The Journal of Antibiotics (2015) 68, 752–756

www.nature.com/ja

NOTE

Evaporative light scattering quantification of natural products possessing a carbon–phosphorus bond

Matthew C Chi and Geoff P Horsman

The Journal of Antibiotics (2015) **68,** 752–756; doi:10.1038/ja.2015.62; published online 20 May 2015

Natural products have had an enormous impact on human health over the last century, and advances in genetics and analytical chemistry are rapidly expanding the frontiers of natural product discovery.[1,2] Research efforts are being further reinvigorated by recently established natural product libraries that lower the barriers-to-entry for researchers interested in testing these molecules in a variety of biological screening platforms.[3–5] Moreover, analytical techniques are increasingly enabling high-throughput library characterization and dereplication to accelerate discovery by focusing only on fractions or organisms possessing desired characteristics.[6–8]

This renewed interest in probing natural product libraries has uncovered a need for absolute and universal quantification of mixtures of natural products in library fractions,[8] and the utility of low-temperature evaporative light scattering detection (ELSD-LT) has recently been demonstrated as an accessible option for this purpose.[9] Along with charged aerosol detection and nano quantity analyte detection, ELSD-LT is a universal detector because signal response is proportional to the weight of the analyte.[10] If less volatile than solvent, the solute particles remaining after low-temperature evaporation may be detected by light scattering, and the resulting peak areas can be used to quantify analytes that do not possess chromophores or ionizable groups. This principle was exploited by Bugni and co-workers to generate a calibration curve with low nanogram limits of quantification (LOQ) for 'universal' quantification of structurally diverse molecules from natural product libraries.[9]

Although the utility of ELSD-LT for low cost and high-throughput quantification of natural product library fractions has been established, the current universal calibration curve reflects a current bias in natural product discovery toward 'typical' compounds from a few natural product classes, such as the polyketides, non-ribosomal peptides and terpenes. However, molecules possessing a C–P bond (phosphonates and phosphinates) are a relatively underexplored class of natural products that have recently attracted renewed interest in part due to the high proportion of these molecules possessing biological activity. In addition, although only ~30 natural C–P compounds have been described, recent bioinformatic analysis suggests that these molecules are much more widespread in nature than previously appreciated.[11,12] In comparison to the 'typical' natural products used to generate the universal calibration curve, C–P compounds generally possess lower molecular weights, higher polarity and often lack a strong UV–visible chromophore (Figure 1). Because ELSD-LT is based upon light scattering from non-volatile molecules, the relatively high polarity and low-molecular volume of C–P compounds may yield anomalous light scattering responses. Herein, we investigate the utility of the previously developed universal calibration curve to quantify C–P compounds and identify conditions suitable for truly universal quantification of structurally diverse natural products. In addition, an HPLC method is described for separating and quantifying C–P compounds in a mixture.

EXPERIMENTAL PROCEDURE

Materials and sample preparation

Compounds **1–6**, **8** and **12–19** were purchased from Sigma-Aldrich (St Louis, MO, USA), and compounds **7**, **9–11** and HPLC grade solvents were purchased from Fisher Scientific (Toronto, ON, Canada). Samples were dissolved to a standard concentration of 10 mg ml^{-1} in either Milli-Q water or HPLC grade methanol depending on solubility. Triplicate serial dilutions generated concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9 and 2.0 ng µl^{-1}.

Sample processing for direct injection

A Shimadzu LC-20AT pump (Shimadzu Corp., Kyoto, Japan) directed solvent at a flow rate of 1.00 ml min^{-1} to a Shimadzu SIL-20ACHT autosampler that was connected to a Shimadzu ELSD-LTII evaporative light scattering detector using 75 cm of 0.127 mm i.d. tubing. The ELSD was operated using N_2 pressure = 50 p.s.i., AD2 sampling = 10 Hz and gain = 8. ELSD drift tube temperature was set to 30, 50 or 70 °C, and the isocratic mobile phase was either 10 or 90% methanol in water. For each sample dilution, 1 µl was injected to yield triplicates of each injection quantity, and the ELSD-LTII response peak areas and peak heights were obtained by autointegration in the Shimadzu LCsolution version 1.25 software (Shimadzu Corp.).

Sample processing for hydrophilic interaction chromatography (HILIC)

Flow from a Shimadzu LC-20AT pump was maintained at 0.25 ml min^{-1} and directed to a Shimadzu SIL-20ACHT autosampler, from which output flow

Department of Chemistry and Biochemistry, Wilfrid Laurier University, Waterloo, ON, Canada
Correspondence: Professor GP Horsman, Department of Chemistry and Biochemistry, Wilfrid Laurier University, Waterloo, ON N2L 3C5, Canada.
E-mail: ghorsman@wlu.ca
Received 3 March 2015; revised 20 April 2015; accepted 24 April 2015; published online 20 May 2015

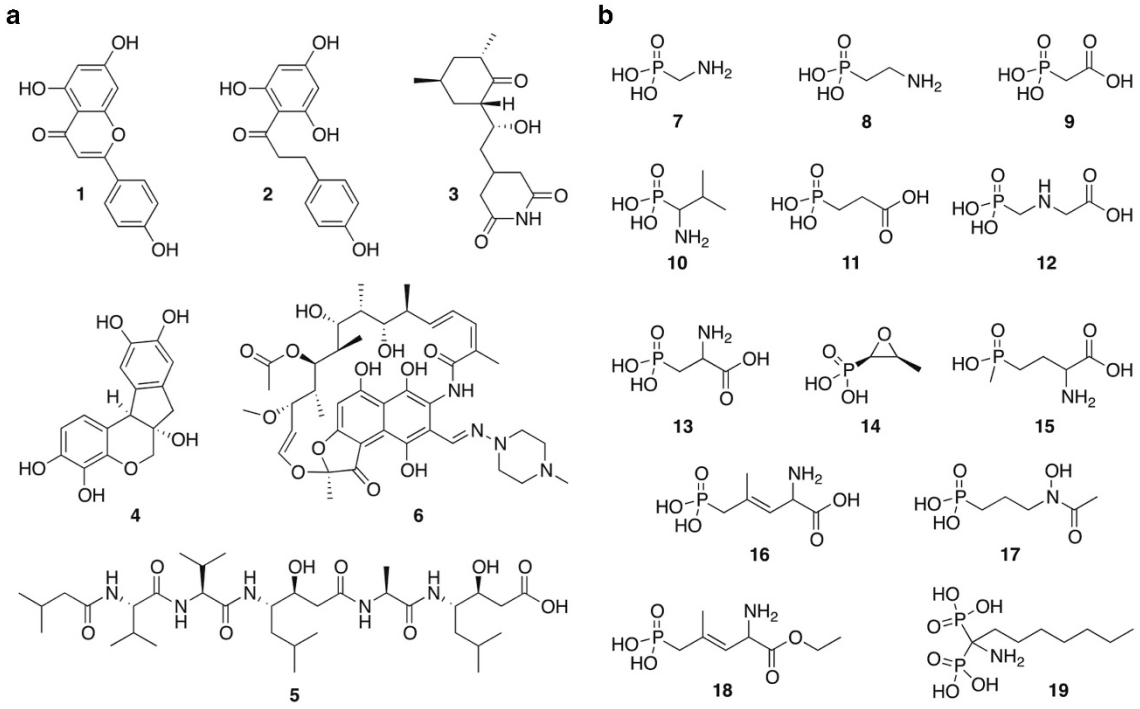

Figure 1 Structures of compounds used in this study. (**a**) Compounds representative of 'typical' natural product classes used to construct the previously described universal calibration curve for low-temperature evaporative light scattering detection quantification: apigenin (**1**), phloretin (**2**), cycloheximide (**3**), hematoxylin (**4**), pepstatin A (**5**) and rifampicin (**6**). (**b**) Phosphonates and a phosphinates used as representative C–P compounds: (1-amino-1-methylpropyl) phosphonic acid (**7**), 2-aminoethylphosphonic acid (**8**), phosphonoacetic acid (**9**), (1-amino-1-methylpropyl)phosphonic acid (**10**), 3-phosphonopropanoic acid (**11**), glyphosate (**12**), DL-2-amino-3-phosphonopropionic acid (**13**), fosfomycin (**14**), glufosinate (**15**), CGP-37849 (**16**), FR-900098 (**17**), CGP-39551 (**18**), (1-amino-1-phosphono-octyl)phosphonic acid (**19**).

passed into the Shimadzu CTO-20AC column oven at 40 °C equipped with an Alltima HP HILIC column (3 µm, 150 × 2.1 mm, Grace Davison Discovery Sciences, Deerfield, IL, USA) before entering the ELSD-LTII detector. Separation was achieved via gradient elution starting at a ratio of 95:5 of solvent A (0.1% trifluoroacetic acid in acetonitrile) to solvent B (water) and increasing solvent B to 20% over 17 min. At 18.5 min, the column was returned to 5% B for the remaining 1.5 min. The ELSD-LTII parameters were set as described above but with the drift tube temperature set at 50 °C.

Constructing calibration curves

Calibration curves for each compound at each of the six conditions (three temperatures and two solvents) were constructed by plotting the log(ELSD response area) versus the log (injection amount), with standard deviations included as error bars. An averaged, or 'universal', calibration curve was constructed for 'typical' compounds and C–P compounds separately at each condition by plotting the average responses for all 'typical' or C–P compounds with standard deviations as error bars.

LOQ determination

LOQ values were determined based on the amount of analyte that would yield a peak height equal to 10 times the standard deviation of the blank (baseline noise) at each condition, and was calculated from calibration curves for each compound using peak height instead of peak area (Supplementary Figure 1). The standard deviations were calculated in Excel (Microsoft, Redmond, WA, USA) from blank injections of mobile phase solvent.

Statistical comparisons of calibration curves

Statistically significant differences between 'typical' and C–P compound calibration curves were evaluated by calculating degrees of freedom with the Welch–Satterthwaite equation[13] and using the Welch's *t*-test[13] (Equation 1) to calculate a *t*-value for each injection quantity, where $\overline{x}_1$, s_1 and n_1 represent the average ELSD response, variance and number of 'typical' compounds tested, respectively; similarly, $\overline{x}_2$, s_2 and n_2 are the same parameters for C–P compounds.

$$t = \frac{|\overline{x_1} - \overline{x_2}|}{\sqrt{\left\{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}\right\}}} \quad (1)$$

RESULTS AND DISCUSSION

Comparison of C–P and 'typical' compounds under universal quantification conditions

Representative 'typical' compounds were tested (Figure 1a, compounds **1**–**6**) under the standard conditions to determine whether an identical calibration curve could be obtained. Although the 90% methanol mobile phase and 50 °C evaporation temperature were identical to those previously reported, the injection volume was reduced from 10 µl to 1 µl to improve reproducibility and enable accurate comparisons between 'typical' and C–P compounds. Reduced injection volumes provided a constant ELSD-LT response that was independent of sample solvent (Supplementary Figure 2). This was critical for comparing the two groups of compounds because methanol was used to dissolve 'typical' compounds, whereas the C–P compounds were dissolved in water. Lower injection volumes also abolished solvent peaks that appeared at higher volumes (Supplementary Table 1). For each of the six compounds, the previously described standard conditions of 90% methanol and 50 °C were used to generate a linear calibration curve in the log-log form that is routinely used for analysis of ELSD data.[14–16]

The resulting calibration curves possessed comparable slopes but smaller y-intercepts (Figure 2a, short dashes) than previously reported (Figure 2a, long dashes),[9] presumably due to the solvent signal observed at higher injection volumes (Supplementary Table 1). The LOQ values were also comparable or slightly lower than those previously reported (Table 1).

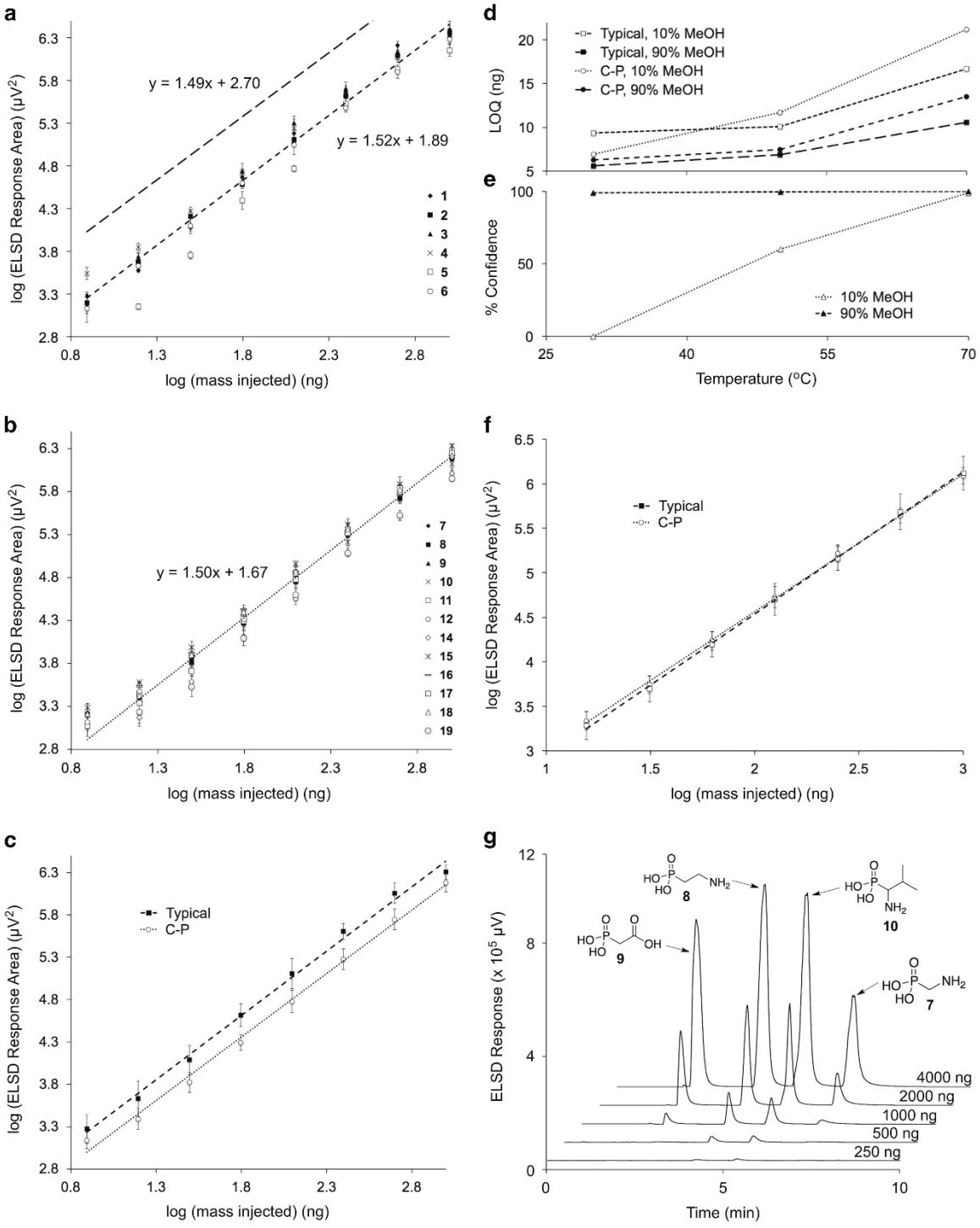

Figure 2 Evaporative light scattering detection (ELSD) analyses of C–P and 'typical' natural products. (**a**) Average calibration curve of 'typical' compounds **1**–**6** generated in this paper (short dashes) compared with the previously reported[9] universal calibration curve (long dashes) at 50 °C and 90% methanol. (**b**) Average calibration curve of C–P compounds **7**–**19** at 50 °C and 90% methanol. (**c**) Comparison of 'typical' and C–P compound calibration curves at 50 °C and 90% methanol. (**d**) Limit of quantification values for both classes of compounds at three different temperatures (30, 50 and 70 °C) and two different mobile phase solvents (10 and 90% methanol in water). (**e**) % Confidence that the 'typical' and C–P compound calibration curves are different under each condition based on the Welch's *t*-test (Supplementary Table 2).[13] (**f**) The condition of 10% methanol and 30 °C that provides a new 'universal' calibration curve representative of both 'typical' and C–P compounds. (**g**) HPLC chromatograms for hydrophilic interaction chromatography separation of different amounts of C–P compounds **7**–**10**.

Table 1 Comparison of molecular weight, enthalpy of evaporation[17] and the LOQ values determined for each compound

			LOQ (ng)					
			90% MeOH mobile phase			*10% MeOH mobile phase*		
Compound	*Mol. Wt.*	*Enthalpy of Evap. (kJ mol^{-1})*	*30°C*	*50°C*	*70°C*	*30°C*	*50°C*	*70°C*
'Typical' natural products								
Apigenin (**1**)	270.24	86.8	6.24	7.20	8.70	12.2	10.9	15.7
Phloretin (**2**)	274.27	84.1	5.40	6.91	10.9	11.0	11.1	19.0
Cycloheximide (**3**)	281.35	87.4	3.93	5.65	12.1	7.72	8.55	18.0
Hematoxylin (**4**)	302.28	91.3	3.16	4.84	6.40	4.88	6.33	9.03
Pepstatin A (**5**)	685.89	164.9	9.28	10.2	13.23	10.1	12.9	19.2
Rifampicin (**6**)	822.94	142.9	5.65	6.42	12.0	10.2	10.6	19.6
AVERAGE	**439.50**	**109.6**	**5.61**	**6.88**	**10.6**	**9.36**	**10.1**	**16.7**
C–P compounds								
AMP (**7**)	111.04	66.3	5.30	6.73	12.2	7.23	10.6	21.0
2-AEP (**8**)	125.06	65.6	3.69	5.00	11.3	5.60	10.0	18.2
P-Ac (**9**)	140.03	82.8	5.81	8.84	13.6	9.86	14.7	19.4
1A1MP (**10**)	153.12	59.7	6.06	6.03	13.5	5.77	12.9	25.1
3-PP (**11**)	154.06	78.9	6.73	5.99	14.0	8.12	16.5	23.2
Glyphosate (**12**)	169.07	79.7	4.16	5.42	11.5	7.15	12.1	19.8
DL-2A3P (**13**)	169.07	81.8	9.18	10.5	16.7	9.34	13.4	20.0
Fosfomycin (**14**)	182.02	64.4	10.7	10.7	19.3	8.73	14.8	31.9
Glufosinate (**15**)	198.16	86.6	5.24	3.89	10.1	5.29	10.5	17.8
CGP-37849 (**16**)	209.14	87.2	6.27	6.77	11.1	7.00	9.58	14.0
FR-900098 (**17**)	219.11	77.6	6.40	8.28	14.0	5.99	9.62	20.9
CGP-39551 (**18**)	237.19	75.7	5.72	7.00	8.98	4.65	9.02	13.4
P-Oct (**19**)	289.21	87.5	6.31	12.0	19.1	5.35	8.69	30.6
AVERAGE	**181.25**	**76.5**	**6.27**	**7.47**	**13.5**	**6.93**	**11.7**	**21.2**

Abbreviations: Evap., evaporation; LOQ, limit of quantification; Mol. Wt., molecular weight.
Averages are calculated for both 'typical' and C–P natural product classes.

After generating a calibration curve of 'typical' natural products, we evaluated its utility for quantifying C–P compounds. Thirteen representative C–P compounds, of which three (**16**, **18** and **19**) were synthetic (Figure 1b, compounds **7**–**19**), were analyzed under the same conditions used to quantify 'typical' compounds, and a linear fit to the log-log plot was obtained (Figure 2b) but with higher LOQ values than for 'typical' compounds (Table 1). Significantly, because C–P compounds elicited a smaller average ELSD response compared with the 'typical' compounds (Figure 2c), use of the current universal calibration curve could significantly underestimate the quantity of C–P compounds in a sample. For example, an ELSD response area of 10 000 μV^2 would underestimate the quantity of C–P compounds injected by ~60%. In summary, the sample selection bias of the current calibration curve toward 'typical' natural product classes has rendered it inaccurate for quantifying C–P compounds, which require a dedicated calibration curve.

Searching for conditions that enable truly universal quantification

To address the shortcomings of the current calibration curve and avoid generating dedicated calibration curves for each natural product class, we elected to search for conditions that could accurately quantify both sets of compounds using a single curve. The pursuit of such truly universal conditions was initiated by determining LOQ values under a variety of evaporation temperatures and mobile phase solvents. Specifically, evaporation temperatures of 30, 50 and 70 °C were combined with an aqueous mobile phase containing either 90 or 10% methanol to achieve six different analysis conditions for each of the 19 compounds. LOQ values under each condition are summarized in Table 1 and provide a useful indicator of ELSD-LT sensitivity for a given compound. As expected, sensitivity increases at lower temperatures and increasing solvent volatility. However, there is no obvious correlation between LOQ and either molecular weight or enthalpy of evaporation under the conditions used, even though ELSD is thought to work best for analytes with molecular weights >270.[8] To identify conditions that elicit similar ELSD-LT response for 'typical' and C–P compounds, the LOQ values were plotted as a function of temperature and solvent (Figure 2d). The intersecting lines on this plot revealed that the two classes of molecules have similar LOQ values between 30 and 50 °C using 10% methanol, suggesting that these conditions might generate a truly universal standard curve. Full calibration curves under each condition were then constructed to determine if truly universal quantification could be achieved (Supplementary Figure 3), and statistical analysis was performed to determine at what confidence interval the two curves could be considered significantly different (Supplementary Table 2). Although all 90% methanol conditions resulted in significantly different calibration curves (≥99% confidence level), the 'typical' and C–P compound curves converged as the temperature decreased (Figure 2e) such that they were statistically indistinguishable at 30 °C (Figure 2f and Supplementary Figure 3). In summary, conditions of 30 °C and 10% methanol mobile phase eliminate differences in ELSD-LT response between 'typical' and C–P compounds and

therefore allow truly universal quantification of structurally diverse natural products with a single calibration curve.

Separation and quantification of C–P compounds in a mixture

In addition to estimating the total quantity of natural products in an extract, relative composition can be estimated by chromatographic separations before ELSD-LT. Specifically, the high polarity of C–P compounds relative to typical natural products provides an opportunity to separate the two classes. We used HILIC to separate several C–P compounds with similar polarity and molecular weights (Figure 2g), although with reduced sensitivity. For example, the LOQ for 2-aminoethylphosphonic acid (**8**) increased to 145 ng, or about 30-fold compared with direct injection (Supplementary Figure 1). Interestingly, although the column efficiently retained polar C–P compounds, many 'typical' natural products rapidly eluted to facilitate estimates of the relative composition of two natural product classes (Supplementary Figure 4). In summary, HILIC separation coupled to ELSD-LT enables rapid and low-cost quantitative estimates of polar versus 'typical' natural products in polar fractions, albeit with reduced sensitivity compared with direct injection, and techniques such as ^{31}P NMR and mass spectrometry could be added to identify C–P compounds.

In conclusion, the relatively small and polar C–P compounds generate a significantly different ELSD-LT response relative to the more widely studied natural product classes such as polyketides and non-ribosomal peptides. Although accurate quantification of C–P compounds can be achieved with a dedicated calibration curve, reduced evaporation temperature and organic solvent composition can yield identical calibration curves for C–P and 'typical' natural product groups, enabling truly universal quantification with a single calibration curve. Moreover, although the LOQ values are >10-fold higher, HILIC chemistry can efficiently separate C–P compounds from one another and from 'typical' compounds to generate additional compositional information. Overall, these results demonstrate the potential of ELSD-LT for inexpensive and high-throughput quantification of natural product libraries possessing C–P compounds.

ACKNOWLEDGEMENTS

This work was funded by the Natural Sciences and Engineering Research Council of Canada (Discovery Grant and Research Tools and Instruments Grant to GPH, and Undergraduate Student Research Award to MC) and Wilfrid Laurier University.

1 Cragg, G. M. & Newman, D. J. Natural products: A continuing source of novel drug leads. *Biochim. Biophys. Acta BBA - Gen. Subj.* **1830**, 3670–3695 (2013).
2 Newman, D. J. & Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **75**, 311–335 (2012).
3 Thaker, M. N. *et al.* Identifying producers of antibacterial compounds by screening for antibiotic resistance. *Nat. Biotechnol.* **31**, 922–927 (2013).
4 Xie, P. *et al.* Biosynthetic potential-based strain prioritization for natural product discovery: a showcase for diterpenoid-producing actinomycetes. *J. Nat. Prod.* **77**, 377–387 (2014).
5 Ren, Y. *et al.* Constituents of an Extract of Cryptocarya rubra housed in a repository with cytotoxic and glucose transport inhibitory effects. *J. Nat. Prod.* **77**, 550–556 (2014).
6 Johnson, T. A. *et al.* Natural product libraries to accelerate the high-throughput discovery of therapeutic leads. *J. Nat. Prod.* **74**, 2545–2555 (2011).
7 Hindra *et al.* Strain prioritization for natural product discovery by a high-throughput real-time PCR method. *J. Nat. Prod.* **77**, 2296–2303 (2014).
8 Ito, T. & Masubuchi, M. Dereplication of microbial extracts and related analytical technologies. *J. Antibiot. (Tokyo)* **67**, 353–360 (2014).
9 Adnani, N., Michel, C. R. & Bugni, T. S. Universal quantification of structurally diverse natural products using an evaporative light scattering detector. *J. Nat. Prod.* **75**, 802–806 (2012).
10 Hutchinson, J. P. *et al.* Comparison of the response of four aerosol detectors used with ultra high pressure liquid chromatography. *J. Chromatogr. A* **1218**, 1646–1655 (2011).
11 Metcalf, W. W. & van der Donk, W. A. Biosynthesis of phosphonic and phosphinic acid natural products. *Annu. Rev. Biochem.* **78**, 65–94 (2009).
12 Yu, X. *et al.* Diversity and abundance of phosphonate biosynthetic genes in nature. *Proc. Natl. Acad. Sci. USA* **110**, 20759–20764 (2013).
13 Zar, J. H. *Biostatistical Analysis* 4th edn. (Prentice Hall, Upper Saddle River, NJ, USA, 1999).
14 Dixon, R. W. & Peterson, D. S. Development and testing of a detection method for liquid chromatography based on aerosol charging. *Anal. Chem.* **74**, 2930–2937 (2002).
15 Holzgrabe, U., Nap, C.-J. & Almeling, S. Control of impurities in L-aspartic acid and L-alanine by high-performance liquid chromatography coupled with a corona charged aerosol detector. *J. Chromatogr. A* **1217**, 294–301 (2010).
16 Vervoort, N., Daemen, D. & Török, G. Performance evaluation of evaporative light scattering detection and charged aerosol detection in reversed phase liquid chromatography. *J. Chromatogr. A* **1189**, 92–100 (2008).
17 *SciFinder*; Chemical Abstracts Service: Columbus, OH. Avialable at https://scifinder.cas.org (accessed on 14 October 2014); calculated using ACD/Labs software, version 11.02; ACD/Labs 1994-2015.

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)