 11 small molecules. They found the NQAD exhibited the lowest detection limits but had poorer reproducibility than CAD.

A total of 12 volatile bases with poor UV chromophores were chosen for this study. The study set includes common chemical reagents such as ammonia, ethylenediamine, and hydrazine that are used in a variety of chemical industries including pharmaceutical processing, where there is currently a need for improved methods to determine residual levels in solvent streams and final products. While GC analyses of these compounds are sometimes feasible, this approach is often difficult or impractical as amines sometimes exhibit poor peak shape on many GC columns, and two of the compounds have no (ammonia and hydrazine) flame ionization detector response. In addition, an extraction step is often required for aqueous sample matrices. Besides GC, ion chromatography (IC) is sometimes used for analysis of these compounds [32–34], but IC equipment is not as commonly accessible in analytical labs. Consequently, there is significant need for a direct HPLC method that can afford rapid and convenient analysis for these compounds, and it is advantageous to have one method that can detect all species (UV absorbing/non-absorbing and volatile/non-volatile) in a single analytical run.

2. Materials and methods

2.1. Reagents

Deionized water was obtained from a Hydro point-of-use water purification system (Durham, NC, USA). HPLC grade acetonitrile (MeCN), glacial acetic acid, pyruvic acid, oxalic acid dehydrate, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), diethylamine, triethylamine, and 1,1,3,3-tetramethylguanidine (TMG) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Hydrochloric acid (37 wt% in H_2O), formic acid, heptafluorobutyric acid (HFBA), N,N-diisopropylethylamine (DIPEA), ethylamine (70 wt% in H_2O), ammonium hydroxide (28 wt% NH_3 in H_2O), ethylenediamine, 1,4-diazabicyclo[2.2.2]octane (DABCO), morpholine, N-methylmorpholine, anhydrous hydrazine, piperazine, methylamine (2.0 M solution in methanol), isobutylamine, 1,8-diazabicycloundec-7-ene (DBU), N,N-dimethylhydrazine, and primidone were obtained from Sigma–Aldrich (St. Louis, MO, USA). All reagents were 99% purity or higher except pyruvic acid, hydrazine, and N,N-dimethylhydrazine which were 98%.

2.2. Instrumentation

An Agilent 1100 HPLC with a variable wavelength UV detector was connected in series to either the NQAD or CAD. The NQAD QT-500 was manufactured by Quant Technologies (Blaine, MN, USA), and the Corona CAD was purchased from ESA Biosciences (Chelmsford, MA, USA). Data was acquired and processed using Atlas Chromatography Data System (Version 8.2 Thermo Electron Corporation, PA, USA).

A zic-*p*HILIC, 5 μm, 150 mm × 4.6 mm column from SeQuant was used for all separations. Other columns used for method development purposes were: Waters xBridge C18, 3.5 μm, 150 mm × 4.6 mm; Phenomenex Luna HILIC, 3 μm, 150 mm × 4.6 mm; Phenomenex Luna NH_2, 3 μm, 150 mm × 4.6 mm; Thermo Hypercarb, 5 μm, 100 mm × 4.6 mm.

2.3. Methods

All method development experiments were performed using the NQAD. Unless otherwise noted, NQAD parameters were N_2 gas pressure = 29 psi, gain = 20×, evaporator temperature = 35 °C, and filter = 5 s. Corona CAD parameters were N_2 gas pressure = 35 psi, filter = medium, and range = 100 pA. Unless otherwise noted, relevant HPLC parameters were as follows: injection volume = 5.0 μL, column temperature = 40 °C, and flow rate = 1.0 mL/min.

A test set of nine volatile, monoprotic bases (ammonia, hydrazine, methylamine, ethylamine, morpholine, diethylamine, triethylamine, isobutylamine, and DIPEA) were separated within 10 min using isocratic, hydrophilic interaction liquid chromatography (HILIC) conditions on the zic-*p*HILIC column. The mobile phase was 0.2 mM HCl in TFA/MeCN/H_2O (0.04:60:40, v/v/v). This is referred to as method 1 in the text.

Three volatile, polyprotic bases (ethylenediamine, piperazine, and DABCO) were separated within 15 min under isocratic HILIC conditions on the same column using a mobile phase of TFA/MeCN/H_2O (0.4:70:30, v/v/v). This is referred to as method 2 in the text.

3. Results and discussion

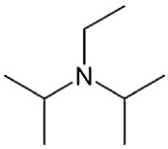
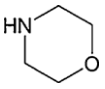
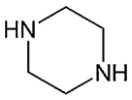
3.1. Analyte and mobile phase modifier properties

Analytes and mobile phase modifiers were purposefully selected for this study with a wide range of molecular properties to ascertain whether any generalizations can be established between chromatographic conditions and aerosol detector sensitivity. Vapor pressures, pK_a, molecular weights, and either molecular structures or formulas of each studied analyte/modifier are provided in Table 1a for bases and Table 1b for acidic modifiers. The volatility of these compounds varied significantly. Ammonia, methylamine, ethylamine, and hydrochloric acid are gases at ambient temperature and pressure with vapor pressures greater than 1000 mm Hg at 25 °C; whereas oxalic acid, TCA, piperazine, and DABCO are all solids at ambient temperature and pressure with vapor pressures less than 5 mm Hg at 25 °C. Acidities and basicities also spanned several orders of magnitude. The ranges of pK_a were from <0 to 4.79 for the acidic modifiers and 8.18–10.98 for the conjugate acids of the basic analytes. Molecular weights spanned approximately one order of magnitude. Both monoprotic and polyprotic acids and bases were included.

3.2. Mobile phase modifier screening

Flow injection analysis (FIA) was used to efficiently screen mobile phase modifiers. For the analysis of bases, eight acidic mobile phase modifiers (acetic acid, formic acid, TCA, TFA, HFBA, pyruvic acid, oxalic acid, and hydrochloric acid) were evaluated. Mobile phases of MeCN/H_2O (1:1, v/v) were individually prepared containing 1.0 mM of each modifier. Solutions of each analyte at a concentration of 1.0 mM were prepared in MeCN/H_2O (1:1, v/v) diluent. To better promote dispersive mixing between the injection plug and mobile phase, the injection volume was decreased to 3.0 μL. Other instrument conditions were as noted in Section

Table 1a
Molecular properties of volatile bases used in the study.

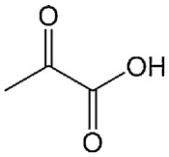
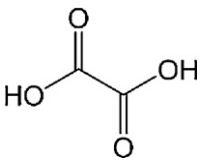
Compound	Structure or molecular formula	Vapor pressure[a] (mm Hg)	pK_a [b]	MW (g/mol)
Ammonia	NH_3	5992.5	9.24	17.03
Methylamine	CH_3NH_2	3965.3	10.66	31.06
Ethylamine	$CH_3CH_2NH_2$	1132.3	10.64	45.08
Diethylamine	$NH(CH_2CH_3)_2$	218.4	10.76	73.14
Triethylamine	$N(CH_2CH_3)_3$	56.1	10.62	101.19
Hydrazine	H_2NNH_2	20.7	8.18	32.05
Isobutylamine	$(CH_3)_2CHCH_2NH_2$	143.9	10.72	73.14
N,N-diisopropylethylamine (DIPEA)		11.6	10.98	129.24
Morpholine		10.4	8.97	87.12
Ethylenediamine	$NH_2CH_2CH_2NH_2$	15.8	9.89, 6.77	60.10
Piperazine		4.0	9.55, 5.30	86.14
1,4-Diazabicyclo[2.2.2]octane (DABCO)		1.2	8.19, 4.20	112.17

[a] Vapor pressures calculated at 25 °C using ACD labs, version 11.0.
[b] pK_a of conjugate acids from ACD labs, version 11.0.

2. It should be pointed out that no appreciable differences were observed for select analytes (DABCO and ethylamine) that were prepared using mobile phase as diluent compared to the nominal diluent. Screening results are provided in Table 2.

When either no modifier or weakly acidic modifiers such as acetic acid and formic acid were used, none of the studied volatile bases produced a significant response that would be considered detectable (a very small peak was observed for most injections including those of the diluent blank). TFA, HFBA, TCA, pyruvic acid, oxalic acid, and HCl modifiers all produced detectable responses for all studied analytes; although, the improvement in response for ammonia was only significant when TCA, oxalic acid, and HCl were present in the mobile phase. It is interesting to note that the polyprotic bases (ethylenediamine, piperazine, and DABCO) generally exhibited the highest responses. This was most dramatic when TFA modifier was used, where the average peak area for the monoprotic bases was 99 while it was 1090 for the polyprotic bases.

Table 1b
Molecular properties of acidic mobile phase modifiers used in the study.

Compound	Structure or molecular formula	Vapor pressure (mm Hg)[a]	pK_a [b]	MW (g/mol)
Formic acid	HCOOH	36.5	3.74	46.03
Acetic acid	CH_3COOH	13.9	4.79	60.05
Trichloroacetic acid (TCA)	CCl_3COOH	0.2	0.09	163.39
Trifluoroacetic acid (TFA)	CF_3COOH	96.2	0.05	114.02
Heptafluorobutyric acid (HFBA)	$CF_3(CF_2)_2COOH$	9.8	0.37	214.04
Pyruvic acid		1.0	2.65	88.06
Oxalic acid		<0.001	4.28, 1.38	90.03
Hydrochloric acid	HCl	>7600	<0	36.46

[a] Vapor pressures calculated at 25 °C using ACD labs, version 11.
[b] pK_a of acids from ACD Labs, version 11.0.

Table 2
Influence of mobile phase modifier on NQAD response for volatile bases.[a]

Analyte	Acidic modifier								
	None	Acetic	Formic	TFA	HFBA	TCA	Pyruvic	Oxalic	HCl
									Peakarea
Ammonia	0	1	1	7	7	61	10	227	147
Hydrazine	1	1	2	40	109	399	81	609	409
Methylamine	1	2	2	96	199	371	109	412	350
Ethylamine	1	3	2	85	178	367	99	383	305
Diethylamine	0	1	2	149	233	493	148	551	455
Triethylamine	1	1	1	50	125	420	241	783	534
Isobutylamine	2	2	1	138	279	603	197	688	566
DIPEA	1	2	1	164	364	763	334	955	686
Morpholine	3	3	2	162	360	786	276	931	749
Ethylenediamine	3	4	4	1238	1072	1285	234	1092	859
Piperazine	2	3	3	868	839	1202	311	1283	1017
DABCO	1	3	1	1016	1255	1009	274	1259	929
Noise[b]	0.004	0.004	0.005	0.005	0.004	0.007	3.146	0.005	0.004

[a] 1.0 mM of each analyte individually measured via flow injection analysis using MeCN/H_2O (1:1, v/v) mobile phases containing 1.0 mM of acidic modifier.
[b] Background noise measured using peak-free region of baseline approximately three peak-widths wide.

An optimal modifier has the greatest response for the largest number of analytes while at the same time exhibiting minimal background noise. Generally, oxalic acid produced the largest increase in NQAD response, followed by HCl. The background noises when using HCl and oxalic acid modifiers were only slightly higher than without modifier; although, it is important to remember that the modifier concentrations were only 1.0 mM. In the case of pyruvic acid, the background noise was several orders of magnitude higher than the other modifiers, and this most likely was due to non-volatile impurities present in this reagent. Since TFA produced a substantial response for many of the analytes and is a commonly used HPLC modifier that is compatible with many columns, it was also included for further development along with HCl and oxalic acid.

3.3. Effect of modifier concentration

The dependence of NQAD detector response on modifier concentration was studied by FIA for three modifiers: TFA, HCl, and oxalic acid. Analytes were individually injected using an injection volume of 3.0 μL at a concentration of 1.0 mM in MeCN/H_2O (1:1, v/v) diluent. The concentrations of each modifier were varied from 0 to 10 mM while holding all other conditions constant. All other instrument conditions were as described in Section 2. Results of these experiments are presented in Fig. 1. Improvement in peak areas reached a plateau at an oxalic acid concentration of 1 mM; however, S/Ns rapidly decreased above 0.5 mM due to increasing noise. HCl and TFA modifiers only exhibited slight increases in noise with concentration. The reason for this disparity in modifier performance may likely be due to the significantly lower vapor pressure of oxalic acid compared to HCl and TFA. Peak areas and S/Ns continued to improve for both HCl and TFA as the concentrations were increased; although, the improvement was better for TFA. Using at least a 10 mM concentration of TFA resulted in similar peak areas and S/Ns as HCl for all analytes except ammonia. This finding is especially useful since TFA has broader column and system compatibility than HCl.

3.4. Column selection

The basic analytes selected for this study were highly polar and difficult to retain by conventional reversed phase conditions. Furthermore, to enable salt formation and subsequent detection, these analytes need to be charged at the mobile phase pH. Possible choices for chromatography modes were reversed phase with ion pairing, HILIC, ion exchange, and mixed mode. The first two modes were examined in this study.

Reversed phase chromatography was initially investigated with TFA as a volatile ion-pairing agent on the xBridge C18 column. Retentivity and specificity were acceptable for less polar analytes, such as triethylamine, DIPEA, and isobutylamine, but ammonia, hydrazine, methylamine, and ethylenediamine eluted at or near the void volume even at 97% aqueous conditions. By substituting a more hydrophobic ion-pairing agent, HFBA, retention increased for all analytes, but ammonia and hydrazine still eluted in the void.

The Hypercarb column, a porous graphite carbon stationary phase, was also studied under reversed phase conditions with HFBA since high affinities have been demonstrated for polar, charged compounds such as amino acids [35]. Retention for all analytes was further increased relative to the xBridge C18 column. However, ammonia and hydrazine were still weakly retained, and an interfering peak in the diluent blank was observed regardless of the brand of MeCN or water source.

Several HILIC columns were explored next: Luna NH_2, Luna HILIC, and zic-*p*HILIC. A gradient of TFA/MeCN/H_2O (0.1:90:10, v/v/v to 0.1:60:40, v/v/v) in 20 min was used to screen columns. For the Luna NH_2 column, analytes were not retained, presumably due to electrostatic repulsion between the positively charged analytes and amino group of the bonded phase. Analytes were retained by the Luna HILIC column, but peak shapes were not ideal and resolution was poor. On the zic-*p*HILIC column, peak shapes were acceptable (USP tailing factors < 2.0), and analytes were well retained. In fact, the doubly charged, polyprotic bases (ethylenediamine, DABCO, and piperazine) did not elute off the column at the initial gradient conditions. These analytes eluted when the TFA concentration was increased to 0.4% by volume.

Aerosol-based detectors are sensitive to column bleed, and the zic-*p*HILIC column exhibited the lowest background noise. This was most likely due to its use of a polymeric support, as opposed to silica, and is consistent with the findings of Huang and coworkers, who used this column for the analysis of non-volatile counterions [9].

A major advantage of using HILIC with aerosol-based detection is higher sensitivity due to improved nebulization efficiency from the high organic mobile phases required [1]. An additional advantage for small molecule pharmaceutical applications is that HILIC often results in high selectivity and short run times (under isocratic conditions) for polar analytes since most small molecule drug substances and related impurities are poorly retained and unlikely to interfere. Based on these points and data from the exploratory

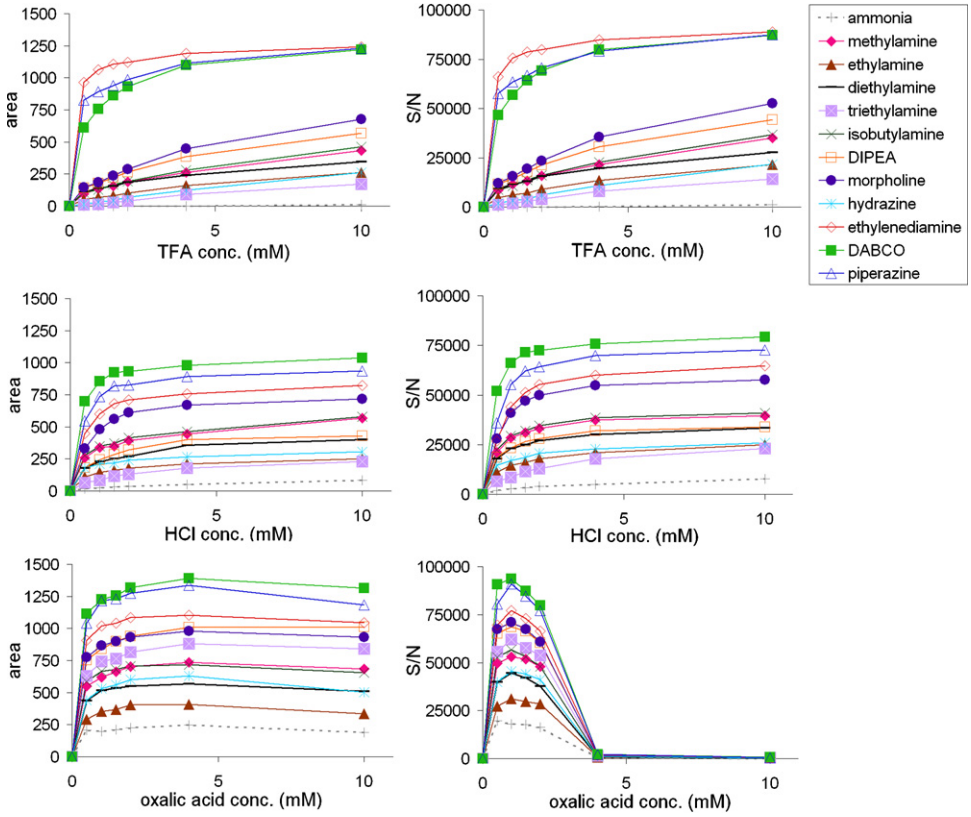


Fig. 1. Effect of mobile phase modifier concentration on NQAD response for analytes individually injected via flow injection analysis at a concentration of 1.0 mM in MeCN/H_2O (1:1, v/v) diluent using 3.0 μL injection volume.

gradient runs, the zic-*p*HILIC column was chosen for further development.

3.5. Mobile phase optimization

The concentration of modifier is directly proportional to the analytes' elution time on the zic-pHILIC column. A concentration of 0.4% by volume of TFA was found to be necessary to elute polyprotic bases while many monoprotic bases co-eluted. Since HILIC often exhibits slow gradient re-equilibration, isocratic methods were deemed more desirable. Because of the significantly stronger stationary phase interaction of the polyprotic bases, two isocratic methods were required.

For the monoprotic bases, all analytes were separated within 10 min using a mobile phase of TFA/MeCN/H_2O (0.04:60:40, v/v/v) and a column temperature of 40 °C. Ammonia was not detected under these conditions. Addition of a small concentration (0.2 mM) of HCl greatly improved ammonia's response as shown in Fig. 2. The addition of HCl did not significantly affect the response or the retention time of the other analytes.

Note that in Fig. 2, analytes are displayed at a relatively high concentration of 0.1 mg/mL. For typical impurity level concentrations (e.g., 0.025 mg/mL and lower), baseline resolution was easily achieved for all analytes. If necessary, resolution could be further improved by increasing the MeCN concentration or decreasing the TFA concentration at the expense of run time.

For the polyprotic bases, a mobile phase of TFA/MeCN/H_2O (0.4:70:30, v/v/v) and column temperature of 40 °C resulted in baseline resolution of all analytes within 15 min on the zic-*p*HILIC column. Similar to the monoprotic bases, elution was observed to be foremost dependent on the TFA concentration and to a slightly less extent on the MeCN concentration; in addition, the elution order was in decreasing hydrophobicity, which is typical of HILIC.

3.6. Influence of NQAD evaporator temperature on analyte sensitivity

In general, increasing an aerosol-based detector's evaporator temperature reduces the response of semi-volatile analytes while

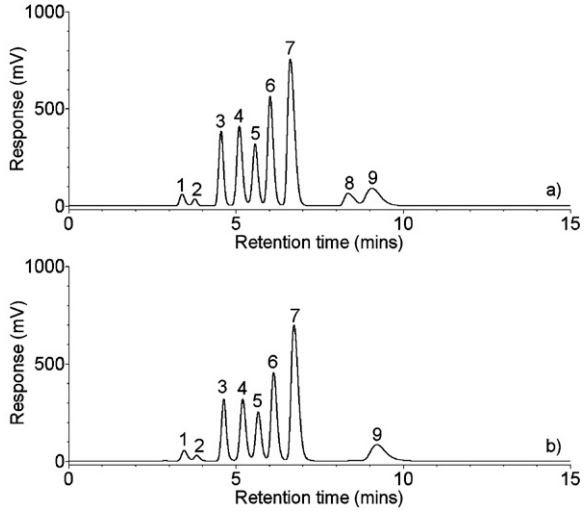


Fig. 2. Separation of singly charged volatile bases on zic-*p*HILIC. Addition of 0.2 mM HCl to mobile phase enables detection of ammonia peak. Each analyte at 0.1 mg/mL using MeCN/H_2O (1:1, v/v) diluent; 1 = DIPEA, 2 = triethylamine, 3 = diethylamine, 4 = isobutylamine, 5 = morpholine, 6 = ethylamine, 7 = methylamine, 8 = ammonia, 9 = hydrazine; (a) mobile phase = 0.2 mM HCl in TFA/MeCN/H_2O (0.04:60:40, v/v/v); (b) mobile phase = TFA/MeCN/H_2O (0.04:60:40, v/v/v).

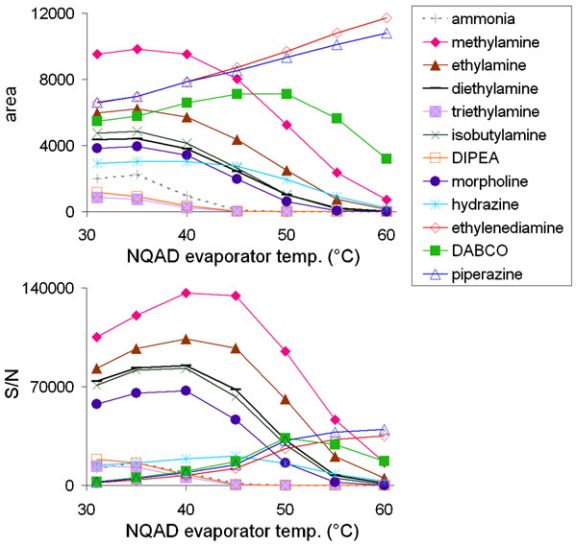


Fig. 3. Effect of NQAD response versus evaporator temperature for volatile bases, each at a concentration of 0.1 mg/mL. Method 1 used for monoprotic bases, and method 2 used for polyprotic bases.

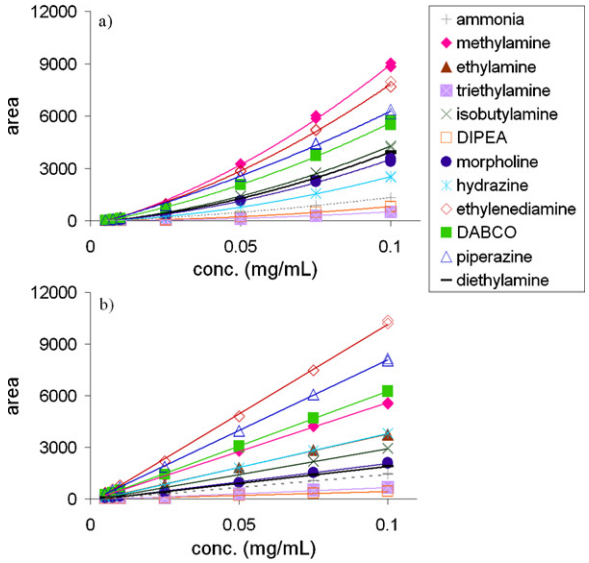


Fig. 4. Concentration versus peak area plot for (a) HPLC-NQAD data fitted by 2nd order polynomial and (b) HPLC-CAD data fitted by linear regression. Method 1 used for monoprotic bases, and method 2 used for polyprotic bases.

at the same time enhancing the response of non-volatile analytes due to a reduction in noise [36]. Here the effect of evaporator temperature on NQAD response was studied using the optimized HPLC conditions as described above. Results are shown in Fig. 3. The minimum operating temperature of the NQAD used in this study (QT-500) was ambient temperature (~31 °C). Evaporator temperature was then increased in 5° increments from 35 to 60 °C while holding all other parameters constant.

Peak areas for the singly charged, volatile amines (except DIPEA and triethylamine) reached their maxima at an evaporator temperature of 35 °C. DIPEA and triethylamine, which are both tertiary amines, appear to have maxima less than 31 °C. At 60 °C, the responses for ammonia, DIPEA, triethylamine, and morpholine were nearly zero. For two of the analytes (ethylenediamine and piperazine), peak areas trended upwards as evaporator temperature increased, while the peak area for DABCO reached a maximum at 50 °C. This behavior points to a substantial reduction in volatility after salt formation with TFA for the doubly charged bases.

Monoprotic bases exhibited optimal evaporator temperatures <31–40 °C while the polyprotic bases exhibited optimal evaporator temperatures of 50 °C for DABCO and >60 °C for piperazine and ethylenediamine. For subsequent experiments, the evaporator temperature was set at 35 °C to allow a more direct comparison with the CAD, which does not provide evaporator temperature control.

3.7. Figures of merit for the analysis of volatile bases

Figures of merit obtained for analysis of volatile bases using either the NQAD or CAD are presented in Tables 3a and 3b, respectively. Note that the monoprotic bases were analyzed using method 1 and polyprotic bases (ethylenediamine, DABCO, and piperazine) were analyzed using method 2.

Limits of detection (LODs) were experimentally determined by triplicate injections of solutions serially diluted from 0.005 mg/mL to 0.0001 mg/mL. The measured signal-to-noise ratios (S/Ns) versus concentration were plotted for each analyte, and a 2nd order polynomial fit was used to determine the concentration at which the S/N was 3. The lowest LODs were measured for the doubly charged bases, while ammonia, DIPEA, triethylamine, and hydrazine had the highest LODs. Detection limits were on par with those reported for non-volatile analytes using the two detectors [10,25,31]. Moreover, the detection limit for the non-volatile analyte, sodium, in a sodium chloride solution was also measured via serial dilution using the conditions from method 1, and the LOD was determined to be 1 ng on the CAD and 3 ng on the NQAD.

The CAD exhibited greater band broadening than the NQAD as measured by the peak width at half height, $W_{1/2}$. On average, $W_{1/2}$ was 40% higher for the CAD than the NQAD, yet the increased band broadening did not result in substantially different detection limits than those of the NQAD. For all analytes, LODs were higher from the NQAD than the CAD (on average, 2.3 times higher). Hutchinson and coworkers actually found better performance when comparing detection limits from the NQAD when the organic content of the mobile phase was high, while differences were negligible at 100% aqueous conditions [31]. Multiple studies have reported lower LODs from both the NQAD and the CAD when compared to the ELSD, which is an older technology [26,30,37–39]. However, direct comparisons between the NQAD and CAD may be more strongly dependent on analyte properties and method conditions.

Instrument precision was measured by performing six replicate injections of a 0.1 mg/mL solution of each analyte and was found to be similar for both detectors. The %RSD of the peak areas was <2.0% for all analytes except DIPEA, which was 2.1% on the CAD and 0.9% on the NQAD.

Plots of the NQAD and CAD response versus concentration are provided in Fig. 4. Three injections were performed at each level spanning a range of 0.005–0.1 mg/mL. Least squares linear regression was used to fit the CAD data while a 2nd order polynomial was required to fit the NQAD data in order to obtain correlation coefficients of at least 0.99. The slope of the NQAD plot was shallow (less sensitive) at low concentrations and becomes steeper (more sensitive) at higher concentrations. For small ranges of less than one order of magnitude, least squares linear regression can also be performed on the NQAD data.

Primidone, a neutral, small molecule drug substance, was used as a model compound for spiked recovery experiments. For both methods, the primidone peak eluted in the void volume without interfering with any analytes, and this behavior is typical of other proprietary small molecule pharmaceutical compounds that have been analyzed (data not shown). Example chromatograms are shown in Fig. 5 for both detectors using method 2.

Table 3a
Figures of merit for analysis of volatile bases by HPLC-NQAD.

Analyte[a]	RT (min)	$W_{1/2}$ [b] (s)	LOD[c] (μg/mL)	LOD[c] (ng)	Precision[d] (%RSD)	Goodness of fit[e] (r)	% Recovery[f]
DIPEA	3.5	10.4	4.6	23	0.9	0.9996	107.0
Triethylamine	3.9	9.7	5.5	27	1.4	0.9995	112.5
Diethylamine	4.6	9.9	1.4	7	1.0	0.9996	92.6
Isobutylamine	5.2	11.5	1.9	9	1.0	0.9996	97.4
Morpholine	5.7	11.9	1.7	8	1.9	0.9993	100.1
Ethylamine	6.2	12.0	1.1	5	1.1	0.9995	95.1
Methylamine	6.8	14.4	0.7	3	1.2	0.9995	93.8
Ammonia	8.6	20.9	2.5	13	0.8	0.9993	100.6
Hydrazine	9.3	29.6	2.9	15	1.1	0.9996	89.4
DABCO	9.1	32.4	0.7	3	1.4	0.9996	106.0
Piperazine	10.9	48.1	0.5	3	1.5	0.9993	103.1
Ethylenediamine	13.1	54.4	0.8	4	1.5	0.9996	80.2

[a] Monoprotic bases were analyzed via method 1 while polyprotic bases were analyzed via method 2.
[b] $W_{1/2}$ measured for 0.1 mg/mL level.
[c] Detection limit experimentally determined when S/N = 3 (average of 3 injections).
[d] Precision of peak areas from 6 replicate injections at 0.1 mg/mL level.
[e] Correlation coefficient from least squares regression fit of 2nd order poly. (range = 0.005–0.1 mg/mL).
[f] % Recovery for 0.5% of analyte spiked into 5 mg/mL of primidone.

Table 3b
Figures of merit for analysis of volatile bases by HPLC-CAD.

Analyte[a]	RT (min)	$W_{1/2}$ [b] (s)	LOD[c] (μg/mL)	LOD[c] (ng)	Precision[d] (%RSD)	Linearity[e] (r)	% Recovery[f]
DIPEA	3.3	13.4	1.7	8	2.1	0.9965	106.1
Triethylamine	3.7	17.5	1.8	9	1.9	0.9931	95.2
Diethylamine	4.4	13.2	0.7	4	0.9	0.9991	92.1
Isobutylamine	4.9	16.2	0.8	4	0.7	0.9996	85.2
Morpholine	5.3	16.7	0.9	4	1.4	0.9990	100.2
Ethylamine	5.8	17.1	0.6	3	0.5	0.9998	89.6
Methylamine	6.4	19.9	0.3	2	0.4	0.9999	88.1
Ammonia	8.0	23.2	1.9	10	1.3	0.9976	96.5
Hydrazine	8.6	38.8	1.0	5	0.7	0.9996	73.2
DABCO	9.4	45.2	0.3	2	0.5	0.9999	106.8
Piperazine	11.3	65.5	0.3	1	0.8	0.9999	101.4
Ethylenediamine	13.8	85.8	0.3	1	0.9	0.9995	87.1

[a] Monoprotic bases were analyzed via method 1 while polyprotic bases were analyzed via method 2.
[b] $W_{1/2}$ measured for 0.1 mg/mL level.
[c] Detection limit experimentally determined when S/N = 3 (average of 3 injections).
[d] Precision of peak areas from 6 replicate injections at 0.1 mg/mL level.
[e] Correlation coefficient from linear least squares regression (range = 0.005–0.1 mg/mL).
[f] % Recovery for 0.5% of analyte spiked into 5 mg/mL of primidone.

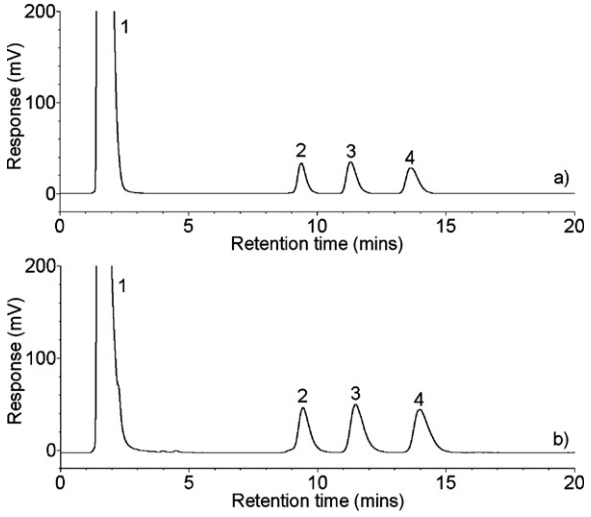


Fig. 5. Comparison of HPLC-NQAD and HPLC-CAD for separation of doubly charged volatile bases. Each compound at a level of 0.5% relative to 5 mg/mL of primidone in methanol diluent; 1 = primidone, 2 = DABCO, 3 = piperazine, 4 = ethylenediamine; (a) HPLC-NQAD; (b) HPLC-CAD. HPLC conditions per method 2.

Analytes were spiked at an impurity level concentration of 0.5% relative to 5 mg/mL of primidone. Methanol was used as diluent for this experiment due to the higher solubility of primidone in methanol compared to acetonitrile and water. No peak distortion was observed from the mismatch of mobile phase and diluent solvents. The average percent recoveries were 98% and 93% from the NQAD and CAD, respectively. All recoveries were within 70–130%, which is acceptable for impurity analysis in the pharmaceutical industry. The lowest recoveries were observed for hydrazine and ethylenediamine, and this may point to higher oxidative susceptibility. Approaches to stabilize these analytes might be necessary for more complex matrices.

3.8. Influence of physicochemical properties on analyte detectability and sensitivity

The pK_a of the mobile phase modifier or analyte is a major determinant of the ability to form a detectable salt. This is supported by data in Table 2 showing no discernable responses for any analytes when using acetic acid (pK_a = 4.79) and formic acid (pK_a = 3.74) modifiers whereas moderate to high responses were obtained when using mobile phase modifiers with pK_a < 3. To further test this claim, two organic superbases, TMG and DBU (pK_a of 13.6 and 11.82 for the conjugate acids, respectively [40]), were analyzed by FIA using previously described method conditions, and the responses were high for all modifiers except acetic and formic

acids (average peak areas of 904 and 1093, respectively). A moderate response (peak area of 662) was even observed for DBU when using formic acid as modifier.

A second property that appears to influence sensitivity is the number of ionizable groups. From the data in Table 2, use of oxalic acid as modifier, which was the only polyprotic acid in the study, resulted in the highest responses for all monoprotic basic analytes. In addition, the three polyprotic basic analytes had high responses with all modifiers except formic and acetic acid. Furthermore, the optimal NQAD evaporator temperature (from Fig. 3) was significantly higher for all polyprotic bases, suggesting that the salt particles formed during aerosolization were physically more stable.

Lastly, steric effects (i.e., accessibility of the ionizable site) also appeared to influence sensitivity. This was most apparent in the cases of the two hindered amines, DIPEA and triethylamine, which had the highest detection limits. Yet there was no significant difference in pK_a compared to secondary amines, diethylamine and isobutylamine.

One additional experiment was performed to test these observations. The responses for two sterically hindered, less basic analogs of morpholine and hydrazine were measured using the FIA conditions previously described. Due to their lower basicity and steric hindrance, it was expected that they would exhibit lower response. This was found to be the case. For N-methylmorpholine, responses were lower for all modifiers tested compared to morpholine (average peak areas of 205 and 408, respectively). For N,N-dimethylhydrazine, responses were markedly lower for all modifiers tested compared to hydrazine (average peak areas of 30 and 206, respectively).

4. Conclusion

HPLC with aerosol-based detection, such as CAD or NQAD, was extended to the analysis of volatile bases via formation of low volatility salts of these analytes with mobile phase additives. By screening a large test set of compounds, three physicochemical properties that influence detectability and sensitivity were apparent. First, detectability was directly related to the difference in pK_a between the acidic modifier and conjugate acid of the volatile base. Second, the number of ionizable sites on the modifier or analyte was proportional to response. Although, the free acid form of the modifier should exhibit high vapor pressure to minimize background noise. Third, compounds with sterically hindered ionizable groups had lower response.

Based on these principles, two isocratic, HILIC methods were developed: one for monoprotic bases and one for polyprotic bases. TFA modifier was employed for both methods, as it was able to greatly enhance detectability of most analytes. For the case of ammonia, a small concentration (0.2 mM) of HCl was also needed to detect this compound. The measured LOD (1–27 ng), precision ($\leq$2.1%), and recovery (70–130%) indicated that these methods were appropriate for impurity analysis. These methods should prove attractive to chemists in a wide range of industries where volatile bases with poor UV chromophores need to be analyzed. This should be especially useful where the complexity of the sample matrix, availability of instrumentation, or analysis requirements rule out more conventional technology such as GC or IC.

Acknowledgements

This work was supported by the Merck New Technology Research Licensing Committee (NTRLC) which provided funding to purchase the instrumentation. We are also grateful to Christopher Welch for careful manuscript review.

References

[1] T. Gorecki, F. Lynen, R. Szucs, P. Sandra, Anal. Chem. 78 (2006) 3186.
[2] K. Mojsiewicz-Pienkowska, Crit. Rev. Anal. Chem. 39 (2009) 89.
[3] J.P. Hutchinson, J. Li, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, J. Chromatogr. A 1217 (2010) 7418.
[4] R. Cohen, W. Stevens, Eastern Analytical Symposium, Somerset, NJ, 2009.
[5] M.D. Lantz, D.S. Risley, J.A. Peterson, J. Liq. Chromatogr. Rel. Technol. 20 (1997) 1409.
[6] D.S. Risley, B.W. Pack, LC–GC N. Am. 24 (2006) 776.
[7] C. McClintic, D.M. Remick, J.A. Peterson, D.S. Risley, J. Liq. Chromatogr. Rel. Technol. 26 (2003) 3093.
[8] U. Holzgrabe, C.J. Nap, S. Almeling, J. Chromatogr. A 1217 (2010) 294.
[9] Z. Huang, M.A. Richards, Y. Zha, R. Francis, R. Lozano, J. Ruan, J. Pharm. Biomed. Anal. 50 (2009) 809.
[10] K. Zhang, L. Dai, N.P. Chetwyn, J. Chromatogr. A 1217 (2010) 5776.
[11] C. Elfakir, P. Chaimbault, M. Dreux, J. Chromatogr. A 829 (1998) 193.
[12] K. Petritis, H. Dessans, C. Elfakir, M. Dreux, LC–GC Europe 15 (2002) 98.
[13] C. Schonherr, S. Touchene, G. Wilser, R. Peschka-Suss, G. Francese, J. Chromatogr. A 1216 (2009) 781.
[14] R. Diaz-Lopez, D. Libong, N. Tsapis, E. Fattal, P. Chaminade, J. Pharm. Biomed. Anal. 48 (2008) 702.
[15] C. Brunelli, T. Gorecki, Y. Zhao, P. Sandra, Anal. Chem. 79 (2007) 2472.
[16] A. Soman, M. Jerfy, F. Swanek, J. Liq. Chromatogr. Rel. Technol. 37 (2009) 1000.
[17] A. Blazewicz, Z. Fijalek, M. Warowna-Grzeskiewicz, M. Jadach, J. Chromatogr. A 1217 (2010) 1266.
[18] D. Kou, G. Manius, S. Zhan, H.P. Chokshi, J. Chromatogr. A 1216 (2009) 5424.
[19] K. Takahashi, S. Kinugasa, M. Senda, K. Kimizuka, K. Fukushima, T. Matsumoto, Y. Shibata, J. Christensen, J. Chromatogr. A 1193 (2008) 151.
[20] I. Marquez-Sillero, E. Aguilera-Herrador, S. Cardenas, M. Valcarcel, J. Chromatogr. A 1217 (2010) 1.
[21] L. Novakova, D. Solichova, P. Solich, J. Chromatogr. A 1216 (2009) 4574.
[22] R.W. Dixon, D.S. Peterson, Anal. Chem. 74 (2002) 2930.
[23] P.H. Gamache, R.S. McCarthy, S.M. Freeto, D.J. Asa, M.J. Woodcock, K. Laws, R.O. Cole, LC–GC N. Am. 23 (2005) 150.
[24] S. Fekete, K. Ganzler, J. Fekete, J. Chromatogr. A 1217 (2010) 6258.
[25] J. Olsovska, Z. Kamenik, T. Cajthaml, J. Chromatogr. A 1216 (2009) 5774.
[26] K. Takahashi, S. Kinugasa, R. Yoshihara, A. Nakanishi, R.K. Mosing, R. Takahashi, J. Chromatogr. A 1216 (2009) 9008.
[27] U. Holzgrabe, C.-J. Nap, T. Beyer, S. Almeling, J. Sep. Sci. 33 (2010) 2402.
[28] L.B. Allen, J.A. Koropchak, Anal. Chem. 65 (1993) 841.
[29] L.B. Allen, J.A. Koropchak, B. Szostek, Anal. Chem. 67 (1995) 659.
[30] J.A. Koropchak, C.L. Heenan, L.B. Allen, J. Chromatogr. A 736 (1996) 11.
[31] J.P. Hutchinson, J. Li, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, J. Chromatogr. A 1218 (2011) 1646.
[32] I. Poels, L.J. Nagels, Anal. Chim. Acta 440 (2001) 89.
[33] R.W. Slingsby, J. Chromatogr. 371 (1986) 373.
[34] F. Qu, M. Shifen, Microchem. J. 63 (1999) 317.
[35] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, J. Chromatogr. A 870 (2000) 245.
[36] R. Pennanec, LC–GC Europe 17 (2004) 44.
[37] L.M. Nair, J.O. Werling, J. Pharm. Biomed. Anal. 49 (2009) 95.
[38] H.Y. Eom, S.-Y. Park, M.K. Kim, J.H. Suh, H. Yeom, J.W. Min, U. Kim, J. Lee, J.-R. Youm, S.B. Han, J. Chromatogr. A 1271 (2010) 4347.
[39] N. Vervoort, D. Daemen, G. Torok, J. Chromatogr. A 1189 (2008) 92.
[40] T. Ishikawa, in: T. Ishikawa (Ed.), Superbases for Organic Synthesis: Guanidines, Amidines, Phosphazenes and Related Organocatalysts, John Wiley & Sons, Ltd., West Sussex, UK, 2009.