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Quantitation and linearity for particle-beam liquid chromatography–mass spectrometry

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ABSTRACT

Quantitative performance for a particle-beam liquid chromatography–mass spectrometry system is evaluated with particular attention to non-linear behavior at low concentrations. A mathematical model for the non-linear behavior is proposed and shown to be in agreement with experimental data. The effects of 10 high-performance liquid chromatography mobile phase additives and 24 analytical probes on the linearity are shown. Although certain combinations of probes and additives show improved linear response, no single additive appears to completely alleviate the non-linear behavior as has been suggested by earlier work.

INTRODUCTION

Since the commercial introduction several years ago of particle-beam (PB) liquid chromatography (LC)–mass spectrometry (MS) systems, the technique has rapidly gained acceptance and popularity making it one of the most widely used techniques for combining the disparate techniques of LC and MS. This popularity is due to the technique's ability to yield either classical library-searchable electron impact (EI) spectra or solvent independent chemical ionization (CI) spectra and its ease of use relative to other LC–MS interfaces.

The technique has gone through a series of stages typical of all new analytical techniques. Initially describes as MAGIC LC–MS (monodispersed aerosol generation interface combining LC and MS) [1], the technique was met with some skepticism and largely overshadowed by thermospray LC–MS. Immediately following the introduction of the first commercial interface systems, a great deal of exc' .ement was generated as the initial results in the analytical community indicated a great potential for the technique in a number of areas, such as environmental and pharmaceutical analysis requiring quantitative analysis of analytes coupled with sufficient qualitative information to ensure positive identification. Naturally, as the LC–PB-MS became more widely used, the limitations of the system became recognized. The primary

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limitations of the system were limited sensitivity, dependence of quantitative performance on high-performance LC (HPLC) conditions and limited linearity. The first two of these limitations prompted the introduction of a second generation of commercial instrumentation resulting in significantly improved sensitivity (5–10-fold for the current generation of instrumentation relative to its predecessor) and improved performance over a wider range of HPLC operating conditions (particularly for aqueous mobile phases). The non-linear behavior, however, is still to be addressed. The non-linear behavior was first described by Bellar *et al.* [2], and has since been described by McLaughlin *et al.* [3] and Kim *et al.* [4]. Bellar *et al.* [2] described the phenomenon as a "carrier effect" referring to the appearance of increased ion abundances for coeluting compounds. The described mechanism involves coeluting compounds or mobile phase additives "carrying" analyte particles through the PB momentum separator resulting in an increased transfer efficiency. The addition of mobile phase additives, such as ammonium acetate was shown to improve sensitivity and linearity with the implication that such an approach could alleviate the non-linear behavior. Similarly, Kim *et al.* [4] showed that the addition of 0.4 m*M* malic acid lead to significantly improved sensitivity and linearity in the analysis of Alar.

The purpose of the current work is to demonstrate that while the addition of mobile phase additives can have positive effects on both sensitivity and linearity for various analytes, the effect is both sample and additive dependent and there is currently no "magic bullet" additive which leads to linear behavior under all conditions.

EXPERIMENTAL

Instrumentation

An HP1090 HPLC system with ternary solvent delivery system, autosampler, column oven and filter photometric detector (Hewlett-Packard, Palo Alto, CA, USA) was used as a pumping system throughout this work. The flow was set at 0.2 ml/min of methanol–water (50:50) (with additives as noted below). Injections of 1–2 μl were made in a flow injection analysis (FIA) mode.

An HP 59980A PB interface coupled to an HP5988 quadrapole mass spectrometer with high mass option and high-energy dynode (HED) detector was used for all the MS work. The PB nebulizer was operated at 30–60 p.s.i. helium inlet pressure, as determined by a standard optimization procedure. The PB desolvation chamber was operated at 60°C. The MS source temperature was 250°C and the MS analyzer was held at 100°C. The MS electron multiplier was run at approximately 200 V above autotune values and the HED voltage was set at 7 kV. All data acquisition was done in selected ion monitoring (SIM) mode of 70 eV EI ionization, monitoring the ions of interest for selected analytical probes.

The HPLC and the PB interface were connected using approximately 50 cm of 0.12 mm I.D. × 0.50 mm O.D. flexible stainless-steel capillary and a 0.5-μm low-dead-volume precolumn filter (Upchurch Scientific, Oak Harbor, WA, USA).

The system was controlled using a custom prototype data system running under Microsoft Windows running on an HP Vectra QS16 computer. All data manipulation was performed using Statgraphics version 3.0 (STSC, Rockville, MD, USA). The theoretical modelling was performed using MathCad version 2.5 (Math Soft, Cambridge, MA, USA).

Chemicals

HPLC-grade methanol was obtained from Burdick & Jackson (Baxter Healthcare Corp., Muskegon, MI, USA). HPLC-grade water was prepared inhouse starting from distilled water and further processing the solvent with a Water-I (Barnstead, Boston, MA, USA) solvent-purification system until the resistivity was greater than 18 MΩs/cm.

The mobile phase additives studied are listed in Table I and were obtained from Aldrich (Milwaukee, WI, USA). The analytical probes were obtained from either Aldrich or from Sigma (St. Louis, MO, USA). The chemical structures of the probes are shown in Fig. 1.

Techniques

The bulk of this work is based on two separate series of experiments. The first examined the effect of sample type and mobile phase additives on linear behavior. The second experiment examined the so called "carrier effect" by injecting coeluting compounds and evaluating any increased ion abundances.

The linearity studies were conducted as follows. Serial dilutions of the analytical probe samples listed in Table II (and shown in Fig. 1) were made at twelve levels from stock solutions at the 1000 ng/μl level in methanol. The resulting concentrations were 1000, 750, 500, 250, 125, 62.5, 31.25, 15.625, 7.81, 3.90, 1.95 and 0.97 ng/μl. For each different mobile phase additive evaluated, new dilutions were prepared using the mobile phase as the diluent. Mobile phases were prepared using methanol–water (50:50) plus the additive at 0.1 *M* concentration. The mobile phase plus additive were premixed and used as a single HPLC mobile phase channel. The samples were then run in triplicate in SIM mode, monitoring the ions listed in Table II. The samples were run as 2-μl injections in order from lowest to highest concentration to avoid any cross-contamination or carry-over. The peak height data based on the integration were then subjected to least squares linear regression to evaluate the linear performance. For some combinations of mobile phase additive and analytical probe the poor sensitivity resulted in less than four concentration data points for the linear regression. These data were rejected from further consideration. Due to the large number of combinations of mobile phase additives and analytical probes, the entire experiment required several months to complete. In order to account for day-to-day variations in instrument performance, a 20-ng benzidine sample was run in SIM mode every morning and later used to normalize peak height data.

As a subset of the above experiment, a number of mobile phase additives were run at different concentrations and/or different pH values to estimate these effects.

TABLE I

MOBILE PHASE ADDITIVES

Ammonium acetate	Ammonium bicarbonate
Ammonium formate	Ammonium thiocyanate
Ammonium citrate	Trifluoracetic acid (TFA)
Ammonium oxalate	Triethylamine
Ammonium tartrate	Ethylamine

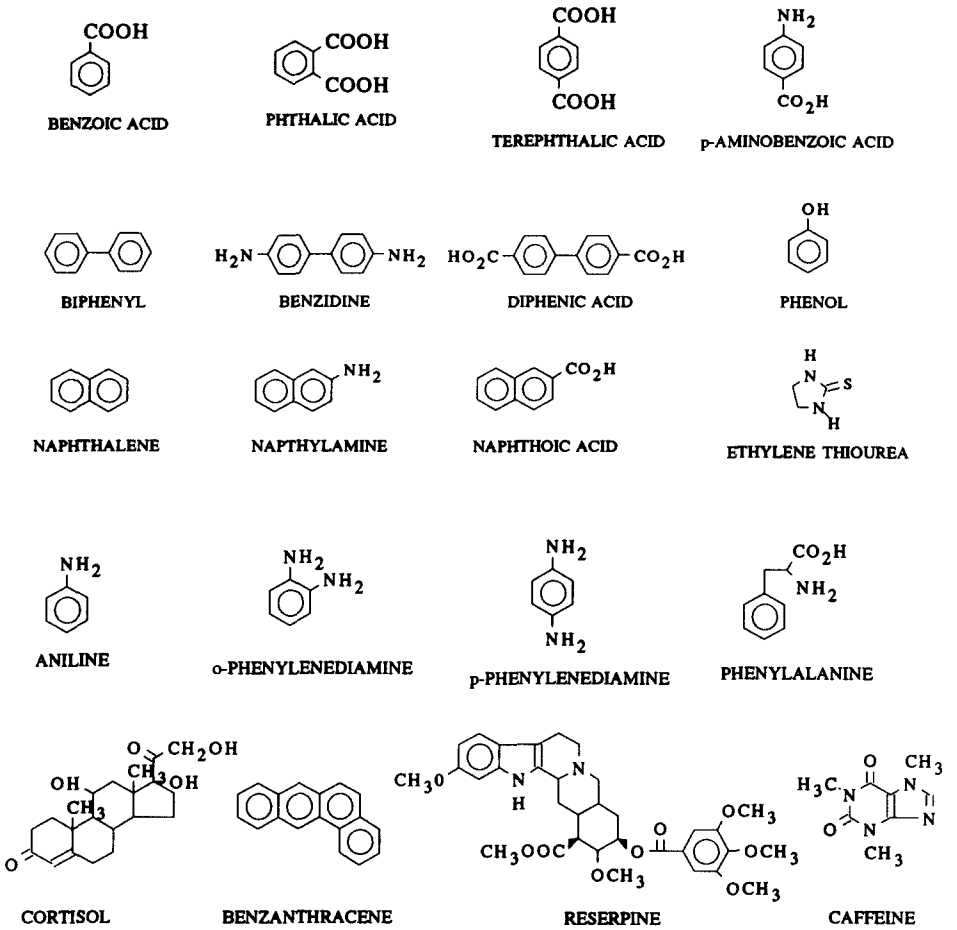

Fig. 1. Chemical structures of analytical probes used in linearity study.

TABLE II

LIST OF ANALYTICAL PROBES

Sample	Mol.wt.	Ions monitored	Sample	Mol.wt.	Ions monitored
Benzoic acid	122	105,122	Phenol	94	94
Aniline	93	93	*o*-Nitrophenol	139	109,139
p-Aminobenzoic acid	137	120,137	*p*-Nitrophenol	139	109,139
Phthalic acid	166	77,105	2,4-Dinitrophenol	184	124,184
p-Phenylenediamine	108	80,108	Picric acid	229	180,229
o-Phenylenediamine	108	80,108	Diphenic acid	242	197,242
Terephthalic acid	166	71,166	Benzidine	184	184
Phenylalanine	165	74,91,120	Biphenyl	154	154
Naphthoic acid	172	127,172	Reserpine	608	365,608
Naphthylamine	149	128,149	Caffeine	194	109,194
Naphthalene	128	128	Ethylenetiourea	102	73,102
2,3-Benzanthracene	228	71,228	Cortisol	363	163,302

For the carrier effect studies, samples were prepared at 20 and 50 ng/μl concentrations either alone in the mobile phase (plus additive) or in the presence of 1000 ng/μl cortisol as carrier in the mobile phase (plus additive). While monitoring the characteristic ions for the probe, the samples were run first injecting 1 μl five times without the carrier and then 1 μl five times with the carrier.

RESULTS AND DISCUSSION

The PB interface is shown schematically in Fig. 2 and operates as follows: the effluent from the HPLC enters the system through a coaxial pneumatic nebulizer which generates an aerosol. The aerosol passes through a desolvation chamber which is held at approximately 200 Torr and 60°C. As the droplets are desolvated the more volatile components (such as the HPLC solvent) evaporates leaving the less volatile components (*e.g.* analyte) to condense into desolvated particles. At the end of the desolvation chamber, a mixture of helium gas, solvent vapor and desolvated analyte particles enters a two-stage momentum separator. The momentum separator consists of three parts; a nozzle and two skimmers. The vapor, gas and particles exit the nozzle at supersonic velocities. The heavier particles have significantly higher momentum relative to the vapor and gas molecules and consequently pass through the momentum separator and into the mass spectrometer source volume. The lighter gas and vapor molecules have less momentum than the particles and can be pumped away to exhaust. This process results in analyte enrichment relative to the mobile phase and a pressure reduction from a pressure of approximately 200 Torr in the desolvation chamber to 5–10 Torr in the first momentum separator stage to <0.5 Torr in the second momentum separator stage to $1\text{–}2 \cdot 10^{-5}$ Torr in the mass spectrometer source manifold. After the particles in the particle beam enter the MS source, they strike the heated source wall, are vaporized and ionized by EI or CI. For the current work, all spectra were EI.

Theoretical model

In an attempt to more fully understand the phenomenon involved in the nonlinear behavior, a mathematical model was proposed and evaluated to predict the characteristics of the response factors and linear performance. The model is based on hypothesizing that the PB interface has a particle size cutoff level below which small particles are pumped away in the momentum separator (or otherwise lost in the

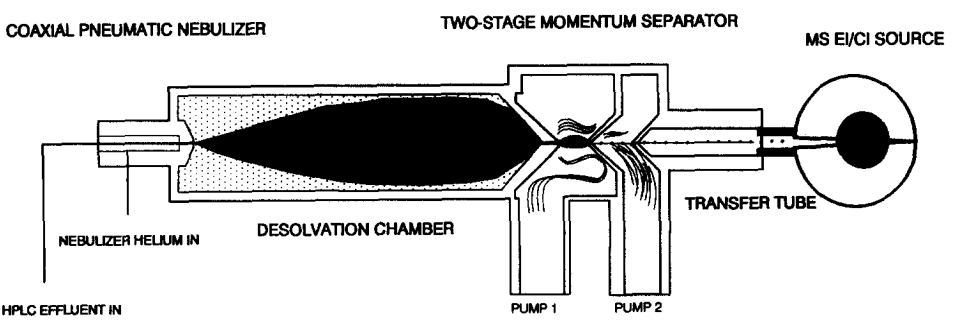

Fig. 2. Schematic of particle-beam LC–MS interface.

system) and above which the larger particles are transferred quantitatively into the MS source. Although the use of such an abrupt high pass filter analogy is an over simplification, the general concept is reasonable if one considers small particles in the limit of one molecule per particle. The entire purpose of the particle beam interface is to separate these vapor molecules from the larger particles.

Given an initial aerosol entering the PB desolvation chamber with a given droplet size distribution (assumed to be normal), it is possible to calculate the resulting desolvated particle diameter if the further assumptions of solid spherical particles with densities identical to their respective bulk materials are made. Again these assumptions are simplifications since it has been shown [5] that the particles may have a number of different non-spherical forms. However, for this model it is assumed that the size and mass of the resulting desolvated particle depend only on the initial droplet size, the sample concentration and the sample bulk density. As the sample concentration is reduced, the resulting particle size is reduced as well. At some point, the mean of the particle distribution begins to pass the hypothetical cutoff level. This results in a reduction in response factor going eventually to zero as shown in Fig. 3A. This, in turn, results in a calibration plot with a characteristic linear range at the

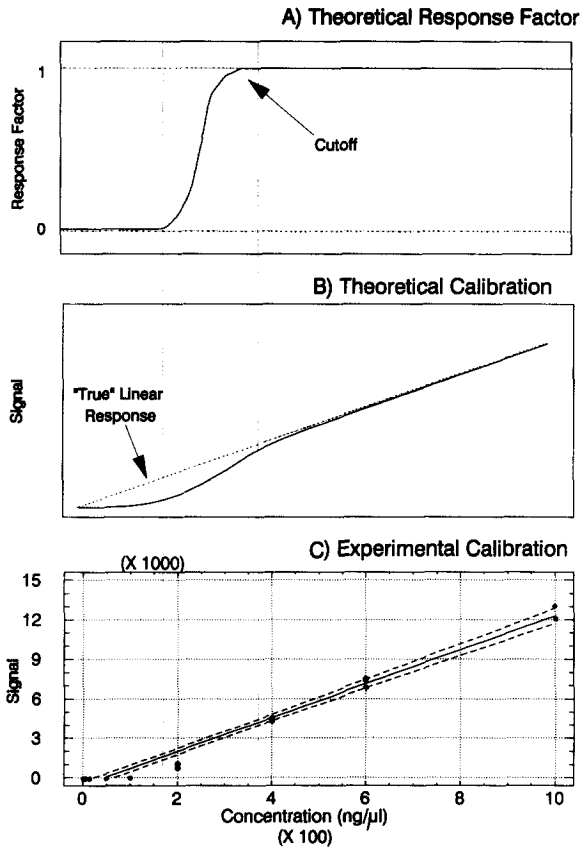

Fig. 3. Theoretical model for non-linear behavior in LC–PB-MS. (A) Calculated response factor, (B) calculated calibration plot, (C) example of experimental calibration plot for *p*-phenylenediamine.

higher concentrations and a deviation from linearity at the lower levels, as shown in Fig. 3B. Although it is not possible to get a direct comparison with real data without further information about the actual physical cutoff level, comparison with the data in Fig. 3C show that experimental data are consistent with the behavior proposed by this model.

Effect of mobile phase additives

As noted in the introduction, several researchers have suggested that the addition of semivolatile compounds to the mobile phase can show improvements in both linearity and sensitivity. In light of the model described above, it is clear that, in principle, this should be true. The addition of some mobile phase modifier will have the effect of increasing the overall concentration of material in each aerosol droplet, and consequently will increase the resulting desolvated particle size. This improvement, however, will depend on the ability of the probe and the additive to interact in such a way that neither is evaporated and pumped away in the system. It is demonstrated below, that although it is possible to make substantial gains in both linearity and sensitivity, it is not universally the case that addition of a modifier will lead to improvements.

The results of the least squares linear regression analysis, as expressed as the coefficient of variation (r^2), for the combinations of mobile phase additives and analytical probes, is shown in Table III. Table IV shows the Δr^2 values which are obtained by subtracting the r^2 value with mobile phase additive from the r^2 value with no additive. Brief examination of Table IV shows that there are both positive and negative values throughout the table indicating that in some cases (positive values) there is an improvement in linearity and in others (negative values) there is a degradation in performance. For none of the mobile phase additives is there an overall improvement in all sample cases.

This can be evaluated more systematically through analysis of regression (ANOVA) studies. The results of ANOVA for the effect of mobile phase additives are shown in Table V and depicted graphically in the "box and whisker" plot in Fig. 4. From the ANOVA table, we can conclude that at the 98 confidence limit, the mobile phase additives do have a statistically significant effect. The F value of 2.1 indicates the significance of this effect as the ratio of the "between additive" means square and the "within additive" mean square. In the "box and whisker" plot shown in Fig. 4, each box and whisker represents all of the analytical probes run for a given mobile phase additive, the center line in each box indicates the mean while the edges of the box are the 50 percentile and the whiskers are the range extremes. The additional points represent actual data points. Although the mean in each case is near 0 (no effect), there is a range of responses for different combinations of additive and probe. (Compare this plot to the similar discussion concerning the effect of analytical probes below.) Fig. 5 shows a similar box and whisker plot for the effect of additive on sensitivity.

As an example consider Fig. 6 in which the linearity for *p*-phenylenediamine is shown with no additive (A), 0.1 M ammonium acetate (B) and 0.1 M ammonium oxalate (C). In this case, the addition of the additives does result in a significant improvement in performance. Note the characteristic deviation from linearity at low levels in A which improves in B with acetate and almost disappears in C with oxalate.

TABLE III

COEFFICIENTS OF DETERMINATION (r^2) FOR ADDITIVE PROBE MATRIX

All additives at 0.1 *M* (except as noted in text) and adjusted to pH 7.0 if possible. Blank values represent insufficient data.

	No additive	Ammonium acetate	Ammonium formate	Ammonium citrate	Ammonium oxalate	Ammonium tartrate	Ammonium bicarbonate	Thio-cyanate	TFA	Triethyl-amine	Ethyl-amine
Benzoic acid	83.7				98.29					93.23	90.32
Aniline	99.25	99.48	99.95		99.57					76.62	99.74
p-Aminobenzoic acid	92.92	95.65	98.99	99.86	99.4	99.9	87.29	31.55	98.19	73.01	96.18
Phthalic acid	95	96.58	99.51		99.96		99.27	62.02	93.97	99.86	98.5
p-Phenylenediamine	75.17	72.01	90.71	98.72	99.93	99.84	94.48	99.5	99.74	98.59	93.5
o-Phenylenediamine	78.49			99.74	99.07	99.7	76.46	59.68	66.71	34.85	93.83
Terephthalic acid	98.5	98.59	99.93	99.75	99.92	84.84	99.8	99.44	99.25	98.57	97.6
Phenylalanine	87.05		89.14	73.84	99.51	99.21	98.57	96.85	86.05	91.11	92.4
β-Naphthoic acid	89.07	93.87		99.42	98.93	90.57	96.91		93.31	90.54	83.06
β-Naphthylamine	92.2	83.36	74.71	99.62	97.15	95.85			82.26	93.67	97.78
Naphthalene	99.9	94.75	99.95		99.92	97.39	98.78	99.7	99.77	99.33	99.62
2,3-Benzanthracene	98.86	99.63	99.87	99.88	99.84		99.76	93.76	98.36	98.05	98.75
Phenol	99.79	99.41	89.57		98.09		99.32	88.61	92.81	87.68	99.68
o-Nitrophenol	67.13				63.43		99.26		98.48	85.96	95.15
p-Nitrophenol	92.39	18.48	56.63	88.56	99.17	99.74	96.7			97.94	95.52
2,4-Dinitrophenol	48.5	93.23	94.91	91.25	96.49	75.25					97.25
Picric acid	99.09		91.83	99.2	93.52	99.78	99.75		97.7	99.35	98.37
Diphenic acid	95.58		97.83	83.54	95.06	98.79	95.13	95.01	94.92	94.14	95.81
Benzidine	99.57	76.02	99.31	94.21	99.88	99.53	99.6	93.61		99.65	98.33
Biphenyl	99.7	99.86			99.57	95.02	99.38	98.11	99.85	98.12	99.54
Reserpine	95.93	99.18	99.77	97.28			96.16	97.06	98.23	95.28	98.55
Caffeine	99.78	98.31	98.77	92.02		97.2	97.44	98.59	90.81	98.25	98.64
Ethylene thiourea	98.2	98.56	99.65		95.85		95.41	98.41	96.48	97.18	98.21
Cortisol	99.34	99.05	99.92		88.67	98.83	99.63	98.41	98.37	99.02	98.7

TABLE IV

DIFFERENCES IN COEFFICIENTS OF DETERMINATION (r^2) FOR ADDITIVE/PROBE MATRIX

All additives at 0.1 *M* (except as noted in text) and adjusted to pH 7.0 if possible. Blank values represent insufficient data.

	Ammonium acetate	Ammonium formate	Ammonium citrate	Ammonium oxalate	Ammonium tartrate	Ammonium bicarbonate	Thio-cyanate	TFA	Triethyl-amine	Ethyl-amine
Benzoic acid				14.59					9.53	6.62
Aniline	0.23	0.7		0.32					−22.63	0.49
p-Aminobenzoic acid	2.73	6.07	6.94	6.48	6.98	−5.63	−61.37	5.27	−19.91	3.26
Phthalic acid	1.58	4.51		4.96		4.27	−32.98	−1.03	4.86	3.5
p-Phenylenediamine	−3.16	15.54	23.55	24.76	24.67	19.31	24.33	24.57	23.42	18.33
o-Phenylenediamine			21.25	20.58	21.21	−2.03	−18.81	−11.78	−43.64	15.34
Terephthalic acid	0.09	1.43	1.25	1.42	−13.66	1.3	0.94	0.75	0.07	−0.9
Phenylalanine		2.09	−13.21	12.46	12.16	11.52	9.8	−1	4.06	5.35
β-Naphthoic acid	4.8		10.35	9.86	1.5	7.84		4.24	1.47	−6.01
β-Naphthylamine	−8.84	−17.49	7.42	4.95	3.65			−9.94	1.47	5.58
Naphthalene	−5.15	0.05		0.02	−2.51	−1.14	−0.2	−0.13	−0.57	−0.28
2,3-Benzanthracene	0.77	1.01	1.02	0.98		0.9	−5.1	−0.5	−0.81	−0.11
Phenol	−0.38	−10.22		−1.7		−0.47	−11.18	−6.98	−12.11	−0.11
o-Nitrophenol				−3.7		32.13		31.35	18.83	28.02
p-Nitrophenol	−73.91	−35.76	−3.83	6.78	7.35	4.31			5.55	3.13
2,4-Dinitrophenol	44.73	46.41	42.75	47.99	26.75					48.75
Picric acid		−7.26	0.11	−5.57	0.69	0.66		−1.39	0.26	−0.72
Diphenic acid		2.25	−12.04	−0.52	3.21	−0.45	−0.57	−0.66	−1.44	0.23
Benzidine	−23.55	−0.26	−5.36	0.31	−0.04	0.03	−5.96		0.08	−1.24
Biphenyl	0.16			−0.13	−4.68	−0.32	−1.59	0.15	−1.58	−0.16
Reserpine	3.25	3.84	1.35			0.23	1.13	2.3	−0.65	2.62
Caffeine	−1.47	−1.01	−7.76		−2.58	−2.34	−1.19	−8.97	−1.53	−1.14
Ethylene thiourea	0.36	1.45		−2.35		−2.79	0.21	−1.72	−1.02	0.01
Cortisol	−0.29	0.58		−10.67	−0.51	0.29	−0.93	−0.97	−0.32	−0.64

TABLE V

EFFECT OF ADDITIVES ON LINEARITY

D.F. = Degrees of freedom.

Source of variation	Analysis of variance			
	Sum of squares	D.F.	Mean square	*F*-ratio
Between additives	6237	10	623	2.1
Within additives	70 729	239	297	
Total (corr.)	76 966	249		

Fig. 7 shows the residuals for the linear regressions shown in Fig. 6. Although in Fig. 7A (no additive), Fig. 7B (0.1 *M* ammonium acetate) and Fig. 7C (0.1 *M* ammonium oxalate) there is a clearly discernable deviation from linear performance, note that the magnitudes of the deviations decrease more than 30-fold from no additive to oxalate.

Table VI shows the normalized peak heights for 600-ng injections for the combinations of mobile phase additives and analytical probes. By examining the ANOVA results (Table VII) and the corresponding box and whisker plot (Fig. 8), it can be seen that over the entire set of probes there is not a statistical difference in signal heights that can be attributed to the additive. However, in several specific cases, the sensitivity is improved by using a mobile phase additive, and in particular, the use of acetate and oxalate lead to significant improvements. This is further reinforced by examining the peak intensities shown in the calibration plot in Fig. 6 which show an approximately 6-fold increase comparing A (no additive) to C (0.1 *M* ammonium oxalate).

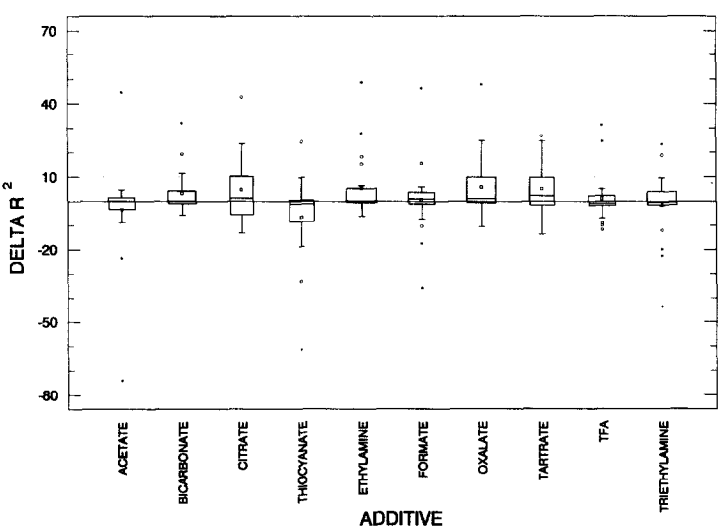

Fig. 4. Effect of additive on linearity; box and whisker plot. Delta R^2 = Differences in coefficient of determination (r^2) relative to a mobile phase with no additive.

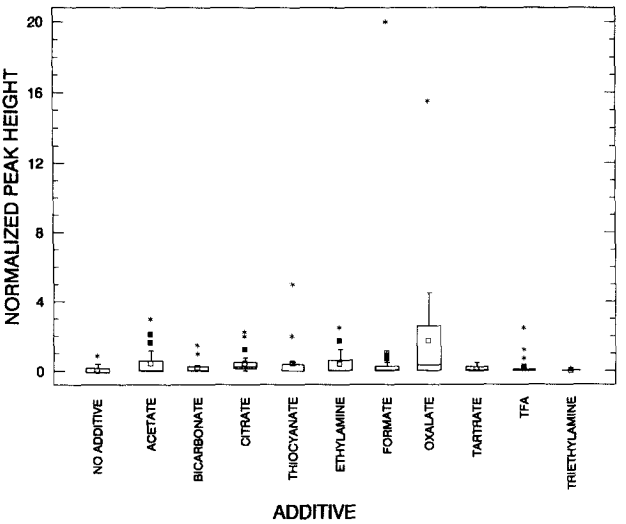

Fig. 5. Effect of additive on sensitivity; box and whisker plot. Normalized peak heights are normalized with respect to daily instrument response and benzidine run with no additive.

Effect of analytical probes

The effects of the analytical probes on linearity and sensitivity are similar to the effects of the mobile phase additives. Results of ANOVA on the Δr^2 values in Table IV are shown in Table VIII along with corresponding box and whisker plot in Fig. 8. Again, although the results are mixed, it is possible to say that at the 95% confidence limit, the character of the analytical probes does have a statistically significant effect. In fact, examining the F-value of 6.4 shows that this effect is more significant than that of the mobile phase additives.

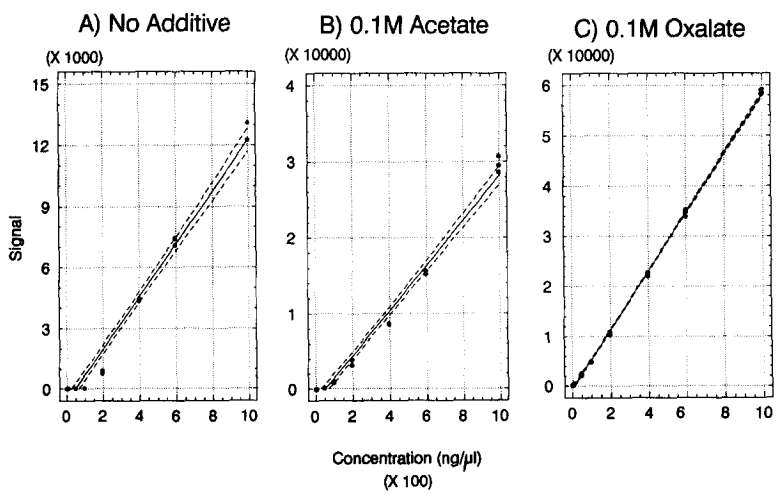

Fig. 6. p-Phenylenediamine calibration plot with (A) no additive, (B) 0.1 M ammonium acetate and (C) 0.1 M ammonium oxalate.

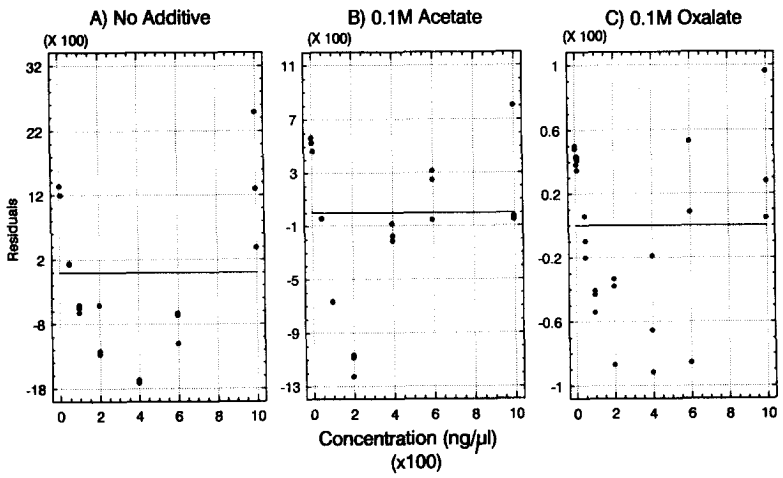

Fig. 7. Residuals for regression plots shown in Fig. 6 (A) No additive, (B) 0.1 *M* ammonium acetate and (C) 0.1 *M* ammonium oxalate.

Although comparisons between thermospray and particle-beam LC–MS [6,7] have suggested that the range of response factors is less for particle beam than thermospray resulting in a more uniform response, of course the response factors are not totally uniform. This is evident in the data shown in Table VI.

By comparing subsets of the analytical probes, it is possible to characterize some structural effects on a preliminary basis. It should be noted that this work is still in progress and the following analysis is not taking into account any other physical or thermodynamic characteristics of the probes or modifiers. In particular, by comparing three sets; (benzoic acid, aniline and *p*-aminobenzoic acid), (biphenyl, benzidine and diphenic acid), and (naphthalene, naphthylamine and naphthoic acid), it is possible to compare acids and bases. In general, for the acidic modifiers, the acids and neutral probes work better than the basic probes whereas the basic probes improve for basic modifiers. This is perhaps not surprising, but it does suggest more chemical interaction than simply physical. Similarly, by comparing the subsets (benzoic acid, phthalic acid and therephthalic acid), (aniline, *p*-phenylenediamine and *o*-phenylenediamine) and (phenol, *o*-nitrophenol, *p*-nitrophenol, 2,4-dinitrophenol and picric acid), one can compare the effect of 1, 2 or 3 polar substituents. To some extent this overlaps a comparison of relative positioning of aromatic substituents that can be obtained by comparing the subsets (*o*-nitrophenol and *p*-nitrophenol), (*o*-phenyl-

TABLE VII

EFFECT OF ADDITIVES ON SENSITIVITY

Source of variance	Analysis of variance			
	Sum of squares	D.F.	Mean square	*F*-ratio
Between additives	56	10	5.59	1.79
Within additives	698	223	3.13	
Total (corr.)	754	233		

TABLE VI

NORMALIZED PEAK HEIGHTS FOR ADDITIVE PROBE MATRIX

All additives at 0.1 *M* (except as notes in text) and adjusted to pH 7.0 if possible. Data normalized with respect to benzidine/no additive.

	No additive	Ammonium acetate	Ammonium formate	Ammonium citrate	Ammonium oxalate	Ammonium tartrate	Ammonium bicarbonate	Thio-cyanate	TFA	Triethyl-amine	Ethyl-amine
Benzoic acid	0.018	0.042	0.001	0.250	1.320				0.001		0.007
Aniline	0.001	0.001	0.007	0.002	0.007			0.001			0.002
p-Aminobenzoic acid	0.150	0.140	0.750	0.500	2.750	0.500	1.500	0.009	0.750	0.150	1.000
Phthalic acid	0.022	0.088	0.033	0.250	0.279		0.008	0.011	0.090	0.007	0.225
p-Phenylenediamine	0.250	0.306	20.000	0.248	2.750	0.235	0.039	2.000	1.250	0.017	0.225
o-Phenylenediamine	0.001	0.003	0.250	0.222	2.301	0.078	0.002	0.004	0.038		0.002
Terephthalic acid	0.250	0.787	1.000	1.250	4.500	0.250	0.217	5.000	0.750	0.025	1.250
Phenylalanine	0.175	0.800	0.182	0.750	4.000	0.163	0.239	0.500	0.063	0.009	0.750
β-Naphthoic acid	0.250	0.375	0.057	0.143	2.400	0.033	0.250	0.050	0.097	0.018	0.500
β-Naphthylamine	0.001	0.002	0.001	0.022	0.389	0.003		0.005	0.011		0.002
Naphthalene	0.003	0.009	0.010		0.020	0.002	0.002	0.003	0.004	0.001	0.006
2,3-Benzanthracene	0.250	1.165	0.500	2.250	15.545	0.250	0.250	0.150	0.250	0.018	0.500
Phenol	0.001	0.003	0.001	2.000	0.009	0.002	0.001	0.001	0.002		0.002
o-Nitrophenol		0.001			0.006	0.001					0.001
p-Nitrophenol	0.001	0.012	0.036	0.250	0.416	0.131	0.001		0.005	0.001	0.012
2,4-Dinitrophenol	0.001	0.034	0.004	0.171	0.332	0.024			0.000		0.007
Picric acid	0.001	0.011	0.001	0.204	0.064	0.072	0.008	0.001	0.004	0.001	0.014
Diphenic acid	0.002	0.030	0.002	0.500	0.351	0.249	0.005	0.004	0.035	0.006	0.050
Benzidine	1.000	2.127	0.750	0.500	4.250	0.500	1.000	0.250	2.500	0.125	2.500
Biphenyl	0.002	0.006	0.001	0.021	0.007	0.001	0.001	0.001	0.001		0.004
Reserpine	0.004	0.014	0.001	0.013	0.013		0.003	0.002	0.015		0.013
Caffeine	0.500	3.000	0.247	0.199	0.003	0.032	0.239	0.500	0.001	0.050	1.750
Ethylene thiourea	0.225	1.658	0.196	0.250	0.224	0.023	0.250	0.500		0.025	1.000
Cortisol	0.050	0.276	0.022	0.013	0.039	0.006	0.038	0.024	0.001		0.150

TABLE VIII

EFFECT OF PROBES ON LINEARITY

Source of variance	Analysis of variance			
	Sum of squares	D.F.	Mean square	*F*-ratio
Between probes	30 304	23	1317	6.4
Within probes	46 662	225	207	
Total (corr.)	76 966	248		

enediamine and *p*-phenylenediamine) and (phthalic acid and terephthalic acid). In terms of the number of substituents there is not a significant difference in linearity based on number of substituents (for these compounds). The substituted compounds do behave more linearly than their unsubstituted parents. In almost all cases the *para*-substituted examples behave more linearly than the *ortho* substitution. For the three pairs given as examples, the *para*-substituted compound also gives significantly larger signals.

Carrier effect experiments

The non-linear behavior observed for LC–PB-MS can also be seen as an increase in signal for coeluting peaks. In the presence of a coeluting, compound, an analyte may exhibit more efficient transport and therefore yield a larger signal than in the absence of the coeluting "carrier" compound. An example of this is shown in Figs. 9 and 10. In Fig. 9, 10 ng *p*-phenylenediamine is injected alone and then together with 1000 ng cortisol using mobile phases with (A) no additive, (B) 0.1 *M* ammonium acetate and (C) 0.1 *M* ammonium oxalate. It can be clearly seen that the 0.1 *M* oxalate reduces the magnitude of the "carrier effect" relative to the no additive case.

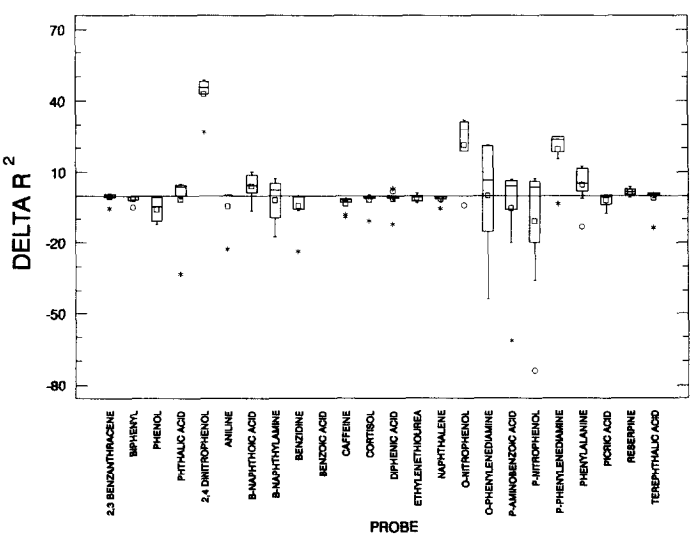

Fig. 8. Effect of probe on linearity box and whisker plot. Delta R^2 = Differences in coefficient of determination (r^2) relative to a mobile phase with no additive.

Fig. 10 represents an identical experiment except that the *p*-phenylenediamine is at 50 ng level. In this case, even in the absence of any additive, the increase in signal due to the "carrier effect" is relatively minor compared to the 10-ng case. This is because at 50 ng, *p*-phenylenediamine is just beginning to exhibit non-linear behavior. At levels above this, the non-linearity becomes less significant. In essence, at this level, *p*-phenylenediamine is its own carrier.

The effect of coeluting peaks on analyte signal intensities has raised questions concerning the use of isotopically labelled internal standards for quantitation. Although it has yet to be demonstrated, it seems clear that the use of such coeluting standards should be a viable approach to quantitation since the effect of coeluting compounds is to decrease the reduction in the response factor due to non-quantitative transfer processes. It should be noted that ion abundances can only increase to the point at which 100% of the analyte is being transferred into the ion source. Thus, depending on the level of the internal standard, the linear response of the analyte should be more or less improved. We hope to demonstrate this in future work.

CONCLUSIONS

In conclusion, it has been demonstrated that the so-called "carrier effect" and the non-linear behavior of LC–PB-MS are based on the same phenomenon which is consistent with a "high pass filter" model.

It has been shown that effect can be mitigated in some cases though the use of semivolatile mobile phase additives, but that this does not result in improvement in all cases. The effectiveness of this approach depends on the chemical characteristics of both the additive and of the analyte. This strongly suggests that the phenomenon involves a chemical interaction rather than a simple physical process.

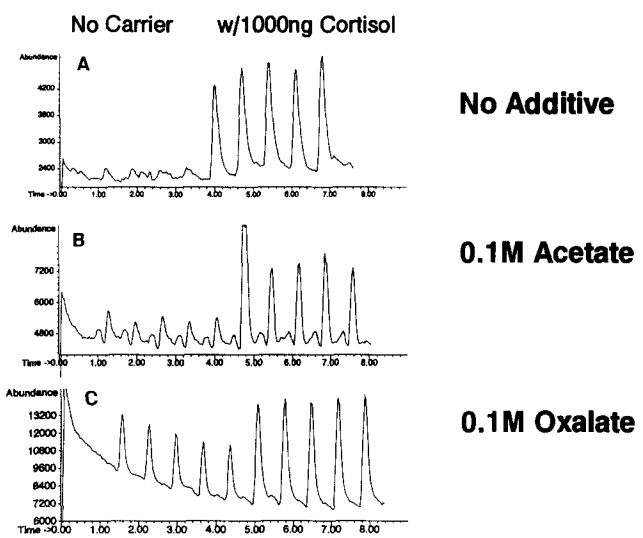

Fig. 9. Carrier effect for 10 ng *p*-phenylenediamine for (A) no additive, (B) 0.1 *M* ammonium acetate and (C) 0.1 *M* ammonium oxalate. The first five injections contain only 10 ng *p*-phenylenediamine, the second five contain 10 ng *p*-phenylenediamine coeluting with 1000 ng cortisol as carrier. Time in min.

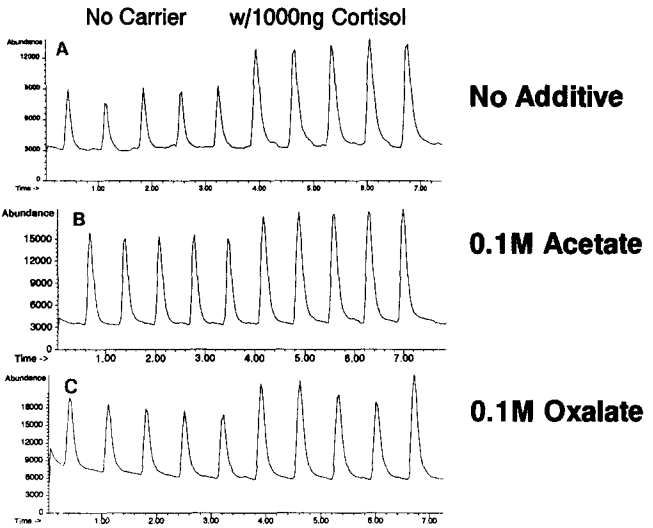

Fig. 10. Carrier effect for 50 ng *p*-phenylenediamine for (A) no additive, (B) 0.1 *M* ammonium acetate and (C) 0.1 *M* ammonium oxalate. The first five injections contain only 50 ng *p*-phenylenediamine, the second five contain 50 ng *p*-phenylenediamine coeluting with 1000 ng cortisol as carrier. Time in min.

In future work, we hope to further investigate and characterize the nature of the chemical interactions with the aim of providing useful criteria for choosing appropriate mobile phase additives for a given analysis.

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