

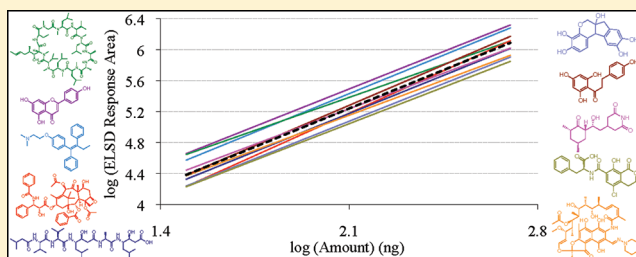
Universal Quantification of Structurally Diverse Natural Products Using an Evaporative Light Scattering Detector

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Supporting Information

ABSTRACT: A lack of good methods for absolute quantification of natural products has limited the accuracy of high-throughput screening. Many currently used methods for quantification are either too slow or not amenable to the structural diversity of natural products. Recent developments in low-temperature evaporative light scattering detectors (ELSD-LT) have overcome several historical limitations of ELSDs, including analyte decomposition and low sensitivity. Primarily, ELSDs have been used for relative quantification and detection of compounds that lack a UV chromophore. In this study, we employ an ELSD-LT for absolute quantification of natural products. Calibration curves were constructed using a weighted least-squares analysis for a diverse set of natural products and other compounds. An average calibration curve was evaluated for the “universal” quantification of natural products. Optimization of ELSD-LT hardware and parameters improved sensitivity and throughput and established the utility of ELSD-LT for quantification of large natural product libraries.



Natural products, or semisynthetic compounds inspired by natural product scaffolds, have been effective therapeutics, particularly as anticancer and anti-infective agents.¹ Natural products exhibit unique structural characteristics that are complementary to combinatorial libraries.² Despite decades of prolific natural product drug discovery, many large pharmaceutical companies have terminated natural product research programs for a variety of reasons including limited financial return as well as technical limitations in progressing compounds into clinical trials.³ Ongoing efforts toward improving methods for natural product isolation, screening, and structure elucidation aim to revive global efforts in natural products drug discovery.

High-throughput screening (HTS) platforms serve to detect therapeutically relevant natural products. In order to reduce false positives/negatives, sample concentrations must be prepared within the concentration threshold defined by each screen.⁴ Natural products are often screened as mixtures; therefore, minor components can easily be below detectable limits.⁵ The challenge of quantifying natural products has limited the ability to accurately screen samples, especially when using microscale screening libraries.

Complications due to structural diversity and low throughput have limited many methods for quantification of natural products. Molinski and co-workers quantified nanomole quantities of natural products by integrating ¹³C satellite peaks of deuterated solvents using NMR spectroscopy.⁶ While the NMR approach was useful and accurate, sample preparation and analysis time limit the feasibility of this method for large numbers of natural products. Alternatively, the use of an evaporative light scattering detector (ELSD) has shown great success for quantification of combinatorial libraries, but has not been investigated to the same extent for natural products.^{7,8} Potential difficulties with

ELSD stem from the correlation between the size and shape of a compound and the amount of light scattering.⁹ Nonetheless, Fang et al. have demonstrated a modest error associated with quantification of structurally diverse combinatorial libraries using an ELSD.⁸ However, the use of a “universal” calibration curve for natural products quantification has not been reported.

Recent improvements in ELSD technology have alleviated several historical limitations and have enabled additional applications of an ELSD for natural products quantification. Previous reports of melting and/or decomposition of analytes¹⁰ have been overcome with the development of a low-temperature evaporative light scattering detector (ELSD-LT). Typically, ELSD-LT instruments operate from ambient to 80 °C, and the use of temperatures closer to ambient has alleviated melting and decomposition problems. ELSD sensitivity was previously limited to 1–50 ng injections at best;¹¹ however, 200 pg injections of fructose have been detected using a modern ELSD-LT.¹²

Recently, the charged aerosol detector (CAD) was introduced as a competing mass-dependent detector. In contrast to the light scattering detection of an ELSD, a CAD generates a response signal based on the amount of charged analyte particles detected. Vervoort et al. compared the performance of a traditional ELSD versus a CAD when coupled to reversed-phase liquid chromatography and reported that the CAD provided marginally better sensitivity, reproducibility, and calibration curve linearity.¹³ To our knowledge, a thorough performance evaluation of a CAD versus a modern ELSD-LT has not yet

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been completed. Schiesel et al. utilized a CAD for “universal” quantification of impurities with unknown structures from nutritional infusion solutions.¹⁴ Eight compounds were used to construct standard calibration curves, which yielded a relative standard deviation (RSD) of 21% and 28% for the slope and *y*-intercept, respectively. As a comparison, a RSD of 7% and 11% was calculated for the slope and *y*-intercept, respectively, for the 10 calibration curves constructed in our study. While the CAD shows promise as an alternative mass-dependent detector, our study applies the more economical ELSD-LT to high-throughput “universal” quantification.

The number of publications employing an ELSD has continued to increase.¹⁵ A search for “ELSD” within the *Journal of Natural Products* showed 107 published journal articles between the years of 1999 and 2011, signifying the importance of this detection technique. Within natural products research, ELSDs have been used primarily for detection of compounds lacking a UV chromophore and for relative quantification within an HPLC chromatogram. Recently, ELSDs have been incorporated into natural product library generation platforms.^{16–22} Crews and co-workers have constructed a high-throughput LC-MS-UV-ELSD-based method for generating natural product libraries that are amenable to HTS.²³ They identified a new compound, aignopsanoic acid B, that lacked a UV chromophore and was detected only by an ELSD. Furthermore, Quinn and co-workers recently employed a coupled RP HPLC-UV-ELSD in the development of a two-step fractionation method for generating libraries containing natural products with lead- and drug-like physicochemical properties. Subsequent HTS of 202 983 lead-like enhanced fractions yielded 60 active compounds against a malaria screen and 58 against human African trypanosomiasis.²⁴ Increased automation has greatly increased the feasibility of generating large natural product libraries. Quantification of libraries after fractionation in a high-throughput fashion using ELSD could provide direct benefits for subsequent HTS. Additionally, the application of ELSDs within natural product research is increasingly apparent, but methods for absolute quantification of natural products using an ELSD have not been investigated. Absolute quantification of natural products would support library generation and significantly improve sample preparation for HTS by allowing minor components to be enriched. Herein, the use of an ELSD-LT for absolute quantification of natural products is described. An average calibration curve was constructed using a representative set of standards and evaluated as a “universal” calibration curve for structure-independent quantification of natural products. In order to make absolute quantification of natural products amenable to large natural product libraries ELSD hardware and parameters were optimized to improve sensitivity and throughput.

In order to evaluate an ELSD-LT for “universal” quantification of natural products, a diverse set of 10 naturally occurring and other compounds was selected that represented a range of scaffolds (Table 1). In particular, compounds that crossed a broad spanning molecular weight range were selected to investigate their suitability for quantifying compounds of unknown structure. Overall, the goals of this study were to evaluate an ELSD-LT for quantification with no knowledge of the structure with sufficient accuracy to support screening efforts, and to evaluate the linearity of quantification. Additionally, throughput was increased to demonstrate feasibility of quantifying large natural product libraries for HTS.

Table 1. Compounds Used as Standards for Quantification Using an ELSD

name	molecular weight (g/mol)
apigenin	270.24
phloretin	274.27
cycloheximide	281.35
hematoxylin	302.28
tamoxifen	371.51
ochratoxin A	403.81
pepstatin A	685.89
rifampicin	822.94
paclitaxel	853.91
cyclosporin A	1202.61

Prior to calculating calibration curves for each of the 10 compounds, solvent composition effects were investigated. Using rifampicin, ELSD responses were analyzed using increasing concentrations of methanol in water. Consistent with previous results, the ELSD response was dependent on solvent composition and increased with increasing methanol (Figure 1).²⁵

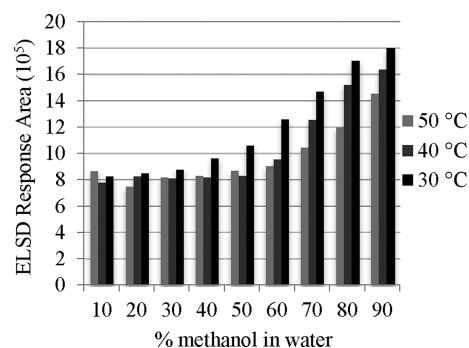


Figure 1. Effects of solvent composition and evaporation temperature on ELSD response.

However, increasing the ELSD evaporation temperature minimized the effect, but not sufficiently to quantify peaks using gradient elution without a scaling factor. For the purposes of this study, the ELSD temperature was kept at 50 °C to minimize potential problems with melting or decomposition.

Minor differences in solvent composition that could occur with 96-well plates created using gradient elution were minimized by operating the ELSD at 50 °C for the remaining studies. Importantly, a major goal was to determine feasibility and accuracy for quantifying large natural product libraries in 96-well plates. Due to variations in ELSD response with varying solvent compositions, a defined solvent composition (90% MeOH) was used for direct injection of each compound quantified by ELSD. Overall, this one simple step removed variations observed during gradient elution and greatly simplified quantification.

In order to construct calibration curves for each of the 10 compounds, samples were prepared such that an injection volume of 10 μ L was used for each concentration. The injection volume was maintained to prevent any discrepancies in solvent composition. A coupled HPLC–ELSD system was used to analyze the samples. HPLC flow was directly connected to an ELSD-LT. A low-flow nebulizer was used in the ELSD to increase sensitivity. While a low-flow nebulizer could operate using flow rates as low as 0.04 mL per minute, a flow of 1 mL

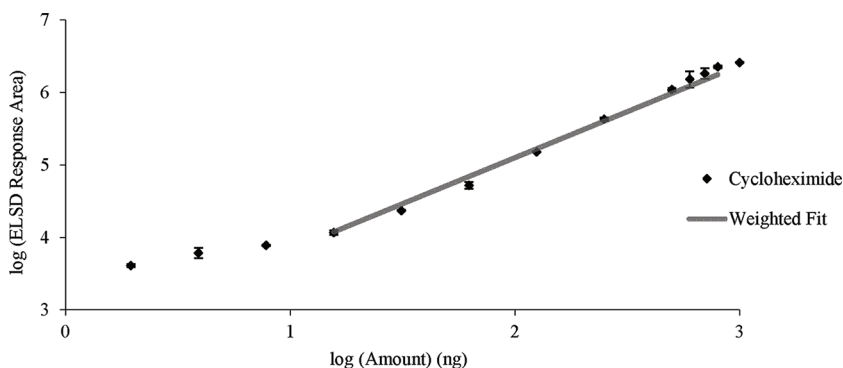


Figure 2. Calibration curve of cycloheximide.

per minute was chosen to maintain peak shape, decrease run time, and still remain below the maximum flow rate that was tolerated by the low-flow nebulizer. As a reference, a high-flow nebulizer for the ELSD-LTII used in this study can handle flow rates between 0.2 and 2.5 mL per min.

The ELSD hardware and parameters were optimized to increase sensitivity and throughput. The original tubing connecting the autosampler to the ELSD was 100 cm long and had a 0.3 mm internal diameter (i.d.) and was replaced with 26 cm of 0.127 mm i.d. tubing. Decreasing the length and i.d. of the tubing yielded sharper peaks, improved the detection limits from 14 ng to 3 ng, and reduced acquisition times to 0.17 min per sample. Additionally, the rinse volume and the rinse dip time were decreased, while the sampling speed was increased (see Experimental Section). After optimizing hardware and internal parameters, the total recycle time (injection to injection) was reduced from 1 min to 0.62 min per sample. Samples were directly injected from 96-well plates. A 12-plate Shimadzu rack changer was installed to accommodate large natural product libraries. A batch containing twelve 96-well plates, with 80 filled wells per plate (960 samples total), was analyzed in 10.2 h.

Calibration curves were constructed for each compound in Table 1 by plotting log(ELSD response area) versus log(amount). Consistent with previous research, a log/log plot was necessary to yield a linear calibration curve.²⁶ A weighted least-squares fit was determined for each standard (Supporting Information, Figures S1–S9). As an example, the weighted least-squares fit for cycloheximide is shown in Figure 2. Additionally, cycloheximide was used as a representative compound to determine the limit of quantification (LOQ), limit of linearity (LOL), and the linear dynamic range. Normalized residuals were calculated (eq 1), where y_{wtd} is the weighted response area, y is the average measured response area, and σ is the standard deviation. Normalized residuals were used to define the LOQ and LOL (see Experimental Section). The LOQ and LOL of cycloheximide were 15.6 and 700 ng, respectively, which defined the lower and higher limits of the linear dynamic range. The corresponding slope of the calibration curve represents the sensitivity of the calibration curve. For all 10 standards, the slope and y -intercept for each calibration curve, as well as the LOQ, were determined (Table 2).

$$\text{normalized residuals} = \frac{y_{\text{wtd}} - y}{\sigma} \quad (1)$$

Calibration curves for the 10 standards were used to construct a “universal” calibration curve to evaluate quantification

Table 2. Calibration Curve Data for Each of the 10 Standards and the Average Calibration Curve

compound	LOQ (ng)	slope	y -intercept
apigenin	15.6	1.38	2.60
phloretin	7.81	1.49	2.16
cycloheximide	15.6	1.31	2.49
hematoxylin	15.6	1.38	2.18
tamoxifen	7.81	1.42	2.46
ochratoxin A	15.6	1.34	2.22
pepstatin A	31.3	1.40	2.23
rifampicin	7.81	1.29	2.44
paclitaxel	15.6	1.56	1.90
cyclosporine A	15.6	1.22	2.83
average		1.49	2.70

of natural products independent of structure. For comparison, the overlapping linear regions between 31.3 and 500 ng of each calibration curve were plotted (Figure 3). The ELSD response area corresponding to each data point was averaged for all 10 standards to provide an average “universal” calibration curve.

The maximum error associated with quantification using the “universal” calibration curve was investigated. The standard deviation (σ) of the standard compounds at each concentration was used to calculate the maximum error (see Experimental Section) (Table 3). Smaller error was previously reported using a single calibration curve for quantification of combinatorial libraries;⁸ however, the natural products quantified in our study contained compounds with a broader molecular weight range. A maximum error of 31% suggests that if the “universal” calibration curve was used to determine 10 ng of a natural product with an unknown scaffold, the true quantity would range between 6.9 and 13.1 ng. For the purposes of sample preparation prior to HTS, this level of error is acceptable.

Five compounds were evaluated at concentrations within the linear region of the “universal” calibration curve to determine the error associated with “universal” quantification. Four of the compounds were used to create the “universal” calibration curve, and one, deferoxamine (also known as desferrioxamine) mesylate was not used to establish the “universal” calibration curve. Rifampicin and cycloheximide were selected because the slopes and intercepts for their calibration curves were similar to the “universal” calibration curve. Conversely, apigenin and ochratoxin A were chosen because their slopes and intercepts showed the largest deviation from the “universal” calibration curve (Figure 3 and Table 2). The average error from “universal” quantification of the four standards and deferoxamine mesylate was 19.3% and 24%, respectively.

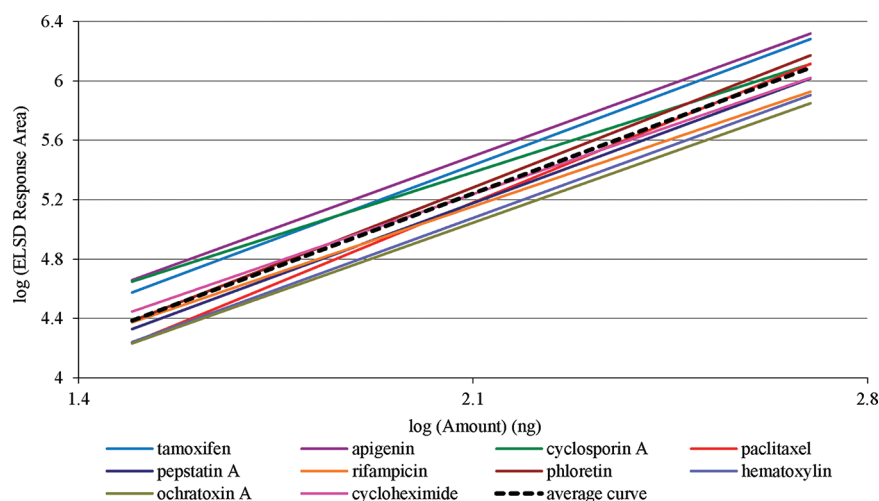


Figure 3. Comparison of standard calibration curves and construction of average “universal” calibration curve.

Table 3. Average Error (%) Associated with Quantifications of All 10 Standards for Each Amount Injected using the “Universal” Calibration Curve

quantity (ng)	positive error (%)	negative error (%)
500	27.1	−21.3
250	31.0	−23.7
125	31.9	−23.6
62.5	29.0	−22.5
31.3	31.3	−23.8

Natural product libraries often contain mixtures of compounds; therefore, the error associated with using the “universal” calibration curve for mixtures was investigated. Similar to the rationale above, mixtures of rifampicin and cycloheximide were used as well as mixtures of apigenin and ochratoxin A. The two sets of samples were prepared as both pure samples and mixtures. The average calibration curve was used to quantify each sample. The error associated with the experimental versus the true amount (250 ng) was calculated for each sample (Table 4). Both sets of mixtures displayed a comparable error to the pure samples, which suggests that the average calibration curve could be used to quantify mixtures within natural product libraries with acceptable accuracy for screening purposes.

In conclusion, the use of an ELSD-LT for quantification of structurally diverse natural products was evaluated. An average “universal” calibration curve provided structure-independent quantification of natural products with modest error. Depending on amounts to be quantified, parameters could be changed to achieve a different linear dynamic range. For our purposes, we focused on a concentration range that would be useful for ongoing projects. Optimization of sensitivity and throughput validates this method for high-throughput quantification of natural product libraries. The method presented in this study provides several advantages compared to current methods of real-time splitting of HPLC flow to an ELSD. The effect of gradients on quantification was eliminated using isocratic conditions. Also, the injection amount could be precisely controlled to minimize the amount of material directed to the ELSD, compared with online analysis, where the split ratio is typically constant. Furthermore, the described method provided an alternative strategy for quantification of fractionated or peak

Table 4. Error Associated with Pure Samples Versus Mixtures

mixture	experimentally determined (ng) ^a	error (%)
rifampicin	238	4.8
rifampicin:cycloheximide (3:1)	231	7.6
rifampicin:cycloheximide (1:1)	230	8.0
rifampicin:cycloheximide (1:3)	243	2.8
cycloheximide	251	0.4
ochratoxin A	202	19.2
ochratoxin A:apigenin (3:1)	239	4.4
ochratoxin A:apigenin (1:1)	295	18.0
ochratoxin A:apigenin (1:3)	354	41.6
apigenin	383	53.2

^aFor each sample a total of 250 ng was analyzed. The experimental quantification was performed using the “universal” calibration curve.

natural product libraries. In particular, offline quantification of libraries reduces data analysis and puts fewer constraints on constructing appropriate splits. Overall, this study confirmed that ELSD quantification was suitable for quantification of libraries for HTS independent of structure, and the method allows minor components to be enriched prior to screening.

EXPERIMENTAL SECTION

Sample Preparation. Ochratoxin A, rifampicin, apigenin, tamoxifen, paclitaxel, cyclosporin A, phloretin, deferoxamine mesylate, and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hematoxylin and cycloheximide were purchased from Fisher Scientific (Hampton, NH, USA) and A.G. Scientific, Inc. (San Diego, CA, USA), respectively. Samples were prepared in MeOH, EtOH, or CH₃CN depending on solubility at concentrations from 0.20 to 100 ng/ μ L.

Sample Processing. HPLC flow from a Shimadzu LC-20AT pump (Shimadzu Corp., Kyoto, Japan) was directed to a Shimadzu SIL-20ACHT autosampler that was directly connected to a Shimadzu ELSD-LTII evaporative light scattering detector with a low-flow nebulizer. ELSD parameters were set as follows: N₂ pressure = 350 KPa, temperature = 50 °C, gain = 8 (maximum gain = 12), flow rate = 1 mL/min. Samples were subjected to isocratic mobile phase of methanol and water (90%) at a flow of 1 mL/min for an acquisition time of 0.17 min. ELSD response area was obtained through auto-integration of peaks by Shimadzu LCSolution version 1.25 software.

Constructing Calibration Curves. Serial dilutions yielded samples in 0.200, 0.390, 0.780, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, and

100 ng/ μ L solutions. A volume of 10 μ L was directly injected into the HPLC flow, and the HPLC outflow was directed into ELSD-LTII without splitting. Each sample was injected in triplicate, and response peak areas were transformed and averaged. Plots of log(ELSD response area) versus log(amount) were constructed to yield calibration curves.

Weighted Least-Squares Analysis and Limit of Quantification. A weighted least-squares fit was constructed for each standard. Normalized residual values were calculated using eq 1. Data points that had residuals greater than 3σ (3 times the standard deviation) from the true mean value, or clearly deviated from the linear curve, were determined as outliers and were omitted from the weighted least-squares analysis by assigning no weight. The LOQ was defined as the lower limit of linearity.

Cycloheximide Limit of Linearity. Samples of cycloheximide were prepared in concentrations of 50.0, 60.0, 70.0, 80.0, and 90.0 ng/ μ L. Volumes of 10 μ L were directly injected into the ELSD. The detector was saturated when injected with 900 ng of cycloheximide. Using residuals, an injection of 800 ng was determined to be nonlinear; however, 500–700 ng was linear. The LOL of cycloheximide was determined to be 700 ng.

Constructing "Universal" Calibration Curve. The transformed average ELSD areas for all standards were averaged at each sample concentration. σ was calculated for each average. The average points were plotted as log(ELSD response area) versus log(amount). A weighted least-squares analysis was performed to generate a line for the "universal" calibration curve.

Calculating Potential Error for "Universal" Calibration Curve. Positive and negative error were calculated for the average ELSD response area for each injection quantity. The σ was added or subtracted, respectively, to each average value. The "universal" calibration curve yielded a quantity for each average ELSD response area. Experimental values were transformed and divided by the true injection quantity.

Quantification of Four Standards and Deferoxamine Mesylate Using the "Universal" Calibration Curve. Quantities of 250 ng of rifampicin, cycloheximide, ochratoxin A, and apigenin were injected. Experimental quantities were calculated using the "universal" calibration curve. Error values were calculated to be 4.8, 0.4, 19.2, and 53.2%, respectively. Injection quantities of 100, 200, and 400 ng of deferoxamine mesylate yielded errors of 9.2, 24.3, and 39.7%, respectively, following quantification using the "universal" calibration curve.

Analysis of Mixtures versus Pure Samples. Samples of rifampicin, cycloheximide, apigenin, and ochratoxin A were injected in quantities of 250 ng. Mixtures were prepared in ratios of 1:3, 1:1, and 3:1.

Optimization of ELSD Parameters. Tubing length was shortened from 100 cm to 26 cm. Tubing with 0.3 mm i.d. was replaced with 0.127 mm i.d. tubing. Rinse volume was reduced from 200 μ L to 50 μ L. Sampling speed was increased from 5 μ L/s to 10 μ L/s. Rinse dip time was reduced from 2 s to 1 s.

■ ASSOCIATED CONTENT

📄 Supporting Information

Calibration curves for each of the nine remaining compounds used in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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