The determination of the best separation conditions for a mixture of preservatives of varying polarity using HPLC: An ACELL experiment.[1]

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Introduction

Chromatography is a fundamental component of most analytical chemistry units in undergraduate science programs. Many gas chromatography (GC) and high performance liquid chromatography (HPLC) experiments tend to focus on the quantitative aspect of the technique (determination of benzoic acid in coca cola or ethanol in wine) [1-3]. However, these experiments do not actively develop students understanding of the separation processes involved and method development (ie development of best separation conditions) is limited due to time constraints. To address these issues we have been active in developing experiments that promote understanding of the separation mechanisms involved [4,5].

This aims of the experiment described here are to 1. extend and develop students understanding of the mechanisms of separation in reversed phase (RP) chromatography 2. provide students with some experience in method development and 3. expose students to appropriate tools that can facilitate method development.

Students are required to determine the best separation conditions (ie mobile phase conditions) to separate a complex mixture including the non-polar parabens (esters of para-hydroxybenzoic acids) based preservatives and the polar preservatives, sorbic acid and benzoic acid using RP-HPLC system. The introduction of an ion-pairing reagent is necessary to resolve the mixture. A modelling package, DryLab®, is used to simulate the separation and determine the best conditions (ie the amount of pairing reagent required to resolve the mixture).

Preservatives were chosen as it has immediate relevance and also because the commonly used preservatives fall into two categories, the non-polar and closely related parabens and the polar organic acids. The parabens are easily resolved by reversed phase HPLC and but the acids and the parabens are difficult to resolved simultaneously by RP-HPLC, hence the need for an ion-pairing reagent.

Acknowledgements

The original idea for this experiment came from a paper by Dr. Mary Boyce and Dr. Evadne Spickett entitled ‘Separation and Quantification of Preservatives Using Ion Pair HPLC and CZE: An Extended Investigation of Separation Mechanisms’ [5]. The experiment has been adapted to include a modelling component and the capillary zone electrophoresis (CZE) component has been removed. The authors wish to thank: Mr Mark Bannister, laboratory technician from Edith Cowan University, for his help in setting up this experiment. In addition, thanks must also go to the members of the ACELL team for useful feedback and suggestions.

Educational Template

Section 1 - Summary of the Experiment

1.1 Experiment *Title*

The determination of best separation conditions for a mixture of preservatives of varying polarity using HPLC.

1.2 Description of the Experiment

In this experiment students are required to determine the best separation conditions (ie mobile phase conditions) to separate a complex mixture including the non-polar paraben based preservatives and the polar preservatives, sorbic acid (2,4-dihexenoic acid) and benzoic acid using RP–HPLC system. The main aims of the experiment are to 1. promote student understanding of the mechanisms of separation in chromatography, in this case RP and ion pair (IP)-RP separations and 2 to expose students to modern tools, in this case a modelling package, used to facilitate the process.

1.3 Course Context and Students’ Required Knowledge and Skills

At ECU our students complete a Bachelor of Technology (Applied & Analytical Chemistry) Degree. This degree has a significant TAFE component in first and second year and as a result our chemistry majors have good hands on instrumentation skills. In third year, our students complete several analytical chemistry units at ECU, one of which is Analytical Chemistry I* where the main emphasis is on chromatographic techniques including HPLC, gas chromatography and capillary electrophoresis. The theory of chromatography is covered in some detail and includes a discussion on RP and IP separations. In the laboratory, students complete introductory HPLC based experiments that focus on proper use and maintenance of HPLC instrumentation, quantitative measurements and the recording and generating of data such as retention times, peak widths, etc.

[1] The complete documentation for this experiment is freely available on the APCELL web site [www.apcell.org]. It includes the educational template, a set of student notes, demonstrator notes and technical notes to allow ready implementation into a new laboratory.

The experiment described here is one of a few extended activities that students complete in the final weeks of semester. Therefore, it is assumed that students will have the skills (with some help if required from the demonstrator) to operate the pump system, UV-VIS detector and the HPLC software. In other words students are not generally preoccupied with how to inject the sample or get the computer to take in the data.

**Some students will be taking chemistry as a supporting major and will not have done the TAFE component and therefore introductory HPLC experiments are vital for these students.*

1.4 Time Required to Complete

Prior to Lab	30min for reading
In Laboratory	3 hours for laboratory work
After Laboratory	2 hours for analysis of results, report writing and answering questions.

Providence

The original source of this experiment is a paper by Dr. Mary Boyce and Dr. Evadne Spickett entitled 'Separation and Quantification of Preservatives Using Ion Pair HPLC and CZE: An Extended Investigation of Separation Mechanisms' [5]. Two main modifications have been made from the original paper; inclusion of modelling component using DryLab® software and the removal of capillary zone electrophoresis component.

Other Comments

Although butyl paraben (butyl-4-hydroxy-benzoate) is also a common non-polar preservative, we have not used it in this experiment due to time constraints. Butyl paraben is the heaviest of the parabens and would have taken over 10min to come off the column.

Section 2 – Educational Analysis

Learning Outcomes *What will students learn?*	**Process** *How will students learn it?*	**Assessment** *How will staff know students have learnt it?* *How will students know they have learnt it?*

Theoretical and Conceptual Knowledge

Students will gain a deeper understanding of the separation mechanism in RP chromatography.	Students will run a mixture containing polar and non-polar preservatives on a C18 column using a polar mobile phase and identify the peaks on the resulting chromatogram. The separation and elution order of the non-polar preservatives provide a classic example of how such solutes are resolved by RP chromatography. The inability to resolve the polar solutes under the same conditions also highlights the limitations of the RP conditions.	Students are required to label the peaks in the chromatograms recorded and hence explain the elution order observed. The students will discuss and explain their results with the demonstrator. The demonstrator can then probe students understanding and clarify any misconceptions the students may have.
Student will learn how separation mechanisms can be cleverly manipulated to provide better resolution – in this case the addition of a pairing reagent to the mobile phase to separate the polar preservatives.	Students will add pairing reagent (at different concentrations) to the mobile phase in an attempt to resolve the coeluting polar solutes. They will determine the identity of the peaks recorded for each chromatogram recorded by running individual standards of the solutes.	Each student group will determine the elution order and identify the components separated under the various conditions. The students will discuss and explain the elution order observed with the demonstrator. The demonstrator will also probe students understanding. In addition students are required to complete post lab questions which are assessed and returned to students for feedback.
Students get an opportunity to apply and consolidate theory learnt in lectures in the laboratory environment	Students completing the experiment must use separation and chromatography theory to explain their results.	The feedback received from the laboratory write-up and the final examination mark.

Scientific and Practical Skills

Provide students with more experience in the use of HPLC instrumentation	Students are required to vary the mobile phase, condition the column between changes in mobile phase and carry out a number of separations in completing this experiment	Students will build on instrumentation skills already developed. Students will concentrate on explaining their results rather than how to use the injection loop or the HPLC software.
Expose students to the use of a chromatographic modelling package.	Students will use the modelling package to determine best separation conditions for the mixture under investigation. Students will take their experimental data, insert it into the modelling package, model the separation and hence predict best separation conditions. They will then test the predicted conditions experimentally.	Students will complete a simulation and obtain best separation conditions for their data.
Recording, extracting and calculating relevant scientific data including retention times and peak widths.	Students will use appropriate formulas to work.	Students will obtain 'reasonable' data typical for HPLC.
Students will better appreciate how the quality of resolution will be compromised by budget (eg. time and chemical costs).	The modelling package will allow students to view the resolution and retention times of the analytes under different conditions. While best resolution might be achieved at longer run times, students will also be able to determine conditions that support short run times but with poorer resolution.	What "best separation conditions" means will be discussed by the students and demonstrator – are all analytes of interest quantitatively, will a short run time resolve the key analyte of interest – what is the cost of the separation.

Generic Skills

Students will further develop their report writing skills.	Students will be asked to prepare a clear, well-structured, formal report.	The report will be marked according to the criteria given in experimental notes and written feedback will be provided with respect to those criteria.
Students will get the opportunity to participate in group discussions and work as a team.	Students will be encouraged to discuss questions 'in the lab' and their results within the group prior to writing up the final report.	The demonstrator will actively participate in students' discussions. As part of the scientific report students will be required to give a written account (as part of the formal laboratory report, which is assessed) of the questions discussed during the laboratory session.
Students will develop new and industry relevant computer skills.	Students will use, DryLab®, a computer package to determine best separation conditions.	Be familiar with a HPLC modelling package.

Section 3 – Student Learning Expertise

Explanatory notes to Student Learning Experience

This experiment was presented at the ACELL workshop in 2002. The exercise and associated documentation was revised, incorporating suggestions from workshop participants. The version presented here includes further changes in response to the 2002 student feedback, and to comments from the ACELL referees.

Did this experiment help you to understand the theory and concepts of the topic? If so, how, or if not, why not?

S1: I didn't fell it increased/improved my prior understandings.
S2: Yes, helped clarify my understanding of mobile/stationary interaction.
S3: Yes.
S4: Yes - experiment was based on theory rather than learning analytical/instrumental techniques.
S5: Most definitely. The demonstrator was terrific at asking leading questions.
S6: Yes.

3.2 How is this experiment relevant to you in terms of your interests and goals?

S1: Not particularly relevant – Not my area of expertise/interest. However, found it interesting to do something different.
S2: Not relevant, but it was fun to think.
S3: It broadened my outlook. Not directly relevant to me.
S4: Interested in food and instrumentation so very relevant experiment.
S5: I want to incorporate HPLC experiments in Second Year analysis of biological molecules.
S6: Very – formulation analysis.

3.3 Did you find this experiment interesting? If so, what aspects of this experiment did you find of interesting? If not, why not?

S1: Yes. Conceptually interesting. Good to have discussion.
S2: Interesting because students should be able to see industrial/employment relevance.
S3: I am surprised it's aimed at second half of 3rd year. I'd see it as a good early HPLC experiment followed by a more demanding one.
S4: Yes – learning a different way of separating compounds.
S5: I am an organic chemist, so it was very interesting (structure-polarity relationships, etc).
S6: How the instrument works. What is good separation. Software is cute. Bit expensive.

3.4 Can the experiment be completed comfortably in the allocated time? Is there time to reflect on the tasks while performing them?

S1: Ran a bit short of time in making $sol^{n}s$, conditioning.
S2: I think it's suitable for a 3 hour lab but 90 minutes was really rushed.
S3: I think so.
S4: We were pushed for time in out 90 minutes. Yes – because it takes time for each run so you have time to reflect and discuss.
S5: Hard to tell without doing the experiment in full.
S6: Yes.

3.5 Does this experiment require teamwork and if so, in what way? Was this aspect of the experiment beneficial?

S1: Not necessarily. Discussion in the group was helpful.
S2: Teamwork helps to get ideas out.
S3: Not really!
S4: Not required at all but we discussed results amongst the group.
S5: The discussions were extremely beneficial. I would have been less confident doing it on my own.
S6: Discussing separation theory.

3.6 Did you have the opportunity to take responsibility for your own learning, and to be active as learners?

S1: Yes.
S2: It was a bit easy to "cruise" and let more vocal members of the group to continue talking.
S3: A bit – demonstrator took control.
S4: Yes.
S5: Yes, needed to take risk to make suggestions.
S6:

3.7 Does this experiment provide for the possibility of a range of student abilities and interests? If so, how?

S1: Don't really know. Haven't thought about it.
S2: I think not-too-enthusiastic students can hide in the group and vocal students can dominate.
S3: I feel not very demanding for very bright students. It's really routine analytically.
S4: Yes – actual experiment wasn't difficult – more the interpretation but this (I assume) could be taken away from the lab and worked through.
S5: Yes.
S6: Not really.

3.8 Did the laboratory notes, demonstrators' guidance and any other resources help you in learning from this experiment? If so, how?

S1: Didn't get much opportunity to read them. Can't really comment.
S2: Didn't really get to see.
S3: The demonstrator was very pleasant and enthusiastic which was more than half the enjoyment. I might find it harder with a frumpy demonstrator or no demonstrator.
S4: Yes – demonstrator guided us along.
S5: N/A.
S6: Yes example chromatograms.

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Table 2.
Effect of reactant concentrations on the chemiluminescence reaction of manganese(VII) at pH 2.0 with sodium borohydride

NaBH 4/M	Manganese (VII)/M			
	0.0001	0.0005	0.001	0.005
0.001	Dull	Dull	Not Obs.	Not Obs.
0.005	Dull	Brighter	Brighter	Not Obs.
0.01	Dull	Brightest	Brighter	V. Dull
0.05	Dull	Brightest	Bright	V. Dull
0.1	Dull	Brightest	Bright	Not Obs.

Table 3. Effect of pH on the chemiluminescence reaction of manganese(III) with sodium borohydride (0.05M)

Manganese (III)/M	Observations
0.0001	V. Dull
0.0005	Bright
0.001	Brightest
0.005	Brightest
0.01	Bright

Table 4.
Effect of pH on the chemiluminescence reaction of manganese(IV) with sodium borohydride (0.05M

Manganese (IV)/M	Observations
0.001	Not Observed
0.005	Very Dull
0.001	Dull

Conclusions

The reactions of manganese (III), (IV) or (VII) with sodium borohydride are some of a small group of purely inorganic chemiluminescent reactions. Whilst not as spectacular in intensity as the oxidation of white phosphorus or the combustion of carbon disulfide in nitrous or nitric oxides, it is our opinion that this simple demonstration is considerably superior to the oxidation of siloxane. As a demonstration it is devoid of toxic or corrosive by-products and sufficiently cheap to make it readily accessible to high school classrooms.

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3.9 Are there any other features of this experiment that made it a particularly good or bad learning experience for you?

S1: I am easily distracted, so I found the small amount of "hands on" work to be not engaging enough. This may just be me, however.

S2: Like practicing, injecting, purging, conditioning column.

S3: This kind of experiment is one where I would need to take my own time to work on the machine etc. In this way, group work is quite frustrating – I wanted to go slowly at one point, and they rushed ahead.

S4: Nice clear demonstration – can see on paper that peaks separate.

S5: No time to comment.

S6:

3.10 What improvements could be made to this experiment?

S1: No real comments/suggestions

S2: Actually using real life samples eg. cosmetic or food.

S3: Don't know.

S4: None.

S5:

S6:

3.11 Other Comments

Demonstrator was v.good and explained things clearly and concisely.

Not sure if the design of the experiment is meant to be so demonstrator-centred. As a student, that would drive me nuts!

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