

Toward Single-Calibrant Quantification in HPLC. A Comparison of Three Detection Strategies: Evaporative Light Scattering, Chemiluminescent Nitrogen, and Proton NMR

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There is an urgent need for detection technologies that enable accurate and precise quantification of solutions containing small organic molecules in a manner that is rapid, cheap, non-labor-intensive, readily automated, and without a requirement for specific analyte standards. We provide a theoretical analysis that predicts that the logarithmic nature of the working domain of the evaporative light-scattering detector (ELSD) will normally bias toward underestimation of chromatographically resolved impurities, resulting in an overestimation of analyte purity. This analysis is confirmed by experiments with flow injection analysis (FIA) and gradient reversed-phase high performance liquid chromatography (RP-HPLC). Quantification is further compromised by the dependence of response parameters on the matrix composition and hence on the retention time of the analyte. Attempts were made to ameliorate these problems by using the response surface of a single compound to calibrate throughout the HPLC gradient. A chemiluminescent nitrogen detector (CLND) was also used in a similar manner, and the performance of the two techniques were compared against those of each other and that of a reference standard technique. A protocol for this purpose was developed using proton nuclear magnetic resonance (¹H NMR) and the ERETIC method to enable quantification by integrating proton signals. The double-blind comparison exercise confirmed molar nitrogen CLND response to be sufficiently stable and robust across a methanol gradient to be used with a single external nitrogenous calibrant to quantify nitrogen-containing compounds of known molecular formula. The performance of HPLC-CLND was very similar to that of NMR, while that of HPLC-ELSD was seen to be significantly worse, showing it to be unsuitable for the purpose of single-calibrant quantification. We report details and experience of our use of RP-HPLC-CLND-MS to characterize and quantify small amounts of solutions of novel compounds at nominal levels of 10mM in microtiter plate (MTP) format.

Modern pharmaceutical research programs produce and screen hundreds of thousands of new chemical entities (NCEs)

per annum. Before they can be used either as synthetic intermediates or for initial biological testing, NCEs must be shown to have a minimal standard of purity to ensure they are fit for the business purpose. At the time of initial synthesis (often sub-milligram amounts in MTP format), the identity of many impurities will be unknown and so it will not be possible to economically establish their response factors for typical HPLC detectors used in high-throughput analysis mode. In these circumstances, purity assessments are often made on the basis of relative "Area Percents" using detectors such as UV, ELSD, and MS, in the knowledge that there is potential for response factors of unknown impurities to differ significantly from those of each other and of the target compound. Accordingly, purity assessments are inevitably compromised to some degree.

Rapid and sustained improvements in automated library synthesis and robotic screening capabilities are fueling an increasing demand for accurate quantification capabilities as part of normal QC protocols. In particular this is driven by the need to ensure the integrity of the structure–activity relationships (SAR) that critically input into drug discovery research programs. The requirement for improved high-throughput, automated quantification is self-evident; however, this is difficult to achieve in practice. While it is now completely practical to automate high-throughput purification, for example, by mass-directed preparative chromatography,¹ the gravimetric weighing of large numbers of products is slow, labor-intensive, and only sufficiently accurate for products in excess of low milligram amounts. Dissolution of these products to a nominated concentration for screening also assumes complete solubility in the chosen solvent and that the mass is composed totally of target analyte. This is not always the case, furthering the need for dependable solution concentration measurement techniques² to facilitate accurate potency values and reliable SAR

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data. Alternative approaches are required that are rapid, sensitive, accurate, precise, and specific. The term specificity not only suggests the use of a chromatographic "front end" but also implies that due account must be taken of residual water, solvents, salts, silica, and additives that may be present as a result of synthetic and purification procedures. Ideally chromatographic separation from related impurities accompanies expression of target compound peak area as an amount.

UV and mass spectrometric (MS) detectors are only capable of performing this role to a limited extent. When a set of compounds has a common chemical scaffold upon which minimal chemical diversity is expressed, then extinction coefficients at particular wavelength(s) or ion potentials at specific value(s) of m/z may on occasion be expected to converge toward reasonably constant values. A well-characterized compound from the series may then be suitable for use as a common quantitative calibrant. However, in the majority of cases, the extent of chemical diversity within a library, regardless of any conserved structural elements, is so large that gross variations in extinction and ionization properties are present, and so the assumption of a common response factor results in severe quantitative errors.

Our efforts toward obtaining a universal quantitative detection regime have been focused upon the evaporative light-scattering detector (ELSD) and the chemiluminescent nitrogen detector (CLND). Both of these detectors have been used for a number of years to aid quantification and purity assessment in conjunction with high-throughput chemistry programs.^{3–17}

ELSD was originally introduced in 1966 by Ford and Kennard,¹⁸ and the theory of its operation was developed by Charlesworth,¹⁹ Stolyhwo et al.,^{20,21} and Mourey and Oppenheimer.²² It has been used extensively for qualitative and quantitative analysis of several classes of compounds, especially poorly chromophoric materials.^{21,23–28} Quantitative work has employed well-characterized standards, either of the specific analyte, or of closely related compounds, and the latter practice has perforce been followed when using ELSD to quantify combinatorial libraries. These are

normally comprised of chromophoric compounds, and one attraction of ELSD is that it generally provides a more uniform response than does UV. Thus, when standards that were sufficiently representative of the whole library were employed, quantification errors of as low as 10–20% were found for limited sample sets.^{3,6} Fang et al.⁸ applied the ELSD detector to 15 small molecule libraries using 90 standard compounds, obtaining an average error of 18.5%, and later work from the same group¹¹ demonstrated improved quantification over that obtained using UV at 214 nm. However, larger errors were obtained when standards were less representative of the library,⁸ and reduced ELSD response may be obtained from low molecular weight compounds.^{6,11} These considerations are less important later in the drug development process when target molecules are normally available in sufficient quantities and purities for use as quantitative reference standards for the chosen detection scheme (e.g., in metabolic and pharmacokinetic studies with LC-MS-MS).

Since it was first used with HPLC in 1992 by Fujinari and Courthaudon,²⁹ CLND has found rapid application in the areas of combinatorial chemistry^{4,5,7,9,12,16,17} and metabolic studies.¹³ Taylor et al.⁵ found the absolute error for a set of chemically and structurally diverse compounds averaged approximately $\pm 10\%$. Shah et al.⁹ used flow injection analysis (FIA) in conjunction with CLND and MS to analyze up to 1000 compounds per day.

Unlike ELSD, CLND can provide molar information, although to convert CLND data into an amount of analyte, it is necessary to know the molecular weight and number of nitrogen atoms in the molecule. These data are normally available from the registered structure and may be confirmed with concurrent MS techniques, which unless already required for other purposes can be argued to inflate the cost of individual measurements well above those that use a single ELSD. However, CLND has greater instrumental complexity than ELSD and so has potential for higher maintenance overheads. Diminished CLND response can occur for compounds where adjacent nitrogen atoms may give diatomic nitrogen during combustion.³⁰ CLND is incompatible with nitrogenous chromatographic solvents and additives, and baseline chromatographic separation is necessary for accurate quantification. In this context supercritical fluid chromatography with carbon dioxide and methanol may for some cases be an attractive option.^{31,32} For these reasons, comparisons between the performances of CLND and ELSD are particularly relevant.

Allgeier et al.¹⁶ compared the performance of four compounds using ELSD and CLND, and Yurek et al.¹² described the development and use of a combined HPLC-PDA-ELSD-CLND-TOFMS system for identification and quantification of samples from

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combinatorial libraries, finding a correlation $r = 0.94$ between the two measurements for a set of 35 similar library samples. A double-blind study comparing quantification by NMR and by LC-CLND was performed by Lewis et al.⁷ who found both techniques to be generally accurate to within $\pm 5\%$. Additionally, Hamper et al.³³ and Pinciroli et al.³⁴ have used ^1H NMR to quantify small libraries, and recent work by Yan et al.³⁵ compared quantification by UV, ELSD, CLND, and NMR, concluding that the former two techniques provide a measure of relative purity, while NMR and LC-MS-CLND are capable respectively of medium- and high-throughput quantitative (absolute) purity determinations. Our initial evaluation of CLND indicated that molar nitrogen response is sufficiently stable and robust to consider it in preference to ELSD as a high-throughput quantitative tool without the need to weigh any materials other than a single primary reference standard such as caffeine or indole.

In this report, we provide a short theoretical analysis to illustrate the limitations of using ELSD areas either as indicators of chemical purity or as the basis of single-calibrant quantification procedures for multiple analytes. We document our evaluation of an experimental protocol³⁶ designed to allow convenient use of a single ELSD calibrant to enable quantification across a broad range of chemical diversity and report the application of the ERETIC³⁷ Method in Proton NMR as a “Gold Standard” to compare single-calibrant ELSD and CLND based quantifications of a set of 117 samples having three different core scaffolds. Finally, we report further experiments designed to demonstrate some potential consequences of using ELSD without appropriate analyte-specific calibration.

THEORY

Evaporative Light-Scattering Detection. Although the logarithmic nature of the main working regime of the ELSD is well-known, the extent to which the suppressive effect at low levels of analyte can cause quantitative errors is poorly appreciated. The area response A of the ELSD is known to be related to the mass M of the analyte by the following relationship³⁸

$$A = aM^b \quad (1)$$

where a is the response factor and b is the coefficient of regression of the response curve. Considering the case of two compounds denoted 1 and 2, eq 1 may be restated as

$$\log A_1 = \log a_1 + b_1 \log M_1 \quad (2)$$

$$\log A_2 = \log a_2 + b_2 \log M_2 \quad (3)$$

$$\log(A_2/A_1) = \log a_2 - \log a_1 + b_2 \log M_2 - b_1 \log M_1 \quad (4)$$

In an idealized situation the two compounds may exhibit the same response factors a and coefficients b , and eqs 2 and 3 may be rewritten as

$$\log(A_2/A_1) = b \log(M_2/M_1) \quad (5)$$

Suppose the object of the experiment is to measure the absolute level of compound 2 in compound 1 as a percentage of the total

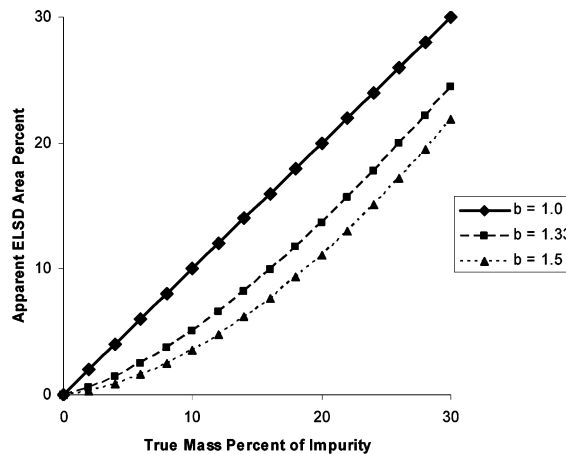


Figure 1. Apparent (Area%) levels of an impurity in a major component that eq 7 predicts would be obtained by HPLC using ELSD. The assumption was made that both major and minor components have identical values of response factor a and coefficient of regression b . The effect of the value of b is shown.

sample so that $M_2 + M_1 = 100$, then the areas returned by the ELSD will vary as a function of exponent b :

$$A_2/A_1 = [M_2/(100 - M_2)]^b \quad (6)$$

Setting $A_2 + A_1 = 100$, the area percent of compound 2 is A_2 when

$$A_2 = 100[M_2/(100 - M_2)]^b / \{1 + [M_2/(100 - M_2)]^b\} \quad (7)$$

This relationship is illustrated in Figure 1, which shows that underestimations of purity will increase with increasing exponent b , the value of which is expected to be 1.33 in an idealized situation of pure Mie scattering³⁹ (note that for detectors such as UV, CLND, and MS where one normally works in a linear response domain, b would be unity).

It is seen that an impurity present at 10% m/m would appear to be present at only 5% even in the ideal set of circumstances where a single (i.e., Mie) light-scattering mechanism existed and both the main compound and its impurity had identical response factors. This suppression effect produces greater relative error as the true amount of impurity diminishes; impurities present at 1% m/m will only contribute 0.2% of total area. Compounds will therefore generally appear to be of inflated purity if assessed by an uncalibrated Area% HPLC-ELSD method. Even this idealized

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situation rarely applies when working with NCEs containing unknown levels of unidentified impurities. Nebulization will normally result in a variety of crystal morphologies and scattering mechanisms, so in general $a_1 \neq a_2$ and $b_1 \neq b_2$. Variations among the values for parameters a and b will be significant even for closely related compounds. Thus, for example Fang et al.⁸ obtained calibration curves from six standard compounds selected from within a library and found slope b values between 1.41 and 1.47 and intercept $\log a$ values between 2.02 and 2.18. This small difference in $\log a$ represents a 45% difference in response factor a .

In the general case where $a_1 \neq a_2$ and $b_1 \neq b_2$, eq 4 has no unique solution for M_1 and M_2 for any given set of values for A_1 , A_2 , a_1 , a_2 , b_1 , and b_2 . Unlike “linear” detectors such as UV where $b_1 = b_2 = 1.00$, the area ratio A_2/A_1 in ELSD depends not only on the ratio M_2/M_1 but also upon the absolute values of M_2 and M_1 . Different amounts of material can be delivered either as a more concentrated solution or as greater volume of the same solution, so ELSD area ratio is predicted to change with injection volume.

It is clear that strictly relevant calibration is required to enable quantitative use of ELSD.

ERETIC Method in Proton NMR. Recent advances in NMR have made quantification by “proton counting” an attractive option. The ERETIC (**E**lectronic **R**Eference **T**o access **I**n-vivo **C**oncentrations) method in NMR was introduced in 1997 by Barantin et al.³⁷ as a way of determining absolute concentrations. It is based upon the addition of an artificial radio frequency (rf) reference signal, to the observed NMR spectrum, of whatever sample is being measured. Full control of the frequency (position), amplitude (size), and phase of the synthetic signal, simultaneous with the signals from the physical sample, enables accurate integral referencing and comparison for quantification of all components in the measured spectrum. This straightforward method, which obviates all of the careful considerations and the contamination of the sample associated with an internal standard, has allowed routine application of the ERETIC technique in NMR for structure verification and quantification in high-throughput drug discovery. Hence, in our laboratories many tens of thousands of samples per annum are routinely verified and quantified by NMR.

EXPERIMENTAL SECTION

Materials. Fluticasone propionate was available in-house, dexamethasone was purchased from Avocado Research Chemicals Ltd., Heysham, UK, and doxazosin mesylate and dofetilide were from Apin Chemicals, Abingdon, UK. The other materials shown in Figure 10, dimethyl sulfoxide (DMSO), and formic acid (96%) were obtained from Sigma-Aldrich, Gillingham, UK. The deuteriodimethyl sulfoxide (DMSO- d_6) was obtained from CIL Inc, Andover, MA. Research samples used in the course of this work were made available from current synthetic programs at GSK, Stevenage, UK, either as crystalline solids or as solutions in DMSO. N,N -Dimethylformamide (DMF) and HPLC grade acetonitrile (MeCN) were purchased from Fisher Scientific UK Ltd., Loughborough, UK. Ultra gradient grade methanol (MeOH) was obtained from Romil Ltd., Cambridge, UK. Water was purified through an ELGA water purification system (High Wycombe, UK).

Instrumentation. Flow injection analysis (FIA) and HPLC were performed at 40 °C on Agilent HP1100 Series instruments equipped with diode-array detection (Agilent Technologies, Wald-

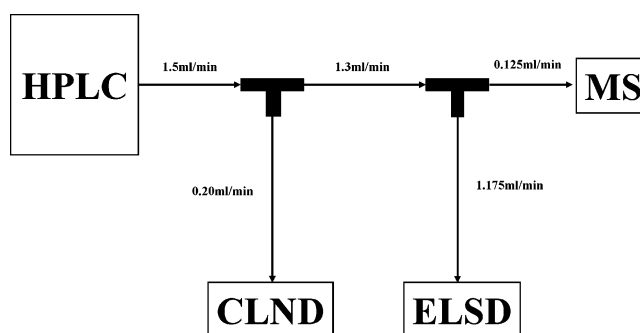


Figure 2. Schematic diagram of the HPLC-ELSD-MS-CLND system employed. The ELSD used was the PL Model 1000 with nebulizer at 50 °C and evaporator at 70 °C. The HPLC was equipped with a diode array detector.

bronn, Germany). The static mixers were replaced with A-330 Semi-Prep filters (Anachem, Luton, UK) to provide a low dwell volume configuration. One system was kept for exclusive use with CLND and was not exposed to nitrogenous solvents such as acetonitrile either before or during this work. This system was also equipped with an Agilent Model 1100 G2255A WP handler with deepwell stack and G2254A Autointerface to a G1367A WPALS. The Model 8060-C CLND (Antek Instruments Inc., Houston, TX) instrument was controlled using Antek Model 8060 Test Software version 1.06 and operated with a furnace temperature of 1050 °C, ozone and oxygen settings at 250 mL/min, and inlet and makeup helium flows at 50 mL/min. Two ELSD instruments were used: a Polymer Labs (Church Stretton, UK) Model 1000, with nebulization and evaporation temperatures set as indicated in the text, and nitrogen nebulizing gas at 0.4 L/min, and a Polymer Labs Model 2100 with nebulization and evaporation respectively set at 26 and 28 °C, and nitrogen at 1.4 L/min. The HPLC-ELSD-MS-CLND system utilized the Model 1000 ELSD with nebulizer at 50 °C and evaporator at 70 °C and is shown schematically in Figure 2. It incorporated an Agilent 1100 MSD in +ve mode to acquire TIC and SIM mass spectral data. This combination of instruments was controlled using Agilent Chemstation version 10.1. A separate Model-1100 HPLC-DAD-ELSD combination operating under MassLynx V3.5 control was also used to acquire FIA data.

The ^1H NMR measurements were carried out at a magnetic field strength of 14.1 T on a Bruker AV600 FTNMR spectrometer equipped with a 1 mm diameter triple nucleus ($^1\text{H}/^{13}\text{C}/^{15}\text{N}$) Z-gradient probe with automatic tuning and matching. Each spectrum was acquired under the control of the program XWIN-NMR 3.5 (Bruker Biospin, Karlsruhe, Germany) with a simple 30° observation pulse on one channel and the ERETIC signal (set at -1 ppm) on the second channel, into 65 536 data points. The pulse repetition rate was 5.7 s to allow for sufficient T_1 relaxation of the solute and a total of 64 transients per sample were acquired to build up signal to noise. The resultant free induction decay (FID) was Fourier-transformed with an apodization line broadening of 1 Hz, phased, baseline-corrected, and integrated relative to the ERETIC signal.

The ERETIC signal integral area was calibrated by measuring a series of standard samples of known solute concentrations, each produced by careful weighing and measured solvent volume dissolution. Once calibrated, the ERETIC signal conditions, the

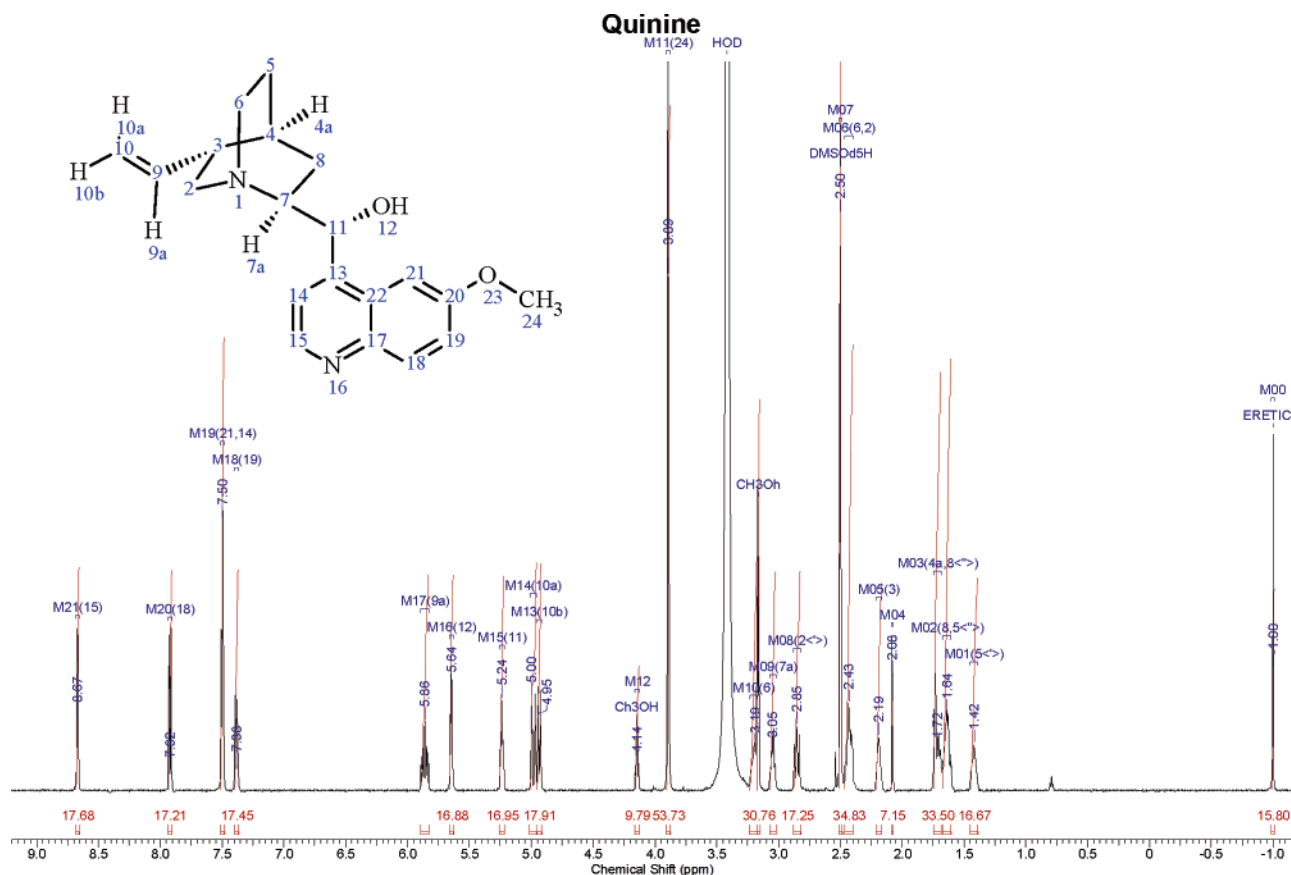


Figure 3. Proton NMR spectrum of quinine at 600 MHz, showing ERETIC signal at -1 ppm with its integral set to the calibrated value (15.8) and hence the average single-proton solute signal gives a direct readout of 17.6 mM for that sample.

frequency, power, shape, and phase were kept constant for each sample measured. The reference integral for the ERETIC was set to the calibrated value and the concentration of the solute was obtained by identifying a single-proton signal and reading its integral directly. In cases where no single proton was accessible, then either sums of two or three proton multiplets were identified and their integrals divided by 2 or 3, respectively, to give the absolute concentration of that particular sample. It is important to recognize that the process of integral reference value setting and single-proton signal detection may be automated. However, in this study, for absolute reliability of analysis, manual inspection and interpretation were employed. As an example, the second sample of the test compound quinine is shown in Figure 3.

“Blind” Quantification Comparisons. A set of 20 acidic, basic, and neutral compounds (Table 1) representing a cross section of compounds of pharmaceutical interest was chosen for this study. The set contained a variety of compounds of molecular weight 165–340 and calculated boiling points (at 760 Torr) of approximately 300–600 °C. Each compound was prepared accurately in DMSO- d_6 at two randomly selected concentrations in the range 1–20 mM. The solutions were then assigned random codes and divided into two aliquots for independent quantification by NMR and by HPLC-ELSD-MS-CLND. Raw data was processed prior to decoding and comparison with known concentrations.

The instrumental configuration shown in Figure 2 was employed to obtain the HPLC-ELSD-MS-CLND data. The column used was a 20×2.0 mm $3 \mu\text{m}$ Luna C18 Phenomenex Mercury MS LC/MS cartridge, eluted with a gradient of 0 to 100% “B” in

5.0 min, where “A” = 0.1% formic acid in water and “B” = 0.1% formic acid in methanol. The flow rate was 1.5 mL/min and UV was monitored at 254 nm. One microliter aliquots of calibration standards (5–30 mM) of 5-fluorocytosine (5-FC) in DMSO- d_6 were injected at approximately 0.93 min intervals (determined by autosampler cycle time) throughout the gradient in order to establish a three-dimensional response surface of the nonretained 5-FC as a function of its concentration and the momentary eluant composition (expressed as elution time). 5-FC calibration curves (log Area vs log Amount for ELSD and Area vs Amount for CLND) were obtained at each 5-FC elution point in the gradient. The slope and intercept of the calibrations were expressed as functions of the 5-FC elution times. Analyte retention times were input into these equations, enabling derivation of the relevant response coefficients and subsequent conversion of an analyte peak area into the analyte amount. These values represent the amount of analyte calculated on the common basis of it possessing the same response coefficients as the single-calibrant 5-FC would possess at the retention time of the analyte. This process allowed correction to be made both for changes in response coefficients with eluant composition and for changes in split ratios occurring during the gradient. Calibration standards of indole in methanol/water were also run, and CLND results were also calculated with respect to the response of indole at a single-eluant composition.

Flow Injection Analysis Using ELSD. FIA was performed using a flow rate of 1 mL/min of mixtures of water and MeCN plus 0.05% formic acid. (Note that MeCN was used for this exercise in order to study ELSD response in high-throughput

Table 1. True and Experimentally Determined Concentrations of Samples Quantified “Blind” by ERETIC NMR, HPLC-ELSD, and HPLC-CLND

compound	concentration (mM)					
	true	NMR	ELSD ^a	ELSD ^b	CLND ^c	CLND ^d
sulpiride	4.0	4.0	2.3	2.9	3.8	3.3
	17.3	18.1	7.4	10.8	15.2	14.7
dibucaine	12.2	14.0	6.3	4.1	12.1	11.8
	18.4	21.6	9.7	6.3	17.2	17.1
<i>N</i> -acetylprocainamide	3.3	3.6	2.8	2.8	3.2	2.8
	7.8	8.5	7.0	6.6	7.1	6.7
furosemide	7.7	8.4	5.3	5.9	6.6	6.0
	8.6	8.4	6.0	7.0	7.4	6.9
sumatriptan	12.0	12.6	9.6	9.4	10.5	10.0
	13.7	14.3	11.3	10.5	12.2	11.8
benzocaine	9.1	9.3	0.0	0.0	8.6	7.5
	13.1	13.4	0.0	0.0	12.5	11.4
pindolol	1.3	1.1	0.9	0.2	1.9	1.2
	5.9	6.0	4.7	1.3	5.6	4.9
<i>N</i> -benzoyl-L-tyrosine ethyl ester	11.1	11.4	8.2	9.6	10.2	9.0
	16.3	16.6	12.2	13.5	14.5	13.3
quinine	16.3	15.8	13.7	9.5	13.1	12.6
	18.3	17.6	14.9	11.7	15.1	14.6
2,4,6-triphenylaniline	10.4	10.2	8.4	5.7	10.1	7.9
	12.4	12.3	9.4	5.6	11.2	8.8
viloxazine	6.0	5.3	1.1	0.0	5.4	4.1
	12.1	11.6	2.7	0.0	10.2	8.9
chlorpropamide	8.5	8.9	5.2	3.3	7.4	6.8
	9.6	9.9	6.0	1.8	8.5	7.9
caffeine	5.9	6.3	4.3	0.1	5.2	5.0
	11.0	11.3	9.5	0.6	10.4	10.2
<i>N</i> -benzyl-4-chloro-5-sulfamoylanthranilic acid	8.0	8.2	6.0	6.7	7.5	6.9
	16.7	18.3	13.4	ND	14.6	14.0
papaverine	5.0	5.3	5.8	3.2	5.3	4.1
	13.2	10.3	7.2	6.0	9.4	8.2
<i>N</i> -[(R)-1-(10-naphthyl)ethyl]phthalamic acid	13.9	15.2	11.6	12.8	12.6	11.3
	19.6	22.1	16.9	ND	17.7	16.4
propranolol	7.0	7.1	4.2	0.0	6.9	5.7
	9.6	9.7	5.1	0.0	9.0	7.8
2-(4-chloro-3-nitrobenzoyl)benzoic acid	7.8	7.5	5.1	5.5	7.6	6.3
	13.3	14.3	9.3	9.7	12.2	10.9
primidone	8.5	8.8	8.3	7.4	8.1	7.6
	10.5	11.3	10.5	9.2	9.7	9.2
tolfenamic acid	1.2	1.3	0.8	0.6	2.7	1.4
	18.6	19.8	14.7	3.6	16.9	14.6

^a Determined with nebulizer at 50 °C and evaporator at 70 °C. ^b Determined with nebulizer at 110 °C and evaporator at 130 °C. ^c Determined using 5-FC as reference standard with correction for retention time. ^d Determined using indole as reference standard without retention time correction. ND = not determined.

applications that did not involve CLND. It was anticipated that each solvent might influence response changes in a specific manner.) A short length of 0.005 in. i.d. PEEK tubing (Anachem) was used in place of an HPLC column. Both ELSD Models 1000 (with nebulizer at 110 °C and evaporator at 130 °C) and the 2100 were employed. Eluant from the DAD was connected directly to the ELSD under test without any splitting of the flow. The compounds indicated in Figure 10 were dissolved in appropriate mixtures of water and MeCN plus 0.05% formic acid. Small amounts of DMF were used where necessary to aid solubility. DMF was transparent to ELSD. Calibration series solutions were accurately prepared for each compound, generally within the range 1.5 µg/mL to 3.2 mg/mL, so that 2.5 µL injections delivered approximately 4–8000 ng to the detector. The matrix solvent was normally water/MeCN (1:1, v/v) plus 0.05% formic acid. ELSD calibration curves were established for each compound at each

of 11 different FIA solvent conditions (MeCN percentages of 0, 10, 20, ..., 100).

HPLC Analysis Using ELSD. Mixtures of selected materials were prepared and quantified using gradient HPLC. Gradient HPLC employed a 50 × 4.6 mm column of 3 µm ABZ+PLUS (Supelco, Sigma-Aldrich) operated at 1.0 mL/min with mobile phase “A” of water plus 0.05% (v/v) formic acid and mobile phase “B” of MeCN plus 0.05% (v/v) formic acid. “B” was 0% for 0.1 min and then linearly programmed to 100% over 4.9 min. Injection volumes were as reported in the Results and Discussion. The DAD was operated at 210–350 nm. Results were compared with the area ratios obtained and those predicted from the calibration curves and retention times.

Statistical Methods. Linear and quadratic regressions were performed using Microsoft Excel 2002 (Microsoft Corp., Redmond, WA) and analysis of residuals from resulting models used

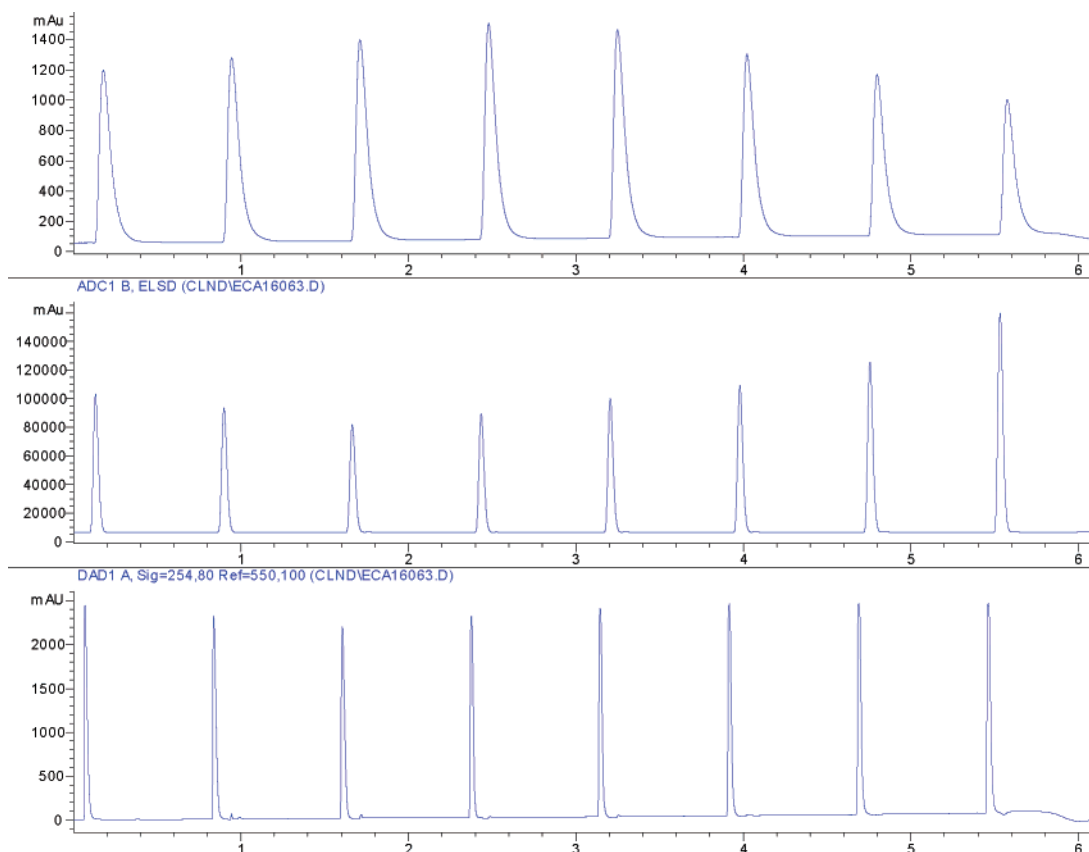


Figure 4. CLND (upper), ELSD (central), and UV (254 nm) (lower) data channels from 8 injections of 15 mM 5-FC made throughout one gradient run as described in the Experimental Section for “Blind” Quantification.

Minitab Release 13.20 (Minitab Inc., PA). Minitab was also used to plot histograms of the absolute errors found for each detector as well as to compare their variances using the Bartlett test. Predicted molecular properties and descriptors, calculated using ACD/Labs software (Advanced Chemistry Development, Inc., Toronto, Canada), were obtained using SciFinder version 2004, Copyright American Chemical Society.

RESULTS AND DISCUSSION

“Blind” Quantification Comparisons. 5-FC³⁶ was chosen as the primary reference standard for this exercise because it contains nitrogen, is reasonably involatile, and is essentially nonretained in rp-HPLC. It therefore possesses favorable CLND detection properties and is readily detectable by ELSD under the chosen conditions in which poorly retained DMSO is transparent. Its lack of retention means that 5-FC elutes at regular intervals when a repeating cycle of injections is made throughout a linear rp-gradient. This is illustrated in Figure 4, which, apart from emphasizing the band dispersion associated with the CLND flow path, illustrates the changes in CLND and ELSD response observed post-split. The DAD is located prior to the flow splitters and the UV response of 5-FC is essentially constant throughout the gradient. Where the flow is split to CLND and ELSD, changes in response may be anticipated as a consequence of changes in split ratio during gradient delivery. Additionally, the response of the ELSD will be dependent on eluant composition insofar as this impacts the particle size distribution of the nebulizate. Both split and composition induced variability can be accommodated by

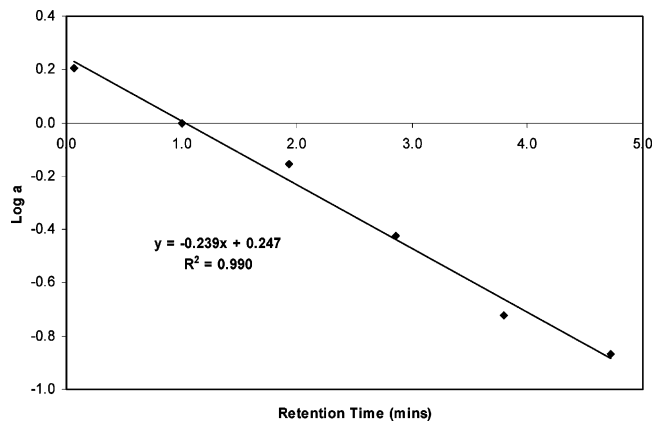


Figure 5. Plot of $\log a$ of 5-FC ELSD response curves as a function of peak retention time. Each point on the graph represents the gradient of an individual calibration curve having $r^2 \geq 0.998$.

empirical description of the dependency of response factors on the elapsed gradient elution time. Figure 5 shows that the value of $\log(\text{response coefficient } a)$ of ELSD calibration curves (y) was linearly related to 5-FC elution times (x) by the equation $y = -0.2394x + 0.2466$. Similarly, it was found that the variation of the value of the intercept b of the same calibration curves as a function of elution time could be described by the equation $y = 0.0727x + 1.3968$ ($r^2 = 0.98$). Use of these equations enabled calculation of ELSD response coefficients a and b at any desired elution time, in turn creating the necessary specific ELSD

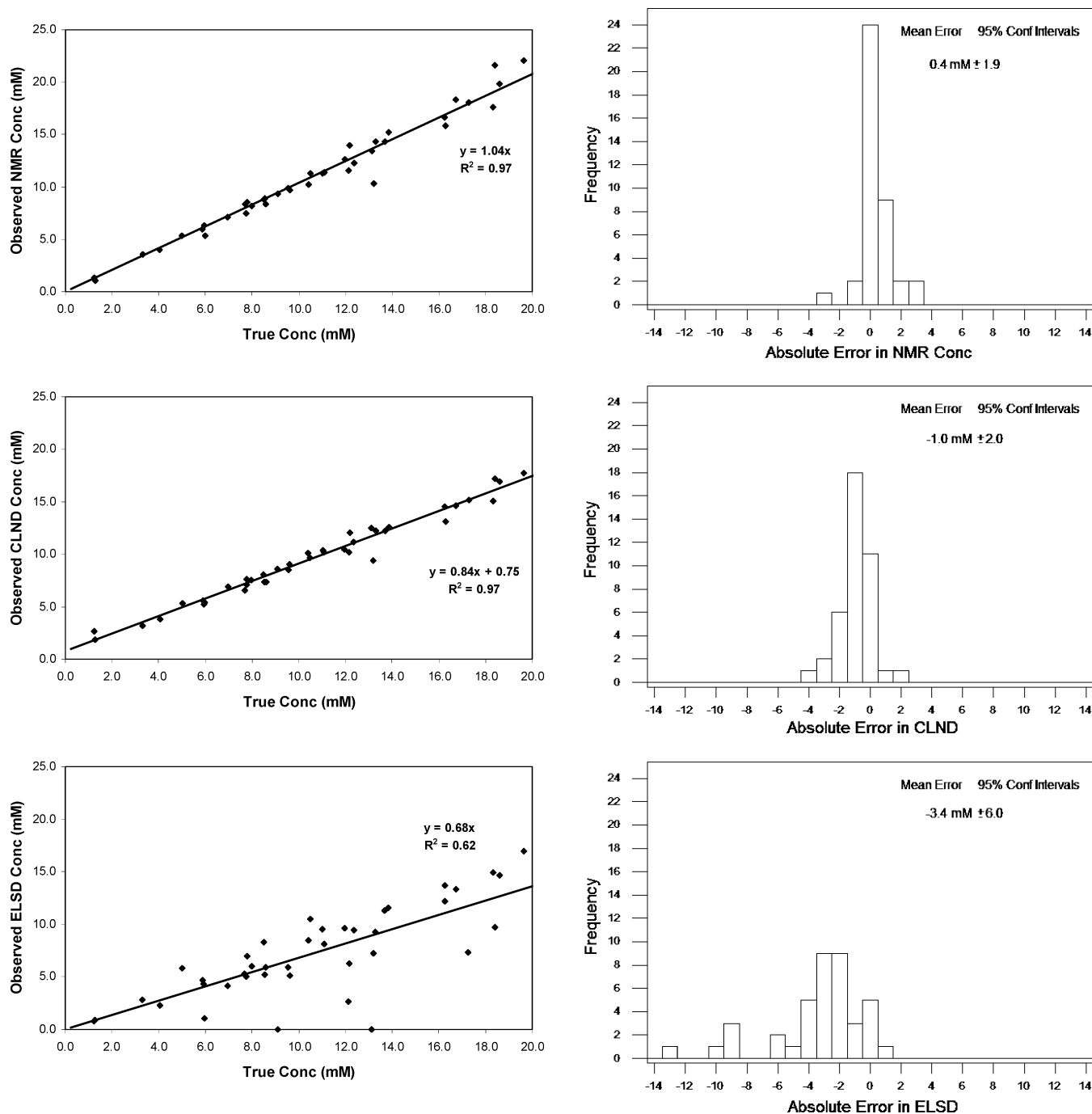


Figure 6. Linear regression models (L) and absolute error distributions (R) of observed versus true concentrations of analytes as determined by NMR (top), HPLC-CLND (center), and HPLC-ELSD (bottom).

response equations to convert peak area into amounts expressed as 5-FC, thereby accommodating apparent net changes in ELSD response of typically 35–55% over the conditions studied. This was achieved conveniently by substitution into eq 2 and rearrangement to provide the expression

$$\log M_1 = (\log A_1 + 0.2394RT_1 - 0.2466) / (0.0727RT_1 + 1.3968) \quad (8)$$

where M_1 is the mass of material 1 eluting in the gradient at retention time RT_1 minutes with peak area A_1 units.

In the case of the CLND, the gradients of calibration curves throughout the HPLC gradient range proved less dependent on retention time (RSD of $\pm 6.4\%$), but this minor dependency was found to be best described by second order polynomials ($y = -0.0046x^2 + 0.0166x + 0.0343$ for calibration curve gradients and $y = 0.324x^2 + 1.053x - 62.5$ for the intercepts). This variation in CLND response is largely attributable to split ratio changes incurred during the gradient and will in turn be a function of tubing dimensions.³¹

The results obtained by NMR, ELSD, and CLND after calculation and decoding are shown in Table 1. The HPLC-CLND data

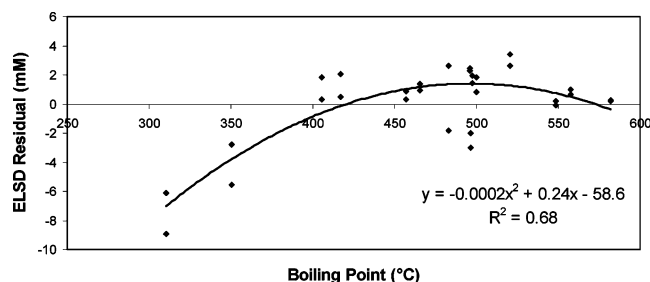


Figure 7. Plot of HPLC-ELSD residuals as a function of estimated boiling point at 760 Torr of 15 analytes.

using indole as reference standard are slightly less accurate than those using 5-FC and data derived from use of the latter standard is used henceforward so as to enable a more direct comparison with HPLC-ELSD.

The data in Table 1 were used to build three linear regression models (using Excel) of the true concentration versus the observed concentration for each of the detectors studied (see Figure 6). Strong correlation between the true concentration of the compounds studied and the results generated using NMR and CLND was observed (r^2 of 0.97 in both cases). The calculated results using NMR and CLND were very comparable (regression analysis of the CLND results versus the NMR results produced an even higher r^2 of 0.99). The ELSD results, however, were poorly correlated with the true concentrations having a much lower observed r^2 of 0.62. A histogram (using Minitab) of the absolute error (difference between the true concentration and that calculated by the detector) has been plotted for the three detectors adjacent to each linear regression model in Figure 6. The NMR and CLND histograms are similar—the errors are normally distributed around the target concentration with ± 2 mM 95% confidence intervals (± 2 standard deviations). The distribution of the ELSD errors is much more variable (± 6 mM 95% confidence intervals), less accurate (mean absolute error = -3.4 mM), and clearly skewed with more compound concentrations being underestimated than overestimated as predicted by theory. A three-group Bartlett test for equal variance was performed on the NMR, CLND, and ELSD absolute errors. The variance of the ELSD data was determined to be significantly higher than that of the other two detectors with a certainty of $>99\%$.

The residuals from the ELSD linear regression model in Figure 6 were plotted against a wide range of properties and descriptors including molecular weight, predicted (using ACD/Labs software) vapor pressure, and predicted (also using ACD/Labs software) boiling point (at 760 Torr). Correlation between the ELSD residuals and molecular weight and the predicted boiling point of each compound was observed. In general one may expect decreased molecular size and increased volatility to tend to reduce ELSD response, and indeed in another experiment with nebulizer at 110°C and evaporator at 130°C the ELSD data was somewhat worse, with little or no response obtained for some of the more volatile compounds. The relationship between boiling point and the ELSD residuals provided the most descriptive model and can be observed in Figure 7. Boiling points of 15 of the 20 compounds have been predicted and plotted against the ELSD residuals from the linear regression model. A quadratic relationship can be fitted with r^2 of 0.68. One of the compounds (dibucaine, predicted boiling point of 496°C) was poorly predicted by the model and if the two

residuals due to dibucaine are removed then a much better model is observed (r^2 of 0.81). With the exception of this one unusual compound there is no significant lack of fit (i.e., the model is more significant than the variability between the two residuals observed for each compound). Using the model in Figure 7, it can be concluded that the accuracy and variability in ELSD measurements improves as the boiling point increases from 300 to 490°C . The fitted regression line appears to plateau at $\sim 490^\circ\text{C}$, suggesting that compounds with boiling points at or above this temperature tend to respond to the ELSD detector in a broadly similar fashion. Despite this, the mean absolute error of compounds with predicted boiling points of greater than 490°C is still poor (mean absolute error = -2.8 mM).

We have applied these quantitative approaches to samples derived from high-throughput synthetic programs: in one exercise some 474 samples representing widely varying structural types were identified and quantified by HPLC-CLND-MS and NMR. In contrast to the study described above which used model compounds possessing nonadjacent nitrogen atoms, the synthetic analytes were not of uniform high purity and the possibility of coelution of the target compound with nitrogenous impurities in HPLC-CLND was high. Additionally, the sample set contained examples where the analyte had adjacent nitrogens and reduced CLND response would be expected. In view of these possibilities the degree of correlation ($r^2 = 0.75$) between the two methods was considered to be reasonable. In a further study, 117 samples (all nominally 10 mM in DMSO in MTP format) were determined in “blind” fashion by HPLC-ELSD and HPLC-CLND-MS and compared to those obtained by the “Gold Standard” reference method of NMR. This sample set had low chemical diversity as all the samples had been elaborated on one of only three different chemical scaffolds. The results are shown in Figure 8. CLND is seen to clearly outperform ELSD, which tends to underestimate the amounts present in a manner that was largely independent of chemical scaffold. We believe that the agreement between CLND and NMR data is acceptable considering that the materials concerned are, unlike the 20 selected standards, of variable purity (20–100%). In these exercises both the NMR proton assignments and the integration of HPLC peaks of variable size, number, and quality were more difficult than for the idealized cases used to demonstrate that NMR and CLND provide similar results for pure samples. In 78% of the set of 117 samples shown in Figure 8 the value obtained by CLND was within 20% of that obtained by NMR, whereas for ELSD, only 15% of ELSD measurements were in similarly close agreement with NMR.

Analysis and quantification with HPLC-CLND-MS has been continuously used for several months in our laboratories to support various aspects of synthetic and screening programs. The technology has proved sufficiently reliable for use in a high-throughput environment, and minimal downtime has been experienced. Long-term repeatability of the variability of the indole calibrant peak area is about $\pm 10\%$.

Flow Injection Analysis (FIA) and HPLC Analysis Using ELSD. The experiments with “blind” quantification increased our concerns that ELSD was not an appropriate tool for work of this nature. No matter what correction routine and detector is employed, single-calibrant detection schemes require that all analytes have similar response coefficients to those of the

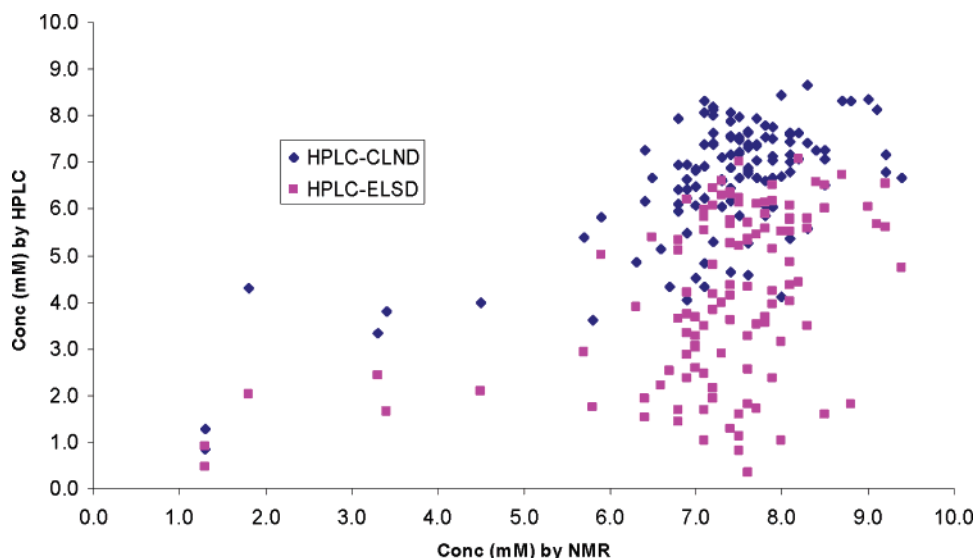


Figure 8. Comparison of results of "blind" quantification of 117 samples using HPLC-CLND and HPLC-ELSD with the values obtained using NMR with the ERETIC method.

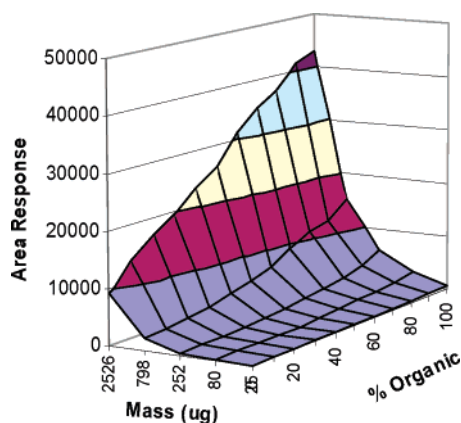


Figure 9. Three-dimensional surface representing calibration curves for 25–8000 ng dexamethasone obtained using FIA in 11 different MeCN/H₂O mixes as described in the Experimental Section.

reference compound. This fundamental requirement is largely fulfilled by CLND, but the variability of the ELSD results indicates considerable diversity among ELSD response factors. Furthermore, the statistical analysis presented above provides good evidence to suggest that compounds of low boiling point have particularly poor response characteristics. Our practice has been to run high-throughput ELSD applications at moderate temperatures to ensure DMSO transparency and to avoid clogging, so collection of response factors at different operating temperatures is of interest.

FIA was used to study the response factors of a set of diverse compounds that included a number of steroids of similar structure and molecular weight that would be expected to have similar response functions in ELSD. Figure 9 illustrates the set of 11 calibration curves (Area vs Mass as a function of %MeCN) obtained for dexamethasone with the low-temperature Model PL2100, plotted as a three-dimensional surface. Overall response increases with increasing organic percentage. There is a general trend for the ELSD response coefficient $\log a$ to increase with increasing acetonitrile concentration, as shown in Figure 10. It is noteworthy that this behavior contrasts with that seen in Figure 5 for HPLC-CLND-ELSD where the matrix solvent was methanol/

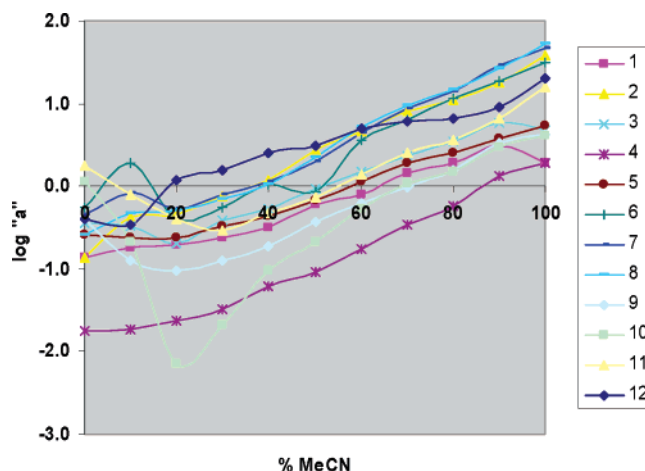


Figure 10. Plot of gradients ($\log a$) of FIA calibration curves of 12 compounds as a function of the matrix composition. Key: 1 = 5-fluorocytosine, 2 = doxazosin mesylate, 3 = 6,7-dimethoxy-2,4-quinazolidinedione, 4 = amitriptyline·HCl, 5 = camphor-*p*-tosylhydrazide, 6 = hydrocortisone, 7 = dexamethasone, 8 = cortisone, 9 = adrenosterone, 10 = 9-phenylanthracene, 11 = dofetilide, and 12 = fluticasone propionate.

water mixes, and $\log a$ decreases with methanol concentration. However, in the corresponding curves for calibration graph intercept values b (data not shown), a decrease of b with organic is seen in the case of acetonitrile and an increase with methanol so the overall effect for both solvents tends to be compensatory. Thus, the overall ELSD response (e.g., as seen in Figure 4) of a given compound varies less than might be anticipated from consideration of $\log a$ values alone. Nevertheless, Figure 10 emphasizes the diversity of response factors displayed by different compounds and it is this diversity that renders unreliable the use of ELSD single calibrant work. Consistent with the previously described association of ELSD residuals with calculated boiling points is the observation that the compounds with highest values of $\log a$ in Figure 10 tend to be relatively involatile materials such as steroids and doxazosin, while the compound with the lowest $\log a$, amitriptyline, is the most volatile. This association between volatility and ELSD response is also reinforced by the observation

that operation at ambient temperature with the Model 2100 consistently provided 3–10 times greater sensitivity than the Model 1000 which for this experiment was operated with nebulizer at 110 °C and evaporator at 130 °C. However, we found that even when the Model 2100 is used at ambient temperatures the diversity of response factors was similar to that obtained at higher temperatures with the Model 1000, and so the viability of single-calibrant quantification will remain poor. Our findings are consistent with those of the recent study which introduced the calibration routine with 5-FC. Mathews et al.³⁶ found that, provided the analytes are not volatile, single-calibrant ELSD gave close approximations of the nominal analyte concentration. However, in several instances, significant errors (>20%) are evident from the data reported, and these tend to be associated with smaller, more volatile analytes. The most significant source of error was acknowledged to be the variability of ELSD response characteristics between analytes with different physicochemical properties. The data reported here supports the view that CLND response is largely independent of analyte physicochemical properties, in particular volatility, and as such represents a better choice of detector than ELSD for single-calibrant work with most nitrogen-containing analytes.

The effects of diverse ELSD coefficients on uncalibrated quantification protocols were briefly evaluated for binary mixtures by substitution of real response coefficients into the equations governing the detection process and then comparing the resultant predicted area ratios with those obtained by HPLC-ELSD. The low-temperature Model 2100 was used for this purpose. In a typical experiment a solution of 5-FC and cortisone at 395 and 329 $\mu\text{g/mL}$ respectively was prepared in 50% MeCN + 0.05% formic acid. Injections of 2.5, 5, and 10 μL were made using the gradient HPLC method. Eluant composition at peak retention times was calculated using the system dwell volume, interstitial column volume, and gradient profile. Interpolated values of $\log a$ and b were calculated from FIA data and combined with the mass M of each compound injected to predict cortisone/5-FC peak area ratios using eq 4. The ratios predicted were 2.19, 1.75, and 1.40, respectively, and the corresponding observed ratios were 2.09, 1.51, and 1.38. The true mass ratio is known to be 0.83. The UV peak area ratio remained constant at 0.30 ± 0.02 SD. It is seen that unless the analyst can calibrate raw peak area ratios by knowing the response coefficients of each compound, and not simply those of a single calibrant, then serious errors in quantification will normally occur. The dependence of relative peak area on the absolute amounts of materials in the ELSD is a direct consequence of the exponential nature of the detector's response (i.e., $b \neq 1.00$) and was predicted as discussed under "Theory". Similar behavior was observed (Figure 11) even for fluticasone propionate (FP,) and dexamethasone, which have similar chromophores and ELSD response coefficients. The ELSD ratios change with absolute amount injected, whereas because of the similar chromophores of the two compounds, the UV ratios were generally within 25% of the true ratios. ELSD percentage errors can be very large; a mixture that represented a hypothetical contamination of a sample of FP with 10% dexamethasone would appear to show only about 2% contamination on an uncorrected Area% basis. This behavior (Figure 11) is entirely consistent with that predicted by theory as illustrated in Figure 1. The extent to which ELSD can be used

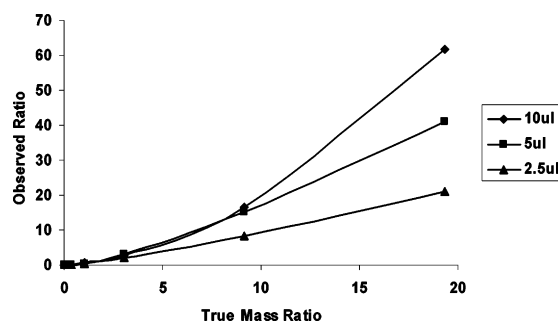


Figure 11. Plot of observed dexamethasone/FP HPLC-ELSD peak area ratio versus true dexamethasone/FP ratio for 10, 5, and 2.5 μL injections of mixtures containing a constant total steroid composition of 115 ± 2 $\mu\text{g/mL}$.

for quantification of libraries will depend on the range of response coefficients of the compounds present, and this in turn will depend on the amount of chemical diversity explored within the library. In general, small molecule libraries may be more susceptible to this because they will show more response diversity arising from differences in volatility. Analyte-specific calibration is essential for accurate quantitative work with ELSD.

Taken overall, our results confirm the viability of CLND for quantification in HPLC applications since it provides results of high accuracy and precision. However, it does not represent an ideal tool for widespread use within the industry as it is significantly more complex, expensive, and resource-dependent than traditional detectors such as UV diode array. Significant practical issues would need to be addressed in order to deploy it routinely on all high-throughput applications in the industry. Our opinion is that it may most effectively be exploited as a standard calibrant technique against which subsequent UV quantifications can be compared until reference materials become available. Nussbaum et al.¹⁵ have shown that CLND may be used to determine relative UV response factors. CLND-based calibration of the UV detector modules of existing HPLC investment is an economically and technically attractive possibility to significantly improve the accuracy of high-throughput HPLC quantification technology.

CONCLUSIONS

Three techniques for quantification of NCEs without analyte-specific calibration have been carefully compared and it has been found that both the ERETIC method in NMR and CLND, which essentially function as specific atom counters, provide results of high accuracy and precision in the 1–20 mM concentration range. Best results are obtained with manually evaluated NMR, and we have used this to validate the use of the particularly convenient combination of CLND with pre-existing HPLC and MS systems in a high-throughput research environment. CLND results show marginal improvements if appropriate corrections are made for apparent response changes that occur during gradient HPLC as a result of changes in flow delivered to respective detectors downstream of the flow splitters.

HPLC-ELSD proved significantly less able than HPLC-CLND to provide reliable quantitative data in single-calibrant protocols. Rigorous correction both for the logarithmic nature of its response, and for the effects of gradient eluant composition on the response coefficients, was insufficient to obtain accurate results, despite

expression on a common basis against a single calibrant. This was shown to be due to the diversity of response factors shown in ELSD, even between similar compounds. There is evidence that associates increased inaccuracy with lower values of calculated boiling point. Increasing the ELSD evaporation and nebulization temperatures increased the extent of the errors obtained.

We have confirmed theoretical predictions that relative area measurements in ELSD, either within or between runs, will underestimate the smaller peak if the results are interpreted as representing the % m/m of components present. This overestimation of sample purity is a direct consequence of the nonlinear nature of ELSD response. The effect will adversely affect business critical applications such as impurity measurements and the

estimations of concentration using calibrants different from the analyte.

It is concluded that quantitative work with ELSD, even on a relative area basis, requires analyte-specific calibration if the possibility of significant error is to be avoided. CLND, however, is entirely suitable for single-calibrant quantification of nitrogenous analytes that do not contain adjacent nitrogen atoms. There is however, no detection scheme that permits truly universal quantitative detection, and so further development of novel detection strategies should be encouraged.

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