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Performance of charged aerosol detection with hydrophilic interaction chromatography

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ABSTRACT

The performance of the charged aerosol detector (CAD) was investigated using a diverse set of 29 solutes, including acids, bases and neutrals, over a range of mobile phase compositions, particularly with regard to its suitability for use in hydrophilic interaction chromatography (HILIC). Flow injection analysis was employed as a rapid method to study detector performance. CAD response was 'quasi-universal', strong signals were observed for compounds that have low volatility at typical operating (room) temperature. For relatively involatile solutes, response was reasonably independent of solute chemistry, giving variation of 12–18% RSD from buffered 95% ACN (HILIC) to 10% ACN (RP). Somewhat higher response was obtained for basic compared with neutral solutes. For cationic basic solutes, use of anionic reagents of increasing size in the mobile phase (formic, trifluoroacetic and heptafluorobutyric acid) produced somewhat increased detector response, suggesting that salt formation with these reagents is contributory. However, the increase was not stoichiometric, pointing to a complex mechanism. In general, CAD response increased as the concentration of acetonitrile in the mobile phase was increased from highly aqueous (10% ACN) to values typical in the HILIC range (80–95% ACN), with signal to noise ratios about four times higher than those for the RP range. The response of the CAD is non-linear. Equations describing aerosol formation cannot entirely explain the shape of the plots. Limits of detection (determined with a column for solutes of low k) under HILIC conditions were of the order of 1–3 ng on column, which compares favourably with other universal detectors. CAD response to inorganic anions allows observation of the independent movement through the column of the cationic and anionic constituents of basic drugs, which appear to be accompanied by mobile phase counterions, even at quite high solute concentrations.

1. Introduction

An important problem for high performance liquid chromatography (HPLC) is the limited choice of detectors that respond to compounds containing no UV/VIS chromophores. Charged aerosol detection (CAD) is a relatively new type of detector developed for use in HPLC over the last 10 years [1]. About 100 publications concerning the detector have appeared to date (e.g. [2–4]). The detector seems very suitable for the analysis of some pharmaceuticals and compounds of biomedical significance, at least in the reversed-phase (RP) mode [5], however, more detailed study is necessary to further understand its properties. Its response is dependent on the formation of aerosol particles (see Fig. 1), similar to techniques such as evaporative light scattering detection (ELSD) [6] and condensation nucleation light scattering detection (CNLSD) [7]. This dependence results in a response which is supposedly independent of solute molecular structure, giving a signal for any compound that is able to form stable aerosol particles. Therefore, CAD is potentially suitable for impurity analysis, particularly in pharmaceutical development where measurement by UV or mass spectrometry (MS) requires the use of standards that may be unavailable for unknown impurities. In CAD, the aerosol particle becomes charged through collision with positively charged nitrogen gas [8], which differs from MS interfaces which generate molecular ions rather than charged particles [9]. The present work aims to study the performance of the CAD, and investigate to what extent it may fulfil the requirements of a universal detector, particularly with regard to its use in hydrophilic interaction chromatography (HILIC). Clearly some factors influencing CAD behaviour are already understood, although commercial instruments have some differences from the prototype described by Dixon and Peterson [1]. These differences are sometimes ignored

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in the literature in discussions of the mechanism of operation of commercial instruments [10,11]. Nevertheless, the process in both may involve transfer of charge from the sheath gas (e.g. nitrogen) to the solute particles (see Fig. 1), which is distinct from the more direct exposure of the corona discharge to the eluent as occurs in atmospheric pressure chemical ionisation (APCI) sources used in mass spectrometry. As CAD response (along with that of all aerosol detectors) depends on the formation of solid particles, it is limited to solutes that have low volatility at the operating temperature. However, few studies have investigated in detail any relationship between volatility and detector signal. The ability to differentiate between solute and mobile phase determines the detection limit, which has been quoted as 0.1–1 ng sample on-column [2,12]. Salt buffers are often critical additives to HPLC mobile phases in any separation mode, but are potentially detrimental to CAD performance. In HILIC, salt buffers can lead to better peak shape than simple acid solutions [13–15], thus we wished to investigate their influence on CAD sensitivity. Furthermore, as with other aerosol-based detectors, detector response is dependent on organic solvent content. While changing detector response with organic solvent concentration has been investigated for its detrimental effect on response uniformity in gradient elution [16–18], high organic concentrations as used in HILIC may be advantageous for sensitivity as it should facilitate desolvation of particles in the CAD. Aerosol-based detectors are known to produce non-linear calibration curves [19], which can arise for different reasons in different detectors. For instance in the ELSD, it is due to both the non-linearity of aerosol formation and a change in detection mechanism with the size of aerosol particles [20]. The mechanism of detection in CAD is more straightforward than ELSD [5], and CAD calibration curves can be close to linear over small concentration ranges [8]. The detailed mechanism that causes non-linearity of CAD calibration curves and their profile has not been described to date. Detector response for aerosol-based detectors is believed to be mostly independent of solute chemistry [5]. However this factor has also not been investigated in much detail with respect to CAD for a sufficiently broad selection of solute structures.

Approximately 50% of drug active pharmaceutical ingredients (API) are salts [21], and many salt counter ions do not contain chromophores. An important benefit of CAD is the ability to detect solutes which do not contain chromophores, and thus it should respond to these counterions [22].

2. Experimental

2.1. Chemicals and reagents

A set of 29 probe compounds comprising acids, bases and neutrals (as used in a previous study [23]) was obtained from Sigma Aldrich (Poole, UK) and used as probes. Structural and physicochemical data are provided in Table 1. Log D values were calculated as the average from three different software packages: ACD version 12.0 (ACD Labs, Toronto, Canada), Marvin (Chem Axon, Budapest Hungary) and MedChem Designer (Simulations Plus, Lancaster, USA). Standards were diluted in the exact mobile phase from stock solutions typically at 10,000 mg/L made up in 50% ACN containing 0.1% FA. ACN (HPLC gradient grade), ammonium formate (AF), formic acid (FA) (LCMS grade), ammonium acetate (AA) and acetic acid (HPLC grade), were purchased from Fisher Scientific (Loughborough, UK).

2.2. Equipment and methodology

A Thermo UltiMate 3000 Rapid Separation Liquid Chromatography system was used for all experiments, comprising a quaternary pump, diode array detector (DAD) and either a Corona Ultra or Corona Veo CAD, with Chromeleon 7.2 software (Thermo, Germering, Germany). The CAD is a destructive detector, therefore the DAD and CAD detectors were connected in series in some experiments, with flow first through the DAD. Thermo Viper tubing (0.13 mm ID) was used as connection tubing. Data collection rates were 100 Hz for both DAD and CAD, due to narrow peak widths (typically 1 s at half height in flow injection analysis (FIA)). The Corona Ultra nebuliser (cross flow design similar to that used in atomic absorption spectrometry) was controlled at 22 °C with the evaporator tube at ambient temperature, while the Veo (concentric flow design similar to those used in mass spectrometry) nebuliser was at ambient temperature and the evaporator tube set to 30 °C. The Veo had a power function (PF) designed to 'linearise' data, which was set to either 0.67 (this simulates 'off'), 1.00 (the default) or 1.2 (optimised setting using experimental data, see below).

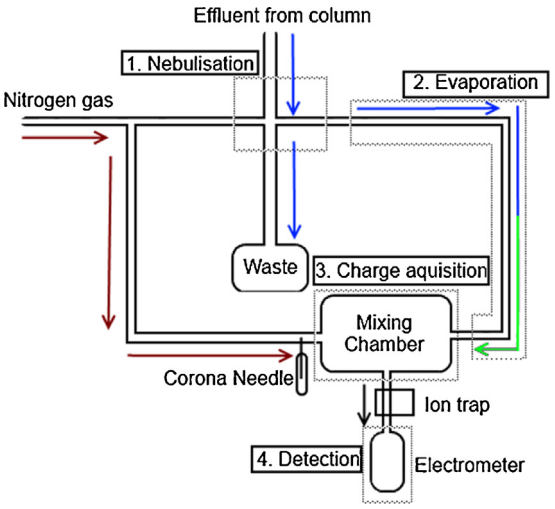


Fig. 1. Simple schematic of CAD operation.

An ethylene bridged hybrid (BEH) amide column (150 × 4.6 mm, particle size = 3.5 μm, Waters, Milford, USA) was used for determination of the detection limit, linearity and for the salt separation experiments. An Atlantis bare silica column (250 × 4.6 mm ID, particle size = 5 μm, Waters) was used for some salt composition experiments. The mobile phase was ACN-5 mM ammonium formate or ammonium acetate buffer (80:20, w/w) unless otherwise stated. The pH meter was calibrated in aqueous buffers and formic or acetic acid was used to adjust the aqueous portion to $_{w}{}^{w}$pH 3 or 5. Solutions at $_{w}{}^{w}$pH 6.8 were unadjusted 5 mM ammonium acetate. Care is necessary as pH calibration buffers can be a major source of non-volatile contaminants in the mobile phase.

In flow injection analysis (FIA), narrow bore tubing (75 μm × 1100 mm) was used in place of the chromatographic column to maintain sufficient backpressure. Samples for FIA were prepared at a concentration of 300 mg/L; injection volumes were 1 μL unless otherwise stated. Flow rate was 1 mL/min.

For calculation of retention factors, toluene is generally used as a void volume marker in HILIC with UV detection [14], but is too volatile for use with the CAD. Naphtho [2,3-a] pyrene appeared to be a suitable alternative for CAD.

3. Results and discussion

3.1.1. Detection limits (HPLC)

When applied to the impurity profiling of amino acid mixtures in nutritional infusion bags, CAD limits of quantitation (LOQ) were reported at 10 ng on-column (1 μg/mL; 10 μL injection) [22]. The

Table 1
Identities and physico-chemical characteristics of test compounds.

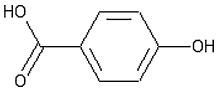
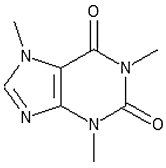
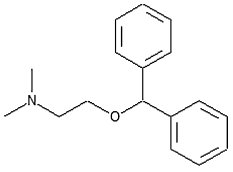
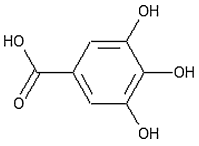
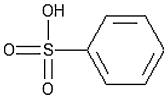
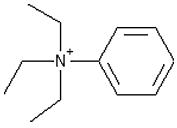
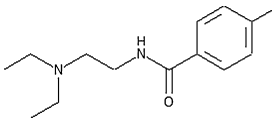
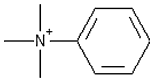
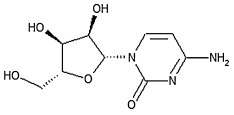
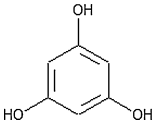
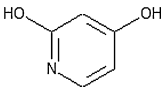
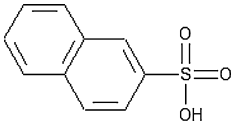
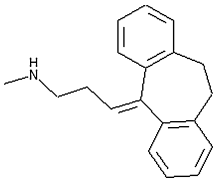
Solute	Structure	MW	MW salt	Log D (pH 3)[a]	BP (°C)	MP (°C)
4-Hydroxybenzoic acid		138	–	1.46	336[b]	213.5
Caffeine		194	–	−0.41	178	238
Diphenhydramine hydrochloride		255	292	−0.013	344[b]	168[d]
3,4,5-Trihydroxy benzoic acid (THBA)		170	–	0.6	501[b]	261.5
Benzenesulfonic acid (BSA)-Na salt		158	180	−2.35	319[c]	65.5
Benzenetriethylammonium chloride (BTEAC)		192	228	−1.67	445	191
Procainamide hydrochloride		235	272	−2.38	422[b]	167[d]
Trimethylphenylam-monium chloride (TMPAC)		136	172	−2.01	[e]	247
Cytidine		243	–	−3.51	546[b]	225
Phluroglucinol		126	–	0.39	331[b]	204.5
2,4-Dihydroxypyridine		111	–	−0.4	510[b]	274
Naphthalene-2-sulfonic acid-Na salt (2-NSA)		208	230	−1.09	392[c]	124.5
Nortriptyline hydrochloride		263	300	0.94	403[b]	214[f]

Table 1 (*Continued*)

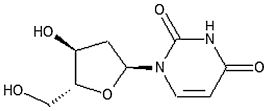
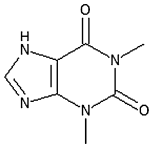
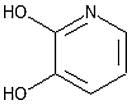
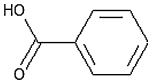
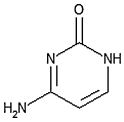
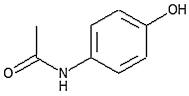
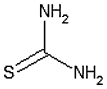
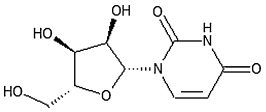
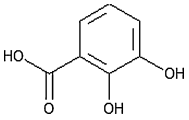
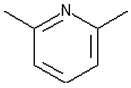
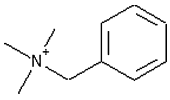
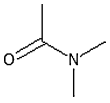
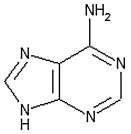
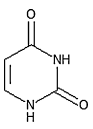
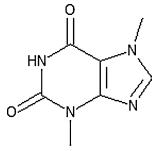
Solute	Structure	MW	MW salt	Log D (pH 3)[a]	BP (°C)	MP (°C)
2′-Deoxyuridine		228	–	−1.49	519[c]	165
Theophylline		180	–	−0.31	454[b]	272
2,3-Dihydroxypyridine		111	–	−0.62	441[b]	245
Pyridine		79	–	−0.9	115	−41.6
Benzoic acid		122	–	1.65	249	122.4
Cytosine		111	–	−2.86	283[c]	322.5
Paracetamol		151	–	0.58	388[b]	169.75
Thiourea		76	–	−0.82	187[b]	177
Uridine		244	–	−2.06	556[c]	165
2,3-Dihydroxybenzoic acid		154	–	0.86	344	205
2,6-Dimethylpyridine		107	–	−1.11	144	−5.8
Benzenetrimethylammonium chloride (BTMAC)		150	186	−2.18	[e]	239
N,N-Dimethylacetamide		87	–	−0.48	166	−20
Adenine		135	–	−1.62	554[b]	220
Uracil		112	–	−0.97	367[c]	335

Table 1 (*Continued*)

Solute	Structure	MW	MW salt	Log D (pH 3)[a]	BP (°C)	MP (°C)
Theobromine		180	–	−0.65	483[c]	357

[a] Average log D from three packages (see Section 2).
[b] Predicted at 760 mmHg using ACD labs program (see Section 2).
[c] Predicted from [42].
[d] Value from [41].
[e] Data not available.
[f] Value from [36].

authors used a signal to noise ratio of 5:1 to determine the LOQ [22], whereas common practice is to use a S:N of 10. Ramos et al. reported CAD limits of detection (LOD) 4 times lower than ELSD for analysis of membrane phospholipids by normal phase HPLC [24]. Hutchinson et al. reported that the LOD for 11 solutes was over 5 times smaller using CAD compared with ELSD [19]. Detection limits for an acid, neutral and basic solute in our experiments are shown in Table 2 for a typical HILIC mobile phase (5 mM ammonium formate pH 3 in 80% ACN). A signal to noise ratio of 3 was used as LOD and 10 for LOQ. The LOD of 1–3 ng and LOQ of 5–9 ng (both on column, 1 μL injections) compare favourably with other 'universal' detectors such as refractive index (LOD ~1 μg on column [25]) and ELSD (LOD 1–100 ng on column [5,11]). While the data in Table 2 was recorded for the BEH column, using the same mobile phase we did not observe serious noise or bleeding with the Atlantis column, as reported by Jia et al. [26]. Table 2 (and indeed most of our work) was based on use of an acidic mobile phase, whereas Jia et al. used unbuffered ammonium acetate that has approximately neutral pH in aqueous solution. This higher pH might have caused some dissolution of silica and thus noise in the CAD.

3.1.2. Calibration curves (HPLC)

It has been reported that over wide ranges of analyte concentration, (e.g. 1–1000 ppm) CAD response is non-linear, while over narrow ranges of analyte concentration, it is quasi-linear [8]. Using FIA, Hutchinson et al. investigated CAD calibration using sucralose, amitriptyline, dibucaine and quinine at concentrations of 1 μg/mL to 1 mg/mL (25 μL injections) [18]. Although not commented on by the authors, their data suggest a low quasi-linear range below approximately 0.05 mg/mL and an upper quasi-linear linear range between 0.4 and 1.0 mg/mL. Fig. 2 shows calibration plots for the acid BSA, the base nortriptyline and the neutral uridine over the range 1–1000 mg/L, using a BEH amide column with the CAD (Fig. 2a) and for UV detection (Fig. 2b). Hutchinson et al. [18] reported maximum reliable CAD response at 70% ACN, and recommended this concentration for applications requiring maximum sensitivity. Retention is often poor at this ACN concentration in HILIC; a typical range for HILIC is 70–95% ACN. We therefore selected 80% ACN for our study. UV detection shows excellent linearity over the entire range (lowest R^2 0.9995, for Nortriptyline). Our results indicate also a lower quasi-linear range (1–100 mg/L) and an upper quasi-linear range (400–1000 mg/L) for CAD. The calibration curves appear to be sublinear (concave to the *x*-axis). The consistent general shape of CAD calibration curves (Fig. 2a; also [18]) is perhaps described by some empirical formula, however no such interpretation has been attempted to date.

Table 2
Detection limits for charged aerosol detection in HILIC conditions. HPLC, mobile phase 80% ACN, 5 mM ammonium formate pH 3.

Solute	LOD/mg per L	LOQ/mg per L
BSA	3	9
Uridine	2	6
Nortriptyline	1	5

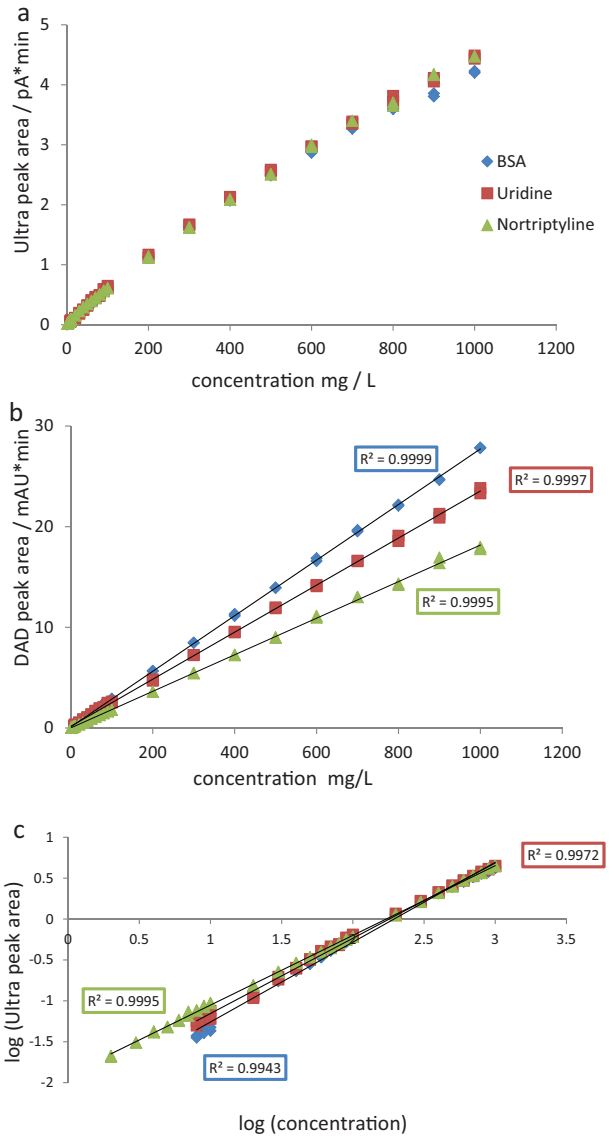


Fig. 2. Peak area vs. concentration for a neutral (Uridine), acid (BSA) and base (Nortriptyline) (a) CAD Ultra, (b) DAD and (c) log/log CAD Ultra (HPLC, mobile phase 80% ACN, 5 mM ammonium formate pH 3).

The particle size d_p as described for ELSD (1) is given by the equation:

$$d_p = d_D \left(\frac{c}{\rho_p} \right)^{1/3} \quad (1)$$

where d_D is the diameter of the droplet, c is the analyte concentration and ρ_p is the density of the particle. The aerosol formation step should be similar in ELSD and CAD. It was assumed by Charlesworth that aerosol particles are approximately spherical for ELSD [27], as confirmed by fundamental studies [28]. Dixon and Peterson assumed this for their prototype aerosol detector [1]. The authors of that study reported that the prototype's detector sensitivity (defined as the gradient of the calibration curve) was lower for solute particles with diameters greater than 10 nm, i.e. the gradient of a plot of response vs. concentration is steep at low concentrations and becomes shallower at higher concentrations. The surface area of a sphere is given by:

$$A = 4\pi r^2 \quad (2)$$

It follows that:

$$A = d_D^2 \pi \left(\frac{c}{\rho_p} \right)^{2/3} \quad (3)$$

Thus, the surface area of the particle is theoretically proportional to solute concentration via the power equation (3). Therefore an increase of solute concentration results in a larger particle surface area and higher detector response. The exponent of (3) is not unity. This theory assumes that adsorption of charged nitrogen onto the particles is a linear (Langmuirian) relationship with surface area. Equation (4) simplifies the relationship between CAD response and analyte concentration (c). Plotting CAD response vs. concentration should yield a non-linear curve with a fractional exponent of 2/3. This relationship is in agreement with work from the manufacturer of the charged aerosol detector [29].

$$\text{CAD Response} \propto c^{2/3} \quad (4)$$

Taking logs gives (5), a simple linear relationship, where log of the coefficient a becomes the intercept and the slope is 2/3.

$$\log(\text{CAD Response}) = \frac{2}{3} \log c + \log a \quad (5)$$

Fig. 2c shows log/log calibration plots for Nortriptyline, BSA and Uridine. Linearity was very good, much-improved compared to the raw data (Fig. 2a) giving R^2 values of 0.994–1.000; other authors have noted similary good linearity of these log/log plots [24,30]. The gradient of these plots ranged from 0.853 to 0.976, (as expected from the sublinear nature of Fig. 2a) which clearly is larger than the value of 2/3 expected from equation (4). Chaminade et al. reported gradients of CAD log/log calibration plots between 0.79 and 1.11 for membrane phospholipids using normal phase separation [24]. Nevertheless, log/log plots seem a pragmatic way to calibrate the detector. Newer CAD models such as the Corona Ultra RS and Corona Veo contain an in-built 'power function' feature, which is intended to 'linearise' data. This function appears to be based broadly on the arguments presented above, although its operation is proprietary. The use of a user-inputted power function of 1.2 on the Corona Veo gave linearity similar to the log/log plots discussed.

We investigated the possible effect of solute density on detector response as it is a factor in equation (3), but no apparent relationship was indicated (results not shown).

Table 3
Peak areas of BTEAC, BTEABr and BTEAI by FIA and HPLC. Mobile phase 80% ACN, 5 mM ammonium formate pH 3.

Compound	Peak area of $BTEA^+$ (FIA)	Peak area of $BTEA^+$ (HPLC)	Proportion of compound as $BTEA^+$
BTEAC	1.70	2.89	84%
BTEABr	1.51	2.58	71%
BTEAI	1.39	2.28	60%

3.2. Response universality and uniformity

3.2.1. Flow injection analysis

FIA is a rapid method of determining detector response, avoiding any problems of interference from the column (e.g. irreversible adsorption of part of the injected solute). Problems have been reported of poor reproducibility of peak area at low solute concentrations by FIA [17]. It appears that this problem may be related to disturbances shown in blank injections of pure mobile phase. These were minimised by using analyte concentrations of 300 mg/L, which gave blank disturbances that were very small in comparison with the analyte signal.

3.2.2. Effect of solute salt composition on response (HPLC; FIA)

To obtain net neutrality, ionised solutes must be associated with a counterion. This counterion is usually assumed to originate from the mobile phase, although it is conceivable that the counterion from the injected salt is involved, dependent on solute concentrations and mobile phase conditions. To investigate this further, the chloride, bromide and iodide salts of the quaternary ammonium compound benzyltriethylammonium (BTEA) were prepared at 300 mg/L of salt (e.g. 300 mg/L of BTEAC), and individually separated by HPLC using a BEH Amide column (80% ACN, 5 mM AF pH 3) (Fig. 3a–c). The salts were also analysed by FIA, with identical mobile phase. For analysis by HPLC, peak areas for the same injected mass of each salt decreased for the cationic moiety in the order BTEAC > BTEABr > BTEAI (Table 3). This result is in agreement with the cationic part of the salt contributing a decreasing fraction of the total mass as the anion gets larger (chloride to iodide). For HPLC analysis, the result indicates that the solute cation may be accompanied by formate anions during passage through the column, as the injected solute cation and anion clearly separate on the column (Fig. 3a–c). Note also the different retention times of chloride, bromide and iodide in these Figures. However, the same pattern of detector response was found by FIA, which involves no separation process. The standards were first diluted from the stock solutions (10,000–300 mg/L) with the FIA/HPLC mobile phase and injected into the same solution. This suggested that the large excess of sample diluent formate anions (see Section 2) and mobile phase buffer anions (5 mM AF pH 3) largely replace the solute (halide anions) in solution, which may influence the subsequent formation of aerosol particles. Thus the solute halide ions may have little contribution to the overall response even in FIA. A loading study for nortriptyline hydrochloride was carried out to further investigate the separation of the anion and cation in the HPLC process, up to much higher concentrations than those used in FIA. The salt was dissolved in the exact mobile phase at concentrations from 100 to 10,000 mg/L; separations were carried out on the BEH Amide and also on an Atlantis silica column. Fig. 3d and e show distinct peaks for the nortriptylinium cation and the chloride anion at all concentrations on the amide and bare silica columns respectively. In the separation of amino acids by electrostatic repulsion-hydrophilic interaction chromatography (ERLIC), Alpert [33] showed a symmetrical peak for arginine when the solute was dissolved in mobile phase −10 mM triethylaminephosphate (TEAP) pH 2 in 70% ACN, using a Polywax LP column. The peak

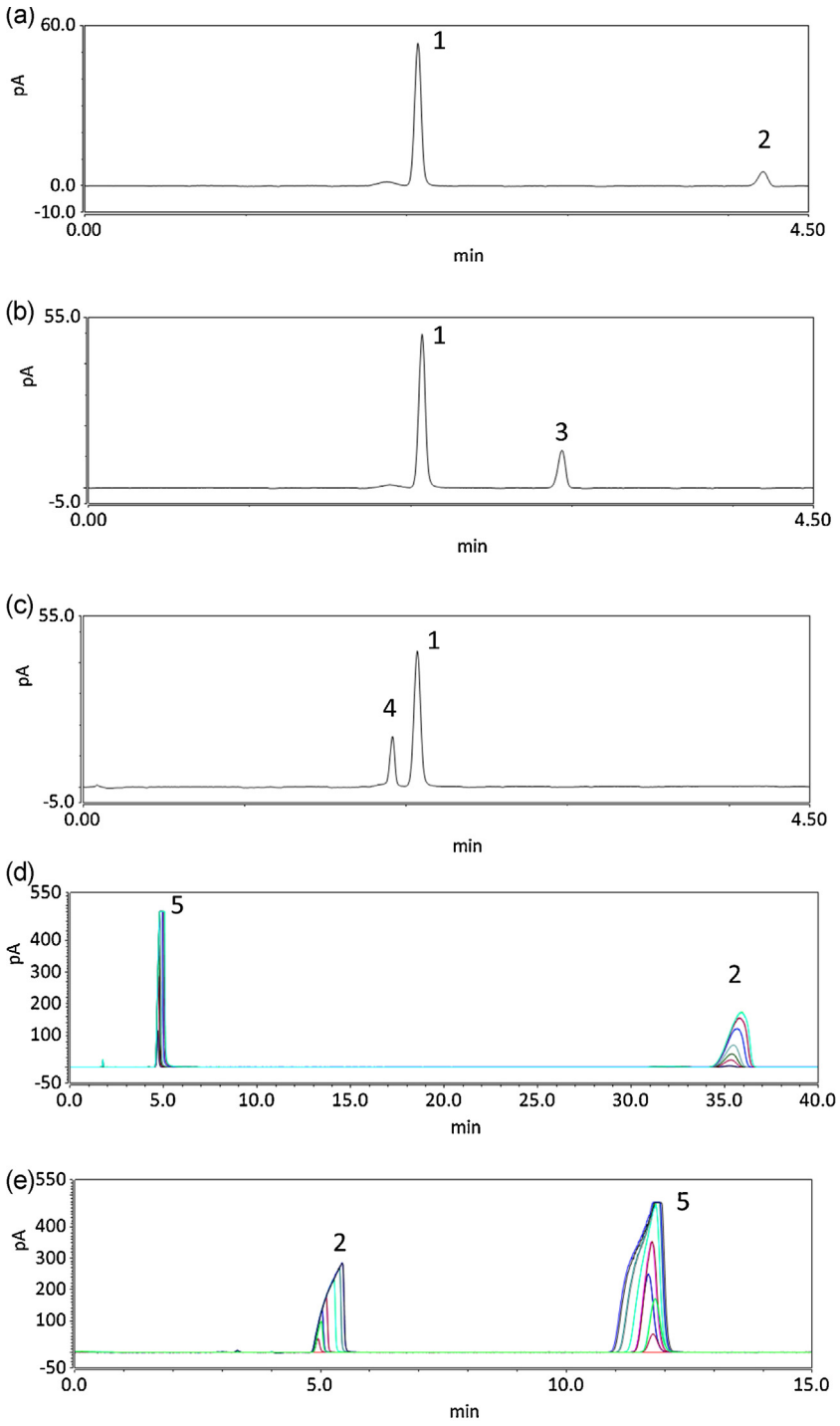


Fig. 3. HILIC-CAD separation and detection of the salts (a) benzyltriethylammonium chloride, (b) benzyltriethylammonium bromide, (c) benzyltriethylammonium iodide; (d)–(e) nortriptyline hydrochloride. Peak identities 1 = benzyltriethylammonium, 2 = chloride, 3 = bromide, 4 = iodide, 5 = nortriptylinium. HPLC, mobile phase 80% ACN for (a)–(c), 95% ACN for (d), 90% ACN for (e) all containing 5 mM ammonium formate pH 3, Atlantis column for (e), BEH Amide column for all others. Nortriptyline hydrochloride concentration 100–10,000 mg/L, injection volume 10 μL, others 300 mg/L, injection volume 1 μL.

was attributed to arginine phosphate. However, when the solute was dissolved instead in triethylaminemethylphosphonate (TEA-$MePO_3$) an additional peak appeared at earlier retention time, with a continuum evident between the peaks. It was suggested that the earlier peak was due to arginine molecules that had retained $MePO_3$ as the counterion, while some slow counterion exchange takes place with the mobile phase. Our data in Fig. 3 show a different behaviour, as they suggest independent migration of the solute anion and cation through the column. Clearly, the result is likely to be influenced by the exact combination of solute, mobile phase buffer (and their concentrations), and stationary phase used. Fig. 3d shows detector overload for the modestly-retained nortriptyline (k = 1.9) above 20 μg sample load on the BEH amide column, but no evidence of detector overload for the well-retained chloride (k = 21) even at 100 μg sample load. The chloride peak continues to increase in size even at the highest sample loads. Fig. 3e using the Atlantis column shows detector overload for the nortriptylinium cation only above 80 μg sample load and none for the chloride

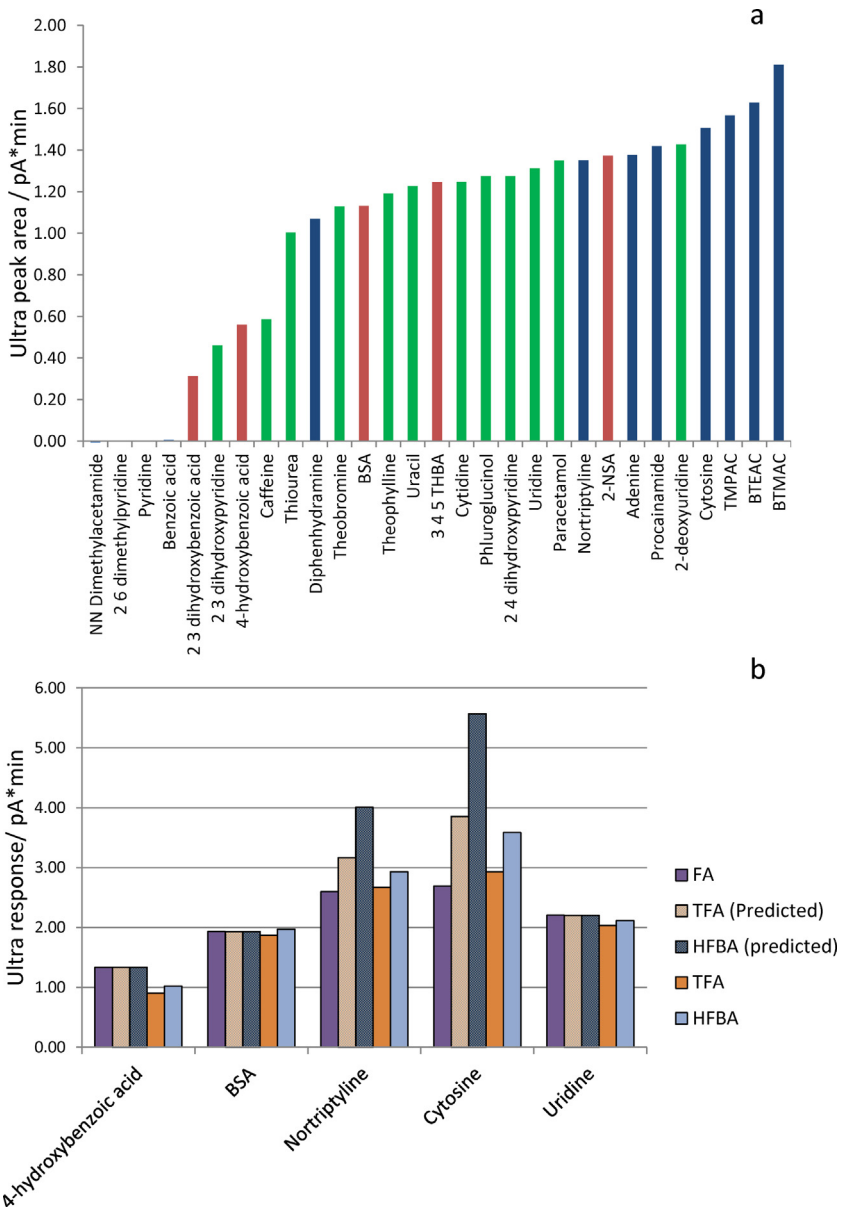


Fig. 4. (a) CAD response for 29 compounds (FIA, mobile phase 80% ACN, 5 mM ammonium formate pH 3). Blue = bases, red = acids, green = neutrals. (b) CAD Ultra data in dilute acids for the bases nortriptyline and cytosine, acids BSA and 4-HBA and the neutral Uridine (FIA, 80% ACN, FA (0.1% v/v) vs. TFA (0.2%) vs. HFBA (0.345%)). Predicted values from ratios explained in 3.4.3 in hashed-line bars. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

anion, with peaks continuing to behave independently. These data suggest a detector dynamic range of 1 ng to over 20 μg, i.e. over 4 orders of magnitude. The results indicate that even at the highest concentrations studied, a plateau in the chloride response is not attained, suggesting that no association of chloride with nortriptyline occurs. The elution order of solute anion and cation reversed when switching from the BEH Amide (Fig. 3d) to the Atlantis bare silica column (Fig. 3e), due to much stronger cation exchange retention of nortriptyline on the Atlantis column [23].

3.2.3. *Response uniformity (FIA)-dependence on solute and mobile phase buffer*

CAD peak areas were measured for injection of 300 ng of the 29 compounds in 80% ACN, 5 mM AF pH 3using FIA (Fig. 4a). The relative standard deviation (RSD) of the response for 21 compounds (omitting 8 with no or low response N,N-dimethylacetamide to caffeine) was 14% in this mobile phase, which shows reasonable uniformity considering the diverse structures of the compound set. Greater response uniformity for CAD in comparison with UV detection is also seen in Fig. 2a and b. Response appeared somewhat higher for basic compounds (shown in blue) than neutrals (green), albeit with some overlap (Fig. 4a). This observation is unexpected and unreported to date, as the production of physical particles by aerosol-based detectors should be independent of solute chemistry. Ionogenic compounds are often available in their salt form (e.g. Nortriptyline 300 mg/L was prepared as 300 mg/L of Nortriptyline HCl salt). While neutral compounds would not be expected to interact strongly with mobile phase buffer constituents in particle formation, this is clearly a possibility for ionogenic compounds, and may be responsible for the differences in response.

The mean response for the same 21 compounds was compared for additives commonly used in HILIC in addition to ammonium formate (AF) including formic acid (0.100%, v/v) (FA), ammonium acetate (AA), trifluoroacetic acid (0.200%, v/v) (TFA), and

Table 4
Peak areas and uniformity of response for 21 compounds in a selection of HILIC mobile phases using Flow Injection Analysis.

	Mobile phase									
	95% ACN 5 mM AF pH 3	80% ACN 5 mM AF pH 3	50% ACN 5 mM AF pH 3	10% ACN 5 mM AF pH 3	80% ACN 5 mM AF pH 5	80% ACN 5 mM AA pH 5	80% ACN 5 mM AA pH 6.8	80% ACN FA (0.1% v/v) [a]	80% ACN TFA (0.2% v/v) [a]	80% ACN HFBA (0.345% v/v) [a]
Mean Ultra response/pA min	1.83	1.37	1.21	0.37	1.37	1.39	1.37	1.24	1.14	1.38
Uniformity of response (RSD)	12%	14%	15%	18%	13%	12%	14%	13%	23%	30%

[a] Acids FA, TFA and HFBA molar concentration each 26.5 mM.

heptafluorbutyric acid (0.345%, v/v) (HFBA) (Table 4). The acid solutions were equimolar (26.5 mM). The mean response at 80% ACN concentration did not appear to be greatly affected when changing the pH of the salt buffer, or simple acid modifiers. However, the spread of response to the individual compounds was greater in mobile phases of equimolar TFA and HFBA (23% and 30% RSD, respectively) than for the other mobile phases. For the particular case of *ionised* solutes, association with mobile phase counterions of increasing mass might be expected to produce an increase in CAD response, giving some explanation of these data. Thus for analysis of cationic solutes, the response might increase in the order formate, trifluoroacetate (TFA) and heptaflurobutyrate (HFBA) whose anions have molar mass 45, 113 and 213, respectively. Koupparis and co-workers [31] found that the response for the hydrophilic antibiotic Amikacin using ELSD increased for TFA over FA, further increasing for higher concentrations of TFA. In addition, they evaluated HFBA and nonafluoropentanoic acid (NFPA), claiming that their ELSD responses increased in relation to the mass of the anion of these strong acids. However, the concentration of acid was not kept constant between datasets, either in terms of v/v or molar concentration. If response indeed increases in proportion to the added mass of a heavier anion, the ratio of response for strongly basic solutes, e.g. nortriptyline should be in the order nortriptyline formate:nortriptyline trifluoroacetate:nortriptyline heptafluorobutyrate 1.00:1.22:1.54. To investigate this hypothesis, FIA was performed in FA, TFA and HFBA each at a concentration of 26.5 mM. The compounds used were the stronger base nortriptyline, the weak base cytosine, neutral uridine as a control, the strong acid benzenesulfonic acid and the weak acid 4-HBA. A solute concentration of 600 mg/L was used, which is somewhat higher than used above because of higher baseline disturbances from TFA and HFBA compared to FA. The results (Fig. 4b) show that nortriptyline response did not follow the predicted ratios, with response of 1.00:1.03:1.13 for the FA, TFA and HFBA, respectively. The weak base cytosine followed the same trend, with response increasing in the ratio 1.00:1.09:1.33 for FA, TFA and HFBA but not in line with the predicted increase of 1:1.43:2.07.

It is apparent from Fig. 4a that neutral solutes have broadly lower response than ionised basic solutes, although the cause of this is unexplained to date. There have been no published reports of mobile phase pH affecting small molecule solute CAD response, therefore the weak acid 4-HBA was expected to also be unaffected by choice of acid buffer. However, Fig. 4b shows a decrease in response for 4-HBA in both TFA and HFBA compared with FA (33% decrease in TFA compared to FA). TFA and HFBA are capable of neutralising 4-HBA under the conditions used; decreasing the degree of ionisation of 4-HBA in the stronger acid would result in a predominantly neutral form of the solute reaching the detector. BSA, which is deprotonated at all pH values, was unaffected by the choice of acid buffer. Uridine, which is neutral under these conditions showed small reductions in response in TFA and HFBA (less than 10% reduction in peak area). Khandagale et al. described the CAD solute plug as a 'plume' [32], which travels within the detector after nebulisation. The plume has only a finite period of time to undergo all the processes required to produce a peak in the CAD (Fig. 1). We estimated the detector residence time at ~1 s using an effective detector volume of 14 μL reported by the manufacturer [3] and a flow rate of 1 mL/min. The process of forming aerosol particles by evaporation of aerosol droplets is possibly analogous to crystal formation from bulk solution. Ionic solids have much higher melting points than solids of neutral compounds and it is well-known that optimum growth rates for ionic crystals are at least a factor of 10 times greater than for molecular crystals, due to the high strength of coulombic intermolecular interactions relative to weaker vander-Waals and London dispersion forces [34]. Perhaps ionogenic solutes are better able to form stable aerosol particles within this short time window, compared to neutrals which are held together by weaker interactions.

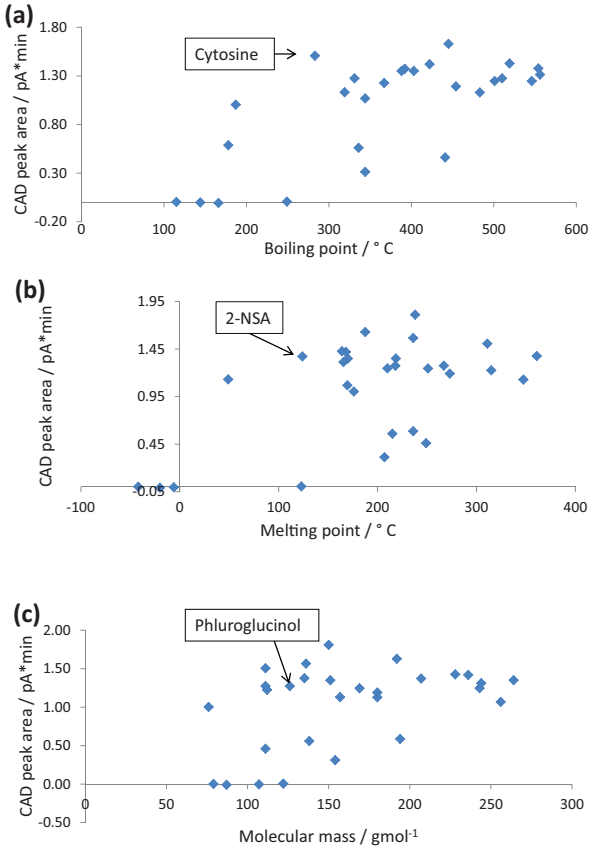


Fig. 5. CAD response for 29 compounds, plotted against (a) boiling point, and (b) melting point; (c) molecular mass (FIA, conditions as per Fig. 2).

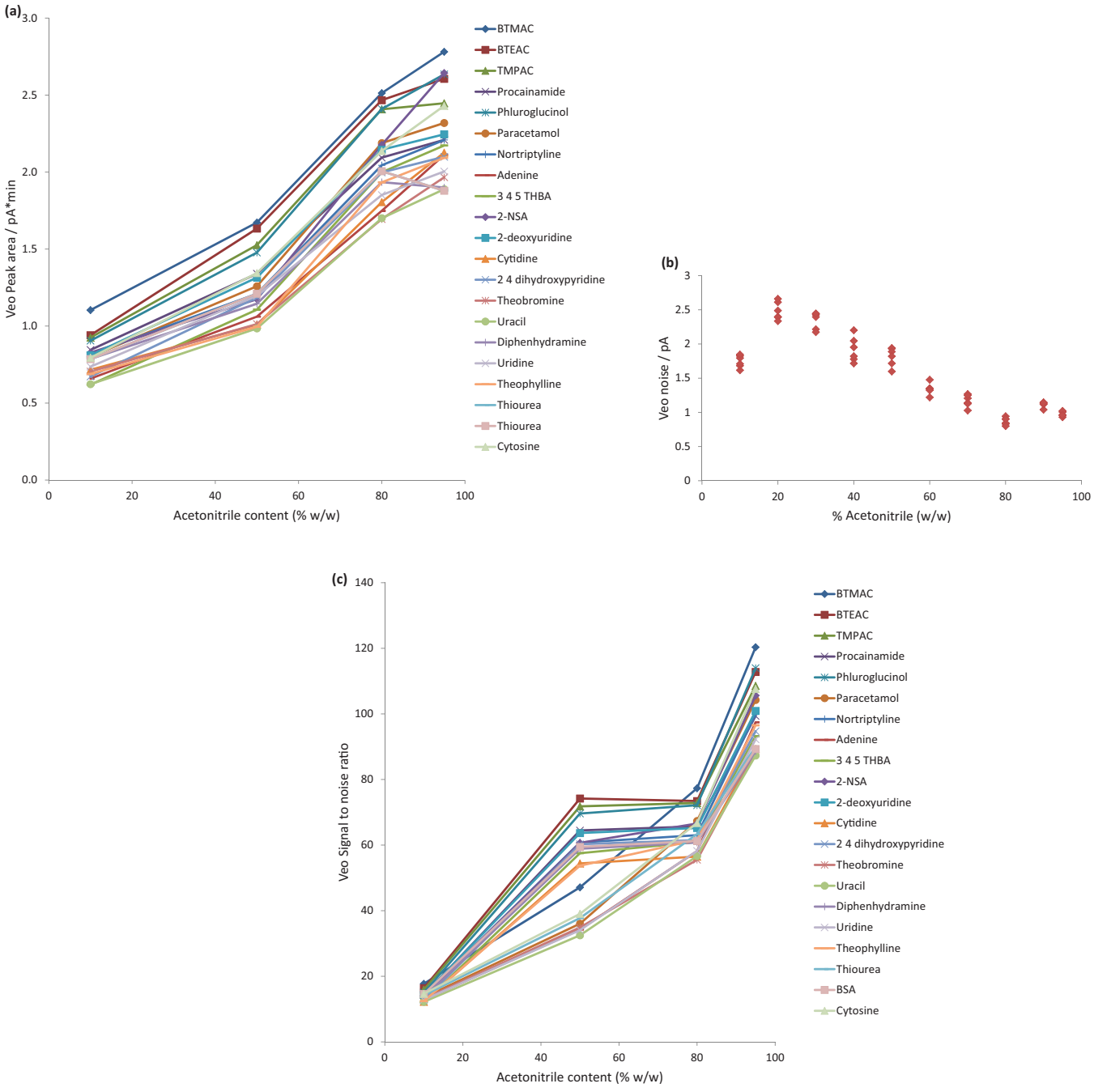


Fig. 6. Effect of organic solvent content on (a) peak area, (b) signal to noise ratio and (c) noise (FIA, mobile phase 10–95% ACN, other conditions as per Fig. 2).

3.2.4. *Effect of solute volatility on response*

The CAD response using FIA for the set of 29 diverse compounds (Fig. 4a) indicates that eight gave low or no response, three of which were liquids at room temperature (pyridine, 2,6-dimethylpyridine and N,N-dimethylacetamide). Solutes which respond poorly in CAD are too volatile to form stable aerosol particles [3]. Some relationship between solute volatility and response might be expected. Compounds that respond to CAD in general are those which have higher boiling point, melting point or molecular mass (Fig. 5a–c respectively), which are typical indicators of solute volatility. However, there are clear exceptions. 2,3-dihydroxybenzoic acid (bp 344 °C, mp 205 °C) gives only a third of the response of diphenhydramine (bp 344 °C, mp 168 °C). Benzoic acid has an appreciably high boiling point at 249 °C and molecular mass of 122 g/mol but gave no response whatsoever; thiourea is smaller with even lower boiling point (bp 187 °C, MW 76 g/mol, mp 177 °C) but its CAD response was strong. However, benzoic acid has a low mp (122 °C) and is known to be volatile, sufficiently so that its analysis by headspace GC-MS is possible [35]. The exceptions make a definitive cut-off point for a response difficult to predict purely from physico-chemical indicators of solute volatility. A minority group of four solutes including caffeine and 4-hydroxybenzoic acid (4-HBA) responded in CAD, but with peak areas ca. 40% lower than the strong CAD responders. The bp and mp points of these solutes are diverse. Indeed the data for 4-HBA (bp 336 °C, mp 214 °C), 2,3-dihydroxypyridine (bp 441 °C mp 245 °C), and 2,3-dihydroxybenzoic acid (bp 344 °C, mp 205 °C) overlap with those for strong responders. Caffeine is frequently referred to as 'semi-volatile' [36]. It has a sublimation point (bp Table 1) of 178 °C, considerably below its melting point of 238 °C [37] which may

explain its behaviour, although its sublimation temperature is still well above the detector settings. The CAD nebuliser is set to room temperature by default, and the evaporation process is also endothermic. Therefore this definition perhaps does not apply to CAD. It seems that experimental measurement (e.g. by FIA) is necessary to confirm response.

Fig. 5a–c show clearly that no relationships are apparent between response and melting point, molecular mass, and boiling point, with correlation coefficients close to zero. This result however fits with the claim of reasonably uniform response for non-volatile substances for the CAD. For ELSD, which might be expected to show similar effects, conflicting findings have been published on the effect of MW on detector response [6,20].

3.2.5. Effect of organic modifier (FIA)

Haddad and co-workers showed that CAD signal increases in proportion to the organic solvent concentration of the mobile phase for acetonitrile, acetone, isopropylalcohol and methanol [12,18]. These previous studies considered only peak areas and not the effect on uniformity of response or signal to noise ratio. Fig. 6a shows a plot of peak area vs. organic solvent concentration for 10–95% ACN. CAD response is roughly proportional to ACN concentration, in good agreement with earlier reports [12,18]. Our data show peak areas under typical HILIC conditions (70–95% ACN) roughly twice that of typical RPLC conditions (10–50% ACN). Excessively large droplets are removed by droplet selection inside the nebuliser (Fig. 1). It is possible that highly-aqueous mobile phases produce excessively large droplets by condensation, which are removed in the nebuliser, resulting in reduced CAD response. Smaller droplets in greater numbers are formed with the lower viscosity, density and surface tension of highly organic eluents [16], which perhaps explains the better transport efficiency in these conditions.

Table 4 shows that with AF pH 3 as buffer the uniformity of response was slightly improved in HILIC conditions compared with RPLC. Moreau found CAD background current was higher for organic solvents compared to water [38], attributable to their dry residue content (typically 2 ppm); it is therefore conceivable that the high ACN content might also cause high noise with HILIC mobile phases. We measured noise over a 30 min period after system stabilisation (Fig. 6b); noise was lower in higher ACN concentrations. As a result, the response of the CAD (measured in terms of S/N) was further improved (Fig. 6c) under HILIC conditions compared with the improvements in terms of crude solute peak area. It is possible that while organic solvents can indeed be a source of particulates, the fine aerosol particles are sufficiently small in organic-rich mobile phases [9] that they are poorly detected.

3.2.6. Effect of elevated temperature (FIA)

The simpler Corona Ultra design allows thermostatting of the nebuliser from 18 to 35 °C (with the objective only to prevent freezing when using normal phase solvents); the temperature of the evaporator tube is at ambient. Using HILIC mobile phase, the Veo had a temperature range of 27–88 °C for the evaporator tube. Fig. 7 shows CAD peak areas for the 29 test compounds at 30, 60 and 80 °C at high acetonitrile content (90% ACN). The effect of elevated temperature on the noise was small (not shown). There is clearly no advantage in using high evaporation temperatures for the majority of solutes: signal drops off dramatically in many cases, due to volatilisation of the solute. Nevertheless, evaporation temperature can be a tool for distinguishing analyte from background based on volatility, and it is possible that optimising this temperature in smaller increments (e.g. 5 °C) could be beneficial in some cases. Compounds such as caffeine which give moderate CAD response show a dramatic reduction in response at higher temperatures. Xanthine derivatives theobromine and theophylline, which

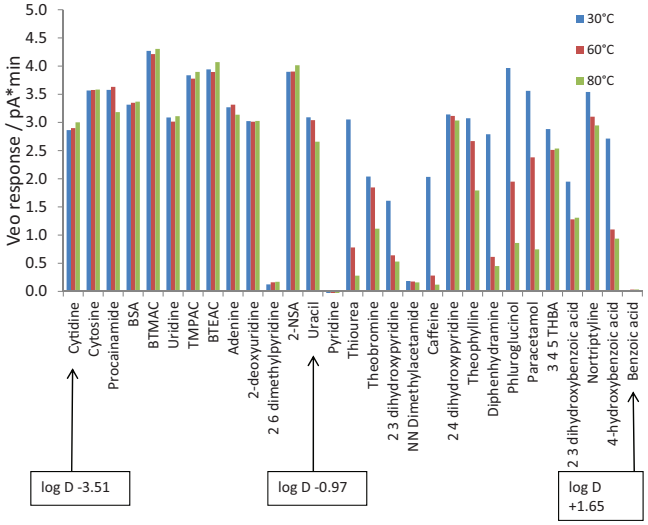


Fig. 7. Effect of elevated temperatures on Veo response in order of log *D* (−ve on left, +ve on right) (FIA, mobile phase 90% ACN, other conditions as per Fig. 2). Log *D* values were the average from three software packages (see Section 2) Blue = 30 °C, Red = 60 °C, Green = 80 °C. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

are structurally similar to caffeine, perform well at low temperatures, but also had low CAD response at temperatures of 60 °C and above. The base procainamide maintains good CAD peak area at the elevated temperature of 60 °C, whereas the base diphenhydramine shows a drop-off comparable to the xanthine derivatives. Procainamide is more hydrophilic than diphenhydramine, and this result suggested a possible relationship with solute log *D* values. Thus the data were plotted in order of increasing (more positive) log *D* for hydrophobic solutes from left to right. Solutes on the left side of the plot (negative log *D* values), maintained good CAD peak areas even up to 80 °C. Solutes on the right side of the plot, (positive log *D* values), showed drastic reduction in peak area at higher temperatures. It is possible that the hydrophobic solutes are lost more readily as the ACN evaporates first from the aqueous organic mixture, whereas hydrophilic solutes can be solvated by the remaining aqueous liquid. Fundamental aerosol studies by Reid et al. showed that hydrophilic/hydrophobic mixtures in aerosols can form a biphasic droplet [28]. To gain thermodynamic stability, hydrophobic components form surface lenses (partially engulfed structures), due to the relative surface tensions of the two phases [28]. Such a system can perhaps favour migration of hydrophobic components to the surface of aerosol droplets, and at high temperatures lead to their evaporation. The above confirms that low evaporation temperatures are required for optimal CAD performance, as loss of signal can be dramatic for a variety of solutes at higher temperatures. This effect was also observed with the equivalent salt-buffered mobile phase at 10% ACN (data not shown).

3.3. Analysis of salts (HPLC)

Zhang et al. separated and detected 25 typical pharmaceutical salt counter ions by HILIC–CAD [39] using gradient elution and a ternary solvent system. The authors reported separation of each ion from each other but did not comment on the separation of anion and cation for single salts. A mixture of inorganic salts was analysed by HILIC-CAD using a BEH Amide column (Fig. 8). The CAD was able to detect group (I) and (II) metals, common halides, and nitrate. There was good separation of cations from their corresponding anions. With the exception of chloride and nitrate, these ions are generally UV-transparent. The retention order of these salts is interesting, with cations retaining longer than anions (Fig. 8), probably due to

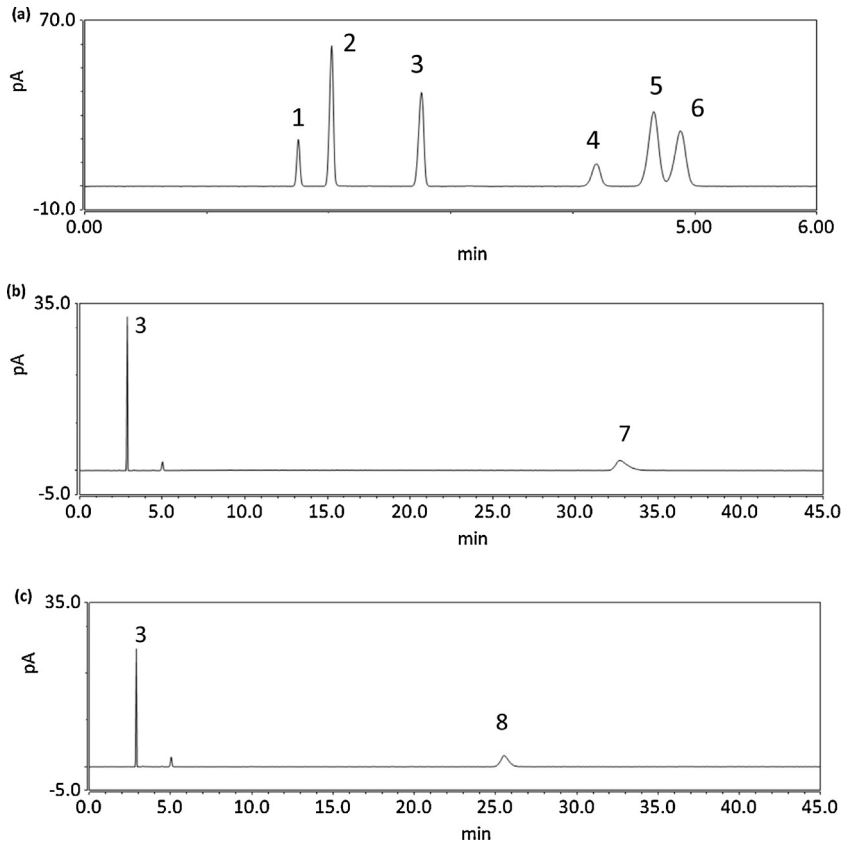


Fig. 8. HILIC separation and CAD detection of (a) a mixture of inorganic salts, (b) calcium chloride, (c) magnesium chloride. Peak identities 1 = iodide, 2 = nitrate, 3 = chloride, 4 = potassium, 5 = sodium, 6 = lithium, 7 = calcium, 8 = magnesium (HPLC, mobile phase 70% ACN, 5 mM ammonium formate pH 3, BEH Amide column).

ionic retention of cations and repulsion of anions on ionised silanol groups, which exist on all silica based-columns [23]. HILIC retention for ionogenic solutes is due to a mixture of ion-exchange and partition mechanisms [14,40]. This application demonstrates the potential application of the CAD in the pharmaceutical industry.

4. Conclusion

The charged aerosol detector (CAD) is a quasi-universal detector for HPLC. Flow injection analysis (FIA) was shown to be a rapid method for assessing the performance of the CAD, as long as baseline disturbances encountered in this method were taken into account. Volatile compounds cannot form stable aerosol particles and give no response. Some compounds (e.g. Caffeine) had response ca. 40% lower than the average, and can be classed as 'semi-volatile' compounds although they were not readily identifiable as such from physico-chemical data (e.g. bp or mp). Response from solute-to-solute was not truly uniform; however over the diverse range of solutes tested the uniformity of response was as low as 12% RSD. The on column detection limit (1–3 ng) and limit of quantitation (5–9 ng) compared favourably to published LOD and LOQ for universal detectors. The detector's dynamic range was over 4 orders of magnitude (1 ng to over 20 μg sample loads) for modestly-retained solutes, which supports data described by the manufacturer. Calibration curves generated by HPLC for three diverse solutes were all non-linear over three orders of magnitude, supporting earlier findings performed by FIA [17,18]. A theoretical explanation based on the findings of the manufacturers of CAD [29] was unable to describe the shape of typical CAD calibration curves. Possible solutions are calibration over narrow concentration ranges, plotting log/log calibration curves and use of an inbuilt 'power function'. These were equally effective at producing linear calibration. The precise workings of the 'power function' are proprietary information of the instrument manufacturer, so there may be reticence to its widespread use. HILIC was found to have excellent compatibility with CAD: uniformity of response was improved over RPLC conditions, signal under HILIC conditions was approximately twice as high and signal to noise around 4 times higher. Interestingly, the CAD signal appeared somewhat higher for basic compounds than neutral compounds. The uniformity of response was less with TFA and HFBA than with AF buffers (23% RSD, 30% RSD and 14%, respectively). Weakly acidic solutes exhibited lower CAD responses in these buffers than for the formate and salt buffers; conversely weak bases exhibited increased CAD response. It is possible that increasing the mass of buffer counterions may improve the response to solute ions of opposite charge, which was investigated principally using stronger bases (cationic solutes) and different mobile phase acid anions. When maintaining constant molar concentration of the acid, detector response showed some increase, but not in proportion to the weight of the acid anion. Furthermore, the mobile phase pH produced by the different acids can change the degree of ionisation for weakly acidic and basic solutes, which may affect their response. Low evaporation temperatures are recommended for general use. However, hydrophilic solutes gave good response at higher evaporation temperatures (up to 80 °C) as they may be retained longer in the aqueous portion of the aerosol particles. The CAD has many potential applications in the pharmaceutical industry, for instance in the monitoring of inorganic anions that can be separated as counterions of basic drugs. It appears that solutes injected as salts dissociate and travel through the column as separate cationic and anionic entities up to high concentrations, presumably accompanied by counterions from the mobile phase.

Data availability

The authors confirm that all data underlying the findings are available without restriction. All relevant data are contained within the paper.

Acknowledgement

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