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Evaluation of Evaporative Light-Scattering Detector for Combinatorial Library Quantitation by Reversed Phase HPLC

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A quantitation study using reversed phase HPLC with UV and evaporative light-scattering detector (ELSD) was conducted on 90 library standards selected from 15 small molecule combinatorial libraries (six standards from each library). This study assessed the quantitation errors using a single calibration curve for rapid purity analysis of combinatorial libraries. The average quantitation error of six standards from one library at 200 μM by UV was 13.4%, 20.6%, and 60.3%, at 214, 220, and 254 nm, respectively. By ELSD, the average quantitation error of these six standards at 200 μM was only 7.7%. Applying this ELSD calibration curve to 84 standards from 14 structurally diverse libraries, an average quantitation error of 16.4% was obtained. The average quantitation error of all 90 standards from 15 libraries using 15 calibration curves was 18.5%.

Introduction

Combinatorial chemistry has made it possible to produce libraries of hundreds or thousands of structurally related compounds simultaneously.[1−6] These compound libraries are evaluated in high throughput bioassay screens for specific drug targets. By this approach, the drug discovery process can be significantly accelerated. To assess the quality of the library compounds used in the biological screening, it is essential to have a measure of their purity. However, for a library containing 5000 compounds designed to have maximum structural difference from one another, it is intrinsically difficult to analyze the purity of these compounds using a single high throughput method.

Mass spectrometry (MS) is an essential element in combinatorial library analysis. It has been used in flow injection analysis (FIA/MS) and also in conjunction with HPLC (HPLC/UV/MS) analysis to identify products and verify structures.[7−10] Since compounds within a library generally have significant differences in ionization efficiency, MS is generally not suitable for purity analysis. HPLC with UV detection has typically been used to qualitatively assess product purity and yield. Because significant differences in molar absorptivity may exist between library compounds, this method cannot be used for quantitation without well-characterized reference standards.

Alternative detection methods such as evaporative light-scattering detection (ELSD) and chemiluminescent nitrogen detection (CLND) have been introduced for quantitation of combinatorial library compounds. ELSD responds to the amount of material (mass) rather than absorptivity or ionization efficiency; therefore, it is a nonselective detector for relatively nonvolatile compounds.[11,12] CLND responds to the nitrogen content; therefore, it is a selective detector only for nitrogen-containing compounds.[13,14] Both of these methods make it possible to quantitatively analyze libraries with a single standard.[13,15]

Although ELSD coupled with MS is an important tool for assessing purity in combinatorial libraries, only few papers have been published on the subject. Kibbey[15] described ELSD quantitation for a series of steroids, hydantoins, and protected amino acids using a single external standard by normal phase HPLC. Not surprisingly, Kibbey found the optimal quantitation accuracy for each series was achieved with structurally related standards. The average quantitation error was approximately ±10%. Hsu[16] has recently reported a direct library quantitation using a known amount of peptide as an internal standard to estimate the concentration of a reaction product based on the area percentages of the two chromatographic peaks in ELSD. The deviation of this estimation is about 20%. In both publications, a limited number of compounds with minimum structural variation were used.

In our synthesis of 5000-compound libraries marketed for general high throughput screening, we independently synthesize, purify, and characterize six representative compounds (standards) for each library. The analytically pure standards are used to generate HPLC/UV calibration curves. As part of the quality control process, we then quantitatively analyze and report the purity of six reference samples from the combinatorial library. Even though this purity analysis is accurate, it is time-consuming and impractical for a large number of standards. It is our interest to explore a "universal" detector; so all library compounds could be analyzed with reasonable accuracy without well-characterized reference standards. In this article, we report our evaluation of ELSD for quantitation by reversed phase HPLC based on 90 standards selected from 15 diverse libraries. We made one calibration curve based on six standards from one library and applied this curve to 84 standards from 14 diverse libraries to obtain the quantitation error of each standard. We also examined the quantitation error of ELSD across

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Table 1. Curve-Fit Statistics of the Six Standards from LIB 1 in UV and ELSD

		214 nm			220 nm			254 nm			ELSD		
name	MW	slope	intercept	R^2	slope	intercept	R^2	slope	intercept	R^2	slope	intercept	R^2
Cali-A	558.7	7.24E−03[a]	6.7E−02	0.998	6.18E−03	2.3E−02	1.000	6.09E−03	1.7E−02	1.000	1.413	2.160	1.000
Cali-B	452.6	5.03E−03	7.8E−02	0.998	3.67E−03	2.5E−02	0.998	5.18E−04	1.4E−03	0.999	1.432	2.144	0.999
Cali-C	412.5	4.01E−03	1.7E−02	1.000	4.01E−03	1.7E−02	1.000	4.05E−03	1.5E−02	1.000	1.418	2.028	0.999
Cali-D	466.6	6.54E−03	7.7E−02	0.998	5.89E−03	2.9E−02	0.999	2.82E−03	3.4E−03	1.000	1.415	2.050	1.000
Cali-E	515.7	5.88E−03	−1.1E−02	1.000	5.11E−03	−3.4E−03	1.000	6.50E−03	−2.2E−02	1.000	1.472	2.016	1.000
Cali-F	549.6	5.55E−03	8.2E−02	0.992	3.53E−03	2.8E−02	0.998	7.77E−04	2.9E−03	1.000	1.437	2.039	1.000
Cali-all		5.72E−03	4.9E−02	0.998	4.73E−03	2.0E−02	0.999	3.46E−03	2.5E−03	1.000	1.465	2.182	0.993

[a] Read as 7.24×10^{-3}.

Table 2. Relative Response Factors of LIB 1 Standards

name	214 nm	220 nm	254 nm	ELSD
standard_A	1.90	1.67	11.92	1.02
standard_B	1.46	1.03	1.00	1.05
standard_C	1.00	1.10	8.01	1.03
standard_D	1.74	1.62	5.51	1.01
standard_E	1.36	1.31	11.73	1.02
standard_F	1.53	1.00	1.52	1.00

diverse chemotypes by applying 15 calibration curves to all 90 standards.

Results and Discussions

A hydroxyproline library, LIB 1, consisted of 6336 compounds with three diversity dimensions. The six standards were selected based on specific criteria to represent the range of functionality found in the combinatorial library. The standards were synthesized, purified, and characterized by NMR (^{1}H and ^{13}C), mass spectrometry, and CHN analysis. Their calibration curves were generated at multiple UV wavelengths and in ELSD. The molecular weights of the standards varied from 412 to 558.

Curve-fit statistics of the six standards (Cali-A to Cali-F) from library LIB 1 by UV at 214, 220, and 254 nm and by ELSD are summarized in Table 1. Each UV calibration curve was generated by plotting peak area ratio versus concentration from a series of concentrations from 31 to 500 μM (see Experimental Section for detail). An ELSD calibration curve was produced by plotting of log(peak area) of ELSD signal versus log(μg/mL). The slopes of the six calibration curves had relative standard deviation percentages (%RSD) of 19.8, 24.4, and 74.0 with UV detection at 214, 220, and 254 nm, respectively. These numbers indicate that the responses among the six standards were quite different and these differences became much larger at longer wavelength (254 nm vs 214 nm). With ELSD, the slopes of these six calibration curves ranged from 1.413 to 1.472 and gave a %RSD of 1.6. This clearly indicated minimal response variation among these standards with ELSD. The relative response factors listed in Table 2 varied from 1 to 1.9, 1 to 1.7, and 1 to 12 with UV at 214, 220, and 254 nm, respectively, and only from 1.00 to 1.05 with ELSD.

To illustrate the magnitude of potential errors in determining purity, one calibration curve (Cali-all) was generated from the six standards from LIB 1 at each UV wavelength and in ELSD. Cali-all for UV was generated by plotting the average peak ratio of the six standards versus concentrations, while Cali-all for ELSD was generated from all data points of the six standards in a single log(peak area)/log(μg/mL) plot. These four Cali-all curve-fit statistics are listed in the bottom row of Table 1.

Table 3. Quantitation Errors (%) Resulting from Cali-all Compared to Individual Calibration Curves for Standards at 200 μM Concentration

name	214 nm	220 nm	254 nm	ELSD
standard_A	−18.4	−30.9	−78.8	−7.2
standard_B	9.6	22.0	84.2	−11.8
standard_C	32.7	15.5	−19.5	10.8
standard_D	−16.6	−25.4	17.7	8.9
standard_E	2.7	−5.6	−84.9	−3.5
standard_F	0.2	24.5	76.8	4.2
average	13.4	20.6	60.3	7.7

The relative percentage errors resulting from the quantitation of each standard for LIB 1 at 200 μM using each calibration curve versus using Cali-all$_{\text{LIB 1}}$ are summarized in Table 3. These quantitation errors varied from 0.2 to 32.7%, 5.6 to 30.9%, and 17.7 to 84.9%, at 214, 220, and 254 nm, respectively. The average quantitation errors were 13.4% at 214 nm, 20.6% at 220 nm, and 60.3% at 254 nm. This indicates those quantitation errors for LIB 1 become substantially higher at higher wavelength. These results clearly demonstrate that quantifying product purity by UV peak area ratio is not a reliable method.

However, the quantitation errors with ELSD were much smaller for each standard from LIB 1 and varied from 3.5% to 11.8% at 200 μM. The average quantitation error was only 7.7%, approximately half of the error at 214 nm and one-eighth of the error at 254 nm. These results clearly demonstrate that ELSD gave much less error than UV detection for determining purity.

Using ELSD, the calibration curve obtained from all six standards generally offered better results over a calibration curve made from a single standard. The quantitation errors for the six standards using a single-compound calibration curve and Cali-all$_{\text{LIB 1}}$ are summarized in Table 4. For example, Cali-A was applied to standards B through F and gave quantitation errors ranging from 3.6% to 18.0% with an average of 8.7%. Cali-E gave the minimum quantitation error of 7.3%, which was better than 7.7% from Cali-all, while Cali-C gave the maximum error of 12.6%.

To expand the applicable scope, the Cali-all$_{\text{LIB 1}}$ was applied to quantify the six standards from LIB 2, a sister library of LIB 1 with the same core structure but different diversity elements. The quantitation errors of LIB 2 six standards at 200 μM using their own calibration curves versus Cali-all$_{\text{LIB 1}}$ are summarized in Table 5. We are happy to

Table 4. Quantitation Errors (%) of Six Standards from LIB 1 at 200 μM Concentration in ELSD

name	Cali-A	Cali-B	Cali-C	Cali-D	Cali-E	Cali-F	Cali-all
standard_A	–	−3.9	21.6	18.7	3.3	12.0	−7.2
standard_B	3.6	–	26.1	23.0	8.2	16.6	−11.8
standard_C	−18.0	−20.5	–	−2.5	−13.2	−7.1	10.8
standard_D	−15.8	−18.6	2.5	–	−11.7	−5.0	8.9
standard_E	−3.9	−7.4	16.9	14.1	–	8.0	−3.5
standard_F	−11.0	−14.2	8.3	5.6	−7.4	–	4.2
average	8.7	10.8	12.6	10.7	7.3	8.1	7.7

Table 5. Quantitation Errors Resulting from Using Cali-all$_{LIB1}$ Compared to Individual Calibration Curves for Six Standards from LIB 2 at 200 μM Concentration

name	MW	error (%)
standard_A	594.7	−11.6
standard_B	482.6	−10.6
standard_C	370.4	5.2
standard_D	517.2	−0.6
standard_E	457.5	15.3
standard_F	608.3	11.6
average		9.2

see that the average quantitation error was 9.2%, which was very close to 7.7% obtained from the six standards of library LIB 1.

With the encouraging results, we then applied Cali-all$_{LIB\ 1}$ to an additional 78 standards (molecular weights ranging from 300 to 630) from 13 libraries. These libraries were selected to represent a wide range of structural diversities. They include nonaromatic heterocycles, such as hydrofurans, isoxazolines and oxadiazolidinone; aromatic heterocycles, such as indoles, quinolines, oxadiazoles, and isoxazoles; and other important classes of compounds such as aryl sulfonamides, aryl amides, and guanidines.

The ELSD quantitation errors of each standard using their own calibration curves versus Cali-all$_{LIB\ 1}$ are summarized in Table 6. Of the 90 standards listed in Table 6, the error ranged from 0.2% to 43.7%. It is clear that the quantitation error varied from standard to standard. However, the variation of the average quantitation error of six standards from each library ranged from 7.5% to 34.6%. The average quantitation error of all 90 standards from 15 libraries using Cali-all$_{LIB\ 1}$ was 16.5%.

The same quantitation calculation was carried out for the 90 library standards using each ELSD Cali-all from each of the remaining 14 libraries. The average quantitation errors of six standards from each library using 15 Cali-all curves are summarized in Table 7. Cali-all$_{LIB\ 7}$ gave the minimum error of 14.2% for all 15 libraries. The smallest error of 5.6% was obtained from library LIB 14, 13.2% was from library LIB 7, and the largest error of 26.5% was from library LIB

Table 6. Quantitation Errors (%) Resulting from Using Cali-all$_{LIB1}$ Compared to Individual Calibration Curves for 90 Standards from 15 Libraries at 200 μM Concentration

name	LIB 1	LIB 2	LIB 3	LIB 4	LIB 5	LIB 6	LIB 7	LIB 8	LIB 9	LIB 10	LIB 11	LIB 12	LIB 13	LIB 14	LIB 15
standard_A	−7.2	−11.6	18.5	−31.5	16.2	−35.2	18.9	−42.9	5.0	−4.1	−29.8	7.3	−3.5	3.2	2.5
standard_B	−11.8	−10.6	14.7	−40.6	13.2	20.6	−28.6	−26.0	5.9	35.5	−22.8	−2.1	23.6	−7.7	13.0
standard_C	10.8	5.2	25.4	−42.5	−27.7	−29.6	−17.8	−29.5	8.4	−15.8	−20.8	4.0	−13.0	−20.6	−15.9
standard_D	8.9	−0.6	10.9	−31.3	22.0	−35.3	−9.9	−26.1	27.3	17.8	−16.9	−8.7	−2.5	−7.1	−10.5
standard_E	−3.5	15.3	17.6	−17.0	−1.9	−43.4	−17.2	−3.5	21.9	18.4	−16.8	−12.0	35.4	−11.8	5.5
standard_F	4.2	11.6	−0.2	−24.9	5.2	−43.7	−13.3	−25.2	5.5	−5.9	−16.4	11.1	4.4	−5.2	20.0
average	7.7	9.2	14.5	31.3	14.3	34.6	17.6	25.5	12.3	16.3	20.6	7.5	13.7	9.2	11.2

Table 7. Average Absolute Quantitation Errors (%) of Six Standards from 15 Libraries Resulting from 15 Cali-all$_{library}$ Compared to Individual Calibration Curves

library name	ELSD Cali-all of														
	LIB 1	LIB 2	LIB 3	LIB 4	LIB 5	LIB 6	LIB 7	LIB 8	LIB 9	LIB 10	LIB 11	LIB 12	LIB 13	LIB 14	LIB 15
LIB 1	7.7	7.9	11.4	24.0	8.9	22.8	8.3	21.1	14.1	9.3	17.6	7.7	9.5	8.8	7.8
LIB 2	9.2	9.4	12.0	24.9	10.2	24.0	9.7	22.2	13.5	11.0	18.6	9.2	11.0	10.8	9.3
LIB 3	14.5	13.5	7.0	34.2	13.0	37.0	17.4	33.2	7.4	8.4	27.1	14.5	9.3	21.1	14.2
LIB 4	31.3	34.1	43.5	0.4	39.3	7.9	20.5	7.3	49.4	41.6	7.5	31.3	42.2	21.8	32.6
LIB 5	14.3	14.0	12.6	27.0	14.3	27.7	15.6	25.3	14.1	12.9	21.6	14.3	13.3	17.8	14.3
LIB 6	34.6	36.8	44.2	2.7	40.7	13.4	26.5	13.9	48.7	42.7	15.7	34.6	43.1	27.3	35.6
LIB 7	17.6	19.3	29.3	15.0	21.4	15.0	13.2	12.8	30.1	25.9	9.5	17.6	25.4	11.3	18.3
LIB 8	25.5	28.2	36.9	4.8	33.2	6.3	16.3	6.0	42.8	35.3	6.8	25.5	35.9	17.8	26.7
LIB 9	12.3	10.5	7.9	33.1	8.1	32.9	17.5	30.9	9.3	7.2	27.1	12.3	7.3	18.9	11.6
LIB 10	16.3	16.6	18.7	29.3	16.9	30.3	16.1	27.4	18.7	17.9	22.5	16.3	17.8	17.0	16.4
LIB 11	20.6	23.1	35.5	8.0	26.5	7.4	13.1	5.6	38.0	31.7	3.3	20.6	31.3	11.6	21.6
LIB 12	7.5	7.7	15.7	23.2	7.9	25.6	7.2	21.7	15.2	11.5	15.2	7.5	10.6	9.0	7.6
LIB 13	13.7	13.9	20.8	28.8	13.8	31.9	15.3	27.7	19.4	17.6	21.0	13.7	16.9	16.1	13.8
LIB 14	9.2	10.8	22.7	17.3	13.1	17.8	5.6	14.8	24.1	18.6	9.9	9.2	18.0	4.9	9.9
LIB 15	11.2	10.3	11.1	25.4	11.1	23.8	10.6	22.4	14.0	10.8	19.3	10.5	11.1	11.7	10.5
average	16.4	17.1	22.0	19.9	18.6	21.6	14.2	19.5	23.9	20.2	16.2	16.3	20.2	15.1	16.7

6. Cali-all$_{LIB\ 9}$ gave the maximum error of 23.9% for all 15 libraries. The smallest error of 7.4% was from library LIB 3, 9.3% was from library LIB 9, and the largest error of 49.4% was from library LIB 4. The error discrepancy for each library varied from 0.4% to 49.4%. However, this discrepancy was much smaller and varied only from 14.2% to 23.9% when all 15 libraries were taken into consideration. The average quantitation error of 15 Cali-all curves to 15 libraries was 18.5%.

The quantitation error of Cali-all to its own library varied only from 0.4% to 17.9% (numbers on the diagonal in Table 7). The average quantitation error was 9.5% for these 15 libraries.

From both Tables 6 and 7, one can see that the variation in quantitation error is quite large when one standard or one library was considered. However, the variations in average quantitation error of all 90 standards or all 15 libraries were much smaller. One can use one calibration curve made from standards from one library to quantify the rest of the library or several other libraries and expect about 20% quantitation error on average for each compound. Therefore, ELSD can be used as a "universal" detector for rapid purity analysis in combinatorial chemistry for small molecule libraries with about 20% errors on average. For most of the libraries, ELSD will give a smaller error than UV.

Conclusion

For this set of libraries, ELSD has been demonstrated to provide more uniform responses for compounds of small molecule libraries in comparison with UV. A single calibration curve in ELSD gives an average quantitation error of 18.5% for 90 standards selected from 15 libraries with different structures. These experiments indicate that ELSD can be used as a "universal" detector for rapid quantitation in combinatorial chemistry with about 20% quantitation error on average across diverse structural classes.

Experimental Section

Six standards from each library were synthesized, purified, and characterized by NMR (^{1}H and ^{13}C), mass spectrometry, and CHN elemental analysis. All standards were weighed to the nearest 0.01 mg on an AT261 DeltaRange analytical balance (Mettler Toledo, Columbus, OH). A 1.00 mM stock solution of each standard was prepared in either methanol or acetonitrile. HPLC separation was performed on a HP1100 system (Hewlett-Packard, Palo Alto, CA), which consists of a vacuum degasser, binary pump, autosampler, column compartment, and a diode array detector. A SEDERE 55 (S. E. D. E. R. E., Alfortville Cedex, France) evaporative light-scattering detector was connected to the outlet of the UV detector. The ELSD signal was collected through an 35900E interface (Hewlett-Packard, Palo Alto, CA) and processed on an HP Chemstation.

Reversed phase HPLC was carried out on a C18 column (3.0 × 100 mm, 5 μm, 100 Å) from Phenomenex (Phenomenex, Torrance, CA) at 40 °C with a flow rate of 0.5 mL/min. Two mobile phases (mobile phase A: 99% water, 1% acetonitrile, 0.05% TFA; mobile phase B: 1% water, 99% acetonitrile, 0.05% TFA) were employed to run a gradient condition from 0% B to 100% B in 6.0 min, 100% B for 2.0 min, and reequilibrate at 0% B for 2.0 min. An injection volume of 10 μL was used. ELSD drift tube temperature was 40 °C, gain was set at 7, and nitrogen flow rate was 2.0 L/min.

Calibration Curve. A serial dilution of the1.00 mM stock solution yielded 31, 62, 125, 250, and 500 μM solutions. A 125 μM solution of one standard from each library was used as the external standard (ES) for the quantitation of that library. The samples were analyzed in HPLC/UV/ELSD in the following order: solvent, ES, 0, 31, 62, 125, 250, and 500 μM solution. For the UV signal, the peak area at each concentration was divided by that of ES to give the peak ratio. A plot of peak area ratio of compound to ES vs concentration yielded the calibration curve. For the ELSD signal, the concentration was converted into μg/mL. A plot of log(peak area) versus log(μg/mL) yielded a linear curve for each standard (at an injection volume of 10 μL, these corresponded to a mass range of 0.1 to 3 μg injected). The correlation coefficient constants square (R^2) were > 0.99 for all calibration curves.

Measurement of 200 μM Standard. A 200 μM solution of each standard was prepared in triplicate. These samples were analyzed by HPLC/UV/ELSD in the following order: solvent, ES, solvent, sample1, sample2, and sample3. The concentrations calculated from the calibration curves gave accuracy better than 5% and a precision better than 1%.

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