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# From multi-step enzyme monitoring to whole-cell biotransformations: development of real-time UVRR spectroscopy

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## ABSTRACT

Process Analytical Technologies (PAT) are used within industry to give real-time measurements of critical quality parameters, ultimately improving the quality by design (QbD) of the final product and reducing manufacturing costs. Spectroscopic and spectrophotometric methods are readily employed within PAT due to their ease of use, compatibility towards a range of sample types, robustness and their multiplexing capabilities. We have developed a UV resonance Raman (UVRR) spectroscopy approach to quantify industrially-relevant biotransformations accurately, focusing on nitrile metabolising enzymes: nitrile hydratase (NHase) and amidase *versus* nitrilase activity. Sensitive detection of the amide intermediate by UVRR spectroscopy enabled discrimination between the two nitrile-hydrolysing pathways. Development of a flow-cell apparatus further exemplifies its suitability towards PAT measurements, incorporating *in situ* analysis within a closed system. Multivariate curve resolution-alternating least squares (MCR-ALS) was applied to the UVRR spectra, as well as off-line HPLC measurements, to enable absolute quantification of substrate, intermediate and product. Further application of hard modelling to MCR-ALS deconvolved concentration profiles enabled accurate kinetic determinations, thus removing the requirement for comparative off-line HPLC. Finally, successful quantitative measurements of *in vivo* activity using whole-cell biotransformations, where two *Escherichia coli* strains expressing either NHase (transforming benzonitrile to benzamide) or amidase (further conversion of benzamide to benzoic acid), illustrates the power, practicality and sensitivity of this novel approach for multi-step and with further refinement we believe multiple micro-organism biotransformations.

## INTRODUCTION

Nitrile hydrolysing enzymes, namely, nitrilases, nitrile hydratases (NHase) and amidases, are extensively used within chemical synthesis in industry owing to their high selectivity and activity as well as their broad substrate specificity.[1] These nitrile metabolising enzymes work at ambient temperature(s) and pressure(s) and can offer enantioselective control, meaning they are attractive alternatives within pharmaceutical processes. At present, acrylamide (>30,000 tons per year), nicotinamide (Vitamin $B_3$, >6,000 tons per year), (*R*)-mandelic acid (a drug precursor), pyrazinamide (an antituberculosis agent) and 5-cyanovaleramide (a herbicide intermediate) are largely synthesised using nitrile hydrolysing biocatalytic routes.[2-7] There has also been increasing interest in the detoxification of wastewater containing toxic nitriles of anthropogenic origins using nitrile hydrolysing enzymes.[7,8]

Due to the appealing nature of these biocatalysts, different techniques to monitor the progression of these biotransformations have been investigated. On-line techniques (in contrast to off-line) are often more desirable as they directly monitor the reaction in real-time, providing rapid, continuous feedback. As such, sample manipulation, extraction or (partial) purification is no longer required prior to analysis, reducing the error by minimizing the need for sample transfers and handling. Furthermore, real-time reaction monitoring greatly improves the efficiency and accuracy of the overall process within PAT (process analytical technology), an essential component of QbD (quality by design).[9,10] Fluorometric and colorimetric assays are examples of on-line techniques that have been widely studied to monitor nitrile hydrolysis. Examples of colorimetric assays include those that rely on changes in pH,[11-14] whereas spectrophotometric assays have involved monitoring ferrous and ferric ions,[15,16] as well as NADH consumption when paired with an amidase and glutamate dehydrogenase.[17] Whilst these techniques are advantageous over other more time consuming and labour-intensive off-line techniques, such as HPLC, NMR or GC-MS or LC-MS, a major limiting factor is the requirement of a fluoro- or chromo-genic substrate, along with limited diagnostic and structural information.[18,19] Consequently, there is an increased effort towards the emergence of new and improved high-throughput screening (HTS) methods within biocatalysis and *in situ* PAT techniques. Raman spectroscopy offers considerable potential as a physicochemical technique for monitoring real-time enzymatic catalysed biotransformations. However, 'normal' Raman scattering is often too weak, limiting its application, yet enhancements of $10^3 – 10^5$ are attainable when deep-UV (244 nm in this case) excitation is employed. In UV resonance Raman (UVRR) spectroscopy the laser frequency coincides with the electronic transitions of the molecules under investigation, specifically aromatic and conjugated systems, thus enhancing the Raman response.[20,21]

We recently demonstrated that UVRR could successfully monitor the real-time progression of biotransformations using either NHase or xanthine oxidase (conversion of hypoxanthine to xanthine, followed by xanthine to uric acid).[22] UVRR achieved results that were in agreement with off-line HPLC analysis, but with a >30-fold reduction in acquisition time (20 s measurement time for UVRR *versus* >10 min for each HPLC run). Ultimately, rapid and sensitive detection of these analytes was possible by exploiting aromatic functionality which is inherent to their structure.

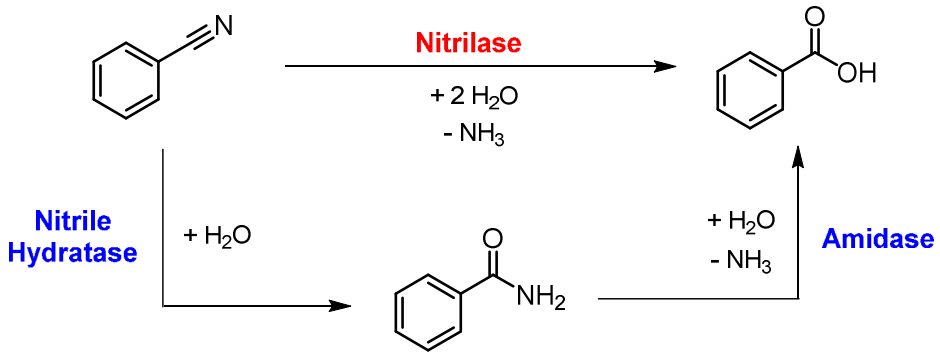


Figure 1. Enzymatic conversion of benzonitrile to the corresponding carboxylic acid (benzoic acid) and/or carboxamide (benzamide).

In this study, we further investigate the application of UVRR to discriminate quantitatively between nitrile metabolising enzymes. Nitrile catabolism comprises of two distinct pathways: (1) direct generation of carboxylic acids *via* nitrilase; and (2) a two-step process whereby nitrile hydratases catalyse the formation of an amide, which is subsequently hydrolysed by an amidase to give the corresponding carboxylic acid (Figure 1).[1] Consequently, discrimination between these pathways until now has been problematic as they generate the same final product, resulting in only semi-quantitative or qualitative analysis. We describe how UVRR spectroscopy can be used in real-time to easily distinguish between the two pathways, without the requirement of additional fluoro- or chromo-genic substrates, enabling full recovery of the reaction sample whilst affording quantitative analysis. Furthermore, a flow-cell apparatus has been developed to reduce signal-to-noise interference, further demonstrating its amenability for use in industrial processes (closed system enabling direct *in situ* measurements). Finally, investigations were extended to include whole-cell biotransformations (*in vivo*), omitting protein purification steps thus further increasing the overall speed and utility of this technique.

## EXPERIMENTAL

**Materials.** All chemical reagents were of analytical grade and used with no additional purification unless otherwise stated. A nitrile hydratase (EC 4.2.1.84) construct was kindly provided by the Uwe Bornscheuer group (Greifswald University),[23] and an amidase (EC 3.5.1.4) synthetic gene was purchased from Genewiz (New Jersey, USA). Full details of cloning, expression and protein purification is described within the Supporting Information.

**Reaction sample preparation.** The starting reaction mixture contained either benzonitrile or benzamide (final concentration $1.25 \times 10^{-2}$ M) in potassium phosphate buffer ($2.5 \times 10^{-2}$ M, pH 7.2). Reactions were performed using either purified enzyme (*in vitro*) or *Escherichia coli* whole-cells (resting state) which expressed either NHase or amidase protein (*in vivo*). For reactions involving purified enzymes, NHase + amidase ($6.0 \times 10^{-6}$ M and $3 \times 10^{-6}$ M, respectively) were added to the reaction mixture to initiate the biotransformation. For whole-cell biotransformations, NHase or amidase (500 µL or 250 µL respectively, of concentrated whole-cells in buffer) were introduced into the reaction mixture or a combination of the two (375 µL of NHase and 25 µL of amidase of whole-cells in buffer). See Supporting Information, supplementary methods for further information.

**UVRR Instrumentation.** UVRR was performed using a Renishaw Raman 1000 system (Renishaw, Wotton-under-edge, Gloucestershire, UK). Approximately ~0.2 mW of power was delivered to the sample using a Lexel Model 95 ion laser (frequency doubled) emitting at 244 nm.

**Reaction set-up.** UVRR monitoring of biotransformations were performed using a flow-cell set-up. In brief, a quartz flow-cell was focussed beneath the UVRR microscope objective, which was connected to the reaction reservoir using tubing (Figure S1). Continuous stirring of the reaction mixture was achieved using a peristaltic pump, along with a stirrer bar agitating the reaction reservoir (the location at which enzyme is added and HPLC samples removed). A total reaction volume of 10 mL was used, UVRR analysis was performed using a 20 s acquisition time at various intervals (mainly every minute). In addition, every 2-3 min 20 µL of the reaction mixture was removed and quenched with MeOH (180 µL), before being subjected to comparative HPLC analysis.

## RESULTS AND DISCUSSION

Multi-step enzyme biocatalysis

To explore if UVRR could distinguish between the two nitrile metabolising pathways, we investigated the more complex, two-step pathway combining both a nitrile hydratase and an amidase. A flow-cell apparatus was developed and optimized to reduce signal-to-noise interference by incorporating a quartz flow-cell at the site of UVRR analysis (images of the set-up are shown in Figure S1). Benzonitrile was investigated as the starting material (SM), which produces both benzamide as the intermediate (I) and benzoic acid as the final product (P) in the presence of NHase and amidase (Figure 1). Characteristic UVRR peaks for each analyte are highlighted in Figure 2 (see Table S1 in the Supporting Information for tentative band assignments). Benzamide and benzoic acid produce very similar UVRR responses yet subtle differences are seen, most noticeably, one can observe the peak shift from 1413 to 1389 $cm^{-1}$ (benzamide to benzoic acid) and the weak peak at 847 $cm^{-1}$ which is absent in both benzonitrile and benzamide but present within the benzoic acid product.

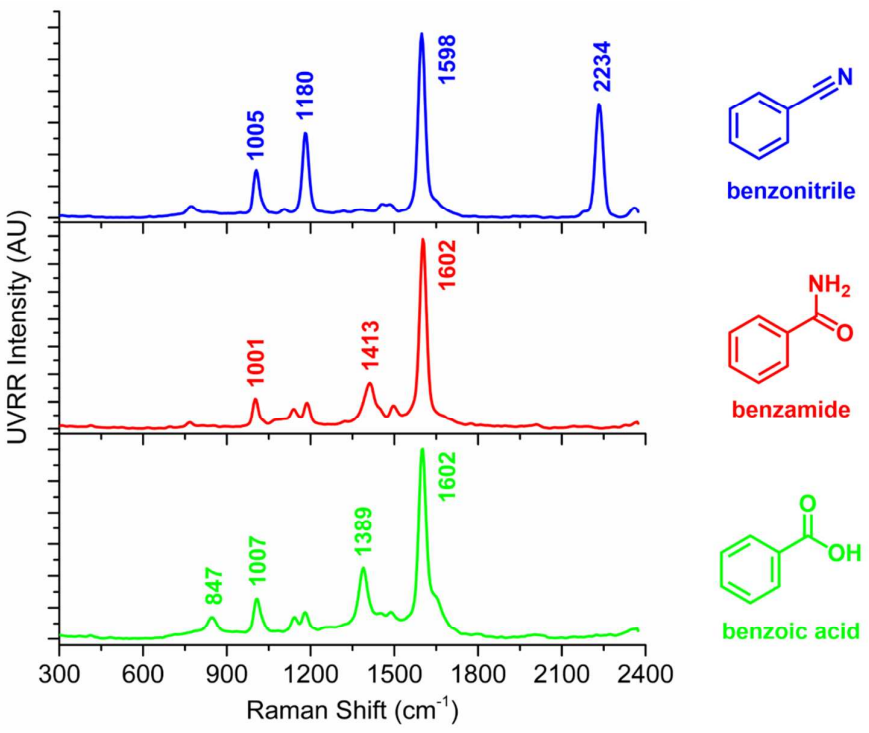


Figure 2. Average UVRR spectra ($n$ = 2) of each analyte under investigation: benzonitrile (blue), benzamide (red) and benzoic acid (green). Spectra were obtained at 1.1 x $10^{-2}$ M in potassium phosphate buffer (2.5 x $10^{-2}$ M, pH 7.2), using conditions and concentrations that were representative of the initial reaction mixtures, with characteristic peaks identified (see Table S1 for assignments). UVRR spectra were obtained for 20 s with baseline correction and normalisation applied (see the Supporting Information, "Data processing" for full details).

Initially we looked at the conversion of benzonitrile to benzamide and subsequently to benzoic acid using NHase and amidase *in vitro* (purified enzymes), with comparative HPLC measurements acquired to confirm that the *in situ* UVRR measurements were accurate. Multivariate curve resolution-alternating least squares (MCR-ALS) is a soft-modelling method, used mathematically to deconvolve an instrumental response into the pure contributions of individual components present within a mixture. The input of pure spectra of each component of the mixture is an initial requirement, and the iterative method can then deconvolve the complex mixture and provide concentration profiles of each component. [24,25] To benchmark this approach HPLC measurements of the same samples allows the comparison of the known analyte concentrations with the predicted UVRR concentrations *via* MCR-ALS modelling, thus serving as external validation (an overview of the spectral pre-processing and MCR-ALS process is shown in Figure S2). One can also compare how well the pure analyte spectra (as initially inputted into the model) and the resolved MCR-ALS spectra agree with one another and in this case the real and deconvolved were congruent (Figure S3).

It is important to note that UVRR spectra were acquired every minute throughout the monitoring process, whereas HPLC aliquots were taken less frequently (2-3 minute intervals). Time points consisting of both HPLC and UVRR measurements were used as the training set for the MCR-ALS model, with only UVRR measurements used as the test set. Figure 3 shows the resolved concentration profiles, illustrating very good agreement between the HPLC and UVRR predictions. The MCR-ALS model correctly recognized all three components within the reaction mixture, despite their spectral similarities. Thus, enabling distinction between the two-step pathway (NHase + amidase) *versus* nitrilase catalysis (no intermediate). Regression-coefficient values ($R^2$) demonstrate the proportion of variability within a data set, as accounted for by the statistical model (MCR-ALS). Generally, $R^2$ values closer to 1 demonstrate excellent fit and correlation between the HPLC measured and UVRR predicted concentrations. Results are provided in Figure S4 for all three analyte components – benzonitrile (SM), benzamide (I) and benzoic acid (P) – with their corresponding $R^2$ values: 0.9637, 0.9076 and 0.9895, respectively, indicating very good agreement between the two techniques.

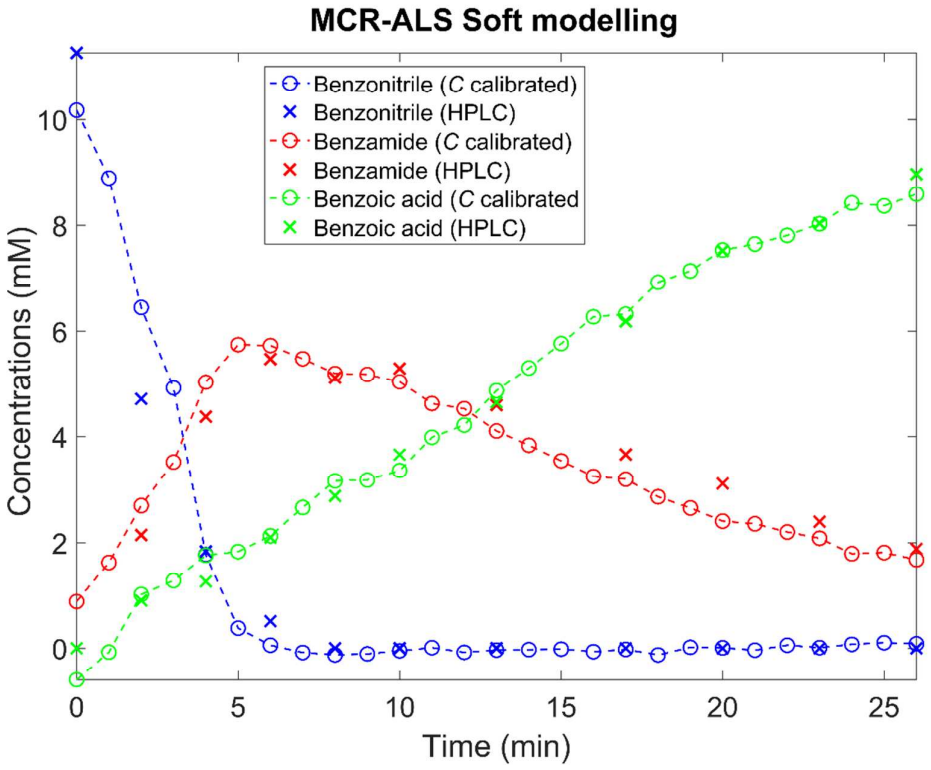


Figure 3. An MCR-ALS model was applied to the UVRR data where it successfully deconvolved spectra into its pure components for the biotransformation using pure enzymes. This figure shows the reaction dynamics from the real-time UVRR measurements (denoted by circular symbols) and off-line HPLC data (denoted by cross symbols) as a function of time for the conversion of benzonitrile (SM; blue) to benzamide (I; red) to benzoic acid (P; green), catalysed by NHase and amidase, correspondingly.

To complement the incorporation of UVRR within on-line PAT processes further, we wanted to explore the application of hard modelling (HM),[26,27] as this would remove the requirement for external calibration (*i.e.*, the additional HPLC measurements). The multivariate curve resolution-alternating least squares-hard modelling (MCR-ALS-HM) process is shown in Figure S5. In brief, assuming that the two steps of the biotransformations both followed a first-order reaction, the resolved concentration profiles from MCR-ALS were used to determine the reaction rate constants $k_1$ and $k_2$ using the kinetic models as shown in Figure S6. The concentrations of the reactants at any time points during the reaction were then derived using the estimated $k_1$, $k_2$ and the known initial concentration of the starting material (*i.e.*, benzonitrile). The MCR-ALS-HM results are shown in Figure 4 along with comparative HPLC results to highlight their consistency. One can see that the results are once again in good agreement, particularly for the benzonitrile (SM). However, benzamide and benzonitrile HM concentration profiles do not agree with the HPLC calibration concentrations quite as well. The MCR-ALS-HM approach assumes that 100 % of the SM (*i.e.*, benzonitrile) is converted to product(s) (*i.e.*, benzamide + benzoic acid). Upon further inspection of

the total concentration throughout the monitoring process, we experience significant fluctuations in total mass, consequently causing a disparity between the HPLC and MCR-ALS-HM results. The total analyte concentration at various timepoints (as calculated by HPLC) is shown in Table S2. After the initial timepoint, a substantial drop in the total concentration occurs and a third of the starting concentration is unaccounted for, which subsequently increases again with increasing time (final timepoint concentration = 96 % of starting concentration). We hypothesise that these variations in the total concentration is a consequence of reduced solubility of the intermediate (benzamide), resulting in partial insolubility before being converted to benzoic acid (product) where it is soluble once again due to the reduced concentration of benzamide. As the MCR-ALS and HPLC results (Figure 3) were in such good agreement with one another, we can conclude that both techniques (UVRR and HPLC) are detecting this reduction in concentration and it is a true effect. Unfortunately, the HM results cannot adjust for this mass imbalance, thus we see a discrepancy between the MCR-ALS-HM and HPLC results (Figure 4). Nevertheless, we can calculate reaction rate constants using the MCR-ALS-HM model and these constants were $k_1$ = 0.3316 and $k_2$ = 0.0797 for NHase and amidase, respectively (the equations for kinetic calculations are shown in Figure S6).

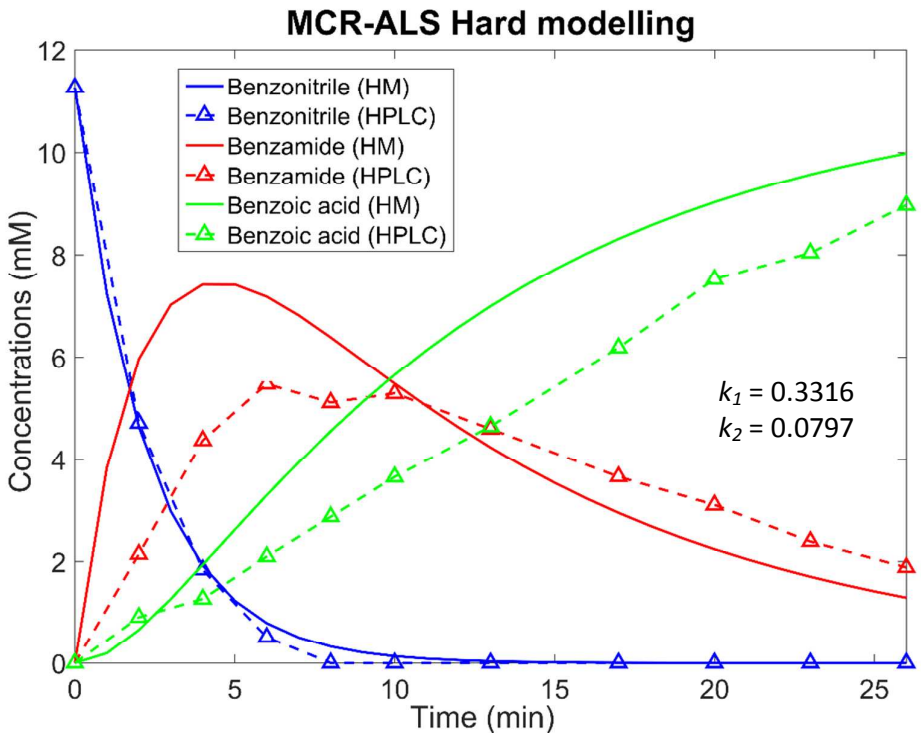


Figure 4. Multivariate curve resolution-alternating least squares-hard modelling (MCR-ALS-HM) results show good agreement with HPLC measured concentrations. Calculated kinetic rates of each enzyme were $k_1$ = 0.3316 and $k_2$ = 0.0797 for NHase and amidase, respectively.

Whole-cell biotransformations incorporating multi-step enzyme biocatalysis

In addition to the *in vitro* pure enzyme assessment above, we wanted to perform this biotransformation using *in vivo* conditions using whole-cells. Omitting protein purification steps would increase the overall efficiency and speed of this real-time monitoring approach, further proving its suitability within industrial processes. NHase and amidase were expressed within *E. coli* BL21 (DE3) cells using optimised expression conditions (see Supporting Information section 'Protein expression and purification'). Once cells were induced for protein expression and allowed to grow overnight, the cells were harvested by centrifugation and re-suspended in potassium phosphate buffer prior to use within the reaction. To ensure that bacterial cells were in the resting state and no further growth was experienced, optical density (OD) measurements at 600 nm were performed throughout the monitoring process. $OD_{600}$ measurements were relatively consistent throughout the 60 min period and thus we conclude that there was no significant further bacterial growth (see Supporting Information section '$OD_{600}$ measurements' and Table S3). Initially we performed two separate whole-cell biotransformations: the first contained *E. coli* expressing NHase for conversion of benzonitrile (SM) to benzamide (P), and the second biotransformation with benzamide (SM) used *E. coli* expressing amidase, generating benzoic acid (P). In order to avoid photo-damage of these *E. coli*, as shown in Figure S1, a peristaltic pump was used to flow the bacteria reaction mixture continuously through the quartz flow-cell that was used for collection of UVRR spectra.

The first one-step, *in vivo* biotransformation studied was the conversion of benzonitrile to benzamide using *E. coli* cells expressing NHase activity. Consistent with our previous investigations, both UVRR and HPLC measurements were taken and MCR-ALS analysis performed. The MCR-ALS resolved profiles for starting material and product can be observed in Figure S7, along with the predicted concentration profiles and known HPLC concentrations comparisons of each analyte (Figure S8). The resolved MCR-ALS spectrum of benzamide (P) was less successful than benzonitrile (SM), reflected in their $R^2$ values of 0.7286 and 0.9514, correspondingly. This is likely due to the *E. coli* background response coinciding with the benzamide product peaks within the region of 1200 – 1800 $cm^{-1}$. Despite this the MCR-ALS modelled concentrations *versus* HPLC results (Figure 5) are evidently in very good agreement with one another and the UVRR approach can successfully quantify the whole-cell catalysed hydrolysis of benzonitrile to benzamide.

As with the pure enzyme reactions hard modelling was further applied to these results, however as previous, the reaction experienced a reduction in the total concentration (assuming benzamide insolubility) which had a substantial effect on MCR-ALS-HM. The final reaction timepoint ($t$ = 18 min)

had a 38 % mass loss when compared to the starting concentration, thus rendering the MCR-ALS-HM results inaccurate (MCR-ALS-HM kinetic equations are shown in Figure S9).

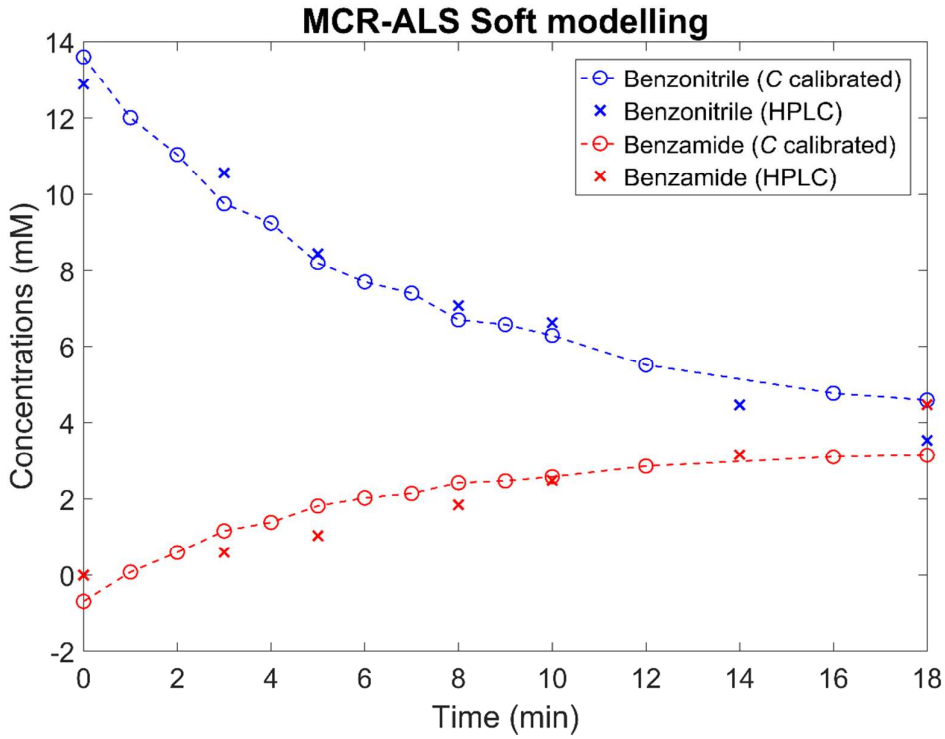


Figure 5. MCR-ALS model applied to the whole-cell biotransformation of benzonitrile (SM, blue) to benzamide (P, red) using NHase-containing *E. coli* cells. Figure shows the reaction dynamics from the real-time UVRR measurements (denoted by circular symbols) and off-line HPLC data (denoted by cross symbols) as a function of time.

Next, the second step of the biotransformation (benzamide to benzoic acid) was monitored in the presence of amidase-expressing *E. coli* cells. The increased expression of amidase *in vivo*, relative to NHase, enables a much lower quantity of cells to catalyse the reaction (which we estimated to be 1:15), hence the *E. coli* background does not overtly dominate the UVRR response and characteristic peaks are observed. Thus, the MCR-ALS soft modelling approach was applied to the UVRR and HPLC data and were shown to be in very good agreement with one another ($R^2$ = 0.9439 and 0.9862 for benzamide and benzoic acid, Figure S10 and S11). Figure 6A illustrates the similarity between the two methods. The total concentration remains stable throughout, so MCR-ALS hard modelling was applied to the data, removing the requirement of comparative HPLC (MCR-ALS-HM kinetic models shown in Figure S9). Results of the MCR-ALS-HM are shown in Figure 6B, where one can see that the hard modelling results (represented by the solid lines) are highly comparable to the off-line HPLC

measured concentrations (dashed lines), enabling an accurate measurement of the kinetic rate, identified as $k = 0.1986$.

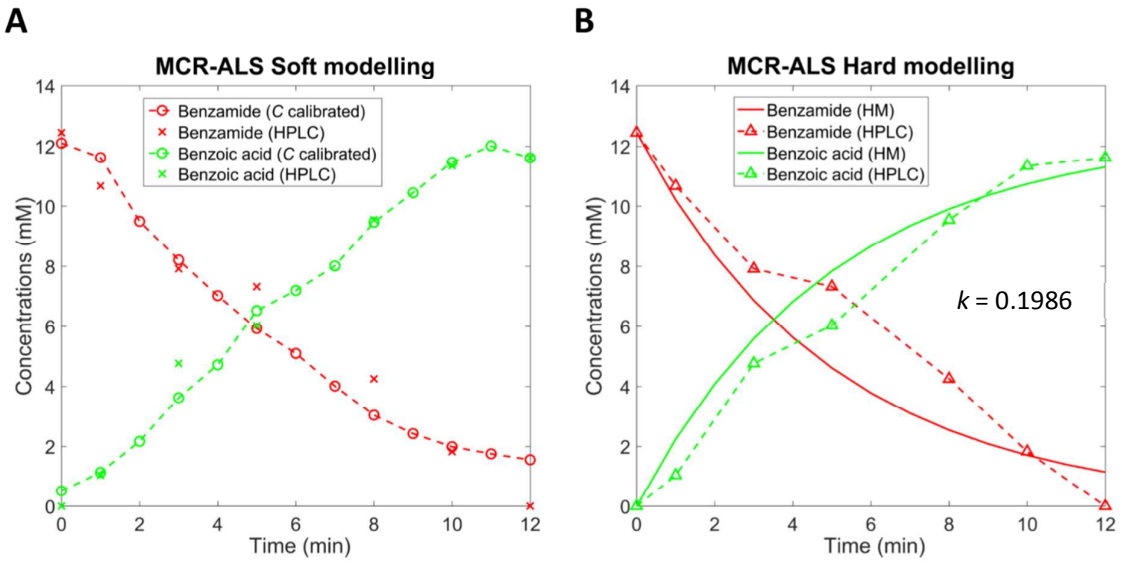


Figure 6. Modelling results for amidase-expressing bacterial whole-cells catalysing the conversion of benzamide (SM) to benzoic acid (P). **(A)** MCR-ALS soft modelling results and **(B)** MCR-ALS hard modelling results, kinetic rate was calculated as $k = 0.1986$.

Finally we combined the two different *E. coli* together. As discussed above the relative expression levels of NHase were very low in comparison to that of amidase, and this required a much larger quantity of NHase-containing *E. coli* cells within the reaction mixture. We established using HPLC (data not shown) that the 15:1 ratio of NHase:amidase enabled detectable concentrations of benzamide before its subsequent hydrolysis to benzoic acid. Unfortunately, despite this optimisation to see the intermediate, *in vivo* UVRR reaction monitoring in the presence of NHase + amidase (15:1 ratio) was unsuccessful due to the high density of *E. coli* cells. Figure S12a shows the corresponding UVRR spectra over the reaction time course, one can easily identify the decreasing nitrile peak at 2234 $cm^{-1}$, indicative of benzonitrile (SM) consumption. However, the bacterial cells produce a UVRR response which has peaks coinciding with those characteristic of benzamide and benzoic acid (1200 – 1800 $cm^{-1}$, see Figure S12b) which meant that subsequent MCR-ALS modelling was unsuccessful. Despite trying to include the biomass peak profile within the MCR-ALS model (as an additional individual component) the deconvolution into four individual components (SM, I, P and biomass) was still unsuccessful. We believe that if the NHase expression had been higher that we would have been able to achieve an *in vivo* biotransformation using multiple organisms; however we are yet to find a clone that has sufficiently high expression at this time.

## CONCLUSIONS

We have successfully demonstrated how UVRR can be used in a real-time, label-free and rapid (20 s) manner to discriminate between nitrile-metabolising pathways (NHase + amidase *versus* nitrilase) by observation of the amide intermediate. Combining on-line UVRR measurements with off-line HPLC analysis, MCR-ALS modelling could be implemented to quantify each of the components accurately within the reaction. High $R^2$ values demonstrate that the two analytical techniques are in very good agreement with one another. The similarity of the UVRR responses of the intermediate (benzamide) and product (benzoic acid) does not appear to impact the model's ability to distinguish them, further indicating the sensitivity and selectivity of this combined UVRR + MCR-ALS approach. In contrast to previous open pot reactions where products and substrates may evaporate,[22] the development of a flow-cell apparatus enables collection of UVRR measurements through a quartz flow-cell, highlighting its suitability within PAT processes and facilitating measurements within a closed system. To complement the application of a closed system further, hard modelling was combined with MCR-ALS (MCR-ALS-HM) as to remove the requirement of external HPLC measurements, with results shown to be in good agreement with off-line HPLC (validation). However, fluctuations in the total concentration (arising from reduced solubility of the intermediate) reduced the accuracy of the model as the mass of the system was no longer in equilibrium.

Finally, to extend our investigations and the applicability of this approach towards industrial development, we introduced *in vivo* conditions by using *E. coli* whole-cells which expressed either NHase or amidase activity. Omitting protein purification is attractive as it increases the overall speed, enabling faster turnover of the desired product. Low-level expression of NHase *in vivo* and the consequent requirement of a high density of bacterial cells led to unsuccessful detection and discrimination of the individual analytes when performed in the presence of both NHase and amidase bacterial cells. However, separating the two individual biotransformations proved to be very successful. MCR-ALS soft modelling and hard modelling approaches could be applied to the amidase-catalysed reaction, owing to its high-level expression (*in vivo*) and avoidance of solubility interference. We are confident that these results showcase the sensitivity and accuracy of UVRR spectroscopy for *in situ*, continuous measurements of enzyme activity, and its suitability for implementation within industry.

**SUPPORTING INFORMATION**

Details of: (i) supplementary methods; (ii) instrumentation and data processing, (iii) optical density (OD600) measurements; and (iv) protein expression and purification are provided in the SI. Provide are the UVRR instrument set-up (Figure S1) and UVRR band assignments for analytes (Table S1). For enzyme biocatalysis: the overall MCR-ALS processes (Figure S2) along with deconvolved spectra (Figure S3) and results of modelling (Figure S4) are included as is the extension to MCR-ALS-HM (Figure S5) with concentration constraints (Table S2) and the implementation of the kinetic modelling (Figure S6). For whole-cell biotransformations: OD600 measurements are included (Table S3), results of MCR-ALS deconvolution (Figure S7) and quantifications (Figure S8), the extension to kinetic MCR-ALS-HM modeling is proposed (Figure S9), along with the results of deconvolution (Figure S10), quantification (Figure S11) and UVRR spectra (Figure S12). Finally, details of cloning, expression and protein purification protocols are provided (Figure S13-14).

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