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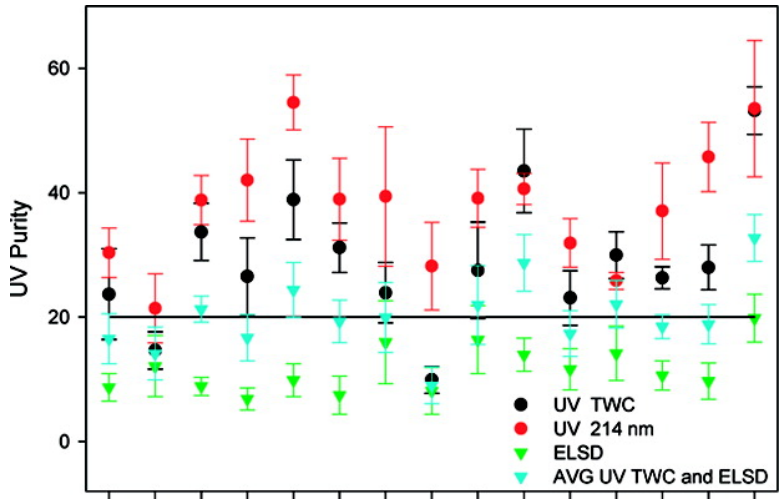

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Dual Detection Approach to a More Accurate Measure of Relative Purity in High-Throughput Characterization of Compound Collections

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The accurate determination of compound purity is crucial for characterizing library purity, monitoring the stability of storage compounds, and obtaining meaningful high-throughput screening results. However, current high-throughput techniques for the determination of compound purity are inadequate. We evaluated on-line chromatography detectors, including UV_{TWC}, UV_{214}, and ELSD detectors, in a series of studies of 233 compound mixtures prepared with known compositions. Results indicate that both UV_{TWC} and UV_{214} overestimate the minor component in a mixture whereas ELSD underestimates the minor component. An average of UV_{TWC} and ELSD purities gives a more accurate measure of the relative purity for a wide range of compounds in various purity ranges. This technique was applied to 959 compounds from our compound collection to more accurately determine their relative purity.

The study of interactions between small molecules and proteins or cells is a critical undertaking in drug discovery and chemical biology research. These molecular probes, coming from parallel synthesis, natural products isolation, or one-at-a-time synthesis, are stored in compound repositories. Due to the postpurification processing and the continuous degradation in storage, the purity of compounds needs to be repeatedly surveyed using high-throughput analytical techniques during their screening and storage life cycle. On the other hand, the determination of compound purity is also essential for quality control of compound library synthesis and production. However, the reported relative purity of a compound is often an uncertain number without knowing the details of how it was measured. For example, the purity of the same sample can be reported to have a higher than its true purity by UV_{254} or evaporative light scattering detector (ELSD) detection methods or a lower value by UV_{214} or total wavelength content, 210–400 nm (UV_{TWC}), methods.

Several techniques that are commonly used to determine the quantitative purity of a compound of interest are not well suited to a high-throughput environment. The use of standards to generate calibration curves for analysis of each compound in a compound library is not feasible due to the lack of authentic compound standards. Flow-injection NMR analysis[1] has been shown to quantify samples accurately and recover most of samples. However, the time required for spectral interpretation is still a bottleneck. Elemental analysis is another accurate technique.[2] However, it is time-consuming and requires too much sample; therefore, it is not suitable for compound collections that contain compounds with microgram quantity. Chemiluminescent nitrogen detection (CLND)[3−9] is compatible with a medium-throughput operation, but it is not widely used.

Due to difficulties in obtaining quantitative purity of compounds in repositories or libraries, relative purity is often an acceptable measure for compound quality considering the fact that the relative purity is very close to the true quantitative purity if compounds are chromatographically purified.[9,10] One commonly used technique for determining compound relative purity is LC/UV/ELSD/MS.[10−19] This method is fast and requires little sample. Compounds and their impurities are separated on a reverse-phase HPLC column, which can take only a couple of minutes in the case of ultraperformance liquid chromatography (UPLC). The compounds are identified by their mass-to-charge ratio using mass spectrometry and the purity of the compound of interest can then be determined by UV at a single wavelength or over a range of wavelengths with a photodiode array detector, or by ELSD.

Although these detection methods are widely used, each has its own unique drawbacks that make the use of a single detector problematic. Although mass spectrometry is an excellent technique for determining the identity of a compound, the ion current is highly dependent on the ionization efficiency of the compound and therefore mass spectrometry does not yield accurate purity information. UV absorbance at a single wavelength does not always yield accurate purity either, as the absorbance of compounds is wavelength dependent. For example, purity measured by UV absorbance at 254 nm is often overestimated because many impurities may have no absorption at this wavelength.[10] UV detection using a PDA allows for analysis over a wide range of wavelengths and is better than a single wavelength UV detector. However, some compounds do not have a UV chromophore and therefore are invisible to the PDA. ELSD

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Table 1. Commercial Compounds Used in the Study

number	compound name
1	hydrocortisone acetate
2	hydrocortisone
3	cortisone acetate
4	cortisone
5	progesterone
6	cyproterone acetate
7	mifepristone
8	dexamethasone
9	prednisone
10	Fmoc-ornithine(Alloc)
11	Fmoc-glutamic acid(OcHx)
12	Fmoc-phenylglycine
13	Fmoc-O-*t*Bu-serine
14	Fmoc-isoleucine
15	Fmoc-lysine(Alloc)
16	Fmoc-phenylalanine
17	2′,3′-O-isopropylideneadenosine
18	*N*-(α)-tosyl-L-arginine methyl ester
19	phthalylsulfacetamide
20	warfarin
21	perphenazine

does not require that the compound contain a UV chromophore, but detection does depend on the volatility of the compound. Highly volatile compounds are not detected, and low molecular-weight compounds (<300 Da) are commonly invisible or underrepresented in this detector.[10,11] Therefore, the question is if there is no single accurate detector for measuring compound purity, does a combination of two detectors accomplish the task?

The keys for high-throughput compound quality control (usually using LC/MS/UV/ELSD) are to rapidly confirm the identity of the compound of interest and to determine its relative purity. If these are not accurately measured, then the biological testing with the compound will yield ambiguous and unreliable results. The identity of the compound is determined by online mass spectrometry. The single quadrupole mass spectrometer used for these studies has unit resolution and is well suited for high-throughput analysis and is able to identify the compound of interest. In cases where multiple compounds in a mixture have similar masses, a high-resolution mass spectrometer or use of another detection method such as NMR may be necessary for compound identification.

Here, we investigate the accuracy of purity determinations of 233 compound mixtures with known composition using UV_{214}, UV_{TWC}, and ELSD detection (for experimental conditions, see the Supporting Information). Twenty-one compounds (Table 1) were used to prepare these mixtures based on their drug-like properties and their structural diversity. These compounds were mixed into "impure" samples with known amount of an "impurity". The mixtures were prepared such that each compound was present as 20, 40, 60, or 80% of the total concentration. Solutions were dissolved in DMSO to a total concentration of 1.0 mM. The injection volume is 1 μL, resulting in 1.0 nmol of total compound being injected. The relative purity of the compound of interest was determined by measuring the peak area and dividing it by the area of all nonsolvent peaks present in the chromatogram.

UV Relative Purity. Sample UV_{214} and UV_{TWC} chromatograms for a solution containing 60% compound no. 2 (hydrocortisone) and 40% compound no. 21 (perphenazine) are shown in Figure 1. There is a larger signal-to-noise ratio

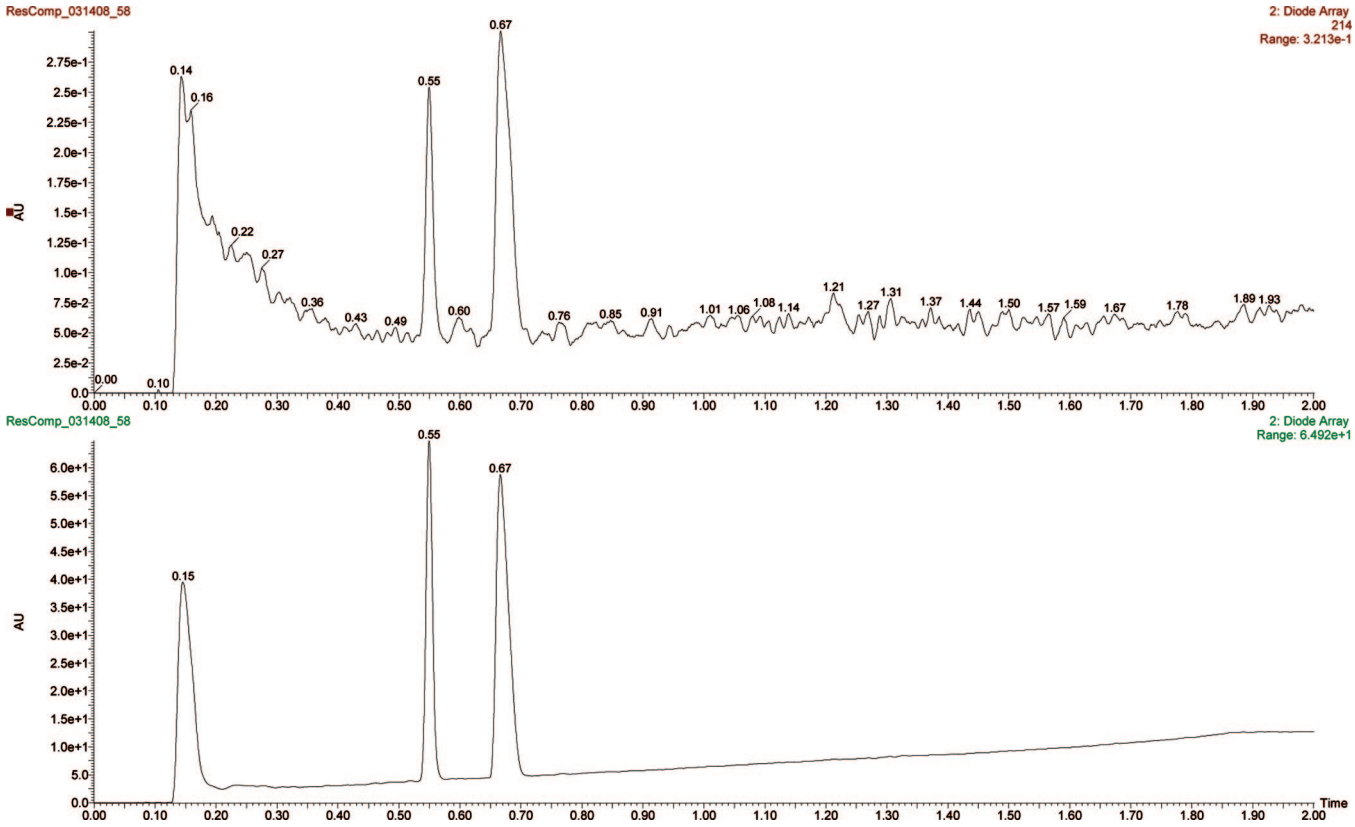

Figure 1. Chromatograms for a solution containing 0.6 mM compound no. 2 and 0.4 mM compound no. 21 with UV_{214} (top) and UV_{TWC} detection (bottom).

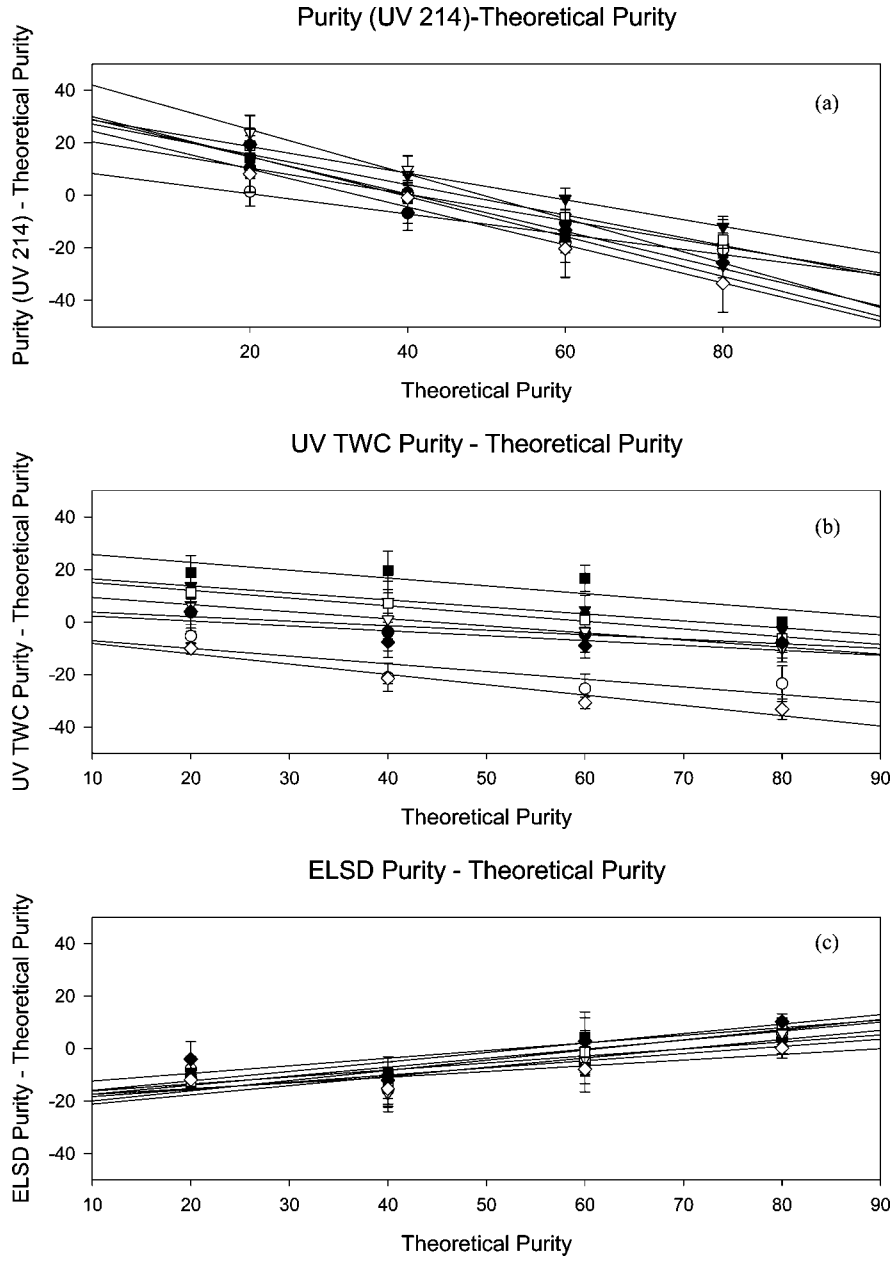

Figure 2. Difference between theoretical sample purities and purities measured by (a) UV_{214}, (b) UV_{TWC}, and (c) ELSD for 233 mixtures. Each point represents the average purity difference for all mixtures containing one of compound nos. 1−9 or 10−16 and one of compound nos. 1 and 17−21.

for the peaks in the UV_{TWC} chromatogram than for UV_{214}, suggesting a higher sensitivity in UV_{TWC} detection mode.

The difference between the theoretical sample purities and those measured by UV_{214} and UV_{TWC} are given in Figure 2a and b. Each point represents the average purity from mixtures containing an "impurity" and a compound from nos. 1−9 or 10−16 (Table 1). There is a wide range of observed UV purities for mixtures which have the same theoretical purity, likely due to the use of diverse impurity compounds which contain different UV chromophores. Mixtures that contain 20% of an impurity compound are estimated to have 25.9 ± 10.2% impurity by UV_{TWC} and 34.4 ± 9.3% by UV_{214}, with the error representing two standard deviations. UV_{TWC} appears to yield more accurate purities than UV_{214} with similar error although the precision is lower for UV_{TWC}. It is also noted that departures from theoretical purities as measured by UV_{214} or UV_{TWC} show a downward trend as the compound of interest becomes a major component. The trend is opposite in the case of ELSD detection. The downward trend in UV detection and upward trend in ELSD detection are expected. When the compound of interest becomes the major component in a mixture, its purity is underestimated by UV_{214} or UV_{TWC} methods which overestimate the minor components in a mixture. Similarly, when the compound of interest is the major component, ELSD will overestimate its purity by underestimating the minor components in the mixture.

The observed UV and ELSD purities for the minor (20%) component compound in the mixtures are shown in Figure 3. All 15 of the UV_{214} purities and 13 of 15 UV_{TWC} purities are greater than 20%. Additionally, the UV_{214} purities are higher than the UV_{TWC} purities in 13 of 15 cases. Both methods appear to overestimate the amount of the minor component, with UV_{214} even less accurate than UV_{TWC}.

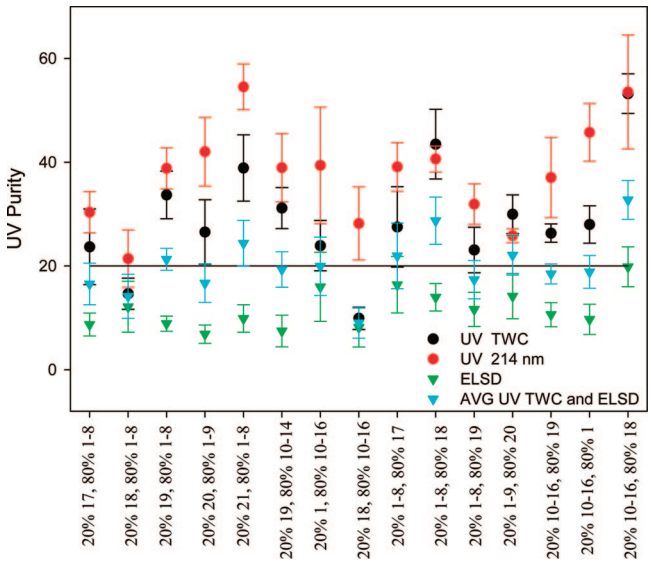

Figure 3. UV_{TWC}, UV_{214}, and ELSD purities of 233 mixtures that contain 0.2 mM (20%) of one compound. Each point represents the average purity for all mixtures containing one of compound nos. 1−9 or 10−16 and one of compound nos. 1 and 17−21.

If too much sample is injected, the PDA detector may become saturated and the response will become nonlinear, resulting in an observed underestimation of the major component or the apparent overestimation of the minor component compound. In order to remove this ambiguity and confirm that we are not saturating the UV detector, four compounds at a total concentration of 0.5 mM were analyzed at different injection amounts (Figure 4). The data in all four plots appears to be linear up to at least an injection of 2.0 nmol. In this work, we used a maximum injection amount of 1.0 nmol, which appears to be within the linear range of the detector and indicates that the underestimation of the most abundant component of a mixture is not due to UV detector saturation.

ELSD Relative Purity. The difference between the theoretical sample purities and those measured by ELSD for solutions containing known amounts of a compound of interest and an impurity compound are given in Figure 2c. There is less scatter in the ELSD purities than there was for the UV_{TWC} values. Whereas the UV results **overestimate** the amount of the minor component compound, ELSD **underestimates** this amount. This trend is also seen in Figure

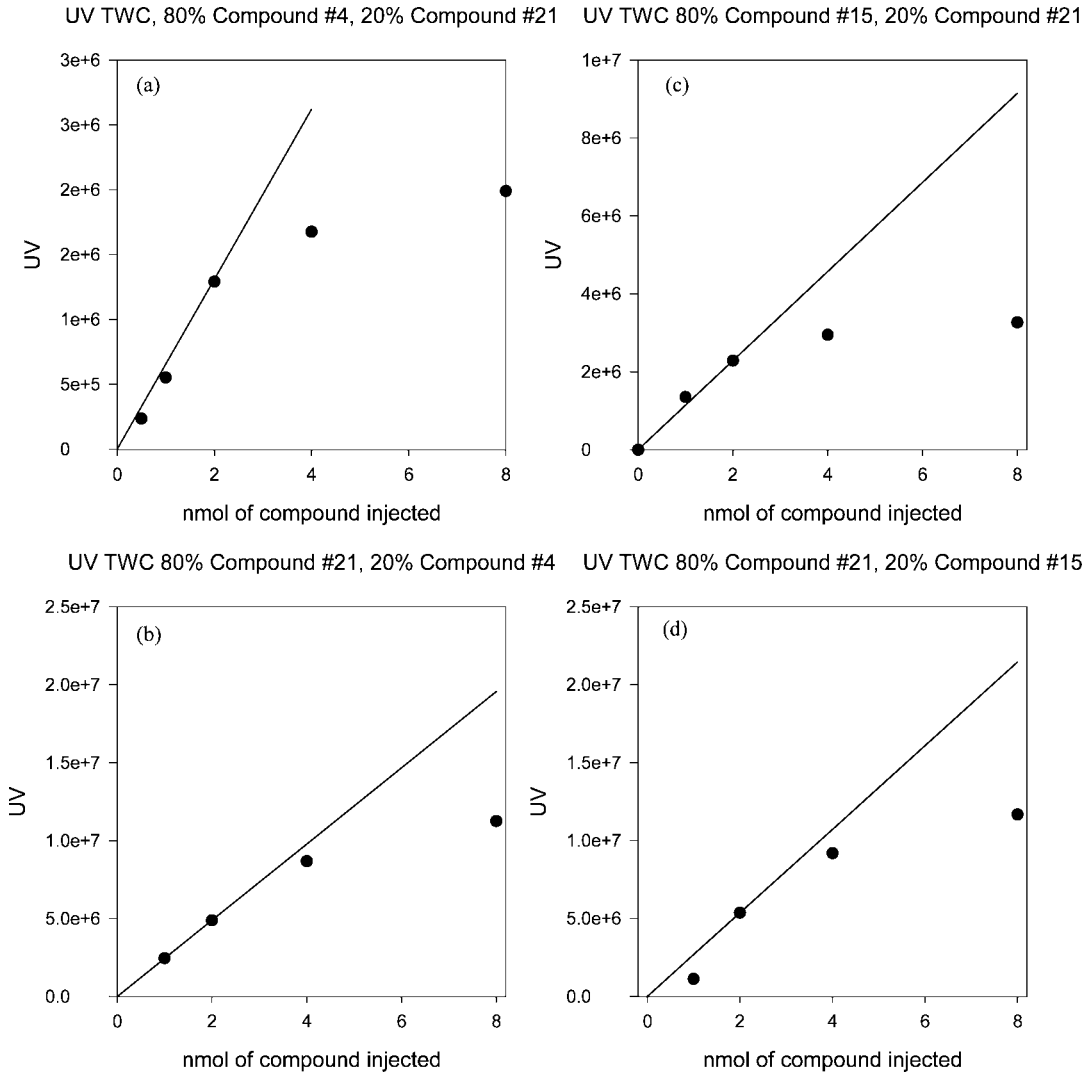

Figure 4. UV_{TWC} absorbance for (a) compound no. 4, (b) compound no. 21, (c) compound no. 15, and (d) compound no. 21 in mixtures containing 80% of the compound plotted versus the amount of compound injected. A linear fit for the small injection volume data is shown to indicate the curvature at large injection volumes.

Table 2. Comparison of Purities from Individual Detectors to the Average of the UV_{TWC} and ELSD Purities for Compounds Containing 20% or 40% of a Minor Component

detector	average percent purity	theoretical
UV_{214}	37.68 ± 9.45	20
UV_{TWC}	28.73 ± 10.73	20
ELSD	11.32 ± 4.62	20
Avg UV_{TWC} and ELSD	**20.06 ± 6.59**	**20**
UV_{214}	46.42 ± 10.01	40
UV_{TWC}	42.19 ± 15.50	40
ELSD	34.40 ± 10.88	40
Avg UV_{TWC} and ELSD	**38.79 ± 12.86**	**40**

3, as all 15 mixtures yield ELSD purities below the theoretical amount of 20%. The mixtures that contain 20% of the impurity compound are estimated to have 9.7 ± 4.4% impurity compound by ELSD versus 25.9 ± 10.2% impurity by UV_{TWC} and 34.4 ± 9.3% by UV_{214}. This ELSD phenomenon has been seen previously[11,30−32] and is believed to be due to the detector having a logarithmic response, meaning that a sample which contains twice as much of a compound will not show twice the response by ELSD.[1,2] The lack of variation in the ELSD purities despite the wide range of impurity compounds studied is due to ELSD signal not being dependent on the compound containing a UV chromophore. It should be noted that all compounds used here have molecular weights above 300 Da. If smaller and/or volatile compounds were used, there may be a larger disparity in the ELSD data.

Combining UV and ELSD Purities. The data from Figures 2 and 3 indicate that UV overestimates the amount of the minor component compound present whereas ELSD underestimates this. A method which combines the results from these detectors should yield results that more accurately determine the relative amount of impurity. Figure 3 shows that the average values of UV_{TWC} and ELSD purities are close to the theoretical purity. Additional comparison between the average of the UV_{TWC} and ELSD purities and the values from the individual detectors is provided in Table 2. The average of the UV_{TWC} and ELSD purities is more accurate for both the 20% and 40% purity mixtures than any individual detector.

The advantages of the proposed purity measurement method are that it is compatible with common equipments

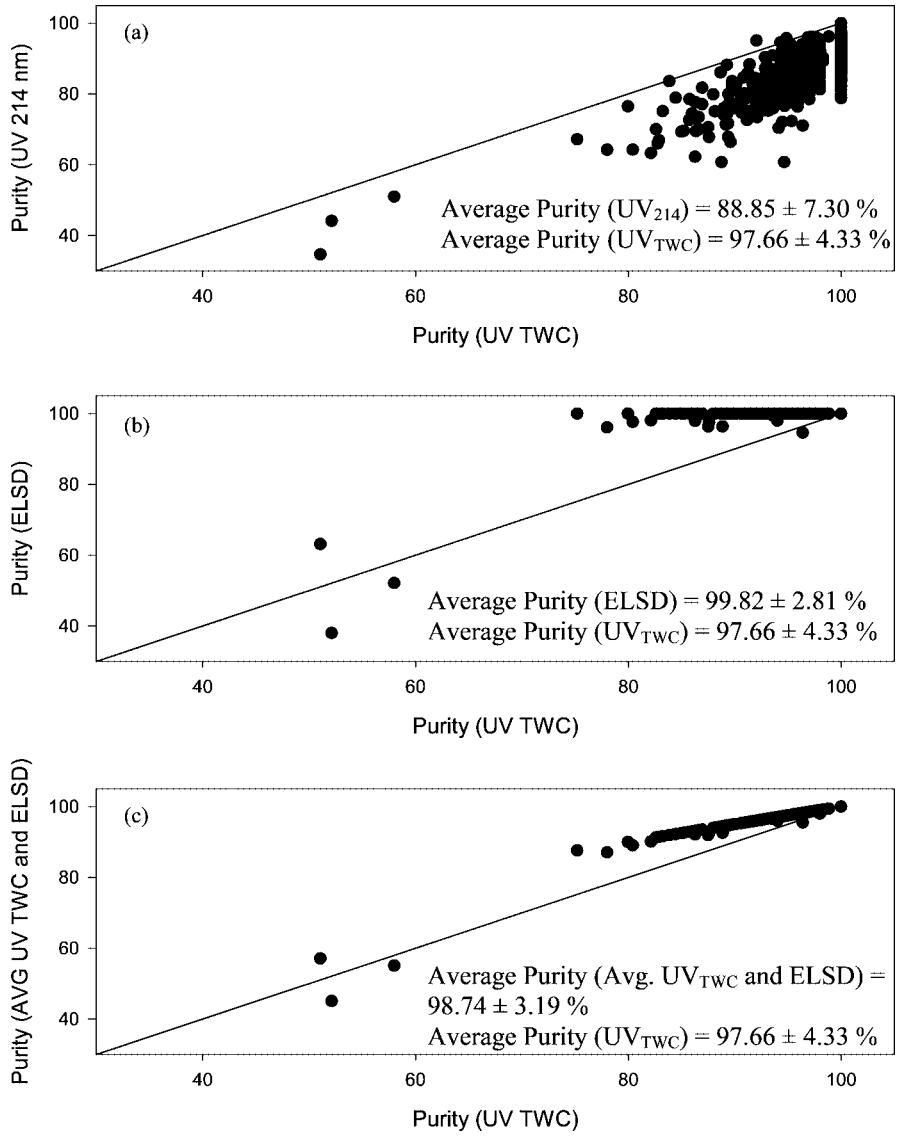

Figure 5. Purities for 959 storage compounds. (a) Purity UV_{214} vs UV_{TWC}. (b) Purity ELSD vs Purity UV_{TWC}. The solid line represents where the two purities values are equal. Compound no. 21 (top) UV_{214}. (bottom) UV_{TWC}.

in most high-throughput analysis laboratories and it could be made even faster by shortening the HPLC method, using multiple LC/UV/ELSD/MS instruments, using UPLC or by incorporating a multiplex-electrospray (MUX) source. Although this method cannot be applied to compounds that are not UV active or to volatile or semivolatile compounds, most pharmaceutically relevant compounds have molecular weights above 300 Da and are UV active.

Test Case: Compounds from Collections. A collection of 959 compounds from our compound repository was analyzed by LC/MS/UV/ELSD in order to determine the purity of the compounds. The purity determined by UV_{TWC}, UV_{214}, ELSD, and that for the average of UV_{TWC} and ELSD are shown in Figure 5. Data show that UV_{214} values are lower than UV_{TWC} measurements (below the line) and ELSD values are higher (above the line) than UV_{TWC}. The average of UV_{TWC} and ELSD is slightly above the line. These values are more accurate because it is known that UV_{TWC} is always underestimating the purity.

Trends seen in the compound collection are also similar to those seen for the standard mixtures. The purity by UV_{TWC} is greater than or equal to that by UV_{214} for 955 of 959 compounds, and the purity by ELSD is greater than or equal to that by UV_{TWC} in 956 of 959 cases. Additionally, there is less scatter in the ELSD data than either of the UV methods. The results from the standard mixtures indicate that UV underestimates purity while ELSD overestimates purity for the major component compound and that the average of the two values provides a more accurate and consistent value than either method alone. The average of the UV_{TWC} and ELSD purities for this library is $98.74 \pm 3.19\%$. This value is likely more accurate than either the UV_{TWC} purity ($97.66 \pm 4.33\%$) or the ELSD purity ($99.82 \pm 2.81\%$).

Relative purity determined by current high-throughput methods cannot reflect the true purity of compound collection and libraries. Purity measured by all single wavelength or PDA-based UV detections overestimate it while that from ELSD detection underestimate it. Our results indicate that the average of the UV_{TWC} and ELSD purities is by far the best measure for relative purity over a wide range of purities for many types of compounds. Although a further investigation of this method with a wider range of compounds is needed, this purity measure provides a more accurate measure of the relative purity of compounds from compound collections and synthetic libraries.

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Supporting Information Available. Details of the experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Keifer, P. A. *Drug Discovery Today* **1997**, *2*, 468–478.
(2) Serna, F.; Garcia, F.; de la Pena, J. L.; Calderon, V.; Garcia, J. M. *J. Polym. Res.* **2007**, *14*, 341–350.
(3) Yan, B.; Zhao, J.; Leopold, K.; Zhang, B.; Jiang, G. B. *Anal. Chem.* **2007**, *79*, 718–726.
(4) Kerns, E. H.; Di, L.; Bourassa, J.; Gross, J.; Huang, N.; Liu, H. L.; Kleintop, T.; Nogle, L.; Mallis, L.; Petucci, C.; Petusky, S.; Tischler, M.; Sabus, C.; Sarkahian, A.; Young, M.; Zhang, M. Y.; Huryn, D.; McConnell, O.; Carter, G. *Comb. Chem. High Throughput Screen.* **2005**, *8*, 459–466.
(5) Peake, D. A.; Duckworth, D. C.; Perun, T. J.; Scott, W. L.; Kulanthaivel, P.; Strege, M. A. *Comb. Chem. High Throughput Screen.* **2005**, *8*, 477–487.
(6) Corens, D.; Carpentier, M.; Schroven, M.; Meerpoel, L. *J. Chromatogr. A* **2004**, *1056*, 67–75.
(7) Yurek, D. A.; Branch, D. L.; Kuo, M. S. *J. Comb. Chem.* **2002**, *4*, 138–148.
(8) Fitch, W. L.; Szardenings, A. K.; Fujinari, E. M. *Tetrahedron Lett.* **1997**, *38*, 1689–1692.
(9) Letot, E.; Koch, G.; Falchetto, R.; Bovermann, G.; Oberer, L.; Roth, H. J. *J. Comb. Chem.* **2005**, *7*, 364–371.
(10) Yan, B.; Fang, L. L.; Irving, M.; Zhang, S.; Boldi, A. M.; Woolard, F.; Johnson, C. R.; Kshirsagar, T.; Figliozzi, G. M.; Krueger, C. A.; Collins, N. *J. Comb. Chem.* **2003**, *5*, 547–559.
(11) Fang, L. L.; Pan, J. M.; Yan, B. *Biotechnol. Bioeng.* **2000**, *71*, 162–171.
(12) Loughlin, J.; Phan, H.; Wan, M.; Guo, S.; May, K.; Lin, B. W. *Am. Lab.* **2007**, *39*, 24-+.
(13) Li, Y. F.; Wang, Y.; Qu, H. B.; Cheng, Y. Y. *Chromatographia* **2008**, *67*, 293–297.
(14) Pereira, A. S.; Schelfaut, M.; Lynen, F.; Sandra, P. *J. Chromatogr. A* **2008**, *1185*, 78–84.
(15) Lu, Y.; Yu, K.; Qu, H. B.; Cheng, Y. Y. *Chromatographia* **2007**, *65*, 19–24.
(16) Yu, Q. T.; Qi, L. W.; Li, P.; Yi, L.; Zhao, J.; Bi, Z. M. *J. Sep. Sci.* **2007**, *30*, 1292–1299.
(17) Liu, L.; Lu, Y.; Shao, Q.; Cheng, Y. Y.; Qu, H. B. *J. Sep. Sci.* **2007**, *30*, 2628–2637.
(18) Zhang, D.; Yang, L.; Yang, L.-X.; Wang, M.-Y.; Tu, Y.-Y. *Yao Xue Xue Bao* **2007**, *42*, 978–81.
(19) Zhao, J.-k.; Wang, D.-s.; Duan, S.-r.; Wangzsn, J.-x.; Bai, J.; Li, W.-l. *Nan Fang Yi Ke Da Xue Xue Bao* **2007**, *27*, 1830–3.
(20) Ferreira, I.; Eca, R.; Pinho, O.; Tavares, P.; Pereira, A.; Roque, A. C. *J. Liquid Chromatogr. Related Technol.* **2007**, *30*, 2139–2147.
(21) Nadarassan, D. K.; Chrystyn, H.; Clark, B. J.; Assi, K. H. *J. Chromatogr. B* **2007**, *850*, 31–37.
(22) Havlikova, L.; Matysova, L.; Novakova, L.; Solich, P. *J. Pharm. Biomed. Anal.* **2006**, *41*, 671–675.
(23) Platzer, D. J.; White, B. A. *J. Pharm. Biomed. Anal.* **2006**, *41*, 84–88.
(24) Dongre, V. G.; Shah, S. B.; Karmuse, P. P.; Phadke, M.; Jadhav, V. K. *J. Pharm. Biomed. Anal.* **2008**, *46*, 583–586.
(25) Shabir, G. A. *J. Liquid Chromatogr. Related Technol.* **2008**, *31*, 428–442.
(26) Webster, G. K.; Jensen, J. S.; Diaz, A. R. *J. Chromatogr. Sci.* **2004**, *42*, 484–490.
(27) Hsu, B. H.; Orton, E.; Tang, S. Y.; Carlton, R. A. *J. Chromatogr. B* **1999**, *725*, 103–112.
(28) Kibbey, C. E. *Molecul. Divers.* **1996**, *1*, 247–258.
(29) Fang, L. L.; Wan, M.; Pennacchio, M.; Pan, J. M. *J. Comb. Chem.* **2000**, *2*, 254–257.
(30) Lane, S.; Boughtflower, B.; Mutton, I.; Paterson, C.; Farrant, D.; Taylor, N.; Blaxill, Z.; Carmody, C.; Borman, P. *Anal. Chem.* **2005**, *77*, 4354–4365.
(31) Lane, S.; Boughtflower, B.; Mutton, I.; Paterson, C.; Farrant, D.; Taylor, N.; Blaxill, Z.; Carmody, C.; Borman, P. *Lc Gc Europe* **2006**, *19*, 161-+.
(32) Stolyhwo, A.; Colin, H.; Martin, M.; Guiochon, G. *J. Chromatogr.* **1984**, *288*, 253–275.

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