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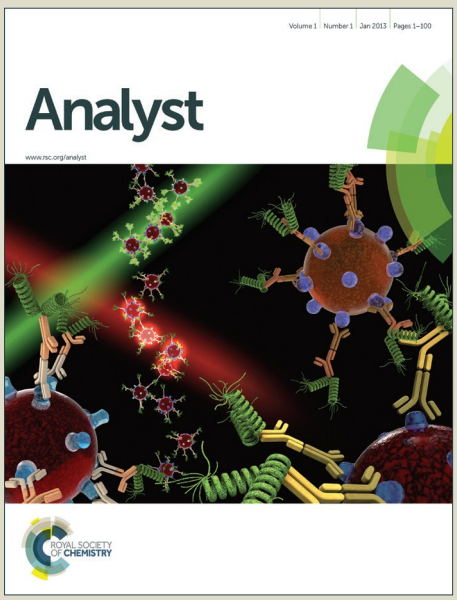

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Rapid and sensitive monitoring of biocatalytic reactions using ion mobility mass spectrometry (IM-MS)

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The combination of stable isotope labelling with direct infusion ion mobility mass spectrometry (IM-MS) enabled qualitative and quantitative monitoring of biocatalytic reactions with reduced analysis times, enhanced sensitivity and µL-level assay volumes. The new approach was demonstrated by applying to both lipase and monooxygenase enzymes, including multi-substrate screening.

The past two decades have witnessed a dramatic increase in the range of enzyme candidates that can be used as biocatalysts in industrial organic synthesis; this increase has arisen through a combination of massive genomic data providing natural sequences together with directed evolution techniques that generate thousands of variants with novel unnatural activities.[1] To fully exploit this resource of available biocatalysts, there is an urgent need for high-throughput assays to track target biotransformations, a common bottleneck in biocatalyst development pipelines.[2,3] Many common biotransformations are not amenable to sensitive spectrophotometric assays and screening is often limited to low-throughput analytical techniques, such as nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), and liquid or gas chromatography coupled with mass spectrometry (HPLC/GC-MS(MS)).[4-6] Direct MS-based analysis is an attractive option and has been demonstrated for enantioselective chemical reactions, including Pd-catalysed allylic substitutions and organocatalytic Diels-Alder reactions[7]. Isotope labeling strategies are immensely powerful in conjunction with MS and have been used for the relative quantification of proteins, using metabolic enrichment[8,9] or at the peptide level with SILAC approaches,[10] although the latter are not compatible with high-throughput analysis. Label-assisted mass spectrometry can accelerate reaction discovery[11] suggesting significant potential for biocatalytic reaction screening.

The separation capability of ion mobility mass spectrometry (IM-MS), coupled with enhancement in ion transmission from S-wave technology makes it possible to perform direct infusion of complex mixtures, removing an HPLC stage.[12] Subsequent target identification in direct infusion MS data from complex mixtures is still limited by ion suppression as well as the dynamic range of the analyser, indicating the need for an additional analytical validator step. The combined use of IM and accurate mass measurement has previously shown that isotopically labeled species possess identical drift times as their unlabeled twin; isotopes have negligible effect on the physicochemical properties that govern the mobility of an ion. [13] In this work, we demonstrate a rapid assay for biotransformations by coupling isotope labelling of the biocatalytic substrate with IM-MS. The basic workflow of our approach is shown in Figure 1. Light/heavy isotope substrate mixtures (either supplemented with deuteriated substrates, or exploiting the natural isotopic abundance of chlorine or bromine atoms) were subjected to biotransformation. Samples were then filtered and directly analysed by IM-MS.

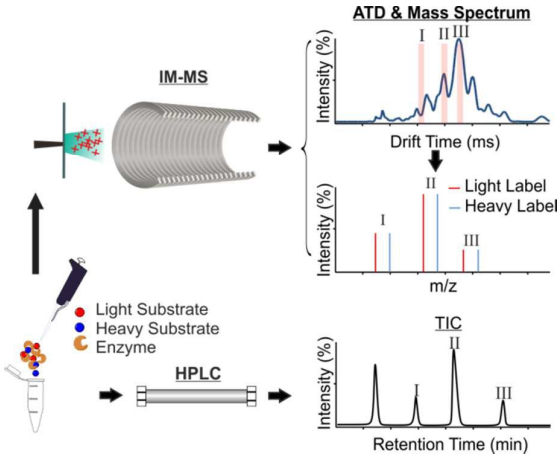

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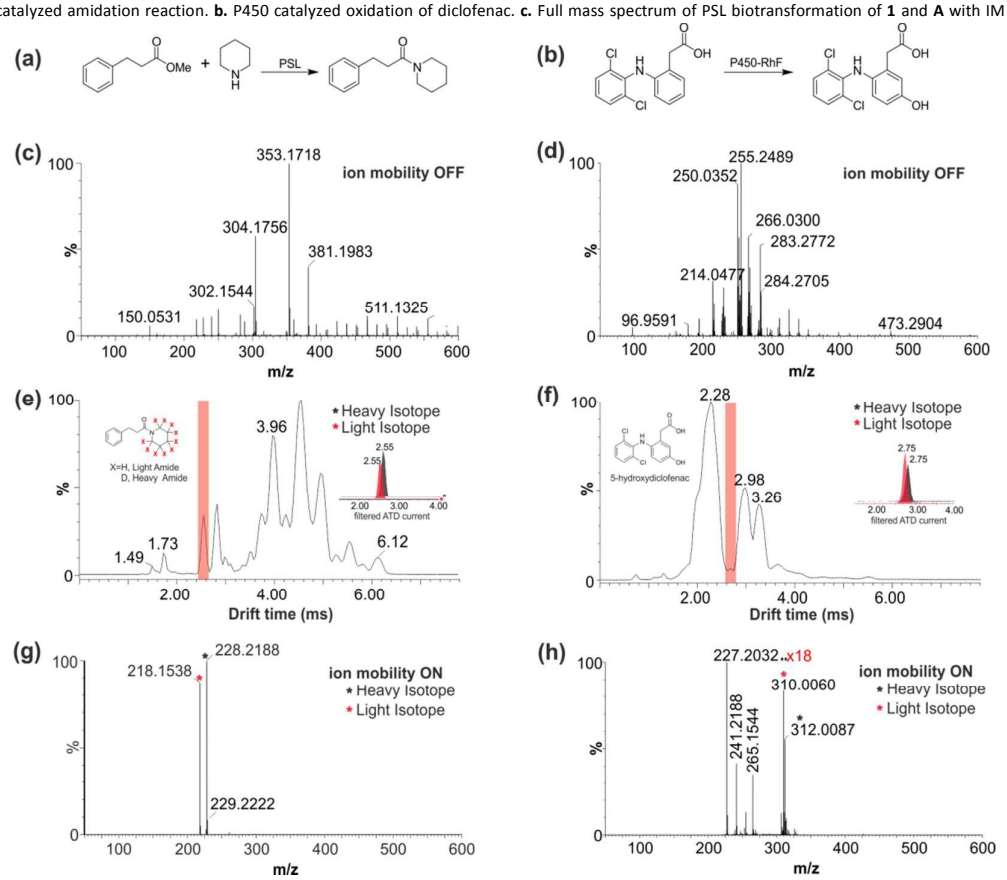
Figure 1. Overview of the experimental workflow of the present method in comparison to LC separation. Crude samples from biotransformations were centrifuged to remove solids and the supernatant injected directly into the IM-MS via a nanoESI source.

Figure 2. a. PSL catalyzed amidation reaction. **b.** P450 catalyzed oxidation of diclofenac. **c.** Full mass spectrum of PSL biotransformation of **1** and **A** with IM function OFF. **d.** Full



mass spectrum of P450 biotransformation of diclofenac with IM function OFF. **e.** ATD chromatography of PSL biotransformation utilizing IM. The peak of target amide **1A** (DT = 2.55 ms) was indicated with a red shadow in the spectrum. **g.** ATD chromatography of P450 biotransformation utilizing IM. The peak of 5-hydroxydiclofenac (DT = 2.75 ms) was indicated with a red shadow in the spectrum. **h.** Full mass spectrum of PSL biocatalytic reaction sample in DT = 2.55 ms with IM function ON, light and heavy forms of the target compound are labeled with red and black asterisks respectively. **i.** Full mass spectrum of P450 biocatalytic reaction sample in DT = 2.75 ms with IM function ON, light and heavy forms of the target compounds are labeled with red and black asterisks respectively.

The IM separation step also serves to decrease dramatically the chemical noise of crude biotransformation samples. Replacing HPLC separation with IM provides substantial reduction in both analysis time (milliseconds vs. minutes)[14] and in liquid handling volumes (µL vs. mL) and minimizes the use of solvents, all contributing to a significant reduction in the cost of the process. Additionally, the compatibility of IM with a "twin peak approach" outlined above ensures very high confidence in target identification. This workflow was applied to two distinct biotransformations. Firstly to lipase-catalysed amidations (Figure 2a), which are of interest as green alternatives for some of the most frequently used chemical reactions in the pharmaceutical industry. Secondly, we demonstrate the flexibility of our approach by applying it to the cytochrome P450 catalyzed oxidation of a commonly used therapeutic, diclofenac (Figure 2b). Both reactions have previously been monitored by chromatographic approaches,[15,16] which suffer from low throughput and require significant amounts of material.

For the amidation reaction, 1:1 mixtures of protiated/deuteriated substrates were used to track the reaction, in what we term a twin-substrate approach. Twinned MS signals corresponding to the heavy/light amide product were used to identify successful transformations. Initial work focused on the reaction of methyl 3-phenylpropionate (**1**) with a 1:1 mixture of labeled and unlabeled piperidine (**A** / D_{11}-**A**), catalyzed by lipase PSL from *Pseudomonas stutzeri*. Despite centrifugation, dilution and filtration, amidation samples were still very heterogeneous, due to organic soluble impurities extracted from the crude PSL protein preparation. This is evident from the full mass spectrum (Figure 2c) where impurities mask signals for the target amide **1A** (m/z 218.1539 and m/z 228.2188 for unlabeled/labeled). With the inclusion of IM separation (Figure 2e) the quality of the mass spectrometry data is improved dramatically (Figure 2g). As light and heavy forms of the amide are seen to have identical drift times (DT = 2.5 ms), integrating the areas of the arrival time distribution (ATD) corresponding to light/heavy product amides generates a ratio of 0.8:1, close to the initial 1:1 ratio of starting materials. Taken in context, this provides high confidence for product detection, even in cases of low signal. Using a standard quantitation assay we demonstrate linearity from 10 nM to 10 µM for detection of the amide **1A** (Figure S1). This new method is found to be three orders of magnitude more sensitive than HPLC-UVD which exhibited a limit of detection for the amide **1A** of ~50 µM (Figure S2).

Next, we investigated the P450 oxidation[17] of the nonsteroidal anti-inflammatory drug diclofenac, a biocatalytic reaction that is important for biotechnology, drug metabolism and environmental analysis.[18] Cytochrome P450-RhF from *Rhodococcus* sp. NCIMB 9784[19] catalyses this oxidation with high selectivity to yield 5-hydroxydiclofenac (Figure 2b). Instead of relying on a twinned product signal derived from a deuteriated substrate to confidently identify the product, the natural isotope ratio ($^{35}Cl/^{37}Cl$) of the chlorine atoms of diclofenac was used, and the product was identified by a pattern of light/heavy isotope peaks: dominant peaks appear at m/z 310.0060 ($^{35}Cl/^{35}Cl$) and m/z 312.0087 ($^{35}Cl/^{37}Cl$), while the weakest peak ($^{37}Cl/^{37}Cl$) is not shown due to the lower abundance. Although the overall signal was very low (Figure 2h), the product could be clearly detected and this highlights the power of our method. The experimental ratio of $^{35}Cl/^{37}Cl$ 5-hydroxydiclofenac was 0.78 (Figure 2f), also close to the theoretical value 0.64. As in the previous example, the diagnostic twinned peaks that are distinguished when IM-MS is used (Figure 2h) provide greater confidence in assignment than achieved from a mass spectrometry analysis alone (Figure 2d).

Accurate quantification of the lipase-catalysed amidation biotransformation was demonstrated by using only labeled amine in the reaction, thereby only generating labeled amide D_{10}-**1A**. Introduction of a set quantity (reflecting 20% conversion) of unlabeled **1A** to the first stage of sample dilution (post reaction) allowed accurate quantification of the labeled D_{10}-**1A** derived from the biotransformation. The ratios of light/heavy twin peak intensities were used to calculate conversions. Experiments run in parallel on HPLC-UVD matched well to results from nanoESI IM-MS (Figure S3). Using this technique we were able to accurately track the progress of the PSL catalyzed reaction over the course of 27 h (Figure S4). This demonstrates a high-throughput quantification dimension to our reported assay system.

In addition, our method was applied to the screening of multiple substrates in one-pot reactions. 1:1 mixtures of the light/heavy amine **A** (**A** / D_{11}-**A**), or ester **1** (**1** / D_6-**1**), were set against panels of esters (**1-5**) and amines (**A-E**), respectively (Figure 3), using PSL as the biocatalyst. 0 h, 5 h and 23 h reaction time points were monitored in all cases (Figure 4a-b). The filtered ATD chromatography with theoretical m/z of light/heavy amides shows the respective trends of the PSL catalyzed reactions (Figure 4c-d). Twin peaks of successful amide transformations were rapidly identified with high confidence (Figure 4e-f), with the now established transformation yielding **1A** (DT = 2.5 ms) being used as a positive control. Esters **3** and **5** were found to yield amides **3A** and **5A** (DT = 2.1 ms and 2.5 ms, respectively)**,** and amines **B** and **E** yielded amides **1B** and **1E** (DT = 3.3 ms and 3.5 ms, respectively). In all cases detected amides featured twin peaks with mass shifts of 10 Da for amides derived from a 1:1 mixture **A** / D_{11}-**A**, and 6 Da for amides derived from a 1:1 mixture **1** / D_6-**1**. The mass errors of light and heavy isotope labeled amides were less than 10 ppm in all cases. For the amides **5A** (DT = 2.8 ms) and **1E** (DT = 3.5 ms), two sets of twin peaks were observed. The second peaks in each pair were identified as the sodium adducts of **1A** (light form m/z 240.1345, heavy form m/z 250.1983) and **1B** (light form m/z 295.1805, heavy form m/z 301.2184), respectively. The protonated forms have different arrival times than the sodiated forms, and

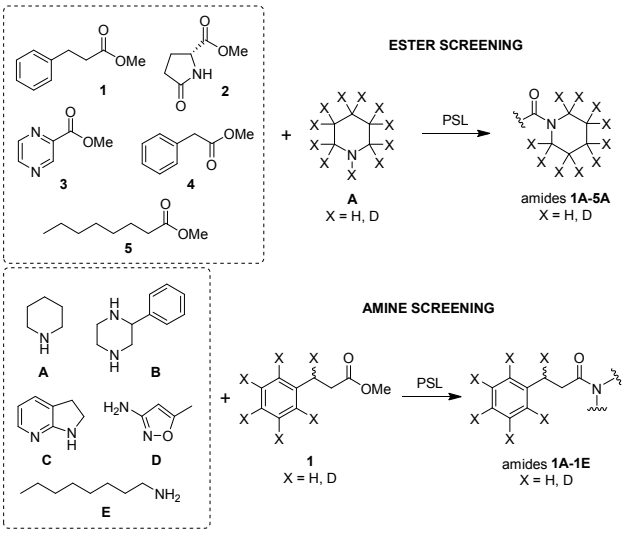


Figure 3. One-pot multi-substrate screening of esters and amines using the nanoESI IM-MS approach. The numbering of product amides is based on the combination of the corresponding ester (**1-5**) and amine (**A-E**).

deconvolution of the MS from the ATD allows unambiguous identification of each product whether protonated or sodiated.

The robustness of our system was demonstrated by screening a panel of lipases for amidation reactions: PSL from *Pseudomonas stutzeri*, CalB from *Candida antarctica*, PFL from *Pseudomonas fluorescens*, and PFL5963 an in-house recombinantly expressed lipase from *Pseudomonas fluorescens*. Accurate conversion efficiencies for all of these enzymes were determined through the use of the nanoESI IM-MS quantification method described above, and compared well with parallel HPLC analysis (Figure S3).

The use of a stable isotope/MS based system to screen a biocatalyst has been previously reported, making use of ESI-MS to monitor the kinetic resolution of racemic compounds or prochiral substrates.[20-22] In contrast, the method reported here focuses on analysis of crude samples of biotransformations that have been challenging to assay in a high-throughput manner, as well as providing a single analytical platform (IM-MS) to monitor the reaction. The addition of IM allows for the dramatic reduction of signal to noise ratio (increased sensitivity), permitting the use of crude samples yet maintaining a high-throughput timeframe. The one-pot multi-substrate screening protocol reported here provides immediate benefit in terms of throughput to biocatalyst development. Compared to HPLC-UVD, our method is found to be several orders of magnitude more sensitive and considerably faster. The method presented is semi-quantitative, and can be modified to a highly accurate quantitative approach with the use of internal standards of the unlabeled product compound. Importantly, the nanoESI-IM-MS method has the potential to be universally applied to any reaction system that involves mass change, where analysis of crude samples is necessary and low conversions are anticipated. Given the substantial gain in both speed and sensitivity, the method represents a step change in methodology for high-throughput analysis of biotransformation products.

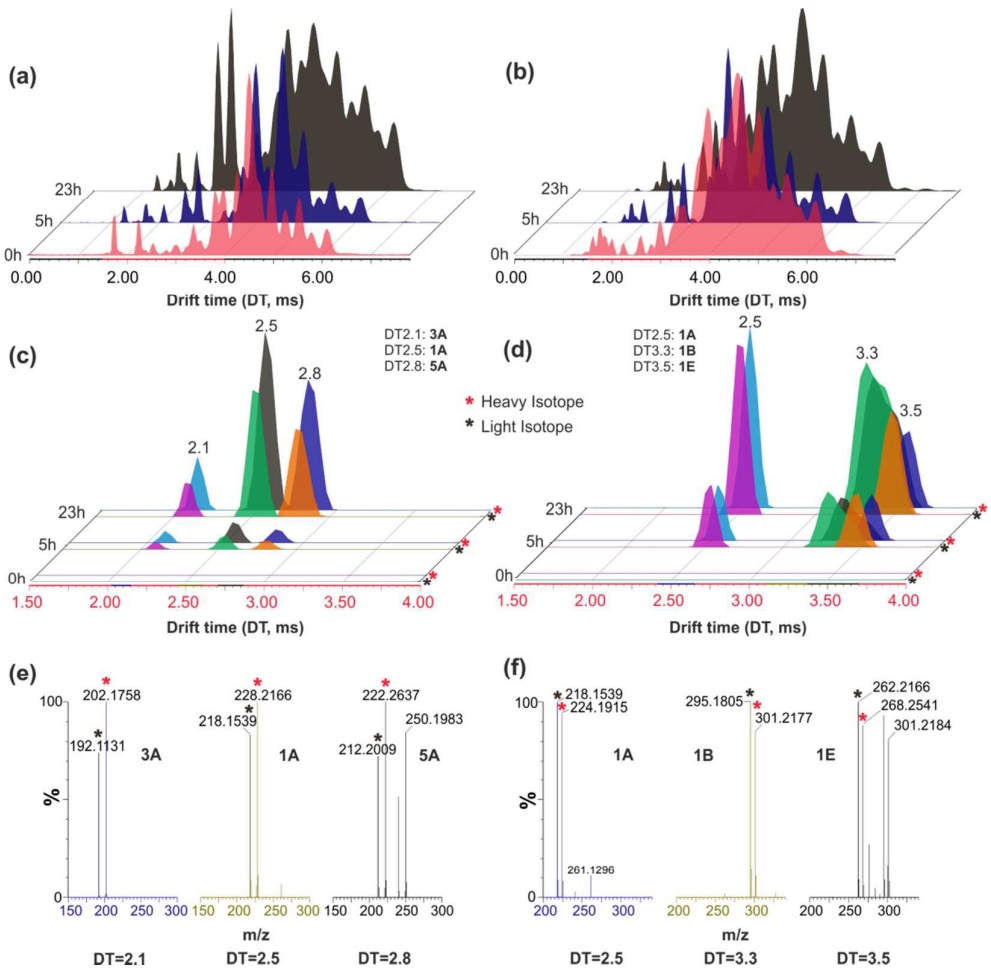


Figure 4. One-pot approach for multiple substrate screening with the biocatalyst PSL. (a) ATD chromatographs of the reaction of **1-5** with **A**, at times 0 h, 5 h and 23 h. (b) ATD chromatographs of the reaction of **1** with **A-E**, at 0 h, 5 h and 23 h. (c, d) Filtered ATD chromatograms with theoretical mass to charge ratio of putative amides at 0 h, 5 h and 23 h. (e) Full mass spectra of the products of the reaction of **1-5** with **A** at DT = 2.1 ms (**3A**), 2.5 ms (**1A**) and 2.8 ms (**5A**). The light and heavy form of each amide appear as equal intensity twin peaks with mass shift of 10 Da. (f) Full mass spectra of the products of the reaction of **1** with **A-E** at DT = 2.5 ms (**1A**), 3.3 ms (**1B**) and 3.5 ms (**1E**). The light and heavy form of each amide appear as equal intensity twin peaks with mass shift of 6 Da. Light and heavy forms of the target amides are labeled with black and red asterisks respectively.

Acknowledgements

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