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An Improved Spectrophotometric Method for Toluene-4-Monooxygenase Activity

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Abstract: Monooxygenases, an important class of enzymes, have been the subject of enzyme engineering due to their high activity and versatile substrate scope. Reactions performed by these biocatalysts have long been monitored by a colorimetric method involving the coupling of a dye precursor to naphthalene hydroxylation products generated by the enzyme. Despite the popularity of this method, we found the dye product to be unstable, preventing quantitative readout. By incorporating an extraction step to solubilize the dye produced, we have improved this assay to the point where quantitation of enzyme activity is possible. Further, by incorporating spectral deconvolution, we have, for the first time, enabled independent quantification of the two possible regioisomeric products: 1-naphthol and 2-naphthol. Previously, such analysis was only possible with chromatographic separation, increasing the cost and complexity of analysis. The efficacy of our improved workflow was evaluated by monitoring the activity of a toluene-4-monooxygenase enzyme from *Pseudomonas mendocina* KR-1. Our colorimetric regioisomer quantification was found to be consistent with chromatographic analysis by HPLC. The development and validation of a quantitative colorimetric assay for monooxygenase activity that enables regioisomeric distinction and quantification represents a significant advance in analytical methods to monitor enzyme activity. By maintaining facile, low-cost, high-throughput readout while incorporating quantification, this assay represents an important alternative to more expensive chromatographic quantification techniques.

Introduction

Enzyme engineering has enabled the generation of biocatalysts capable of driving conversions that are challenging with conventional, small-molecule catalysts.[1] In particular, monooxygenases (MOs) have been extensively developed for biotechnology because of their ubiquity and broad reactivity.[2] In nature, these enzymes perform diverse oxygen insertion reactions. These range from methane to methanol conversions (methane monooxygenases (MMOs)) in bacteria to drug metabolism in humans (cytochrome P450).[2–4] Because of the diversity of substrates that these enzymes react with and the ability to insert an oxygen atom into an inert C–H bond, MOs have been the subject of extensive engineering, expanding their substrate scope to include conversions that remain difficult for synthetic chemists such as epoxidation.[5,6] Though improvements to MO stability,[7] regiospecificity,[8,9] and enantioselectivity[10] are critical for their industrial applications, the enzyme engineering process can generate highly active mutants that drive undesired side reactions. A key example is MO regiospecificity; these enzymes can insert oxygen-containing moieties at multiple substrate sites.[11] The lack of insertion site specificity is especially problematic for aromatic substrates, which can yield between 2–6 regioisomers for a substrate like naphthalene, reducing the yield of the desired product and increasing the complexity of subsequent separations.[11,12] Thus, effective tools to identify optimized mutants must provide more information than simply total converted product, namely quantification of individual regioisomers.[13,14]

The gold standard for such analysis is gas or liquid chromatography to separate products combined with identification by mass spectrometry and/or quantification by UV–visible absorbance.[1,15] These methods are accurate, quantitative, and capable of multi-analyte detection and quantification. However, they necessitate costly instrumentation and extensive sample preparation, limiting their broad utility. Instead, many have turned to simple colorimetric assays to monitor enzyme activity.[16–21] Colorimetric assays provide a rapid, simple, inexpensive method to monitor activity. For qualitative analyses, visual inspection is often sufficient. Thus, in many cases, *no* equipment is required. If quantitative measurements are needed, small, portable, inexpensive instruments are available.[22–24] Additionally, these assays generally require minimal training and are easy to perform, making them broadly available, even to those without scientific training. The main downsides to these methods are minimal, including the need for larger sample volumes than are required for chromatog-

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raphy and the potential for interference from the sample matrix.

Due to these key advantages, several colorimetric techniques have been developed to monitor MO activity. Most have been used for decades, including a prevalent assay involving MO hydroxylation of naphthalene. This assay yields colorless naphthol products.[16] These products are then reacted with a diazonium dye precursor (tetrazotized *o*-dianisidine) to produce an intense purple azo dye (Figure 1a).[16] This chemistry has been an essential screening tool for MO activity.[25–27] However, this assay is rarely used quantitatively because of its sensitivity to timing and sample matrix.[26] Further, current readout methods cannot quantify individual naphthol regioisomers, preventing the evaluation of the enzyme regiospecificity without additional chromatographic analysis (Figure 1b).[16,26]

Despite the popularity of this assay to monitor protein activity, challenges are often described in the literature. In addition to these reported limitations with this assay, we found that the dye product of this reaction is insoluble in aqueous matrices, making activity detection difficult and naphthol quantification impossible. Because of the utility and broad applications of this assay, we sought to improve it to enable quantitative and consistent results. Our strategy centered on improving the stability of the dye product. Beyond the direct applications to the assay described here, working to stabilize the products of colorimetric assays will generally improve workflows in the chemical and biochemical community. Such improvements will also circumvent costly and time-consuming analytical measurements, an improvement that will benefit the entire chemical field. As the fields of enzyme discovery and engineering continue to grow, so does the need for assays that balance speed and simplicity with data quality. Here, we describe key improvements to the workflow of this assay to monitor naphthol production as well as incorporate deconvolution of the absorbance spectra to not only quantify the total naphthol produced but also the amount of each regioisomer generated.

Results and Discussion

The development of an improved method for naphthol quantification was motivated by challenges encountered with reproducing reported results from published protocols.[16,26,28] Specifically, precipitate formation was observed following azo coupling, making accurate quantification of the dye product by UV–Vis spectroscopy impossible. Thus, initially, efforts were focused on solubilizing and stabilizing the dye product (Figure 1b(*ii*)). As the dye was not soluble in aqueous solutions, the incorporation of an extraction into an organic solvent was investigated, and an ethyl acetate extraction step was found to be critical to maintain a soluble, stable dye. Upon spectroscopic analysis of the ethyl acetate-extracted dye, an unexpected advantage of this strategy was discovered; distinct spectral features were observed for 1-naphthol and 2-naphthol products, both of which are formed from MO-catalyzed hydroxylation of naphthalene. These features were sufficiently distinct that each regioisomer could be independently quantified from composite spectra containing both naphthol dye products. This innovation not only addresses the traditional instability of the assay but also demonstrates the powerful insights offered by properly optimized colorimetric methods.

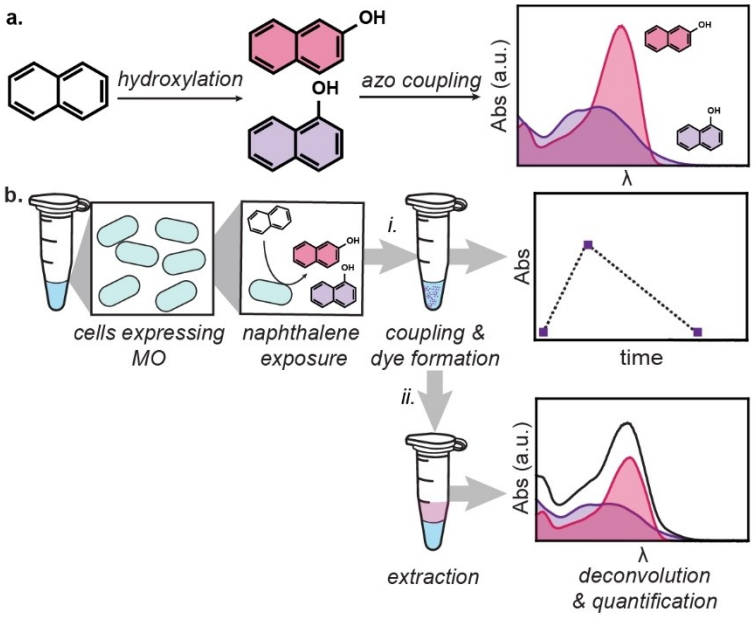

Figure 1. Workflow for the colorimetric quantification of naphthols. **a**. Naphthalene is converted to naphthol by many monooxygenases (MOs); naphthols can then react with diazonium salts via aromatic substitution to yield brightly colored dyes with distinct spectra. **b**. Original workflow in which tetrazotized *o*-dianisidine is directly added to the aqueous medium containing naphthol prior to measurement (*i*). In the optimized workflow reported here (*ii*), the dye is extracted into ethyl acetate for improved stability. Deconvolution of UV–Vis spectra can then be used to quantify regioisomers.

Challenges with the Existing Protocol: Aggregation and Insolubility

The prevalent colorimetric method to monitor the activity of enzymes such as MOs is based on a protocol first reported nearly four decades ago involving the conversion of a non-native substrate (naphthalene) to naphthol.[16] Both the 1-naphthol and 2-naphthol regioisomers can be generated with this reaction (Figure 1a).[11] Because both naphthalene and naphthol are colorless, subsequent conversion of the colorless products to highly-colored dyes through a reaction with a dye precursor enables colorimetric determination of enzymatic activity.[16] Specifically, the naphthol enzymatic product undergoes an electrophilic aromatic substitution with tetrazotized *o*-dianisidine to yield a colored product that is readily detected by UV–Vis spectroscopy.[16,29] In the standard workflow, this product must be measured within two minutes of the dye formation before the dye begins to degrade and precipitate (Figure 1b).[16,26,30] In the literature, the 1-naphthol product is reported to form significantly faster than the 2-naphthol, with the workflow therefore purported to be specific for the 1-naphthol product.[16] If a single product is formed, it can be quantified based on a reported extinction coefficient with Beer's law.[16,27,30] Thus, in theory, this method enables easy, rapid quantitation of MO enzymatic activity, but in practice, we observed significant inconsistencies and irreproducibility based on this workflow.

Azo dye formation was initially tested using 1- and 2-naphthol standards. Surprisingly, both reactions rapidly yielded dye, contradicting conventional knowledge about the regioselectivity of the colorimetric measurement.[16] A more immediate concern, though, was the observation of precipitate formation following dye precursor coupling for both naphthols. After centrifugation and collection of the precipitate, nearly all characteristic absorption features of the dyes were eliminated (Figure 2a, b). Further, the minimal remaining signal observed between 300–500 nm is also present in samples containing only tetrazotized *o*-dianisidine (Figure S1). The dye precursor reactivity with both naphthol regioisomers combined with the insolubility of the product rendered the assay non-quantitative. Additionally, the short window recommended for measurement prevents high-throughput monitoring with the assay. Thus, we first aimed to address precipitation as the cause of the signal degradation. Then, we address the unique reactivity of each regioisomer.

Improving Upon the Existing Assay

Despite the initial challenges, the fundamental azo-coupling chemistry is robust and easy to perform.[31,32] Therefore, the coupling reaction was preserved and efforts were centered on improving the post-coupling steps. The apparent instability of the azo dye products was initially addressed. As visible

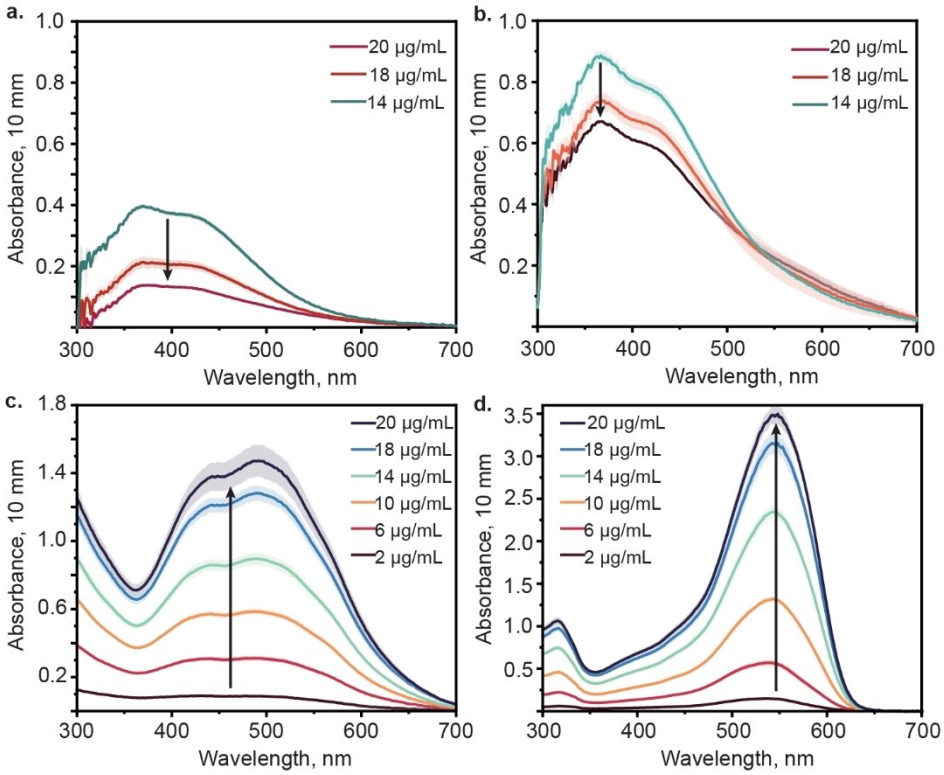

Figure 2. Dye conversion from existing and optimized workflows. UV–Vis spectra from the published workflow for **a**. 1-naphthol standards and **b**. 2-naphthol standards. UV–Vis results from optimized workflow for **c**. 1-naphthol standards and **d**. 2-naphthol standards. Shaded error indicates sample standard deviation (n = 2 for a, b; n = 3 for c, d).

precipitate formed after several minutes of the dye product being in an aqueous solution, we evaluated alternative solvents. Adjusting the solution pH with acetic acid was unsuccessful, as was the addition of miscible co-solvents including ethanol and acetonitrile (Figure S2).[28]

Because maintaining an aqueous environment (even with co-solvents present) led to precipitation, liquid-liquid extraction to transfer the dye to an organic solvent was next evaluated. Ethyl acetate was initially tested for extraction, as it is a prevalent solvent for such processes in organic chemistry (Figure 1b(*ii*)).[33] Though the addition of an extraction step increased the complexity of the workflow, the common, non-hazardous, and inexpensive solvent preserves the assay's use as a rapid, inexpensive method. Importantly, the quality and reproducibility of the data were similarly improved with the maintenance of distinct spectral features and no visible precipitate following centrifugation of the extracted samples (Figure 2c, d). Reaction of the dye precursor with 1-naphthol yielded a bimodal spectrum with absorption maxima of similar intensities at 441 ± 6 nm and 487 ± 5 nm. For 2-naphthol, a single absorbance maximum at 543 nm $\pm$ 5 nm was observed. The only comparison available for our spectra is for aqueous samples, in which the absorbance maxima are shifted and instability is reported.[26] A consistent spectrum was observed from the reaction of naphthol with the dye precursor and no precipitate was observed in the ethyl acetate. Thus, the key challenge observed with this workflow, the dye precipitation, was circumvented through extraction. We therefore incorporated this extraction protocol into the workflow for improved consistency.

Despite the improvements afforded by extraction, calibration curves from naphthol titration were non-linear, preventing quantification (Figure S2). Two potential causes of non-linearity were investigated: 1) insolubility of the dye in ethyl acetate at high concentrations; or 2) varying reaction rates for each naphthol regioisomer impacting absorbance. As no dye precipitate was visible in the ethyl acetate, we eliminated the first option as a cause. We then focused on the second possibility based on our observation that both 1- and 2-naphthol react appreciably with the dye precursor. This observation contradicts literature reports, but we consistently observed reaction of the dye precursor with 2-naphthol. Though the reaction was slower than with the 1-naphthol regioisomer, it also produced a more intensely colored dye product.[16] Thus, we anticipated that insufficient reaction time because of the ethyl acetate extraction led to incomplete conversion of the 2-naphthol produced. Incomplete reaction would explain the non-linearity in the standard curves. To evaluate the impact of reaction time on dye formation, ethyl acetate extraction was performed at varying timepoints following dye precursor addition (Figure S3). Though a slight decline in absorbance was noted with later extraction times for 1-naphthol, when 2-naphthol was evaluated independently, dye intensity increased significantly at later extraction times. These results validated our prior observations and supported our hypothesis that the sluggish reactivity of 2-naphthol led to issues with quantification. Upon standardizing the length of the reaction to a window between four and eight minutes, the linearity of the titration improved and the error between trials decreased (unoptimized: Figure S2, optimized: Figure 2c, d). Thus, our inability to yield quantitative results was addressed simply by optimizing the reaction time with the dye precursor.

As conventional applications of this and similar colorimetric assays are often limited to single-wavelength absorbances, the linearity at 510 nm and 550 nm of naphthol standards was evaluated using ethyl acetate extraction. Measurement at 510 nm is effective for 1-naphthol, and 550 nm is effective for 2-naphthol. Though the wavelengths used for analysis differ from those previously reported (528 nm or 530 nm[16,27,30]), the selected wavelengths maximize sensitivity for both the 1-naphthol and 2-naphthol dye conjugates. At both wavelengths for both naphthols, linear standard curves are obtained ($R^2 \geq 0.99$). The incorporation of an extraction step, optimized reaction times, and absorbance measurements that yield linear standards enable quantification of naphthol from simulated samples with known quantities of reactant.

Validation of Quantification

With a method to generate quantitative results from UV–Vis measurements, our improved assay was next benchmarked against rigorous but resource-intensive HPLC-UV analysis.[26,28] By HPLC, 2-naphthol and 1-naphthol elutions are distinct and separated by 0.4 minutes in an isocratic 55:45% water-acetonitrile method. Quantification from the resultant spectra yield highly linear standard curves ($R^2 > 0.999$) (Figure 3a, b), as compared to an $R^2 \geq 0.99$ for the single-wavelength colorimetric readout. The comparable linearity of these methods highlights the importance of our method, as it is significantly less labor-intensive and requires less costly and time-consuming analysis than the HPLC strategies.

Despite the advantages of our improved workflow, measurement at a single wavelength is insufficient to clearly distinguish between 1- and 2-naphthol, as is readily accomplished by HPLC. The inability to independently quantify each regioisomer would limit the utility of this assay for experiments evaluating biological systems, as MOs are known to produce both isomers concurrently.[11] Though single-wavelength measurements limit the information that can be derived from a regioisomeric mixture, when full spectra are obtained, spectral differences between the 1- and 2-naphthol were hypothesized to enable individual regioisomer quantification through proper spectral deconvolution.

Deconvolution

To further improve upon our analytical method, we then sought to develop a deconvolution strategy to quantify the naphthol regioisomers independently in a mixed solution. To accomplish this goal, each single-species spectrum was simplified into individual gaussian components. Peak amplitude, wavelength, and width were optimized using a non-linear least squares

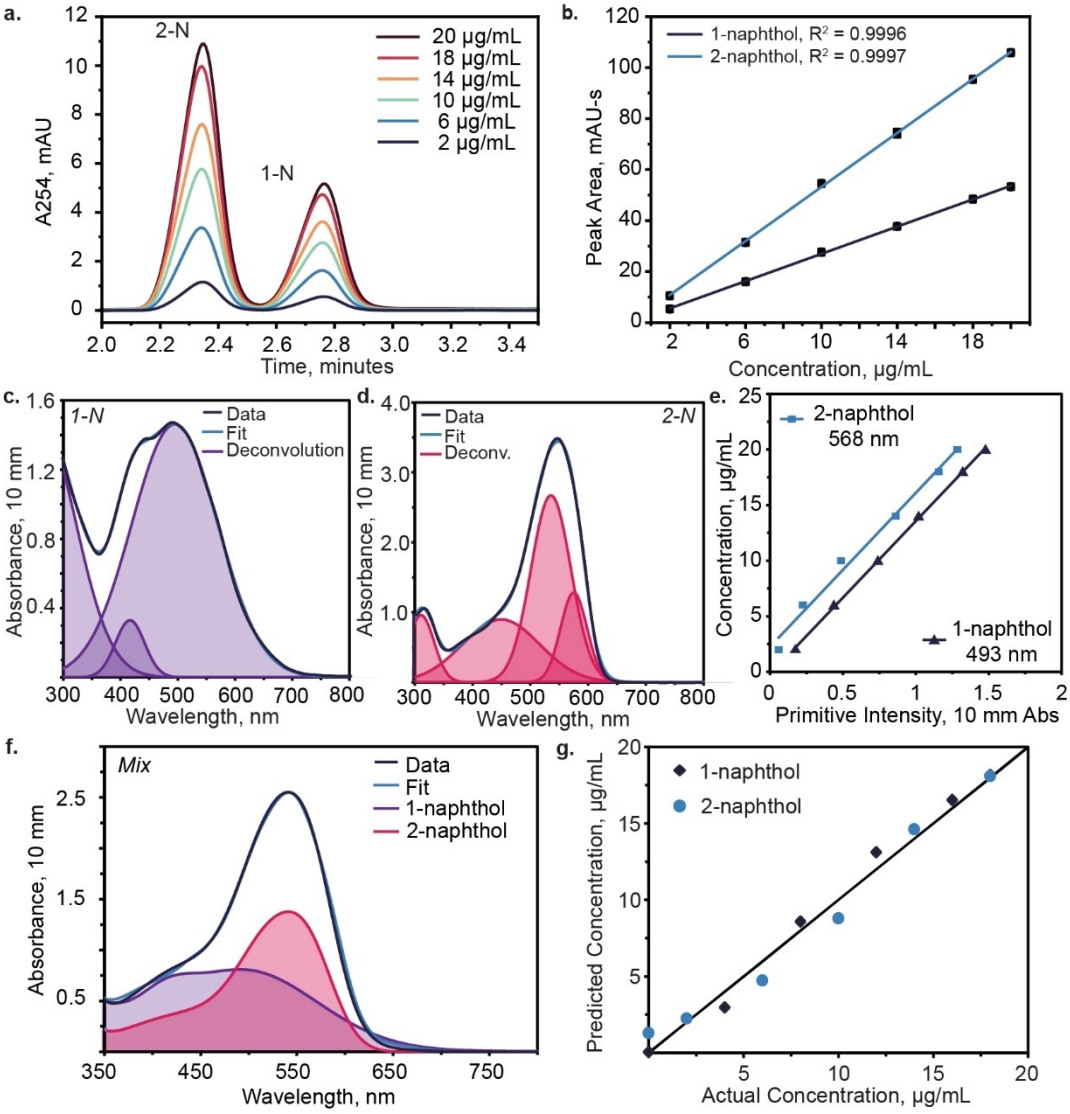

Figure 3. Deconvolution of sample spectra for mixed sample quantification. **a**. Representative HPLC-UV traces for regioisomer quantification (2-N is 2-naphthol and 1-N is 1-naphthol). **b**. Linear standard curves for HPLC in mixed samples. **c**. The spectra for 1-naphthol were deconvoluted using three gaussian primitives with the fit overlay and experimental data for a 20 µg/mL sample. **d**. Deconvoluted spectra for 2-naphthol were fit using four primitives with fit overlay and experimental data for a 20 µg/mL sample. **e**. All fit primitives were assessed for linearity with concentration. Regressions for the peak primitive for each species are shown ($R^2 = 0.9995$ for 1-N and $R^2 = 0.9891$ for 2-N). **f**. Deconvolution for a mixed sample. **g**. Predicted concentrations for deconvoluted mixed samples yield accurate results across the tested concentration range of 0–18 µg/mL.

approach (Figure S4). Three primitives were sufficient to deconvolute the 1-naphthol spectrum (Figure 3c), while four were optimal for 2-naphthol (Figure 3d). The amplitude of the optimized dominant primitive was highly linear with the naphthol concentration (Figure 3e). Using the primitives, functions were developed to reproduce pure 1- or 2-naphthol spectra. A mixed sample was anticipated to be separable into a linear combination of the 1- and 2-naphthol functions (Figure 3f).

To evaluate the efficacy of our deconvolution strategy, mixed standards were prepared using 1- and 2-naphthol mixed to final concentrations similar to the single-species standards. Relative amplitudes, widths, and peak wavelengths were fixed to averages obtained from the standards, providing a single characteristic spectrum for each isomer. The spectra from mixed samples were fit using a linear combination of the two individual spectra (Figure S7). Though qualitative relative abundances of the regioisomers of the individual components can be determined through measurement of absorbance at wavelengths where there is minimal overlap between the 1- and 2-naphthol spectra, the spectral overlap between the regioisomers is sufficient to generate significant error using this strategy. In contrast, our deconvolution and linear combination procedure enabled quantification of the concentration of each isomer (maximum relative error $< 12\%$) (Figure 3g). Thus, the stable colorimetric assay combined with proper spectral analysis facilitated, for the first time, regioisomer quantification in mixed samples without requiring chromatographic separation. Our

method provided similar results to HPLC without the time-consuming and costly workflow.

Biological Relevance

The performance of the improved workflow was impressive in synthetic samples containing no interfering or confounding contaminants. However, biological matrices, such as those in which MOs natively function, are inherently complex. Matrix components could inhibit the reaction with the dye precursor, interfere with the extraction, or disrupt quantification. Thus, ensuring that the assay performed equivalently for biological samples was essential to fully validate the improved workflow. The regiospecificity of enzymatic oxidation is of substantial interest,[11,28] especially as alternatives to chemical catalysts for precise syntheses.[8,11,28] Soluble diiron MOs such as toluene *ortho*-monooxygenase (TOM), toluene/*ortho*-xylene monooxygenase (ToMO), toluene *para*-monooxygenase (TpMO), and toluene-4-monooxygenase (T4MO) are prevalent enzymes for chemical conversions and each have different regiospecificities for the generation of 1- or 2-naphthol.[11,34] T4MO from *Pseudomonas mendocina* KR-1 was selected as a model enzyme due to its established basal regiospecificity of 52% 1-naphthol and 48% 2-naphthol, with prior engineering efforts focusing on single amino acid mutations for altering selectivity (Figure 4a, b).[11,35,36] The ability of this enzyme to generate nearly equivalent amounts of 1- and 2-naphthol is an ideal system in which to test the overall workflow and spectral deconvolution.

Biological Validation

The T4MO enzyme was overexpressed in *Escherichia coli* using two concentrations of IPTG inducer, and cells with no inducer added were evaluated as negative controls (Figure 4c, d).[37,38] The regiospecificity of the enzyme was evaluated by both HPLC as well as our colorimetric assay. Similar selectivity towards 2-naphthol was computed using both methods, and no naphthol formation was observed in the uninduced samples, validating our workflow with complex, biological samples (Figure 4d). As a final control, the MO was actively repressed with phenylacetylene, an established MO inhibitor (Figure S8, S9).[39] As expected, no signal was observed from these samples. Even in complex biological samples, our improved colorimetric workflow enabled quantitative determination of MO regioselectivity consistent with HPLC measurements and with no off-target reactivity.

Conclusion

Colorimetric measurements are optimal for fast, inexpensive, high-throughput analysis of MO enzymatic activity. Established methods involve the bioconversion of naphthalene to naphthol, followed by reaction of the naphthol with a dye precursor. Such methods have been used for the past four decades but suffer from dye product instability and insolubility. By incorporating an extraction step to move the dye product into organic solvent, we resolve these key challenges. Further, we approach the capabilities of the gold standard analytical method, HPLC, to quantify regioisomers through the incorporation of a deconvolution method for the 1- and 2-naphthol dye products. Finally, we demonstrate the performance of this improved workflow through the quantification of 1- and 2-naphthol generated by a native MO expressed in *E. coli*. Future work will involve the expansion of the assay to additional MO enzymes and classes of enzymes.[11,40] When full spectral information is obtainable, multiple reaction products can be simultaneously monitored and quantified, circumventing key challenges in this space.

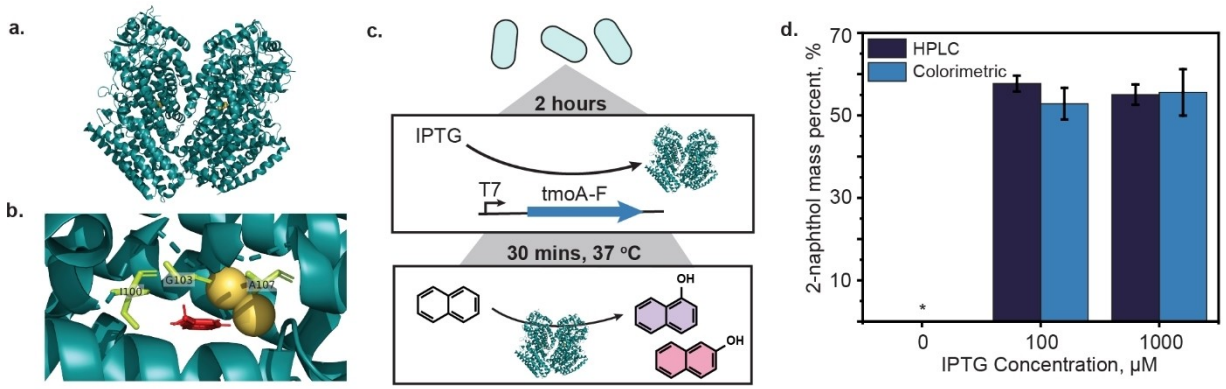

Figure 4. Biological validation of the workflow and deconvolution. **a**. Crystal structure for the hydroxylase subunit of toluene-4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR-1 [PDB: 5TDS] (35, 36). **b**. Magnification of the T4MO active site. Point amino acid mutations (yellow: I100, G103, A107) are known to affect naphthalene oxidation regiospecificity (11, 35, 36). **c**. Workflow for T4MO expression and naphthalene exposure in *E. coli*. **d**. Regiospecificity determination using HPLC and colorimetric readout for cultures induced with IPTG at varying concentrations. Error bars represent the standard deviation for n = 3 replicates.

Experimental Section

Azo-coupling for standard curve generation: 1-naphthol and 2-naphthol stocks were prepared in anhydrous ethanol to a final concentration of 20 mg/mL. For quantification experiments, a 1 : 1000 dilution was prepared in phosphate-buffered saline (PBS; 5 mM sodium phosphate, 50 mM sodium chloride, pH 7.0) to a working solution concentration of 20 μg/mL for both naphthols. For individual standards, dilutions were prepared from the 20 μg/mL stock in triplicate. For mixed species samples used to evaluate deconvolution, appropriate volumes of the stock solutions and PBS diluent were combined. The dilutions were maintained at 0 °C. Fresh 0.2 % (w/v) Fast Blue B/tetrazotized *o*-dianisidine solution was prepared prior to each experiment in MQ at 4 °C. 80 μL of 0.2 % tetrazotized *o*-dianisidine stock was added to 2 mL of the naphthol standard in PBS. Following mixing, the reaction was incubated at 0 °C for 4 mins. 2 mL of reagent grade ethyl acetate was then added to the samples. The samples were vigorously vortexed, followed by centrifugation at 4,000 rcf for 5 mins to facilitate liquid-liquid phase separation. UV–Vis absorbance of the resultant ethyl acetate solution was measured by NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) in a 10 mm quartz cuvette.

For samples replicating the published workflow, the coupling was performed at ambient temperature in a rich medium, lysogeny broth (LB), and the dye was not extracted. Following coupling, the reaction was centrifuged at 16,100 rcf for 4 mins, and the supernatant was removed and analyzed by UV–Vis (NanoDrop One) in a 10 mm cuvette. In cases where acetic acid stabilization was evaluated, glacial acetic acid was added to samples to a final concentration of 16 % v/v prior to centrifugation at 16,100 rcf.[28]

Chromatographic separation of naphthols: An Agilent 1260 Infinity II system (Agilent Technologies, Santa Clara, CA) with a photodiode array detector was used for chromatographic separation of 1- and 2-naphthol. An isocratic method of 45 % acetonitrile/55 % water and a Zorbax SB-C18 column (2.1 × 50 mm, 1.8 micron) column were used at a flow rate of 0.2 mL/min and 30 °C. Spectra were monitored at 254 nm, with peak integration performed in ChemStation (Agilent Technologies). For biological samples, ethyl acetate extracts were prepared as described and filtered through a 0.22 μm PTFE filter prior to analysis.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Data Availability Statement

All data are contained within the manuscript.

Keywords: analytical chemistry · cloning · colorimetric assay · enzyme · HPLC · monooxygenase · regiospecificity · UV–Vis spectroscopy

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