Accelerating Students’ Learning of Chromatography with an Experiential Module on Process Development and Scale-Up

Mingrou Xie¹, Pavan Inguva¹,², Wenqian Chen¹*, Nicholaus Prasetya¹, Andrew Macey¹, Peter DiMaggio¹, Umang Shah¹, Clemens Brechtelsbauer¹*

¹Department of Chemical Engineering, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom
²Institute of High-Performance Computing, Agency for Science, Technology and Research (A*STAR), 1 Fusionopolis Way, #16-16 Connexis, Singapore 138632, Singapore

ABSTRACT
The objective of the presented module is to train students with no background in process development and scale-up of chromatographic processes to a high level of competency within forty contact hours. The key pedagogical approach is ‘progression’ where students’ capabilities are gradually built up with appropriate scaffolding provided at each stage of their learning. The module is broken up into three steps with each step covering a different aspect of chromatography. Knowledge gained in one step is the foundation for work in the next. In the first step students investigate several chromatographic column packing materials and perform a solvent selection process. Design of Experiment (DOE) to systematically vary process parameters for method development is introduced in the second step. In the last step, students use a preparative-LC system to perform a larger scale separation. Students explore different scale-up scenarios, including volume fraction collection and column overloading. Pedagogic outcomes of the module were determined through surveys, interviews and personal interaction during the study. Results clearly indicate that students engaged well with the module while meeting overall learning objectives. The module is equally suitable for third or fourth year university students or industry practitioners unfamiliar with chromatography as part of continuing professional development.
GRAPHICAL ABSTRACT

KEYWORDS
Second-Year Undergraduate, Upper-Division Undergraduate, Continuing Education, Analytical Chemistry, Chemical Engineering, Laboratory Instruction, Hands-On Learning / Manipulatives, Inquiry-Based / Discovery Learning, Chromatography, Separation Science

INTRODUCTION
Chromatographic separation techniques are highly valued in the fine chemicals and pharmaceutical industry\(^1\) due to their ability to provide reproducible results and readily meet purity specifications. In addition, chromatography is also heavily used as an analytical technique in almost any chemical related industry. This widespread use of chromatographic techniques, therefore, presents a strong justification to train students in using them during their undergraduate course. Particularly for chemical engineers, developing an early appreciation of both the separation and analytical aspects of chromatography would be of immense use for their later careers. Students entering higher education may have had some exposure to chromatography during secondary education in the form of separating ink on paper using a solvent\(^3\). The teaching challenge then
becomes to design a module that simultaneously builds up students’ capability with the right scaffolding, whilst fostering independent learning and exploration.

There are many examples of teaching high performance liquid chromatography (HPLC) as an analytical tool to chemistry students. These modules typically use items which would be both accessible and interesting to students such as tea\textsuperscript{3}, plant extracts\textsuperscript{4} or dairy\textsuperscript{5}. With these model systems, the various analytical aspects are then explored, such as quantitative analysis of species and measurements of antioxidant activities\textsuperscript{4}. Thin-Layer Chromatography (TLC), valued for its simplicity and cost\textsuperscript{6}, is another chromatographic analytical tool frequently taught to and used by undergraduates\textsuperscript{7}. Such experiments incorporate TLC amongst other analytical techniques of accessible systems such as caffeinated beverages\textsuperscript{8} or use TLC as the analytical step following other activities such as synthesis or extraction\textsuperscript{9,10}.

The use of preparative chromatography for teaching purposes has been rather limited. One case study\textsuperscript{11} incorporated preparative HPLC as a means of exposing upper-division students to its capability for separating and recovering high value compounds. In that case, a valuable vegetable extract was desired, and students were tasked to perform all steps of the process, from extraction to polishing by preparative HPLC. This provided an authentic learning experience to students as the desired compound was not readily available from suppliers. Another example of introducing preparative chromatography was described by Hahn et al., 2015\textsuperscript{12} where upper-division students were tasked to model a bio-separation process and subsequently separate a protein mixture using preparative ion-exchange chromatography.

Modules that focus on teaching chromatography as an analytical tool usually target lower-division students such as 2\textsuperscript{nd} year undergraduates. They emphasize developing competencies in the use of these analytical techniques over the engineering aspects such as optimising process parameters. The experiments on preparative chromatography on the other hand are less accessible to lower-division students, as they require prior knowledge and advanced experimental skills to carry out such a task. This presents an opportunity for educators to design a chromatography module which enables students to seamlessly develop from novice to competent user level by providing appropriate support and scaffolding. The experiential learning module described in this paper takes on this challenge and
provides a systematic framework for teaching chromatography with an engineering focus on separation and purification, thus up-skilling students in both the analytical and separation aspects of chromatography.

**COURSE OVERVIEW**

This module was offered as one of multiple options that 2nd year undergraduate students could choose from for their practical laboratory component of the year. Students taking this module would have completed a general chemistry course, an introductory course in MATLAB as well as a first-year laboratory course which trains essential experimental skills such as safely handling chemicals, operating equipment and recording / analysing data. By the time students complete the module, they would have also attended a co-current course on non-ideal thermodynamics. Details on the resources available to students during the module can be found in the pedagogy section. The module is broken down into three steps with step one, the introductory step, being at a difficulty level that is immediately accessible to the student and each step progressively becoming more challenging as they are exposed to new and increasingly complex material. The 40 contact hours are distributed over three weeks, with each step taking one week. In this module, students are provided with a sample of degraded aspartame and are tasked to employ chromatographic techniques to purify and recover the aspartame. The reasoning for choosing aspartame as a model system is described in the methods section below.

Step one tasks students to use TLC to analyse a sample of degraded aspartame that is to be purified. They identify the degradation pathway and attempt to find a suitable solvent composition for good separation. In step two, students need to develop a suitable method on an analytical HPLC system to quantitatively analyse the sample. They also need to explore various process conditions such as composition, mobile-phase flowrate and column temperature. In the last step, students utilize a preparative HPLC system to perform the separation at a larger scale and optimize the system.

**EXPERIMENTAL METHODS**

**Choice of Chemical System**

Aspartame is a dipeptide and is commonly used as an artificial sweeter. It is known to decompose in certain conditions, forming various degradation products. It is frequently used in educational
settings, focusing on its synthesis and degradation analysis such as kinetics and products.

Aspartame thus provides an excellent model system for the experiment due to the availability of information. It also provides realistic training from a fine chemicals separation and industrial applicability perspective. The partially degraded sample provided to students for analysis was prepared at pH 7 with a 0.01M phosphate buffer, causing the degradation product to be 3-carboxymethyl-6-benzyl-2,5-diketopiperazine (DKP).

**Thin Layer Chromatography – Step 1**

TLC was chosen for the basis of step one as it is conceptually and operationally similar to what students would be familiar with as previously discussed. Both normal (TLC Silica gel 60 F$_{254}$, Merck KGaA) and reverse phase (TLC Silica gel 60 RP-18 F$_{254{s}}$, Merck KGaA) TLC plates were provided for students to choose from. The students’ choice of TLC plates and the effect they have on the resultant separation helped reflect the significance of using different packing in chromatography, in this case highly polar and highly non-polar stationary phases, respectively.

For reference against the partially degraded sample of aspartame, individual solutions of 0.1 g/L concentration were prepared for pure aspartame, phenylalanine, aspartic acid and DKP. These were spotted onto (8 x 4 cm) TLC plates with 1μL capillary tubes alongside the partially degraded sample. The TLC plates were then developed in solutions of varying water and acetonitrile (ACN) ratios, from 10% v/v water to 90%. Students appreciated the use of TLC as a quick method for narrowing the range of desired solvent ratios before scaling up to analytical and preparative HPLC.

To enable independent inquiry, multiple common visualising options were also made available for students to choose from. These included the use of chloranil, ninhydrin, iodine, potassium permanganate and a 254 nm UV lamp. Students were expected to select the appropriate visualizing agent and be able to explain the reason for their efficacy in spot visualization of different compounds.

**Analytical HPLC – Step 2**

A Shimadzu LC-2030C 3D Plus Prominence-i HPLC system with a Photodiode Array (PDA) Detector set to collect absorbance data at wavelengths of 220 and 254 nm and equipped with a C18 column (Capcell Pak 250 mm x 4.6 mm, 5μm, Shiseido) was used. The mobile phase, which is the same across
Steps 2 and 3, consists of water with 0.1% v/v trifluoroacetic acid (TFA) and acetonitrile. Students were provided with the same degraded solution from Step 1 for analysis.

To point students in the right direction during the limited laboratory time, a template for a simple full factorial design of experiments (DoE) to optimize the separation was provided in the handout for Step 2. Through the template, students could vary three variables – mobile phase flowrate, column temperature and solvent composition – in order to optimize the separation. A possible criterion for adequate separation would be a peak resolution of ≥1.5.17 Variable bounds especially on the solvent composition are provided so that students will not be overwhelmed during method development. Students are also encouraged to explore the concepts of DoE and other important aspects of HPLC further through the provision of guiding questions in the handout.

**Preparative HPLC – Step 3**

An Agilent ProStar preparative HPLC system was used for separation. The system consisted of an Agilent ProStar 410 Autosampler, Agilent 1200 Series Manual Injector with a 5mL sample loop, Agilent ProStar Multiwave Length Detector (MWD), Agilent 440-LC Fraction Collector and an Agilent Zorbax SB-C18 column with dimensions 21.2mm X 250mm and 7µm particle size. Due to calibration issues with the Agilent system HPLC pump, an alternative Knauer Preparative Pump 1800 was used. The solvents were premixed, gravity filtered and degassed using an ultrasonic bath. Analysis of the sample before and after separation was carried out using analytical HPLC to determine the purity and recovery of the separation process.

The initial estimate for the flowrate and injection volume to be used on scale-up was estimated by equations (1) and (2).18

\[
Flow_{\text{Prep}} = Flow_{\text{Ana}} \times \left( \frac{D_{\text{Column}, \text{Prep}}}{D_{\text{Column}, \text{Ana}}} \right)^2 \times \left( \frac{D_{\text{Particle}, \text{Prep}}}{D_{\text{Particle}, \text{Ana}}} \right)
\]  

(1)

\[
V_{\text{Inj}, \text{Prep}} = V_{\text{Inj, Ana}} \times \left( \frac{L_{\text{Column}, \text{Prep}} \times D_{\text{Column}, \text{Prep}}^2}{L_{\text{Column}, \text{Ana}} \times D_{\text{Column}, \text{Ana}}^2} \right)
\]  

(2)

The subscripts Prep and Ana refer to the preparative and analytical systems respectively, Flow is the mobile phase flowrate, \(D_{\text{Column}}\) is the column diameter, \(D_{\text{Particle}}\) is the particle size, \(V_{\text{Inj}}\) is the injection volume and \(L_{\text{Column}}\) is the column length.
Detailed project handouts and experimental procedures are included in the supporting information.

HAZARD AND SAFETY PRECAUTIONS

All of the chemicals for this project were chosen for their non-toxic nature, however they are still hazardous when inhaled or ingested. In addition, some chemicals are irritants to skin and eyes. Therefore, personal protective equipment (PPE) including lab coat, safety goggles and disposable gloves should be worn during the experiment in order to prevent direct contact with the test fluids. Some of the common chromatographic solvents are flammable and hence must be handled and stored away from sources of heat and ignition.

Another hazard is the possible glassware breakage during sample preparations. All sample preparations is to be carried out in fume hoods fitted with drip trays to account for secondary containment.

Detailed activity and chemical risk assessments for the project are included in the supporting information.

EXPERIMENTAL RESULTS

Thin Layer Chromatography – Step 1

The effect of solvent composition and polarity of the stationary phase on retention factors can be seen from Fig 1. Retention factors are a means of normalizing the separation of the components with the distance travelled by the solvent front and serve as an indication of the polarity of a compound relative to other compounds and stationary and mobile phases.

\[
Retention\ Factor\ R_f = \frac{d_i}{d_s}
\]  

Here, \( d_i \) is the distance travelled by the component \( i \) while \( d_s \) is the distance travelled by the solvent front.

High \( R_{f,\text{Normal}} \) values were reported for solvents below 70% v/v acetonitrile, corresponding to polar functional groups on molecules which form strong intermolecular interactions with the more polar mobile phase. Above this solvent composition the retention factor, \( R_{f,\text{Normal}} \), decreased sharply.
Similarly for reverse phase at high % v/v acetonitrile, $R_{f,Reverse}$ for aspartic acid and phenylalanine decreased significantly while $R_{f,Reverse}$ for Aspartame increased. It would be beyond the scope of this project to discuss the non-equilibrium effects and complex interactions between compound molecules, stationary and mobile phases\textsuperscript{19}, nonetheless the TLC results offer students a greater appreciation of the complexity behind even a simple TLC system.

With regards to the degradation pathway, aspartame degrades in two ways: hydrolysis to form its constituent amino acids\textsuperscript{16} or cyclization to form DKP\textsuperscript{15}. From Fig.1, the degraded sample did not
contain the constituent amino acids, which serves as evidence against hydrolysis as the pathway of degradation. However, there was also a lack of positive confirmation of DKP as the degradation product. As DKP weakly absorbs at 254 nm, it was not detectable under a 254 nm UV lamp. Other visualization agents, such as chloronil, ninhydrin, iodine and potassium permanganate also did not produce a visible spot. Chloronil and ninhydrin are suitable visualization agents for compounds with amine groups, but the cyclization reaction forms an amide bond which prevents visualization. A more sophisticated visualization agent or technique would be needed to visualize DKP, which is beyond the scope of this course. Instead, analytical HPLC in the next step would be required for positive confirmation of DKP.

**Analytical HPLC – Step 2**
Spectrums produced by the analytical HPLC system feature two main peaks, as shown in Fig.2. By running separate calibration curves with aspartame and DKP solutions of increasing concentrations, the first peak was identified as DKP and the second one as that of aspartame. The extent of degradation could also be determined by quantitative analysis using the calibration curves to determine the DKP and aspartame concentrations in the sample.

From Fig.3, we can see that increasing temperature decreases retention times. Higher column temperatures reduce the viscosity of the mobile phase and affect interactions between analytes and the stationary phase. This results in a reduction in retention time. Acetonitrile is a stronger elution solvent for the two analytes, reducing retention times with increasing acetonitrile compositions. As a result of faster elution times, reduced peak widths and increased peak symmetry were also observed. However, increasing acetonitrile compositions decreased the separation between aspartame and DKP as shown in Fig.2. The degree of separation between two analytes is quantitatively measured by the peak resolution, which can be calculated with LabSolutions software.
Figure 2. Spectrum at 220 nm for flow rates of 1 ml/min (a: 20% v/v ACN, 25°C; b: 20% v/v ACN, 45°C; c: 30% v/v ACN, 25°C).
To determine optimal operating conditions, students would need to weigh the importance of reducing solvent usage by reducing elution time, against the need for good separation. This dilemma highlights two common chemical engineering considerations of cost vs. product purity. An ideal operating condition which minimizes solvent usage but has good peak resolution, for instance, might

Figure 3. Influence of temperature, acetonitrile composition and flowrate on retention time (top: DKP; bottom: aspartame).
be a solvent composition of 20-25% v/v acetonitrile at higher temperatures of 45°C and flowrates of 1.5 ml/min.

Preparative HPLC – Step 3

The following quantities were defined for characterising the performance of the preparative HPLC method.

\[
\text{% Recovery} = \frac{C_{\text{APM,Volume Fraction}} \times V_{\text{Mobile Phase}} \times (t_f - t_i)}{C_{\text{APM,Batch}} \times V_{\text{Inj,Prep}}} \tag{4}
\]

\[
\text{Overall Method Efficiency} = \frac{(C_{\text{APM,Batch}} \times V_{\text{Inj,Prep}}) \times \text{% Recovery}}{V_{\text{Mobile Phase}} \times t_{\text{Total}}} \tag{5}
\]

\(C_{\text{APM,Volume Fraction}}\) is the concentration of aspartame in the volume fraction collected and is experimentally determined, \(C_{\text{APM,Batch}}\) is the aspartame concentration in the degraded solution batch, \(V_{\text{Mobile Phase}}\) is the mobile phase flowrate, \(t_f\) and \(t_i\) are the start and end time for the collection of the volume fraction, and \(t_{\text{Total}}\) is the total run time per sample injected. The overall method efficiency has units of [mg][mL]-1 and can be understood as the solvent consumption of the method per unit aspartame recovered.

From the scale-up rules (equations (1) and (2)), we determine \(Flow_{\text{prep}} = 29.7\) mL/min and \(V_{\text{Inj,Prep}} = 0.212\) mL. As the objective was separation of larger quantities of aspartame, simultaneous concentration and volume overloading was carried out. Flowrates of 20 and 30 mL/min were tested. Higher flowrates were not possible due to the high-pressure requirements exceeding the pump specifications. A higher concentration of aspartame (5mg/mL before degradation) was used and \(V_{\text{Inj,Prep}}\) ranging from 2 to 5mL was used.

To optimize the separation method, a recovery of 100% was assumed for calculations to determine the most efficient method. A sample containing 2.114 mg/mL aspartame was tested. The mobile phase composition, flowrate and sample injection volume were varied. Methods which did not provide baseline separation between DKP and APM peaks were treated as having zero method efficiency. These results can be seen in Fig.4 with the color of the datapoint corresponding to the method efficiency as per the color bar. Once the most efficient method was selected, the volume fractions were collected to calculate the actual method efficiency.
Figure 4. Optimisation of Prep-LC Method
The Prep-LC system enables students to see a variety of chromatographic phenomena associated with overloading. The first noticeable feature is peak broadening and increase in tailing which are characteristics of volume overloading\(^1\). In addition, the DKP peak which elutes earlier exhibits peak splitting at lower %ACN in the mobile phase. This can be understood as a function of the increased mass-loading which leads to non-linear equilibrium effects between the stationary and mobile phase\(^2\).

The peak splitting is mitigated as the %ACN in the mobile phase increases as can be seen in Fig.5 which corresponds to an increase in the solvent elution strength.

The current system can cope with the maximum injection volume of 5 mL and potentially higher solute concentrations, but this was avoided to mitigate possible clogging issues. As shown in Fig.4, increasing the %ACN does improve method efficiency as the retention time decreases, but it simultaneously reduces peak resolution, as can be seen in Fig.5, which can affect the purity of the fraction collected. The mobile phase flowrate does not significantly change the method efficiency, but it does reduce method run-time. Hence the following conditions were tested for the actual method efficiency: 20\% ACN, 30 mL/min flowrate and 5 mL injection volume. Higher method efficiencies could
potentially be obtained by using solvent compositions of approximately 22% ACN. However, tasking
students to comprehensively optimize the method efficiency yields marginal pedagogical benefits. The
actual task of optimization consists of carrying out more runs which eventually becomes repetitive. A
basic optimization demonstrates the core principles that the module strives to teach while not
overburdening the students. From an experiential learning standpoint, such an approach also
provides students with an authentic experience as they need to learn to deliver a completed work
package that has been reasonably optimized within both time and resource constraints: it can’t be
perfect, it just has to be good enough.

The final experiment was performed in triplicates using another degraded sample. The overall
method efficiency was $0.0648 \pm 0.0035 \text{ mg/mL}$ with a recovery of $92.1 \pm 4.98 \%$. A source of
variability arises from the manual operation of the injection valve which can result in a slight shift in
the elution time and correspondingly affect the quantity of aspartame recovered in the specified time
fractions.

PEDAGOGY
The pedagogical philosophy underpinning this module can be understood through a constructivist lens which has found broad application in scientific and engineering education, particularly in practical modules. Vygotsky characterizes the learner as someone who actively constructs their knowledge, with this construction taking place at the learner’s ‘zone of proximal development’ by interactions with fellow students and teaching staff. This interaction can be activated through the provision of a well-structured course which includes, for instance, adequate communication and meetings between learners and teachers and providing a sequence of planned activities. This suitably guides students with the teacher acting as a facilitator whilst simultaneously providing them with the independence and autonomy to pursue their own approach. We believe that such an approach is key to enabling students to achieve a high degree of competency in a complex subject matter within a relatively short time frame.

Figure 6. Students Zone of Proximal Development at the Beginning of Step 1

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The zone of proximal development is shown in Fig. 6. The entire module can be thought of as an iterative activity that expands the domain of knowledge and activities that students can confidently do unaided. Each step, therefore, has been structured to simultaneously build up students’ capability in the objective for that step and sets the context for the next step. As such, each step is appropriately placed in the learner’s zone of proximal development. For instance, using analytical HPLC before the module starts may be in the ‘out of reach’ zone, but by the end of step 1 (TLC), HPLC techniques now firmly lie in the learner’s zone of proximal development.

Support and Scaffolding Provided to Students

The module was first introduced in 2015 and has since been in continuous development with input from students who passed this module, graduate teaching assistants, laboratory technicians, as well as the module leader. From discussions with these stake holders, “pinch points” were identified over the years and an adequate support structure was put in place to overcome them. The module strives to facilitate independent learning and exploration by students, which is a significant shift away from a ‘cookbook’ style of lab experiments to a more open ended and self-directed approach. This may be initially intimidating to students and as such, appropriate scaffolding and support needs to be provided along the way. Examples of the support given to students include:

1) Handouts, which contain a problem statement and possible sequence of experiments to conduct for each step. These handouts contain references to relevant literature and are kept deliberately brief to prompt students to review quoted papers themselves.

2) Training resources were provided for reference. Training videos on the operational aspects of the course such as setting up and running the TLC and the HPLC systems and comprehensive standard operation procedures (SOPs) were prepared. Students are encouraged to view these videos and familiarize themselves with the SOP before the start of the lab session so that they would have adequate operational knowledge and would be able to make full use of their laboratory time instead of focusing on operational issues. These materials were developed by senior students involved in the module development process. This helped to make operation of the equipment much more accessible to students.28
3) Frequent review sessions with their project supervisor, a GTA. These provide an opportunity to review the work already carried out and to plan next steps. Students are strongly encouraged to pursue ideas beyond the provided suggestions from the handout. These ideas, together with the proposed workflow for the week are agreed upon with the GTA. The scheduling of these meetings is up to the students, thus increasing their ownership of the project. In addition, there are also several informal interaction opportunities during a lab session where students can raise any issues they encountered e.g. during the DoE for optimization.

**Evaluation of Learner Outcome**

A voluntary anonymous survey to understand students’ response to the module was carried out after the project concluded. Students had time and space to reflect and respond to the survey at their own pace. Out of the 11 students who participated in this module, n=10 responded to this survey and the results are presented below.

The survey results strongly indicated that students found the module structure logical with each step well situated in their zone of proximal development. This is further supported by almost all students indicating they consider themselves confident to use TLC and analytical HPLC in the future. Lastly, students also mostly indicated that the level of support and scaffolding provided during the course was adequate for their learning.
CONCLUSIONS

A module structure where junior students are taught fundamental aspects of chromatography and are progressively trained to use preparative LC for chemical separations was successfully developed with positive feedback from learners. The key to enabling students to learn effectively in such a rapid manner is to ensure that each step of the course is suitably placed in their zone of proximal development and to provide them with adequate support throughout the course. Each step therefore has two objectives; the first is to provide a good foundation of the topic to be covered and the second is to set the context for the next part of the course. This short course format is well suited to higher education as well as continuing professional development in industrial practice.

ASSOCIATED CONTENT

The associated content contains complete experimental instructions for carrying out the practical project of the aspartame degradation problem, including:

Figure 7. Chromatography Module Survey Results
1. Student handouts (problem definition, step 1, step 2, step 3) (PDF)
2. Activity risk and chemical hazard assessments (ARAF, COSHH) (PDF)
3. Standard operating procedures for the thin layer and high-performance liquid chromatography (TLC/HPLC SOP) (PDF)

This comprehensive set of documents allows interested readers to fully replicate the project in their own setting.

**AUTHOR INFORMATION**

**Corresponding author:**
Dr Clemens Brechtelsbauer
*E-mail: c.brechtelsbauer@imperial.ac.uk

Dr Wenqian Chen
*E-mail: wenqian.chen06@imperial.ac.uk

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