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Mobile Tool for HPLC Reaction Monitoring

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Abstract:

A mobile HPLC reaction monitoring tool consisting of a cart-mounted microfluidic HPLC instrument equipped with a tethered, automated sampling and dilution module is described. Several examples of the use of the instrument for carrying out reaction progress analysis are presented. Reaction aliquot size is typically only a few microliters, allowing extensive sample monitoring from small volume reactions. Reaction quenching is possible, and aliquot dilution is adjustable, with suitable precision and accuracy even at hundredfold dilution. A sampling capillary with a chemically inert stainless steel fritted terminus allows sampling from some heterogeneous reactions. The sampling interval is adjustable, from a minimum of about 4 min, upwards. Visualization of an ongoing or completed study as either stacked "waterfall" chromatograms or as graphs of integrated peak areas (or any derived function, such as percent ee or percent conversion) vs time affords the process chemist valuable information on reaction kinetics and a useful record of reaction progress over time. While online HPLC analysis has been known for some time, the compact and mobile nature of this instrument renders it especially useful for carrying out reaction progress monitoring in the laboratory environment.

Introduction

Online/inline analysis is a powerful tool for monitoring organic reactions during process development and scaleup. The kinetic information obtained from such studies allows improved monitoring and control of reactions during routine production and also facilitates the design of robust processes and the understanding of reaction mechanisms. By eliminating the need for manual sampling and off-line analysis, online/inline analysis greatly reduces the labor requirement for reaction monitoring. The technique is especially useful in situations where some of the reaction components are labile or where the reaction matrix may be hazardous to the operator. Blackmond has recently commented on the value of reaction progress analysis in developing a greater understanding of organic reactions,[1] and the recent use of online/inline spectroscopic tools such as React IR, UV, and Raman probes as well as calorimetry by process chemists has led in many instances to improved understanding of reaction mechanism, which in turn may lead to better chemical manufacturing processes.[2–13]

In general, online reaction monitoring using HPLC provides more specific information than online spectroscopic techniques. For example, online HPLC analysis can be used to simultaneously monitor a variety of minor impurities, and can even be used for monitoring enantiopurity, both challenges for existing online spectroscopic techniques. While online chromatographic analysis has been known for some time,[14,15] most of the applications in this area have been focused on bioprocesses and ion analysis, with many examples confined to pilot plant applications where custom designed and permanently installed equipment are used. Online HPLC analysis is rarely used by the typical chemist "at the bench"; this is despite the fact that off-line HPLC, along with NMR, is by far the most commonly used analytical tool for reaction progress analysis among organic process research chemists. When considering the benefits of a recently developed microfluidic HPLC system (small size, fast run times, and extremely low mobile phase usage and waste generation), we were struck with the possibility of using such an instrument for a mobile HPLC reaction monitoring system. We reasoned that such a tool could replace the sporadic off-line HPLC analysis typically performed by process chemists to monitor reaction progress, allowing more complete data coverage while eliminating the need for scientists to carry out tedious sampling, dilution, HPLC analysis, data transfer, and graphing functions. Ideally, such a tool would operate in the background, requiring only minimal input from

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the chemist, who could ignore results when nothing of interest is observed, but would have a complete record of the reaction when something interesting, useful, or important emerges.

In this study, we report the design, development, initial testing, and subsequent modifications of a mobile HPLC reaction monitoring system. This project was a collaborative effort between a pharmaceutical process research group and an instrument supplier, where detailed knowledge of the needs of the end user provided by the pharma group was helpful in guiding the system engineering, software development, and product design expertise of the instrument company, ultimately leading to a commercial device for carrying out a much needed function to support process research investigations. We present several examples of the use of the mobile tool for performing online HPLC analysis in support of pharmaceutical process research.

Experimental

The prototype HPLC reaction monitoring instrument (AliquotMobile) described in this study is based on the ExpressLC-100 microfluidic HPLC instrument. (Eksigent Technologies, Dublin, CA), with a number of modifications that are described in detail in the results and discussion section. A commercial version of the instrument is now available as the ExpressRT-100.

Model compounds, *p*-bromophenol, 1,1′-bi-2-naphthol, propiophenone, acetophenone, triethylamine, and acetic anhydride) were obtained from Aldrich Chemical Company, Milwaukee, WI. The HPLC solvents, acetonitrile, and water were obtained from Sigma-Aldrich, St. Louis, MO. Compounds depicted in Figures 8, 9, and 10 are from ongoing projects under investigation in these laboratories.

Results and Discussion

Initial considerations based on the perceived needs of process research chemists for a mobile online sampling tool led us to the identification of a number of important features that the instrument should possess. At the outset, mobility was a key design feature, since the instrument would be shared by a number of users and should consequently be movable from hood to hood. The ability of the instrument to perform fast chromatography and to allow a fast sampling interval was deemed important, as this feature would dictate the utility for characterizing faster reactions. The ability to quench or dilute samples with diluents other than HPLC mobile phase was deemed important, as this would allow for the possibility of reaction quenching by addition of specific reagents and would allow for adjusting sample polarity for appropriate dissolution of sample and optimal chromatography. Similarly, an adjustable wide range of dilution was deemed important, so as to be able to draw samples from either concentrated or dilute reactions and to dilute appropriately for optimal chromatography. Small sampling volume was considered to be important owing to the fact that many reactions in the early stages of process research investigations, particularly at the high throughput screening stage, are carried out in very small volumes, often a milliliter or less. Resistance to sample clogging—the ability to sample

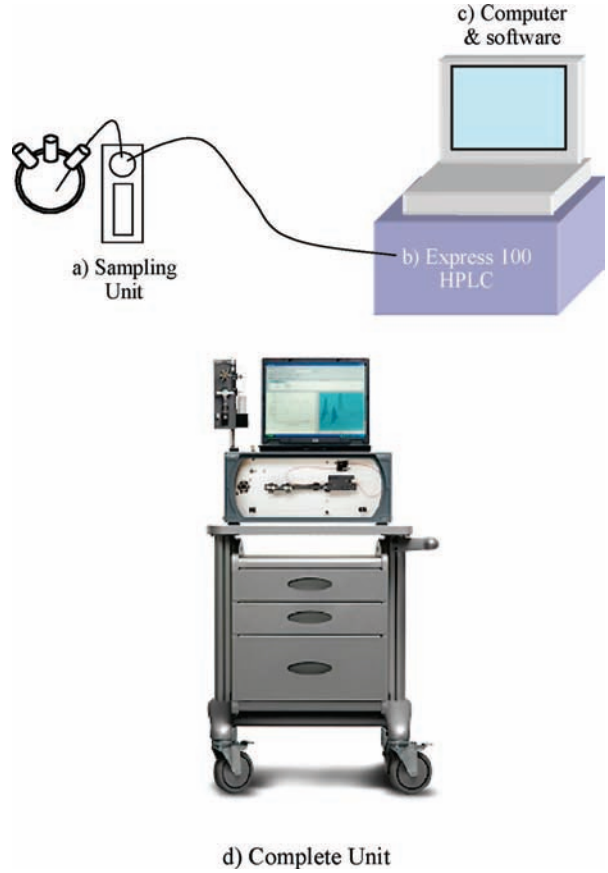

Figure 1. **Schematic illustrating components of the AliquotMobile online HPLC reaction monitoring tool. (a) Sampling and dilution unit. (b) Eksigent ExpressLC-100 microbore HPLC. (c) Computer and software. (d) Photograph of the entire unit.**

from heterogeneous reactions—was also considered to be an important criterion, as completely homogeneous reactions constitute only a small fraction of the reactions typically studied in a process research environment. The ability to sample from reactions at a wide range of temperatures or from reactions under pressure was also considered to be an important, but perhaps less easily achieved objective.

Initial design considerations based on this set of desired features led to the general design depicted in Figure 1, which was chosen for the initial prototype. The problems of extreme temperature range and sampling from heterogeneous reactions and from pressurized reaction vessels were not addressed in the initial design and development stage. We reasoned that a simple prototype without these added features would still be of considerable value to process chemists and that additional capabilities could be incorporated at a later date, if needed. The system is comprised of three main components: (a) a microbore HPLC system capable of very rapid gradient separations, (b) a compact sampling unit that performs inline sampling and dilution functions, and (c) a computer and system software.

The HPLC instrument (ExpressLC-100,[16] Eksigent Technologies, Dublin, CA) used in the monitoring system is a relatively compact, high performance, microbore system with

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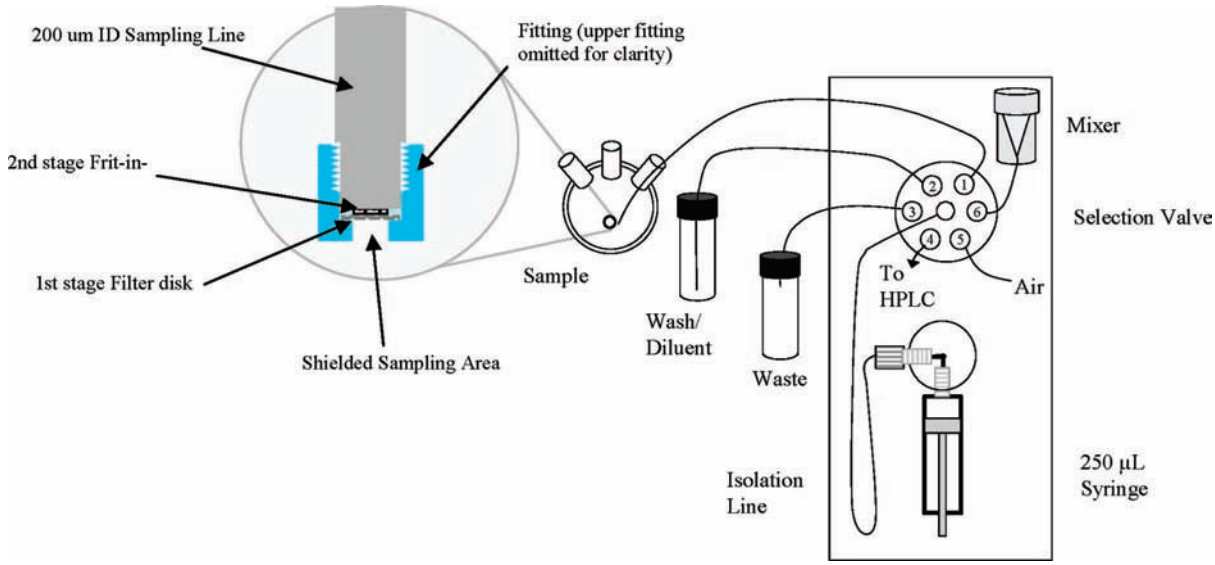

Figure 2. **Sampling unit schematic for the inline HPLC reaction monitoring tool.**

characteristics well suited for carrying out fast chromatographic analysis.[17–20] The compact nature of the HPLC makes it well suited for use in the mobile reaction monitoring HPLC system. Hardware components (pumps, flow meters, valves, column, linear array UV detector, etc.) are integrated into a single compact unit with width, depth, and height dimensions of 19 in. × 18 in. × 8 in., respectively. The HPLC system offers the widely recognized performance advantages of microbore chromatography.[21] Unlike previous HPLC pumping systems for the 1–10 μL/min range, continuous feedback from mobile phase flow meters to the high-pressure pumps provides very reliable and reproducible flow gradients.

The sampling unit and associated software was designed and developed specifically for this organic reaction monitoring project. With dimensions of roughly 2.5 in. × 6 in. × 8 in., the sampling unit is small enough to be mounted on the standard 0.5 in. support rods typically found in an organic laboratory fume hood. A schematic diagram of the sampling unit is shown in Figure 2. The unit consists of a miniature syringe pump, an analytical-quality six-position valve, and a mixing chamber. Wetted parts such as tubing and fittings are made of borosilicate glass, fused silica, or premium fluoropolymers. Exceptions include the disposable sampling filters that are made of PEEK or 316L stainless steel and the analytical valve that has DuraLifeIITM coatings on wetted parts.

The sampling unit is controlled through electronic communications via RS-232 and synchronized control of multiple units is enabled via RS-485. Several inputs and outputs are included on the unit for control of additional devices such as pressure transducers, selection valves, and solenoids. The sampling module also includes programmable auxiliary analog and digital input/output (I/O) connections.

The three main components in the unit (syringe, valve, and mixer) plus ancillary devices and flexible software allow a wide range of custom methods for sampling and diluting. However, typical operation of the sampler proceeds as follows:

1. A 10–20 uL potion of sample is withdrawn from a reaction vessel through a two-stage particle filter.
2. A portion of the sample is metered out and delivered to the mixer.
3. A metered amount of a solvent is delivered to the mixer to quench and dilute the sample.
4. The contents of the mixer are mixed.
5. The mixer contents are delivered to the injection valve on the HPLC.
6. The mixer and nearby tubing are rinsed in preparation for the next sample.

Sample dilution is a very common task in both analytical and industrial process environments. Thus, numerous dilution approaches have been developed and are widely applied. In developing this HPLC reaction monitoring system, many dilution approaches were considered as were the advantages and limitations of each approach. We evaluated a number of dilution strategies, including continuous flow dilution with precision pumps, membrane dilution, cascade dilution, and a variety of methods based on dispersion.[22] We settled on a design that offered the benefits of mechanical simplicity and compact size, while providing consistent dilution across the wide range of samples encountered, with good accuracy and precision.

Software was created to allow control of dilution and sampling interval and duration using a method approach analogous to that typically used for HPLC instrument control. In addition, software was created to enable visualization of the results of an online monitoring experiment using either stacked "waterfall" chromatograms or integrated peak areas vs. time, or a variety of math functions obtained from the integrated areas

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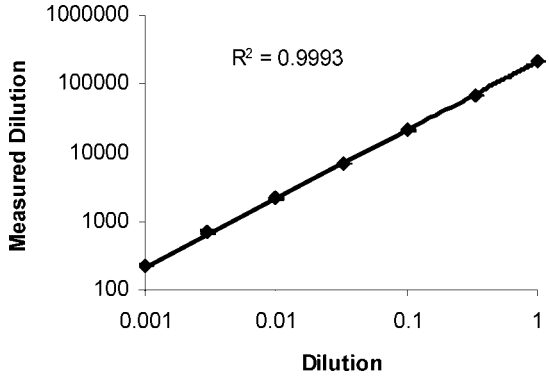

Figure 3. **Dilution precision and approximate linearity using the prototype AliquotMobile HPLC reaction monitoring tool. Dilutions at 0.001, 0.003, and 0.01 (1000×, 333×, and 100×) were obtained by sampling from a standard solution of 10 μL/mL acetophenone in 80:20 acetonitrile:water, while dilutions at 0.3 and 1 (3.3× and 1×) were obtained by sampling from a standard solution of 0.1 μL/mL acetopnenone in 80:20 acetonitrile:water. The conditions were as follows: (column) WakoSil 3 μm C_{18} 0.3 mm × 50 mm; (mobile phases) 0.1% aqueous trifluoroacetic acid (aq), 0.085% TFA in acetonitrile (org); (gradient) 10:90 50:50 aq/org to 10:90 aq/org in 2 min; (flow rate) 4 μL/min; (detection) UV @ 242 nm; (injector settings) 60 nL injection.**

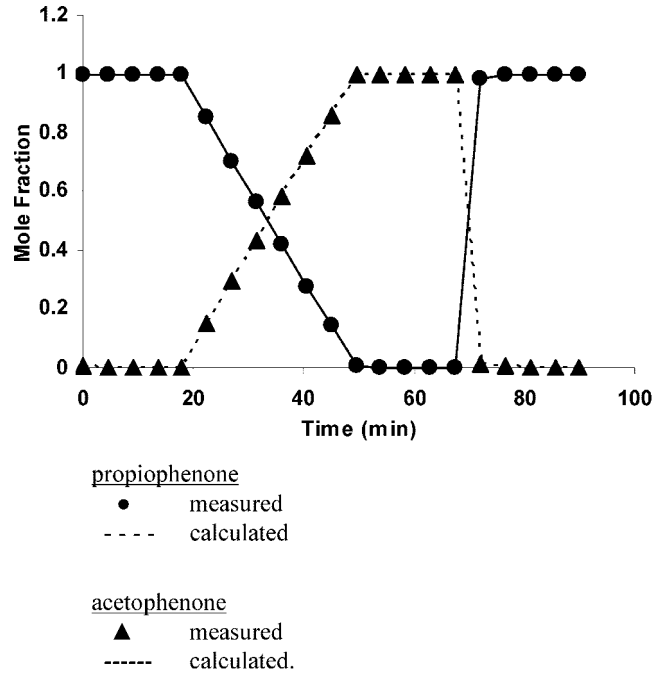

Figure 4. **Performance of the prototype instrument in determining the mole fraction of a set of eight standard mixtures of acetophenone and propiophenone using manual movement of the sampling capillary from vial to vial. HPLC and sampler conditions were those described in Figure 3.**

(liquid chromatography area percent (LCAP), percent conversion, percent ee, etc.)

With the initial prototype in hand, we set out to evaluate the performance of the instrument using a set of straightforward experiments, initially involving simply sampling from solutions of standard mixtures to determine dilution precision, linearity, and response time. Figure 3 shows dilution linearity and approximate precision for 1 to 1000× dilutions of acetophenone solutions in 80:20 acetonitrile/water. To maintain an optimal area count for the diluted samples, a 10 μL/mL of acetophenone in 80:20 acetonitrile:water was used for 0.001× and 0.003× dilution, 1 μL/mL for 0.01× and 0.03× dilution and 0.1 μL/mL for 0.1×, 0.3×, and 1× dilutions. Five or more samples at each concentration were averaged and used for precision estimates shown on the graph. Data was fitted to a line using a weighted least-squares fit. The reciprocal of the standard deviations for each dilution were used as weights. The excellent R^2 value of 0.9993 indicates satisfactory linearity over this dilution range.

The ability of the system to respond adequately to changing reaction conditions was next examined. For our initial tests, concentrations of two compounds (propiophenone and acetophenone) were varied across a series of eight vials. As indicated in the graph in Figure 4, the relative mole fractions of the two compounds in the eight vials were 0, 1/7, 2/7, 3/7, 4/7, 5/7, 6/7, and 1. To mimic the course of a reaction, multiple samples were acquired from the first vial, followed by manually moving the sampling probe to consecutive vials. To mimic a sharp concentration change, multiple samples of the last vial were followed by samples from the first. The sampling system tracked very well with the worst error (1.4 %) occurring at the largest concentration transition. This transition of 100–0 percent represents a much worse scenario than would occur in real-world reactions occurring over comparable time scales.

We next used the instrument to investigate a sample where concentration was changing continuously over time. In order to allow convenient adjustment and study, an experimental setup involving syringe pump addition of to a stirred vial (Figure 5a) was used. In these studies, the expected increase in concentration over time was observed as a linear increase in the integrated peak area attributed to the propiophenone peak in successive chromatographic injections.

Similarly, syringe pump addition of a solution of propiophenone to a stirring solution of acetophenone afforded the results shown in Figure 6. In this instance, a more complex curved shape is obtained, as the concentration of propiophenone increases as more solution is added, while the acetophenone concentration decreases through dilution. The ability of the instrument software to express the results of an experiment in terms of peak area (or peak height, retention time, etc.) or any math function derived from combinations of these primary measurements is a very useful feature. For example, analysis of peak area vs time or LCAP (LC area percent vs time, Figure 6a) demonstates a quite smooth appearance. However, Figure 6b, in which a function of the ratio of the two peaks is graphed, clearly reveals the point at which the syringe pump ran out of fluid.

With these preliminary results in hand, we next turned to the exploration of some actual reactions, restricting ourselves to room temperature homogeneous reactions for the sake of simplicity. We found that DMAP catalyzed acylation of phenols using acetic anhydride could easily be monitored by this approach. A waterfall plot showing chromatograms from the acylation of *p*-bromophenol is shown in Figure 7a, with the corresponding plot of LCAP vs time shown in Figure 7b. Interestingly, a decrease in the reaction rate over time can be

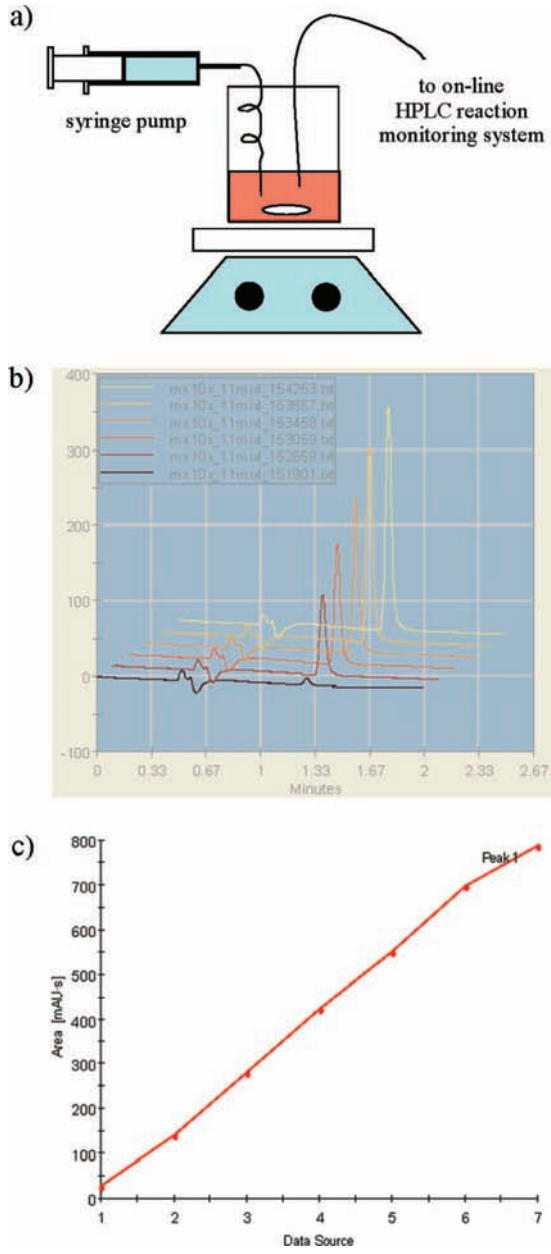

Figure 5. **HPLC timecourse monitoring of the syringe pump addition of solution of propiophenone to acetonitrile/water. (a) Experimental setup; 2 mg/mL solution of propiophenone in 80% acetonitrile/water added via syringe pump (3 mL/h) to 5 mL of solution of 80% acetonitrile/water. (b) Waterfall plot showing stacked chromatograms with increasing peak area vs time. (c) Plot of integrated peak area vs time. HPLC and sampler conditions were those described in Figure 3, with additional conditions of a 0.8 min equilibration time, a 10× sample dilution setting using 80% acetonitrile/water as diluent, and a sampling interval of 4 min.**

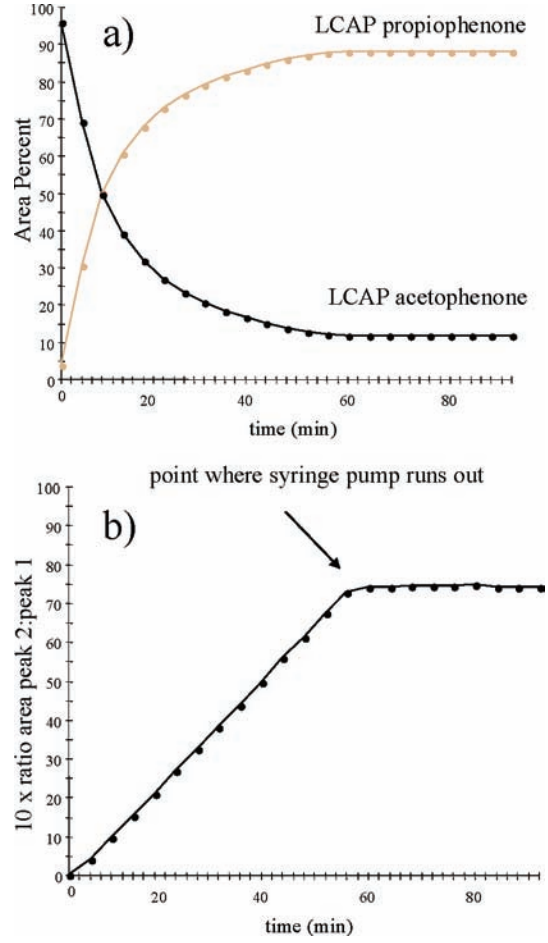

Figure 6. **HPLC timecourse monitoring of the syringe pump addition of a solution of propiophenone to a solution of acetophenone in acetonitrile/water. The conditions were as follows: 1 mg/mL solution of propiophenone in 80% acetonitrile/water added to 1 mL of 0.3 mg/mL acetophenone in 80% acetonitrile/water at a rate of 5 mL/h; HPLC method as in Figure 5. (a) Plot of LCAP vs time. (b) Plot of the function of the ratio of the two peak areas vs time, revealing the point at which syringe pump ran out of liquid.**

noted, presumably resulting from the acidification of the catalyst. Similarly, acylation of the bis-phenol, β-binaphthol, is shown in Figure 7c. In this example, disappearance of starting material can be seen to be very fast, with accumulation of the mono-acyl intermediate ultimately giving way to formation of the doubly acetylated product. Interestingly, extensive sampling of these reactions was possible despite the fact that the reaction volume was only 1 mL.

We next turned to investigations of actual reactions being carried out in a process research environment. Attempts to utilize the instrument for study of heterogeneous reactions led, not surprisingly, to problems with clogging of the sampling line. A variety of possible solutions to the sampling problem were considered and evaluated, ultimately leading to the relatively simple terminal hardware modification of the sampling capillary depicted in Figure 2. In this approach a small filter screen and housing is added at the capillary terminus, effectively blocking the entry of particles larger than the 2 μm screen size of the filter. Using this modified device, sampling from some heterogeneous reactions proved possible, including stirred solutions containing powdered activated carbon. However, reactions in which active precipitation is occurring proved to still lead to significant problems with clogging.

With the modified instrument in hand, we set out to evaluate a variety of routine reactions taking place in the process research environment. In the example presented in Figure 8, a kinetic study of the acid catalyzed introduction of a tetrahydropyranyl

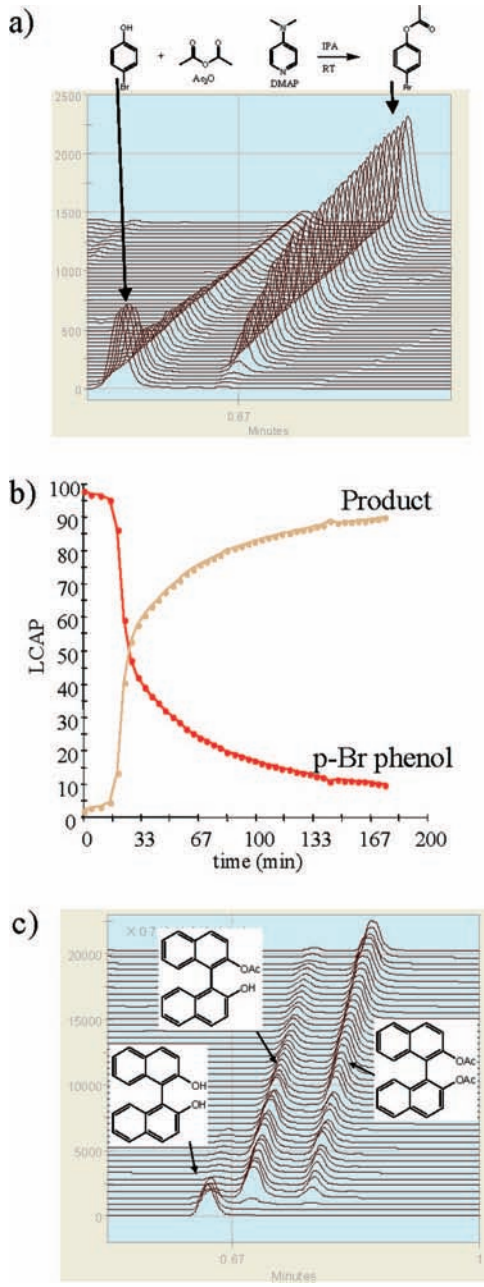

Figure 7. **Monitoring timecourse of DMAP catalyzed acylation of phenols with acetic anhydride. (a) Waterfall plot showing acylation of *p*-bromophenol. (b) Plot of LCAP vs time, showing slowing of the reaction rate over time (presumably owing to acidification of catalyst). (c) Waterfall plot showing acylation of *β*-binaphthol.**

(THP) protecting group onto pyrazole[23] was carried out prior to reaction scale-up. The timecourse plot (Figure 8) clearly shows that that high conversion and yield of the protected heterocycle can be achieved in a reasonable timeframe using equimolar quantities of pyrazole and the protecting reagent (3,4-dihydropyran). Consequently, it was possible to develop a more

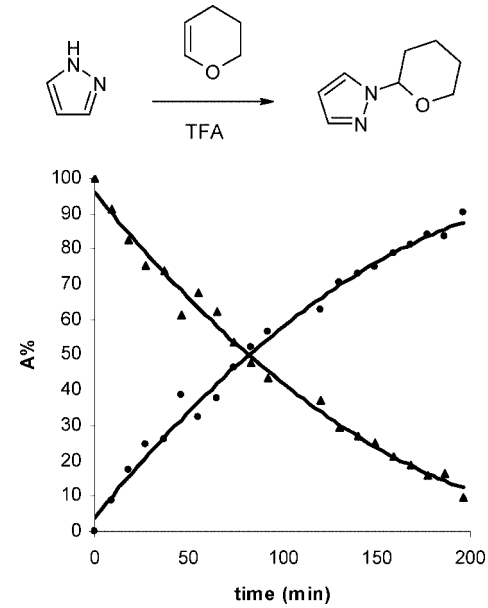

Figure 8. **Reaction timecourse for THP protection of pyrazole. The sampler conditions were as follows: 1000× with acetonitrile. (HPLC conditions) 95:5 0.1% H_3PO_4(aq):acetonitrile to 60:40 in 3 min at 10 *μ*L/min, 210 nm detection.**

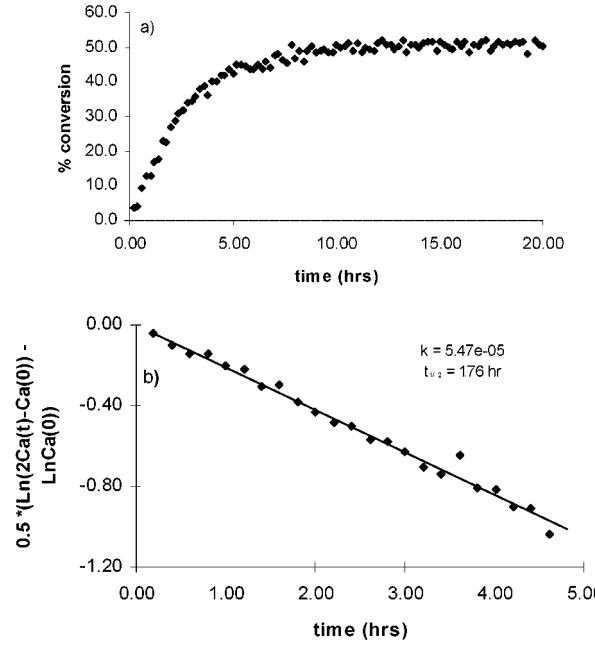

Figure 9. **Racemization of an enantiomerically pure cyclic drug candidate in ethanol. (a) Plot of area percent of the undesired enantiomer vs time. (b) Kinetic plot. The conditions were as follows: (sampler conditions) 30× dilution with 80:20 acetonitrile:water, 45 nL injection; (HPLC conditions) 80:20 0.1% H_3PO_4:acetonitrile to 5:95 0.1% H_3PO_4:acetonitrile in 10 min, flow rate 8 *μ*L/min, 220 nm detection.**

refined version of the original literature procedure in which a 50% excess of 3,4-dihydropyran was employed.

While the pyrazole protection could probably be effectively monitored using IR or other spectroscopic approaches, the example shown in Figure 9 would not be amenable to such approaches. In this example, racemization of a key intermediate in the synthesis of a preclinical candidate was investigated using normal phase chiral chromatography. The results clearly show

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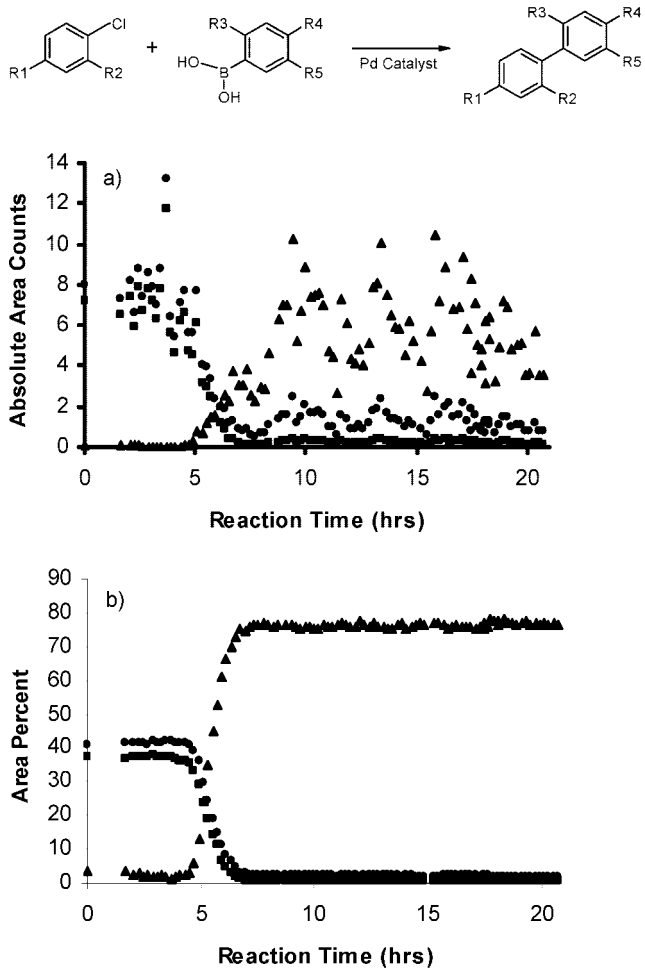

Figure 10. **Monitoring timecourse of a biphasic Suzuki coupling reaction. (a) Raw area counts of the reaction components vs time. (b) Plot of the area percent of the components vs time (● boronic acid starting material, ■ aryl chloride starting material, ▲ coupled product): (sampler conditions) 30× dilution with 80:20 acetonitrile/water, 45 nL injection; (HPLC conditions) 80:20 0.1% H_3PO_4:acetonitrile to 5:95 0.1% H_3PO_4:acetonitrile in 10 min, flow rate 8 μL/min, 220 nm detection.**

the increase in the minor enantiomer over time, to the equilibrium 1:1 mixture of enantiomers. Graphical analysis allows convenient access to rate constant and racemization half-life—key pieces of information for informing process development on this project.

In a more challenging reaction, a biphasic Suzuki coupling reaction taking place in a rapidly stirred mixture of heptane and water was studied. Withdrawing an aliquot from a rapidly stirred biphasic reaction is always somewhat problematic, as considerable variation of the relative amounts of the two phases is possible, especially if the sampling size is small. In the case of the biphasic Suzuki reaction, a significant variability in the absolute peak areas for the two coupling partners and final product were observed (Figure 10a). However, simply expressing the data as LCAP (LC area percent) effectively shows the desired information about the progress of the reaction, effectively normalizing the data (Figure 10b) and allowing the study of an interesting induction period and catalytic onset that was otherwise difficult to follow.

Subsequent use of the HPLC reaction monitoring tool for timecourse analysis of a variety of reactions in the process research environment has shown significant practical value. The design of the instrument continues to evolve to enable more effective coverage of a wider variety of reaction types, but already, the instrument shows considerable promise as a valuable analytical companion for working process chemists.

Conclusion

A mobile HPLC reaction monitoring instrument was developed for carrying out reaction progress analysis. The chromatographic nature of the unit affords versatility, specificity, and sensitivity and gives it distinct advantages over strictly spectroscopic online analytical techniques. Specifically, the ability of the unit to track reaction species with very similar functional groups as well as individual enantiomers was demonstrated in this work. The utility was broader than originally envisioned, with the unit able to provide valuable reaction profiling data for biphasic reactions. While online HPLC analysis has been known for some time, the compact size and microfluidic nature of this instrument makes it ideal for the limited space often found in the laboratory environment. The small sampling amount of the unit allows for small reaction scales, and the low chromatographic flow rate of the chromatograph reduces both solvent and reagent use, important green chemistry considerations. The results reported here suggest that online HPLC analysis may become an important analytical tool in the process chemist's quest for understanding and optimizing process reactions.

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