Development of an Intelligent Heart-cut Liquid Chromatography System for Chiral Analysis of Chemical Processes

Nour Bizzari

A Thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science

Graduate Program in Chemistry

York University

Toronto, Ontario

November 2017

Abstract

Recent advancements in high performance liquid chromatography (HPLC) have encouraged scientists to use multi-dimensional HPLC as a routine process analytical technology (PAT) tool for chiral drug development.[1,2] Multi-dimensional HPLC techniques enable researchers to monitor multiple essential and sometimes, critical quality attributes (CQAs) in parallel. A unique three-column, four-dimension liquid chromatography system employing a two-loop heart-cut strategy is reported. The system is capable of re-circulating effluents from each dimension through a single detector and performing heart-cut operations using a programmable ten-port, two-position heart-cut valve. The first dimension of analysis measures peak ratios of products to reagents while the second dimension offers peak purity of the heart-cut material. The third dimension uses a chiral column to measure the enantiomeric excess (ee) of the chiral analyte and the effluent from the third dimension is re-analyzed via a fourth dimension to determine if any loss of chirality has occurred resulting from the analytical method itself. Performance of the setup was evaluated using the synthesis of Warfarin as a model chemical process.

Acknowledgments

I would like to take this opportunity to thank my supervisor Dr. Michael G. Organ for his invaluable support and assistance throughout the course of my research. I would also like to thank my committee members, Dr. Robert McLaren and Dr. Derek J. Wilson for their guidance throughout these two years. I would also like to acknowledge Dr. Debasis Mallik for his consistent support, and guidance through my research.

Further acknowledgements towards all members in the Organ group, especially Wenyao Zhang, Jee Kwak, Sepideh Sharif, Dr. Greg Price and Dr. Jonny Day, for providing me with unfailing support, encouragement and guidance. Thank you.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

API: Active Pharmaceutical Ingredient

HPLC: High Performance Liquid Chromatography

LC: Liquid Chromatography

PAT: Process Analytical Technology

2D-HPLC: Two-dimensional High Performance Liquid Chromatography

Multi-D HPLC: Multi-dimensional High Performance Liquid Chromatography

CPPs: Critical Process Parameters

QAs: Quality Attributes

CQAs: Critical Quality Attributes

Ee: Enantiomeric Excess

FDA: Food and Drug Administration

DAD: Diode Array Detector

UV-VIS: Ultra-violet Visible Spectrometer

MS: Mass Spectrometry

CSP: Chiral Stationary Phase

PEPPSI: Pyridine Enhanced Pre-Catalyst Preparation Stabilization Initiation

MACOS: Microwave Assisted Continuous Organic Synthesis

D-AAs: Dansylated amino acids

DPEN- 1,2-Diphenylethylenediamine

EDTA-Tri-sodium ethylenediaminetetraacetic acid

RSD- Relative Standard Deviation

List of Schemes

1. Introduction

1.1 Background

Quality control is an important aspect in the manufacturing industry wherein a rigorous set of procedures are implemented in order to monitor and maintain the quality of product output. Product quality is defined by a number of important attributes (e.g. yield, purity, particle size) with pre-set objectives or standards. Some attributes must meet rigorous standards set by various regulatory agencies (e.g., Food and Drug Administration (FDA) in the United States of America or Health Canada in Canada). These attributes are called quality attributes (QAs), and an example of such an important attribute is product purity.[3] Pharmaceuticals, which have the potential to cause serious physiological harm, require extensive testing for product purity in order to meet regulatory standards. On the other hand, there are other sets of attributes (e.g., product yield) that are key towards successful and profitable production of an active pharmaceutical ingredient (API).[3] From a chemical manufacturer's point of view, the objective is simple, to achieve maximum product yield with acceptable purity from a single production run. However, there is no direct correlation between product yield and product purity. The search for optimal production conditions is often conducted through empirically formulated experiments in-line with continuous monitoring via a process analytical technology (PAT) tool.[4] PAT is defined as an in situ analytical tool capable of designing, analyzing, and controlling chemical processes through timely measurements of quality attributes to gain process understanding.[3] The growing use of PAT in process optimization and in manufacturing has sparked interest in new technologies that will not only help with monitoring of a reaction process but also provide users with a feedback of data on the state of the reaction over time.[4] The practice of optimizing and monitoring a reaction process is resource-intensive and requires a significant amount of analytical support in order to screen through a relatively large number of conditions within a short period of time. There is not one PAT tool applicable for all

chemical applications, and so multiple, appropriate analytical tools may have to be coupled to provide timely measurements of chemical processes.[3] As the number of analytical tools that need to be coupled increase in number, automation must be incorporated to provide analytical support and ensure reliability of the operation.[2] Chemical analysis, which deals with the science of measurement to evaluate the fate of a scientific experiment, plays a key role in this pursuit of ideal strategies for chemical production.

High Performance Liquid Chromatography (HPLC) is one of the most widely-used analytical tools for chemical analysis.[4–6] In HPLC, an analytical sample is injected onto a chromatographic medium, and a liquid mobile phase is used to separate various chemical components which are then sent to a suitable detector one component at a time to separate reaction components and to assess purity of a particular component (i.e., the HPLC purity). HPLC technologies have advanced significantly over the decades and the analysis has become relatively complex, but routine.[4–6] The analysis has become complex especially when analyses are conducted on complex mixtures containing a large amount of closely related compounds (i.e. similar fragmentation). In HPLC, such complex mixtures would display poor resolution in the chromatogram; furthermore, for unstable compounds capable of further reacting, or degrading on-column, the analysis may not provide meaningful output of data (i.e. in measurement of HPLC purity) and may require off-line purification techniques that may extend to long periods of time. In addition to this, analytical methods often call for elaborate preparation of a sample prior to injection. This is observed in chemical reactions containing metal; the metal can chelate towards the stationary phase and hinder separation of the reaction mixture. Sample preparation can be tedious and time consuming, and often the preparation varies depending on what type of purity analysis is required in a particular manufacturing process. For example, if the targeted component is a chiral molecule, compound purity as well as optical purity assessment is necessary in order to fully understand the process.

Unfortunately, data for these QAs (i.e., the HPLC purity and the optical purity) do not originate from a single analytical experiment. For optical purity measurements, one must remove all achiral components from the sample prior to performing a chiral HPLC analysis.[7] Quite often, off-line chromatographic purifications (e.g. column chromatography) are conducted for each and every chemical synthesis to obtain sufficient quantities of clean target materials before chiral analysis can be conducted.[7] This process is iterative, time-consuming, and requires multiple layers of analytical validation protocols to confirm if the HPLC purity of the chiral injectable is satisfactory for further chiral analysis.

Multi-dimensional chromatography, which was first developed to analyze target analytes using multiple chromatographic media, has emerged as an important tool for the manufacturing of chiral pharmaceuticals.[1,8,9] A chromatographic dimension refers to an analysis step where information on a QA or verification of an output QA can be obtained (i.e. it is an analytical space where chromatographic separations and analyses are enabled). These analyses can be conducted through one or more columns and one or more detectors. Significant amount of proteome analysis applications have relied on multidimensional separation protocols[10–15]; with few other applications expanded towards enantioseparation of pharmaceutical drugs.[1] Modifications were made to the original multi-dimensional platform to not only increase the resolution of chromatographic separations, but also to acquire data for various types of QAs from a single chromatographic experiment. The ability to collect data for multiple QAs from a single injection is extremely useful, especially for drug manufacturing where more than half of the drugs currently in market are chiral compounds, and nearly 90% are administered as racemates.[8] In drug manufacturing, the product purity and optical purity both play an equal role in defining the fate of the manufacturing process. The multi-dimensional technology for drug analysis employs an achiral chromatographic medium for first dimension analysis and a chiral chromatographic medium (contains a single enantiomer of a

chiral compound to form a Chiral stationary phase (CSP)) for second dimension analysis). In order to conduct a multi-dimensional chiral analysis for a target chiral analyte, the selective transfer of the chiral analyte from the achiral chromatographic medium to the chiral chromatographic medium must be facilitated. When an analyte is sent directly to a detector from an achiral chromatographic medium, the output data constitutes the first dimension analysis, and provides information on product purity, a QA, and if an internal standard is used, product yield, a key QA for the process. When a targeted fraction from the first dimension analysis is diverted to the same detector or a second detector via a chiral chromatographic medium, data for the second dimension analysis, which contains the chiral purity information, is collected. The technique involved in the process of transferring a selected fraction from the first dimension effluent to the second-dimension stream is known as the 'heart-cut' technique.[16] A heart-cut protocol utilizes a transfer valve which first traps a portion of the effluent from the first-dimension stream in a loop and then delivers the trapped sample from the loop to the second-dimension stream.

Veuthey and coworkers reported a liquid chromatography-mass spectrometer (LC-MS) system equipped with a heart-cut mechanism (Figure 1) in 2003 for the analysis of seven process related substances in tablets containing cetirizine as the active ingredient.[17] The authors did not use a conventional two-dimensional chromatography platform for this work. The authors observed that repeated injections of high concentrations of cetirizine deteriorated the performance of the MS detector.[17] Their heart-cut design was employed to reduce interference of the highly concentrated cetirizine by channeling selective portions of the post-chromatography stream to two detectors in parallel located downstream of the heart-cut valve.[17] A schematic outline of the design is shown in Figure 1. Once the heart-cut operation of a chosen segment was completed, there was no provision to recycle the material and repeat

the heart-cut operation in the event the heart-cut operation failed to isolate the chosen segment in desired purity from the first heart-cut attempt.

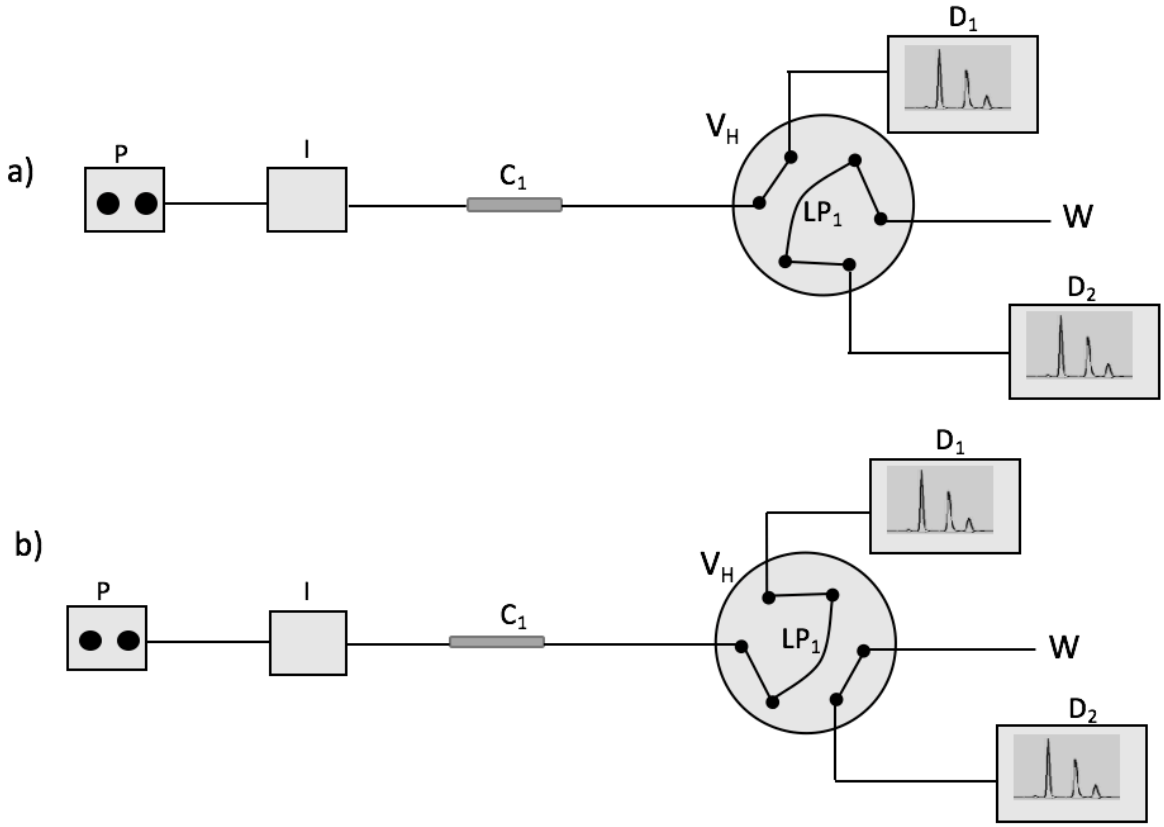


Figure 1: a) Heart-cut design by Veuthey and coworkers for the analysis of cetirizine tablet ingredients with heart-cut valve in communication with UV detector. b) Heart-cut design by Veuthey and coworkers for the analysis of cetirizine tablet ingredients with heart-cut valve in communication with MS detector. Legends: P = HPLC pump, I = Injector, C1 = Chromatographic medium, VH = Heart-cut valve, LP1= loop 1, D1 = First detector (UV), D2 = Second detector (MS), W = Waste

A two-dimensional analytical platform, comprising of an achiral first dimension and a chiral second dimension, was developed by Natalini and his group to quantify dansylated amino acid (D-AAs) levels in cows with mastitis.[18] In their design, the first dimension analysis was performed for the separation of D-AAs and the second dimension analysis was performed for the chiral separation of a particular portion of the first dimension effluent.[18] A six-port heart-cut valve, which was responsible for trapping a portion from the first-dimension stream and transferring the trapped material to the second-dimension stream, was located downstream of a detector responsible for the first dimension analysis.[18] A schematic outline of the design

is shown in Figure 2. A second detector solely responsible for acquiring data for the heart-cut sample was located downstream of the heart-cut valve. The fate of the heart-cut operation was only known after the heart-cut sample reached the second detector. The design did not offer a provision to re-circulate the heart-cut sample in the event the heart-cut operation failed to isolate the desired portion of the effluent from the first-dimension run. One must perform multiple injections and run the entire sequence of both dimension runs in order to perfect the heart-cut operation.

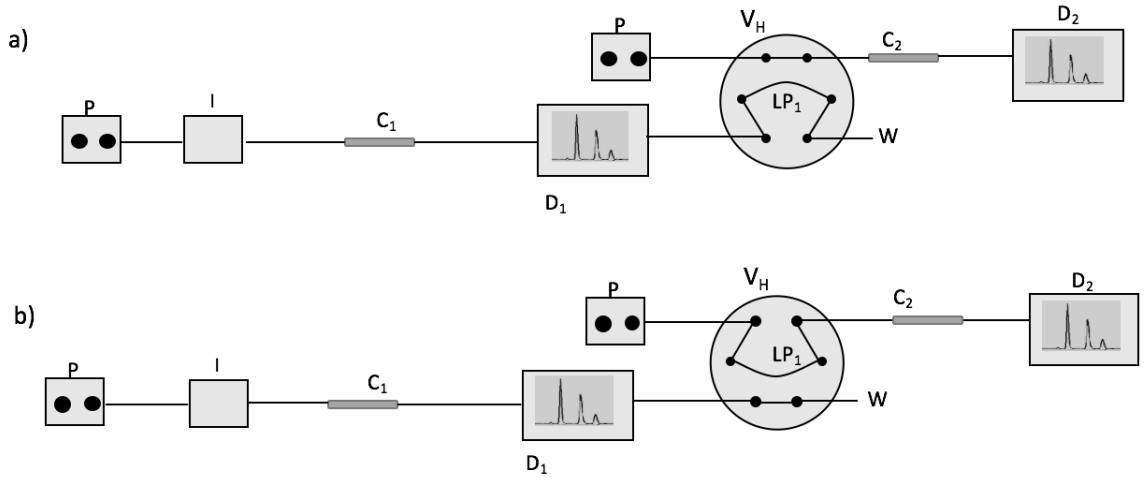


Figure 2: a) Two-dimensional design by Natalini and coworkers for the analysis of dansylated amino acids with heart-cut valve loop in communication with first dimension analysis. b) Two-dimensional design by Natalini and coworkers for the analysis of dansylated amino acids with heart-cut valve loop in communication with second dimension analysis. Legends: P1 =HPLC pump for the first-dimension chromatography, P2 = HPLC pump for the second-dimension chromatography, I = Injector, C1 = Chromatographic medium for the first dimension, C2 = Chromatographic medium for the second dimension, VH = Heart-cut valve, LP1=loop 1, D1 = Detector for the first dimensional analysis, D2 = Detector for the second dimensional analysis, W = Waste

Chetwyn and coworkers also reported a design wherein multi-dimensional analysis was carried out using a heart-cut valve.[19] A schematic outline of their design is shown in Figure 3. In this setup, the first dimension analysis was done using a first-dimension detector placed before the heart-cut valve.[19] Following the heart-cut valve operation, the second dimension analysis was done using a dedicated second detector.[19] The heart-cut valve, which was equipped with two sample holding loops, was capable of diverting multiple heart-cut samples

to the second detector.[19] If the heart-cut method failed to capture a particular portion of the effluent from the first-dimension run (i.e., if the purity of the heart-cut material is less than 100%), a second injection would be needed to rectify the heart-cut operation as there is no provision to bring back the heart-cut sample post-detection. Furthermore, if any comparison of data between the first and the second dimension analysis were to be made, calibration of the two detectors would be required in order to ensure consistency.

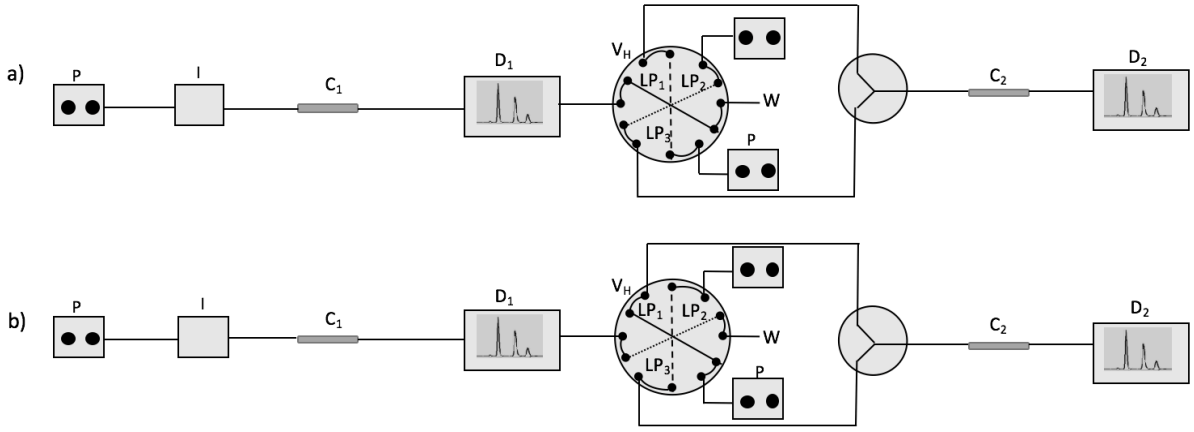


Figure 3: a) Two-dimensional design by Chetwyn and coworkers with heart-cut valve in primary configuration for first dimension analysis. b) Two-dimensional design by Chetwyn and coworkers with heart-cut valve in secondary configuration for second dimension analysis. Legends: P = HPLC pump, I = Injector, C1 = Chromatographic medium for the first dimension, C2 = Chromatographic medium for the second dimension, VH = Heart-cut valve, LP1= loop 1, LP2= loop 2, LP3= loop 3, D1 = First detector (UV), D2 = Second detector (MS), W = Waste

Scientists from Waters Corporation have developed a multi-dimensional platform suitable for chiral drug analysis (Figure 4). The design uses a set of detectors and pumps dedicated for each of the two dimensions. The design is also equipped with a 'trap and purge' mechanism for post heart-cut sample purification.[20] However, the detector for the second dimension is still at the terminal point of the multi-dimensional analysis.[20] The success in the operation of the heart-cut mechanism is reliant on the chemical properties of the trap since there is no provision to re-circulate the heart-cut fraction back into the second dimension chromatographic medium. One must perform multiple injections and run the entire sequence of both dimension runs in order to perfect the heart-cut operation.

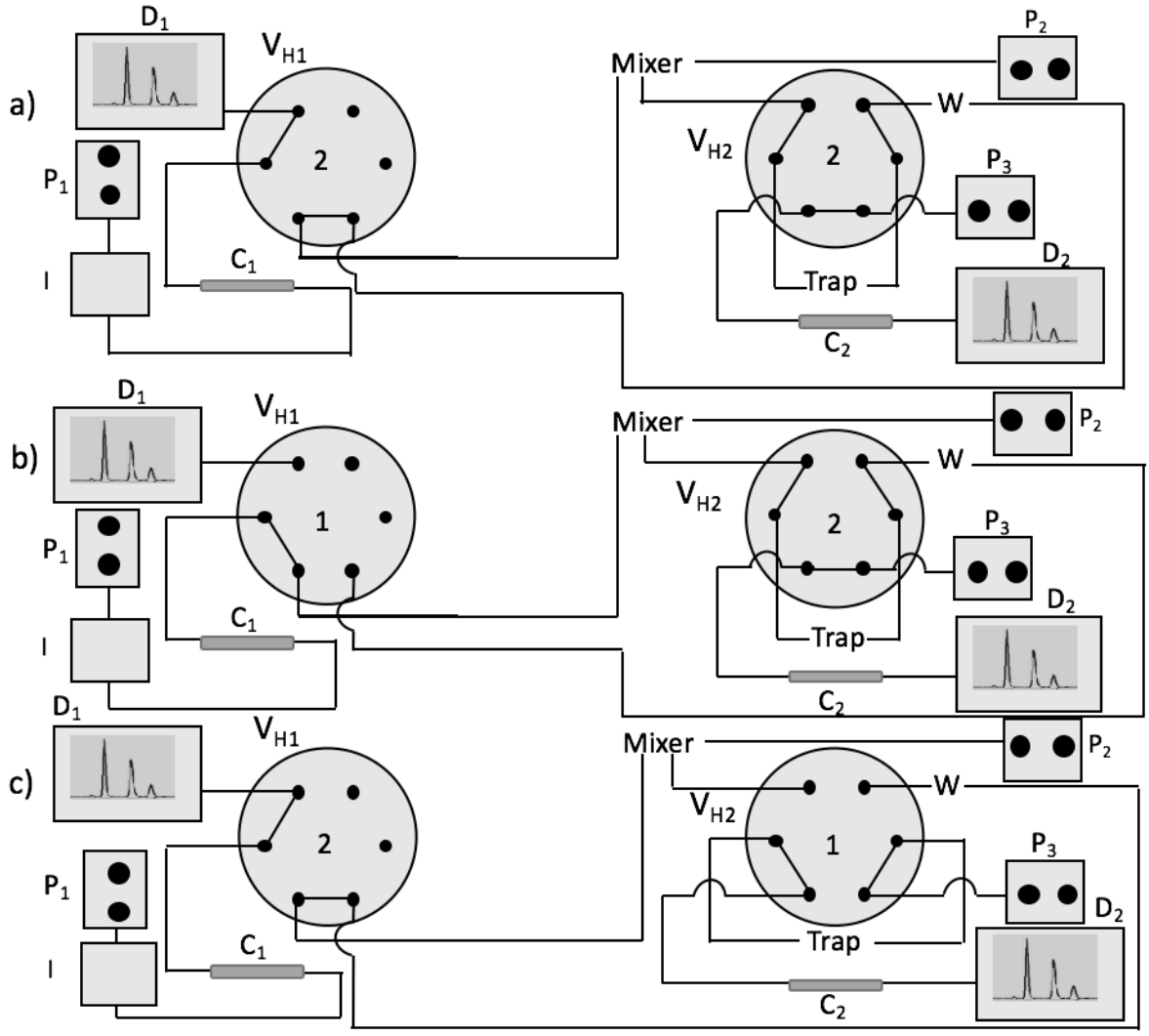


Figure 4: a) Two-dimensional design by Waters Corporation with primary heart-cut valve in position 2. b) Two-dimensional design by Waters Corporation with primary heart-cut valve in position 1 for peak trapping in trap column. c) Two-dimensional design by Waters Corporation with secondary heart-cut valve in position 1. Legends: P1 =Quaternary Solvent Manager, I= Sample Manager, P2 = Isocratic Solvent Manager, P3= Binary Solvent Manager, C1 = Chromatographic medium for the first dimension, C2 = Chromatographic medium for the second dimension, Trap= Trap column, VH1 = Primary Heart-cut valve, VH2 = Secondary Heart-cut valve, D1 = Detector for the first dimensional analysis (Photodiode Array Detector), D2 = Detector for the second dimensional analysis (Tunable Ultraviolet Detector), Mixer= Mixing unit, W = Waste, Boxed ports are not in use.

Pedrazolli Jr. and coworkers investigated chiral separations of pantoprazole in human plasma through a multidimensional HPLC coupled with a restricted access media (RAM) bovine serum albumin (BSA) column and chiral polysaccharide column.[21] The setup shown in Figure 5 offered a means of analyzing biological fluids while reducing overall sample

preparation and analysis time.[21] The RAM column was coupled to prevent the elution of unwanted proteins towards analysis (i.e. solid- liquid extraction to prevent access of matrix components while retaining desired hydrophobic analytes).[21] In the following setup, the RAM column acted as a heart-cut valve and delivered matrix components towards waste and retained analytes were eluted out towards the chiral column.[21] Similar to the other examples above, the setup has no provision of ensuring that the injectable sent towards chiral analysis is sufficiently pure.

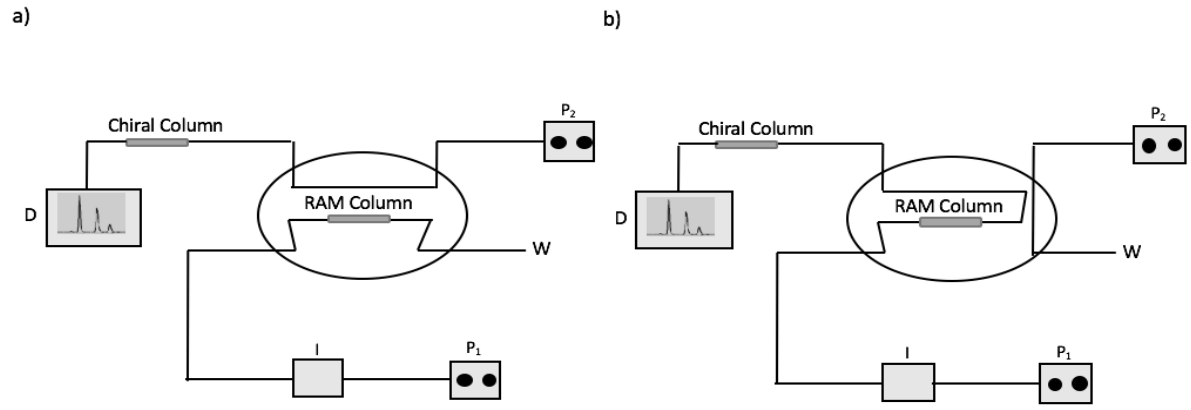


Figure 5: a) Two-dimensional design by Pedrazolli Jr. and coworkers using a RAM column for enantiomeric determination of pantoprazole in fluid communication with RAM. b) Two-dimensional design by Pedrazolli Jr. and coworkers using a RAM column for enantiomeric determination of pantoprazole in fluid communication with chiral column Legends: P1 =HPLC pump for the first-dimension chromatography, P2 = HPLC pump for the second-dimension chromatography, I = Injector, RAM = restricted access media column, D = Detector for the second dimensional analysis, W = Waste

Nik P. Chetwyn and coworkers have developed an automated online multi-heart cutting two dimensional HPLC system with UV-charged aerosol MS detection.[22] The platform shown in Figure 6 was developed to resolve the challenging issues present in pharmaceutical impurity analysis.[22] Their setup comprises of a first dimension, primary column in-line to a second dimension with six orthogonal columns along with an intermediary pair of switching valves with multiple loops for multiple heart-cutting of peaks.[22] They carried out analysis on Genentech proprietary developmental drugs to establish

a proof of concept to their setup.[22] However, the detector for the second dimension is still at the terminal point of the multi-dimensional analysis, and so the setup has no provision of ensuring that the injectable sent towards further analysis is sufficiently pure.

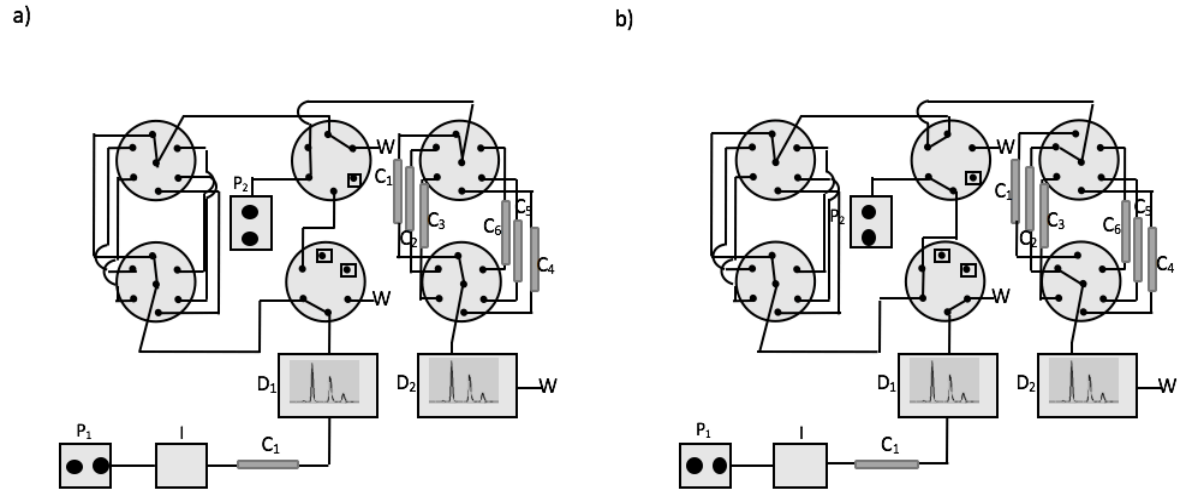


Figure 6: a) Two-dimensional design by Nik P. Chetwyn and coworkers using an UV-charged aerosol MS detection in position 1 for first dimension analysis. b) Two-dimensional design by Nik P. Chetwyn and coworkers and coworkers using an UV-charged aerosol MS detection in position 2 for second dimension analysis (with option of second dimension column from C1-C6). Legends: P1 =HPLC pump for the first-dimension chromatography, P2 = HPLC pump for the second-dimension chromatography, I = Injector, C1 = Chromatographic medium for the first dimension, C1-C6 = Chromatographic mediums for the second dimension, D1 = Detector for the first dimensional analysis, D2 = Detector for the second dimensional analysis, W = Waste, Boxed ports are not in use.

Shalliker and coworkers developed a multidimensional reversed-phase HPLC for isolation of active constituents from a crude extract of Clerodendrum floribundum R. Br.[23] Their system shown in Figure 7 comprises of two dimensions of analysis through two columns run through an isocratic solvent method. The two dimensional HPLC's heart-cut protocol was employed via two six port two position valves intermediary to both dimensions of analysis.[23] Similar to the above examples, the detector for the second dimension is at the terminal point of the multidimensional analysis, and so the setup has no provision of ensuring

that the injectable sent towards further analysis is sufficiently pure.

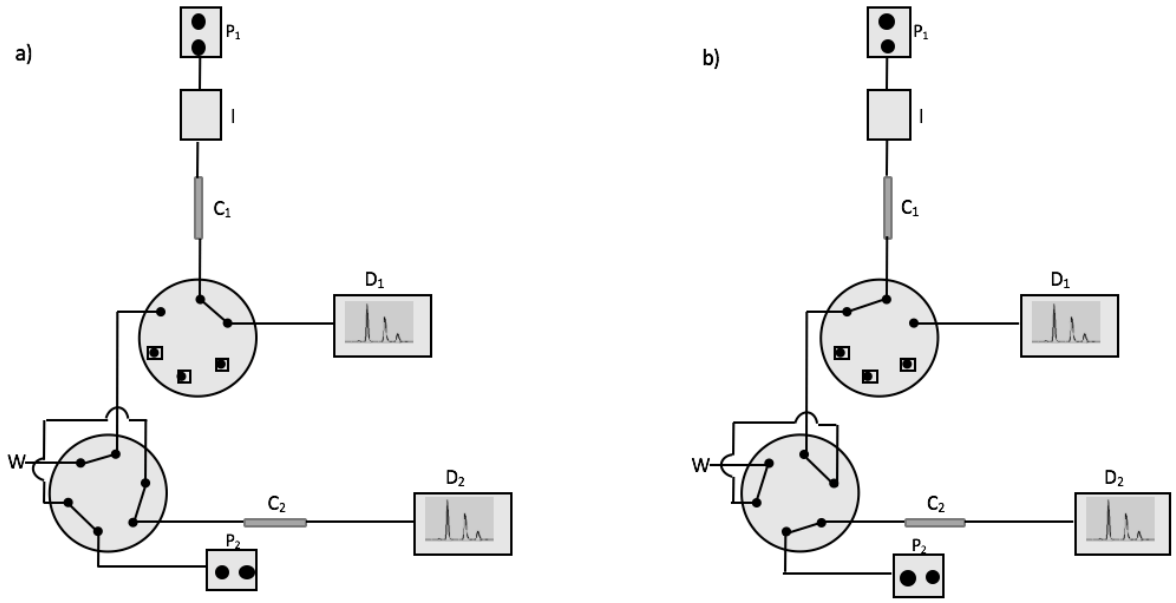


Figure 7: a) Two-dimensional design by Shalliker and coworkers using two six-port two position valves for heart-cutting in position 1 first dimension analysis. b) Two-dimensional design by Shalliker and coworkers using two six-port two position valves for heart-cutting in position 2 second dimension analysis. Legends: P1 =HPLC pump for the first-dimension chromatography, P2 = HPLC pump for the second-dimension chromatography, I = Injector, C1 = Chromatographic medium for the first dimension, C2 = Chromatographic medium for the second dimension, D1 = Detector for the first dimensional analysis, D2 = Detector for the second dimensional analysis, W = Waste, Boxed ports are not in use.

It is important to note here that the quality of the multi-dimensional chiral analysis depends on the accuracy of the heart-cut operation. For example, if the heart-cut method fails to trap the single target analyte of the first-dimension effluent from the achiral dimension, more than two peaks, that is two peaks originating out of the chiral separation of enantiomers in addition to other impurities originating from the imperfect heart-cut event, are likely to appear in the chiral dimension, which would make the chiral dimension analysis cumbersome and possibly error-prone. There is need for a multi-dimensional chromatographic setup that can verify completion of the heart-cut operation before the analysis for the chiral dimension takes place. The promise of such a chromatographic setup that would allow scientists to channel the targeted effluent of one dimension into another with a “confirmed” purity of the injectable, is enormous. This would allow researchers in screening facilities to reduce the risk of making

errors while generating valuable data on synthetic methods. However, incorporation of such a concept calls for the ability to re-circulate a selected portion of the effluent into a chromatographic medium equivalent to the first dimension so that the verification of the heart-cut operation could take place.

Another common dilemma associated with multi-dimensional chiral analysis stems from the possibility of on-column degradation (or racemization) during chromatographic separation. Racemization of a chiral center in the molecule of interest may occur under chromatographic conditions and the current state of the art in the field of multi-dimensional chromatography does not offer a solution to such chromatographic artefacts. Chemists often resort to the highly time-consuming and tedious task of synthesizing optically pure samples of each enantiomer of a target analyte either using an available synthetic procedure or through off-line purification techniques. The practice of making enantiomerically pure analytical samples for bulk manufacturing of the same chiral molecule often comes at a high cost primarily due to the expensive research and development work. The ability to isolate either of the two enantiomers from the chiral dimension stream and re-circulate one of the two enantiomer peaks back into the same chiral chromatographic medium would be extremely valuable. If an enantiomer were recirculated from the second chiral chromatographic run and produces more than one peak in the chromatogram, this would provide users with evidence of on-column degradation. The ratio of peak areas from the second chiral dimension could potentially allow for quantification of the chromatographic artefact from the first chiral dimension analysis.

A multi-dimensional chromatographic system that would allow any portion of the effluent from any chromatographic medium to be re-circulated through the same, or a different chromatographic medium for any number of times would be valuable. For example, when equipped with a heart-cut mechanism, such a setup would allow chemists to verify the accuracy

of the heart-cut operation by recycling a target analyte through the appropriate chromatographic media.

1.2 Aims and Objectives

The goal of the project was to design and test a multi-dimensional chromatographic platform which allows for the measurement of multiple QAs from a single injection. Specifically, the multi-QA analysis was to be tested for the synthesis of chiral compounds.

A preliminary two-dimensional HPLC setup (beta-platform) platform was constructed with the aim of providing information on the heart-cut valve switching mechanism. The first objective of the project was to investigate the basic two-dimensional HPLC capabilities for heart-cutting and valve switching without co-elution of the mobile phases.

The second objective was to implement the re-circulation function of separated effluents representing a specific peak in the multi-dimensional HPLC system.

The third objective was to integrate the analytical platform to an existing flow reactor system to test the effectiveness of the new multi-dimensional design in the flowed synthesis of chiral compounds.[24–28] Ultimately, the design was to be tested with real-life drug syntheses to see if the new design shows promise to be a multi-QA PAT tool.[3]

2. Results and Discussion

2.1 Multi-QA Analysis

In response to the highlighted notion of employing a multi-dimensional setup capable of recirculating target analytes through different chromatographic media, a multi-dimensional chromatographic setup coupled with heart-cut capabilities was developed. The multi-dimensional chromatographic setup would allow for the separation of an analytical mixture using a chromatographic medium and the isolation of a portion of the effluent in a two-position heart-cut valve following the first dimension analysis by a detector located upstream of the valve. The valve, which is capable of establishing fluid communications with multiple numbers of chromatographic media and a detector downstream of the chromatographic media, would send a selected portion of the effluent from the first dimension chromatographic medium to a new chromatographic medium that is identical in stationary phase to the first dimension chromatographic medium. The analysis by the second chromatographic medium would provide information on the accuracy of the heart-cut operation. This is the second dimension of analysis, which would act as a qualifying run for the subsequent chiral analyses in the proposed multi-dimensional setup. Once the heart-cut operation was judged successful (i.e. single peak observed), the heart-cut valve would channel the effluent into a chiral chromatographic medium to provide optical purity of the injected sample. This is the third dimension of analysis for the proposed design. The effluent from the third dimension analysis could be further recycled via the same or an identical chiral dimension to inspect if any on-column degradation took place during the third dimension analysis. This is the fourth dimension of the proposed multi-dimensional analysis. In our lab, this analytical protocol employing the analysis of multiple QAs along with in-line validation of the heart-cut process is called multi-QA analysis. A work-flow of this multi-QA analysis protocol incorporating various chromatographic dimensions and their purposes are shown in Figure 8.

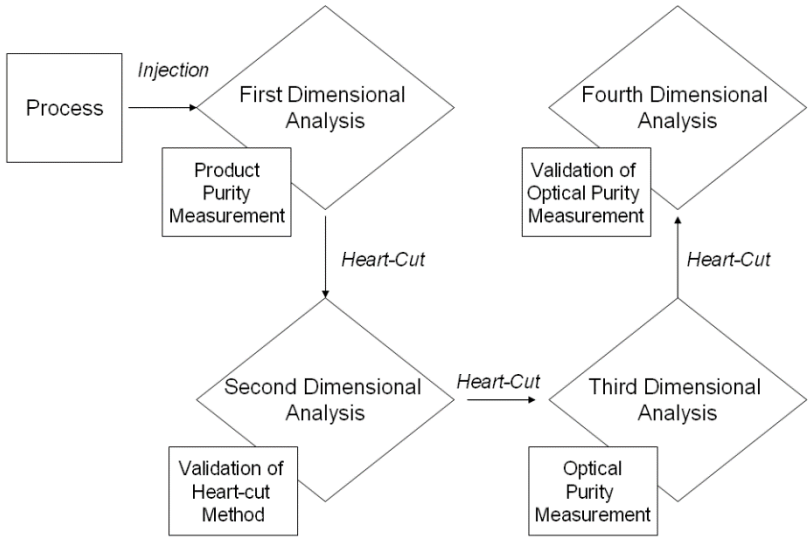


Figure 8: Schematic drawing of proposed novel multi-QA analytical protocol

The schematic drawing of the proposed multi-dimensional chromatographic platform is shown in Figure 9. Sample injection would be facilitated via an auto sampler that would direct the sample into an injector valve (V_I). This injector valve would be in fluid communication with a downstream column selector valve (V_S), which would direct samples through a host of chromatographic media (e.g., C_1, C_2 and C_3). The injected sample would be first flowed into C_1, which would act as the first dimension chromatographic medium and channel the separated effluent stream to a detector (D) located downstream of C_1 via V_S. This completes the first dimension analysis, which would give a measurement of peak areas of the product, reagents, and any byproducts as recorded by detector D. A heart-cut valve (V_H), which is located downstream of D receives the effluent from the first dimension analysis and would trap a selected portion of the effluent for subsequent analyses. Valve V_H would channel the trapped sample to chromatographic medium C_2, which is identical in stationary phase to chromatographic medium C_1. The same detector would be used to confirm if the heart-cut operation by valve V_H successfully trapped a single peak of interest. In other words, the second dimension analysis offers peak purity of the heart-cut sample. In the event the heart-cut operation failed to isolate a single peak of interest, the design is capable of recirculating the

unsuccessfully heart-cut sample back into valve V_H and eliminating peaks that are not of interest. The new heart-cut sample can be recycled through chromatographic medium C_2 again to re-assess the performance of the second heart-cut step. In other words, the design is such that any particular chromatographic medium downstream of valve V_H (e.g., C_2 or C_3 in Figure 9) could receive the same heart-cut sample a several times in order to troubleshoot the heart-cut operation. The strategy could fail however, if the intensity of the observed response from detector D diminishes below the recordable limit of the detector. The stated eventuality might occur if sample is lost due to binding onto the stationary phase, or if the heart-cut loop is not overfilled resulting in the analyte being diluted or if the analyte is too broad, in peak width, resulting in unsuccessful trapping of the entire contents of the peak in the loop. Once the operation in the heart-cut method is deemed satisfactory, the same heart-cut valve streams a desired portion of effluent from the second dimension analysis, the target chiral analyte at this point, towards the chiral chromatographic medium to undergo a chiral separation to give two peaks, each representing a single enantiomer (if both are present) (C_3 in Figure 9). The detector would measure the respective peak areas to provide optical purity (or % enantiomeric excess (%ee)) of the target analyte. This is the third dimension of the entire multi-dimensional analysis. The selected portion of the effluent (single targeted enantiomer) from the third dimension would be then diverted back to the same chiral chromatographic medium (C_3) by valve V_H and re-analyzed to determine if any degradation (i.e., racemization) of the stereocenter has occurred during the third dimension analysis. This constitutes the fourth dimension of analysis. In short, this unique design provides information on product purity through the first dimension analysis and optical purity through the third dimension analysis. Intermediary to these dimensions, the second dimension analysis confirms the quality of results to be acquired from the chiral analysis by confirming the purity of the heart-cut sample prior to its injection into the chiral dimension. The fourth dimension also confirms the quality of

data from the chiral analysis by verifying the stability of the corresponding enantiomers under the chiral chromatographic conditions. A step-by-step schematic outlining the valve actuation and entire multi-QA analytical protocol can be found in the Experimental on page 73.

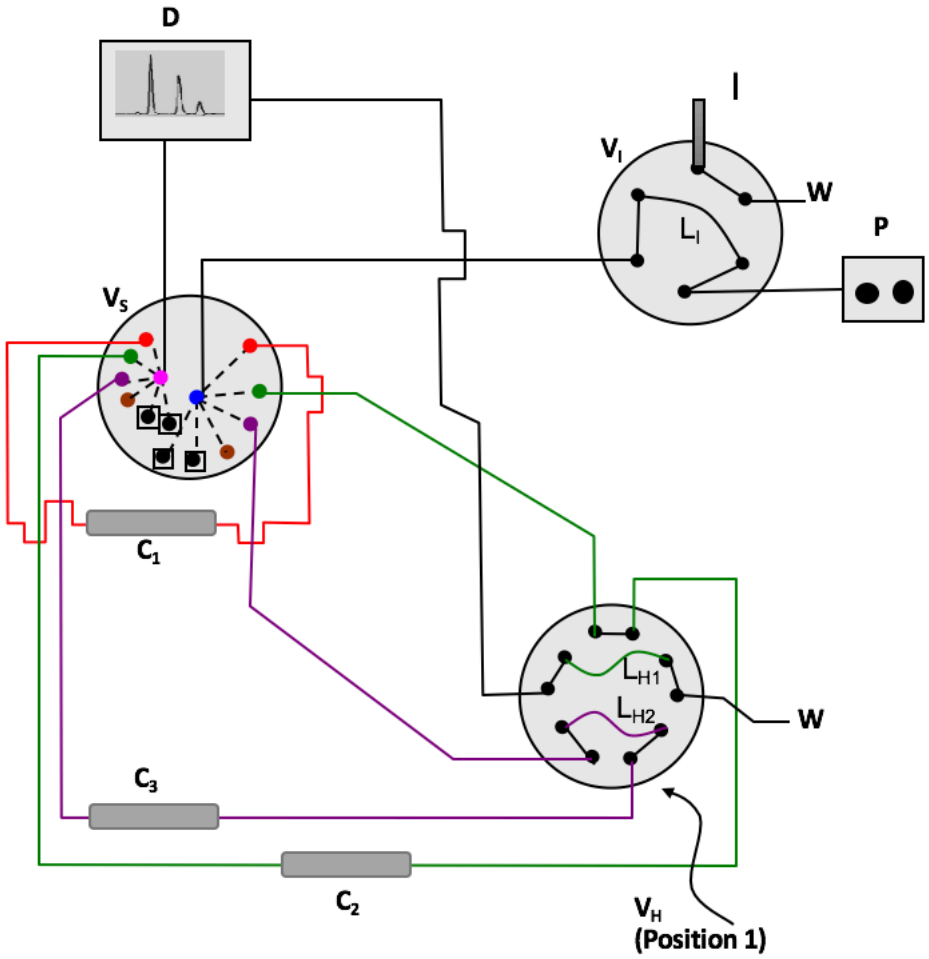


Figure 9: Novel multi-dimensional HPLC analytical platform with heart-cut capability. Legends: P = HPLC pump, I = Injector, C1 = Chromatographic medium for the first dimension, C2= Chromatographic medium for the second dimension, C3= Chromatographic medium for the third dimension, VH = Heart-cut valve, D = Detector (DAD), W = Waste, LI= Injector valve loop, LH1= Heart-cut valve loop 1, LH2= Heart-cut valve loop 2.

The new design is expected to provide a tool for multi-dimensional chiral analysis. The ability to re-circulate a selected portion of the effluent from one dimension to the other dimension for a number of cycles would allow for exhaustive analysis of samples that may have a limited supply (e.g., samples labelled with radio-isotopes or samples with hazardous

properties). Furthermore, any heart-cut sample can be re-circulated through the same chromatographic medium for a multiple number of times depending on the recovery of sample from each chromatographic run. This would allow a new mechanism to perfect a heart-cut method until the method is declared optimal based on a live feed from the detector. The heart-cut method is dependent on several parameters that control the heart-cut valve's actuation times for effective heart-cutting of a target analyte. The valve has to switch back and forth between load and inject positions to successfully trap a selected portion of the effluent. The actuation times cannot be set absolute, but have to be adjusted relative to an absolute reference time which is the time of injection. The absolute reference time is necessary in analysis as it establishes a starting point from which retention time of each eluting peak can be measured from. This measure of retention time t_R is important as based on retention time of a targeted peak, a delay time is to be applied in the controller software so that the heart-cut valve starts to receive the selected portion of the effluent in the heart-cut loop by switching to the load position. This time is termed as the 'open delay'. The time when the valve must switch back to its original position (i.e., the inject position) is termed as the 'close delay'. The difference between these two delay times constitutes the time during which the heart-cut valve resides in the load position. This time is a function of various chromatographic conditions of the previous dimension run, which during the heart-cut operation, remains active. Two parameters that primarily impact the fate of the heart-cut process are the flow-rate of the mobile phase of the active dimension run and the peak-width of the targeted peak of interest. There are also secondary influences, which are of no less importance for a successful heart-cut, including the laminar nature of flow in chromatographic flow-paths and the post-detection rate of diffusion of the target analyte plug in the mobile phase during its journey from the detector to the heart-cut valve. The back-pressure from the chromatographic media in question is also expected to play a role during the actuation of the heart-cut valve. It is important to note that the detection

time needs to be offset by a time based on the swept volume between the detector and the heart-cut valve so the actuation of the heart-cut valve happens exactly when the targeted portion of the effluent enters the heart-cut valve.

In comparison to conventional analytical protocol along with other multidimensional analytical protocol, a multi-QA analytical method developed through a multidimensional HPLC provides effective timely measurements of quality attributes with a confirmation of purity (Table 1). The disadvantages noted will soon be addressed in the future, wherein our detector will be treated as a live feed detector offering live feed monitoring capabilities along with valve actuations of peaks on a peak area basis (i.e. peak trapped within a range of time once its magnitude/ peak height exceeds a set threshold). Furthermore, the largest disadvantage currently present for a variety of multidimensional applications is the difficulty to switch directly between a reverse phase mode of separation in the first dimension to a normal phase mode of separation in the second dimension or vice versa. It is important to maintain the solvent strength across both dimensions of analysis in multidimensional applications and employ a gradient elution method to prevent co-elution of the mobile phase.

Advantages	Disadvantages
• Reaction monitoring • Adjustment of Heart-cut protocol • Multivariate analysis of multiple quality attributes • Recycling through same or different dimensions of analysis • Compliance with other stationary phase media and other detectors • Intelligent System Controller Software • Priming protocol prevents co-elution of mobile phase (i.e. ensures safety of column)	• Actuation on time basis • No live feed detection • Maintenance of solvent strength through all dimensions

Table 1: Advantages vs. Disadvantages of our multi-QA analytical protocol through a multidimensional HPLC

The performance of the setup was initially evaluated using a Buchwald-Hartwig amination reaction in flow. The sampling was done online using an online sampling mechanism developed in the Organ laboratory.[24–28] Later, synthesis of Warfarin was used as a model chiral drug synthesis to evaluate the performance of the proposed multi-dimensional design. The chiral drug syntheses were performed under batch conditions and the samples were analyzed off-line using the proposed multi-dimensional HPLC system.

2.2 Development of a first-generation platform with a heart-cut mechanism (Beta-model)

The first step towards building the proposed multi-dimensional chromatographic platform (Figure 9) was to examine chromatographic properties (e.g., retention time, peak area, width, and

shape) of peaks in the new dimension when a selected portion of the chromatographed effluent from one chromatographic medium is re-circulated through the same or a different chromatographic medium. The selected effluent would travel through various chromatographic modules, which could consist of flow-paths of vastly different internal diameters (e.g., typical movable slits inside the rotor of a heart-cut valve would have an internal diameter of 0.4 mm and a typical flow-path of a detector flow-cell would have a path length of 10 mm) during the multi-dimensional operation. The back-pressure from different chromatographic media could also be different depending on the porosity of the stationary phases and the flow-rate of the mobile phase. A lack of uniformity in chromatographic flow-paths will result in variations in back-pressure that may cause turbulence and pressure fluctuations throughout analysis. Furthermore, this also could lead to undesirable peak broadening due to Brownian motion. A rudimentary two-dimensional HPLC (2D-HPLC) setup equipped with a heart-cut mechanism was first built as a beta-platform to investigate the behavior of when injected samples undergo a heart-cut process of a single peak of interest from one dimension towards another. A schematic diagram of the rudimentary 2D-HPLC setup is shown in Figure 10.

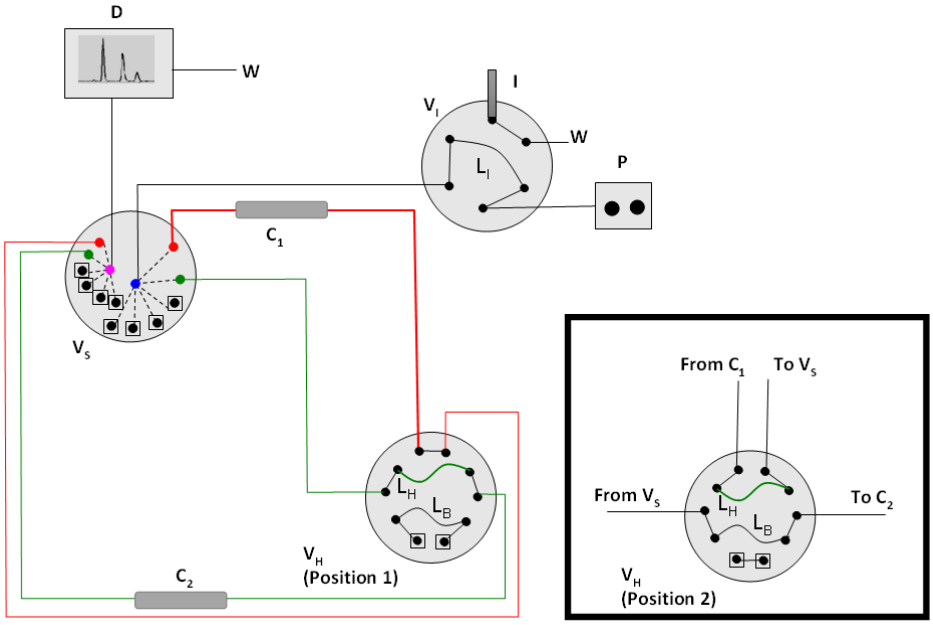


Figure 10: Rudimentary 2D-HPLC setup (beta-platform) for preliminary investigation. The insert shows the second position of the heart-cut valve. Legends: P = HPLC pump, VI=Injector valve, VS = Column selector valve, VH = Heart-cut valve, I = Injector port, LI = Injection loop, LH = Heart-cut loop, LB = Bypass loop, C1 = First dimension chromatographic medium, C2 = Second dimension chromatographic medium, D = Detector, W = Waste

In the beta-platform, a quaternary HPLC pump (P) was connected to a six-port two-position (load and inject) injector valve (V_I), which remained in fluid communication with a fourteen-port six-position column selector valve (V_S). The injector valve was equipped with a standard injection loop (L_I), capable of receiving analytes at the load position of V_I and channeling the injected analyte plug toward V_S from the inject position. Valve V_S was capable of diverting fluids through one of the six available flow-paths; each flow-path comprised of a set of inlet and outlet ports. Fluids travel to and from the valve via the central inlet and outlet ports (shown in blue and pink respectively, Figure 10). Two chromatographic media (HPLC columns C_1 and C_2) were placed in two of the six available flow-paths of V_S in accordance with the connectivity illustrated in Figure 10 (shown in red for C_1 and in green for C_2). The remaining four flow-paths were not used (shown in boxed black circles, Figure 7) at this point. A ten-port, two-position valve (V_H), which was used to perform heart-cut tasks, was situated downstream of the central outlet port of the column selector valve (V_S). Column C_1 was

connected to the first set of inlet and outlet ports of V_S via two adjacent ports on valve V_H (Figure 10). On the other hand, column C_2 was connected to the second set of inlet and outlet ports of V_S via a heart-cut loop (L_H) on V_H. A variable wavelength photo diode array UV detector (D) was connected downstream of the central outlet of V_S. When V_S set fluid communication between pump P and C_1, the fluid moved from V_I to V_H via C_1. Valve V_H was capable of channeling effluent from C_1 directly to detector D in the valve-position shown in Figure 10. Data acquired by detector D constitutes the data for the first dimension analysis.

Only eight out of ten ports of valve V_H were used for this rudimentary 2D-HPLC design. Two loops were mounted on V_H (L_H and L_B, shown in green and black). When V_H was rotated (insert in Figure 10), a portion of the effluent from C_1 flowed into loop L_H, which was the heart-cut loop. During this time, C_2 maintains fluid communication with valve V_S using the second loop L_B, which is a bypass loop. When V_H was rotated back to its original position, a portion of the fluid was isolated in L_H. At this point, V_S was rotated to establish fluid communication between pump P and C_2, and the fluid from L_H moved towards C_2. The effluent from C_2 reached the same detector via V_S. Data collected by detector D from this stream constitutes the data for the second dimension analysis.

Detector D was the terminal point of this beta-design. There was no provision for the effluent to travel through the detector and re-enter into the stream selector flow-paths for further analysis. As mentioned before, the setup was built to assess any impact that peak trapping could have on peak properties, which in turn, could affect analytical requirement such as good run-to-run reproducibility. Separate injections were made to analyze a target analyte from the first and the second-dimension chromatograms. In some cases, the heart-cut valve was physically disconnected from the flow-path to assess the peak broadening effects during the heart-cut step.

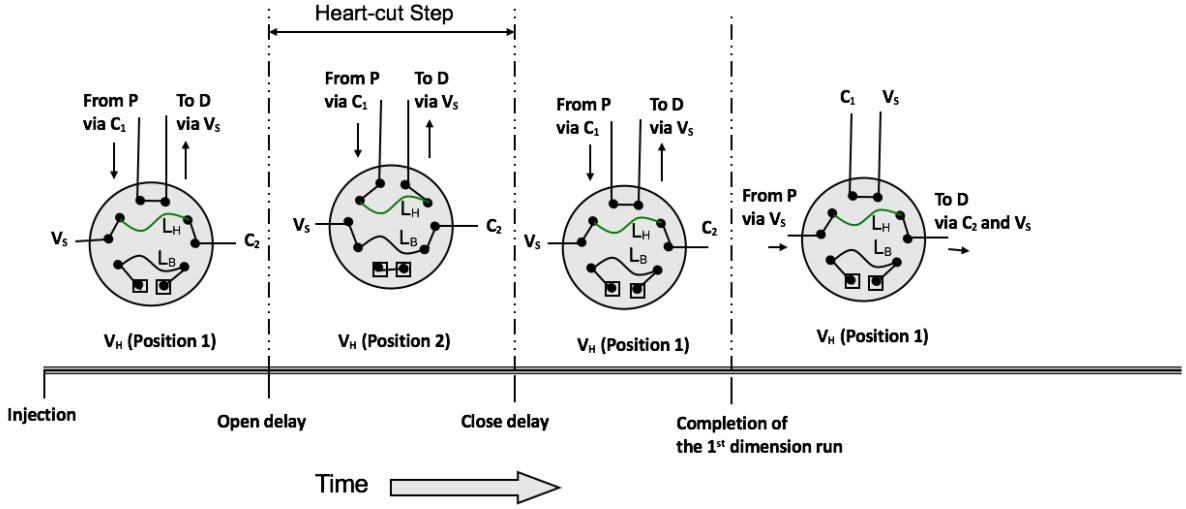


Figure 11: A schematic representation of the 'open delay' and 'close delay' time functions with respect to valve VH positions. The arrows indicate the direction of active flow during the heart-cut operation. Legends: VS = Column selector valve, VH = Heart-cut valve, C1 = First dimension chromatographic medium, C2 = Second dimension chromatographic medium, LH = Heart-cut loop, LB = Bypass loop.

In order to trigger the actuations (i.e., rotation of valve) of V_H, a valve switching protocol was implemented in the controller software. The firmware for the heart-cut operation involved two parameters: 'open delay', a time-function, is set relative to the time of the injection of an analyte at V_I, and 'close delay', a second time-function, is set relative to the open delay time. In other words, the open delay function exposes the heart-cut loop to the first-dimension (C_1) stream. The close delay function on the other hand, sets a time to rotate the heart-cut valve back to its initial position and isolates a portion of the first-dimension stream in L_H. Figure 11 illustrates the fluid movement during the execution of the open and close delay functions in the firmware. In an ideal world, these two time-functions can be calculated based on a simple mathematical relationship between flow-rate of the mobile phase for the chromatographic run in question and swept volumes of relevant flow-paths between detector D and the heart-cut valve V_H. Detector D measures retention time, a time at which a certain peak of interest reaches the point of detection in D. When a calculated off-set time is subtracted from the retention time value, the time at which a targeted peak reaches V_H, can be estimated. An arbitrarily chosen analyte (2-chloropyridine) was used to examine this hypothesis. A

sample was injected in C_1, a reverse phase C18 HPLC column, to measure the retention time of the analyte from the first dimension analysis. A separate second injection of the same analyte was made, this time with the heart-cut valve actuated to trap a portion of the first-dimension stream in a 10 μL heart-cut loop (L_H). Valve V_H and V_S were rotated appropriately to move the heart-cut sample from L_H to detector D via C_2. For the preliminary study, C_2 was also an identical C18 reverse phase HPLC column. The outcome of the heart-cut method was evaluated based on the presence or absence of the analyte peak in the second-dimension chromatogram. Unfortunately, the peak was not observed in the second-dimension chromatogram. Small volume loops, which often come with narrow internal bore size, are ideal for trapping a narrow segment of the first-dimension stream. However, the estimation of open and close delay functions proved to be extremely difficult using small volume loops. Alternatively, a larger sample holding loop (100 μL), which is commercially available only in wider bore sizes (0.02" bore) can be used for heart-cut purposes. It is important to note here that the wider bore flow-path could affect the quality of the second-dimension data. In using a wider bore size and larger loop volume there is the potential risk of transferring over peaks aside from the target analyte; in contrast to this, a smaller loop volume and narrower bore size, would heart-cut only a 10μL portion of the target peak towards the second dimension and so the risk is minimized. Generally, increasing the diameter or length of tubing (i.e. connections) will also contribute to band broadening due to the dispersion / Brownian motion through the flow-path. On one hand, the velocity of the effluent would drop at the entry of a wider-bore flow path (i.e. changing diameters of flow path can affect the velocity of the sample plug) heart-cut loop making the heart-cut actuations more error-tolerant. Increasing the diameter of a capillary line would result in more space for the sample plug to disperse/ expand and thus reduce the flow rate (i.e. less shear). Fortunately, when the 100 μL loop was used for the heart-cut trapping, the peak was observable in the second-dimension chromatogram. Throughout the heart-cut trapping

protocol, the target analyte peak was trapped with the goal to overfill the heart-cut loop with the entire contents of the target analyte trapped. Fortunately, the larger heart-cut loop allowed us to trap the analyte in question. However, a potential concern was whether significant dilution or band broadening of the trapped peak will occur when parked in the heart-cut loop. An experiment was conducted to observe for the dilution effect on how long the peak can remain parked in the loop until it is broad. A target analyte was injected towards a column for the first dimension analysis and was heart-cut towards the second dimension analysis. Prior to switching towards the second dimension analysis, the target analyte was left in the loop until the first dimension analysis and priming time (further discussed on page 45) were complete; once complete, the target analyte eluted out. A small, albeit significant variation in the retention time [RSD 0.5%] of the target analyte was observed during the first dimension analysis (i.e. when sample is run through same primary column multiple times). This was of concern since the open and close delay times were set prior to the start of the chromatographic run involving the heart-cut step. Fortunately, the variation (0.02 minutes) was significantly less than the time allowed between the open and close delay functions (1 minute) for the 100 μL loop. This experiment not only exhibited the success of heart-cut protocol in trapping a target analyte but also exhibited little to no peak broadening within the range of 0.2-0.3 min at most.

2.2.1 Evaluation of peak properties using the two-dimensional beta-setup

The success of the heart-cut experiment subsequently led to an evaluation of peak properties arising from the heart-cut operation. In order to test for peak broadening, the same sample of 2-chloropyridine was run through the first-dimension chromatographic medium (C18 HPLC column) with the heart-cut valve set in the bypass mode (i.e., bypassing the heart-cut loop and sending the sample to the detector). The peak width of the target analyte was found to be 0.3 min. Next, the same experiment was performed without the heart-cut valve physically being a part of the chromatographic path. The peak width of the same injectable was

0.2 min. The peak width variation was judged to be manageable since the detectability of peaks by the software remained unaffected with or without the presence of the heart-cut valve in the chromatographic path. Peak broadening was a potential concern as the heart-cut valve was placed behind the detector and, thus, elongating the flow path through additional capillaries and a wider bore loop- these all would contribute towards Brownian motion (longitudinal diffusion) of the target analyte's molecules within the flow path. Furthermore, varying the diameters of the flow path lines will result in pressure fluctuations throughout the lines and may also contribute to peak broadening that may be evidently observed in the output chromatogram. The same set of experiments were performed, this time using the first and the second dimension setup (i.e., using two identical C18 HPLC columns in accordance with the 2D-HPLC schematic presented in Figure 10). The peak properties were found to vary in peak width, height and symmetry, but showed little variation in the peak area between dimensions due to the heart-cut operation (Table 2). However, peaks were found to retain their Gaussian shape allowing the integrated software to easily register all analyzed peaks by data processing algorithm of the controller software.

Peak properties	**First dimension analysis; the heart-cut loop bypassed (min)**	**Second dimension analysis; injection from the heart-cut loop (min)**
Peak width	0.11	0.21
Peak area	858.8	868.8
Peak height	123.3	58.1
Peak symmetry	0.75	0.37

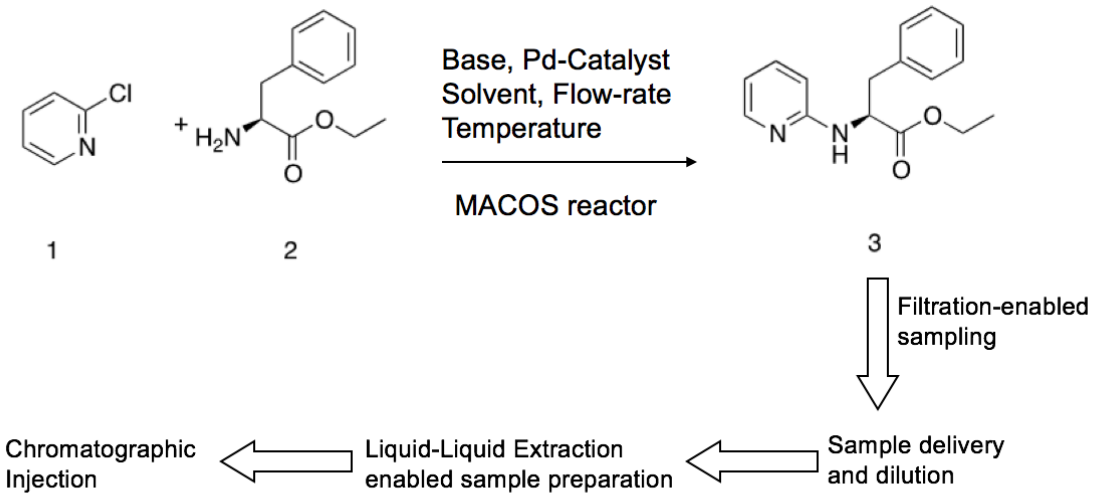
Table 2: A comparison of peak properties with and without the heart-cut step included in the two-dimensional chromatographic method.

Pressure fluctuations, measured by an inline pressure sensor, during the switching of the heart-cut (V_H) valve were found to be negligible (less than 1 bar) whereas switching of the stream selector valve (V_S) caused pressure to increase by more than 1 bar leading to small blips being registered in the chromatogram during. Ideally, this pressure fluctuation should be reduced, and if possible, completely eliminated so the identification of real peaks by the data processing algorithm remains unambiguous (i.e. can differentiate between real sample peaks

and pressure fluctuations). However, a pressure mitigation mechanism requires physical alterations to the chromatographic flow-paths in question. Fortunately, this pressure-mediated artefact is small and predictable (i.e., the data processing software could identify the blips based on the actuations of V_S recorded by the controller software). The heart-cut mechanism was tested under various repetitive chromatographic conditions to conclusively identify and discount the pressure-related artefacts from the chromatographic analysis. The detector was set at a wavelength of 254 nm where all target chemical components were observable at high absorbance values; the impact from the pressure fluctuation artefacts were rendered far less observable in comparison to the target chemical components in our analyses.

2.2.2 Evaluation of the beta-setup using a Buchwald-Hartwig Amination reaction

With a functional 2D-HPLC platform in hand, a model reaction was chosen to test the heart-cut mechanism. The reaction chosen for this study was a Buchwald-Hartwig amination under flow conditions. Professor Michael Organ from York University developed a flow-cell-free sampling device that is capable of sampling from a microwave assisted high pressure flow reactor. This technology is commonly termed as Microwave-Assisted Continuous-flow Organic Synthesis (MACOS).[29] The MACOS reactor was first integrated to the 2D-HPLC setup using a previously-reported sampling technology.[28] Details of this integration process, which was comprised of troubleshooting on sampling, sample delivery, and sample preparation steps, can be found in the experimental section. Once a suitable sample preparation mechanism was in place, attention was turned toward optimizing the model reaction so that monitoring two process quality attributes (in this case, product yield and optical purity of a chiral product) could take place in a real-life laboratory situation.



Scheme 1: Buchwald-Hartwig amination of 2-chloropyridine using a palladium pre-catalyst in flow. Filtration enabled –sampling was employed via a 10-port valve with an in-line filter and it is a sampling mechanism that was developed for flow synthesis in the Organ lab.

In this model reaction, 2-chloropyridine (**1**) was reacted with *L*-phenyl alanine ethyl ester (**2**) in the presence of a base and a suitable palladium catalyst (Scheme 1). The Pd-PEPPSI (Pyridine Enhanced Pre-Catalyst Preparation Stabilization Initiation) pre-catalyst system, which was also developed by Professor Michael Organ from York University was proven to be a highly efficient pre-catalyst for such reactions.[24] In order to monitor the progress of this reaction under flow conditions, a reverse-phase chromatographic method using anisole as an internal standard was developed (Figure 12). The second-dimension (chiral) separation, of product **3**, was attempted first using a reverse-phase chromatographic condition using a KH_2PO_4 /K_2HPO_4 buffer at pH 5.8. The chromatographic medium (C_2) for this dimension was a protein-based ES-OVM HPLC column. Unfortunately, chiral separation was found to be irreproducible, despite thorough screening of buffer conditions and experimenting with various reverse phase solvent systems. The peak from one enantiomer was found to be excessively broad, and the peak identification method in the data processing software failed to reliably identify the peak. Work on this chiral separation, which was heavily reliant on pH-dependent structural behavior protein-based stationary medium, was found to be time-consuming, at least

for the purpose of testing the beta-platform. Parallel separation work using a ChiralPAK IA HPLC column was successful (resolution: 3.6) using normal phase elution conditions (hexane/iso-propanol) (Figure 13). Unfortunately, ChiralPAK IA column, which uses a carbohydrate-based stationary phase for chiral separations, cannot tolerate any aqueous conditions (i.e. water or any mobile phase composition comprising more than 50% alcohol in normal alkane as the primary solvent). This restriction prevented the use of reverse phase conditions for the first dimension analysis. Consequently, C_1 was switched from the C18 HPLC column to a CN HPLC column, which is known to function under normal phase chromatographic conditions. A representative sample of compound **3** with a known optical purity was analyzed using the 2D-HPLC setup. The optical purity from the two-dimensional beta-setup was compared to that from a Nuclear Magnetic Resonance (NMR) Spectroscopy technique using a shift reagent.[30] The ratio between two enantiomers was reported as 2:1 by NMR. The same ratio from the current beta-setup was found to be 67.58: 32.42 (2.08:1).

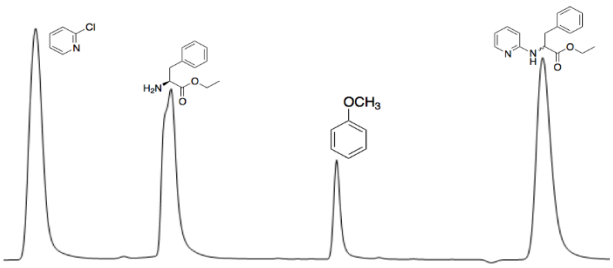


Figure 12: First dimension chromatogram of Buchwald Hartwig reaction mixture with anisole as the internal standard.

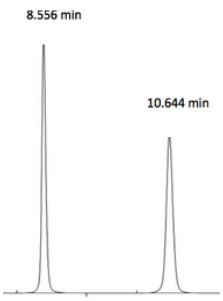


Figure 13: Chiral separation of product 3 via the ChiralPAK IA column under normal phase conditions

The next step was to integrate the 2D-HPLC setup to a previously reported sampling mechanism so that a representative sample could be transferred from the MACOS reactor to the 2D-HPLC platform in an automated manner. It is important to note that the model reaction involved a transition-metal mediated cross-coupling. The presence of colloidal palladium particles in the injection matrix could affect the performance of the analytical column due to chelation. The metal ions could ligate to a portion of the analyte to form soluble metal complexes, which could have different UV-absorption properties (leading to detection issues) and chromatographic affinities (leading to retention time variability). Run-to-run reproducibility could potentially deteriorate due to binding of the stationary phase to the metal ion. Initially, injections were made directly from the reactor output without any sample purification, however run-to-run reproducibly was poor during those experiments. Removal of palladium ions from the samples was attempted using an automated liquid-liquid extraction (LLE) technique with various palladium scavenging agents. Ultimately, a wash with an aqueous solution of tri-sodium ethylenediaminetetraacetic acid (EDTA) solution was found to be effective for sample preparation. Relevant details on the sampling and sample preparation work can be found in the Experimental section.

With a functional sampling and sample preparation strategy for the 2D-HPLC in hand, the model Buchwald-Hartwig amination reaction was attempted in flow using three positive-displacement pumps. The flow synthesis method comprised of one pump moving reagent **1** and the base, the second moving reagent **2** and catalyst, and the third moving the internal standard, all as solutions in DME at a net flow-rate of 75 μL/min. Cesium carbonate was first chosen as the base. A microwave generator, which is a part of the MACOS reactor platform, was set to heat the flowed reagents at 75°C. A 33 μL sample was sent from the flow reactor to the analytical platform to measure conversion to product **3** and its optical purity. Although the sampling mechanism was enabled with slurry handling capabilities, the reagent pumps were

not equipped with suitable technology to pump such slurries and cesium carbonate was also found to precipitate in the reagent syringe itself. Consequently, delivery of the base to the reactor was unreliable. This was also evident from the chromatogram which showed no formation of product **3**. Soluble sodium phenolate was used as an alternative base; however, this base had a poor shelf life (i.e. precipitation occurred in the syringe when the batch of the base was more than a day old). Also, there was no observable product formation. Several attempts were made using a variety of Pd-PEPPSI pre-catalysts, and different bases. A summary of reaction trials can be found in Table 3. Ultimately, a significantly more reactive pre-catalyst, (Pd-PEPPSI-^{i}Hept^{Cl}(η^3-cinnamyl)), which was found to activate at 75 $^{\circ}$C under flow conditions, was used and 3 was obtained in 78% yield. With product conversion information in hand, optical purity of the material was measured from a second injection using the heart-cut method. Enantiomeric excess (ee) was found to be very low (<1%). This was expected since strong bases like sodium tert-butoxide are known to epimerize the chiral center of product **3**.

Reaction Condition	Outcome	Possible reason
Reagents **1**, **2**, cesium carbonate, Pd-PEPPSI-iPent, 75° C, 75 μL/min flow-rate	No product	Precipitation of cesium carbonate in pump
Reagents **1**, **2**, sodium phenolate, Pd-PEPPSI-iPent, 75° C, 75 μL/min flow-rate	No product	Instability of sodium phenolate under flow conditions
Reagents **1**, **2**, sodium tert-butoxide, Pd-PEPPSI-iPent, 75° C, 75 μL/min flow-rate	No product	Pre-catalyst may not have sufficient time for the activation
Reagents **1**, **2**, sodium tert-butoxide, Pd-PEPPSI-iPent with Li-isopropoxide activation, 75° C, 75 μL/min flow-rate	No product	Pre-catalyst may not have sufficient time for the activation
Reagents **1**, **2**, sodium tert-butoxide, Pd-PEPPSI-iPent^{Cl}-o-Picoline, 75° C, 75 μL/min flow-rate	No product	Pre-catalyst may not have sufficient time for the activation
Reagents **1**, **2**, sodium tert-butoxide, Pd-^{i}Hept^{Cl}(η^3-cinnamyl), 75° C, 100 μL/min flow-rate	78% conversion into a racemic product mixture	Racemization was due to the use of a strong base
Reagents **1**, **2**, sodium tert-butoxide, Pd-^{i}Hept^{Cl}(η^3-cinnamyl), 80° C, 100 μL/min flow-rate	Unstable reaction temperature	Possible interference between the heat source (microwave) and the metal ion in solution

Table 3: Summary of results from various attempts of the model Buchwald-Hartwig amination in flow.

The purpose of using the model Buchwald-Hartwig amination reaction was to quickly assess whether the multi-dimensional platform could be used to measure two quality attributes in parallel and optimize a chemical process that gives a chiral product. The next logical step in this direction was to maximize conversion to product **3** by increasing the reaction temperature or by reducing the reaction flow-rate. Unfortunately, the temperature of the reactor was found to be unstable above 80°C due to a technical issue with the microwave reactor. An increase in palladium pre-catalyst loading, which could also increase the product conversion, led to some anomaly in the heating mechanism possibly due to the interference of excessive metal ions with microwave. In some cases, the temperature was found to unexpectedly rise within the reactor and syringe placed in-line to the microwave reactor. The sampling valve failed to collect the contents of the reaction mixture. The hypothesis made was that the valve, which was mostly made of stainless steel, may have acted as a heat sink in this case. Lowering the flow-rate was also found to be problematic. The reagent pumps, which move all reagents towards a common

union, did not work well when the flow-rate was very low. Instead of conducting further research to identify the issues relating to the flow reactor technology, a different model reaction was chosen, this time using the multi-dimensional platform to move towards a multi-QA analysis protocol.

2.3 Development of the ultimate multi-dimensional chromatography platform for multi-QA analysis

A fundamental difference between the two-dimensional beta-platform (described in the previous section, Figure 10) and the proposed multi-dimensional platform (Figure 9) stems from the ability to re-circulate a portion of the chromatographed effluent post-detection. A detailed version of the schematic presented in Figure 9 can be found in Figure 14. The detector is no longer the terminal (i.e. after the second dimension of analysis as observed in our beta-platform) of the chromatographic design. The detector (D) outlet was connected to the heart-cut valve (V_H) instead of to waste (W). Valve V_H, in turn, was connected to a set of chromatographic media to be used for multi-dimensional analysis. Inter-connectivity of modules was changed from the schematic shown in Figure 6 in order to support the concept of multiple recycling of chromatographed effluent in the new design. Four of the six available flow-paths of the column selector valve (V_S) were used. The first flow-path (shown in red) was connected to a chromatographic medium (C_1), which was responsible for the first dimension analysis. The second and the third flow-paths (shown in green and purple, respectively) were connected to two chromatographic media (C_2 and C_3 respectively) via the heart-cut valve (V_H). Valve V_H was equipped with two heart-cut loops (L_{H1} and L_{H2}, shown in green and purple), which could establish fluid communications to C_2 via L_{H1} and to C_3 via L_{H2}. L_{H2} is shown to have a fluid communication with C_3 in Figure 14. A portion of the effluent from C_1 could be moved to L_{H1} in this position of V_H by pump P. When valve V_H was rotated to the other position (shown in the insert of Figure 14), the material in L_{H1} could be moved towards C_2 for analysis and another portion of the effluent from C_1 could be trapped in L_{H2}. When valve V_H was rotated

back to its original position (i.e., the position of V_H shown in main schematic of Figure 14), the second portion in L_{H2} could be moved toward C_3 for analysis. It is important to note that the portion of the effluent from the first dimension analysis that was not trapped in L_{H1} or L_{H2} flowed to waste from either position of V_H.

Upon injection at V_I, V_S was set to flow the injected sample to C_1. The data collected during this time constitutes the first dimension analysis. A portion of the first dimension effluent was trapped in L_{H1} until the remaining portion of the first dimension analysis was completed using L_{H2} of V_H. Upon completion of the first dimension run, V_S was rotated to establish fluid communication between pump P and C_2. The heart-cut material trapped in L_{H1} was sent to C_2 for analysis. The data acquired during this time constitutes the second dimension analysis. A selected portion of the second dimension stream was isolated in L_{H2} using a similar strategy. Once the chromatographic run for the second dimension analysis was completed, V_S was rotated to set up a fluid communication between pump P and C_3. The material in L_{H2} was then sent to C_3 for a third dimension analysis. It is important to remember that V_H could channel any portion of the effluent from detector D to either of the two loops (green L_{H1} and purple L_{H2}) and recycle the trapped portion either through C_2 or C_3. For example, if a portion of the fluid from the third-dimension stream was to be re-analyzed by C_3 again, V_H and V_S could be configured appropriately to do that task. Depending on the purpose of that analysis a new set of data could be generated by the detector to obtain a fourth dimension analysis. The entire operation was to happen from a single injection at V_I.

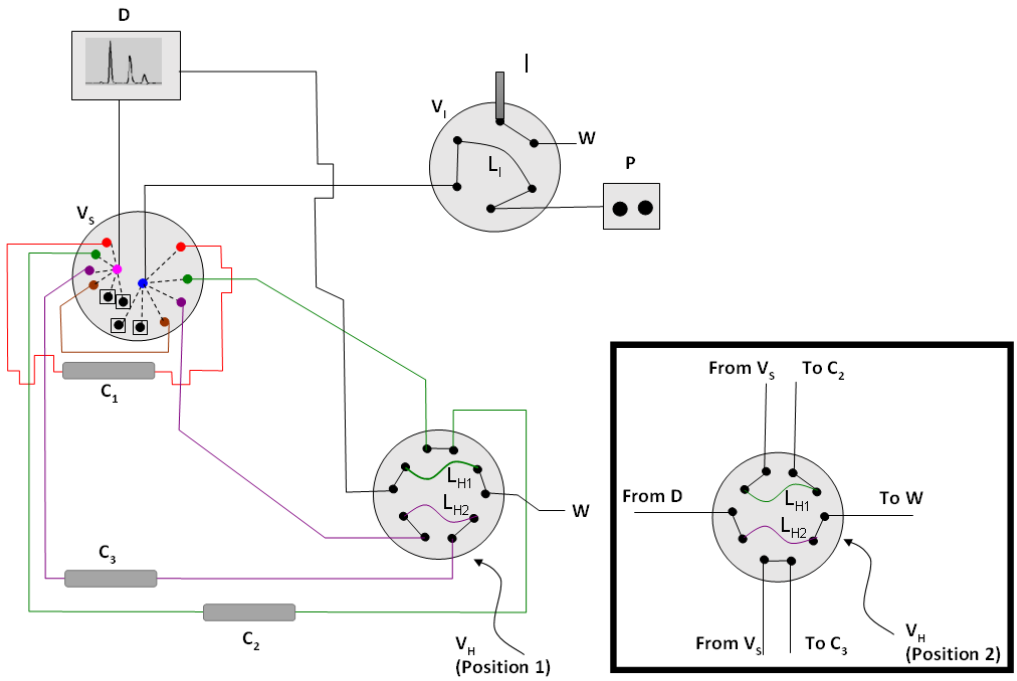


Figure 14: Novel multi-dimensional setup with heart-cut capabilities and priming line. The insert shows the second position of the heart-cut valve. Legends: P = HPLC pump, VI=Injector valve, VS = Column selector valve, VH = Heart-cut valve, I = Injector port, C1 = First dimension chromatographic medium, C2 = Second dimension chromatographic medium, C3 = Third dimension chromatographic medium, LI = Injection loop, LH1 = First heart-cut loop, LH2 = Second heart-cut loop, D = Detector, W = Waste.

To test the entire setup, a sample of Warfarin was injected into valve V_I of the new design. The first dimension-chromatographic medium (C_1) was a CN HPLC column, which was suitable for normal phase chromatographic conditions. Two 250 μL loops with wider bore size were used as the heart-cut loops to ensure that all of the selected portion of the effluent was transferred during the heart-cut process. An identical cyano HPLC column was used as C_2. A heart-cut protocol (i.e., optimized for open and close delay functions) for the first and the second dimension analyses were successfully implemented. Focus then shifted toward the third and the fourth dimension analysis. Keeping the objective of chiral analysis in mind, ChiralPAK IA was chosen as C_3. Chromatographic conditions (i.e., the mobile phase) for the first two dimensional analyses were expected to be significantly different from the one for the chiral analysis. It is important to note that only one HPLC pump and one detector were employed for the entire multi-dimensional design leading to sharing of flow-paths for the mobile phases in

different dimensions of analysis. Switching of chromatographic conditions between any two dimensions would not be accurate unless the chromatographic conditions for the dimensions in question are identical. In order to ascertain that the chromatographic fluid in the shared flow-path is in steady state before the start of a particular dimension of analysis, a restricted union path was connected to the fourth available flow-path of valve V_S (shown in brown in Figure 14). When a particular dimension of analysis was completed, valve V_S was switched to the fourth flow-path (i.e., the restricted union path) to drive all effluents from the previous dimension of analysis out of the shared flow-path before starting a new dimension of analysis. The strategy was verified by comparing retention times of a chosen target analyte (Warfarin, in this case) with varied amount of priming time between any two chromatographic dimensions. The priming time is defined in our setup as the time required to switch from one mobile phase separation method to another without co-elution of the mobile phase that could hinder the separation of samples (i.e. it is the time the column selector valve is in fluid communication with the restricted union path). The average priming time within the operational range of chromatographic flow-rates using the above-mentioned C_1, C_2 and C_3 HPLC columns was found to be 30 min. Chromatographic methods were developed for the achiral and chiral dimension analysis using the cyano and ChiralPAK IA HPLC columns. The priming strategy was incorporated in-between dimensions of the multi-dimensional chromatographic method to ensure steady states were achieved prior to the start of a new dimension. A racemic sample of Warfarin was recycled through C_1 (CN HPLC column) and three times through C_2 (a second CN HPLC column) to examine peak recovery in a four-dimensional chromatogram (Figure 15). Various peak properties of the control analyte are presented in Table 4. A chosen impurity (methyl m-nitrobenzoate) was added to test the performance of the first heart-cut step, sole purpose of which was to purify a targeted peak in the injected sample for subsequent analyses. The impurity was carefully chosen such that it

does not react with warfarin or any of the starting reagents. Percent recovery between first two dimensions (peak areas from row 1 and 2) was found to be 58%. The similar comparison between the second to the third (peak areas from row 2 and row 3) and the third to the fourth (peak areas from row 3 and row 4) were found to be 68% and 60%, respectively. Overall, on an average approximately 60% of a chromatographic peak was recovered between two dimensions. A part of this peak loss could be from the inability to capture all of the effluent containing the peak of interest during the heart-cut step. The logic in the decision-making process of the data processing algorithm is built around the ability to identify a chromatographic peak and calculate its purity based on the peak area in the dimension in question. Variation in peak areas between dimensions did not factor in the calculations as long as the peak properties were in agreement with the peak identification qualifiers set in the data processing algorithm. It is also important to note that the signal-to-noise ratio of the fourth-dimension peak was high enough for the sample to be further recycled if there was an analytical need. Peak symmetry was also found to be satisfactory for accurate identification of a target peak by the data processing algorithm.

Warfarin peak from various dimensions	**Retention Time (min)**	**Peak area (mAu*min)**	**Peak height (mAu)**	**Percent recovery$^{\Delta}$**	**Peak symmetry**	**s/n ratio**
1st dimension	37.2	1082.19	1036.29	-	1.19	8767.99
2nd dimension	48.3	632.50	921.03	58	2.93	5898.72
3rd dimension	53.1	424.80	479.26	67	1.14	3069.43
4th dimension	56.41	255.78	410.88	60	2.90	14477.75

Table 4: A comparison of peak properties of Warfarin from multiple recycling iterations in the second dimension of the multi-dimensional chromatographic design. Δ Calculated from the ratio of peak areas from the first to the second dimension between two consecutive dimensional analyses.

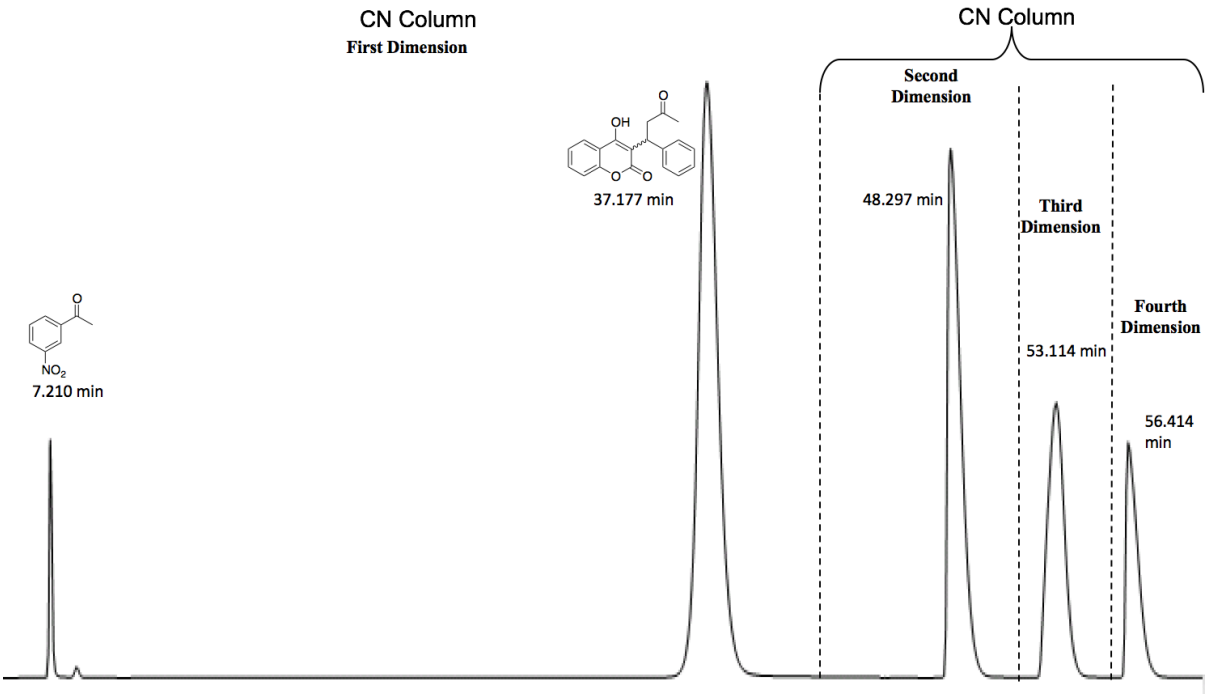
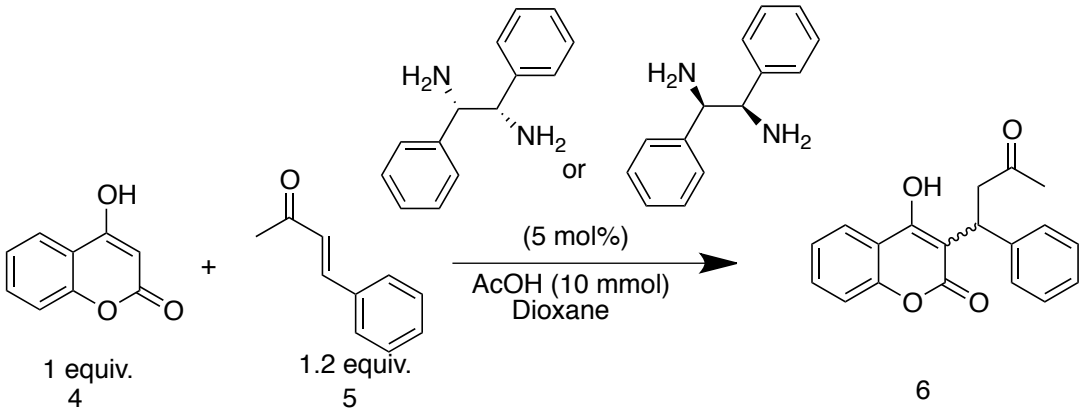


Figure 15: Chromatogram from multiple recycling of Warfarin for the study of its peak properties in various chromatographic dimensions

2.3.1. Evaluation of the multi-dimensional setup using synthesis of Warfarin

With the heart-cut protocol for the multi-dimensional setup validated, testing of the multi-dimensional setup for the parallel analysis of multiple quality attributes began. Synthesis of Warfarin was chosen as a model reaction for this purpose. Warfarin, which is an active pharmaceutical ingredient for an anti-coagulant drug Coumadin™, has one chiral centre in it.[31] The synthesis of Warfarin via an asymmetric Michael Addition reaction is well documented in the literature and several chiral catalysts including those derived from amino acid-based imidazolidines[32] and 1,2-diphenylethanediamine[31,33] have been reported in the literature. The reaction has been examined largely in batch as the reaction is reported to be problematic in flow due to the poor solubility of 4-hydroxycoumarin. The reaction of 4-hydroxycoumarin (**4**) with trans-phenyl butenone (**5**) in the presence of chiral 1,2-diphenylethanediamine to give Warfarin (**6**) is presented in Scheme 2. Percent product conversion to 6 and optical purity were chosen as the two quality attributes to monitor. A multi-dimensional chromatographic method

was needed first in order to study the synthesis of product 6 using this multi-QA analytical platform.



Scheme 2: Synthesis of Warfarin in batch

2.3.1.1. Development of a multi-dimensional chromatographic method for analytical study of Warfarin synthesis

A racemic sample of product 6 was first used to develop a chromatographic method for all four dimensions of analysis. A mixture of hexanes and iso-propanol (95:5 by volume ratio) was found to be optimal for the first two achiral dimensions, which were done using two identical cyano HPLC columns. The chiral analyses (the third and fourth dimensions) were done with a mixture of hexanes and iso-propanol (80:20 by volume ratio) using a ChiralPAK IA HPLC column. The priming strategy was successfully implemented to ensure all three HPLC columns were at steady state prior to the start of each dimension analysis.

As seen during the evaluation of the beta-setup, pressure fluctuations during the switching of the column selector valve (V_S) were noticeable. To simplify the peak identification process by the data-processing algorithm, a time-programmed variable-wavelength detection method was used. The first dimension analysis was done at 254 nm wavelength while the remaining dimensions were analyzed at 214 nm. An external standard calibration curve was constructed using the variable-wavelength detection protocol. Once all aspects of the chromatographic method were judged satisfactory, an analytical sample from a

trial sample from a batch synthesis of 6 was injected to obtain a full four-dimensional chromatogram (Figure 16). The portion of the chromatogram representing the first dimension analysis showed three peaks wherein the first peak was compound **5** (8.4 min), second peak was compound **4** (9.9 min), and the last peak was the chiral product Warfarin (compound **6**, 38.1 min). The HPLC purity (i.e., the percent product conversion to 6) was found to be 17%. The portion of the chromatogram representing the second dimension analysis showed a single peak of **6** at 45.2 min. Appearance of the warfarin peak as a single peak confirmed that the open and close delay parameters for the first heart-cut step were optimal and the purity of the heart-cut sample was satisfactory for a meaningful chiral analysis in later dimensions. The verified Warfarin peak was recycled using a second heart-cut step for subsequent chiral analyses. Two peaks were observed at 89.7 min and 100.4 min for the two enantiomers of Warfarin in the chiral dimension (i.e., the third dimension). The enantiomer at 100.4 min was recycled through the chiral column (C_3) after a third heart-cut. The targeted enantiomer appeared at 121.1 min in the fourth dimension portion of the chromatogram. The ratio of peak areas from both enantiomers were found to be equal, which was consistent with the injection of the racemic sample. Appearance of a single peak in the fourth dimension portion of the chromatogram suggested that there was no on-column degradation of the enantiomer product (i.e., no on-column racemization).

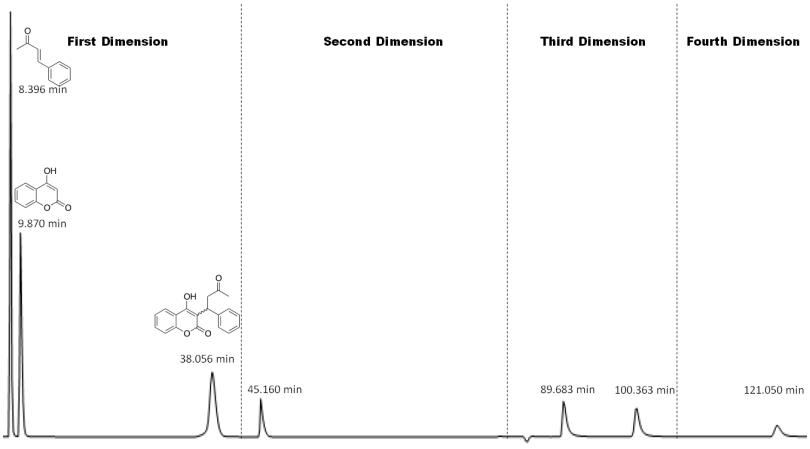


Figure 16: A sample multi-dimensional chromatogram from the synthesis of Warfarin

2.3.1.2. Reaction optimization for the synthesis of Warfarin using the multi-dimensional multi-QA analytical platform

With satisfactory validation data from the multi-QA setup in hand, a batch reaction for the synthesis of product 6 was set up using (1S,2S) -(-)-1,2-Diphenylethylenediamine ((S, S)-DPEN) as the chiral catalyst according to the reaction condition shown in Scheme 2. The presented four-dimensional chromatogram below displays the output results of an aliquot taken after 24 hours of the reaction (Figure 17). The product conversion was found to be just 16 % with an optical purity of 79 %. This chromatogram proved that the strategy to obtain multi-QA data from a single injection using the multi-dimensional setup of Figure 14 is achievable.

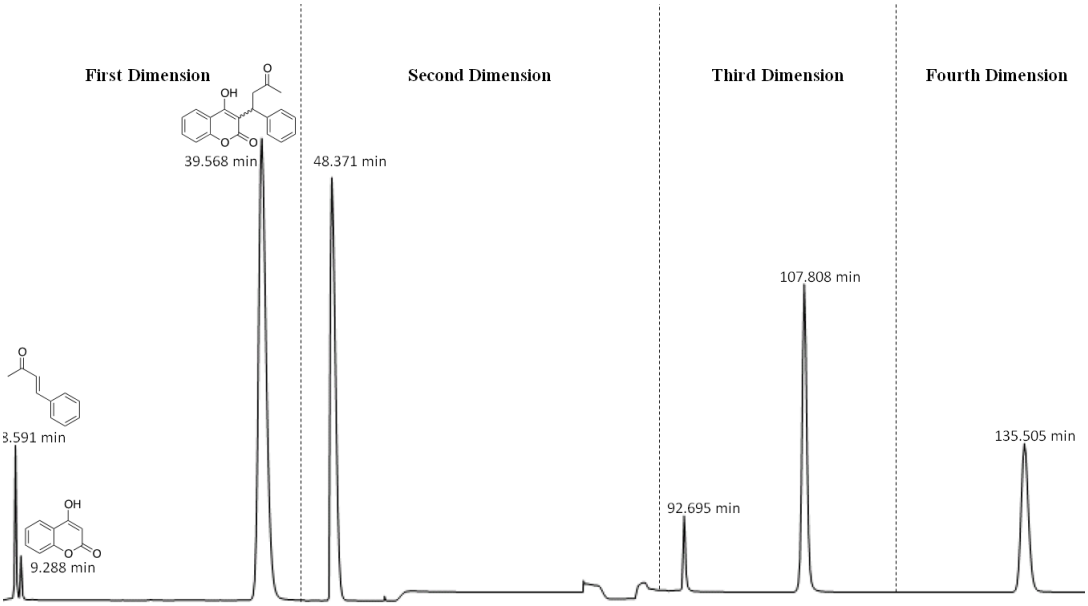


Figure 17: Multi-dimensional chromatogram from the synthesis of Warfarin using S, S-DPEN as the chiral catalyst.

A slightly larger scale reaction was set up and aliquots were manually collected from time to time to monitor the progress of the reaction (i.e., percent product conversion and optical purity) over a week. If the reaction was allowed to stir for a prolonged period of time, the reaction could proceed in the forward direction (i.e., higher conversion to product **6**), although optical purity of **6** could be compromised over time. The ability to obtain multiple number of quality attributes could mitigate the risk of losing product purity (e.g., optical purity in this case) while monitoring product conversion over time. The multi-QA analytical platform

successfully reported both percent product conversions and optical purity from all samples as shown in Figure 18. The formation of product **6** was found to increase overtime (approximately 5% per day) and the product was found to retain its enantiomeric purity (range: 75 - 82 %) in the reaction mixture over seven days.

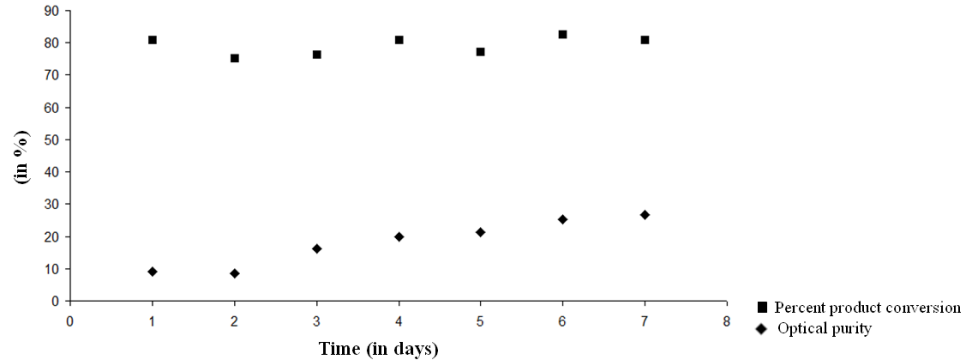


Figure 18: Multi-QA results from the monitoring of Warfarin synthesis using S, S-DPEN as the chiral catalyst for 168 h.

The synthesis of Warfarin was also done using (1R,2R) -(-)-1,2-Diphenylethylenediamine ((R, R)-DPEN) as the chiral catalyst. As expected, the other enantiomer of Warfarin (i.e., the first eluting peak in the third dimension portion of the multi-dimensional chromatogram), was found to be the major product this time (Figure 19). The minor enantiomer (i.e., the second eluting peak in the third dimension portion of the chromatogram) was recycled into the fourth dimension to check for on-column racemization of the chiral product **6**. Further examination on the peak recycling of the first major enantiomer in this experiment was conducted in a later experiment and yielded inconclusive results, which is discussed on page 47. In this experiment, upon recycling of the minor enantiomer, no degradation was observed in the fourth dimension portion of the chromatogram. A similar seven-day experiment was also done to monitor the progress of Warfarin synthesis from the R, R-DPEN catalyst (Figure 20).

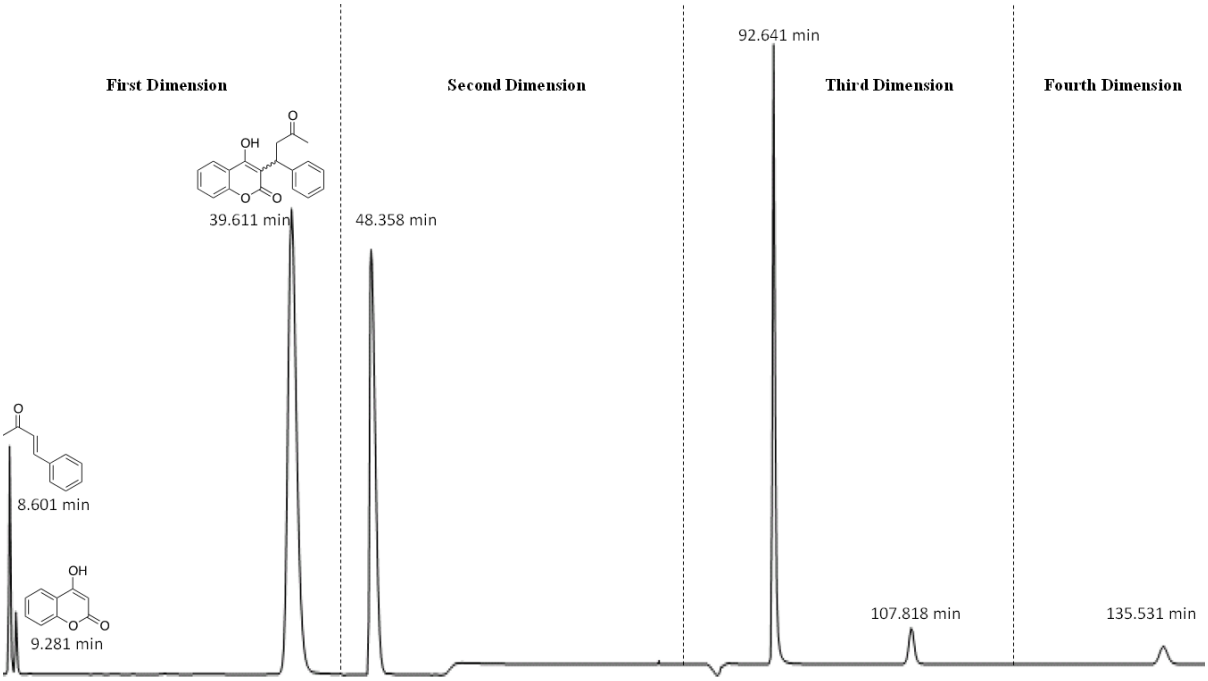


Figure 19: Multi-dimensional chromatogram from the synthesis of Warfarin using R, R-DPEN as the chiral catalyst.

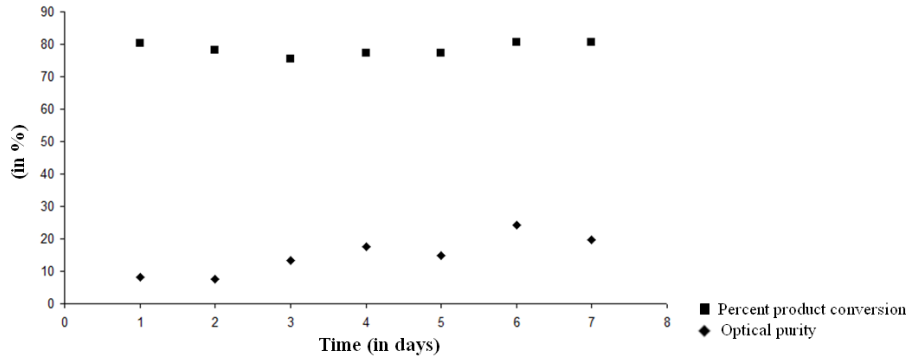


Figure 20: Multi-QA results from the monitoring of Warfarin synthesis using R, R-DPEN as the chiral catalyst for 168 h.

Once sufficient amount of product **6** was isolated from the scale-up reaction using the S, S-DPEN catalyst, product **6** was also purified using conventional preparative scale chromatographic techniques. The purity of compound **6** was confirmed by standard HPLC technique and the purified sample was injected directly into the ChiralPAK IA column to compare optical purity obtained by the multi-QA HPLC technique to the one obtained from the standard one-dimensional chiral HPLC analysis, which is the current state of the art. The

results were found to be identical. The optical purity was also determined using NMR spectroscopic and polarimetric techniques. Comparison of data are presented in Table 5. Stability of the chiral centre in product 6 during off-line purification, which may lead to the epimerization, could have been a concern. It is important to note here that preparative-scale purification prior to chiral analysis is a standard and mandatory practice since the results from available chirality assessment techniques (standard HPLC or polarimetry) are very sensitive to the purity of the analyte. In fact, the ability to conduct chiral analysis without incorporating a preparative-scale laboratory purification step is one of the principal advantages of the presented multi-QA analytical platform. It is also important to note that any chromatographic artefacts which may have originated from the first (purification) and the third (analysis) dimension chromatographic analyses, were identified in the second and the fourth dimension chromatographic analyses by the data processing algorithm to validate analytical measurements.

Analytical technique	Chiral purity
One dimensional HPLC	86 %
Polarimetry	90 %
The new multi-dimensional multi-QA HPLC	86 %

Table 5: Comparison of optical purity from various techniques including the multi-QA platform.

An attempt was made to examine the reaction when a reaction condition is changed to optimize for the Percent conversion. This experiment was done as additions made to a reaction vessel may change the contents of the reaction mixture in a way that upon analysis retention time shift and even possible unforeseen impurities or byproducts may be observed. An attempt was made to make Warfarin using 5 mol% of lithium acetate (LiOAc) as an additive according to a literature procedure .[31] Product conversion from two consecutive batch runs using LiOAc were found to be low (18%), but consistent. Enantiomeric excess of product **6** was found to be 86 %. The chromatogram is shown in Figure 21.

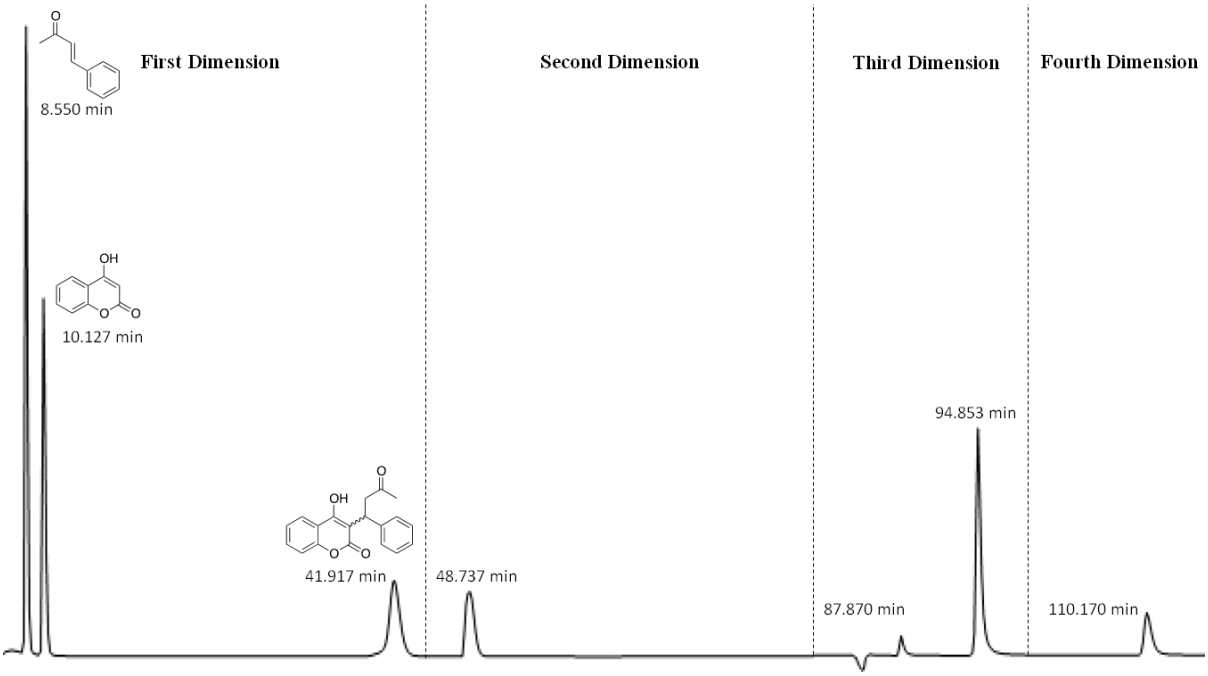


Figure 21: Multi-dimensional chromatogram from the synthesis of Warfarin using S, S-DPEN as the chiral catalyst using LiOAc as an additive.

The analyses of all four dimensions were done using the minor enantiomer (i.e., the second eluting peak from the third dimension portion of the chromatogram in Figure 19) during the experiment with the reaction involving the RR-DPEN catalyst. Recycling of the major enantiomer, which was the first eluting peak in the third dimension portion of the chromatogram, posed a peak identification issue in the data processing software. Figure 22 illustrates an example of a multi-dimensional chromatogram when attempts were made to recycle the major enantiomer (i.e., the first peak of the third dimension portion of the chromatogram). The overlap of peaks occurred between the third and the fourth dimension peaks as the fourth dimension heart-cut process was carried out while the column selector valve was still in fluid communication with the chiral column responsible for analysis of both the third and fourth dimension analysis. Ideally, the workflow of the multi-dimensional method was to carry out the execution of one chromatographic dimension at a time (i.e. each dimension was to be treated as a separate analysis space where an output QA or verification of an output QA is determined). A closer inspection of the schematic in Figure 14 revealed a possible

scenario wherein such overlaps may occur. The design presented in Figure 14 did not allow for the fourth-dimension run to be delayed until the third-dimension run was completed. When valve V_H was rotated to isolate the Warfarin enantiomer in question in L_{H2} during the third and the final heart-cut (Figure 14), pump P immediately established fluid communication with L_{H2} (Figure 14) and commenced the fourth dimension run as soon as the third heart-cut step was completed. In other words, the rotation of V_H to complete the third heart-cut task was responsible for the immediate start of the fourth dimension. In many cases, this phenomenon would not necessarily pose a chromatographic issue (i.e., the overlap of peaks from two different dimensions), especially when the last two dimensions involve chiral resolution. Separation of chiral peaks are generally viewed as difficult and the distance between two enantiomeric peaks (i.e., the chiral resolution of peaks) are generally small. In certain cases, when the chiral resolution is fairly large and the retention time of the first eluting peak is significantly small, such overlaps may result. The phenomenon is further explained pictorially in Figure 23.

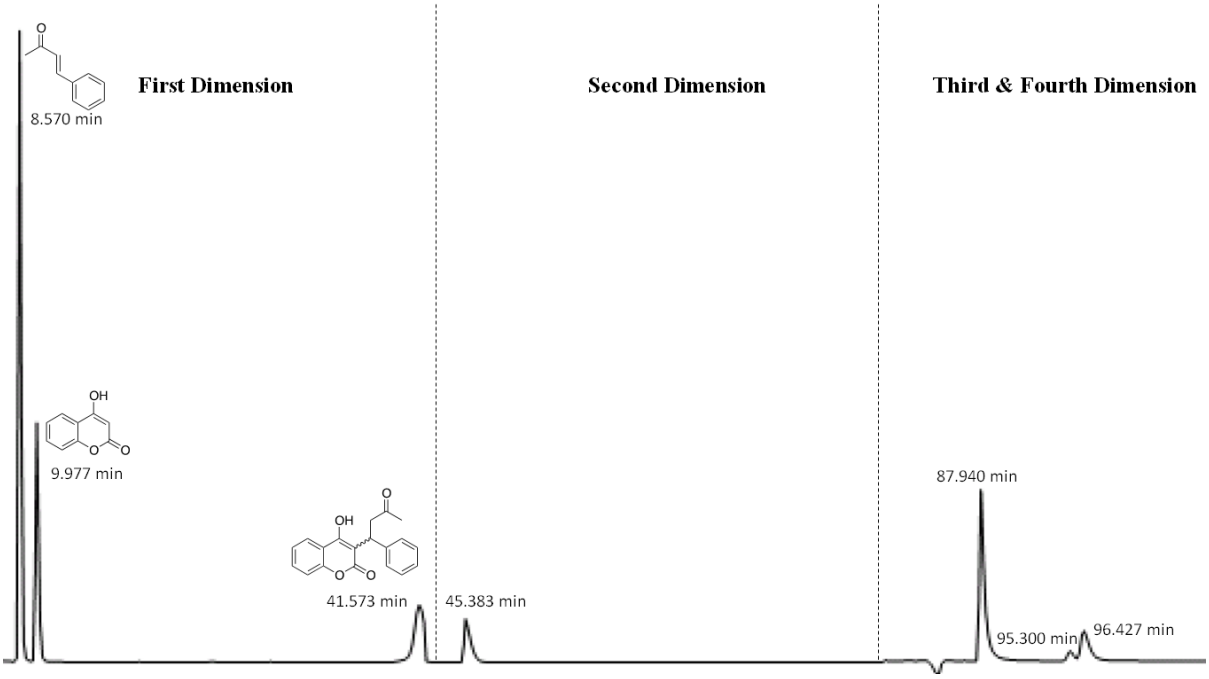


Figure 22: Multi-dimensional chromatogram from the synthesis of Warfarin showing overlap of peaks when the second eluting enantiomer from the third dimension was subjected to heart-cut.

The middle chromatogram in Figure 23 shows the time at which a heart-cut step took place (the vertical line). The top chromatogram represents the chiral resolution of a mixture containing both enantiomers without any heart-cut step. If there is no provision to park the heart-cut sample, the first eluting peak from the chiral separation, if recycled, may re-elute before the elution of the second peak from the chiral separation (i.e., top chromatogram). This is because the retention time of the first eluting peak in the top chromatogram is significantly low and the distance (by time) between both peaks (top chromatogram) is significantly large. First step towards rectifying this matter was to identify all three peaks with certainty beyond the second dimension portion of the multi-dimensional chromatogram. Once the peaks were correctly identified, elution of appropriate peaks can be delayed to eliminate overlapping of peaks. An example from a real chromatogram is shown in segment A of Figure 24, where such an overlap of peaks between 105 and 107 minutes were registered. One of the two peaks in the overlapping zone represents the recycled peak for the fourth dimension. The design of the multi-dimensional setup was slightly modified to examine if one of the peaks can be delayed for unambiguous identification of peaks for the multi-dimensional analysis. Figure 25 illustrates the modified multi-dimensional design.

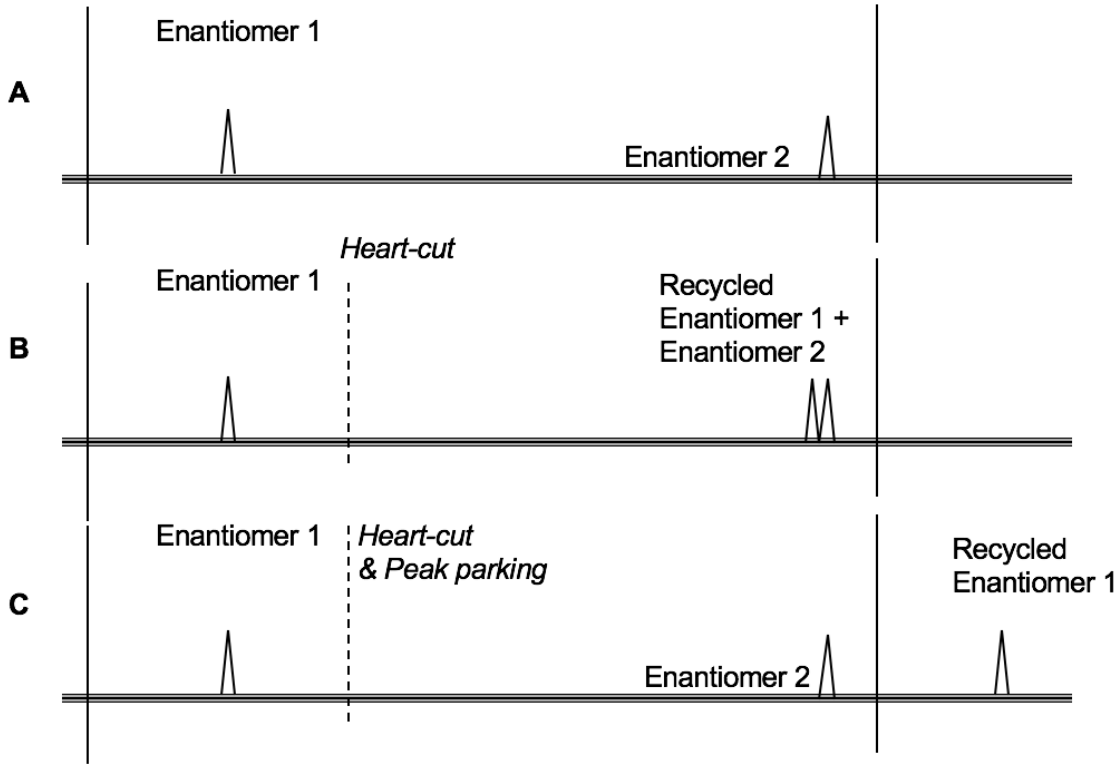


Figure 23. A schematic comparison of the third and the fourth dimension chromatograms when earlier eluting peak from the third-dimension chromatogram was recycled for the fourth dimension analysis. A: A schematic presentation of the third dimension chromatogram without the heart-cut step for the fourth dimension analysis. B: The third and the fourth dimension chromatograms from the heart-cut done using the multi-dimensional design presented in Figure 12. C: The third and the fourth dimension chromatograms using options to park the heart-cut peak until the third dimension analysis is completed.

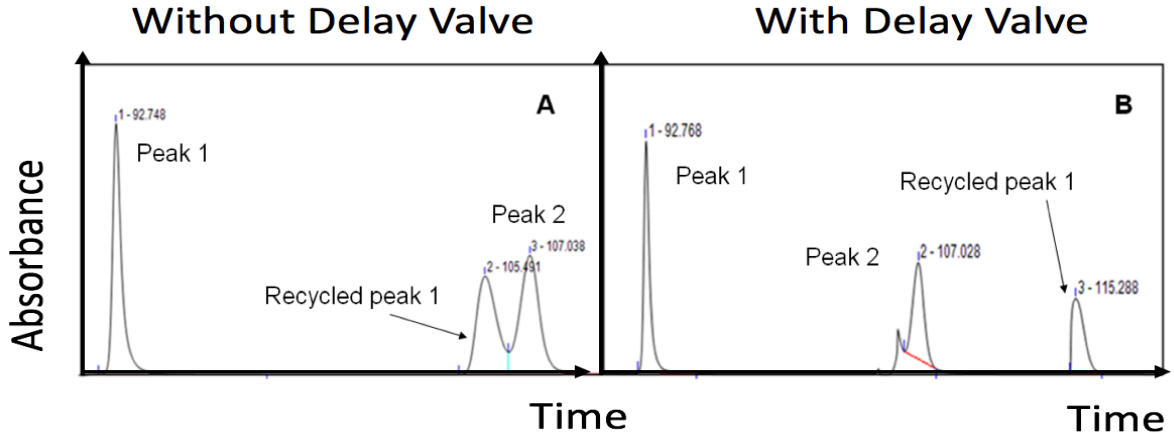


Figure 24: a) Multi-dimensional chromatogram from the synthesis of Warfarin using designs without the delay valve (V_D) where peak 1 at 92.7 min is our first eluting enantiomer that was recycled and found at 105.5 min and peak 2 at 107 min is our second eluting enantiomer b) Multi-dimensional chromatogram from the synthesis of Warfarin using designs with the delay valve (V_D) where peak 1 at 92.7 min is our first eluting enantiomer that was recycled and found at 115.3 min and peak 2 at 107 min is our second eluting enantiomer

A six-port valve two-position valve (V_D), in which a sample could be parked for a period of time until the previous dimension run is completed, was included downstream of C_3 (Figure 25). This delay valve (V_D) could have been put between D and V_H to park a chromatographic peak immediately after its detection. However, the swept volume of the flow-path between the detector D and V_H was a critical parameter that dictated open and close delay time functions. Alteration of this flow-path may require some additional optimization, which could take some time, in the heart-cut firmware. For this reason, the flow-path between D and V_H was kept untouched during the preliminary investigation of peak parking methods. During the third dimension analysis, valve V_D, which was also equipped with a peak parking loop (L_P), was kept in the valve-position shown in Figure 25. After the first eluting peak was recycled in C_3, the effluent carrying the peak was delayed in L_P until the third dimension run was completed. The timing of this particular peak capture in L_P was accomplished by trial and error. No automation was planned for this particular step using this design. The trapped peak in L_P was ultimately released after the third dimension analysis and flowed to detector D using the fourth available flow-path of valve V_S (shown in pink in Figure 25) to obtain an overlap-free third dimension analysis (segment B of Figure 25). Not all of the targeted peak was delayed in the elution order. The purpose of this exercise was to examine if the peak parking strategy could be accurately executed (i.e., precisely targeting a peak from an overlapped chromatographic region) using the current firmware. It is also important to note that the retention time of the targeted peaks vary from run to run due to the continuing modifications made in the hardware and the firmware of the current design. The order in which the peaks appear in all four dimensions of the multi-dimensional chromatogram is, however, predictable. The retention time labels on the chromatogram were generated by the data processing software based on a time-counter referenced to the time of injection at V_I. Retention times for the subsequent dimensions were found to be consistent based on the actuations of V_S, V_H, and V_D.

The retention time labels on the chromatogram were not used to track peaks since the controller software was capable of tracking peaks from all dimensions using the valve actuation information within the software. Ultimate objective of the project would involve actuations of all chromatographic valves based on a live feed from the detector. In that case, tracking of chromatographic peaks based on valve actuation information within the software could be accomplished.

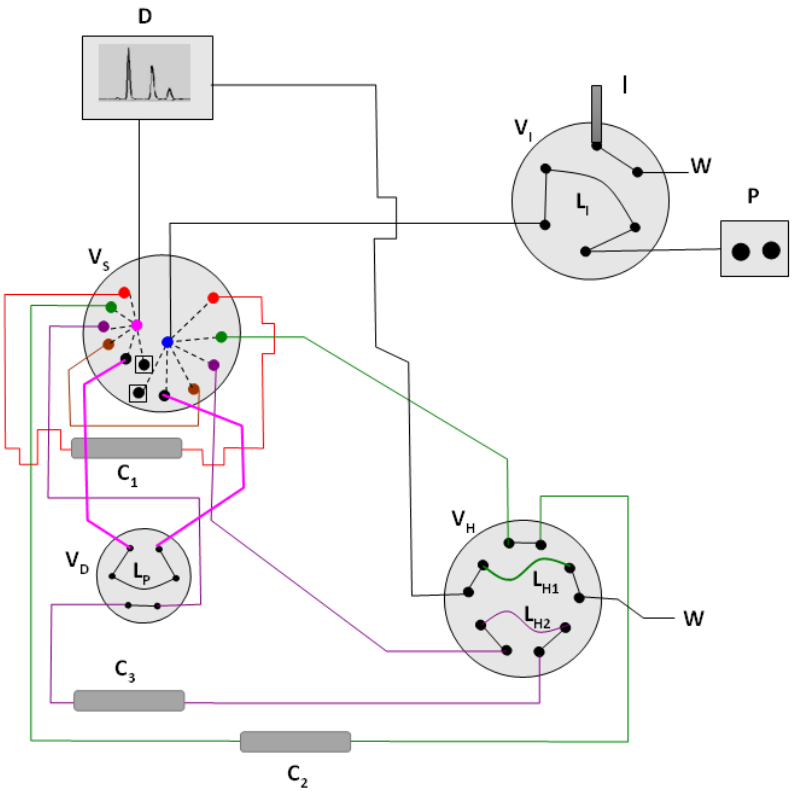
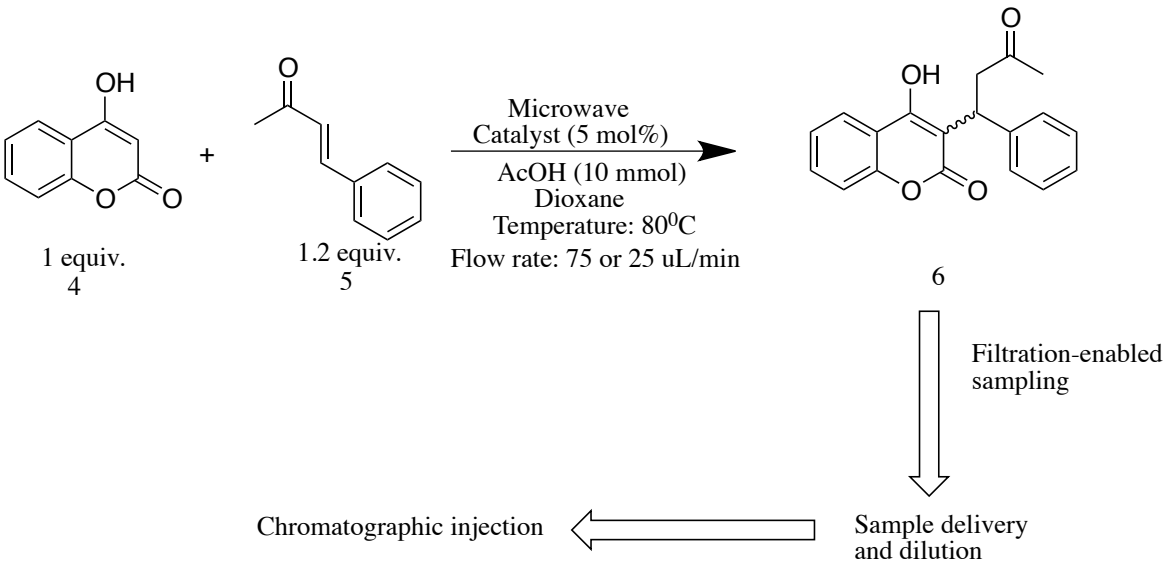


Figure 25: Chromatographic design used to investigate the peak-overlapping scenario. *Legends: P = HPLC pump, V_I=Injector valve, V_S = Column selector valve, V_H = Heart-cut valve, V_D = Delay valve, I = Injector port, L_I = Injection loop, L_{H1} = First heart-cut loop, L_{H2} = Second heart-cut loop, L_P = Delay loop, C_1 = First dimension chromatographic medium, C_2 = Second dimension chromatographic medium, C_3 = Third dimension chromatographic medium, D = Detector, W = Waste.*

2.3.1.3. Reaction optimization for the synthesis of Warfarin in flow using the multi-dimensional multi-QA analytical platform

A prospect of using the multi-QA platform for flow synthesis of Warfarin was also explored. Scheme 3 shows the flow condition in which reagent **4** and **5** were flowed from two separate positive-displacement pumps to a union that was connected to a flow reactor equipped

with a sampling device. The reaction was flowed at two different flow-rates. In both cases, conversion to product **6** was very low (Table 6). Although optical purity of the output was not high, the chromatographic data from the multi-QA analytical setup proved that the parallel analysis of multiple process quality attributes is possible, even when a very low quantity of the sample is available for analysis.



Scheme 3: Synthesis of Warfarin in flow carried out through a microwave reactor upstream from a 10-port sampling valve coupled with in-line filtration capabilities for sampling of heterogeneous mixtures. The sampling mechanism for flow synthesis was developed in the Organ lab. [28]

Flow Rate (µL/min)	**Residence time (min)**	**Enantiomeric excess (%)**	**Conversion to product (%)**
25	60	72	4
75	20	66	2

Table 6: Results from the synthesis of Warfarin in flow

3. Conclusion

In the past, chemists would optimize a reaction one QA at a time, meaning that they would initially screen through various input process parameters to optimize for percent conversion of the reaction. Once that QA is optimized for, the chemist would then follow up to optimizing the reaction to enhance the target product's purity. However, there is no clear relationship between both percent conversion and optical purity, thus optimizing for one QA at a time may result in dwindling the second QA. This form of optimization and process development faces an inherent risk where potential bias of one QA over the other may occur. In response to this inherent risk, several industries couple process optimization protocols with PAT tools capable of monitoring the state of the reaction at various points of time. Furthermore, the PAT tool offers analysis of the reaction mixture where desired QAs can be attained. A new multi-dimensional PAT tool with heart-cut capabilities was developed in the Organ lab capable of analyzing for multiple QAs. A beta-platform was constructed comprising of two dimensions for analysis of two QAs, % conversion and optical purity. The beta platform was coupled to an in-house developed flow system called MACOS with a novel sampling valve coupled with in-line filtration capabilities. The Buchwald Hartwig Amination reaction was chosen as the model reaction and provided valuable insight into the machinations of the heart-cut protocol and how to prevent co-elution of the mobile phase through the addition of a priming line. The novel multi-dimensional tool's capabilities were tested on an asymmetric model reaction, the synthesis of Warfarin. Integrated with the multi-dimensional and heart-cut protocol was a novel analytical method called multi-QA analysis that was capable of analyzing for % conversion and optical purity along with in-line verification of HPLC purity and enantiomer stability. The unique ability of recycling a targeted chromatographic peak through the same or different chromatographic media multiple times can play an important role in parallel analysis of various critical and essential process quality attributes. Artefacts from various laboratory purification techniques can be avoided since the new design does not require any off-line purification means

of injectable into the HPLC or either chromatographic dimension. This can be a significant advantage in chiral drug synthesis. Any chromatographic artefact from the analytical process itself can also be identified and, possibly quantified or eliminated in future generation of the presented design. Future generations of the multi-QA design could be also equipped with a better pressure mitigation strategy towards a complete PAT-solution for chiral drug manufacturing.

4. Experimental

4.1. General description of the experimental setup

Two-dimensional (beta) and multi-dimensional chromatography setups were built from an Agilent 1260 HPLC system. Additional valves were purchased from Vici Valco Instruments Co. Inc. and CTC analytics AG for the modification of the Agilent single-dimensional chromatography system into the ultimate multi-QA analytical setup (Figure 25). Details of the analytical setups can be found in section 4.1. Sampling was done using a previously reported technique, which is termed as Information-based Rapid & Intelligent Sampling (IRIS). Analytes from the IRIS system (i.e. filtration-enabled sampling via a 10-port valve with an in-line filter)[28] were delivered to a Gerstel MPS (Multi-purpose sampler) auto-sampler for automated sample preparation and injection.

A controller software developed in-house was used to coordinate actuations of all devices for the chromatographic setup. Details of the controller software can be found in section 4.2.

Performance of the analytical setups were tested under flow as well as batch conditions. The experimental details of the batch and the flow reactions can be found in section 4.3.

4.1.1 Experimental details pertaining to 2D-HPLC (beta) and multi-D HPLC setup

Two-dimensional beta-setup was constructed in accordance with Figure 7 shown in the previous section. Multi-dimensional setup, which was built to test the ultimate multi-QA analysis, was set up as shown in Figure 25 in the previous section.

HPLC systems comprised of a sampling module, an autosampler, which was responsible for sample delivery and sample preparation, and a chromatography module. The chromatography module comprised of an HPLC pump, a detector, and multiple chromatographic media (HPLC columns) connected to a set of two-position valves (column selector valve V_S, and heart-cut valve V_H). Six basic steps were executed from the controller

software to complete the entire analytical task. They are: sampling, sample delivery, sample preparation, injection, chromatography analysis, and data-processing.

4.1.2. Experimental details for sampling

Sampling and sample preparation from the batch synthesis of Warfarin reactions were done manually. A 33 μL aliquot was taken from the reaction mixture and diluted with 1467 μL of Dioxane HPLC-grade in a glass vial. The 1500 μL of analytical sample was placed on a tray and injected using an 80 μL injector syringe mounted on the Gerstel auto-sampler. Typically, a 10 μL of the analytical sample was injected for chromatographic runs.

Sampling from the flow reaction was done using a 10-port sampling valve coupled with in-line filtration capabilities.[28] This valve, which was integrated to the Gerstel autosampler, comprised of two ten-port valves. A schematic of sampling system is presented in Figure 23. The first valve (V_{IRIS1}), which was purchased from CTC Analytics [Model # 03-01A], was equipped with an inline filter (F) and a sampling loop (L_S). Filter F was cleaned by a positive-displacement pump (P_F), which was purchased from New Era Pump Systems, Inc. [Model # NE-1000]. V_{IRIS1}, which was controlled from the controller software using a proprietary multi-position algorithm, was connected to a flow reactor (R). The second valve (V_{IRIS2}), which was equipped with an internal standard delivery loop (L_I) and an air-sandwich delivery loop (L_A), was in fluid communication with V_{IRIS1}. This valve was also purchased from Vici Valco Instrument Co. Inc. [Model # 12X-0010H]. Two positive-displacement pumps (P_I and P_A), which were purchased from New Era Pump Systems Inc [Model # NE-1000], were used to introduce 100 μL of an internal standard solution and an air-plug in the IRIS sampling line. A small plug of air was used to transport analytical samples from L_S to the multi-QA analytical platform (A); the air acted as a sweep to ensure entire contents of the sample loops were delivered towards analysis regardless of viscosity differences between diluents. A fourth positive-displacement pump (P_D), which was purchased from Gerstel GmbH, was operated

from the auto-sampler controller for the delivery of the analytical plug to analysis, A. The controller software of the multi-QA platform sent necessary signals to the auto-sampler and the sampling system to isolate a 33 μL of the effluent from reactor R in L_S and deliver the trapped sample to the multi-QA platform through a sample delivery mechanism, which is described in the next section.

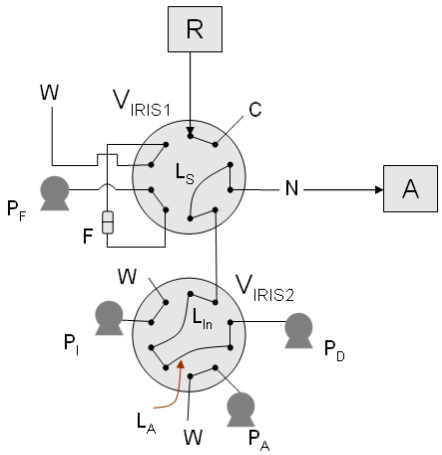


Figure 26: Sampling system for collection and transfer of samples from a flow reaction. *Legends: R = Flow reactor, C = Bulk product collection module, A = Multi-QA analytical system, V_{IRIS1} = Filtration-enabled sampling valve, V_{IRIS2} = Internal standard and air-sandwich valve, F = Inline filter, L_S = Sampling loop, L_{In} = Internal standard loop, L_A = Air-sandwich loop, P_F = Filter back-flush pump, P_I = Internal standard pump, P_A = Air supply pump, P_D = Sample delivery pump, N = Injector syringe, W = Waste*

4.1.3. Experimental details for sample delivery

The sample delivery mechanism was only used during the analysis of samples from the flow reactor (R). For batch reactions, samples were manually placed on the sample tray of the auto-sampler.

As mentioned in the previous section, a 33 μL of analyte was extracted from the flow reactor using V_{IRIS1} and moved by pump P_D to an injector syringe (N) mounted on the auto-sampler. The auto-sampler injector syringe (N) was equipped with a side-port, which could establish fluid communications between injector syringe N and sampling loop L_S of valve V_{IRIS1} of the IRIS system through a sample transfer line. During sample delivery, the controller

software sent necessary signals to the auto-sampler controller to move the injector syringe plunger away from the side-port (Figure 27). A portion of the fluid (1100 μL) in the sample transfer line was first sent to waste using the auto-sampler. The next 1500 μL segment of the fluid in the sample transfer line was collected in a sample vial for analysis. A 2000 μL segment of fluid in the sample transfer line was sent to waste to reduce carry-over.

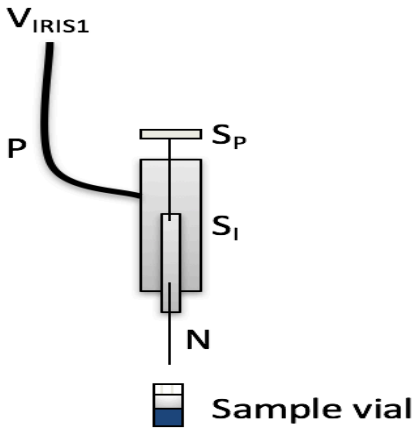


Figure 27: Sample delivery system for flow and batch. *Legend: V_{IRIS1}=IRIS Sampling valve, S_P=Syringe plunger, S_I= Injector Syringe, N=Injector Needle, P= Side port*

4.1.4. Experimental details for sample preparation

All sample preparation tasks for sampling from batch reactions were done manually. Sample preparation for flow sampling, which was done using the Sampling system, was executed from the Gerstel auto-sampler. Analytical samples were prepared using two sample preparation functions.

Simple dilution of the analytical sample, which was used for reactions not containing any metal ions, was achieved by controlling the volume of the transport solvent that also acted as the diluent. All vials used in reactions requiring simple dilutions without extraction, were diluted to 1500 μL.

Liquid-liquid extraction (LLE) technique was used for analytical samples that require removal of metal ions or any other chemical agents, which could harm the analytical system. During LLE, analytes were delivered to a 10 mL sample vial by the IRIS system using ethyl acetate as the transport solvent. The auto-sampler was used to deliver 1.5 mL of an aquous solutiuon of tri-sodium EDTA to the same vial. LLE was done using a programmable agitator device mounted on the auto-sampler. Agitation was done at 250 rpm for 1 min. Upon completion of the agitation, the biphasic mixture was allowed to settle for 1 min and the injector syringe was used to make 10 μL injection to the chromatography platform.

4.1.5. Experimental details for chromatography analysis

A schematic of the latest analytical platform is shown in Figure 25. The injector valve (V_I), which was a six-port two-position CTC valve with a vertical injection port (I), was mounted on the auto-sampler. Valve V_I was equipped with a 20 μL injection loop (L_I), and connected to a column selector valve (V_S), which was a fourteen-port six-position stream selector valve and purchased from Agilent Technologies. Valve V_S was in fluid communication with a ten-port six-position heart-cut valve (V_H), which was purchased from Vici Valco Instruments Co. Inc. A six-port two-position delay valve (V_D), which was purchased from Vici Valco Instruments Inc. Co., was in fluid communication with valve V_S and V_H. An Agilent 1260 HPLC system with a built-in vacuum degasser was used as the chromatography system. Quaternary HPLC pump of the chromatography system was connected to valve V_S, which was also in fluid communications with three HPLC columns (C_1, C_2, and C_3) hosted in an Agilent 1290 thermostatted column compartment (TCC). An Agilent 1260 diode array detector (PDA) was used as the detector. All chromatographic modules were interconnected in accordance with Figure 22 by stainless steel tubing of 0.03″ internal diameter. Waste lines were connected by PEEK tubing (internal diameter: 0.004″).

In the two-dimensional beta-platform, first-dimension column (C_1) was a 2.1 mm x 150 mm Poroshell C18 column (particle size: 2.7 μm), which was purchased from Agilent

Technologies. The second-dimension reverse phase column (C_2) was a 2 mm x 150 mm chiral column with an ES-OVM column (particle size: 5 μm), which was also purchased from Agilent Technologies. The mobile phase used in the first dimension comprised of a mixture of acetonitrile and water, and the mobile phase used in the second chiral dimension comprised of a Gomori buffer (KH_2PO_4/K_2HPO_4) at pH 5.8, acetonitrile, and water. HPLC grade acetonitrile and water were purchased from Millipore Sigma. For normal phase chromatography using the two-dimensional beta-setup, a 4.63 mm x 150 mm Poroshell CN columns (particle size: 5 μm), which was purchased from Agilent Technologies, was used.

In the multi-dimensional HPLC setup, the first-dimension column (C_1) was a 4.63 mm x 150 mm Poroshell CN column (particle size: 5 μm). The second-dimension column was a 3 mm x 50 mm Poroshell CN column (particle size: 2.7 μm), which was also purchased from Agilent Technologies. The third-dimension column (C_3) was a 10 mm x 250 mm chiralPAK IA column (particle size: 5 μm), which was purchased from Chiral Technologies Inc. The mobile phase used across all three dimensions comprised of a mixture of HPLC grade hexanes and iso-propanol, which were purchased from Millipore Sigma. Shown below are two tables presenting important parameters in the method development for multi-QA analysis of Warfarin.

Separation Method Total Run Time 170 min								
Column Gradient		Solvent Gradient						
Time (min)	Column	Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)	Flow rate (mL/min)	Pressure (bar)
0	4	0	0	0	5	95	0.5	600
42	5	55	0	0	5	95	0.5	600
55	3	60	0	0	20	80	0.5	600
80	2	80	0	0	20	80	0.5	200
140	3	140	0	0	20	80	0.5	200
		145	0	0	5	95	0.5	600

Table 7: A multi-QA analysis separation method developed for Synthesis of Warfarin in batch
Legend: Column 4 is the first dimension CN column, Column 5 is the second dimension CN column, Column 3 is the priming line, Column 2 is the third dimension ChiralPAK IA column.

Heart-cut Method Enabled Starting Position at A					
Number of 2D Recycle	0	Run Time 1D (s)	2520	Wavelength Change	
Number of 3D Recycle	1	Run Time 2D (s)	4800	Time (s)	2040
	Open Delay (s)	Close Delay (s)			
1D	2241	60			
2D	2894	60			
3D-RCY	6414	60			

Table 8: A multi-QA analysis heart-cut method developed for Synthesis of Warfarin in batch *Legend: 1D= heart-cut sequence from the first dimension towards the second dimension, 2D= heart-cut sequence from the second dimension towards the chiral dimension, 3D-RCY= heart-cut recycle sequence in the chiral dimensions, Run time 1D= run time of first dimension, Run time 2D= starting time of chiral dimension, Wavelength change= time at which detection wavelength is changed.*

4.1.6. Experimental details for data processing

Raw analytical data acquired from the Agilent detector was processed by Chromeleon software from Thermo-Fisher Scientific. The processed data was relayed to the controller software of the multi-QA platform for decision-making purposes. Peak identification criteria were tested first in the data processing method of Chromeleon chromatography data system (CDS). The criteria were later embedded in the controller software through Chromeleon software development kit (SDK). Various peak parameters (e.g., peak area, signal-to-noise ratio, peak symmetry, baseline, etc.) were used to build a custom peak qualifier method for appropriate identification of relevant chromatographic peaks in the multi-dimensional chromatogram. A team of engineers were responsible for the development of the peak identification strategy. Purpose of this project, which was discussed in this thesis, was to design

and test the chromatographic setups using the peak identification strategy. Definitions of peak properties are described below:

Peak Asymmetry (A_S): a factor used to determine whether a peak is tailing (if Asymmetry is greater than 1) or fronting (if Asymmetry is less than 1).

$$Equation\ 1: A_S = \frac{RW_{5\%} + LW_{5\%}}{2 * LW_{5\%}}\ or\ A_S = \frac{RW_{10\%}}{LW_{10\%}}$$

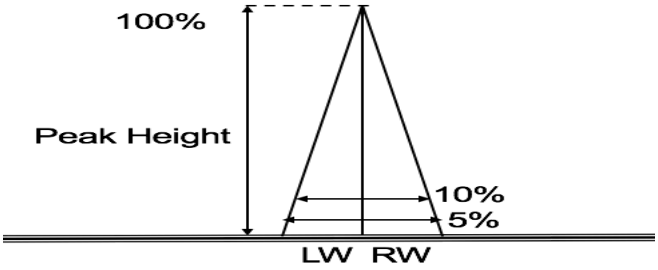


Figure 28: A schematic depicting the Peak Asymmetry Factor Calculation. *Legend: $RW_{5\%}$ = Right peak width in 5% of the peak height, $LW_{5\%}$= Left peak width in 5% of the peak height and $RW_{10\%}$= Right peak width in 10% of the peak height, $LW_{10\%}$= Left peak width in 5% of the peak height*

Peak Recovery: a factor used to quantify the heart-cut transfer efficiency of a target peak from one dimension to the other.

$$Equation\ 2: \%Recovery = \frac{Peak\ area\ of\ target\ analyte}{Peak\ area\ of\ heartcut\ analyte} \times 100\%$$

Peak width: a time-based factor that provides a measure of the base width of a peak and qualitative assessment of the peak broadness

Resolution: a measure of separation efficiency between two peaks (generally R>1.5 is deemed sufficient)

$$Equation\ 3: R = 2\left|\frac{t_{Rpeak1} - t_{Rpeak2}}{W_{peak1} + W_{peak2}}\right|$$

where t_{Rpeak1}= retention time of the first eluting peak, t_{Rpeak2}= retention time of the second eluting peak, W_{peak1}= peak width of the first eluting peak, W_{peak2}= peak width of the second eluting peak.

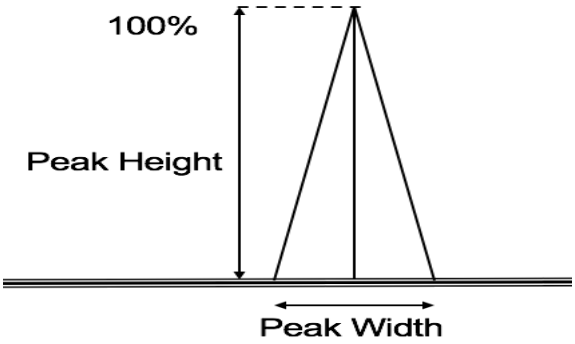


Figure 29: A schematic depicting the Peak Width of a chromatogram peak

Analytical data from the Chromeleon CDS was used to calculate *Percent conversion to product* using a mathematical formula shown below. This number was generated automatically by the controller software for making decision on a chemical reaction condition.

$$Equation\ 4: \%Conversion = \frac{Final\ Concentration\ of\ starting\ material}{Initial\ Concentration\ of\ starting\ material} \times 100\%$$

Optical purity was measured based on *Enantiomeric excess (%ee)*, which was calculated using the following formula:

$$Equation\ 5: \%ee = \frac{|R - S|}{R + S} \times 100\%$$

Data for the *Percent conversion to product* calculation was generated using an external standard calibration curve for the synthesis of Warfarin.

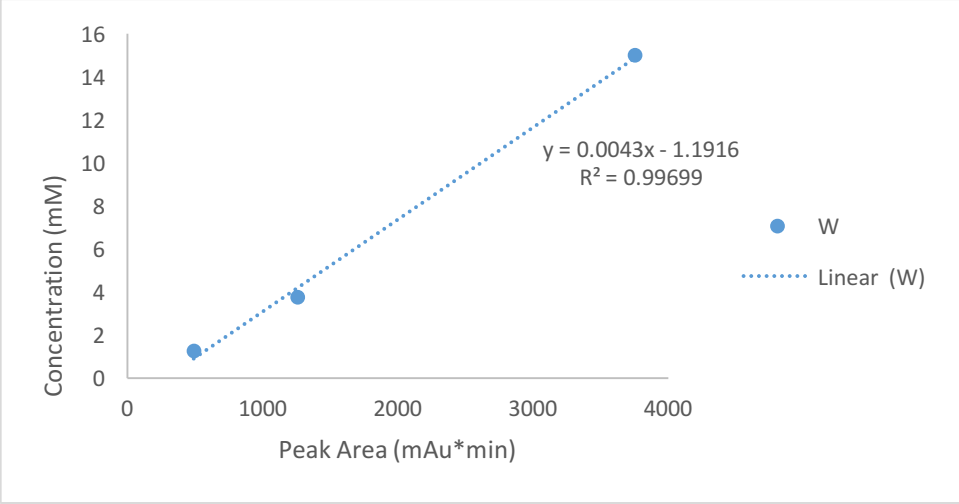


Figure 30: External Standard Calibration Curve of Warfarin

Data for *Product yield* was calculated using an internal standard calibration curve for the analysis of flow samples from a Buchwald-Hartwig reaction.

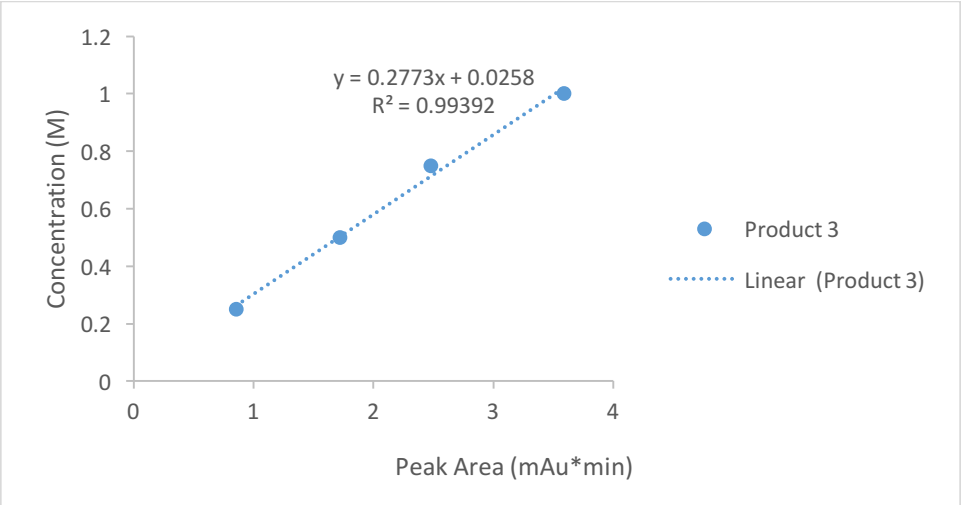


Figure 31: Internal Standard Calibration Curve of Product 3

4.2. Development of the controller software

The system controller, which ultimately controls all hardware of the entire multi-QA setup, is a beta-version and developed in-house by a team of engineers. The auto-sampler, which was controlled from Maestro software platform of Gerstel GmbH, was integrated to the HPLC system, which was controlled by ChemStation software from Agilent Technologies. Instructions for all analytical tasks (from injection to detection) were sent to a job queue inside Chromeleon software, which, in turn, relayed that information to the auto-sampler and HPLC controllers. The in-house system controller had direct controls over the IRIS system, which was responsible for sampling and sample delivery tasks. Valves V_H and V_D were also controlled by the system controller itself. Figure 32 shows a summary of software hierarchy to execute the entire multi-dimensional chromatography task.

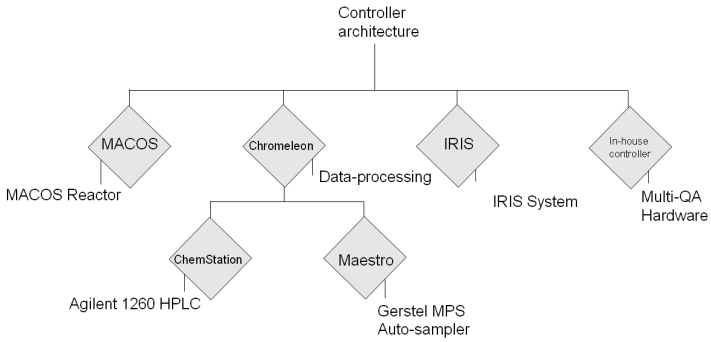


Figure 32: Workflow of software commands from the in-house system controller to various synthetic and analytical modules.

4.3 Multi-QA Analytical Protocol

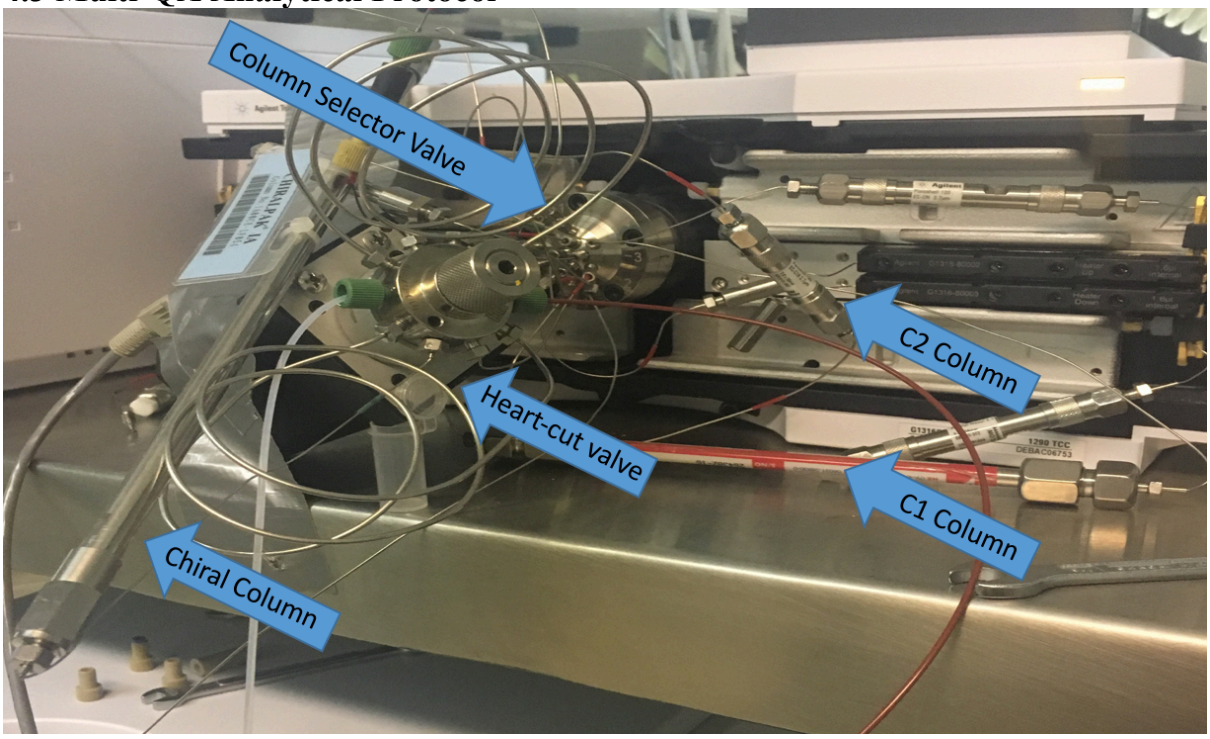


Figure 33: Image of the Heart-cut valve integrated in line to the Column selector valve

Shown below are detailed step by step animated images of both the entire multi-QA analytical protocol via the novel multi-dimensional analytical platform along with the step-by-step valve heart-cut valve actuation protocol between dimensions.

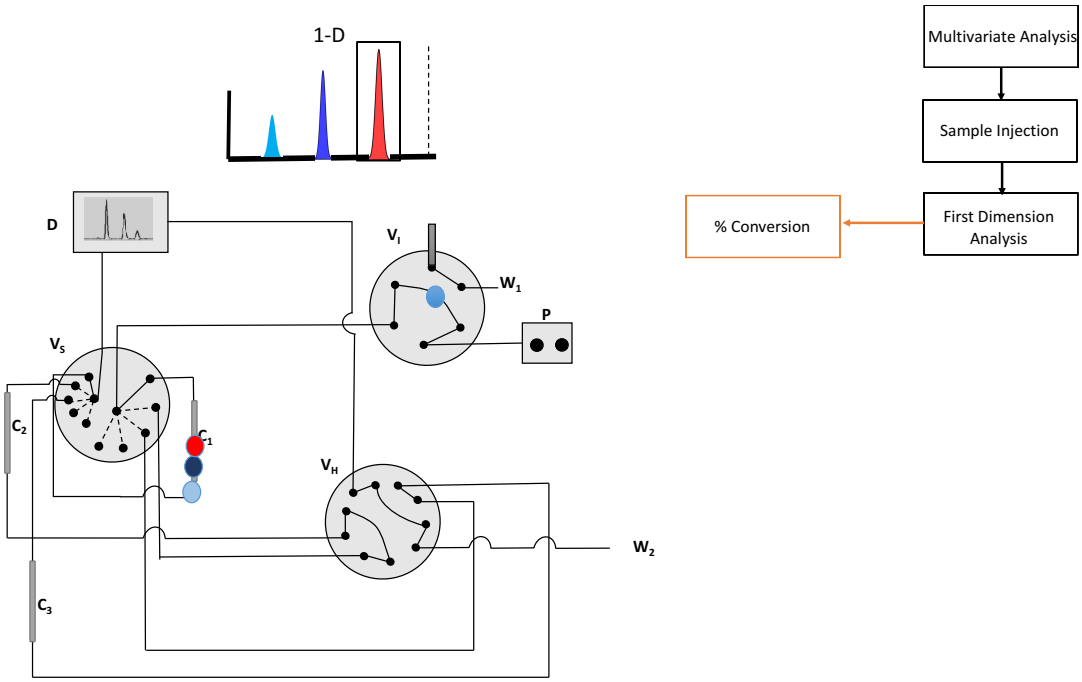


Figure 34: First dimension Analysis through C_1. *Legends: P = HPLC pump, I = Injector, C_1 = Chromatographic medium for the first dimension, C_2= Chromatographic medium for the second dimension, C_3= Chromatographic medium for the third dimension, V_H = Heart-cut valve, D = Detector (DAD), W = Waste*

Sample injection is facilitated through the injector valve V_I and is directed to the column selector valve V_S that is in fluid communication with our first column C_1 for first dimension analysis and resolution of the reaction mixture. The output QA from the first dimension analysis is HPLC purity or if chromatogram was run in-line with a calibration curve of the reaction mixture then the % conversion is attainable.

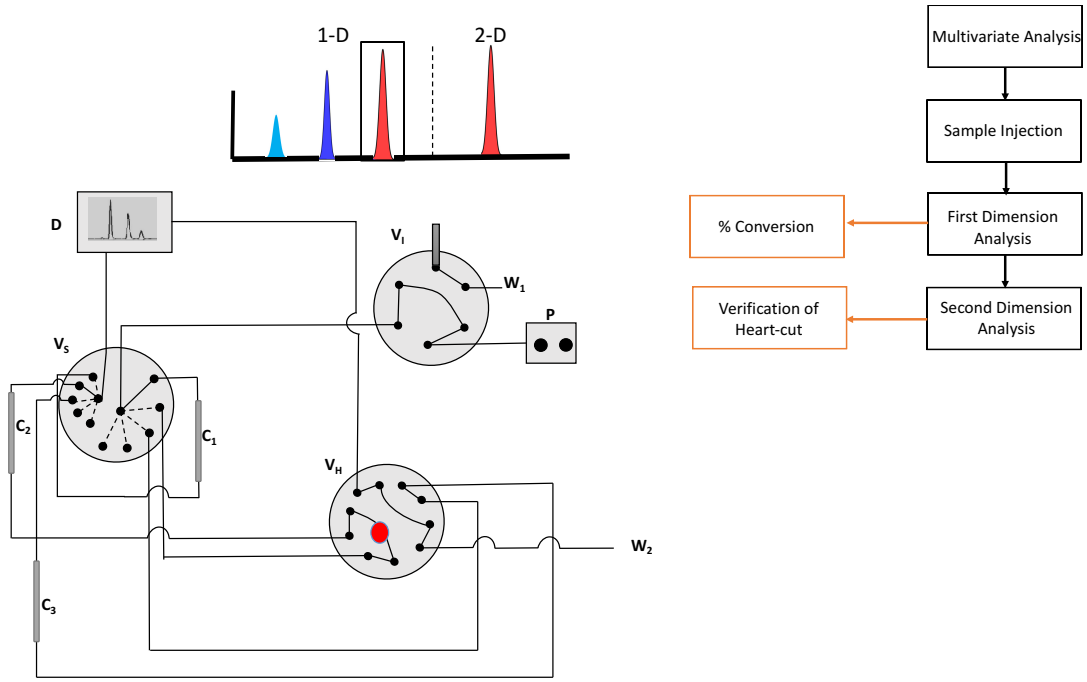


Figure 35: Second dimension Analysis through C_2. *Legends: P = HPLC pump, I = Injector, C_1 = Chromatographic medium for the first dimension, C_2= Chromatographic medium for the second dimension, C_3= Chromatographic medium for the third dimension, V_H = Heart-cut valve, D = Detector (DAD), W = Waste*

The boxed red peak in the first dimension chromatogram is our target chiral product requiring further analysis on its optical purity. Prior to optical purity measurement (i.e. chiral resolution), the target red peak was heart-cut via the heart-cut valve V_H towards the second column C_2 (same stationary phase as C_1) to ensure the injectable towards chiral analysis is sufficiently pure.

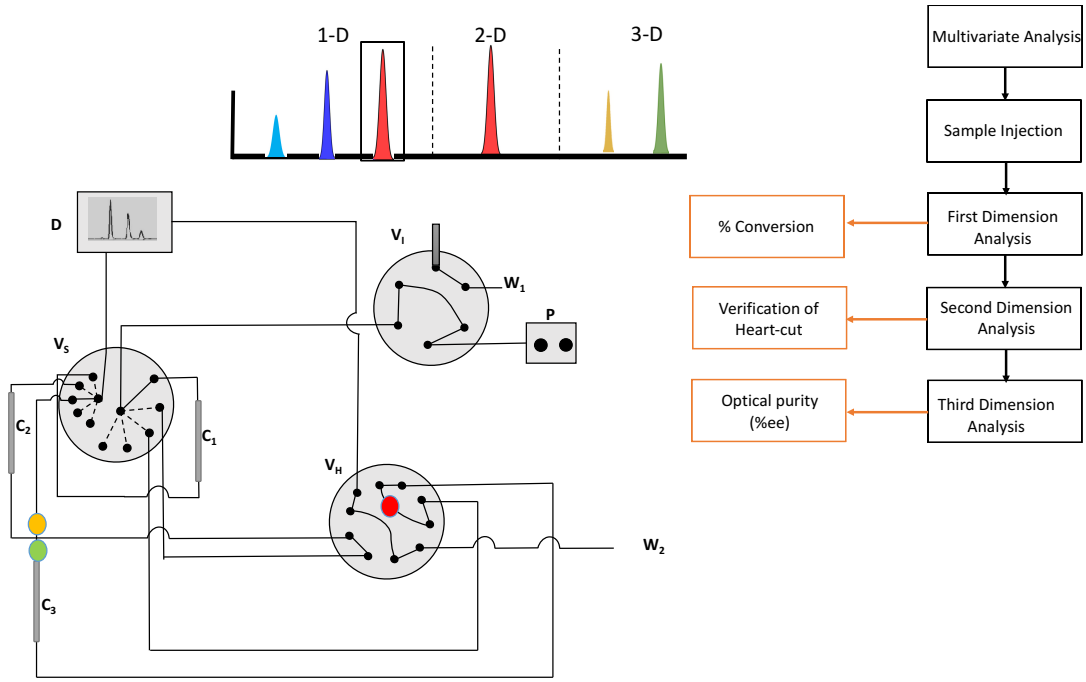


Figure 36: Third dimension Analysis through C_3. *Legends: P = HPLC pump, I = Injector, C_1 = Chromatographic medium for the first dimension, C_2= Chromatographic medium for the second dimension, C_3= Chromatographic medium for the third dimension, V_H = Heart-cut valve, D = Detector (DAD), W = Waste*

Once second dimension analysis is complete and has concluded that target analyte is sufficiently pure (i.e. single peak is observed) the analyte is heart-cut once more through the heart-cut valve and is directed towards the chiral column C_3. In the chiral column, the isolated chiral sample will resolve down to its two enantiomers wherein the optical purity /

enantiomeric excess can be determined.

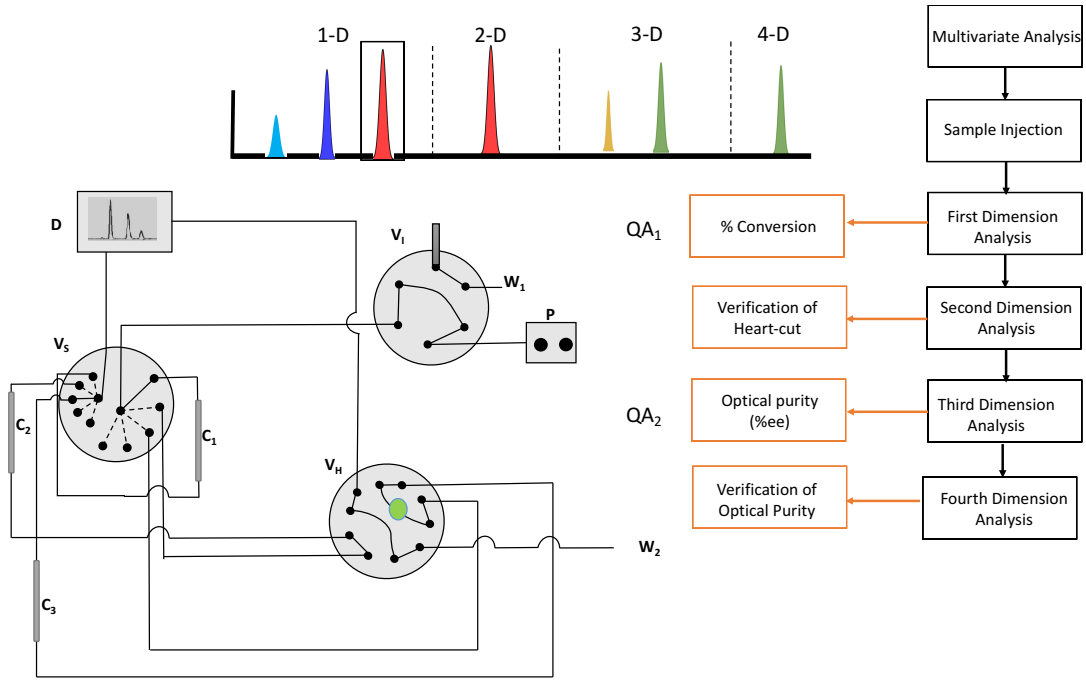


Figure 37: Fourth dimension Analysis through C_3. *Legends: P = HPLC pump, I = Injector, C_1 = Chromatographic medium for the first dimension, C_2= Chromatographic medium for the second dimension, C_3= Chromatographic medium for the third dimension, V_H = Heart-cut valve, D = Detector (DAD), W = Waste*

To ensure no interconversion between both resolved enantiomers has occurred on-column, one of the two enantiomers was recycled through the heart-cut valve once more towards C_3.

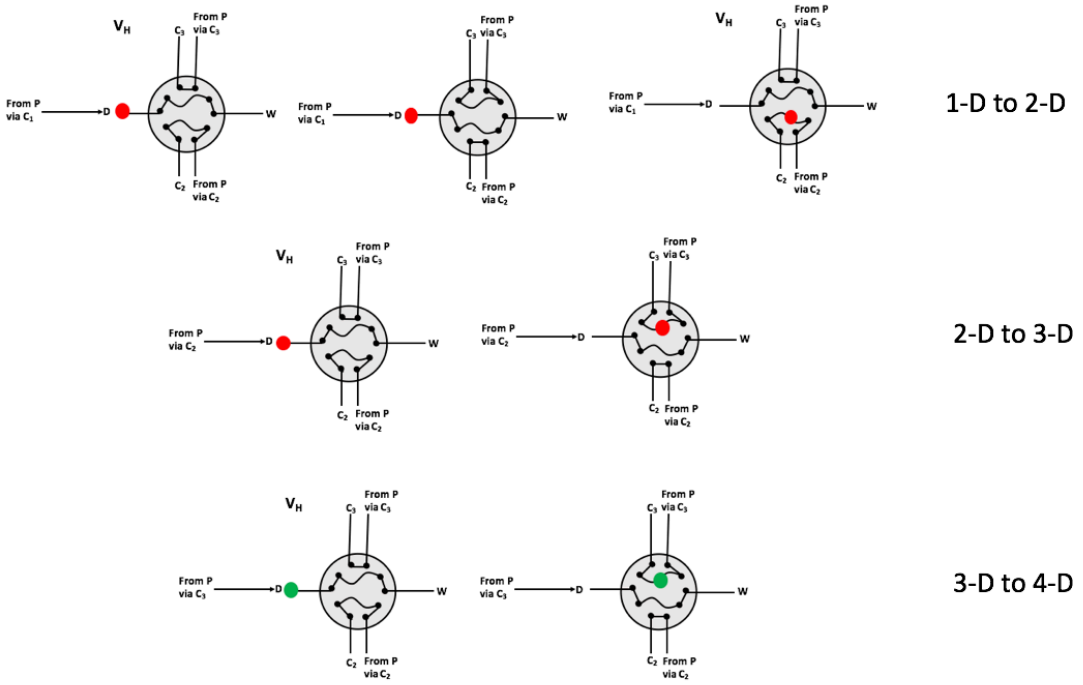


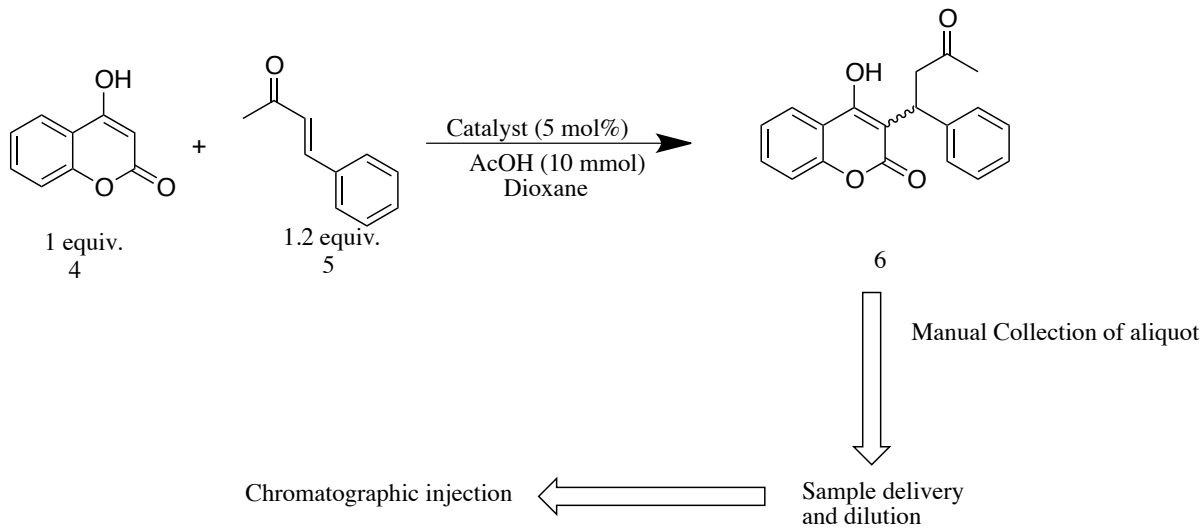
Figure 38: Heart-cut valve actuation protocol across all four dimensions. *Legends: P = HPLC pump, C_1 = Chromatographic medium for the first dimension, C_2= Chromatographic medium for the second dimension, C_3= Chromatographic medium for the third dimension, V_H = Heart-cut valve, D = Detector (DAD), W = Waste*

Shown above is a step by step schematic of the valve actuation protocol used to facilitate heart-cut across all dimensions through our multi-QA analytical protocol. The open delay time is set prior to heart-cut implementation at this registered time the flow path towards the loop is open to allow for the analyte to enter, and then a close delay time is registered to close the flow path and trap the analyte in the heart-cut loop of interest. The valve is actuated accordingly to ensure entry and exit of the analyte towards the target loop of interest (i.e. dependent on which column or dimension of analysis the analyte is transferred towards). For the heart-cut to be facilitated, a preliminary injection of the reaction mixture must be facilitated to obtain a measure of the target analyte's retention time and peak width (Baseline to baseline time measurement of a peak). Based on this information, the size of the heart-cut loop should be set accordingly to accommodate the volume of the target analyte peak in the event the heart-cut is facilitated with the aim to trap the entire contents of the peak (i.e. which was the case in our setup).

4.4 General description of the synthetic setup

The synthesis of Buchwald-Hartwig amination reaction was done in flow. Three positive-displacement pumps (purchased from New Era Pump Systems, Model # NE-1000) were fitted with 12 mL plastic syringes containing 10 mL of reagent **1** and base, 10 mL of reagent **2** and catalyst, and 10 mL of internal standard, anisole. Individual flow-paths from the syringes were connected to a common union, which was in fluid communication to a 1 mL glass reactor. The glass reactor was placed inside a flow microwave reactor, which was purchased from a company formerly known as WaveCraft AG [Model # Arrhenius I]. The microwave reactor was controlled from an in-house controller, which was integrated to the Microwave, WaveCraft controller. The reaction mixture was flowed from the reactor to a bulk product collector via the IRIS system as shown in Scheme 1.

In contrast to the Buchwald Hartwig amination reaction, the synthesis of Warfarin was done in batch as shown in Scheme 4. To a round-bottom flask equipped with a magnetic stir-bar were added 4-Hydroxycoumarin (162 mg, 1mmol), acetic acid (572 μL, 10 mmol), benzylideneacetone (175 mg, 1.2 mmol), and catalytic amounts of either (1S, 2S) -(-)-1,2-Diphenylethylenediamine ((S, S)-DPEN) (11 mg, 5 mol%) or (1R, 2R) -(+)-1,2-Diphenylethylenediamine ((R, R)-DPEN) (11 mg, 5 mol%) in 2 mL 1,4-Dioxane at room temperature. The reaction mixture was allowed to stir at room temperature for 24 hours. Reaction progress was monitored by HPLC (95 Hexane: 5 2-Propanol) as described above. A 33 μL of aliquot was taken from the reaction mixture every 24 hours and diluted with 1467 μL of 1,4-dioxane in a 1500 μL sample vial. The sample vial was placed on the tray of the Gerstel Autosampler for HPLC injections.



Scheme 4: A schematic of the synthesis of Warfarin in batch

4.5 Aqueous Work-up of Warfarin for NMR and Polarimetry

4-Hydroxycoumarin (162 mg, 1mmol), acetic acid (572 μL, 10 mmol), benzylideneacetone (175 mg, 1.2 mmol), and catalytic amounts of either (1S, 2S) -(−)-1,2-Diphenylethylenediamine ((S, S)-DPEN) (11 mg, 5 mol%) or (1R, 2R) -(+)-1,2-Diphenylethylenediamine ((R, R)-DPEN) (11 mg, 5 mol%) were added to an oven-dried flask. 1,4-Dioxane (2mL) was then added and the mixture stirred at room temperature for 24 hours. The Reaction progress was monitored by Thin Layer Chromatography (TLC) (5% Methanol: 95% Dichloromethane). Once complete, the reaction mixture was filtered, and extracted with ethyl acetate. The combined organic phases were dried with anhydrous sodium sulfate, filtered, and the solvent evaporated. The crude product was then purified by column chromatography (Methanol/Dichloromethane) to give warfarin as (60 mg, 20%).

The Flash chromatography was performed through a Biotage using a SNAP-50g silica gel cartridge. TLC was carried out on silica gel pre-coated glass plates with detection by UV light. NMR analyses for Warfarin were recorded in DMSO-d_6 at 400 MHz via an available Bruker NMR instrument. Polarimetry was carried out using a Na_D lamp at a single wavelength of 589nm for detection (path length=1dm). For Polarimetry analysis, a 3.3 mg/1mL Warfarin solution in 2-propanol was prepared and taken for quantitative analysis of its observed rotation.

Bibliography

(1) Guttman, A.; Varoglu, M.; Khandurina, J. *Drug Discov. Today* **2004**, *9* (3), 136–144.
(2) Erni, F.; Frei, R. W. *J. Chromatogr. A* **1978**, *149* (Supplement C), 561–569.
(3) Bordawekar, S.; Chanda, A.; Daly, A. M.; Garrett, A. W.; Higgins, J. P.; LaPack, M. A.; Maloney, T. D.; Morgado, J.; Mukherjee, S.; Orr, J. D.; Reid, G. L.; Yang, B. S.; Ward, H. W. *Org. Process Res. Dev.* **2015**, *19* (9), 1174–1185.
(4) Hinz, D. C. *Anal. Bioanal. Chem.* **2006**, *384* (5), 1036–1042.
(5) Lindon, J. C.; Nicholson, J. K.; Wilson, I. D. *Hyphenated Tech. LC their input Biosci.* **2000**, *748* (1), 233–258.
(6) Conroe, K. E. *Chromatographia* **1975**, *8* (3), 119–120.
(7) Sekhon, B. S. *Int. J. PharmTech Res.* **2010**, *2* (2), 1584–1594.
(8) Nguyen, L. A.; He, H.; Pham-Huy, C. *Int. J. Biomed. Sci.* **2006**, *2* (2), 85–100.
(9) Hoke, S. H.; Morand, K. L.; Greis, K. D.; Baker, T. R.; Harbol, K. L.; Dobson, R. L. M. *Int. J. Mass Spectrom.* **2001**, *212* (1–3), 135–196.
(10) Wagner, K.; Miliotis, T.; Marko-Varga, G.; Bischoff, R.; Unger, K. K. *Anal. Chem.* **2002**, *74* (4), 809–820.
(11) Valentine, S. J.; Kulchania, M.; Barnes, C. A. S.; Clemmer, D. E. *Int. J. Mass Spectrom.* **2001**, *212* (1–3), 97–109.
(12) Link, A. J. *Trends Biotechnol.* **2002**, *20* (12), s8–s13.
(13) Wagner, Y.; Sickmann, A.; Meyer, H. E.; Daum, G. *J. Am. Soc. Mass Spectrom.* **2003**, *14* (9), 1003–1011.
(14) Delahunty, C.; Yates, J. R. *Methods* **2005**, *35* (3 SPEC.ISS.), 248–255.
(15) Wu, Q.; Yuan, H.; Zhang, L.; Zhang, Y. *Anal. Chim. Acta* **2012**, *731*, 1–10.
(16) Deans, D. R.; Chemical, I. 18–22.
(17) Rudaz, S.; Souverain, S.; Schelling, C.; Deleers, M.; Klomp, A.; Norris, A.; Vu, T. L.; Ariano, B.; Veuthey, J. L. *Anal. Chim. Acta* **2003**, *492* (1–2), 271–282.
(18) Ianni, F.; Sardella, R.; Lisanti, A.; Gioiello, A.; Terzo, B.; Goga, C.; Lindner, W.; Natalini, B. *J. Pharm. Biomed. Anal.* **2015**, *116*, 40–46.
(19) Venkatramani, C. J.; Wigman, L.; Mistry, K.; Chetwyn, N. *J. Sep. Sci.* **2012**, *35* (14), 1748–1754.
(20) Li, Z.; Hong, P.; Mcconville, P. *Effective Determination of Pharmaceutical Impurities by Two Dimensional Liquid Chromatography Method conditions*; Milford, 2017.
(21) Cass, Q. B.; Degani, A. L. G.; Cassiano, N. M.; Pedrazolli, J. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2002**, *766* (1), 153–160.
(22) Zhang, K.; Li, Y.; Tsang, M.; Chetwyn, N. P. *J. Sep. Sci.* **2013**, *36* (18), 2986–2992.
(23) Wong, V.; Sweeney, A. P.; Shalliker, R. A. *J. Sep. Sci.* **2004**, *27* (1–2), 47–52.
(24) Hoi, K. H.; Organ, M. G. *Chem. - A Eur. J.* **2012**, *18* (3), 804–807.
(25) Somerville, K.; Tilley, M.; Li, G.; Mallik, D.; Organ, M. G. *Org. Process Res. Dev.* **2014**, *18* (11), 1315–1320.
(26) Tilley, M.; Li, G.; Savel, P.; Mallik, D.; Organ, M. G. *Org. Process Res. Dev.* **2016**, *20* (2), 517–524.
(27) Sauks, J. M.; Mallik, D.; Lawryshyn, Y.; Bender, T.; Organ, M. **2014**, *18* (11), 1310–1314.
(28) Kwak, J. S.; Zhang, W.; Tsoy, D.; Hunter, H. N.; Mallik, D.; Organ, M. G. *Org. Process Res. Dev.* **2017**, *21* (7), 1051–1058.
(29) Comer, E.; Organ, M. G. *J. Am. Chem. Soc.* **2005**, No. 4, 31–33.
(30) Sharif, S.; Mitchell, D.; Rodriguez, M. J.; Farmer, J. L.; Organ, M. G. *Chem. - A Eur. J.* **2016**, *22* (42), 14860–14863.
(31) Yang, H. M.; Li, L.; Jiang, K. Z.; Jiang, J. X.; Lai, G. Q.; Xu, L. W. *Tetrahedron* **2010**, *66* (51), 9708–9713.

(32) Halland, N.; Hansen, T.; Jørgensen, K. A. *Angew. Chemie - Int. Ed.* **2003**, *42* (40), 4955–4957.
(33) Wong, T. C.; Sultana, C. M.; Vosburg, D. A. *J. Chem. Educ.* **2010**, *87* (2), 194–195.