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Thermodynamics and Kinetics of Small Molecule Interactions with Human Hair Keratin

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Abstract

Keratin is a ubiquitous class of proteins with extreme importance to the textiles and hair care industries. The molecular behaviour between keratins and small molecules of interest is poorly understood, prompting the need for studies to better understand interactions between small molecules and keratin.

In this work solid-state ^1H spin-lattice (T_1) and spin-spin (T_2) NMR techniques have been developed to evaluate interactions between small molecules and hair fibres, and understand how these interactions can inform the level of damage in these materials using ^{13}C Cross Polarisation/Magic Angle Spinning NMR. The sorption and desorption of different molecules has also been studied using High Performance Liquid Chromatography and Dynamic Vapour Sorption.

The key findings from this work include a reproducible method to measure molecular interactions within human hair using NMR. This study also demonstrates that molecular behaviour in hair can be predicted from the molecular properties of the active chemicals in hair. Increasing moisture content and damage in hair samples both result in increased interactions with small molecules. This work suggests that NMR relaxation times can be used as a measure of damage in hair. A novel HPLC method was devised to measure real-time loss of dye actives from dyed hair, allowing desorption kinetic analysis to be performed. Finally, hysteresis in hair water sorption isotherms was examined by reducing the crosslink density in hair samples. It was determined that hysteresis in water sorption isotherms results from the

swelling properties of the hair fibre, with low crosslinked density associated with reduced hysteresis and early onset of moisture induced glass transition events in hair.

This work has developed three robust experimental methods; using relaxation properties to interrogate molecular interactions and hair damage, and also using HPLC to measure desorption from keratin fibres. The foundations these methods have built will greatly improve understanding of small molecular behaviour in keratins and how keratin structure affects these interactions. This work has significant applications to product formulation for both the hair care and textiles industries.

Statement of Originality

I hereby certify that the content of this thesis is my own work, carried out in the Department of Chemistry and the Department of Chemical Engineering of Imperial College London between October 2017 and November 2021. All external assistance, collaboration, and any information derived from other sources have been acknowledged appropriately.

Susannah Molisso

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My dad passed in March 2020, and my grandmother developed dementia shortly after. I miss you both so much all the time, and wish you could have both read (or pretended to read) this thesis.

List of Abbreviations

AHT	4-amino-2-hydroxytoluene
CMC	Cell Membrane Complex
CP	Cross Polarisation
CPMG	Carr-Purcell-Meiboom-Gill
CSA	Chemical Shift Anisotropy
DVS	Dynamic Vapour Sorption
HDAP	1-hydroxyethyl-4,5-diamino pyrazole
HPLC	High Performance Liquid Chromatography
IF	Intermediate Filaments
KAP	Keratin Associated Protein
KIF	Keratin Intermediate Filament
MAS	Magic Angle Spinning
MEA	Monoethanolamine
MW	Molecular weight
NMR	Nuclear Magnetic Resonance
RH	Relative Humidity
SS-NMR	Solid-state Nuclear Magnetic Resonance
TGA	Thermogravimetric Analysis

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Chapter 1 - Introduction

1.1 Introduction

The haircare and textiles industries have a combined value of over £70 billion each per year in the UK alone.^{1,2} Keratin is the protein which forms the major substituent of both human hair and wool, making it an important protein to understand both in terms of its kinetics, and its structure activity relationships.

A successful haircare product results in the modification of hair keratin to a desired outcome. This can include modifications that rely on structural changes to keratin such as: changing hair fibre shape with permanent waving or chemical straightening, and surface modification to prevent lipid loss.^{3,4} Or, modifications can rely on chemical interactions e.g., with dye molecules to change hair colour, silicones for improved texture, or protein deposition for increased hair strength.^{5,6}

With such a reliance on the understanding of how keratin responds to a chemical for these modifications, it is surprising that after over fifty years of research the interactions between keratin and these actives are still not well understood. A major complication is the highly heterogeneous nature of keratins, which contain both amorphous and crystalline fractions.⁴ Interactions of small molecules vary across the fibre length,⁷ and between fibres of different origins, making any interaction difficult to predict.

The advancement of keratin science underpins the future progress of both the haircare and textile industries, demonstrating that understanding how small molecules interact with keratin is of high research importance.

To understand the difficulties in this task, this chapter will give an introduction into the highly complex keratin structure, with a focus on human hair keratin.

Virgin hair is a standard terminology used historically in the hair care industry. However, this author believes that using 'virgin' as a synonym for 'not damaged' in this context, is a relic of out-dated beliefs relating to the status of virginity in wider society. Therefore, going forward in this thesis, 'virgin' hair will be referred to as 'untreated hair'.

1.2 Keratin

1.2.1 Diversity of keratins

Keratins are a class of fibrous proteins which are found in abundance in biological systems and nature, in comparison to other intermediate filaments (IFs).⁸ In cells keratins function in important cellular processes, such as acting as intermediate filaments for the structure of cytoskeleton in eukaryotic cells, and taking part in transmembrane signalling processes.^{9,10} In nature, keratins are found in all vertebrates as structural components of complex hierarchal macrostructures in the form of wool, quill, nail, feather, hoof and hair.¹¹

1.2.2 The α and β -structure of keratin

Keratin proteins are found in both an α -helical and β -sheet structure. In the α -helical form, two keratin strands wrap together using electrostatic and hydrophobic interactions, and disulphide bonds to form a coiled coil.¹²

To form this particular geometry, a repeating sequence of seven amino acids is needed.¹³ This heptad (abcdefg) requires the a and d amino acids to be non-polar, including residues such as leucine and isoleucine.¹⁴ The heptad sequence of polar and non-polar amino acids allows the formation of a hydrophobic core in the centre of the repeat (Figure 1.1).^{11,15}

The coiled coil is further stabilised by electrostatic interactions between the positively charged g residue (e.g., lysine, arginine, or histidine) and the negatively charged e residue (e.g., aspartic acid or glutamic acid).^{14,16} The coiled coil structures have a 47 nm long crystalline central rod domain which are capped by globular N- (amino) and C- (carboxyl) terminals.^{8,17,18}

In contrast to the strong α -helical structure, keratin can also form a β -sheet pleated molecular structure, where β strands link through hydrogen bonding in antiparallel chains to give rise to the pleated sheet formation.¹² α -keratin can be stretched to transition into β -keratin, though this mechanism is not well understood.¹⁹

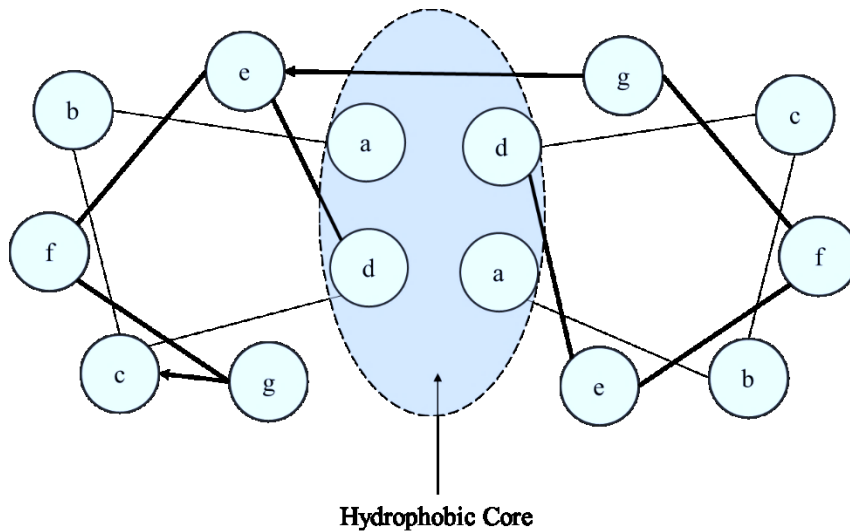


Figure 1.1 The heptad sequence of amino acids in a coiled coil of keratin, highlighting the central hydrophobic core of a and d amino acids. This figure is adapted from Chemical Society Reviews, Popescu and Höcker¹¹

1.2.3 Keratin Intermediate Filament's and microfibrils

Keratin coiled coils assemble to form higher order structures, including keratin intermediate filaments (KIF's), which are the building block of hair fibres. The assembly from coiled coils to KIF's is not well understood. First dimers aggregate in an antiparallel coordination to form four stranded protofibrils, with a 2 nm diameter.^{12,18} These protofibrils are stabilised by ionic interactions between the charged residues on the α -helical backbone and also the overlapping of the N- and C- terminals.^{20,21} Protofibrils then polymerise to groups of 8-16 coiled coils to create a KIF, with a diameter of approximately 7 nm.¹² KIF's can be acidic (Type 1) or basic (Type II), and a 1:1 ratio of both KIF's form heterodimers which assemble into microfibrils.^{11,18}

1.3 Hair structure

1.3.1 Structure of hair

Hair has a complex and highly hierarchal structure which is 50-100 μm in diameter.²² It can be considered primarily as a two-phase model with crystalline regions of α - or β -keratin embedded in an amorphous matrix.^{4,23} The resilience and stability of the hair fibre is due to the large content of cysteine residues, which form S-S crosslinks (cystine) throughout the fibre.¹¹

This complex structure can be separated into three main sections; the cuticle, a protective layer to the hair shaft, the inner cortex, and the medulla which is absent in most hair types. Figure 1.2 shows a simplified flow diagram to show how keratin proteins assemble to form the complete hair structure.

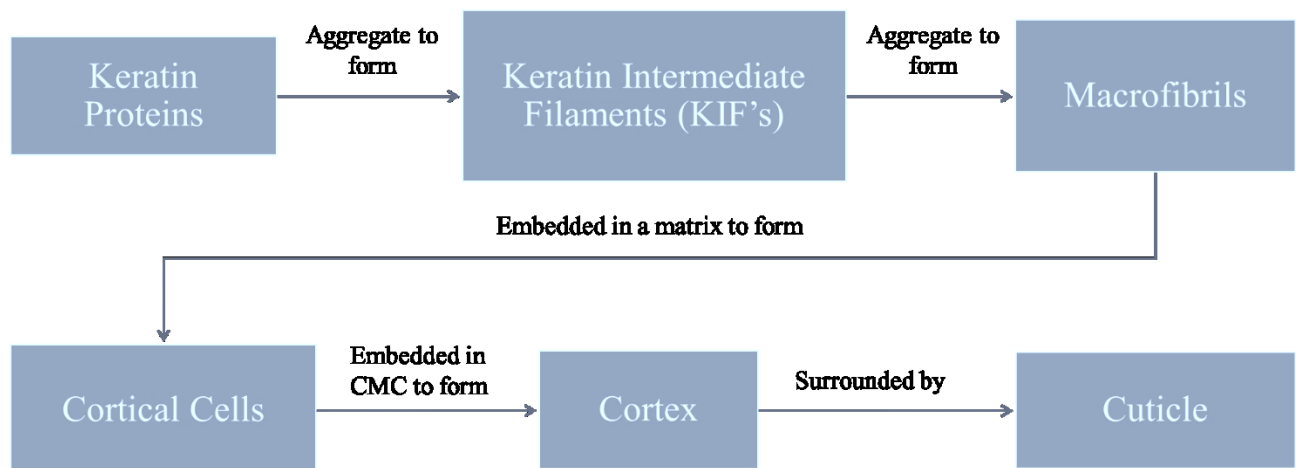


Figure 1.2 A flow diagram showing the simplified hierarchal assembly of the full hair fibre from initial keratin proteins.

This section will go into further detail, briefly breaking down the main features of a hair shaft, cuticle, cortex, and the medulla.

1.3.2 The Cuticle

The outermost layer of hair is the cuticle, which functions as a chemically resistant barrier to protect the hair shaft from chemical and physical stress.

It is comprised of 0.5 μm thick 'disordered' overlapping flat cells, which in 9-10 sheets, gives the cuticle a thickness of approximately 5 μm .^{22,24} The substructure of this cuticle from outwards in, can be described in 3 layers; the epicuticle, the exocuticle, and the endocuticle which sits on an inner layer of the cell membrane complex (CMC).^{11,25} A simplified cross-section of the whole cuticle structure is given in Figure 1.3.

The 3 nm thick epicuticle is the surface of the hair fibre, coated in 18-methyl eicosanoic acid (18-MEA) through thioester linkers which affords the layer high hydrophobicity.^{26,27} The epicuticle sits on the exocuticle which can be divided into the A-layer and B-layer.

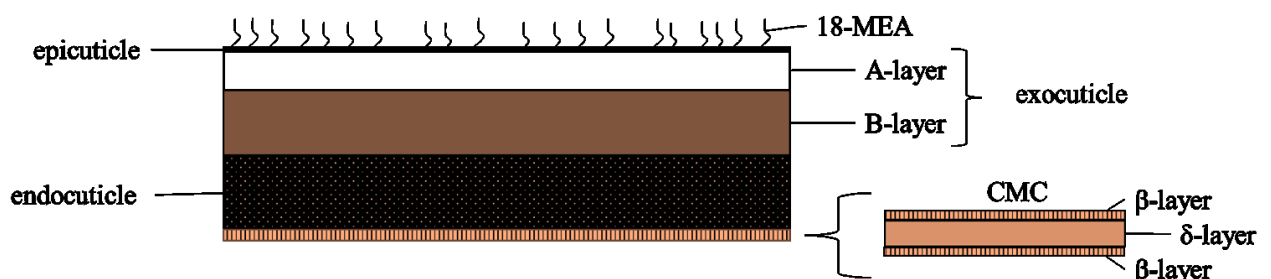


Figure 1.3 A simplified cross-section of the cuticle of human hair. The cuticle layers are shown from the outer epicuticle to the CMC. The CMC can then be divided into a further 3 sublayers.

The 100 nm thick A-layer is the most highly cross-linked region of hair, having both disulphide bonds (35% cystine content) and isopeptide crosslinks, imparting great resilience to solubilisation from reducing agents.⁴ In contrast, the 150 nm thick B-layer has only a 15% cystine content.²⁸ Underneath this layer lies the endocuticle which sits on a thin layer of 'intercellular cement' called the cuticle-cuticle CMC. The endocuticle has a thickness which varies from 50-300 nm, with a low cystine content of approximately 3%.^{4,28} This makes this layer most prone to swelling with few crosslinks present to restrict it.

The cuticle-cuticle CMC is made of cellular membranes and adhesives which act as the 'glue' for cuticle cells. It is made of three layers, a central 15 nm δ -layer which lays in between two 5 nm lipid β -layers.^{4,29,30} The δ -layer has proteins with little crosslink content, while the upper β -layer contains covalently bound 18-MEA. The lower β -layer also contains covalently bound fatty-acids, but due to the difficulty in isolating these layers, their contents remain not well understood.^{4,26}

1.3.3 The Cortex

The cortex comprises of 70-90% of the total mass of human hair.⁴ It has less sulphur content than the cuticle, containing spindle shaped cortical cells which are embedded in the cortex-cortex CMC.^{4,11}

The cortex-cortex CMC is similar to the cuticle-cuticle CMC, but with lipids which are non-covalently bonded. The lipids in the cortex-cortex CMC are found mostly in disordered monolayers and bilayers, with a preferred orientation parallel to the surface of hair.³¹

Cortical cells are approximately 50-100 μm long and 1-6 μm thick, with axes arranged parallel to the axis of the hair shaft.^{4,32} Cortical cells have been shown to twist around each other to

form a 'cortical yarn' which has been proposed as another reason for the strength of the hair shaft.²⁵ Three types of cortical cell can be present: orthocortical with low cystine content, mesocortical with medium cystine content, and paracortical with high cystine content.^{4,11} The segregation and relative amounts of these cells help determine the shape and wave of human hair.⁴

Cortical cells themselves are comprised of 5-8 macrofibrils surrounded by a disorganised matrix, containing cytoplasmic and nuclear remnants.^{4,11} The matrix has a high disulphide bond content, filled with keratin associated proteins (KAP's). The matrix has been shown to have high stretching ability at low temperatures which is uncommon for a typical crosslinked polymer, suggesting that there is a large degree of orientation along the hair shaft axis.³³ Macrofibrils are arranged in a spiral formation in the cortical cell, with each microfibril being made up of 500-800 microfibrils, with a diameter of approximately 7.4 nm.^{25,34}

Macrofibrils can be seen as a two-phase structure where microfibrils pack together in whorl-like or near hexagonal configurations, and are embedded in a high sulphur amorphous inter-microfibrillar matrix.^{35,36} While the content of the matrix is not well understood, it has been shown to contain phosphorylated and high sulphur proteins which allows a high S-S bond content, and also high levels of cross-linking to occur between the matrix and cysteine residues on the rod domain in the microfibrils.^{27,37}

Microfibrils themselves account for 60% of the total cortex mass.³⁸ The microfibrils are in turn are comprised of KIF's forming the crystalline domain of hair.^{4,11} For each cortical cell, there are approximately 20,000 KIF's present.³⁹

1.3.4 The Medulla

In white and coarser hair types, a section of hair called the medulla can be found.³¹ This region can be continuous or discontinuous in the core of the hair fibre.⁴⁰ It is low in cystine content, and contains cells which are spherical hollow vacuoles, making this region largely empty.^{4,5,41} The medulla also contains lipids, yet the composition of these lipids varies greatly depending on hair origin. The exact composition is still poorly understood due to the poor solubility of the medulla, and it's resistance to alkali and keratolytic agents.^{4,40}

In summary of this section, Figure 1.4 shows the full structure of the hair fibre.

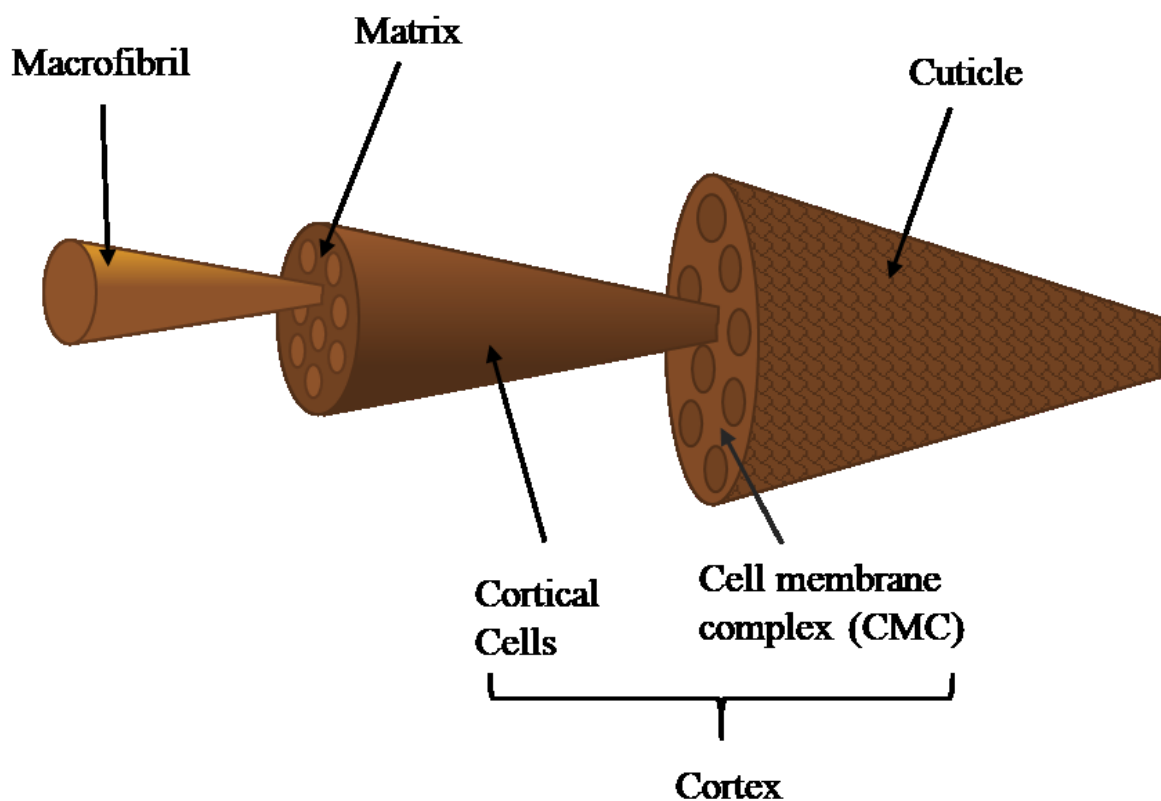


Figure 1.4 The simplified structure of hair, showing the main structural features and hierarchal substituents. This figure is adapted from the Textile Research Journal, M. Feughelman.⁴⁰

1.3.5 Chemical composition

While hair is mostly comprised of α -keratin, it also contains water, lipids, pigments, and trace metals.⁴

Human hair has a total moisture content of around 15%, with approximately 11% of that being more labile water molecules and approximately 4% being more strongly bound water molecules.⁴² Like most biological materials, water plays an important role in the function and mechanical properties of the hair fibre.^{4,43} How water partitions into different populations in hair is also of significant research interest. The identification of populations interacting with a protein has been explored extensively in literature, with interacting water populations having distinct spin-lattice (T_1) and spin-spin (T_2) relaxation times to that of the mobile surrounding hydration layers.⁴⁴ 'Bound' water is associated with the keratin structure, having similar rotational correlation times to keratin, while 'free' water is not directly associated with keratin structure, making it more mobile than the bound population.^{45,46} The concept of 'bound' and 'free' water populations will be discussed in further detail in Chapter 3.1.2.

Hair fibres are composed of 1-9% lipid.⁴⁷ The surface of hair has covalently bound MEA, which accounts for approximately 40% of the total lipids in hair.²⁶ The rest of the hair fibre contains other lipids including cholesterol, ceramides, and fatty acids such palmitic, stearic, and oleic acid.^{4,26} Hair also contains sebaceous lipids originating from the scalp, which are typically branch chained fatty acids.²⁶

The elemental composition of hair breaks down to 49.0% carbon, 14.5% nitrogen, 30.0% oxygen and 3.0% sulphur.⁴⁷ Less than 1% of the fibre contains trace elements, largely originating from sweat deposits, but the most commonly found metals - calcium and

magnesium - are absorbed from tap water into hair. ⁴ Trace metals including iron and copper are found in areas with high carboxylic acid content such as the endocuticle, CMC and medulla.^{4,48}

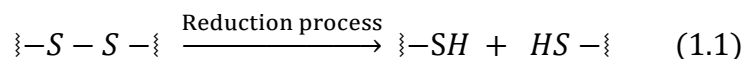
Pigments in the hair fibre have the highest metal content than other parts of the fibre.⁴ Melanin pigments are formed in the hair follicle, all resulting from the initial amino acid tyrosine, and give the colour to the hair fibre. Grey and blonde hairs have less pigment, and become more sensitive to light radiation.⁴ Metal ions in hair pigments are also important in the degradation of hydrogen peroxide to form hydroxyl radicals, which are necessary for bleaching and hair dyeing processes.⁴⁹

The exact amino acid content is not well defined in hair due to variations between fibres. Various studies have shown however that hair contains high amounts of arginine, cysteine, glutamic acid, and serine.^{4,47}

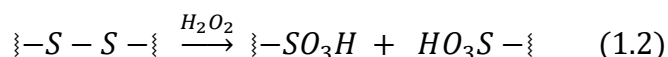
1.3.6 Disulphide bonds

Cystine is present in reduced or oxidised forms in proteins, and some success has been had using ¹³C NMR to distinguish between these states.^{50,51}

The cross-linking of cysteine residues forms disulphide bonds in hair. This bond is responsible for the high stability, rigidity and insolubility of hair and keratin fibres.^{4,52} This bond can be broken through reduction processes - which are usually found in hair treatments such as permanent waving - to form thiols. (Equation 1.1). ⁴



Cleavage of the disulphide bond can also occur through oxidation such as bleaching to form cysteic acid (Equation 1.2).⁴



As disulphide bonds are integral to the conformation of hair proteins, matrix and cuticle, reduction and oxidation of these crosslinks results in the damage and reduced stability of the fibre.⁵³

Disulphide bonds have been identified in the crystalline and amorphous domains of hair with different chemical shifts in ¹³C Cross Polarisation/Magic Angle Spinning NMR spectra.⁵⁴ The crystallinity of ordered regions in keratin may also result from the ordering of the repeated β-bend structure of disulphide bonds, which forms a hydrophobic environment in the matrix.⁵⁴

The disulphide bond content of a hair fibre has also been shown to have great effect on the fibres water sorption abilities, with a reduction in disulphide bonds leading to reduced sorption and increased fibre permeability.⁵⁵

The swelling capability of the hair matrix also suggests that the disulphide bonds are intramolecular as opposed to intermolecular, resulting in the matrix to behave like a crosslinked gel.^{27,35}

1.3.7 Variability between fibres

While the hair fibre itself is incredibly heterogeneous, the difference between fibres can vary greatly, making analysis of hair difficult and unpredictable.²⁸ The two main classes of variability are variability between fibres of different origins, and fibres of the same origin.

What is macroscopically clear, is that hairs of different ethnic origins are visibly different. It was once thought that the microstructures of these different hair types were similar, but has since been revealed that ethnic variations include differences in thermal degradation properties, water content, cross-sectional shape, pigmentation, keratin transverse architecture, and lipid orientation.^{8,56–58}

Within hair fibres of the same ethnic origin the variabilities are wide ranging. There are variations between the packing of coiled coil forms of keratin between hair samples, and variations in amino acid content due to genetics.^{4,8} In the larger structures, the thickness of the cuticle varies greatly, leading to variations in the diameter of hair fibres which has been thought to correlate to the variations in lipid content of hair fibres.^{4,31}

This variability between fibres means that analyses on hair (including in this work) are largely performed using batch sources of hair (multiple donors, same hair type and ethnic origin), to average the variability between fibres and get an analytical result that represents the overall behaviour of a particular type of hair.

1.4 Characterisation tools

1.4.1 Solid-state Nuclear Magnetic Resonance (SS-NMR)

Nuclear Magnetic Resonance (NMR) is often used to characterise biological systems, but in the case of large insoluble proteins, such as keratin, solid state NMR is needed.^{59–61} Due to the homogeneous and complex nature of hair and other keratins, static NMR provides broad and hard to interpret spectra. Magic Angle Spinning (MAS) is therefore integral to reduce the broadness of signals and achieve clear spectra from keratins. The theory and physics behind NMR spectroscopy will be explained in detail in Chapter 2.1, this section will instead focus on the applications of this versatile method.

¹³C CP/MAS spectra recorded using SS-NMR has been used to characterise numerous types of keratins including horse hoof, horse hair, myoglobin, cytochrome C, human hair, human hair melanin, mouse KIF's, gecko keratin, rhino and more commonly wool.^{62–65}

These ¹³C CP/MAS spectra have been essential to the mapping of amino acids in keratin. Figure 1.5 shows a ¹³C CP/MAS spectra for untreated brown Caucasian hair, and the assigned peak designations are found in Table 1.1. Clear signals can be seen from the α -carbon of the amino acids between approximately 53 and 60 ppm. Peaks at 10-48 ppm represent the alkyl components of the amino acid side chains, with the peak at 40 ppm corresponding to the β -carbon of the leucine residue and also cross-linked cystine.^{52,65,66} Finally, the carbonyl signals for all amino acids are found at 172-176 ppm.^{65,67,68}

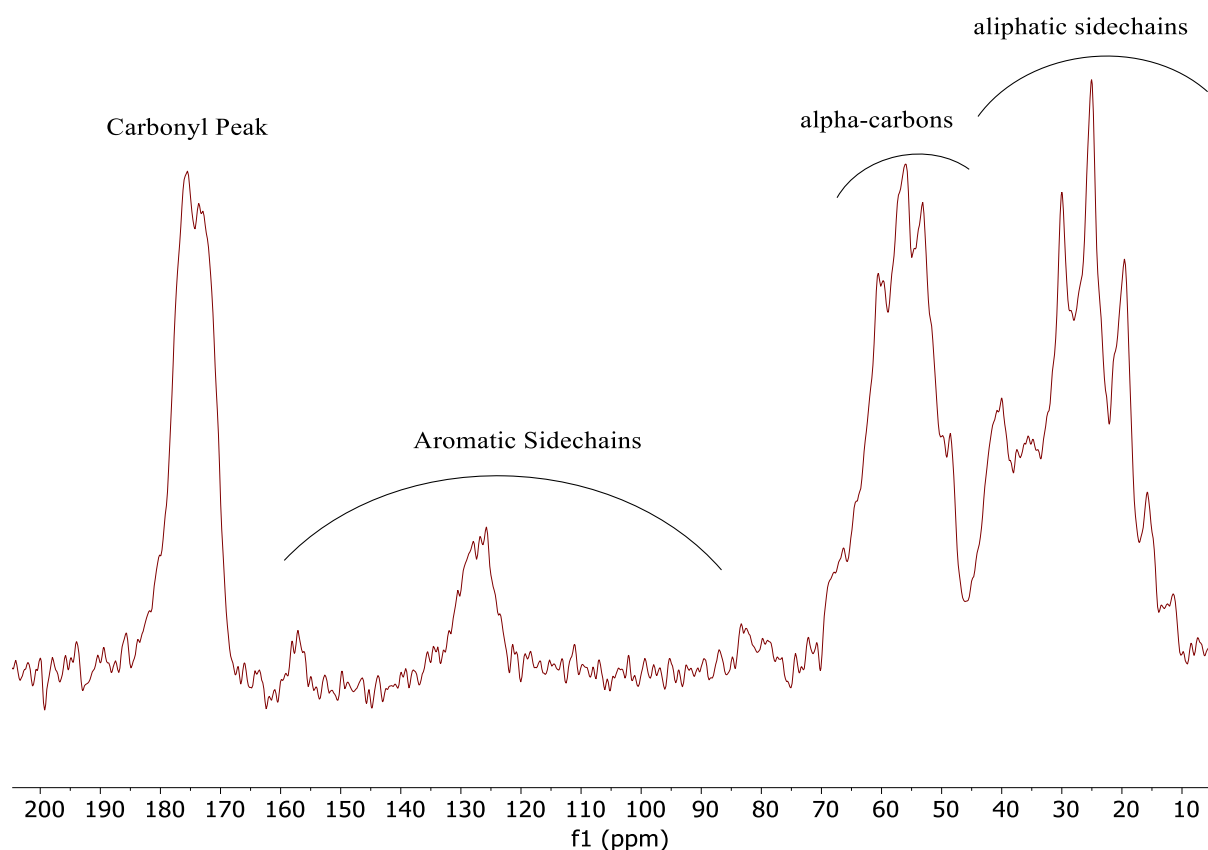


Figure 1.5 The ^{13}C CP/MAS spectrum of brown untreated Caucasian hair, with major features annotated. This spectrum was recorded using a solid-state 200 MHz NMR spectrometer.

The carbonyl peak is particularly useful in determining the α -helix and β -sheet keratin content in hair.^{52,65,67–70} The broad carbonyl signal can be deconvoluted to two peaks, with the downfield peak representing the α -helix content and the upfield peak representing the β -sheet and coiled-coil keratin content.

As well as recording the amino acid content, NMR can examine other structural properties of keratins, including KIF structure, disulphide bond distribution, and KAP structure.⁷¹ The microstructure of KIF's has been further examined, by using spin-lattice T_1 relaxation times to demonstrate the flexibility of side chains of coiled coil keratin rod domains.^{10,67}

Table 1.1 Chemical peak assignment of the ^{13}C CP/MAS spectra of untreated Caucasian hair in Figure 1.5.⁶⁵ *indicates peaks not present in this sample, but commonly found in other hair samples.

Approximate chemical shift (δ ppm)	Assignment
11	δ -CH ₃ of isoleucine
15	β -CH ₃ of alanine (αh)
19	γ -CH ₃ of valine
24.5	δ -CH ₃ of leucine and δ -CH ₂ of proline
*26	β -CH ₂ of cystine ⁵²
29	β -CH ₂ of glycine (αh), β -CH ₂ of trans proline
*32	β -CH ₂ of cis proline, β -CH of valine (βs), β -CH ₂ of glutamic acid (βs)
35	β -CH ₂ of lysine (βs), β -CH ₂ of asparagine (αh)
39	β -CH ₂ of leucine (αh), β -CH ₂ of crosslinked cystine ⁶⁷
48	δ -CH ₂ of proline
53	α -CH of glutamine (βs) ⁵² , α -CH of lysine (βs), α -CH of alanine (αh)
56	α -CH of serine (βs), α -CH of leucine (αh)
60	α -CH of phenylalanine (αh), α -CH of tyrosine (αh), α -CH of proline
66	β -CH ₂ of serine (βs) and β -CH of threonine (βs)
120-130	Aromatic CHs of phenylalanine and tyrosine
158	O-C of tyrosine
172-176	Carbonyl for all amino acids

Water is incredibly important in biological systems, with water content determining the functional and mechanical properties of proteins.^{61,62,72} In keratins, NMR has been used to examine the anisotropy of absorbed water, and the characteristics of water in different domains, in particular the restriction of water molecules through inter-keratin bonding, which prevents water from freezing in keratin fibres.^{62,73,74}

NMR is particularly helpful in determining the structural changes to keratin which occur from damage. The carbonyl peak in the ¹³C CP/MAS spectra has been used extensively to examine the effect of pressure, heat, permanent waving, and dehydration on the α -helix and β -sheet content in keratinous materials.^{52,54,69,75} Dissolution of keratins using ionic liquids and urea has been observed, while the effects of acids has also been investigated.^{76–78} KIF's have been shown to be disrupted between pH 6 and 7, with acids reducing the crystalline content of keratin and increasing the amorphous content.^{77,78} Damage from hair treatments such as permanent waving has been shown to include the degradation of α -helix structure to random coil, and incomplete re-oxidation of disulphide bonds.⁷⁰

1.4.2 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is a versatile tool which has been used extensively to study the properties of hair. In particular, TGA methods have been used to measure the moisture content of hair, melting and degradation of keratin, and how modifications to the fibre or even ethnic origin affect these properties.^{42,56,79–83}

When hair is heated from 30°C to 150°C, mass loss is related to absorbed water, while water involved in hydrogen bonding is lost at temperatures above 150°C.^{56,80,81,84,85} At 130°C in

wool, the scaffold structure of KIFs degrade.⁷⁵ In hair however, while KIF denaturation begins at 140°C, total degradation occurs in the range of 210-260°C, largely due to the rigidity of the matrix which prevents the unfolding of α -keratin helices.^{80,86} The matrix itself undergoes pyrolysis at 230°C, other organised structures degrade at temperatures up to 400°C, and complete fibre degradation is observed to temperatures up to 700°C.^{80,86,87}

The denaturation of α -keratin varies greatly depending on the fibre's water content and if it has undergone any chemical treatments.⁸⁰ Denaturation temperatures can be depressed significantly with increasing water content, and also can be affected by heating in the presence of water, or by changing the heating ramp rate.^{81,84,87} In damaged hair samples, a second mass loss stage is observed after KIF's degrade from 140°C. This second step occurs between 160-170°C, and is resultant of disorganisation in the matrix which occurs from chemical/thermal damage.⁸⁸

1.5 Damage to hair keratin

1.5.1 Chemical Damage

On the molecular level, the main causes of damage to hair results mainly from the reduction and oxidation of disulphide bonds.^{4,11} The reduction of disulphide bonds leads to considerable changes in the fibre such as the α -keratin melting point, mechanical weakening and decreased hair breaking stress.^{4,40,80,89} Chemical damage includes acid and alkali treatments, photochemical damage with UV radiation and common cosmetic hair treatments.

Acid and alkali treatment leads to significant hair damage, which increases the water content of hair the hair fibre by increasing its ability to swell.^{11,47,90} Acid treatment leads to

degradation of the matrix and KIFs, increasing the amorphous content in hair.^{77,78,91} Cystine cleavage is optimal in the acid pH region of 4-6.⁴ Alkaline degradation also includes KIF destruction, removal of 18-MEA from the cuticle surface, oxidation of amino acids, and peptide hydrolysis.⁴

Photochemical damage from UV exposure leads to hair sample becoming more hygroscopic, with damage being accelerated in bleached samples.⁷⁹ UV damage also causes reduced tensile strength.⁹² While initial cuticle 'fusion' as a result of UV damage causes a decrease in the hair surface area, further UV damage causes pore generation, which increases the surface area and indicates severe damage.⁹²

Cosmetic treatments such as permanent waving disrupts the α -helical structure in the microfibrils, and leads to random coil formation through reduction of disulphide bonds.⁷⁰ Chemical Straightening (or relaxing), by using heat and chemicals such as glyoxylic acid formaldehyde, leads to reduced moisture content, cuticle damage, and decreasing break stress.^{42,93} Bleaching processes are also incredibly damaging to hair, causing the formation of hydroxyl radicals which causes significant damage, in particular to the cuticle.⁹⁴ Bleached hair has reduced water content and reduced tensile strength, through the creation of pores in the fibre.^{42,92} Radical reactions from the bleaching process also oxidise 15-25% of disulphide crosslinks in hair, and remove much of the protective 18-MEA on the cuticle surface.^{4,5} This radical process is mediated by metal ions from tap water (e.g. Mg^{2+} , Ca^{2+}) which lead to the degradation of H_2O_2 to a wide range of reactive oxygen species including hydroxyl radicals, perhydroxyl radicals, and superoxide anions. These radicals cause severe damage to the CMC by destroying thioester bonds between cuticle cells, cleaving the disulphide bonds throughout the fibre, and resulting in fractures and the formation of split ends in hair.^{4,94,95}

1.5.2 Physical Damage

The most daily source of damage to hair originates from weathering and friction from actions such as brushing.⁴ Other physical damage includes mechanical damage from stretching which creates defects causing hair to break, and α - to β -keratin transitions.^{39,69} High temperatures cause irreversible damage which reduces tensile strength and increases the random coil content of keratin.^{39,69,75,86,96} Random coil formation is also seen when the hair fibre is dehydrated with the reduction of disulphide crosslinks and loss of α -helix structure.^{39,52}

1.5.3 Current techniques to measure damage in hair

The ability to sensitively quantify damage in hair is of great importance to industry, to understand how much a treatment affects hair and how far products can go to repair incurred damage. There is therefore a wide range of analytical tools to quantify damage in hair.

Some of the most commonly used within industry and research include the measurement of mechanical properties of a hair fibre such as tensile strength and fibre torsion analysis to determine rigidity.^{30,93,97–99}

Differential Scanning Calorimetry (DSC), a thermo-analytical technique, is also widely used to measure the melting behaviour of α -keratin crystallites. The melting point of these crystallites has been shown to be influenced by damage in the fibre throughout literature.^{30,42,79,80,87,93,100}

Another thermo-analytical technique, TGA, is often used to examine water content, which alongside the use of Dynamic Vapour Sorption (DVS) methods (DVS will be discussed in greater detail in Chapter 2.4), can be used to evaluate damage in terms of hydration, with damaged hairs having a reduced moisture content.^{30,93,101}

Analysis of amino acid content is frequently examined using Fourier-Transform Infrared Spectroscopy (FTIR) - a non-destructive analytical tool. FTIR can be used to measure cysteine acid content, a by-product caused by oxidative damage and destruction of disulphide bonds. While these measurements are single fibre analysis only, they provide a sensitive chemical measure of damage in the hair itself.^{7,93,98,102} Other amino acids have been used as markers of damage, e.g., the increased presence of lanthionine with heat damage.⁹⁸ Amino acid analysis can also be undertaken by measuring the total loss of protein from the fibre after damage, where loss increases with damage, and particular fragments can be identified using mass spectrometry.^{97,102-104}

Visually, damage to the hair cuticle is frequently observed using scanning electron microscopy (SEM). SEM is routinely used to evaluate cuticle damage from a multitude of causes: bleaching, relaxing, heat and dyeing.^{41,79,93,97,102,103,105}

This is not an exhaustive list of analytical techniques used by researchers to measure hair keratin damage. However, the drawbacks to many of these common analytical techniques result from the inherent heterogeneity of the hair fibre. Single fibre analysis tools require many repeat experiments to achieve a large enough sample size for a meaningful analysis. Techniques such as DSC, protein loss measurements and mechanical tests result in the destruction of hair fibres and prevent further analysis. There is therefore continual development in damage measurement procedures for use within industry and research.

Some of these more uncommon techniques include measuring hair porosity using gas sorption, infrared remote sensing of hydration levels of in vivo hair and electron paramagnetic resonance (EPR) for detection of radicals created through damage.^{92,101,106}

1.6 Interactions with small molecules

The interactions of keratinous materials such as hair are of great interest to research and industry alike. Understanding how keratins interact with their environment can help inform on their biological role in cells, and also how to better formulate hair care products. In particular it is important to understand how keratins interact with small molecules such as fragrances, dyes, solvents, and other molecules of interest.

The mass uptake of a solvent either results in dissolution of the material or swelling. Swelling informs on biological structure, whether that be the ability of the fibre to swell, or the composition as affected by a solvent to cause swelling.⁹⁰ In keratins it can be a two-stage process. The first stage is a concentration dependant Fickian diffusion which reaches an equilibrium uptake.¹⁰⁷ Fickian diffusion is observed as a linear absorption of a solvent into a material as a function of time, which is then followed by a plateau region of sorption.¹⁰⁸ The concentration (C) of a solvent in a material can be calculated at time t using Fick's second law:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad (1.3)$$

Where D is the diffusion coefficient. After this first Fickian stage of diffusion, a second stage may occur which results from intermolecular rearrangement due to the absorbed solvent.¹⁰⁹

The uptake and retention of small molecules in hair, like in other materials is highly dependent on the adsorbent. Polar molecules can use water in materials as a diffusion

pathway out of pores, to increase evaporation from a material, and reduce the compounds retention.¹¹⁰ Polar molecules may also be able to form hydrogen bonding with the material to overcome weaker dispersive forces which can increase retention – if bonding sites are available.¹¹¹ There may be competition for binding sites if water is present in the sample, making the relative humidity an important consideration.¹¹² This short discussion on a simple factor, such as polarity, demonstrates that characterising interactions between small molecules and materials is very complex.

What complicates this further, is that biomaterials such as human hair have such high heterogeneity, that its own individual properties are not easy to categorise. Therefore, the properties of a small molecule itself can be used to investigate the properties of a material. Surface area and surface energies of a material have been commonly investigated with the interactions between polymers and gases.^{113,114} Surface properties can be investigated with sorption of molecules with different functional groups, and pore sizes in materials can be investigated via molecule retention, with bulky molecules unable to enter pores and small adsorbates being lost easily from the material.^{110,111} Sorption can also be used to examine internal mechanics of a biological material such as such as cell wall modulus.¹¹⁵

Hair sorption, swelling, and surface area have been recorded throughout literature.^{22,55,116–119} There is however still great room for further characterisations of interactions which may help form predictive models which will benefit industry and research alike. New approaches are therefore needed to sophisticatedly characterise the interactions of small molecules with hair. Chapter 8.1 will go into further detail into classical theory behind solvent uptake in hair.

1.7 Aim and Approach

While there is a great amount of literature related to keratins, a large focus of this has been structural analysis and property changes, with a lesser focus underlying the physics of the interactions themselves.

There are two main aims of this thesis; to establish new methods to analyse interactions with keratin, and to understand fundamental sorption processes in hair. A multi-technical approach will be used including solid state NMR, high performance liquid chromatography (HPLC) and dynamic vapour sorption.

The interactions of small molecules and hair will be examined with SS-NMR, observing how humidity impacts on these interactions. Water will be further examined to observe how water mobilities in hair keratin can inform on hair damage.

Small molecule sorption in hair will be first examined using an inverse liquid chromatography method to measure active desorption from dyed hair. Finally, the sorption of water will be investigated using DVS, to determine the fundamental role of hair swelling in water sorption isotherms, and its influence on hysteresis.

The next chapter will explore the key theory required for the understanding of this thesis. Each subsequent results chapter will include: an introduction, methods section, additional theory where relevant, and discussion.

Chapter 2 - Theory

2.1 Nuclear Magnetic Resonance

2.1.1 General NMR Theory

2.1.1.1 Spin in the microscopic level

This section details the basic principles behind NMR and how it can be applied to the solid state. Nuclear magnetic resonance relies on the inherent angular momentum of the nuclei called spin (I). Angular momentum in atoms is resultant from the electron spin, the proton spin, neutron spin and the orbital angular momentum. In most molecules the sources of angular momentum in the molecular ground state result from the rotation of the molecule and its nuclear spins.¹²⁰ Angular momentum is quantized, with different rotational states (J). Each J has $2J + 1$ sublevels, which take the quantum number M_J . The energy of J is also quantised:

$$E_J = BJ(J + 1) \quad (2.1)$$

Where B is a rotational constant. Outside the magnetic field, all states with the same J value but different M_J value have the same energy, and are therefore degenerate.¹²⁰

Spin is an intrinsic property of a particle and is a form of angular momentum, not related to the rotational properties of the particle. Depending on the number of protons and neutrons in a nucleus, the nuclear spin can take integer and non-integer values. i.e., $^1\text{H} = 1\text{p} = I = 1/2$. Particles with a spin value I have $(2I + 1)$ sublevels. The total angular momentum (S_{tot}) for particles with spin is:

$$S_{tot} = [I(I + 1)]0.5\hbar \quad (2.2)$$

where $\hbar = h/2\pi$, where h is the Planck constant.

The direction of the spin angular momentum is called the spin polarization axis. Particles with spin have polarization axes pointing in all possible directions, and so the polarization is isotropic.

However, in the presence of a magnetic field (B_0) the spin polarization axis precesses around the magnetic field. This precession arises from angular momentum being a vector. The vector is twisted by the magnetic field to reorientate it along the magnetic field; however, this twisting produces a torque which moves the vector around the axis of the B_0 . The precession of individual magnetization vectors results in a distribution of vectors in a cone with an angle of 54.7° with respect to B_0 , with the sum of all these vectors being the net magnetization. If the spin polarization is at 90° to the magnetic field axis, the precession forms a flat disk around B_0 (Figure 2.1). The precession around B_0 is at the Larmor frequency (ω_0) which is proportional to the strength of B_0 (Equation 2.3). The Larmor frequency is described in terms of cycles per second (Hz) or rad s^{-1} , and γ is the gyromagnetic ratio ($\text{rad s}^{-1} \text{T}^{-1}$).

$$\omega_0 = B_0 \gamma \quad (2.3)$$

In a magnetic field the degeneracy in spin states is broken, resulting in different energy spin states, called Zeeman splitting, a mechanism which makes NMR possible (Figure 2.2).¹²¹

^1H - the most common spin active nuclei - has a I of $+1/2$. This results in 2 degenerate spin states. In the presence of B_0 the two spin states split to form $+1/2$ (α) and $-1/2$ (β). The energy levels of these states are quantised, with the lowest energy spin state (α) aligning with the B_0 , and the higher energy spin state (β) aligning against B_0 . The energy between nuclear ground states (Equation 2.4) and nuclear excited states is large and therefore excited states may not be accessible.

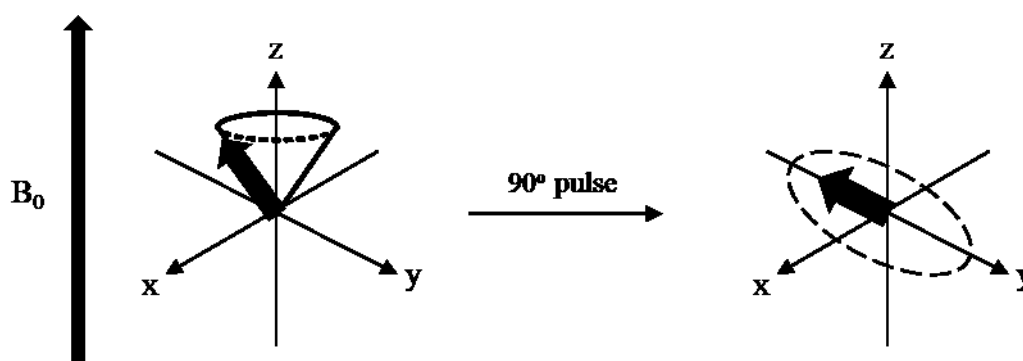


Figure 2.1 Precession of a net magnetization vector (large arrow), comprised of multiple polarization axes in a sample, from a cone to a flat disk after a 90° NMR pulse.

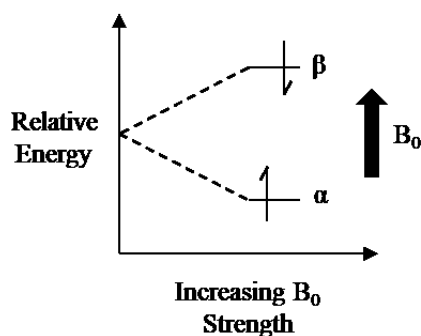


Figure 2.2 The Zeeman Effect: degenerate spin states separate in a magnetic field (B_0) with the spin aligned against the B_0 having the highest energy.

$$\Delta E = \gamma B_0 \hbar \quad (2.4)$$

However, the Zeeman splitting between the same nuclear ground state is small, and therefore can be probed with NMR. Therefore, isotopes where the nuclear ground state with $I = 0$ cannot be probed with NMR. Due to the dependency on ΔE on B_0 , larger magnetic fields increase the gap between spin states, increasing the spin excess in the ground state and the Larmor frequency.

2.1.1.2 Spin on the macroscopic level

At a macroscopic level, the spin properties of these spin-active nuclei allow them to be manipulated with transient electromagnetic fields using R.F. radiation. The overall magnetisation vector in the z-axis (M_z^0) can be excited to align orthogonally to the magnetic field using a transverse oscillating transient magnetic field. The precession here (See Figure 2.1) is at its largest, and this rotation produces a rotating magnetic field which, in presence of

a wire coil, induces an oscillating voltage. It is this electric current which is recorded as the NMR signal.¹²⁰ As the precession gets smaller as the angular momentum relaxes back to the direction of the magnetic field, the current decays – this is called a free induction decay (FID).¹²² The FID is a complex wave, made of all the different precessions of the individual nuclei in the sample. A Fourier transform is a mathematical function which is used to deconvolute the cosine and sine waves which form the FID, plotting the intensity against the frequency that these waves occur. The resultant frequency spectrum is what forms a typical NMR spectrum of a sample.

The return to equilibrium (M_z^0) of a sample which produces an FID, occurs through two mechanisms: spin-lattice relaxation (T_1) and spin-spin relaxation (T_2). These will be discussed further in sections 2.1.3 and 2.1.4.¹²³

2.1.2 Chemical Shift and line broadening

The chemical shift of a nucleus is the resonance frequency of a nucleus in respect to a chemical standard in a magnetic field. Shielding from the magnetic field is provided by electrons which surround the nucleus, allowing the nucleus to require a lower applied R.F. to induce resonance.

As the chemical shift of a nucleus depends on its electron distribution, it is also influenced by surrounding atoms.¹²⁴ As these distributions are uneven, when B_0 is applied, nuclei have different degrees of shielding. This anisotropy can be described by a chemical shift tensor. This anisotropy also means that the chemical shift of a nucleus is dependent on its orientation in the magnetic field - this is called chemical shift anisotropy (CSA).

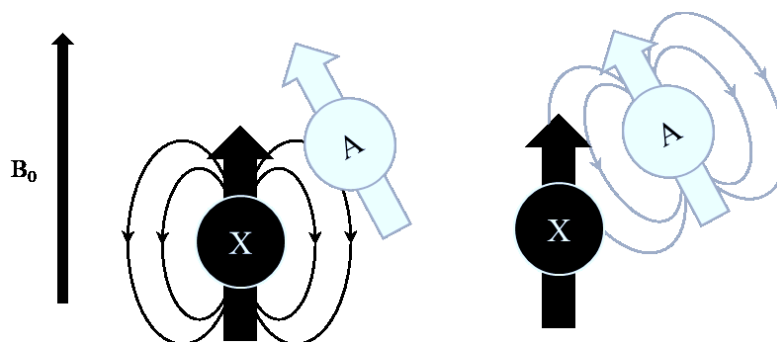


Figure 2.3 Dipolar interactions in an AX system, where the magnetic field of the spins act upon each other. The large arrows (black for spin X, blue for spin A) demonstrate the overall magnetisation vector for the spins, with magnetic fields indicated by circular arrows.

For liquids, the averaging motions of tumbling molecules reduce the CSA, while in solids this becomes a larger issue. The two main interactions that affect chemical shift are dipolar and quadrupolar interactions.

Dipolar interactions result from nuclear spins creating magnetic fields which interact with the nuclear spin of neighbouring nuclei. Dipolar interactions can be described by evaluating a heteronuclear AX spin system. In an AX system, A and X have different gyromagnetic ratios, and therefore produce different magnetic fields (Figure 2.3).

If the magnetization vector for X is aligned with B_0 in the z axis, the resonance of X is determined by the magnetic field in the z direction, which is the sum of B_0 and the magnetic field of A in the z direction (B_A).

Due to the different spins of A - causing two different precession frequencies for X - the splitting of X is dependent on B_A . Dipolar coupling is therefore dependent on the separation (r) and the angle (θ) of the magnetic vectors, and the magnetic fields of both.¹²⁴

Quadrupolar interactions arise from quadrupolar nuclei with $I > \frac{1}{2}$ having an electric quadrupole moment. An electric quadrupole moment results from a non-uniform charge distribution in the nucleus, which causes an electric field gradient. The electric quadrupole moment of the nucleus depends on the orientation of the electric field gradient to the magnetic field.

The lowest energy state occurs when the more negative points of the magnetic moment lie in plane of B_0 , whereas orientation perpendicular to B_0 creates a high energy state. This perpendicular orientation is an anisotropic interaction which produces line splitting. Quadrupolar interactions can result in spin relaxation when modulated by molecular tumbling. Fast spin relaxation causes the quadrupole orientation to flip rapidly, so that any coupled nucleus to the quadrupole has a resonance frequency which changes with each change in orientation. This causes significant line broadening.^{124,125}

Chemical shift anisotropy, dipolar and quadrupolar interactions all result in line broadening in the NMR spectra. As all of these interactions have an angular dependence $(3\cos^2(\theta)-1)$, line broadening effects are particularly apparent in solid state NMR, where there are no averaging motions of molecules which is found in solutions state NMR. Magic Angle Spinning (MAS) is used to counteract these effects, by spinning the sample at 54.7° to reduce splitting, and improve the quality of spectra.

2.1.3 Spin-lattice Relaxation

2.1.3.1 Origin of spin-lattice relaxation

As described previously when a sample is placed in B_0 it experiences Zeeman Splitting. Spin-lattice (T_1) relaxation allows the spins to flip among the different energy levels, therefore creating the Boltzmann population differences which are needed to make an NMR experiment, and a net magnetization vector. As the spin populations reach thermal equilibrium, they release energy to the environment.¹²⁰

The mechanism of nuclear spin relaxation lies in magnetic interactions – importantly dipolar coupling. As a molecule tumbles, r and θ vary causing the dipolar interaction to vary rapidly. Therefore, nuclear spins experience time dependent local magnetic fields, which if at the Larmor frequency can induce radiationless transitions which return the spins to equilibrium (See Equation 2.4). The rate of relaxation is therefore dependent on the probability that a nuclear spin is at the Larmor frequency – this probability is related the spectral density $J(\omega)$ which is highly dependent on the rotational correlation time (τ_c) of the molecule.

2.1.3.2 Relation between the rotational correlation time and spectral density

The rotational correlation time (τ_c) can be understood by visualising molecules at the north pole of a sphere. Due to the random motions of a sample, nuclei undergo a ‘random walk’ due to collisions with other nuclei, such that their position and orientations change from when $t = 0$. When $t > 0$, molecules in the sample will have moved from their initial positions, but if $t \approx 0$, they will not have distributed far, remaining mostly in the top half of the sphere. When $t \gg 0$, random collisions and movements will distribute the molecules all around the sphere. τ_c is the root-mean-square movement of the molecules around 1 radian, i.e., it is

related to the average time taken for these molecules to distribute about 1 radian around the sphere.¹²⁴ We can consider τ_c^{-1} as approximately the root-mean-square rotational frequency for the sample.

The range of frequencies for a sample can be described by the spectral density $J(\omega)$, which is proportional to the probability of one of the molecules having a certain frequency. For slow tumbling large molecules, it is clear that $J(\omega)$ is highest at frequencies of $< \tau_c^{-1}$, as this is related to molecules which have not travelled 1 radian during time τ_c . Consequently, the $J(\omega)$ for large molecules will be lowest at frequencies $> \tau_c^{-1}$ at time τ_c . For small molecules, which distribute quickly around the sphere, $J(\omega)$ will be low for all frequencies as all orientations and locations are fully randomised.

2.1.3.3 Impact of rotational correlation on T_1 relaxation times

As T_1 relaxation occurs when a nucleus is oscillating at the Larmor frequency (ω_0), the probability of finding a nucleus at ω_0 is directly related to the τ_c of the molecule. Molecules with very slow tumbling (large molecules, e.g., proteins) have long relaxation times as $J(\omega)$ is highest at frequencies $< \omega_0$. Molecules with very fast tumbling (small molecules) have long relaxation times due to the aforementioned low $J(\omega)$ across all frequencies. Where $\tau_c = 1/\omega_0$, $J(\omega_0)$ is highest, so these molecules have the highest relaxation rates. This accounts for the v shaped graph for relaxation shown in Figure 2.4.^{123,124}

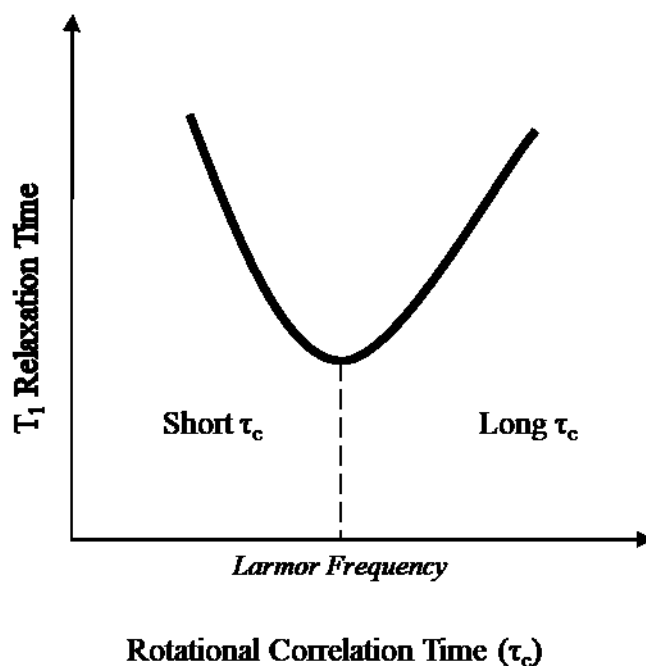


Figure 2.4 The impact of the rotational correlation time of a molecule on its T_1 relaxation. For small molecules such as water (left hand of graph), fast rotation leads to longer relaxation times. As rotation slows, the relaxation times get shorter. When a molecule has a τ_c equivalent to its Larmor frequency, the T_1 relaxation time is at a minimum.

Due to T_1 dependence on the Larmor frequency, T_1 relaxation varies greatly with the magnetic field strength B_0 , increasing with stronger local fields. It is also distance dependent (r^6). T_1 is therefore sensitive to internuclear distance and molecular structure. The dipolar field produced by a spin is proportional to the square of its gyromagnetic ratio, therefore the T_1 relaxation is proportional to the multiplication of these squares for the two nuclei (Equation 2.5):

$$\frac{1}{T_1} = \left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_I^2 \gamma_S^2 \hbar^2 \tau_c}{r^6} \quad (2.5)$$

Nuclear spin relaxation is slow as the magnetic fields are feeble and the spectral density at the Larmor frequency is always small. Energy is released or absorbed by the spin system as it relaxes. The relaxation couples the spins to the motion of the molecules and therefore provides a pathway for exchange of energy between the spin system and its surroundings. T_1 relaxation times give an insight into how molecules interact within a system, allowing the strength of interaction of non-covalent bonding to be established.¹²³ These interactions with the environment take the form of dispersive interactions, induced dipole interactions or hydrogen bonding.

2.1.3.4 T_1 relaxation in an NMR experiment

In a macroscopic sense, T_1 relaxation is seen as a breakdown of precession around B_0 and a loss of energy in the sample. T_1 relaxation is associated with longitudinal relaxation of the z-component of the overall magnetisation vector for the sample. This relationship is expressed as a time dependent exponential shown in Equation 2.6:¹²⁰

$$M_z(t) = M_{eq} \left(1 - \exp\left(-t/T_1\right) \right) \quad (2.6)$$

After one T_1 time constant, the recovery of longitudinal magnetisation reaches 63% of its original equilibrium value.

Inversion recovery experiments are used to measure T_1 values. The pulse sequence for this is shown in Figure 2.5. An 180°_x pulse is applied to invert M_z^0 . After a set time period (τ), a 90°_x

pulse is applied to along the x-axis to allow for signal collection in the y-axis (Figure 2.6). Varying τ times are used to measure the magnetization recovery over time, resulting in an exponential plot of signal intensity over time, with many scans at the set intervals to improve signal acquisition. This can be fitted using Equation 2.6 to calculate the T_1 time for the sample.

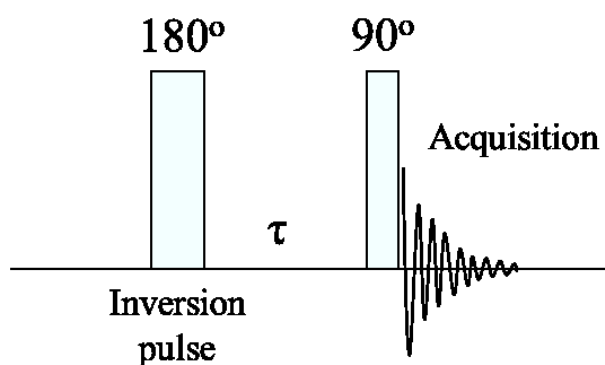


Figure 2.5 The pulse sequence for a T_1 inversion recovery experiment. The blocks denote the sequence of pulses with the pulse type written on top. Acquisition occurs with the production of an FID after the 90° pulse.

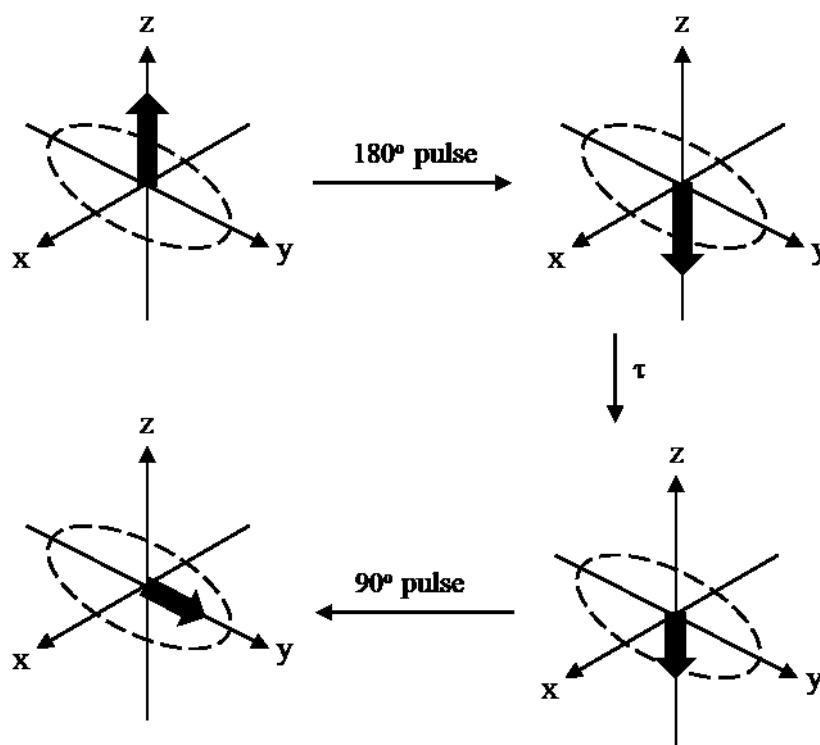


Figure 2.6 An axial representation of the T_1 inversion recovery sequence, where the large black arrow is the net magnetization vector. A 180° pulse inverts the magnetization vector, which relaxes over time τ . A 90° pulse moves the vector into the y axis for measurement.

2.1.4 Spin-spin Relaxation

While T_1 relaxation is the relaxation of spins to thermal equilibrium, spin-spin (T_2) relaxation is associated with a loss of phase coherence after spins align with B_0 . Phase coherence requires a consistent polarization of nuclear spins. As the spins precess around B_0 , and experience varying local magnetic field differences, the spins lose their coherence.¹²⁰

The T_2 relaxation time relates to the time taken for a nucleus to exchange magnetisation energy to other nuclei in the sample. This is strongly governed by the rotational correlation time of the nuclei. This relaxation results in the loss of magnetisation, and therefore intensity of the NMR signal, in the transverse x-y plane. The T_2 time is the time needed to relax 37% of

the x and y components, and is usually faster than the T_1 time. The transverse relaxation is related to the $M_{x,y}$ magnetization vector with the following exponential relationship: ^{120,126–}

128

$$M_{x,y}(t) = M_{x,y}(0)e^{\frac{-t}{T_2}} \quad (2.7)$$

To measure the T_2 spin-spin relaxation time, a series of spin echo sequences are used. After an initial 90° pulse the magnetisation vector is orientated to the x-y plane. Over time there is loss of phase coherence, and so subsequent 180° pulses are applied to return spins to phase (Figure 2.7).

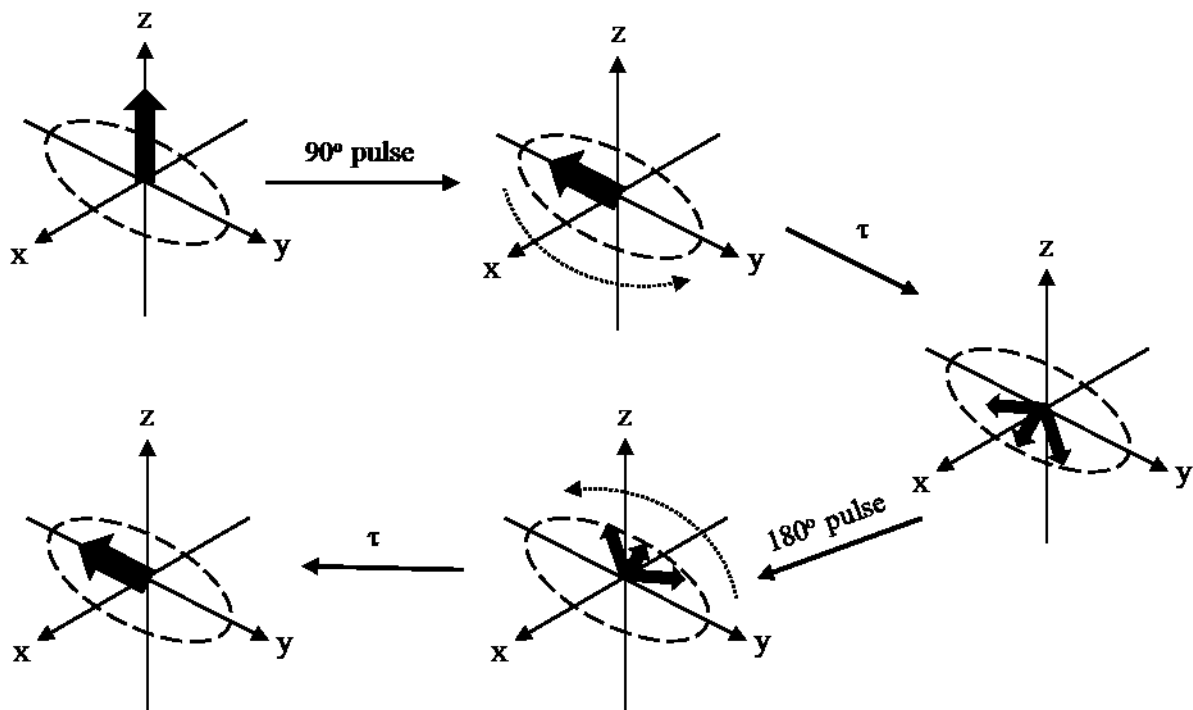


Figure 2.7 An axial representation of the T_2 recovery sequence. The large black arrow is the net magnetization vector for the sample. After the vector is moved into the y axis with a 90° pulse, phase coherence is lost leading to multiple vectors. After a 180° pulse the vectors regain coherence.

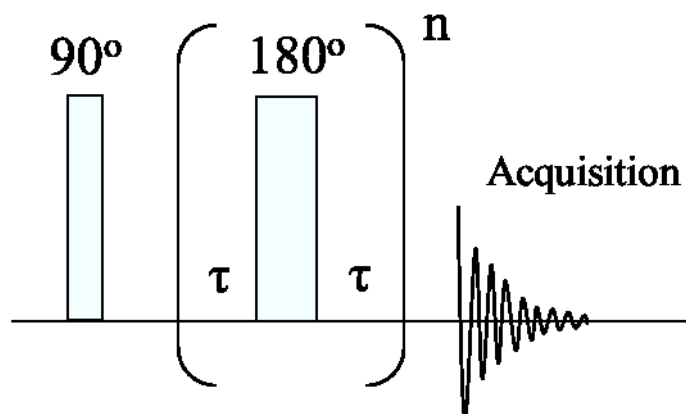


Figure 2.8 The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to measure T_2 relaxation. The blocks denote pulses with the pulse type written on top. Acquisition occurs with the production of an FID after a 180° pulse.

The Carr-Purcell-Meiboom-Gill (CPMG) experiment (Figure 2.8) is used to measure the T_2 relaxation time with multiple delay times (τ) and 180° pulses.

2.1.5 Cross polarisation techniques

Cross polarisation is a technique used often to improve signal quality in SS-NMR. The method relies on mutual spin flips; a process where two coupled spins undergo opposite transitions in spin states simultaneously. This magnetization transfer conserves the overall polarization and the energy of the system. For uncoupled heteronuclear pairs, R.F. irradiation is used to generate mutual spin flips, between abundant nuclei, such as ^1H , and rare nuclei such as ^{13}C .

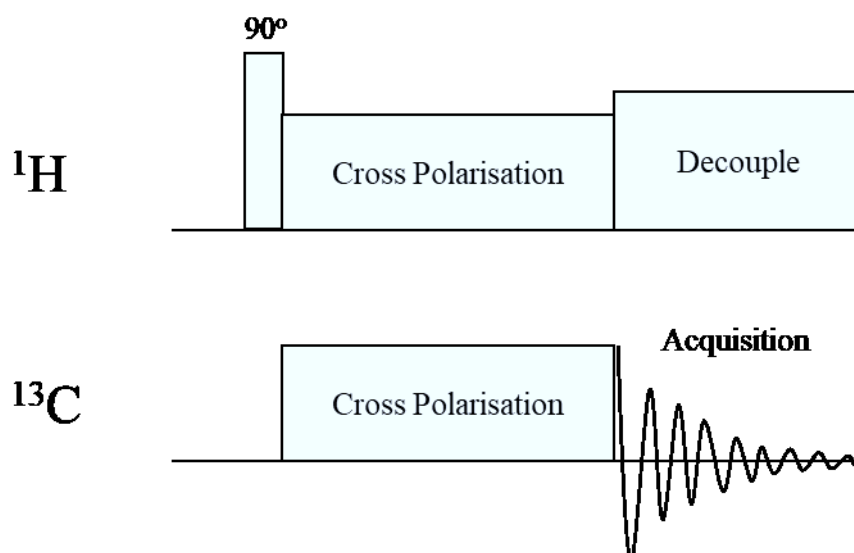


Figure 2.9 The Hartmann-Hann cross polarisation sequence between ^1H and ^{13}C for improved ^{13}C signal. The blocks denote pulses with the pulse type written on top, with pulses for radiating ^1H nuclei on the top, and ^{13}C nuclei on the bottom. Acquisition occurs for the ^{13}C signal as the proton signal decouples.

This results in an increased polarization in the rare spins, making it equal to that of the abundant spins, and enhancing the signal of the rare spin. This cross-polarisation technique was first developed by Hartmann and Hahn in 1962, with the sequence shown in Figure 2.9.^{129,130}

2.2 High Performance Liquid Chromatography

In liquid chromatography, a liquid mobile phase is investigated using a solid stationary phase. The separation of components in the mobile phase is achieved through the properties of the chosen stationary phase. The mobile phase is separated due to compound size or affinity to the stationary phase.

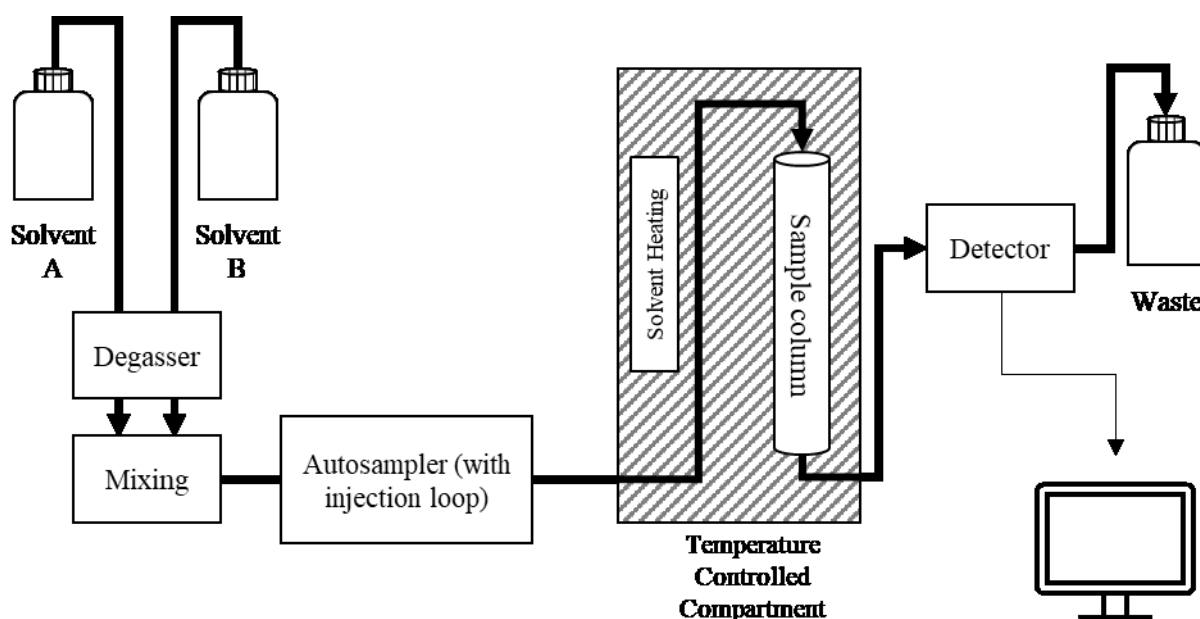


Figure 2.10 A simplified HPLC schematic adapted from Ylä-Mäihäniemi *et al.*¹³¹ Bold arrows indicate solvent flow through the system.

HPLC systems rely on the principles of liquid chromatography, while incorporating high pressures in solvents to speed up the experimental procedure. In traditional HPLC the separated compounds can be identified using mass spectroscopy, UV absorption or by other spectrometric capabilities present in the HPLC system. A schematic depicting a simplified HPLC system is shown in Figure 2.10.

Two reservoirs are filled with solvent, acting as the mobile phases. A high-pressure pump is used to generate a specific flow rate of the two solvents, passing them through a degasser, and then to a mixing unit where the solvents can be mixed to different ratios. Alternatively, only one solvent is used. The now singular mobile phase then passes through an autosampler, where the sample, or series of liquid samples, are injected into the continuously flowing phase. The mobile phase enters a temperature-controlled compartment where it is heated to the desired temperature, and then passes through a HPLC column. This column – the

stationary phase – is packed with material needed for effective separation, and is often pre-packed for use. A detector after the column measures the desired properties (MS, UV, fluorescence etc.) to identify the eluting compounds from the samples as they separate. This creates a chromatogram for the experiment, where each peak represents a different compound. The mobile phase can then be fractioned or sent to waste.¹³²

HPLC can also be used for inverse liquid chromatography, where the medium under investigation is the stationary solid phase, and not the mobile solvent phase. In these experiments the columns are packed with the sample under investigation by hand. These techniques allow measurements of sorption isotherms, allowing the retention of the solvent in the sorbent to inform on the properties of the solid phase. This has been used in literature to examine competitive binding in proteins, and protein-protein interactions.^{133–135}

2.3 Thermogravimetric Analysis

Thermogravimetric analysis is a gravimetric technique where the mass of a sample is measured while the temperature is changed. This technique is often used to analyse water content, and also degradation of a sample with associated mass loss being recorded at different thermal events.

2.4 Dynamic Vapour Sorption

2.4.1 DVS systems

Dynamic Vapour Sorption (DVS) is a gravimetric technique used to measure the mass of a solvent vapour adsorbing onto a sorbent. Figure 2.11 demonstrates a simplified schematic of a DVS system. The sample under investigation is placed in a microbalance pan, where the

mass is continually recorded - in reference to a known reference balance pan - to a resolution of 0.1 μg . The sample pan is located in a humidity and temperature-controlled chamber, which can be adjusted with computer control. To investigate the sorption properties of the sample, a chosen solvent is added to a reservoir. Under computer control, the liquid solvent (either water, or organic solvent) from the reservoir is vaporised and mixed with either nitrogen or dry air to create a certain vapour partial pressure, or percent relative humidity (%RH). A mass flow controller regulates the flow rate of the gas in sccm. The vapour composition is measured using a Speed of Sound sensor, which can accurately determine the %RH by measuring how the speed of sound in the vapour changes. This gaseous mix is flown over the sample and the mass of the adsorbing sample is measured throughout to give the mass of solvent vapour absorbed in real time.¹³⁶ This technique is often used to measure sorption and desorption isotherms, as well as sorbent surface area.^{137,138}

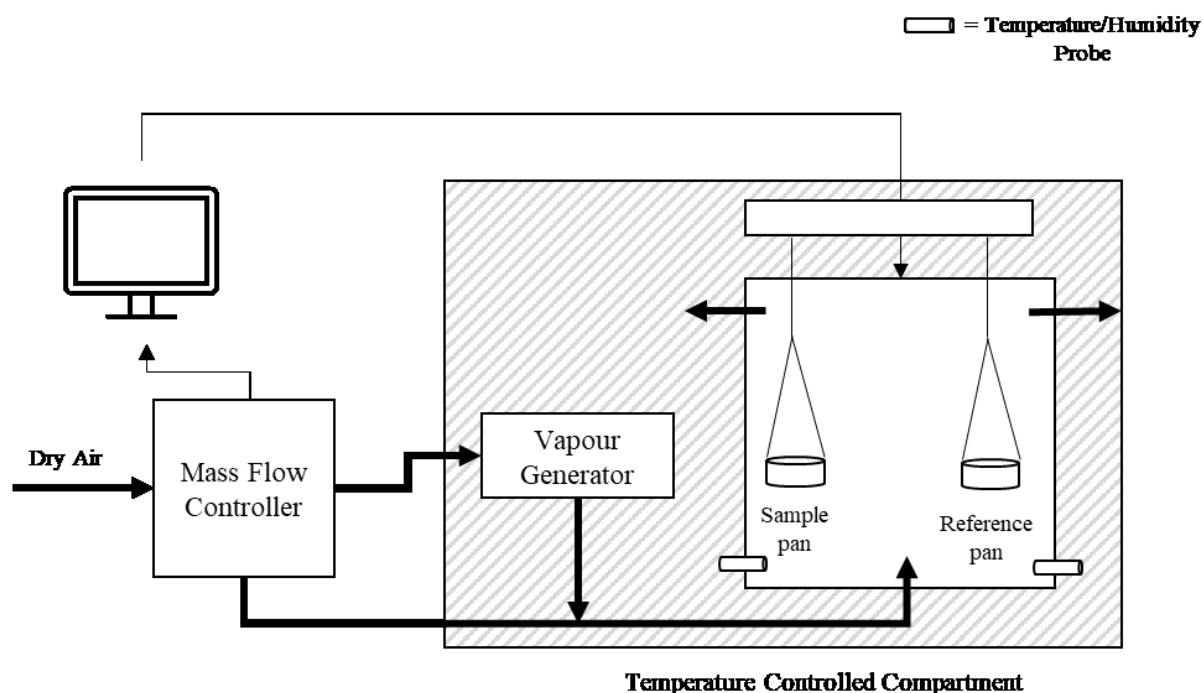


Figure 2.11 A simplified Dynamic Vapour Sorption system schematic; the bold arrows show the direction of gas flow through the system, from the mass flow controller to the sample pan.

The DVS can be programmed to change the vapour concentration, %RH, and the temperature that the samples are exposed to, to suit a desired measurement protocol. Two DVS systems were used in this work, a single chamber DVS (DVS-Advantage, Surface Measurement Systems Limited, UK) and a five chamber DVS (DVS-Endeavour, Surface Measurement Systems Limited, UK).

2.4.2 Adsorption isotherms

The six isotherms in the (International Union of Pure and Applied Chemistry) IUPAC classification scheme that can be used to describe adsorption of a gas on a solid are shown in Figure 2.12. These are based off the classical Brunauer isotherms and are used to describe numerous sorption behaviour.¹³⁹

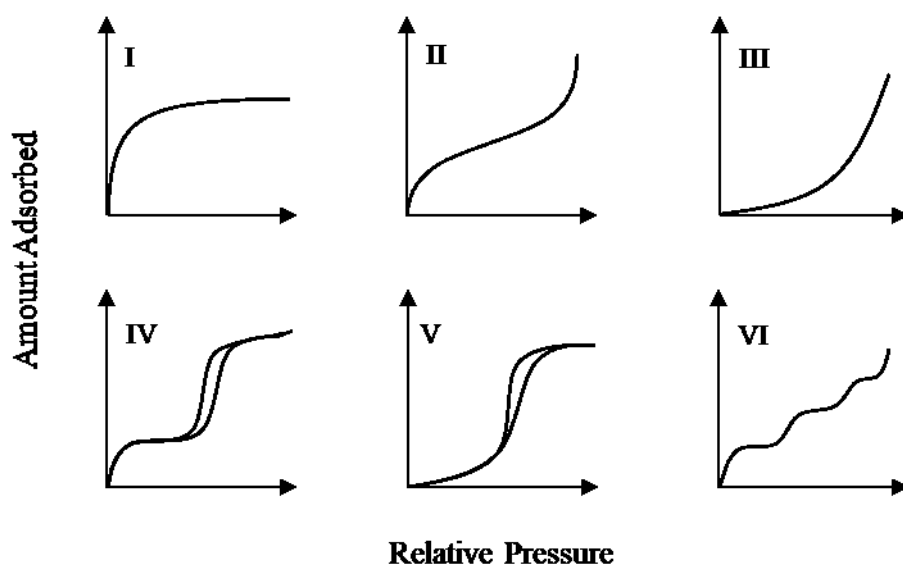


Figure 2.12 The six types of adsorption isotherms in the IUPAC classification scheme. This figure is adapted from Adv. Colloid Interface Sci, M. D Donohue.¹³⁹

Type I isotherms show adsorption onto microporous structures which reach a limiting value.

¹³⁹ Type II and type III isotherms represent adsorption on a macroporous material. These isotherms form with an initial adsorption of a monolayer followed by further multilayers of adsorbent being adsorbed to the surface. Type IV and V also involve multilayer adsorption but also include capillary condensation.¹⁴⁰ Capillary condensation occurs when the probing gas condenses inside the materials pores. The properties of the meniscus that forms in these pores dictate that the liquid will evaporate at lower vapour pressures, giving the characteristic condensation hysteresis.¹⁴¹ Type VI indicates that adsorption can occur as a multistep process.¹³⁹ A greater discussion of the modelling of isotherms, in particular for Type IV isotherms, will be presented in Chapter 8.

Chapter 3 - Investigating small compound interaction and partitioning in α -Keratin using ^1H NMR Spin-Lattice (T_1) relaxation times

3.1 Introduction

3.1.1 NMR of keratins

Keratins such as hair and wool are the foundation of the global haircare and textile industries. In the UK alone, these industries are purported to be worth over £70 billion a year.^{1,2} Surprisingly, the interactions between small molecules and keratinous materials such as hair are still poorly understood, despite their wide-ranging applications in numerous other fields. These other interests include the regeneration of keratin from feather and wool biomass,^{142,143} and innovating the use of keratins as antioxidants,^{144,145} nanocomposite hair protection,¹⁴⁶ hydrogels,¹⁴⁷ adsorbents for waste dye and oils in water,^{148–150} and for use as components in electronic devices.¹⁵¹

NMR has been a vital tool to understand these small molecule interactions, with a focus on the spin-lattice (T_1) and spin-spin (T_2) relaxation times of water in keratins. The dependence of T_1 and T_2 relaxation processes on water mobility and local environment allows these

measurements to be used to examine how water mobility changes in proteins, and what that can reveal about the protein structure itself. Relaxation methods are still one of the most common ways of interrogating these water-protein interactions, being used to examine lysozyme,¹⁵² keratin hydrogels,¹⁵³ serum albumins,^{154,155} and soya protein.¹⁵⁶ In diagnostic medicine, the sensitivity of water to local changes in structure is exploited in Magnetic Resonance Imaging, demonstrating how the local environment of water molecules can lead to dramatic differences in magnetic properties.^{157,158}

3.1.2 T_1 and T_2 relaxation in keratins

For a protein such as keratin in water, the overall relaxation of the water molecules is defined as a weighted average of the molecules interacting directly with the protein, and those of the bulk solution.¹⁵⁷

Daszkiewicz *et al.* first measured the presence of a water population separate to that of the bulk solution; this population behaved rigidly, and interacted with the protein in an ‘irrotational way’.⁴⁶ These water molecules were reported to have similar rotational correlation times to the protein itself, and have distinct differences in mobility to water molecules in the surrounding hydration layers of the protein, which belonged to the bulk water solution.⁴⁶

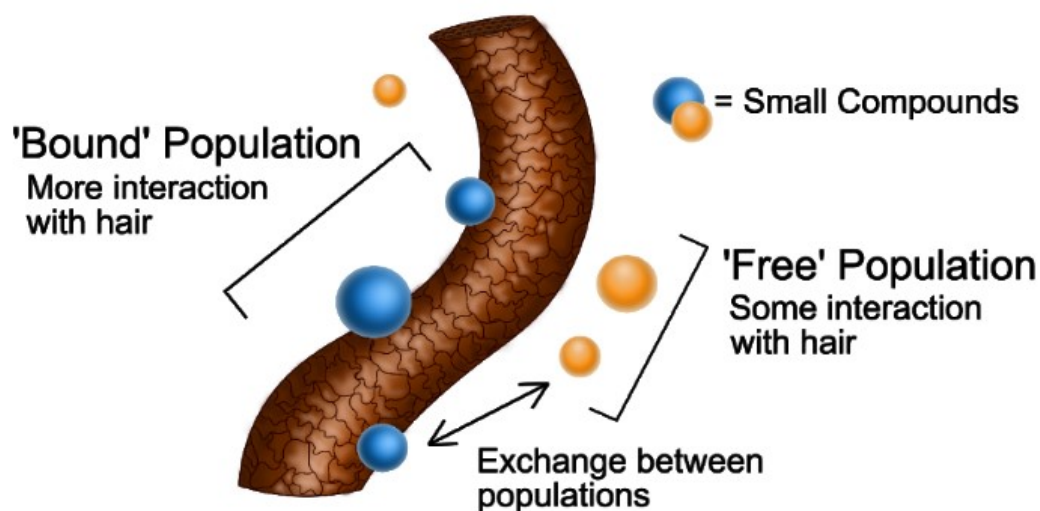


Figure 3.1 A depiction of a hair fibre showing a two-phase model of small molecule populations identifiable by SS-NMR. Orange spheres represent a free population, and blue spheres represent the bound population of the same solvent. The bulk population of solvent has no interactions with hair (image courtesy of Marylyn Molisso).

Further research demonstrated that this model was a simplification. The ‘irrotational’ water molecules identified by Daszkiewicz *et al.* have been classified further to another two different water regimes. The first is a fast-relaxing regime which relates to an immobile ‘bound’ population associated with the keratin structure itself.^{45,46} The second, a slow relaxing regime relating to a labile ‘free’ population, is not directly associated to the keratin structure, allowing these water molecules greater mobility.^{60,65,159,160} Whilst these populations are distinct, they undergo some exchange (Figure 3.1).⁷³

Most historical NMR studies of water keratin have involved wool. Feughelman & Haly first defined a five-phase model of water in keratins, determining that water molecules can form 1-4 interactions with keratin directly, or resemble the previously mentioned ‘free’

population.⁴⁵ However, most studies have been limited to the measurement of the broader concept of 'bound' and 'free' populations, demonstrating the T_1 and T_2 times of water in wool have a dependence on multiple factors.

Lynch *et al.* determined the temperature dependence for both the T_1 and T_2 ^1H relaxation times of water in wool, also demonstrating that the 'bound' populations are 'non-freezable'.^{73,161} Using T_2 relaxation data, it was determined that while ^1H T_2 times of absorbed water increased with increasing water content of the fibre, above a certain hydration the T_2 value plateaus. This suggested a separate proton phase with a shorter T_2 value exists, further supporting the model of a tightly 'bound' water population which undergoes exchange with the 'free' water in the fibre.¹⁶¹ Lynch & Haly used T_2 relaxation to examine anisotropy of water in wool fibres, showing that T_2 times had a dependence on the orientation of the fibre to the direction of the NMR magnetic field.⁶² Liff *et al.* proposed that the partitioning of D_2O in wool, as observed using ^2H NMR, suggested the existence of two different morphological regions of wool.¹⁶²

Of course, this alludes to the fact that changes in relaxation behaviour may be resultant of the structural properties of the keratin fibre. Semmence demonstrated the reduction of ^1H T_1 times of free water in hair damaged by chemical and cosmetic treatments. It was proposed that damage resulted in increased porosity of the fibre, allowing greater exchange between the bound and free populations, and increased interaction between the free population and the hair fibre with increased penetrability.⁷

While a two-population model, and the five phase Feughelman & Haly model are undoubtedly oversimplifications, there must be caution when applying multi-exponential fits to relaxation data. The high heterogeneity of keratins has led to multi-exponential fits being used to model

the relaxation behaviour of water, resulting in a Gaussian distribution of relaxation times.^{74,162} This multi-population inference may indeed result instead from the intrinsic heterogeneity of the keratins themselves, and not representative of discrete structural phases.¹⁶² This consideration is further supported by measurements of water in hair by Clifford & Sheard, where the two most immobile bound populations measured by ^1H T_1 experiments contributed to 90% of the overall relaxation behaviour, while only making up 10% of the total water populations identified.⁷⁴

3.1.3 Aims

In this Chapter, ^1H T_1 relaxation measurements are used for the first time to examine the populations of a series of model compounds which are commonly found in personal care formulations, acting as fragrances or solvents.¹⁶³ While the majority of chemical species found in formulations can be much larger in size and more complex,¹⁶⁴ the model compounds chosen in this work have relatively simple structures, providing a foundation for future work. These small molecules are methanol, acetic acid, ethyl acetate, benzyl acetate, and isoamyl acetate (Figure 3.2). These compounds were selected for their relative structural simplicity, commercial relevance, and range of their partition coefficient (LogP) and molecular weight (MW) values. The effect of the LogP and MW of these model compounds on relaxation in hair was examined by measuring changes between the solvent in bulk solution, and solvent saturated in hair. NMR methods are particularly suited to interrogating molecular interactions due to the high sensitivity of the measurements.

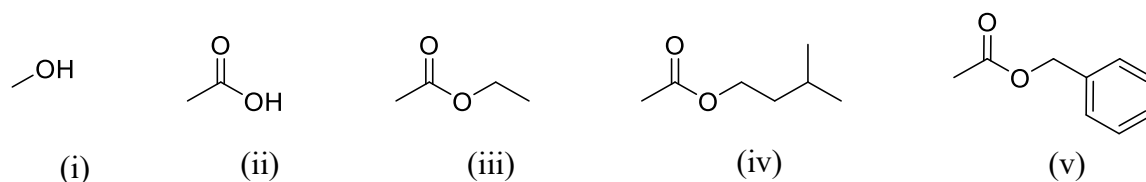


Figure 3.2 The structures of compounds investigated, from left to right; (i) methanol, (ii) acetic acid, (iii) ethyl acetate, (iv) isoamyl acetate and (v) benzyl acetate. All compounds are commercially relevant, being used as ingredients in personal care products. 163

T_1 relaxation has a high dependency on molecular motion, allowing subtle differences in molecular bonding to be identified. The ability of NMR to be performed on a significant quantity of hair (in comparison to single fibre experiments often used in industry) allows a more representative measurement of the average fibre behaviour to be analysed. ^1H T_1 times are often an order of magnitude longer than the respective T_2 times, falling between seconds and milliseconds. This allows a larger detection range for changes in T_1 times. This Chapter aims to take a step forward in understanding how the properties of a small molecule affect its interactions with hair.

3.2 Methods and Materials

Untreated hair (European brown Caucasian, batch source) was used in all experiments, provided by Procter & Gamble as a gift. All hair used in this Chapter, and elsewhere in this thesis, was obtained from healthy volunteers.

Hair was chopped finely to lengths of approximately 0.5 cm and dried over several days over P_2O_5 to achieve a constant dry mass. Dried hair was then soaked for approximately 16 hrs in the chosen compound to reach full saturation. P_2O_5 , ethyl acetate, acetic acid and methanol

were purchased from Sigma-Aldrich, benzyl acetate and isoamyl acetate were purchased from Tokyo Chemical Industries. Unbuffered de-ionized water was used.

Rotors (4 mm, ceramic) were loaded with damp fibres (axially). Static measurements were taken at room temperature using a solid-state Bruker Avance 200 MHz NMR spectrometer.

The inversion recovery experiment pulse sequence is shown in Equation 3.1.

$$d1 - p1(180^\circ) - d2 - p2(90^\circ) - \text{FID} \quad (3.1)$$

Where the initial delay time ($d1$) was 4 s, the $p1$ and $p2$ pulses had lengths of 4 μs and 8 μs , respectively. Measurement of the inversion recovery curves were achieved with 32 recovery delay times ($d2/\tau$) with times varying between 1 ms – 30 s. 16 scans were performed at each delay time with a spectral width of 250 ppm/50 kHz.

All ^1H NMR static spectra for solvents and solvents in hair are shown in the Appendix (Chapter 10.1).

3.3 Results & Discussion

3.3.1 Fitting inversion recovery curves of small compounds in hair

As the solvents are present in great excess to protons present in hair, static NMR experiments were performed with the confidence that contribution from hair protons would be negligible in this work.

As described in section 3.1.2, the apparent multi-exponential relaxation of compounds in hair may not be representative of discrete structural phases. This work therefore uses a minimum fitting approach to prevent an overexpression of phases, and confirming the existence of

bound and free populations of different solvents in hair. To determine the existence of these two phases, ^1H T_1 recovery curves were fit to either Equation (3.2):

$$A(\tau) = A_0 - B\exp(-\tau/T_{1\text{Solv}}) \quad (3.2)$$

Or a two-component variant Equation (3.3):

$$A(\tau) = A_0 - B_b\exp(-\tau/T_{1\text{Hb}}) - B_f\exp(-\tau/T_{1\text{Hf}}) \quad (3.3)$$

Where $A(\tau)$ is the NMR peak area at time τ , A_0 is the peak area at $\tau = 0$, B is a pre-exponential factor (B_b is a pre-exponential factor for the bound fraction of compound, and B_f for the free fraction) and $T_{1\text{Solv}}$, $T_{1\text{Hb}}$, $T_{1\text{Hf}}$ are defined as the ^1H T_1 times (s) for the bulk solvent, bound population, and free population of compound in hair, respectively.

Table 3.1 details the calculated T_1 times and R^2 values for all compounds investigated, showing both one and two component fits for solvent in hair. Successful fits were determined where $R^2 > 0.99990$.

Table 3.1 The ^1H T_1 times of solvents, and solvents in hair fitted to either a one or two-component fit to reach $R^2 > 0.99990$, where T_{ISolv} , T_{1Hb} , T_{1Hf} are the ^1H T_1 times (s) for the bulk solvent, bound population, and free population of compound in hair, respectively. (Error = 1σ from $n = 3$)

	Solvent		Solvent in Hair				
	One-component fit		One-component fit		Two-component fit		
	^1H T_{ISolv} (s)	R^2	^1H T_{1H} (s)	R^2	^1H T_{1Hb} (s)	^1H T_{1Hf} (s)	R^2
Water	2.73	0.99996	0.65 ± 0.08	0.99764 ± 0.00032	0.10 ± 0.00	1.05 ± 0.09	0.99996 ± 0.00000
Methanol	3.12	0.99997	0.83 ± 0.15	0.99928 ± 0.00021	0.45 ± 0.06	1.36 ± 0.22	0.99995 ± 0.00001
Acetic Acid	2.03	0.99996	0.63 ± 0.09	0.99924 ± 0.00012	0.23 ± 0.04	0.78 ± 0.11	0.99998 ± 0.00001
Ethyl Acetate	3.20	0.99990	2.53 ± 0.07	0.99904 ± 0.00023	0.51 ± 0.04	2.95 ± 0.06	0.99999 ± 0.00001
Benzyl Acetate	2.43	0.99991	1.97 ± 0.18	0.99930 ± 0.00053	0.47 ± 0.05	2.28 ± 0.09	0.99999 ± 0.00001
Isoamyl Acetate	2.00	0.99998	1.73 ± 0.11	0.99966 ± 0.00010	0.38 ± 0.06	1.92 ± 0.10	0.99999 ± 0.00000

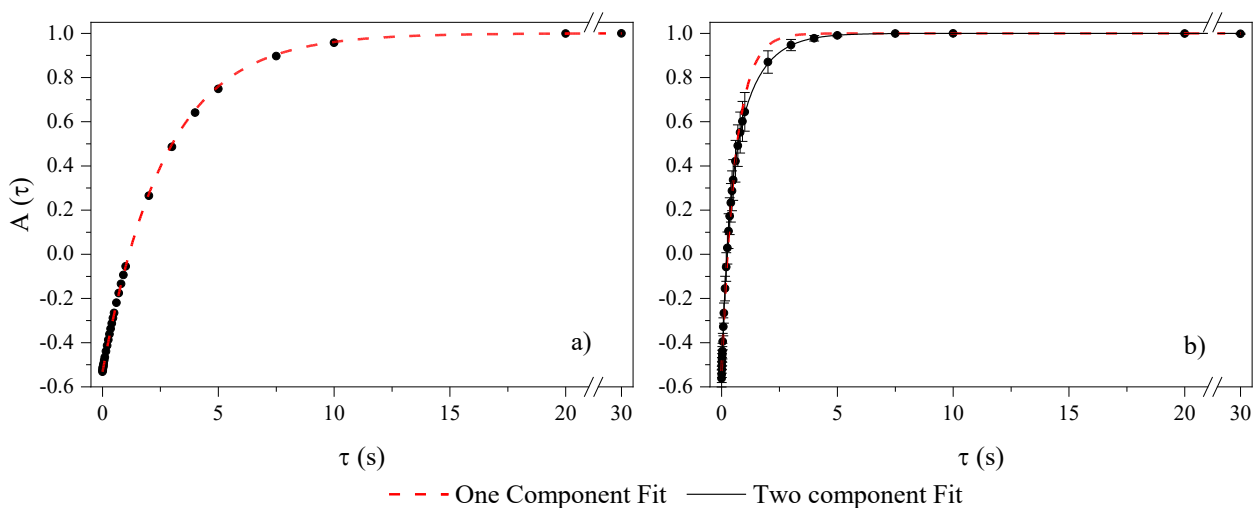


Figure 3.3 The ^1H inversion recovery curves of a) bulk water and b) water in hair demonstrating successful one-component exponential fit to bulk water and a successful two-component fit for water in hair. (Error bars= 1σ from $n = 3$)

Figure 3.3 shows a successful one-component fit for bulk water, whilst demonstrating the unsuitability of a one-component fit to model the ^1H T_1 relaxation curve for water in hair.

The inversion recovery curves for all experiments are displayed in Figure 3.4 and Figure 3.5.

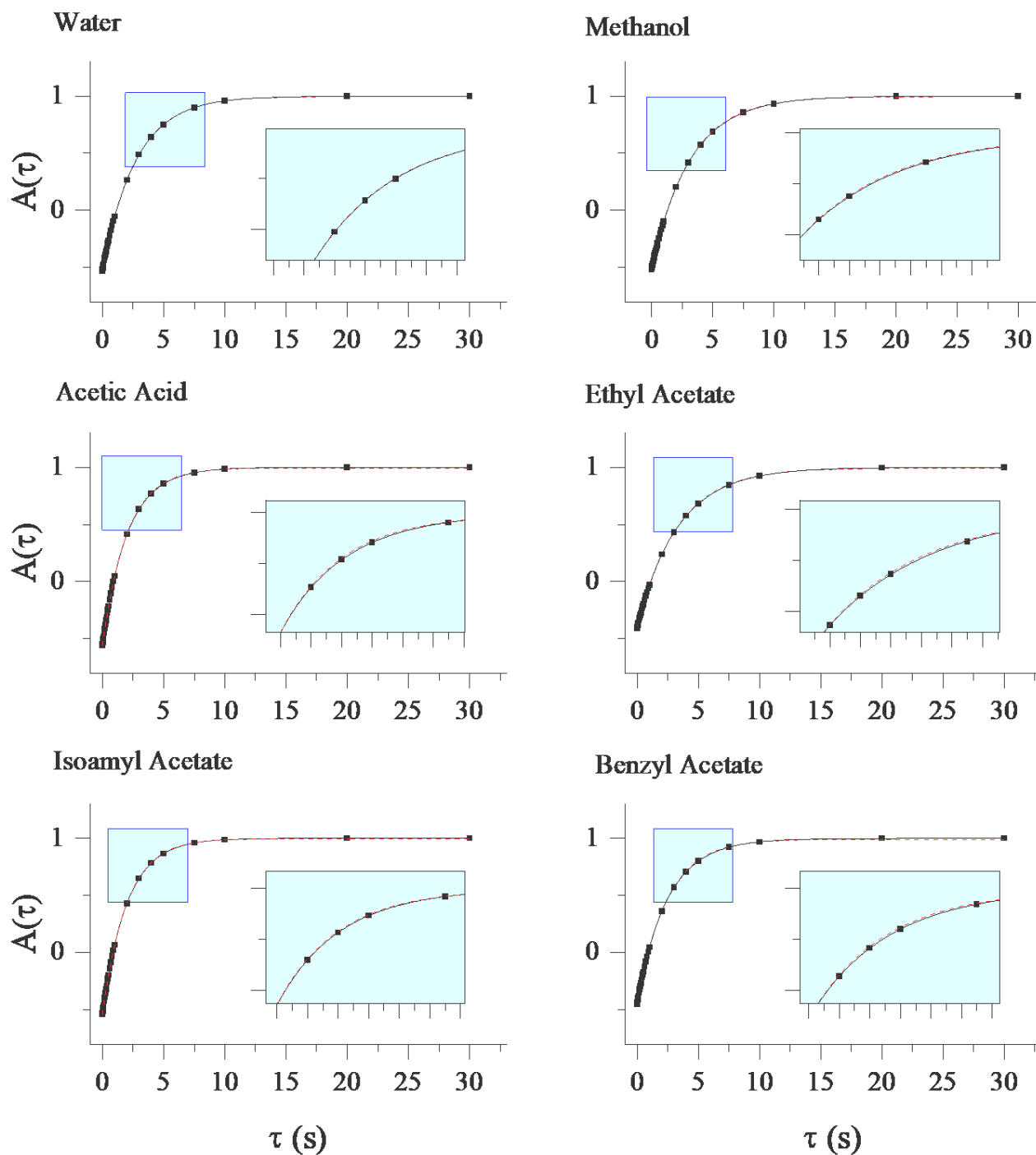


Figure 3.4 The inversion recovery curves for water, methanol, acetic acid, ethyl, isoamyl and benzyl acetate in hair. Curves are fitted to a one (---) and two component (—) fit, with inset showing no deviations between fits, confirming a one-component fit is suitable to model the solvents T_1 relaxation behaviour.

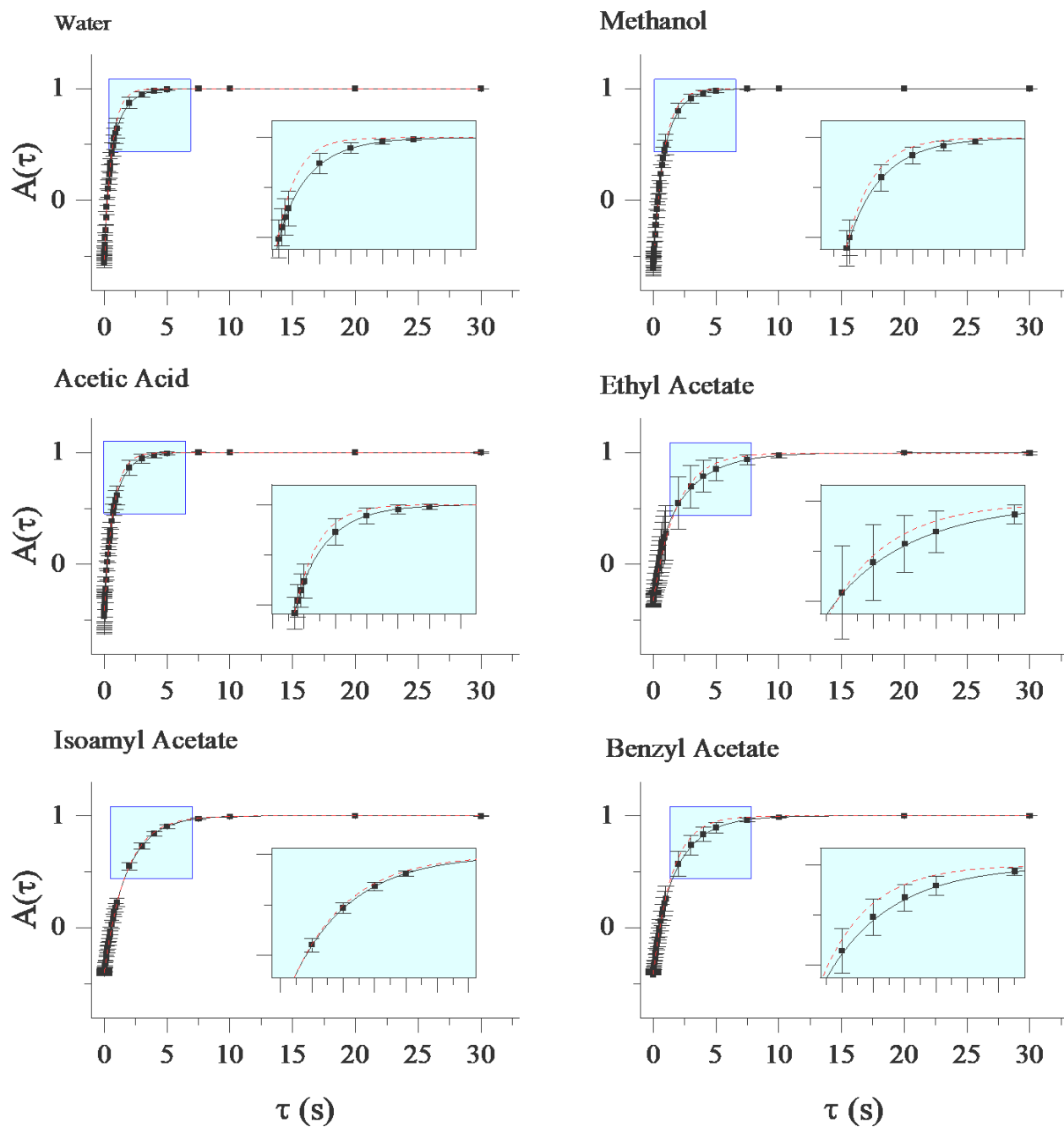


Figure 3.5 The inversion recovery curves for water, methanol, acetic acid, ethyl, isoamyl and benzyl acetate in hair. Curves are fitted to a one (---) and two component (—) model, with inset demonstrating the validity of a two-component fit. R^2 values are found in Table 3.1 (Error bars= 1σ from $n = 3$).

The data in Figure 3.5 can also be plotted linearly to further highlight the deviation from a single exponential fit. This is achieved by plotting this data as $\ln(1-A_\tau/A_0)$ vs t , where a linear plot indicates relaxation has one associated $^1\text{H } T_1$ relaxation time. For solvents in hair, these graphs are shown in Figure 3.6, demonstrating that one $^1\text{H } T_1$ time is insufficient to describe the relaxation behaviour of the solvents measured in hair.

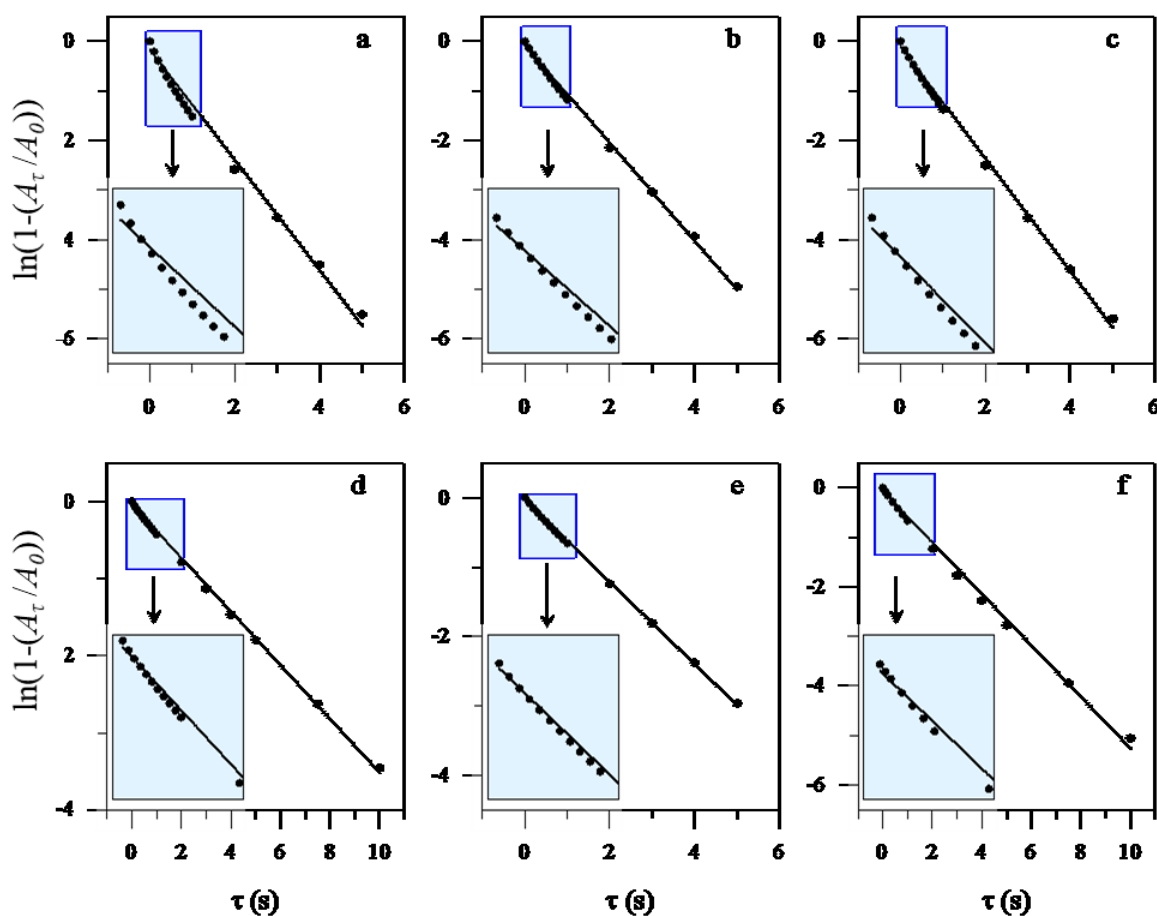


Figure 3.6 Linearised forms of the inversion recovery curves from Figure 3.5 for a) water b) methanol c) acetic acid d) ethyl acetate e) isoamyl acetate and f) benzyl acetate in hair. Inset are expanded sections of the graph data to demonstrate deviations from linearity, and that solvents in hair do not have only one $^1\text{H } T_1$ time.

To further confirm the need for a two-component fit, the NMR spectra themselves can be analysed. During the experiment, there is a time point where the longitudinal magnetization has zero intensity - this is called τ_{null} . For a one-component system, this can be observed, unless the sampling of data falls outside of this time point. As the peak relaxes at one rate, consecutive spectra will show negative intensity 'flipping' to the positive region (Figure 3.7a). In two-component systems where each component has a different T_1 value, the τ_{null} is unobservable as they relax at different rates. Instead, using water in hair as an example, two peaks relaxing through the τ_{null} are observed, each representing a distinct water population with different relaxation times (Figure 3.7b).

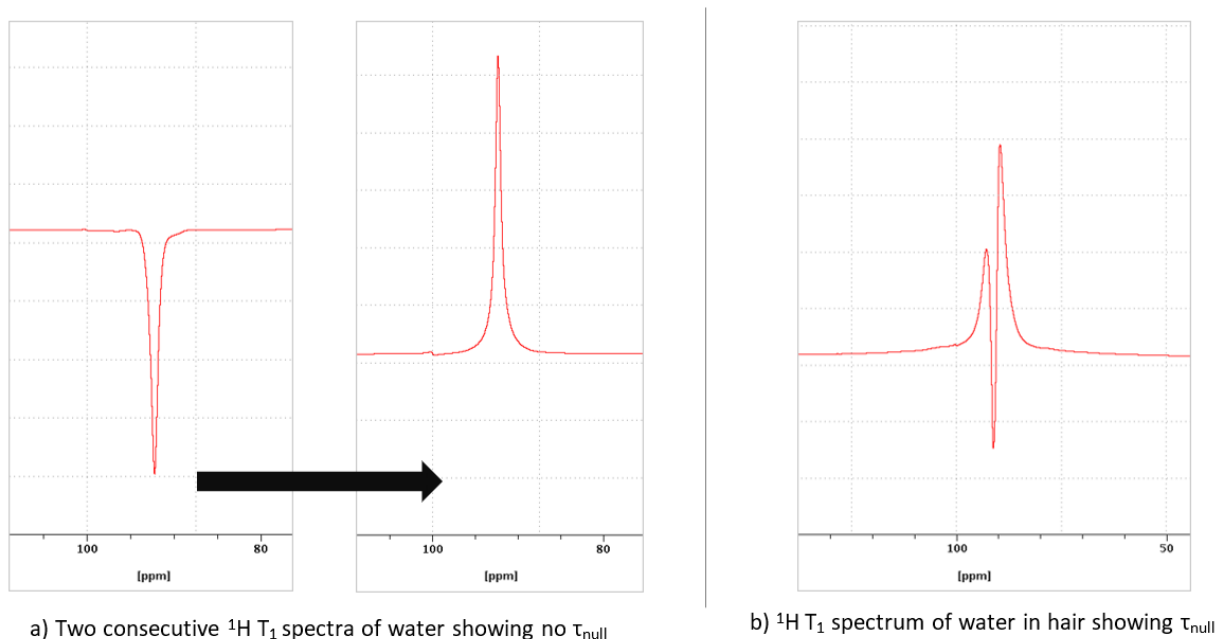


Figure 3.7 a) Two consecutive spectra (left to right) of a ^1H T_1 experiment with water; the spectrum relaxes with one component, seen as one peak. b) A spectrum of a T_1 experiment with hair soaked in water, the spectrum relaxes through τ_{null} with two peaks, showing a two-component relaxation mechanism.

3.3.2 Normalising T_1 times to determine extent of compound interaction with hair

As shown in Table 3.1, fitting the T_1 recovery curves for the solvents in hair result in two distinct T_1 times. These times are both shorter than the $T_{1\text{Solv}}$ times. The reduction in these T_1 times is a consequence of the reduced mobility of the solvents once in the hair fibre. This reduction in mobility may arise from electrostatic and dipole interactions with the fibre relating to higher proximity to keratin, indicating greater penetration of the compound into the fibre. Mobility reduction may also result from hydrogen bonding between compounds with the ability to form hydrogen bonds, and hydrophilic amino acid side chains in keratin, such as lysine, arginine, serine, glutamine and cysteic acid. Increased interactions with the fibre through proximal interactions or bonding will consequently result in shorter T_1 relaxation times.

The shortest time ($T_{1\text{Hb}}$) therefore represents a ‘bound’ population with greater interactions with the hair fibre. The longer time ($T_{1\text{Hf}}$) represents a ‘free’ population, with fewer intermolecular interactions with hair.

Changes in compound mobility, and consequently the changes between $T_{1\text{Solv}}$ and $T_{1\text{H}}$ values, inform on the extent of compound interaction with hair. Each compound has a unique $T_{1\text{Solv}}$ time, therefore normalising the $T_{1\text{H}}$ times to the associated $T_{1\text{Solv}}$ of the compound allows a comparison of changes in mobility. This allows a quantification of interaction. Here, changes between $T_{1\text{Solv}}$ and $T_{1\text{H}}$ are given numerically as a ratio between $T_{1\text{Hb}}$ or $T_{1\text{Hf}}$ and $T_{1\text{Solv}}$ (in Equations 3.4 and 3.5).

$$\text{Change in } T_1 \text{ relaxation for the bound population} = \frac{T_{1\text{Hb}}}{T_{1\text{Solv}}} = \Delta T_{1\text{Hb}} \quad (3.4)$$

$$\text{Change in } T_1 \text{ relaxation for the free population} = \frac{T_{1\text{Hf}}}{T_{1\text{Solv}}} = \Delta T_{1\text{Hf}} \quad (3.5)$$

The $\Delta T_{1\text{H}}$ ratios can therefore be used as a quantitative measure of interaction between a particular compound population and the hair fibre. Small values demonstrate a greater change in relaxation time, indicating reduced mobility associated with increased interaction between a compound and hair.

The values for $\Delta T_{1\text{Hb}}$ and $\Delta T_{1\text{Hf}}$ for the compounds investigated are displayed in Table 3.2. Where compounds have a high degree of hydrogen bond forming ability, such as water, methanol, and acetic acid, they consequently have the lowest $\Delta T_{1\text{Hb}}$ and $\Delta T_{1\text{Hf}}$ values out of the set of compounds investigated. As previously mentioned, hydrogen bonding would allow greater immobility of the compounds. The impact of hydrogen bonding ability is especially evident in the free population, where those with extensive hydrogen bonding ability have $\Delta T_{1\text{Hf}}$ values approximately 2-3 times smaller than those without. The molecular weight of the compounds also appears to be an influencing factor, with the larger acetates having $\Delta T_{1\text{Hf}}$ values close to $\Delta T_{1\text{Solv}}$.

Table 3.2 The ratios of ΔT_{1Hb} and ΔT_{1Hf} for compounds in hair, calculated using Equations 3.4 and 3.5 (Error = 1σ from $n = 3$). Lower values of ΔT_{1H} indicate stronger interactions with hair.

	ΔT_{1Hb}	ΔT_{1Hf}
Water	0.10 ± 0.00	0.38 ± 0.03
Methanol	0.14 ± 0.02	0.43 ± 0.07
Acetic Acid	0.11 ± 0.02	0.38 ± 0.06
Ethyl Acetate	0.16 ± 0.01	0.92 ± 0.02
Isoamyl Acetate	0.19 ± 0.02	0.94 ± 0.04
Benzyl Acetate	0.19 ± 0.03	0.96 ± 0.05

Table 3.3 The values of LogP and molecular weight for the model compounds investigated.

	LogP ¹	Molecular Weight
Water	-1.78	18.02
Methanol	-0.71	32.04
Acetic Acid	-0.28	60.05
Ethyl Acetate	0.71	88.11
Isoamyl Acetate	2.12	130.19
Benzyl Acetate	1.93	150.18

¹ LogP values were provided by Procter and Gamble, calculated using Advanced Chemistry Development Labs ACD/LogP software.

3.3.3 Impact of MW and LogP on ΔT_{1Hb} and ΔT_{1Hf} values

The ΔT_{1Hb} and ΔT_{1Hf} values can be used to examine the relationship between chemical parameters, such as MW and LogP (Table 3.3), to keratin interaction.

The ΔT_{1Hb} and ΔT_{1Hf} values demonstrate an apparent sigmoidal relationship to MW (Figure 3.8a). With limited data points available, this is only a preliminary model, and so the trendlines are provided in Figure 3.8 as a suggestion of a sigmoidal relationship.

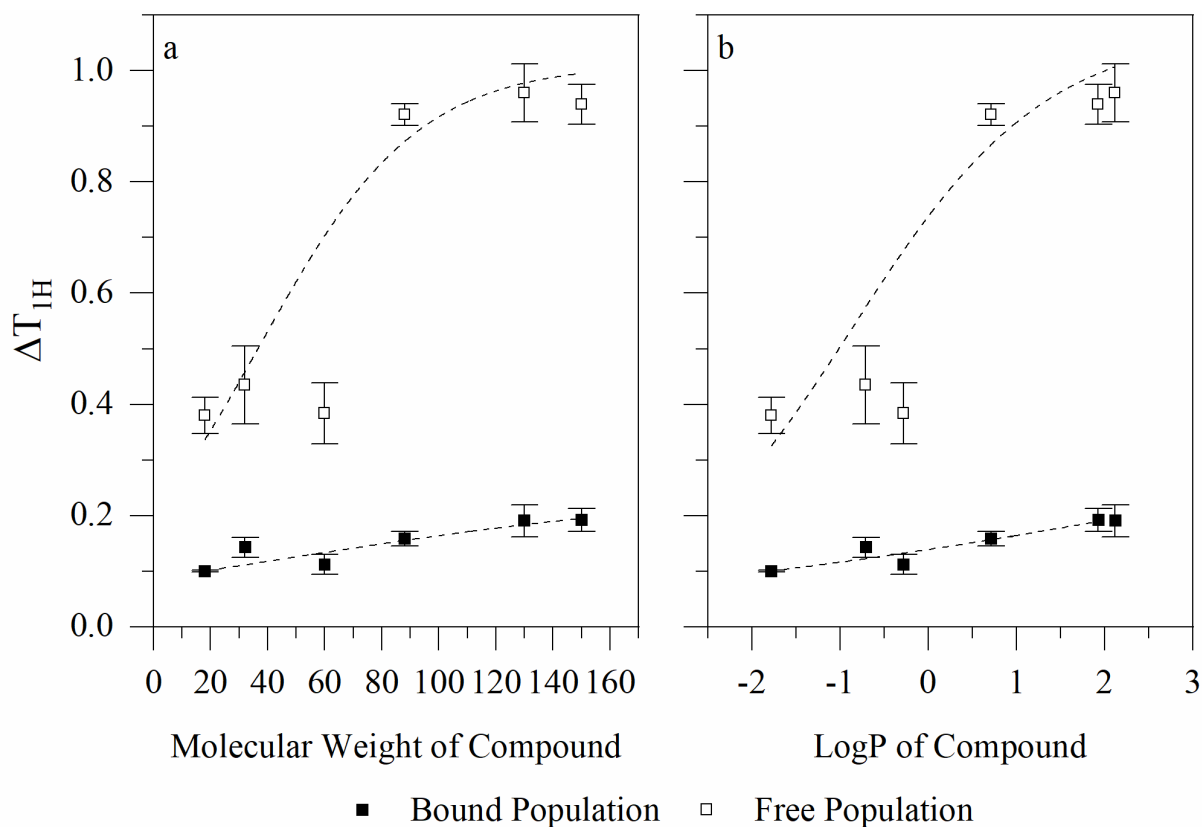


Figure 3.8 The effect of a) MW and b) LogP on the ΔT_{1Hb} and ΔT_{1Hf} values of the compound in hair. Compounds with low MW and low LogP values have smaller ΔT_{1Hb} and ΔT_{1Hf} values, indicating more interactions with hair keratin. This trend is demonstrated with a line to guide the eye. (Error bars= 1σ from $n = 3$)

We would expect to see such a sigmoidal relationship; where above a given MW, compounds can no longer penetrate as successfully into the hair fibre and will have reduced interactions. A step-like relationship also reflects that there is a limit to the immobilization a compound can have with strong interactions; compounds which have a MW below a certain size which allows significant penetration into the fibre, will have similar immobilisations and so ΔT_{1H} values will plateau.

This relationship or step-like function indicates that between MW 60-88, there is a critical mass for compounds which prevents strong interactions with the hair fibre. Simply, smaller molecules will be able to penetrate the hair cortex better than larger ones. As magnetization transfer through dipolar mechanisms is distance dependent ($1/r^3$), smaller molecules which can penetrate further into the hair fibre will have significantly increased interactions and reduced mobility.²⁰

Figure 3.8b demonstrates a similar trend with for the changes in ΔT_{1Hf} values with respect to the LogP values of a compound. As previously mentioned, more hydrophilic compounds, which can form interactions such as hydrogen bonding, ionic and electrostatic interactions with amino acid side chains, will have reduced ΔT_{1Hf} values. A definitive cut off is seen when comparing ΔT_{1Hf} values with MW (at approx. MW > 88), whereas when examining the effect of LogP a plateau in changes of ΔT_{1Hf} values has still not been reached. The MW of a compound may therefore be a better indicator of the 'critical' range between 'strong' and 'weak' interactions of a compound with hair.

For the bound populations, the effect of LogP and MW has a minimal effect on ΔT_{1Hb} values. This can be understood as when the bound population has formed sufficient interactions with

hair to impede its mobility, the variation in LogP or MW properties of the bound population will not have a great impact, as the compounds are all sufficiently immobilised.

Notably, a major outlier to the trendlines indicated in the ΔT_{1Hf} values in Figure 3.8 is acetic acid (MW = 60, LogP = -0.28). As the only – albeit weak - acid investigated, it is likely to impact property changes on the hair fibres during its saturation. This was observed visually after the 16-hour saturation time, with fibres becoming lighter in colour, and more brittle. This is likely to have led to an apparent increase in interaction for acetic acid, whereas it is more reflective of the damage caused by acetic acid, allowing better penetration into the fibre and access to binding sites. In this work, a binding site is defined as a charged and/or hydrogen bonding amino acid side chain of amino acids such as lysine, arginine, serine, glutamine and cysteic acid.

In this work the compounds with low MW values also have low LogP values. This results in the inability to determine the importance, or indeed separate, the contributions of MW or LogP to small molecule interactions with hair. It is also a great simplification to suggest that compound interactions with hair can be predicted solely with one or two chemical parameters. Nonetheless, given the highly heterogeneous nature of hair, these results are highly surprising and encouraging. The observation of trends for simple characteristics such as MW and LogP, suggest that a multiparameter model for predicting small molecule interactions with hair is a strong possibility. The existence of such a model would be important in improving the understanding of the complex relationships between small molecules and keratins. This makes this work a great step forward in this area of research.

3.3.4 Relative sizes of free and bound populations

The pre-exponential factors B_b and B_f calculated from fitting recovery curves to Equation 3.3 reflect the relative population sizes of the bound and free populations of compound in hair, respectively. The relative populations sizes for the bound and free populations were calculated as:

$$\text{Relative Population size} = \frac{B_{b/f}}{(B_b + B_f)} \quad (3.6)$$

Equation 3.6 was used to calculate the relative sizes of the bound and free populations for all compounds investigated (Table 3.4).

Table 3.4 The relative population sizes for the bound and free populations of compound in hair, calculated used Equations 3.3 & 3.6. (Error bars= 1σ from $n = 3$)

	Relative population size	
	Bound Population	Free Population
Water	0.35 ± 0.04	0.65 ± 0.04
Methanol	0.31 ± 0.06	0.69 ± 0.06
Acetic Acid	0.19 ± 0.02	0.81 ± 0.02
Ethyl Acetate	0.09 ± 0.01	0.91 ± 0.01
Benzyl Acetate	0.06 ± 0.01	0.94 ± 0.01
Isoamyl Acetate	0.09 ± 0.05	0.91 ± 0.05

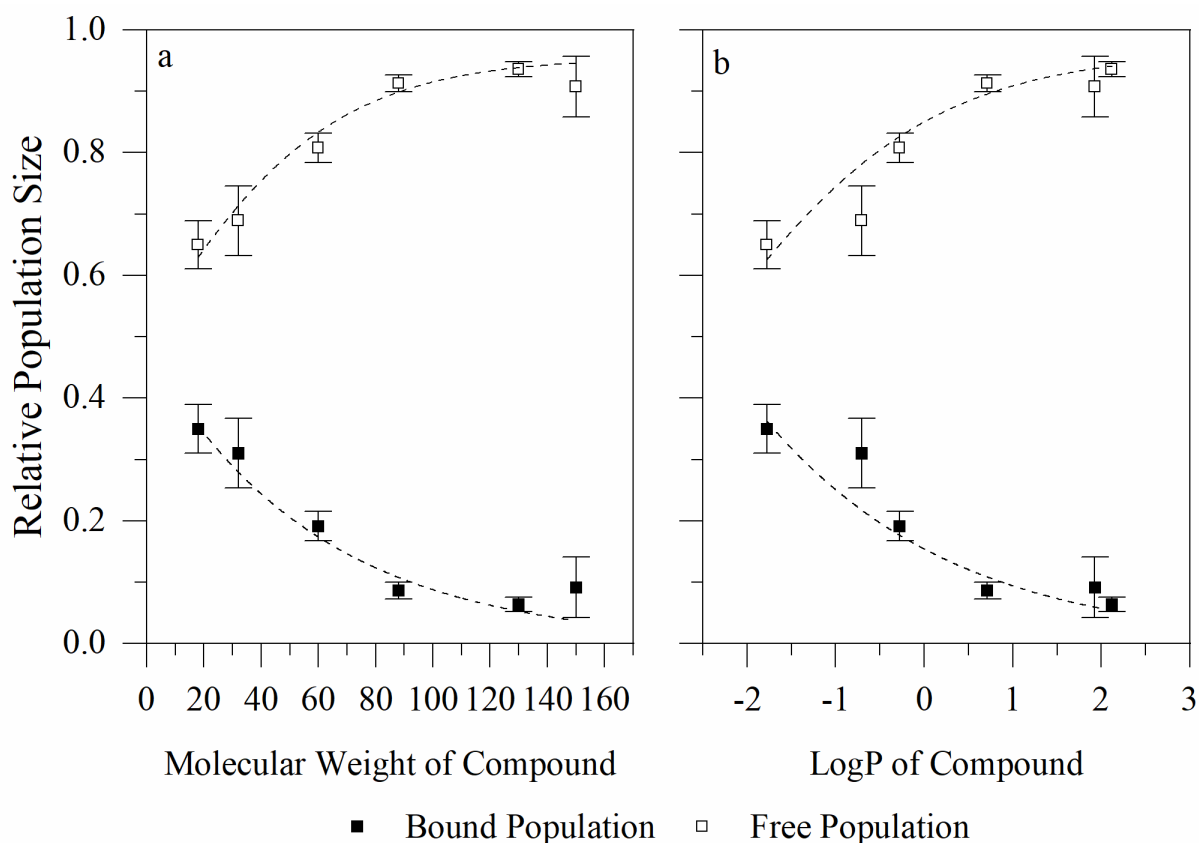


Figure 3.9 The effect of a) MW and b) LogP on the population sizes of a compound in hair. The size of the bound populations increases with low MW and low LogP values. Conversely, the size of free populations increases with high MW and high Log P values. This trend is demonstrated with a line to guide the eye. (Error bars= 1σ from $n = 3$)

The relative size of the free population is in excess of the bound population for every compound examined. Due to the saturation process of the hair fibres, and the limited binding sites available in hair, this excess is expected. The effect of the MW and LogP values of the compounds on their relative population sizes is shown in Figure 3.9.

The relative size of the bound population of a compound in hair increases with low MW and low LogP values. For compounds with the opposite properties (such as ethyl, isoamyl & benzyl acetate), the bound population only accounts for less than 10% of the total population. This

makes the overall behaviour of these compounds governed by the behaviour of the free population, in this case, having weak interactions with hair.

As previously discussed, molecules with high MW will not penetrate the hair fibre as successfully as smaller molecules, resulting in inability to access binding sites and resulting in a larger free population size. Conversely, molecules with lower MW will be able to penetrate the hair cuticle better than larger molecules, having the ability to access these binding sites and increasing these compounds relative bound population sizes. While access to binding sites may be a limiting factor, the compounds ability to successfully interact with said binding sites, through hydrogen bonding, ionic or electrostatic interactions (e.g., water, methanol, and acetic acid), will also contribute to the size of the relative bound population, as shown in Figure 3.9b.

This information is important to the practical applications of this work, as the largest population size will dominate the overall solvent behaviour in hair. This work has demonstrated that for compounds with $MW > 60$ and $LogP > 0$, the free population behaviour dominates. For compounds with $MW < 60$ and $LogP < 0$, the behaviour of the bound population cannot be ignored, having substantial contribution to the overall $^1H\ T_1$ properties and interaction of the compound in hair.

3.4 Expanded solvent range

With the previously discussed limitations attributed to the small sample of model compounds investigated, preliminary work took place to expand this range to see if the established relationships would hold. The compounds investigated for this preliminary work included: limonene, propyl acetate, butyl acetate, n-pentyl acetate and n-hexyl acetate (Figure 3.10 and

Table 3.5). Similar to the initial set investigated, these all appear in product formulations as fragrances.¹⁶³

Due to the nature of this preliminary work being used to merely confirm the relationships established in previous sections (and $n = 1$ sample size for the majority), the individual properties of these compounds and their impact on hair interactions will not be discussed. A 10% estimated error is included in all ΔT_1 calculations, based on average errors found in the previous section. (See Appendix 10.2 for T_{1H} values and fits, ΔT_1 values and Relative population Sizes).

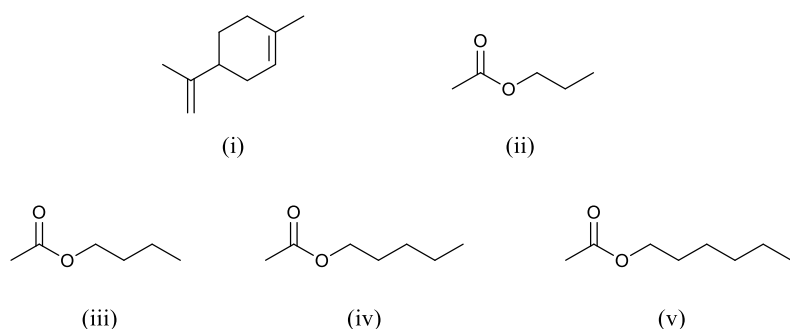


Figure 3.10 The structures of additional compounds investigated: (i) limonene, (ii) propyl acetate, (iii) butyl acetate, (iv) n-pentyl acetate and (v) n-hexyl acetate.

Table 3.5 The values of LogP and molecular weight for the expanded range of compounds investigated.

	LogP ²	Molecular Weight
Limonene	4.45	136.24
Propyl Acetate	1.24	102.13
Butyl acetate	1.77	116.16
n-pentyl acetate	2.30	130.19
n-hexyl acetate	2.83	144.21

The relationships between ΔT_1 values and the effect of MW and LogP (Figure 3.11) of a compound has been modelled with a simple sigmoidal function (Equation 3.7) to evaluate the R^2 values of the apparent relationship.

$$y = \frac{a}{1 + \exp(-k(x - x_c))} \quad (3.7)$$

Where a is the amplitude of the curve, x_c the centre, and k a coefficient. As a preliminary model, with not enough data points, this section will not evaluate the physical implications of these constants, and instead use this model to assess the validity of the proposed sigmoidal relationship.

² National Center for Biotechnology <https://pubchem.ncbi.nlm.nih.gov> Accessed July 2021.

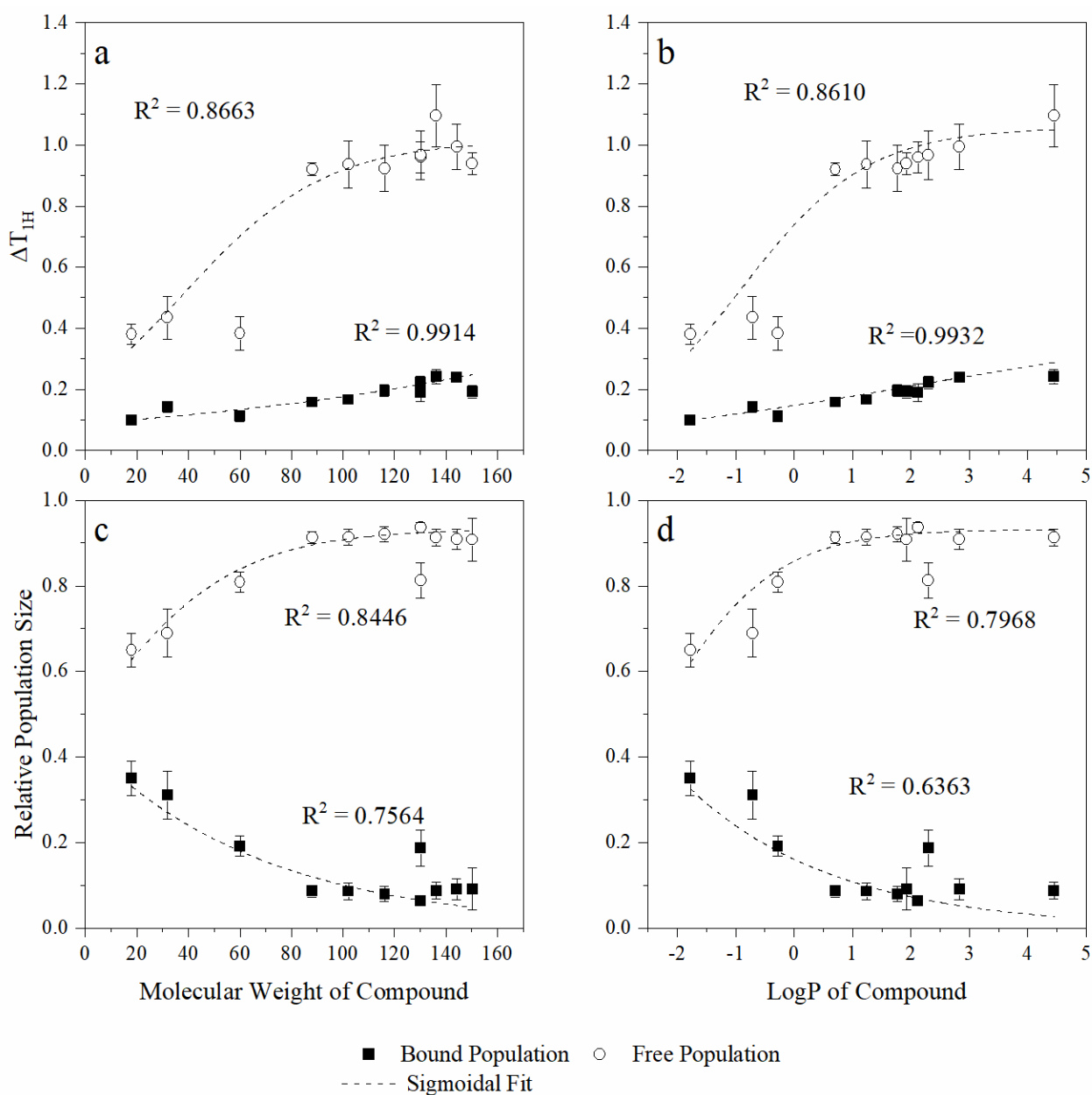


Figure 3.11 The effect of a) MW and b) LogP on the ΔT_I values of a compound, and the effect of c) MW and d) LogP on the relative bound and free population sizes of a compound. Trends are demonstrated with a sigmoidal relationship (Equation. 3.7) . (Error bars= 1σ from $n = 3$ for water, methanol, acetic acid, ethyl, benzyl and isoamyl acetate, n-hexyl acetate, and a 10% estimated error are applied to values for limonene, propyl, butyl and n-pentyl acetate, where $n = 1$.)

The R^2 values are high considering the heterogeneity of hair fibres. This further supports the validity of the observed relationships seen in previous sections. In turn, this provides confidence for the success of future work to develop quantitative structure-activity relationship (QSAR) models.

3.5 Conclusion

In this Chapter, the static ^1H T_1 relaxation times for a series of model small molecular compounds were measured in untreated hair. For the first time in literature, two populations of compound were identified for methanol, acetic acid, ethyl acetate, isoamyl acetate and benzyl acetate. The rigid bound population was identified with shorter relaxation times ($T_{1\text{Hb}}$) and the labile free populations with longer relaxation times ($T_{1\text{Hf}}$). Comparing the solvent populations in hair to bulk solvent was used as a measure of interaction between the compound and hair. Small values of $\Delta T_{1\text{Hb}}$ and $\Delta T_{1\text{Hf}}$ indicated reduced mobility and increased interactions with the hair fibre. Compounds with low molecular weights and low LogP values have greater interactions with hair. The relative sizes of the bound and free populations were also determined, demonstrating that for large, high LogP compounds, the free population of the compound dominates the overall behaviour of the compound. For smaller, hydrophilic compounds, the size of the bound population increases considerably and becomes more relevant to understanding the total behaviour of the compound in hair.

Future work should explore the use of Magic Angle Spinning (MAS) experiments. Potential points of contact between a compound and hair could be examined by measuring the ^1H T_1 times of the proton environments within a specific compound. The proton environment with

the lowest $\Delta T_{1\text{Hb}}$ and $\Delta T_{1\text{Hf}}$ value may indicate a point of contact between the compound and a binding site in hair.

Hair is an incredibly heterogeneous fibre and the determination of trends for these populations are incredibly surprising, despite the small sample size compounds investigated. This work has provided a foundation for further studies, with studies on a wider range of compounds having the potential to form quantitative structure-activity relationship (QSAR) models. Whilst such a model would be a great step forward in understanding the behaviour of small molecules in human hair, this method can also be applied to other large insoluble proteins, whose interactions cannot be examined using traditional solution state NMR.

The data in this Chapter was utilised to publish the following paper:

Molisso S, Williams D. R., Ces O, Rowlands L. J., Marsh J. M., Law R. V. Molecular interaction and partitioning in α -Keratin using ^1H NMR Spin-spin (T_2) relaxation times. *Journal of the Royal Society Interface* **2021**

Chapter 4 - Effect of humidity on small compound interactions

4.1 Introduction

4.1.1 Importance of water in hair keratin

The previous Chapter discussed the interactions between small molecules and hair, where hair was fully dried to remove water. However, in ambient conditions hair has a significant water content absorbed from moisture present in the air. Table 4.1 displays the approximate moisture content in hair at different relative humidity's (%RH), as compiled by Robbins.⁴ With hair able to take up to 32% by weight of water,⁴ it is vital to understand how the presence of water affects hair interactions with small molecules.

The uptake of water, even at ambient conditions, will result in the swelling of the hair fibre. Hair swelling increases the diameter of the fibre, whereas the change in length is negligible.⁹⁰ The endocuticle layer of the cuticle is most affected by water content and swells the most, with the δ -layer thickness increasing by up to 10% from relative humidity's above 45 %RH.^{4,22}

Table 4.1 The water content of untreated brown human hair at different %RH, adapted from Robbins (where n = 5).⁴

%RH	Approximate Water Content of Hair (%)
29.2	6.0
40.3	7.6
50.0	9.8
65.0	12.8
70.3	13.6

For keratins such as wool, until 80 %RH volume swelling is not proportional to water sorption, as water molecules can fill voids in the hair structure, resulting in lower swelling than expected.¹⁶⁵

Water content also has an impact on the structural properties of hair keratin, regarding its thermal and mechanical behaviour. The increasing moisture content of hair results in the early onset of α -crystallite melting, leading to lower degradation temperatures.^{166,167}

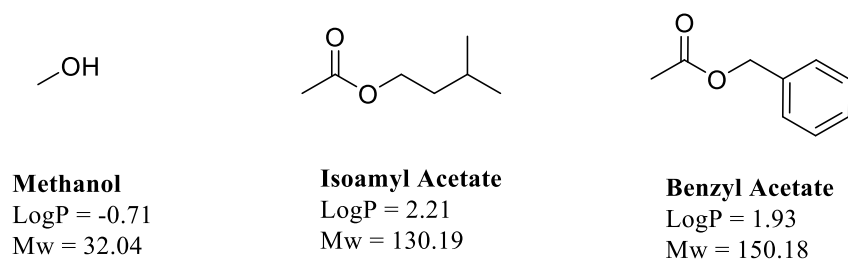


Figure 4.1 The structures and properties of compounds investigated, from left to right; methanol, isoamyl acetate and benzyl acetate.

Water molecules act as a plasticiser in the matrix, leading to the melting of amorphous fractions of α -keratin in hair at lower temperatures than hair's typical glass transition temperature (T_g). Hair with a 0% water content was shown to exhibit a T_g at 144°C, whereas a hair sample with a 15% water content had a corresponding T_g at approximately 40°C.¹⁶⁸ This is further demonstrated by the greater extension ability of keratins before they break, as %RH increases.¹²

With these numerous variables, it is not adequate to only understand small molecule behaviour in dry hair; the impact of varying moisture content must also be examined.

4.1.2 Aims

In this work, the relaxation methods developed in the last Chapter were used to investigate hair that was conditioned to relative humidity's of 0.00 %RH, 7.75 %RH, 32.70 %RH and 97.10 %RH. Solid state Magic Angle Spinning (MAS) NMR was used to examine the change in ^1H spin-lattice (T_1) relaxation of methanol, isoamyl acetate and benzyl acetate in conditioned hair (Figure 4.1). Overall, this work aims to examine the impact of moisture content of hair on its interactions with small molecules.

4.2 Methods and Materials

Untreated hair (European brown Caucasian, batch source) was used in all experiments, provided by Procter & Gamble as a gift. Hair was chopped finely to lengths of approximately 0.5 cm before preconditioning. Hair was preconditioned to 0.00, 7.75, 32.70 and 97.10 %RH using saturated salt solutions. Deionized water was loaded with salt and kept stirring at 40°C until the solution reached saturation. Once the solutions were cooled, they were placed in the bottom of a glass desiccator (with desiccant removed) to generate the desired humidity.

Table 4.2 Salts used to make saturated solutions and the humidity's generated.¹⁶⁹

Saturated salt solutions used	%RH
LiBr	7.75
MgCl ₂ .6H ₂ O	32.70
K ₂ SO ₄	97.10

Table 4.2 displays which salts were used to get the appropriate relative humidity. Hair was conditioned at different relative humidity for 3 days before being soaked in solvent for 16 hours.

For diffusion experiments, samples of soaked hair were extracted from a batch of hair soaked in solvent, at different time intervals between (5-960 min) for measurement. To prepare hair at 0.00 %RH, hair was dried over several days using P₂O₅.

P₂O₅, methanol, lithium bromide, magnesium chloride hexahydrate and potassium sulphate were purchased from Sigma-Aldrich, benzyl acetate and isoamyl acetate were purchased from Tokyo Chemical Industries. Unbuffered de-ionized water (Milli-Q) was used.

Rotors (4 mm, ceramic) were loaded axially with damp fibres. MAS measurements were taken at room temperature using a solid-state Bruker Avance 200 MHz NMR spectrometer, with a spinning frequency of approximately 6 kHz. This frequency was needed to provide adequate

splitting of peaks, to allow clear resolution of the CH₃ peak present in all three solvents. This allowed measurements of T_1 times without influence from water. The inversion recovery experiment is described in detail in Chapter 3.2. Example ¹H MAS NMR spectra are shown in Figure 4.2, Figure 4.3 and Figure 4.4 for solvents in hair at 7.75 %RH to demonstrate clear separation of solvent and water peaks.

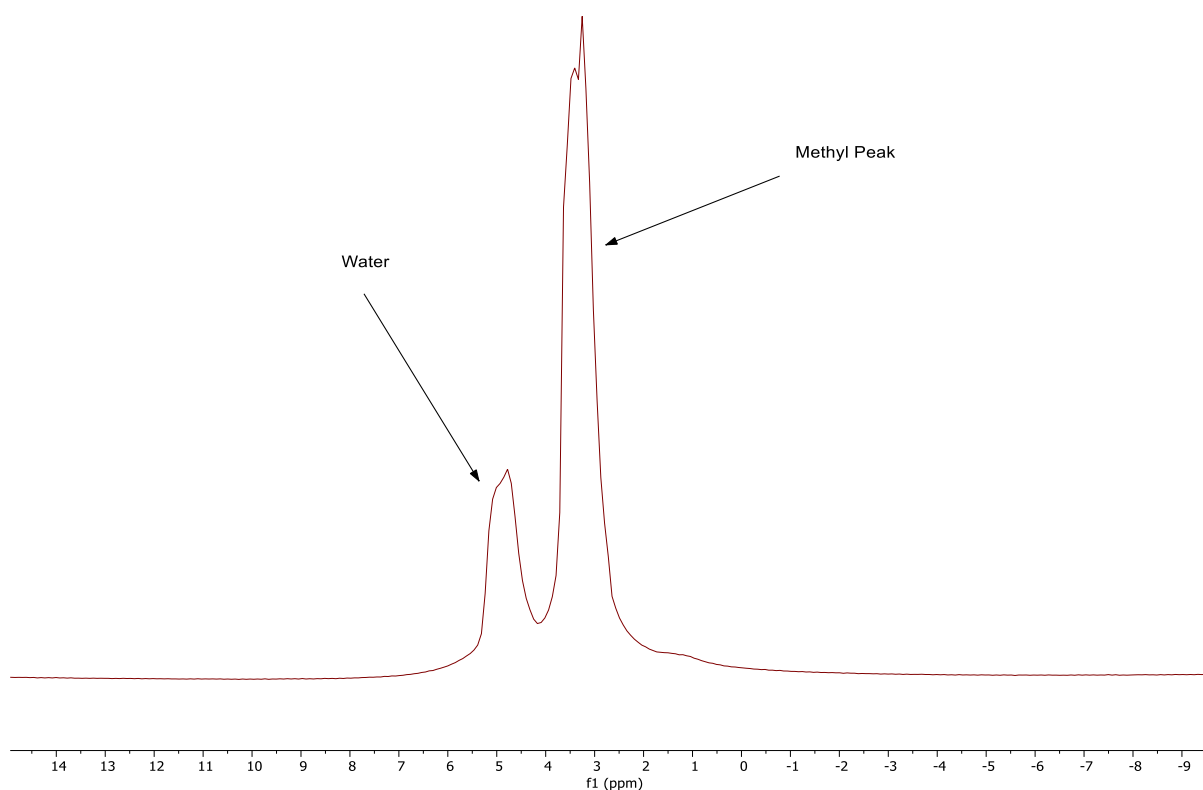


Figure 4.2 ¹H MAS NMR spectra for methanol in hair conditioned to 7.75 %RH. Highlighted are the water and methyl peaks.

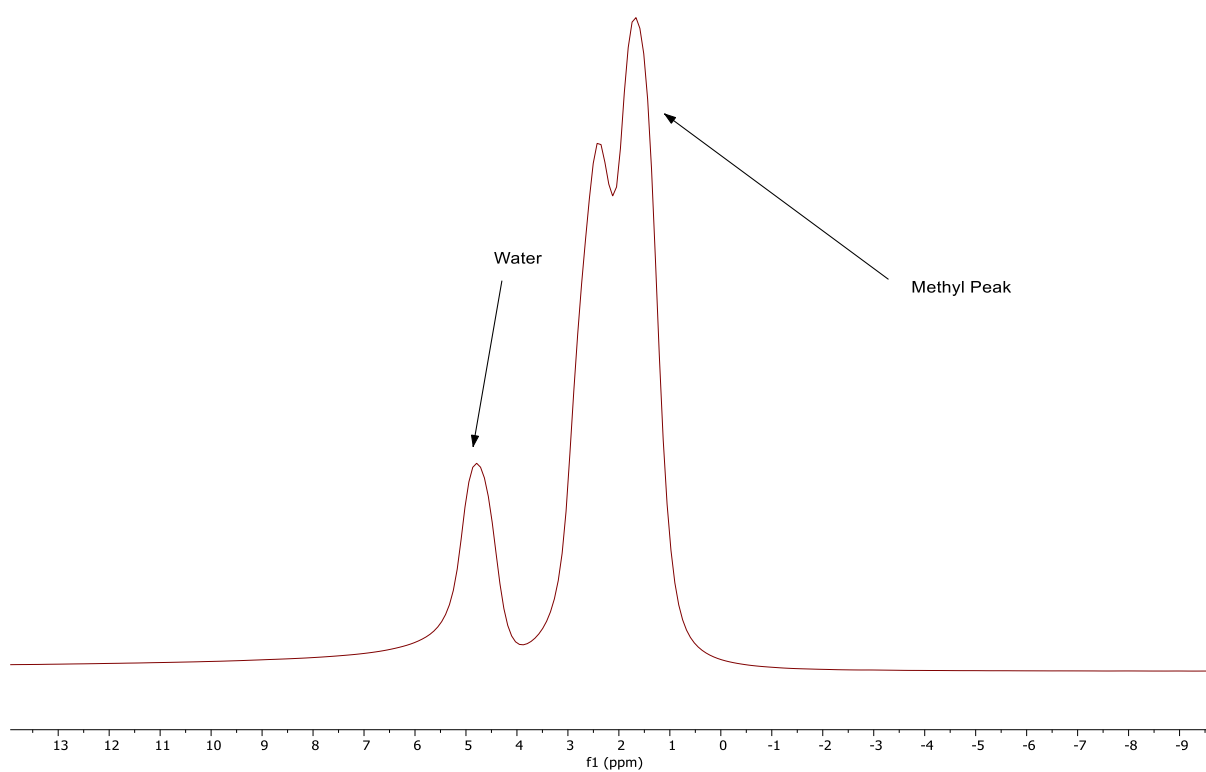


Figure 4.3 ^1H MAS NMR spectra for isoamyl acetate in hair conditioned to 7.75 %RH.

Highlighted are the water and methyl peaks.

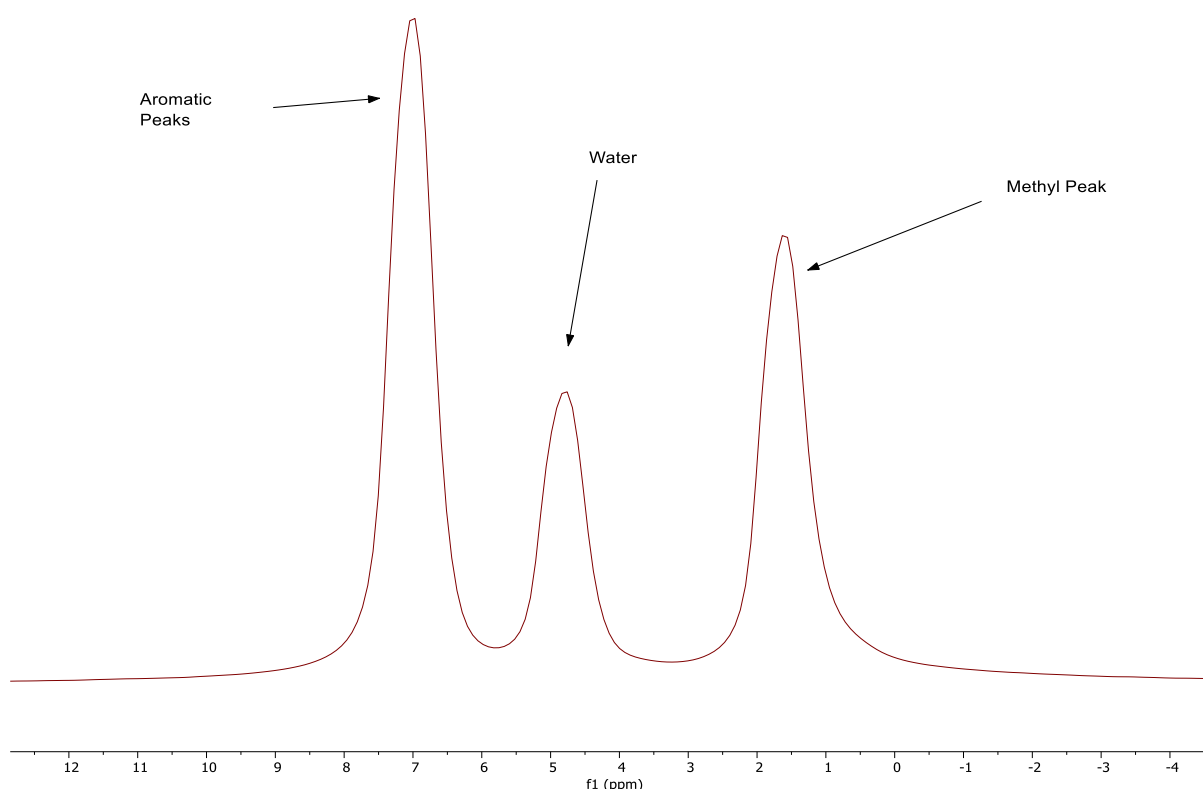


Figure 4.4 ^1H MAS NMR spectra for benzyl acetate in hair conditioned to 7.75 %RH.

Highlighted are the water and methyl peaks.

4.3 Results

To determine the existence of bound and free populations of solvent in hair, the MAS ^1H T_1 recovery curves for the CH_3 peak of the solvents were fit to either Equation (3.2) or the two-component variant (3.3). Table 4.3 details the calculated T_1 times and R^2 values for the CH_3 group of methanol, benzyl acetate and isoamyl acetate, using a single component fit to reach $R^2 > 0.9990$ (fits achieved using *Origin2020*).

Table 4.4 shows the T_1 times for CH_3 groups of solvent in hair, conditioned at different humidity's, including the one and two-component fits.

Table 4.3 The ^1H T_1 times of solvents for the CH_3 group. Solvents were fit to a one-component fit to reach $R^2 > 0.9990$, where $T_{1\text{Solv}}$ is the ^1H CH_3 T_1 time (s) for the bulk solvent.

Solvent		
One-component fit		
	^1H CH_3 $T_{1\text{Solv}}$ (s)	R^2
Methanol	2.94	0.99923
Benzyl Acetate	1.82	0.99979
Isoamyl Acetate	1.90	0.99991

Table 4.5 displays the same parameters for solvents in hair which were measured at different soak times, to understand how diffusion into the fibre affects the relative population sizes and relaxation times. For the two-component fits designated with '*' where $R^2 < 0.99950$, linear graphical representations are shown in Figure 4.2 demonstrating a two-component fit is still appropriate.

Table 4.4 The ^1H T_1 times of CH_3 groups of solvents in hair. Solvents were fit to a one-component or two-component fit to reach $R^2 > 0.99950$, where $T_{1\text{Hb}}$ and $T_{1\text{Hf}}$ are the ^1H T_1 times for the bound and free populations of solvent in hair. For the two-component fits designated with “*” where $R^2 < 0.99950$, linear graphical representations are shown in Figure 4.2, demonstrating a two-component fit is still appropriate. (Methanol 97.10 %RH: error = 1σ from $n = 2$, all other measurements have an estimated 10% error.)

	Humidity Hair conditioned to	One-component fit		Two-component fit		
	(%RH)	$^1\text{H } T_{1\text{H}} \text{ (s)}$	R^2	$^1\text{H } T_{1\text{Hb}} \text{ (s)}$	$^1\text{H } T_{1\text{Hf}} \text{ (s)}$	R^2
Methanol	0.00	1.11 ± 0.11	0.99729	0.50 ± 0.05	1.53 ± 0.15	0.99999
	7.75	0.78 ± 0.08	0.99878	0.41 ± 0.04	1.28 ± 0.13	0.99996
	32.70	0.41 ± 0.04	0.99168	0.03 ± 0.00	0.50 ± 0.05	0.99946*
	97.10	0.43 ± 0.03	0.99641 ± 0.00194	0.04 ± 0.01	0.49 ± 0.05	$0.99943 \pm 0.00020^*$
Benzyl Acetate	0.00	1.59 ± 0.16	0.99873	0.97 ± 0.10	2.68 ± 0.27	0.99980
	7.75	0.71 ± 0.07	0.99373	0.07 ± 0.01	0.91 ± 0.09	0.99936*
	32.70	0.54 ± 0.05	0.99357	0.06 ± 0.01	0.69 ± 0.07	0.99948*
	97.10	0.35 ± 0.04	0.99553	0.03 ± 0.00	0.41 ± 0.04	0.99951
Isoamyl Acetate	0.00	1.52 ± 0.15	0.99929	0.87 ± 0.09	2.17 ± 0.22	0.99990
	7.75	1.27 ± 0.13	0.99926	1.01 ± 0.10	2.71 ± 0.27	0.99989
	32.70	0.47 ± 0.05	0.98560	0.03 ± 0.00	0.62 ± 0.06	0.99930*
	97.10	0.43 ± 0.04	0.97706	0.03 ± 0.00	0.63 ± 0.06	0.99840*

Table 4.5 The ^1H T_1 times for CH_3 groups of solvents in hair soaked for different times. Solvents were fit to a one or two-component fit to reach $R^2 > 0.99950$, where $T_{1\text{Hb}}$ and $T_{1\text{Hf}}$ are the ^1H CH_3 T_1 times for the bound and free populations of solvent in hair. For fits designated with '*' where $R^2 < 0.99950$, linear graphical representations are shown in Figure 4.2, showing a two-component fit is needed. (Methanol 97.10 %RH: error = 1σ from $n = 2$, all other measurements have an estimated 10% error.)

Soak Time		One-component fit		$\pm$		
	(min)	^1H $T_{1\text{H}}$ (s)	R^2	^1H $T_{1\text{Hb}}$ (s)	^1H $T_{1\text{Hf}}$ (s)	R^2
Methanol in 97.10 %RH hair	5	0.46 ± 0.05	0.99815	0.12 ± 0.01	0.57 ± 0.06	0.99997
	180	0.42 ± 0.04	0.99812	0.06 ± 0.01	0.47 ± 0.05	0.99913*
	270	0.45 ± 0.05	0.99885	0.08 ± 0.01	0.51 ± 0.05	0.99965
	960	0.43 ± 0.03	0.99641 ± 0.00194	0.04 ± 0.01	0.49 ± 0.05	$0.99943 \pm 0.00020^*$
Benzyl Acetate in 7.75 %RH hair	5	0.53 ± 0.05	0.99749	0.12 ± 0.01	0.63 ± 0.06	0.99996
	10	0.57 ± 0.06	0.99877	0.11 ± 0.01	0.66 ± 0.07	0.99996
	90	0.56 ± 0.06	0.99880	0.12 ± 0.01	0.66 ± 0.07	0.99997
	450	0.56 ± 0.06	0.99865	0.11 ± 0.01	0.66 ± 0.07	0.99996
	960	0.70 ± 0.07	0.99397	0.06 ± 0.01	0.88 ± 0.09	0.99936*
Benzyl Acetate in 97.10 %RH hair	5	0.29 ± 0.03	0.99947	0.05 ± 0.01	0.32 ± 0.03	0.99993
	90	0.28 ± 0.03	0.99913	0.03 ± 0.00	0.30 ± 0.03	0.99973
	180	0.28 ± 0.03	0.99933	0.04 ± 0.00	0.31 ± 0.03	0.99979
	960	0.35 ± 0.04	0.99553	0.03 ± 0.00	0.41 ± 0.04	0.99951

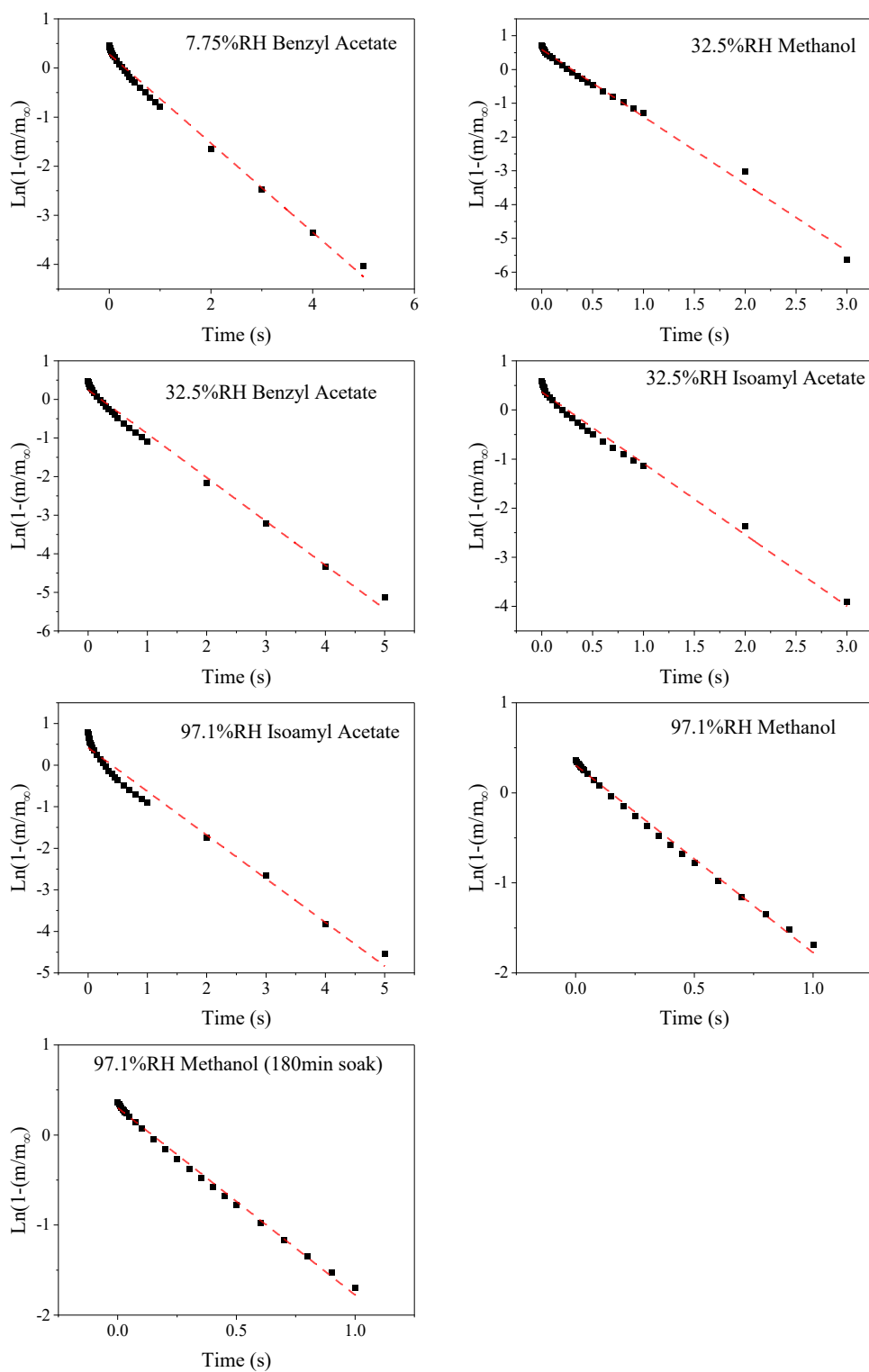


Figure 4.5 Linearized graphs for the relaxation data of selected experiments for solvents in hair, demonstrating a linear fit cannot fully describe the relaxation process, even where R^2 values of a two-component fit is below 0.99950.

4.4 Discussion

4.4.1 Effect of Humidity on small molecule interactions

To examine the impact of hair moisture content on solvent interactions, hair was preconditioned to 0.00, 7.75, 32.70 and 97.10 %RH to give varying moisture contents. Hair was then saturated in either methanol, benzyl acetate or isoamyl acetate to examine the change in interactions with the hair fibre measured by the ^1H T_1 relaxation experiments.

Table 4.1 displays the $\Delta T_{1\text{H}}$ values for the solvents in hair conditioned to different %RH, calculated using Equations 3.4 and 3.5 from Chapter 3. Relative population sizes are also displayed, calculated using Equation 3.6.

Across all solvents and populations, the $\Delta T_{1\text{Hb}}$ values decrease as hair is exposed to increasing humidity's. This demonstrates that solvents can interact more strongly with hair which has a higher moisture content, likely due to the fibres more swollen state at higher %RH. It is important to note, that this is a highly complex system, and water in hair will be lost during the soaking of hair in solvent. It is hard to draw firm conclusions from this dynamic system, and the following discussion will therefore be limited to the evidence available.

For the bound population (Figure 4.6), all solvents experience similar reductions in ^1H T_1 times where hair has been conditioned ≥ 32.70 %RH, with $\Delta T_{1\text{Hb}}$ values being relatively unchanged. This suggests at higher moisture contents of the hair fibre, the additional swelling of hair does not have an impact on solvent penetration into the fibre. These trends are shown with a line to guide the eye.

Table 4.6 Values of ΔT_{1Hb} , ΔT_{1Hf} , and relative population sizes for CH₃ groups of compounds in hair conditioned to %RH, calculated using Equations 3.4, 3.5 and 3.6 (Methanol 97.10 %RH: error = 1 σ from n = 2, all other measurements have an estimated 10% error)

	Humidity Hair conditioned to (%RH)	ΔT_{1Hb}	ΔT_{1Hf}	Relative Population Size	
				Bound Population	Free Population
Methanol	0.00	0.17 $\pm$ 0.02	0.52 $\pm$ 0.05	0.38 $\pm$ 0.04	0.62 $\pm$ 0.06
	7.75	0.14 $\pm$ 0.01	0.44 $\pm$ 0.04	0.41 $\pm$ 0.04	0.59 $\pm$ 0.06
	32.70	0.01 $\pm$ 0.00	0.17 $\pm$ 0.02	0.20 $\pm$ 0.02	0.80 $\pm$ 0.08
	97.10	0.01 $\pm$ 0.00	0.17 $\pm$ 0.02	0.11 $\pm$ 0.03	0.86 $\pm$ 0.03
Benzyl Acetate	0.00	0.53 $\pm$ 0.05	1.47 $\pm$ 0.15	0.47 $\pm$ 0.05	0.53 $\pm$ 0.05
	7.75	0.07 $\pm$ 0.01	0.50 $\pm$ 0.05	0.17 $\pm$ 0.02	0.83 $\pm$ 0.08
	32.70	0.03 $\pm$ 0.00	0.38 $\pm$ 0.04	0.18 $\pm$ 0.02	0.82 $\pm$ 0.08
	97.10	0.02 $\pm$ 0.00	0.23 $\pm$ 0.02	0.16 $\pm$ 0.02	0.84 $\pm$ 0.08
Isoamyl Acetate	0.00	0.46 $\pm$ 0.05	1.15 $\pm$ 0.12	0.35 $\pm$ 0.04	0.65 $\pm$ 0.07
	7.75	0.53 $\pm$ 0.05	1.42 $\pm$ 0.14	0.74 $\pm$ 0.07	0.26 $\pm$ 0.03
	32.70	0.02 $\pm$ 0.00	0.33 $\pm$ 0.03	0.24 $\pm$ 0.02	0.76 $\pm$ 0.08
	97.10	0.02 $\pm$ 0.00	0.33 $\pm$ 0.03	0.30 $\pm$ 0.03	0.70 $\pm$ 0.07

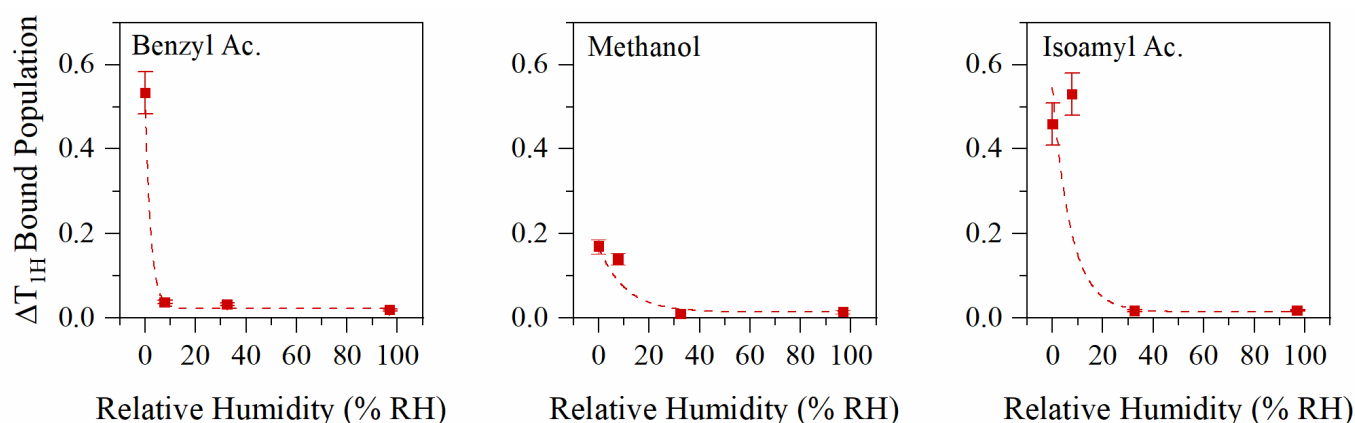


Figure 4.6 Changes in ΔT_{1H} for the bound population of solvent with increasing %RH conditioning. As the moisture content of the hair increases, the interactions between solvent and hair increases. Trendlines are demonstrated with a line to guide the eye. (Methanol 97.10 %RH : error = 1σ from $n = 2$, all other measurements have an estimated 10% error)

From the trendlines shown in Figure 4.6, there is a significant difference from the dry to 'wet' state for both acetates, suggesting that the pre-swelling of the fibre allows better penetration of the larger molecules, whereas methanol is not affected as strongly by the increased access to the fibre. As a swelling agent in hair, methanol is likely to be able to diffuse to sites for binding even in dry states, with limited benefits from any pre-swelling occurred through preconditioning.¹⁷⁰ In this work, a binding site is defined as a charged and/or hydrogen bonding amino acid side chain of amino acids such as lysine, arginine, serine, glutamine and cysteic acid.

For benzyl acetate (MW: 150.18, LogP: 1.93) this reduction in relaxation times occurs at lower moisture contents than for isoamyl acetate (MW:130.19, LogP: 2.21).

It has been recorded that a low %RH, hair water sorption is not proportional to the swelling of the fibre,¹⁶⁵ suggesting that when hair is conditioned to 7.75 %RH there is a higher water

content in comparison to the expected swelling in the fibre. This suggests that at this %RH the hydrophobic interactions have a greater impact on interactions with hair than the size of the compound, as seen in benzyl acetate having greater interactions than isoamyl acetate, as it is less hydrophobic while having a larger MW.

The changes in the free populations are greater (Figure 4.7), with ΔT_{1Hf} values decreasing by approximately 5 times for all solvents, demonstrating large increases in interactions as the moisture content of hair increases. The trends are similar for the bound populations, and likely due to the same reasons for changes in the bound population; swelling of the hair fibre allows conformational changes which affords access to binding sites not found in the hair's dry state.

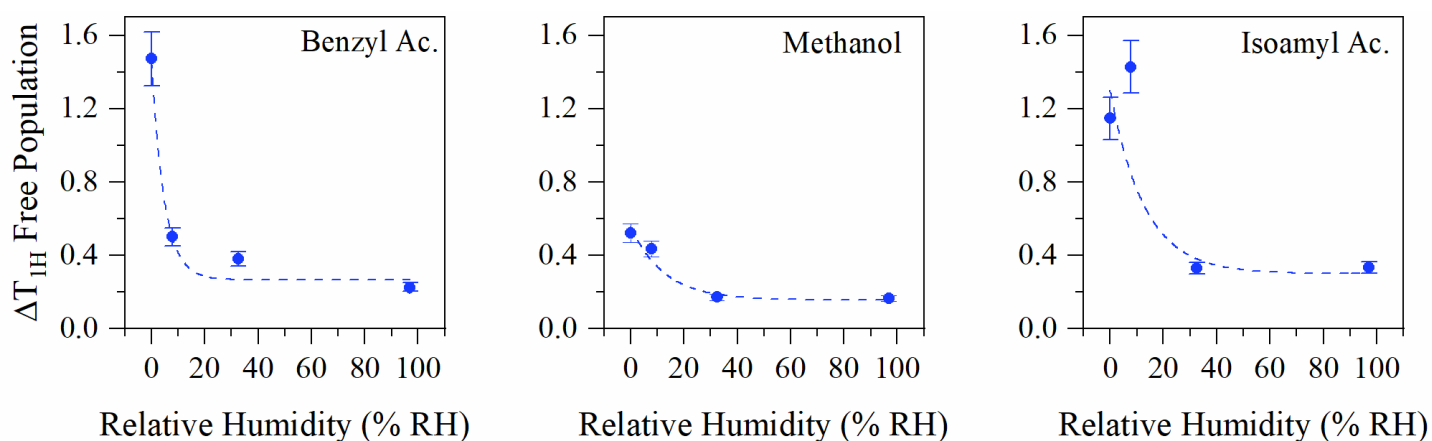


Figure 4.7 Changes in ΔT_{1Hf} for the free population of solvent with increasing %RH conditioning. As the moisture content of the hair increases, the interactions between solvent and hair increases. Trendlines are demonstrated with a line to guide the eye. (Methanol 97.10 %RH : error = 1σ from $n = 2$, all other measurements have an estimated 10% error)

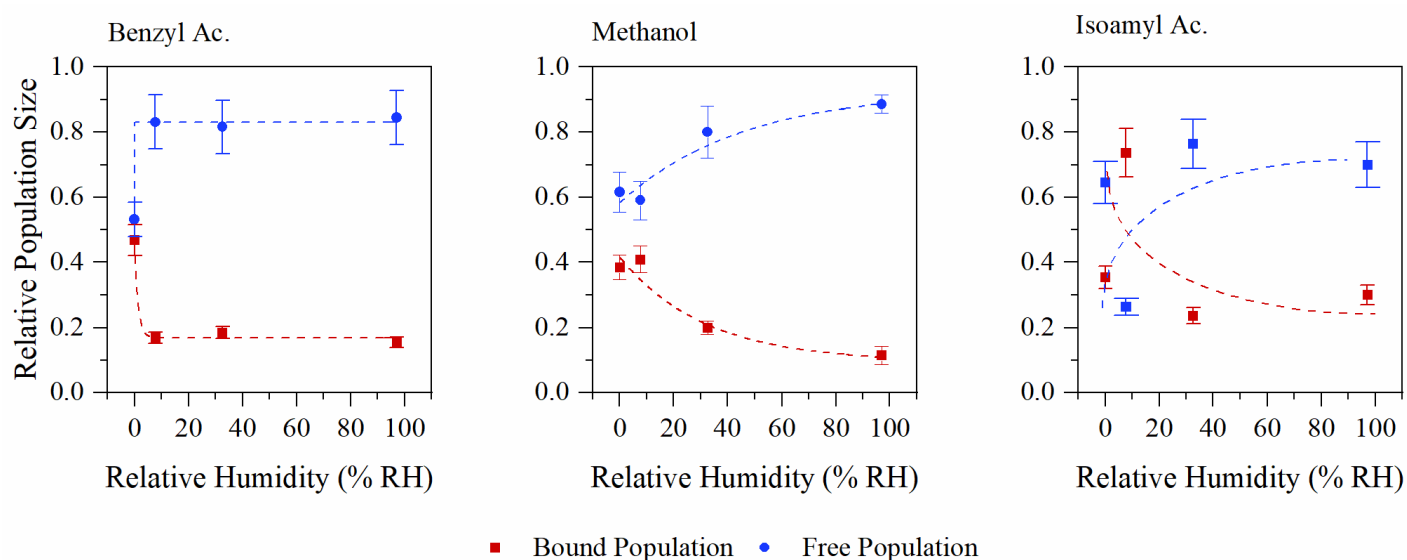


Figure 4.8 Changes in the relative population sizes for the free and bound populations of solvent in hair as moisture content in hair increases. Trendlines are demonstrated with a line to guide the eye. (Methanol 97.10 %RH: error = 1σ from $n = 2$, all other measurements have an estimated 10% error)

The changes in the bound and free relative population sizes are shown in Figure 4.8. For all solvents, as hair moisture levels increase, the bound population size decreases and the corresponding free population size increases. This is observed in benzyl acetate, where even hair conditioned to 7.75 %RH leads to a bound population size drop from 0.47 to 0.17.

The water content of hair allows better penetration of larger molecules to reach strong binding sites, leading to reduced T_1 times. However, the presence of water may strongly limit the total availability of these sites, whether that is through preferential binding of water, or the presence of water preventing benzyl acetate diffusing through certain pathways due to hydrophobic interactions. For isoamyl acetate, there is an increase in population size at 7.75 %RH, in contrary to what is seen in the other solvents. A wider solvent range would help

establish the cause of this increase, and confirm the trends observed. This increase may also be due to the difficulties in separation of the methyl solvent peak from other peaks in the spectra (Figure 4.3). A higher spinning frequency may resolve the peaks further in future experiments, or selective labelling of the isoamyl acetate may be necessary.

For methanol, there is also a reduction in the bound population which is more gradual than that found in benzyl acetate. Methanol may be more successful at competing with water for binding sites, but at 80 %RH all amino groups in hair are already saturated with water, leaving fewer binding sites, and therefore reducing the relative population size for the bound population of methanol.¹⁶⁵

In Chapter 3, it was noted that bound population contributions to the relaxation properties of a solvent in hair should be taken into consideration for smaller more hydrophilic molecules, however this preliminary work suggests that relative free population sizes remain relatively similar for all compounds as the moisture content of the fibre increases.

4.4.2 Time dependent saturation of solvent in humidified hair

4.4.2.1 Overall results

The last section discussed the swelling of the hair fibre at different %RH and how that may result in increased interactions for larger molecules. To determine whether the changes in interaction between solvent and hair were due to the swelling of the hair fibre at different humidity's, time resolved experiments were undertaken to measure the T_{1H} times of benzyl acetate and methanol at 7.75 and 97.10 %RH. The T_{1H} time of the CH₃ group of the solvent in hair was measured between 5 minutes and 16 hours to observe what change in interaction occurred. The further a molecule can penetrate hair (as allowed by its state of swelling) the

more reduced its relaxation times become over time - if the increase in interaction was indeed due to swelling of the fibre. Swelling mediated diffusion of the solvent into hair may therefore be seen in the changing T_{1H} times. Table 4.7 displays the ΔT_{1H} values for the solvents in hair conditioned to different %RH and measured at different soak times. These parameters were calculated using Equations 3.4 and 3.5 from Chapter 3. Relative population sizes are also displayed, calculated using Equation 3.6.

For brevity in this section, hair conditioned to 7.75 %RH will be abbreviated to '7.75 %RH hair', and hair conditioned to 97.10 %RH will be abbreviated to '97.10 %RH hair'.

Table 4.7 The ^1H T_1 times for CH_3 groups of solvents in hair soaked for different times. Solvents were fit to a one-component or two-component fit to reach $R^2 > 0.99950$, where $T_{1\text{Hb}}$ and $T_{1\text{Hf}}$ are the ^1H CH_3 T_1 times for the bound and free populations of solvent in hair. (Methanol 97.10 %RH, $t = 960$ min: error = 1σ from $n = 2$, all other measurements have an estimated 10% error)

	Soak Time (min)	$\Delta T_{1\text{Hb}}$	$\Delta T_{1\text{Hf}}$	Relative Population Size	
				Bound Population	Bound Population
Benzyl Acetate in 7.75 %RH hair	5	0.07 ± 0.01	0.35 ± 0.04	0.23 ± 0.02	0.77 ± 0.08
	10	0.06 ± 0.01	0.36 ± 0.04	0.12 ± 0.01	0.88 ± 0.09
	90	0.06 ± 0.01	0.36 ± 0.04	0.13 ± 0.01	0.87 ± 0.09
	450	0.06 ± 0.01	0.36 ± 0.04	0.13 ± 0.01	0.87 ± 0.09
	960	0.03 ± 0.00	0.48 ± 0.05	0.16 ± 0.02	0.84 ± 0.08
Benzyl Acetate in 97.10 %RH hair	5	0.03 ± 0.00	0.17 ± 0.02	0.08 ± 0.01	0.92 ± 0.09
	90	0.02 ± 0.00	0.16 ± 0.02	0.08 ± 0.01	0.92 ± 0.09
	180	0.02 ± 0.00	0.17 ± 0.02	0.08 ± 0.01	0.92 ± 0.09
	960	0.02 ± 0.00	0.23 ± 0.02	0.16 ± 0.02	0.84 ± 0.08
Methanol in 97.10 %RH hair	5	0.04 ± 0.00	0.19 ± 0.02	0.19 ± 0.02	0.81 ± 0.08
	180	0.02 ± 0.00	0.16 ± 0.02	0.10 ± 0.01	0.90 ± 0.09
	270	0.03 ± 0.00	0.17 ± 0.02	0.10 ± 0.01	0.90 ± 0.09
	960	0.01 ± 0.00	0.17 ± 0.02	0.11 ± 0.03	0.86 ± 0.03

4.4.2.2 Time dependent saturation of benzyl acetate at 7.75 and 97.10%RH hair

Initially, the ΔT_{1Hb} for the bound population at 7.75 %RH is higher than for the bound population in the equivalent experiment using 97.10 %RH hair (Figure 4.10). This is likely due to the greater swelling of 97.10 %RH hair which allows greater initial access to the hair fibre as previously discussed. Figure 4.6 shows the changes in ΔT_{1H} values and relative population sizes for the bound and free populations of benzyl acetate over time in 7.75 %RH hair. As mentioned previously, water molecules during low %RH conditioning steps will fill void spaces before hair swelling becomes significant.¹⁶⁵

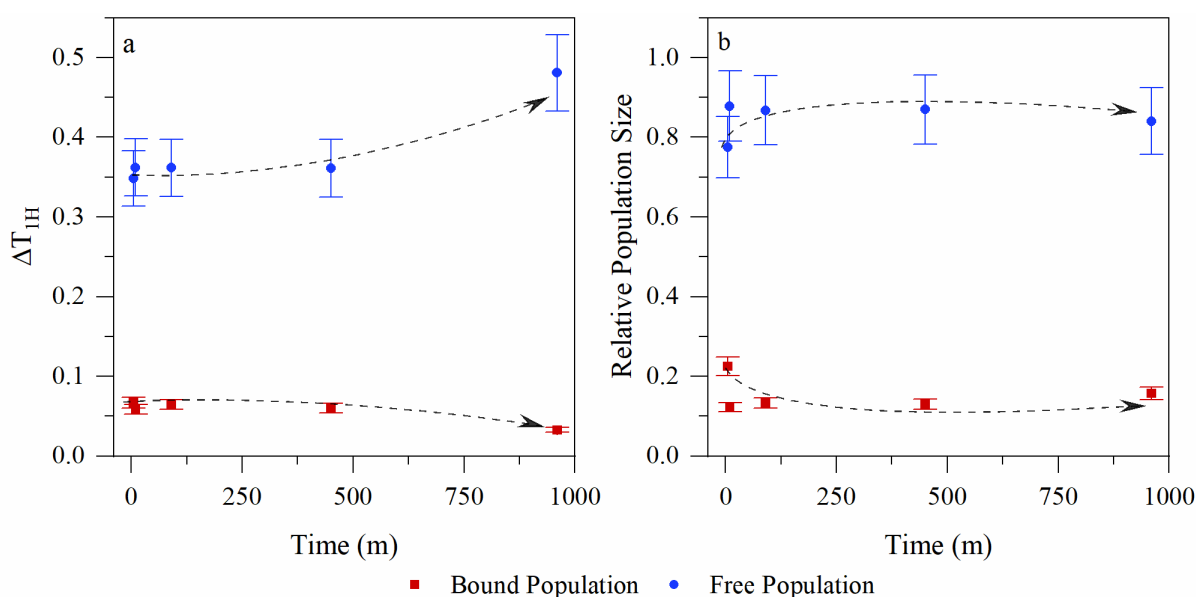


Figure 4.9 Changes in the a) ΔT_{1H} values and b) relative population sizes for the free and bound populations of **benzyl acetate** in hair conditioned to **7.75 %RH**, with increasing soak time. Trendlines are demonstrated with a line to guide the eye. (Measurements have an estimated 10% error).

However, in 7.75 %RH hair, the bound population increases its interaction with the hair fibre over time, whereas in 97.10 %RH hair, the bound population of benzyl acetate remains consistent. For 7.75 %RH hair there is less void space filling by water, allowing better diffusion of bound population, leading to reduced T_1 times over time. An explanation is that these void spaces are initially quickly filled, which is reflected in the increased relative population size of the free population after ten minutes (Figure 4.6), which then plateaus.

The increasing ΔT_{1Hf} values and decreasing ΔT_{1Hb} values while population sizes remain consistent, suggest that these populations are behaving independently to each other and may be found in different regions of the fibre, where the partitioning into each region remains consistent, but their individual mobilities undergo change.

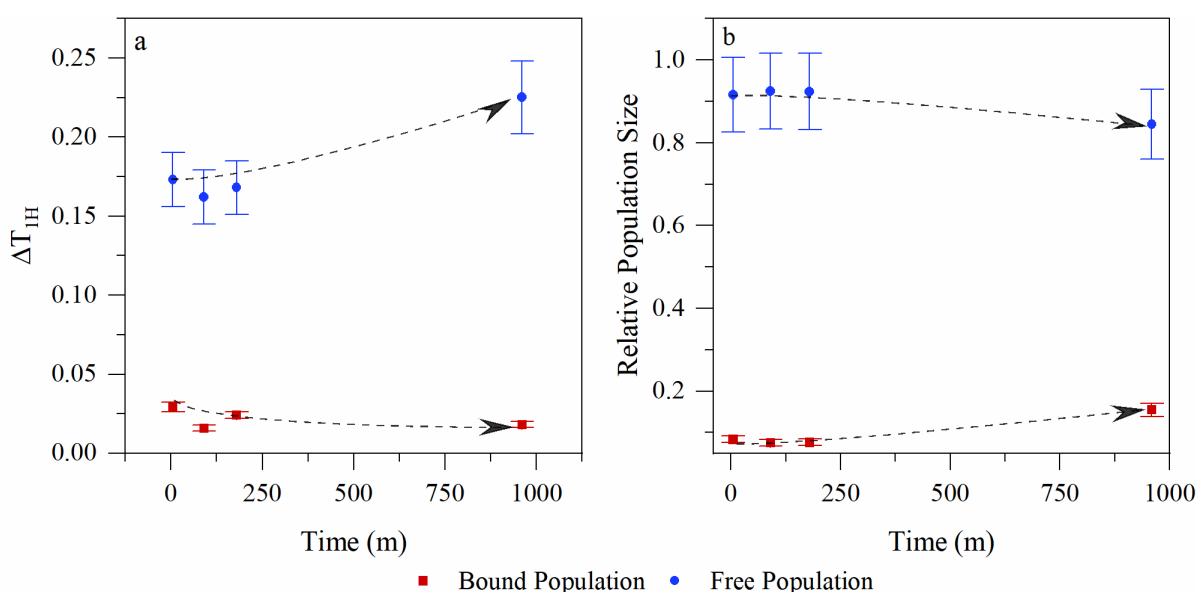


Figure 4.10 Changes in the a) ΔT_{1H} values and b) relative population sizes for the free and bound populations of **benzyl acetate** in hair conditioned to **97.10 %RH**, with increasing soak time. Trendlines are demonstrated with a line to guide the eye. (Measurements have an estimated 10% error).

In 97 %RH hair, the fibre has swelled enough to allow a strong interaction, but now all void spaces are likely filled. This results in no change to the ΔT_{1Hb} values of the bound population, as sites for stronger interactions are fully saturated by water. In contrast to the 7.75 %RH hair, the relative bound population size in 97.10 %RH hair increases over time, with the relative free population size subsequently decreasing (Figure 4.10). The implication of this is that over time, some of the free population migrate to stronger binding sites e.g., a charged and/or hydrogen bonding amino acid side chain of amino acids such as lysine, arginine, serine, glutamine and cysteic acid. This results in increasing the size of the bound population. These molecules which 'migrate' are likely to have had stronger interactions than the rest of the free population, perhaps residing in pores, resulting in their 'migration' or diffusion further into hair resulting in an average T_1 time increase for the rest of the free population.

This conclusion supports the hypothesis that it is the initial fibre swelling state which allows greater interactions. Conformational changes in the fibre, resulting from increased moisture content, allow populations of solvent in hair to 'migrate', whereas in drier hair samples, this process is not accessible.

4.4.2.3 Time dependent saturation of benzyl acetate and methanol at 97.10 %RH

In the previous section, there was evidence to suggest swelling and diffusion occurring in 97.10 %RH hair. To confirm, methanol, a smaller more hydrophilic solvent was also used in a time-resolved experiment with 97.10 %RH hair. The changes in ΔT_{1H} values and relative population sizes for the bound and free populations of methanol in 97.10 %RH hair are displayed in Figure 4.11.

For the free population, an equilibrium ΔT_{1Hf} is achieved after only 5 min of soak time, within error. For the bound population of methanol, the ΔT_{1Hb} values decrease slightly. While interactions with the bound population of methanol and 97.10 %RH hair increase, the relative population size decreases. As discussed previously, water will have fully saturated amino acid binding sites at this %RH,¹⁶⁵ therefore, the increase in the bound population interactions suggests successful competition is occurring between methanol for those amino acid sites.

As more methanol diffuses from the bulk solvent into the free population - which does not change in interaction strength over the course of the experiment – the relative free population size increases, leading to a decrease in the relative bound population size.

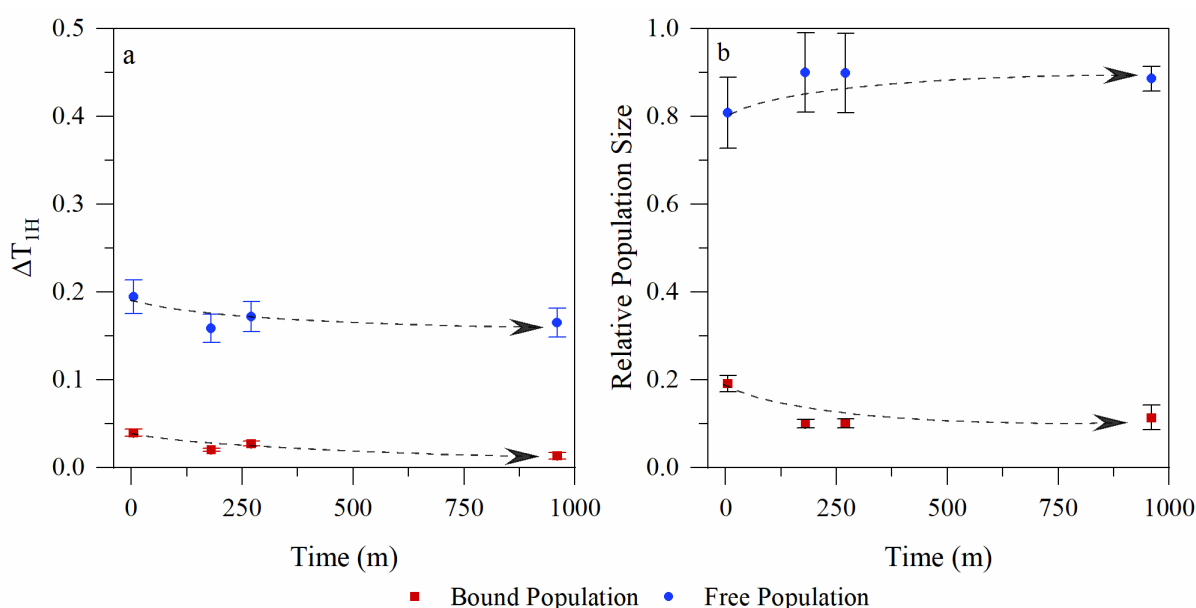


Figure 4.11 Changes in the a) ΔT_{1H} values and b) relative population sizes for the free and bound populations of *methanol* in hair conditioned to **97.10 %RH**, with increasing soak time. Trendlines are demonstrated with a line to guide the eye. ($t = 960$ min: error = 1σ from $n = 2$, all other measurements have an estimated 10% error).

Overall, this data suggests that there are two processes occurring. First, methanol in the bound population successfully competes with water at saturated amino acid binding sites. Secondly, more methanol is able to diffuse into the fibre increasing the size of the free population. This is contrasting to benzyl acetate in 97.10 %RH hair, where diffusion occurs from the free population to the bound population. Methanol is a solvent which extensively swells hair,¹⁷⁰ and this may be responsible for its apparent difference in diffusion behaviour.

4.5 Summary and conclusion

The effect of humidity on interactions between solvents and water is complex. In this investigation, the solvents investigated all showed an increase in interaction compared to hair which had been prepared at 0.00 %RH. The increase in interaction suggested that the swelling of the hair fibre allowed greater penetration of larger solvents to active sites in hair for binding and increased interactions. At low %RH hair, there is evidence to suggest that the LogP of a molecule may impact its interaction with hair as the fibre becomes more hydrophilic with more water present, but has not undertaken significant swelling.

To examine the effect of swelling, time dependent saturations of solvent in humidified hair was examined. As compounds diffuse into hair over time, interactions with hair increase. Comparing the changes in benzyl acetate mobility over time at 7.75 %RH and 97.10 %RH hair, demonstrated that diffusion between the free population to the bound population was possible at higher moisture content fibres, whereas low moisture content fibres have separated populations. This difference supports the hypothesis that swelling allows stronger interactions between small compounds and hair.

Methanol was also examined, which gave evidence to support that the swelling ability of methanol may be instrumental in its interactions with the hair fibre. This suggests that other compounds with the ability to swell hair may have strong interactions which may not be predicted by LogP and MW alone. Acetic acid was shown to be an outlier in the trends discussed in Chapter 3, and its swelling ability may also be the cause of this increased interaction.¹¹⁶

Clearly, this is a complex phenomenon, and a greater range of solvents and humidity's should be explored in future work. These initial results do suggest however that in more ambient conditions, the size of a molecule may have more impact on its interaction with the hair fibre than its LogP, with the swelling of the fibre being of great importance. Understanding these interactions is integral to predicting how small compounds will partition and interact with hair fibres.

Chapter 5 - Quantifying heat and pH

damage in keratin using ^1H spin-spin (T_2)

times of water and ^{13}C CP/MAS spectra

5.1 Introduction

The changes in structures of fibrous proteins such as keratin are challenging and difficult to characterise. Keratins are abundant in biology, as filaments providing structures in cells, and in the macromolecular in wool, nail, and hair.⁴ It is important to understand how keratin structures can be affected by damage, and understanding the extent of damage in keratinous materials is necessary to predict changes in structure and overall response to environmental and chemical stressors.

One of the major challenges in examining damage to keratins is their high complexity and heterogeneity. The main classical techniques to examine hair keratin can be put into two categories: single fibre analysis such as scanning electron microscopy (SEM) and X-ray diffraction,^{58,171} and destructive techniques which can be thermo-analytical or mechanical.^{80,172} Both have their pitfalls. Single fibre analysis does not account for the extreme heterogeneity between hair fibres, while destructive techniques – in their nature – prevent further analysis of the fibre. Chapter 1.5.3 goes into more detail on the current approaches to measuring and quantifying damage in hair.

Nuclear magnetic resonance (NMR) provides an array of sensitive and non-destructive techniques to probe hair keratin structure and interactions.^{66,67,78,161} While the static NMR spectra of keratins such as hair provide broad and often complex spectra, using Magic Angle Spinning (MAS) techniques has allowed many ¹³C Cross Polarisation/MAS spectra of keratins to be acquired, giving great insight into the amino acid content and the extent of order of keratin, notably the alpha helix, beta sheet and random coil conformations.^{65,69}

The carbonyl peak for keratin found at approximately 175 ppm, has often been used as an estimate of molecular order in hair samples.^{52,67,69,70} This broad peak can be broken down to two contributions from the downfield α -keratin/ordered region, and the upfield disordered region comprising of β -keratin and random coil amorphous contributions. This has been used extensively to examine the effect of pressure, heat, and dehydration in keratinous materials.^{52,67,69,70}

One particular advantage of NMR is the ability of the technique to analyse water in keratin, which like most proteins, is fundamental to its mechanical properties.^{67,75} The spin-spin (T_2) relaxation times of water in keratins have been used historically to examine the partitioning of water.⁷² A two-component system is seen in many biopolymers with both fast and slow relaxation components, representing a 'rigid lattice regime' and an 'motional regime'.⁶⁰ In keratins, this technique has mostly been utilised in wool, with ²H relaxation times being used to measure the distribution of D₂O in the fibre, identifying two morphological phases in the wool cortex.¹⁶² Other relaxation experiments have been used to examine the clustering effect of water molecules, and even the anisotropic nature of water in wool keratin, demonstrating the deep insight that these relaxation times can offer.^{62,161} In hair keratin, Gaussian

distributions of different populations of water have been identified, each with different spin-lattice (T_1) relaxation times.⁷⁴

The interactions between these water populations and their keratin environment underpins their relaxation times - which are strongly associated with the molecules mobility - resulting in the relaxation times being incredibly sensitive to changes in environment. This relationship offers the opportunity to measure how damage affects keratin structure through water mobility, providing a sensitive and non-destructive alternative to more traditional methods.

In the previous chapter, spin-lattice (T_1) times were used to measure changes in relaxation times for different molecules. However, when looking at the changes in mobility impacted by keratin damage on a single molecule, such as water, a method with higher sensitivity is needed. T_2 relaxation has a higher sensitivity to molecular mobility, meaning changes in the mobility of the water molecules will be clearly identified with high resolution measurements. Therefore, in this work the changes in mobility for water molecules in damaged hair samples have been quantified using ^1H spin-spin (T_2) times. Small changes in the environment as a result of keratin damage will be clearly identified by the changes in relaxation time for water in comparison to water in untreated samples. The T_2 times identified will give an average relaxation time for water in each sample, allowing a total average damage to the fibres to be assessed. This is particularly useful in hair analysis where damage to hair fibres can lead to a different level of damage across different regions of the fibre. Single fibre measurements may therefore be misleading if not enough hair samples are analysed.

The efficacy of this method to quantify damage was confirmed by measuring the relative content of α -keratin in the sample, which was ascertained using ^{13}C CP/MAS.

5.2 Methods

5.2.1 Preparation of damaged hair samples

As received untreated Caucasian hair (brown, batch source) was a gift provided by Procter & Gamble, and used in all experiments. For acid treatments (pH 1-6), hydrochloric acid solutions were prepared via serial dilutions from 1M HCl (aq) with Milli-Q water. Alkali solutions (pH 8-13) were prepared via serial dilutions with 1M NaOH (aq). Hair (0.08g, chopped to lengths of 0.5 cm) were soaked in the acid or alkali solutions (2 ml) for 24 hrs at room temperature. The solutions were neutralised with either dilute HCl (aq) or NaOH (aq), over a few hours until a stable pH 7 solution was achieved. This was to ensure that the hair samples were fully neutralised, as any charged species within the hair fibres would have a great impact on the experimental results. Neutralised samples were washed with Milli-Q water. For the samples damaged by heat, hair (cut to 3 cm lengths) was heated for 30 s at 50, 100, 150, 200, or 230°C, using a ghd - Original Styler Professional Ceramic hair straightener. Samples were then cut to lengths of 0.5 cm. All samples were incubated in excess water for 16 hours before ^1H T_2 measurements.

Samples were prepared in collaboration with Charlotte E Trott (MSci). NaOH and HCl were purchased from Sigma Aldrich. Buffered de-ionized water (Milli-Q) was used throughout.

5.2.2 ^1H T_2 Measurements of water in damaged hair

All NMR experiments were conducted using a solid-state Bruker 200 MHz NMR Spectrometer. The ^1H CPMG $90^\circ_x\text{-}\tau\text{-}180^\circ_y\text{-}\tau\text{-}$ sequence was conducted in a static environment with 32-time steps (4 scans each) with an echo time measured up to 10 s. Damp hair fibres were loaded axially in a 4 mm ceramic rotor.

^1H T_2 measurements were performed in collaboration with Charlotte E Trott (MSci).

5.2.3 ^{13}C Cross Polarisation/Magic Angle Spinning (CP/MAS) NMR acquisition

^{13}C CP/MAS NMR spectra were acquired with the minimum number of scans being 30,000 and a spinning frequency of ≈ 6 kHz. For these experiments, dry hair samples (0.08 g, chopped to lengths of 0.1 cm) were soaked in Milli-Q water for a few minutes before data acquisition, with damp fibres being loaded axially into 4 mm ceramic rotors. Previous experiments demonstrated no significant difference between wet and dry ^{13}C CP/MAS spectra of untreated hair samples, therefore the addition of water here to improve spinning of the samples was not expected to impact the spectra acquired.

5.3 ^1H T_2 measurements of water in damaged samples

5.3.1 ^1H T_2 data analysis

Spin-spin relaxation (T_2) is strongly governed by the rotational correlation time of the nuclei, and so informs on the relative mobility of nuclei in the sample.

The Carr-Purcell-Meiboom-Gill (CPMG) $90^\circ_x\text{-}\tau\text{-}180^\circ_y\text{-}\tau\text{-}$ experiment was used to measure the T_2 times of water in samples, with the phase coherence decay following the exponential relationship described in Equation 2.7. ^{126–128}

$$M_{x,y}(t) = M_{x,y}(0)e^{\frac{-t}{T_2}} \quad (2.7)$$

Where τ is the time step between NMR pulses.

In a complex polymer system, such as hair keratin, a multi-exponential can be used fit to different components, to identify different populations of substrate in hair. The efficacy of a multi-component fit has been questioned however, suggesting that multi-exponentials may reflect the heterogeneity of the fibre itself and not distinct populations of water.¹⁶² In this work, a two-component fit was used using Topspin 3.2 software, and also a Python program created in-house (Charlotte E Trott, MSci). Figure 5.1 demonstrates a fit using the aforementioned Python program, using Equation 3.3.

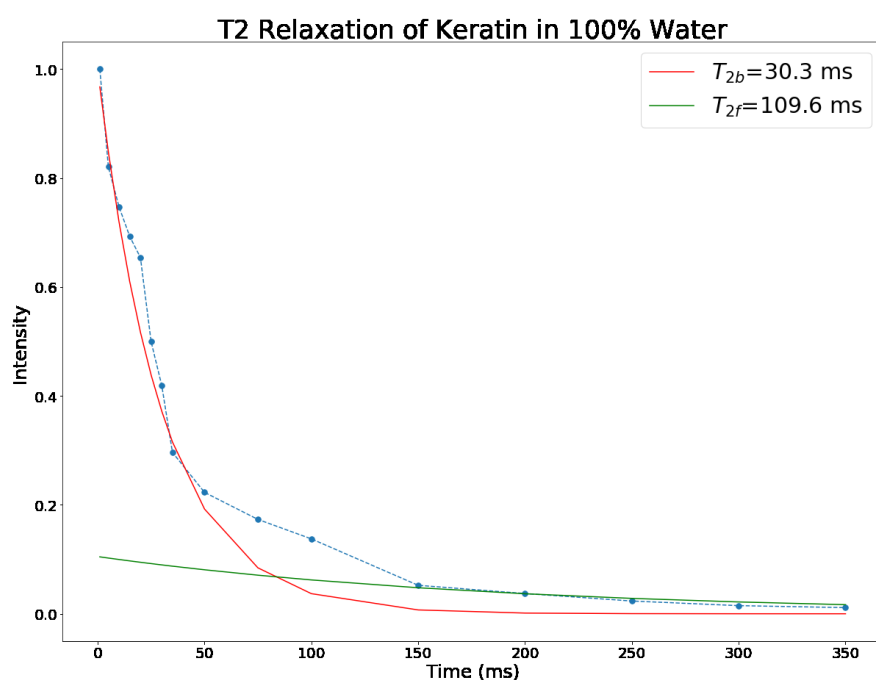


Figure 5.1 Fitting of T_2 relaxation of water in hair, achieved using an in-house Python program.

Fitting and figure provided by Charlotte E Trott (MSci).

Two ^1H T_2 times (T_{2H}) were identified for the water in hair, with both times shorter than that of bulk water. When small molecules such as water interact with hair, it can form hydrogen

bonds, electrostatic and dipole interactions. This results in reduction of the mobility of the water molecules, lowering the rotational correlation time, and leading to shorter T_2 relaxation times. Therefore, in the two-component fit, the longest component was assigned as the free population (T_{2Hf}), with fewer interactions with hair and high mobility, and the shorter component the bound population (T_{2Hb}), with greater interactions with hair and lower mobility. These values give an insight into the dynamics of water in the keratin system, helping explore how damage changes the keratin structure.

The T_2 times recorded represent an average time for each population. The dynamic changes between bound and free populations can be contrasted to the relaxation time of the bulk water solvent in the system, to examine how much the mobility of these molecules has changed. In this work, the relaxation values the T_{2Hb} and T_{2Hf} were converted into ratios (ΔT_{2Hb} and ΔT_{2Hf} respectively) to examine the extent of this change in mobility (Equation 5.1).

$$\frac{{}^1H\ T_{2H}\ of\ water\ in\ hair}{{}^1H\ T_2\ of\ bulk\ water} = \Delta T_{2H} \quad (5.1)$$

Low ΔT_{2Hb} values indicate a significant reduction in mobility between water molecules in the bulk water and those interacting with keratin. Given the exchange between water molecules in the free population and the bulk water, low ΔT_{2Hf} values show that more water molecules from the bulk water in the system are able to interact with keratin, giving the average population decreased mobility on the NMR timescale.

5.3.2 ^1H T_2 damage results

Heat was applied to hair at different temperatures to damage the keratin fibres to different degrees. Hair was also treated at different pH levels to examine the damage caused in both alkali and acidic environments. A sample for hair treated with pH 14 was not acquired, due to the complete destruction of the fibre at this high alkalinity.

Table 5.1 The values for ΔT_{2Hb} and ΔT_{2Hf} calculated for water in damaged hair samples (Error bars= 1σ from $n = 3$)

Treatment used to damage hair		ΔT_{2Hb}	ΔT_{2Hf}
pH	1	0.02 ± 0.00	0.27 ± 0.01
	2	0.02 ± 0.00	0.26 ± 0.03
	3	0.02 ± 0.00	0.34 ± 0.01
	4	0.01 ± 0.00	0.33 ± 0.01
	5	0.02 ± 0.00	0.41 ± 0.00
	6	0.02 ± 0.00	0.42 ± 0.01
	7	0.02 ± 0.00	0.41 ± 0.01
	8	0.02 ± 0.00	0.42 ± 0.02
	9	0.02 ± 0.00	0.41 ± 0.01
	10	0.02 ± 0.00	0.36 ± 0.03
	11	0.02 ± 0.00	0.30 ± 0.05
	12	0.02 ± 0.00	0.20 ± 0.01
	13	0.02 ± 0.00	0.17 ± 0.04
	14	0.02 ± 0.00	0.13 ± 0.00
Temperature ($^{\circ}\text{C}$)	25	0.02 ± 0.00	0.41 ± 0.01
	50	0.02 ± 0.00	0.38 ± 0.01
	100	0.02 ± 0.00	0.30 ± 0.00
	150	0.02 ± 0.00	0.24 ± 0.00
	200	0.02 ± 0.00	0.20 ± 0.00
	230	0.02 ± 0.00	0.17 ± 0.00

After damage was applied, the hair was soaked in water and the ^1H $T_{2\text{H}}$ times of the water was measured, and the corresponding $\Delta T_{2\text{H}}$ values calculated using Equation 5.1. These values are displayed in Table 5.1. It is clear in this table that all values for $\Delta T_{2\text{Hb}}$ are unchanging with different damage treatments. (Also seen in Figure 5.2).

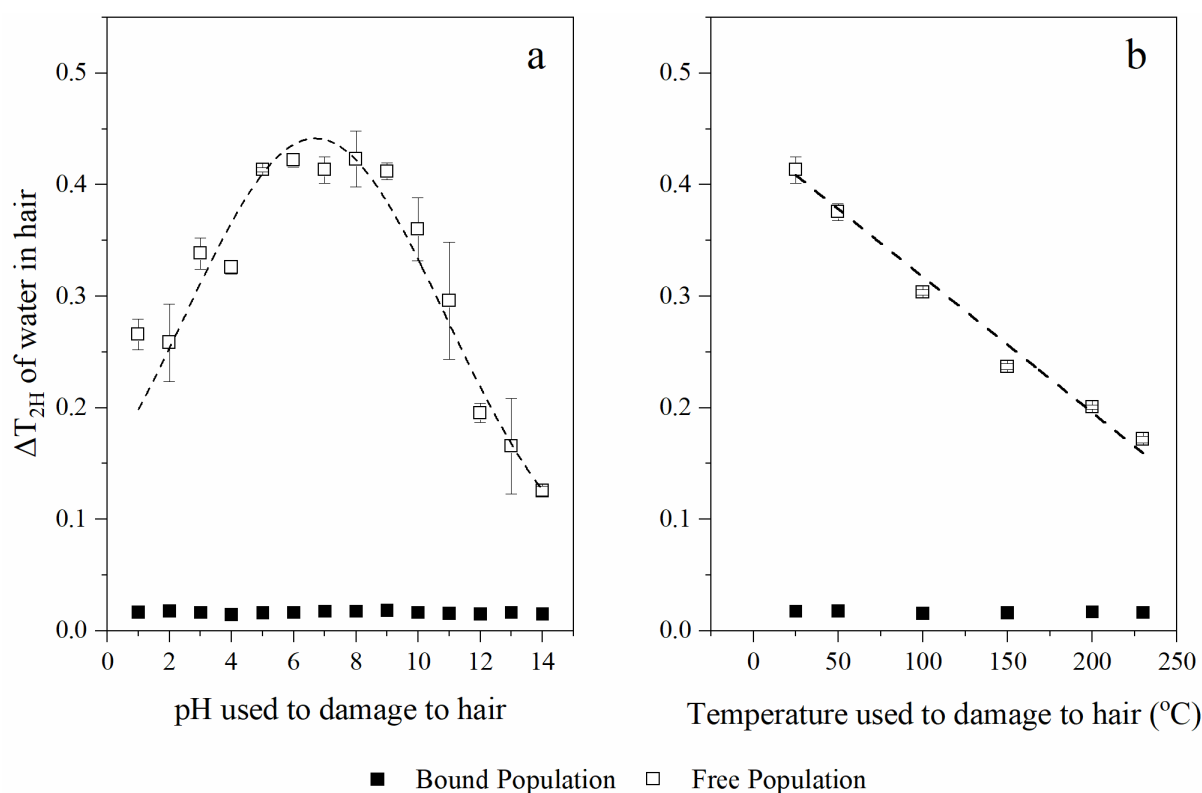


Figure 5.2 The $\Delta T_{2\text{Hb}}$ and $\Delta T_{2\text{Hf}}$ values for hair damaged using a) pH treatment and b) temperature. There is no significant change in $\Delta T_{2\text{Hb}}$ values of water in damaged hair, while $\Delta T_{2\text{Hf}}$ values show a gaussian relationship with pH damage, and a linear relationship to thermal damage. Trendlines given as a line to guide the eye. (Error bars= 1σ from $n = 3$)

This is understandable – damage causes the decomposition of the hair cortex, allowing the hair to swell further and for molecules to bind to active sites that may not have been accessible before. However, as T_2 times are an average of the population, more water molecules being able to access these sites for binding will not change the average T_2 time of this population. Examination of the free population show a wider change in ΔT_{2Hf} values and therefore are of better use to examine the effect of keratin damage on water.

5.3.2.1 pH damage

The ΔT_{2Hf} values for the free population of water in hair decreases with pH treatments between pH 5-1 (Figure 5.2a). Acid hydrolysis of keratins is thoroughly reported in literature, with irreversible changes in structure below pH 2.^{4,11,47} pH treatment between pH 4-6 has been shown to be optimal for cystine cleavage, and more acidic conditions associated with the complete destruction of tryptophan residues, and partial decomposition for threonine, tyrosine, phenylalanine, and arginine.⁴ This decomposition creates carboxylic acid and amino groups which can be used in hydrogen bonding between water and hair, reducing the ^1H T_2 times of the water molecules through reduced molecular rotation. Acid hydrolysis will also result in the hair fibres being able to swell more, with removal of proteins from the cortex-cortex CMC, allowing the fibre to take up increased water volumes, and have greater access to active sites for water than in the dry state.^{4,90}

The water molecules which have the highest mobility, and highest ΔT_{2Hf} values, lay in the range of pH 5 to pH 9. This demonstrates a relatively stable zone for keratin between this range. The knowledge of this stable pH zone is useful for formulating treatments for hair. From this work, a range of weakly acidic and alkali pH's can be used for chemical reactions without damage to the hair fibres.

Above pH 9, a reduction in ΔT_{2Hf} values is measured, associated with alkali damage. The decrease in values for the alkaline treatments is steeper than for that of acid treatment, suggesting that alkali treatment has a greater effect on hair than acidic treatments, even at both extremes in pH. Hair is known to be damaged in both high and low pH values, resulting in the hydrolysis of proteins, however, hair has been reported to be more sensitive to alkali treatment than acid.^{4,11,47} In alkali conditions lanthionyl residues are formed, there is hydrolysis of ester and thioester groups of the CMC and removal of 18-MEA from the hair surface. At high pH, cystine bonds are also broken as in acid hydrolysis, however peptide bond hydrolysis can also occur.⁴ This results in more extensive swelling of hair above pH 10, which can be observed in Figure 5.2a, with ΔT_{2Hf} for pH's above pH 10 being lower than hair treated to extreme acid conditions.

5.3.2.2 Temperature damage

When we examine the ΔT_{2Hf} values against increasing temperature, we see that the ΔT_{2Hf} values reduce (Figure 5.2b). As expected, when the hair fibres become more damaged, more water enters the fibres through increased swelling. Swelling allows greater penetration of water into keratin, the proximity effects of this diffusion reducing the water molecules mobility and therefore their T_{2Hf} times. This trend shows significant differences between each 50-30°C step suggesting that the sensitivity of this method is high, or that these increases in temperature cause substantial damage in the hair fibre. Damage to hair cuticle has been recorded with exposure to hair drying temperatures as low as 47°C, demonstrating that this method may be sensitive to changes in the cuticle, which is often only observed by using SEM methods.¹⁷³ At 90°C, the cuticle has been observed to be partially stripped, at 150°C melting of α -crystallites in hair is observed and at 210°C we would expect the onset of complete

degradation of the hair fibre.^{80,81,173,174} All these thermal events correlate to statistically significant decreases in ΔT_{2Hf} of water in damaged hair samples, further supporting the sensitivity and validity of this method to measure damage in hair keratin.

These results also show that some surprising conclusions can be drawn between the two damaged hair groups. When the results from both temperature and pH damage are compared, it can be shown that 30 s of exposure to 100 – 150°C on hair, has the same impact as 24 hr exposure to extreme acid treatment. 30 s exposure to 230°C is equivalent to 24 hr exposure to extreme alkali treatment. This indicates that even short exposure to heat can be incredibly damaging to hair. It may also be indicative of the differences in damage caused by different treatments, with the thermal treatments affecting the cuticle more directly. With the cuticle being the barrier into the hair fibre, it is understandable that direct damage via a hair straightening iron will result in great changes in the ability of the hair fibre to absorb and interact with water molecules.

5.4 Quantifying damage in keratin using ^{13}C CP/MAS spectra

5.4.1 ^{13}C CP/MAS spectra of damaged hair samples

The ^{13}C CP/MAS NMR spectra of thermally damaged samples were recorded. The ^{13}C CP/MAS spectra for untreated hair and hair treated to 230°C is shown in Figure 5.3. From the spectra, three main changes can be observed. First, the carbonyl peak at approximately 174 ppm (labelled a in Figure 5.3) becomes narrower, and the peak moves more downfield with increasing temperature.^{52,67,69,70} This is expanded in Figure 5.4 for better comparison, demonstrating the changes in the peak, as the doublet reduces to a singlet associated with more disordered keratin.

Another spectral feature is the peaks in both the α -C and carbons in the aliphatic regions of the amino acids becoming broader with increasing temperature. With degradation, the samples increase in amorphous content which results in the significant line-broadening of the ^{13}C NMR signals observed.⁶⁵ Finally, at point b (Figure 5.3) there is a reduction in the peak at 40 ppm, associated with the resonance for crosslinked for the β -CH₂ of crosslinked cystine,⁶⁷ indicating the breaking of disulphide bonds with increased temperature.⁵² Full peak assignment can be found in Table 5.2.

Table 5.2 Assignments of the chemical peaks in the ^{13}C CP/MAS spectra of different hair samples⁶⁵

Approximate chemical shift (δ ppm)	Assignment
11	δ -CH ₃ of isoleucine
15	β -CH ₃ of alanine (α h)
19	γ -CH ₃ of valine
24.5	δ -CH ₃ of leucine and δ -CH ₂ of proline
29	β -CH ₂ of glycine (α h), β -CH ₂ of trans proline
32	β -CH ₂ of cis proline, β -CH of valine (β s), β -CH ₂ of glutamic acid (β s)
35	β -CH ₂ of lysine (β s), β -CH ₂ of asparagine (α h)
39	β -CH ₂ of leucine (α h), β -CH ₂ of crosslinked cystine ⁶⁷
48	δ -CH ₂ of proline
53	α -CH of glutamine (β s) ⁵² , α -CH of lysine (β s), α -CH of alanine (α h)
56	α -CH of serine (β s), α -CH of leucine (α h)
60	α -CH of phenylalanine (α h), α -CH of tyrosine (α h), α -CH of proline
66	β -CH ₂ of serine (β s) and β -CH of threonine (β s)
120-130	Aromatic CHs of phenylalanine and tyrosine
158	O-C of tyrosine
172-176	Carbonyl for all amino acids

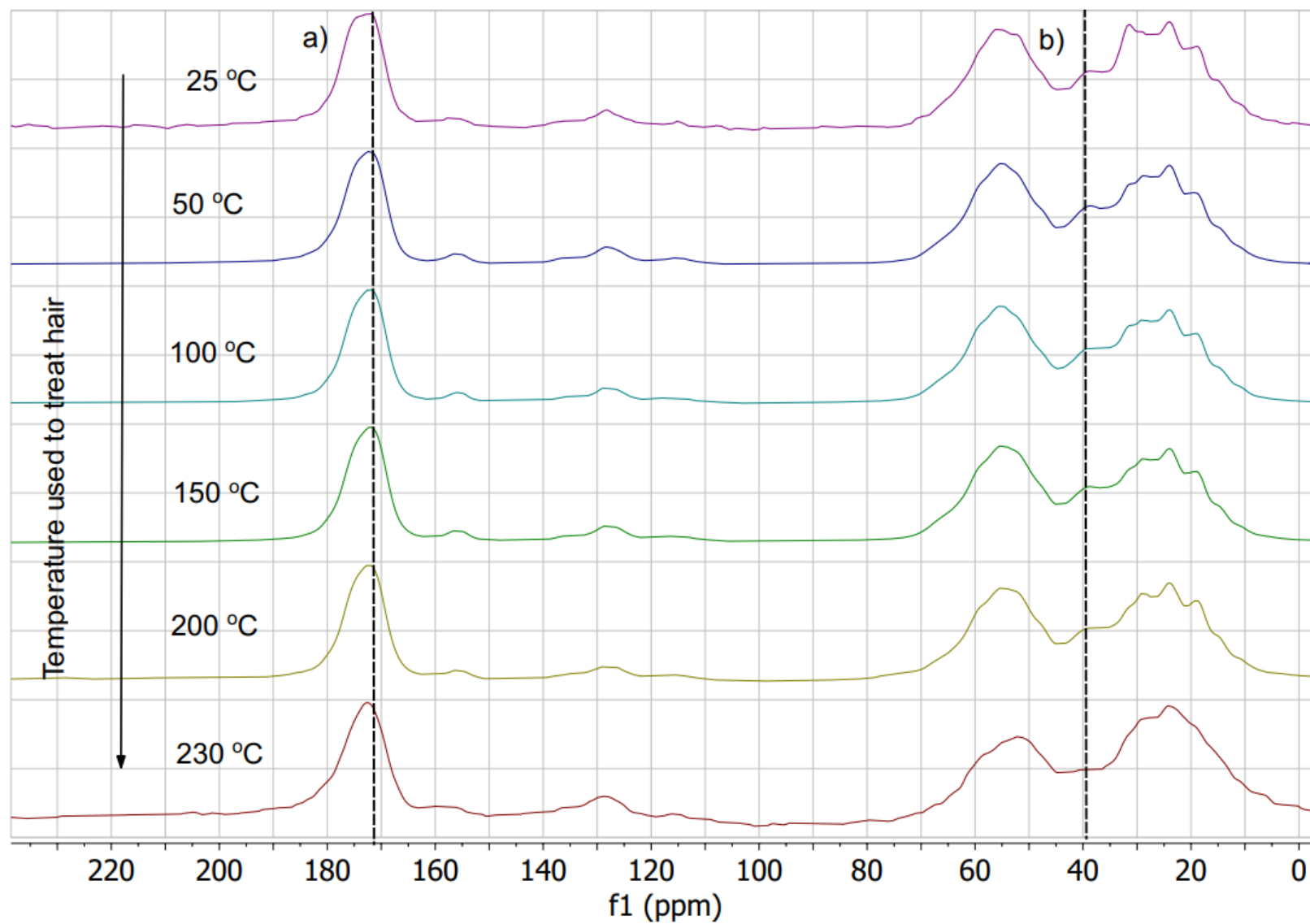


Figure 5.3 The ^{13}C CP/MAS spectra for hair samples treated with heat damage up to 230°C. Dotted lines show peaks at approximately a) 170 ppm for the carbonyls, and b) 40 ppm for $\beta\text{-CH}_2$ of crosslinked cystine.⁶⁷

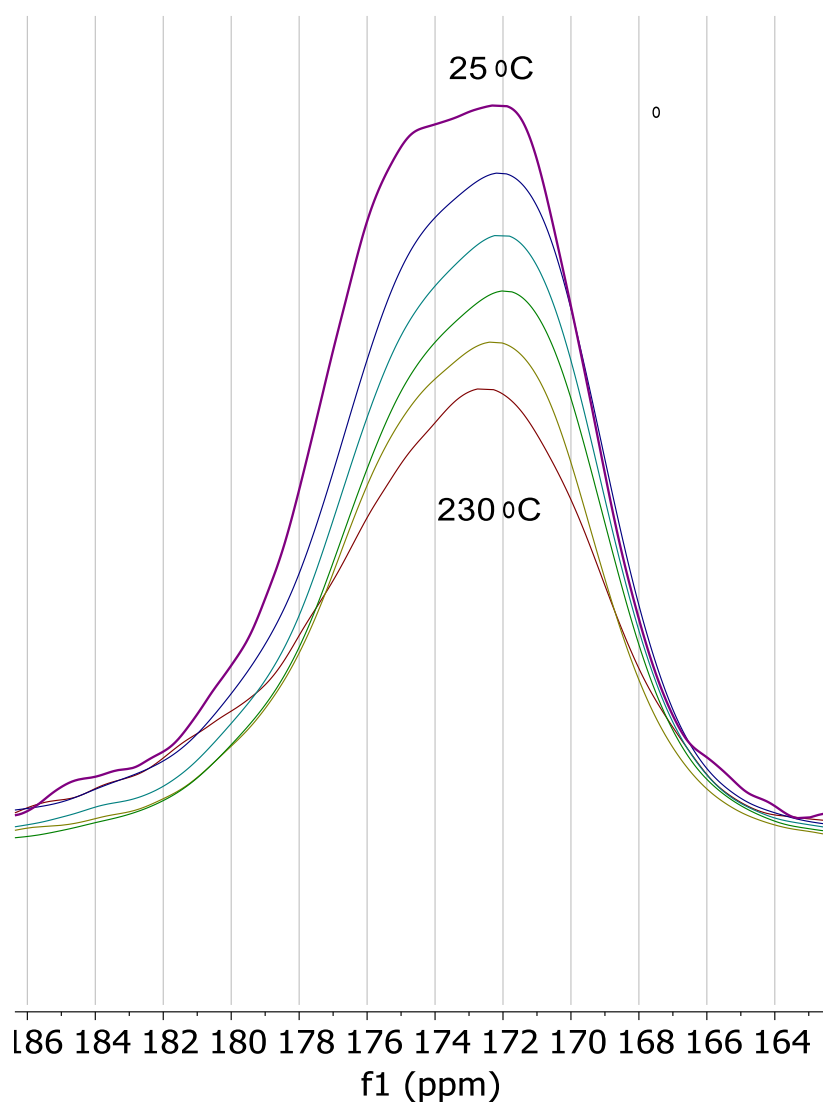


Figure 5.4 Expanded section of the carbonyl region of the spectra found in Figure 5.3 (spectra scaled for clarity). From top to bottom the temperature applied to hair increases, demonstrating the doublet found in spectra of untreated hair (25°C) reducing to a broader singlet in hair treated to 230°C. This is demonstrating a decrease in α -keratin content.

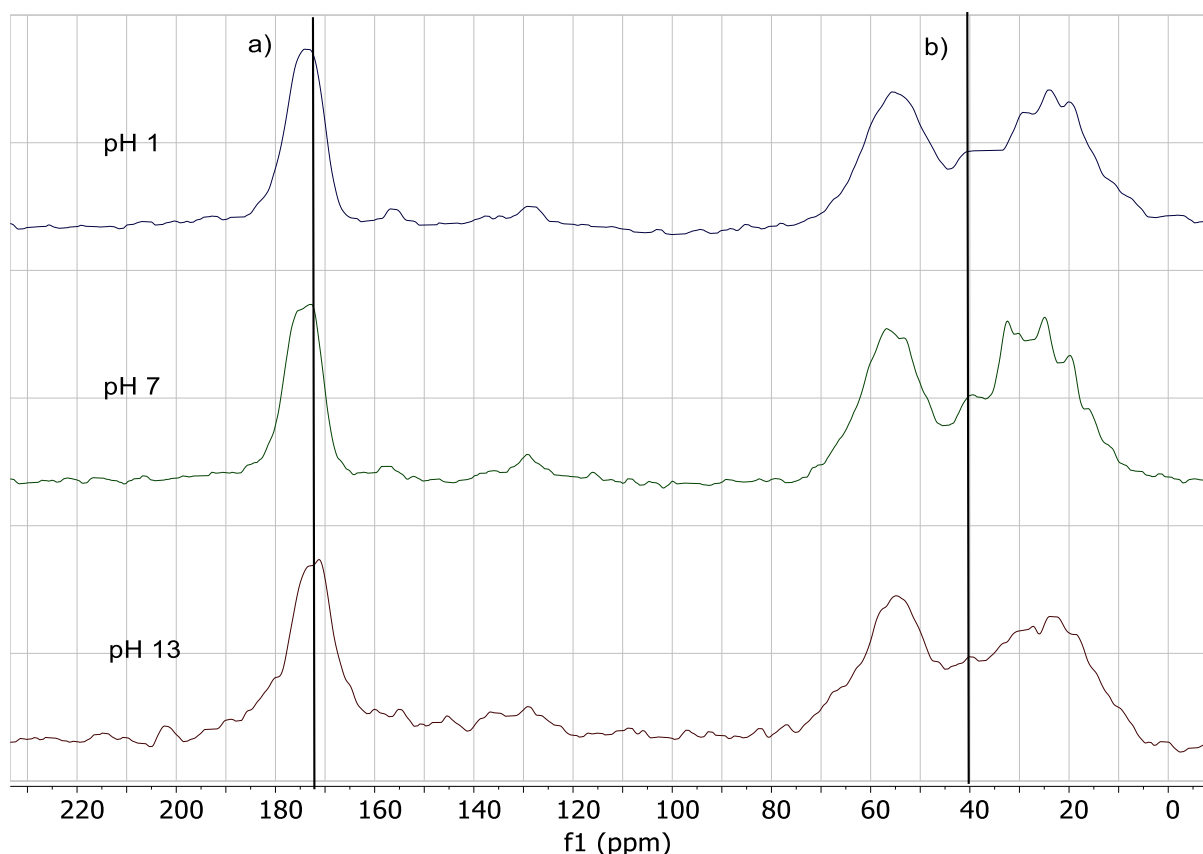


Figure 5.5 The ^{13}C CP/MAS spectra for hair samples treated with pH 1 and pH 13, with an untreated sample (pH 7) for reference. Dotted lines show peaks at approximately a) 170 ppm for the carbonyls, and b) 40 ppm for $\beta\text{-CH}_2$ of crosslinked cystine⁶⁷.

Two ^{13}C CP/MAS spectra were recorded for samples treated with pH 1 and pH 13 solutions (Figure 5.5). Similar to the thermally treated spectra, there is a change in the carbonyl region (Figure 5.5a) with narrowing of the peak in the pH 1 sample, and a sharp peak becoming observable in the pH 13 sample where the disordered keratin content is increasing. The peak representing the $\beta\text{-CH}_2$ of crosslinked cystine (Figure 5.5b) is significantly reduced in both damaged samples, indicating reduction of disulphide bonds in the samples. In the aliphatic regions for the damaged samples, there is suppression of signals, which could be resultant of line broadening, or from hydrolysis of amino acids via the pH treatments. A reduction of the

peak at 40 ppm indicates breaking of disulphide bonds, as would be expected in these treatments.⁵²

5.4.2 Relative α -Keratin content of samples

As previously described, the carbonyl region in the ^{13}C spectra can be analysed to provide an indication of α -keratin content of the samples.^{52,67,69,70} Damaged hair samples should show a decrease in α -keratin, as transitions via hydrolysis or temperature denaturation increase the content of coiled coil and β -sheet keratin.

To better quantify the damage in the treated samples, the carbonyl peaks for these samples were analysed using *Mestrenova 14.1.2* software to calculate the full width half maximum (FWHM) of both the peaks related to ordered and disordered keratin (Figure 5.6).

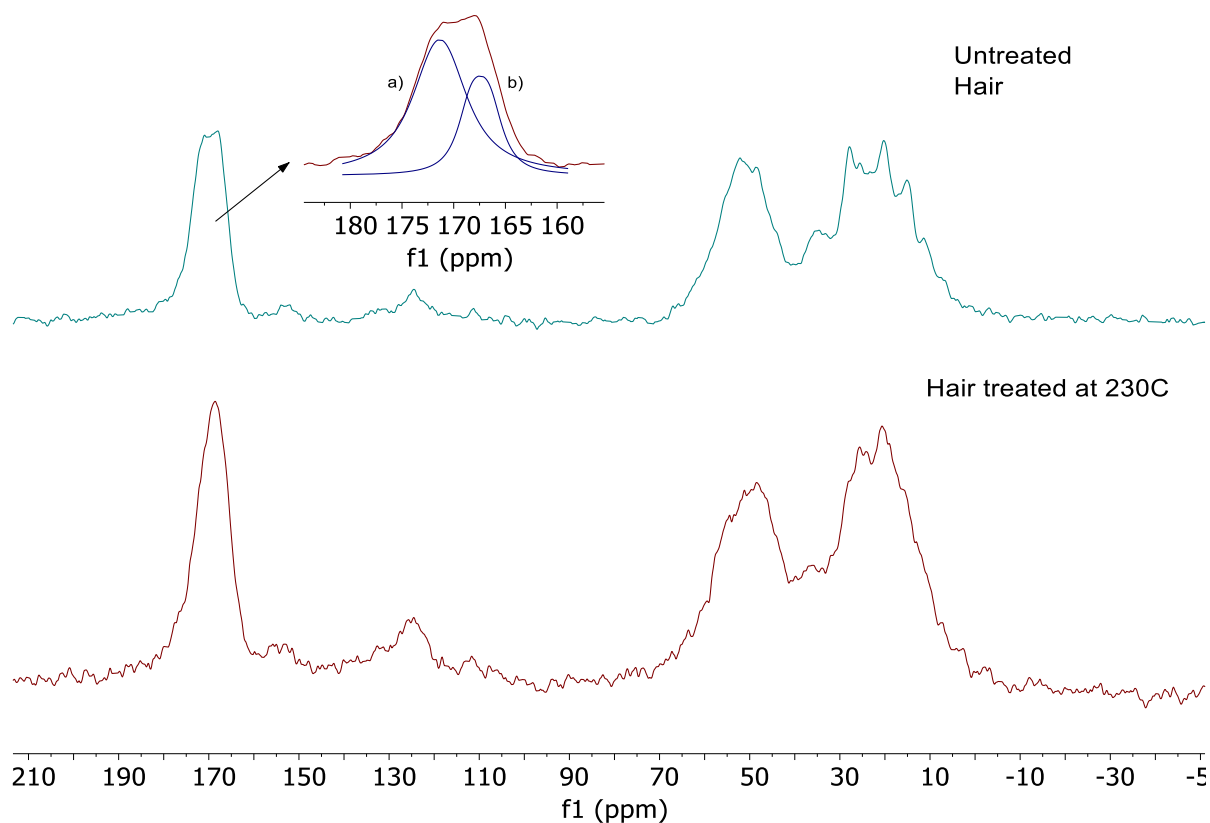


Figure 5.6 The ^{13}C CP/MAS NMR spectra for untreated, and hair treated to 230°C . Inset shows the line fitting for the untreated hair carbonyl peak, showing a) the ‘ordered’ peak and b) the ‘disordered’ peak.

To examine how these two components changed across all the experiments, a ratio was made between the FWHM of the ordered peak and the disordered peak. This ratio was called the relative α -keratin content and was used as a measure of order in the samples, and therefore can be used as a measure of damage, shown in Equation 5.2.

$$\frac{\text{FWHM of ordered peak}}{\text{FWHM of disordered peak}} = \text{Relative } \alpha - \text{Keratin Content} \quad (5.2)$$

Table 5.3 displays the Relative α -Keratin content for each sample analysed, the associated error results from three different analyses of the spectra, used to prevent any error occurring from manual phasing.

From Table 5.3 it can be seen that as heat treatment increases for different hair samples the relative α -keratin content decreases, demonstrating an increase in the disordered content in the hair fibre.

Table 5.3 The Relative α -Keratin content for certain damaged hair samples, calculated from the ^{13}C CP/MAS spectra. (Error= 1σ from $n = 3$).

Treatment used to damage hair		Relative α -Keratin content
Temperature ($^{\circ}\text{C}$)	25	1.27 ± 0.04
	50	1.17 ± 0.06
	100	1.10 ± 0.04
	150	1.04 ± 0.03
	200	0.95 ± 0.02
	230	0.90 ± 0.04
pH	1	1.04 ± 0.02
	13	0.90 ± 0.01

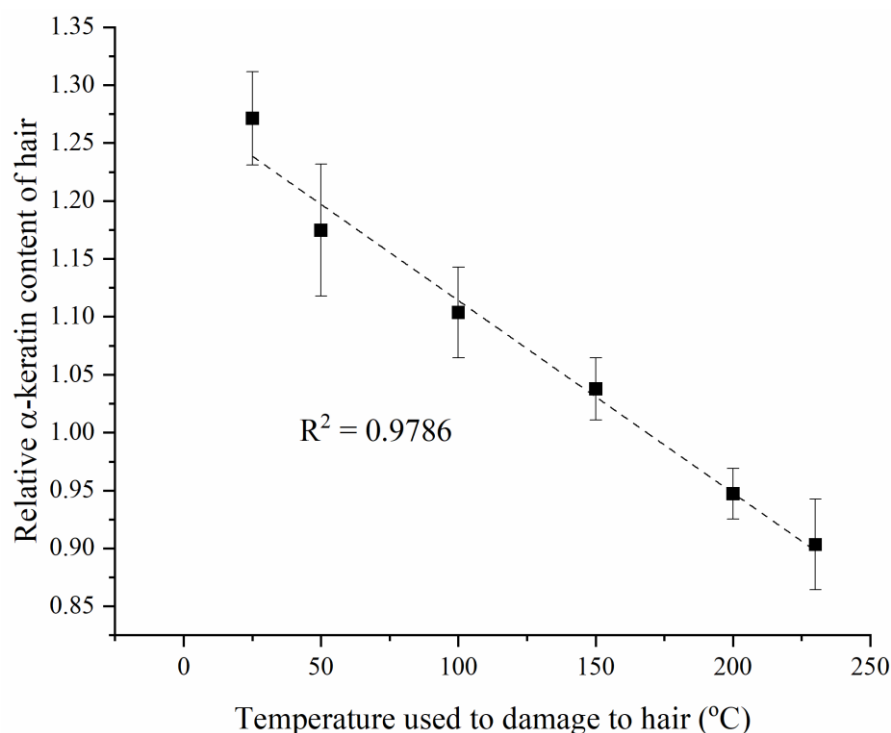


Figure 5.7 The Relative α -keratin content of hair samples damaged with heat, plotted against the temperature used to damage them, showing a strong negative correlation. (Error= 1σ from $n = 3$).

To determine if the relative α -keratin content was a suitable measure of damage, the calculated relative α -keratin content was plotted against the temperature used to damage the samples (Figure 5.7). A linear fit ($y = mx + c$) shows that there is a strong negative correlation, demonstrating as the temperature used to damage hair increases, the relative α -keratin content decreases. This confirms that the carbonyl peak of the ^{13}C NMR spectra can be successfully used to measure damage in samples.

While the effect of heat is well known to cause damage in the fibres, ^{4,11,80,86,96} this is the first time a correlation has been shown between heat damage and relative α -keratin content.

5.5 Correlating ^1H T_2 times of water in damaged hair with ^{13}C CP/MAS spectra

The $\Delta T_{2\text{Hb}}$ values of the damaged samples are unvarying (Table 5.1), so the ^1H $\Delta T_{2\text{Hf}}$ values of the damaged samples were compared to relative α -keratin content (Table 5.3). A positive correlation would confirm that the T_2 measurements of water in hair are a good measure of damage in keratin (Figure 5.8).

There is a strong positive correlation between $\Delta T_{2\text{Hf}}$ values of water and relative α -keratin content of the hair samples, this is reflected with a linear fit ($y = mx + c$) R^2 value of 0.9645. The more ordered the keratin is, the more mobile the water in the sample.

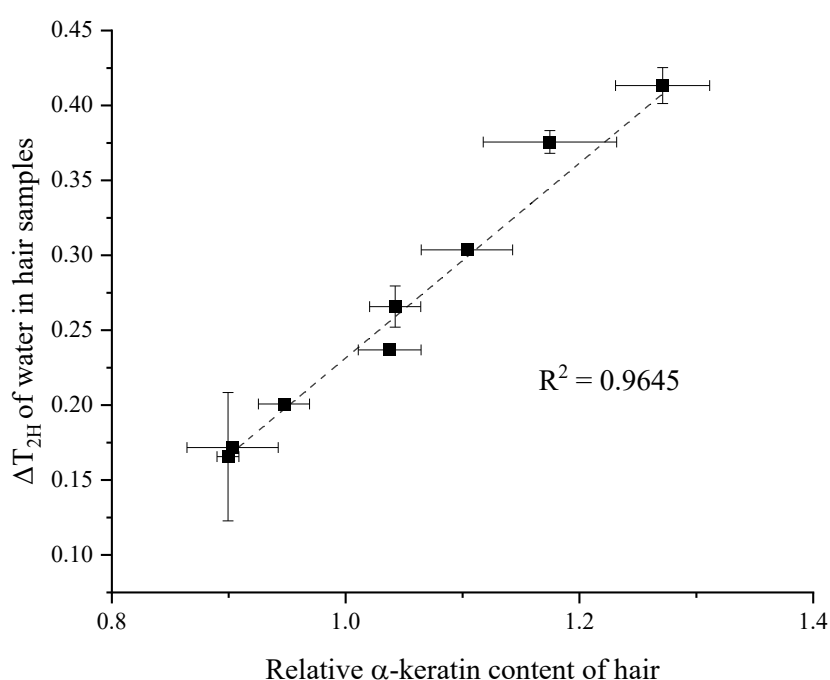


Figure 5.8 The positive correlation between $\Delta T_{2\text{Hf}}$ times of water in damaged hair, and the measured relative α -keratin content of the samples. (Error bars= 1σ from $n = 3$).

Conversely, the more damaged and disordered the sample becomes, the more the water molecules interact with the hair sample and reduce their rotational correlation times, leading to reduced ΔT_{2Hf} values. These results demonstrate that ΔT_{2Hf} values can be successfully used as a measure of damage in hair keratin, making this a straightforward non-destructive method to examine the extent of damage in keratin.

5.6 Conclusion

The sensitive relationship between the ^1H T_2 relaxation of water molecules and their keratin environment, allows the extent of damage in keratin to be probed through the mobility of water in the sample. This work demonstrates that ^1H T_2 relaxation times can be used to quantify damage in hair which has been treated to different thermal, acidic and alkali conditions.

Hair samples were damaged to different degrees using thermal and pH treatments. The ^1H T_2 times of water in these samples was measured to examine the mobility of the bound and free populations of water in the hair. The free population showed the greatest difference, with decreasing ΔT_{2H} values as hair became more damaged, demonstrating the reduction of mobility in the water molecules as they were able to access more binding sites.

The use of ^1H T_2 measurements has been shown to be a successful non-destructive method to measure damage in hair. This is further corroborated with ^{13}C CP/MAS data, successfully demonstrating the link between increased amorphous content in alpha keratin and the mobility of water molecules.

Chapter 6 - A new inverse liquid chromatography method to measure the real-time kinetics of small molecule dye desorption from keratin fibres

6.1 Introduction

6.1.1 High Performance Liquid Chromatography

Liquid chromatography methods using High Performance Liquid Chromatography apparatus have become a standard in the indirect measurements of many compounds in hair, including toxins, heavy metals, and drugs.^{175–179} These methods have also been used to measure the content of dyes in dyed hair fibres, with successful methods being achieved through extraction or measurement of aqueous washes of the dyed hair.^{180,181}

Whilst the measurement of total dye content in hair has been achieved, the use of liquid chromatography to directly measure the kinetics of loss for small compounds such as dye and alkalizers in hair has not been reported.

The kinetics of dye sorption has been thoroughly recorded in literature.^{119,182–188} The measurements of dye diffusing from a dye bath to hair has been achieved by measuring the

dye bath spectrophotometrically; as the concentration of the dyebath decreases, the concentration of dye in hair must therefore increase.¹⁸³

This indirect method has been used to determine the sorption behaviours of not only dye molecules, but other species including metal ions and surfactants into different classes of keratins such as wool and human hair.^{184,189,190}

Dye desorption kinetics has been investigated very rarely, despite its great importance to industry and research. Visual colour graded ratings are used widely in industry, while more quantitative spectrophotometric measurements have been taken of dye molecules diffusing from cotton and chitosan to desorption solutions to ascertain dye loss kinetics.^{187,191–193}

These static, and often indirect measurements of dye loss from materials, do not accurately reflect the real-world scenarios of dye loss through the washing of hair.¹⁸⁴

6.1.2 Hair Dye formulations

Dyeing hair requires a complex product formulation containing a range of chemical species. Understanding the loss and retention of this range of chemicals in the formulation by hair is vital to comprehend their impact on the hair fibre. Permanent hair dye formulations contain three key components: dye precursors, oxidants and alkalisers. Common dye precursors - such as *p*-diamines and *p*-aminophenol's - undergo radical reactions with electron-rich aromatic dye couplers in hair to form the final dye compounds.¹⁹⁴ Hydrogen peroxide (H₂O₂) plays an important role in decolourising the melanin pigment to lighten hair,^{49,95} and by bleaching pre-existing synthetic hair colour.^{4,89} The hydrogen peroxide also acts as an oxidant in radical reactions between dye precursors and electron-rich aromatic dye couplers in hair to form the final dye compounds.^{89,194} The final component of hair dyes are alkalisers. Alkalisising agents

such as ammonium hydroxide (NH_4OH) and monoethanolamine (MEA) are necessary to keep the alkalinity at a suitable range (pH 8-10) to sustain these radical reactions to form the dye compounds.^{6,194} The increase in pH also promotes hair swelling, which results in the lifting of cuticle scales to aid diffusion of dyestuffs into the fibre.⁴

6.1.3 Damage from dye formulations

Section 1.5.1 described in detail damage that occurs to a hair fibre resulting from cosmetic treatments. In summary, the major source of damage from the hair dyeing process is the reduction of disulphide bonds by H_2O_2 .^{94,95} The reduction of these integral disulphide bonds reduces the α h keratin melting point, which leads to the mechanical weakening of the fibre and decreased breaking stress.^{4,40,80,89} Further to this, damage from H_2O_2 has also been shown to cause increased protein loss from the fibre and cuticle damage.^{103,195,196}

The presence of alkalisers has been suggested to disrupt electrostatic bonding between KIF's, resulting in further damage to hair.^{100,197} MEA in particular has been shown to reduce the breaking stress and elastic modulus of hair, and while ammonia causes less damage, both result in significant protein loss in comparison to untreated hair at 50°C.⁹⁷ The impact of alkalisers in reducing the α h keratin melting point and increasing the mechanical weakening of the fibres, was found to be reversible with dialysis in deionised water.¹⁰⁰ It was also found that the properties of untreated hair could be recovered, suggesting that the effect of these ions in hair may be more important to overall hair structure than the destruction of disulphide bonds.^{100,197}

6.1.4 A Novel HPLC method to measure dye desorption

The complex behaviour of small molecule desorption from hair and the effects of fibre structure on these processes needs to be investigated, however there is no standardised method to measure the loss of small chemical actives from human hair in a direct real-time method. This current work addresses this problem with a novel inverse liquid chromatography method to directly measure the loss of a dye and MEA from a column packed with coloured hair. The use of this new HPLC method includes the following benefits:

- a) a consistent flow of DI water to remove dye from hair, mimicking 'real life' washing scenarios. The dialysis method used by Marsh *et al.* was static and took many days,¹⁰⁰ where this method reduces the diffusional boundary surrounding the hair fibre to prevent suppression of diffusion stages from the inside of the fibre.^{182,183}
- b) direct and automated UV measurements of the eluent water allows constant monitoring of dye loss which results in an incredibly detailed account of the total dye desorption process.
- c) a reliable temperature-controlled environment for the experiments to take place in, allowing further analysis of kinetics.

Untreated hair was treated with a formulation containing MEA, 4-amino-2-hydroxytoluene (AHT) and 4,5-diamino-1-(2-hydroxyethyl)pyrazole (HDAP) to form a red dye product AHT-HDAP through oxidative coupling *in situ* (Figure 6.1).

The loss of AHT-HDAP and MEA in deionised water was measured at 25, 30, 40, 50 and 60°C. The effective diffusion constants of desorption were calculated, as well as pseudo-first and second order rate kinetics.

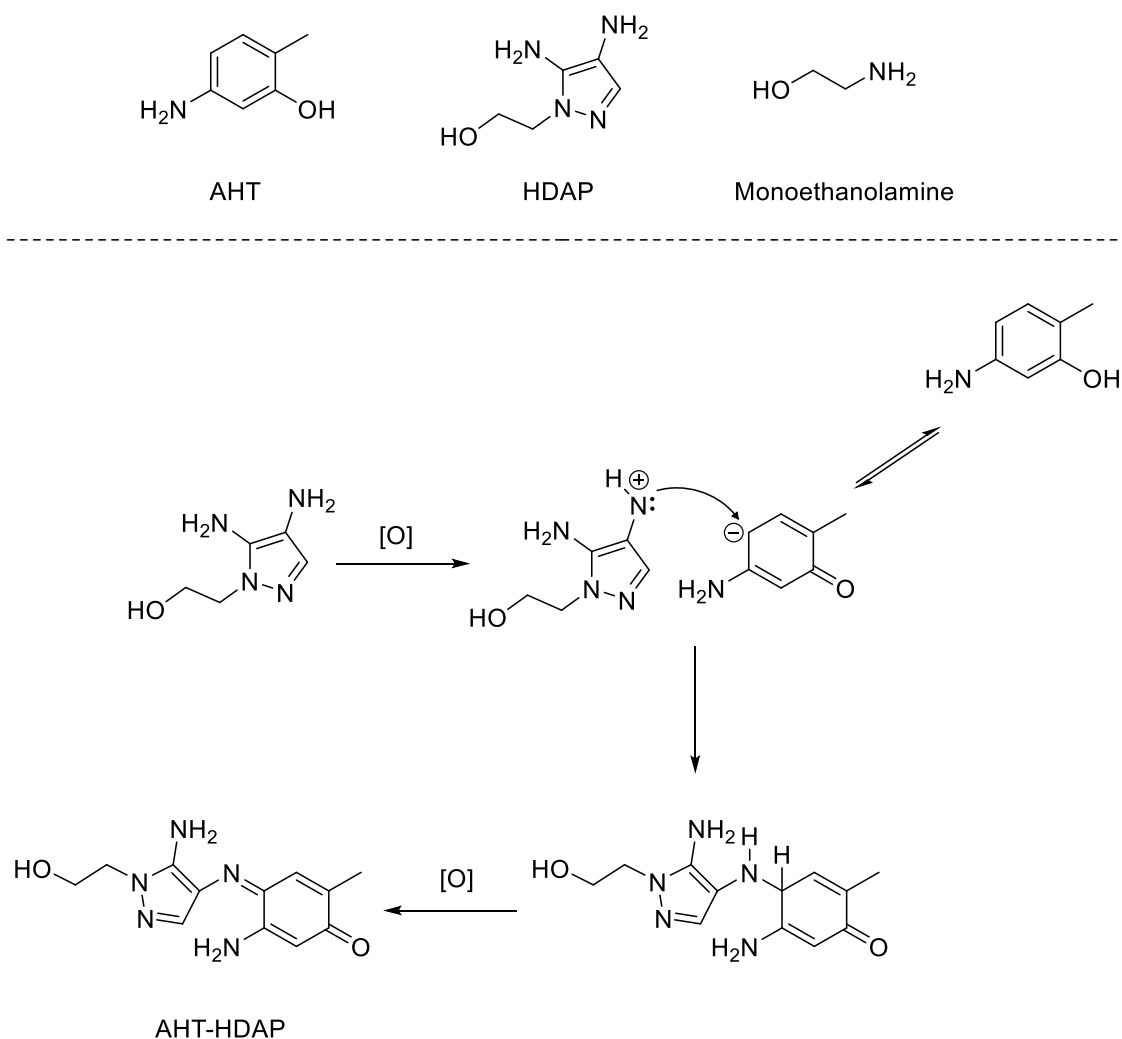


Figure 6.1 The structures of 4-amino-2-hydroxytoluene (AHT) and 4,5-diamino-1-(2-hydroxyethyl)pyrazole (HDAP), and monoethanolamine (MEA), showing the oxidative coupling reaction between AHT and HDAP to form AHT-HDAP dye, with H_2O_2 acting as the oxidant.

The method is a direct and reliable alternative to other liquid state desorption measurement techniques, with a wide range of applications to other materials to increase the understanding of not only dye processes but other small molecule interactions with hair.

6.2 Methods and Theory

6.2.1 Preparation of dyed hair

Untreated Caucasian hair (natural white, batch source) was provided by Procter & Gamble as a gift. A hair dye solution was formulated using hydrogen peroxide, monoethanolamine, 4-amino-2-hydroxytoluene (AHT) and 4,5-diamino-1-(2-hydroxyethyl)pyrazole (HDAP) using a formula provided by Procter & Gamble. Dye structures can vary greatly, as can their reactions. AHT-HDAP was therefore used as a relatively simple structured dimer, whilst also being a non-reactive dye, simplifying its interactions with hair.¹⁹⁴ 4 g swatches of untreated hair were dyed with the dye formulation for 30 min with frequent manual stirring. Hair was thoroughly rinsed for 1 min in cold tap water and combed to remove any tangles, before allowing to air dry at room temperature. H₂O₂ was purchased from VWR International Ltd, AHT and MEA from TCI Europe, and HDAP purchased from Fluorochem Ltd.

6.2.2 Inverse Liquid Chromatography Experiments

Dyed hair was coiled to resemble a yarn, and threaded axially through a standard steel ¼" internal diameter, 2" long HPLC column. The ends of the hair were then cut, resulting in a column containing, at its centre, approximately 0.15 g of dyed hair with a length of ≈ 2" (Figure 6.2).

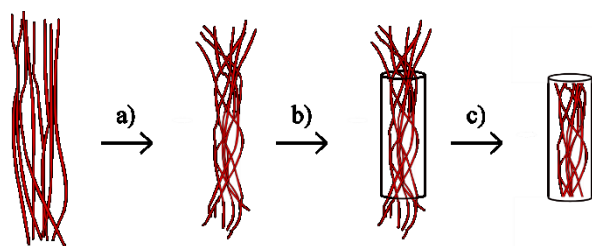


Figure 6.2 Schematic depicting the packing of dyed hair into a HPLC Column: a) hair is coiled to form a yarn, b) the yarn is threaded through the column, and c) extruding ends are cut off.

Experiments were performed using the Shimadzu HPLC Prominence System. DI water was used as the desorption medium, being run continuously through the vertical column at 2 ml/min for 2 hours at a controlled temperature of 25, 30, 40, 50 or 60°C. A pair of SPD-20A UV-VIS detectors were configured before and after the HPLC column,¹³¹ to measure the main absorption peak of AHT-HDAP dye at 477 nm (Figure 6.3). As MEA does not absorb in the UV, a different method was required for its detection. A GC-MS method described by Ali *et al.* was attempted but due to the low concentrations of MEA expected to diffuse from the hair fibres, it was found to be unsuccessful.⁹⁷ As an alkaliser, the change in pH was used instead as an indication of MEA loss. To measure the change in pH, water eluting from the column was fractionated at set times for further analysis. pH measurements were made using a pH meter (Hanna). As MEA is a weak base, the Brønsted-Lowry theory can be used to calculate the approximate MEA concentration. First the concentration of hydroxide ions can be calculated using the recorded pH in Equation 6.1:

$$[OH^-] = 10^{-(14-pH)} \quad (6.1)$$

With the value of $[OH^-]$ calculated, the concentration of MEA can be estimated using the Equation 6.2, where C_b is the concentration of MEA base, and K_b is the basic dissociation constant:

$$C_b = [OH^-]^2 / K_b \quad (6.2)$$

To confirm that the pH changes were resultant of MEA loss, control experiments with untreated hair were also performed (See Appendix 10.3).

All experiments were performed on a dry column e.g., hair was packed dry, which allowed the loss of dye and MEA to be measured from $t = 0$ by eluting water through the column. The dead volume times of the columns varied due to small variations in hair fibre packing volumes. The dead volume time was assigned when the first UV signal in detector B (Figure 6.3) was recorded. The average dead volume time was recorded as 1.53 ± 0.44 min with a median time of 1.48 min.

The amount of AHT-HDAP dye and MEA (mg) diffusing from hair sample were subsequently calculated from the UV and pH measurements (See Section 6.3.1.1) and finally normalised to the mass of hair present in the column (mg/g). This guaranteed that the influence of column packing variability was minimal, while allowing the repeatability of the experiments to be examined.

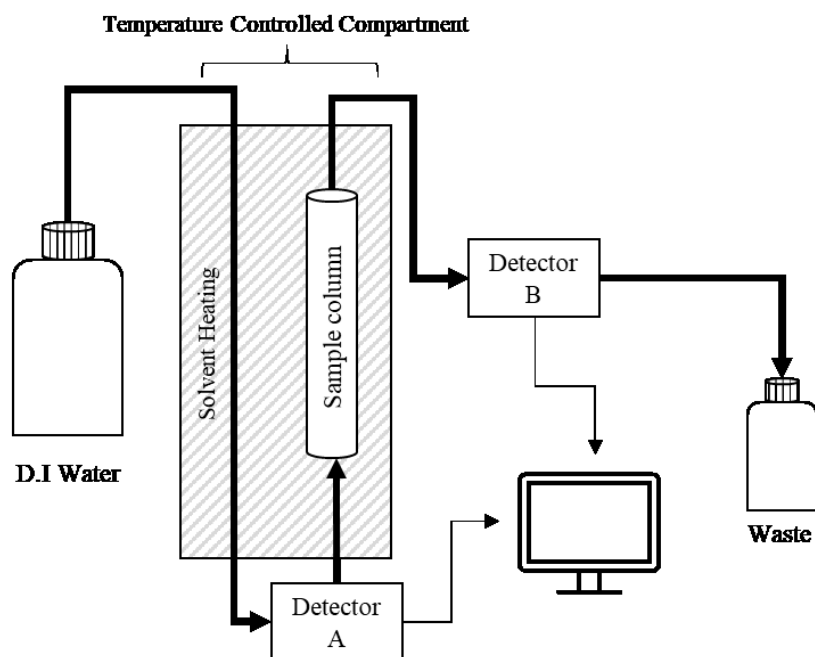


Figure 6.3 A simplified HPLC schematic for the HPLC experiments, demonstrating the two-detector configuration. Figure adapted from Ylä-Mäihäniemi *et al.*¹³¹

6.2.3 Diffusion models in Hair

Fick's Second Law of diffusion applied to a cylinder can be used to model diffusion of a solute into hair. Fick describes radial diffusion into a homogeneous cylindrical object as:¹⁹⁸

$$\frac{\partial C}{\partial t} = \frac{1}{r} \frac{\partial}{\partial r} \left(rD \frac{\partial C}{\partial r} \right) \quad (6.3)$$

where C is the concentration of solute in the object (cylinder) at time t , r is the radius of the cylinder, D is the diffusion coefficient. Hair is not homogeneous, and being highly heterogeneous, the solute will partition into various regions of the hair fibre. A more comprehensive model of diffusion is therefore needed. Wang *et al.* proposed a detailed

theoretical model for effective diffusion in hair. To model this complex solute partitioning, the concentration of solute in hair (C_h) was described as: ¹⁹⁹

$$C_h = \theta_w C_w + \theta_l C_l + \theta_k C_k \quad (6.4)$$

Where C_w , C_l and C_k represent the solute concentration in three regions: free water in internal pore domains, cell membrane complex (CMC) lipid and keratin, respectively. θ denotes the volume fraction for each of these regions in hair. Equations (6.3) and (6.4) can be combined, and applying further approximations for solute mobility, partitioning and binding in hair, Wang *et al* obtained the following equation: ¹⁹⁹

$$\frac{M_t}{M_\infty} = 1 - \exp\left(-2.405^2 \frac{D_e}{R^2} t\right) \quad (6.5)$$

M_t is the mass of solute (mg/g) at time t , M_∞ is the equilibrium mass uptake, D_e is the effective diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$), and R is the radius of hair. Equation (6.5) resembles other models found in the literature, with the added benefit of accounting for the partition behaviour of solutes into hair, making this approach an appropriate model for the desorption behaviour under investigation. ^{118,184} This model was applied to the AHT-HDAP and MEA curves of loss, where R was approximated to be $35\mu\text{m}$, ⁹⁷ with M_∞ being the equilibrium mass loss.

6.2.4 Kinetic Model for solute sorption in hair

The pseudo-first and pseudo-second order kinetic rate equations have been used extensively in literature to describe sorption isotherms for different keratins.^{119,149,187,189,190,200} The pseudo-first order rate equation first proposed by Lagergren (Eqn. 6.6) and the pseudo-second order rate equation (Eqn. 6.7) are as follows:^{201,202}

$$\log(Q_e - Q_t) = \log(Q_e) - \left(\frac{k_1}{2.303}\right)t \quad (6.6)$$

$$\frac{t}{Q_t} = \frac{1}{k_2 Q_e^2} + \left(\frac{1}{Q_e}\right)t \quad (6.7)$$

Q_e (mg/g) is the equilibrium mass of absorbed solute, Q_t (mg/g) is the mass of solute absorbed at time t (min), k_1 (min^{-1}) and k_2 ($\text{g mg}^{-1} \text{min}^{-1}$) are the pseudo-first and pseudo-second order rate constants, respectively. Constants derived from Equation (6.7) are frequently used to calculate the constant h , which is the rate of dye loss when t approaches zero:

$$h = k_2 Q_e^2 \quad (6.8)$$

Desorption data for both AHT-HDAP and MEA were fit to the linearised versions of Equations (6.6) and (6.7) to evaluate goodness of fit and to calculate k_1 , k_2 and h values.

6.3 Results and Discussion

6.3.1 Desorption of AHT-HDAP at 25, 30, 40, 50 & 60°C

6.3.1.1 AHT-HDAP extinction coefficient determination

AHT-HDAP absorbs light with a maximum absorbance at 477nm. This makes it suitable for UV detection using HPLC. To determine the concentration of AHT-HDAP in samples from the UV data, the extinction coefficient for AHT-HDAP in DI water was determined by making a calibration curve from serial dilutions of AHT-HDAP in water and measuring the absorbance of the solutions (Figure 6.4). The extinction coefficient was determined by the gradient of the line, which is related to concentration and absorbance by the Beer-Lambert law (Equation 6.9).

$$A = \epsilon cl \quad (6.9)$$

where A is absorbance, ϵ is the extinction coefficient, c is concentration and l is pathlength.

The data provided a good fit with an R^2 value of 0.9987.

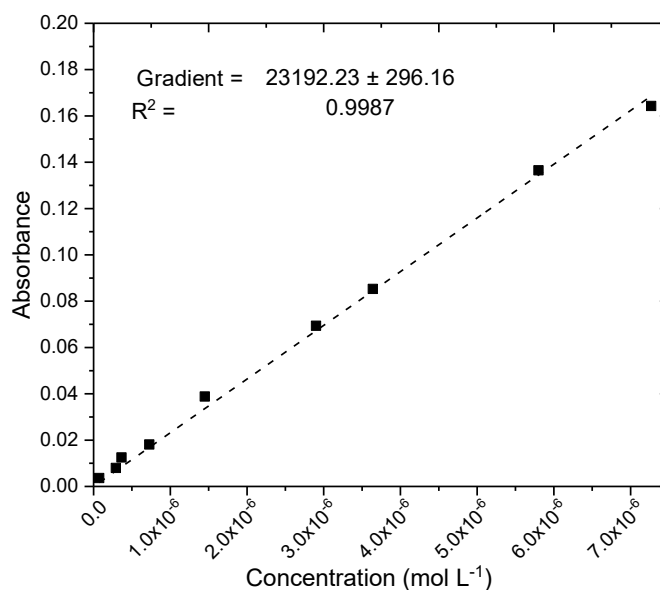


Figure 6.4 The UV-Vis calibration curve for AHT-HDAP dye in DI water. AHT-HDAP pigment provided by Procter & Gamble.

From the gradient ϵ (477nm) was determined to be $23192.23 \text{ M cm}^{-1}$. This data is within good range of the recorded values in ethanol of ϵ (477nm) = $18900.00 \text{ M cm}^{-1}$.

6.3.1.2 UV Chromatographs

The UV measurements for AHT-HDAP dye loss from hair reported large desorption curves across the 120-minute experiments, which reach a concentration/absorbance plateau toward the end of the experiment (Figure 6.5). Total loss of dye from hair (e.g., 100%) was not achieved for any of the experiments in 120 minutes, however there is substantial data available for kinetic analysis.

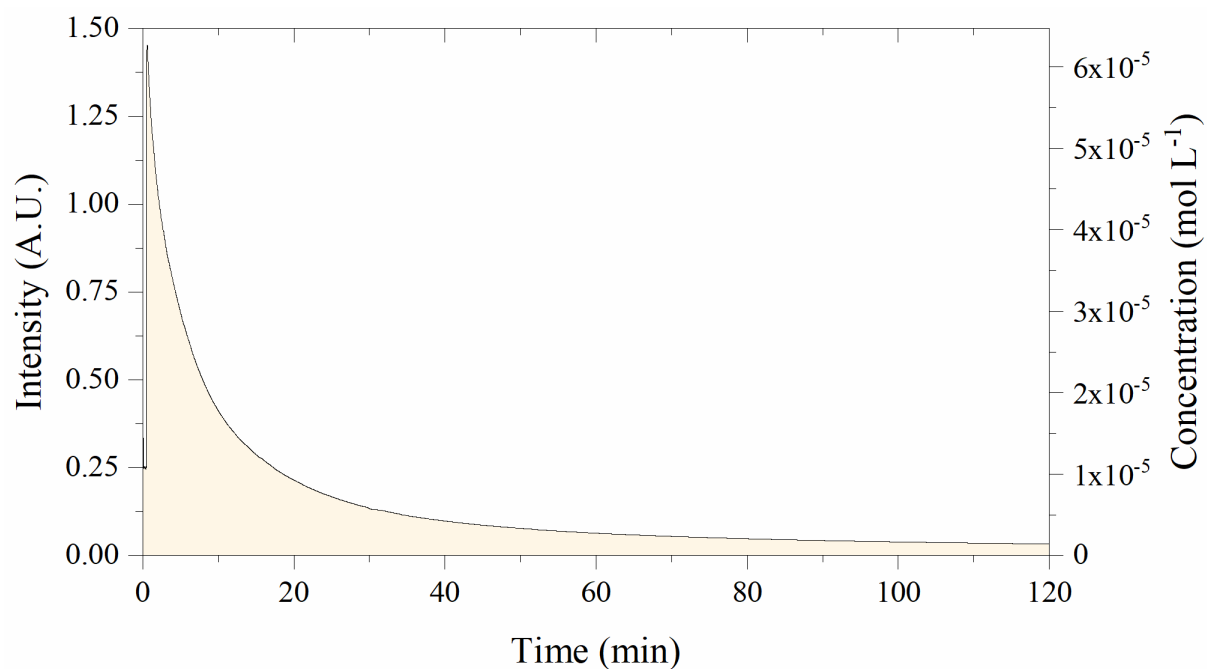


Figure 6.5 The full UV chromatograph for AHT-HDAP dye desorption from hair at 60°C, over 120 min.

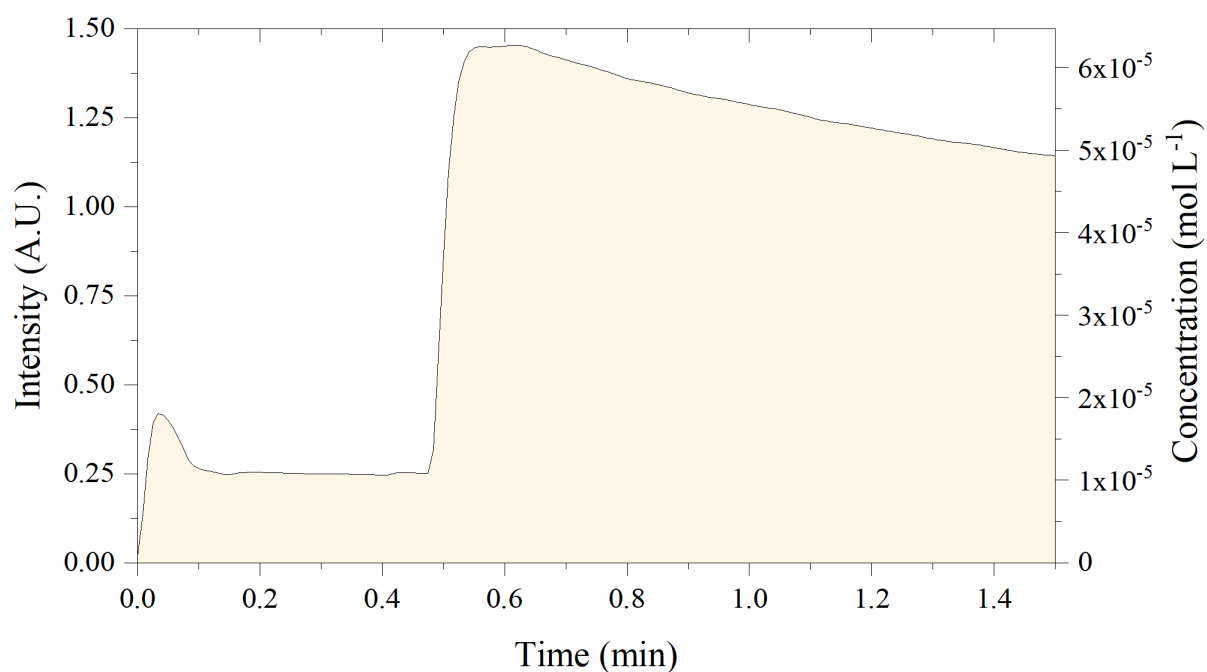


Figure 6.6 The initial desorption peak, taken from Figure 6.5, which is seen across all UV-Vis desorption curves for all samples.

An initial peak can be seen in all the UV chromatographs before the main desorption peak (Figure 6.6). To examine the scientific relevance of these peaks the effective diffusion coefficients for the initial loss across all temperatures were calculated using Equation (6.5). The effective diffusion coefficients showed no relationship with temperature, with high errors for coefficients calculated for experiments undertaken at the same temperature (Figure 6.7). This initial dye loss (<2 minutes) may be attributed to rapid desorption of AHT-HDAP dye molecules on the surface of the hair fibre. During the dye process, AHT-HDAP is formed inside the fibre itself in the presence of metal ions in the hair. While the hair is swollen however, dye may also desorb from the fibre, leading to dye remaining on the surface of the fibre after the brief rinsing time.

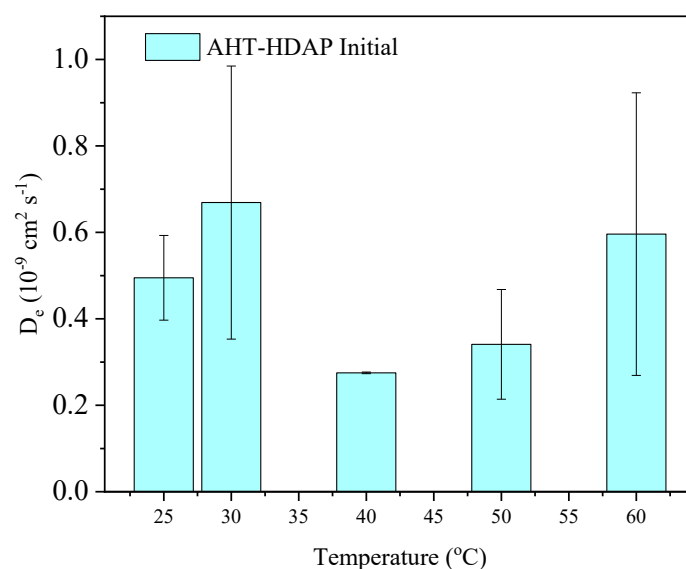


Figure 6.7 Effective diffusion constants D_e for initial loss of AHT-HDAP dye. Initial loss showed no statistical differences in D_e between experiments performed at different temperatures. (Error = 1σ , from $n = 2$)

A longer rinsing time may lead to removing absorbed dye from the hair, preventing the true measurement of loss at $t=0$. These molecules may be assumed to have limited interaction with the fibre bulk, and therefore can be dismissed from further analysis.

6.3.1.3 AHT-HDAP desorption curves

The mass of AHT-HDAP dye loss was calculated from the UV desorption curves for temperatures between 25°C and 60°C. These are shown in Figure 6.8.

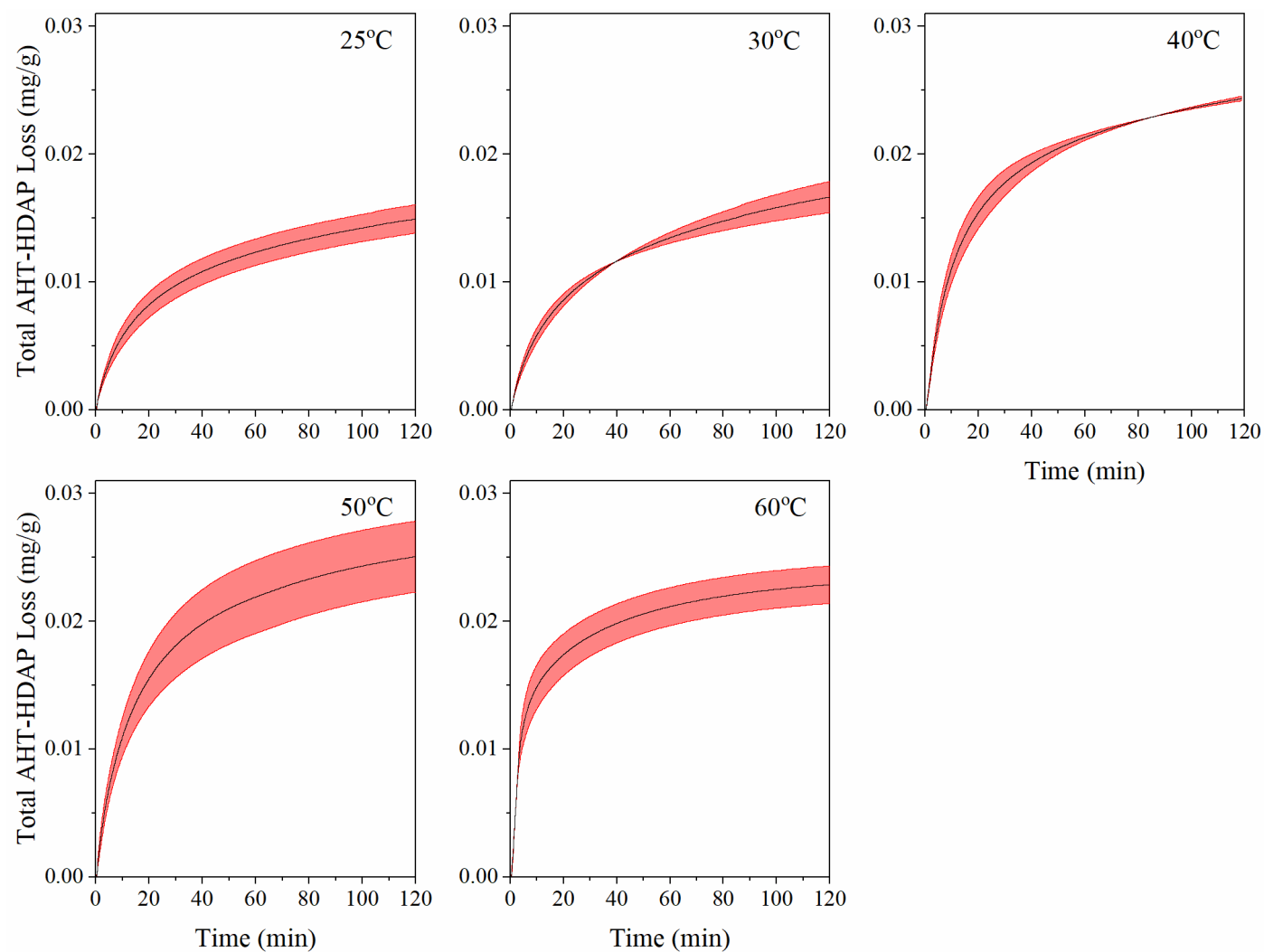


Figure 6.8 The mass of AHT-HDAP dye diffusing from dyed hair over time in DI water, at temperatures of 25, 30, 40, 50 and 60°C. (Error bars = 1 σ from $n = 2$)

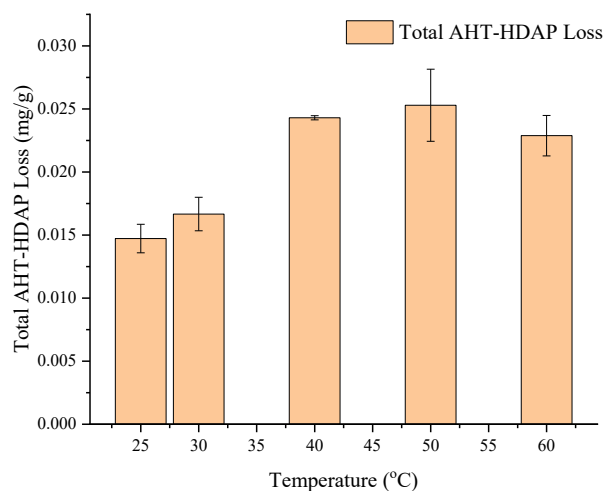


Figure 6.9 The total mass of AHT-HDAP dye desorbed from dyed hair at 25, 30, 40, 50 and 60°C. (Error bars = 1σ from $n = 2$)

The error bars in Figure 6.8 demonstrate a good reproducibility of the data ($n=2$) with larger errors occurring at temperatures greater than 40°C. This temperature dependent structural changes which may be responsible for these increased errors will be discussed in a later Section. Experiments performed at temperatures greater than 40°C have an increased total mass of dye lost from the fibre, which suggests improved solubility of the dye into the DI water (Figure 6.9).

6.3.2 Desorption of MEA at 25, 30, 40, 50 & 60°C

While measuring the loss of AHT-HDAP dye from the hair fibres, samples of the eluting water was collected, and the pH recorded. These pH values were used to estimate the total mass of MEA being desorbed. The loss of MEA from hair was calculated for temperatures between 25°C and 60°C. These are shown in Figure 6.10.

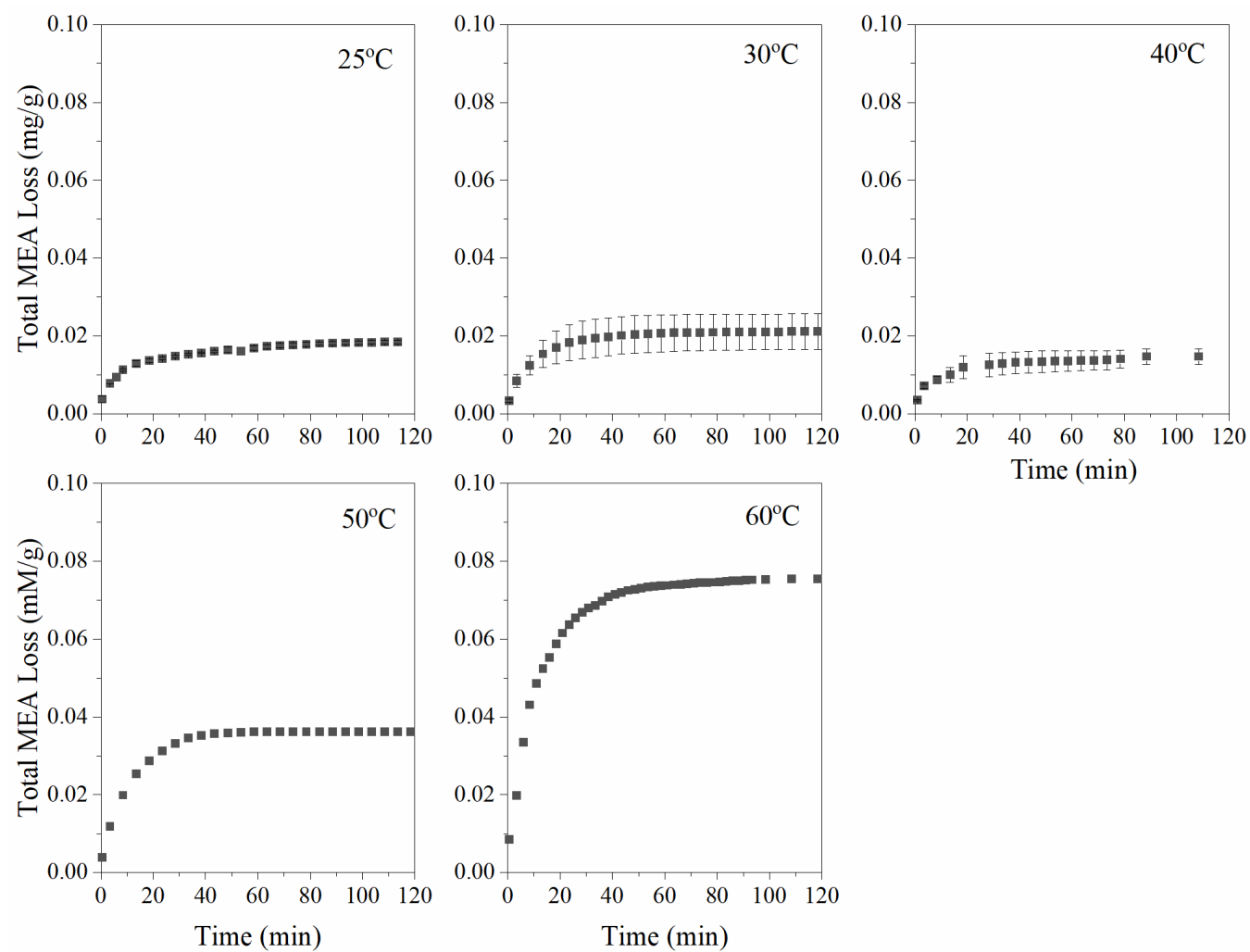


Figure 6.10 The mass of MEA diffusing from hair over time in DI water, at temperatures of 25, 30, 40, 50 and 60°C. (Error bars = 1σ from $n = 2$, for 25-40°C, $n = 1$ for 50-60°C)

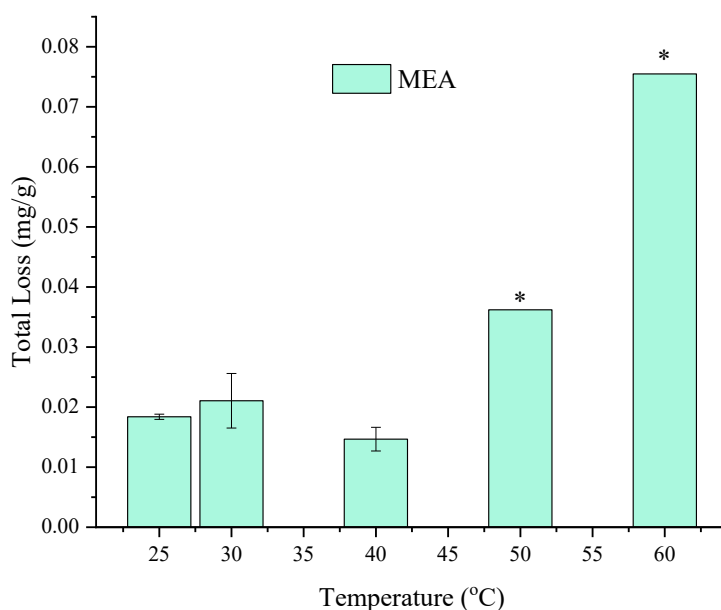


Figure 6.11 The total mass of MEA dye desorbed from hair at 25, 30, 40, 50 and 60°C. (Error bars = 1σ from $n = 2$, * $n=1$).

The MEA mass losses are similar for temperatures below 50°C. For all temperature at approximately 20 minutes the MEA loss reaches a plateau. In terms of scale, the amount of MEA being removed from hair is significantly larger than the amount of AHT-HDAP being lost. Without uptake data of MEA and AHT-HDAP into hair, a discussion between relative amounts of each compound loss cannot be undertaken. However, the kinetics of loss can be compared. The total MEA mass loss is similar between 25-50°C, with a significant increase in MEA loss at 60°C, pointing to a strong structural change imparted from this increase in temperature (Figure 6.11). The relatively rudimentary method for MEA loss of using a simple pH meter resulted in poor measurements with a $n=1$ data set for 50 and 60°C. This may also account for the unclear trend to mass loss under 50°C.

6.3.3 Effective diffusion of small molecules from hair

6.3.3.1 Effective Diffusion fitting

By using Equation 6.5, plotting $\ln(1-M_t/M_\infty)$ versus t yields a straight line with a slope of $2.405^2(D_e/R^2)$ (data used where $M_t/M_\infty < 0.9$ for AHT-HDAP and < 0.95 for MEA due to lack of data points). Fitting the experimental data for both AHT-HDAP and MEA desorption data necessitated that two fitted lines were needed to accurately describe both species loss (Figure 6.12).

These two lines clearly support the supposition that there are two distinct stages of dye diffusion. Fits were achieved using a computational model, where R^2 for a successful fit > 0.999 (PWL2, *OriginPro 2020*). Figure 6.13 demonstrates the use of the same two line fit for MEA desorption data at 30°C. A two-stage diffusion model agrees with work from Hang *et al.*, where dye loss from cotton was successfully modelled with a two-component exponential fit.¹⁹³

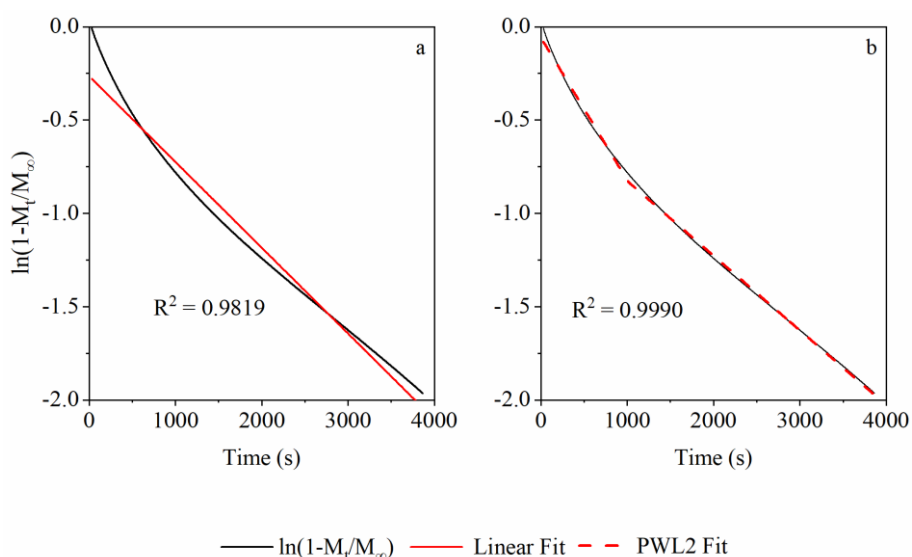


Figure 6.12 a) Poor fit of a single straight line to linearised data of AHT-HDAP loss at 30°C b) A two line (PWL2) fit which more accurately models the same data.

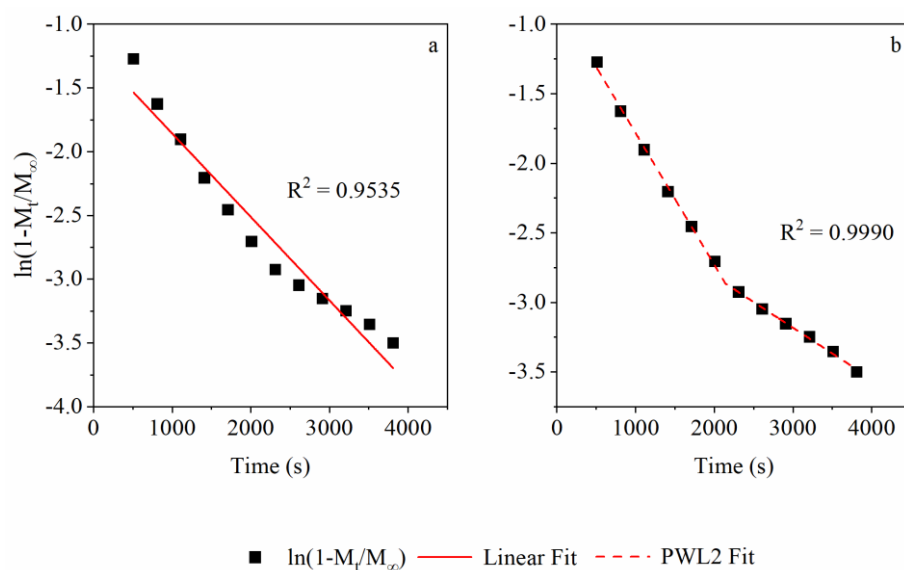


Figure 6.13 a) Poor fit of a single straight line to linearised data of MEA loss at 50°C b) A two line (PWL2) fit which more accurately models the same data.

6.3.3.2 Effective Diffusion coefficients for AHT-HDAP and MEA desorption

The values of D_e AHT-HDAP and MEA loss are shown in Figure 6.14. All values of D_e are in the order of 10^{-10} to $10^{-9} \text{ cm}^2 \text{ s}^{-1}$. These values are within the commonly reported range of recorded diffusion coefficients ($10^{-10} \text{ cm}^2 \text{ s}^{-1}$) found in literature for absorption of small molecules in hair.^{97,199}

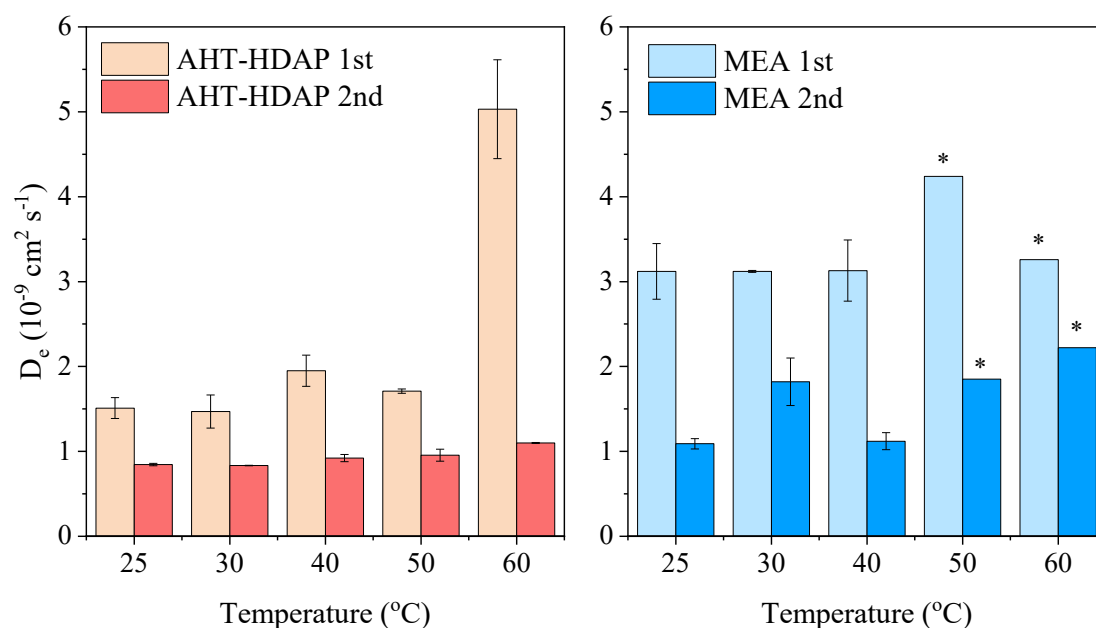


Figure 6.14 The effective diffusion coefficients for (left) AHT-HDAP dye and (right) MEA for the primary and secondary stages of loss from dyed hair for 25-60°C. (Error = 1σ from $n = 2$, * $n = 1$).

The primary stage of loss relates to a fast stage of both AHT-HDAP and MEA loss that occurs within the first 20 min of the HPLC experiment. This process is thought to be related to molecules adsorbed onto the cuticle or present in the pores of the cuticle. For AHT-HDAP, the D_e values for the primary stage increase slightly at 40°C and then much more rapidly at 60°C. The onset of glass transition for hair and wool saturated in water has been recorded at temperatures as low as 35°C depending on water content.^{168,203} With the onset of glass transition, from approximately 35°C for the CMC in both the cuticle and cortex of the hair samples, they will undergo increasing plasticisation, which will lead to the observable increase in diffusion rates. From these results, it may be presumed that full plasticisation has occurred by 60°C. The secondary stage of loss, which is associated with molecules in the hair cortex, also exhibits an increase in D_e for AHT-HDAP loss. Overall, the D_e values do not change as

significantly across the different temperatures in comparison to the primary loss process. This suggests that the dye populations in each stage are being eluted from different regimes in the hair fibre. The plasticisation effects may also be responsible for higher D_e values at higher temperatures, but with the slower diffusion resulting from dye molecules diffusing from the cortex to the cuticle and then finally undertaking transcellular diffusion to the fibre surface for removal.¹⁸⁶ It is also important to note that AHT-HDAP dye is a small dimer (MW = 261.29), whereas other dye molecules can form lengthy polymer chains in hair.²⁰⁴ It would be expected that the effective diffusion would be greatly affected by the size of dye used to treat hair, and perhaps there would be a less significant temperature dependence in the primary stage of dye loss with large polymers.

Two stage diffusion of MEA into has been previously observed by Ali *et al.*⁹⁷ In the data collected from the MEA experiments, while a two-stage diffusion process is clearly seen, the values calculated have large errors and show no determinable trend. It may be that at higher temperatures than currently investigated, a significant increase in loss could be observed. This loss could be attributed to the hydrogen bonding ability that MEA possesses from its amine and alcohol group, allowing many bonds to be formed with amino acids in the hair fibre.

Another interpretation from the data presented in Figure 6.14 is that the loss of dye and alkaliser in these experiments are kinetically independent. However, it is likely that the measurement method, as previously mentioned, is insufficient for these experiments. This further suggests that a better pH measurement system or alternate analytical method for MEA should be used in future experiments. For example, a pH measurement module can be fitted to many HPLC systems which will result in more accurate *in situ* pH measurements.

6.3.4 Pseudo-first and second order analysis of small molecule desorption from hair

6.3.4.1 Choosing a suitable kinetic model

AHT-HDAP and MEA desorption were fitted to pseudo-first and pseudo-second order kinetics, using Equations (6.6) and (6.7) with all parameters being displayed in Table 6.1 and Table 6.2.

For both solutes, the modelled equilibrium mass loss calculated from the pseudo-first order kinetic model (Q'_e) was always under the true Q_e values. This suggests that a pseudo-first order kinetic fit is not appropriate for modelling the data. Using the pseudo-second order fit, the Q'_e values are on average larger than the Q_e values. As a plateau was not reached for most of the solute desorption's, the pseudo-second order model values are acceptable. The R^2 values for the pseudo-second order fit ($1.000 \leq R^2 \leq 0.995$) are also higher than pseudo-first order values ($0.995 \leq R^2 \leq 0.917$). This also suggests a pseudo-second order kinetic fit is more suitable to model the desorption data.

Table 6.1 Kinetic parameters for the pseudo-first and second order fitting of AHT-HDAP dye loss at 25, 30, 40, 50 and 60°C. Q'_e values indicate modelled equilibrium mass loss. $t_{1/2}$ values for averaged $Q_e/Q'_e = 0.5$ are also included.

T (°C)	Q_e (mg/g)	Pseudo 1st order parameters			Pseudo 2nd order parameters				
		Q'_e (mg/g)	k_1 (min ⁻¹)	R^2	Q'_e (mg/g)	h (mg/g min)	k_2 (g mg ⁻¹ min ⁻¹)	R^2	$t_{1/2}$ (min)
25	0.015 ± 0.001	0.012 ± 0.000	0.022 ± 0.004	0.990 ± 0.003	0.017 ± 0.001	0.0009 ± 0.0015	3.138 ± 0.219	0.998 ± 0.001	20.3
30	0.017 ± 0.001	0.014 ± 0.003	0.025 ± 0.002	0.995 ± 0.005	0.019 ± 0.002	0.0009 ± 0.0015	2.547 ± 1.035	0.998 ± 0.002	23.8
40	0.025 ± 0.005	0.017 ± 0.002	0.030 ± 0.001	0.984 ± 0.013	0.027 ± 0.001	0.0019 ± 0.0004	2.762 ± 0.815	1.000 ± 0.001	14.5
50	0.025 ± 0.003	0.018 ± 0.002	0.060 ± 0.033	0.991 ± 0.001	0.028 ± 0.004	0.0018 ± 0.0003	2.397 ± 0.188	1.000 ± 0.000	15.4
60	0.023 ± 0.002	0.011 ± 0.000	0.033 ± 0.000	0.985 ± 0.006	0.023 ± 0.002	0.0043 ± 0.0010	8.330 ± 1.013	0.999 ± 0.001	4.4

Table 6.2 Kinetic parameters for the pseudo-first and second order fitting of MEA loss at 25, 30, 40, 50 and 60°C. Q'_e values indicate modelled equilibrium mass loss. $t_{1/2}$ values for averaged $Q_e/Q'_e = 0.5$ are also included.

T (°C)	Q_e (mg/g)	Pseudo 1st order parameters			Pseudo 2nd order parameters				$t_{1/2}$ (min)
		Q'_e (mg/g)	k_1 (min ⁻¹)	R^2	Q'_e (mg/g)	h (mg/g min)	k_2 (g mg ⁻¹ min ⁻¹)	R^2	
25	0.018 ± 0.000	0.013 ± 0.002	0.042 ± 0.004	0.969 ± 0.013	0.020 ± 0.000	0.003 ± 0.000	6.596 ± 0.175	0.999 ± 0.001	6.4
30	0.021 ± 0.005	0.014 ± 0.003	0.062 ± 0.010	0.994 ± 0.005	0.022 ± 0.005	0.004 ± 0.002	8.727 ± 0.233	1.000 ± 0.000	6.6
40	0.015 ± 0.002	0.007 ± 0.000	0.034 ± 0.011	0.917 ± 0.039	0.015 ± 0.002	0.003 ± 0.001	9.986 ± 2.560	0.995 ± 0.003	4.8
50	0.036	0.030	0.090	0.990	0.038	0.008	5.141	0.999	8.0
60	0.075	0.045	0.054	0.979	0.081	0.012	1.876	0.999	7.7

Solute desorption measurements start when the hair has a non-equilibrium water content, and continue when hair has is fully saturated with water (≈ 2.5 min).²⁰⁵ The pseudo-second order kinetic model can be applied to assess both the equilibrium and non-equilibrium behaviour of hair, where k_2 represents the equilibrium behaviour. As the h value is associated with the rate of loss as t approaches zero, it can be used to represent the non-equilibrium regime of solute loss.

6.3.4.2 Pseudo-second order analysis of AHT-HDAP loss from hair

Average values of h and k_2 calculated for AHT-HDAP desorption data for each temperature are shown in Figure 6.15. The h values (Figure 6.15a) representing the rate of loss at non-equilibrium conditions increase with increasing temperature. This increase in rate is likely resultant of the increased solubility of AHT-HDAP at higher temperature.

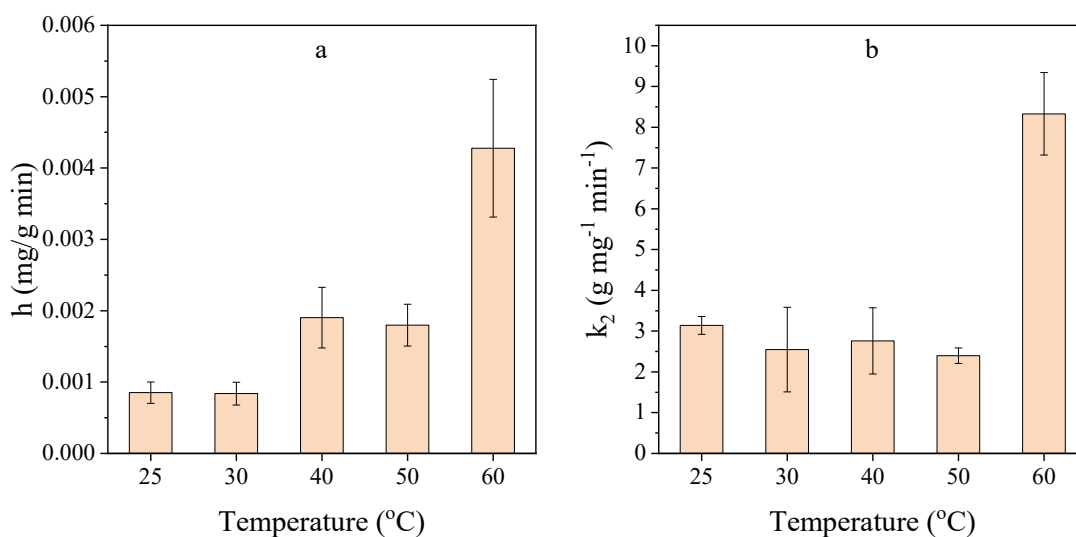


Figure 6.15 The values of a) h and b) k_2 for AHT-HDAP loss from dyed hair from at 25-60°C. (Error bars = 1σ from $n = 2$). Both h and k_2 show a temperature dependence.

As the temperature increases above 30°C, the epicuticle and other areas of the hair fibre will begin to swell allowing improved diffusion.¹⁸⁵ The k_2 values (Figure 6.15b) remain stable until 60°C, which may be due to cuticle lifting and glass transition effects at high temperatures.^{168,185,203} Other significant structural changes have been recorded for wool in literature at high temperatures. At approximately 60°C wool saturated in water has demonstrated the early onset of keratin denaturation, and a reduction in the KIF associated specific volume.^{39,206,207} The contribution of these structural changes may lead to increased AHT-HDAP loss at 60°C.

The AHT-HDAP loss at different temperatures can be normalised using the modelled equilibrium loss values Q'_e calculated from the pseudo-second order kinetic fits (Figure 6.16). Plotting the normalised values against $t^{1/2}$ can help further show differences between the data sets. From this Figure, it can be clearly seen the step changes between 25-30°C, 40-50°C and 60°C, further supporting the notion that significant structural changes occur at these temperatures.

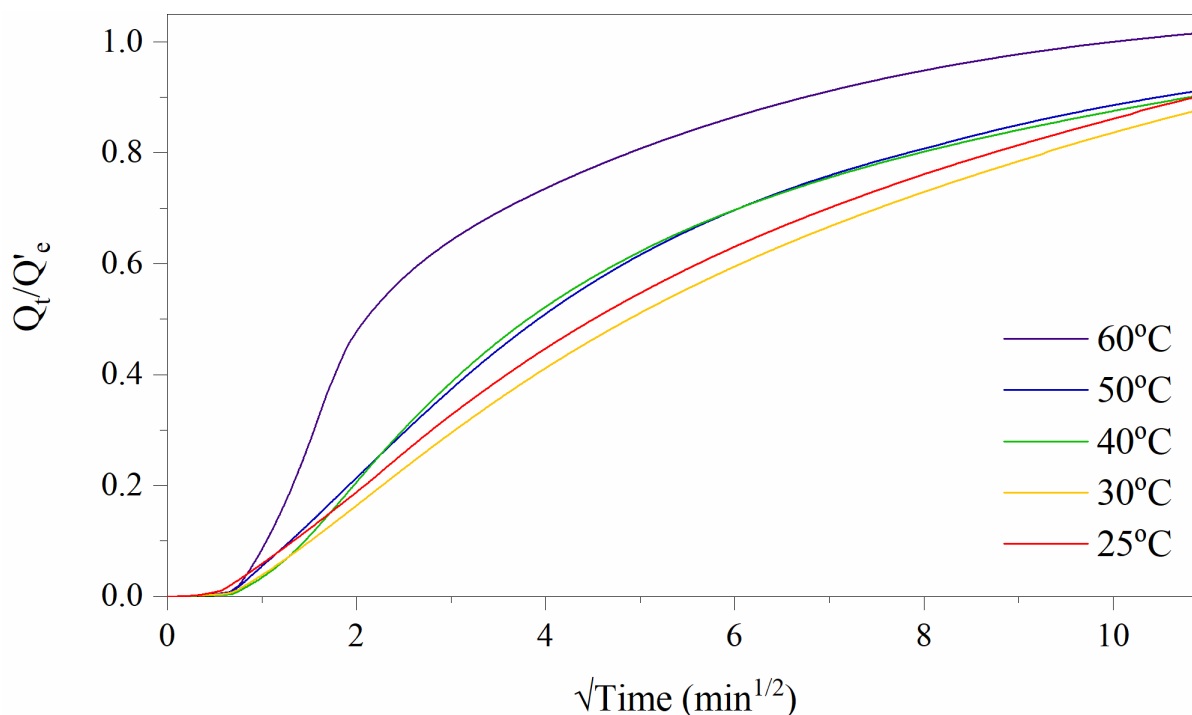


Figure 6.16 AHT-HDAP dye loss data normalised with predicted equilibrium mass values (Q'_e). Q_t/Q'_e values are plotted against $\sqrt{\text{Time}}$. Clear kinetic differences are observed as temperature increases.

Table 6.1 also display the $t_{1/2}$ values for the experiments ($Q_t/Q'_e = 0.5$). As expected, these $t_{1/2}$ times are shorter for temperatures above 30°C ($t_{1/2}$ at 40°C = 14.5 min versus 20.3 min at 25°C). At 60°C the $t_{1/2}$ time is significantly shorter at 4.4 min.

6.3.4.2 Pseudo-second order analysis of MEA loss from hair

Average values of h and k_2 calculated for the MEA desorption data are shown in Figure 6.17. Unlike AHT-HDAP, MEA is readily soluble in water. It is therefore more likely that the increase in the h values (Figure 6.17a) are a result of the aforementioned temperature dependant swelling effects.¹⁸⁵

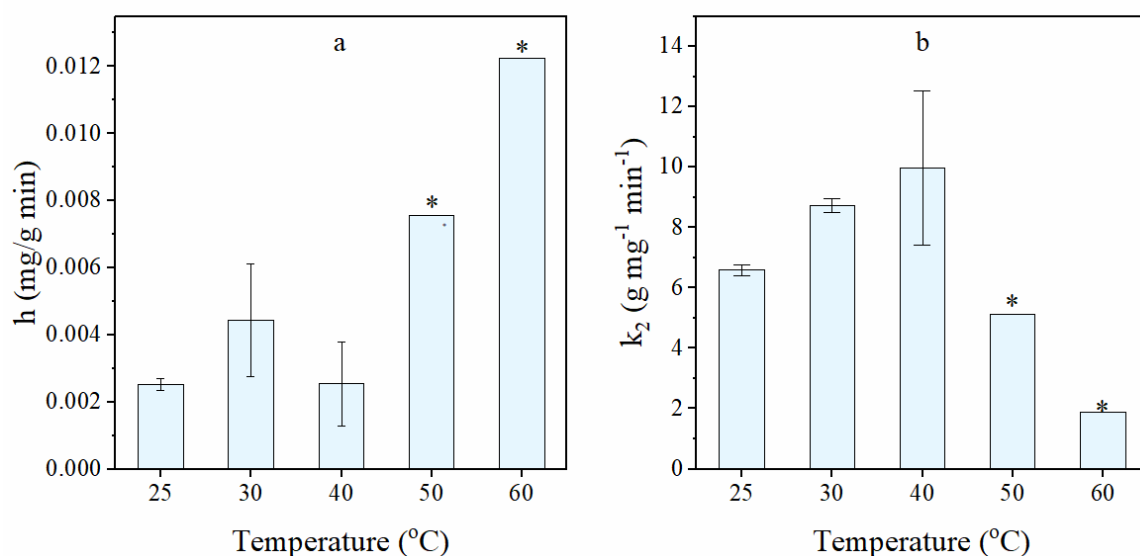


Figure 6.17 The values of a) h and b) k_2 for MEA loss from dyed hair from at 25-60°C. (Error bars = 1σ from $n = 2$, * $n = 1$)

The k_2 values (Figure 6.17b) increase until 40°C, where the rate rapidly decreases. With a large error bar for 40°C and the absence of repeats for desorption experiments at 50 and 60°C, it is impossible to ascertain whether this trend reflects a real-life phenomenon or simply measurement error which has been discussed extensively already in this Chapter.

Figure 6.18 shows the normalised MEA loss at different temperatures. Similarly, to other analysis, any temperature dependence is not clear. The $t_{1/2}$ values from Table 6.2 appear to also show no significant difference between the different temperature regimes. For AHT-HDAP loss at 50°C the $t_{1/2}$ time of 15.4 min is over double the $t_{1/2}$ time MEA loss at only 25°C – 6.4 min. This suggests that AHT-HDAP is retained better in the hair fibre, which may result from its larger size and reduced solubility in DI water in comparison to MEA.

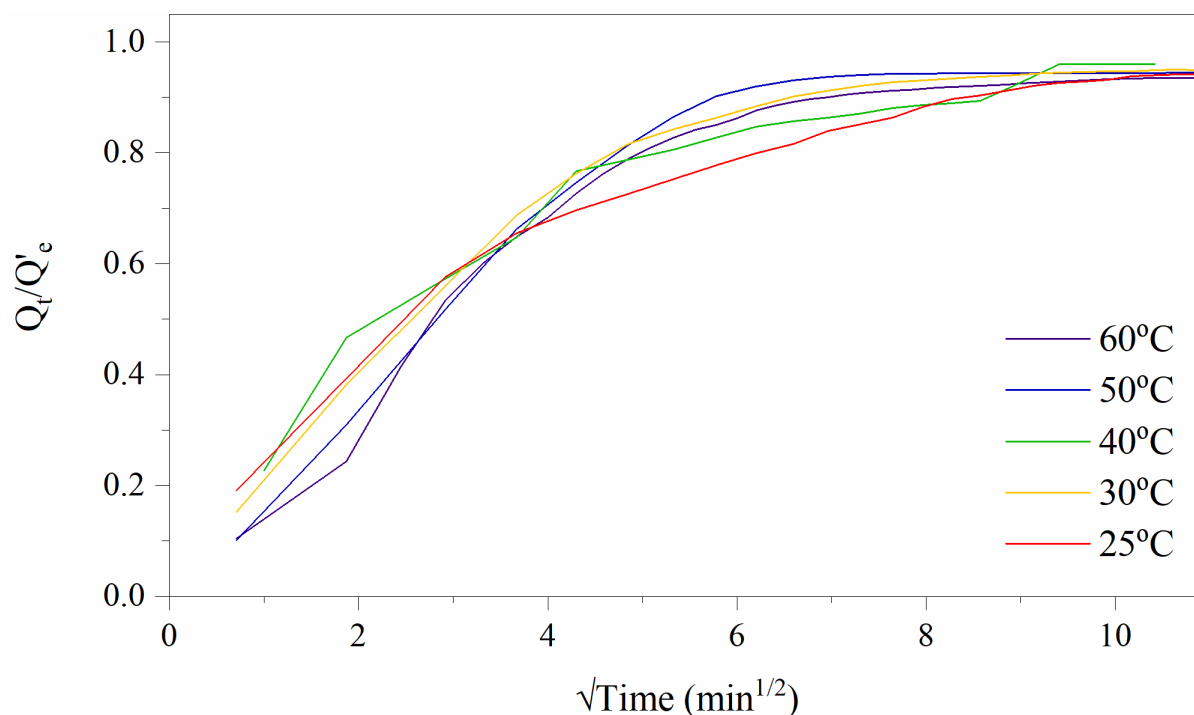


Figure 6.18 MEA desorption data normalised with predicted equilibrium mass values (Q'_e). Q_t/Q'_e values are plotted against $\sqrt{\text{Time}}$, demonstrating no significant trends in MEA desorption at different temperatures.

6.4 Conclusion

This Chapter has described the development of a novel inverse liquid chromatography method using HPLC. Using this method, the desorption kinetics of AHT-HDAP dye and MEA, a common alkaliser in dye formulations, from hair were analysed. The dye desorption was measured directly using UV measurements, in contrast to previous indirect methods described in literature. Temperature control abilities allowed these experiments to take place across 25, 30, 40, 50 and 60°C.

Effective diffusion coefficients were calculated for both AHT-HDAP and MEA demonstrating a two-stage diffusion process consists of an initial fast primary stage which is argued to be from

the hair cuticle and porous regions, and a slow secondary stage attributed to diffusion from the cortex region of the hair fibres.

Both MEA and AHT-HDAP desorption followed pseudo-second order desorption kinetics, where for AHT-HDAP the rate of dye loss increased significantly at 60°C. The kinetic analysis of MEA did not provide any clear trend, suggesting that either the measurement process was not accurate or robust enough, or that the desorption of MEA follows a more complex process. Future work should examine this further with a pH detection system inbuilt into the HPLC to prevent error for example.

The effect of temperature is complex, and this HPLC method demonstrates the ability to observe structural changes in keratin resulting from temperature by monitoring the active loss rates from the fibre.

Overall, this work has proven the success of this method. This straightforward method can be applied to measure desorption from hair any other chemical active, provided the HPLC system has appropriate species detector modules (such as UV/Vis, pH detectors or mass spectrometer detector). HPLC systems are used widely already across many analytical laboratories making the use of this method very accessible.

This method is a significant step forward in understanding the complex process of small molecule desorption from keratin, and the impact of temperature on these processes. This topic has not been investigated thoroughly in literature, further highlighting the importance of this work to industry and research alike.

Chapter 7 - The structural and kinetic impacts of hair dye on keratin

7.1 Introduction

7.1.1 Repeated dye procedures: hair damage and toxicity

The hair colourants industry grew to be worth £334 million in 2020 in the UK.²⁰⁸ In 2017 69% of women in the UK coloured their hair,²⁰⁹ with 38% of all adults having dyed hair in 2020 with increased personal usage.²⁰⁸ A consumer who has treated their hair with a dye will subsequently treat their hair multiple times to regain the desired colour following the initial dye procedure, as often as every 4-6 weeks, as suggested by hair colourant brands.²¹⁰ This treatment level is a considerable amount of exposure to chemicals present in hair dye formulations, resulting in extensive research into the repeated use of hair dyes on both hair fibre damage,^{94,204,211,212} and potential biotoxicity.^{4,80,94,95,103,212–216}

As described in previous Chapters, the most significant damage from hair dyes arises from the destruction of disulphide bonds in hair.^{4,11} Hydrogen peroxide in hair dye formulations acts as an oxidiser, forming hydroxyl radicals in the presence of metal ions which start the radical initiated reactions for dye formation. These hydroxyl radicals oxidise 15-25% of the total S-S bonds in hair, leading to the mechanical weakening of the fibre and reduced stability of α -keratin.^{4,5,40,80,89} The CMC is affected through the destruction of thioester bonds between cuticle cells, resulting in an observed 'bulging' of the CMC after multiple bleach treatments.

^{95,217} In particular, the cuticle undergoes significant damage due to the high concentration of metal ions present in its layers.^{103,104,218,219} Damage results in pore formation in the cuticle,⁹² destruction of the protective 18-MEA lipid layer on the surface of the cuticle,^{5,220} and reduction of total cuticle area as observed by scanning electron microscopy.⁹⁴ The effect of this cuticle damage leads to a reduced water content, reduced tensile strength, and increased protein loss.^{42,92,213} Bleaching has been shown to not only increase the cysteic acid concentration in hair, but to lead to methionine oxidation and tryptophan reduction.^{195,220} Bleached hair is also well known to take up more metal ions e.g., copper, from tap water, resulting in further oxidative damage radical formation from H₂O₂.⁹⁴ The increased ion content in the cuticle leads to greater protein loss in subsequent dye procedures, and increased damage in the cuticle.^{217,219,221} The overall surface area of the cuticle is shown to decrease overall with repeated treatments, as small pores which are formed - which initially increase surface area - collapse with subsequent treatments.⁹² Alkalizers, another main component of hair dyes has also been shown to interrupt electrostatic bonding between KIF's resulting in more internal damage,^{100,197} increased swelling of the hair fibres in water,²²² and protein loss.⁹⁷

The potential link to cancer resulting from use of semi-permanent or permanent hair dye on the scalp has also been investigated thoroughly, demonstrating potential mutagenic properties of these dyes, including links to breast cancer in women.^{204,211,213–215} Understandably, there is considerable interest in how to reduce use of these dyes, with the substituted use of natural dyes, chelates or conditioners.^{6,94,204,213}

To improve dye product formulations which reduce hair damage and toxic exposure, it is important to understand the changing kinetics of dye loss from hair undergoing repeated

treatments, and how this is related to hair damage. There is limited, if any, literature in the public domain on how repeated dye treatments affect the kinetics of small molecule dye loss from hair.

7.1.2 Aims

The HPLC methodology described in Chapter 6 provides a quantitative way of measuring the loss of small actives in hair. This methodology can be used to understand the changing kinetics of active loss from hair undergone repeated dye treatments. By dyeing hair repeatedly with the AHT-HDAP dye formulation described in Chapter 6, this Chapter will investigate the effect of the hair dye and subsequent dye loss on keratin structure and how repeat dye treatments effects the kinetics of chemical species loss

This work is important for understanding how to create more sustainable dye formulations, which reduce overall hair damage and toxic exposure in consumers.

7.2 Materials and Methods

7.2.1 Preparation of dyed hair

Preparation of dyed hair samples has been previously described in Chapter 6, Section 6.2.1. Untreated white Caucasian Hair was provided by Procter & Gamble as a gift. Hair was dyed with 4-amino-2-hydroxytoluene (AHT), 4,5-diamino-1-(2-hydroxyethyl)pyrazole (HDAP), using monoethanolamine (MEA) as an alkalizer and hydrogen peroxide (H_2O_2) as an oxidant, to produce the red dye product AHT-HDAP.

H_2O_2 was purchased from VWR International Ltd, AHT and MEA from TCI Europe, and HDAP purchased from Fluorochem Ltd.

7.2.1 HPLC experiments

HPLC experiments were performed on dyed hair at 40°C following the protocol described in Chapter 6, Section 6.2.2.

To examine the effect of dye extraction on hair damage, a dyed hair sample where the AHT-HDAP dye was fully extracted was prepared using a 25 hr HPLC extraction at 40°C. This extracted hair sample was used for damage assessment.

For the re-dyeing experiments, after an initial HPLC column experiment undertaken at 40°C on dyed hair, the hair sample was re-dyed with the AHT-HDAP formula. This procedure was repeated 3 more times to make 5 dye and column cycles. pH and UV data were collected from each column, and the resulting MEA and AHT-HDAP loss from the keratin packed columns were calculated.

7.2.3 Solid state ^{13}C cross polarisation / magic angle spinning experiments

Approximately 0.08 g of untreated, dyed hair, and extracted hair, were chopped into 0.1 cm lengths, and soaked in water for a few minutes. Damp fibres were packed tightly into 4 mm ceramic NMR rotors. The ^{13}C CP/MAS NMR spectra were acquired using a solid-state Bruker Avance III 200 MHz NMR spectrometer, with the minimum number of scans being 30,000 and a spinning frequency of ≈ 6 kHz. Line fitting of the spectra was achieved using *MeStreNova 14.1.2* software. Analysis was conducted three times for each spectrum to average any error encountered in manual phasing of the spectra.

7.2.4 Preliminary confocal image acquisition

A range of images for different hair samples were obtained using a Leica SP8 Confocal microscope. A single hair was taped to a glass slide for use on the microscope stage. The 65 mW Ar laser (469 nm) was used for the image acquisition.

7.3 Impact of dye loss on hair structure

7.3.1 Introduction

To better interpret the results from repeated dye procedures, structural changes in hair fibres that occur after dye loss were examined. Hair samples that were investigated included untreated white hair, dyed hair, and extracted hair (Figure 7.1). Confocal images and ^{13}C CP/MAS spectra were recorded for the three samples to examine what differences could be observed.



Figure 7.1 An image of untreated hair, dyed hair and extracted hair.

7.3.2 Preliminary Confocal Images of hair

To visually examine cuticle damage and changes after different treatments, electron microscopy (SEM) is often used to give high resolution images allowing cuticle area, distances between cuticle scales, and even angle of cuticle lift to be evaluated.^{41,79,93,97,102,103,105} Confocal microscopy is a fast albeit less powerful microscopy method, but can equally provide a visual assessment to how the surface of the cuticle responds to different treatments. To examine how the HPLC experiment was affecting the hair fibres, preliminary confocal images were taken of fibres which had undergone different treatments (Figures 7.2 - 7.4).

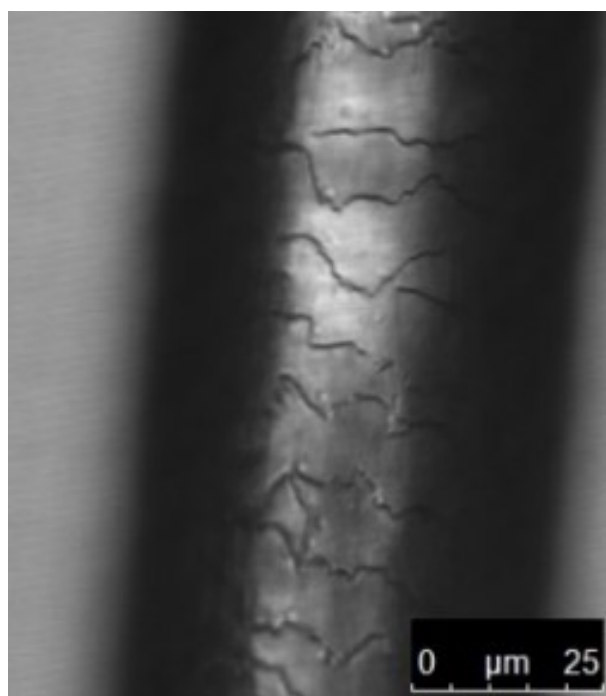


Figure 7.2 Preliminary confocal image of untreated hair, as received. Undamaged cuticle, flush to the surface of the hair fibre can be observed in this image. As image resolution was not optimised, the scale in this image is misleading.

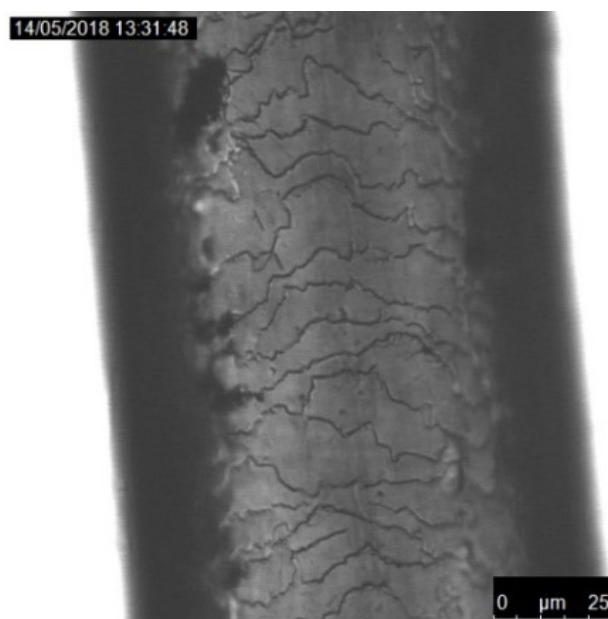


Figure 7.3 Preliminary confocal image of dyed hair, treated with AHT-HDAP dye formulation.

This image shows breakage of the cuticle, with loss of cuticle represented by dark spots.

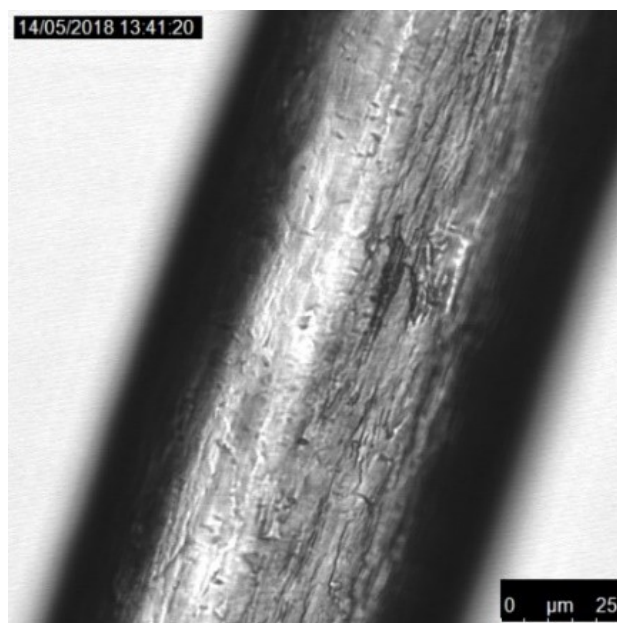


Figure 7.4 Preliminary confocal image extracted hair - dyed hair having undertaken 25 hr of HPLC treatment at 40°C. Striations in the image show complete cuticle stripping.

In the image of untreated hair (Figure 7.2) large scales which are flush to the surface of hair can be seen. This image is similar to images of untreated hair recorded in literature using SEM, demonstrating no damage to the hair sample.^{11,196,223}

However, in the image of dyed hair (Figure 7.3) the cuticle scales are smaller in their surface area, suggesting degradation of the cuticle. This type of change in cuticle structure is often found in damaged samples, where treatments result in cuticle lifting and breaking of cuticle scales.^{105,223}

For extracted hair (Figure 7.4) the traditional cuticle scale structure is no longer apparent, suggesting complete stripping of the cuticle scales, as observed in hair samples where the cuticle has been eroded.^{196,224} While complete stripping of the cuticle is not representative of what would happen to the cuticle after only 2 hours of extraction, it does suggest the flow of water and the pressure of these HPLC extractions causes significant damage to the cuticle. It can therefore be interpreted that consumer washing of dyed hair would likely also lead to some cuticle scale loss.

7.3.3 ¹³C CP/MAS NMR experiments

¹³C CP/MAS NMR spectra were recorded for untreated hair, dyed hair, and extracted hair (Figure 7.5). Peak assignment is found in Table 7.1

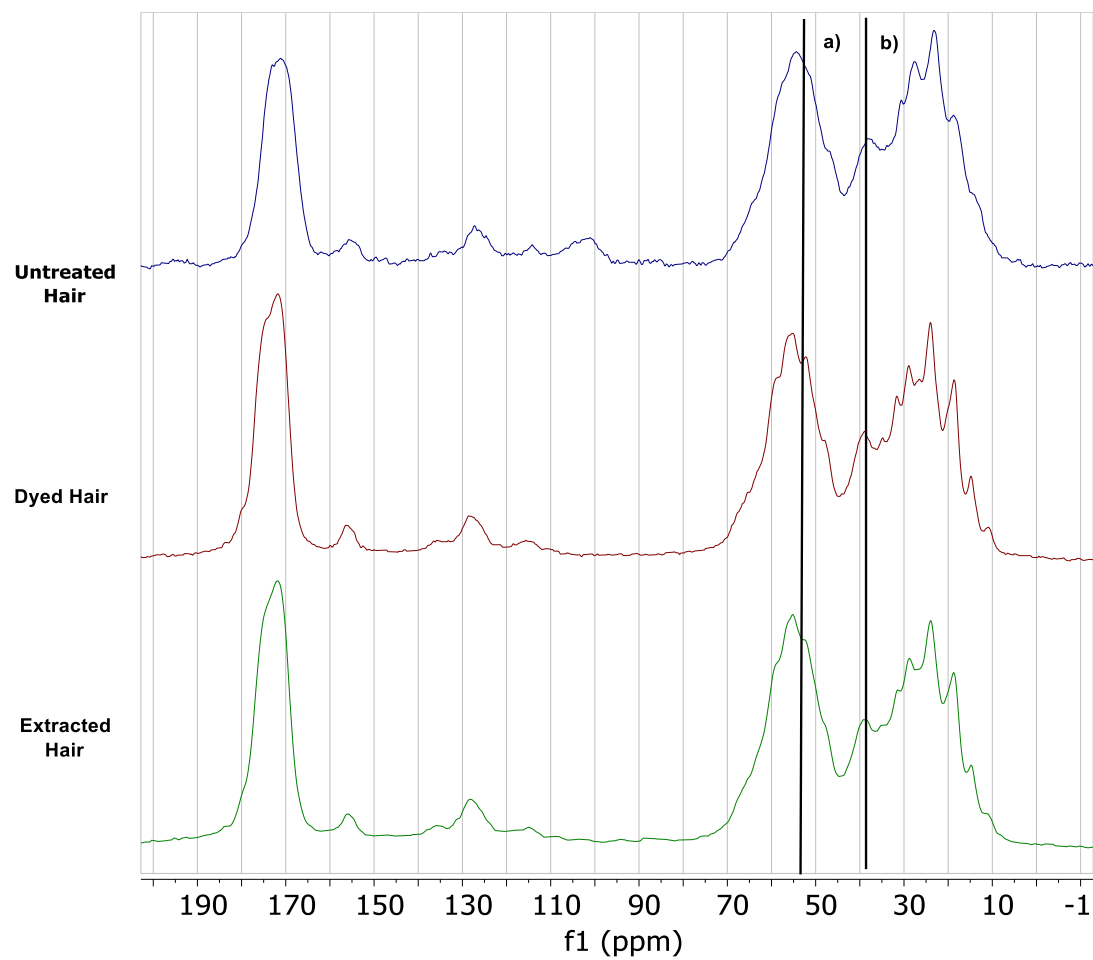


Figure 7.5 The ^{13}C CP/MAS spectra for the three hair samples investigated. Dotted lines show peaks for a) crosslinked cystine at approximately 40 ppm, and b) β s glutamine at approximately 53 ppm.

Table 7.1 Chemical peaks assignments in the ^{13}C CP/MAS spectra of the hair samples investigated. Not all of these peaks may be visible in each spectrum.⁶⁵

Approximate chemical shift (δ ppm)	Assignment
11	$\delta\text{-CH}_3$ of isoleucine
15	$\beta\text{-CH}_3$ of alanine (αh)
19	$\gamma\text{-CH}_3$ of valine
24.5	$\delta\text{-CH}_3$ of leucine and $\delta\text{-CH}_2$ of proline
26	$\beta\text{-CH}_2$ of cystine ⁵²
29	$\beta\text{-CH}_2$ of glycine (αh), $\beta\text{-CH}_2$ of trans proline
32	$\beta\text{-CH}_2$ of cis proline, $\beta\text{-CH}$ of valine (βs), $\beta\text{-CH}_2$ of glutamic acid (βs)
35	$\beta\text{-CH}_2$ of lysine (βs), $\beta\text{-CH}_2$ of asparagine (αh)
39	$\beta\text{-CH}_2$ of leucine (αh), $\beta\text{-CH}_2$ of crosslinked cystine ⁶⁷
48	$\delta\text{-CH}_2$ of proline
53	$\alpha\text{-CH}$ of glutamine (βs) ⁵² , $\alpha\text{-CH}$ of lysine (βs), $\alpha\text{-CH}$ of alanine (αh)
56	$\alpha\text{-CH}$ of serine (βs), $\alpha\text{-CH}$ of leucine (αh)
60	$\alpha\text{-CH}$ of phenylalanine (αh), $\alpha\text{-CH}$ of tyrosine (αh), $\alpha\text{-CH}$ of proline
66	$\beta\text{-CH}_2$ of serine (βs) and $\beta\text{-CH}$ of threonine (βs)
120-130	Aromatic CHs of phenylalanine and tyrosine
158	O-C of tyrosine
172-176	Carbonyl for all amino acids

After dyeing, there is a clearer difference in resolution between aliphatic peaks and α -carbon peaks in the dyed hair and HPLC treated hair, in comparison to the untreated sample. This suggests increased segmental mobility due to a lower degree of crosslinking.⁶⁵ The carbonyl peak (172-176 ppm) also appears to have changed after treatment, indicating a higher level of disorganised keratin content.

Chapter 5 demonstrated the use of ^{13}C CP/MAS NMR to quantify damage in hair by measuring the relative α -keratin content of the samples (Equation 5.2).

$$\frac{FWHM \text{ of ordered peak}}{FWHM \text{ of disordered peak}} = \text{Relative } \alpha - \text{keratin} \quad (5.2)$$

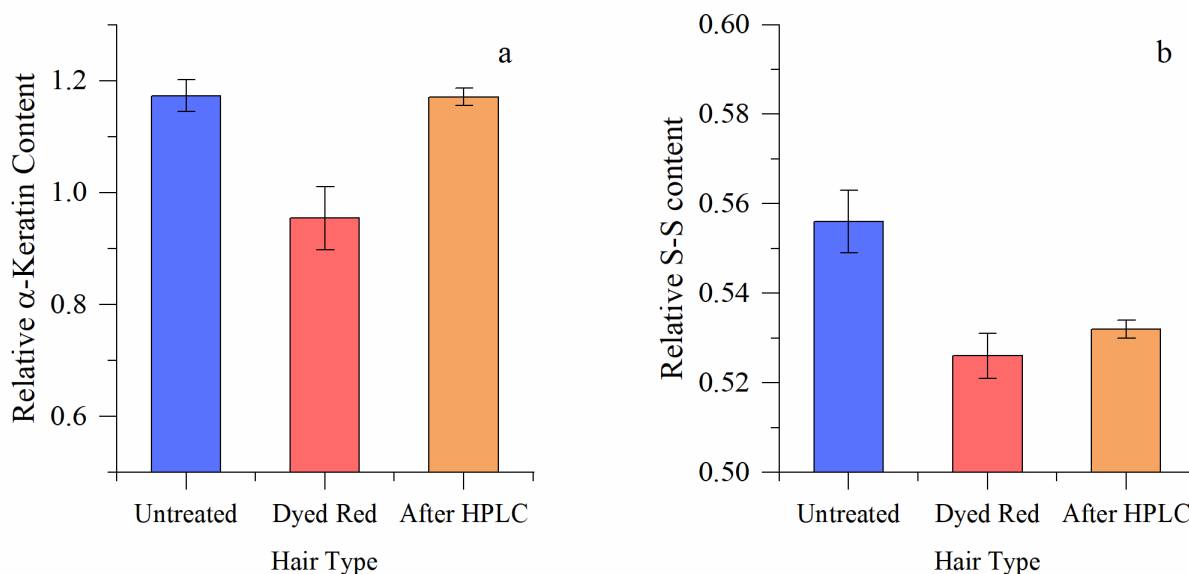


Figure 7.6 Results for the a) relative α -keratin content and b) relative S-S content from analysis of the ^{13}C CP/MAS spectra for the three hair samples investigated.

The carbonyl peaks of the ^{13}C CP/MAS spectra for the untreated, dyed, and reduced samples were analysed, and the Relative α -Keratin content calculated (Figure 7.6a). From these calculations, there is a significant reduction in the relative α -keratin content of hair fibres once it has been dyed. After the dye has been extracted using the HPLC method, the relative α -keratin content returns to approximately that of untreated hair. This agrees with work done by Marsh *et al.*, where it was demonstrated that removal of alkalizer and ions from dyed hair with dialysis helped recover thermal properties of untreated hair samples, e.g., the α h-keratin melting temperature during DSC experiments.¹⁰⁰ The extraction process using the HPLC method will remove any MEA from the dyed hair, and also removed metal ions taken up by hair during washing with tap water. Without these charged species interrupting electrostatic interactions within the hair fibre, the extracted hair has a recovered α -keratin content similar to that of untreated hair.

Changes in amorphous content in the hair fibres can also be observed by changes in intensity of the peak at 53 ppm, associated with resonances for α -CH of glutamine (β s), α -CH of lysine (β s) and α -CH of alanine (α h). As glutamine is one of the most common amino acids in keratinous materials,⁵² an increase in this peak intensity can likely indicate an increased level of β -sheet glutamine and in turn a higher amorphous content of keratin. The increase of this peak is observed in the dyed hair sample in comparison to untreated hair, and decreases in extracted hair, further confirming the relative α -keratin content measurements. It is important to note here that for consumers washing hair with tap water, the removal of charged species will not be effective and further charged metal ions e.g., copper, will be absorbed by the hair cuticle.²²¹ Repeated dye procedures would therefore further decrease the relative α -keratin content in hair, weakening the fibre and resulting in further damage.

The peak at approximately 39 ppm in the spectra in Figure 7.5 correlates to the resonances of β -CH₂ of leucine (α h), and β -CH₂ of crosslinked cystine.⁶⁷ Dyeing procedures using H₂O₂ oxidise disulphide bonds in hair, so this peak can be used as a measure of disulphide bond breakage. To quantify a relative S-S bond content, a reference peak must be used due to the inherent differences in the spectra intensities across the three samples. The peak at approximately 24 ppm is associated with resonances of δ -CH₃ of leucine and δ -CH₂ of proline. As these resonances are not dependent on keratin conformation, and thus should not change with chemical treatment, this peak can be used as a reference. Therefore, the quantification of crosslinked cystine in hair samples can be expressed as the Relative S-S content:

$$\frac{{}^{13}\text{C } \beta - \text{CH}_2 \text{ of crosslinked cystine intensity}}{{}^{13}\text{C } \delta - \text{CH}_3 \text{ of Leu and } \delta - \text{CH}_2 \text{ of Pro intensity}} = \text{Relative S - S content} \quad (7.1)$$

The relative S-S content was quantified for the samples investigated as shown in Figure 7.6b. There is a reduction in disulphide bond content between untreated and dyed samples (0.549 and 0.523 respectively), resulting from the oxidative damage incurred from the dye procedure. After extraction, the relative S-S content is not significantly changed. This result is expected as the oxidation of disulphide bonds via H_2O_2 results in cysteic acid which is not expected to react and reform disulphide bonds during extraction.⁴

7.4 Effects of repeated treatment on hair

7.5.1 Loss of AHT-HDAP and MEA from repeated dye procedures

Hair was dyed red using AHT-HDAP dye and underwent an HPLC experiment desorption described in Chapter 6 at 40°C to measure the loss of AHT-HDAP and MEA from the sample. The same hair sample was retrieved from the HPLC column and four more cycles of dyeing, and measuring of active loss was performed on the same sample. This resulted in 5 data sets. For the first cycle, data recorded in Section 6.3 was used and collated to achieve a better understanding of the initial starting point.

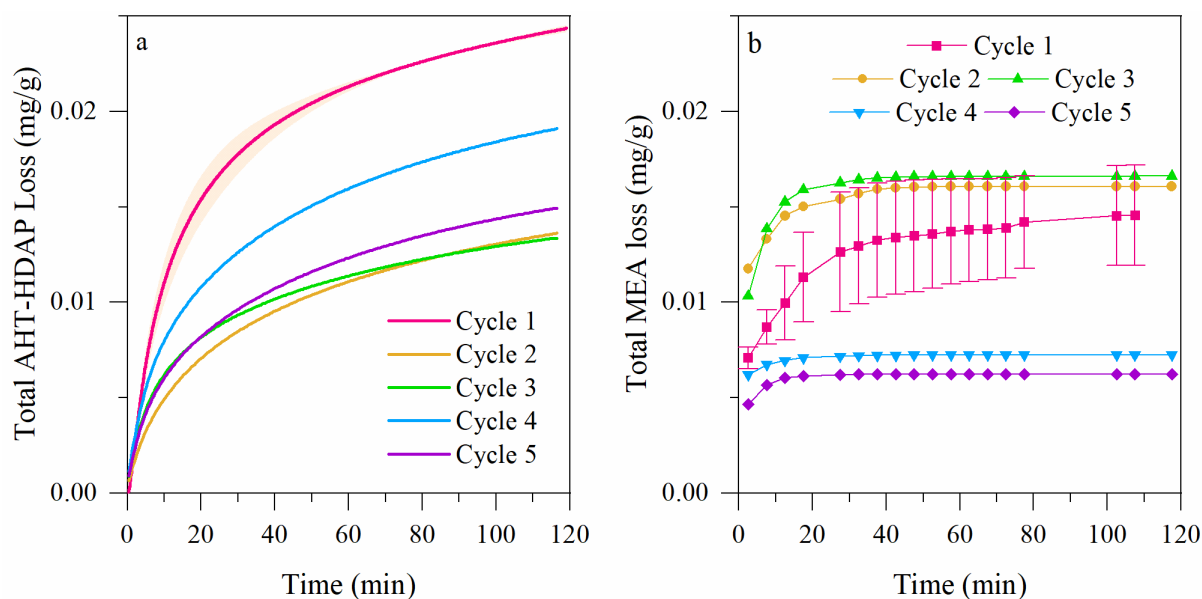


Figure 7.7 a) The loss of AHT-HDAP dye over time from hair after multiple re-dyeing treatments. b) Loss of MEA for the same experiments (Error = 1σ for cycle 1 from $n = 2$).

Figure 7.7a displays the total loss of AHT-HDAP dye exiting the column over 5 different dye cycles on the same sample. No cycle reaches a plateau, with an appreciable drop in total dye loss in subsequent cycles.

Figure 7.7b shows the total loss of MEA during the HPLC experiments for the five different cycles. The first three cycles have a similar total loss of MEA within error. Subsequent cycles have total dye loss reduced by a third, while taking less time to reach a plateau. However, without knowing how much AHT-HDAP dye and MEA is present in the fibres at $t = 0$, it cannot be ascertained whether the changes in total small molecule loss are due to uptake differences or retention differences of the treated fibres. The kinetics must therefore be looked at more closely to understand this process.

7.5.2 Effective diffusion over multiple treatments

The effective diffusion coefficients (D_e) for AHT-HDAP and MEA loss were calculated for each cycle using the same method described in Chapter 6.3.3, where $R^2 > 0.999$. Figure 7.8 shows the changes in effective diffusion of AHT-HDAP dye and MEA for the two calculated diffusion stages over the redyeing experiments. All values of D_e are in the order of 10^{-10} to 10^{-9} $\text{cm}^2 \text{s}^{-1}$. These values are within the expected range for diffusion coefficients (10^{-10} $\text{cm}^2 \text{s}^{-1}$) for small molecules in hair.^{97,199} As only one experiment was performed for cycles 2-5, an estimated 10% error has been included in all further calculations.

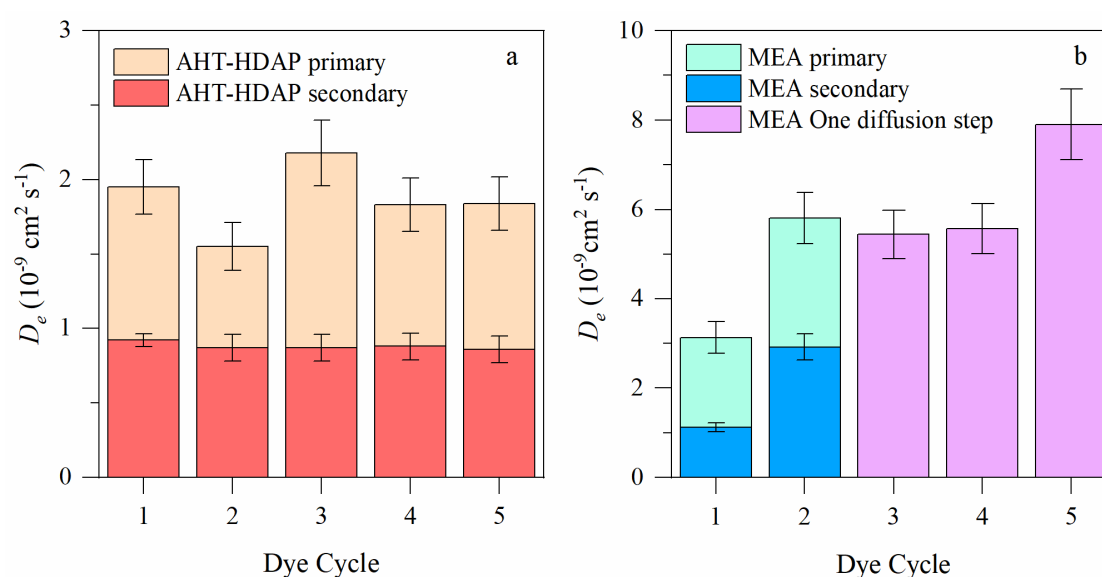


Figure 7.8 The effective diffusion coefficients for a) AHT-HDAP dye and b) MEA for the primary and secondary stages of loss from dyed hair over 5 dye cycles. For MEA at the third dye cycle only one diffusion step is identified (Error = 1σ for cycle 1 from $n = 2$, a 10% error is included for other cycles.)

Diffusion of AHT-HDAP remained a two-stage process across each cycle. For the primary stage of diffusion, D_e values for AHT-HDAP loss vary minimally across the five cycles. With the included estimated error, it can be concluded that there is no significant change in D_e for this stage. The secondary stage of AHT-HDAP loss also remains consistent across each cycle.

AHT-HDAP dye is likely to be formed in the cuticle rather than the cortex: copper, which catalyses the oxidative reactions for dye formation is found mostly in the outer layers of the cuticle.^{104,218,219} Formation of hydroxyl radicals will therefore result in greater damage to the cuticle, which in turn leads to increased copper sorption in the cuticle from tap water in subsequent dye cycles.²²¹ As the cuticle becomes more damaged, it absorbs more metal ions which results in dye formation occurring mostly in the cuticle. The sequential damage results in a decrease in the total area of cuticle through formations of holes from cuticle breaking, as observed in confocal images taken of dyed and extracted hair.¹⁰³

While diffusion values do not change with repeated cycles, Figure 7.7a demonstrates that total dye loss does decrease with each cycle. Diffusion of AHT-HDAP dye from hair must therefore occur from the cuticle; reducing cuticular surface area with each dye cycle will result in lower total dye loss but no significant changes in diffusion values for the remaining cuticle. Further, AHT-HDAP dimers formed in the cuticle may have difficulty diffusing to the cortex of the hair fibre due to their size: previous studies have demonstrated that within 30 min (the dyeing time length in these experiments) bulky dye molecules have yet to migrate to the cortex during a dye procedure.¹⁸⁸ While the previous chapter attributed the secondary stage of dye loss to dye loss from the cortex, there must be a significant contribution from the cuticle in this stage, possibly from the endocuticle or CMC layers of the cuticle (Figure 1.3).

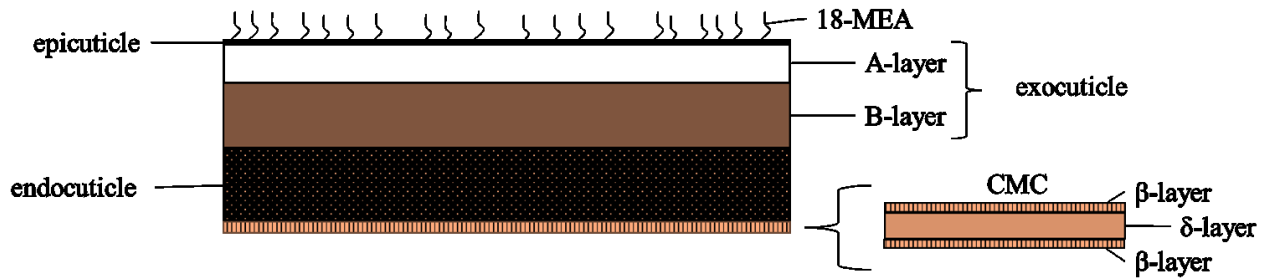


Figure 7.9 A simplified cross-section of the cuticle of human hair. The cuticle layers are shown from the outer epicuticle to the cell membrane complex (CMC). The CMC can then be divided into a further 3 sublayers.

For MEA (Figure 7.8b), diffusion in both the primary and secondary stages of loss increase significantly during the second cycle. From the third cycle only one diffusion rate is observed (Figure 7.10), indicating a significant change is occurring in the hair fibre.

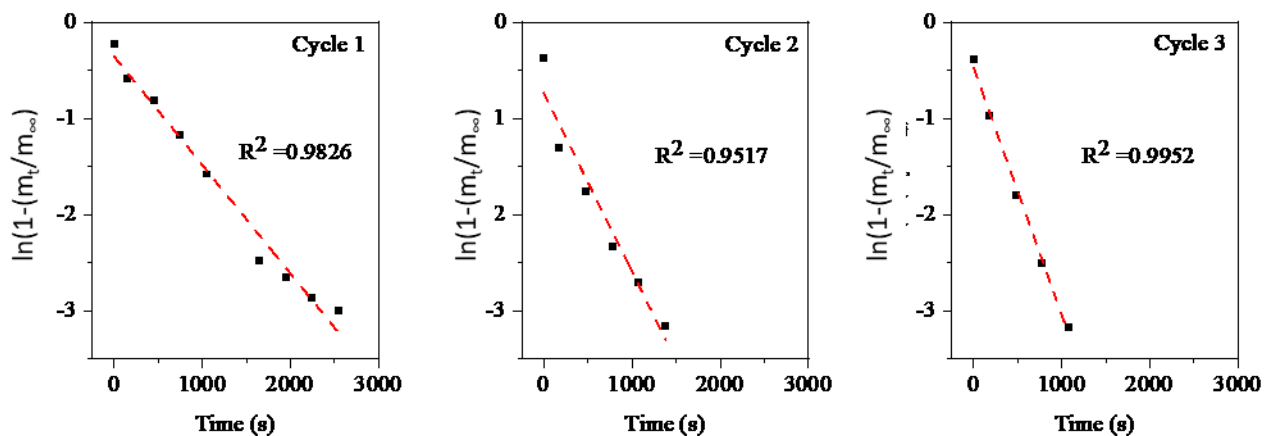


Figure 7.10 Linear fits of MEA loss data from cycles 1 to 3, showing that a linear fit (--) does not accurately describe the behaviour in cycles 1 and 2, however for cycle 3 there is clearly one stage of diffusion, and not two.

This change in diffusion processes, correlates with the reduction in MEA loss after cycle 3 (Figure 7.7b), with the plateau for total MEA loss being reached at a faster rate – as reflected in the increased D_e values for cycle 5. If the diffusion stages are attributed to the cuticle (primary) and cortex (secondary), with the loss of cuticle through damage and stripping, the secondary stage of diffusion may now be dominating the overall behaviour of MEA loss from the fibre. Further, this suggests that MEA is more strongly associated with the cortex, and minimally associated with the cuticle, as loss of cuticle only increases diffusion, which is to be expected if the cuticle is a diffusional barrier for the majority of the MEA residing in the cortex. This hypothesis is supported by studies which have demonstrated that MEA penetrates into the hair cortex within the first few minutes of hair being saturated in a MEA solution.²¹² A reduced total loss of MEA may therefore reflect reduced uptake during the dye procedure.

7.5.3 Kinetic Analysis of multiple treatments

AHT-HDAP and MEA desorption over each cycle were fitted to pseudo-first and pseudo-second order kinetics, using Equations (6.6) and (6.7). All parameters are shown in Table 7.2 and Table 7.3. Similar to previous analysis in Chapter 6, all cycles exhibit a superior fit with a pseudo-second order kinetic model with R^2 values for the pseudo-second order fit ($1.000 \leq R^2 \leq 0.995$) being higher than pseudo-first order values ($0.973 \leq R^2 \leq 0.888$).

Table 7.2 Kinetic parameters for the pseudo-first and second order fitting of AHT-HDAP dye loss over 5 dye cycles. Q'_e values indicate modelled equilibrium mass loss. $t_{1/2}$ values for $Q_e/Q'_e = 0.5$ are also included. (Error = 1σ for cycle 1 from $n = 2$, a 10% error is included for other calculations.)

Cycle	Q_e (mg/g)	Pseudo 1st order parameters			Pseudo 2nd order parameters				$t_{1/2}$ (min)
		Q'_e	k_1	R^2	Q'_e	h	k_2	R^2	
		(mg/g)	(min ⁻¹)		(mg/g)	(mg/g min)	(g mg ⁻¹ min ⁻¹)		
1	0.025 ± 0.005	0.017 ± 0.002	0.030 ± 0.001	0.984 ± 0.013	0.027 ± 0.001	0.0019 ± 0.0004	2.762 ± 0.815	1.000 ± 0.001	14.0 ± 2.7
2	0.014	0.016 ± 0.002	0.034 ± 0.003	0.888	0.017 ± 0.002	0.0007 ± 0.0001	2.302 ± 0.230	0.996	26.0 ± 2.6
3	0.013	0.013 ± 0.001	0.035 ± 0.004	0.894	0.017 ± 0.002	0.0011 ± 0.0001	3.871 ± 0.387	0.998	13.7 ± 1.4
4	0.019	0.020 ± 0.002	0.035 ± 0.004	0.909	0.022 ± 0.002	0.0010 ± 0.0001	2.100 ± 0.210	0.996	22.1 ± 2.2
5	0.015	0.016 ± 0.002	0.034 ± 0.003	0.905	0.018 ± 0.002	0.0007 ± 0.0001	2.258 ± 0.226	0.997	25.0 ± 2.5

Table 7.3 Kinetic parameters for the pseudo-first and second order fitting of MEA loss over 5 dyeing cycles. Q'_e values indicate modelled equilibrium mass loss. $t_{1/2}$ values for $Q_e/Q'_e = 0.5$ are also included. (Error = 1σ for cycle 1 from $n = 2$, a 10% error is included for other calculations.)

Cycle	Q_e (mg/g)	Pseudo 1st order parameters			Pseudo 2nd order parameters				
		Q'_e	k_1	R^2	Q'_e	h	k_2	R^2	$t_{1/2}$ (min)
		(mg/g)	(min ⁻¹)		(mg/g)	(mg/g min)	(g mg ⁻¹ min ⁻¹)		
1	0.015 ± 0.002	0.007 ± 0.000	0.034 ± 0.011	0.917 ± 0.039	0.015 ± 0.002	0.003 ± 0.001	9.986 ± 2.560	0.995 ± 0.003	2.5 ± 0.0
2	0.016	0.004 ± 0.000	0.085 ± 0.009	0.953	0.016 ± 0.002	0.014 ± 0.001	53.489 ± 5.349	1.000	1.5 ± 0.2
3	0.017	0.004 ± 0.000	0.099 ± 0.010	0.973	0.017 ± 0.002	0.011 ± 0.001	39.012 ± 3.901	1.000	2.1 ± 0.2
4	0.007	0.001 ± 0.000	0.073 ± 0.007	0.957	0.007 ± 0.001	0.017 ± 0.002	316.642 ± 31.664	1.000	<0.5
5	0.006	0.000 ± 0.000	0.072 ± 0.007	0.900	0.006 ± 0.001	0.013 ± 0.001	320.620 ± 32.062	1.000	1.34 ± 0.13

As described in the previous chapter, due to hair fibres reaching full water saturation after approximately 2.5 min,²⁰⁵ the h parameter can be used to assess the non-equilibrium behaviour of the hair fibre, while k_2 is the rate constant for the hair fibre at equilibrium.

Values for h and k_2 for both AHT-HDAP are displayed in Figure 7.11. Initial rates of loss (h) reduce after the first cycle, and remain relatively consistent within the estimated 10% error applied. This may reflect that fracturing of the cuticle allows better penetration of AHT-HDAP dye into pores and cracks in the cuticle. Cuticle stripping will unveil other cuticle layers, e.g., the A-layer. The A-layer is rich in disulphide and isopeptide bonds.⁴ Increased interactions between AHT-HDAP dye and the A-layer via electrostatic/ionic interactions or even hydrogen bonding may lead to these surface dye molecules binding more strongly than in untreated hair samples, resulting in decreased h values.

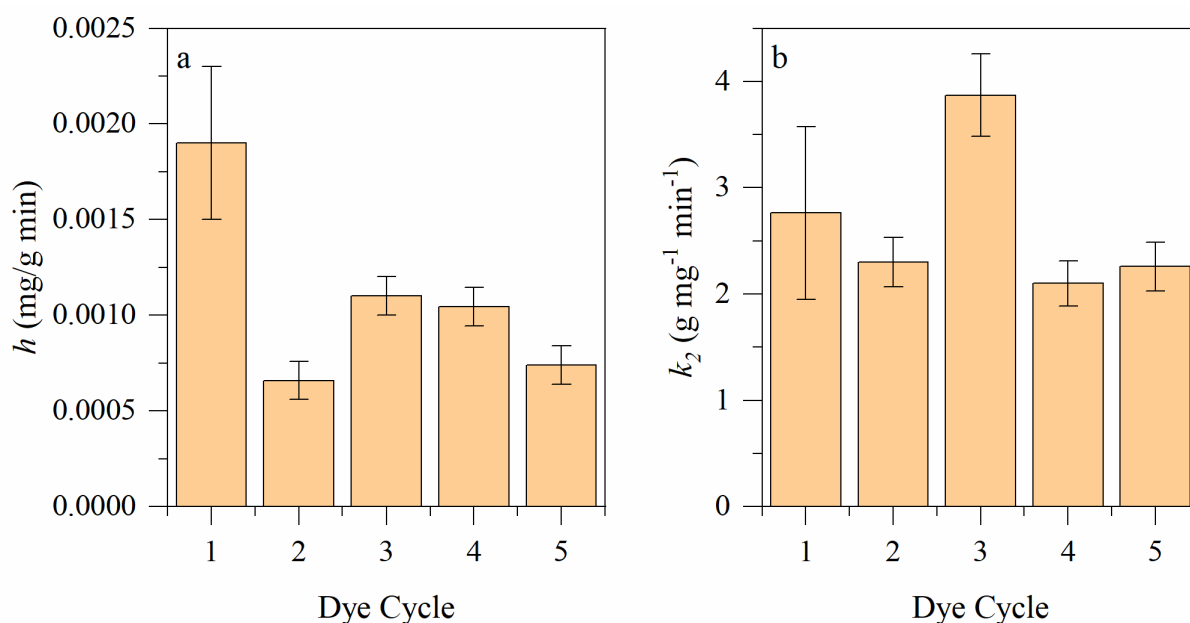


Figure 7.11 Values of a) h and b) k_2 for loss of AHT-HDAP dye from five dye cycles. (Error = 1σ for cycle 1 from $n = 2$, a 10% error is included for other cycles.)

This decrease in initial rates of loss is not seen in the diffusional analysis. As h is representative of the rate of loss ≈ 0 , it is a more sensitive measure of initial loss, which may not be reflected in the diffusion analysis for the primary stage of dye loss across a longer time frame. Values of k_2 remain relatively consistent within error. This agrees with the diffusional analysis of the previous section, where diffusion coefficients remained unchanged after repeated cycles.

Figure 7.12 displays the kinetic parameters for MEA loss over the five cycles. The h values increase rapidly after the first cycle and the k_2 values increase over 30 times by the 4th cycle.

As previously discussed, this loss of cuticle will allow much faster loss of MEA from the damaged fibres; this will affect both the initial and bulk loss of MEA from the hair fibre with cuticle stripping removing a barrier for diffusion. The kinetic analysis suggests that between the 3rd and 4th cycle, a significant proportion of the cuticle has been stripped, resulting in the aforementioned increases in k_2 values for the 4th and 5th cycles.

Some MEA will also be present in any remaining cuticle. As previously mentioned, cuticle damage will result in the uncovering of different cuticle layers. MEA is a hydrophilic molecule, and in the cuticle is likely to reside in the highly hydrophilic layers such as the A-layer rather than the hydrophobic 18-MEA coated epicuticle. With loss of the epicuticle, the A-layer will be exposed, and loss of MEA will increase, resulting in increases in the h value.

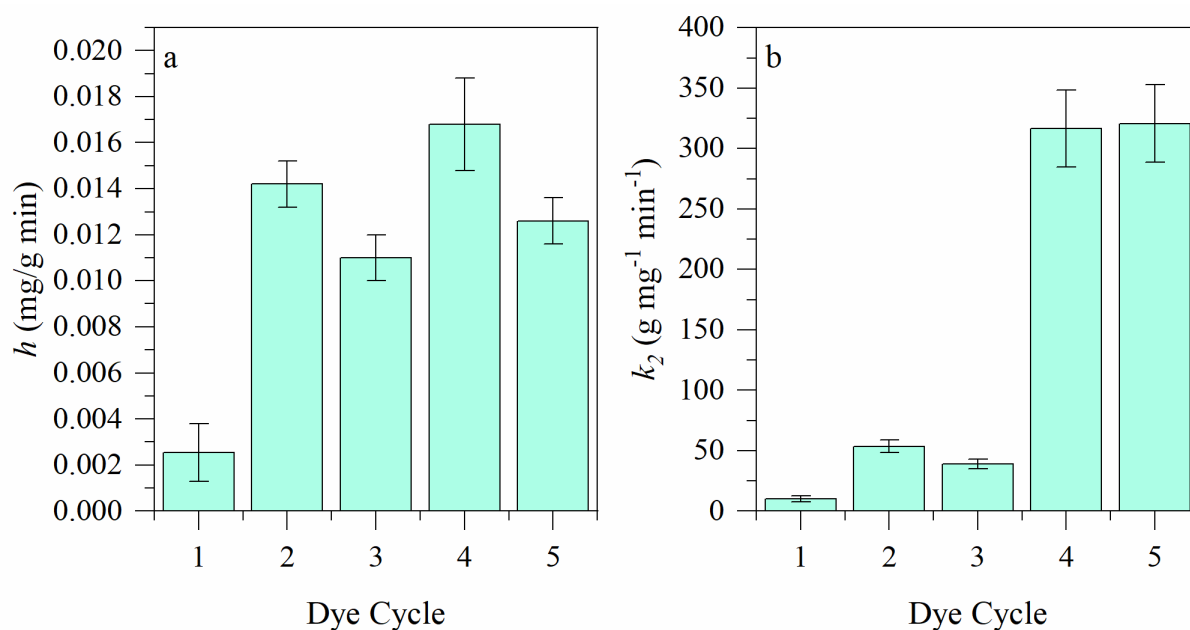


Figure 7.12 Values of a) h and b) k_2 for loss of MEA from five dye cycles. (Error = 1σ for cycle 1 from $n = 2$, a 10% error is included for other cycles.)

The AHT-HDAP and MEA loss across the five cycles can be normalised using the modelled equilibrium loss values Q'_e calculated from the pseudo-second order kinetic fits (Figure 7.13). Table 7.2 and Table 7.3 also display the $t_{1/2}$ values for the experiments ($Q_t/Q'_e = 0.5$).

For AHT-HDAP loss, no discernible trend can be identified from the plot (Figure 7.13a). The $t_{1/2}$ values show there is an overall increase in time taken for $Q_t/Q'_e = 0.5$, except for cycle 3. Without repeats, it cannot be ascertained whether this behaviour is an experimental anomaly. Overall, this analysis suggests that dye loss has slowed, reflecting the decreases in h for the 2nd-5th cycles.

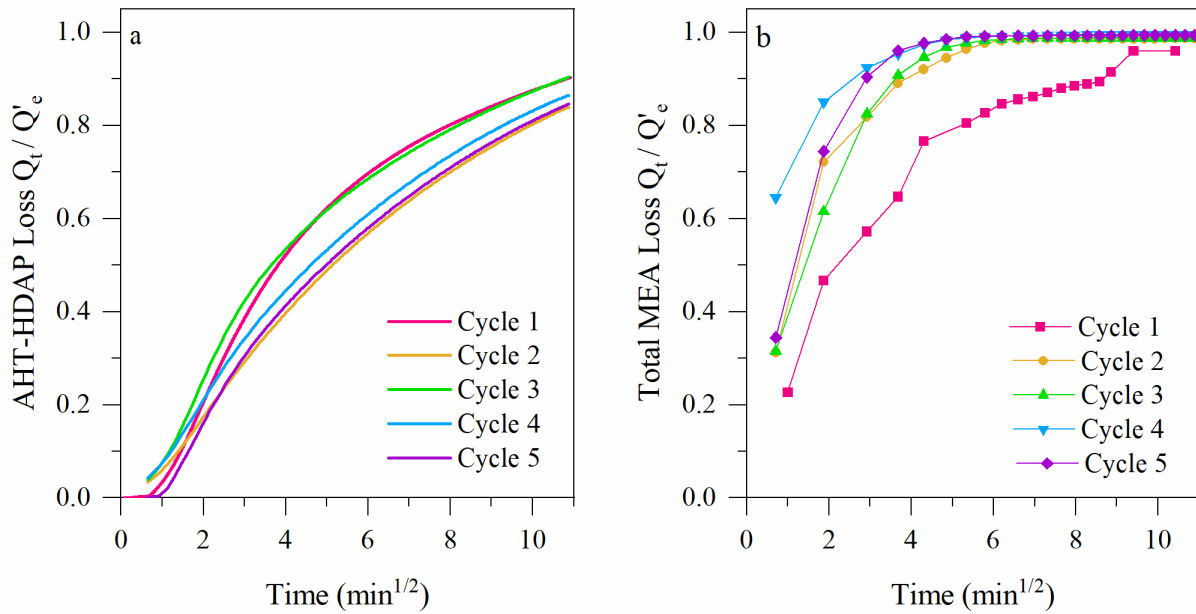


Figure 7.13 a) AHT-HDAP dye and b) MEA loss data over five dyeing cycles normalised with predicted equilibrium mass values (Q'_e). Q_t/Q'_e values are plotted against $\sqrt{\text{Time}}$.

When examining MEA loss however (Figure 7.13b) there is a clear difference between cycle 1 and subsequent cycles, with an average decrease in $t_{1/2}$ times. This again supports the hypothesis that MEA loss increases with subsequent dye procedures due to incurred damage.

7.5 Conclusion and future work

This Chapter has attempted to understand the effects of multiple dye procedures. Preliminary investigations to understand damage accrued from dyeing included:

a) Confocal Microscopy of hair fibres

- Confocal microscopy was used to show that the dye procedure results in degradation of cuticle with a smaller cuticle scale surface area.
- Fully extracted hair showed complete stripping of cuticle, demonstrating cuticle scale loss during extraction/washing processes.

b) ^{13}C CP/MAS NMR of hair fibres

- ^{13}C CP/MAS NMR spectra was used to show a reduced relative α -keratin content and increased amorphous content in dyed hair samples, in comparison to untreated hair. Dyed hair also showed reduced S-S bond content.
- After extraction of dye actives using the HPLC method, the relative α -keratin content increased to that of untreated levels, likely due to removal of MEA and ions which interrupt electrostatic and ionic bonding in keratin.¹⁰⁰ However, the S-S bond content remained unchanged after extraction.

With this background information, a single sample of dyed hair underwent 5 cycles of dyeing and HPLC extraction of actives with DI water at 40°C.

The effective diffusion of AHT-HDAP dye loss did not change significantly across both primary and secondary stages of loss, while total AHT-HDAP loss was reduced. This suggests that dye loss is strongly associated with the cuticle, which reduces in area via damage in subsequent cycles.

For MEA only a single fast stage of diffusion was recorded after the third dye procedure. This indicated that significant cuticle damage and loss had occurred, with diffusion from the cortex dominating the overall loss of MEA from hair. MEA is therefore more strongly associated with the cortex. This hypothesis was further supported by kinetic analysis, where rates of loss increased 30-fold for MEA dye; with removal of the cuticle through damage, MEA has a reduced diffusional barrier and therefore diffusion increases.

From this work it is clear that where an active resides in the hair fibre is of great importance to its changing kinetics of loss during repeated dye procedures.

There are many future experiments that could confirm and expand the conclusions made in this work:

- Confocal imaging of hair fibres after each HPLC treatment (fibres were obtained for this study, but image acquisition was not completed due to Covid access limitations)
- Some studies were performed using porcupine quill cuticle as a model for the hair cuticle to examine the role of the cuticle in small active loss. This was a promising yet complicated line of inquiry; however due to the time constraints of the project repeated experiments were not completed.
- HPLC experiments on de-cuticled hair to confirm preferential binding of MEA to the cortex.

Overall, this work in this Chapter has shown that multiple dye treatments lead to increased loss of actives associated with the cortex, due to depletion of the cuticle. The reduced cuticle surface area will reduce the total amount of actives associated with the cuticle (AHT-HDAP) present; however, the kinetics remain relatively unchanged.

Chapter 8 - An investigation into hysteresis in hair water sorption isotherms

8.1 Introduction

8.1.1 Introduction on water sorption in keratin

The water content and water sorption behaviour of keratins such as hair is intrinsically linked with its mechanical behaviour and physiochemical properties^{42,43}. It is surprising that the water sorption behaviour of hair is still poorly understood despite it having been investigated for over 50 years. This Chapter aims to examine the role of the disulphide bond content, a crosslinking feature of keratin systems and hair fibres, on its water sorption isotherms.

Hair keratin is known to take up or sorb a large amount of water.⁴ As previously described, keratins such as hair exhibit water sorption isotherms which resemble classic type IV isotherms which are typical for gas adsorption in microporous materials. However, hair is not known to have any significant micropore volume or characteristics. While chemical treatments and damage to hair are known to affect its water sorption abilities,^{30,42,80} the characteristic that remains constant within all keratin water sorption profiles is the significant hysteresis that occurs between the sorption and desorption branches of the isotherm.^{4,36}

The physics of this hysteresis process is not well understood, and the fundamental understanding of the water sorption isotherms in hair is also not clear. Due to the high heterogeneity of hair fibres, it can be considered as an amorphous protein-based biopolymer

with crystalline fibular domains.^{4,23,36} This biopolymer classification allows the potential application of classical polymer models for water sorption to be applied, to further understand the thermodynamics and physics of the keratin-water system. The next section will discuss a few of these models, and their limitations when applying to hair-based keratins.

8.1.2 Modelling water sorption in keratins

8.1.2.1 The Brunauer Emmet and Teller Equation

It is common for all sigmoidal shaped isotherms, not just those associated with gas adsorption, to be attributed to multilayer sorption phenomena, with the first theoretical equation made to analyse these isotherms being the Brunauer, Emmet and Teller (BET) sorption equation.²²⁵ It is classically used with inert gas phase species such as N₂ at 77K to estimate the surface areas of porous materials and has become the experimental method of choice for these determinations. This model is used to describe a two-stage mechanism where first a monolayer is formed through adsorption, and further adsorption forms a multilayer. It has also been adapted by some researchers for analysing water sorption isotherms where:²²⁵

$$v(a_0) = \frac{v_{mB}c_B a_0}{(1 - a_0)(1 + (c_B - 1)a_0)} \quad (8.1)$$

where, in the case of water sorption isotherms, $v(a_0)$ is the amount of water sorbed per gram of sorbent at the water activity a_0 , v_{mB} the monolayer value per gram of sorbent, and c_B an

energy constant which relates to the difference in free energy between the water in the monolayer and water in liquid state.^{225,226}

This BET equation is used to calculate monolayer values, which in turn has good correlation to the number of polar side chains of the sorbent.²²⁶ It has therefore become a standard in evaluating monolayer values at low %RH, in part due to its simplicity, and also its approval from the International Union of Pure and Applied Chemistry (IUPAC) in using N₂ to measure the surface area of a material.²²⁷

When modelling the sorption behaviour of keratins however, at humidity's above 50 %RH, this model fails. This region is where keratin-water systems follow more of a Flory-Huggins type model, making it more akin to polymer liquid mixtures.²²⁶

The Flory-Huggins equation for polymers in water is as follows:²²⁸

$$\ln a_1 = \ln v_1 + v_2 + \chi_1 v_2^2 \quad (8.2)$$

where a_1 is high water activity, v_1 is the volume fraction of water, v_2 the volume fraction of polymer and χ_1 the Flory-Huggins interaction parameter. A negative χ_1 value corresponds to attractive interactions which lead to mixing of water and polymer. At high water activity or relative humidity's, the interaction parameter increases with increasing water content for keratins, and is modelled well.²²⁸ However, Flory-Huggins model has been shown to lack in accounting for volume changes in heterogenous structures such as hair, making it only applicable over a short water activity range.²²⁸

Another key issue with this model in the analysis of keratins is the lack of consideration to water uptake into the bulk of the fibre. While there may be multilayer formation of water molecules at the surface of the hair fibre, hair surface areas are small, being calculated using the BET method as approximately 0.2-0.5 m²/g.^{92,117,229} Multilayer absorption cannot therefore account fully for the sorption properties of hair, which absorbs up to 20-25% by weight of water at 90 %RH.^{30,83,93,118,212}

8.1.2.2 The Guggenheim Andersen and de Boer Equation

A closely related model to the BET equation is the Guggenheim Andersen and de Boer (GAB) sorption equation:

$$v(a_0) = \frac{v_{mG}c_Gka_0}{(1 - ka_0)(1 + (c_G - 1)ka_0)} \quad (8.3)$$

Where v_{mG} is the GAB calculated monolayer capacity, and c_G the analogue to the energy constant c_B . The key assumption in this model is that subsequent layers beyond the initial monolayer have the same sorption state, which is different to that of bulk water. For this behaviour, an addition constant k is included as a measure of the energy difference between these two states.²²⁷

The GAB equation is popular due to its close relation to the BET equation and applicability to a wider range of %RH values.¹¹⁸ The GAB equation has been applied to keratins,^{55,118,230} but still remains a simplistic model for a complex material, not able to describe the hysteresis phenomena. From a simple mathematical perspective, the introduction of an additional fit

parameter within this equation virtually guarantees improved levels of fit with experimental data compared to the BET model, but it does not guarantee any improved fundamental correctness in the physics for the model.

8.1.2.3 Keratin specific water sorption models

Some models have been created to apply directly to keratin-water systems. Rosenbaum built upon the Flory-Huggins model to add a compressibility term to account for volumetric changes in hair induced by moisture uptake, however this model has not been thoroughly examined.^{228,231} In this theory of sorption, the initial portion of the isotherm is associated with water saturation of chemical sites with high affinity such as polar groups/ionic groups, whereas high %RH water sorption is associated with uptake at weaker sites, including swelling stresses, and condensation.²³¹

A similar approach is modelled with the Watt and D'Arcy model for wool. This model includes two types of monolayer absorption, including three interaction terms in total.²³² The first term relates to monolayer absorption at strong hydrophilic sites (<20 %RH), the second term relating to monolayer absorption to weaker sites (<40 %RH), and a third term for multilayer formation after 60 %RH. This model is in good agreement with hydrogen-deuterium exchange methods in another complex biopolymer, wood, which determined that the quantity of accessible hydroxyls reaches a maximum at 60 %RH.²³³

There is some consensus in a three-mechanism/three term model for water sorption in hairs dependency on humidity, with Zviak suggesting that:²³⁴

- below 5 %RH water binds strongly to specific hydrophilic groups in amino acid side chains

- between 5 and 75 %RH water is absorbed into weaker sites with hair or to previously bound water molecules
- above 75 %RH clusters of water molecules are formed

For the all the fitting successes of the aforementioned models, none go far in explaining hysteresis in hair, or satisfactorily validating the sorption mechanism they propose. The next Section will discuss the current understanding of hysteresis in complex biomaterials such as hair.

8.1.3 Hypotheses for hysteresis in water sorption isotherms

8.1.3.1 Classical causes of hysteresis

There are many hypotheses for why water vapour hysteresis occurs in water sorption isotherms in polymers. These include:

- hysteresis arising from irreversible water sorption
- capillary condensation in pores²³¹
- pore deformation by sorbate²³⁵
- slow equilibration of sorbent^{231,236}
- relaxation of sorbent structure
- formation of hydrogen bonds²³⁴
- greater access to active sites upon moisture uptake^{42,44}

The next section will look at the historical and current explanations for hysteresis in more complex biological materials.

8.1.3.2 Hysteresis in complex biological materials such as cellulose or wool

One of the earliest explanations for hysteresis in wool-water systems was proposed by Smith in 1947.⁴⁴ Since then, numerous studies on different biological materials such as wool, wood and cellulose have made some way in confirming his hypothesis that the ability of the fibre to swell corresponds to the presence of hysteresis in isotherms.^{44,115,237–240}

Smith proposes that the greater proportion of bound water in desorption isotherms that result in hysteresis, arises from swelling of the fibre during the sorption stages of the isotherm.⁴⁴ Water vapour diffuses through the CMC and endocuticle into amorphous regions of keratins and then forms hydrogen bonds with amide groups.⁴ Deuterium-hydrogen exchange studies in wool have demonstrated that sidechains of the crystallites (asparagine and glutamine) are accessible to water, allowing crystallites to swell.²⁴¹ This swelling results in breaking of hydrogen bonds in the keratin structure, to form bonds with water.^{44,165,236} Free energy is lost from water in the gas phase, which is then transferred to the solid, resulting in conformational changes only otherwise accessible at higher temperatures, with some energy dissipating.^{231,237,238} Changes in keratin protein conformations has indeed been observed with varying degrees of hydration.²⁴²

These structural changes are associated with a somewhat 'glassy' state transitioning to a 'rubbery' state.^{228,243} This transition is not a sharp transition as seen for glass transition temperatures (T_g) in other pure polymers, instead different segments of the glassy state become unfrozen/mobilised at different moisture contents.^{228,243}

During the desorption phase, water is lost, but as the material now passes through to a glassy state, the glassy structure cannot undergo conformational changes to fill these void volumes

once occupied by water, resulting in “static voids”, as proposed by Rosenbaum.²²⁸ The conformational changes that can occur result in expenditure of free energy available for water to become gas, but some of this free energy is also dissipated. This means that not all potential transfer of water to gas occurs, leading to a higher bound fraction in of water in the desorption isotherm, resulting in hysteresis.²³⁷

This thermodynamic model was confirmed with computational studies of hysteresis in cellulose-water systems performed by Chen *et al.*²⁴⁴ During sorption cellulose-cellulose bonds break from swelling and water-cellulose bonds form. Due to the strong energy associated with cellulose-water hydrogen bonds, the cellulose-cellulose bonds do not reform at the same %RH they were broken at, resulting in hysteresis.²⁴⁴

Physical barriers, as well as thermodynamic barriers, may also trap water in complex materials: a collapsing structure upon shrinking,⁴⁴ or glass formation preventing diffusion.^{239,245}

8.1.3.3 The impact of swelling and crosslinks on hysteresis

Overall, the ability of a material to swell increases sorption by breaking structural interactions, creating an amorphous structure through conformational changes, and even creating new porosity in some materials.^{235,246,247} All of these factors suggest a material able to significantly swell will have a ‘glassy state reformation’ occurring at lower %RH and reducing hysteresis if indeed, these conformational changes are responsible for hysteresis.²³⁷

This hypothesis has been proven experimentally: crosslinked celluloses and hydrogels with lower amorphous content demonstrate greater hysteresis than non-crosslinked counterparts.^{247,248} Kim *et al.* demonstrated that reduced backbone connectivity in lysozyme

(i.e., lysozyme versus an amino acid mixture with same composition as lysozyme) showed greater sorption and less hysteresis, related to an increase in solvent-accessible surface area at high %RH.²⁴⁹

It is well understood that the reduction of crosslinks density in polymers allows further swelling and entry of water to allow plasticisation,¹⁶⁵ whereas a perfectly rigid protein simulation was shown to have no hysteresis,²⁵⁰ demonstrating again that swelling ability is key in allowing plasticisation.

The swelling ability of a polymer therefore directly influences T_g heavily. It has been directly observed that the onset of T_g and the onset of hysteresis correlate,^{228,243} with no hysteresis observed at all in materials such as hydrogels and mucins above their moisture induced T_g .^{245,251,252} Such observations provide key evidence of the intrinsic link between hysteresis and T_g . Hysteresis should therefore be temperature dependent; with the ability at elevated temperatures to cross through T_g at lower moisture contents, hysteresis in isotherms should reduce. This has been directly measured in hair keratin water systems, further highlighting the role of glass transition in hair hysteresis.²⁵³

8.1.3.4 The Vrentas-Vrentas model

In 1991 Vrentas and Vrentas devised a model for sorption which highlighted the importance of the T_g in sorption behaviour for polymers.²³¹ While originally devised for the sorption of organic vapours in non-polar polymers, it was then applied to water sorption in hydrophilic pharmaceuticals.²³¹

The significant improvement of the Vrentas-Vrentas equation mirrors the model proposed by Rosenbaum by considering free volume changes below glass transition temperature due to

plasticisation.²²⁸ Above the T_g for a polymer when the material is rubbery, the model reduces to the Flory-Huggins model at high %RH, meaning the isotherm can model both the glass and rubber states of polymer sorption.^{231,243} For wool, the Vrentas-Vrentas model was used to successfully model experimentally determined isotherms from Watt and D'arcy.^{228,232} However, Mavraki demonstrated that sorption behaviour still could not be fully described by the Vrentas-Vrentas equation, even with an inclusion of a T_g term.²⁵⁴

8.1.4 Glass transition in hair

For such a heterogeneous material such as human hair, the existence of a well-defined glass transition temperature has been controversial. However, while hair is extremely heterogeneous, depression of T_g in keratins as a function of water has been shown to allow T_g at ambient temperatures, with wool exhibiting a T_g at 15°C when wet.^{243,253}

Yet, the presence of hysteresis in hair at even 90 %RH demonstrates that not all amorphous content is plasticised by water, and that in fact hair may have multiple T_g 's relating to mobility of different regions in the fibre.^{228,243,254} These regions are likely to be the more amorphous regions of the CMC and matrix; X-ray diffraction has demonstrated that water does not significantly affect the structure of the crystalline regions of KIF's.^{35,234} This diffuse onset of T_g is often seen in proteins,²³¹ suggesting it may be more correct to refer to 'onset' of glass transition events in hair, than defining a T_g at a specific temperature.

The ability of a keratin fibre to swell is therefore intrinsically linked to its T_g , and hysteresis properties. Swelling in keratins is limited by inter-microfibrillar linkages, with more direct bonding between protein molecules in the matrix at low humidity's.¹⁷² To significantly change

swelling properties, and in turn the onset of T_g in hair, covalent bond breaking in the fibre must be achieved.

8.1.5 Aims

This Chapter examines the role of swelling and its potential contribution to hysteresis in keratin water sorption isotherms. Semmence demonstrated that chemical treatments such as perming, relaxing and bleaching resulted in increased lateral swelling of hair fibres in water in comparison to untreated hair.⁷ As swelling in hair is largely governed by disulphide bonds and electrostatic interactions between KIFs, in this study hair samples were reduced to decrease the disulphide bond content using thioglycolic acid, the active ingredient in perms, which Semmence reported to result in the highest swelling of the fibres.⁷ The disruption of electrostatic bonding in hair was achieved by dyeing hair samples using a commercial method, a process which has been shown to significantly affect electrostatic interactions in hair.¹⁰⁰

Finally, Dynamic Vapour Sorption (DVS) was used to characterise the water sorption isotherms. Damage in the samples was further characterised using solid-state Nuclear Magnetic Resonance (NMR) and thermogravimetric analysis (TGA). The reduced keratin samples in this work were prepared for methylation in collaboration with Kitty Lu as a part of her MSc studies, who also performed the TGA, and DVS experiments reported here.

8.2 Materials and Methods

8.2.1 Preparation of hair samples

All hair used in this work was natural white untreated Caucasian hair, originating from a batch source and provided as a gift from Procter and Gamble.

8.2.1.1 Methylated hair

To examine the role of swelling in the hysteresis of hair, it was important to reduce hair to different levels to examine how the changing fraction of reduced disulphide bonds affect the hairs properties. Nishikawa *et al.* devised a straightforward method for the reduction and subsequent methylation of S-S sites in hair, and this method was adapted in this work.⁵⁴

The methylation step in the synthesis scheme was integral to prevent the oxidation of the reduced –SH sites which are known to reform disulphide bonds over time.⁴ Approximately 2 g of hair was chopped into 0.1 cm lengths. Hair was reduced with 7% (wt/wt) ammonium thioglycolic acid (pH 9.6) in sealed vials at 45°C for lengths of 5 min, 10 min, 15 min and 20 hrs (Figure 8.1).

20 hr of reduction was reported to lead to full reduction of the disulphide bonds in hair.⁵⁴ Samples were rinsed with DI water five times and soaked in 0.1 M potassium nitrate. The pH was then adjusted to 4.0 using 0.1 M HCl. The newly formed thiol groups were then methylated with iodomethane (MeI) for 24 hrs, in the dark, stirring at room temperature.

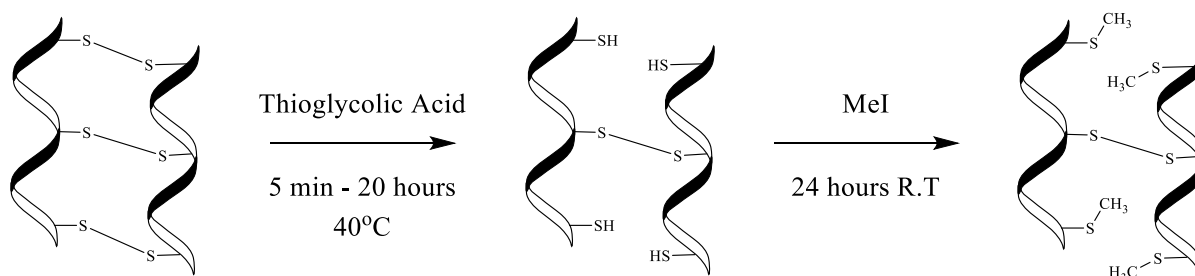


Figure 8.1 The reaction scheme for reduction and methylation of untreated hair samples, to reduce overall S-S content.

Repeated centrifugation (20°C, 2500 rpm, 10 min) removed excess MeI from the samples which were subsequently washed with DI water. Thioglycolic acid was removed via vacuum for the first 5 days at 50°C, and then 4 more days at 75°C.

Ammonium Thioglycolate (ca. 60% in water) was purchased from TCI Europe, potassium nitrate from Scientific Laboratory Supplies Ltd., HCl from Sigma-Aldrich, and MeI from Fisher Scientific Ltd.

8.2.1.2 Preparation of dyed hair

Preparation of dyed hair samples has been previously described in Chapter 6, Section 6.2.1. Hair was dyed with 4-amino-2-hydroxytoluene (AHT), 1-hydroxyethyl-4,5-diamino pyrazole (HDAP) to produce the red dye product AHT-HDAP.

H₂O₂ was purchased from VWR International Ltd, AHT and MEA from TCI Europe, and HDAP purchased from Fluorochem Ltd.

8.2.2 Solid state ¹³C cross polarisation / magic angle spinning (CP/MAS) NMR spectra

Approximately 0.08 g of treated hair samples were chopped to 0.1 cm lengths and soaked in water for a few minutes. Damp fibres were packed tightly into 4 mm ceramic NMR rotors. The ¹³C CP/MAS NMR spectra were acquired using a solid-state Bruker Avance III 200MHz NMR spectrometer, with the minimum number of scans being 30,000 and a spinning frequency of ≈6 kHz. Line fitting of the spectra was achieved using *MeStreNova 14.1.2* software. Analysis

was repeated three times for each spectrum to average any error encountered in manual phasing of the spectra.

8.2.3 Thermogravimetric measurements

The TA Instruments Q500 TGA instrument was used to record the thermogravimetric curves of the samples investigated. To remove loosely bound water, hair was conditioned for 24 hrs at 40°C. ≈ 10 mg of sample cut to lengths of 0.1 cm were loaded into platinum pans. The heating program proceeded as follows:

1. 30°C temperature held for 5 min to remove any ambient water.
2. Heating ramp to 180°C at a rate of 5°C/min.
3. 180°C temperature held for 20 min.
4. Temperature reduced to 30°C at a rate of 5°C/min.

All measurements were performed under nitrogen, with a 60 ml/min flow rate.

8.2.4 Water sorption isotherm measurements

Water sorption isotherms for the reduced samples were obtained at 25°C using a DVS Endeavour from Surface Measurement Systems (London, UK), while isotherms for the dyed and untreated samples were measured using a DVS Advantage from Surface Measurement Systems (London, UK). 20-30 mg of sample chopped to 0.1 cm lengths were placed into DVS pans and conditioned at 0 %RH for 1400 min, until an equilibrium dry mass was reached.

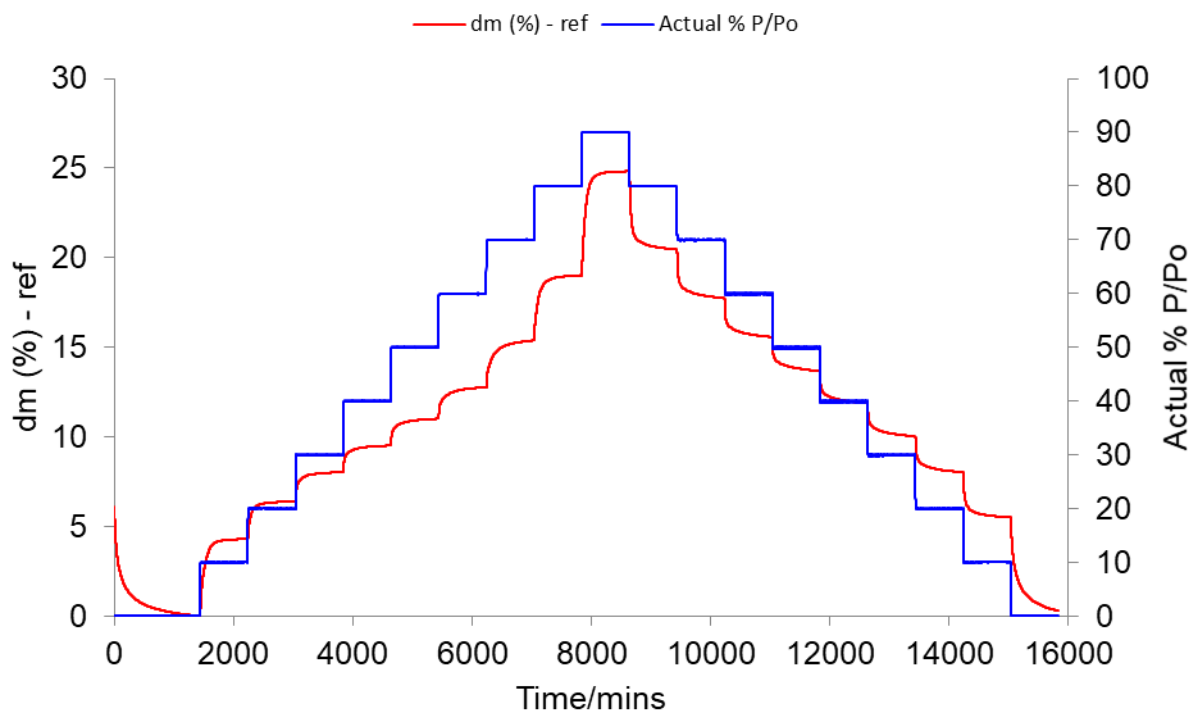


Figure 8.2 A DVS water isotherm for untreated hair, including a 24-hour drying step *in situ* at the start. The red line shows the change in hair mass with respect to the starting mass of hair. The blue line denotes the required humidity as % P/P₀.

The humidity was increased by 10 %RH step increases, each step lasting 800mins to allow for full mass equilibrium, which allows hysteresis to be fully characterised.^{231,236} After reaching 90 %RH, the humidity was decreased back to 0 %RH in 10 %RH steps (Figure 8.2). The total experimental procedure lasted 11 days. Experiments were conducted with a carrier flow rate of 200 sccm. The equilibrium values of mass at each humidity step were used to calculate the sorption and desorption branches of the isotherms for each hair sample.

8.3 NMR Verification for methylated samples

To confirm the success of the reduction process, ¹³C CP/MAS NMR was used to examine the extent of methylation in the samples. Figure 8.3 shows the ¹³C CP/MAS NMR spectra for the dyed, untreated, 5 min, 10 min, 15 min and 20 hr reduced samples. Table 8.1 shows peaks

found within the different spectra of all the hair samples. The peak at approximately 15 ppm denotes the site of methylation in the treated samples⁵⁴. The reduced samples all have an increased peak at 15 ppm compared to the untreated sample, with the 20 hr reduction showing the highest increase in the peak intensity. The differentiation between 5, 10 and 15 min reduced samples is not clear.

Table 8.1 Assignments of the chemical peaks in the ^{13}C CP/MAS spectra of different hair samples⁶⁵

Approximate chemical shift (δ ppm)	Assignment
11	δ -CH ₃ of isoleucine
15	β -CH ₃ of alanine (α h)
19	γ -CH ₃ of valine
24.5	δ -CH ₃ of leucine and δ -CH ₂ of proline
29	β -CH ₂ of glycine (α h), β -CH ₂ of trans proline
32	β -CH ₂ of cis proline, β -CH of valine (β s), β -CH ₂ of glutamic acid (β s)
35	β -CH ₂ of lysine (β s), β -CH ₂ of asparagine (α h)
39	β -CH ₂ of leucine (α h), β -CH ₂ of crosslinked cystine ⁶⁷
48	δ -CH ₂ of proline
53	α -CH of glutamine (β s) ⁵² , α -CH of lysine (β s), α -CH of alanine (α h)
56	α -CH of serine (β s), α -CH of leucine (α h)
60	α -CH of phenylalanine (α h), α -CH of tyrosine (α h), α -CH of proline
66	β -CH ₂ of serine (β s) and β -CH of threonine (β s)
120-130	Aromatic CHs of phenylalanine and tyrosine
158	O-C of tyrosine
172-176	Carbonyl for all amino acids

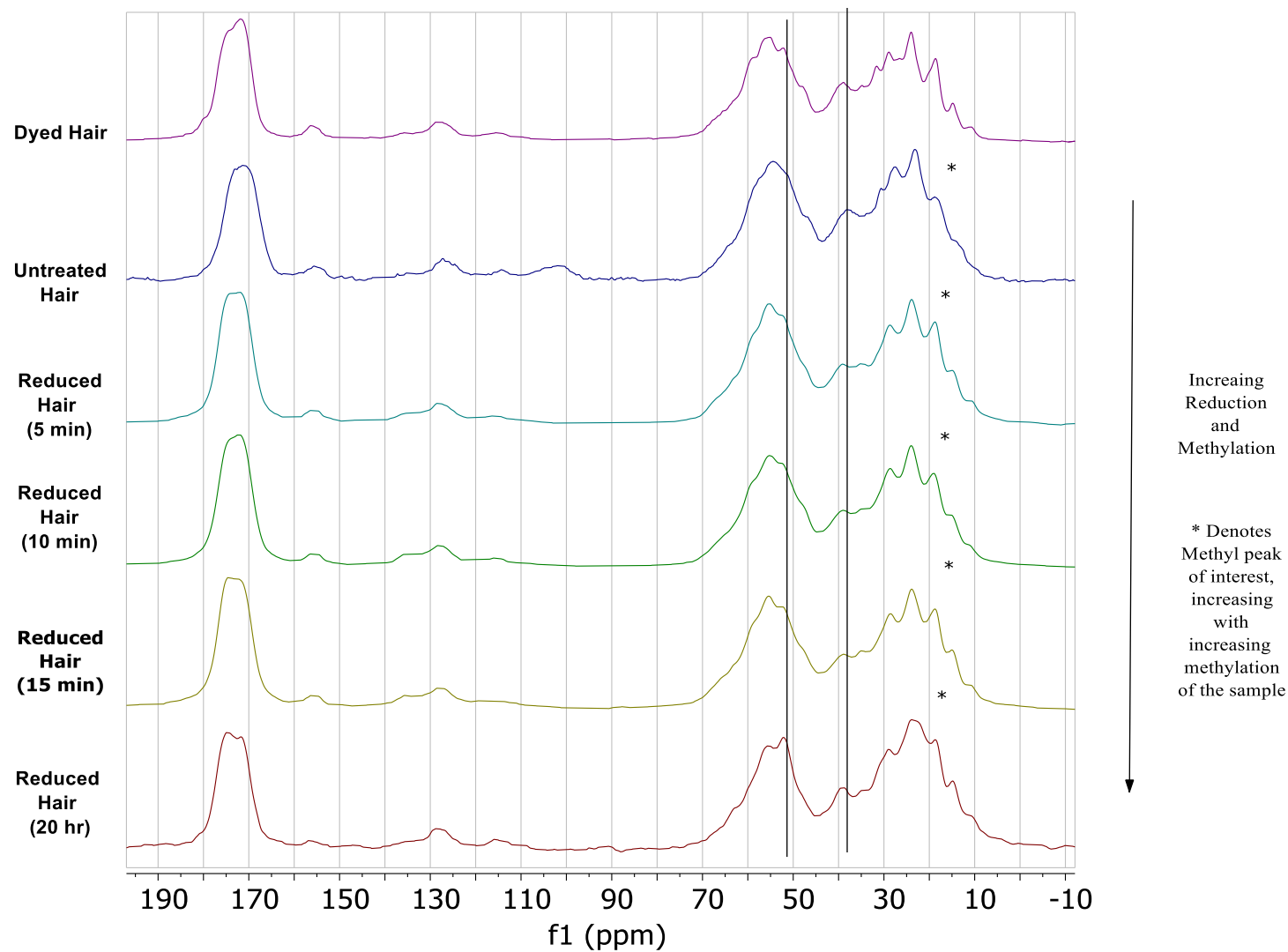


Figure 8.3 The ^{13}C CP/MAS spectra for dyed hair, untreated hair, and the reduced and methylated samples. * indicates the methyl peak at approximately 15 ppm, which increases with increasing methylation. Dotted lines show peaks at approximately 53 ppm for α -CH of glutamine (β s),⁵² and 40 ppm for β -CH₂ of crosslinked cystine.⁶⁷

As the methylation quantification relies on the natural abundance of ^{13}C in the methyl groups, small differences in methylation may not be clear. ^{13}C labelling of the methyl iodide would better show up in the samples to better distinguish the degree of methylation between samples.

Other spectral features of interest can inform on structural changes occurring in the keratin structure. At approximately 53 ppm, resonances for $\alpha\text{-CH}$ of glutamine (βs), $\alpha\text{-CH}$ of lysine (βs) and $\alpha\text{-CH}$ of alanine (αh) are found. Glutamine is one of the most common amino acid residues in keratin, making up 12.1% of the total amino acid content in wool.⁵² The increase of this peak therefore indicates a greater presence of β -sheet glutamine and in turn, that there is a higher amorphous content of keratin in the dyed and reduced samples.

The peak at approximately 39 ppm correlates to the resonances $\beta\text{-CH}_2$ of leucine (αh) and the $\beta\text{-CH}_2$ of crosslinked cystine.⁶⁷ Reduction of this peak indicates breaking of disulphide bonds and the success of the reduction procedure. As discussed in Chapter 7, the relative S-S content can be calculated as a ratio of this peak, and the peak at approximately 24 ppm associated with resonances of $\delta\text{-CH}_3$ of leucine and $\delta\text{-CH}_2$ of proline (Equation 7.1) Due to the lack of α -helix or β -sheet keratin conformational dependence on this peak, it was chosen as a reference and would not be expected to change with chemical treatment of the hair fibres.

$$\frac{{}^{13}\text{C } \beta - \text{CH}_2 \text{ of crosslinked cystine intensity}}{{}^{13}\text{C } \delta - \text{CH}_3 \text{ of Leu and } \delta - \text{CH}_2 \text{ of Pro intensity}} = \text{Relative S - S content} \quad (7.1)$$

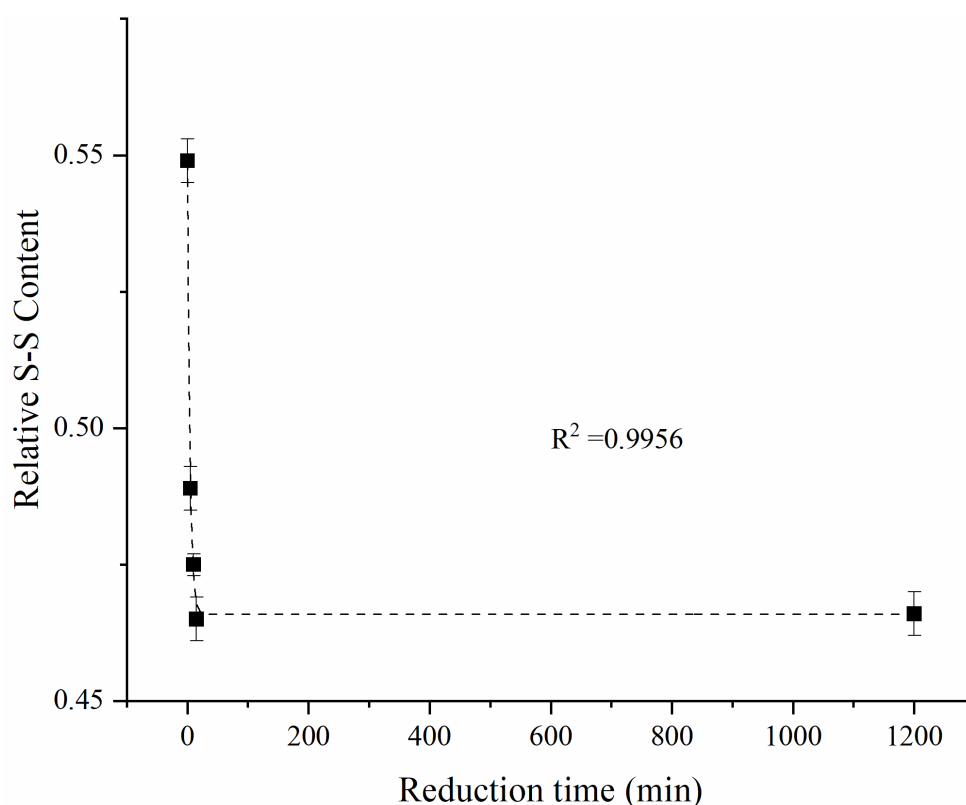


Figure 8.4 The relative S-S content for untreated hair and the reduced and methylated samples as a function of reduction time. As reduction time increases there is an exponential decay in disulphide bond content in the samples. (Error bars = 1σ , where $n = 3$).

The relative S-S content of each hair sample was plotted against the reduction time for the experiments to observe the success of reduction (Figure 8.4). From these results there is a clear exponential relationship between reduction time and disulphide bond content, with most of the disulphide bonds being broken after 15 min of reduction.

The reduction process primarily affects the disulphide bonds present in the hair. To compare the effect of damage between the methylated samples and untreated hair, dyed hair was also investigated. This hair was treated with the AHT-HDAP dye solution previously described in Chapter 6. The damage in this hair will include other oxidative damage from the hydrogen peroxide present in the dye procedure and interruption of ionic bonds from charged chemical

species in the dye formulation.¹⁹⁷ However, this dyeing will be a good comparison for how the methylation of the samples have affected the behaviour of the reduced samples.

8.4 Measuring Damage in the treated hair samples

Before examining the effect on hair treatment on the hysteresis profiles for the samples, experiments were undertaken to further understand the damage caused to the hair samples through the dyeing and reduction treatments. This further informs on any differences in the water sorption profiles.

8.4.1 NMR Damage

Chapter 5 demonstrated the use of ^{13}C CP/MAS NMR to quantify damage in hair by measuring the Relative α -Keratin content of the samples (Equation 5.2). The carbonyl peaks of the ^{13}C CP/MAS spectra for the untreated, dyed, and reduced samples were analysed, and the Relative α -Keratin content calculated to determine the amorphous content of the fibres. These were then correlated to the relative S-S content for the samples, showing a strong positive correlation (Figure 8.5).

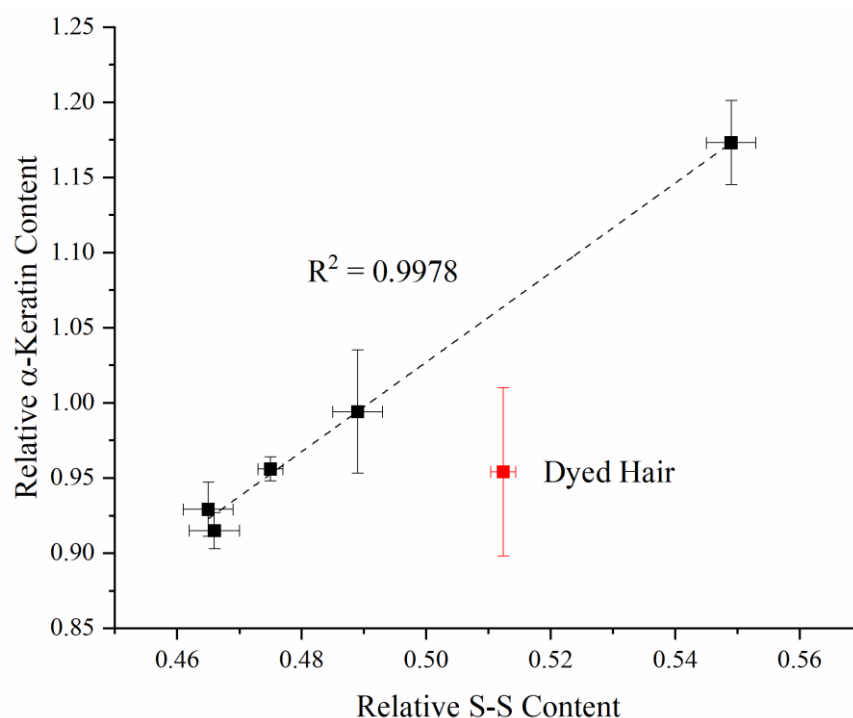


Figure 8.5 The Relative α -keratin content for the samples investigated, correlated to their relative S-S content. (Error bars = 1σ , where $n = 3$). The data point for dyed hair is not included in this linear fit, as additional metal ions and alkalisers present in the fibre will reduce the relative α -keratin content through disruption of electrostatic interactions in the fibre, without reducing the relative S-S content. .

With increasing bond breaking in the samples, the amorphous content of hair will increase, as is reflected in Figure 8.5, with lower relative α -keratin contents. An increased amorphous content will allow increased swelling in the damaged samples, and a potential depression in the onset of T_g for glassy regions in the hair fibres. The outlier in this graph is for the dyed hair sample, and has not been included in the linear fit. Dyed hair also contains MEA and traces of metal ions, which have been shown to interrupt the electrostatic and ionic interactions within keratin, leading to a greater disordered content.¹⁰⁰ This may result in the lower Relative α -

Keratin content in the dyed hair fibre whilst not having corresponding reduced relative S-S bond content.

8.4.2 Bound water content in the hair samples

Thermogravimetric Analysis (TGA) was used to measure the water content of the investigated hair fibres. The untreated, dyed, and reduced hair samples (a TGA curve was not obtained for the 15 min reduced sample) were conditioned overnight at 40°C to remove loosely bound water. This allows the water content measurement to correspond to the bound water present in the samples, which is less liable to variation than loosely bound water in ambient conditions.

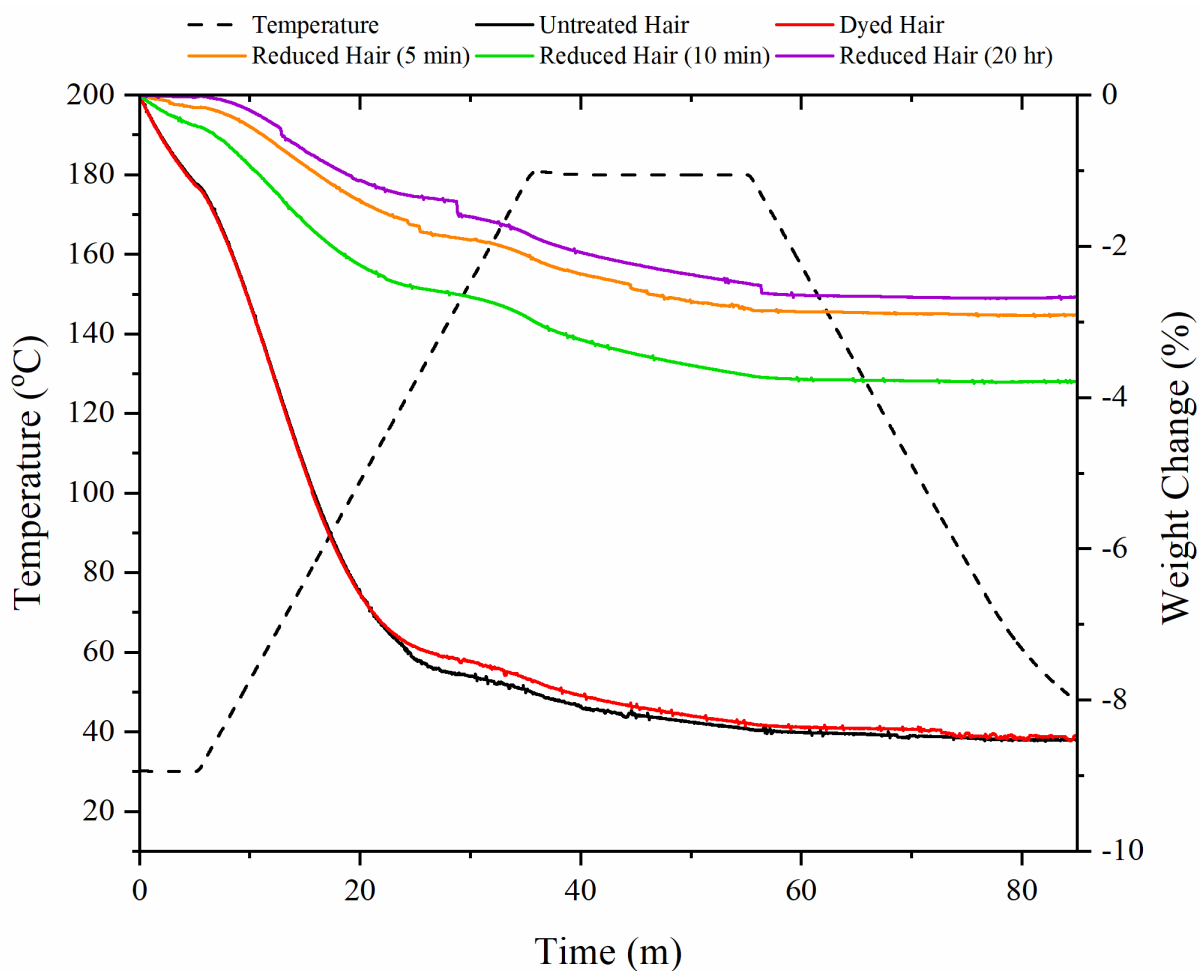


Figure 8.6 The mass loss curves of bound water for the untreated, dyed, and reduced samples measured using TGA. Reduced samples have less bound water content than untreated and dyed samples. An additional mass loss step for damaged samples at $\approx 160^{\circ}\text{C}$ indicates increased keratin disorder.

The samples were then heated to 180°C to remove all the water from the samples without completely degrading the hair fibres, which occurs from 210°C .⁸⁰

The TGA curve for untreated hair demonstrates the characteristic mass loss profile for keratin fibres (Figure 8.6).^{80,88} Up to $130\text{--}140^{\circ}\text{C}$, the mass loss is related to loss of water dissolved in the samples, while above 140°C , mass loss is due to the denaturation of α -keratin.⁸⁰ From this temperature onwards, there is a plateau in the mass loss profile. For the 20 hr reduced

hair sample, jumps in weight changes are likely due to experimental error and not associated with any true thermal events.

Across all the damaged samples, there is a more pronounced second stage of mass loss – between 160°C and 170° C. The treatments of these hair samples caused damage which lead to disorganisation in the hair matrix, through destruction of disulphide bonds and also disruption of ionic bonds in the dyed sample.¹⁰⁰ The disorganisation has been proposed to lead to different melting points of the matrix, correlating to the second mass loss event observed.⁸⁸

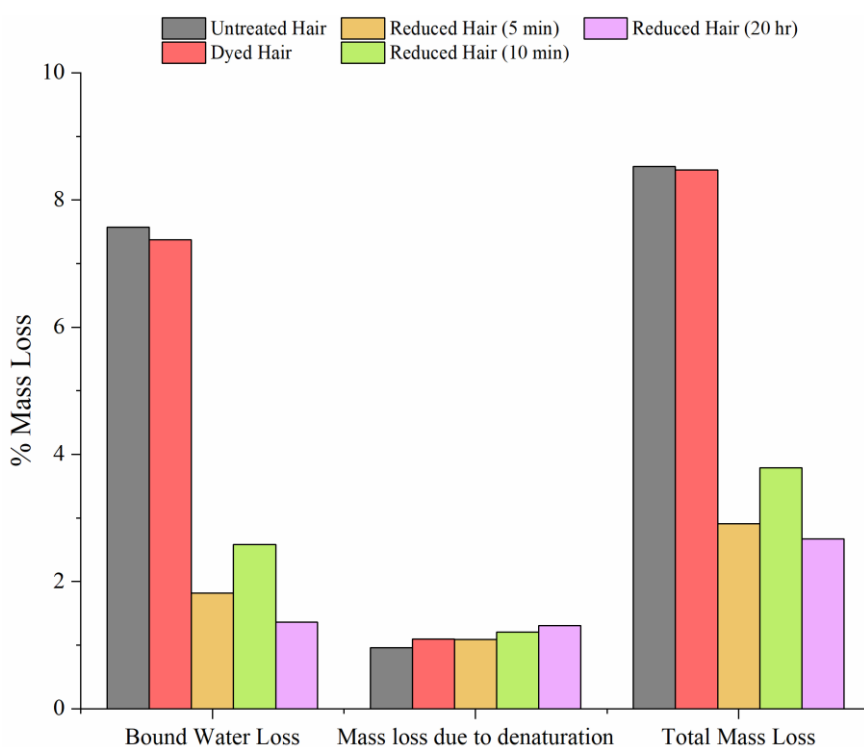


Figure 8.7 The % mass loss for the hair samples, calculated from the TGA curves. This graph shows the mass loss attributed to loss of dissolved bound water, mass loss due to denaturation of alpha keratin and total mass loss in the samples.

Figure 8.7 shows the contributions to mass loss in the hair samples during the TGA experiments, relating to mass loss due to bound water, desorption, and denaturation. In comparison to the untreated hair, dyed hair has a reduced water content, agreeing to some reports examining water content in damaged hair.⁸⁰ Methylated samples show a dramatic decrease in total bound water content; this reduction could result from the methylation of the samples, making the hair more hydrophobic and reducing their ability to absorb moisture from the air. Increasing methylation does not seem to correlate with reducing water content, with the 15 min reduced sample having the lowest water content, instead of the expected 20 hr sample. This again points to the heterogeneity of the fibres not allowing for clear resolution between the reduced samples.

The mass loss due to denaturation is increased in the damaged samples, with the reduced samples having the highest mass loss in this stage. Disruption of α -keratin from treatments will generate less stable amorphous keratin content, and so with increasing damage from treatments would result in a higher mass loss as the amorphous keratin melts. This agrees with the ^{13}C NMR analysis which measured lower relative α -keratin contents in damaged fibres.

8.5 Measuring hysteresis in water sorption isotherms

8.5.1 Water Sorption Isotherms

Figure 8.8 shows the water sorption isotherms for untreated, dyed and the 20 hr reduced hair samples. These isotherms show the typical isotherm behaviour for keratins.¹³⁹

The untreated and dyed hair samples show relatively similar sorption profiles. In the reduced samples however, the lower sorption of water at low %RH suggests a reduction in availability

of hydrophilic groups.^{228,232,234} The methylating procedure is not specific; nucleophilic amino acid sidechains (e.g., glutamine, lysine, tyrosine etc.) may have also been methylated to some degree, that, while not clearly identifiable in the ^{13}C CP/MAS NMR spectra, may have a significant impact on water sorption. Between the reduced samples, there is little variation in either sorption or desorption. From the NMR analysis it has been established that the differences between these samples are not easy to distinguish – this inability could be down to error in the procedure, or that the heterogeneity of the fibres is preventing subtle differences in the hair properties to be clearly noticed.

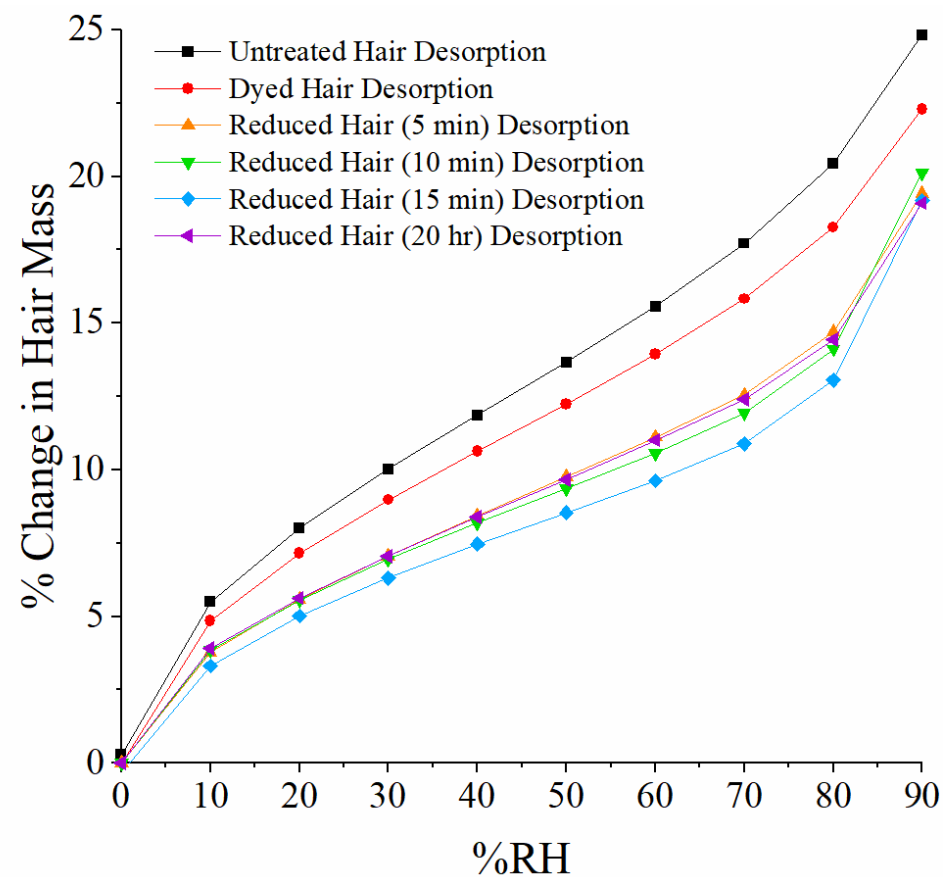
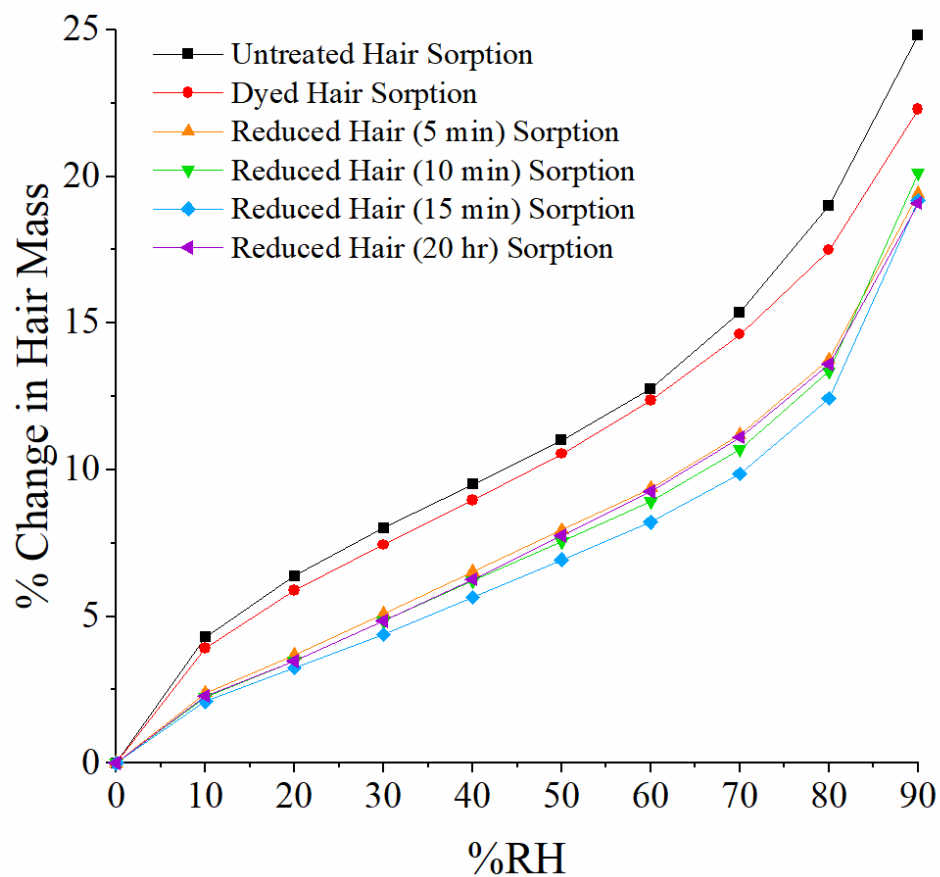


Figure 8.8 The sorption (left) and desorption (right) curves for untreated, dyed, 5 min, 10 min, 15 min and 20 hour reduced hair samples between 0 and 90 %RH. Damaged samples have decreased water sorption in comparison to untreated hair, with reduced samples being most significantly affected.

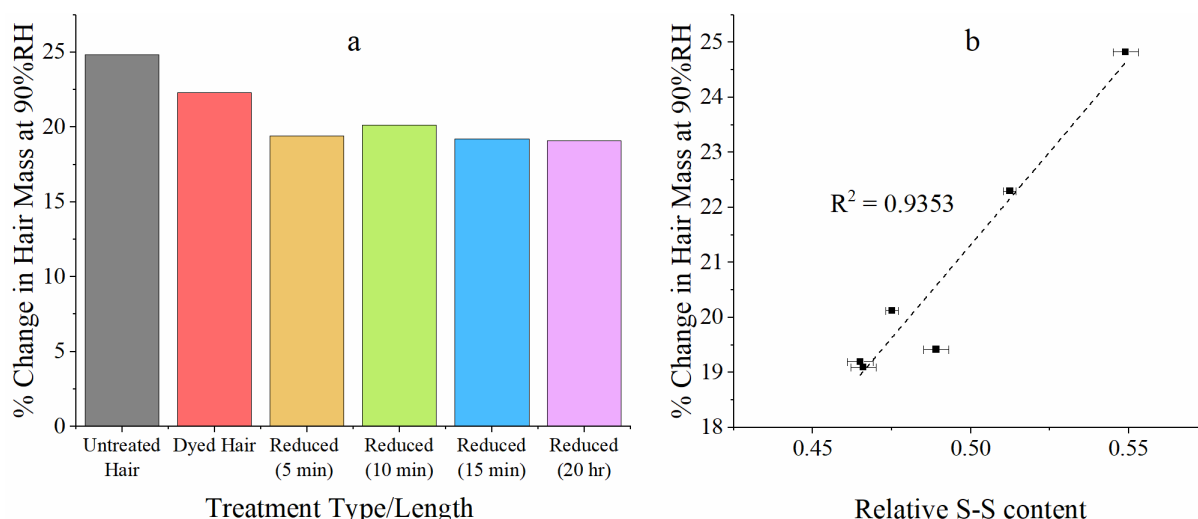


Figure 8.9 a) Total mass uptake at 90 %RH from water sorption and b) total uptake plotted against the relative S-S bond content of the hair samples. Samples with reduced S-S content take up less water. (Error bars for S-S content = 1σ , where $n = 3$).

All damaged samples show a reduction in total sorption capacity at 90 %RH, with lower % changes in hair mass (Figure 8.9a). Untreated hair had a greater water sorption while the reduced sample have on average 6% less uptake. There is less variation between these reduced samples which could be due to the heterogeneity of the fibres.

Damaged hair, and complex materials with lower crosslink densities (such as dextrans and resilin-mimetic gels) are normally attributed to having increased water sorption.^{212,247,255} This is in contrast to the correlation from these results that increasing S-S content results in increased water sorption (Figure 8.9b). However, there has been recorded sorption isotherms where bleached samples had a decreased water sorption, as is seen in the dyed hair sample which has been treated by bleach during the dye procedure.²³⁰ The reduced sorption in reduced samples could be resultant of the methylation procedure making the hair more hydrophobic and less absorbing of water (as previously discussed). Therefore, Figure 8.10b could be explained simply in terms of the decreases in native S-S crosslink resulting in the

creation of new hydrophobic domains. While it might be anticipated that lower cross link densities would result in greater water sorption, these decreases in cross-link density also correspond to increases in hydrophobicity – which appears to be the dominant effect in total water sorption capacity.

8.5.2 Hysteresis

The differences between the sorption and desorption isotherms of water were calculated and plotted as hysteresis graphs in Figure 8.10. There are very distinct hysteresis profiles between untreated and damaged hair samples, in both terms of the magnitude of hysteresis, and the %RH at which the maximum hysteresis is observed. This information in itself is surprising; observations in hysteresis for hair of different ethnic origin and different chemical treatment showed no significant differences in hysteretic behaviour.^{83,253}

Above 30 %RH, untreated hair has the highest hysteresis values, with all damaged samples having lower hysteresis values. Dyed hair has lower hysteresis values than untreated hair across all %RH values. This reduction in hysteresis with reduced crosslinking from damage agrees with other studies, where reducing crosslinks in wood, lysozyme and bamboo all resulted in a reduction in the magnitude of hysteresis.^{239,248,249,256} It is the first time however that this effect has been directly observed in hair keratin.

Whilst ¹³C CP/MAS NMR has demonstrated that the reduced samples have lowest S-S contents, at lower %RH the reduced samples have the highest hysteresis values. This may be associated with the reduction of hydrophilic sites available for sorption, however the occurrence of maximum hysteresis at low %RH values may also indicate changes in the onset for T_g in the hair samples.

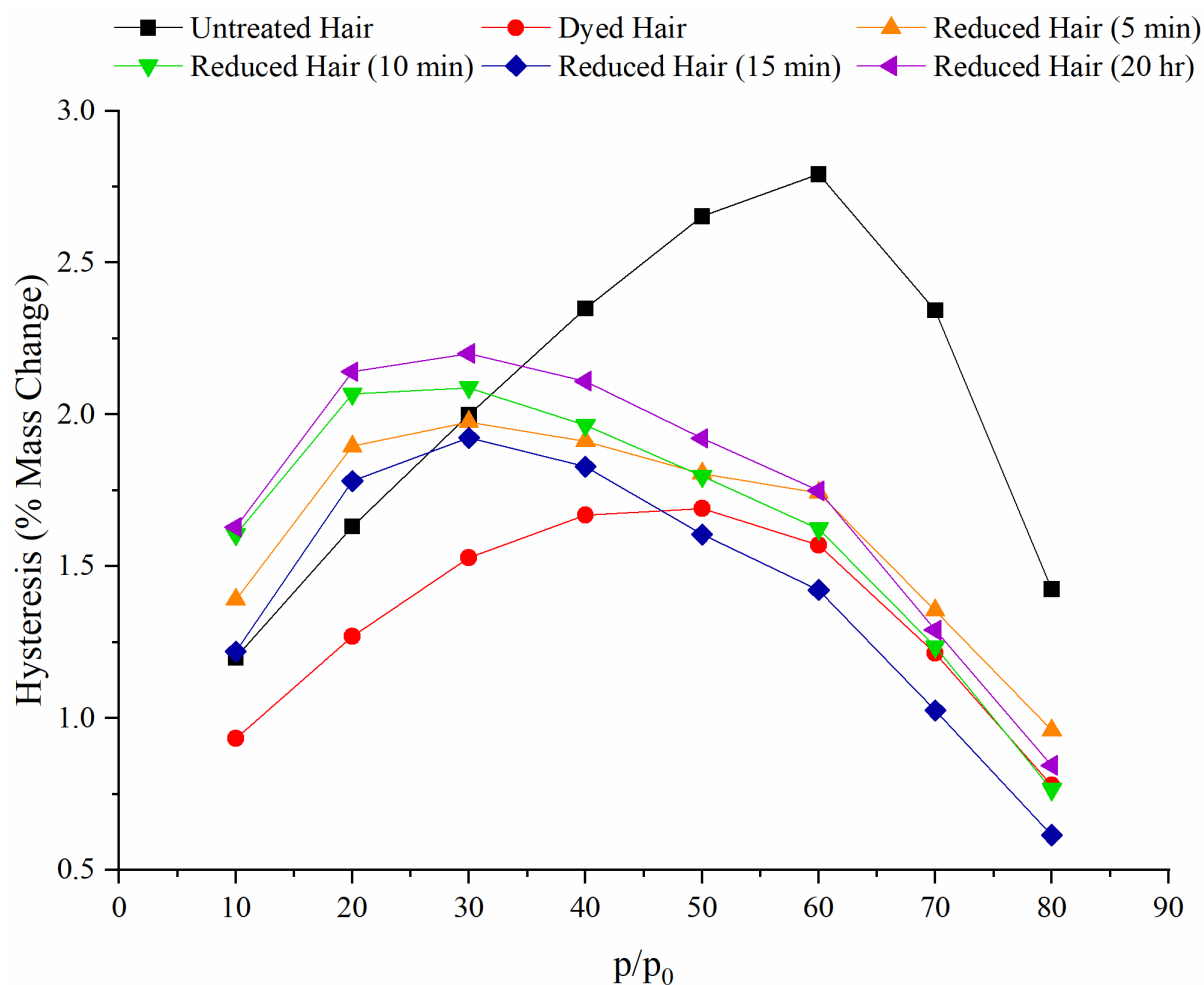


Figure 8.10 The hysteresis plots for the untreated, dyed, and reduced hair samples. Reduced hair samples have greater hysteresis at low p/p_0 , while untreated hair has the greatest hysteresis at higher p/p_0 . The p/p_0 for maximum hysteresis has therefore moved to lower p/p_0 values as hair becomes more damaged.

As discussed in previous sections, and observed by many different groups within different polymeric materials; when a material undergoes a glassy to rubbery transition, hysteresis is no longer observed in the water sorption isotherms.^{228,243,245,251,252}

Hair can be considered to have multiple different glassy domains which melt/mobilise at different temperatures,^{228,243,254} therefore the reduction in hysteresis values may correlate to

the onset of T_g for these regions. NMR studies performed by Kadir *et al.* demonstrated a reduction of rigid domains in the hair matrix by reduction of disulphide bonds.²⁵⁷ The reduction of these rigid/glassy domains would therefore allow melting of different areas in the matrix to occur at lower relative humidity, which may be reflected in the hysteretic behaviour observed in this study. The onset of glass transition events in hair would therefore coincide with the highest hysteresis value point. A reduction of crosslinks is well known to reduce the T_g of a polymer, and so the increased hysteresis values of reduced hair samples at low %RH indicate a drastic reduction in the onset of T_g events in the samples.

From the maximum hysteresis values for the samples, there is a clear difference in the steepness of the reduction in hysteresis as it reaches 0 for 90 %RH. This may be related to the ‘disorganisation’ of keratin as measured in TGA, for reduced and dyed hair. A wider range of melting events related to keratin disorder may lead to a wider onset region for the glassy to rubbery transition.²³¹

To confirm that the occurrence of maximum hysteresis was related to T_g , and knowing that a reduction in crosslinks reduces T_g , the %RH where maximum hysteresis occurs was calculated using model isotherms for all hair samples. The sorption and desorption isotherms were fit to cubic polynomials using *OriginPro 2020*. The average R^2 value for the cubic fits was 0.995 ± 0.003 , with a median value of 0.996. The differences between these isotherms were calculated to obtain a model analytical hysteresis curve. The 1st derivative of the hysteresis curves was taken to find the maximum value where $y = 0$. The %RH where maximum hysteresis occurs for the samples are displayed in Table 8.2 with the corresponding ^{13}C CP/MAS measured relative S-S content.

This data was plotted in Figure 8.11, demonstrating that as disulphide bond content decreases, the maximum hysteresis values occur at lower relative humidity. This confirms that lower S-S density allows the onset of T_g to occur at lower %RH, therefore reducing hysteresis at higher %RH. This correlation agrees with work performed by Ruike *et al.*, where more pronounced hysteresis was observed at lower %RH for dextrans with reduced crosslink densities.²⁵⁵

Table 8.2 The %RH where maximum hysteresis occurs for the hair samples, with their ^{13}C relative S-S content. (Error bars for S-S content = 1σ , where $n = 3$).

<i>Hair Sample</i>	<i>Max. Hysteresis occurrence (%RH)</i>	<i>Relative S-S content</i>
Untreated	53.33	0.549 ± 0.004
Dyed Red	44.05	0.524 ± 0.002
Reduced (5 min)	37.93	0.489 ± 0.004
Reduced (10 min)	34.50	0.475 ± 0.002
Reduced (15 min)	34.77	0.465 ± 0.004
Reduced (20 hr)	35.04	0.466 ± 0.004

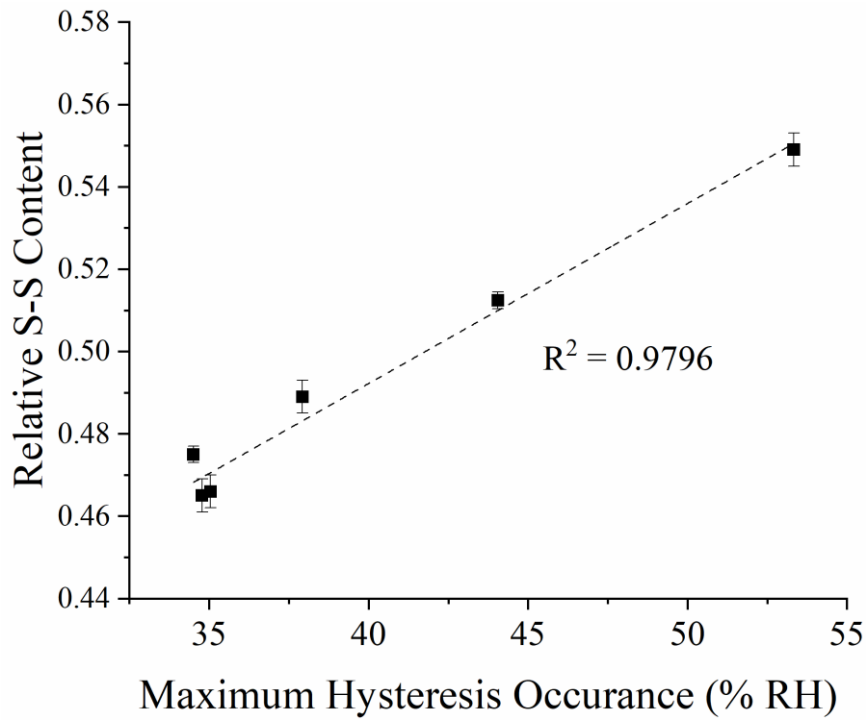


Figure 8.11 Correlations between the relative S-S content of a hair sample and the occurrence of maximum hysteresis in water sorption isotherms. Maximum hysteresis is observed at lower %RH with samples which have reduced S-S crosslink density. (Error bars for S-S content = 1σ , where $n = 3$).

8.5.3 Diffusion coefficients for sorption and desorption

To further examine the effects of disulphide bond content on hysteresis, the diffusion coefficients for water at each humidity step of the DVS experiments were calculated. This was achieved by using Fick's diffusion equation applied to a cylinder (Equation 8.1):⁵⁵

$$\frac{Ct}{C_{eq}} = 4 \left(Dt / \pi r^2 \right)^{1/2} \quad (8.1)$$

where C_t is the concentration of water at time t , C_{eq} is the equilibrium concentration of water absorbed into the fibre at each humidity step, D is the diffusion coefficient and r is the radius of hair. Here, the radius of the hair fibre has been approximated as 35 μm .⁹⁷ However, it is important to note that the different treatments on hair will affect the individual radius of the fibres separately, making these calculations only an approximation of the diffusion coefficients.

The values for (C_t/C_{eq}) were plotted against $(t/r^2)^{1/2}$. The gradient of linear regions where C_t/C_{eq} were less than 0.5, were used to calculate D using Equation 8.2:⁵⁵

$$D = \left(\frac{\pi}{16}\right) \text{gradient}^2 \text{ cm}^2\text{s}^{-1} \quad (8.2)$$

For the sorption isotherms, the diffusion coefficients are displayed in Figure 8.12a. The diffusion coefficients are in order of 10^{-10} - $10^{-9} \text{ cm}^2\text{s}^{-1}$, which is in the same orders of magnitude found elsewhere in water sorption isotherms for hair.^{212,253}

To understand the changes in D , the diffusion in untreated hair must be considered. For untreated hair samples, diffusion is controlled by two factors. At 40-50 %RH water diffuses through the cell membrane complex (CMC) to the cortical cells.^{55,258} Above 60 %RH this water begins to condense, causing swelling of the cortical cells and constricting the CMC, and resulting in a reduced D which can be observed for the untreated sample investigated (Figure 8.12a).^{55,258}

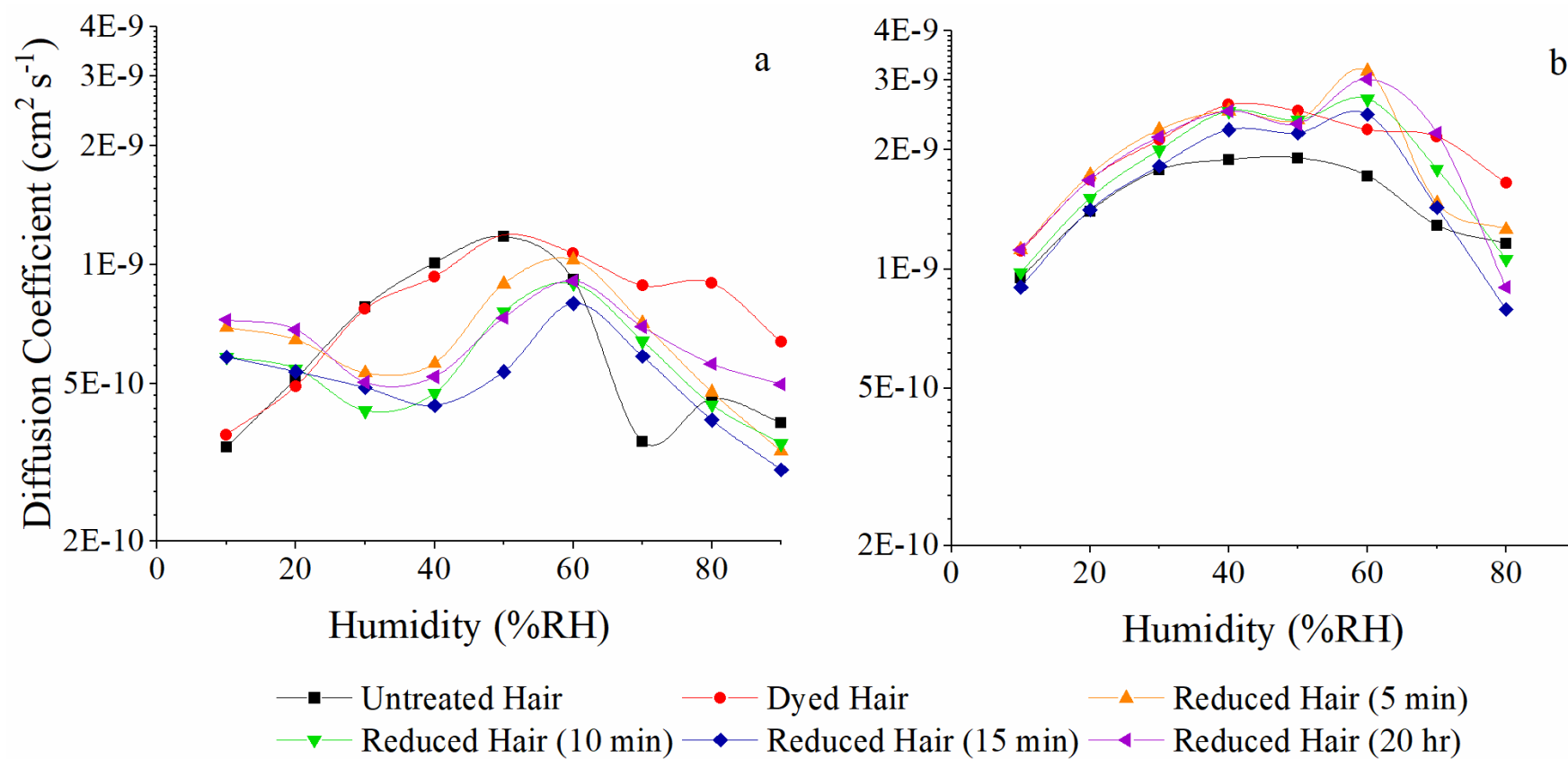


Figure 8.12 The diffusion coefficients for a) water sorption and b) water desorption for different hair samples. Higher diffusion coefficients are associated with water desorption.

In the damaged samples there are higher D values above 50 %RH compared to the untreated sample. This suggests that there is less constriction of the CMC which could be resultant of the reduction in disulphide bonds leading to the ability of the hair fibres to swell more.

For the desorption isotherms (Figure 8.12b), diffusion is on average ten times faster than during sorption, a common characteristic in keratin desorption isotherms.¹¹⁸ Diffusion of water out of untreated hair is fastest at 40-50 %RH, probably related to loss of water from the cortical cells. In the damaged hair samples, more bimodal shapes to the diffusion data are observed, which could represent the dual diffusion from both the cortical cells at 40-50 %RH,²⁵⁸ but also demonstrating shrinkage of the swollen CMC.²⁵⁵ Ruike *et al* demonstrated that the ability of dextrans to reduce in diameter upon drying was intrinsically linked with their diffusion properties. The reduced crosslinked dextrans were observed to shrink more significantly during drying in the desorption isotherms, reducing the distance required for water to diffuse to the surface.²⁵⁵ The ability of the reduced hair fibres to shrink in this work, may have resulted in diffusion coefficients to remain high at higher %RH, whereas for the more highly crosslinked samples such as untreated and dyed hair, significant shrinking will not be observed during water loss, therefore reducing the rate of diffusion of water as distances to the surface of the fibre do not undergo significant change.²⁵⁵ High values of D may be considered as an indication of efficiency in structural relaxation from the swollen to dry state. Therefore, this diffusional analysis supports the hypothesis that reduced hysteresis is observed with faster relaxation of a material during desorption.^{231,236,255}

The data shown in Figure 8.12a, and Figure 8.12b was fitted to a fifth order polynomial using *OriginPro 2020*. These fits had an average R^2 value of 0.9387 ± 0.0231 with a median value of 0.9448.

The maximum D values for both the sorption and desorption profiles of each hair sample was calculated, with the associated %RH. This data is plotted in Figure 8.13 with the relative S-S content of the samples to observe the effect of disulphide bond content on the rate of diffusion.

In the sorption isotherms (Figure 8.13a), there is a strong negative correlation between the relative S-S content and the %RH where fastest diffusion occurs. This data demonstrates that where the fibres are more damaged, with fewer disulphide bonds, there is higher permeability and fast diffusion at higher %RH as the CMC is less constricted by the swollen cortical cells.

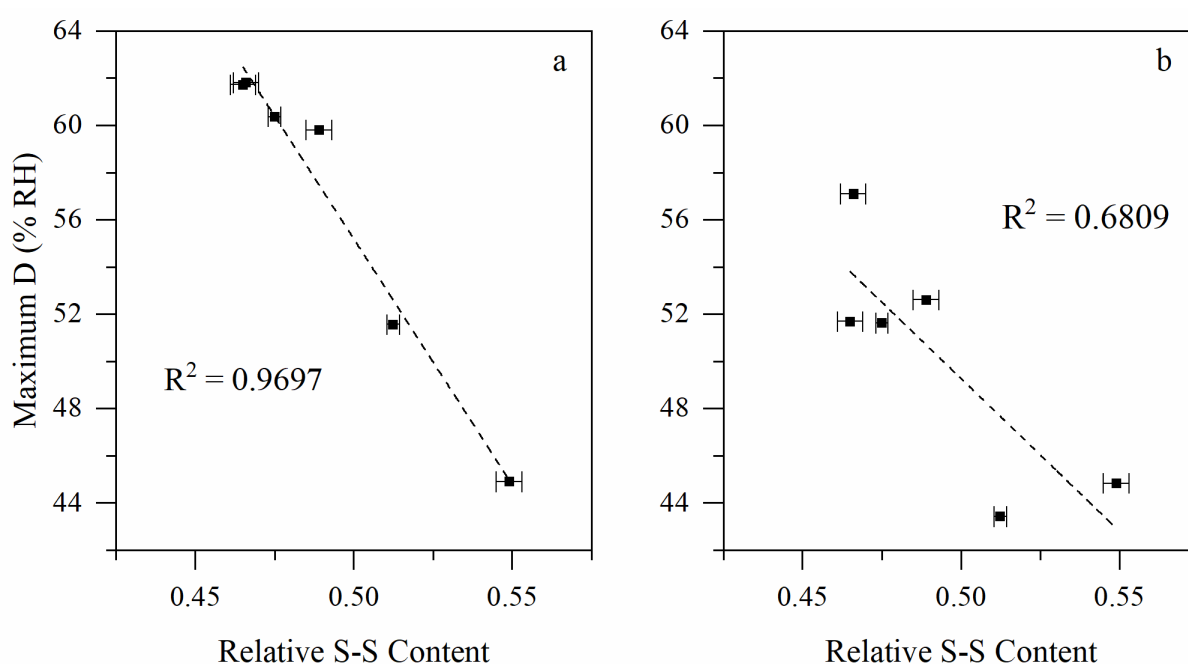


Figure 8.13 Maximum diffusion coefficients occur at higher %RH for a) water sorption and b) water desorption in samples with reduced S-S content. This indicates the better ability of the fibres to swell during sorption, and relax during desorption. (Error bars = 1σ , where $n = 3$).

For the desorption profiles (Figure 8.13b), there is also a correlation between maximum diffusion occurring at higher %RH and relative S-S content. This supports the hypothesis that the damaged hair samples are able to relax faster, resultant of their more amorphous content, and resulting in reduced hysteresis.

8.6 Conclusion

To investigate the impact of the hair fibres ability to swell on their hysteretic properties, hair samples were created with reduced disulphide bond contents through reduction and dye procedures.

^{13}C NMR was used to confirm the reduction process and quantify the relative disulphide bond content in the hair, and identified increased amorphous content of keratin for damaged hair samples. TGA methods were used to demonstrate increased disorder in damaged samples, and a dramatic increase in hydrophobicity of reduced samples, likely resulting from the methylation procedure.

DVS was used to measure the water sorption and desorption isotherms of these samples, demonstrating the distinct differences in the isotherms between untreated and reduced hair samples. Increasing hydrophobicity from methylation was determined to have resulted in a reduction of hydrophilic sites for water binding. The hysteresis curves for all samples were calculated, showing dramatic differences between damaged and untreated hair samples. Damaged samples had reduced hysteresis, with the occurrence of maximum hysteresis, and in turn the onset of T_g events, correlating directly to the disulphide bond content of the fibres. Lower relative S-S content was associated with maximum hysteresis values occurring at lower %RH.

The diffusion coefficients for water at all steps of the sorption and desorption isotherms were also calculated. This demonstrated that in damaged samples the rate of diffusion remained high at higher %RH, as the CMC in damaged samples were allowed to swell, preventing swollen cortical cells from reducing the rate of diffusion into the CMC. Therefore, samples with lower S-S bond content had greater rates of diffusion at higher %RH, in comparison to untreated hair. Diffusion constants in the desorption profiles demonstrated that hair fibres with reduced crosslinks were able to relax more efficiently, allowing faster diffusion at higher %RH values in comparison to untreated hair samples.

Overall, this work has shown that hysteresis is indeed dependent on the swelling abilities of the hair fibre, which is largely governed by the extent of damage and reduction of disulphide bonds in the hair. The ability of a fibre to swell is related to its crosslink density and amorphous content, which governs the onset of T_g , and the ability of the fibre to relax during desorption. Fibres with reduced crosslinks showed a higher amorphous content, depressed T_g onset at lower %RH and faster relaxation properties, all resulting in distinctly different hysteretic profiles to untreated hair.

While previous studies have demonstrated greater swelling of chemically treated hair fibres,⁷ future work should directly measure the diameter of swollen fibres used in this work to confirm this theory, which due to the time constraints of this project was not undertaken.

Chapter 9 - Overview and Future work

9.1 Overview

Hair is a highly complex and heterogeneous material. With over fifty years of investigations into keratins, there is still much more to be understood in how keratins interact with small molecules. This thesis has aimed to address this by examining two key areas:

1. The quantification of small molecule interactions with keratin, and how environment affect these interactions (i.e., humidity and structural changes).
2. Examining keratin sorption processes by a) the development of methods to measure active desorption from fibres and b) improving the understanding of how keratin structure affects the sorption and desorption of small molecules in hair – with a focus on water and hysteresis.

^1H spin-lattice (T_1) relaxation methods were applied to understand how small molecules interact with hair. Two populations of a range of solvents were identified in hair, with bound populations having stronger interactions with hair, and free populations having weaker interactions with hair. Reduced relaxation times indicate reduced mobility and stronger interactions with the fibre. Molecules with low LogP and low MW values had increased interactions with keratin, and a larger relative bound population size. Increasing moisture content of the hair fibre led to increased interactions with small molecules across both bound and free populations, due to the swelling of hair allowing better penetration into the hair

fibre. This leads to the MW of a molecule to have more significance on its interactions with hair than the LogP of a molecule. These trends are incredibly surprising considering the highly heterogeneous nature of hair keratin.

^1H spin-spin (T_2) relaxation of water molecules in damaged hair samples was used to examine the impact of thermal, acidic and alkali treatments on keratin structure, and how this affects small molecule interactions. With more damage, water molecules can better penetrate the hair fibre and form stronger interactions, reducing the mobility of the water molecules. The relative amount of α -keratin in the damaged samples were calculated by recording their ^{13}C CP/MAS NMR spectra. This demonstrated that the reduction in mobility of water in damaged hair samples correlated to a reduced relative α -keratin content. These results support the use of this ^1H T_2 method to quantify damage in hair samples, as a non-destructive alternative to current damage measurement techniques.

The loss of small molecules from keratins has great importance to the hair care and textiles industry, but as of yet, there is no standard method to measure the real-time loss of actives from hair. A HPLC method was devised to measure the real-time desorption of alkalizer and dye from dyed hair. This method identified a two-stage diffusion process for small molecule loss and allowed diffusion constants and kinetic parameters to be analysed, determining a thermal dependence on the rate of active loss.

Understanding how repeated dye procedures affect hair damage and small molecule loss from hair, is important to formulate products which reduce toxic exposure and hair damage in consumers. Confocal imaging and solid-state NMR was used to examine the extent of damage from dye and dye extraction on hair fibres. Dye procedures reduced the total disulphide content in the hair sample, and also the relative α -keratin content of the hair,

which could be recovered through small molecule extraction with HPLC. With this background, HPLC measurements were used to investigate how the kinetics of active loss in dyed hair is affected by multiple dye treatments. There is increased loss of alkalizer which is mainly found in the cortex, associated with depletion of the cuticle. Kinetics of dye loss however remained unchanged. These results demonstrate that the location of an active in hair is key to understanding its kinetic behaviour.

The sorption of actives in hair leads to a characteristic hysteresis which can be measured by DVS. To examine the role of swelling in the hysteresis of water sorption isotherms, hair was damaged via reduction and dyeing to measure the impact of reduced crosslink density on hysteresis. Damage in samples was quantified using ^{13}C CP/MAS NMR and TGA. Hysteresis was found to be dependent on the swelling ability of the hair fibre, which was directly correlated to its crosslink density and amorphous content. A reduced crosslink density allows the onset of T_g at lower %RH, and allows more efficient relaxation of the fibre during desorption. This resulted in damaged samples having distinctly different hysteretic properties to untreated hair.

9.2 Future Work

9.2.1 Immediate work

Some steps that can be taken immediately to confirm conclusions made in this work include the use of pH/MS detectors in the HPLC system to confirm loss of MEA, and prevent error from hand held pH meter readings. Confocal imaging should be used to confirm cuticle stripping in repeat dye cycle experiments, with results being acquired quickly as samples have already been generated. Confocal imaging should also be used to examine damaged samples

measured by ^{13}C NMR, to examine how cuticle surface area correlates to peaks of interest (e.g., the carbonyl peak and peaks relating to S-S content) within the spectra.

Chapter 8 investigated the role of swelling in the hysteresis in water sorption isotherms. To further support the conclusions drawn in this Chapter, the radius of fully hydrated samples used in this work should be measured. X-ray diffraction could also be employed here to examine how inter-keratin distances change during swelling, which has been utilised previously to measure swelling in damaged hair.⁷

9.2.2 Long term studies

Due to the novel nature of this work, a significant portion of the research in this thesis provides a foundation for future studies.

One of the aims from this work, was to examine how alkalizer loss affects hair structure after it was proposed to be the cause for customer dissatisfaction in hair 'feel' after hair is dyed. The HPLC methodology was devised for this, with the ability to form hair samples at different time points during dye loss for further analysis. This would be a straightforward procedure, which was not achieved due to the time constraints of this project. Derived hair samples should be analysed with ^{13}C CP/MAS NMR, DVS methods to examine water sorption properties, confocal or other more powerful microscopy methods to analyse cuticle surface area, and TGA to understand the changes in both water content, and melting points of keratin.

There are also indications in the confocal analysis of hair which has undergone the HPLC process, that the HPLC system can be used to easily and efficiently create large quantities of de-cutilised hair, which has often been formed through single fibre hand sanding.

This work has also shown the ability of SS-NMR to examine small molecule interactions with keratins. A larger solvent range was used to prove that small molecule behaviour in hair followed a trend related to the solvents MW and LogP values. The effect of moisture content in hair was also examined, and this should be expanded to be used as an extra model parameter. With a wider range of compounds, more relationships can be elucidated to form a quantitative structure-activity relationship (QSAR), which has great potential for improving the formulation process in the hair care and textiles industries.

Another step forward in this work would be to perform experiments at dilutions of solvents found in hair care products. This can be achieved by selectively labelling fragrance/solvent compounds, i.e., with ^2H , so that in their water/solvent dilutions the interactions of the compound of interest can be measured independently.

From the studies presented in this thesis, it is clear that the relationships between keratins and solvents are highly complex. However, the foundation provided by this work suggests that successful progress can be made to understand not only the behaviour of small compounds and hair, but molecular interactions with other fibrous proteins/ keratins which are traditionally harder to analyse with current spectroscopic methods.

Chapter 10 - Appendix

10.1 ^1H NMR Spectra for solvents and solvents in hair (Chapter 3)

All solvent in hair spectra are scaled for comparison, with their scaling factors given in each spectra.

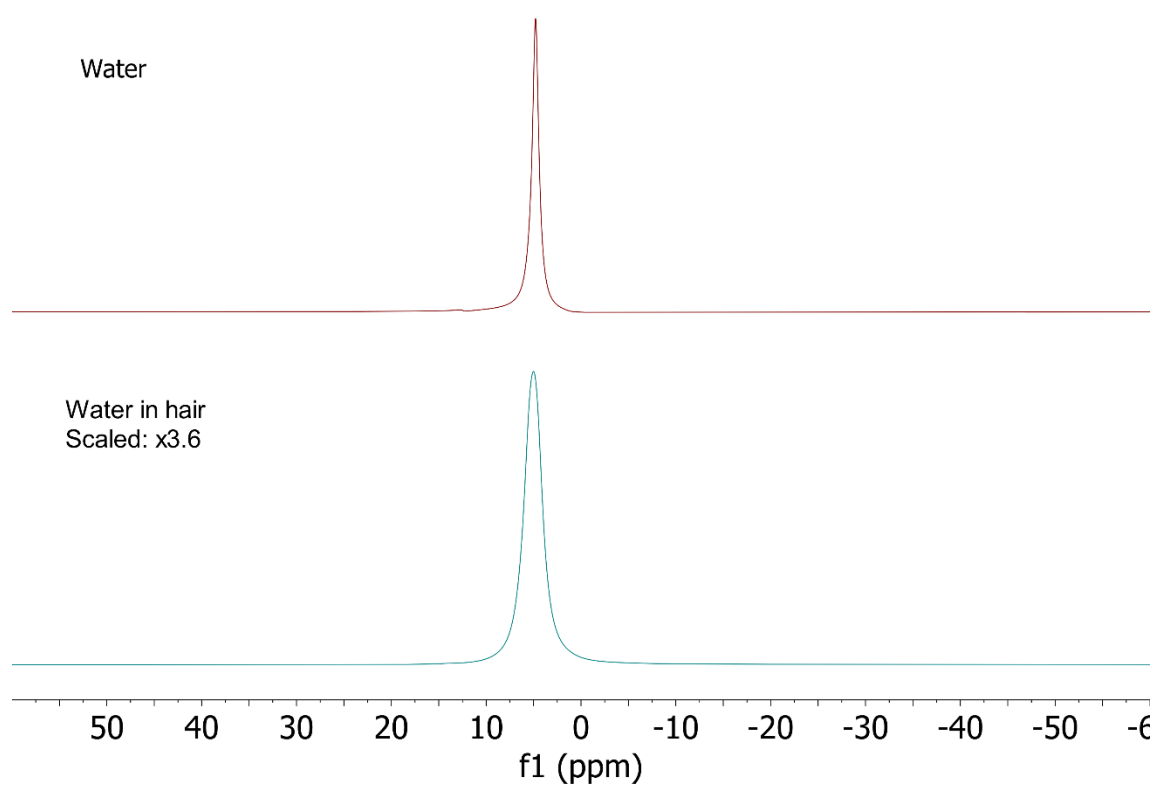


Figure 10.1 ^1H NMR spectra of water (top) and water in hair (bottom)

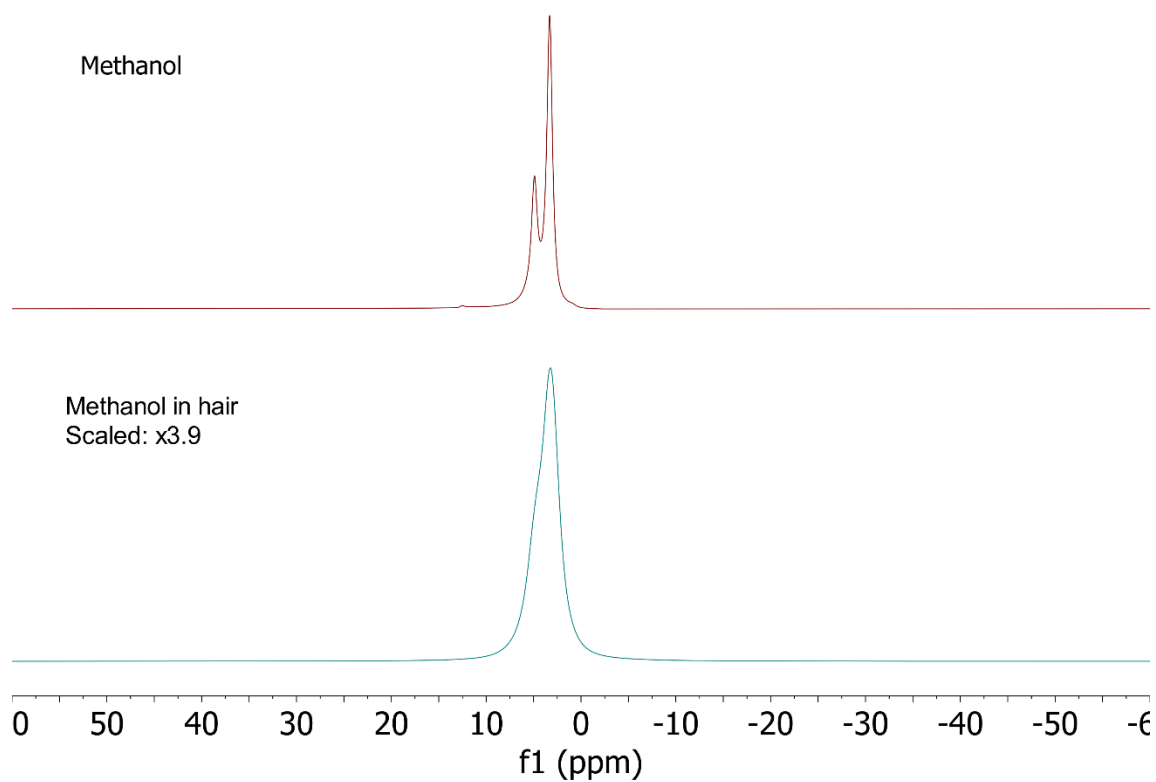


Figure 10.2 ^1H NMR spectra of methanol (top) and methanol in hair (bottom)

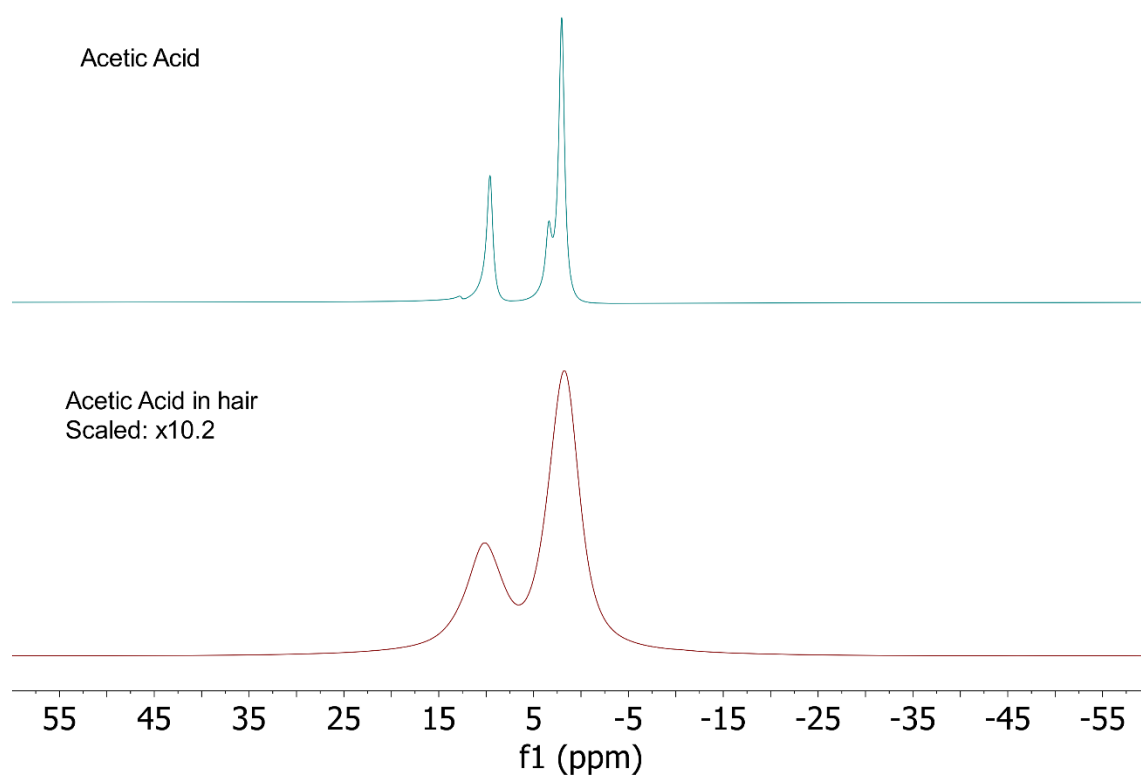


Figure 10.3 ^1H NMR spectra of acetic acid (top) and acetic acid in hair (bottom)

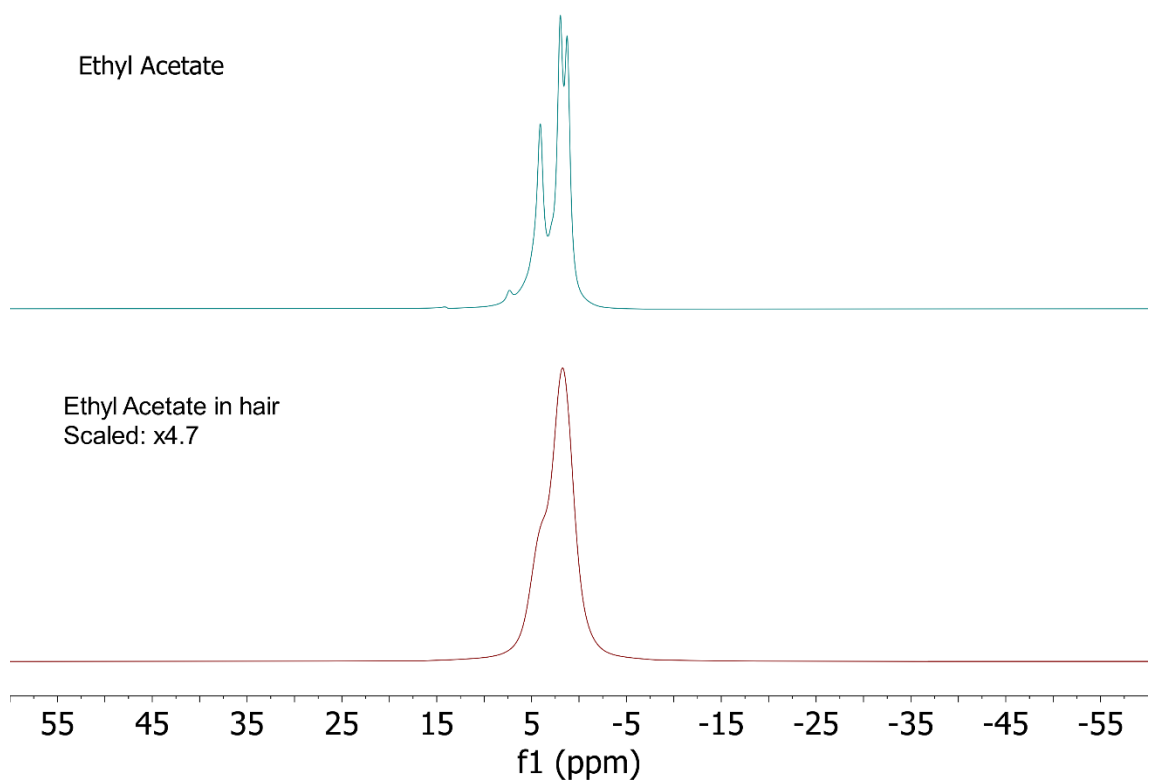


Figure 10.4 ^1H NMR spectra of ethyl acetate (top) and ethyl acetate in hair (bottom)

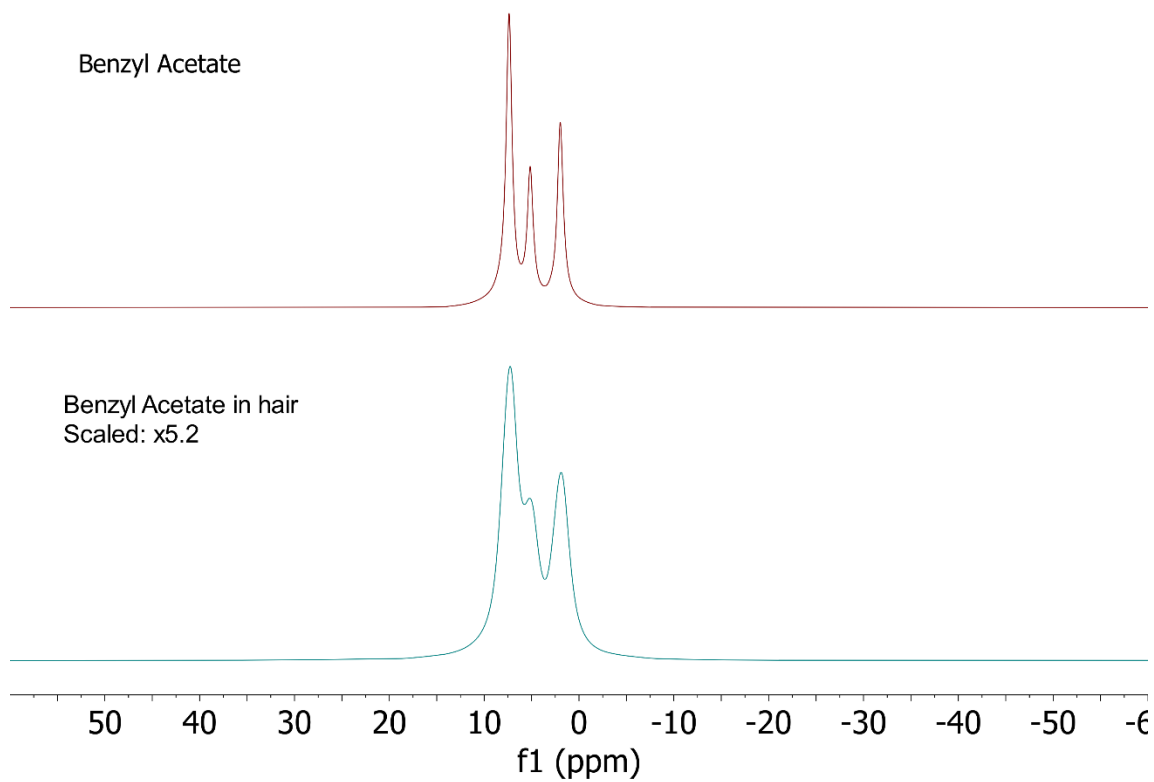


Figure 10.5 ^1H NMR spectra of benzyl acetate (top) and benzyl acetate in hair (bottom)

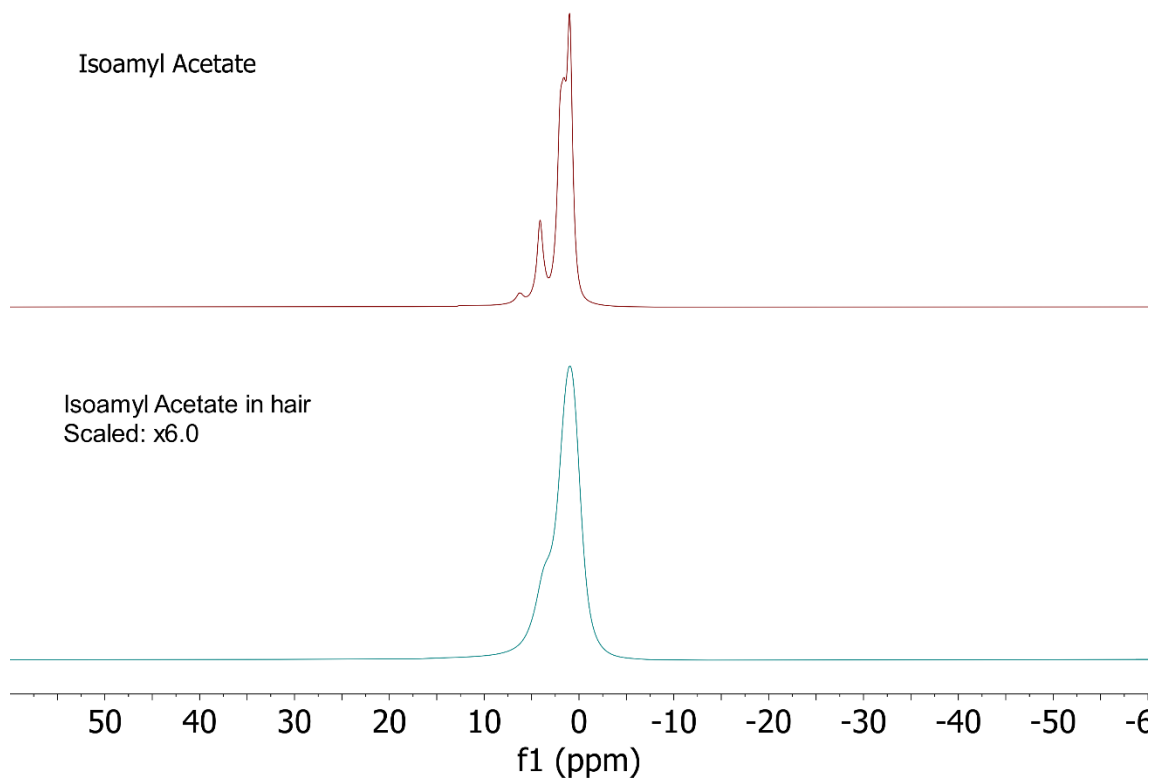


Figure 10.6 ^1H NMR spectra of isoamyl acetate (top) and isoamyl acetate in hair (bottom)

The peak broadening once the solvent is in hair demonstrates the interaction with the hair fibres; restricted mobility creates anisotropy of molecular orientations within the sample which contributes to line broadening in the ^1H NMR spectra.

10.2 $\Delta T_{1\text{H}}$ values and Relative population Sizes for expanded solvent range (Chapter 3)

Table 10.1 lists the ^1H T_1 times for the expanded solvent range investigated in Chapter 3, including solvents in hair displaying the one and two-component fits and the associated R^2 values.

Table 10.1 The ^1H T_1 times of solvents, and solvents in hair fitted to either a one or two-component fit to reach $R^2 > 0.99990$., where $T_{1\text{Solv}}$, $T_{1\text{Hb}}$, $T_{1\text{Hf}}$ are the ^1H T_1 times (s) for the bulk solvent, bound population, and free population of compound in hair, respectively. (Error = 1σ from $n = 2$ for n-hexyl acetate, *estimated errors calculated from errors of solvents in Chapter 3.3.)

	Solvent		Solvent in Hair				
	One-component fit		One-component fit		Two-component fit		
	^1H $T_{1\text{Solv}}$ (s)	R^2	^1H $T_{1\text{H}}$ (s)	R^2	^1H $T_{1\text{Hb}}$ (s)	^1H $T_{1\text{Hf}}$ (s)	R^2
Limonene*	1.79 ± 0.02	0.99994 ± 0.00002	1.72 ± 0.17	0.99961 ± 0.00001	0.44 ± 0.04	1.95 ± 0.20	1.00000 ± 0.00000
Propyl Acetate*	2.40 ± 0.02	0.99998 ± 0.00002	1.94 ± 0.19	0.99922 ± 0.00001	0.40 ± 0.04	2.25 ± 0.23	0.99999 ± 0.00000
Butyl acetate*	2.23 ± 0.02	0.99998 ± 0.00002	1.82 ± 0.18	0.99954 ± 0.00001	0.44 ± 0.04	2.06 ± 0.21	0.99999 ± 0.00000
n-pentyl acetate*	1.81 ± 0.02	0.99998 ± 0.00002	1.37 ± 0.14	0.99890 ± 0.00001	0.40 ± 0.04	1.75 ± 0.18	0.99999 ± 0.00000
n-hexyl acetate	1.70 ± 0.02	0.99997 ± 0.00002	1.44 ± 0.12	0.99949 ± 0.00001	0.41 ± 0.01	1.69 ± 0.13	0.99999 ± 0.00000

Table 10.2 Values of ΔT_{1Hb} and ΔT_{1Hf} for compounds in hair, calculated using Equations 3.3.3 and 3.3.4, and the relative population sizes for the bound and free population of compound in hair, calculated used Equations 3.3.2 & 3.3.6. . (Error = 1σ from $n = 2$ for n-hexyl acetate, *estimated errors calculated from errors of solvents in Chapter 3.3.)

	ΔT_{1Hb}	ΔT_{1Hf}	Relative population size	
			Bound Population	Free Population
Limonene*	0.24 ± 0.03	1.10 ± 0.11	0.09 ± 0.01	0.91 ± 0.01
Propyl Acetate*	0.17 ± 0.02	0.94 ± 0.08	0.09 ± 0.01	0.91 ± 0.01
Butyl acetate*	0.20 ± 0.02	0.92 ± 0.08	0.08 ± 0.01	0.92 ± 0.01
n-pentyl acetate*	0.22 ± 0.02	0.97 ± 0.08	0.19 ± 0.03	0.81 ± 0.03
n-hexyl acetate	0.24 ± 0.00	0.99 ± 0.07	0.09 ± 0.02	0.91 ± 0.02

Table 10.2 shows the calculated ΔT_{1Hb} and ΔT_{1Hf} for compounds in hair, and the relative sizes for the bound and free populations.

10.3 Confirming pH changes result from presence of MEA during HPLC (Chapter 6)

As other methods to determine MEA directly from the DI water eluent in the HPLC experiments were unsuccessful, other methods were used to ensure any pH changes were only due to the presence of MEA. Figure 10.7 shows the pH measured from two experiments at 25°C, demonstrating that pH changes were not recorded in untreated hair. For the untreated sample, the pH value plateaus at 6.9, the pH of DI water.

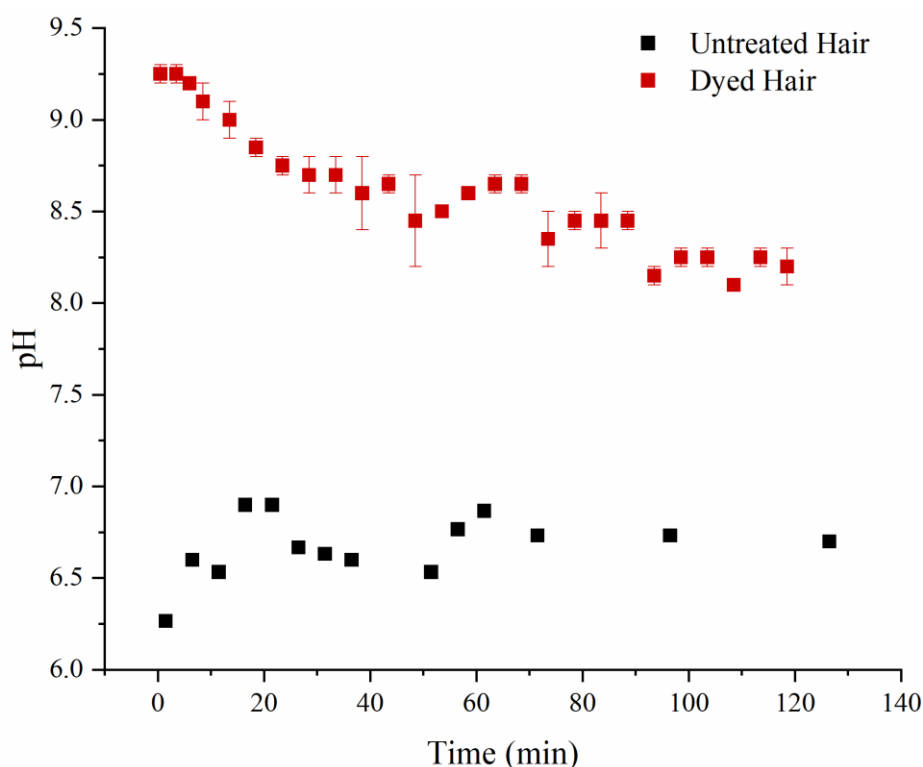


Figure 10.7 The pH of DI water passing through untreated hair and dyed hair at 25°C. pH does not change in untreated hair, demonstrating that pH changes are resulting from actives in the dyed hair fibre; MEA.

A similar experiment should be undertaken with hair just treated with MEA, to fully isolate the source of the pH changes, as well as other methods discussed in Chapter 6. As pH changes were larger at higher temperatures, another experiment was used to determine that the temperature alone did not impact pH values. Figure 10.8 shows a temperature cycling experiment where pH loss from untreated hair was measured, first at 60°C until the pH value plateaued, then the temperature was dropped to 25°C and back to 60°C, demonstrating no changes in pH measurements. This demonstrates that pH measurements are independent of temperature and that the pH changes are not reversible. This further confirms that the changes in pH are resultant from active loss from the fibre.

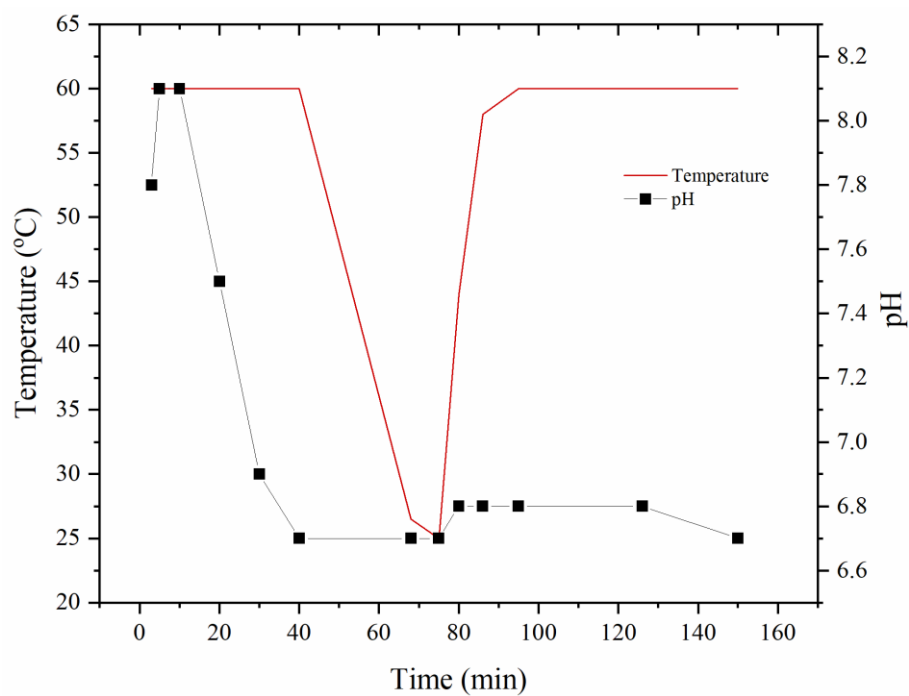


Figure 10.8 The pH of water passing through untreated hair as the temperature is changed.

The pH does not increase significantly when the temperature increases, proving that pH change from HPLC extraction is an irreversible process.

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