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RESEARCH ARTICLE

An ESI-MS Method to Determine Yield and Enantioselectivity in a Single Assay

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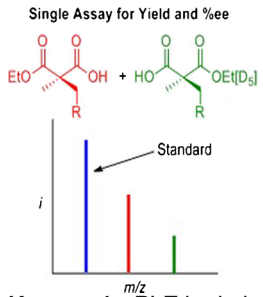

Abstract. A mass spectrometry assay is presented here that allows for the simultaneous determination of yield and enantioselectivity in a single analysis. The assay makes use of molecules that are structurally similar to the analytes of interest as standards. The assay predicts the yields of the reactions reasonably well and with little error. For example, in the pig liver esterase catalyzed hydrolysis of one prochiral malonate, the yield predicted by the assay was 72%, while larger scale isolated reaction yields were within 5% of this value. This assay provides a fast method to determine yield and enantioselectivity in one analysis. The strengths and limitations of this method are discussed.

Key words: PLE hydrolysis, ESI-MS, LCMS, Yield assay, Enantioselectivity assay

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Introduction

A wide range of screening methods have been developed in order to accurately assess the enantiomeric excess (ee) of a catalytic reaction. Common methods of measuring ee of a reaction include polarimetry and various chromatographic approaches [1]. Additionally, nuclear magnetic resonance (NMR) spectroscopy is often used to measure enantioselectivity [2–7]. Mass spectrometry has been used to determine % ee [8] and high-throughput methods have been developed using liquid chromatography mass spectrometry (LC MS) [9–15]. An alternative ESI-MS technique was developed by Masterson in which enantiomerically enriched but not enantiomerically pure probe molecules are used to assay pig liver esterase (PLE) hydrolysis reactions for enantioselectivity [16]. Each of the assays has its own advantages and disadvantages.

Although the methods discussed above provide an excellent route to determine the enantioselectivity of a reaction in a quick and efficient manner, none of the reported assays are able to provide information regarding the yield of the reaction. Many times, a reaction that provides good enantioselectivity will be performed on a larger scale and analyzed. It is has been found that although the % ee may be synthetically useful, the chemical yield may not be high enough to warrant a large scale synthesis. For example, Masterson et al. reported obtaining a half-ester from PLE hydrolysis in 90% ee with the addition of 30% *iso*-propanol co-solvent. However, when this reaction was scaled up to produce the desired product, it was isolated in <10% yield [16]. This introduces a need for quantification of both reaction yield and enantioselectivity in a quick and efficient manner.

Two of the more common quantitation methods used today are NMR and MS. Quantitation by MS is often accomplished through use of an internal standard (IS) [17]. In this method, quantitation is measured using the intensities of the analyte and standard. Typical internal standards should have a similar ionization response factor to the analyte of interest, similar chromatographic retention time, and the isotopic distribution of the two signals should not overlap [18, 19]. MS has been widely used in protein quantitation, drug and related metabolite quantitation, and various other applications [17, 20, 21]. However, measurement of reaction yield is not typically carried out using mass spectrometry. Quantitative NMR (qNMR) has most commonly been utilized to assess the purity and concentration of a particular species [22–25]. NMR quantitation can also be accomplished through the use of an external standard or through the electronic reference to access in vivo concentrations (ERETIC) method [26].

To our knowledge, there are only a few assays that can determine yield and enantioselectivity in a single analysis. Various optical strategies have been employed as a rapid and efficient method to determine both concentration and % ee in a single assay [27–35]. NMR assays have been developed to determine

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both concentration and enantioselectivity of a reaction [36]. Petucci has described a GC/MS method in which both enantioselectivity and percent conversion are determined for the hydrolysis of ethyl-2-methyl-4,4,4-trifluorobutyrate with various enzymes [37]. Here, the percent conversion is calculated using the area of the product relative to the sum of the areas of product and starting material. Although this method gives a good relative yield of the reaction, the absolute yield of the reaction is not determined. Additionally, this method does not account for the response factor of the detector, which may cause the actual yield of the reaction to be different than the measured conversion.

Although several of the above described assays allow for the simultaneous calculation of concentration (or conversion) and enantioselectivity, many of the methods described were developed for a specific type of reaction. Additionally, many of the methods report the *relative conversion* of substrate to product rather than a percent yield of the reaction. Herein we discuss an improvement to our previously reported ESI mass spectrometry method that allows for the determination of both enantioselectivity and absolute reaction yields of PLE hydrolysis reactions through a single analysis [16].

Experimental

All materials were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as received unless otherwise described below. Optima LCMS grade methanol and water were purchased from Fisher Scientific (Waltham, MA, USA). Autosampler vials and caps were purchased from VWR (Radnor, PA, USA). PLE isoenzymes were obtained from Enzymicals AG (Greifswald, Germany). Phosphate buffer (pH 7.4, 0.1 N) was prepared in the laboratory by placing 14.2 mL of phosphoric acid in a beaker with 2.0 L distilled water and adjusting the pH to 7.4 with NaOH pellets.

Mass Spectrometry (General Procedure)

Mass spectrometry was carried out using a ThermoFisher LXQ ESI-Ion trap mass spectrometer (Waltham, MA, USA) coupled to a ThermoFisher Accela HPLC system. The Accela auto sampler was set to inject 2 μL of solution onto a Hypersil Gold Reverse Phase (RP) HPLC column (50 × 2.1 mm, ThermoFisher). The solvent system was 60:40 MeOH/H_2O (v/v) at a flow rate of 100 μL/min. The solvents each contained 1% acetic acid and approximately 10 small crystals of NaCl per liter as charging agents. The mass spectrometer was set to detect in selected-ion monitoring (SIM) mode and the intensities of the ions were used to determine enantioselectivity and yield of the reactions.

Enzyme Assays (General Procedure)

A stock solution of the diester "probe" was prepared by dissolving the diester of interest in *iso*-propanol (*i*-PrOH). This solution was pipetted into a set of Eppendorf tubes such that the final amount of analyte in each tube was 1.5 mg. The *i*-PrOH was evaporated under a stream of nitrogen gas prior to the assay. Once the *i*-PrOH had been evaporated, 1 mL of pH 7.4 phosphate buffer was added to each tube. Stock solutions of the various PLE isoenzymes were prepared in 3 M $(NH_4)_2SO_4$. An aliquot of the PLE solution under study was added to each tube (0.5 units total). The Eppendorf tubes were placed into an Eppendorf Thermomixer (Hamburg, Germany) for 3 d, at 25°C and 1400 RPM mixing rate. After this time period had elapsed, a 200 μL aliquot of a stock solution of the standard was added to each tube and mixed. A 400 μL aliquot of this solution was placed in an autosampler vial and analyzed by HPLC MS as previously described. The standard solutions were added in enantiomerically enriched form; however, the % ee of the standard was not measured in the assay.

Preparation of a Standard Curve – Ionization Efficiency

For each set of product and standard, a standard curve was prepared to determine the response factor of the instrument. Stock solutions of each product were prepared by placing the desired acid-ester product in a 50 mL volumetric flask and dissolving in pH 7.4 phosphate buffer. Stock solutions of each standard were prepared by placing the standard in a 25 mL volumetric flask and dissolving in pH 7.4 phosphate buffer. The concentration of acid-ester solution **1** was 13.9 mM. The concentration of acid-ester solution **2** was 12.9 mM. The concentration of acid-ester solution **3** was 9.8 mM. The concentration of standard solution **4** was 9.9 mM. The concentration of standard solution **5** was 14.9 mM. The concentration of standard solution **6** was 9.9 mM.

A series of 10 solutions was prepared to determine the response factor of the instrument. These solutions were prepared so that the molar ratio of acid-ester products to standard in each solution ranged from 0.1 to 1.1. Tables showing the amounts of acid-ester product solution and standard solution used to prepare each solution in the series are given in the Supplemental Information as Tables 6–8 [ESM1]. The solutions were then analyzed by mass spectrometry as outlined previously. The relative intensity of the acid-ester products, referred to as $I_{products}$, and relative intensity of the standard, $I_{standard}$, were recorded. To determine the instrument response factor, $I_{products}/I_{standard}$ was plotted against the ratio of [products]/[standard]. The slope of the line represents the ionization response factor of the instrument and was taken into account when the yield calculations were performed. Each solution was prepared in triplicate and analyzed. The data was plotted as the average of the three analyses and the error bars represent the standard deviations. Complete data tables for each of the assays are provided in the Supplementary Materials as Tables 9–11.

Data Analysis

The data obtained was imported into Xcalibur software. The ion chromatogram was smoothed using the boxcar fitting, 15 points. The data was averaged and the intensities of the analyte and standard recorded. The enantioselectivity of the reactions was calculated according to Masterson [14].

The percent yield was obtained by first calculating the concentration of the products in solution, denoted by [products]. The concentration of products was calculated using the linear relationship that exists between [products]/[standard] and $I_{products}/I_{standard}$ obtained from preparation of a standard curve. This relationship is shown in Equation 1. In this case, y is the ratio of $I_{products}/I_{standard}$, m is the slope of the line obtained from the standard curve (response factor), and x is the ratio of concentrations [products]/[standard].

$$\frac{I_{products}}{I_{standard}} = m\frac{[products]}{[standard]} + b \quad (1)$$

The equation can be rearranged to determine the concentration of the product in solution shown in Equation 2.

$$[products] = \frac{[standard]\left[\left(\frac{I_{products}}{I_{standard}}\right) - b\right]}{m} \quad (2)$$

The number of moles in solution was calculated by multiplying the concentration by the total volume of solution. Finally, the yield was calculated by dividing the moles in solution by the number of starting moles and multiplying by 100 to obtain the % yield, shown in Equation 3.

$$\%\,Yield = \frac{moles\,in\,solution}{initial\,moles} * 100\% \quad (3)$$

Synthesis of Probes and Standard Molecules

Synthetic procedures for preparation of ethyl diesters, half esters, and probes were very similar to those described in literature. The preparation of the methyl diesters, half esters, and probes were prepared according to modified literature procedures, using dimethyl methyl malonate in place of diethyl methyl malonate [16, 38–40]. Full synthetic detail is provided in the Supporting Information [ESM1].

Applicability of the Assay

Several larger scale reactions were performed to determine if this small scale assay could predict isolated yields for reactions on a larger scale. Each reaction was performed in triplicate. For the reaction, 100 mg of the analyte was placed into a beaker containing 100 mL of pH 7.4 phosphate buffer. The reaction was monitored by a radiometer analytical automatic titrator. The titrator was set to maintain the pH at a constant 7.4, and add 1.0 M NaOH as the reaction progressed. The reaction was deemed to be complete once one equivalent of 1.0 M NaOH had been added to the solution. The solution was worked up according to standard procedure as described below, and the percent yield determined by gravimetric analysis.

Results and Discussion

Choice of Standard

The analytes of interest, shown in Figure 1 as **1-3**, are all products of PLE hydrolysis reactions. The standards, molecules **4-6**, were all chosen because of structural similarity to the analytes. Each of the standards differs from the corresponding analyte by one methylene unit, corresponding to a difference of 14 mass units. Chromatographic separation between the analyte and standard is not necessary because the MS is able to differentiate between the two masses. Each set of analyte and standard should have similar ionization properties because of structural similarities.

Ionization Efficiency

A set of standard curves was prepared as previously described using the chosen acid-ester product and its corresponding standard. For product **1,** there was a linear correlation (R^2 = 0.9936) between the concentration and intensity, and the slope of the standard curve was calculated to be 1.7. For product **2**, a linear correlation between concentration and intensity was also observed (R^2 = 0.9968) with a slope of 1.6. The standard curves for these acid-ester products are shown in Figure 2.

For the third series, the methyl acid ester (**3**) was the acid-ester product chosen and the ethyl acid-ester was used as the standard (**6**). After preparing a standard curve, a linear correlation was observed between the concentration and the intensity (R^2 = 0.9896) with a slope of 0.8. A slope of less than 1.0 was observed because of the lighter species, in this case the methyl acid-ester products, being in the numerator. This indicates that the methyl acid-ester products do not ionize as efficiently as the ethyl acid-ester standard.

In all three cases, the ethyl acid-ester ionized more efficiently compared with the methyl acid-ester, as indicated by the slope of the line of the standard curve. A value closer to 1.0 for the slope would indicate that both the standard and acid-ester products ionize with the same efficiency. However, the

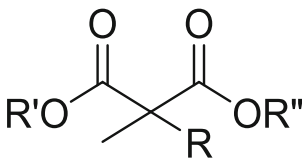

Figure 1. General structure for the half-ester products, standards, and probes. Half-ester products **1** R′ = Et, R = –CH_2NPhth, R″ = H; **2** R′ = Et, R = –$(CH_2)_3$NPhth, R″ = H; **3** R′ = Me, R = –CH_2Ph, R″ = H; Standards **4** R′ = Me, R = –CH_2NPhth, R″ = H; **5** R′ = Me, R = –$(CH_2)_3$NPhth, R″ = H; **6** R′ = Et, R = –CH_2Ph, R″ = H. Probes **7** R′ = Et, R = –CH_2NPhth, R″ = Et[D_5]; **8** R′ = Et, R = –$(CH_2)_3$NPhth, R″ = Et[D_5]; **9** R′ = Me, R = –CH_2Ph, R″ = Me[D_3]

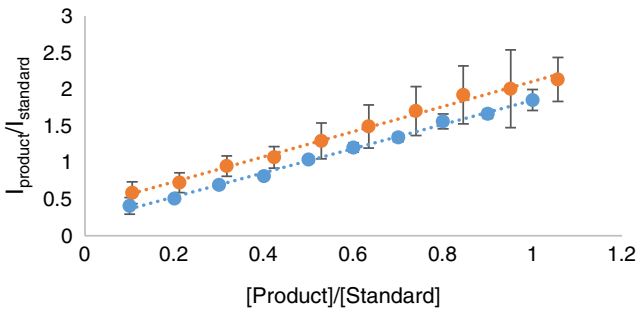

Figure 2. Standard curve for acid-ester products **1**/standard **4** is depicted by the orange line and has a slope of 1.7 and an R^2 value of 0.9936. Standard curve for acid-ester products **2**/standard **5** is depicted by the blue line and has a slope of 1.6 and an R^2 value of 0.9968

variation in ionization efficiency can be taken into account when calculating the yield values from the assay. Other groups have previously reported that the ionization efficiency was improved when the chain length of an alkyl substituent increased [41, 42]. Yang et al. reported that the ionization efficiency increased upon lengthening of the alkyl chain in the derivatization of amino acids. Yang proposed that by lengthening the alkyl chain, the hydrophobic nature of the molecule was increased leading to a higher concentration of the derivatized amino acid at the droplet surface in the ESI-MS causing the higher ionization efficiency of the molecules containing longer alkyl chains [42]. Our results are in agreement with those of Yang.

Results of Mass Spectrometry Assay

Yield studies were first conducted with probe **7**. Previously, co-solvent studies were conducted with this substrate and PLE isoenzymes [38]. However, using the previous assay, only enantioselectivity data was obtained and data regarding yield of the reactions was not available.

All ions were detected as sodium adducts [M + 23]. The ratio of relative intensities of ***(R)*-1** to ***(S)*-1** were used to calculate the enantioselectivity of the reactions, based on our previously reported assay [16]. In this case, the % ee was determined to be –30%, indicating the *(S)* enantiomer was predominantly formed. The ratio of the sum of ***(R)*-1** and ***(S)*-1** to **4** was used to calculate the total concentration of **1** in the solution. In the example in Figure 3, the yield was determined to be approximately 60% using the relative intensities of the acid-ester products and standard. Figure 3 displays the total ion chromatogram (TIC) and reconstructed ion chromatograms (RIC) for each of the detected ions. Interestingly, the retention time of **1** (2.19 minutes) was slightly longer than the retention time of **4** (1.81 min).

The concentration of each acid-ester product was converted into a percent yield for each reaction. The percent yields and enantioselectivities for the hydrolysis reactions of probe **7** are

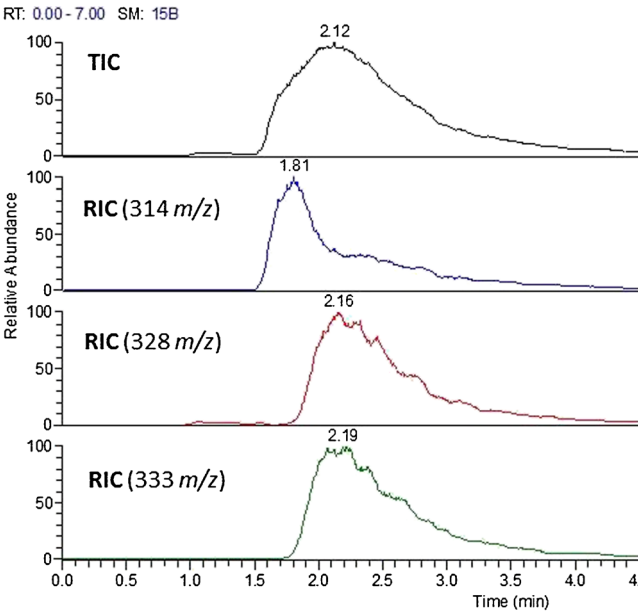

Figure 3. Total ion chromatogram (TIC) and reconstructed ion chromatogram (RIC) for each of the ions

shown in Table 1. Each yield and % ee is the average of three reactions (SD = standard deviation). With the exception of PLE 1, all of the enzymes gave acceptable yields for the hydrolysis reaction (>50%). Crude PLE produced the highest yield, with a ~72% yield, and PLE 1 produced a disappointingly low yield of approximately 18%. The other isoenzymes (PLE 2-6) all produced similar yields of approximately 60%. The enantioselectivity for these hydrolysis reactions is consistent with previously reported data [38]. Crude PLE, PLE 1, and PLE 2 all produced predominantly the *(R)*-enantiomer, whereas PLE 3, PLE 4, PLE 5, and PLE 6 all produced predominantly the *(S)*-enantiomer. Interestingly, PLE 1 provided high enantioselectivity (90% ee, *R*-enantiomer) but disappointingly low yield (~18% yield). Additionally, it is interesting to note that crude PLE, which gave the highest yield (~72%), only provided the *(R)*-enantiomer in 23% ee. Crude PLE is known to contain various ratios of PLE 1–6 so this may account for the low enantioselectivity in the hydrolysis of probe **7** with crude PLE [43].

It has been previously reported that the number of methylene units in the side chain alters the enantioselectivity of the PLE hydrolysis reaction [39]. For example, Banerjee et al. reported that the enantioselectivity of the hydrolysis reaction was improved as the number of methylene units in the side chain increased from one to three in the crude PLE hydrolysis of several prochiral malonic diesters. However, increasing the number of methylene units above three caused a decrease in the enantioselectivity [39]. In the previous study, only crude PLE was used. Probe **8** was selected for the next assay to determine if the isoenzymes would produce high yield in addition to producing high enantioselectivity. A similar assay to the one previously described was performed with probe **8**. Once the reactions were complete, an aliquot of the standard solution **5** was added to each reaction.

Table 1. Percent Yield and Enantioselectivity Data for the Hydrolysis of Probes **7**, **8**, and **9**

	Probe **7**				Probe **8**				Probe **9**			
	% Yield		% ee		% Yield		% ee		% Yield		% ee	
	% Yield	SD	% ee	SD	% Yield	SD	% ee	SD	% Yield	SD	% ee	SD
Crude PLE	71.7	6.0	23.7	0.3	30.5	14.8	98.0	0.7	71.0	11.0	-39.7	6.4
PLE 1	17.9	0.3	90.0	5.2	2.6	0.4	93.4	1.4	11.0	1.8	46.9	12.2
PLE 2	63.5	4.4	69.1	0.9	62.3	4.6	90.5	0.7	13.0	3.4	58.1	6.3
PLE 3	59.6	7.5	–30.2	0.7	49.6	1.6	97.3	0.1	82.0	5.0	21.6	0.3
PLE 4	61.6	6.3	–48.0	0.7	50.3	2.0	93.8	0.5	26.0	9.7	36.1	2.0
PLE 5	61.5	3.3	–53.0	0.8	53.2	0.6	94.7	0.6	80.0	3.3	–58.9	0.7
PLE 6	59.7	1.9	–42.1	1.1	47.1	1.2	98.1	0.3	67.0	9.4	–45.3	0.8

All values are the average of three replicates.
Positive % ee denotes predominantly *(R)* enantiomer. Negative % ee denotes predominantly *(S)* enantiomer.

Table 1 shows the calculated yields and enantioselectivity for the hydrolysis reactions of probe **8**. PLE 2 displayed the highest yield of approximately 62%. Isoenzymes 3–6 all displayed very similar yields of approximately 50%. Crude PLE and PLE 1 gave very low yields, with only 30% and <3% yield, respectively. The enantioselectivities of the hydrolysis reactions with **8** were also determined, as displayed in Table 1**.** Surprisingly, all of the isoenzymes produced the *(R)*-enantiomer as the predominant product. Even more interesting, each enzyme gave greater than 90% enantioselectivity for the hydrolysis of probe **8**. This high enantioselectivity may be accounted for by looking at the active site model for pig liver esterase of Jones et al. [44]. Banerjee et al. have reported a similar trend in enantioselectivites when the number of methylene units were increased [39]. They have proposed that because of the longer side chain length, the molecule is better able to fit into the hydrophobic large pocket of the enzyme and thereby increases the enantioselectivity of the reaction.

Lastly, the assay was performed with probe **9.** In this assay, the ethyl acid-ester molecule (**6**) was used as the standard whereas the methyl acid-ester (**3**) was the analyte. Additionally, from previous work, the diethyl ester provided moderate enantioselectivities with some of the isoenzymes, and we were interested to see how the methyl diester compared [38]. The yields and enantioselectivities of the hydrolysis reactions of **9** are displayed in Table 1. PLE 3 and PLE 5 provided the highest yields of approximately 80%. Crude PLE and isoenzyme 6 provided approximately 70% yield. Isoenzymes 1 and 2 gave very poor yields, with only approximately 10% yield. It is clear that a range of yields are observed for the hydrolysis reactions with the various isoenzymes and probe **9**, ranging from approximately 80% yield with PLE 3 and 5 to approximately 10% with PLE 1 and PLE 2. Furthermore, the data are consistent within each set with only small standard deviations for the three reactions. These data indicate that the assay is able to predict the yields of reactions with low yield as well as those with high yield. Crude PLE, PLE 5, and PLE 6 all gave the *(S)*-enantiomer predominantly, whereas PLE 1, PLE 2, PLE 3, and PLE 4 gave the *(R)*-enantiomer predominantly. The highest enantioselectivity observed was 50% ee for PLE 2 and PLE 5. Several of the other enzymes, such as crude PLE, PLE 1, and PLE 6, showed enantioselectivities slightly lower than 50%. PLE 3 and PLE 4 provided poor enantioselectivity. However, it is known that the diethyl ester provides poor enantioselectivity upon hydrolysis with crude PLE [38].

The yields for three separate hydrolysis reactions were determined through the use of a standard that was structurally similar to our analyte of interest. In all three cases, PLE 1 provided very poor yield (<20%). However, the assay was able to predict this small yield with very low SD. Likewise, the yields of the other reactions were determined with low SD.

Ability of the Assay to Accurately Predict Reaction Yields

As illustrated above, the yields and enantioselectivities for three different hydrolysis reactions were determined using the mass spectrometry assay. Reactions were performed to determine if the assay would accurately predict the isolated yields of hydrolysis reactions performed on a preparative scale. Each hydrolysis reaction was performed using 100 mg of the starting probe in pH 7.4 phosphate buffer and crude PLE. After the reaction was complete, the product was extracted from the buffer and worked up according to typical procedures. The yield was determined by gravimetric analysis. Each hydrolysis was performed in triplicate and the average isolated yield and standard deviation are displayed in Table 2. The yields obtained from the assay are shown for comparison.

In the hydrolysis of **7** with crude PLE, the isolated yield was determined to be 66%. The assay predicted the yield of the reaction to be approximately 72%. While the

Table 2. Comparison of Isolated Yields and Assay Yields for Hydrolysis with Crude PLE

Substrate	Assay yield		Isolated yield		% Error
	% Yield	SD	% Yield	SD	
7	71.7	6.0	66.4	15.7	7.4
8	30.5	14.8	28.5	8.1	6.6
9	71.0	11.0	80.7	5.0	13.6

The yields are the average of three reactions. The % error is the error between the isolated yield and assay yield. The average % error for all the reactions is 9.2%.

isolated yield is slightly lower, the assay gave a good approximation of the yield. It is important to note that the products of the larger scale reactions were extracted, filtered, and purified before the yield of the reaction was determined. The assay determined the yield without performing any of these manipulations. The isolated yield may be lower than the predicted assay yield because of the addition of these steps.

The isolated yield was determined to be approximately 29% for the hydrolysis of **8** with crude PLE. This is in good agreement with the assay prediction of 30% yield. This yield is significantly lower than the yield obtained from hydrolysis of **7**. The assay is able to distinguish high yields from low yields and gives reasonable approximations of the isolated yields of the larger scale reactions.

In the hydrolysis of **9** with crude PLE, the assay predicted the yield to be approximately 71%. The average isolated yield was determined to be 86%. This is a higher yield than predicted by the assay; however, it is within an acceptable range.

The assay is able to provide an approximation of the isolated yield for the larger reactions. Although the isolated yields were found to vary slightly from the yields determined by the assay, they were all within the experimental error of the assay. For all substrates, the predicted yields from the assay are all within reasonable agreement with the isolated yields determined from the larger scale reactions.

Conclusions

This assay allows for the simultaneous determination of yield and enantioselectivity, eliminating the need for scale up reactions to determine the reaction yield. This method will be a useful tool to screen reaction conditions for combinations that provide both high enantioselectivity and isolated yield. Only reactions that provide high enantioselectivity and yield would be performed on a preparative scale, saving time and materials. Although only the PLE hydrolysis reaction was studied here, this method can be extended to screen the outcomes of other reaction types. Typical analysis times were 7 min per sample including time for column flushing and equilibration. Under these conditions, the analysis of up to 204 samples could be completed in a 24-h period through the use of an autosampler.

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