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Monitoring Enzymatic Reactions by LC/Single Quad to Gain Insights on Reaction Mechanisms

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Introduction

Reaction monitoring is important during a synthesis process to identify intermediates and products, as well as track the completion of a reaction. Obtaining fast and reliable results is paramount for making quick decisions. Typically, reactions are monitored using an LC method, but a single quadrupole (SQ) mass spectrometer can be added to increase productivity, sensitivity and selectivity. With the addition of a single quadrupole, co-eluting compounds can be detected separately, and ionizable compounds that do not contain a chromophore or absorb poorly can be detected. With a single quadrupole, compounds that may not have standards, such as reaction intermediates, can be identified. For this study, an enantioselective enzymatic hydrolysis of butyric ester derivatives was monitored using an LC/MSD iQ and a Diode Array Detector.

A 13-minute LC/MS method was developed with UV and mass detection. Several ions, based on the expected products, were monitored on the SQ using selected-ion-monitoring (SIM) and a scan between 100-500 *m/z* was collected in parallel to detect any reaction intermediates to gain insights on reaction mechanisms.

Figure 1. Agilent LC/MSD iQ coupled to a 1290 Infinity II LC System

Experimental

Instrumentation

- 1290 Infinity II Binary Pump (G7120A)
- 1290 Infinity II Vialsampler (G7129B)
- 1290 Infinity II MCT (G7116B)
- 1290 Infinity II DAD (G7117B)
- LC/MSD iQ (G6160A)

Data acquisition and analysis was performed using Agilent's OpenLab CDS 2.4 Software. OpenLab CDS provides full compliance features that support data integrity with US FDA 21 CFR Part 11, EU Annex 11, and other similar regulations.

LC Method		
Column	Poroshell 120 EC-18 2.1x100 mm, 1.9 µm at 40°C	
Flow rate	0.500 mL/min	
Solvent A	0.1% Formic Acid in H_2O	
Solvent B	0.1% Formic Acid in ACN	
Gradient	Time	%B
	0.0 10.0 11.0 11.2 13.2	5 90 90 5 5 (post time)
UV Signal	210, 254, 275 nm	
Inj. Vol.	1 µL	

Table 1. 1290 Infinity II LC Method

MS Parameters		
Scan (200 ms)	100-500 *m/z*	
SIM (15 ms/ion)	177 *m/z* 194 *m/z* 199 *m/z*	247 *m/z* 264 *m/z* 269 *m/z* 159 *m/z*
Fragmentor	70 V	
Gas Temperature	325 °C	
Gas Flow	11 L/min	
Nebulizer Pressure	35 psi	
Capillary Voltage	4500 V	

Table 2. LC/MSD iQ Mixed Scan/SIM Mode Method

Enantioselective Enzymatic Hydrolysis

The reaction monitored was an enantioselective enzymatic hydrolysis as shown in Figure 2. The starting reactants are a racemic mixture from a previous step in a larger reaction. Two different enzymes (L3 and E2) were selected and reactions were monitored across several days after the start of the reaction. Aliquots at different time points were taken directly from the reaction vessel and passed through a C18-SPE cartridge with cold ether, effectively trapping the enzyme and stopping the reaction.

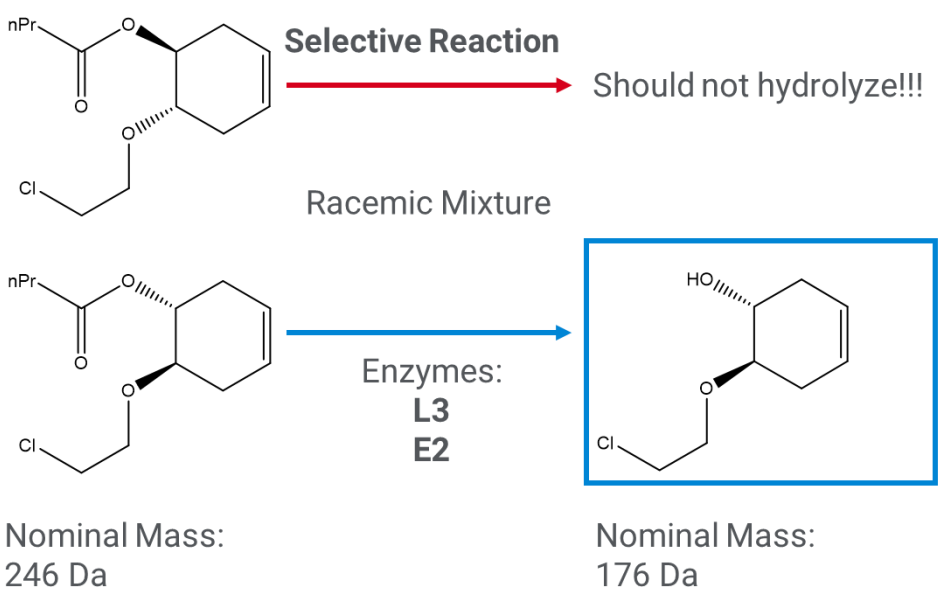

Figure 2. Enantioselective enzymatic hydrolysis reaction carried out in this study

No Compounds Detected in the UV

The reaction compounds most likely do not contain a chromophore, necessitating the need for mass detection. Figure 3 shows a UV isoabsorbance plot of the reaction at 115.5 hours. No compounds were detected across the entire UV range. The wide band is from the absorption of the organic solvent, acetonitrile. Figure 4 shows MS scan and SIM TIC chromatograms of the same sample where both the reactant and product are clearly detected, along with a number of byproducts.

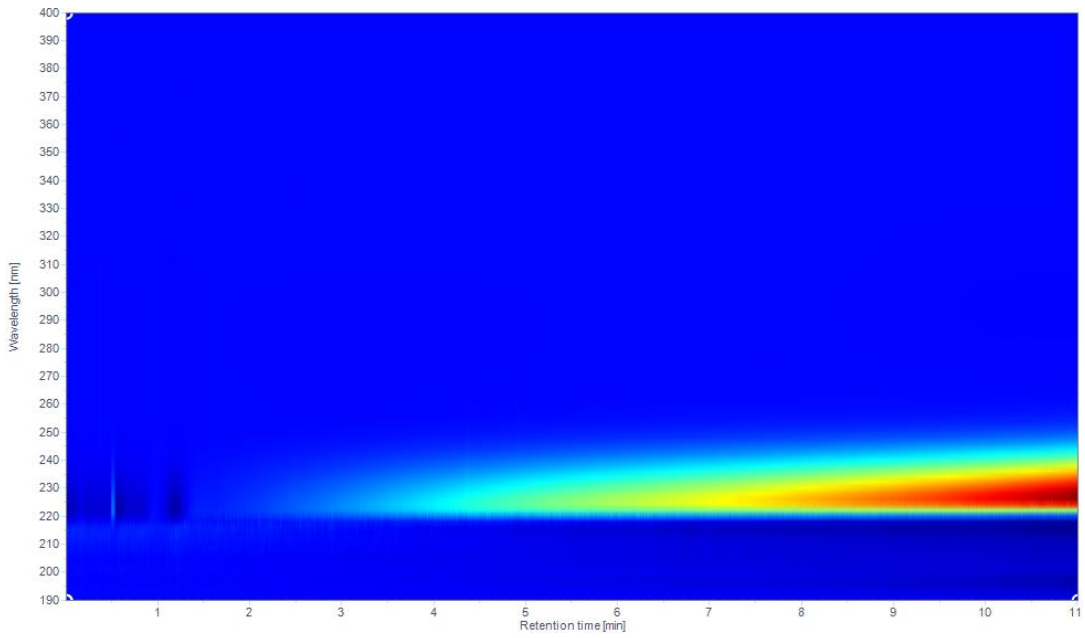

Figure 3. UV isoabsorbance plot of the reaction vessel contents after 115.5 hours

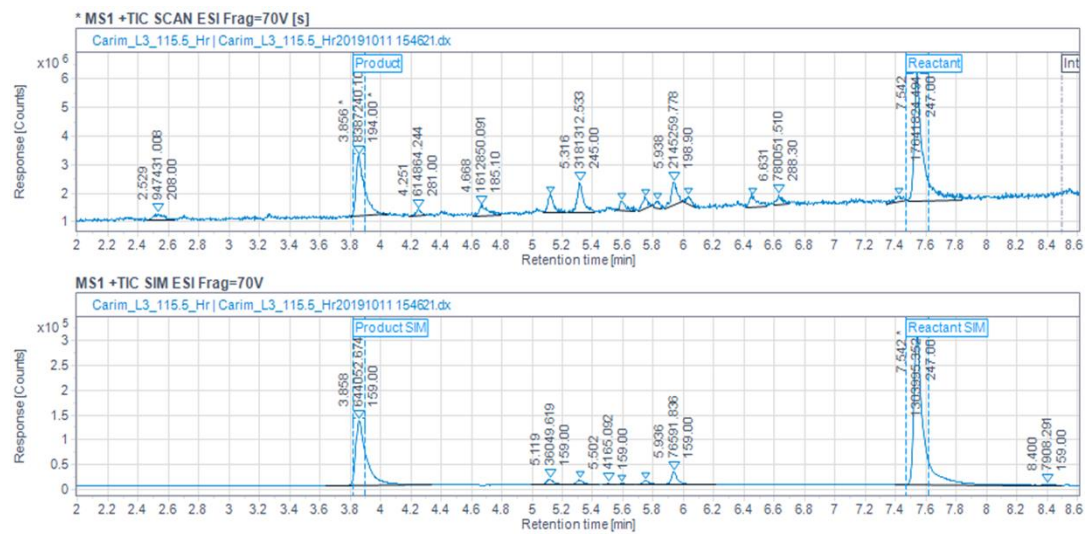

Figure 4. MS scan and SIM TIC chromatograms of the reaction vessel contents after 115.5 hours.

The reactant and product peaks were also monitored in SIM mode with an additional ion at *m/z* 159 which corresponds to a water loss from the product. Several peaks were detected between the product and reactant that contained *m/z* 159, indicating that they are coming from the reaction; possibly as intermediates to the product.

Mass Detection Can Lead to Insights in Reaction Mechanisms

Figure 5 shows MS spectra of the product and reactant peak before and after the addition of NH_4F to the mobile phase. The reactant was detected at *m/z* 247 $[M+H]^+$ and 269 $[M+Na]^+$ while the product was detected at *m/z* 194 $[M+NH_4]^+$ and 199 $[M+Na]^+$. A fragment ion was detected at *m/z* 159 with the product and is formed by a water loss from the unstable $[M+H]^+$ ion. This was confirmed by adding 0.5 mM of NH_4F to the aqueous phase which shows only the $[M+NH_4]^+$ ion, thus stabilizing the product.

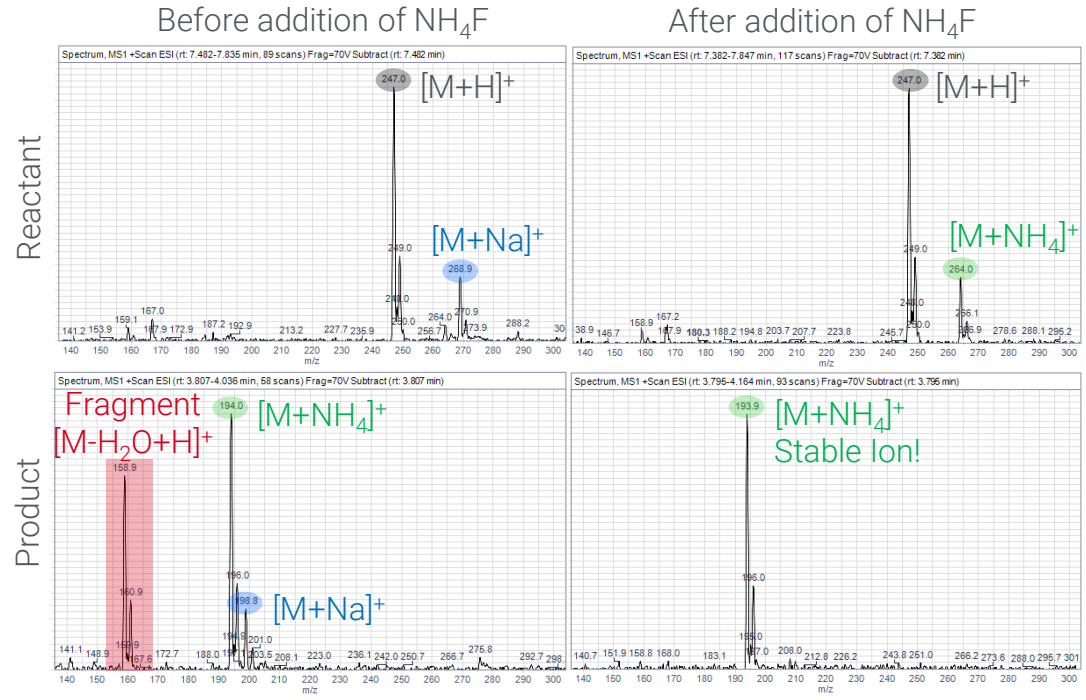

Figure 5. MS Spectra of the reactant peak (7.542 min) and product peak (3.856 min), before (left side) and after (right side) the addition of 0.5 mM NH_4F to the aqueous solvent

Results and Discussion

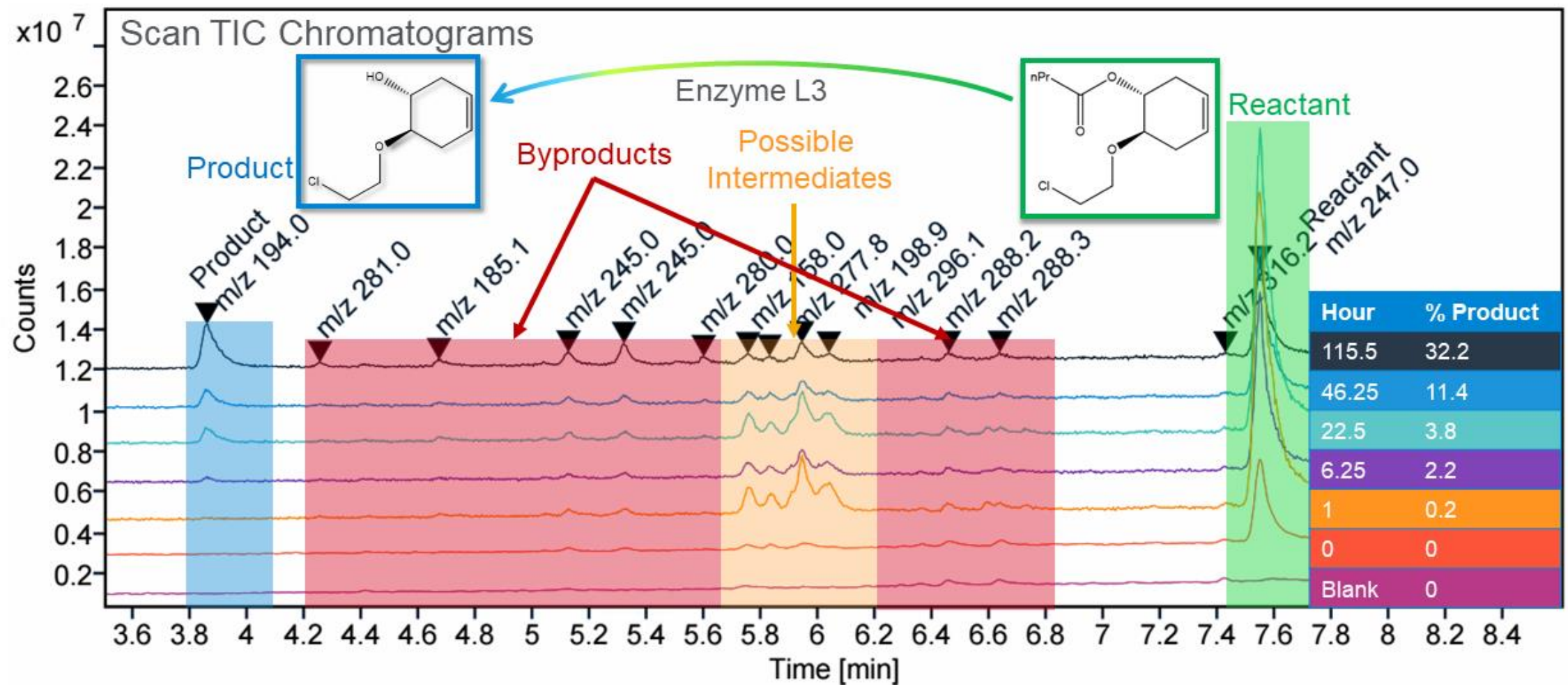

Figure 6. Stacked scan TIC chromatograms of aliquots from the reaction vessel for enzyme L3 during the course of the reaction. Highlighted regions indicate peaks corresponding to: product in blue, byproducts in red, possible intermediates in orange, and reactant in green. A table color coding the TIC to time points of the reaction is inlayed on the right.

Identifying Byproducts and Intermediates from Scan MS Chromatograms

Scan TIC chromatograms at various time points during the reaction with enzyme L3 can be seen in Figure 6. Before 6.25 hours, no product was detected but several peaks appeared between 5.7 and 6.2 minutes. These peaks were classified as intermediates because their abundance begins to decrease and fluctuate with the detection of the product and they all contain an ion at *m/z* 159. Several other peaks whose abundance increased overtime with the product were classified as byproducts.

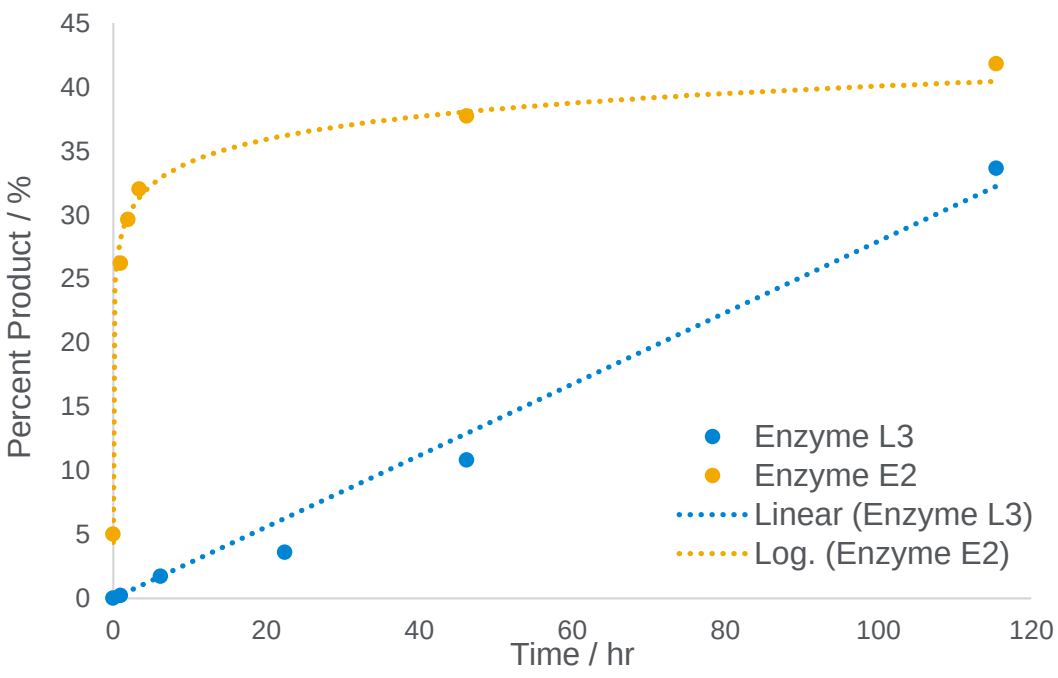

Figure 7. Percent product during reaction for enzymes L3 (blue) and E2 (orange)

Reaction Rates lead to Quick Decisions

A plot of percent product as a function of time for each enzyme can be seen in Figure 7. The percent product should only reach 50% due to the enantioselective nature of the reaction. Within 24 hours it can be seen that the E2 enzyme is much faster than L3 with a relatively logarithmic reaction rate versus linear, respectively.

Conclusions

- An enantioselective enzymatic hydrolysis reaction was monitored for two different enzymes, L3 and E2
- No compounds were detected in the UV signal, necessitating the need for mass detection
- The LC/MSD iQ detected compounds using a mixed mode scan/SIM method
- Products and reactants were detected along with several unknown byproducts and intermediates
- The L3 enzyme produced products much faster than the E2 enzyme

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