Development of a rapid UHPLC method for the in-process determination of coupling reagents used in peptide synthesis

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ABSTRACT

The following thesis details the extensive development of a rapid liquid chromatography method for the in-process determination of peptide coupling reagents used in peptide synthesis. The determination of peptide coupling reagents, additives and associated by-products is important during peptide synthesis to ensure the concentration of these products are below the threshold of toxicological concern in the final peptide.

A number of column technologies and mobile phases were evaluated for the separation of the analytes using an ultra-high performance liquid chromatography system and, a 15 minute method for the simultaneous determination of fourteen peptide coupling reagents, additives and by-products was established. This method was determined to be selective and capable of accurately quantitating the amount of TMU, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, Oxyma Pure, COMU, DIU, DIC, PyBOP, and TCTU in the presence of two peptides from Ipsen Manufacturing Ireland LTD.

Analytical method validation was performed as per ICH guidelines for specificity, accuracy, linearity, precision, detection limit, quantitation limit and robustness. This rapid UHPLC method is directly transferable onto LC-MS and the intended application of this method is for evaluation of development peptides in Ipsen Manufacturing Ireland LTD.

ABBREVIATIONS

AAA	Amino acid analysis
ACN	Acetonitrile
AcOH	Acetic acid
AOMP	5-(1H-7-azabenzotriazol-1-yloxy)-3,4-dihydro-1-methyl-2H-pyrrolium hexachloroantimonate
API	Active pharmaceutical ingredient
BEH	Bridged ethane hybrid
BOC	tert-butoxycarbonyl
BOP	Benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate
BPMP	5-(1H-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl,2H-pyrrolium hexachloroantimonate(BDMP),2,6-Bis{[bis(2-pyridylmethyl)amino]methyl}-4-methylphenol
Bzl	Benzyl
6-ChloroHOBt	6-Chloro-1-hydroxybenzotriazole dehydrate
Cl	Chlorine
COMU	1-((1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-Dimethylamino-Morpholinomethylene)) Methanaminium Hexafluorophosphate
cm	centimetre
CV	Coefficient of Variation
CZE	Capillary zone electrophoresis
C_{18}	Column having octadecyl chains of C atom
C_8	Column having octyl chains of C atom
C_4	Column having butyl chains of C atom
DCC	N,N'-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	N,N'-Diisopropylcarbodiimide
DIU	1,3-Dimethylurea
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DOE	Design of experiments
EDC	1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide
Exp	Experiment
Fmoc	9-fluorenylmethyloxycarbonyl
g	Gram

GC	Gas chromatography
GLP-1	Glucagon-like-peptide-1
GMP	Good Manufacturing Practice
GPC	Gel permeation chromatography
HATU	2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	O-Benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluoro-phosphate
HCTU	(2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate)
HETP	Height equivalent to a theoretical plate
HMPA	Hexamethylphosphoramide
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
H_2O	Water
IARC	International Agency For Research On Cancer
IC	Ion chromatography
ICH	The International Conference on Harmonisation
IMIL	Ipsen Manufacturing Ireland LTD.
k*	Retention factor
kg	kilogram
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
L	Litre
LD_{50}	lethal dose$_{50}$ - the dose that kills half of the tested population in an animal model
LC_{50}	lethal concentration$_{50}$ - concentration of a chemical in air that kills half of the tested population in a given time
LOD	Limit of detection
LOQ	Limit of quantitation
LPPS	Liquid phase peptide synthesis
LTD	Limited
MeOH	Methanol
mg	milligram
min	Minutes
ml	Millilitre
mm	millimetre

mM	millimolar
M	Molar
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
μL	Microliter
μm	Micrometer
$NaHCO_3$	Sodium bicarbonate
nm	nanometer
NMP	N-methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
ODS	Octadecylsilane
Oxyma pure	Ethyl (hydroxyimino)cyanoacetate
PAM resin	Phenylacetamidomethyl resin
PDA	Photodiode-array
PEEK	Polyetheretherketone
PEG	Polyethylene glycol
pH	negative logarithm of H^+ concentration
pK_a	Ionisation constant
psi	per square inch
PyAOP	7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PyClocK	6-chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate
PyCloP	chlorotripyrrolidinophosphonium hexafluorophosphate
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PyBrOP	Bromo-tris-pyrrolidino phosphoniumhexafluorophosphate
PyOxim	Ethyl-cyano(hydroxyimino)acetato-O^2)tri-1-pyrrolidinylphosphonium hexafluorophosphate
RP	Reversed phase
RP-HPLC	Reversed-phase high performance liquid chromatography
Rs	Resolution
RSD	Relative standard deviation
SCID	Severe Combined Immunodeficiency disease
SEC	Size exclusion chromatography
S/N	Signal to noise ratio
SFC	Supercritical fluid chromatography
SPPS	Solid phase peptide synthesis

TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
tBu	tert-butyl
TCTU	O-(6-Chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TLC	Thin layer chromatography
TFA	Trifluoroacetic acid
TMU	Tetramethylurea
TOTU	O-(Cyano(ethoxycarbonyl)methylenamino)-1,1,3,3-tetramethyluronium tetrafluoroborate
TTC	Threshold of toxicological concern
UN	United Nations
UPLC	Ultra performance liquid chromatography
UHPLC	Ultra high performance liquid chromatography
USP	United States Pharmacopeia
UV	Ultra violet
UV-VIS	Ultra violet – visible
α	Alpha
β	Beta
v/v	Volume by volume
v/w	Weight by volume
w/w	Weight by weight
%	Percent
<	Less than
>	Greater than
ºC	Degrees Celsius
Å	Angstrom

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Chapter 1

Literature Review

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1.1   Overview of peptides and proteins

Peptides and proteins are co-polymers of amino acids that are covalently linked through a peptide bond [1]. They differ from each other by the number and sequence of the constituent amino acids [1]. Those with low molecular weights, typically consisting of less than 50 amino acids, are called peptides [2]. Peptides composed of fewer than 20 amino acid residues are known as oligopeptides while those with more than 20 but less than 50 amino acid residues are known as polypeptides [3]. The term protein describes a macromolecule incorporating more than 50 amino acid residues [3]. Peptides and proteins are made up of a possible combination of 20 proteinogenic α-amino acids [3]. Nineteen of the standard α-amino acids are composed of a central α-carbon atom to which a carboxyl group, an amino group, a hydrogen atom and a side chain R are attached [4]. The R represents a side chain specific to each amino acid [4]. The 20th standard α-amino acid, proline, differs from the other amino acids as its amino group is secondary, formed by ring closure between the R group and the amino nitrogen as shown in Figure 1.1 [2]. Proline is an amino acid that confers rigidity in a peptide or protein, as rotation about the carbon atom is not possible [2]. All of the 20 amino acids, with the exception of glycine, are chiral, due to the presence of at least one stereogenic carbon and they all belong to the L-stereochemical series [5].
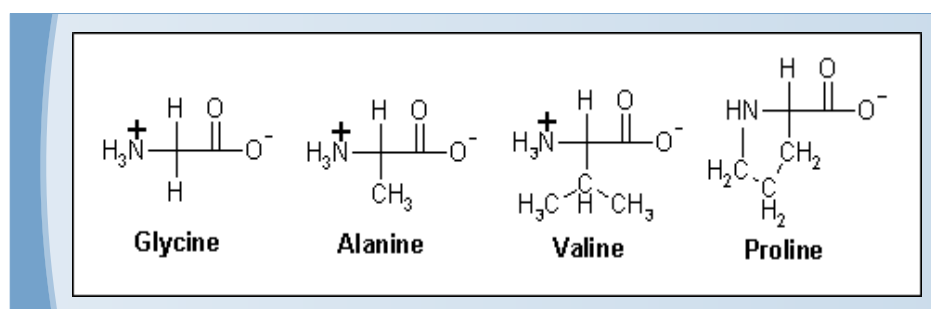
Figure 1.1 *Example structures of several standard amino acids* [2]

Peptides and proteins have well defined three dimensional structures [2]. These structures are made up of a primary structure, which represents the sequence of amino acids and a secondary structure, which is the folding of the amino acid chain into patterns such as α-helix and β-pleated sheets, as shown in Figure 1.2 [2]. The α-helix pattern is a rod-like structure formed when the amino acid chain twists into a right-handed helical conformation [2]. The β-pleated sheets pattern is the formation of two or more polypeptide chains lining up side by side [2]. The backbones of the peptides and proteins then bend and fold to form their tertiary structure [6]. Some proteins, unlike peptides, can be composed of several polypeptide chains and this is referred to as a quaternary structure [2].
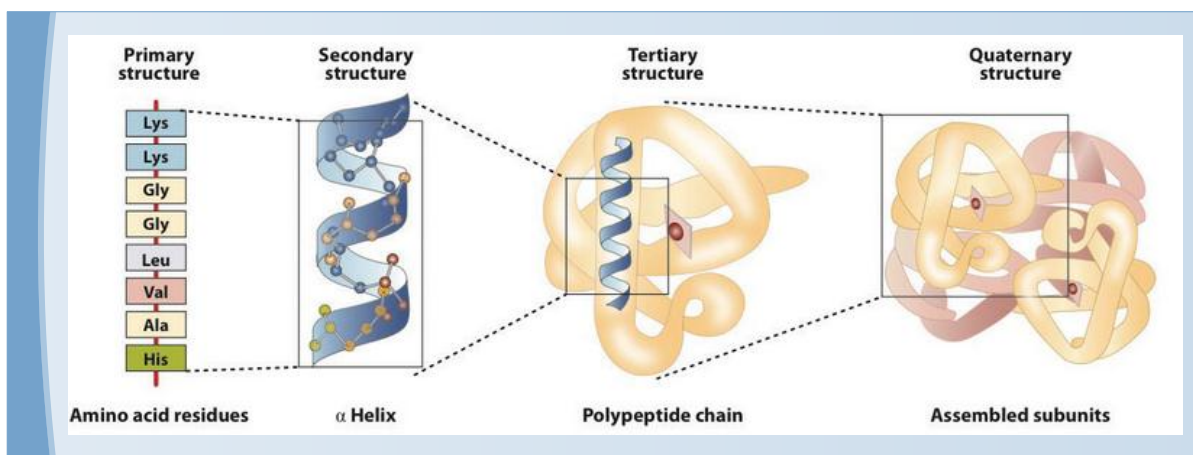
Figure 1.2 *Levels of structures of peptides and proteins* [6]

Peptides and proteins play a key role in a variety of biological and physiological processes in living organisms [7]. They act as hormones and neurotransmitters in intracellular communication, act as antibiotics in the immune system and are involved in the transport of numerous substances through biological membranes [7]. Examples of this can be seen with insulin and glucagon, which are used to regulate the level of glucose in the blood [7,8]. Insulin, the first peptide to be administrated therapeutically, decreases the level of blood glucose by increasing its uptake into the liver where it is stored as glycogen and broken down by glucagon [8,9]. The amino acid sequences that make up peptides and proteins control and direct all aspects of cellular functions and coordinate most intercellular communication within living organisms [10]. Peptides and proteins are the only class of biological molecules that offer such a range of chemical diversity and demonstrate the potential for addressing a growing range of medical challenges [10].

1.2 The use of peptides as drug candidates

Peptides and proteins can influence endocrine, neurological, immune and enzymatic processes with high specificity [3]. It is for this reason that peptides and proteins are being used therapeutically in areas such as regulation of fertility, control of pain, cancer therapy, and the stimulation of growth, cardiovascular problems, and mental illness [3]. Peptide-based drugs tap into the direct hard wiring of human physiology, yielding substantial benefits for drug therapies [11]. Current therapeutic peptides include glucagon-like-peptide-1 (GLP-1) and somatostatin analogues [9]. GLP-1 has insulin-releasing properties, suppresses glucagon levels and also delays gastric emptying for the treatment of diabetes while somatostatin analogues are used for the treatment of cancer and acromegaly [9].

Peptide drug candidates rely on the activation or inhibition of a biochemical process, by the specific recognition of, and interaction with, natural receptors [3]. Peptides have numerous advantages as drug candidates due to their high potency, minimized drug-drug interactions, low toxicity and biological diversity [12]. Peptides have the potential to penetrate deeper into tissues in comparison to

proteins and antibodies due to their smaller size [13]. Peptides also demonstrate advantages over small molecules due to greater efficacy, selectivity, specificity, and reduced half-life, which means few peptides accumulate in tissues [13]. They are composed of naturally occurring or metabolically tolerable amino acids and are generally non-toxic [10]. Therapeutic peptides are mainly receptor agonists and therefore only small quantities of peptide are required to activate the target receptors [13]. Agonist drugs activate receptors by eliciting a biological effect which can be stimulatory or inhibitory, and in contrast, antagonist drugs are agents that block-receptor-mediated effects elicited by hormones, neurotransmitters or agonist drugs by competing for the receptor occupancy [14]. The proportion of a peptide drug available for binding is influenced by the amino acid composition and sequence, the peptide length and peptide flexibility, as well as characteristics such as solubility, pH and the peptide formulation [15,16].

Peptides as drug candidates are not without their disadvantages. They demonstrate low oral bioavailability, which is the rate and extent to which the active ingredient is absorbed from a drug product [13,15]. Peptides also demonstrate a short half-life in the body due to degradation by proteolytic enzymes of the digestive system and are rapidly removed from the circulation system by the liver and kidneys [13]. Their hydrophobicity means they have restricted ability to cross physiological barriers and their high conformational flexibility results in the lack of selectivity for interactions with receptors or targets [13]. Lack of specificity for the target or receptors can result in activation of several targets, leading to side effects and possible immunogenic effects [13]. Enzymes such as peptidases are involved in the rapid degradation of peptides in the body [13]. The lumen of the small intestine is the largest threat to degradation of peptides as it contains gram quantities of the peptidase enzymes [13]. The epithelial cell membranes also contain over 15 peptidases which are specific to degrade both peptides and proteins [13]. As a result, peptides are administered by subcutaneous, intramuscular or intravenous routes to avoid the gut barrier and typically, patients require chronic self-injection [10,13]. Alternative routes of administration and new synthetic strategies are critical for the use of peptides as drug candidates [13].

1.3 Advances in the use of peptides as drug candidates

In the 1980s, pharmaceutical companies focused on small molecules rather than peptides due to their demonstrated advantages in-vivo stability and pharmacokinetics [17]. However in the late 1990s, the unexpected toxicity and cross-reactivity of small molecule drugs turned the focus back to peptides, which typically demonstrate a low toxicity profile. [17]. Now in the 21st century, peptide-based drug targets are being identified at a rapid pace. The number of peptides going into clinical trials has increased from approximately 1.2 % of drugs per year in the 1970s, to approximately 16.8 % of drugs per year in the 2000s [18]. This increase is due to advances in the administration of peptides such as the development of peptides encapsulated in biodegradable polymers in the 1980s, which

resulted in peptide injections only being required at extended intervals [10]. This was a significant milestone for increasing the acceptability of peptides as drug candidates [10]. Alternative routes of delivery are still a focal point of research in peptides including inhaled, buccal, intranasal and transdermal routes of administration [9].

Although formulation and application systems are important to improve peptide drug candidate possibilities, a major strategy in peptide chemistry is directed towards the chemical modification of peptides to increase their chemical and enzymatic stability and to also increase their activity and selectivity towards the receptor [19]. Peptidomimetic modifications are typically used to overcome the unattractive pharmacological properties of native peptides, turning a peptide structure into a non-peptide drug, providing a more conformationally constrained and thus, more stable peptide [17,19]. Typical peptidomimetic approaches used are pseudo-peptides and peptide bond modification, in which the peptide bonds have been replaced with other chemical groups, known as an amide bond surrogate [17]. The amide bond surrogates possess three-dimensional structures similar to the peptide, but they differ significantly from peptides in relation to polarity, hydrogen bonding capability and acid-base character [17]. The amide bond surrogates aim to completely prevent protease cleavage of the amide bond and therefore result in increasing resistance of peptides to degradation and elimination and also increasing their selectivity and bioavailability [13,17]. The development of unnatural amino acids and new synthetic strategies to produce cyclised peptides are also valuable tools developed to overcome the drawbacks of peptide therapeutics [18] Cyclised peptides demonstrate improved chemical stability and thus extend the biological half-life compared to their linear counterpart [20]. Conjugation of a peptide with a fatty acid or polyethylene glycol (PEG) derivative can result in increased half-life, increased bioavailability and also result in more specific binding [12]. PEG consists of a repeating chain of ethylene oxide, and once bound to the peptide; each sub-unit of the PEG becomes tightly associated with two or three water molecules, rendering the peptide more water soluble [21]. The globular structure of PEG protects the peptide from proteolytic degradation and can also aid in drug delivery [21].

1.4   Sources of peptides

Natural sources of peptides, such as extraction from humans and plants, can provide a great variety of peptides, however miniscule amounts can typically only be isolated [3,22]. This is partially due to the low concentration of peptide mediators in some tissues or limited availability of human tissue sources [19]. Contamination of tissue with pathogenic viruses also restricts the use of natural sources for the isolation of peptides [19]. As a result, chemical synthesis, recombinant technology, cell-free expression systems or enzymatic synthesis are other approaches typically used to generate peptides for drug candidates [13]. Each of these techniques has their advantages and disadvantages but generally, the size of the peptide determines the most suitable technology for its production [7,13].

Recombinant technology is currently used for the manufacture of a small number of peptides due to demands of significant personnel input into process development, production, quality assurance and regulatory affairs [10]. However, it is expected that recombinant technology will play an increasingly important role in the future of peptide manufacturing due to larger quantities being required [10]. Insulin, composed of approximately 50 amino acids, was formally obtained from porcine and bovine pancreatic tissue [3]. Sensitisation became an issue with patients as pig and beef insulin is not identical to human insulin and as a result, human insulin was subsequently prepared commercially by recombinant DNA technology [3]. Immunological incompatibilities of peptide drugs from animal sources have been observed, and as a result, significant emphasis has been placed on the use of chemical synthesis for the production of peptides [19]. Chemical synthesis is the only technique which permits the use of unnatural amino acids and the production of large quantities of pure peptide [7,13].

1.5   Chemical synthesis of peptides

Chemical synthesis is currently the preferred technique for manufacturing peptides as it offers a much wider chemical diversity than peptides produced recombinantly. This chemical diversity is partially due to the use of unnatural amino acids and pseudo-peptide bonds [13]. Peptide synthesis involves the formation of a peptide bond between the two amino acid segments by a peptide coupling reaction [23]. A peptide coupling reaction involves the activation of the carboxylic acid moiety of an amino acid which then reacts with the amine moiety of another amino acid forming a peptide bond as demonstrated in Figure 1.3 [24].
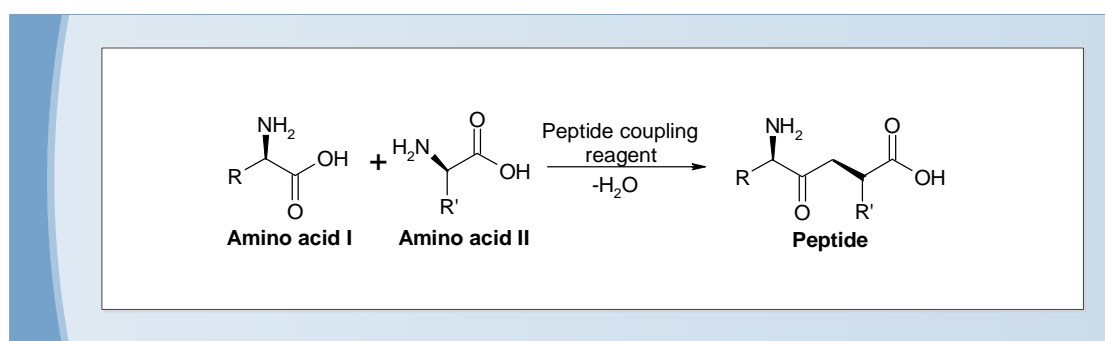
Figure 1.3 *Peptide coupling reaction scheme* [25]

In order to activate the carboxylic acids, peptide coupling reagents are required which generate compounds such as active esters, carbonic anhydrides or acid chlorides [26]. Different strategies have been employed to aid the formation of a peptide bond, usually involving protection, activation, coupling and deprotection steps [23]. During the formation of a peptide bond, one specific amide bond is desired but there is the potential for three amide bonds to form [27]. Therefore protecting groups are used for all functional groups except those that are involved in the specific

amide bond formation to ensure they do not react and only the desired amide bond is formed [27]. As demonstrated in Figure 1.4, two amino acids with protecting groups P_1 and side chain protection P_2, will react to yield a fully protected dipeptide bond in the presence of the coupling reagents [8]. The protecting group (either P_3 or P_4) can be removed, depending on whether the polypeptide chain is to be extended at the N-terminus or the C-terminus [8].
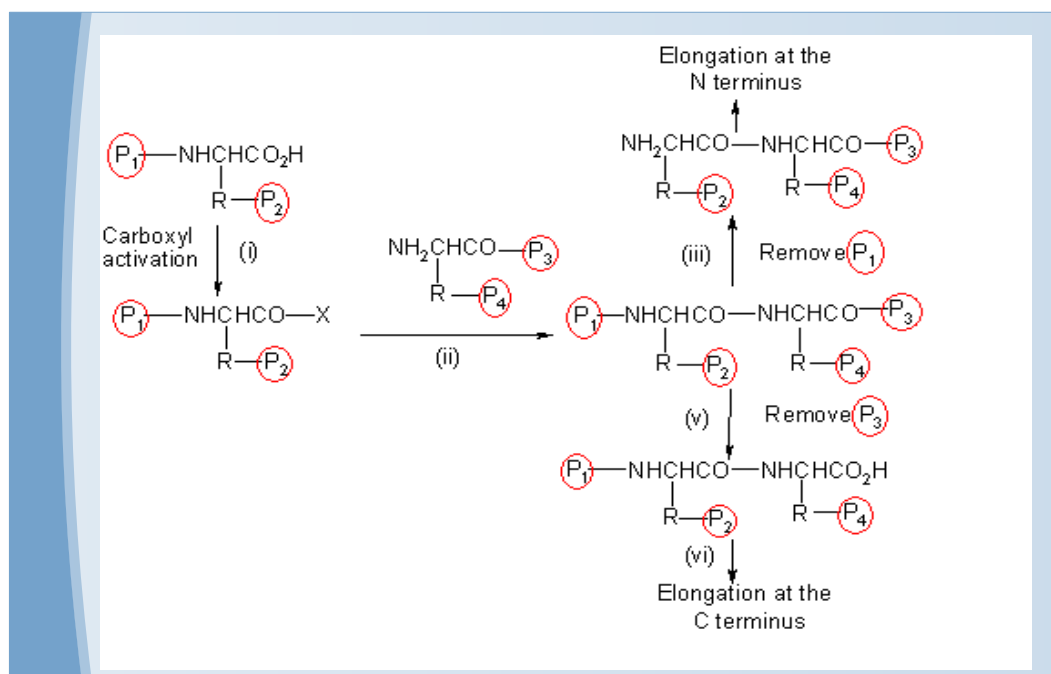
Figure 1.4 *Schematic of peptide synthesis* [8]

The protecting groups are a crucial part of peptide synthesis, the absence of which will result in the possible formation of a mixture of di-, tri- and polypeptides [5]. The protecting groups need to have the ability to be selectively removed during the peptide synthesis [8]. There are three type of protecting groups, N-terminus, the C-terminus, and the side chain protecting groups [5]. N-terminus protecting groups are typically urethane based, such as tert-butoxycarbonyl (Boc) group, which is typically removed with moderately strong acid, and 9-fluorenylmethyloxycarbonyl (Fmoc) group, which is removed under base condition. The N-terminus protecting groups are typically temporary as they are removed in order to grow the peptide, which generally proceeds in the C-N direction [5]. Side chain protecting groups are called 'permanent' protecting groups as they are never removed during the synthesis process and are usually based on benzyl (Bzl) or tert-butyl (tBu) groups [5]. In relation to the C-terminus protecting group, this is dependent on what type of peptide synthesis approach is used [5]. Chemical synthesis involves either a solid phase peptide synthesis (SPPS) or a liquid phase peptide synthesis (LPPS) approach. For SPPS, the C-terminal protecting group is a polymer and in LPPS, tert-butyl esters, benzyl esters and phenyl esters can all be used as protecting groups [5].

1.5.1   *Liquid phase peptide synthesis (LPPS)*

Liquid phase peptide synthesis (LPPS) is the controlled formation of a peptide by the coupling of amino acids in solution [5]. Isolation, purification and characterisation are required after each amino acid addition in LPPS; therefore the process is considerably longer than SPPS [5]. This process can prove advantageous when large quantities are targeted and also, any unwanted side products, such as incomplete deprotection or coupling reactions, are easily detected during the isolation and characterised after each step [12,5]. This process is not suitable for small peptides as it would result in a considerable investment in time and energy for minimal yield and also, the solubility of the peptide decreases with increasing chain length, which can result in intermediates that are so insoluble that they render their chemical reactions effectively impossible [5,28]. Attempts have been made to overcome the problems associated with the classical peptide synthesis in solution and led to the invention of solid phase peptide synthesis (SPPS) [5].

1.5.2   *Solid phase peptide synthesis*

Solid phase peptide synthesis was discovered by Bruce Merrifield in 1963, where he covalently attached an amino acid to an insoluble support and elongated a peptide chain from the support—bound residue [28]. The theory of SPPS involves growing the peptide on an insoluble support in which the by-products are removed after each step [7]. The LPPS problems are no longer applicable in SPPS, as the isolation of intermediates is no longer required and the problem of poor solubility of the intermediates is removed as the growing peptide remains on the insoluble support until the synthesis has been completed [5]. Linkage of the peptide to the insoluble support must be strong enough to ensure it does not break during the course of subsequent peptide synthesis, but still have the ability to be cleaved at the end of the process to liberate the final peptide [8]. The insoluble support must be chemically inert to all of the reagents and solvents used during the peptide synthesis and must not interact physically, with them or the peptide itself [5]. The insoluble support is typically a cross-linked polystyrene material and the cross-linking results in the polymer being insoluble in organic solvents [27]. The polymers, known as resin, typically come in the form of small beads [27]. The polystyrene support beads are typically 20-50 μm in diameter but are swollen in organic solvents such as dichloromethane (DCM) or dimethylformamide (DMF) [29]. The insoluble support must have an appropriate functional group or be capable of functionalisation, to which the amino acid or a linker is attached [28]. A linker can be used which acts as a bifunctional spacer to connect the first amino acid to the solid support, providing more flexibility to modify the properties of the peptide-resin anchorage [5,28]. An example of a linker is 4-(bromomethyl)phenylactic acid which is incorporated into an aminomethyl polystyrene solid support, giving rise to phenylacetamidomethyl resin (PAM resin) [5].

The main SPPS strategy is sequential synthesis which involves the stepwise addition of amino acids to achieve the final peptide sequence [13]. It involves repetitive coupling and deprotection steps to introduce the amino acids as shown in Figure 1.5 [28].
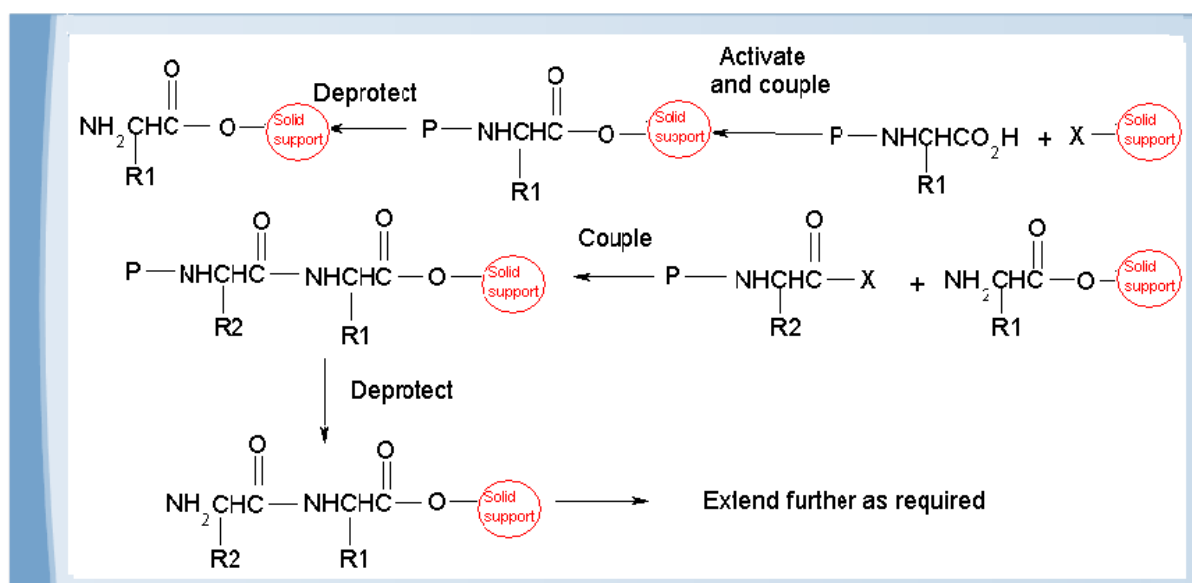
Figure 1.5 *Schematic of solid phase peptide synthesis* [8]

Once the desired peptide length is achieved, the peptide is cleaved from the solid support [28]. Sequential synthesis is a very fast and efficient way to synthesise peptides, however the growing peptide can fold over onto itself, or aggregate with a neighbouring chain which can result in poor yield or a truncated peptide, and therefore careful monitoring of the process is required [30]. SPPS has a wide variety of applications for drugs on the market today; however its limitations make the assembly of large peptides particularly challenging [5]. The peptide remains on the solid support for the entire process and if a coupling reaction fails to go to completion, then the final product will contain deletion peptides, which tend to be difficult to remove [8]. This problem is typically overcome by the confirmation of the completion of each coupling reaction during the synthesis process [28]. The unreacted primary amines react with reagents such as ninhydrin and bromophenol blue, which can be monitored qualitatively or quantitatively [28]. A result of approximately 100 % completion reaction is required for each amino acid coupling to ensure a high yield following the peptide synthesis process [8]. If a low yield is achieved for each coupling reaction then the accumulative yield would be detrimental to large peptide sequences and could result in a significantly low yield at the end of the synthesis process [5]. These problems have resulted in the use of the production of large peptides and proteins being produced chemically by another mode of SPPS, known as convergent synthesis [5].

Convergent synthesis is the formation of independent peptide sequences that are then cleaved from the solid support, purified and characterised, and linked together by condensation in solution to form the final peptide product [13,5]. It utilises the advantages of both solution phase and solid phase

peptide synthesis, with regards to purification and characterisation of intermediates throughout a more rapid process [5]. Convergent synthesis is typically used for peptide sequences that contain > 50 amino acid residues and is advantageous for repetitive sequences and hydrophobic peptides [13]. It generally gives higher overall yields than sequential synthesis and involves the manipulation of small and easily handled peptide fragments [27]. When construction of the target peptide is complete, cleavage and purification are performed as per sequential peptide synthesis [5].

Regardless of the type of chemical synthesis used, the formation of the peptide bond is crucial for obtaining an efficient and economic peptide production process [31]. Success in peptide synthesis is highly dependent on the coupling strategy employed [32]. A fast peptide synthesis is desired during peptide manufacture, and this is affected by the rate of amino acid acylation and is heavily dependent on the properties of the coupling reagents [33]. The synthesis of peptides is a well-established process; however the combination of the 20 proteinogenic amino acids and the increasing number of unnatural amino acids makes each peptide synthesis unique, requiring closer attention to each amino acid coupling and coupling reagents employed [31].

1.6 Peptide coupling reagents

A good coupling reagent is one that works with a high efficiency for a wide variety of peptides, can be used in both solid and liquid phase synthesis and can also be used in stoichiometric quantities [31]. The coupling reagent should demonstrate high stability, result in minimal side reactions and produce by-products that can be completely removed by solvent extraction [31]. Ideally they will be reasonably priced, have a long shelf life and involve a chemistry process that is adaptable for up-scaling [31]. The ideal peptide coupling reagent and its by-products must be safe for the user and the environment [34].

Highly effective peptide coupling reagents are essential in peptide synthesis; otherwise the formation of impurities can occur, which can result in an increase in the time required for the purification of the peptide [31]. Peptide racemisation is a problem related to the coupling reaction [31]. Racemisation is a process that can occur on the C-terminal amino acid residues during a coupling reaction where the chiral α-carbon is converted from the L form to a D/L form mixture [25,27]. Racemisation is a serious problem in peptides as the biological activity of most peptides is critically dependent on the stereochemistry [8]. It is possible that a change in one amino acid residue in a peptide from the L to the D form can result in the compound being biologically inactive [8]. The use of additives, the use of solvents with low dielectric constant, and the reduction in the time of pre-activation of the carboxylic acid will all reduce the formation of racemisation peptides [31]. Deletion peptides, which lack one or more residues, are impurities that can also occur if the coupling reagent is not effective [31]. Truncated peptides, where the N-terminus is irreversibly blocked preventing further elongation, occurs if the resin is over-dried and terminated peptides can also occur in the presence of

acetic acid, trifluoroacetic acid or guanidine derivatives from aminium salts [31]. Impurities formed during peptide synthesis are often difficult to detect as they display similar chromatographic properties as the peptide, therefore it is critical to optimise the coupling reactions [31]. Purification by preparative HPLC has been proven to be a time consuming step in the peptide manufacture process, therefore optimisation of the coupling reagents is critical to increase the crude purity going into purification [12]. Each reagent is classified into several different types, namely carbodiimides, onium salts such as phosphonium and uronium/aminium salts, and immonium coupling reagents [25].

1.6.1   Carbodiimide peptide coupling reagents

The era of peptide coupling reagents began in 1955 with the use of dicyclohexylcarbodiimide (DCC) [31]. DCC belongs to a group of coupling reagents known as carbodiimides, which also includes diisopropylcarbodiimide (DIC) and 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC), the structures of which are outlined in Figure 1.6 [31].
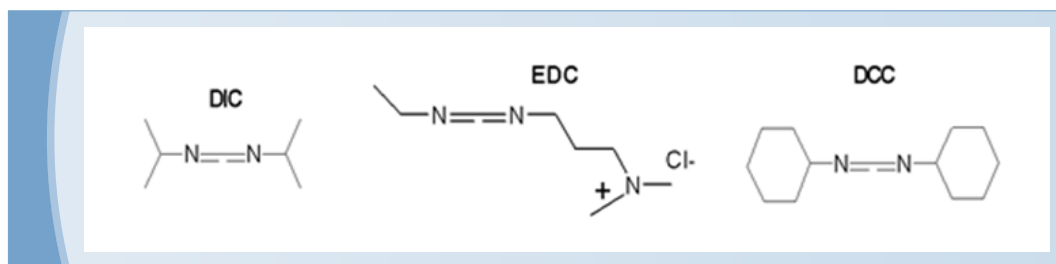
Figure 1.6 *Structures of some carbodiimide peptide coupling reagents* [35]

Carbodiimides contain two nitrogen atoms, which are weakly alkaline, and this triggers a reaction with the carboxylic acid of the amino acid to form O-acylisourea [35]. The carbodiimides reagents are relatively inexpensive and their active species, O-acylisourea is moderately reactive, as seen in the schematic for a carbodiimide reaction in Figure 1.7 [25]. Carbodiimide coupling reactions are usually carried out with ratios of N-protected amino acid to the carbodiimide coupling reagent of 2:1 in the presence of dimethylformamide (DMF) or dichloromethane (DCM) [5]. They are associated with high racemisation and low yields due to the formation of N-acylurea, as shown in Figure 1.7, which has poor reactivity [31]. DCC is incompatible with Fmoc/tBu protecting groups in solid phase chemistry due to the insolubility of dicyclohexylurea in common solvents such as DMF and DCM, in which it precipitates from the reaction mixture [31,36]. However, DCC has been proven to be a very useful reagent for solution phase peptide reactions [37].
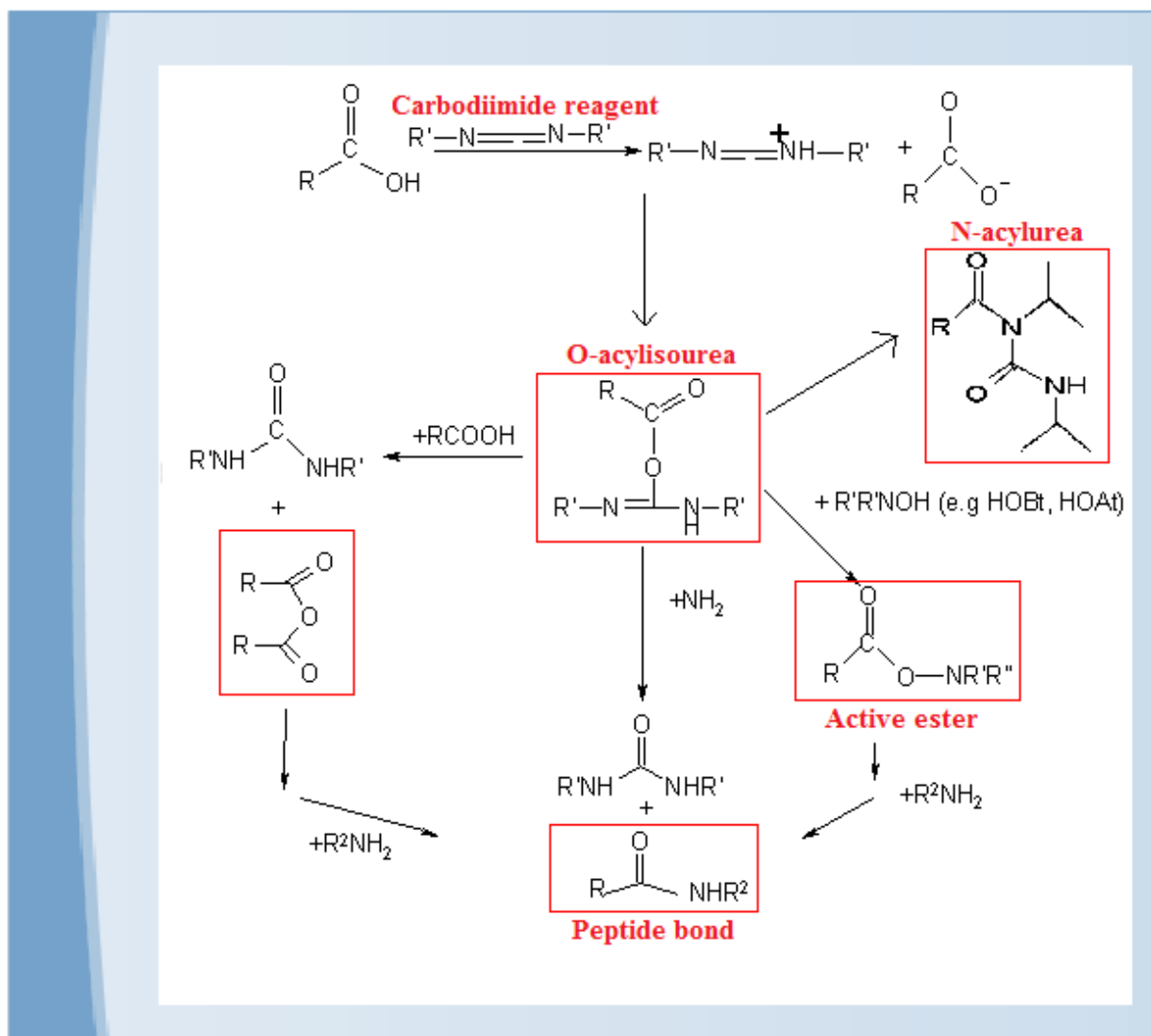
Figure 1.7 *Mechanism of peptide bond formation through carbodiimide activation* [38,39]

1.6.1.1 Benzotriazole additives for carbodiimide reactions

In the 1970's, 1-hydroxybenzotariazole (HOBt) was introduced as an additive to the carbodiimide reactions to suppress racemisation and the formation of side reactions [31]. Adding an equivalent of HOBt, results in reaction of HOBt with the O-acylurea formed in the carbodiimide reaction, to form OBt active esters [26]. The OBt active esters are less reactive than O-acylisourea formed with DCC, and therefore are less prone to racemisation and are more stable [35]. Alongside reducing racemisation, HOBt was also proven to be a rate enhancer for the reaction [24]. However, HOBT with DCC also yielded undesired by-products such as diazetidine [26]. In 1994, another racemisation suppressant was developed, 1-hydroxy-7-azabenzotriazole (HOAt), which was demonstrated to be more efficient than HOBT in relation to yield and racemisation suppression [26]. This was reportedly due to the anchimeric assistance caused by the pyridine ring present in HOAt [23]. The nitrogen on the HOAt also provides a neighbouring group effect that can increase reactivity

and reduce racemisation [24]. Another additive, 6-chloro-1-hydroxybenzotriazole (6-ChloroHOBt) was also used as an additive to the carbodiimide reactions, providing a good compromise between HOAt and HOBt with regards to reactivity and price [35].
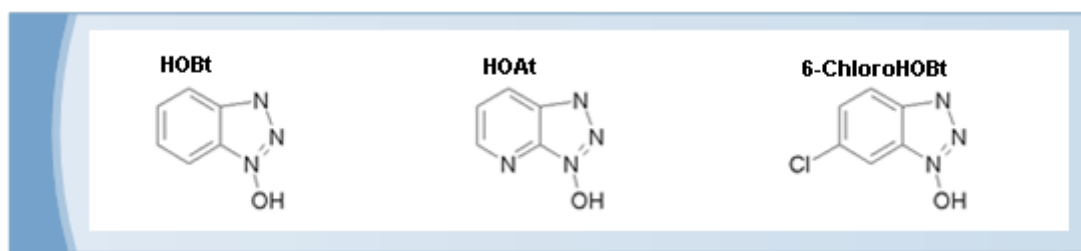
Figure 1.8 *Structures of some benzotriazole additives* [25,31]

Unfortunately, carbodiimide coupling reagents have been shown to demonstrate skin irritating properties and some of the benzotriazole based additives have also been proven to cause skin irritation, as well as contact dermatitis, sensitisation and an allergic reaction in the respiratory tract [31]. The United Nations (UN) reclassified HOBt as a desensitized explosive and the material can no longer be shipped economically, however HOBt hydrate can be shipped safely and is used in its place [40]. HOAt was also determined to be very unstable with high sensitivity to friction and electrostatic discharge and therefore has a risk of burning or exploding. These safety developments have been the driving force for the extensive research into alternative, non-hazardous peptide synthesis reagents [40].

1.6.2 Onium salts as peptide coupling reagents

Onium salts, mainly aminium/uronium and phosphonium, were developed based on 1H-benzotriazoles of HOBt, HOAt and 6-ChloroHOBt [26]. These have become the preferred choice for liquid and solid phase synthesis as they are much safer than the carbodiimide coupling reagents with benzotriazole additives [32]. Onium coupling reagents based on HOAt were reported to be superior to those based on HOBt in terms of efficiency and control of racemisation [26]. Derivatives based on 1H-benzotriazoles of 6-ChloroHOBt have also been demonstrated to be less hazardous and more reactive than HOBt [32]. They generate 6-chloro-1-benzotriazolyl esters which are more reactive than OBt esters due to the increased acidity of 6-ChloroHOBt relative to HOBt [40]. There are two types of onium salt coupling reagents, Aminium/Uronium salts and Phosphonium salts, as detailed below.

1.6.2.1 Aminium/Uronium salts

The most powerful onium salts are the aminium/uronium salts based on the HOBt/HOAt system [26,32]. These salts are prepared by reaction with the 1H-benzotriazoles with the chloroformamidinium salt derived from a urea, such as tetramethylurea (TMU) [41]. The carbon skeleton structure has a determining role in the efficiency of the reagent for the activation step [42].

Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), based on HOBt, is an uronium salt that was discovered in 1978, followed by various HBTU analogues such as O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) as shown in Figure 1.8 [24]. A hexafluorophosphate and tetrafluoroborate anion is used as a non-nucleophilic counter ion in uronium/aminium reagents, the tetrafluoroborate salts are more soluble [24]. The main difference between the two coupling reagents, HBTU and TBTU, is the counter ion, which has no significant influence on the coupling rate or racemisation [24]. 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), developed in 1993, is based on 1H-benzotriazoles of HOAt [33]. It is the most reactive aminium salt and has even been viewed as the most efficient coupling reagent available for peptide synthesis [43]. However, its use is minimal in industry due to its expensive cost and its use is often reserved for very difficult couplings [33,43]. HBTU/TBTU in the presence of 6-ChloroHOBt is a recommended alternative to the expensive HATU [44].

2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), established in 2002, and O-(6-chloro-1-hydrocibenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TCTU) are aminium salts based on 6-ChloroHOBt [34]. HCTU/TCTU reagents are more reactive and less hazardous than HBTU/TBTU salts, due to the presence of a chlorine atom that stabilises the structure [36]. HCTU demonstrates efficiency close to HATU, without the associated cost, and results in higher purity peptides than HBTU and TBTU [33]. There are limits to using the uronium/aminium salts as peptide coupling reagents [32]. Their high reactivity may lead to side reactions, usually during slow couplings such as cyclisations or in the introduction of hindered residues [32]. Uronium/aminium salts can react with the hindered carboxylic components leading to a guanidine derivative which can terminate the peptide sequence [36]. This guandinylation is particularly problematic when carboxyl activation is slow, such as cyclisation reactions [45]. Uronium/aminium reagents are generally less stable than phosphonium reagents (discussed in Section 1.6.2.2) in the presence of base. [24]. A detailed discussion as to the advantages and disadvantages of the coupling reagents (i.e. coupling efficiency, etc.) is outside the scope of this thesis because no synthetic work was performed during this thesis. The structures of some aminium peptide coupling reagents are outlined in Figure 1.9.
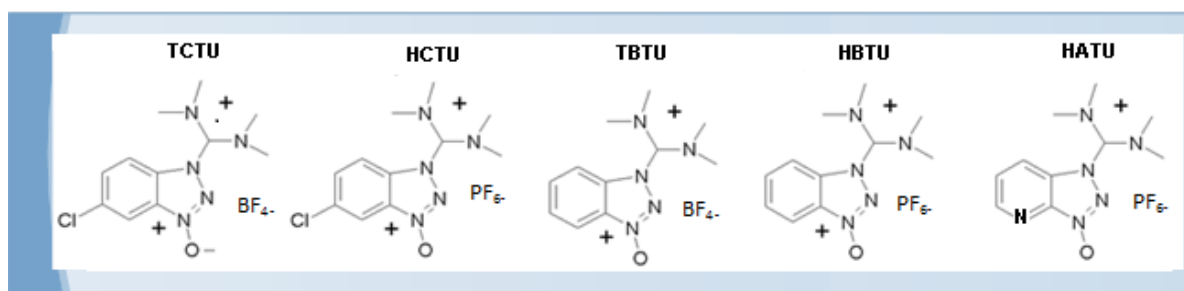
Figure 1.9 *Structures of some aminium peptide coupling reagents* [24,25,32]

1.6.2.2   Phosphonium salts

The second group of onium salts based on 1H-benzotriazoles of HOBt and HOAt are phosphonium salts [26]. They differ from other onium salts in the nature of the electrophilic core as they have a positively charged phosphorus centre [32]. Phosphonium salts demonstrate similar reactivity to the aminium/uronium salts; however they do not undergo peptide termination in excess [32]. Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) was the first HOBt-phosphonium salt developed, however its use is limited due to respiratory toxicity and carcinogenicity [26]. It is an excellent coupling reagent but hexamethylphosphoramide (HMPA), a toxic compound, is formed as a by-product [35]. A pyrrolidino derivative of BOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop) was then developed [26]. This is a useful peptide coupling reagent for the activation of hindered amino acids, where its aminium analogue would result in the formation of guanidine derivatives, and terminate the peptide chain [31]. Alongside PyBOP, chlorotripyrrolidinophosphonium hexafluorophosphate (PyCloP) and bromo-tris-pyrrolidino phosphoniumhexafluorophosphate (PyBrOP) were developed in order to prevent the generation of undesirable HMPA [35]. 7-azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP), derived from HOAt, is the most reactive phosphonium salt [31]. Both PyBOP and PyAOP coupling reagents can be used in excess in a coupling reaction but unfortunately there are limitations with using these phosphonium salts also, PyAOP is relatively expensive and PyBOP, its cheaper counterpart, demonstrates lower reactivity [36]. 6-chloro-benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyClocK) is a phosphonium salt of 6chloroHOBt, which is ideal for difficult or hindered reactions where the carboxyl activation is slow [32]. The structures of some phosphonium peptide coupling reagents are outlined in Figure 1.10. The stability of phosphonium salts can be correlated to their reactivity [36]. PyBOP and PyClocK are more stable and less reactive than PyAOP [36]. PyClocK was reported to perform better than PyBOP in terms of racemisation control and efficiency [36]. However, in the absence of base, the hydrolysis of PyClocK was demonstrated to be more significant than PyBOP [26].
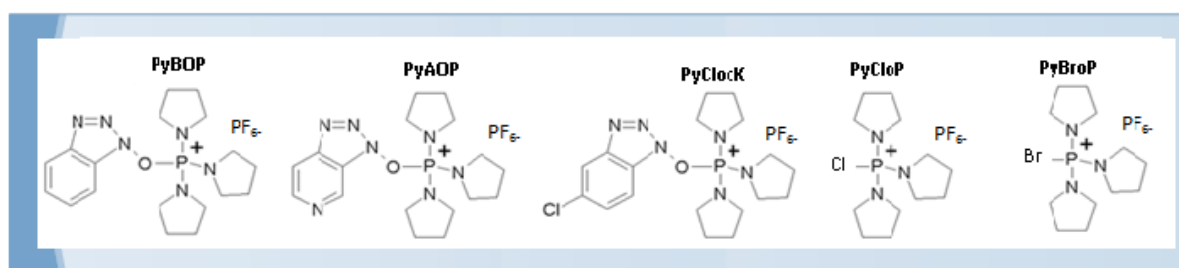
Figure 1.10 *Structures of some phosphonium peptide coupling reagents* [25,46]

1.6.3   Oxyma based additives

Ethyl 2-cyano-2-(hydroxyimino) acetate (Oxyma pure), established in the 1970's, can be used in place of HOBt in carbodiimide-mediated coupling reactions [32,40]. Oxyma pure derivatives have demonstrated higher stability than the benzotriazole derivatives, HATU and HBTU [47]. Oxyma pure is a less hazardous compound in comparison to its explosive counterpart benzotriazole-based reagents, however thermal stability of this compound is relatively low in comparison to HOBt hydrate and HOAt [46]. Oxyma pure demonstrates a remarkable capacity to inhibit racemisation, as well as high coupling efficiency [40,46]. The structure of Oxyma pure can be seen in Figure 1.11.

1.6.4   Oxyma based uronium salts

1-((1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-Dimethylamino-Morpholinomethylene)) Methanaminium Hexafluorophosphate (COMU) is a third generation of uronium-type coupling reagent based on Oxyma pure and a morpholino carbon skeleton [47]. The presence of the morpholino moiety (as shown in Figure 1.11) has an impact on the polarity of the carbon skeleton and therefore influences the stability, solubility, and reactivity of the reagent [47].
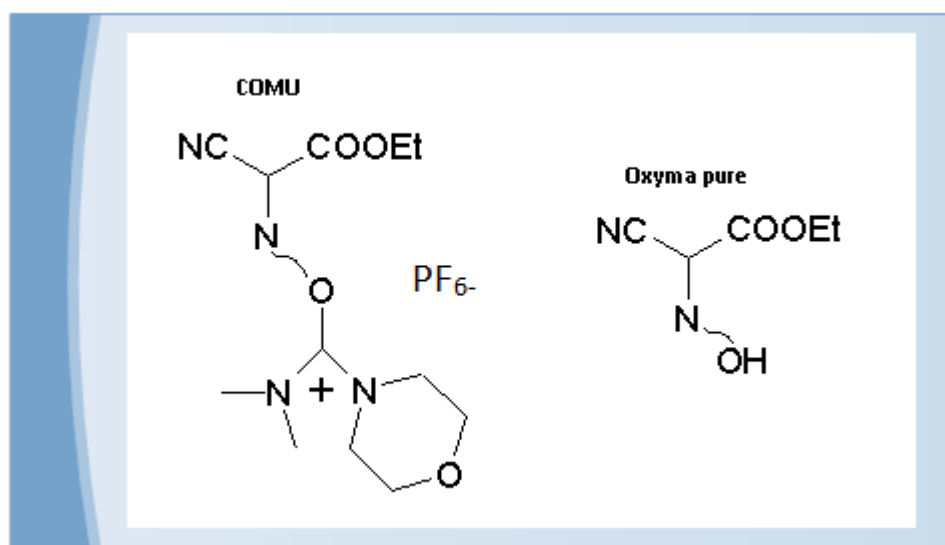
Figure 1.11 *Structures of Oxyma pure and COMU* [47]

The morpholino carbon skeleton acts as a proton receptor, and the Oxyma moiety acts as a leaving group to provide a superior, and safe amide formation [47]. The proton acceptor moiety allows the use of one equivalent of base during coupling, resulting in reduced racemisation without impacting yield or reaction rate [48]. The by-products of COMU are water soluble and easily removed [23]. Like Oxyma pure, COMU is a less hazardous compound in comparison to benzotriazole-based reagents, and is less likely to cause an allergic reaction such as contact dermatitis or asthma [48]. It is reported that COMU has high solubility in most usual solvents, and is applicable for both solid and liquid phase synthesis [48]. Overall, it is reported to be more efficient than benzotriazole-based

reagents in terms of racemisation suppression, stability, solubility, and coupling effectiveness, however this is dependent on the peptide sequence and amino acids used [49]. COMU is used in the same way as PyBOP, HATU, and HBTU, but it generates an ester of Oxyma pure instead of benzotriazolyl esters [40].

O-(Cyano(ethoxycarbonyl)methylenamino)-1,1,3,3-tetramethyluronium tetrafluoroborate (TOTU), another coupling reagent based on Oxyma pure, exhibits many similar properties to COMU [40]. It has high reactivity, solubility, stability and low explosivity [40]. The by-products are water soluble, making TOTU ideal for liquid phase synthesis [40].

Ethyl-cyano (hydroxyimino) acetato-O^2) tri-1-pyrrolidinylphosphonium hexafluorophosphate (PyOxim) is a cost effective alternative to COMU [45]. It has been reported to combine high reactivity and solubility with moderate stability, to make an ideal coupling reagent [50]. Like COMU, it generates Oxyma pure esters and it mediates coupling with low racemisation [45]. PyOxim demonstrates excellent solubility in dimethylformamide (DMF) and N-methyl-2-pyrrolidone (NMP), along with low potential for causing allergic reactions [45].
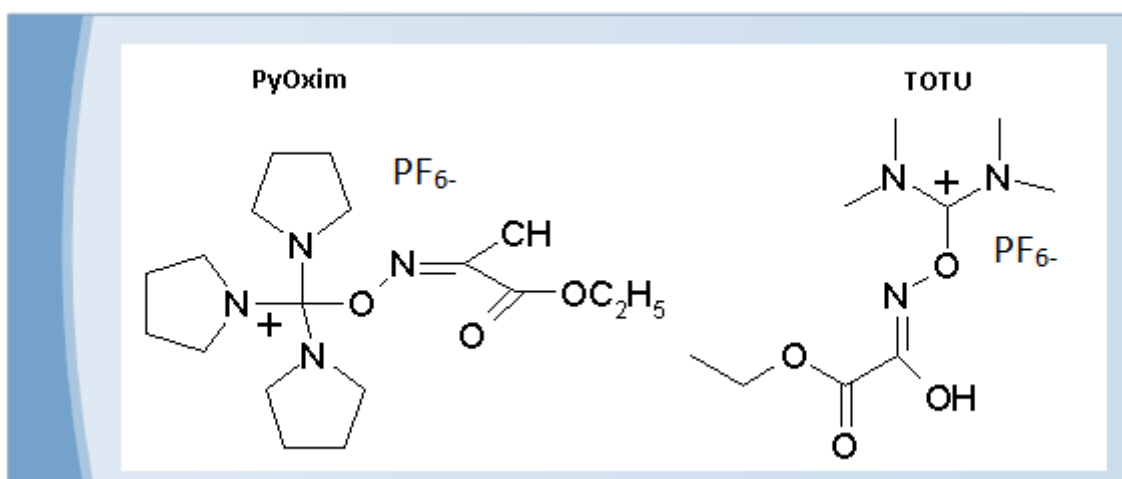
Figure 1.12 *Structures of PyOxim and TOTU* [24]

1.6.5   *Immonium reagents*

Immonium reagents are peptide synthesis reagents which are prepared via modifications of uronium reagents [25]. The structural distinction is due to the replacement of the amino group of the central atom in uronium with a hydrogen, an alkyl or an aryl group [25]. The HOBt and HOAt immonium coupling reagents include 5-(1*H*-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl 2*H*-pyrrolium hexachloroantimonate (BDMP), 2,6-Bis{[bis(2-pyridylmethyl)amino]methyl}-4-methylphenol (BPMP) and 5-(1H-7-azabenzotriazol-1-yloxy)-3,4-dihydro-1-methyl 2H-pyrrolium hexachloroantimonate (AOMP) [51]. These reagents demonstrate rapid reaction speed, low racemisation and good yields and they have been shown to be more efficient then the HOBt and HOAt- derived uranium/aminium and phosponium salts [51].
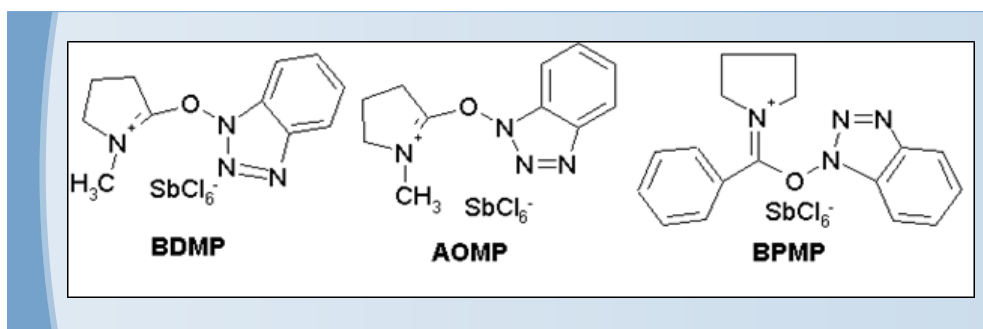
Figure 1.13 *Structures of HOBt-based and HOAt-based immonium type coupling reagents* [51]

1.6.6 Cleavage, deprotection and isolation of the peptide

On completion of the synthesis of the peptide, the peptide needs to be cleaved from the resin and the protecting groups need to be removed from the side chains [52]. The cleavage conditions are critical; they need to be sufficiently vigorous to remove the peptide from the resin and, at the same time, sufficiently mild to allow sensitive structural features to survive [5]. The chemical method employed to cleave the peptides depends on the nature of the cleavable linker attaching the peptide to the support, the nature of the protecting groups and also the reactive properties of the unprotected side-chains [5,52]. Acidolysis is typically the process used and this involves treating the bound peptide residue with acid [5]. Strong acid such as liquid hydrogen fluoride is used for peptides synthesised using the Boc/Bzl approach and a weaker acid, such as trifluoroacetic acid is used for peptides synthesised with the Fmoc/tBu approach [5]. As the side chain-protecting groups are removed during the cleavage, the reaction solution is rich in potent electrophilic alkylating species, which potentially lead to the alkylation of susceptible residues [5]. In order to prevent this occurring, appropriate scavengers, such as anisole and thiol derivatives, must be added to the reaction, isolating the electrophiles and therefore, minimising modification or destruction of the sensitive amino acids [3].

On completion of cleavage of the peptide from the resin, purification of the peptide solution is required to remove side products arising from modification of amino acid side chains, and deletion peptides which arise from incomplete couplings [8]. Purification methods typically utilize various modes of chromatography such as ion exchange chromatography, gel permeation chromatography and preparative reversed phase high performance liquid chromatography (RP-HPLC) [53]. The amount of purification required depends on the purity of the crude peptide being purified and the desired purity of the final peptide product. In sequential SPPS, the amount of purification depends on the number of amino acids, the more amino acids present, the more purification it requires [29]. RP-HPLC is predominantly used for the purification of peptides due to its resolution power, demonstrating the

ability to separate polypeptides that differ by a single amino acid [54]. The use of RP-HPLC for the preparative purification of peptides is discussed in further detail in Section 1.7.2.

In the presence of water, peptides can degrade through hydrolysis or other chemical reactions due to the molecular mobility allowed within a liquid state [55]. As a result, once the desired peptide purity is achieved in purification, the aqueous peptide solution is typically lyophilised, which is a more stable way of storing a peptide [55]. Lyophilisation is a process used to preserve a peptide by freezing the aqueous solution and then applying a pressure to allow the frozen material to sublime directly from the solid phase to the gas phase [55,56].

1.7   Analytical evaluation of peptides

1.7.1   *The use of analytical chemistry for the purification and characterisation of peptides*

Analytical chemistry is the key to peptide synthesis as no synthetic endeavour can be considered complete until the product has been adequately purified and subjected to a battery of analytical tests to verify its structure [29]. Analytical chemistry plays an important role in the preparative purification of peptides as well as the evaluation of the homogeneity and covalent structure of the peptide [29]. Identity confirmation by nuclear magnetic resonance spectroscopy (NMR spectroscopy), mass spectrometry (MS) and amino acid analysis (AAA), alongside peptide content and purity determination by high performance liquid chromatography (HPLC) are all requirements for analytical evaluation of peptides [57]. Analysis of the counter ion (e.g. acetate, hydrochloride or trifluoroacetate, etc.) and determination of moisture content are also used to yield information on peptides, and the results are typically used for the determination of mass balance of the peptide content [57]. Appearance, solubility, residual solvents by gas chromatography, capillary zone electrophoresis (CZE) and specific optical rotation are optional tests that can provide additional information on the peptide [57]. These techniques each have advantages and limitations, such as HPLC, which can be used for purification and assessment of heterogeneity but does not yield structural information [29]. Another example is amino acid analysis which accurately quantitates the amount of peptide and provides molar ratio of amino acid, however it does not distinguish heterogeneous species, and it is less reliable for some unstable amino acids such as cysteine and the compositional accuracy decreases as the peptide gets larger [29]. Despite their limitations, each analytical technique used provides unique information that contributes to the evaluation process [29]. Chromatography is the basis of the main analytical chemistry methods used for the purification, identification and quantitation of peptides.

1.7.2 *Reversed phase - High performance liquid chromatography for the evaluation of peptides*

In reversed phase chromatography (RP-HPLC), a non-polar stationary phase is used in conjunction with polar, largely aqueous mobile phases and this makes up between 70 % and 80 % of all HPLC applications [58]. This mode of liquid chromatography is so named because the elution conditions are essentially the reverse of the normal phase chromatography [29]. This mode of HPLC plays a critical role in analysing and purifying peptides due to its resolution ability [54]. RP-HPLC has the ability to separate peptides of nearly identical sequences, which may differ by a single amino acid, for both small peptides and large polypeptides [54].

Reversed phase high performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity [59]. RP-HPLC separations depend on the hydrophobic binding of the analyte from the mobile phase to the immobilised hydrophobic n-alkyl ligands attached to the stationary phase or sorbent as shown in Figure 1.14 [59].
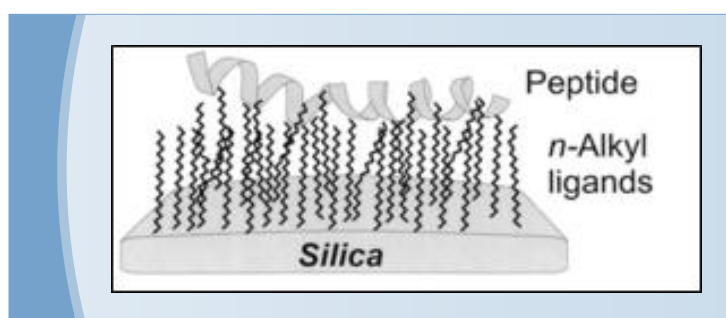
Figure 1.14 *Schematic representation of the binding of a peptide to an RP-HPLC silica-based stationary phase* [59]

The mixture containing the analyte is applied to the stationary phase in the presence of aqueous buffers and the analyte is eluted by the addition of organic solvent to the mobile phase, either isocratically or by gradient elution [59]. The analyte continuously partitions between the mobile phase and the hydrophobic stationary phase, however polypeptides are too large to partition in between the mobile and stationary phase and therefore they absorb to the hydrophobic surface once they enter the column [54]. Only one part of the polypeptide, known as the 'hydrophobic foot', binds to the stationary phase [54]. The 'hydrophobic foot' of a polypeptide can vary depending on the amino acid sequence and conformational properties and therefore, differences in the hydrophobic foot results in the separation of the polypeptides [54].The polypeptides remain bound to the hydrophobic surface until the concentration of organic modifier reaches the critical concentration that causes desorption, where the peptide is eluted from the column [54]. The adsorption/desorption of polypeptides typically occurs at the top of the column, which means the column length does not impact the separation and therefore, 5 cm -15 cm columns are typically used [54]. Small peptides desorb faster than polypeptides and as a result, the column length can impact the separation of smaller peptides and

therefore, longer columns of 15 cm -25 cm in length are typically used [54]. The diameter of a RP-HPLC column is typically 4.6 mm but this is increased for purification by RP-HPLC and reduced if the LC is coupled with MS [54]. The diameter of the column does not affect peak resolution but it does affect sample loading, solvent usage and detection sensitivity [54]. Gradient elution is typically preferred for RP-HPLC because peptides are very sensitive to organic modifier percentage. Isocratic separation is therefore impracticable for peptide separations because very small differences in the percentage organic modifier typically result in very significant changes in retention and therefore gradient elution results in a better separation of peptides and associated impurity peptides [54]. The analytes are eluted in order of increasing molecular hydrophobicity, with the more polar solutes eluting first [60,59].

The RP-HPLC packing materials are generally based on micro particulate porous silica which is chemically modified by a derivitized silane bearing an n-alkyl hydrophobic ligand [59]. The type of n-alkyl ligand significantly influences the retention time of peptides [59]. The ligand is usually a linear alphatic hydrocarbon of eighteen (C_{18}), eight (C_8) or four (C_4) carbons, with other ligands such as phenyl offering different selectivity [54,59]. Although the effect of the ligand structure is not fully understood, a number of factors including the ligand chain length, relative hydrophobicity and the degree of exposure to surface silanols all play a role in the retention process [59]. As a general rule, retention times of analytes are longer the more carbon atoms the bonded ligand contains [60]. C_{18} columns are generally preferred for peptides and small proteins less than 5,000 Daltons, whereas proteins greater than 5,000 Daltons or very hydrophobic polypeptides are best suited for C_4 columns [54]. C_8, phenyl and C_4 columns offer different hydrophobicity properties and therefore these columns offer different selectivity for some peptides [54]. Short alkyl groups such as C_8 have been demonstrated to better separate polar samples, whereas long chains are better for non-polar substances [60]. Most of the surface of a porous packing is contained within the pores and the pore size of a column determines the ability of the analyte to access the pores [61]. The pore diameter of a column should exceed the hydrodynamic radius of the analyte by a factor of four or more [61]. For reversed phase separations of small molecules, column packing with small pores (60-120 Å) is typically used. For small molecules and peptides, 100-150 Å is typically used and for polypeptides and many proteins, 200-300 Å column pore size is used [61].

The mobile phase generally consists of mixtures of aqueous buffer solutions with various water-miscible organic solvents [60]. Aqueous solutions containing trifluoroacetic acid (TFA), formic acid and ammonium acetate and organic modifiers such as acetonitrile, isopropanol and methanol are typically used [60]. Water is the weakest mobile phase for RP-HPLC and the more water present, the slower the elution of the analyte [60]. RP-HPLC is typically the mode of choice for analysis of peptides at crude stage, analysis during purification and for the analysis of the final API and is by far the most common technique for the purification of peptides [28].

1.7.2.1 The use of RP-HPLC in peptides manufacture

RP-HPLC is used in the purifying and characterising of peptides. Post cleavage of the product peptide from the solid support, purification is performed on a preparative reversed phase HPLC [8]. Analytical RP-HPLC is then used to analyse the purification fractions [8]. HPLC methods for purity determination of peptides must enable the separation and determination of the most common impurities in peptides, such as enantiomers, deletion sequences and products of deamidation or acetylation [57].

A significant challenge in peptide manufacturing is the lack of harmonized guidelines across different continents on the level of allowed impurities present in peptide therapeutics [10]. For this reason, most peptide manufacturers apply a stringent approach on impurities, with limits for individual, unidentified impurities of less than 0.1 % [10]. Without vigorous characterisation and evaluation of potential impurities during peptide manufacturing, impurities can be overlooked, often to reappear at a later stage of the process, potentially leading to recalls or impacts on patient's health and safety [11]. On registration of new drug substances, the actual and potential organic impurities most likely to arise during the synthesis, purification, and storage need to be evaluated [62]. This evaluation of potential impurities should be based on sound scientific appraisal of the chemical reactions involved in the synthesis, impurities associated with raw materials that could contribute to the impurity profile and possible degradation products [62].

The ability to optimise and control a peptide synthesis process is highly dependent on the analytical HPLC methods employed [30]. It is critical the correct methods are chosen to evaluate the peptide and RP-HPLC plays a vital role in this process.

1.7.3 Evolution of ultra-high performance liquid chromatography

As HPLC is the dominant analytical technique in labs worldwide, researchers have put significant focus on increasing the speed of HPLC, driven by the sheer volume of samples in laboratories [63]. Faster separation can lead to higher throughput and time savings on analysis and also in method development [63]. The underlying principle behind the evolution of UHPLC is the Van Deemter equation, which describes the relationship between linear velocity (flow rate) and height equivalent to a theoretical plate (HETP).

$$H = Ad_p + \frac{BDm}{u} + \frac{Cd_p^2 u}{Dm}$$ [64]

H represents the height equivalent to a theoretical plate (HETP), d_p represents the particle size of the column packing material, u represents the linear velocity of the mobile phase, Dm outlines the analyte diffusion coefficient and A-C are constants [64]. A relates to the eddy diffusion, the multiple flow paths through a column, which is impacted by particle size distribution and uniformity of the packed bed [65]. B relates to the molecular diffusion and C describes the coefficient of mass transfer, which

reflects the time it takes for the analyte to diffuse in and out of the stationary phase [64,65]. C is directly impacted by the mobile phase velocity due to the fact that higher mobile phase velocities interfere with the equilibrium between the analyte, mobile phase and stationary phase. As a result, the longer the path an analyte has to travel within the pores of the stationary phase, the more detrimental the effect of the mobile phase flow rate will be on the column efficiency [65]. The path a solute has to travel within the pores of a stationary phase particle can be reduced by using smaller size particles because smaller particles have shorter diffusion path lengths and, therefore, are less affected by increases in mobile phase velocity [65]. The Van Deemter equation shows that efficiency varies with velocity and the optimum velocity occurs at linear velocities that are much lower than those typically used with particle size of 3.5-5 μm as demonstrated in Figure 1.15 [64].
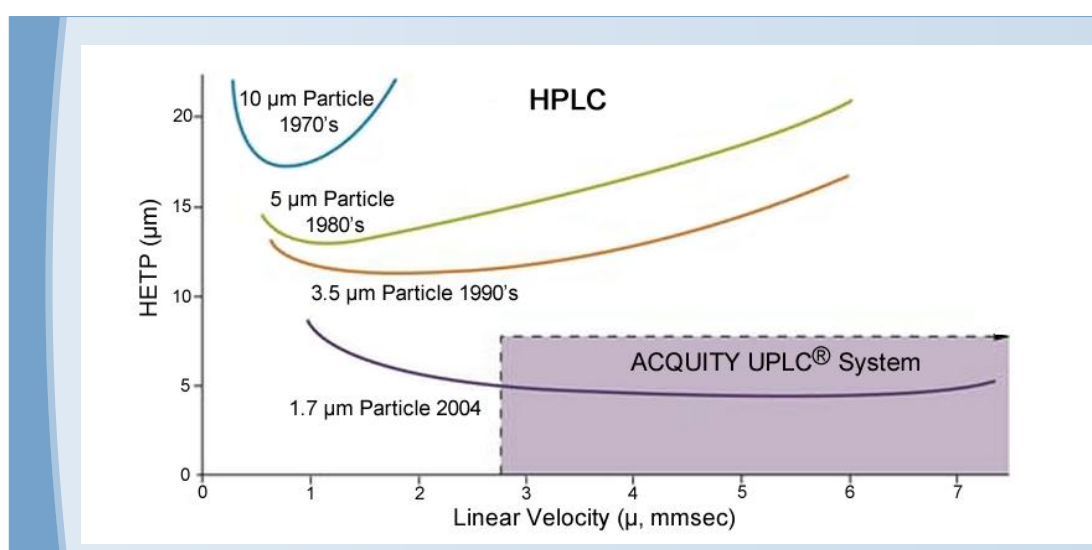
Figure 1.15 *Van Deemter plot demonstrating the effect of particle size on column efficiency (Waters Corporation)* [63]

A decrease in particle size to < 2.5 μm, from the conventional 5 μm particle size, will result in a significant gain in efficiency [63]. By using smaller particles, peak capacity, which is the number of peaks that can resolved per unit time of chromatography, and the speed of the separation can be pushed to new limits [66]. Increased sensitivity will also occur as a result of increased efficiency, due to the band spreading reduction across the separation process, resulting in sharper chromatographic peaks [66]. Resolution of a chromatographic separation is proportional to the square root of efficiency (N), which is inversely proportional to particle size. As a result, decreasing the particle size by a factor of approximately three (from 5 μm for HPLC to 1.7 μm for UHPLC), results in an increase in plate count and resolution by the square route of approximately three [67]. A drawback to smaller particles is higher system pressure, as pressure is inversely proportional to the square root of the particle size [67]. In order to overcome this problem and take advantage of the increased speed,

superior resolution and sensitivity afforded by smaller particles, improved instrumentation and column technology had to be developed [67].

1.7.3.1   *Development of sub-2 μm particles and UHPLC columns*

The design and development of sub-2 μm particles was a significant challenge in the evolution of UHPLC [63]. High efficiency, non-porous 1.5 μm particles were already available but they suffered from poor retention and poor loading capacity due to low surface area [63]. To maintain similar retention and loading capacity to HPLC, porous particles that can withstand high pressures must be used for UHPLC [66]. Silica based particles are used in some UHPLC columns, as they demonstrate good mechanical strength. However, these columns also have disadvantages in terms of limited pH range (pH 2.0 – 7.5) and tailing during analysis of basic analytes [63]. Hybrid particles, which incorporate carbon in the form of methyl groups, were then developed in 2000, which demonstrated good mechanical strength, high efficiency and they operated at a wide pH range [63]. Waters developed a bridged ethane hybrid (BEH) particle, which involved bridging methyl groups to the silica, allowing the particle to withstand the pressure required for UHPLC [63]. Fused-core technology was developed in 2007, which involved superficially porous particles composed of solid inner core as shown in Figure 1.16 [68]. These fused core particles offer a shorter diffusion path as the inner core of solid fused silica is impenetrable by analytes, minimising peak broadening [68].
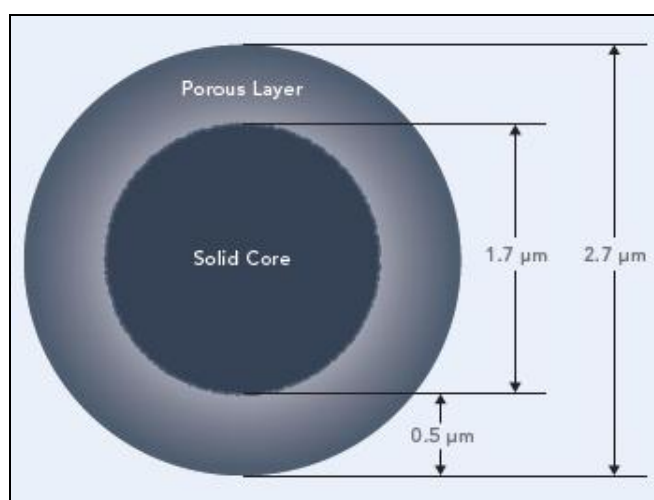
Figure 1.16 *Fused-core particle technology* [65]

In order to be able to pack the small particles into reproducible and rugged columns, the packed bed in the column must be uniform and the interior surface of the column hardware must be smoother to facilitate packing of the small particles into the columns [66,63]. Also, the end frits of the column must be able to retain the small particles [66]. Particles for sub-2 μm range require extremely fine frits with porosity of about 0.2 μm, in comparison to frit porosity of 0.5 μm -1 μm which are typically required for particles in the 3 μm range [69]. A drawback of UHPLC is the occurrence of viscous heating or frictional heating where friction occurs between different fluid layers inside the

column at high flow rates and pressures [70]. Smaller column diameters are a requirement with UHPLC to avoid the effect of frictional heating [63]. Further improvement on efficiency can be obtained by increasing column length as plate count is also proportional to column length [67]. Significant developments in sub-2 μm and column technology has been made over the last decade and currently there is a wide variety of stationary phases packed with sub-2 μm particles, in over 80 chemistries from multiple providers [68].

1.7.3.2 Development of UHPLC instruments

In order to take advantage of the sub-2 μm particles, the HPLC instrumentation needed to be improved in order to withstand the increased pressures associated with the reduced particle size [66]. A conventional HPLC typically demonstrates a pressure limit of 400 bars but this pressure limit does not suffice for the use of sub-2 μm particles [64]. For the use of smaller particles, a solvent delivery system that can deliver solvents at high pressures up to 70 bars and compensate for solvent compressibility across a range of potential pressures must be used [66]. This system must have an injection valve that can protect the column from experiencing extreme pressure fluctuations and, low volume injections, with minimal carryover, are required to accommodate the increased sensitivity benefits [66]. The detector must also have a high sampling rate to capture enough data points (up to 100 Hz) across the peak to perform accurate and reproducible recognition of the analyte peak and the detector flow cell must have minimal dispersion to preserve the efficiency of the separation, such as that observed on UHPLC (Waters, Dublin, Ireland) with a detector flow cell volume of 500 nL [66,71]. The total system volume must also be reduced, in comparison to HPLC, to ensure the UHPLC separation is maintained throughout the chromatographic process [66].

In 2004, a UHPLC system that met all of these requirements was made commercially available from Waters Corporation, known as Acquity UPLC system [63]. This instrument consists of a binary solvent manager that uses two individual serial flow pumps to deliver a parallel binary gradient mixed under pressure [67]. The systems also include UV-Vis and PDA detectors with new electronics and firmware to support high data rates required for UHPLC [67]. The detector cell in the Acquity systems consist of a light guided flow cell equivalent to an optical fibre and the system has a 1,000 bar pressure limit [67]. Multiple other UHPLC systems have since been developed and UHPLC technology is now used worldwide, alongside and as a superior replacement of the conventional HPLC.

1.7.4 Analytical evaluation of coupling reagents during peptide synthesis

During the manufacture of peptides, the synthetic process involves a series of couplings, washes, de-protections followed by additional washes. It is estimated that most triazole-based coupling reagents, such as HATU, HBTU, HOBt, PyBOP, PyAOP, and TBTU bind to the solid

support during peptide synthesis and are removed during piperidine washes [28]. In the event of a poor coupling, the triazole-based reagents bind to the free amino acids and these bound triazole-based reagents are then assumed to be removed by a concentrated base wash, after coupling [28]. So Yeop Han and Young-Ah Kim stated that a triazine moiety, such as that observed in HCTU, 6-Chloro-HOBt, TCTU and PyClocK, demonstrates a weak basicity and the by-products and excess coupling reagents are assumed to be washed out with dilute acid [25]. Fluorous Technologies Incorporated (Pittsburgh, USA) state that HOBt and DIEA salts are effectively removed by aqueous $NaHCO_3$ wash, whereas Ludmila G. Peeva, *et al.* used a washing step for the removal of excess reagents via constant volume diafiltration [72,73]. Alan R. Katritzky *et al.* stated that the benzotriazole generated was readily removed by washing the organic extracts with dilute aqueous sodium carbonate during workup [74]. As outlined above, typically the peptide coupling reagents are assumed to be washed out during peptide synthesis. If the coupling reagents are still present post synthesis, the purification process of peptides (involving column chromatography) is assumed to remove the excess coupling reagents. It is for this reason that very little literature can be found on the analysis of peptides for the presence of coupling reagents used in the process.

During a development peptide synthesis campaign in Ipsen Manufacturing Ireland LTD, this assumption was proved to be incorrect. 6-ChloroHOBt was shown to be present all the way through the synthesis process, throughout the purification fractions and was present in the final, lyophilised peptide. The presence of 6-ChloroHOBt post-synthesis was a surprising result as the 6-ChloroHOBt has a high solubility in DMF (>366 mg/mL) and 4 washes of 10 volumes of DMF were performed during the synthesis, followed by several purification runs. On investigation of the synthetic approach, increasing the number of DMF washes did not reduce the carryover of 6ChloroHOBt. The purification strategy was also examined and it was discovered that 6ChloroHOBt was eluting alongside the peptide, even though they were separated on a HPLC analytical method when standards were prepared separately. An isocratic hold at the beginning of the purification run allowed the sufficient separation of the peptide and 6ChloroHOBT to allow removal of the 6-ChloroHOBt to below the specification in the final API. This problem was suspected to be due to a pH effect in which the 6ChloroHOBt was bound to the peptide. As a result, we can no longer assume the coupling reagents have been washed out during the synthesis process, regardless of their solubility.

1.7.5   *Toxicity of coupling reagents*

A genotoxic compound is defined as a compound that has the potential to damage DNA at any level of exposure and such damage may lead to the development of a tumour [75]. Based on the safety data sheets (SDS), HOAt, PyBOP, DIU, TBTU and COMU are not identified as human carcinogens by the International Agency for Research on Cancer (IARC) but there is no data available for their acute or reproductive toxicity [76 - 80]. This is also the case for HCTU, HBTU, PyBrOP,

HOBt, Oxyma pure, and 6-ChloroHOBt [81 - 86]. TMU is also not identified as a human carcinogen by IARC, however the oral lethal dose$_{50}$ (LD$_{50}$) in rats was determined as 794 mg/kg and the dermal LD$_{50}$ was determined as 3,160 mg/kg in rabbits [87]. LD$_{50}$ is defined as the dose that kills half of the tested population in an animal model, whereas LC$_{50}$ describes the concentration of a chemical in air that kills half of the tested population in a given time (usually 4 hours) [88]. LD$_{50}$ is measured in milligram (mg) per kilogram (kg) of body weight and LC$_{50}$ is measured in mg per litre [88]. The lower the defined amount per kg or per litre, the more toxic the compound, as outlined in Table 1.1. TMU is characterised as a toxicity rating of 4, slightly toxic, which means approximately 600 ml of the compound would be the lethal dose for man.

Table 1.1 *Toxicology classes - Hodge and Sternar scale* **[89]**

		Routes of Administration			
		Oral LD$_{50}$	**Inhalation LC$_{50}$**	**Dermal LD$_{50}$**	
Toxicity Rating	Toxicity term	(single dose to rats) mg/kg	(exposure of rats for 4 hours) ppm	(single application to skin of rabbits) mg/kg	Probable Lethal Dose for Man
1	Extremely Toxic	1 or less	10 or less	5 or less	1 grain (a taste, a drop)
2	Highly Toxic	1 -50	10-100	5 – 43	4 ml (1 tsp.)
3	Moderately Toxic	50-500	100-1000	44-340	30 ml (1 fl. oz.)
4	Slightly Toxic	500-5000	1000-10,000	350-2810	600 ml (1 pint)
5	Practically Non-toxic	5000-15,000	10,000-100,000	2820-22,590	1 litre (or 1 quart)
6	Relatively Harmless	15,000 or more	100,000	22,600 or more	1 litre (or 1 quart)

IARC have not identified TCTU as a human carcinogen but there is also no data for reproductive toxicity and the oral lethal dose$_{50}$ (LD$_{50}$) in rats was determined > 200 mg/kg, characterising the material as moderately toxic [90]. DIC is also not identified as a human carcinogen by IARC and an invitro genotoxicity study confirmed this [91]. However, the inhalation lethal concentration$_{50}$ (LC$_{50}$) was determined 4h – 0.105 mg/L (ppm) in rats, characterising the material as extremely toxic. Unfortunately, there is no toxicity or carcinogenicity available for PyClocK [92].

Typically therapeutic peptides are administrated at doses between 50 μg and 50 mg [10]. If a coupling reagent was present in the peptide product, the quantity would only be present as a very small fraction of the 50 μg to 50 mg dose administrated to patients and therefore, unless the LD_{50} and LC_{50} results outline the reagent as a 1 or 2 toxicity rating, like DIC, then the reagents shouldn't present an issue in terms of toxicity to patients. However, in the absence of carcinogenicity or toxicity data, a threshold of toxicological concern (TTC) has been developed to define a common exposure level for any unstudied chemical that will not pose a risk of significant carcinogenicity or other toxic effects [75]. This TTC value is estimated as of 1.5 μg/person/day for a lifetime intake and 120 μg/person/day for intake over ≤ 1 month, which is considered to be associated with an acceptable risk (excess cancer risk of < 1 in 100,000 over a lifetime) for most pharmaceuticals [93]. This is extremely conservative given that the current lifetime cancer risk in the population is over 1 in 4 [93]. From the threshold value of 1.5 μg/person/day, a permitted level of the presence of each coupling reagent can be calculated based on the expected daily dose [75]. For example, if a peptide is administered in a dose of 20 mg/week, the %w/w of genotoxic impurity allowed for a daily dosage of 2.86 mg is calculated as follows:

$$\% \text{ w/w of genotoxic imp allowed relative to a 20 mg dose per week of Peptide} =$$

$$\frac{Max.\,allowed\,genotoxic\,impurity\,/\,day\,(mg)}{Dosage\,of\,Peptide\,ad\min istered\,/\,day\,(mg)}\,x\,\frac{100}{1}$$

$$\%w/w\text{ of genotoxic impurity allowed} = \frac{0.0015\,(mg)}{2.86\,(mg)}\,x\,\frac{100}{1} = 0.05\%\,w/w$$

Figure 1.17 *Example calculation for determining % w/w of genotoxic impurity allowed for a 20 mg dose of peptide per week*

1.7.6 *Ipsen Manufacturing Ireland LTD. approach to the analysis of peptide coupling reagents*

Ipsen Manufacturing Ireland Limited (LTD.), manufacture three active pharmaceutical ingredients (API), including Lanreotide acetate, Triptorelin acetate and Triptorelin Pamoate, for commercial supply. These peptides have been produced for several years, with hundreds of batches manufactured each year. All impurities > 0.1 % have been identified for the commercial peptides and peptide coupling reagents do not appear to be present in the final API. The analysis of peptide coupling reagents is performed by HPLC on primary reference standards but is not performed on a routine basis for every API batch. Ipsen Manufacturing Ireland LTD. also manufactures API for drug

substances and drug products in development for clinical trials and toxicological studies. Typically, minimal information is known about the manufacture process of development compounds in Phase 1 and 2 clinical trials. As a result, all raw materials used during the manufacturing process are monitored throughout the manufacturing campaign, including peptide coupling reagents. HPLC methods are used for the analysis of peptide coupling reagents within Ipsen Manufacturing Ireland LTD.

1.7.7   *Use of HPLC for analysis of coupling reagents:*

For the analysis of peptide coupling reagents in the APIs, all the peptide coupling reagents must be separated sufficiently from the active substance and from each other so that their concentrations can be reliably measured. Because of the range of polarities between the reagents and the APIs, multiple HPLC methods of run time greater than 60 minutes are required. This results in multiple HPLC systems set up for the in-process analysis throughout an API manufacturing campaign, involving significant amount of resources and time. In order to reduce separation times in the HPLC method without reducing the quality of the separation requires generating higher resolving power per unit time [64]. Whilst the resolution between individual analytes in a particular sample may be increased by improving selectivity or retention, the best general approach to increasing resolving power is to increase separating efficiency, such as that done in ultra-high performance liquid chromatography (UHPLC) [64].

1.8   Aim of the thesis

The aim of this thesis was to develop a rapid liquid chromatography method for the in-process determination of fourteen peptide coupling reagents used in peptide synthesis. This method was required because studies at Ipsen Manufacturing Ireland LTD. have revealed that some peptide coupling reagents may in fact not be fully removed from the peptide product during peptide manufacture. The novelty of this body of work lies in the fact that the simultaneous chromatographic separation of these reagents has heretofore not been demonstrated in the literature. It is likely that this is due to the erroneous assumptions made in the peptide synthesis industry regarding the clearance of these reagents during peptide manufacturing, as described in Section 1.7.4. The analytes chosen were either used in the manufacture of some commercial peptides at Ipsen Manufacture Ireland LTD., or were under investigation for the manufacture of peptides in development at the time of this study. This thesis involves the selection of a commercially available stationary phase and a mobile phase that is compatible with mass spectrometry to evaluate peptide coupling reagents in peptides provided by Ipsen Manufacturing Ireland LTD. This thesis is divided into two separate chapters. Chapter 2 deals with the development of a reversed phase HPLC assay for the selected reagents and it involves stationary phase selection and optimisation of mobile phase composition. Chapter 3 then goes on to describe the validation of the analytical method using standard method performance criteria such as

sensitivity, linearity, robustness, etc. Although UV detection was used for the method described in this thesis, nevertheless, a mobile phase system which was compatible with mass spectrometric detection was developed in order to maximise the potential utility of the method.

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Chapter 2

Development of a UHPLC method for the analysis of peptide coupling reagents, additives

and associated by-products during peptide synthesis

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2.1 Introduction

The determination of peptide coupling reagents, additives and associated by-products is important during peptide synthesis to ensure the concentration of these products are below the threshold of toxicological concern in the final peptide. Several publications have dealt with the development of these compounds as coupling reagents but none specifically focus on the detection of these compounds in the final peptide. This is due to the assumption that they are washed out during peptide synthesis as discussed in Section 1.7.4, however research at Ipsen Manufacturing Ireland LTD. has proven this assumption to be incorrect.

TMU, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, PyClocK, Oxyma Pure COMU, DIU, DIC, PyBOP, PyBrOP, and TCTU are peptide coupling reagents, additives and associated by-products commonly associated with peptide synthesis. In order to monitor these products during a peptide synthesis campaign, it was necessary to develop a quantitative detection method. This method will subsequently be used in Ipsen Manufacturing Ireland LTD., for evaluating all stages of the manufacture of development peptides to ensure the removal of the compounds.

Historically in Ipsen Manufacturing Ireland LTD., HPLC was previously the method of choice for the analysis of peptide coupling reagents in API samples, but due to the wide range of polarities between the reagents and the APIs, multiple HPLC methods of run time greater than 60 minutes were in place. This resulted in multiple HPLC systems set up for in-process analysis throughout a peptide manufacturing campaign, involving significant amount of resources and time. In order to reduce separation times in a HPLC method without reducing the quality of the separation, a higher resolving power is required per unit time and the best general approach to increasing resolving power is to increase separation efficiency, by decreasing particle size and using ultra-high performance liquid chromatography (UHPLC), as discussed in Chapter 1, Section 1.7.3. UHPLC is a relatively new mode of separation science which builds upon well-established principles of liquid chromatography, using sub-2μm porous particles. These particles operate at elevated mobile phase linear velocities to produce rapid separations with increased sensitivity and resolution.

The work presented in this chapter involves the development of a rapid liquid chromatography method for the in-process determination of fourteen peptide coupling reagents used in peptide synthesis. The analytes chosen are either used in the manufacture of some commercial peptides or are under investigation for the manufacture of peptides in development in Ipsen Manufacturing Ireland LTD. A number of column technologies and mobile phases were evaluated for the separation of the analytes. Therefore the aim of this chapter is to select a commercially available stationary phase with a mobile phase that is compatible with mass spectrometry to evaluate peptide coupling reagents in peptides provided by Ipsen Manufacturing Ireland LTD., in a run time of less than 30 minutes.

2.2   Experimental

The study was first initiated with a well-defined set of peptide synthesis reagents, additives and associated by-products which were identified as being of specific interest to Ipsen Manufacturing Ireland LTD. The initial set of compounds included Peptide 1, TMU, HOAt, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, PyClocK, Oxyma Pure and COMU. Therefore, for the most part, the following experimental section is written in chronological order and the individual experiments were conducted using the range of materials previously listed (and referred to hereafter as 'Sample set A').

Midway through the method development, a further five reagents were added to the set of materials under investigation in order to widen the scope of the method. These additional five reagents were identified as further potential materials that could be used by Ipsen Manufacturing Ireland LTD., in the future and so their inclusion in the study (albeit midway through method development) represents an effort to 'future-proof' the method. As a result, the final method would be applicable for the use of a much broader range of peptide coupling reagents, additives and associated by-products as dictated by emerging future trends in peptide synthesis in Ipsen Manufacturing Ireland LTD.

In some cases, the inclusion of the new reagents to the sample set under study necessitated that previous experiments be revisited and further optimised to reflect the new sample set (referred to hereafter as 'Sample set B') and comprising of the reagents from 'Sample set A' (as listed above) and also DIU, DIC, PyBop, PyBrOP, and TCTU. For the sake of clarity, Table 2.1 sets out the chronological order of method development experiments, clearly indicating which sample set was under investigation i.e. 'Sample set A' or the more comprehensive 'Sample set B'. This table is therefore intended as a reference guide to assist the reader.

Table 2.1 *Chronology of method development indicating materials under investigation*

Section	Experiment	Sample set
2.2.3	Solubility studies	B
2.2.4	Determination of optimum detection wavelength	B
2.2.5	Evaluation of columns and mobile phase systems	A
2.2.6	Design of experiments	A
2.2.7	Initial evaluation of optimum buffer pH and column temperature	A
2.2.8	Re-optimisation of optimum pH using 10 m*M* ammonium formate	A
2.2.9	Evaluation of flow rate for optimum separation	A
2.2.10	Re-optimisation of column temperature	B
2.2.11	Evaluation of final percentage acetonitrile required for the gradient	Peptide 1
2.2.12	Re-optimisation of optimum buffer concentration	B
2.2.13	Evaluation of chromatographic gradient profiles	B

Note:

Sample set A - Peptide 1, TMU, HOAt, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, PyClocK, Oxyma Pure and COMU.

Sample set B - Peptide 1, TMU, HOAt, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, PyClocK, Oxyma Pure, COMU, DIU, DIC, PyBop, PyBrOP, and TCTU.

2.2.1 Reagents and standards

HPLC grade acetonitrile (J.T Baker) and LC-MS grade acetonitrile (Ocon Chemicals) were purchased from Sigma Aldrich (Dublin, Ireland). Methanol was obtained from Labscan (Dublin, Ireland). Purified water was obtained from a Millipore Milli-Q water (H_2O) purification system (EMD Millipore Corporation, MA, USA). HPLC grade ammonium formate, acetic acid, ammonium acetate, formic acid, phosphoric acid, sodium phosphate, dimethylformamide (DMF), sodium hydroxide, trifluoroacetic acid (TFA) and ammonium hydroxide were all purchased from VWR (Dublin, Ireland). All active pharmaceutical peptides and diisopropylurea were obtained from Ipsen Manufacturing Ireland LTD. Tetramethylurea (Fluka), diisopropylcarbodiimide (99 %, Aldrich), TBTU (Aldrich), HOBt (Aldrich) and HBTU (Aldrich) were purchased from Sigma Aldrich (Dublin, Ireland). PyBOP, HCTU, PyClocK and PyBrOP were all Novabiochem products and were obtained from Merck (Darmstadt, Germany). COMU, Oxyma pure and TCTU were all purchased from Luxembourg Biotechnologies Ltd. (Rehovet, Israel). 6-ChloroHOBt was obtained from Apollo Scientific Ltd. (Cheshire, United Kingdom) and HOAt was obtained from Acros Organics (Geel, Belgium).

2.2.2 Instrumentation

Chromatographic separations were carried out on numerous LC instruments to maximise lab productivity. Chromatographic separations were performed on a Waters H-Class (quaternary pump) UPLC and a Waters Acquity (Binary pump) UPLC, equipped with a TUV detector and FTN sample manager (Waters, Dublin, Ireland). A Waters 2695 separations module HPLC, equipped with a PDA

detector, was also used (Waters, Dublin, Ireland). The data was acquired via Waters Empower 2 software. Weighing was performed on a Mettler Toledo XP205 analytical balance and a Mettler Toledo XP6 microbalance (Mason Technology, Dublin, Ireland). The pH meter used was a Mettler Toledo SevenMulti pH meter (VWR, Dublin, Ireland).

2.2.3   *Solubility studies*

Solubility studies were performed to determine the optimum solvent to dissolve all peptide coupling reagents, additives and associated by-products. The solubility studies were carried out in water (H_2O), 0.1 M acetic acid (AcOH), methanol (MeOH) and acetonitrile (ACN). Solubility determination was performed by weighing 0.1 g of material into a 10 mL volumetric flask and adding increasing volume increments (100 µL, 1000 µL, 3 mL and 10 mL) of solvent. If samples did not dissolve after the addition of 10 mL of solvent, then the diluent volume was increased to 100 mL with 6 hours of sonication, or if necessary, 1000 mL of diluent with 24 hours of sonication. In all cases, sonication and visual inspection of sample solution was performed at ambient temperature.

2.2.4   *Determination of optimum detection wavelength*

Each reagent was evaluated between 210 nm and 400 nm on a photodiode array detector to determine the optimum detection wavelength for liquid chromatography (LC) analysis. This was performed by dissolving each reagent in 'Sample set A' in the optimum solvent, determined as per Section 2.2.4, and performing analysis on a Waters Acquity system with a PDA detector using a BEH C18 100 mm x 2.1 mm column. A multi-step gradient was employed as detailed in Table A1 in Appendix 1.

Each additional coupling reagent in 'Sample set B' that is not in 'Sample set A' was evaluated between 210 nm and 400 nm on a photodiode array to determine the optimum detection wavelength for LC analysis. This was performed by dissolving each reagent in optimum solvent and analysing on a Waters 2695 HPLC system with a PDA detector, using an YMC-ODS-AM 250 mm x 4.6 mm x 2.1 mm column as per the gradient detailed in Table A2 in Appendix 1.

2.2.5   *Evaluation of columns and mobile phase* systems

Using the optimum sample diluent of 80 % H_2O, 10 % MeOH and 10 % ACN, and the optimum detection wavelength of 220 nm, a mix containing 'Sample set A' was analysed using multiple different mobile phase systems outlined in Table 2.2 and a series of columns outlined in Table 2.4. For columns 2, 9, 11 and 13, each reagent from 'Sample set A' was individually analysed on each mobile phase system in addition to the mixture of reagents analysed on all other columns.

Table 2.2   *Mobile phase systems under evaluation*

Mobile phase system number	Mobile phase A	Mobile phase B
A	0.1 % TFA	0.08 % TFA in ACN
B	0.1 % TFA	0.08 % TFA in MeOH
C	0.1 % Formic acid	0.08 % Formic acid in ACN
D	0.1 % Formic acid	0.08 % Formic acid in MeOH
E	10 mM Ammonium acetate pH 4.0	Acetonitrile
F	10 mM Ammonium acetate pH 4.0	Methanol
G	10 mM Ammonium formate pH 3.0	Acetonitrile
H	10 mM Ammonium formate pH 3.0	Methanol
I	10 mM Sodium phosphate pH 2.0	Acetonitrile
J	10 mM Sodium phosphate pH 2.0	Methanol
K	10 mM Sodium phosphate pH 7.0	Acetonitrile
L	10 mM Sodium phosphate pH 7.0	Methanol

Table 2.3 outlines the multi-step gradient applied to each column. A detection wavelength of 220 nm and an injection volume of 5 μL were employed for all experiments.

Table 2.3 *Comparison table for columns under investigation and the corresponding gradient profiles*

Columns	Gradient profile used (Appendix 1)
1,2,3,4	A3
5,6	A4
7,8,9	A5
10,11	A6
12	A7
13,14	A8
15	A9
16	A10
17	A11

Note: All subsequent sections of the Experimental were analysed on Column 15 (YMC Triart 100 mm x 2.0 mm, 1.9 μm) using the optimum diluent of 80 % H_2O, 10 % MeOH and 10 % ACN, the optimum detection wavelength of 220 nm and an injection volume of 5 μL, unless otherwise specified. All chromatograms were evaluated based on standard chromatographic criteria.

Table 2.4 *Columns under investigation (including detailed physical specifications)*

Column number	Column	Manufacturer	Phase	Particle size (μm)	Dimensions	Pore size	Ligand type	pH range	Temp limits	Surface area	Carbon load	Capping
1	Acquity HSS C18 [94]	Waters	HSS	1.8 μm	100 x 2.1 mm	100Å	Trifunctional C18	1-8	Max 45 °C	230 m²/g	15 %	End capped
2	Acquity HSS T3 [94]	Waters	HSS	1.8 μm	100 x 2.1 mm	100 Å Å	Trifunctional C18	2-8	Max 45 °C	230 m²/g	11 %	End capped
3	Agilent Zebra SB C18 [95]	Agilent	Zorbax SB	1.8 μm	100 x 2.1 mm	80 Å Å	C18	1-8	Max 90 °C	180 m²/g	10 %	Non-end capped
4	Acquity HSS C18 SB [94]	Waters	HSS	1.8 μm	100 x 2.1 mm	100 Å Å	Trifunctional C18	2-8	Max 45 °C	185 m²/g	8 %	Non-end capped
5	Acquity BEH300 C4 [95]	Waters	BEH	1.7 μm	100 x 2.1 mm	300 Å Å	Monofunctional C4	1-10	Low pH=80 °C High pH=60 °C	185 m²/g	8 %	Non-end capped
6	Acquity BEH Phenyl [94]	Waters	BEH	1.7 μm	100 x 2.1 mm	130 Å Å	Trifunctional C6 Phenyl	2-11	Low pH=80 °C High pH=60 °C	185 m²/g	15 %	End capped
7	Acquity BEH C18 Shield [94]	Waters	BEH	1.7 μm	50 x 2.1 mm	130 Å Å	Monofunctional embedded polar group C18	1-12	Low pH=50 °C High pH=45 °C	230 m²/g	17 %	End capped
8	Acquity BEH C18 [94]	Waters	BEH	1.7 μm	50 x 2.1 mm	130 Å Å	Trifunctional C18	1-12	Low pH=80 °C High pH=60 °C	185 m²/g	18 %	End capped
9	Phenomenex Kinetex [96]	Phenomenex	C18	1.7 μm	50 x 2.1mm	100 Å Å	C18	1.5-10	60 °C	200 m²/g	12 %	End capped
10	Agilent Eclipse Plus C18 [95]	Agilent	Eclipse plus	1.8 μm	50 x 4.6 mm	95 Å	C18	2-9	Max 60 °C	160 m²/g	9 %	Double
11	Agilent Eclipse Plus C8 [95]	Agilent	Eclipse plus	1.8 μm	50 x 4.6 mm	95 Å	C8	2-9	Max 60 °C	160 m²/g	7 %	Double
12	Halo C18 [65]	Advanced Materials technology	C18	2.7 μm (1.7μm solid core)	50 x 2.1mm	90 Å Å	C18	2-9	Max 90 °C	150 m²/g	10 %	End capped
13	YMC Ultra HT Hydrosphere C18 [97]	YMC	YMC Hydrosphere C18	2 μm	50 x 3mm	120 Å Å	C18	2-8	Max 50 °C	330 m²/g	12 %	End capped
14	YMC Ultra HT Pro C18 [97]	YMC	YMC Pro C18	2 μm	50 x 3mm	120 Å Å	C18	2-8	Max 60 °C	330 m²/g	17 %	End capped
15	YMC Triart C18 [98]	YMC	YMC Triart C18	1.9 μm	100 x 2.0mm	120 Å Å	C18	2-8	Low pH=70 °C High pH=50 °C	330 m²/g	20 %	Multi staged hybrid groups
16	Acclaim RSLC 120 C18 [99]	Dionex	C18	2.2 μm	100 x 2.1mm	120 Å Å	C18	2-8	Max 60 °C	300 m²/g	18 %	End capped
17	Hypersil gold [100]	Thermo	C18	1.9 μm	100 x 2.1mm	175 Å Å	C18	1-11	Max 60 °C	220 m²/g	10 %	End capped

2.2.6   Design of experiments

After the optimum stationary phase and mobile phase were selected, further optimisation was performed on the composition of the mobile phase, pH of the mobile phase, system flow rate, column temperature and gradient change. Modde 9 software was used to compile an experimental design space where all of these parameters were varied at the same time. Evaluation of chromatograms was performed for each of the 19 experiments outlined in Table 2.6 and in addition, each reagent was also individually injected for experiment N11 and N15. Table 2.5 outlines the multi-step gradient applied to each experiment.

Table 2.5 *Design of experiments and corresponding gradient profiles*

Experiments	Gradient profile used (Appendix 1)
N1, N4, N6, N7, N10, N11, N13 and N16	A12
N2, N3, N5, N8, N9, N12, N14 and N15	A13
N17, N18 and N19	A14

Table 2.6 *Parameters evaluated during design of experiments for mobile phase optimisation*

Exp no.	pH	Concentration of buffer (mM) *	Flow rate (mL/min)	Temp (°C)	Mobile phase B change/min (%)
N1	3.8	5	0.3	20	1.2
N2	9.2	5	0.3	20	0.2
N3	3.8	50	0.3	20	0.2
N4	9.2	50	0.3	20	1.2
N5	3.8	5	0.5	20	0.2
N6	9.2	5	0.5	20	1.2
N7	3.8	50	0.5	20	1.2
N8	9.2	50	0.5	20	0.2
N9	3.8	5	0.3	50	0.2
N10	9.2	5	0.3	50	1.2
N11	3.8	50	0.3	50	1.2
N12	9.2	50	0.3	50	0.2
N13	3.8	5	0.5	50	1.2
N14	9.2	5	0.5	50	0.2
N15	3.8	50	0.5	50	0.2
N16	9.2	50	0.5	50	1.2
N17	3.8	27.5	0.4	35	0.7
N18	3.8	27.5	0.4	35	0.7
N19	3.8	27.5	0.4	35	0.7

*Buffer = Ammonium formate

2.2.7 Initial evaluation of optimum buffer pH and column temperature

Further optimisation was performed with 10 m*M* ammonium formate pH 2.8, 3.8 and 4.8, each at column temperatures of 25 °C, 35 °C and 45 °C using the gradient detailed in Table A15 in Appendix 1. Another experiment was then performed using 10 m*M* ammonium formate pH 4.8 with a range of column temperatures from 10 °C to 50 °C using the same multi-step gradient detailed in Table A15 in Appendix 1.

2.2.8 Re-optimisation of buffer pH using 10 mM ammonium formate

Following the addition of more peptide coupling reagents and associated by-products to the study as explained on page 2, the optimum pH range was re-evaluated for the ammonium formate buffer at a range of 2.8 to 4.8 (2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.8, 4.3 and 4.8) . This was carried out by dissolving each reagent in 'Sample set B' in the optimum diluent at a concentration of 0.5 mg/mL and analysing using the multi-step gradient detailed in Table A15 in Appendix 1.

2.2.9 Evaluation of flow rate for optimum separation

After the optimum ammonium formate concentration and pH were selected, further optimisation was performed with a range of flow rates from 0.25 mL/min, 0.30 mL/min and 0.35 mL/min using the gradient detailed in Table A16 in Appendix 1. An injection volume of 10 μL and a column temperature of 25 ℃ were also employed.

2.2.10 Re-optimisation of column temperature

Following the addition of new peptide reagents and the change in optimum pH of ammonium formate, further optimisation was performed with a range of column temperatures from 10 °C to 45 °C, at 5 °C intervals, using the gradient detailed in Table A16 in Appendix 1.

2.2.11 Evaluation of final percentage acetonitrile required for the gradient

An investigation was performed to determine the effect of percentage acetonitrile on the last eluting peak, PyBrOP. This was carried out by dissolving PyBrOP at a concentration of 0.5 mg/mL and analysing on three different gradients with varying final percentages of the acetonitrile. Three multi-step gradients were employed using 10 m*M* ammonium formate pH 3.3 as mobile phase A as detailed in Table A17 (experiment 1), Table A18 (experiment 2) and Table A19 (experiment 3) in Appendix 1.

2.2.12   *Re-evaluation of optimum buffer concentration*

Following the addition of new peptide reagents and the change in optimum pH of ammonium formate, further optimisation was performed on the concentration of ammonium formate in mobile phase A. This was performed with a range of concentrations of ammonium formate from 5 mM to 40 mM (in 5 mM intervals). The multi-step gradient detailed in Table A20 (Appendix 1) was employed for each concentration of ammonium formate at pH 3.3.

2.2.13   *Final optimisation of chromatographic gradient profiles*

An evaluation of the gradient profile and flow rate for the method was carried out by analysing 'Sample set B' on each gradient detailed in Appendix 2.

2.3   Results and discussion

2.3.1   *Assessment of samples for analysis*

HPLC method development typically follows a systematic strategy that includes a series of steps outlined in Figure 2.1.

```
┌─────────────────────────────────────────────┐
│              1. Information on sample,        │
│                 define separation goals       │
│                        ↓                       │
│              2. Need for special HPLC          │
│                 procedure, sample,             │
│                 pretreatment, etc.?            │
│                        ↓                       │
│              3. Choose detector and            │
│                 settings                       │
│                        ↓                       │
│              4. Choose LC method:              │
│                 Preliminary run, estimate      │
│                 best separation conditions     │
│                        ↓                       │
│              5. Optimize separation            │
│                 conditions                     │
│                        ↓                       │
│              6. Check for problems or          │
│                 requirement for special        │
│                 procedure                      │
│          ↙             ↓             ↘        │
│  7a. Recover purified   7b. Quantitative   7c. Qualitative │
│  material               calibration        method         │
│                        ↓                       │
│              8. Validate method for            │
│                 release to routine             │
│                 laboratory                     │
└─────────────────────────────────────────────┘
```

Figure 2.1   *HPLC method development schematic*

Sample information was required in order to define the method development goal and strategy [94]. This information included the chemical structure, molecular weight, UV spectra, solubility of each of the compounds and also the number of compounds present in the sample [8]. It should be noted that this data was generated for the reagents defined earlier as 'Sample set A'. Similar data for 'Sample set B', which includes the additional reagents added to the study, is presented later in this chapter to more accurately reflect the chronology of the method development.

2.3.1.1   Assessment of analyte chemical structure
The chemical structure of each peptide coupling reagent, additive and associated degradant is outlined in Table 2.7.

Table 2.7 *Chemical structure of all peptide coupling reagents, additives and by-products*

Compound	Structure	Detail
HOBt [31,84]		Molecular weight: 135.12g mol^{-1}
HOAt [101,76]		Molecular weight: 136.11g mol^{-1}
6-ChloroHOBt [31,86]		Molecular weight: 169.57g mol^{-1}
HCTU [48,81]		Molecular weight: 413.69g mol^{-1}
Oxyma pure [47,85]		Molecular weight: 142.11g mol^{-1}
TBTU [31,79]		Molecular weight: 321.08g mol^{-1}
PyClocK [46,92]		Molecular weight: 554.84g mol^{-1}
HBTU [31,82]		Molecular weight: 379.3g mol-1
COMU [16,80]		Molecular weight: 428.27g mol^{-1}
TMU [87,102]		Molecular weight: 116.16g mol-1

2.3.1.2   Solubility determination

The solubility study was carried out in H$_2$O, 0.1 M acetic acid (AcOH), methanol (MeOH) and acetonitrile (ACN) as outlined in Section 2.2.3. High organic diluents injected onto a highly aqueous mobile phase can result in the splitting of peaks on the chromatogram and as a result, the ideal diluent would be composed of a small amount of organic solvent. The US Pharmacopeia outlines that the approximate solubility of a substance, evaluated at 20 °C, is described by one of the terms described in Table 2.8.

Table 2.8   *USP solubility definitions* [103]

Definition	Volume of solvent required per 1g of solute
Very soluble	Less than 1 mL of solvent needed to dissolve 1 g solute
Freely soluble	1 mL-10 mL of solvent needed to dissolve 1 g solute
Soluble	10 mL-30 mL of solvent needed to dissolve 1 g solute
Sparingly soluble	30 mL-100 mL of solvent needed to dissolve 1 g solute
Slightly soluble	100 mL-1000 mL of solvent needed to dissolve 1 g solute
Very slightly soluble	1000 mL-10,000 mL of solvent needed to dissolve 1 g solute
Practically insoluble	More than 10,000 mL of solvent needed to dissolve 1 g solute

The reagents in 'Sample set A' were all classified according to the USP solubility definition and the optimum diluent for each reagent is underlined Table 2.9.

The optimum diluent was determined as the solvent composition that dissolved the highest concentration of each of the reagents. The optimum diluent was different for some of the reagents and several reagents demonstrate poor solubility in aqueous solutions. As a result, the order of addition of solvent is important and there was a requirement to initially add the optimum diluent to fully dissolve the reagents, followed by a dilution with an aqueous solution. The optimum solvent was determined as 80/10/10 H$_2$O/ACN/MeOH and the order of addition required was determined as per Table 2.10.

Table 2.9 *Solubility of coupling reagents, additives and associated by-products.*

Compound	Solvent	USP solubility description	Approx solubility concentration
HCTU	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Slightly soluble	1 mg/mL
	ACN	Freely soluble	<u>100 mg/mL</u>
HBTU	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Slightly soluble	1 mg/mL
	ACN	Freely soluble	<u>100 mg/mL</u>
HOBt	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Freely soluble	<u>100 mg/mL</u>
	ACN	Sparingly soluble	10 mg/mL
HOAt	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Soluble	<u>33.33 mg/mL</u>
	ACN	Slightly soluble	1 mg/mL
6-ChloroHOBt	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Very slightly soluble	0.1 mg/mL
	MeOH	Freely soluble	<u>33.33 mg/mL</u>
	ACN	Slightly soluble	1 mg/mL
Oxyma Pure	0.1 M AcOH	Soluble	10 mg/mL
	H_2O	Soluble	10 mg/mL
	MeOH	Very soluble	<u>1000 mg/mL</u>
	ACN	Freely soluble	100 mg/mL
COMU	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Soluble	10 mg/mL
	ACN	Freely soluble	<u>33.33 mg/mL</u>
PyClocK	0.1 M AcOH	Very slightly soluble	0.1 mg/mL
	H_2O	Very slightly soluble	0.1 mg/mL
	MeOH	Slightly soluble	1 mg/mL
	ACN	Freely soluble	<u>100 mg/mL</u>
TBTU	0.1 M AcOH	Freely soluble	33.33 mg/mL
	H_2O	Soluble	10 mg/mL
	MeOH	Soluble	10 mg/mL
	CAN	Freely soluble	<u>100 mg/mL</u>

Table 2.10 *Order of diluent addition for dissolving analytes under investigation.*

Product	Initial diluent	Second diluent	Third diluent
HCTU	ACN	MeOH	H_2O
HBTU	ACN	MeOH	H_2O
HOBt	MeOH	ACN	H_2O
HOAt	MeOH	ACN	H_2O
6ChloroHOBt	MeOH	ACN	H_2O
Oxyma Pure	ACN	MeOH	H_2O
COMU	ACN	MeOH	H_2O
PyClocK	ACN	MeOH	H_2O
TBTU	ACN	MeOH	H_2O

2.3.1.1   Determination of optimum detection wavelength for 'Sample set A'

The type of detector used for the detection of analytes impacts the relative response of sample components in terms of sensitivity, selectivity and baseline noise. Detection affects assay sensitivity via the signal to noise ratio (S/N). As a result, better sensitivity can be achieved by increasing the S/N ratio. Determining the optimum wavelength will maximise the signal for each of the analytes. The wavelength chosen for detection must provide acceptable absorbance by the various analytes in the sample, combined with acceptable light transmittance by the mobile phase [61]. Typically, HPLC method development is carried out with an ultraviolet (UV) detector, however alternative detectors may be required if analytes have minimal or no UV absorbance, analyte concentrations are too low for UV detection or sample interferences are present [61]. Each reagent was therefore evaluated between 210 nm and 400 nm on a photodiode array detector to determine the optimum detection wavelength, as shown in Figure 2.2.

The UV absorbance profiles of the mobile phases that were investigated during the method development were accessed as outlined in Table 2.11. Water is effectively non-absorbing above 180 nm, so this mobile phase component can be ignored [61]. The mobile phase must transmit sufficiently at the wavelength used for detection as baseline noise has been demonstrated to increase significantly when absorbance (AU) of the mobile phase is greater than 0.7 [61]. Less pure solvents can demonstrate a higher UV absorbance and as a result, all reagents used were of HPLC grade or higher [60].
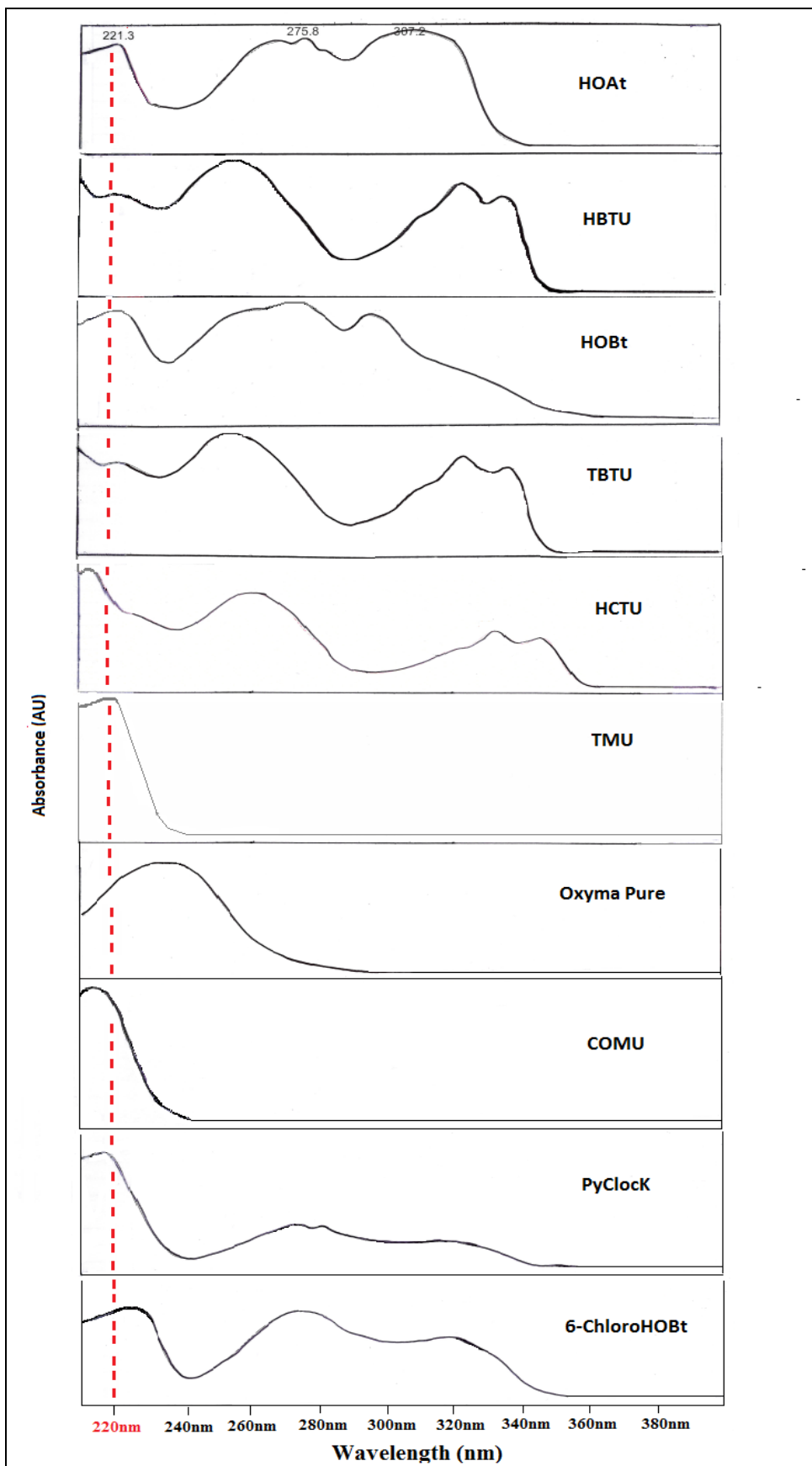
Figure 2.2: *PDA profile of reagents in 'Sample set A'. The red dotted line represents the optimum wavelength.*

Table 2.11 *Absorbance (AU) of mobile phase components at selected wavelengths (nm)* [61,104,105]

Absorbance (AU) at wavelength specified (nm)										
Mobile phase component	**200**	**205**	**210**	**215**	**220**	**230**	**240**	**250**	**260**	**280**
Acetonitrile	0.05	0.03	0.02	0.01	0.01	<0.01	-	-	-	-
Methanol	2.06	1.00	0.53	0.37	0.24	0.11	0.05	0.02	<0.01	-
0.1 % TFA	1.20	0.78	0.54	0.34	0.20	0.06	0.02	<0.01	-	-
0.1 % TFA in acetonitrile	0.29	0.33	0.37	0.38	0.37	0.25	0.12	0.04	0.01	<0.01
10 mM Ammonium acetate	1.88	0.94	0.53	0.29	0.15	0.02	<0.01	0.02	-	-
100 mM Sodium phosphate pH 6.8	1.99	0.75	0.19	0.06	0.02	0.01	0.01	0.01	0.01	<0.01
Ammonium formate	UV cut-off 210 nm (AU > 0.5)								0.05	0.04
0.1 % Formic acid					1.00	0.50	0.10	0.02	-	0.01
0.1 % Formic acid in acetonitrile					1.00	0.50	0.10	0.05	-	0.05

A wavelength of 220 nm was selected based on results outlined in Figure 2.3. At this wavelength, all components will be clearly visible with minimal interference from mobile phase composition. Formic acid demonstrates a high absorbance for at ≤ 220 nm, however this was partially negated by the addition of formic acid in both mobile phase A (0.1 % formic acid) and mobile phase B (0.1 % formic acid in acetonitrile) [104].

2.3.1.2 *Evaluation of multiple columns and mobile phase systems prior to detailed method development.*

Reverse phase chromatography (RPC) is typically the first choice for most regular samples with a vast selection of efficient, stable and reproducible columns [61]. RPC separations are typically carried out with silica-based, bonded-phase columns and the sample retention mainly depends on three characteristics on a column: bonded ligand type, concentration of bonded phase and column surface area [61]. Further effects upon retention and/or peak shape are due to non-specific interactions with the silica substrate (silanols), which can be reduced to some extent by end capping or by addition of mobile phase additives'. Variations to bonded ligand type can result in changes to selectivity of analytes and therefore several types of columns were chosen to be evaluated during the method development. C18 (octyldecylsilane, ODS) columns are particularly useful for the separation of peptides less than 2,000 Daltons and as a result, a lot of the columns chosen for the evaluation in this study were C18 [106]. The choice of the pore size is determined by the molecular weight of the component being analysed and based on Table 2.7, all of the analytes under investigation have a small molecular weight [106]. For the reversed phase separation of small molecules, column packing with

small pores (60-120 Å) is typically used [106]. For small molecules and peptides, 100-150 Å is typically used and for polypeptides and many proteins, 200-300 Å column pore size is used [106]. Therefore, most of the columns chosen for the method development investigation had pore sizes of 80 to 130 Å, with two higher pore size columns included to evaluate the effect of column pore size on the separation of the analytes. This is because wide pore columns can also separate peptides well and often result in different selectivity and resolution [31].

Each column was assessed using the Product Quality Research Institute (PQRI) approach by United States Pharmacopeia (USP) to ensure they were not equivalent. The PQRI approach provides characterisation of every reversed phase column in terms of hydrophobicity (H), steric interaction (S*), hydrogen-bond acidity (A) and basicity (B), and relative silanol ionization or cation-exchange capacity (C) [103]. Column hydrophobicity (H) increases with an increase in total carbon. H also increases for small-pore columns due to the compression of the ends of the alkyl chains. Column steric interactions (S*) increase as the bonded phase becomes more crowded, such as with increased chain length or narrow-pore packing's. Column hydrogen-bond acidity (A), due to non-ionized silanols, increases with column acidity. Column hydrogen-bond basicity (B) arises from various functional groups within the bonded ligand and in general, columns with high B values preferentially retain acidic compounds. Overall, column hydrophobicity has only a minor effect on column selectivity, while S*, A and B have a significant effect on column selectivity. Relative silanol ionization or cation-exchange capacity (C) is dependent on mobile phase pH and therefore the USP program allows a choice of pH = 2.8 (low) or 7.0 (high), whichever value is closest to the actual mobile phase pH and also allows the assessment of acidic and basic analytes [103].

Using the PQRI parameters, each column was therefore compared and their similarities are indicated by an F value as shown in Table 2.12. Columns which have values of F ≤ 3 are very likely to give an equivalent and acceptable separation for any sample. The larger the F value, the greater the difference between the columns [103]. This was used to ensure the columns chosen for method development would demonstrate different selectivity. The Acquity BEH 300 C4 and Acquity BEH C18 have not been classified by UPS-PQRI and therefore no F value could be determined. All F values were determined as > 3 at pH 2.8 for acidic and basic analytes, with the exception of the Halo C18 and the Agilent Zorbax Extend C18, with an F value of 2.53 and the comparison of Agilent Zorbax Eclipse Plus C18 and Phenomenex Kinetix, which demonstrated an F value of 2.65. These columns were then compared at pH 7.0 and the F value for Agilent Zorbax Eclipse Plus C18 and Phenomenex Kinetix was subsequently determined as 9.06. However, the F value between Halo C18 and the Agilent Zorbax Extend C18 was 2.39 and as a result, the Agilent Zorbax Extend C18 column was removed from the method development investigation.

Table 2.12 *Comparison of columns by PQRI approach at pH 2.8 for acidic and basic compounds*

F	Acquity HSS C18	Acquity HSS C18 SB	Acquity HSS T3	Acquity BEH300 C4	Acquity BEH C18	Acquity BEH C18 Shield	Dionex Acclaim 120 C18	Acquity BEH Phenyl	Agilent Eclipse Plus C18	Agilent Eclipse Plus C8	Agilent Zorbax Extend C18	Agilent Zorbax SB C18	Phenomenex Kinetix	Halo C18	YMC Ultra HT Hydrosphere C18	YMC Ultra HT Pro C18	YMC Triart C18	Hypersil gold
Acquity HSS C18	-	37.22	7.08	N/C	N/C	24.2	3.24	23.3	6.42	9.15	6.05	15.58	5.46	6.21	17.95	18.04	9.41	12.91
Acquity HSS C18 SB	-	-	37.04	N/C	N/C	45.11	34.56	26.21	39.5	43.25	38.67	24.68	39.29	37.3	48.84	51.01	41.74	26.54
Acquity HSS T3	-	-	-	N/C	N/C	21.35	7.12	25.84	5.75	7.2	10.76	15.79	3.98	11.48	14.22	15.95	6.33	13.25
Acquity BEH300 C4	-	-	-	-	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C
Acquity BEH C18	-	-	-	-	-	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C	N/C
Acquity BEH C18 Shield	-	-	-	-	-	-	25.97	31.2	22.39	20.21	26.23	26.85	21.48	28.06	20.04	21.88	24.62	22.78
Dionex Acclaim 120 C18	-	-	-	-	-	-	-	22.54	7.91	11.26	7.63	14.33	7.06	6.85	19.71	20.22	10.03	11.89
Acquity BEH Phenyl	-	-	-	-	-	-	-	-	28.31	30.2	26.8	21.57	26.97	25.88	38.11	39.09	31.37	13.77
Agilent Eclipse Plus C18	-	-	-	-	-	-	-	-	-	5.26	6.52	16.11	2.65	8.15	12.33	12.77	5.23	16.14
Agilent Eclipse Plus C8	-	-	-	-	-	-	-	-	-	-	10.51	20.38	4.46	12.37	9.19	9.65	5.72	18.57
Agilent Zorbax Extend C18	-	-	-	-	-	-	-	-	-	-	-	15.37	7.85	2.53	17.77	17	10.53	16.51
Agilent Zorbax SB C18	-	-	-	-	-	-	-	-	-	-	-	-	16.68	14.8	25.74	27.17	19.56	10.83
Phenomenex Kinetix	-	-	-	-	-	-	-	-	-	-	-	-	-	9.19	12.75	13.49	5.22	15.01
Halo C18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19.9	19.28	11.6	16.07
YMC Ultra HT Hydrosphere C18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.38	11.28	25.69
YMC Ultra HT Pro C18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.29	27.27
YMC Triart C18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19.11
Hypersil gold	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

To ensure that all columns were evaluated equally, a gradient for each column was calculated to account for differences in particle size, column length and diameter. For this, a gradient of 0.9 % B change per minute, for a 100 mm (length) x 2.1 mm (diameter) and 1.7 μm (particle size), a flow rate of 0.4 mL/min, was chosen as the standard conditions. The gradient for every other column was then determined manually using the calculations outlined below [108].

1.   To determine flow rate with target column:

$$\text{Flow rate} = \text{Original flow rate} \times \frac{\text{Column diameter}^2 \text{ (target column)}}{\text{Column diameter}^2 \text{ (original column)}} \times \frac{\text{Particle size (original column)}}{\text{Particle size (target column)}}$$

2.   To determine the gradient duration for each step in the original gradient

Gradient volume = Flow rate x Time

Column volume = π x r^2 x Length of column

$$\text{Gradient duration} = \frac{\text{Gradient volume}}{\text{Column volume}}$$

3.   To calculate the time required to get the same gradient duration with the target column:

Gradient step volume = Gradient duration x Target column volume

Gradient step time = Gradient step volume / Flow rate^2

Using the above calculations, a gradient was determined for each specific column dimension as demonstrated below in Table 2.13 and Table 2.14. In this example, a gradient for a 100 mm length x 2.1 mm diameter column with 1.9 μm particle size (Table 2.13) was converted to the equivalent gradient for a 50 mm length x 2.1 mm diameter column with 1.9 μm particle size (Table 2.14).

Table 2.13 *Gradient for a 100 mm length x 2.1 mm diameter column, 1.9 μm particle size*

	Length (cm)	Diameter (cm)	Particle size (μm)	Column volume (mL)	Flow rate (mL/min)	Dwell volume system (mL)
Column	10	0.21	1.9	0.35	0.4	0.4
Gradient	**Step**	Time (mins)	% A	% B	Gradient volume (mL)	Gradient duration (min)
	Step 1	0	95	5	N/A	N/A
	Step 2	5	95	5	2	5.78
	Step 3	55	50	50	22	63.55
	Step 4	59	50	50	23.6	68.17
	Step 5	60	95	5	24	69.33
	Step 6	65	95	5	26	75.1

Table 2.14 *Gradient for* a *50 mm length x 2.1 mm diameter column, 1.9 µm particle size*

	Length (cm)	Diameter (cm)	Particle size (µm)	Column volume (mL)	Flow rate (mL/min)	Dwell volume system (mL)
Column	5	0.21	1.9	0.17	0.4	0.4
Gradient		Target Time (mins)	% A	% B	Target gradient volume (mL)	Gradient duration (mins)
	Step 1	0	95	5	n/a	n/a
	Step 2	2.5	95	5	1	5.78
	Step 3	27.5	55	45	11	63.55
	Step 4	29.5	55	45	11.8	68.17
	Step 5	30	95	5	12	69.33
	Step 6	32.5	95	5	13	75.1

Using the optimum sample diluent, detection wavelength of 220 nm and an equivalent gradient profile, a sample containing a mixture of Peptide 1 and 'Sample set A' was analysed using a series of columns with multiple different mobile phases as outlined previously in Table 2.2 and Table 2.4 (page numbers 47 and 48). For columns 2, 10, 12 and 14, each reagent was individually analysed on each mobile phase system to determine the order of elution of the analytes for each system. The mobile phase components chosen for the evaluation include commonly used reagents such as TFA, formic acid, ammonium acetate, ammonium formate and sodium phosphate. For reproducibility, the pH of any given mobile phase should be +/- 1.0 pH unit above or below the pK_a of the solutes being separated otherwise it could lead to asymmetric peaks that are broad, tail, split or shoulder [106]. Some of the analytes under investigation have low pK_a values, such as HOAt at 3.28, 6-ChloroHOBt at 3.35 and HOBt and Oxyma Pure at 4.60 [108]. However, some of the pK_a/pK_b values of the analytes were unknown and some were quite high, such as TMU at pK_b 12.00 and therefore more than one mobile phase pH needed to be investigated to determine the optimum pH for the analysis [23]. The ionised form of the analytes are more polar and therefore exhibit less retention in RP-HPLC. As a result, the pH needs to be lower than the pK_a of the acidic analytes (ideally < 2 pH units) in order to suppress the ionisation to achieve a good peak shape and good retention. Acetonitrile and methanol were chosen as the organic solvents for the evaluation. Acetonitrile is the most commonly used solvent in RP-HPLC because it is volatile (compatible with mass spectrometric detection), has low viscosity and is practically transparent for UV detection for low wavelengths < 200 nm [107]. Each column/mobile phase system was evaluated in terms of number of peaks resolved, the number of peaks with a resolution with their nearest neighbour > 2, the number of peaks with retention factor (k*) > 2, the number of peaks with tailing factors < 2, and the number of peaks with plate count > 2,000, as shown in Table 2.15, using the Aquity HSS C18 column as an indicative example.

It should be noted however that strictly speaking, efficiency is not defined in chromatographic gradients. In order for efficiency to be calculated, there is an assumption that the mobile phase composition is the same at the start of a peak, the peak apex, and the tail of a peak. In mobile phase

gradients, this is not the case. In fact, the solute molecules at the head of the peak "experience" a slightly lower solvent concentration relative to molecules in the bulk of the peak, and so slow down slightly. Conversely, solute molecules at the tail of the peak experience slightly higher solvent concentration and so move faster than molecules in the centre of the band. This phenomenon is partly responsible for chromatographic zone focussing during mobile phase gradients. The authors fully acknowledge therefore that, strictly speaking, the use of plate count determinations is not accurate from a theoretical standpoint. However, for the remainder of this chapter (and indeed Chapter 3) we have elected to use plate count as a means of evaluating peak width, merely for the sake of simplicity in method development, rather than the more accurate measurement of average peak width across the chromatogram, or a measure of other parameters such as peak capacity.

When retention factor k* is mentioned in this thesis, the reader should not the difference between k (used for isocratic separations) and k* (used for gradient separations). In isocratic separations the retention factor (k*) is a constant value during the separation and depends upon the partition coefficient for the analyte in question. Conversely, in gradient methods, the retention factor (denoted k*) is not constant, but rather, varies throughout the run. The symbol (k*) can be thought of as the average k-value throughout the separation, and is equivalent to the isocratic k-value at the point a band has moved halfway down the column. The k* value is calculated using the following formula:

$$k* = \frac{t_g F}{1.15 S \Delta \Phi V_m}$$

Where t_g is the gradient time (minutes) F is the flow rate, S is a constant determined by the analyte mass (usually 4 for analytes < 500 Da), $\Delta \Phi$ is the change in volume fraction of organic (final %B minus initial % B) and V_m is the column void volume ($\pi r^2 L$ x 0.68).

The score evaluation (number of peaks resolved, the number of peaks with a resolution with their nearest neighbour > 2, the number of peaks with retention factor (k*) > 2, the number of peaks with tailing factors < 2, and the number of peaks with plate count > 2,000) was performed on each column for each mobile phase system and the results are summarised in Table 2.16. The example in Table 2.15 is shown as the first line of data in Table 2.16.

Table 2.15 *Score evaluation of Acquity HSS C18 for each mobile phase system*

Note: This column was arbitrarily chosen for illustrative purposes in order to demonstrate how scores were calculated. The data from the right column is transposed in the first row of Table 2.16.

Mobile phase system	Mobile phase A composition	Mobile phase B composition	Total peaks resolved	No of peaks Rs >2.0	k* range	No of peaks k* > 2	Plate count range	No of peaks plate count >2,000	Tailing range	No of peaks peak tailing ≤ 2.0	Score
1	0.1 % TFA	0.08 % TFA/ACN	10	8	1.45 - 24.93	9	5,500 - 30,500	10	1.01 - 2.32	9	46
2	0.1 % TFA	0.08 % TFA/MeOH	9	7	3.98 - 40.25	9	3,414 - 281,472	9	1.04 - 2.08	8	42
3	0.1 % Formic acid	0.08 % Formic acid /ACN	9	6	2.00 - 23.49	9	5,303 - 51,921	9	1.08 - 5.51	7	40
4	0.1 % Formic acid	0.08 % Formic acid/MeOH	8	4	3.93 - 36.82	8	502 - 25,128	7	1.08 - 2.96	6	33
5	10 mM CH$_3$COONH$_4$ pH 4.0	ACN	8	7	2.41 - 50.36	8	1,072 - 145,841	7	0.92 - 3.73	5	35
6	10 mM CH$_3$COONH$_4$ pH 4.0	MeOH	7	5	3.93 - 80.06	7	177 - 106,221	5	0.91 - 3.92	5	29
7	10 mM NH$_4$HCO$_2$ pH 3.0	ACN	8	6	4.28 - 48.90	8	1,379 - 11,124	7	1.09 - 1.60	8	37
8	10 mM NH$_4$HCO$_2$ pH 3.0	MeOH	9	6	8.13 - 78.00	9	2,643 - 117,748	9	1.07 - 2.51	6	39
9	10 mM NaH$_2$PO$_4$ pH 2.0	ACN	8	5	1.83 - 23.76	7	1,980 - 14,219	7	1.04 - 1.26	8	35
10	10 mM NaH$_2$PO$_4$ pH 2.0	MeOH	8	6	3.87 - 39.14	8	1,043 - 13,953	7	1.01 - 2.34	7	36
11	10 mM NaH$_2$PO$_4$ pH 7.0	ACN	4	1	-0.23- 11.62	3	125 - 4,048	1	0.85 - 2.60	2	11
12	10 mM NaH$_2$PO$_4$ pH 7.0	MeOH	6	4	1.00 - 51.85	4	308 - 3,963	3	1.18 - 1.89	6	23

*Analytes evaluated include 'Sample set A' and Peptide 1

Table 2.16 *Summary of score evaluation for all columns for each mobile phase system*

	Column	1	2	3	4	5	6	7	8	9	10	11	12	No. of mobile phase systems ≥ 40	Total overall score
Columns	Acquity HSS C18	46	42	40	33	35	29	37	39	35	36	11	23	3	406
	Acquity HSS C18 SB	29	37	31	29	30	28	26	23	35	38	19	25	0	349
	Acquity HSS T3	47	43	39	37	42	38	39	40	43	40	29	26	6	463
	Acquity BEH300 C4	35	33	33	31	22	21	24	23	38	33	12	12	0	315
	Acquity BEH C18	36	43	31	37	32	36	30	38	29	39	21	26	1	398
	Acquity BEH RPC18 Shield	33	29	27	32	26	27	26	26	23	28	18	22	0	317
	Acquity BEH Phenyl	30	35	39	30	37	36	38	36	37	44	29	27	1	418
	Agilent Eclipse Plus C18	43	48	41	42	35	36	36	36	40	43	29	30	6	459
	Agilent Eclipse Plus C8	41	49	37	42	25	36	32	40	33	40	33	36	5	444
	Agilent Zorbax SB C18	40	43	31	31	29	29	33	29	28	34	30	33	2	390
	Phenomenex Kinetix	31	31	26	32	31	37	32	33	42	44	26	21	2	386
	Halo C18	33	41	30	37	29	33	31	33	36	33	19	22	1	377
	YMC Ultra HT Hydrosphere C18	39	38	35	37	39	41	35	37	44	39	31	32	2	447
	YMC Ultra HT Pro C18	41	42	39	40	37	42	35	38	41	46	23	27	6	451
	YMC Triart C18	42	40	33	39	43	42	40	37	43	43	35	37	7	474
	Acclaim RSLC 120 C18	39	38	40	39	34	35	29	30	34	39	36	31	1	424
	Thermo Hypersil Gold	39	41	42	43	33	34	40	39	30	30	26	30	4	427
	Mobile phase system total overall score	644	673	594	611	559	580	563	577	611	649	427	460		

*Analytes evaluated include 'Sample set A' and Peptide 1

*The composition of the mobile phase systems can be cross referenced with Table 2.15.

*The odd numbered mobile phase systems use acetonitrile as the organic modifier and the even numbered mobile phase systems use methanol as the organic modifier.

Table 2.16 details the overall score for each column/mobile phase system. Different columns can vary in plate number, band symmetry, retention, band spacing, and lifetime [61]. The maximum possible score was determined as 60, if all 12 peaks within the mix were separated with resolution > 2, peak tailing ≤ 2 and r > 2. Since the conditions being evaluated were not optimised for parameters such as temperature, pH, concentration, etc., at this stage in the developmental work, all column/mobile phase systems with a score of > 40 were deemed acceptable for further evaluation. A score of 40 meant that at least 8 of the peaks evaluated were resolved, most of which met the criteria for resolution > 2, retention factor (k*) > 2, tailing factors < 2, and plate count > 2,000.

The *total overall score* (last column on Table 2.16) was calculated by adding all of the individual scores for a given column. The *mobile phase system total overall score* (last row of Table 2.16) was calculated by adding all individual scores for a given mobile phase system.

On all columns, HOAt, HBTU and TBTU eluted very early and therefore they are the most hydrophilic analytes. Oxyma Pure, 6-ChloroHOBt, PyClocK and the Peptide 1 were the most retained analytes and therefore the most hydrophobic. None of the 204 experiments demonstrated the ability to resolve HBTU and TBTU or 6-ChloroHOBt and PyClocK. HBTU and TBTU can't be resolved because the analytes only differ by their counter ion (as shown in Table 2.7, page 57) and therefore are the same compound when dissolved [109].

The Acquity BEH 300 C4 was the worst performing column in the investigation, achieving the lowest overall score of 315. The column resulted in poor retention of the analytes and as a result, the column demonstrated poor performance with no mobile phases demonstrating a score above 40. This was expected as sample retention typically increases for bonded phases of greater length (C18 > C8 > C3 > C1) [94]. Also, this column was of pore size 300 Å, which is typically used for high molecular weight analytes [106]. Smaller pore size columns were determined to be better for the separation of these low molecular weight analytes. Figure 2.3 shows a sample chromatogram on the Aquity BEH 300 C4 column, clearing showing poor retention relative to an Acquity BEH C18, which was chosen for comparative purposes. Mobile phase system 1 was arbitrarily selected so that a useful comparison can be made.

Acquity HSS C18 SB (total score of 349) and Acquity BEH Shield RP C18 (total score of 317) were also determined as poor performing columns, with no mobile phase systems resulting in a score above 40 for either column. Acquity HSS C18 SB is a non-end capped column and all chromatograms displayed poor peak shape. End-capping is a process which is used to react silica gel silanol groups that may remain after reaction, with a large silylating agent such as octadecyltrichlorosilane [106]. The column is said to be end capped when a small silylating reagent (such as trimethylchlorosilane or dichlorodimethylsilane) is used to react with residual silanol groups on a silica gel-based packing surface [106]. It is used to minimize undesirable adsorption of basic, ionisable, and ionic compounds and therefore, the poor peak shape obtained during the analysis of HSS C18 SB is potentially due to the lack of end-capping [106]. Figure 2.4 shows a sample

chromatogram on the Acquity HSS C18 SB column, clearly showing poor peak shape when compared with an Acquity HSS C18 column, which is end capped. Again, mobile phase system 1 was arbitrarily selected for comparative purposes. For this separation, it was concluded that end-capped columns were demonstrated to be better than non-end-capped columns, as would be expected.
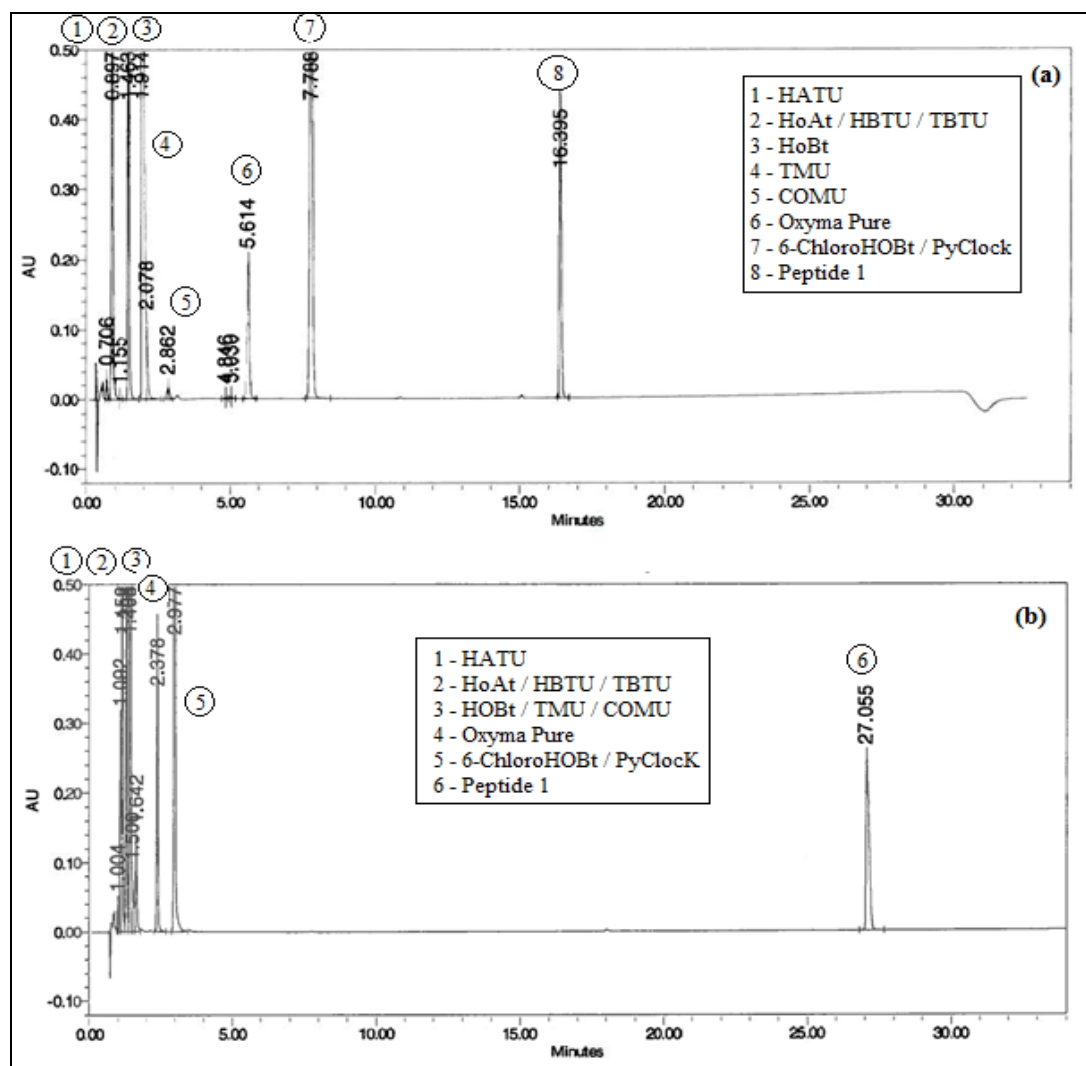
Figure 2.3: *Comparison of retention on (a) Acquity BEH C18 and (b) Acquity BEH 300 C4. (Chromatographic conditions:, Mobile phase A: 0.1 % TFA, Mobile phase B: 0.08 % TFA in acetonitrile, Gradient program: A5 for (a) and A4 in (b) in Appendix 1, Injection volume: 5 µL, Column temperature: 25 °C, Detection wavelength: 220 nm.*
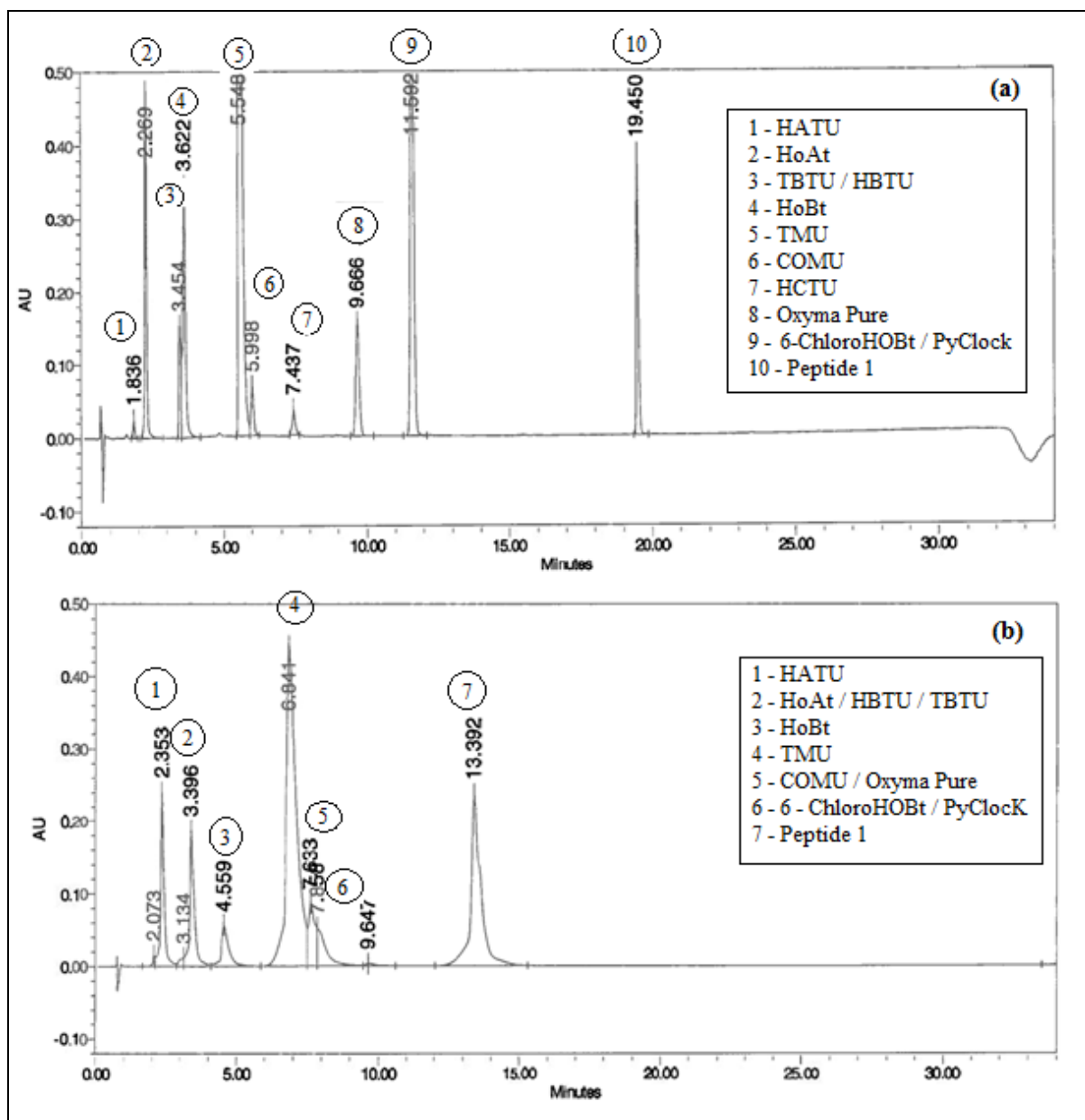
Figure 2.4: *Comparison of retention on (a) Acquity HSS C18 (end capped) column and (b) Acquity HSS C18 SB (non-end capped). (Chromatographic conditions:, Mobile phase A: 0.1 % TFA, Mobile phase B: 0.08 % TFA in acetonitrile, Gradient program: A3 in Appendix 1, Injection volume: 5 µL, Column temperature: 25 ^{o}C, Detection wavelength: 220 nm.*

A comparison of the use of acetonitrile or methanol as the eluting solvent in the mobile phase system was performed. In Table 2.16, the odd numbered mobile phase systems use acetonitrile as the organic modifier, whereas the even mobile system numbering use methanol. Acetonitrile as an eluting solvent resulted in an overall score of 3398 (addition of score of all 102 experiments performed with acetonitrile as the eluting solvent), whereas methanol resulted in an overall score of 3550. This could be due to methanol being the weaker eluting solvent and therefore resulting in better retention of early eluting peaks and a better resolution of closely eluting peaks. Methanol demonstrated better

selectivity for the polar compounds; however sharper peaks were obtained using acetonitrile as observed in Figure 2.5.The order of elution of the reagents is the same for both acetonitrile and methanol, however acetonitrile resulted in the separation of TMU and COMU and also HCTU and Oxyma Pure, whereas they co-eluted with the use of methanol. Acetonitrile was chosen as the optimum eluting solvent for further optimisation studies, due to improved peak shape. Sharp symmetrical peaks are necessary to achieve low detection limits, low relative standard deviation (RSD) between injections and reproducible retention times. For this separation, acetonitrile was demonstrated to be better than methanol for the separation of the analytes.
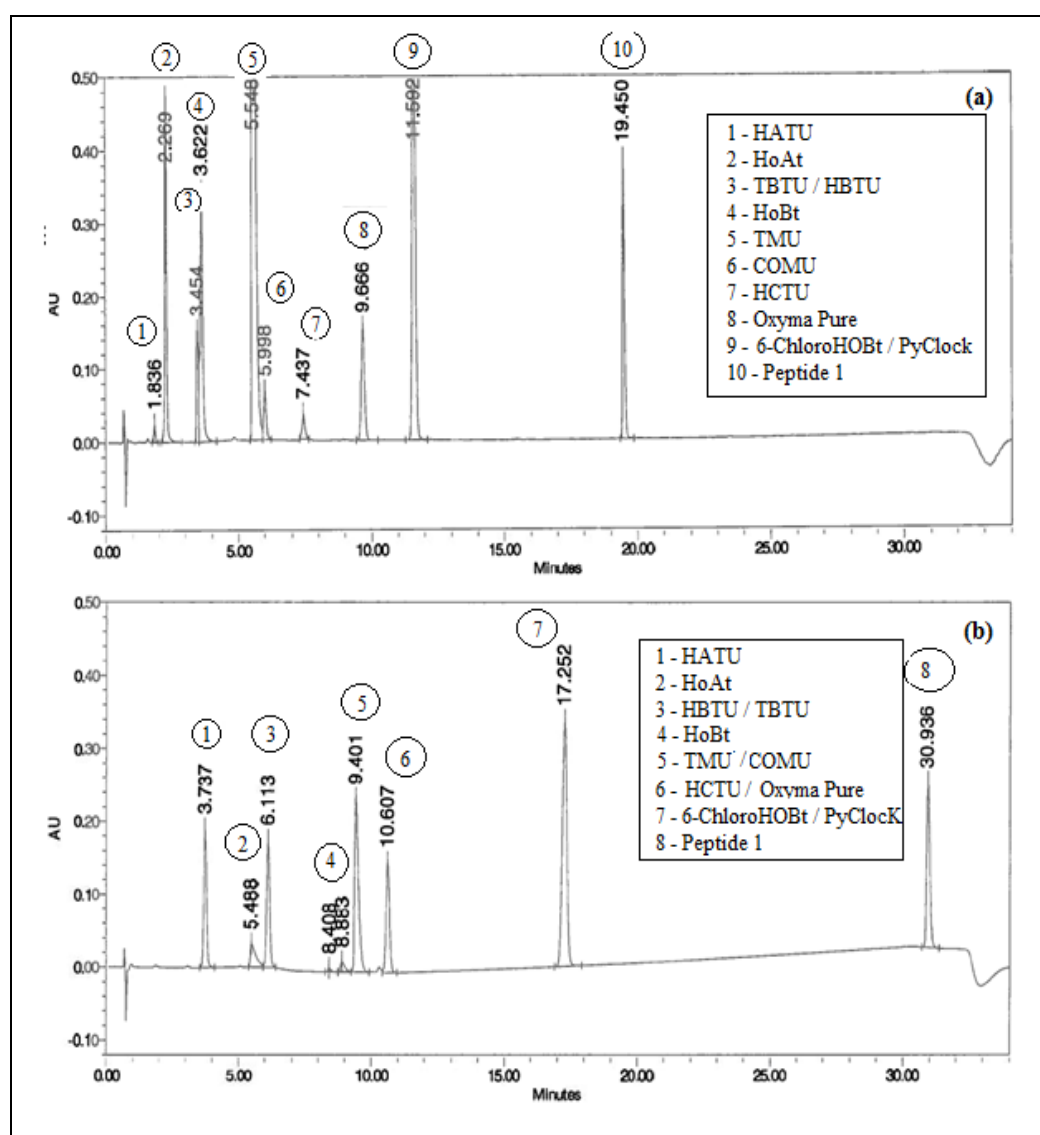
Figure 2.5: *Comparison of peak shape on Acquity HSS C18 using (a) acetonitrile and (b) methanol as organic modifier in mobile phase B. Chromatographic conditions: Mobile phase A: 0.1 % TFA, mobile phase B: 0.08 % TFA in acetonitrile or methanol. Gradient program: A3 in Appendix 1, Injection volume: 5 μL, Column temperature: 25 °C, Detection wavelength: 220 nm.*

The best column was determined as YMC Triart C18, with the greatest overall score of 474 and 7 mobile phase systems with a score > 40. A holistic evaluation of all mobile phases containing acetonitrile by comparing the scores (on the bottom row of Table 2.16) revealed that TFA was the best mobile phase (score of 644), followed by sodium phosphate pH 2 (score of 611), formic acid (score of 594), ammonium formate (score of 563), ammonium acetate (score of 559) and then the worst mobile phase system was obtained for sodium phosphate pH 7 (score of 427). A comparison of sodium phosphate pH 2 and sodium phosphate pH 7 as shown in Figure 2.6, demonstrates the poor retention of some of the analytes at higher pH. This is due to the ionisation of acids (most of reagents in 'Sample set A') and bases as the pH increase, which results in a decrease of retention of acids and increase in retention of bases [61].
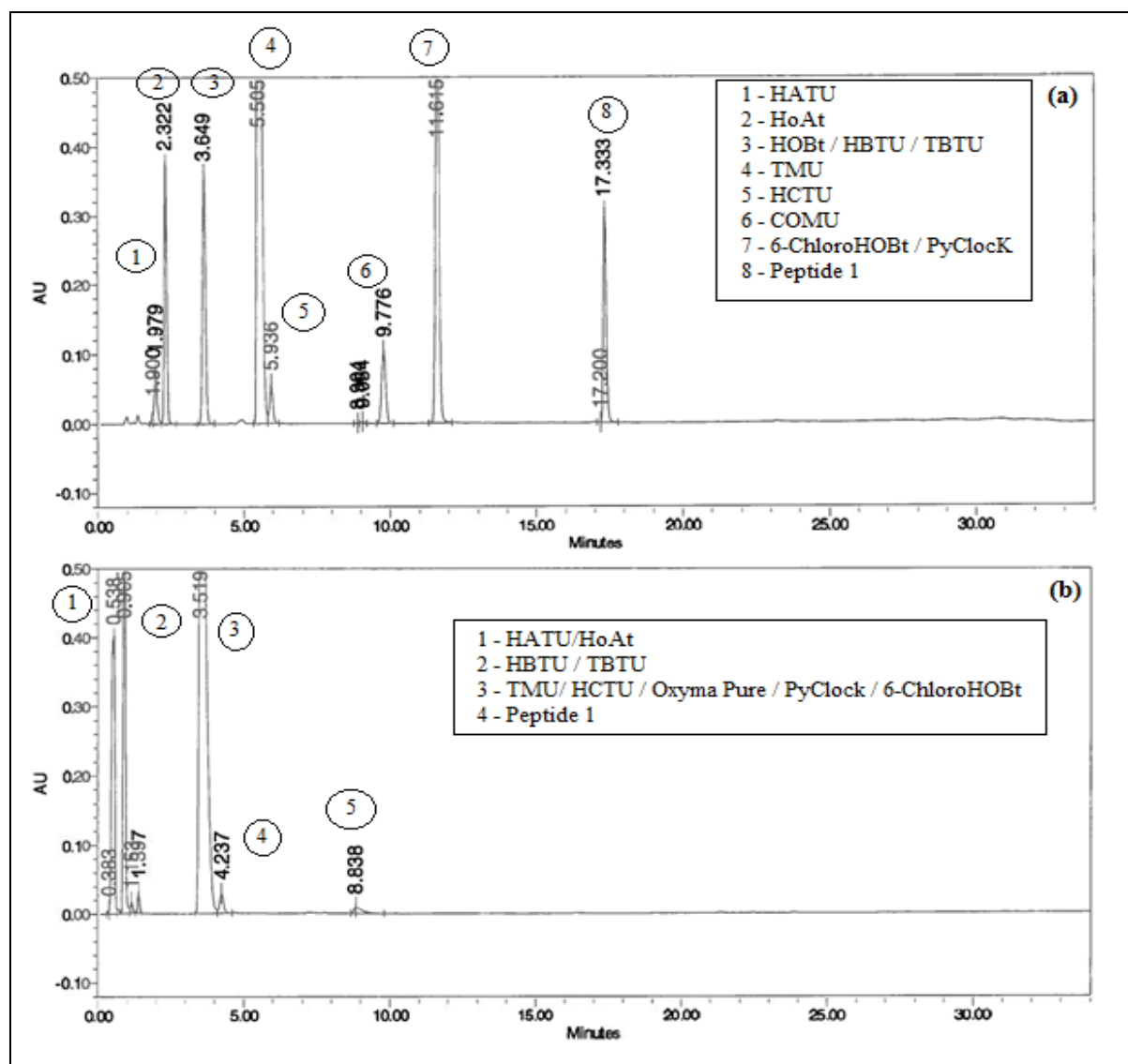
Figure 2.6: *Comparison of retention on Acquity HSS C18 using (a) sodium phosphate pH 2 and (b) sodium phosphate pH 7 as mobile phase A. Chromatographic conditions: Mobile phase B: Acetonitrile, Gradient program: A3 in Appendix 1, Injection volume: 5 µL, Column temperature: 25 °C, Detection wavelength: 220nm.*

All column/mobile phase systems with a score of > 40 were deemed acceptable for further evaluation and therefore this reduced the number of column/mobile phase systems from 204 to 46. There was very little difference between the column/mobile phase systems with scores over 40 and the results did not demonstrate that any particular column/mobile phase system that was superior above all others. Therefore other performance evaluation criteria (i.e. mass spectrometry compatibility) were necessary to distinguish optimum column/mobile phase system over and above the 'scoring system' previously described. The ideal method would be directly transferrable to a mass spectrometer and therefore, the 46 columns/mobile phase systems were further evaluated depending on the mass spectrometry (MS) compatibility of the mobile phase system. TFA and sodium phosphate are not mobile phase components that are used in mass spectrometry and therefore this reduced the number of experiments from 46 to 17, as outlined in Table 2.17. TFA is a volatile mobile phase component however, it can suppress ionization in the LC-MS interface, causing a drop in signal and therefore it is not an ideal for LC-MS [110].The ideal method would be compatible with the mass spectrometer because this would allow the confirmation of the molecular weight of all the compounds. Sodium phosphate is not volatile and can also result in the unwanted formation of sodium adducts.

Table 2.17 *Summary of mass spectrometry compatible column and mobile phase systems*

Column	Mobile phase system	Score
Acquity HSS C18	3	40
Acquity HSS T3	5	42
	8	40
Agilent Eclipse Plus C18	3	41
	4	42
Agilent Eclipse Plus C8	4	42
	8	40
YMC Ultra HT Hydrosphere C18	6	41
YMC Ultra HT Pro C18	4	40
	6	42
YMC Triart C18	5	43
	6	42
	7	40
Acclaim RSLC 120 C18	3	40
Thermo Hypersil Gold	3	42
	4	43
	7	41

Note: Mobile phase 1, 2, 9, 10, 11 and 12 are not eligible for further study since they contain TFA and sodium phosphate respectively. They are therefore excluded from this table.

When acidic and basic samples are present in the sample, it is strongly advisable to control mobile phase pH by adding a buffer [61]. Mobile phase pH can be one of the most important variables in the control of retention in a reversed-phase HPLC separation and therefore is a very powerful tool

for method development [110]. This would therefore eliminate formic acid as a mobile phase, reducing the number of experiments to 9, as seen in Table 2.18.

Since YMC Triart C18 was determined as the best performing column and it was also the column with the most mobile phase systems in the final selection (see Table 2.18 below), this was determined as the best column for further evaluation. This column gave optimum results using both ammonium acetate (mobile phases 5 and 6) and ammonium formate (mobile phases 7 and 8). Both mobile phase systems result in a decreasing baseline with an increasing of amount of acetonitrile. The pK_a of ammonium acetate is 4.8 and 9.2 and the pK_a of ammonium formate is 3.8 and 9.2. The buffer should be used to control pH over a range of pK_a +/- 1.0 and therefore ammonium acetate can be used at pH 3.8 to 5.8 and 8.2 to 10.2 [61]. Ammonium formate can be used at pH 2.8 to 4.8 and also 8.2 to 10.2. This means that although the pH of the mobile phases can be adjusted outside of the range, however there is negligible buffering capacity beyond +/- 1.0 pH unit about the pK_a [110]. Based on the analysis above where the analysis of the analytes at a high pH using sodium phosphate resulted in poor resolution of peaks, ammonium formate and acetonitrile were chosen as the mobile phase to further evaluate for the separation of the analytes, as this mobile phase can be used at a lower pH range, which could be optimum for the separation of the analytes.

Table 2.18 *Summary of optimum mobile phase systems (based upon selected scoring system)*

Column	Mobile phase system	Score
Acquity HSS T3	5	42
	8	40
Agilent Eclipse Plus C8	8	40
YMC Ultra HT Hydrosphere C18	6	41
YMC Ultra HT Pro C18	6	42
YMC Triart C18	5	43
	6	42
	7	40
Thermo Hypersil Gold	7	41

Note: Mobile phase with formic acid alone are excluded from this table

2.3.2   *Design of experiments (DoE)*

After the optimum stationary phase and mobile phase were selected, further optimisation was performed. Modde 9 software was used to compile an experimental design space where all of the selected parameters are varied at the same time. A DoE is a process used to maximise the information obtained regarding the impact of the parameters on the chromatographic separation with the minimum number of experiments. The factors under investigation included the concentration of the mobile

phase, pH of the mobile phase, column temperature, flow rate and gradient change. A buffer concentration of up to 50 mM is adequate for small molecules and therefore the concentration range investigated was 5 mM to 50 mM [111]. The pK$_a$ of ammonium formate is 3.8 and 9.2, therefore these were chosen as the pH values for the investigation [61]. The YMC Triart column has a maximum temperature limit of 50 ºC for high pH mobile phases and therefore the column temperature range chosen for the investigation was 20 ºC to 50 ºC [97]. Column temperature can have a significant impact on the separation of peptides, with reduced retention typically observed for increased temperature alongside changes in selectivity [107]. A flow rate of range of 0.3 mL/min to 0.5 mL/min and a 0.2 % to 1.2 % change in mobile phase B per minute were chosen as the other parameter ranges to be investigated.

The DoE was a full factorial DoE of Resolution V+ design, which is capable of resolving all the main effects and the two factor interactions. In other words, the software can isolate and quantify the effects of each main factor and each two-factor interaction [112]. This DOE study involved 19 experiments (16 + 3 centre points). The centre point experiments were used to ensure there were no external influences such as a system bias, environmental changes, and random skewing. The values of the factors for the centre point experiments were calculated from the average of each particular factor, for example: column temperature was selected as 35 ºC, the average of the addition of 20 ºC and 50 ºC. The responses evaluated were the overall number of peaks resolved, the % of peaks resolved that demonstrate resolution > 2, the % of peaks with k* > 2 and the % of peaks with plate count > 2,000. A resolution of 2 or greater is generally desirable for rugged methods and therefore a minimum resolution of 2 was the criteria set for the development experiments [61]. The number of theoretical plates is a measure of column efficiency and this depends on the elution time and peak width but the number should typically be > 2,000 and therefore a criterion for number of theoretical plates was set at > 2,000 for all method development experiments [61]. The results of each DoE experiment are outlined in Table 2.19.

Table 2.19 *DoE evaluation and corresponding chromatographic performance criteria.*

Exp Name	pH	Conc (mg/mL)	Flow rate (mL/min)	Temp (°C)	% B Change/min	Peaks resolved	R >2 (%)	k*>2 (%)	Plate count > 2,000
N1	3.8	5	0.3	20	1.2	8	62.5	100	87.5
N2	9.2	5	0.3	20	0.2	7	14.29	85.71	14.29
N3	3.8	50	0.3	20	0.2	9	66.67	100	100
N4	9.2	50	0.3	20	1.2	6	66.67	100	66.67
N5	3.8	5	0.5	20	0.2	8	75	100	87.5
N6	9.2	5	0.5	20	1.2	7	14.29	71.42	14.29
N7	3.8	50	0.5	20	1.2	9	55.56	100	77.78
N8	9.2	50	0.5	20	0.2	7	42.86	85.71	57.14
N9	3.8	5	0.3	50	0.2	8	87.5	100	75
N10	9.2	5	0.3	50	1.2	6	33.33	66.67	16.67
N11	3.8	50	0.3	50	1.2	8	75	100	75
N12	9.2	50	0.3	50	0.2	6	33.33	83.33	50
N13	3.8	5	0.5	50	1.2	8	62.5	100	87.5
N14	9.2	5	0.5	50	0.2	6	50	66.67	33.33
N15	3.8	50	0.5	50	0.2	8	62.5	100	87.5
N16	9.2	50	0.5	50	1.2	6	50	66.67	50
N17	3.8	27.5	0.4	35	0.7	9	55.56	100	77.78
N18	3.8	27.5	0.4	35	0.7	9	55.56	100	77.78
N19	3.8	27.5	0.4	35	0.7	9	55.56	100	77.78

A replicate plot was constructed to show the variation among the three centre point experiments (experiments N17, N18 and N19) and to demonstrate the reproducibility of the model. The variation in the replicate plots for retention factor (k*), resolution between critical peak pair, peak plate count and number of peaks resolved demonstrated minimal variation in the entire investigation series. It was therefore concluded that the replicate error would not complicate the data analysis.

A regression model was constructed to evaluate the summary of fit plot to determine if the results for each factor were valid. A 'valid' model has properties (R^2 goodness of fit, Q^2 goodness of prediction, reproducibility) close to 1.0. In this case, the regression plot for number of peaks resolved indicated that the model was acceptable ($R^2 > 0.8$, $Q^2 > 0.5$ and the difference R^2- $Q^2 < 0.2$-0.3), with R^2 of 0.89 and Q^2 of 0.78.
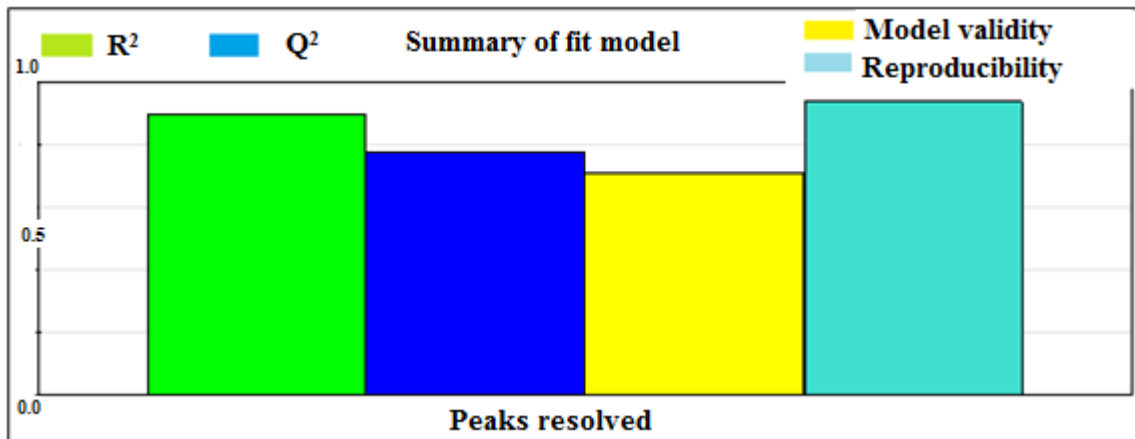
Figure 2.7: *Regression model for number of peaks resolved*

The regression model for the % of peaks with k* > 2 and % of peaks with plate count > 2,000 were also valid with results of R^2 of 0.83 and Q^2 of 0.57, and R^2 of 0.83 and Q^2 of 0.60, respectively. However, the regression model for the % of peaks with resolution > 2 was determined to be invalid with R^2 of 0.56 and Q^2 of 0.04 and therefore this data could not be used to accurately evaluate the impact of the 5 factors on resolution.

A coefficient plot was then compiled to determine the influences, if any, of the 5 factors on the number of peaks resolved, % of peaks with k* > 2 and % of peaks with plate count > 2,000. Figure 2.8 shows the coefficient plot for the response for the number of peaks resolved, indicating that pH had the largest effect on the number of peaks resolved. It had a large negative effect on the number of peaks resolved (i.e. increasing pH led to a reduction in the number of peaks resolved). Column temperature was the next most influential factor with a negative effect. Buffer concentration and flow rate had a smaller positive effect on the number of peaks resolved and a small negative effect was observed for % change in mobile phase B per minute.
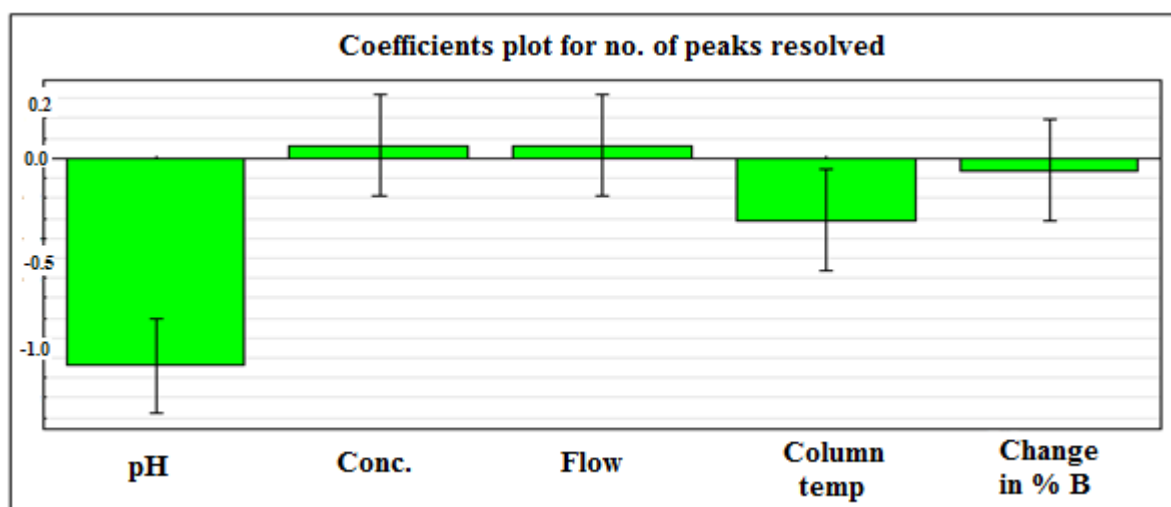
Figure 2.8: *Coefficient plot for number of peaks resolved*

Figure 2.9 shows the coefficient plot for the response for the % of peaks with k*>2, indicating that pH also had a largest negative effect on the % of peaks with k*>2. Column temperature was also the next most influential factor with a negative effect. Flow rate and % change in mobile phase B per minute had a smaller positive effect on the % of peaks with k*>2 and a small positive effect was observed for buffer concentration. The error bars details the ± 95 % confidence intervals for the results of each factor.
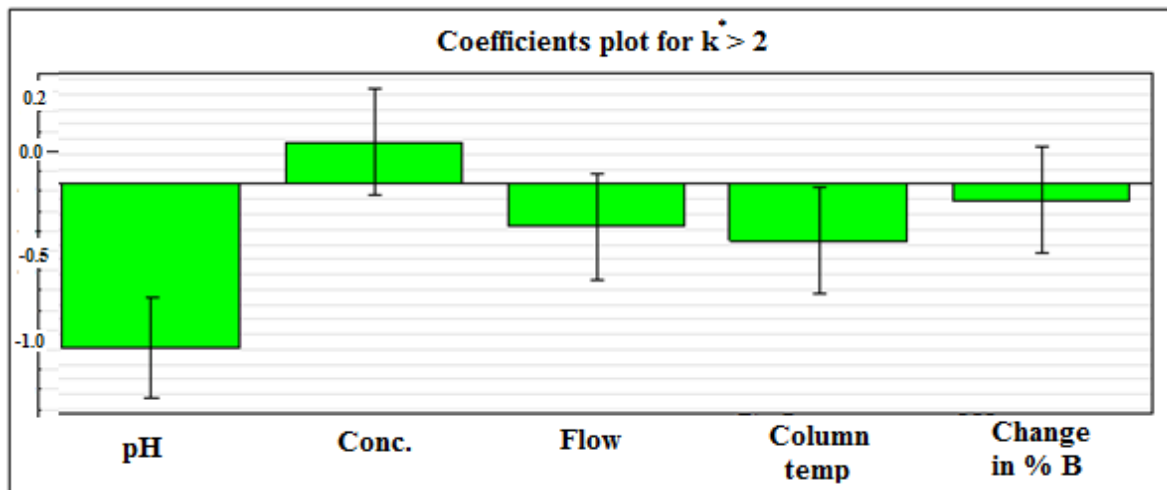
Figure 2.9: *Coefficient plot for the % of peaks with k*>2*

Figure 2.10 shows the coefficient plot for the response for the % of peaks with plate count >2,000, indicating that pH also had a large negative effect on the % of peaks with plate count >2,000. Buffer concentration was the next most influential factor with a positive effect. Column temperature and % change in mobile phase B per minute had a smaller negative effect on the % of peaks with plate count >2,000 and buffer concentration demonstrating a small positive effect.
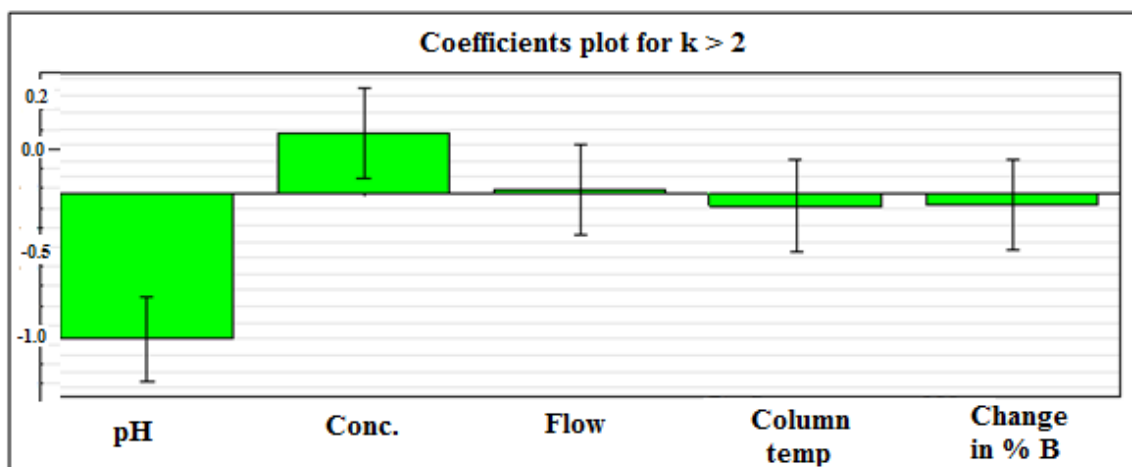
Figure 2.10: *Coefficient plot for the % of peaks with plate count > 2,000*

A contour plot was compiled to indicate/predict the conditions for optimum chromatographic results. The contour plot is a graphical tool, which utilizes the regression coefficients to make predictions of where to position new experiments. Contour plots were used to indicate/predict where the 'optimum' chromatographic results can be attained with respect to each factor. The 3 less influential factors (flow rate, column temperature and change in % B per minute) were held constant and the effect of pH and buffer concentration on all 4 responses are observed in Figure 2.11.
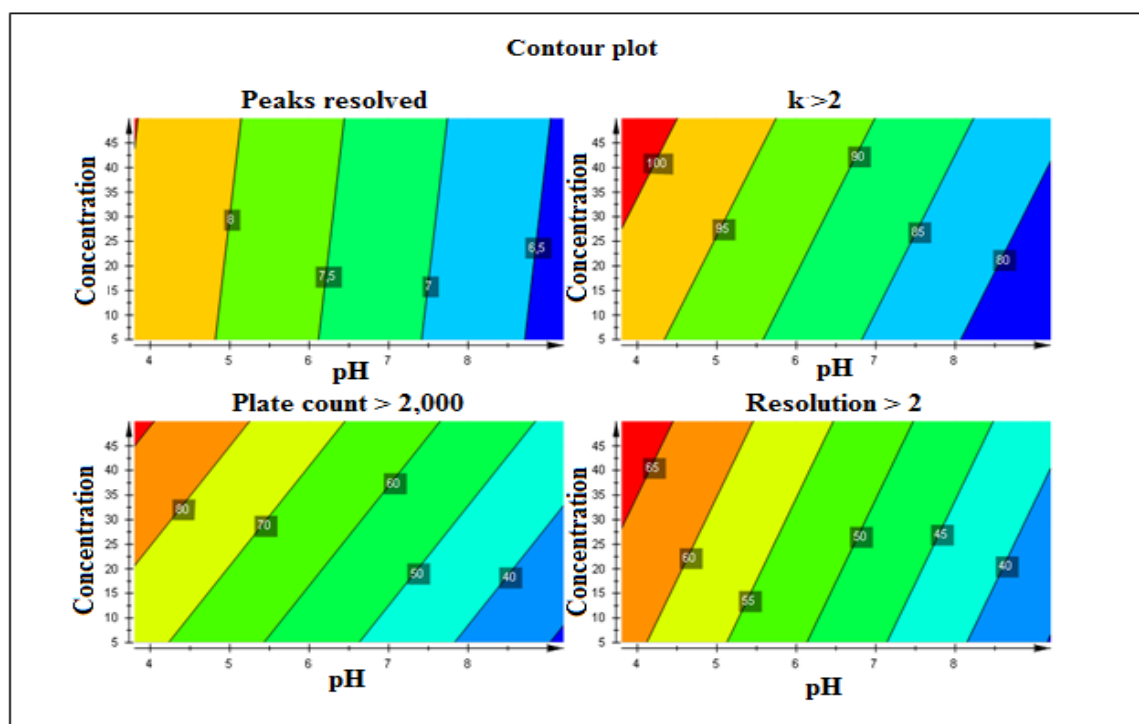
Figure 2.11: *Contour plot for all chromatographic performance criteria with varying pH and buffer concentration. (The flow rate was 0.4 mL/min, column temperature was 35ºC, the % B change/min was set at 0.7 % change/minute).*

The red region is the optimum region in the contour plot demonstrating the highest overall score (i.e. chromatographic conditions which resulted in the greatest number of peaks resolved, greatest number of peaks with a resolution with their nearest neighbour > 2, greatest number of peaks with retention factor (k*) > 2 and the greatest number of peaks with tailing factors < 2). Conversely, the blue region represents the opposite scenario, i.e. poorest chromatographic performance. Based on the three coefficients plots and the contour plots, pH is the most influential on the responses, with a low range pH giving the optimum results. Buffer concentration had a small effect on the responses, with a larger buffer concentration potentially giving the optimum results. The flow rate, column temperature and percentage change in mobile phase B per minute all have minimal influence on the response, however a slower flow rate, low column temperature and a smaller percentage change in mobile phase B per minute potentially gives the optimum results.

2.3.3 Evaluation of optimum pH range and column temperature

Based on the DoE in Section 2.3.2, a low pH range was recommended for the best chromatographic results. The pK_a of ammonium formate is 3.8 and as a result, the pH range that was evaluated was 2.8 to 4.8, within the allowed criteria of +/- 1.0 pH units of the pK_a value [61].
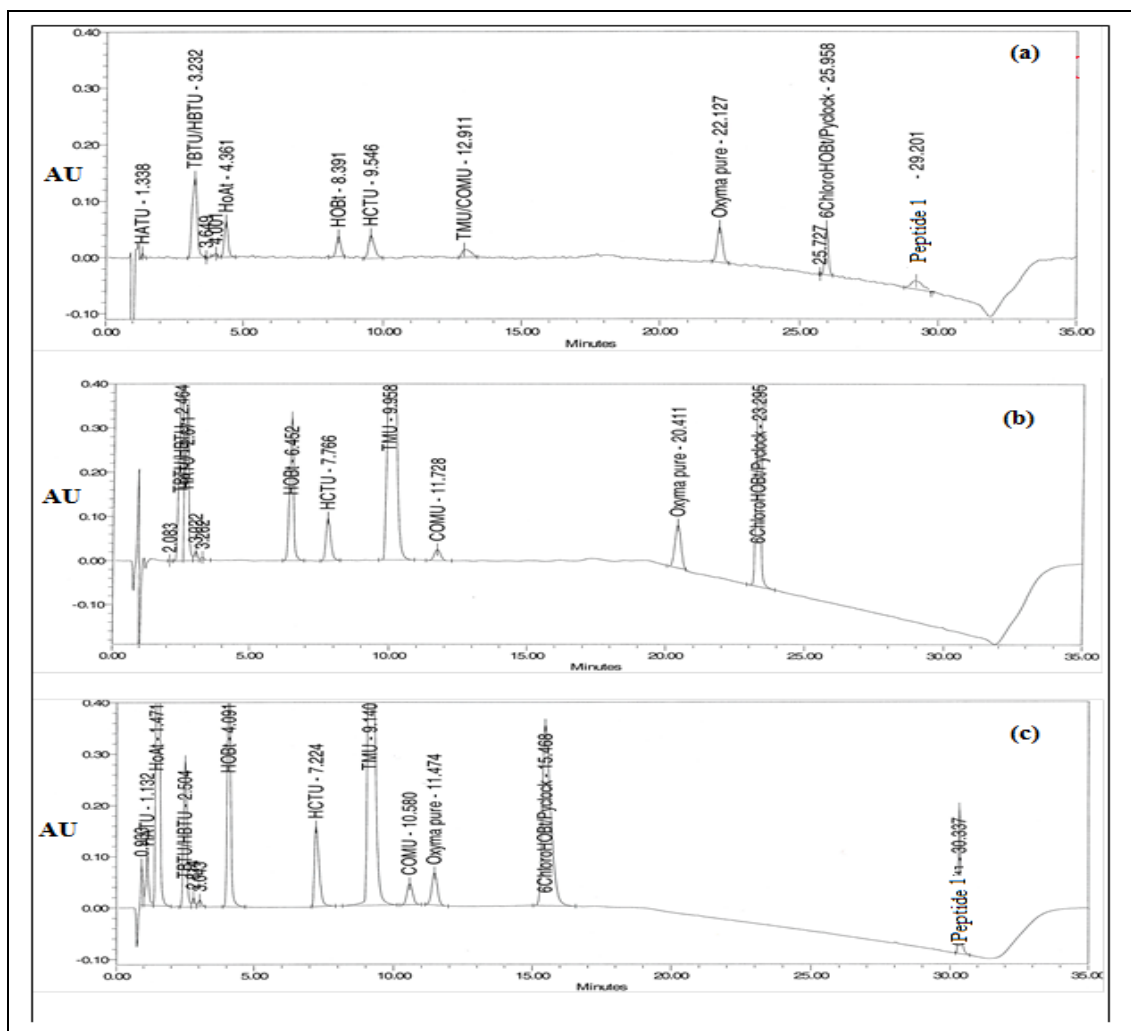
Figure 2.12: *Effect of buffer pH upon retention. Buffer pH was (a) pH 2.8, (b) 3.8 and (c) 4.8, all at a column temperature of 35 °C (Mobile phase: 10 mM ammonium formate and acetonitrile, column: YMC Triart C18, flow rate: 0.3 mL/min, injection volume: 5 µL, detection wavelength: 220 nm and gradient profile: A15 in Appendix 1).*

The DoE outlined that the effect of column temperature was minimal, with lower column temperature potentially giving the optimum results. In order to evaluate the pH range of 2.8 to 4.8 and to further evaluate temperature under these conditions, a mix containing 'Sample set A' was evaluated in terms of resolution, plate count, tailing, retention factor (k*) and absorbance response at pH 2.8, 3.8, and 4.8, each at temperatures of 25 °C, 35 °C and 45 °C. Figure 2.12 demonstrates the effect of change of pH from 2.8 to 4.8 on the separation of the analytes at 35 °C. The retention of early eluting peaks increased as pH decreased, however the higher pH resulted in better resolution of early eluting

peaks. The small changes in pH had a significant impact on the separation due to the analytes being ionisable. The increase in retention at lower pH values was expected because decreasing the pH results in the analytes becoming more non-polar which therefore resulting in better retention. The experiment at pH 2.8 gives a poor absorbance response and noisy baseline relative to higher pH. This is possibly due to the large amount of formic acid added to the mobile phase to achieve the low pH. The experiment at pH 3.8 resulted in the co-elution of early eluting peaks and therefore pH 4.8 was chosen as the optimum pH.

Figure 2.13 demonstrates the effect of temperature 25 °C, 35 °C and 45 °C at a pH of 4.8. The retention of early eluting peaks increased as temperature decreased and the lower temperature was also optimum for the separation of the critical peak pair of TMU and COMU.
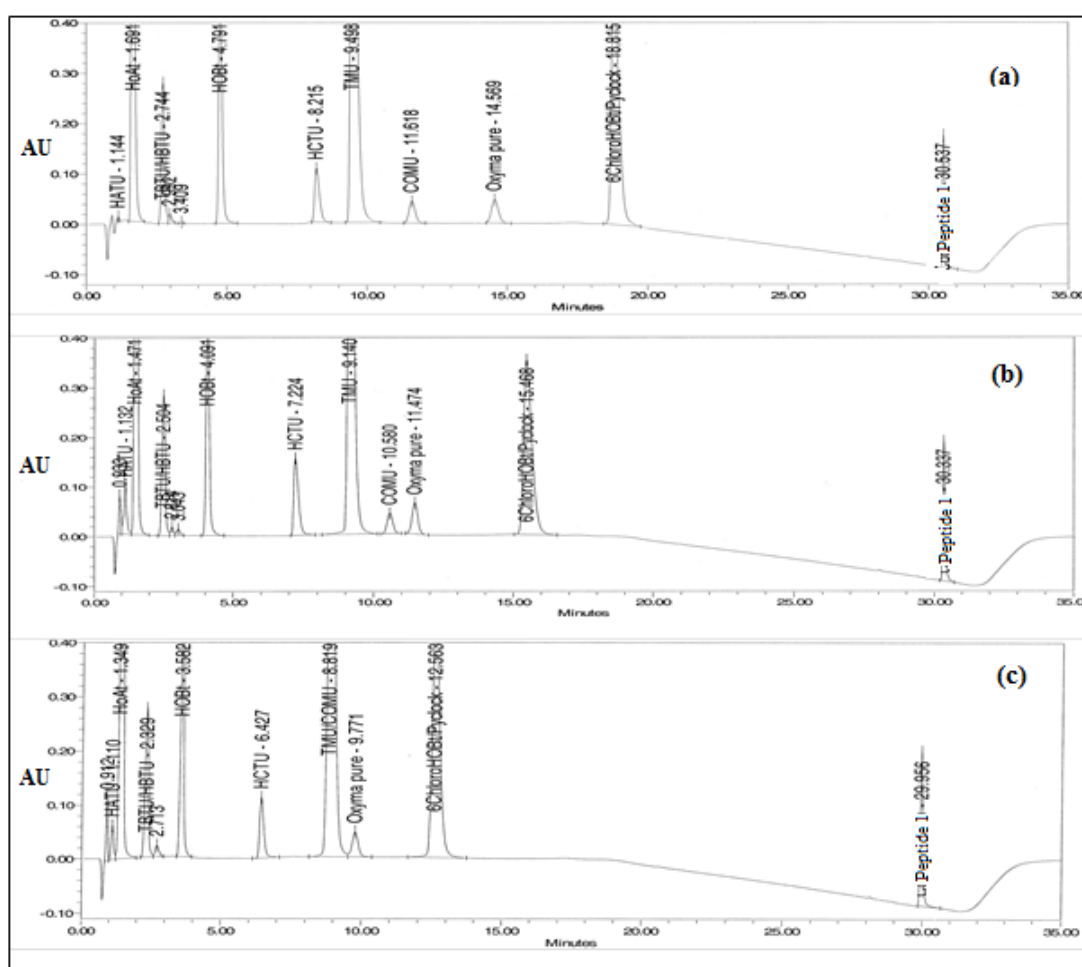
Figure 2.13: *Effect of column temperature upon retention at pH 4.8. Column temperature was (a) 25 °C, (b) 35 °C and (c) 45 °C. (Mobile phase: 10 mM ammonium formate and acetonitrile, column: YMC Triart C18, flow rate: 0.3 mL/min, injection volume: 5 µL, detection wavelength: 220 nm and gradient profile: A15 in Appendix 1).*

2.3.4   Evaluation of column temperature using 10 mM ammonium formate pH 4.8

Using the optimised pH of 4.8 and buffer concentration of 10 m*M*, the entire column temperature range was evaluated from 10 °C to 50 °C (in 5 °C intervals) to ensure 25 °C was the optimum temperature, as determined in Section 2.3.3. Figure 2.14 demonstrates the effect of temperature change and as determined in Section 2.3.3, the retention of the first peak is greater at lower temperatures. The main impact of temperature on the separation is the effect on the critical peak pair. The critical peak pair changes from HCTU and TMU at low column temperature, to COMU and Oxyma Pure at 30 °C, and then to TMU and COMU above 40 °C column temperature. Based on this, the optimum column temperature was confirmed to be 25 °C.
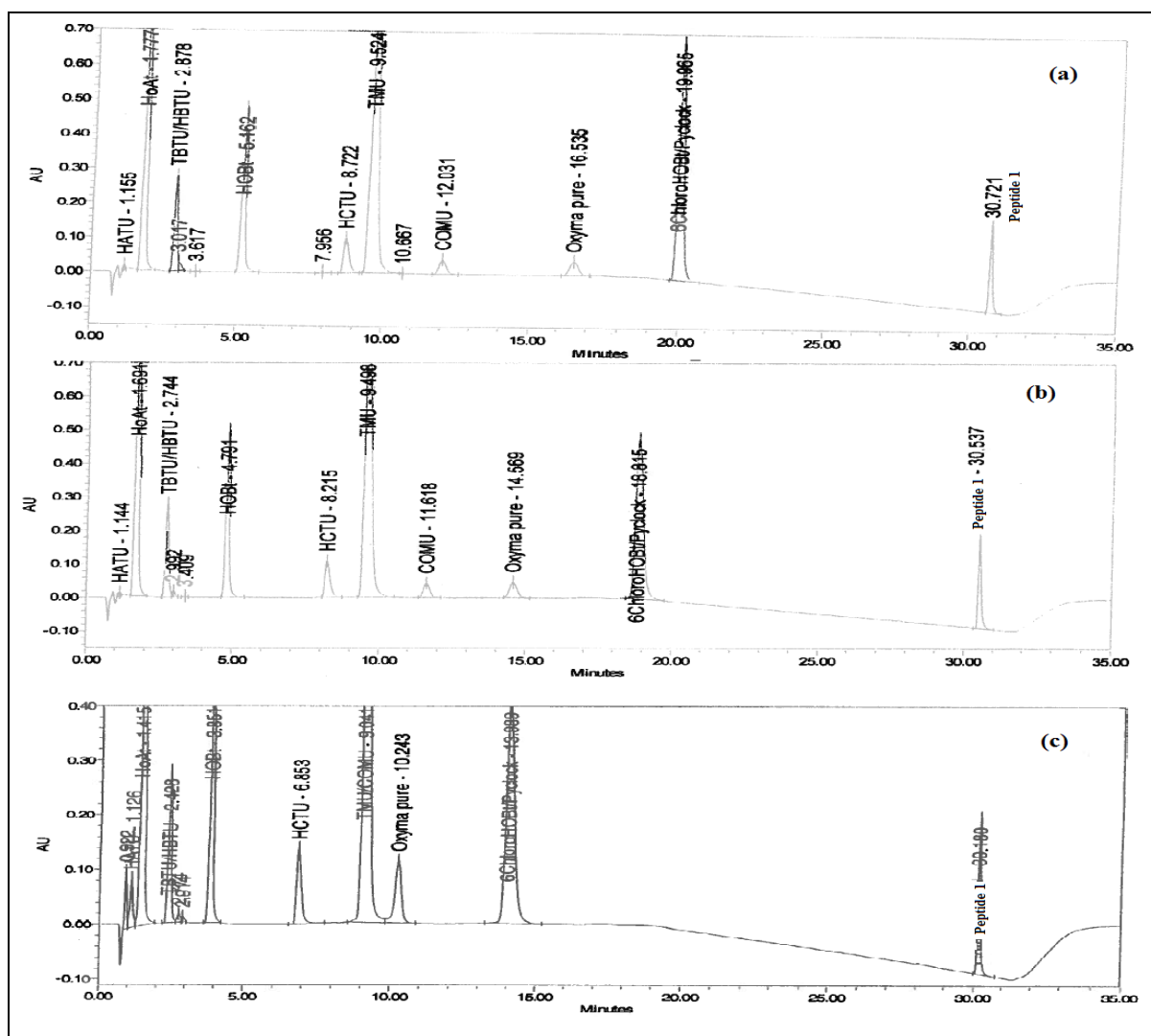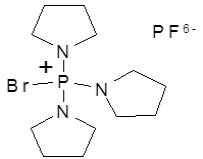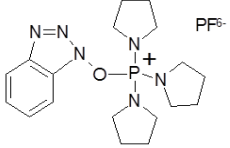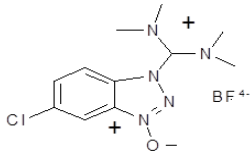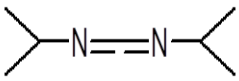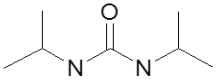
Figure 2.14: *Comparison of column temperature of (a) 15 °C, (b) 25 °C and (c) 40 °C. (Mobile phase: 10 mM ammonium formate and acetonitrile, column: YMC Triart C18, flow rate: 0.3 mL/min, injection volume: 5 μL, detection wavelength: 220 nm and gradient profile: A15 in Appendix 1).*

2.3.5   *Analysis of additional coupling reagents*

During the method development, additional coupling reagents were introduced into the scope of the method development due to investigational work on early development compounds within Ipsen Manufacturing Ireland LTD. Sample information was therefore required on the additional reagents, as per Section 2.3.1. The chemical structure of each additional reagents of 'Sample set B' is outlined in Table 2.20.

Table 2.20 *Chemical structure of additional peptide coupling reagents and by-products added to the study.*

Compound	Structure	Details
PyBrOP [101, 83]		Molecular weight: 466.2g mol^{-1}
PyBOP [48,77]]		Molecular weight: 520.39g mol^{-1}
TCTU [31,90]		Molecular weight: 355.53g mol^{-1}
DIC [101,91]		Molecular weight: 126.2 mol^{-1}
DIU [78,113]		Molecular weight: 144.2 mol^{-1}

The solubility and order of solvent addition to dissolve each of these reagents was assessed as per Section 2.2.3 and the results are outlined in Table 2.21 and Table 2.22. DIC is a solution and was fully miscible in all solutions at each concentration, as determined by a visual inspection.

Table 2.21 *Solubility of additional reagents added to the study*

Compound	Solvent	Description	Approx solubility concentration
TCTU	0.1 M AcOH	Slightly soluble	1 mg/mL
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Slightly soluble	1 mg/mL
	ACN	Soluble	33.33 mg/mL
PyBOP	0.1 M AcOH	Very slightly soluble	0.1 mg/mL
	H_2O	Very slightly soluble	0.1 mg/Ml
	MeOH	Slightly soluble	1 mg/mL
	ACN	Freely soluble	100 mg/mL
PyBrOP	0.1 M AcOH	Very slightly soluble	0.1 mg/mL
	H_2O	Very slightly soluble	0.1 mg/mL
	MeOH	Slightly soluble	1 mg/mL
	ACN	Freely soluble	100 mg/mL
DIU	0.1 M AcOH	Slightly soluble	1 mg/Ml
	H_2O	Slightly soluble	1 mg/mL
	MeOH	Soluble	33.33 mg/mL
	ACN	Sparingly soluble	10 mg/mL

Table 2.22 *Order of diluent addition for dissolving additional reagents.*

Product	Initial diluent	Second diluent	Third diluent
TCTU	ACN	MeOH	H_2O
PyBOP	ACN	MeOH	H_2O
PyBrOP	ACN	MeOH	H_2O
DIU	MeOH	ACN	H_2O

2.3.5.1   Determination of optimum detection wavelength for additional reagents

Each additional reagent was evaluated between 210 nm and 400 nm on a photodiode array to ensure they could be detected at the selected wavelength of 220 nm. Figure 2.15 demonstrates that DIU and PyBOP have minimal absorbance at 220 nm and therefore the wavelength for analysis was changed to 215 nm.
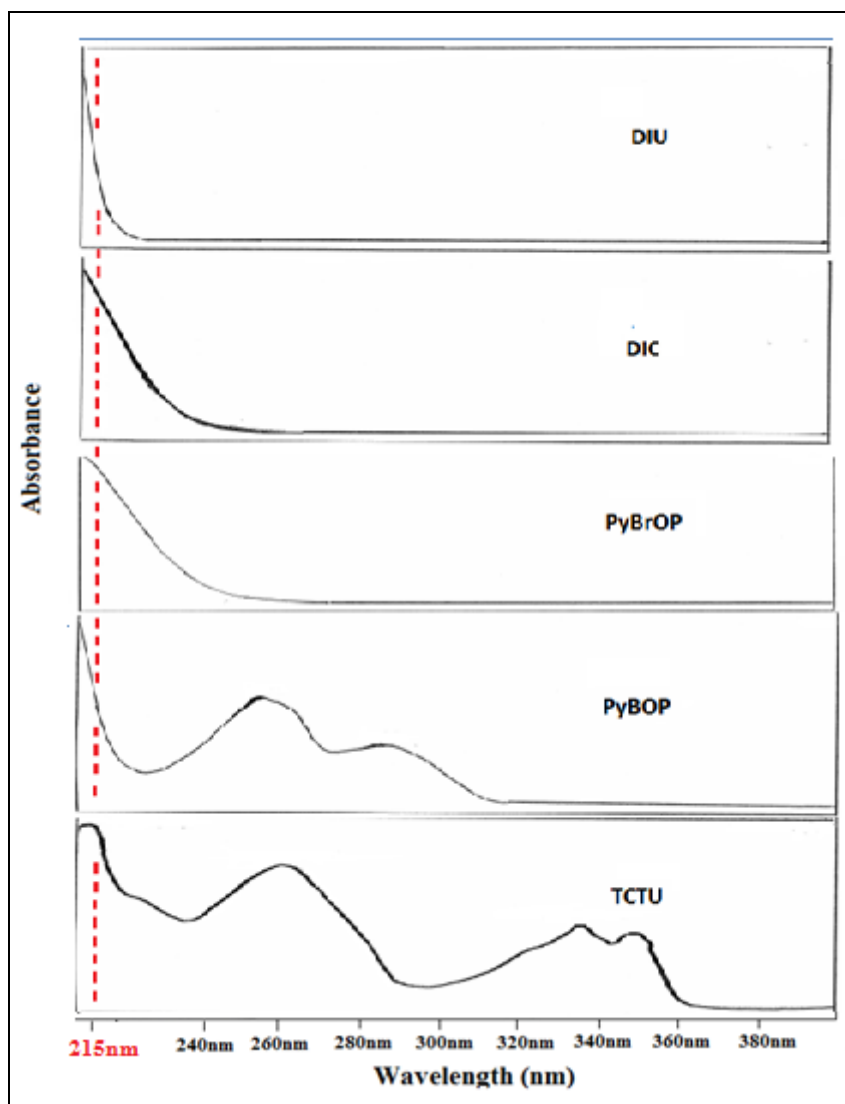
Figure 2.15: *PDA profile of additional analytes. The red dotted line represents the optimum wavelength.*

2.3.6   *Optimisation of buffer pH for separation of 'Sample set B'*

The addition of extra reagents to the study necessitated the re-optimisation of buffer pH. A mix containing 'Sample set B' was evaluated in terms of resolution, plate count, tailing, retention factor (k*) and sensitivity at pH 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.8, 4.3 and 4.8. The pH affects the selectivity and the critical peak pair change and a pH of 3.3 was chosen as the optimum based on the results outlined in Figure 2.16. Table 1 in Appendix 3 details that that lowest resolution (resolution=1.3) was achieved using pH 3.3 (with the exception of pH 3.8, for which resolution could not be calculated), however as observed in Figure 2.16, an increase in the pH resulted in a change of critical peak pair and a significant reduction in k of the first peak. A lower pH than 3.3 resulted in an increase in peak tailing and therefore pH 3.3 was chosen as the best option to meet the chromatographic criteria for both critical peak pairs of TBTU/HBTU and HoAt and HoBt and HCTU.
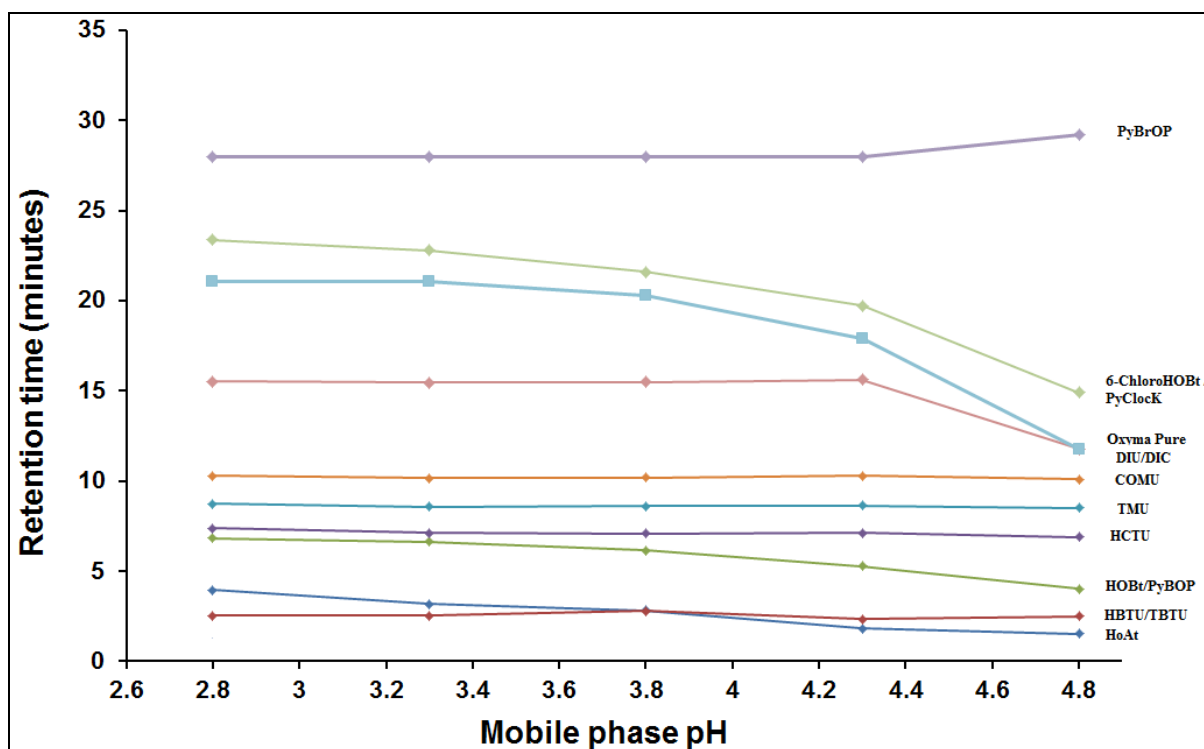
Figure 2.16: *Effect of mobile phase pH upon retention for Sample set B. (Mobile phase: 10 mM ammonium formate and acetonitrile, column: YMC Triart C18, flow rate: 0.3 mL/min, injection volume: 5 µL, column temperature: 25ºC, detection wavelength: 215 nm and gradient profile: A15 in Appendix 1).*

2.3.7   Optimisation of flow rate for Sample set B

The flow rate of the mobile phase was evaluated for the separation of all reagents in 'Sample set B'. A mix containing each reagent in 'Sample set B' were evaluated in terms of resolution, plate count, tailing, retention factor (k*) and absorbance response at flow rates of 0.25 mL/min, 0.30 mL/min and 0.35 mL/min. The higher flow rate resulted in better resolution of the critical peak pair; however the lower flow rate resulted in a better retention of early eluting peaks as shown in Table 2 Appendix 3. As a result, a flow rate of 0.30 mL/min was selected to give the optimum results for both resolution and plate count.

2.3.8   Re-evaluation of column temperature for Sample set B

The column temperature was re-evaluated following the addition of new peptide reagents to determine the impact of temperature on results. Each reagent was evaluated in terms of resolution, plate count and retention factor (k*) at column temperature 10 °C – 45 °C using a 10 m*M* ammonium acetate buffer pH 3.3. Increasing temperature decreased the plate count and k* of the first peak as detailed in Table 3 in Appendix 3. It had limited effect on tailing and resolution of the critical peak

pair was suspected to be better at a mid-range temperature and therefore 25 °C was chosen as the optimum temperature.

2.3.9 Evaluation of final percentage acetonitrile required for the gradient

An investigation was performed to determine the effect of percentage acetonitrile on the last eluting peak, PyBrOP. Three experiments were performed by varying the final percentage of the acetonitrile within the same time frame. Table 4 in Appendix 3 details that the retention time (R_t) of PyBrOP decreased with increased % acetonitrile. Significant tailing was observed for a final % acetonitrile of 30 % (tailing = 2.0). This is possibly due to the elution strength of acetonitrile not being strong enough to elute PyBrOP effectively such that it was slowly eluted from the column, resulting in tailing. There is no difference in tailing between 40 % and 50 % acetonitrile and as a result, a minimum of 40 % acetonitrile was selected as the final percentage acetonitrile to ensure timely elution of PyBrOP.

2.3.10 Evaluation of optimum buffer concentration

The concentration of ammonium formate was evaluated following the selection of optimum pH, flow rate and column temperature. Each reagent was evaluated in terms of resolution, plate count and retention factor (k*) at ammonium formate concentration 5 m*M* to 40 m*M*. Table 5 in Appendix 3 demonstrates that an increase in buffer concentration resulted in an increase in the resolution of the critical peak pair and also an increase in k* value of the first peak. Buffer concentration appears to have minimal effect on plate count and tailing. However, increasing concentration had a significant impact on AU response (from absorbance = 2.4 AU to absorbance = 0.2 AU), with higher concentration resulting in poor AU response. As a result, a concentration range of < 15 m*M* was required to ensure a high AU response was achieved. Since low concentration of ammonium formate resulted in a poor resolution between the critical peak pair, a mid-range concentration of 15 m*M* was chosen to obtain the optimum compromise between both criteria.

2.3.11 Final optimisation of buffer pH for Sample set B (to account for AU response)

The previous evaluation of pH of the mobile phase did not include the assessment of AU response. As a result of the effect of concentration on AU response, the pH of the mobile phase was re-evaluated to ensure the optimum pH has been chosen for all of the investigative parameters. It was decided to widen the scope of the pH to include additional pH data points within the allowed range of 2.8 to 4.8 pH. Table 6 in Appendix 3 demonstrates that increasing pH results in the increase of resolution of the critical peak. A mid-range pH from 3.6 to 3.9 gave the optimum plate count result, with higher and lower pH of five a lower plate count result. A pH of 2.8 to 3.8 resulted in the same k

value for the first peak, with k* value decreasing as pH increases from 3.8 to 4.8. A pH of 3.8 to 4.8 resulted in the same high AU response, with a decrease in AU response value as pH decreases from 3.8 to 2.8. A change in pH had minimal effect on tailing. The most important response was increasing AU because other parameters can be altered by changing the gradient profile and as a result, the optimum pH was determined as 4.2.

2.3.12 Evaluation of chromatographic gradients for separation of peptide synthesis reagents and by-products

Until this point in the chapter (method development following the addition of further reagents to the study), all chromatographic parameters were optimised with the exception of the gradient. As a result, the final step of method development for the separation of Sample set B was gradient optimisation as described below. Using a buffer of 15 mM ammonium formate pH 4.2 as mobile phase A, gradients were varied in terms of hold times and percentage of mobile phase B, to determine the optimum gradient for the resolution of the critical peak pair as well as other chromatographic parameters (adequate retention of the first peak, tailing). The flow rate was also modified within gradients to evaluate the impact of flow rate changes throughout the gradient profile. Table 7 in Appendix 3 demonstrates that the gradient had very little impact on tailing and AU response. The flow rate and acetonitrile concentration had a significant impact on the separation. The optimum results were determined using gradient 12, as detailed in Table 2.30.

Table 2.23 *Final optimised gradient program.*

Time (min)	Flow rate (mL/min)	Mobile phase A	Mobile phase B
Initial	0.275	97 %	3 %
9.00	0.275	97 %	3 %
9.01	0.3	97 %	3 %
12.00	0.3	92.5 %	7.5 %
12.01	0.4	87.5 %	12.5 %
16.00	0.4	60 %	40 %
22.00	0.4	97 %	3 %
23.00	0.4	97 %	3 %
26.00	0.275	97 %	3 %

2.3.13 *Final method for the detection of 14 peptide coupling reagents, additives and associated by-products*

Following investigation of multiple columns, several mobile phase systems and the refinement of the method parameters such as mobile phase concentration, pH, column temperature and gradient flow rate, a UHPLC method was established for the simultaneous determination of peptide coupling reagents, additives and by-products used in peptide synthesis. Coupling reagents HBTU and TBTU differ only by their counter-ion and are therefore detected as the same peak. This is also the same case for HCTU and TCTU. PyBOP is not stable and as a result, it is detected as its more stable by-product HOBt. DIC is also unstable and results in the formation of DIU, which it is detected as. This is also the case for PyClocK, which degrades to its more stable by-product, 6-ChloroHOBt. The critical peak pair of the final method is Oxyma Pure and 6-ChloroHOBt/PyClock, with a resolution of 2.9. The final method is compatible with mass spectrometry and the chromatogram is shown in Figure 2.17.
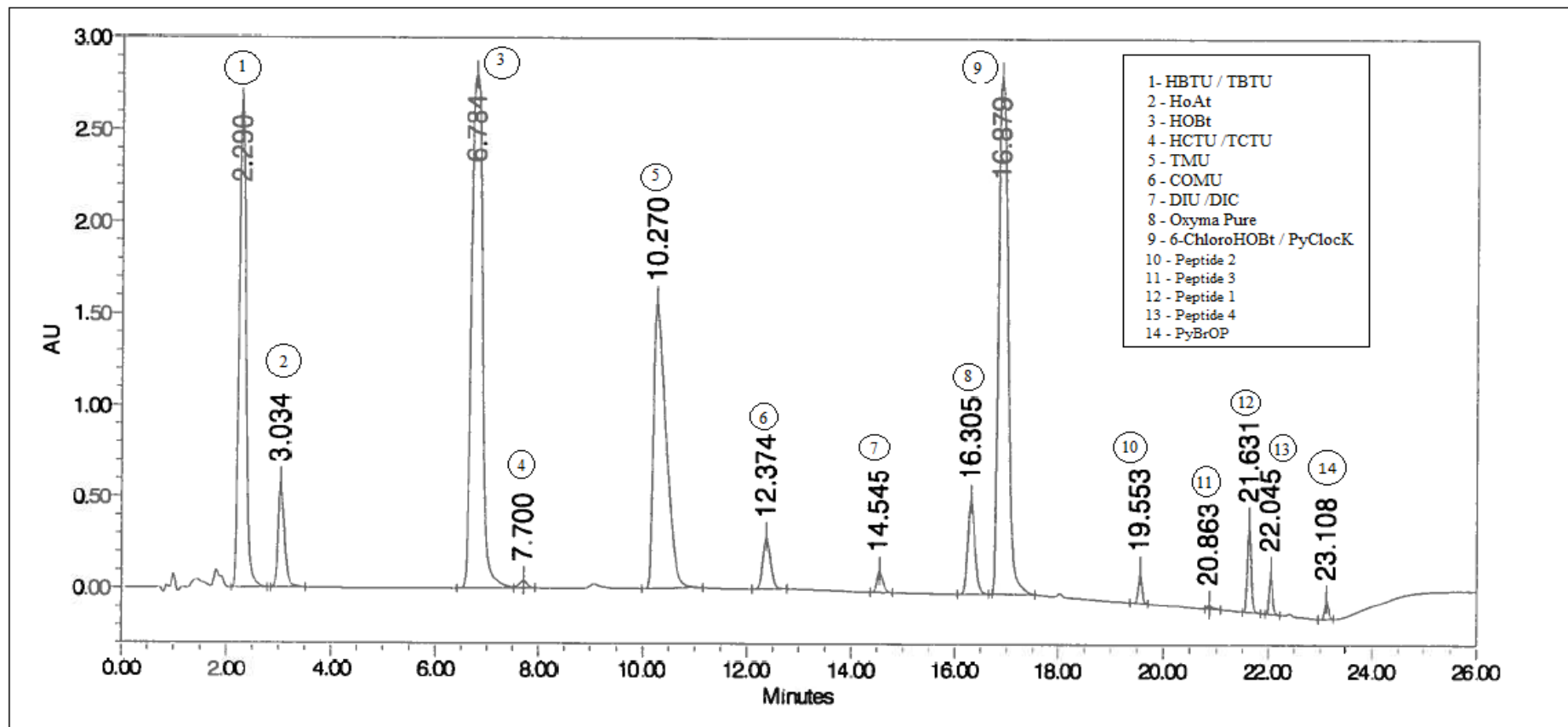
Figure 2.17: *Final optimised separation for the detection of 14 peptide coupling reagents, additives and associated by-products in the presence of 4 IMIL peptides (Column: YMC Triart C18, Mobile phase A: 15 mM ammonium formate pH 4.2, Mobile phase B: acetonitrile, flow rate: 0.3 mL/min, injection volume: 5 µL, column temperature 25ºC, detection wavelength: 215 nm and gradient profile: Table 2.32)*

2.4   Conclusions

In conclusion, a fast, reliable ultra-high performance liquid chromatography method for the simultaneous determination of peptide coupling reagents, additives and by-products used in peptide synthesis, has been developed. Using a YMC Triart reverse-phase UHPLC column with particle size of 1.9 µm, the UV assay can detect 14 commonly used peptide synthesis reagents in the presence of 4 peptides within a run time of 26 minutes. This rapid UHPLC method is directly transferable onto LC-MS and offers significant advantages over current HPLC methods with long run times and methods that can only detect single analytes.

Chapter 3

The validation of a UHPLC method for the analysis of peptide coupling reagents, additives and associated by-products during peptide synthesis

3.1   Introduction

The determination of peptide coupling reagents, additives and associated by-products is important during peptide synthesis to ensure the concentration of these products are below the threshold of toxicological concern in the final peptide. The analytical method used to generate results about the characteristics of drug related samples needs to be reliable and generate accurate results. TMU, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, PyClocK, Oxyma Pure COMU, DIU, DIC, PyBOP, and TCTU are peptide coupling reagents, additives and associated by-products commonly associated with peptide synthesis. In order to monitor these products during a peptide synthesis campaign, a quantitative detection method was developed, as detailed in Chapter 2. The intended application of this method is for evaluation of all stages of peptide manufacture to ensure the removal of these products, and therefore method validation is required to ensure the method can be accurately used to provide precise results. Therefore this chapter presents the validation of a rapid liquid chromatography method for the in-process determination of fourteen peptide coupling reagents used in peptide synthesis. The analytes chosen were either used in the manufacture of some commercial peptides or were under investigation for the manufacture of peptides in development. Analytical method validation is achieved by performing testing on a number of validation characteristics such as specificity, accuracy, linearity, precision, detection limit, quantitation limit and robustness, as per ICH guidelines.

Note: The work described in this chapter can be broadly divided into three discrete sections. Experimental work began with the optimised gradient discussed at the end of Chapter 2. Preliminary investigation then revealed that buffer concentration had a significant effect upon the method sensitivity for several analytes. The first section of this chapter therefore describes efforts to maximise sensitivity by reducing buffer concentration in the mobile phase. Secondly, PyBrOP was removed from the study due to its demonstrated poor performance as a peptide coupling reagent at Ipsen Manufacturing Ireland LTD. This meant that the original gradient (initially 26 minutes long to facilitate late elution of PyBrOP as shown in Figure 2.18 could be significantly reduced. Therefore, the second section of this chapter describes re-optimisation of the original gradient to allow faster run times, which includes studies into the effect of flow rate, buffer pH, etc., upon resolution. The final section of this chapter deals with the validation of the method for all reagents under investigation (without PyBrOP).

3.2 Experimental

3.2.1 Reagents and standards:

All reagents and standards were as described in Chapter 2.

3.2.2 Instrumentation

All instrumentation was as described in Chapter 2.

3.2.3 Effect of buffer concentration upon sensitivity

The evaluation of the detection limit of each reagent was carried out by dissolving each reagent in 'Sample set B' in the diluent at a concentration of 0.5 mg/mL. A 1/1000 dilution was performed on each solution using diluent and each reagent was analysed on a 100 x 2.0 mm, YMC Triart C18 1.9 µm column using ammonium formate buffers 5 mM, 10 mM and 15 mM (pH 4.2) with acetonitrile as a mobile phase B. A multi-step gradient was employed as detailed in Table 3.1.

Table 3.1 *Optimum gradient profile for buffer optimisation.*

Time (min)	Flow rate (mL/min)	Mobile phase A	Mobile phase B
Initial	0.275	97 %	3 %
9.00	0.275	97 %	3 %
9.01	0.3	97 %	3 %
12.00	0.3	92.5 %	7.5 %
12.01	0.4	87.5 %	12.5 %
16.00	0.4	60 %	40 %
22.00	0.4	97 %	3 %
23.00	0.4	97 %	3 %
26.00	0.275	97 %	3 %

Note: This gradient program is the optimum program as determined in Chapter 2.

An injection volume of 10 µL was also employed for each injection. If no peak was present in the 1/1000 dilution injection, a 1/500 dilution, or a 1/100 dilution was performed (if necessary) and signal:noise ratios were calculated and compared.

3.2.4 Adjustment of mobile phase gradient following removal of PyBrOP

Following the removal of PyBrOP from the test mixture, the method gradient was re-evaluated by analysing 'Sample set B' (without PyBrOP) at a concentration of 0.5 mg/mL. The gradients detailed in Table A21 to A30 in Appendix 1 incorporated the use of a YMC Triart C18 100

x 2.0 mm, 1.9 µm column and 5 m*M* ammonium formate pH 4.2 / acetonitrile as mobile phases. The effect of flow rate (0.20 mL/min to 0.55 mL/min) upon the separation was also evaluated using the optimum gradient.

3.2.5   *Further evaluation of optimum buffer pH*

Following the increase of Peptide 1 and Peptide 2 concentrations from 0.5 mg/mL during method development (Chapter 2) to 5 mg/mL, the optimum pH range was re-evaluated for the ammonium formate buffer at a range of 4.3 to 4.8. This was carried out by analysing a sample containing Peptide 1 at a nominal concentration of 5 mg/mL and each of the peptide coupling reagents, additives and by-products at their specification level of 0.1 % w/w. This was performed with a range of ammonium formate pH values from 4.3 to 4.8 using the multi-step gradient detailed in Table A30 in Appendix 1.

Note:

The analytical validation was performed using either a solution of Peptide 1 at nominal concentration (5 mg/mL) as well as each of the analytes at a level of 0.1 mg/mL set (referred to hereafter as 'Test mix A') a or a solution of Peptide 2 at a nominal concentration (5 mg/mL) as well as each of the analytes at a level of 0.1 mg/mL set (referred to hereafter as 'Test mix B') a, unless otherwise stated. The analysis was carried out using the multi-step gradient detailed in Table A30 in Appendix 1.

3.2.6   *Specificity of the analytical method*

The specificity of the analytical method was evaluated by preparing a solution 'Test mix A' and 'Test mix B'. Each individual reagent, a mixture of both peptides with the reagents and a blank injection of the diluent were injected individually using the optimised chromatographic conditions.

3.2.7   *Accuracy of the analytical method*

The accuracy of the method was evaluated at three different concentration levels in the range of 80 – 120 % of 'Test mix A'. Each of the reagents was also individually diluted to a concentration of 0.1 % w/w relative to 5 mg/mL and injected using the optimised chromatographic conditions.

3.2.8   *Precision of the analytical method*

System precision was evaluated from 6 injections of 'Test mix A'. *Repeatability* was evaluated from 6 replicate preparations the above sample preparation. *Intermediate precision* was evaluated from 6 replicate preparations of the above sample, which were analysed and evaluated

independently by two analysts, on different days, using different instrument set ups (difference UHPLC instrument serial numbers and different mobile phase preparation).

3.2.9   Sensitivity of the analytical method

The limit of detection (LOD) of each of the analytes was evaluated by preparing a 0.1 mg/mL solution and performing serial dilutions until a peak with a height of approximately 3 times the height of the baseline was achieved. The limit of quantitation (LOQ) was evaluated by performing serial dilutions of the above sample until a peak with a height of approximately 10 times the height of the baseline was achieved.

3.2.10   Linearity of the analytical method

The evaluation of linearity of the detector response to each of the analytes involved injecting each of the analytes from their limit of quantitation to 120 % of their specification of 0.1 % w/w. In order to evaluate to relative response factor (RRF) of the analytes compared to the peptide products, Peptide 1 and Peptide 2 were evaluated in the range of 50 % to 120 % of their nominal concentration of 5 mg/mL.

3.2.11   Robustness of the analytical method

The robustness of the analytical method was evaluated from duplicate injections of 'Test mix A' on the method with small variations detailed as follows: buffer concentration (2.5 mM, 3.75 mM and 7.5 mM), buffer pH (pH 4.5, 4.55 and 4.7), column temperature (20 °C and 30 °C), flow rate (0.45 mL/min and 0.55 mL/min) and column to column variation (two YMC Triart C18 columns with different serial numbers: 0210002430 and 0210002540).

3.2.12   Solution Stability

The stability of 'Test mix A' was evaluated over a 135 hour period at ambient and refrigerated storage conditions (2-8 °C) by injecting the test mix at specific timed intervals and evaluating changes in peak area.

3.3   Results and discussion

3.3.1   Effect of buffer concentration upon sensitivity.

Usually, evaluation of method sensitivity is performed toward the end of a validation study, and certainly after the optimum chromatographic conditions have been established (mobile phase composition, gradient profiles etc.). However in this case, preliminary observations during method development revealed that the concentration of buffer in mobile phase A (ammonium formate) appeared to have a significant effect upon sensitivity for certain analytes, most notably DIC, DIU and PyBrOP. Therefore it was considered prudent to more fully investigate the effect of buffer concentration and then re-optimise chromatographic conditions as necessary as described in later stages of this chapter.

A known impurity needs to be detected at levels of 0.1 % w/w and as a result, the minimum requirement for limit of detection for each of the reagents is a 1/100 dilution of a 0.5 mg/mL solution, relative to a 5 mg/mL peptide solution. Therefore, a 1/1000, 1/500 or a 1/100 dilution of a 0.5 mg/mL solution of each analyte was made (equivalent to 0.01 % w/w, 0.02 % w/w or 0.1 % w/w respectively) for this study.

The results of investigations into the effect of buffer concentration upon sensitivity are displayed in Figure 3.1 below. For the most part, analyte concentrations were 0.01 % w/w unless otherwise indicated in the figure caption. For most analytes, sensitivity was maximised with 5 m*M* ammonium acetate relative to higher buffer concentrations with the exception of PyBOP (for which 15 m*M* was best) and HBTU, TCTU and TBTU (for which 10 m*M* was best). Additional studies into the effect of detection wavelength were carried out since the original detection wavelength of 220 nm had to be changed to 215 nm with the addition of DIC and DIU reagents to the study due to their poor UV absorbance, (thereby further exacerbating the effect of buffer concentration on sensitivity at lower wavelengths). Reducing the detection wavelength from 220 nm to 215 nm actually improved sensitivity for most analytes: HOBt by 24 %, PyBrOP: 2,400 %, TMU: 65 %, DIC: 184 %, HCTU: 97 %, PyBOP: 10 %, DIU: 143 %, COMU: 100 %, HBTU: 67 % and TCTU: 43 %. There was an observed reduction in sensitivity for other analytes; 6-ChloroHOBt: 26 %, HOAt: 60 %, PyClock: 58 %, Oxyma Pure: 19 % and TBTU: 29 %.

Nevertheless, a mobile phase buffer concentration of 5 mM, with a detection wavelength of 215 nm was selected as optimum conditions for further study since all analytes (including the initially problematic DIC, DIU and PyBrOP) could be readily detected at 0.01 % w/w. Note: these preliminary sensitivity studies were performed in strictly order to optimise chromatographic conditions, which were further modified in the section which immediately follows. Therefore, a more rigorous examination of method sensitivity is presented toward the end of this chapter, after all method optimisation was completed.
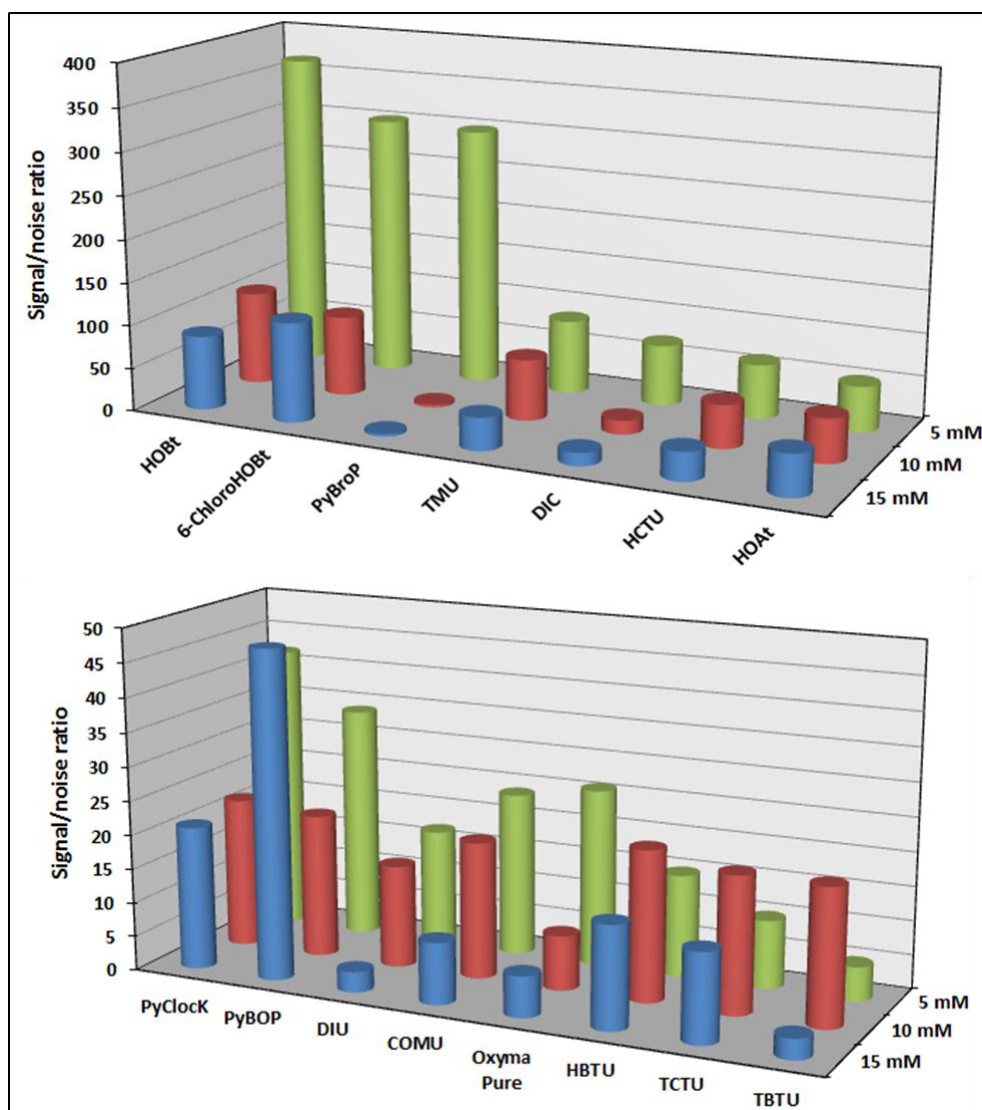
Figure 3.1 *Effect of buffer concentration upon signal:noise ratio for 0.01 % w/w injections of analyte. Note: DIU was injected at 0.02 % w/w and DIC/PyBrOP were present at 0.1 % w/w.*

3.3.2   *Adjustment of mobile phase gradient following removal of PyBrOP*

As can be seen from the final optimised chromatogram displayed at the end of Chapter 2, PyBrOP exhibited significantly later retention relative to any of the other test analytes. This necessitated an extended run time of 26 minutes to ensure that PyBrOP did not co-elute with the peptide APIs. It was therefore fortuitous that Ipsen Manufacturing Ireland LTD., reported poor yield results when PyBrOP was investigated as a peptide coupling reagent. This justified the removal of PyBrOP from the set of analytes under investigation. As a result, there was a considerable scope to significantly reduce the run time from 26 minutes by re-optimisation of the gradient (in terms of hold times and acetonitrile concentrations). Resolution of the critical peak pair (HoAt and HBTU/TBTU),

retention factor of the first peak, tailing and total run time were evaluated for gradients detailed in Table A21 to A30 in Appendix 1, as detailed in Table 3.2.

Table 3.2 *Results of gradient optimisation following the removal of PyBrOP from the mixture*

Gradient	Rs. of critical peak pair	k* of first peak	Tailing - largest result	Run time (mins)
1 (A21)	1.79	2.1	1.8	26
2 (A22)	2.7	0.7	1.8	20
3 (A23)	2.9	2.1	1.7	18
4 (A24)	1.6	1.8	1.5	12
5 (A25)	1.23	1.8	1.4	14
6 (A26)	2.2	2.3	1.4	15
7 (A27)	1.6	2.5	1.3	16
8 (A28)	2.1	2.6	1.4	17
9 (A29)	2.9	2.2	1.5	15
10 (A30)	2.9	2.4	1.6	15

Table 3.2 demonstrates that the reduced run times typically resulted in slightly less peak tailing. Gradient A30 in Appendix 1 was selected as optimum and for the sake of clarity, is presented below in Table 3.3. The corresponding chromatogram is presented below as Figure (b) in Figure 3.2. The total runtime (gradient time) of the method was reduced by 42 % (from 26 minutes to 15 minutes) by the removal of PyBrOP from the test mix of analyse under investigation.

Table 3.3 *Final optimised gradient after removal of PyBrOP from the test mix.*

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.50	97	3
4.00	0.50	97	3
10.00	0.50	92.5	7.5
10.25	0.50	87.5	12.5
11.50	0.50	87.5	12.5
12.00	0.50	60	40
12.50	0.50	97	3
15	0.50	97	3

Gradient optimisation also necessitated an examination of the effect of flow rate upon the separation and resulted are illustrated in Figure 3.2. Specifically, using the optimised gradient, flow rates of 0.2 mL/min to 0.55 mL/min were applied, with lower flow rates (0.2 mL/min, 0.3 mL/min and 0.4 mL/min) resulting in co-elution of early peaks (HOAt and TBTU/HBTU). This is due to

increased retention of peaks at reduced flow rates since the analytes are travelling through the column at a slower pace leading to chromatographic zone broadening and co-elution of closely eluting peaks. The reason for the chromatographic zone broadening is due to unwanted longitudinal diffusion of bands at lower (i.e. non-optimum) flow rates.
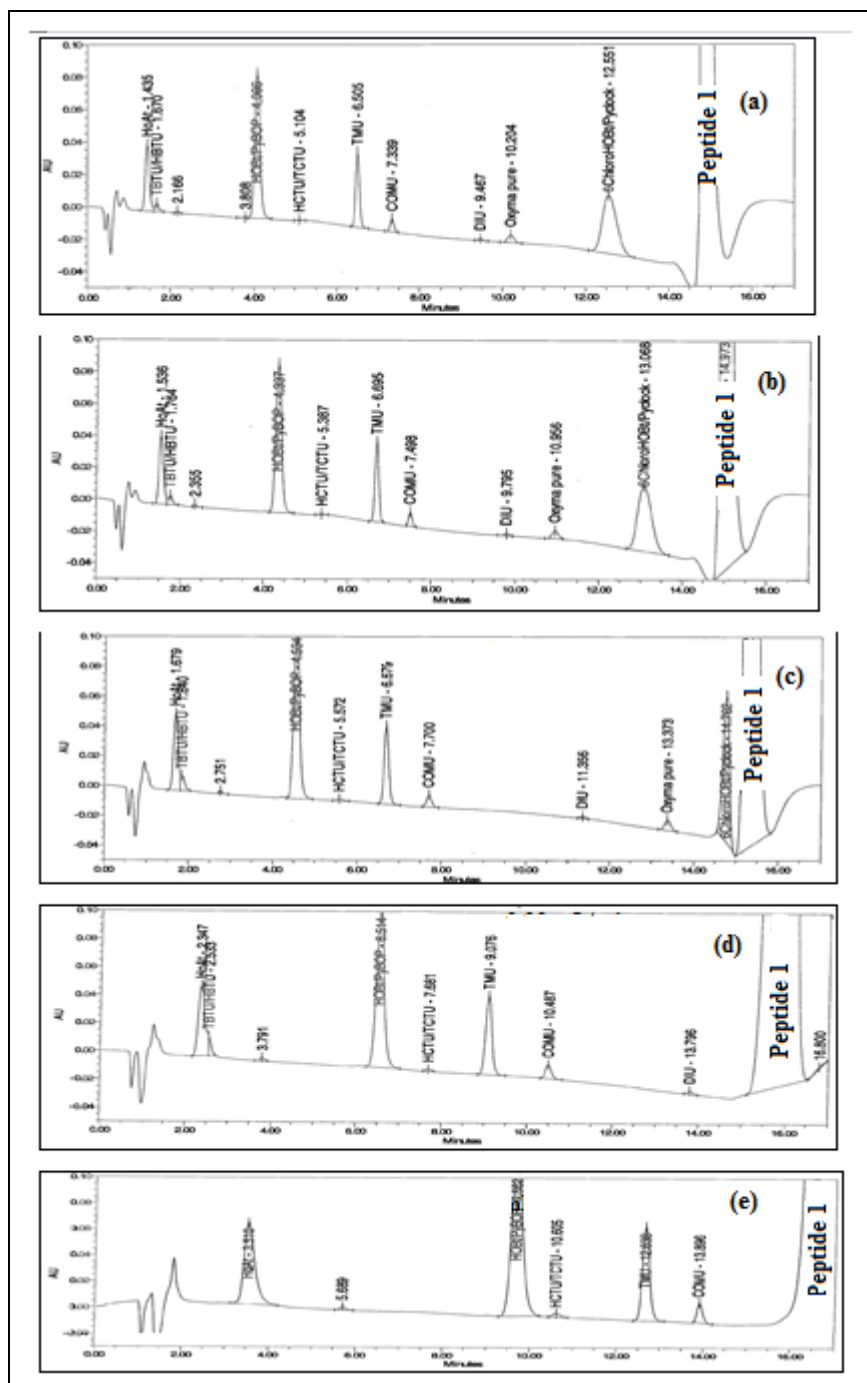
Figure 3.2 *Effect of flow rate upon separation. (a): 0.55 mL/min, (b): 0.50 mL/min, (c): 0.40 mL/min, (d): 0.30 mL/min, (e): 0.20 mL/min. Chromatographic conditions: Column: 100 x 2.0 mm, YMC Triart C18, 1.9 μm d$_p$, Mobile phase A: 5 mM ammonium formate pH 4.2, Mobile phase B: acetonitrile, Gradient program: Table 3.3, Injection volume: 5 μL, Column temperature: 25 ^{o}C, Detection wavelength: 215 nm. Note: DIC is a co-eluting peak with DIU.*

Lower flow rates also resulted in the unacceptably late elution of more strongly retained analytes (6-ChloroHOBt/Pyclock, Oxyma Pure, DIU/DIC), which in extreme cases lead to their co-elution with the main peptide API peak (chromatograms c, d and e in Figure 3.2). An optimum flow rate of 0.50 mL/min was therefore chosen which is illustrated as chromatogram (b) in Figure 3.2, based upon the optimised resolution between HOAt and TBTU/HBTU.

3.3.3 Further evaluation of optimum buffer pH

Peptide 1 and Peptide 2 were evaluated at a concentration of 0.5 mg/mL during method development in Chapter 2, however in order to maximise the quantitation limit for the analytes of interest, the concentration of Peptide 1 and Peptide 2 was increased to 5 mg/mL. Interestingly, this resulted in a decrease in retention for most analytes; 1.2 % for Oxyma Pure, 5.2 % for DIU/DIC, 7.2 % for COMU, 4.8 % for TMU, 6.2 % for HCTU/TCTU and 1.2 % for HOBt/PyBOP. However, the resolution between the critical peak pair (HOAt and TBTU/HBTU) was significantly affected since the reduction in retention time for both peaks was unequal. Specifically, the retention of HOAt decreased by only 3.9 % whereas the retention of TBTU/HBTU decreased by 8.2 % resulting in a loss of resolution between this critical peak pair as shown in Figure 3.3 below. This reduction in retention times occurred due to overloading of the stationary phase with the peptide API.
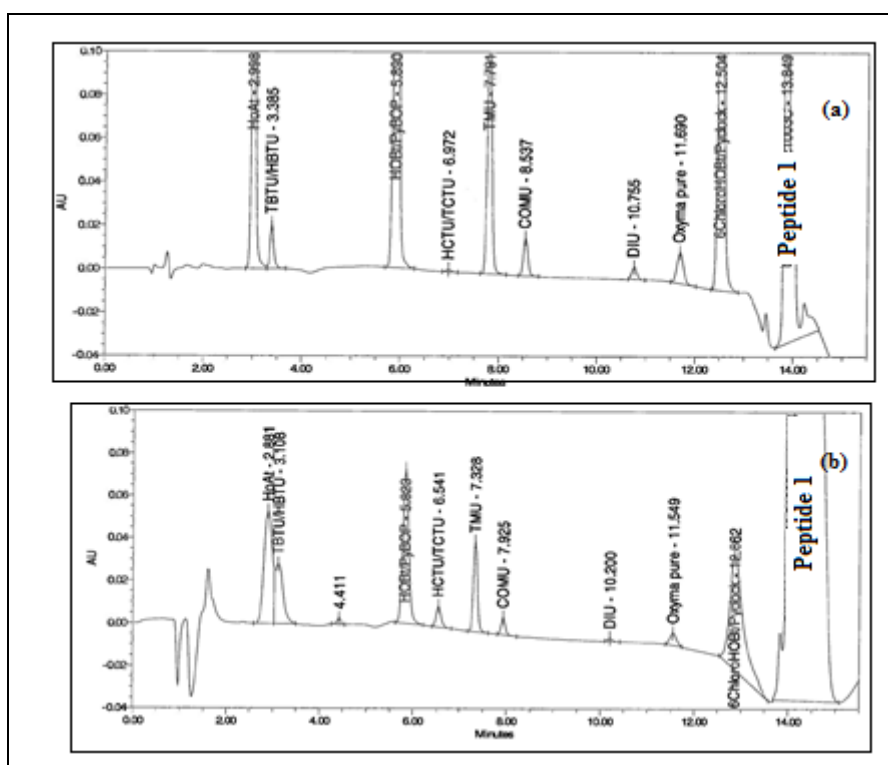
Figure 3.3 *Effect of Peptide 1 concentration upon analyte retention. (a) Mix of reagents with Peptide 1 at 0.5 mg/mL (b) Mix of reagents with Peptide 1 at 5 mg/mL. Chromatographic conditions as in Figure 3.2. Mobile phase flow rate: 0.5 mL/min. Note: DIC is a co-eluting peak with DIU.*

Previous mobile phase pH studies in Chapter 2 indicated that changes in mobile phase pH resulted in selectivity changes for early eluting peaks and so mobile phase pH evaluation was re-visited here. Mobile phase pH was adjusted between pH 4.3 and 4.8 as illustrated in Figure 3.4.
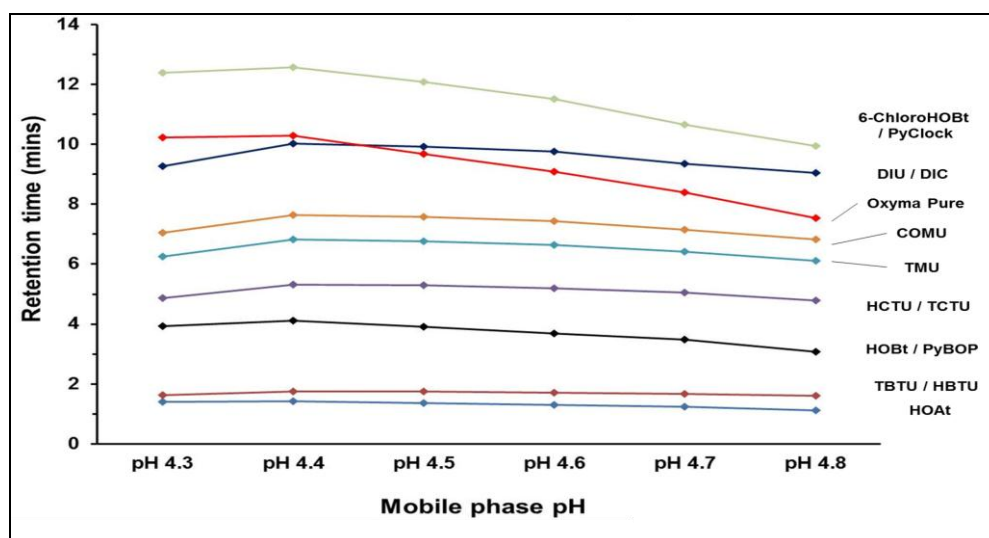
Figure 3.4 *Effect of pH on analyte retention. Chromatographic conditions as per Figure 3.3.*

As can be seen, the critical peak pair at pH 4.3 was HOAt and TBTU/HBTU but with inadequate resolution of < 1.5. Increasing mobile phase pH resulted in a greater decrease in retention of HOAt relative to TBTU/HBTU such that resolution improved correspondingly. Interestingly, increases in mobile phase pH also lead to significant changes in selectivity for the Oxyma Pure and the DIU/DIC peak such that their elution order actually switched at 4.4 – 4.5. This resulted in the emergence of a new critical peak pair in this pH region. Therefore, a mobile phase pH of 4.6 was chosen as optimum resulting in resolution (resolution = 2.3) for the HOAt and TBTU/HBTU peak pair, and (resolution = 3.8) for the Oxyma Pure and DIU/DIC peak pair. The final optimised chromatogram is shown in Figure 3.5 using a mobile phase pH of 4.6. All validation parameters, with the exception of robustness, were evaluated using the chromatographic conditions shown in the caption of Figure 3.5. An injection volume of 5 μL was chosen as the injection volume in order to minimise peak shape distortion for early eluting peaks.
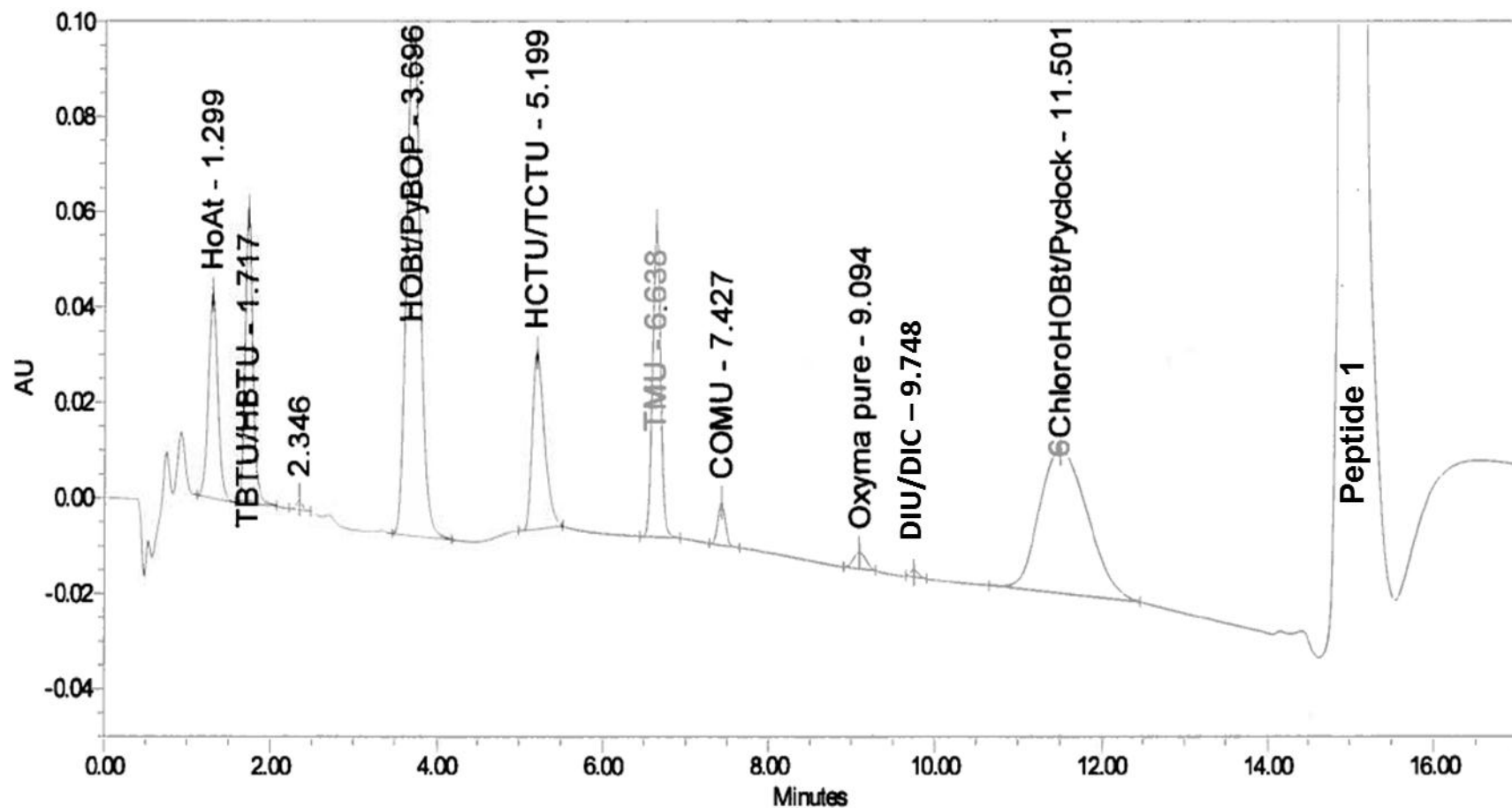
Figure 3.5 *Final optimised separation of fourteen peptide coupling reagents from a selected commercial peptide API. Chromatographic conditions: Column: 100 x 2.0 mm, YMC Triart C18, 1.9 μm d_p, Mobile phase A: 5 mM ammonium formate pH 4.6, Mobile phase B: acetonitrile, Gradient program: Table 3.3, Flow rate 0.5 mL/min, Injection volume: 5 μL, Column temperature: 25 °C, Detection wavelength: 215 nm*

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3.3.4 Validation of analytical method for the determination of peptide coupling reagents, additives and associated by-products

The International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use outlines that a registration application should include documented evidence that the analytical procedures are validated and suitable for the detection and quantification of impurities [115]. The validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose and the ICH has introduced a guideline on how this should be performed [116]. This guideline outlines that the number of validation characteristics that should be considered, depending on the type of method being validated [116]. Analytical methods for testing impurities can be either a quantitative test or a limit test and different validation characteristics are required for a quantitative test relative to a limit test [116]. These validation characteristics include accuracy, precision, repeatability, intermediate precision, specificity, detection and quantitation limit, linearity and range [116].

3.3.4.1 Method validation background

Peptide coupling reagents and by-products are monitored in peptide manufacturing campaigns for development batches and testing of these reagents is also required for the assignation of primary reference standards in two commercial APIs. There is no specification for the peptide coupling reagents and associated by-products in campaigns for clinical batches because the specification is dosage dependent. The specification for peptide coupling reagents and by-products is 0.1% (w/w) for the assignation of a primary reference standard for the two commercial peptide APIs. As a result, the nominal specification was set at 0.1% (w/w) in this validation and this is the specification requirement for known impurities.

The validation was performed on two Ipsen peptides (referred to as "Peptide 1" and "Peptide 2") spiked with reagents. Both peptides were evaluated in the selectivity and linearity sections (which follow hereafter) to demonstrate specificity and to calculate RRF values relative to each peptide, however only Peptide 1 was evaluated in remaining validation parameters.

3.3.5 Specificity of the analytical method

Specificity of an analytical method is the ability to measure an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known degradation products that may be expected to be present in the sample matrix [116]. For chromatographic methods, representative chromatograms demonstrating the discrimination of the peptides from the peptide coupling reagents, additives and associated by-products demonstrates specificity of the analytical method [116]. The specificity acceptance criteria, which states the peak tailing of each peak should be

≤ 2.0, the retention factor (k*) should be ≥ 1.0 and the resolution of all peaks should be ≥ 1.5, were fulfilled and the blank chromatogram was free from interfering peaks, as illustrated in Figure 3.6. The method was determined to be selective with respect to the detection of each of the peptide coupling reagents, additives and by-products in the presence of both Peptide 1 and Peptide 2, as demonstrated in Figure 3.7 and 3.8.
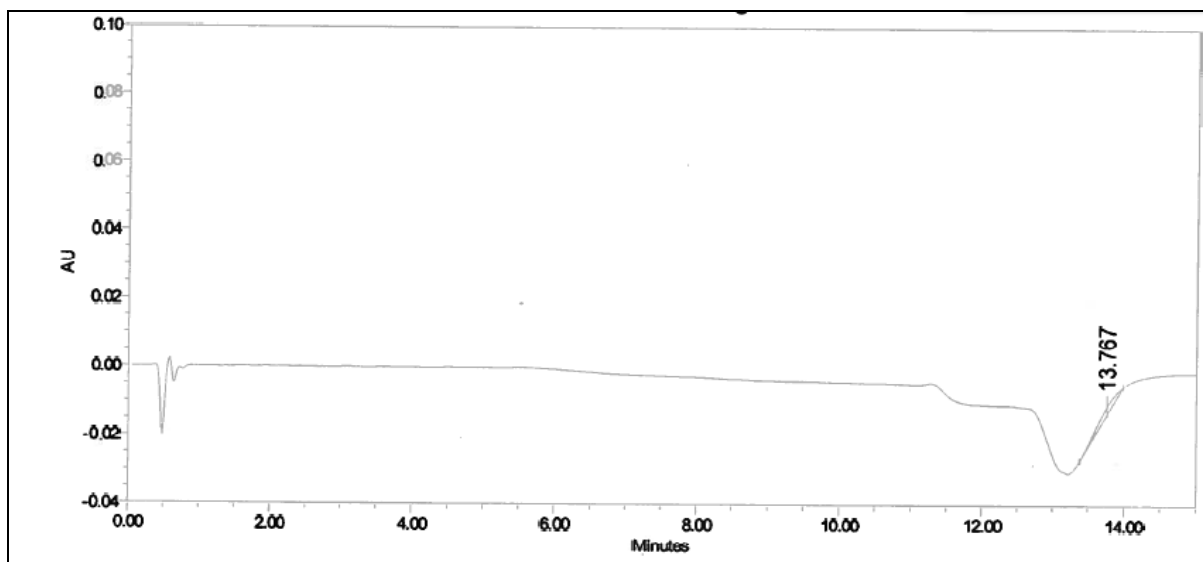
Figure 3.6 *Blank chromatogram demonstrating no interfering peaks present. Chromatographic conditions as shown in Figure 3.5.*
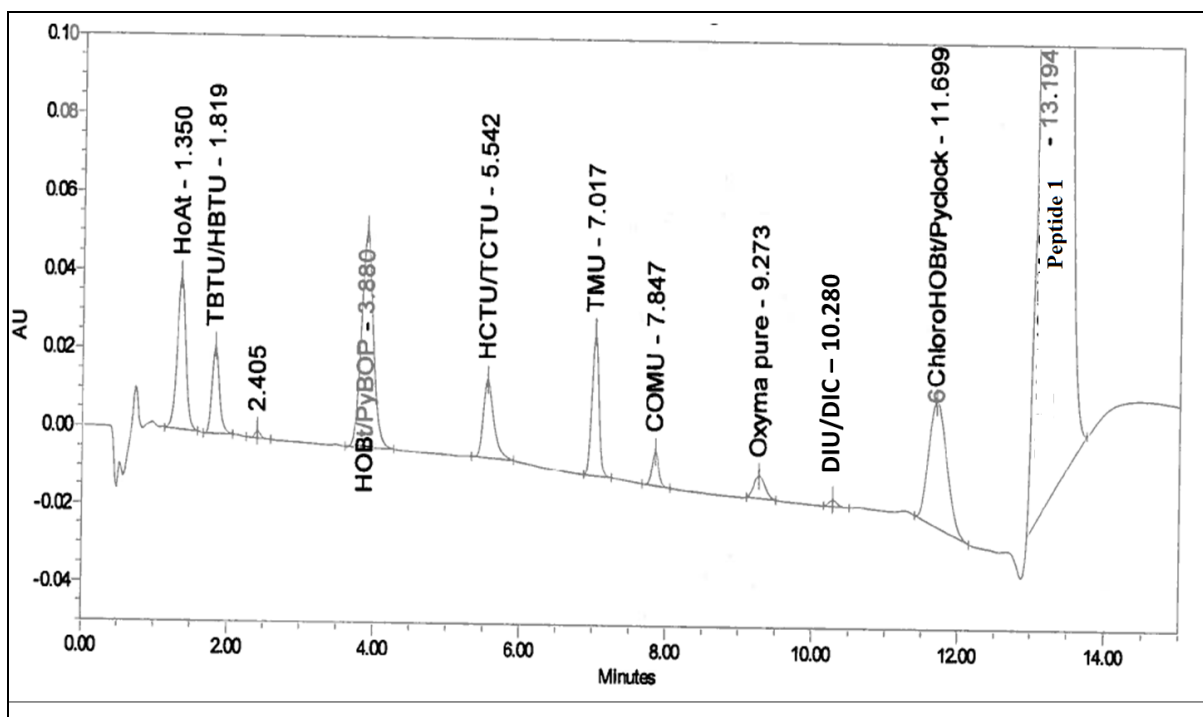
Figure 3.7 *Chromatogram demonstrating specificity of reagents in the presence of Peptide 1. Chromatographic conditions as shown in Figure 3.5. Note: Peptide 1 is labelled as "BIM-23014C".*
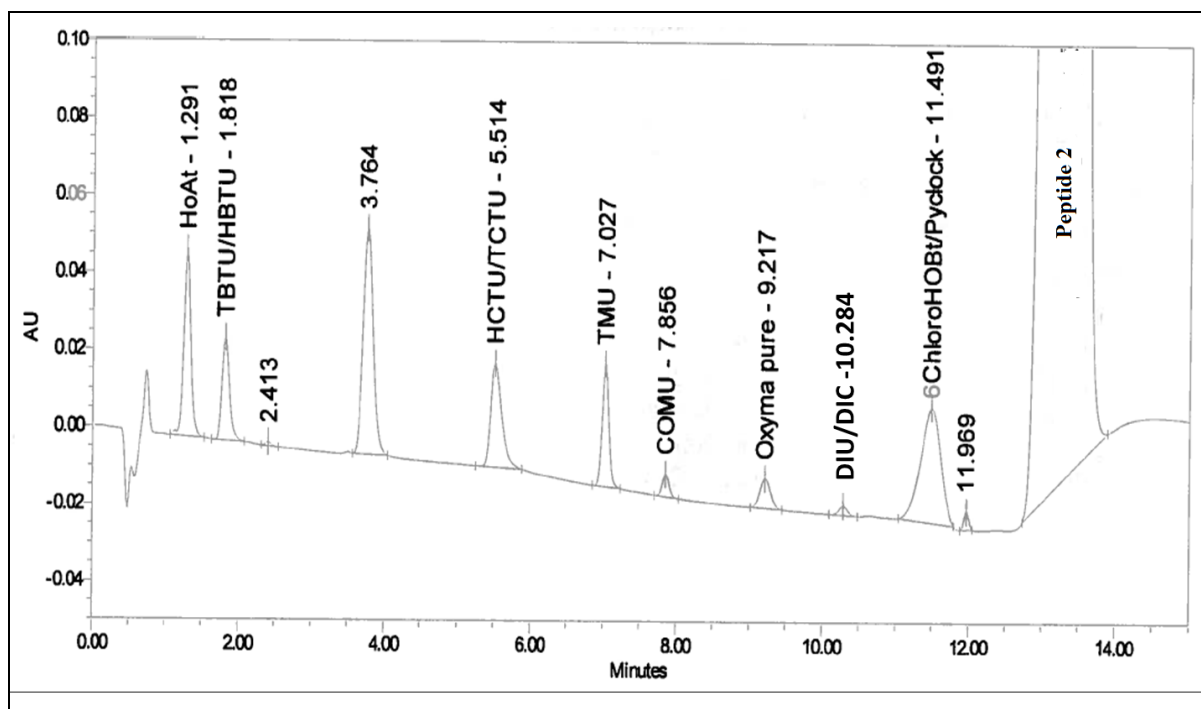
Figure 3.8 *Chromatogram demonstrating specificity of reagents in the presence of Peptide 2. Chromatographic conditions as shown in Figure 3.5. Note: Peptide 2 is labelled as "BIM-21003C".*

3.3.6 Accuracy of the analytical method

The accuracy of an analytical procedure demonstrates the closeness of agreement between the result that is accepted, either as a conventional true result or an accepted reference result, and the result determined [116]. Accuracy is typically demonstrated as percentage recovery where the analyte is spiked into a sample and also individually analysed to determine the effect the sample has on the analyte [114]. The accuracy of each of the reagents was assessed on duplicate preparations of the reagents spiked into Peptide 1 over three ranges. The reagents were also analysed in the absence of Peptide 1 and the results were compared to determine the percentage recovery relative to peak area. All reagents, with the exception of PyClocK, were demonstrated to be accurate within the range of 90.0 % to 107.0 % as shown below in Table 3.4. The method was determined to be inaccurate with respect to PyClocK.

Table 3.4   *Accuracy of each analyte at 80 %, 100 % and 120 % of the specified range*

Accuracy range	80 %	100 %	120 %
TMU recovery	104.5 %	106.2 %	106.0 %
TBTU recovery	97.0 %	99.9 %	100.0 %
HOBt recovery	100.9 %	102.6 %	102.9 %
TCTU recovery	102.0 %	102.1 %	99.2 %
Oxyma Pure recovery	100.2 %	100.8 %	100.4 %
DIC recovery	103.6 %	106.3 %	103.1 %
6-ChloroHOBt recovery	106.0 %	104.9 %	105.0 %
HOAt recovery	98.4 %	99.1 %	98.2 %
HBTU recovery	103.1 %	103.4 %	102.7 %
PyBOP recovery	98.5 %	102.0 %	105.6 %
HCTU recovery	100.9 %	100.3 %	99.2 %
COMU recovery	100.8 %	103.1 %	101.1 %
DIU recovery	93.0 %	91.9 %	90.6 %
PyClocK recovery	24.5 %	28.0 %	37.0 %

3.3.7   *Precision of the analytical method*

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling performed on the same sample under the same conditions [116]. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility and is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements [116]. Repeatability expresses the precision under the same operating conditions over a short interval of time [116]. It is also known as intra-precision and can be assessed using 9 determinations covering a specified range or 6 determinations of the 100 % range [114]. The method was demonstrated to be repeatable with the results for all the reagents within the criteria of ≤ 5.3 % RSD of peak area (Ipsen Manufacturing Ireland LTD. internal specification), as detailed in Table 3.5. Some of the reagents demonstrated a higher % RSD than others and this is due to the stability of the reagents as detailed in Section 3 3.5. Retention time precision was also evaluated for replicate injections of a test mix and found to be ≤ 0.52 % for all analytes.

Intermediate precision expresses within-laboratories variations, such as different days, different analysts and different equipment. The criteria of % RSD of ≤ 5.3 % of peak area was met for all peptide coupling reagents, additive and associated by-products and therefore the method was determined to be precise with respect to variation within the same laboratory. System precision was also assessed to determine the impact of the system on the same sample. The criteria of % RSD of ≤ 5.3 % for peak area was also met for all peptide coupling reagents, additive and associated by-products, as detailed in Table 3.5.

Table 3.5   *Precision, repeatability and intermediate precision results*

Reagent	Precision % RSD	Precision (Retention time) % RSD	Repeatability % RSD	Int. precision % RSD
HOAt	0.4%	0.38%	0.4%	2.3%
TBTU/HBTU	1.4%	0.52%	1.3%	1.5%
HOBt/PyBOP	0.7%	0.23%	0.5%	1.9%
HCTU/TCTU	1.2%	0.38%	1.9%	3.0%
TMU	1.2%	0.22%	0.9%	2.2%
COMU	1.7%	0.23%	1.5%	1.8%
Oxyma Pure	1.4%	0.17%	2.3%	4.5%
DIU/DIC	2.2%	0.20%	5.2%	4.9%
6-Chloro/PyClocK	1.2%	0.08%	2.4%	3.5%

Note: Precision was determined by evaluating peak area, except where indicated above.

3.3.8   Limit of Quantitation (LOQ) of the analytical method

The limit of quantitation is the lowest amount of analyte in the sample that can be quantitatively determined with suitable precision and accuracy [114,116]. The LOQ was determined using a signal to noise approach where the LOQ was determined as the concentration that results in a signal to noise of 10 to 1. The quantitation limit is affected by the accuracy of the sample preparation and also the detector sensitivity at such a low concentration [114]. The LOQ value for each of the peptide coupling reagents, additives and associated by-products is detailed in Table 3.6.

Table 3.6   *Limit of quantitation results*

Reagent	LOQ (% w/w)	LOQ % RSD
HOAt	0.0013	7.5%
TBTU	0.0080	3.6%
HBTU	0.0010	7.7%
HOBt	0.0007	15.8%
PyBOP	0.0400	8.7%
HCTU	0.0080	16.0%
TCTU	0.0080	8.8%
TMU	0.0010	19.0%
COMU	0.0066	6.3%
Oxyma Pure	0.0080	10.2%
DIU	0.0266	16.7%
6-Chloro	0.0050	15.2%
PyClocK	0.0067	15.5%
DIC	0.0500	18.9%

There are significant differences in some of the LOQ values and this is related to the maximum absorbance at the detection wavelength of 215 nm. HOAt demonstrates a very low LOQ of 0.000667 % w/w and this is a reflection on the high UV absorbance at 215 nm as demonstrated in Figure 2.3, whereas DIU has a higher LOQ at 0.0133 % which reflects the low UV absorbance at 215

nm as demonstrated in Figure 2.16. To ensure that the LOQ results are reproducible, six injections of the LOQ concentration of each of the reagents were evaluated. All % RSD results were within the criteria of ≤ 20.0 % RSD for peak area, as detailed in Table 3.6 (Ipsen Manufacturing Ireland LTD. Internal specification).

3.3.9 *Limit of Detection (LOD) of the analytical method*

The detection limit of an analytical procedure is the lowest amount of analyte in a sample that can be detected but not quantitated as an exact value [116]. The detection limit is determined as the concentration amount that results in a peak with a height at least 3 times as high as the baseline noise level measured [116]. The LOD results are not required to be repeatable as the analytes will not be quantified at this concentration. However, LOD reproducibility was assessed as part of the validation for information purposes on six injections of the LOD concentration of each of the reagents. All % RSD results were within the LOQ criteria of ≤ 20.0 % RSD for peak area, as detailed in Table 3.7.

Table 3.7 *Limit of detection results*

Reagent	LOD (% w/w)	LOD % RSD
HOAt	0.0007	5.2%
TBTU	0.0040	3.9%
HBTU	0.0005	5.9%
HOBt	0.0003	9.8%
PyBOP	0.0100	8.4%
HCTU	0.0040	4.0%
TCTU	0.0040	7.9%
TMU	0.0005	9.7%
COMU	0.0040	6.9%
Oxyma Pure	0.0040	5.2%
DIU	0.0133	16.5%
6-Chloro	0.0010	10.2%
PyClock	0.0133	4.8%
DIC	0.0250	8.5%

3.3.10 *Linearity of the analytical method*

The linearity is the ability of the analytical procedure to produce test results which are directly proportional to the concentration range of the analyte in samples within a given range [116]. For all peptide synthesis and degradation products, the linearity was assessed in a range from LOQ to 120 % of the expected specification of 0.1 % w/w, relative to a concentration of 5 mg/mL. The linearity of the Peptide 1 and Peptide 2 was assessed at 50 to 120 % of the peptide concentration of 5 mg/mL. A plot of peak area versus concentration was compiled to demonstrate linearity for each of the reagents and each of the peptides. Table 3.8 details the slope and residual sum of squares. All peptide coupling

reagents, additives and associated by-products meet the criteria of residual sum of squares ≥ 0.99. Peptide 1 and Peptide 2 also meet their linearity criteria of ≥ 0.999.

Table 3.8 *Linearity of analytical method*

Reagent	Linearity range	Slope	Residual sum of squares
HOAt	0.000667 % - 0.12 %	67994387.827	1.00
TBTU	0.004 % - 0.12 %	22008078.925	1.00
HBTU	0.0005 % - 0.12 %	19320030.453	1.00
HOBt	0.000334 % - 0.12 %	87581346.212	1.00
PyBOP	0.01 % - 0.12 %	6322842.105	1.00
HCTU	0.004 % - 0.12 %	35646533.957	1.00
TCTU	0.004 % - 0.12 %	42421895.957	1.00
TMU	0.0005 % - 0.12 %	34269185.825	1.00
COMU	0.004 % - 0.12 %	10892203.050	1.00
Oxyma Pure	0.004 % - 0.12 %	16025780.438	1.00
DIU	0.0133 % - 0.12 %	1942903.897	0.99
6-ChloroHOBt	0.001 % - 0.12 %	82702151.435	1.00
PyClocK	0.0133 % - 0.12 %	5876492.443	1.00
DIC	0.025 - 0.12 %	1560661.017	0.99
Peptide 1	50 to 120 %	5429795.322	0.999
Peptide 2	50 to 120 %	10264856.210	0.999

The relative response factor can be used to correct differences in response between any related substances such as the reagents and the drug substance [114]. The relative response factor is determined by comparing the slope of the related substance to the slope of the drug substance [114]. If a relative response factor is significantly different, such as a difference of 20 %, a correction factor should be applied to the calculation of the concentration of the related substance in the drug substance [114]. If this correction factor is not applied then the results can be grossly overestimated or underestimated [114]. Table 3.9 details the results for the relative response factor results. The relative was determined using the slope of the linearity curve according to the formula - RRF=slope impurity / slope API. In order to account for the relative response factor, the result for the reagent present in the Peptide sample must be multiplied by 1/RRF value.

Table 3.9   *Determination of relative response factors*

Reagent	Slope	Peptide 1 Slope	RRF Peptide 1	Peptide 2 Slope	RRF Peptide 2
HOAt	67994387.827	5576075.550	12.19	25507694.000	2.67
TBTU	22008078.925	5576075.550	3.95	10264856.210	2.14
HBTU	19320030.453	5576075.550	3.46	10264856.210	1.88
HOBt	87581346.212	5576075.550	15.71	10264856.210	8.53
PyBOP	6322842.105	5576075.550	1.13	10264856.210	0.62
HCTU	35646533.957	5576075.550	6.39	10264856.210	3.47
TCTU	42421895.957	5576075.550	7.61	10264856.210	4.13
TMU	34269185.825	5576075.550	6.15	10264856.210	3.34
COMU	10892203.050	5576075.550	1.95	10264856.210	1.06
Oxyma Pure	16025780.438	5576075.550	2.87	10264856.210	1.56
DIU	1942903.897	5576075.550	0.35	10264856.210	0.19
6-ChloroHOBt	82702151.435	5576075.550	14.83	10264856.210	8.06
PyClock	5876492.443	5576075.550	1.05	10264856.210	0.57
DIC	1560661.017	5576075.550	0.28	10264856.210	0.15

3.3.11   Robustness of the analytical method

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small, but deliberate changes in method parameters and provides an indication of its reliability of the analytical method during normal usage [116]. Robustness was assessed on critical parameters of the analytical method such as column variation, column temperature, buffer concentration and pH and also flow rate. The criteria for method robustness states the peak tailing of each peak should be ≤ 2.0, the retention factor (k*) should be ≥ 1.0 and the resolution of all peaks should be ≥ 1.5. Figure 3.9 demonstrates that the method was robust with respect to column to column variation. Columns with different serial numbers were evaluated and all criteria were achieved on both columns. This is the most important robustness parameter as a method must be robust to change in columns as this regularly occurs when a method is in routine use.
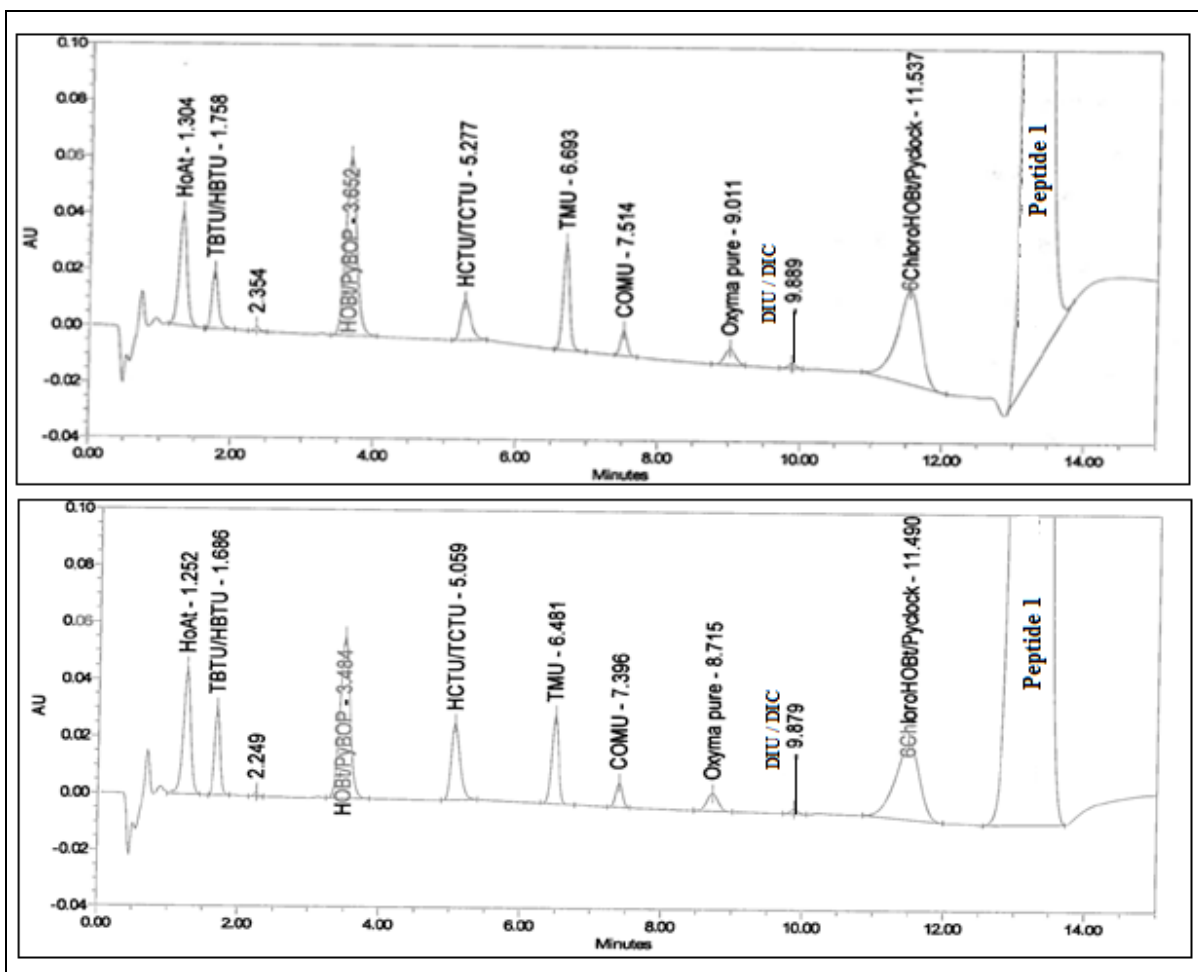
Figure 3.9: *Robustness testing on YMC Triart columns with different serial numbers – (a) Column serial number 0210002430 and (b) Column serial number 0210002540. Chromatographic conditions as shown in Figure 3.5.*

Figure 3.10 demonstrates the robustness of the method to changes in flow rate. The flow rate was assessed at ± 0.05 mL/min and all criteria were achieved at each flow rate. Peak broadening was observed for the 6-ChloroHOBt/PyClocK peak for the higher flow rate of 0.55 mL/min, however all chromatographic performance criteria were achieved. The flow rate is dictated by the UHPLC instrument and it is unlikely that the flow rate will be impacted by as much as ± 0.05 mL/min, however if this did occur, the analytical method is capable of producing reproducible results.
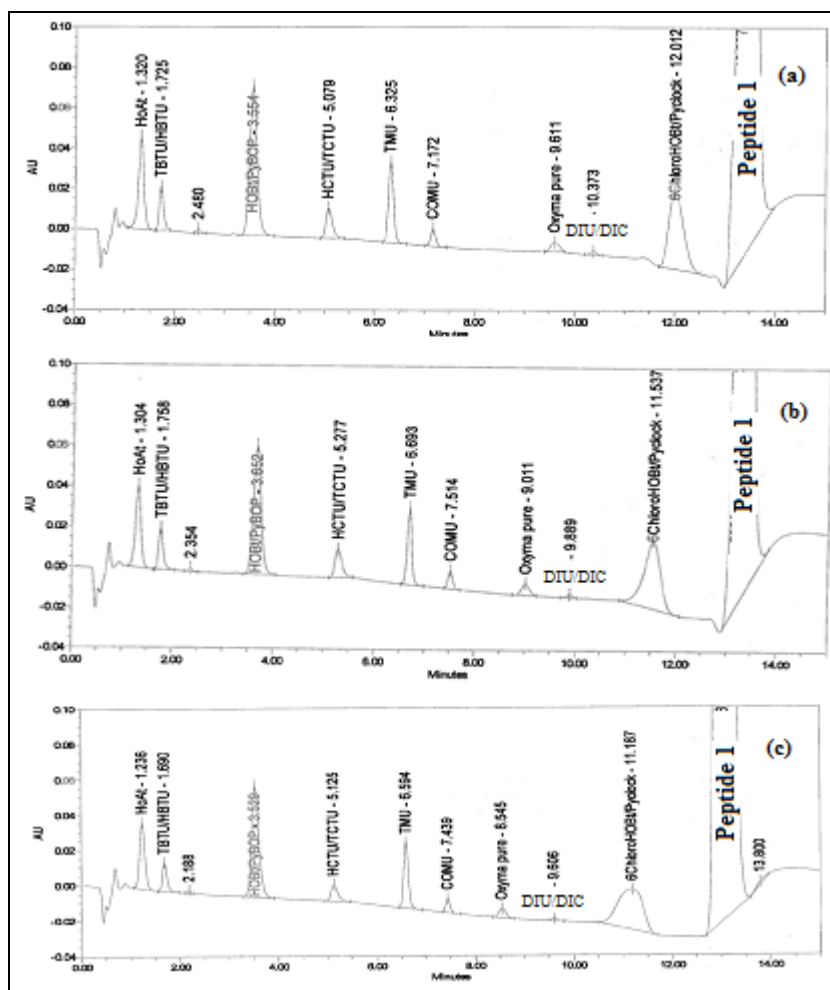
Figure 3.10: *Robustness testing for flow rate variation – (a) flow rate: 0.45 mL/min, (b) flow rate: 0.50 mL/min and (c) flow rate: 0.55 mL/min. Chromatographic conditions as shown in Figure 3.5.*

Figure 3.11 shows the effect of column temperature on the analytical method. The method was assessed using column temperatures of 20 °C, 25 °C (normal conditions) and 30 °C and all chromatographic performance criteria were achieved for each condition. The reduction in column temperature resulted in the critical peak pair of Oxyma Pure and DIU/DIC to elute closer together (resolution = 2.33) and an increase in column temperature has the opposite effect (resolution = 6.12). Peak broadening was also observed for the 6-ChloroHOBt/PyClocK peak at the higher column temperature of 30 °C, however all chromatographic performance criteria were achieved.

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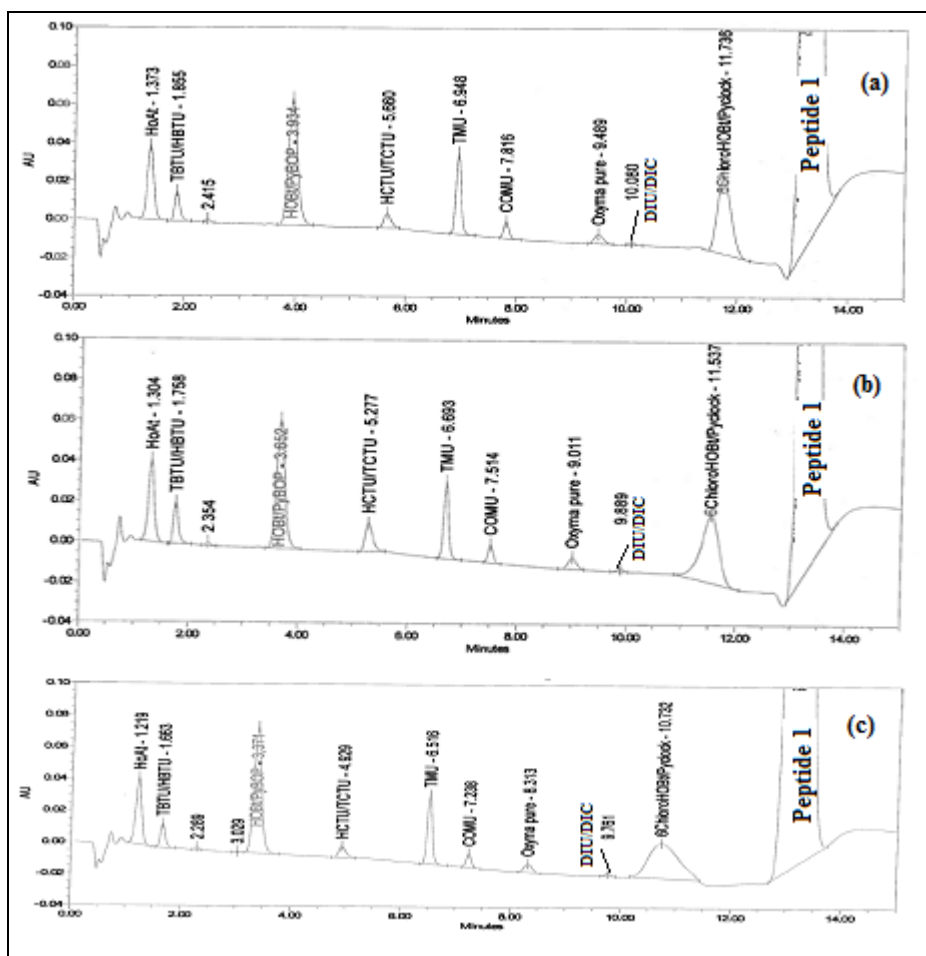
Figure 3.11: *Robustness testing for column column temperature variation - (a) column temperature: 20 °C, (b) column temperature: 25 °C and (c) column temperature: 30 °C. Chromatographic conditions as shown in Figure 3.5.*

Figure 3.12 shows the effect of buffer concentration on the analytical method. The method was initially assessed using an ammonium formate concentration of 2.5 m*M*, 5 m*M* (normal conditions) and 7.5 m*M*. The reduction in ammonium formate concentration to 2.5 m*M* resulted in the critical peak pair of Oxyma Pure and DIU/DIC and HOAt and TBTU/HBTU eluting closer together and the results failed the criteria for resolution ≥ 1.5. As a result, an additional concentration of 3.75 m*M* ammonium formate was added to the robustness evaluation. All criteria were achieved for 3.75 m*M* and 7.5 m*M* ammonium formate. As the method is not robust at a low concentration of 2.5 m*M* ammonium formate, the concentration of the ammonium formate needs to be suitably controlled and a precautionary statement should be included in the method. The buffer concentration effected retention times because the retention of ionisable analytes in this instance is likely governed by a range of different retention mechanisms such as ion exchange interactions with deprotonated silonols on the silica support particle, as well as hydrophobic interactions with the C18 ligand. The buffer acts as a

counter ion to compete with negatively charged silanols which is a phenomenon not typically observed for neutral analytes.
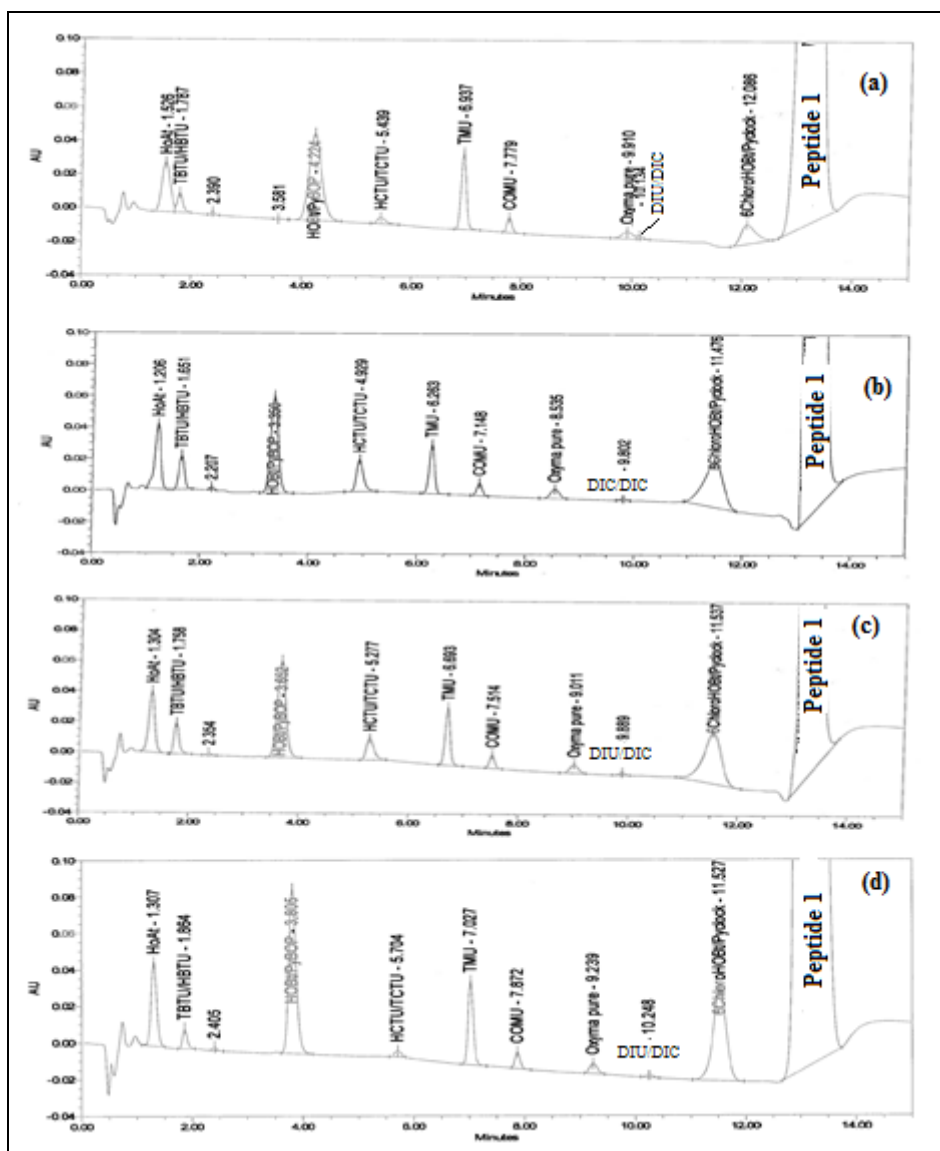
Figure 3.12: *Robustness testing for buffer concentration variation. (a) 2.5 mM ammonium formate, (b) 3.75 mM ammonium formate, (c) 5 mM ammonium formate and (d) 7.5 mM ammonium formate. Chromatographic conditions as shown in Figure 3.5.*

Figure 3.13 demonstrates the effect of buffer pH on the analytical method. The method was initially assessed using ammonium formate pH of 4.5, 4.6 (normal conditions) and 4.7.
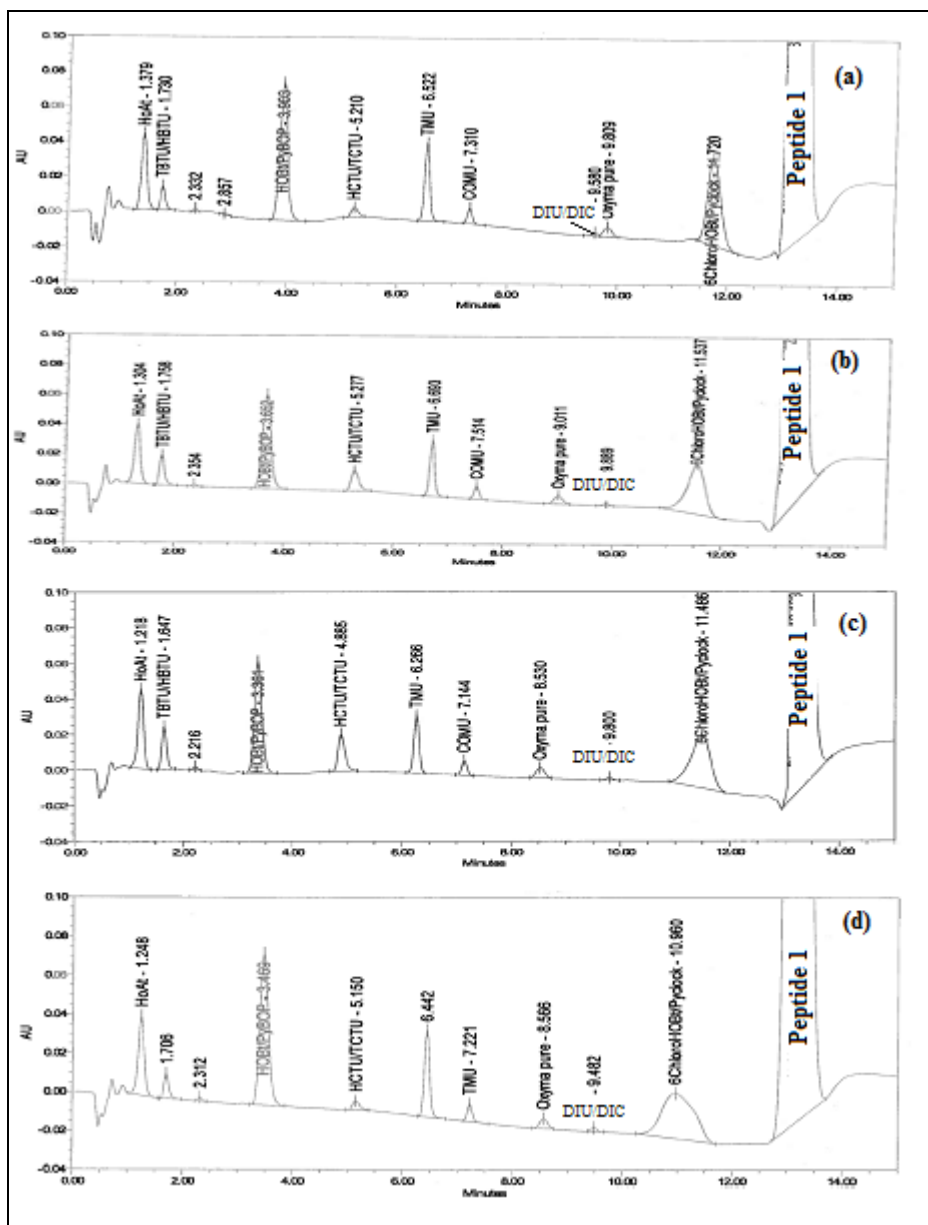
Figure 3.13: *Robustness testing for variation of buffer pH. (a): pH 4.5, (b): pH 4.55, (c): pH 4.6 and (d): pH 4.7. Chromatographic conditions as shown in Figure 3.5.*

The reduction in ammonium formate pH to 4.5 resulted in the critical peak pair of Oxyma Pure and DIU/DIC to elute closer together and the results failed the criteria for resolution ≥ 1.5. As a result, an additional pH of 4.55 was added to the robustness evaluation. All criteria were achieved for pH 4.55 and 4.7 despite a deterioration of peak shape for 6-ChloroHOBt/PyClock at the higher pH. As the method is not robust at a low pH of 4.5, the pH of the ammonium formate also needs to be suitably controlled and a precautionary statement should be included in the method.

3.3.12   Solution stability

In relation to the detection method, the aim is to accurately determine the concentration of peptide coupling reagents, additives and associated by-products in API. As this API is formulated as a drug substance it is important to access the effect of the final concentration of these reagents if left in solution over time. As a result, the API should be assessed for reagents initially after sample make up and also after approximately 72 hours. This will insure the reagents that increase in area count due to degradation of other reagents do not go above the threshold of toxicological concern in the final peptide. Solution stability is usually evaluated by comparison of freshly prepared solutions with those stored at particular conditions for a specific length of time. Therefore, a mixture containing Peptide 1 at a nominal concentration of 5 mg/mL and each of the peptide coupling reagents, additives and by-products at their specification level of 0.1 % w/w was evaluated over a 135 hour period at ambient and refrigerated storage conditions (2-8 °C). For this study, a change in peak area ≤ 5.3 % was considered to be indicative of solute stability.

Firstly, Figure 3.14 and Figure 3.15 both illustrate the stability of each of the reagents at ambient temperature over the evaluation period (with some of the analytes normalized to begin at 100 % for illustrative purposes). HOAt was determined to be stable for approximate 20 hours at ambient temperature, with a % change in peak area of 5.03 %, whereas COMU was stable for up to 16 hours. Conversely, all other solutes were stable for 1 hour or less, with the degradation of TBTU/HBTU and TCTU/HCTU particularly notable over the 135 hour time period as shown in Figure 3.13. As a result of the degradation of these analytes, the peak area of HOBt/PyBOP, TMU, and 6-ChloroHOBT/PyClocK all correspondingly increased significantly within 0.5 hours since TBTU/HBTU and TCTU/HCTU were demonstrated to degrade to these more stable forms as discussed in Chapter 2.
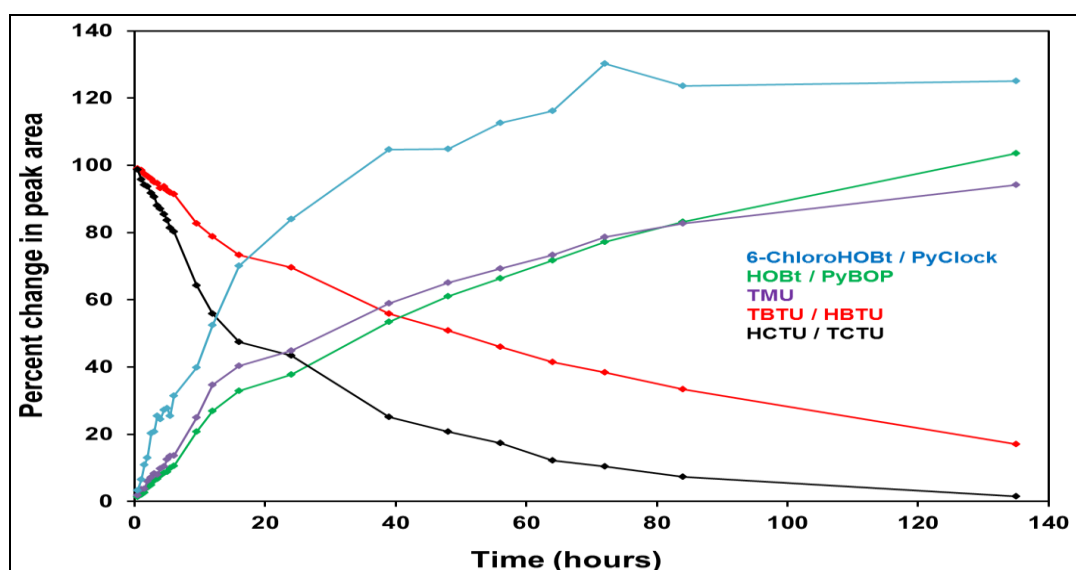
Figure 3.14: *Stability at ambient sample temperature for selected analytes.*
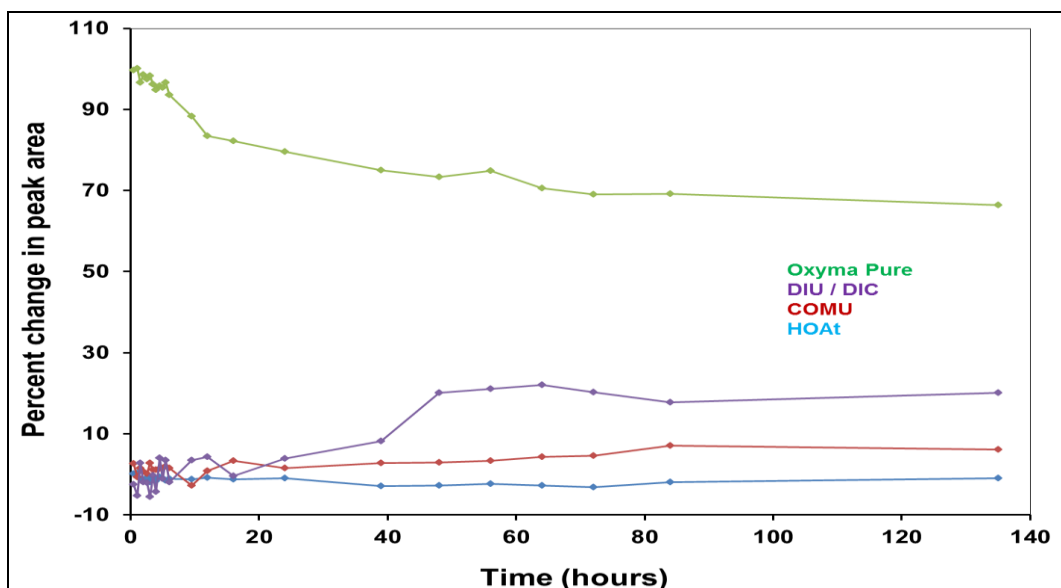
Figure 3.15: *Stability at ambient sample temperature for remaining analytes in the study.*

Figure 3.16 shows a chromatogram comparison for the test mix injected immediately after preparation, and also after 135 hours when stored in the HPLC auto sampler at ambient temperature. For illustrative purposes, arrows are included in the figure to indicate which analytes increase or decrease in peak area over the time period, and this can readily be cross-referenced with the patterns shown in Figure 3.14 and Figure 3.15.
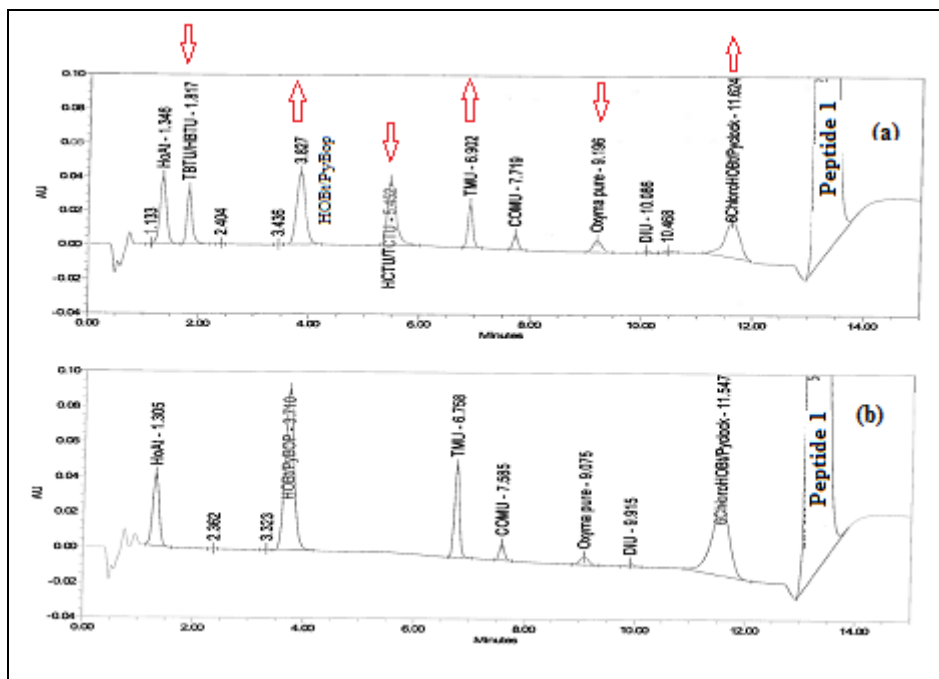
Figure 3.16: *Test mix stability at ambient sample temperature over 135 hours. (a) time-point: 0 hours (b) time-point: 135 hours. Chromatographic conditions as shown in Figure 3.5.*

As a result of the poor solute stability when test solutions were stored in the auto-sampler at ambient temperature, a second study was performed in which solutions were held at 5 $^{\circ}$C and injected at timed intervals. Figure 3.17 and Figure 3.18 clearly illustrate that solution stability (as indicted by a change in peak area of not more than 5.5 %) significantly increased for all analytes with the exception of 6-ChloroHOBt/PyClock for which there was no improvement. The largest improvement in solute stability was observed for DIU/DIC which increased by a factor for 40 when stored at 5 $^{\circ}$C rather than ambient temperatures, followed by TBTU/HBTU and HOAt which both increased by a factor of seven. Although all reagents did appear to degrade over time, even when stored at 5 $^{\circ}$C in the auto sampler, nevertheless the degradation was significantly reduced, clearly demonstrating a distinct advantage of the use of an auto-sampler cooling function during HPLC analysis.
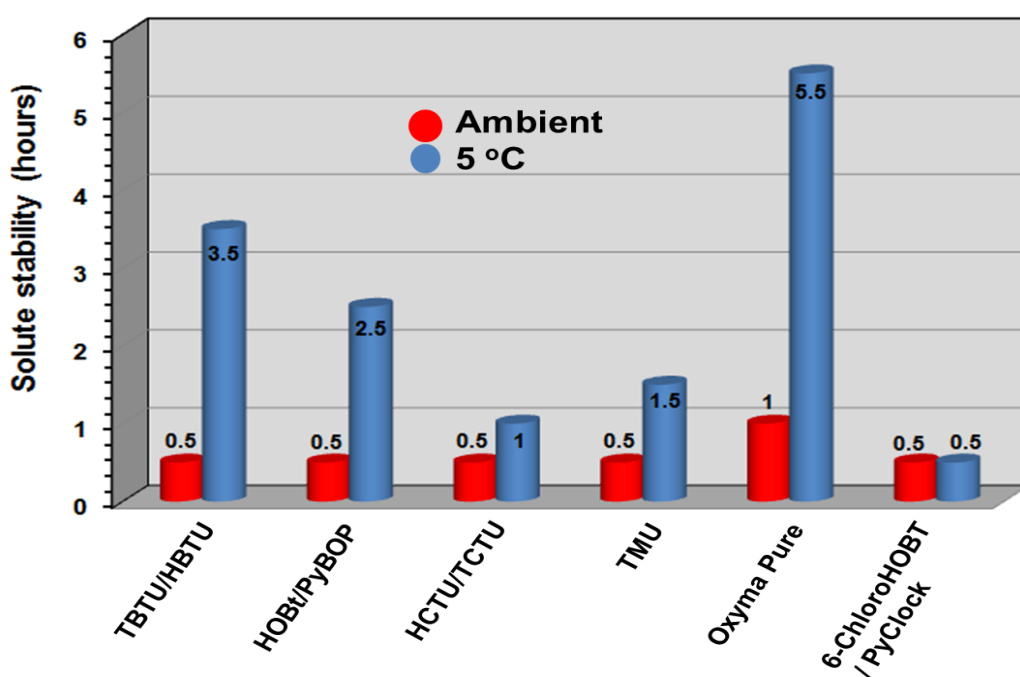
Figure 3.17: *Comparison of solute stability (≤ 5.3 % change in peak area) at ambient temperature and 5 $^{\circ}$C for selected reagents.*
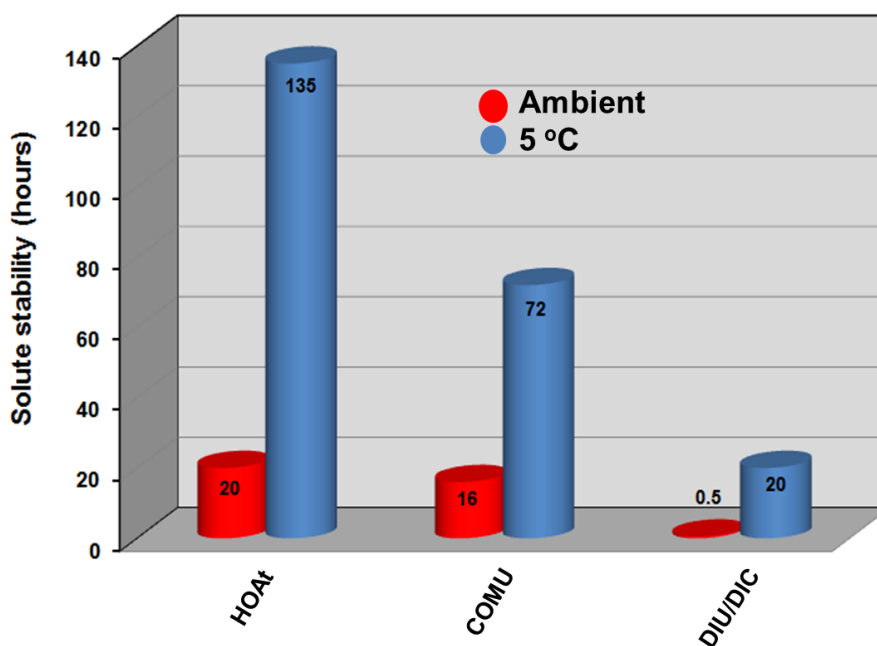
Figure 3.18: *Comparison of solute stability (≤ 5.3 % change in peak area) at ambient temperature and 5 °C for the remaining reagents in this study.*

3.4   Conclusions

A fast, reliable ultra-high performance liquid chromatography method for the simultaneous determination of peptide coupling reagents, additives and by-products used in peptide synthesis, was developed an d validated. Using a YMC Triart reverse-phase UHPLC column with particle size of 1.9 μm, the UV assay can detect 14 commonly used peptide synthesis reagents in the presence of 2 peptides within a run time of 15 minutes. This 15 minute method was determined to be selective and capable of accurately quantitating the amount of TMU, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, Oxyma Pure, COMU, DIU, DIC, PyBOP, and TCTU in the presence of two peptides from Ipsen Manufacturing Ireland LTD. The precise method was determined to be linear for the response of each reagent and both peptides. The method was proven to be robust with respect to variations in the method parameters and it is also capable of reproducibly detecting levels below the required threshold. This rapid UHPLC method is directly transferable onto LC-MS and offers significant advantages over current HPLC methods with long run times and methods that can only detect single analytes.

Chapter 4

Conclusion

4.1   Overall conclusion

In conclusion, a 15 minute ultra-high performance liquid chromatography method for the simultaneous determination of fourteen peptide coupling reagents, additives and by-products used in peptide synthesis, was developed using a YMC Triart reverse-phase UHPLC column with particle size of 1.9 μm with UV detection. The method development involved evaluation of 17 commercially available stationary phase and 12 mobile phase systems. This method was subsequently validated according to ICH guidelines and it was determined to be selective of accurately quantitating the amount of TMU, HOBt, HCTU, HBTU, 6-ChloroHOBt, TBTU, Oxyma Pure, COMU, DIU, DIC, PyBOP, and TCTU in the presence of two peptides from Ipsen Manufacturing Ireland LTD. The method was also determined to be valid for linearity, precision and robustness with an acceptable limit of detection and limit of quantitation value for each reagent.

The determination of peptide coupling reagents, additives and associated by-products is important during peptide synthesis to ensure the concentration of these products are below the threshold of toxicological concern in the final peptide. This method was required because studies at Ipsen Manufacturing Ireland LTD. have revealed that some peptide coupling reagents may in fact not be fully removed from the peptide product during peptide manufacture. The analytes evaluated were either used in the manufacture of some commercial peptides or were under investigation for the manufacture of peptides in development in Ipsen Manufacturing Ireland LTD. This rapid assay replaces multiple HPLC methods of run time greater than 60 minutes for the in-process testing during peptide synthesis and it also has applicability as a release test for the final API. To the best of my knowledge, no chromatographic separation has been developed for all of the reagents evaluated in the scope of this study.

The rapid UHPLC method is directly transferable onto LC-MS and offers significant advantages over current HPLC methods with long run times and methods that can only detect single analytes. It is anticipated that this method will be used in the biopharmaceutical industry, particularly in Ipsen Manufacturing Ireland LTD.

REFERENCES

[1] Hughes, A.B., *Amino acids, Peptides and Proteins in Organic Chemistry*, Volume 4, Wiley-VCH Verlag GmbH & Co. KGaA publications, 2011.

[2] McKee, T., and McKee, J., *Biochemistry: The molecular basis of life*, Firth edition ed., Oxford University Press publications, 2011.

[3] Jones, J., *The Chemical Synthesis of Peptides*. Clarendon Press publications, 1994.

[4] Benoiton, N.L., *Chemistry of peptide synthesis*. Taylor & Francis publications, 2005.

[5] Albericio, F., Giralt, E., Lloyd-Williams, P., *Chemical approaches to the synthesis of peptides and proteins*, CRC Press LLC publications, 1997.

[6] Hill, J.W., Scott, R.J., Ball, D.W., *The Basics of General, Organic, and Biological Chemistry*., Volume 1, Flat Wold Knowledge publications, 2011.

[7] Seebach, D., Kimmerlin, T., *J.Peptide Res*, vol. 65, pp. 229-260, 2005.

[8] Doonan, S., *Peptides and Proteins*. Cambridge, United Kingdom: RSC publications, 2002.

[9] OJM, *An international Journal of Medicine*, vol. 92, no. 1, pp. 1-4, 1999.

[10] http://www.polypeptide.com/assets/002/5188.pdf (accessed 02 July 2013).

[11] http://www.iptonline.com/articles/public/AmericanPeptide.pdf (accessed 02 July 2013).

[12] Ayoub, M., Scheidegger, D., *Chimica oggi/Chemistry today*, vol. 24, no. 24, July/August 2006.

[13] Lisowski, V., Martinez, J., Khrestchatisky, P., Vlieghe, P., *Drug discovery today*, vol. 15, no. 1/2, pp 3-15, January 2010.

[14] Limbird, L.E., *Cell Surface Receptors: A Short Course on Theory and Methods*.: Springer publications, 2005.

[15] Makoid, M.C.,Vuchetich, P.J., Banakar, U.V., *Basic pharmacokinetics*., 1999.

[16] Egleton, R.D., Davis, P.D., *Peptides*, vol. 18, no. 9, pp. 1431-1439, 1997.

[17] Otvos, L., *Peptide-based Drug Design: Methods and protocols*. Humana Press publications, 2008.

[18] Papini A.M., *Peptide chemistry today/Chemistry today*, vol. 30, no. 2, pp22-13, March/April 2011.

[19] Sewald, N., Jakubke,H.D., *Peptides: Chemistry and Biology*.: WILEY-VCH publications, 2009.

[20] Ovadia, O., Greenberg, S., Laufer B, Gilon, C., Hoffman, A., Kessler, H., *Expert Opin. Drug Discov.*, vol. 5, no. 7, pp. 655-671, 2010.

[21] http://www.americanpeptide.com/corp/spciality_chemicals_may2010.pdf (accessed on 02 July 2013)

[22] Ma, X., Wu, C., Wang, W., and Li, X., *Asian Journal of Traditional Medicines*, pp 1-6.

[23] Bofill, J.M., & Albericio, F., *Tetrahedron Letters 40* , pp. 2641-2644, 1999.

[24] http://www.merckmillipore.co.uk/chemicals/showBrochure?id=201010.178 (accessed on 02 July 2013)

[25] Han, S.Y., and Kim, Y. A., *Tetrahedron*, no. 60, pp. 2447-2467, 2004.

[26] Valeur. E., and Bradley, M., *Chem.Soc.Rev.*, vol. 38, pp. 606-631, 2009.

[27] Bailey, P.D., *An introduction to Peptide Chemistry*.: John Wiley & Sons Ltd. publications, 1990.

[28] Kates, S.A., Albericio, F., *Solid-phase synthesis, A practical guide*, first edition, Taylor & Francis publications, 2000.

[29] Grant, G.A., *Synthetic Peptides: A users guide*, W.H Freeman and Company publications, 1992.

[30] Bay, B.L., *Drug discovery*, pp. 587-593, July 2003.

[31] Marder, O., Albericio, F., *Chemistry Oggi/Chemistry today*, pp. 6-11, 2003.

[32] Shvo, Y., Albericio, F., Marder, O., *ChemInform*, vol. 34, July 2003.

[33] http://www.livchem.com/WebRoot/Store/Shops/DemoShop/4BBE/EDFD/B804/1B54/33B1/C0A8/2C05/D8BC/Infofile_Peptidkuppler.pdf (accessed on 02 July 2013).

[34] El-Fahdam, A., and Albericio, F., *J. Pept Sci*, vol. 16, pp. 6-9, 2010.

[35] El-Fahdam, A., and Albericio, F., *Chem. Rev.*, vol. 111, pp. 6557-6602, 2011.

[36] http://www.aapptec.com/custdocs/Coupling-Reagents.pdf (accessed on 10 September 2013).

[37] Prasad, KVSRG., Bharathi, K., Haseena, B. B., *International Journal of Pharmaceutical Sciences Review and Research*, vol. 8, no. 1, pp. 108-119, June 2011.

[38] http://www.sigmaaldrich.com/etc/medialib/docs/Aldrich/Bulletin/al_chemfiles_vol8_no7.Par.0001.File.tmp/al_chemfiles_vol8_no7.pdf (accessed on 31 August 2012).

[39] Montalbetti, C. A.G.N., Falque, V., *Tetrahedron*, vol. 61, no. 46, pp. 10827–10852, Nov 2005.

[40] http://pepnet.com/ShoppingUsers/PdfDocument.aspx?FileName=pyclock.pdf (accessed on 02 July 2013).

[41] Khattab, S.N., Abdul-Ghani, M., Albericio, F., El-Faham, A.," *European Journal of Organic Chemistry*, pp. 1563-1573, 2006.

[42] Fuentes, G., Patel, H., Page, K., Menakuru, M., Park, J.H., Hood, C.A., *Journal of Peptide Science*, vol. 14, pp. 97-101, 2008.

[43] http://www.ptipep.com/rks_images/documents/item_248.pdf (accessed on 02 July 2013).

[44] Joullié, M.M., Lassen, K.M., *ARKIVOC,* Department of Chemistry , University of Pennsylvania, Pennsylvania, 2010.

[45] Funosas, R.S., Prohens, R., Albericio, F., El Faham, A., *Chemistry - A European Journal*, vol. 15, pp. 1904-9416, 2009.

[46] Albericio, F., *Chimica Oggi/Chemistry today*, vol. 26.

[47] Acosta, G.A., El-Faham, A., Albericio, F., Subiros-Funosas, R., *Tetrahedron letters 50*, pp. 6200-6202, 2009.

[48] www.merckmillipore.co.uk/chemicals/showBrochure?id=200907.116 (accessed on 02 July 2013).

[49] Prohens, R., Barbas, R., El-Faham, A., Albericio, F., Subiros-Funosas, R., *Chem. Eur. J*, vol. 15, pp. 9394-9403, 2009.

[50] El-Faham, A., Albericio, F., Subiros-Funosas, R., *Organic & Biomolecular Chemistry,*, 8, pp 3655-3673, 2010.

[51] Li, P., Xu, J.C., *Tetrahedron Letters*, no. 41, pp. 721-724, 2000.

[52] http://www3.appliedbiosystems.com/cms/groups/psm_marketing/documents/generaldocuments/cms_040654.pdf (accessed on 02 July 2013).

[53] http://www.polypeptide.com/assets/001/5078.pdf (accessed on 02 July 2013).

[54] http://wolfson.huji.ac.il/purification/pdf/reversephase/vydachandbookrpc.pdf (accessed on 02 July 2013).

[55] Costantino, H.R., Pikal, M.T., *Lyophilisation of Biopharmaceuticals*. AAPS Press publications, 2004.

[56] http://www.baxterbiopharmasolutions.com/pdf/publications/practical-tech-lyo-genengnews.pdf (accessed on 02 July 2013).

[57] Swietlow, A., Lax,R., *Chimica oggi/Chemistry Today* , pp. 22-24, July/August 2004.

[58] Neue, U.D., *HPLC Columns: Theory, Technology and Practice*, first edition, Wiley-VCH publications, 1997.

[59] Aguilar, M.I., *HPLC of Peptides and Proteins: Methods and Protocols*, Volume 251, Humana Press Inc. publications, 2003.

[60] Meyer, V.R., *Practical High-Performance Liquid Chromatography*, Second edition, John Wiley & Sons Ltd. publications, 1994.

[61] Kirkland, J.J., Glajch, J.L., Snyder, L.R., *Practical HPLC method development*. John Wiley & Sons, Inc. publications, 1997.

[62] http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm127984.pdf (accessed on 02 July 2013).

[63] Swartz, ,M.E., *Journal of Liquid Chromatography & Related Technologies*, vol. 28, pp. 1253-1263, 2005.

[64] Wren, S.A.C.,Tchelitcheff, P., *Journal of Chromatography A*, vol. 1119, pp. 140-146, 2006.

[65] http://www.mac-mod.com/pdf/product-bulletins/088-HALO-Catalog.pdf (accessed on 02 July 2013).

[66] http://waters.com/webassets/cms/library/docs/720000819en.pdf (accessed on 02 July 2013).

[67] Swartz, ,M.E., *Separation science redefined*, May 2005.

[68] Ruta,J., Rudaz, S., Veuthey, J.V., Guillarme, D., *Anal Bioanak Chem*, pp. 1069-1082, 2010.

[69] http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/General_Information/1/t409191.Par.0001.File.tmp/t409191.pdf (accessed on 02 July 2013).

[70] Cabooter, D., Desmet, G., *UHPLC in life sciences*., RSC publications, 2012.

[71] http://www.waters.com/webassets/cms/library/docs/720002260en.pdf (accessed on 02 July 2013).

[72] http://fluorous.com/download/FTI_AppNote_F019099_Pyridinium_Salt.pdf (accessed on 02 July 2013).

[73] Peeva, L.G., Tate, E.W., Leatherbarrow, R.J., Livingston, A.G., So, S., *Chem. Commun.*, vol. 46, pp. 2808-2810, 2010.

[74] Chen, J., Yang, Z., Katritzky, A.R., *Journal of Organic Chemistry,* 60 (17), pp 5638-5642, 1995.

[75] Committee for medical products for human use, "Guideline on the limits of genotoxic impurities ," European Medicines Agency, London, 2006.

[76] http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=IE&language=en&productNumber=445452&brand=ALDRICH&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2F445452%3Flang%3Den (accessed on 02 July 2013).

[77] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=377848&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/aldrich/377848?lang=en&region=IE (accessed on 02 July 2013).

[78] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=741574&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/aldrich/741574?lang=en&region=IE (accessed on 02 July 2013).

[79] http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=IE&language=en&productNumber=12806&brand=FLUKA&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Ffluka%2F12806%3Flang%3Den (accessed on 02 July 2013).

[80] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=712191&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/aldrich/712191?lang=en&region=IE (accessed on 02 July 2013).

[81] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=FLUKA&productNumber=04936&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/fluka/04936?lang=en&region=IE (accessed on 02 July 2013).

[82] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=12804&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/aldrich/12804?lang=en&region=IE (accessed on 02 July 2013).

[83] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=18565&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/aldrich/18565?lang=en&region=IE (accessed on 02 July 2013).

[84] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=FLUKA&productNumber=54804&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/fluka/54804?lang=en&region=IE (accessed on 02 July 2013).

[85] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=233412&PageToGoToURL=http://www.sigmaaldrich.com/catalog/product/aldrich/233412?lang=en&region=IE (accessed on 02 July 2013).

[86] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=IE&brand=ALDRICH&productNumber=39785&PageToGoToURL=http://www.sigmaaldrich.c

om/catalog/product/aldrich/39785?lang=en&region=IE (accessed on 02 July 2013).

[87] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=I E&brand=FLUKA&productNumber=87849&PageToGoToURL=http://www.sigmaaldrich.co m/catalog/product/fluka/87849?lang=en&region=IE (accessed on 02 July 2013).

[88] Aguilera, D., Das, C., Vasquez, H., Zage, P., Gopalakrishnan, V., Wolff, J., Zhang, M., *Anticancer Research*, vol. 27, pp. 35-38, 2007.

[89] Singh, D.K., *Toxicology: Agriculture and Environment: Pesticide Chemistry and Toxicology*.: Bentham Books publications, 2012.

[90] http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=IE&language=en &productNumber=78133&brand=ALDRICH&PageToGoToURL=http%3A%2F%2Fwww.sig maaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2F78133%3Flang%3Den (accessed on 02 July 2013).

[91] http://www.sigmaaldrich.com/MSDS/MSDS/PleaseWaitMSDSPage.do?language=&country=I E&brand=FLUKA&productNumber=38370&PageToGoToURL=http://www.sigmaaldrich.co m/catalog/product/fluka/38370?lang=en&region=IE (accessed on 02 July 2013).

[92] http://www.merckmillipore.com/ireland/life-science-research/pyclock/EMD_BIO-851087/p_5gGb.s1OYaYAAAEjIRl9.zLX?attachments=MSDS (accessed on 02 July 2013).

[93] Muller, L., et al, *Regulatory toxicology and pharmacology*, vol. 44, pp. 198-211, 2006.

[94] http://www.ecs.umass.edu/eve/facilities/equipment/Acquity/UPLC%20column%20Brochure.pdf (accessed on 02 July 2013).

[95] http://www.chromtech.com/Catalog/ProductLine/brochures/Zorbax_Guide.pdf (accessed on 02 July 2013).

[96] http://www.phenomenex.com/webdocument/reversedguide.pdf (accessed on 02 July 2013).

[97] http://www.ymc-europe.com/ymceurope/files/literature/Flyer_YMC-Triart-C18_19_Englisch.pdf (accessed on 02 July 2013).

[98] http://www.ymc.de/ymceurope/files/technicaldatasheets/TD_Fast_LC.pdf (accessed on 02 July 2013).

[99] http://www.dionex.com/en-us/webdocs/4139-DS_Acclaim_120_LPN1413-02.pdf (accessed on 02 July 2013).

[100] http://www.lifescience.ca/DATA/CATALOGUE/51~v~Hypersil_Gold_Technical_Guide.pdf (accessed on 02 July 2013).

[101] Han, S.Y., Kim, Y.A., *Tetrahedron*, no. 60, pp. 2447-2467, 2004.

[102] ://www.merckmillipore.com/is-bin/INTERSHOP.enfinity/WFS/Merck-MY-Site/en_US/-/USD/ViewPDF-Print.pdf?RenderPageType=ProductDetail&CatalogCategoryID=oIyb.s1LuicAAAEWsOEfVhTl&ProductUUID=gYSb.s1OFRsAAAEZxdU80Y70&PortalCatalogUUID=t02b.s1LX0MAAAEWc9UfVhTl (accessed on 02 July 2013)

[103] http://www.usp.org/usp-nf/compendial-tools/pqri-approach-column-equiv-tool (accessed on 02 July 2013)

[104] www.emdmillipore.com/showBrochure?id=200907.345 (accessed on 02 July 2013)

[105] Moldoveanu, S., David V., *Essentials in modern HPLC separations*, Elsevier publications, 2013.

[106] http://www.chem.agilent.com/Library/primers/Public/LC-Handbook-Complete-2.pdf (accessed on 02 July 2013)

[107] Carr, D., *A guide to the analysis and purification of proteins and peptides by reversed-Phase HPLC*. Scotland: Advanced Chromatography Technologies.

[108] http://www.waters.com/waters/downloadFile.htm?lid=10122587&id=10122586&fileName=720003167en&fileUrl=%2fwebassets%2fcms%2flibrary%2fdocs%2f720003167en.pdf (accessed on 02 July 2013)

[109] Matos, J., Moreira, R., Gomes, P., Vale, N., *Tetrahedron 64*, pp. 11144-11149, 2008.

[110] Dolan, J., *A guide to HPLC and LC-MS Buffer Selection*. Advanced Chromatography Technologies.

[111] http://ccc.chem.pitt.edu/wipf/Web/HPLC_RP_Buffers.pdf (accessed on 02 July 2013)

[112] Umetrics academy, *Design of experiments*.: Umetrics AB, 2009.

[113] http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=20084 (accessed on 02 July 2013)

[114] Y.C.Lee, Lam, H., Zhang , X.M., Chan,.C. C., *Analytical method validation and instrument performance verification*. John Wiley & Sons, Inc. publications, 2004.

[115] http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm127984.pdf. (accessed 02 July 2013)

[116] http://www.ich.org/fileadmin/Public Web Site/ICH Products/Guidelines/Quality/Q2 R1/Step 4/Q2 R1 Guideline.pdf. (accessed 02 July 2013)

APPENDIX 1 –METHOD DEVELOPMENT: GRADIENT PROFILES

Table A1

Time (mins)	Flow rate (mL/min)	0.1 % TFA	0.08 % TFA in ACN
0	0.3	100	0
5	0.3	100	0
30	0.3	50	50
32	0.3	50	50
35	0.3	100	0
40	0.3	100	0

Table A2

Time (mins)	Flow rate (mL/min)	0.1 % TFA	0.08 % TFA in ACN
0	1	95	5
5	1	95	5
40	1	50	50
45	1	50	50
48	1	95	5
60	1	95	95

Table A3

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.38	95	5
5.29	0.38	95	5
58.24	0.38	50	50
62.47	0.38	50	50
63.53	0.38	95	5
68.82	0.38	95	95

Table A4

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.40	95	5
5.0	0.40	95	5
55.00	0.40	50	50
59.00	0.40	50	50
60.00	0.40	95	5
65.00	0.40	95	95

Table A5

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.91	95	5
2.50	0.91	95	5
27.50	0.91	50	50
29.50	0.91	50	50
30.00	0.91	95	5
32.50	0.91	95	95

Table A6

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.91	95	5
5.61	0.91	95	5
58.62	0.91	50	50
62.87	0.91	50	50
63.93	0.91	95	5
69.23	0.91	95	95

Table A7

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.25	95	5
3.97	0.25	95	5
43.68	0.25	50	50
46.85	0.25	50	50
47.65	0.25	95	5
51.62	0.25	95	95

Table A8

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.69	95	5
3.34	0.69	95	5
32.75	0.69	50	50
35.10	0.69	50	50
35.69	0.69	95	5
38.63	0.69	95	95

Table A9

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.26	95	5
3.82	0.26	95	5
42.06	0.26	50	50
45.12	0.26	50	50
45.88	0.26	95	5
49.71	0.26	95	95

Table A10

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.31	95	5
6.47	0.31	95	5
71.18	0.31	50	50
76.32	0.31	50	50
77.65	0.31	95	5
84.12	0.31	95	95

Table A11

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.36	95	5
5.59	0.36	95	5
61.47	0.36	50	50
65.94	0.36	50	50
67.06	0.36	95	5
72.65	0.36	95	95

Table A12

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.3	100	0
10	0.3	100	0
35	0.3	65	35
36	0.3	100	0
46	0.3	100	0

Table A17

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.3	97	3
4	0.3	97	3
10	0.3	90	10
17	0.3	80	20
20	0.3	70	30
21	0.3	97	3
25	0.3	97	3

Table A18

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.3	97	3
4	0.3	97	3
10	0.3	90	10
17	0.3	80	20
20	0.3	60	40
21	0.3	97	3
25	0.3	97	3

Table A19

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.3	97	3
4	0.3	97	3
10	0.3	90	10
17	0.3	80	20
20	0.3	50	50
21	0.3	97	3
25	0.3	97	3

Table A20

Time (mins)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B %
0	0.3	97	3
7	0.3	97	3
10	0.3	90.5	9.5
30	0.3	65	35
31	0.3	97	3
35	0.3	97	3

Table A21

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
9.00	0.275	97	3
12.00	0.55	92.5	7.5
16.00	0.55	87.5	12.5
22.00	0.55	60	40
23.00	0.55	97	3
26.00	0.55	97	3

Table A22

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.55	97	3
6.00	0.55	97	3
9.00	0.55	92.5	7.5
13.00	0.55	87.5	12.5
16.00	0.55	60	40
18.00	0.55	97	3
20.00	0.55	97	3

Table A23

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.225	97	3
6.00	0.225	97	3
9.00	0.55	92.5	7.5
13.00	0.55	87.5	12.5
15.00	0.55	60	40
16.00	0.55	97	3
18.00	0.55	97	3

Table A24

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.25	97	3
4.00	0.25	97	3
6.50	0.55	92.5	7.5
9.00	0.55	87.5	12.5
9.50	0.55	60	40
10.00	0.55	97	3
12.00	0.55	97	3

Table A25

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.55	97	3
6.00	0.55	97	3
8.50	0.55	92.5	7.5
11.00	0.55	87.5	12.5
11.50	0.55	60	40
12.50	0.55	97	3
14.00	0.55	97	3

Table A26

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.50	97	3
4.00	0.50	97	3
10.00	0.50	92.5	7.5
11.00	0.50	87.5	12.5
11.50	0.50	60	40
12.00	0.50	97	3
14.00	0.50	97	3

Table A27

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.50	97	3
4.00	0.50	97	3
11.00	0.50	92.5	7.5
12.00	0.50	87.5	12.5
13.00	0.50	60	40
13.50	0.50	97	3
16.00	0.50	97	3

Table A28

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.50	97	3
4.00	0.50	97	3
10.00	0.50	92.5	7.5
13.00	0.50	87.5	12.5
14.00	0.50	60	40
14.50	0.50	97	3
17.00	0.50	97	3

Table A29

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.50	97	3
4.00	0.50	97	3
10.00	0.50	92.5	7.5
10.25	0.50	87.5	12.5
12.00	0.50	60	40
12.50	0.50	97	3
15.00	0.50	97	3

Table A30

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.50	97	3
4.00	0.50	97	3
10.00	0.50	92.5	7.5
10.25	0.50	87.5	12.5
11.50	0.50	87.5	12.5
12.00	0.50	60	40
12.50	0.50	97	3
15.00	0.50	97	3

APPENDIX 2 – FINAL METHOD OPTIMISATION: GRADIENT PROFILES

Gradient 1

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.3	97	3
9.00	0.3	97	3
11.50	0.5	92.5	7.5
17.00	0.5	79	21
21.00	0.5	60	40
22.00	0.5	97	3
25.00	0.5	97	3

Gradient 2

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.3	97	3
9.00	0.3	97	3
11.50	0.5	92.5	7.5
13.50	0.5	79	21
19.00	0.5	60	40
21.00	0.5	97	3
25.00	0.5	97	3

Gradient 3

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.3	97	3
9.00	0.3	97	3
12.00	0.5	92.5	7.5
18.00	0.5	79	21
22.00	0.5	60	40
23.00	0.5	97	3
26.00	0.5	97	3

Gradient 4

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.25	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.5	92.5	7.5
18.00	0.5	79	21
22.00	0.5	60	40
23.00	0.5	97	3
26.00	0.5	97	3

Gradient 5

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.25	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.45	92.5	7.5
18.00	0.45	79	21
22.00	0.45	60	40
23.00	0.45	97	3
26.00	0.45	97	3

Gradient 6

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.25	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.55	92.5	7.5
18.00	0.55	79	21
22.00	0.55	60	40
23.00	0.55	97	3
26.00	0.55	97	3

Gradient 7

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.5	92.5	7.5
18.00	0.5	79	21
22.00	0.5	60	40
23.00	0.5	97	3
26.00	0.5	97	3

Gradient 8

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.45	92.5	7.5
18.00	0.45	79	21
22.00	0.45	60	40
23.00	0.45	97	3
26.00	0.45	97	3

Gradient 9

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.45	92.5	7.5
16.00	0.45	79	21
22.00	0.45	60	40
23.00	0.45	97	3
26.00	0.45	97	3

Gradient 10

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
3.00	0.3	97	3
9.00	0.3	97	3
12.00	0.4	92.5	7.5
16.00	0.4	79	21
22.00	0.4	60	40
23.00	0.4	97	3
26.00	0.4	97	3

Gradient 11

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
3.00	0.55	97	3
9.00	0.55	97	3
12.00	0.55	92.5	7.5
16.00	0.55	79	21
22.00	0.55	60	40
23.00	0.55	97	3
26.00	0.55	97	3

Gradient 12

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.275	97	3
3.00	0.275	97	3
9.01	0.3	97	3
12.00	0.3	92.5	7.5
16.00	0.4	79	21
22.00	0.4	60	40
23.00	0.4	97	3
26.00	0.275	97	3

APPENDIX 3 –METHOD OPTIMISATION: RESULTS TABLES

Table 1 *Effect of buffer pH upon chromatographic performance for Sample Set B.*

pH	Resolution of critical peak pair	Plate count - smallest result	k* of first peak	Tailing - largest result
2.8	1.6	2,152	2.6	2.2
2.9	1.6	2,544	2.5	1.7
3.0	1.6	2,380	2.5	1.9
3.1	1.7	2,387	2.4	1.7
3.2	1.9	2,547	2.4	1.5
3.3	1.3*	829	2.4	1.5
3.8	Not calculated**	Not calculated*	2.4	1.9
4.3	2.6	707	1.6	1.8
4.8	2.5	102	1.2	4.6

*Change in critical peak pair

**Empower software did not automatically calculate result due to interference with a closely eluting peak

Table 2 *Effect of flow rate on chromatographic performance for Sample set B*

Flow rate	Resolution of critical peak pair	Plate count - smallest result	k* of first peak	Tailing - largest result
0.25 mL/min	Not calculated*	3,522	3.3	1.5
0.30 mL/min	1.7	3,147	2.3	1.9
0.35 mL/min	1.9	2,699	2.1	1.5

*Empower software did not automatically calculate result due to interference with a closely eluting peak

Table 3 *Effect of column temperature upon chromatographic performance for Sample set B*

Temperature	Resolution of critical peak pair	Plate count - smallest result	k* of first peak	Tailing - largest result
10 ∘C	Not calculated*	3,148	2.9	1.5
15 ∘C	1.7	3,145	2.7	1.8
20 ∘C	1.7	3,077	2.6	1.6
25 ∘C	1.6	3,023	2.5	1.5
30 ∘C	Not calculated*	2,800	2.4	1.6
35 ∘C	Not calculated*	2,509	2.2	1.5
40 ∘C	Not calculated*	2,288	2.1	1.5
45 ∘C	Not calculated*	1,991	2.0	1.5

Table 4 *Effect of acetonitrile concentration upon retention of PyBrOP.*

% Acetonitrile / time	PyBrOP R_t	PyBrOP tailing
30 % within 20 minutes	21.868	2.0
40 % within 20 minutes	21.311	1.3
50 % within 20 minutes	20.732	1.3

Table 5 *Effect of pH upon chromatographic performance and AU response for Sample set B*

pH	Resolution of critical peak pair	Plate count - smallest result	k* of first peak	Response AU of largest peak	Tailing - largest result
3.2	1.0	1,084	3.5	1.1	1.6
3.3	1.0	1,125	3.5	1.3	1.7
3.4	1.2	1,274	3.5	1.8	2.1
3.6	1.4	2,465	3.5	2.1	2.0
3.8	Not calculated*	Not calculated*	3.3	2.5	2.0
3,9	Not calculated*	1,777	3.1	2.5	2.0
4.2	2.5	1,370	1.7	2.5	2.0
4.4	1.9	1,381	2.1	2.5	2.0
4.6	Not calculated*	1,172	2.0	2.5	2.0
4.8	Not calculated*	768	1.8	2.5	2.1

Table 6 *Results from optimum buffer concentration for separation*

Conc.	Resolution of critical peak pair	Plate count - smallest result	k* of first peak	Response AU of largest peak	Tailing largest re
5 mM	0.4	3,593	3.3	2.4	2.0
10 mM	1.0	1,810	3.5	2.0	2.3
15 mM	0.8	2,235	3.6	1.2	1.9
20 mM	1.3	2,297	3.6	1.0	2.2
25 mM	2.0	2,298	3.7	0.6	2.1
30 mM	2.5	2,254	3.8	0.5	2.0
40 mM	2.4	2,151	3.8	0.2	1.9

Table 7 *Results of gradient evaluation*

Gradient	Rs. of critical peak pair	Plate count - smallest result	k of first peak	Response AU of largest peak	Tailing - largest result
1	0.9	1,403	3.0	2.5	2.2
2	1.1	1,302	2.8	2.5	2.1
3	1.4	1,140	2.2	2.5	2.1
4	1.2	1,885	3.0	2.5	2.1
5	1.1	1,883	3.1	2.5	2.1
6	1.3	1,306	2.8	2.5	2.1
7	1.7	1,299	2.4	2.5	2.0
8	2.0	1,225	2.4	2.5	2.1
9	2.0	1,504	2.5	2.5	2.1
10	2.1	1,427	2.5	2.5	2.0
11	2.7*	1,584	2.6	2.5	1.6
12	2.9	1,530	2.5	2.5	1.6

Note: Gradient profiles detailed on page 10. *Change in critical peak pair