Journal of Chromatography B, 877 (2009) 4133–4139

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Comparison of the sensitivity of evaporative universal detectors and LC/MS in the HILIC and the reversed-phase HPLC modes

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ARTICLE INFO

Article history:
Received 13 August 2009
Accepted 25 October 2009
Available online 30 October 2009

Keywords:
Sensitivity
Response factor
HILIC
RPLC
ELSD
Corona CAD
ESI
Mass spectrometry

ABSTRACT

It was hypothesized that the hydrophilic interaction liquid interface chromatography (HILIC) mode should produce more response than the reversed-phase HPLC mode on detectors with an evaporative component to the detection process. HILIC mobile phases are mostly composed of polar organic solvent and are more volatile than reversed-phase mobile phases. Therefore the more easily evaporated HILIC mobile phases should produce greater sensitivity for those detectors that remove mobile phase by evaporation. The responses of 12 compounds were measured in the reversed-phase mode and the HILIC mode with three detectors: evaporative light scattering detector (ELSD), corona charged aerosol detector (cCAD), and electrospray mass spectrometry (ESI-MS). The compounds studied were very polar compounds that were retained in the HILIC mode. Generally, the HILIC mode was able to achieve greater sensitivity than the reversed-phase mode for these compounds. The increases in sensitivity observed can be attributed to the more volatile HILIC mobile phase. For the ELSD, the HILIC mode produced slightly greater sensitivity than the reversed-phase mode. The cCAD was approximately 10 times more sensitive in the HILIC mode and the ESI-MS was approximately 5–10 times more sensitive in the HILIC mode. There was one instance in the study where a compound produced more response in the reversed-phase mode. Thymine yielded more sensitivity in the reversed-phase mode with the ESI-MS detector. In a given mode of operation, there was significant variation in the measured response factors for all compounds on each detector. While this is not unexpected for the ESI-MS detector, variation in the response factors between compounds indicates that the cCAD and ELSD are not truly universal detectors in the sense that all compounds have identical responses.

1. Introduction

The analysis of very polar compounds by HPLC can be challenging for chromatographers. Originally, column liquid chromatography was discovered by Tswett in what we call today the normal phase mode. While the normal phase mode is well-suited for the analysis of neutral and polar molecules, it is very difficult to directly analyze samples that are ionizable and/or are extracts from biological matrices. Additionally, the typical normal phase solvents are incompatible with atmospheric pressure ionization mass spectrometry (API MS), which is a powerful tool in modern chemical analysis. The reversed-phase mode is well suited for ionizable compounds and biological extracts, however very polar molecules can be poorly retained and frequently have poor peak shape. Several aqueous based chromatographic techniques have evolved over the years and have been applied to the analysis of very polar molecules, including: ion-exchange chromatography, ion pairing chromatography, derivatization and analysis by reversed-phase, mixed mode stationary phases, and hydrophobic interaction chromatography.

Another alternative to these techniques is the hydrophilic interaction liquid interface chromatography (HILIC) mode of HPLC. The term HILIC was first suggested by Alpert in 1990 [1] and in recent years there has been increased interest in HILIC among scientists. A SciFinder search for the term HILIC yields over 400 unique hits, 90% of which have been published since 2004. HILIC is a mode of operation in HPLC that is distinct from the reversed-phase mode and the normal-phase mode. The HILIC stationary phase is a polar stationary phase, such as a silica, diol, or amino phase [2] and the mobile phase is composed of polar solvents and is typically composed of acetonitrile and water, with a greater percentage of organic solvent. The strong eluent in HILIC is water and is present minimally at 2% by volume in the mobile phase. It has been theorized that there is a layer of water present on the surface of the HILIC stationary phase and retention occurs by interaction of polar analytes with this stagnant layer of water [3]. There has been a significant amount of work to determine if retention in HILIC is a partition process or an adsorption process; both mechanisms have been demonstrated

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doi:10.1016/j.jchromb.2009.10.027

for different analytes [3]. The compounds that can be successfully retained by HILIC are very polar, water-soluble compounds that have low solubility in organic solvents, such as acetonitrile. Like the reversed-phase mode, gradient elution is useful when analyzing compounds with wide differences in retentivity. During gradient elution in the HILIC mode, the strength of the eluent is increased by increasing the relative amount of water in the mobile phase over a period of time. Additionally, acidic/basic/salt additives are frequently used to impact retention and selectivity.

The HILIC mode has several advantages over the reversed-phase mode for the analysis of polar compounds. HILIC methods are less susceptible to analyte peak deformations due to the use of strong organic solvents in the sample matrix. Conversely, polar analytes can suffer from retention time shifts and poor peak shape in reversed-phase methods. Additionally, polar analytes can be retained more strongly by HILIC methods compared to reversed-phase methods. This is very important for the analysis of extracts and samples from biological matrices which are very important to the pharmaceutical industry. Some examples of HILIC applications are for analysis of peptides [4,5], carbohydrates [6], and pharmaceuticals [7–9].

In addition to HILIC, there are two additional modes of HPLC that have properties that are similar to HILIC. Aqueous normal phase (ANP) has been demonstrated on silicon hydride materials [10–12]. The second mode is the polar organic mode (PO) for enantiomeric separations [13–16]. Mechanistically these three modes, HILIC, ANP and PO, are distinct from each other, as well as distinct from the reversed-phase mode and the normal phase mode, and execution of these modes can require unique columns. However, what is common to these three modes is that they utilize mobile phases that are predominantly, if not entirely, a polar organic solvent (e.g. acetonitrile). In light of this similarity between the HILIC, ANP and PO modes, the HILIC mode will be the focus for this work. However, the conclusions formed herein for HILIC methods should be equally valid for ANP and PO methods.

While the most popular detector for HPLC is the UV spectrophotometer, chromatographers are increasingly making use of alternate detectors. These alternate detectors are chosen to address a variety of needs that may include: improved sensitivity, improved selectivity, superior gradient compatibility, and poor analyte UV absorbance. Three detectors that can meet a number of these needs are the evaporative light scattering detector (ELSD), the corona charged aerosol detector (cCAD), and the electrospray mass spectrometer (ESI-MS). The ELSD is a fairly mature detector that has been commercially available for many years [7,17,18]. Detection is achieved by first nebulizing the column effluent into an aerosol which then travels down a heated drift tube where the bulk of the mobile phase is evaporated leaving a solid residue of analyte particles. The residue is detected by measuring the intensity of scattered light when the residue traversed the detection window. The ELSD is usually chosen as an alternate detector when the compounds of interest lack a UV chromophore and gradient compatibility is needed. The cCAD is a fairly new technology which has some operational principles that are similar to the ELSD [19–22]. The cCAD also nebulizes the column effluent into an aerosol which then travels down a heated drift tube where the bulk of the mobile phase is evaporated leaving a solid residue of analyte particles. At this point, the similarities with an ELSD end. The analyte residue passes a corona discharge needle that imparts charge to the particles, and detection is achieved by measuring the total charge entering the detection cell over time. The cCAD is chosen as an alternate detector when the compounds of interest lack a UV chromophore, gradient compatibility is needed, or better sensitivity than an ELSD or refractive index detector is needed. ESI-MS is a fairly mature and well known technique. While the specifics of the electrospray process have been the subject of debate [23], there are elements of the ESI-MS detection process that are similar to ELSD and cCAD. The column effluent is sprayed into the electrospray source which is similar to a nebulization process in that the feed solution is dispersed into small droplets. Additionally, the analyte molecules are extracted from the mobile phase droplets. It is well established that ESI-MS has excellent sensitivity, selectivity, gradient compatibility, and no need for UV chromophores.

While the sensitivity of these three detectors (ELSD, cCAD, and ESI-MS) is somewhat different [24–26], the common element of each detection process is that the mobile phase is removed by evaporation/dispersion and detection is performed on the analyte molecules that remain after the evaporation step. Given the evaporative nature of these detectors, we propose that very polar compounds analyzed in the HILIC mode (and similar modes) should produce greater sensitivity than in the reversed-phase mode. The HILIC mode mobile phases are primarily organic solvent and more easily evaporated than reversed-phase mode mobile phases and should therefore produce greater response for the specified detectors. In several MS applications, this notion is considered conventional wisdom [27]. The aim of this research is to compare the sensitivity of the three detectors, cCAD, ELSD, and ESI-MS, in the reversed-phase mode and the HILIC mode for very polar compounds.

2. Experimental

2.1. Reagents

Omnisolve grade acetonitrile was purchased from EM Sciences (Gibbstown, NJ). HPLC grade water was obtained by purifying house DI water with a Millipore Milli-Q system (Billerica, MA). Ammonium formate and formic acid were purchased from Sigma–Aldrich (St. Louis, MO) and were 99% pure. The analyte solutes examined here were also from Sigma–Aldrich and were obtained in high purity grade (Table 1). All of the analytes had higher melting points and negative cLog *P* values (calculated octanol/water partition coefficients) indicating the polar nature of the compound.

2.2. Equipment and conditions

The HPLC system consisted of a 1200 series binary pump, autosampler, column thermostat, and diode array UV detector (Agilent, Palo Alto, CA). The mobile phases were pumped at a flow rate of 1.0 mL/min with a column temperature of 35 °C. The injection volume was set at 5.0 μL. For the reversed-phase mode experiments, the HPLC column was the Waters Atlantis T3 C18 (Milford, MA) and for the HILIC mode experiments the HPLC column was the Phenomenex Luna HILIC (Torrance, CA). Both columns had the dimensions of 150 mm × 4.6 mm with 3.0 μm particle size.

The buffer used was a 10 mM ammonium formate solution in water that had been pH adjusted to 3.0 with formic acid. The reversed-phase mode experiments utilized the Atlantis T3 column and the mobile phase was typically 5% acetonitrile/95% buffer mobile phase (isocratic). The HILIC experiments were operated with the Phenomenex Luna HILIC column and the mobile phase was typically 90% acetonitrile/10% buffer mobile phase (isocratic). Specialized columns, such as the Atlantis T3 used here, are capable of running at 100% aqueous conditions without loss of retention or phase dewetting. For these experiments, the 5/95 acetonitrile buffer was selected as a common mobile phase for the reversed-phase mode as this is the weakest mobile phase that can be utilized on the majority of reverse phase stationary phases while still maintaining reproducible chromatography. There were several instances where the acetonitrile content of

Table 1
Solutes examined in this study. The cLog *P* values were determined by Chemdraw Ultra version 9.0.7. The melting point data were obtained from the Merck Index Online.

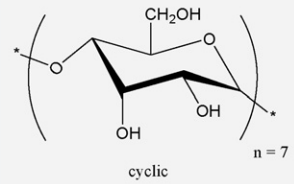
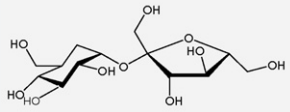
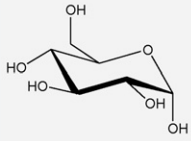
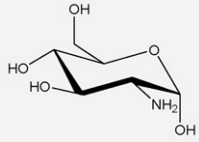
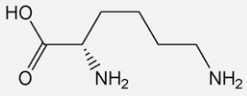
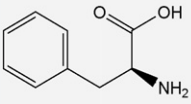
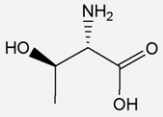
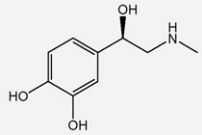
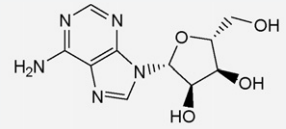
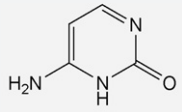
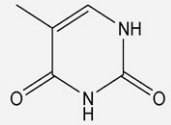
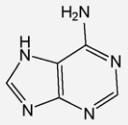
Name	Molecular weight (g/mole)	Melting point (°C)	cLog *P*	Class	Structure
β-Cyclodextrin	1134.4	197[a]	<−4	Saccharide neutral	
Sucrose	342.3	160–186[a]	−2.9	Saccharide neutral	
Glucose	180.1	146–150	−2.2	Saccharide neutral	
Glucosamine	179.2	88	−2.76	Saccharide basic	
Lysine	146.2	224[a]	−3.4	Amino acid zwitterion	
Phenylalanine	156.1	283[a]	−1.6	Amino acid zwitterion	
Threonine	119.1	255[a]	−2.4	Amino acid zwitterion	
Epinephrine	183.2	211–212	−0.7	Hormone and neurotransmitter basic	
Adenosine	267.2	234–235	−2.2	Nucleoside basic	
Cytosine	111.1	320[a]	−1.8	Nucleotide basic	
Thymine	126.1	335–337[a]	−0.6	Nucleotide basic	

Table 1(*Continued*)

Name	Molecular weight (g/mole)	Melting point (°C)	cLog *P*	Class	Structure
Adenine	135.1	110	−0.1	Nucleotide basic	

[a] The listed temperature is the onset of decomposition.

these mobile phases was adjusted so the retention of the analyte was similar in each mode of operation. This was done so the peaks for a given analyte would have similar retention factors (and therefore similar peak widths) in both modes of operation. The intent of varying the mobile phase compositions was to ensure as fair of a comparison as possible between the two modes of operation (comparing the peak area of peaks with similar widths is a more fair comparison than comparing peaks with different widths).

The ELSD was model 3300 from Grace Davison Discovery Sciences (Deerfield, IL). The ELSD drying gas flow rate was set at 1.6 L/min, the drift tube temperature was 60 °C and the gain was set at 4. The cCAD was from ESA Biosciences (Chelmsford, MA) and was operated with a range setting of 100 pA and a filter setting of none. The mass spectrometer was an Agilent 6120 and was operated in the positive ion mode with an electrospray ion source that was operated with the following settings: fragmentor = 70 V, dry gas temperature = 300 °C, dry gas flow rate = 10 L/min, nebulizer gas pressure = 35 psig, capillary voltage = 3000 V. The MS scan was a single ion monitoring (SIM) on the M + H ion for each analyte. Additionally, a 1–10 post-UV detector split was used during the MS experiments. For the MS and ELSD experiments, chromatographic data was obtained with Chemstation software from Agilent. For the cCAD experiments, chromatographic data was acquired from a Thermo A2D with Thermo Atlas chromatographic data system (v8.2, Thermo Electron, USA).

3. Results and discussion

Twelve compounds were analyzed in the reversed-phase mode and the HILIC mode of operation and detected with three different detectors. The compounds (Table 1) were chosen to have a polarity sufficient to allow for retention in the HILIC mode while being poorly retained in the reversed-phase mode. For each compound, linearity solutions were prepared (3–5 levels) in the 1–50 μg/mL range. Generally, mobile phase solutions were used as sample diluent, i.e. reversed-phase mobile phase for the reversed-phase linearity solutions, and HILIC mobile phase for the HILIC linearity solutions. Occasionally the diluents used for the HILIC mode had to be adjusted to improve sample solubility.

The ELSD response follows the relationship:

$$y = ax^b \quad (1)$$

where y is the response (area) and x is the mass injected on-column. The a and b terms are response factors that are related to the nature of the light scattering phenomena. Particles of different size can scatter light by different mechanisms, such as Rayleigh scattering, Mie scattering, reflection and refraction [28]. Consequently, determining the impact of concentration on signal, i.e. sensitivity, is difficult. There is precedence in the literature [29] for using a log–log linearization of data to determine a first-order slope

$$\text{Log}\, y = \text{Log}\, a + b\text{Log}\, x \quad (2)$$

which allows the sensitivity

$$\text{sensitivity} = a \times b \quad (3)$$

to be expressed as the product of the two ELSD response factors. This is the procedure that was utilized for the ELSD data and the results are presented in Table 2. The cCAD has been shown to have a second-order relationship between concentration and response and the manufacturer recommends that a quadratic equation be fit to calibration data. Over narrower concentration ranges, it has been shown that linear fits to calibration data produce acceptable correlation coefficients [19] and the cCAD results in Table 3 bear out this observation. Lastly, API mass spectrometry has been shown to be linear, albeit with somewhat narrow dynamic ranges. The data from the ESI-MS experiments were fit with a first-order relationship and the results are presented in Table 4. In all cases, the response data from the linearity solutions were plotted versus the sample mass injected on column. Generally the correlation coefficients (r) of these linear fits were very good (0.99–1.00).

3.1. Results grouped by detector

The results for the ELSD indicate that better sensitivity can be achieved in the HILIC mode (Table 2, Fig. 1). While the two modes

Table 2
Summary of sensitivity and correlation coefficient (r) results for the ELSD in the HILIC mode and the reversed-phase mode in the 5–500 μg/mL range.

	ELSD HILIC		ELSD reversed-phase	
	Sensitivity	r	Sensitivity	r
Adenine	6486	1.00	3770	1.00
Adenosine	7152	1.00	1689	1.00
Epinephrine	12366	1.00	8653	1.00
β-Cyclodextrin	5825	1.00	4466	1.00
Cytosine	5595	1.00	3412	1.00
Glucosamine	6070	1.00	4438	1.00
Glucose	5036	1.00	5080	1.00
Lysine	9267	1.00	6650	1.00
Phenylalanine	8255	1.00	5324	1.00
Sucrose	6535	1.00	3730	1.00
Threonine	6126	0.99	4699	1.00
Thymine	2130	0.99	2055	1.00

Table 3
Summary of linearity results for the cCAD in the HILIC mode and the reversed mode in the 5–500 μg/mL range.

	cCAD HILIC			cCAD reversed-phase		
	Slope	y-Intercept	r	Slope	y-Intercept	r
Adenine	3809	57.3	1.00	446.2	4.8	1.00
Adenosine	3750	49.6	1.00	311.0	−0.7	1.00
Epinephrine	2889	9.9	1.00	240.0	8.1	0.99
β-Cyclodextrin	2683	22.3	1.00	289.8	14.1	0.99
Cytosine	10064	−22.3	1.00	646.8	13.2	1.00
Glucosamine	3386	28.6	1.00	317.0	3.0	1.00
Glucose	3879	26.0	1.00	344.2	2.8	1.00
Lysine	3606	98.4	1.00	409.6	7.1	1.00
Phenylalanine	3344	−3.5	1.00	501.2	−1.9	1.00
Sucrose	2830	16.9	0.99	260.3	4.3	0.99
Threonine	7713	−55.3	1.00	650.6	12.7	1.00
Thymine	4532	27.3	1.00	801.0	−1.5	1.00

Table 4
Summary of linearity results for the ESI-MS detector in the HILIC mode and the reversed-phase mode in the 1–25 μg/mL range.

	ESI-MS HILIC			ESI-MS reversed-phase		
	Slope	*y*-Intercept	*r*	Slope	*y*-Intercept	*r*
Adenine	99265330	−127.37	1.00	8028892	704.1	1.00
Adenosine	17302810	4954.31	1.00	3008104	1092.2	1.00
Epinephrine	20363	−49.56	1.00	2907	339.4	0.99
β-Cyclodextrin	5950	−27.32	1.00	2547	−26.8	1.00
Cytosine	159492579	22005.31	1.00	10733424	3251.9	1.00
Glucosamine	251134	−1290.90	1.00	3273	−620.2	0.99
Glucose	55623	136.51	1.00	1696	332.5	0.98
Lysine	16326	−918.02	1.00	4588	41.2	1.00
Phenylalanine	129587	−224.79	1.00	10610	−44.5	1.00
Sucrose	630072	2695.23	1.00	263187	−187.6	1.00
Threonine	75564629	3540419	0.98	7404798	72197.96	1.00
Thymine	11314773	152577.2	0.99	13947136	87607.25	1.00

produce similar sensitivity, the HILIC mode is slightly better than the reversed-phase mode. Both glucose and thymine yield nearly identical sensitivity both modes. The improvements to sensitivity when using the HILIC mode are less pronounced compared with the other detectors. One possible reason for this may be due to the design of the ELSD detector used in this study. Several designs for commercially available instruments make use of an impactor in the nebulizer, which filters large droplets out of the sprayed aerosol. Essentially, this reduces the total flow of column effluent into the spray chamber. The ELSD utilized here did not have an impactor as part of the nebulization process. Consequently the total flow from the column is introduced into the drift tube. In contrast, the cCAD has an internal split that directs a significant portion of the nebulized spray to the waste, and the LC/MS experiments used a 1:10 post-column split to reduce the total flow into the ESI source (which is a good practice for LC/ESI-MS). Of all the detectors studied, the ELSD is burdened with the greatest volume of mobile phase to evaporate in order to achieve detection. This may contribute to the overall smaller increase in sensitivity compared to the cCAD and ESI-MS as well as similar sensitivity for glucose and thymine.

The cCAD is much more sensitive for the HILIC applications studied than the reversed-phase applications (Table 3, Fig. 2). All of the response factors determined in the HILIC mode are significantly greater than the response factors from the reversed-phase mode. On average the cCAD is 10 times more sensitive in the HILIC mode than in the reversed-phase mode. This is not unexpected. It has been shown that analyte response in the cCAD is related to the size of the analyte particles after nebulization and drying, and that mobile phases with higher organic compositions produce larger particles in the cCAD nebulizer [22]. Consequently, the sensitivity improvement for the HILIC mode with the cCAD is related to the larger particles that form when nebulizing the organic rich mobile phase. Mobile phase volatility is a component of the aerosol drying process that impacts the size of the analyte particles. Fig. 3 is an example of the synergistic benefits that can be taken advantage of when using HILIC with the cCAD. In this example sucrose has superior retention and response when using the HILIC method. Conversely, when utilizing a reversed-phase method, sucrose is unretained and less signal is obtained.

The ELSD and cCAD are often described as universal detectors that have similar or identical responses for all analytes [18,19,22,25,30]. Generally this is held to mean that all analytes will produce an approximately equal response with no dependence on the chemical properties of the analyte. For the ELSD, there is a significant difference in response among the compounds in both modes (Table 2, Fig. 1) that range from approximately 2000–12,000 area units/μg in the HILIC mode, and ~2000–8000 area units/μg in the reversed-phase mode. Likewise, within each mode of operation, the cCAD also exhibits some variation in response, ~3000–10,000 area units/μg for HILIC and ~200–800 area units/μg for the reversed-phase mode (Table 3, Fig. 2). It may be possible to decrease the range in response factors of the ELSD, thus making the detector have a more universal response, by optimizing the nebulization geometry, gas flow rate and drift tube temperature. However the

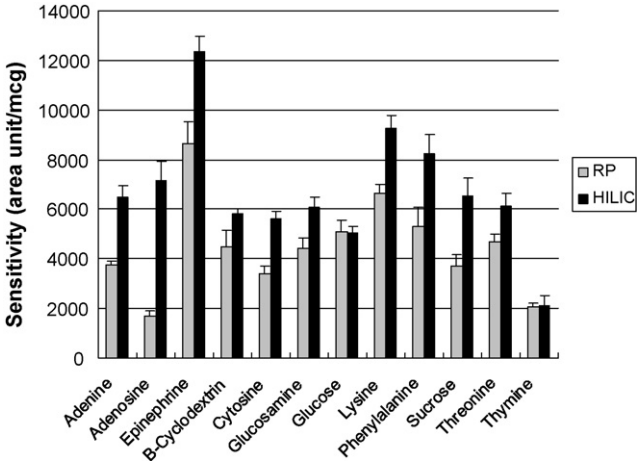


Fig. 1. Comparison of the chromatographic sensitivity between the reversed-phase mode and the HILIC mode for the ELSD. Error bars of 3 times the standard error of regression are included on each data point.

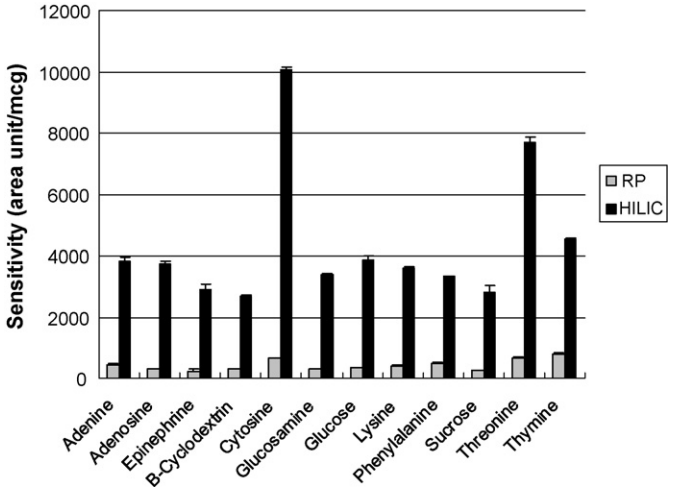


Fig. 2. Comparison of the chromatographic sensitivity between the reversed-phase mode and the HILIC mode for the cCAD. Error bars of 3 times the standard error of regression are included on each data point.

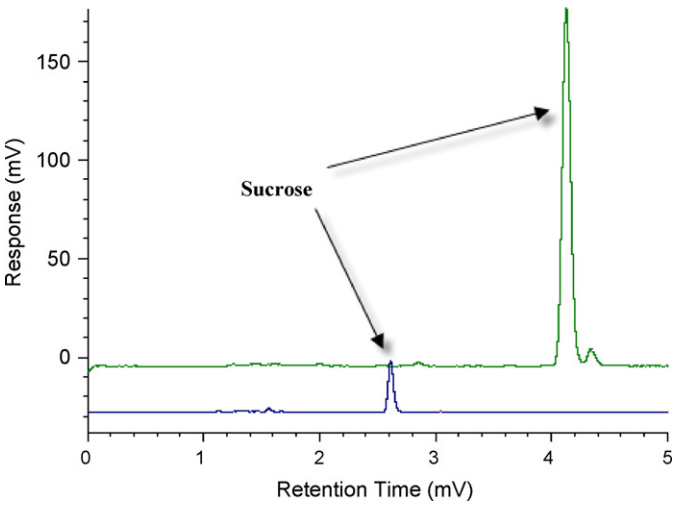


Fig. 3. Comparison of the difference in response for sucrose using the cCAD. The bottom trace is in the reversed-phase mode with a 100% buffer mobile phase and the top trace is in the HILIC mode with a 70% acetonitrile/30% buffer mobile phase. Both chromatograms are from injections of a 100 μg/mL solution preparation.

cCAD does not enable the user to adjust the drying gas flow rate or temperature.

The sensitivity of the ESI-MS detector also benefits from the volatility of HILIC mobile phases. Generally, the compounds examined produced approximately 10 times more signal in the HILIC mode compared with the reversed-phase mode (Fig. 4, Table 4). There is one exception to this observation. While both modes of operation yield excellent sensitivity for thymine, the reversed-phase mode yields slightly greater (20%) response than the HILIC mode. The reason for this is not readily apparent. The peak shape of thymine in both modes is symmetrical and there are no other ionization mechanisms (e.g. sodium or ammonium adduct formation, solvent clustering or fragmentation) evident when the mass spectra are examined. Given the basic nature of thymine and the presence of formic acid in the mobile phase, it is possible that ion transport to the gas phase is not hindered by the presence of water in the reversed-phase mobile phase.

The data in Fig. 4 are presented with a log-based *y*-axis. This is done because the sensitivity factors obtained from the LC/MS

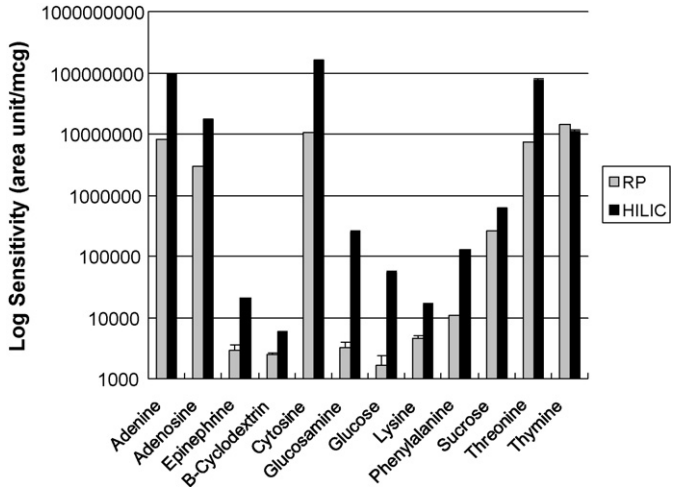


Fig. 4. Comparison of the chromatographic sensitivity between the reversed-phase mode and the HILIC mode for the LC ESI-MS detector. Error bars of 3 times the standard error of regression are included on each data point. Note that the *y*-axis is a log scale.

span several orders of magnitude. This is an important difference between the LC/MS and the other two detectors. As previously mentioned, the ELSD and cCAD are described as universal detectors. In contrast, the LC/MS is not a universal detector. The difference in response between two analytes is related to the ionization efficiency in the API source (ESI or APCI). Consequently a compound that does not ionize well in the ion source can have much lower response than a compound that does ionize well.

3.2. Results by compound classification

The compounds studied can be classified into three groups by their function in nature: amino acids, saccharides, and nucleosides/nucleotides. Another classification scheme is by molecular properties: neutral compounds, basic compounds, and zwitterionic compounds. In either case the compounds studied tend to be grouped together in the same fashion (Table 1). The data from Tables 2–4 was analyzed for any trends by compound classification, however there were no evident correlations. The most prominent trend is those compounds that did yield higher than normal response tended to possess basic nitrogen atoms. The higher response of these basic compounds may be related to the mobile phase buffer used (ammonium formate) or it may be compound specific. Further work is necessary to determine this.

4. Conclusions

For the compounds studied, the HILIC mode was generally able to produce greater response than the reversed-phase mode. The improved sensitivity of the HILIC mode is due to the volatile nature of HILIC mobile phases. These organic solvent rich mobile phases tend to produce greater analyte signal in the ELSD, cCAD and ESI-MS detectors. The response factors measured exhibited a significant level of variability between analytes for all detectors. This indicates that the universal ELSD and cCAD do not always provide a universal response, but are capable of very similar response within a mode of operation. The cCAD and the ESI-MS were capable of much greater response in the HILIC mode. Within the study, several compounds with basic residues produced responses that were greater than average. Future work should include sample sets that contain acidic analytes to determine if there are any functional group effects on sensitivity. Additionally, a variety of high molecular weight analytes should be included to assess any molecular weight effects on sensitivity.

Acknowledgements

The authors gratefully acknowledge the support of Abbott Laboratories for support of this work and Ye Bao gratefully acknowledges the support of the Abbott Laboratories Summer Internship Program.

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