The immunodetection of cocaine for forensic applications

Susan van der Heide

School of Chemistry
University of East Anglia
Norwich, UK
April 2014

A thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy at the University of East Anglia.

Declaration

I declare that the work contained in this thesis submitted by myself for the Degree of Doctor of Philosophy is my work, except where due reference is made to other authors, and has not previously been submitted by me for a degree at this or any other university.

Susan van der Heide

Acknowledgements

"Some people grumble that roses have thorns; I am grateful that thorns have roses."

- Alphonse Karr

First and foremost I would like to thank my primary supervisor Prof David Russell for presenting me with the opportunity to do this PhD in the first place. The continued support, faith, inspiration and kindness provided to me by Prof Russell throughout this study has been invaluable and is hugely appreciated. I am also grateful to my second supervisor Dr. Myles Cheesman for his guidance and technical support during this study.

Financial support from the School of Chemistry, University of East Anglia, the Home Office Centre for Applied Science and Technology (CAST) and Intelligent Fingerprinting Ltd. is much appreciated. I would like to thank Andrew Cunningham at the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and Sheila Hardwick at the Home Office CAST for their continued support throughout this project. The provision of the UK banknotes by Kevin Wills on behalf of the Bank of England, and the Scottish banknotes by the Scottish Crime and Drug Enforcement Agency (SCDEA) are gratefully acknowledged. The 'street' cocaine provided by Steven Hamilton at the Norfolk Constabulary, and the fingerprint samples and oral fluid analysis provided by the kind folk at Intelligent Fingerprinting Ltd. are much appreciated. The technical assistance and sample analyses performed by Dr. Colin MacDonald at the University of East Anglia and Simon Hudson at HFL Sports Science are also hugely appreciated. The work of Paula Garcia Calavia on the analysis of fingerprint samples by cEIA, performed under my guidance as part of her undergraduate summer research project, is gratefully acknowledged.

I would particularly like to thank my past, present, visiting and honorary colleagues in the Russell group; Dr. Maria Jose Marin Altaba, Dr. Girgis Obaid, Dr. Tanya Stuchinskaya, Dr. Oriol Penon, Lawrence Eaglen, Smita Ramma, Paula Garcia Calavia and Elizabeth Shedden. Maria, Lawrence, Girgis, Paula, and Liz – thank you for your friendship and all of the help and support you have given me; for your ideas and input, for proof-reading, for checking my calculations, for the hundreds of sanity-restoring coffees and pep-talks, for your relentless faith in my abilities and even for

laughing at my jokes (and trying to understand my accent). I couldn't have done it without you.

Thank you to all of my friends, both in the UK and overseas, for the support, entertainment, distraction and for keeping me grounded during this PhD study. Tara, Skip, Ali, Justin, Mayo, Ian, Mark, Toni, Sarah, Reg, Andrew and Leish (& co.) – cheers! Thank you to my dance crew - Kim, Linda, and Sarah for your encouragement and motivation, and particularly to my dance teacher Kathy Buller for being such an inspiration.

Most importantly I would like to thank my family and my partner Alex for all of the love and support they have given me throughout this PhD. Thank you for the Skype dates, for developing a sudden interest in nanoparticles, for listening to stories about chemistry (most of which your probably didn't understand but pretended to anyway), for the cards, parcels and little reminders of home, and most of all for having faith in me even when for years I didn't seem to be getting anywhere. I truly couldn't have made it this far without your tireless support, both emotionally and financially, and I am incredibly grateful.

Mama, Pap, Maike (& co.) and Alex – I dedicate this thesis to you.

Abstract

Cocaine on banknotes can be indicative of drug trade; analytical methods which can distinguish 'drug money' from contaminated banknotes in general circulation are crucial in providing incriminating evidence. The development and application of two analytical methods for the detection of cocaine on banknotes are presented in this thesis.

A competitive enzyme immunoassay (cEIA) was developed and used to quantify cocaine in extracts taken from both banknotes and latent fingerprints. The limit of detection (LOD) of 0.162 ng mL^{-1} achieved with the assay was comparable to that of conventional chromatography-mass spectroscopy techniques. When compared to LC-MS for the analysis of ten UK banknote samples, the two approaches produced results with statistically significant similarity. The immunoassay presents a simple and cost-effective alternative to the current techniques for the quantitation of cocaine at forensically significant concentrations.

Secondly, a novel immunodetection method was developed for the location-specific staining of cocaine on banknotes. The method was based on the application of an acrylamide gel matrix for maintaining the position of the cocaine during subsequent immunostaining. The performance of the method was determined using newly-minted UK banknotes as true negative sample matrices, resulting in staining which could be readily distinguished from that on newly-minted notes spiked with cocaine. The method was successfully applied for the detection of cocaine on the surface of six UK banknotes from general circulation. The developed method, for the first time, demonstrates the location-specific immunostaining for the visualisation of cocaine on banknotes.

Finally, a comparison of four different techniques for the functionalisation of cocaine-binding antibody-gold nanoparticles *via* protein or ligand intermediates is also described. The addition of antibody onto the AuNP surface was facilitated by a polyethylene glycol (PEG) linker with a –COOH terminal functional group, an aminated PEG ligand and an SPDP-Protein A/G intermediate. The study showed that the cocaine binding efficacy was the greatest for AuNP functionalised with the Protein A/G intermediate.

Contents

CHAPTER 1

Introduction

The supply and consumption of cocaine is the cause of health, social and economic problems on a global scale. In order to combat the effect of usage and trade, many countries implement laws that allow the seizure of circumstantial evidence associated with cocaine trafficking. Sensitive, specific and reliable analytical methods for the detection of cocaine in different forms and matrices are crucial for the provision of forensic evidence *via* which individuals or groups associated with cocaine trafficking can be prosecuted. Cocaine on paper currency, for instance, can be indicative of drug use or trade and thus used to provide incriminating evidence. Quantitative methods which enable the differentiation between 'drug money' (*i.e.* money which has been in direct contact with cocaine) and cocaine contamination from general circulation, and are in turn suitable for daily analysis in a forensic laboratory are crucial in providing such evidence. It is therefore of interest that a greater range of suitable methods for this type of analysis to be developed.

Cocaine, its effects and origins, methods of production, networks of transport and trade, social consequences and legislation are introduced in this chapter. Current methods for the detection of cocaine in non-biological samples, as well as for the detection of cocaine and metabolites in biological samples of forensic relevance are discussed. Current analytical techniques used for the detection of cocaine on banknotes and its forensic relevance are also outlined in this chapter.

1.1 Cocaine

1.1.1 Synthesis and origin

Cocaine (benzoylmethylecgonine) is a naturally occurring alkaloid found in the leaves of the coca plant, a name that is attributed to any plant of the genus *Erythroxylum* which is native to South America. The *Erythroxylum* genus contains over 200 distinct species, of which only two (*Erythroxylum coca* and *Erythroxylum novogranatense*) contain significant quantities of cocaine.[1,2] The use of coca leaves by the Amara Indians of Peru as a psychoactive substance is known to have occurred as early as 5000 BC, based on the stockpiles of coca leaves found in ancient Peruvian burial sites.[3,4] Coca leaves contain between 0.3 and 1.5 % cocaine, and when taken in the traditional ways of chewing coca leaves or by drinking coca leaf tea this can act as an appetite or thirst suppressor and a mild stimulant.[3] Archeological evidence from 1500 BC also indicates the use of liquid coca-leaf extract as an early anaesthetic during brain surgery.[4] The cocaine alkaloid was first extracted from coca leaves in 1859 by the chemist Albert Niemann, after which cocaine became commercially available and was widely used as a medicinal treatment for pain and fatigue, among other ailments.[5] Social problems resulting from the use of cocaine led to a ban of the sale of the substance without prescription in the US in the early 1900s.[4] Supply and production of cocaine for non-medicinal purposes became internationally prohibited in 1961 through the Single Convention on Narcotic Drugs, which remains in place today.[6]

Cocaine, the chemical structure of which is shown in **Figure 1.1**, is predominantly encountered as an odourless, white, crystalline powder usually in the form of a salt such as cocaine hydrochloride (HCl).[1] Cocaine can be produced in two ways; either synthetically, or naturally by purification from coca leaves.[2] Although four pairs of enantiomers of the cocaine alkaloid are theoretically possible, only one (commonly termed *l*-cocaine) occurs naturally.[1] Cocaine has a pK_a of 8.61 and therefore exists in the protonated form under physiological conditions. Cocaine can be synthesised from ecgonine or by total synthesis *via* the Mannich reaction, although this is prone to

producing optical isomers which are less pharmacologically active than the natural material.[1] Illicit synthetic cocaine is therefore extremely rare and accounts for less than 0.001 % of all cocaine seizures, and typically only occurs as a result of licit drug diversion or illegal prescriptions.[2] Licit cocaine is manufactured for pharmaceutical use as a by-product of the industrial extraction of flavouring agents from coca leaves for use in the food industry.[2] The resulting product is generally of 99.5 % purity or better; conversely, the purity of natural illicit cocaine typically varies from 80 to 97 %.[2] This variation in purity enables pharmaceutical cocaine HCl to be readily distinguishable from its natural illicit counterpart by detailed forensic analysis.[7]

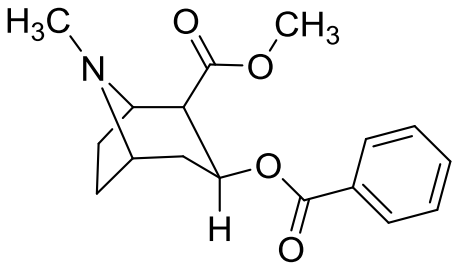

Figure 1.1. The chemical structure of cocaine.[1]

The modern illicit production of cocaine HCl from coca leaves is a three-step process; in the first a crude paste is obtained from coca leaves, followed by purification to a coke base, and finally the conversion of coke base to cocaine HCl as a salt.[2] An interview with a family of coca farmers published by Streatfeild in 2003 describes a large-scale acid-base extraction method similar to that detailed by Casale and Klein, the details of which have apparently changed little since the early 1900s.[2,8] According to the descriptions of the coca farmers, the coca leaves are first dried for half a day. The leaves are then shredded and a small amount of powdered cement or sodium carbonate is applied. The leaf mixture is covered in petroleum or a similar hydrocarbon solvent such as kerosene and soaked for a full day. The solvent is then removed *via* the addition of dilute sulfuric acid (typically battery acid) to create

a two-part phase separation, through which the acidified cocaine free base in the petroleum is extracted. Sodium hydroxide, limestone or lime is then added to increase the pH, whereupon a precipitate forms which can be removed by filtration and dried to give crude coca paste.[8] The production of coca paste is usually performed on-site by the coca farmer, yielding a crude product which contains between 30 and 80 % cocaine, is of a gummy consistency and has a short shelf-life.[2] For this reason it is always immediately turned for processing into coke base.

Coke base is purified from crude coca paste in small-scale clandestine laboratories. The process involves dissolving the coca paste in dilute sulfuric acid, then oxidising any residual impurities by titration against a concentrated solution of aqueous potassium permanganate. The resulting mixture is filtered to remove the manganese oxide precipitate, after which dilute aqueous ammonia is added to precipitate the cocaine free base.[2] The resulting free base is filtered, dried, packaged and sold. It contains between 80 and 95 % cocaine and is commonly turned over to larger and more sophisticated clandestine laboratories which produce cocaine HCl on an industrial scale and to a higher standard than occurs in the previous two processes.[2] Alternatively, it can be sold as ‘crack cocaine’ which has a more granular texture than the salt form, is frequently in the form of nuggets or ‘rocks’ and has a slightly waxy appearance.[9]

The procedures involved in the production of cocaine hydrochloride are varied and depend on the laboratory and the solvents that are locally available.[2] In the classic methodology, coke base is dissolved in diethyl ether and filtered to remove any insoluble impurities that may be present. An equal volume of acetone containing a stoichiometric quantity of concentrated hydrochloric acid is then added to the filtrate and stirred. The hydrochloric acid immediately ion-pairs with the coke base, resulting in cocaine HCL salt which appears as a shiny white and flaky crystalline precipitate. The crystallisation process is allowed to continue for a further 3 to 6 hours after which the product is filtered, dried, pressed, packaged and distributed.[2] The resulting

cocaine HCL typically contains between 80 and 97 % cocaine, as well as numerous impurities which can be detected and traced for forensic purposes.[7]

1.1.2 Trade, use and social consequences

The coca plant is native to the central and Northern Andean Ridge of South America and this remains the region of the world where the cultivation of coca for illicit sale takes place. The majority of this cultivation zone (approximately 60 %) is in Peru, 30 % is in Bolivia and the remainder is distributed throughout Colombia, Ecuador, Venezuala, Brazil, Argentina and Panama.[2,9] As discussed in **Section 1.1.1**, the production of coca paste normally occurs at the site of cultivation, and due to its short shelf life the processing of paste into coke base is also performed in the local area. As a result, over 99 % of coca processing laboratories are located in the three main cultivating countries; Bolivia, Colombia and Peru, although additional laboratories for the production of cocaine HCL from coke base have also been reported in other countries in South America and occasionally in Europe.[10]

The main markets for cocaine are North America and Western Europe, with each accounting for over 40 % and 25 % of global cocaine consumption (at an estimated 470 tonnes), respectively.[10] The two regions combined account for more than 80 % of the global cocaine market, which was estimated at US$88 billion in 2008.[10] From its origins in South America, cocaine is shipped worldwide *via* two main trafficking routes, a schematic of which is shown in **Figure 1.2**. The first route bound for North America travels from Colombia to Mexico or Central America by sea before continuing overland to the US and Canada, while the second bound for Europe travels directly from Colombia or Peru *via* maritime routes, typically in container shipments.[10]

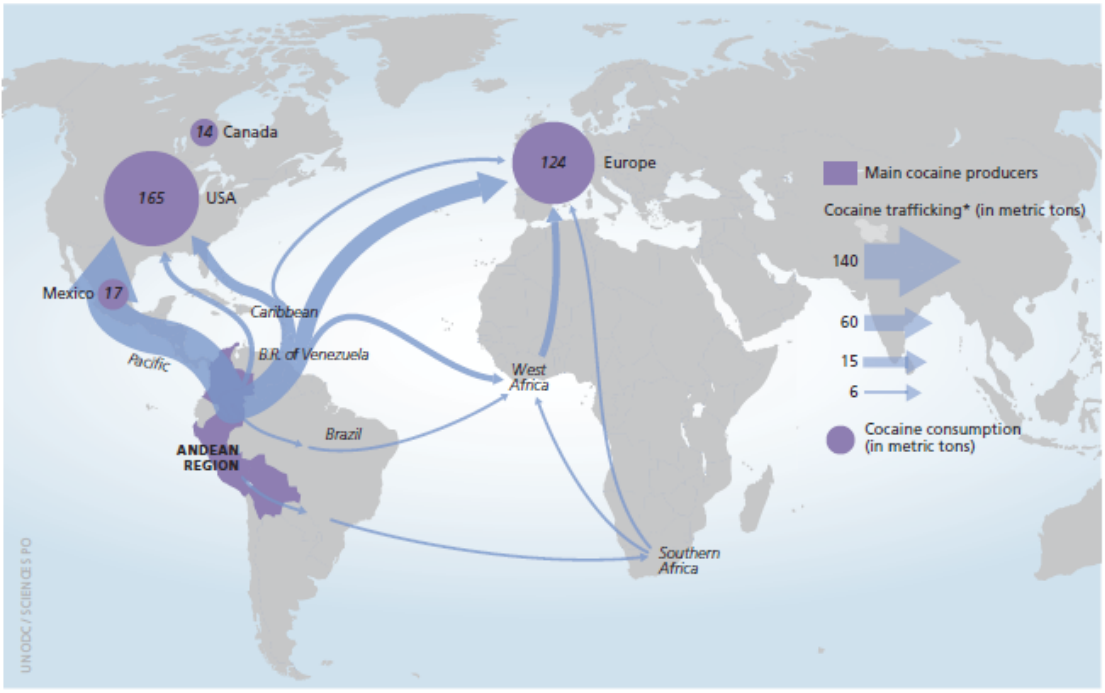

Figure 1.2. Major trading routes for the trafficking of cocaine worldwide (image obtained from the UNODC World Drug Report, 2010).[10]

The annual prevalence of cocaine use in Western and Central Europe has seen a marked increase over the past decade, and has recently plateaued at nearly three times the global average (1.2 %).[11] Cocaine use in the UK is the most abundant out of all of the EU countries at 2.4 %.[10] Cocaine is sold illicitly to 'recreational' users by local dealers in two forms; as cocaine HCl, which is taken by inhalation through the nasal passage (snorting), and 'crack' cocaine or coke base which is typically smoked.[9] At 'street' level, cocaine HCl is supplied as 1 g units wrapped in paper or cling-film wrappers, plastic bags or heat sealed wraps.[9] It is then divided by the user into 'lines' of between 50 and 200 mg of cocaine, which can be inhaled through the nasal passage *via* rolled paper or a straw.[12] Street cocaine is sold in the UK at purities of 40 % to 80 %, and is commonly diluted or 'cut' with additional substances including sugars such as glucose and lactose, analgesics such as procaine or lidocaine which mimic the analgesic properties of cocaine, caffeine which acts to increase stimulant properties as well as mask a reduced drug content, and over-the-counter bulking agents such as baking soda or multi-vitamins.[9,13] Crack cocaine is

commonly sold as 0.2 g ‘rocks’ which can be adulterated with other substances of a similar appearance; the occasional inclusion of toxic substances such as levamisole for this purpose has been documented.[13] Crack cocaine vaporises at 90 °C which enables it to be smoked *via* short pipes made of glass or metal.[14]

Cocaine is a local anaesthetic, a vasoconstrictor and a powerful stimulant, the latter of which gives rise to its psychologically addictive properties which can lead to abuse of the drug.[9] Cocaine administered by insufflation or inhalation is rapidly absorbed through the bloodstream into the central nervous system where it acts upon the dopamine reuptake receptors of neurons.[4] The binding of cocaine to these receptors prevents the clearance of dopamine from the synapses, which in turn results in increased concentrations of dopamine and norepinephrine being present.[4,9] The resulting overstimulation of the dopamine ‘reward’ pathway of the body and brain leads to feelings of well-being and euphoria, competence and a heightened sense of energy and pleasure.[4,9] The effect of the drug is short-lasting (from 30 min to 1 h) and is frequently followed by anxiety, paranoia and restlessness.[9] Although physical dependence to cocaine is uncommon, the drive to re-experience initial euphoric and pleasant sensations can cause users to over-indulge in the drug, particularly due to the increased dosage that is required to obtain the same response after repeated use.[14]

Excessive doses of cocaine can result in numerous long and short-term health problems including tremors and convulsions, hallucinations, agitation and hyperthermia, the latter of which can be particularly acute and dangerous.[15] Severe cardiac adverse events, particularly sudden cardiac death, also become a serious risk at high doses due to cocaine's blocking effect on cardiac sodium channels which can lead to increased blood pressure and life-threatening arrhythmia.[4] Such cardiac conditions can develop as a result of chronic cocaine use along with other numerous adverse effects including neurotoxicity, respiratory conditions, hyperpyrexia and degradation of the septum.[4,14,15] The smoking of crack cocaine, which generates higher blood cocaine concentrations than the snorting of powder cocaine HCl, is

particularly harmful and is commonly associated with psychological dependence.[14] Although the number of cocaine-related deaths each year is relatively low compared to other class A drugs, 'polydrug' use, where cocaine is combined with other drugs such as heroin and alcohol, accounts for significant fatalities.[12]

The social ramifications of cocaine use, as well as the implications of trafficking and trade are widespread and significant.[11] The use of cocaine can lead to unemployment, poverty, crime as a means of drug acquisition, malnutrition, rapidly developing ill health and sometimes death.[14,16] These adverse effects of cocaine use are not restricted to an individual but will spread to families and communities around them; this in turn leads to increases in health problems, violence and crime.[17] At the other end of the scale, the growth of coca plants and the subsequent production of cocaine in clandestine laboratories presents health risks for workers, causes an uptake in cocaine use among members of the local community and this in turn disrupts local economies and social networks.[14,17] Overall the social and economic effects caused by the production or trafficking of cocaine can span entire countries or regions and include lowered productivity, drug-associated turmoil, wide-scale corruption, and skewed economies toward cocaine production and money laundering.[11,17]

1.1.3 UK law enforcement

As stated in Section 1.1.1, the supply and production of cocaine for non-medicinal purposes is internationally prohibited by the Single Convention on Narcotic Drugs, as well as the Convention on Psychotropic Substances and the United Nations Convention Against Illicit Traffic in Narcotic Drugs and Psychotropic substances.[6,18,19] Substances prohibited and penalties for their sale and possession in accordance with the aforementioned treaties are enacted in the UK *via* The Misuse of Drugs Act 1971, in which cocaine is listed as a Class A substance.[20] Implementation of the Act by police and law enforcement agencies is in line with the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) recommendation for an emphasis on harm

minimization.[12,21] This has led to a de-emphasis on the persecution of simple possession in favour of the implementation of public health, social and policing measures for the reduction of small-scale street dealing and acquisitive crimes in the interest of public health and safety.[21] The focus of enforcement agencies is instead on organised crime and cocaine trafficking at higher levels, particularly the groups involved in trafficking and the ways in which they operate.[12,21]

Cocaine is smuggled into the UK through Western Europe, where British organised crime groups based in Spain and the Netherlands dominate the supply chain.[12] Entry of cocaine into the UK occurs *via* four primary modes of transport; by air freight using drug couriers or 'mules' who swallow or conceal cocaine internally; by maritime ports, particularly concealed within domestic or Heavy Goods Vehicles (HGVs) or by small unauthorized vessels; by rail *via* the two international rail links, and finally by parcel post. At the majority of these entry routes, the detection of illicit drugs like cocaine is performed by customs officers of the UK Border Agency, who subsequently seize cocaine and detain the persons caught transporting it as they attempt to cross the border. This is a means of interrupting both the entry and supply chains involved in the trafficking of cocaine. The spectrum of analytical methods for the detection and identification of cocaine currently performed at Heathrow airport, for example, is one of the most comprehensive of its type involving sniffer dogs, X-ray body scanners, heat detectors which show concealed and internalised cocaine as black areas, and trace detection devices such as portable ion-mobility spectrometers for the identification of cocaine on luggage and clothing material.[12]

1.2 The detection of cocaine for forensic evidence

1.2.1 Cocaine detection in non-biological samples

Laboratory procedures for the routine identification of seized cocaine include, but are not limited to; colorimetric indicator tests, immunoassays, high performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), ultraviolet-visible

(UV-Vis) spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, powder X-ray diffraction, gas or liquid chromatography coupled with mass spectrometry (GC-MS or LC-MS), tandem MS (MS^2) and nuclear magnetic resonance (NMR).[1,9,22] Of these, chromatography methods coupled with MS detection such as LC-MS and particularly GC-MS remain the most commonly used. Hyphenated techniques of this kind are based on the initial chromatographic separation of the constituent components of the sample based on their respective retention times, after which identification and the counting of relevant components is performed by MS.[23] Analysis by MS is sensitive, specific and can provide a wealth of forensic information in terms of drug molecule identification and impurity profiling from a single measurement.[1,23]

Recent research in the area of forensic substance identification has become more focussed on the development of portable instruments which enable the rapid, sensitive, and non-destructive analysis of both bulk and microscopic material without the need for any sample preparation.[24] Methods of this nature enable drugs like cocaine to be identified on-site and in a matter of seconds, rather than the hours or days required for external laboratory testing.[1] As discussed in **Section 1.1.3**, this is particularly important in the context of border security as it enables the immediate seizure of known illicit drug substances, as well as the timely charge or release of suspected drug smugglers depending on the results of the test. Advances in techniques such as ion mobility spectrometry (IMS) and Raman spectroscopy have enabled the development of miniaturised instrumentation, resulting in portable or handheld devices which are highly practical in a forensic setting.[25,26]

Raman spectroscopy, for example, is a technique based on the detection of Raman scattering, a term which describes the small fraction of inelastic scattered light that occurs when monochromatic light is applied to a sample.[26] The difference in wavelength between the scattered and the incident light corresponds to an energy shift which in turn relates to the vibrational modes of the target molecule. These vibrational modes are unique and can therefore be used to identify the molecules

that are present.[1] Ryder *et al.* have reported the use of Raman for the quantitation of cocaine in mixed solids containing glucose and caffeine to represent typical cutting agents.[24] The spectrum from cocaine was easily distinguishable from those of other sample constituents, although it was noted that the quantitative accuracy was limited due to a lack of reference spectra.[24] Because of such limitations, Raman spectroscopy is more commonly used for initial substance identification for which it is highly suited; it can for instance be applied for the examination of surfaces, and can analyse through packaging such as glass bottles and plastic bags.[27] This in combination with its recent miniaturisation and the development of enhancement techniques which intensify the relatively weak Raman signal for increased sensitivity, has proven Raman spectroscopy to be highly applicable for the on-site detection of cocaine.[26] The suitability of Raman spectroscopy for on-site analysis was proven by the success of trial studies performed in border control settings such as at an airport, and on seized street samples in different containers.[22,27]

1.2.2 Cocaine detection in biological samples

The chronic or abusive use of drugs like cocaine can lead to serious health, social and economic consequences for the both the user and those around them, as discussed in Section 1.1.2. The testing for cocaine in biosamples occurs in two main contexts; the first is therapeutic, for example during emergency medical intervention or as part of a monitored drug treatment program, and the second is forensic, where detection is required to demonstrate the use or presence of cocaine in association with illegal activity.[9] Cocaine testing can also be performed by an employer to ensure the compliance of employees with drug-related safety regulations. In a forensic setting, the results from toxicological analyses can provide the court with evidence that an individual was under the influence of cocaine during a particular event (such as a robbery, rape, or traffic accident); identification of cocaine and its metabolites in biological specimens therefore needs to be objective and reliable.[9,28]

As mentioned in **Section 1.1.2**, the effects of cocaine are short-lasting even when taken in high dosages. Cocaine has a half-life of only 1 hour due to its rapid metabolism in the bloodstream which occurs *via* the four pathways shown in **Figure 1.3**.[29] The primary route for cocaine metabolism is by spontaneous hydrolysis, which accounts for 80 to 90 % of total elimination and can occur in one of two ways; either non-enzymatically to the primary cocaine metabolite benzoylecgonine, or enzymatically to methylecgonine by the action of esterase enzymes present in blood and tissue.[30] Alternatively, metabolism of cocaine *via* oxidation by mixed function oxidase enzymes can occur, the process of which takes place predominantly in the liver.[30] Recent cocaine use by an individual is normally demonstrated using benzoylecgonine rather than cocaine as an analyte. This is due to the shorter half-life of cocaine itself in blood and urine samples (the detection time for cocaine is typically between 1 and 2 days in blood after a single dose),[29] and its low concentrations in urine where it is present for only a brief period.[1] Benzoylecgonine, on the other hand, is present at high concentrations in urine and can be detected up to 3 days following use.[30] Ecgonine methyl ester has been reported as detectable for up to 7 days after a single dose of cocaine in urine.[29]

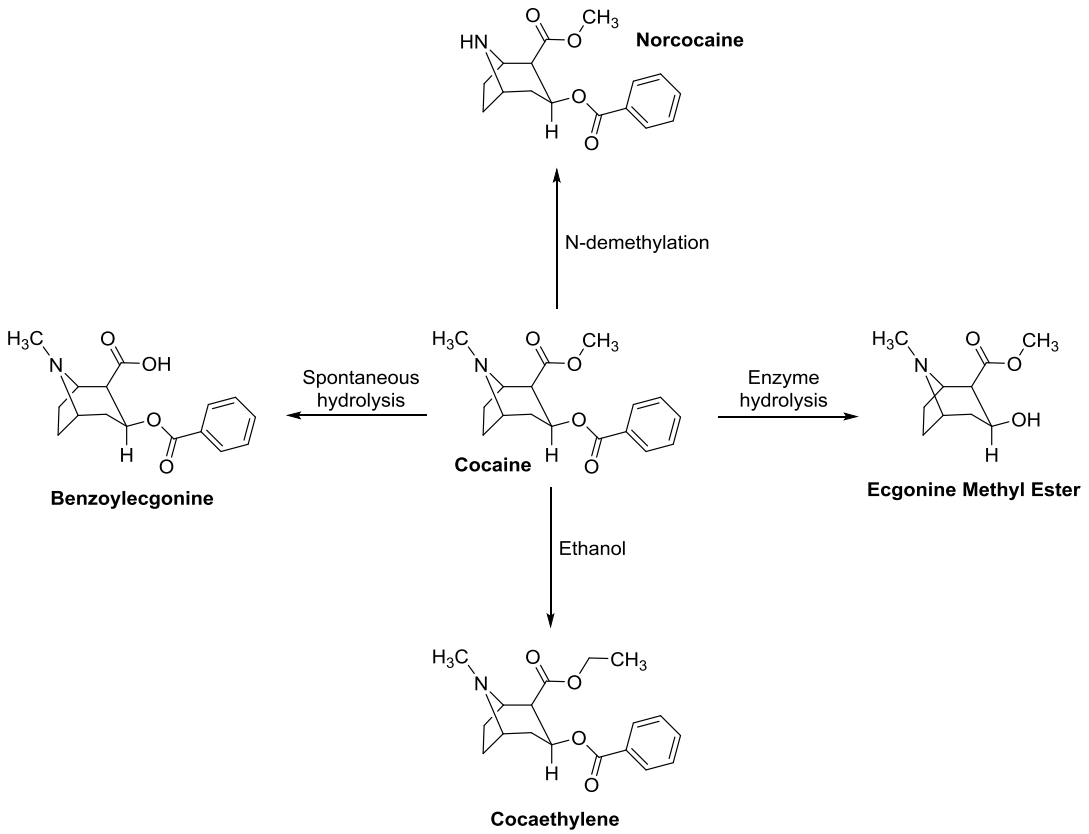

Figure 1.3. The metabolic processing of cocaine in blood.[30]

Various analytical techniques have been used for the detection of cocaine individually, or alongside other illicit substances in a number of different biological matrices.[31] Urine has traditionally been the most common biological specimen however advances in sample preparation methods, chromatography-based separation and detection techniques have nowadays made blood an equally accessible matrix.[1] Due to difficulties in describing recent or current exposure to cocaine, various biological specimens have been incorporated into analytical techniques, including but not limited to hair, sweat, oral fluid, nails, teeth, amniotic fluid, meconium and inner organs.[28] In oral fluid samples, for example, both cocaine and benzoylecgonine are detectable following a single dose of cocaine for up to 12 and 24 hours after use, respectively.[29]

Due to the complexity of biological specimens, sample preparation steps are often required to pre-concentrate the target compound and remove it from potential interferents in the matrix prior to analysis.[28] This is particularly important for post-mortem specimens where by-products of degradation which can interfere with analytical techniques causing false-positive or false-negative results are commonly present.[30] With the exceptions of urine and oral fluid which may require little or no sample preparation, the majority of liquid samples are pre-concentrated by solid-phase or liquid-liquid extraction (SPE and LLE, respectively).[28] Of the two techniques, SPE is the most common due to the flexibility enabled by the range of different cartridges with different properties available. Solid samples such as teeth and tissue specimens need to be pulverised and dissolved, or homogenised prior to extraction.[28] Of all the common matrix types, hair is the most complicated and requires multiple digestion steps prior to extraction; in addition to LLE and SPE, the application of solid-phase micro-extraction (SPME) or ultrasound extraction for this matrix type is common.[32]

Following sample preparation, the identification and quantitation of cocaine by toxicological analysis is performed in two stages: screening and confirmation.[1] Different groups of analytical techniques are applied in each step. The purpose of screening is to identify samples where cocaine is present at quantities of forensic significance prior to further analysis.[33] For example, the presence of negligible concentrations of cocaine (<0.1 ng mL^{-1}) is unlikely to stand up in court as evidence; higher concentrations (*e.g.* >50 ng mL^{-1}), however, provide evidence of recent drug use and could therefore be relevant to a criminal trial.[1] Distinguishing negative from positive samples at the beginning of the analytical process avoids the need to perform costly, sophisticated and labour intensive analyses on an unnecessarily high number of samples. In order to distinguish negative from positive samples, a quantitative analytical method with a 'cut-off' concentration (CO), above which the sample is positive and requires further confirmatory analysis, and below which it is considered negative, is applied.[28] Rapid, specific, sensitive, inexpensive and easy to perform techniques such as thin layer chromatography (TLC) and immunoassays are

commonly used as screening methods.[1] The accuracy of the technique at determining whether a sample falls below the cut-off concentration is crucial for avoiding false negative results, as this will prematurely end the toxicological analysis.[28]

The majority of the immunoassays used for forensic screening purposes target benzoylecgonine as the primary cocaine metabolite, typically alongside other illicit substances or metabolites.[1] Enzyme immunoassays such as the enzyme multiplied immunoassay technique (EMIT) and the enzyme-linked immunosorbent assay (ELISA) are widely used, although immunoassays with different labelling systems such as fluorescence polarization immunoassays (FPIA) and radioimmunoassays (RIA) are also frequently applied.[28] All immunoassays harness the analyte (known as an antigen)-binding capacity of antibodies for their target specificity, the process of which is explained in more detail in **Chapter 3.1.2**. Due to the specificity of the antibody-antigen binding process even in the presence of potential matrix interferents, assays of this nature are highly suited to the detection of trace drug compounds in complex biological samples with only minimal sample preparation.[34] The limit of detection (LOD) of immunoassays, which are typically around 0.1 ng mL^{-1}, fall well below the typical cut-off concentrations of 50 to 300 ng mL^{-1} (for blood and urine) used in forensic analysis.[28] This is illustrated in **Table 1.1**, in which the details of a number of recent studies where immunoassays have been applied for the screening of toxicological specimens are listed alongside their respective cut-off concentrations.[33,35-44] The assay procedure can be readily automated for high-throughput analysis, and the present-day mass manufacturing of drug-targeting assays for forensic purposes enables inexpensive screening particularly compared to sophisticated detection methods such as mass spectrometry (MS).[28]

Table 1.1. The details of recent studies in which immunoassays have been applied for the screening of a range of toxicological specimens. Abbreviated terms are as follows; CO, cut-off concentration; LOD, limit of detection; LOQ, limit of quantitation; PM, post-mortem; extract., extraction; RIA, radioimmunoassay; FPIA, fluorescence polarisation immunoassay; EMIT, enzyme multiplied immunoassay technique; CEDIA, cloned enzyme donor immunoassay; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; SPE, solid-phase extraction; LLE, liquid-liquid extraction; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; LC-MS, liquid chromatography-mass spectrometry.

Ref.	Specimen	Sample prep.	Screen	LOD / ng mL^{-1}	CO / ng mL^{-1}	Sample prep.	Confirmation	LOQ / ng mL^{-1}	CO / ng mL^{-1}
34	Urine	None	RIA // FPIA // EMIT // CEDIA	-	150	SPE	GC-MS	-	15
32	PM blood// tissue	Homogenation // centrifugation	ELISA // RIA	-	300	LLE	GC-MS // $GC\text{-}MS^2$ // HPLC	-	50
35	Hair	Heat extraction	ELISA // EIA	0.05 ng mg^{-1}	0.08 ng mg^{-1}	Ultrasonication// SPE	LC-MS // GC-MS	0.016 // 0.05 ng mg^{-1}	0.1 ng mg^{-1}
36	Sweat	Elution from patch	EIA	< 1	10	Elution // SPE	GC-MS	2	4
37	Meconium	Homogenation	ELISA	-	20 ng g^{-1}	Homogenation	GC-MS	-	20 ng g^{-1}
38	Oral fluid	Elution from collection pad	EIA	0.95	10	Elution from collection pad	GC-MS	7	10
39	Hair	Ultrasonic extract.	ELISA	-	5	SPE	GC-MS	0.03 ng mg^{-1}	0.11 ng mg^{-1}
40	Hair	Ultrasonic formic acid extract.	ELISA	< 0.5 ng mg^{-1}	0.5 ng mg^{-1}	Ultrasonic formic acid extract.	$LC\text{-}MS^2$	0.001 ng mg^{-1}	0.5 ng mg^{-1}
41	Whole blood // urine	None	ELISA	5	150	SPE	GC-MS	-	-
42	PM blood	None	ELISA	10	5	LLE	GC-MS	-	5
43	Oral fluid	None	EIA	10	-	SPE	GC-MS	2.5	8

The confirmatory analysis that follows screening is performed on samples in which the concentration of cocaine is determined as being above the cut-off concentration by the screening method. Confirmation is performed using methods which enable quantitation of drug compounds to high levels of accuracy and specificity. In the same way as for the detection of cocaine in non-biological sample matrices described in **Section 1.2.1**, chromatography separation coupled with mass spectrometry detection techniques remain the gold standard when it comes to the detection of illicit drug compounds in a toxicological setting.[23] As shown in **Table 1.1**, GC-MS is the most frequently applied method for the confirmation of cocaine in a range of biological matrices in the studies shown, however advances in the sensitivity of LC-MS^2 have also increased the number of studies performed using this method.[45-47] The use of HPLC coupled with alternative detection methods such as ultraviolet (UV) and fluorescence (FL), as well as capillary electrophoresis (CE)-based techniques for the quantitative detection of cocaine has also been reported.[28]

Recent research into alternative biological specimens for illicit drug testing beyond traditionally used samples such as blood and urine has led to the development of methods for the analysis of sweat and oral fluid specimens, among others.[39,48,49] Drug compounds such as cocaine are transported by epithelial cells from blood plasma into sweat either by diffusion or *via* the process of active transport (secretion).[50] The presence of cocaine and benzoylecgonine in sweat from regular cocaine users was investigated by Kidwell *et al.* using sweat collection patches analysed by GC-MS.[48,50] Concentrations ranging from 250 to 22,000, and 30 to 2,200 ng per patch were obtained for cocaine and benzoylecgonine, respectively; this notably showed that cocaine was present at higher concentrations than its principle metabolite.[48,50] This phenomenon is thought to occur due to the lipophilic nature of cocaine, which enables it to be more easily transported by the lipid membrane of epithelial cells and thereby causing it to be more readily excreted into sweat than its hydrophilic metabolic counterpart.[48] A study performed by Huestis *et al.* established that cocaine was present in predominance over benzoylecgonine in sweat sampled from volunteer subjects using sweat patches, and was detectable by

GC-MS for up to 48 hours after the drug was administered.[51] The metabolite ecgonine methyl ester was also detected in sweat during this study, at concentrations comparable to those of the benzoylecgonine present in the same samples.[51] The existence of a time lag between the taking of cocaine and its excretion in sweat suggests that detection will not only be indicative of drug use, but also provide a potential window of time during which the drug was taken. This could be important in circumstances where an issue has been raised as to whether or not an individual is in a fit state to, for example, operate machinery or drive a vehicle.

Interest has also grown in recent years in gaining information from latent fingerprints beyond suspect identification *via* the unique ridge pattern that forms the print. Additional circumstantial information in a forensic context, such as the use by or recent exposure of an individual to illicit drugs, could provide a greater link between a fingerprint and a suspect.[52] The mechanism of drug and metabolite compounds in sweat has been harnessed for the detection of cocaine in latent fingerprints using a number of different analytical approaches, and is particularly well-described for prints artificially doped with compounds of interest.[53-56] However, recent advances by Leggett *et al.* using fluorescently labelled, antibody-functionalised magnetic particles have also demonstrated the detection of drug metabolites endogenously produced in the latent fingerprints of drug users.[57-59]

In addition, Rowell *et al.* were able to show isolated patches of cocaine contamination in a fingerprint obtained from an individual being treated at a drug addiction centre using surface assisted laser desorption/ionisation time-of-flight mass spectrometry (SALDI-TOF-MS).[55] The non-homogenous pattern of cocaine contamination in the print was thought to be the result of the subject's fingertip coming into contact with the drug prior to the time of sampling.[55] Analytical methods for the detection of these types of cocaine residues in latent fingerprints could be used in a forensic context, as their presence would provide further evidence of a suspect's involvement in drug use or trade.

1.3 Cocaine on paper currency

1.3.1 Banknotes as forensic evidence

Over the past two decades, interest has grown in the detection of illicit drugs such as cocaine on paper currency. The number of scientific publications relating to the subject rose four-fold in the previous decade, from 1.8 papers per year in 1994 - 2003 to 4.5 papers per year in 2005 - 2007.[60] The presence of background levels of cocaine contamination on the majority of banknotes, discovered using newly developed analytical methods, coincided with an increase in popularity of the drug worldwide and generated a surge of public interest in the matter.[60] Headlines like those shown in **Figure 1.4** featured widely in news reports by the UK media in 2010 - 2011, fuelling this public interest and resulting in further studies into the presence and detection of cocaine on banknotes.

Figure 1.4. Typical examples of the headlines from UK media reporting on the widespread contamination of paper currency with background levels of cocaine, published in 2010 and 2011.[61-65]

The presence of cocaine on banknotes is unsurprising since the notes can be involved in both the use and trade of the drug. For example, a common route of oral self-administration is by insufflation, which can be performed using a rolled banknote. The exchange of cocaine for cash monies by traders is the main route by which contamination occurs, either *via* the handling of banknotes with contaminated fingers, or by placing of banknotes onto a surface on which cocaine was previously present. The latter is described by Ebejer *et al.* as particularly common, as bulk cocaine received by a trader will first be weighed and packaged into smaller portions to be sold, a process performed on a flat surface (such as a table) which is most often the same surface where cash is counted or placed during a sale.[66]

A number of possible mechanisms have been suggested for the adherence of cocaine to banknotes including dissolution in surface inks or grease, or chemical binding.[67,68] The most probable of these adherence mechanisms involves the physical entrapment of small particles in the interstices between the fibres that form a banknote.[69] UK Sterling banknotes, for example, are printed on a blend of cotton and linen rag.[66,67] After a period of use the characteristics of the banknote fibres can change, resulting in a loosening of the meshwork of fibres and the subsequent formation of cavities within the weave of the note.[70] As described in **Section 1.1.2**, cocaine HCl is most commonly traded as a solid, impure crystalline powder. Crystalline cocaine of this nature can become entrapped and retained in the cavities produced by the loosening of banknote fibres over time, illustrated by the improved adhesion of the drug to older banknotes as previously demonstrated.[70-72] The images obtained using a scanning electron microscope (SEM) by Anglada *et al.* and by Roberts *et al.* visually demonstrate the entrapment of cocaine crystals within a meshwork of banknote fibres (**Figure 1.5**).[72,73]

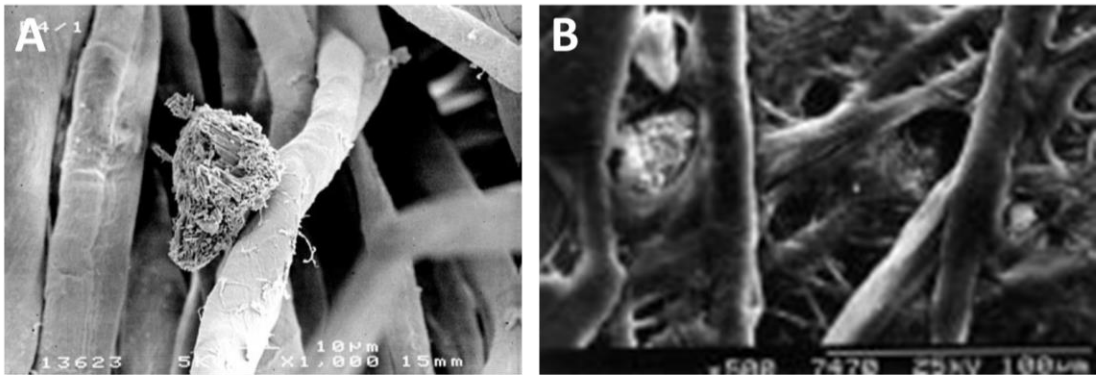

Figure 1.5. SEM images of cocaine crystals in the interstices between the fibres of a banknote; images (A) and (B) were obtained from Anglada *et al.* and Roberts *et al.*, respectively.[72,73]

The low or 'background' levels (*ca.* 1 μg note^{-1}, for example) of cocaine contamination found on the majority of banknotes in general circulation is caused by the indirect transfer of drug crystals from notes which have been in direct contact with the drug *via* the mechanisms described in the previous paragraph. Transfer of this type is known to occur *via* two main routes; physical contact between a contaminated note with other banknotes around it, and by the workings of bank sorting machines. The former is known to occur to a much lesser extent than the latter, which is the route by which the majority of cross-contamination takes place. Transfer from one contaminated note to others within a bundle of banknotes was experimentally demonstrated by Carter *et al.* using the drug diamorphine as a model substance.[66] The study showed that transfer from a single note to its nearest neighbours did not happen readily, was limited to only a small proportion of the total contamination (less than 6 %) and did not spread beyond more than two adjacent banknotes.[66]

In a separate study by the same research group, Carter *et al.* also report the widespread contamination of mock banknotes (made of cotton paper) with illicit substances including cocaine after processing by bank sorting machines.[67] The mock banknotes were sent to a number of different domestic bank branches within the UK for counting in the usual way, after which 60 % were found to be contaminated with

cocaine.[67] The authors noted a diminishing concentration of cocaine throughout a bundle of notes which coincided with the order in which they had passed through the machine. The presence of crystalline material on one of the notes also coincided with the location of black marks on the surface of the note thought to originate from components of the sorting mechanism.[67] These results confirm the presence of cocaine in dust samples obtained from counting machines at a bank in Switzerland as indicative of the cause of cross-contamination.[74]

The presence of illicit substances including cocaine on paper currency is frequently used by police as circumstantial evidence of an individual's involvement in illegal activities such drug trafficking or trade.[66] Where activities of this type are suspected, the results of an analysis of banknotes obtained from the suspect indicating the presence of cocaine can be incriminating.[69] However, presenting cocaine on banknotes as evidence requires the concentrations of cocaine on the confiscated banknotes to be distinguishable from those present in general circulation. The development of analytical methods for the quantitative analysis of cocaine on banknotes has led to efforts being made towards the identification of different degrees of contamination. This in turn would enable 'dirty money', which has recently been in contact with drugs, to be distinguishable from 'background money' which has become inadvertently contaminated by general circulation.[70]

The simplest way to distinguish dirty from background banknotes would be to establish a cut-off concentration in a similar way to the screening methods performed on biological samples, or to set cut-off ranges for quantitative analysis. Cocaine levels below the cut-off would fall within the range of 'background' concentrations, whereas concentrations beyond that range could be associated with direct contact with cocaine. In a study by Dixon *et al.*, 11,983 GB£ banknotes were analysed for cocaine by thermal desorption followed by MS^2 detection.[75] The notes were divided into two groups based on origin; 4826 were case notes seized from alleged drug dealers, and 7157 were from general circulation obtained from banks around the UK. The cocaine contamination was determined based on peak intensity rather than

concentration, as the MS^2 method could not be applied quantitatively due to difficulties in comparing the responses from standard solutions with those of cocaine within banknote matrices. The peak intensity obtained from the banknote samples was subsequently compared using pattern recognition techniques to determine if the distribution of the seized notes was higher than that of the notes from general circulation.[75] The analysis showed a clear difference between the distributions of the two groups of banknotes, where the peak intensity of the case notes was significantly higher than the circulation notes with a few exceptions.[75] The results indicate that the quantitative analysis of cocaine on banknotes is a viable means by which to distinguish the route by which the contamination, whether by direct contact with drugs or *via* general circulation, could have occurred.

Due to variation in the levels of background contamination of cocaine in different countries, further studies of this kind would be needed if a firmer set of cut-off ranges is to be established. This is illustrated in **Table 1.2**, where a summary of the different concentrations of cocaine detected on banknotes from different currencies and countries is shown alongside the methods used for analysis. A difference in the cocaine concentrations from different denominations of the same currency was observed by Zuo *et al.*, who noted that sampled notes of higher denominations such as US$5 and US$10 also had higher concentrations of cocaine on them when compared with US$1 bills. This trend, however, was not observed in other studies of US currency.[76-78] Variation was also observed between banknotes obtained in different cities in a case study performed by Di Donato *et al.* in Brazil, and amongst those from different states in a study by Jourdan *et al.* performed in the US.[78,79]

Table 1.2. A summary of the quantities of cocaine previously detected on banknotes of different currencies, and the methods used for analysis.

Year	Currency	Method	Denomination	Average [cocaine]/ ng note^{-1}	[Cocaine] range/ ng note^{-1}	Ref.
1996	US$	GC-MS	$1 (n = 136)	22,300	0 – 1,327,000	68
1998	US$	GC-MS	$1 (n = 4) $20 (n = 10)	2,860 830	140 – 10,020 0 – 2,990	77
2001	US$	GC-MS	$1 (n = 40)	28,750	0 – 922,720	76
2005	Spanish €	GC-MS/MS	€5 (n = 3) €10 (n = 7) €20 (n = 4) €50 (n = 2)	82,200 281,900 36,900 57,400	1,250 – 234,000 26,800 – 889,000 27,200 – 76,700 47,000 – 67,800	80
2007	Irish €	LC-MS/MS	€5 (n = 20) €10 (n = 10) €20 (n = 10) €50 (n = 5)	18.76 5.07 66.64 14.34	0.09 – 286.66 0.07 – 19.17 0.7 – 576.14 0.25 – 32.47	81
2007	R$	GC-MS	R$1 (n = 46)	51,150	0 – 275,100	79
2008	US$	GC-FID/MS	$1 (n = 5) $5 (n = 5) $10 (n = 7) $20 (n = 6) $50 (n = 4) $100 (n = 4)	0 19,200 10,300 4,200 13,400 2,220	– 4,650 – 49,400 0 – 46,200 0 – 9,210 3,820 – 19,600 0 – 8,870	82
2011	Canary Isl. €	LC-MS/MS	Mixed (n = 120)	Median = 188.48	0.00 - 15,023.1	83
2011	Luxem-bourgish €	LC-MS/MS	Mixed (n = 64)	Median = 106	≤ 2 - 141	70
2013	US$	GC-MS, LC-MS and LC-MS/MS	$1 (n = 630) $5 (n = 628) $10 (n = 678) $20 (n = 870) $50 (n = 688) $100 (n = 680)	2.15 3.62 3.18 1.61 2.26 1.49	0.09 – 20.6 0.08 – 94.7 0.05 – 37.5 0.06 – 12.2 0.06 – 29.0 0.05 – 29.6	78

1.3.2 Methods of analysis

Analytical methods for the detection of cocaine on banknotes, like other forensic analytical methods require a high level of sensitivity, accuracy and robustness in order to detect trace levels of cocaine in the presence of potential interferences in the sample matrix. The level of sensitivity required is illustrated by the low concentrations of cocaine, in the ng to µg range, detected on banknotes as reported by the studies shown in **Table 1.2**. In addition to the low analyte concentration, the analytical method requires a level of specificity that is suitable for the detection of trace levels of cocaine in a complex sample matrix brought about by the nature and function of banknotes.

As detailed in **Section 1.3.1**, Sterling banknotes comprise of cotton and linen rag which is treated with preservatives prior to printing to strengthen the material and minimize degradation.[83] In addition to preservatives, the way in which paper currency is handled and transferred means environmental pollutants such as dust, soil, food particles, sweat, inks, oils, cosmetics and resulting micro-organisms can all be found on the fibres of a banknote.[25] The presence of pharmaceutical substances or compounds deriving from food sources on banknotes is known; of these, caffeine is the most predominant but nicotine, codeine and paracetamol have also been reported at trace levels.[80] In terms of illicit or controlled substances of forensic relevance, the primary cocaine metabolite benzoylecgonine, as well as other drug compounds including 6-monoacetylmorphine (6-AM), tetrahydrocannabinol (THC), morphine, 3,4-methylenedioxymethamphetamine, methamphetamine, heroin, ketamine, and phencyclidine (PCP) have been found on paper currency.[81,84] Along with cocaine as the most prevalent drug on banknotes, heroin and ecstacy have also reportedly been detected on a number of notes at particularly high concentrations, raising concerns about health risks relating to exposure.[85]

In the presence of the potential interferents listed in the previous paragraph within the sample matrix, it is imperative that the analytical technique used for the detection of cocaine is sufficiently specific and robust to ensure its accuracy. GC-MS was in

1989 the first reported method employed for the detection of cocaine on banknotes.[86] A two- or three-step process was a common method for analysis in the reports that followed; in the first step the cocaine is extracted from the banknote using an organic solvent such as methanol, after which the extract is further purified by solid-phase extraction (SPE) before analysis by GC or LC coupled with MS.[68,77] Sample preparation procedures of this kind enable the cocaine to be separated from potential interferents on the banknote, thereby facilitating further specific analysis enabled by the sensitivity of the MS. As demonstrated by the summary of analytical methods shown in **Table 1.3**, methanol remains the most commonly used solvent for cocaine extraction, although the advancement of GC and LC-MS technologies has meant that SPE is sometimes no longer necessary. The advent of chromatography-coupled MS^2 techniques in particular has improved the sensitivity, selectivity and repeatability of analytical methods in this field, thereby enabling quantitative detection of cocaine on banknotes at extremely low levels.[23]

Table 1.3. A summary of recently reported analytical methods for the detection of cocaine on banknotes. Abbreviated terms are as follows; LOD, limit of detection; RSD, relative standard deviation; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; TD-APCI-MS, thermal desorption-atmospheric pressure chemical ionisation-mass spectrometry; IMS, ion-mobility spectrometry; GC-FID/MS, gas chromatography-flame ionisation detection/mass spectrometry, ECL, electrochemiluminescence; LC-MS, liquid chromatography-mass spectrometry, LC-MS/ESI, liquid chromatography-mass spectrometry/electrospray ionisation; extract., extraction; SPE, solid-phase extraction; TD, thermal desorption; ACN, acetonitrile; MeOH, methanol.

Year	Method	Sample treatment	LOD / ng note^{-1}	% RSD	Recovery / %	Ref.
1996	GC-MS	MeOH extract. // SPE	> 90			68
1997	GC-MS	HCl extract. // SPE	1.0			77
	TD-APCI-MS	Vacuum // TD			Semi-quantitative	72
	Immunoassay	None			Semi-quantitative	87
2001	GC-MS	ACN extract. // SPE	1.0 ng mL^{-1}			76
2003	TD-APCI-MS	None			Semi-quantitative	67
2004	Raman	None			Semi-quantitative	88
2005	GC-MS^2	MeOH extract.	0.15	6	101 - 98	80
2006	LC-MS^2	MeOH extract.	0.004	3.69	87	81
	TD-APCI-MS^2	None			Semi-quantitative	75
2007	GC-MS	Aq. extract.				79
	TD-APCI-MS	None			Semi-quantitative	66,89
2008	GC-FID/MS	Ultrasonic aq. extract.	1	< 2	95	82
	IMS	Vacuum	0.2 ng			25
2011	LC-MS^2	ACN extract.	0.003	< 5		83
	LC-MS^2	MeOH extract.	0.004	7.2	65	70
	ECL biosensor	Acetic acid extract.	3.7 pmol L^{-1}	5.2	92	82
2013	GC-MS	MeOH extract.	0.1			78
	LC-MS/ESI		0.1			
	LC-MS^2		0.05			

Although the methods developed using GC-MS and LC-MS for the detection of cocaine on banknotes provide undisputed levels of sensitivity, the disadvantages of using these techniques include the time needed for analysis and the destruction of the banknotes *via* solvent-based extraction systems.[1] These disadvantages have led to the development of alternative methodologies including IMS and TD-MS which enable non-destructive sampling followed by rapid and sensitive detection allowing 50 banknotes to be analysed in a 4 min period.[60] The bulk of the reported studies into the development of MS^2 based techniques for the detection of cocaine on banknotes have been performed by researchers at Mass Spec Analytical Ltd., who developed a series of methods based on the use of thermal desorption and APCI as sampling techniques.[66,67,69,72,75,89,90] In these methods, cocaine is obtained from the banknotes either by trapping dust from the notes in a vacuum filter, or by desorbing the cocaine directly from the note itself into the source of the MS.[69] Cocaine from the vacuum filters yields characteristically protonated molecular ions at *m/z* 304, whereas specific gas-phase ion transitions can be monitored for cocaine at *m/z* 304 to *m/z* 182, and *m/z* 304 to *m/z* 105 from the directly desorbed banknote material.[60] The LOD for this type of technique is dependent on the amount of desorbed material, but is typically around 1 ng and therefore comparative to the sensitivity achieved by chromatography-MS based methods.[72] However, due to the difficulties in comparing the responses from standard solutions with those of particulate materials trapped within banknote matrices, the developed MS^2-based methods cannot be used quantitatively for applications of this nature.[60] Instead, recent studies have focussed on the use of statistical analysis *via* which 'drug money' and banknotes from general circulation can be discriminated based on their respective peak intensities rather than cocaine concentration.[75,90]

IMS, like TD-MS^2, is a highly sensitive technique for the non-destructive and rapid detection of cocaine on banknotes. Samples containing trace compounds for IMS detection are typically applied to filter paper which is then heated to vaporization and ionised by electrons emitted by a ^{63}Ni source. The time required for the resulting ions to drift through an electric field before meeting with a detector, known as 'drift time',

is substance-specific and therefore enables the presence of compounds in the sample to be identified.[60] The method facilitates the detection of cocaine on bulk quantities of banknotes *via* vacuum sampling, which is typically performed by running the sampler down two specific 'lanes' down one side of the banknote prior to analysis.[25] The detection of cocaine can alternatively be performed on individual banknotes by collecting surface swabs or by introducing the banknote directly into the vaporizer unit of the IMS.[25] The latter, performed by Briellmann *et al.* using a specifically modified vaporizer unit, obtained a 1000-fold increase in sensitivity when compared with analysis by vacuum sampling.[74] IMS is a sophisticated method capable of detecting ng quantities of cocaine, however it suffers from the same drawbacks as TD-MS^2 in that due to the nature of desorption sampling techniques it cannot be applied for quantitative measurement.[60,91] Nevertheless due to its inherent versatility, portability and low maintenance requirements it is widely used in security and law enforcement settings and remains the technique of choice for the detection of cocaine on banknotes by UK law enforcement agencies.[12,25,60]

Despite the prevalence of immunoassay-based methods for the detection of cocaine and its primary metabolite benzoylecgonine in biological samples (as described in **Section 1.2.1**), the gold-labelled optically-read rapid immunoassay (GLORIA) is at present one of few immunoassays which have been successfully applied for the detection of cocaine on banknotes.[87] The assay design is based on a lateral flow-style set-up in which the detection of cocaine is facilitated by anti-cocaine antibody-functionalised gold nanoparticles (AuNP). Cocaine on a banknote is sampled by swabbing using the 'collection fleece' part of the device; the fleece is then dipped in water, which immobilises the sample material and transports it toward the detection zone. Cocaine present in a sample will bind to immobilised anti-cocaine antibody in the detection zone, and is subsequently labelled by the addition of the anti-cocaine AuNP which produces a visible white to pink colour change when cocaine is present.[87] The assay consists of a transportable device which enables the detection of ng quantities of cocaine in less than 3 min, although concerns have been raised

over its efficacy and specificity due to reports of false positive results in the presence of cocaine metabolites, pharmaceutical compounds and food additives.[60]

As described in Section 1.2.1, immunoassays and particularly enzyme immunoassays are widely used by forensic toxicologists for the screening of illicit substances such as cocaine in biological specimens. The screening process requires quantitative accuracy in order to establish whether a sample is below the cut-off concentration, which is typically in the order of ng mL^{-1} concentrations. The successful applications for assays of this type for analysing complex forensic samples such as postmortem blood, or homogenised tissue demonstrates their efficacy for the detection of trace drug compounds in complex sample matrices. Banknotes, like biological samples, harbour trace levels of cocaine and present a challenging matrix for forensic analysis; however, the application of enzyme immunoassays for the detection of cocaine on banknotes is limited and does not allow for quantitative analysis. The widespread use of immunoassays for toxicological analysis is due to the fact that they are operator friendly, economical, rapid, sensitive and accurate alternatives to the more sophisticated and expensive hyphenated MS-based methods which dominate the types of analyses currently performed on banknotes. Based on this, the development and application of a quantitative enzyme immunoassay for cocaine extracted from banknotes, as well as the location-specific immunostaining of cocaine on the surface of banknotes will be demonstrated in this study.

1.4 Thesis outline

This thesis describes the development of two novel immunological methods for the quantitative and location-specific detection of cocaine on paper currency. The application of a technique for the detection of cocaine in latent fingerprints, and a comparison of different functionalisation strategies for the synthesis of antibody-gold nanoparticles for the detection of cocaine are also described.

A detailed description of the materials, reagents, instrumentation and experimental procedures used in this study are detailed in Chapter 2. The development of a competitive enzyme immunoassay for the detection of cocaine on paper currency, and its application for the analysis of cocaine extracted from both banknotes and latent fingerprints are described in Chapter 3. The preliminary efficacy of the assay is assessed based on the analysis of ten Bank of Scotland (UK) banknotes obtained from general circulation and the results of which are then compared to those of a parallel LC-MS analysis. The assay is also applied to the detection of cocaine in ten latent fingerprints obtained from five test subjects being treated for the habitual use of drugs, including cocaine, at a methadone treatment clinic; the resulting concentrations are then compared with the cocaine concentrations of corresponding oral fluid samples obtained by GC-MS analysis.

Chapter 4 describes the development of an immunodetection method for the immobilisation and subsequent localised staining of cocaine on banknotes, using an enzyme-labelled anti-cocaine antibody and an acrylamide gel matrix. A comparative study of different semi-permeable hydrogel matrices for the immobilisation of cocaine is initially performed. Optimisation of the in-gel immunodetection method is then carried out using polyacrylamide gel electrophoresis gels, and cotton paper samples as a representation of the banknote sample matrix. The labelling of anti-cocaine antibody with the enzyme horseradish peroxidase is also described. The final optimised method is applied to control samples consisting of newly minted banknotes spiked with ‘street’ cocaine, after which banknotes obtained from general circulation are analysed.

A comparative study of four strategies for the synthesis of antibody-functionalised gold nanoparticles for the detection of cocaine is detailed in Chapter 5. Characterisation of the functionalised particles is performed using transmission electron microscopy, ultraviolet visible spectrophotometry, and gel electrophoresis methods. The cocaine binding efficacy of the resulting nanoparticles, and their suitability for the detection of cocaine in forensic specimens is then determined by

enzyme labelling using a cocaine-horseradish peroxidase conjugate, and by the application of a plate-based immunoassay in which the immobilisation of cocaine is facilitated by a bovine serum albumin protein intermediate.

Finally, conclusions of the findings for all the experiments presented in the thesis are provided in Chapter 6, along with detailed suggestions for future experiments.

1.5 References

(1) Kidwell, D. A.; Athanaselis, S. A. In *Handbook of forensic drug analysis;* Smith, F. P., Ed.; Elsevier Academic Press: San Diego, 2005, 235

(2) Casale, J. F.; Klein, R. F. X. *Forensic Sci. Rev.* **1993**, *5*, 12.

(3) Karch, S. B. *J. Roy. Soc. Med.* **1999**, *92*, 393.

(4) Gold, M. S. *Cocaine*; Plenum Medical Book Company: New York, 1993.

(5) Streatfeild, D. *Cocaine: A definitive history*; Virgin Publishing: London, 2001.

(6) Lande, A. *The Single Convention on Narcotic Drugs;* International Organization, **1961**, *16*, 776.

(7) Moore, J. M.; Casale, J. F. *J. Chromatogr. A* **1994**, *674*, 165.

(8) Streatfeild, D. *Cocaine: An unautharized biography*; Diane Publishing Company: London, 2002.

(9) Jickells, S.; Negrusz, A. *Clarke's Analytical Forensic Toxicology*; Pharmaceutical Press: London, 2008.

(10) United Nations Office on Drugs and Crime. *World drug report 2010;* United Nations Publications, 2010.

(11) United Nations Office on Drugs and Crime. *World drug report 2013;* United Nations Publications, 2013.

(12) House of Commons Home Affairs Committee. *The cocaine trade;* The Stationary Office Ltd.: London, 2010.

(13) Chang, A.; Osterloh, J.; Thomas, J. *Clin. Pharmacol. Ther.* **2010**, *88*, 408.

(14) Allen, D. F.; Jekel, J. F. *Crack: The Broken Promise;* Macmillan Academic and Professional: London, 1991.

(15) Devlin, R. J.; Henry, J. A. *Crit. Care* **2008**, *12*, 202.

(16) Seddon, T. *Brit. J. Criminol.* **2006**, *46*, 680.

(17) Singer, M. *Int. J. Drug Policy* **2008**, *19*, 467.

(18) United Nations. *Convention on Psychotropic Substances,* Vienna, 1971.

(19) United Nations. *United Nations Convention Against Illicit Traffic in Narcotic Drugs and Psychotropic Substances,* Vienna, 1988.

(20) Parliament of the United Kingdom. *Misuse of Drugs Act,* London, 1971.

(21) Dorn, N. *J. Drug Issues* **2004**, *34*, 533.

(22) Hargreaves, M. D.; Page, K.; Munshi, T.; Tomsett, R.; Lynch, G.; Edwards, H. G. *J. Raman Spectrosc.* **2008**, *39*, 873.

(23) Maurer, H. H. *J. Mass Spectrom.* **2006**, *41*, 1399.

(24) Ryder, A. G.; O'Connor, G. M.; Thomas J, G. *J. Raman Spectrosc.* **2000**, *31,* 221.

(25) Dussy, F. E.; Berchtold, C.; Briellmann, T. A.; Lang, C.; Steiger, R.; Bovens, M. *Forensic Sci. Int.* **2008**, *177*, 105.

(26) Izake, E. L. *Forensic Sci. Int.* **2010**, *202*, 1.

(27) Weyermann, C.; Mimoune, Y.; Anglada, F.; Massonnet, G.; Esseiva, P.; Buzzini, P. *Forensic Sci. Int.* **2011**, *209*, 21.

(28) Janicka, M.; Kot-Wasik, A.; Namieśnik, J. *TrAC Trend. Anal. Chem.* **2010**, *29*, 209.

(29) Verstraete, A. G. *Ther. Drug Monit.* **2004**, *26*, 200.

(30) Fenton, J. J. In *Forensic Science: An Introduction to Scientific and Investigative Techniques;* 2nd ed.; James, S. H., Nordby, J. J., Eds.; CRC Press: New York, 2005.

(31) Mali, N.; Karpe, M.; Kadam, V. *J. Appl. Pharm. Sci.* **2011**, *1*, 58.

(32) Harrison, R.; Fu, S. *J. Forensic Investigation* **2013**, *1*, 8.

(33) Moore, K. A.; Werner, C.; Zannelli, R. M.; Levine, B.; Smith, M. L. *Forensic Sci. Int.* **1999**, *106*, 93.

(34) Schutz, H.; Auch, J.; Erdmann, F.; Weiler, G.; Verhoff, M. A. *Arzneim.-Forsch.* **2006**, *56*, 414.

(35) Armbruster, D. A.; Hubster, E. C.; Kaufman, M. S.; Ramon, M. K. *Clin. Chem.* **1995**, *41*, 92.

(36) Musshoff, F.; Kirschbaum, K.; Graumann, K.; Herzfeld, C.; Sachs, H.; Madea, B. *Forensic Sci. Int.* **2012**, *215*, 60.

(37) Spiehler, V.; Fay, J.; Fogerson, R.; Schoendorfer, D.; Niedbala, R. S. *Clin. Chem.* **1996**, *42*, 34.

(38) Marin, S. J.; Merrell, M.; McMillin, G. A. *J. Anal. Toxicol.* **2011**, *35*, 40.

(39) Niedbala, R. S.; Kardos, K.; Fries, T.; Cannon, A.; Davis, A. *J. Anal. Toxicol.* **2001**, *25*, 62.

(40) Lachenmeier, K.; Musshoff, F.; Madea, B. *Forensic Sci Int.* **2006**, *159*, 189.

(41) López, P.; Martello, S.; Bermejo, A.; De Vincenzi, E.; Tabernero, M.; Chiarotti, M. *Anal. Bioanal. Chem.* **2010**, *397*, 1539.

(42) Kerrigan, S.; Phillips Jr, W. H. *Clin. Chem.* **2001**, *47*, 540.

(43) Spiehler, V.; Isenschmid, D. S.; Matthews, P.; Kemp, P.; Kupiec, T. *J. Anal. Toxicol.* **2003**, *27*, 587.

(44) Kim, I.; Barnes, A. J.; Schepers, R.; Moolchan, E. T.; Wilson, L.; Cooper, G.; Reid, C.; Hand, C.; Huestis, M. A. *Clin. Chem.* **2003**, *49*, 1498.

(45) Wood, M.; Laloup, M.; Fernandez, M. d. M. R.; Jenkins, K. M.; Young, M. S.; Ramaekers, J. G.; Boeck, G. D.; Samyn, N. *Forensic Sci. Int.* **2005**, *150*, 227.

(46) Sergi, M.; Bafile, E.; Compagnone, D.; Curini, R.; D'ascenzo, G.; Romolo, F. *Anal. Bioanal. Chem.* **2009**, *393*, 709.

(47) Feng, J.; Wang, L.; Dai, I.; Harmon, T.; Bernert, J. T. *J. Anal. Toxicol.* **2007**, *31*, 359.

(48) Kidwell, D. A.; Holland, J. C.; Athanaselis, S. *J. Chromatogr. B* **1998**, *713*, 111.

(49) Madej, K. A. *TrAC Trend. Anal. Chem.* **2010**, *29*, 246.

(50) Kidwell, D.; Kidwell, J.; Shinohara, F.; Harper, C.; Roarty, K.; Bernadt, K.; McCaulley, R.; Smith, F. *Forensic Sci. Int.* **2003**, *133*, 63.

(51) Huestis, M. A.; Oyler, J. M.; Cone, E. J.; Wstadik, A. T.; Schoendorfer, D.; Joseph Jr, R. E. *J. Chromatogr. B* **1999**, *733*, 247.

(52) Leggett, R.; Lee-Smith, E. E.; Jickells, S. M.; Russell, D. A. *Angew. Chem.* **2007**, *119*, 4178.

(53) Day, J. S.; Edwards, H. G.; Dobrowski, S. A.; Voice, A. M. *Spectrochim. Acta A* **2004**, *60*, 563.

(54) Ifa, D. R.; Manicke, N. E.; Dill, A. L.; Cooks, R. G. *Science* **2008**, *321*, 805.

(55) Rowell, F.; Hudson, K.; Seviour, J. *Analyst* **2009**, *134*, 701.

(56) West, M. J.; Went, M. J. *Spectrochim. Acta A* **2009**, *71*, 1984.

(57) Hazarika, P.; Jickells, S. M.; Wolff, K.; Russell, D. A. *Angew. Chem., Int. Edit.* **2008**, *47*, 10167.

(58) Hazarika, P.; Russell, D. A. *Angew. Chem., Int. Edit.* **2012**, *51*, 3524.

(59) Hazarika, P.; Jickells, S. M.; Wolff, K.; Russell, D. A. *Anal. Chem.* **2010**, *82*, 9150.

(60) Armenta, S.; De la Guardia, M. *TrAC Trend. Anal. Chem.* **2008**, *27*, 344.

(61) *The Telegraph;* 2 December ed.; Telegraph Media Group: UK, 2011, <http://www.telegraph.co.uk/>

(62) Travis, A. In *The Guardian;* 1 December ed.; Guardian Media Group: UK, 2011, <http://www.theguardian.com/>

(63) Doyle, J. In *Daily Mail;* 2 December ed.; Daily Mail and General Trust plc: UK, 2011, <http://www.dailymail.co.uk/>

(64) Hall, R. In *The Independent;* 2 December ed.; Independent News & Media plc: UK, 2011, <http://www.independent.co.uk/>

(65) Camber, R. In *Daily Mail;* 9 January ed.; Daily Mail and General Trust plc: UK, 2010, <http://www.dailymail.co.uk/>

(66) Ebejer, K. A.; Winn, J.; Carter, J. F.; Sleeman, R.; Parker, J.; Körber, F. *Forensic Sci Int.* **2007**, *167*, 94.

(67) Carter, J. F.; Sleeman, R.; Parry, J. *Forensic Sci. Int.* **2003**, *132*, 106.

(68) Oyler, J.; Darwin, W. D.; Cone, E. J. *J. Anal. Toxicol.* **1996**, *20*, 213.

(69) Sleeman, R.; Burton, F.; Carter, J.; Roberts, D.; Hulmston, P. *Anal. Chem.* **2000**, *72*, 397A.

(70) Wimmer, K.; Schneider, S. *Forensic Sci. Int.* **2011**, *206*, 172.

(71) Moss, J.M., M. Sc. Thesis, University of Bristol, UK, 1997.

(72) Roberts, D.; Carter, J.; Sleeman, R.; Burton, I. *Spectrosc. Eur.* **1997**, *9*, 16.

(73) Anglada, F.; Delémont, O.; Guéniat, O.; Esseiva, P. *CHIMIA* **2012**, 66, 346.

(74) Briellmann, T. A., Dittmann, V., Dussy, F., Shwerzmann, T. *Kriminalistik* **2001**, *2*, 4.

(75) Dixon, S. J.; Brereton, R. G.; Carter, J. F.; Sleeman, R. *Anal. Chim. Acta* **2006**, *559*, 54.

(76) Jenkins, A. J. *Forensic Sci. Int.* **2001**, *121*, 189.

(77) Negrusz, A.; Perry, J. L.; Moore, C. M. *J. Forensic Sci.* **1998**, *43*, 626.

(78) Jourdan, T. H.; Veitenheimer, A. M.; Murray, C. K.; Wagner, J. R. *J. Forensic Sci.* **2013**, *58*, 616.

(79) Di Donato, E.; Martin, C. C. S.; De Martinis, B. S. *Quim. Nova* **2007**, *30*, 1966.

(80) Esteve-Turrillas, F. A.; Armenta, S.; Moros, J.; Garrigues, S.; Pastor, A.; de la Guardia, M. *J. Chromatogr. A* **2005**, *1065*, 321.

(81) Bones, J.; Macka, M.; Paull, B. *Analyst* **2007**, *132*, 208.

(82) Zuo, Y.; Zhang, K.; Wu, J.; Rego, C.; Fritz, J. *J. Sep. Sci.* **2008**, *31*, 2444.

(83) Luzardo, O. P.; Almeida, M.; Zumbado, M.; Boada, L. D. *J. Forensic Sci.* **2011**, *56*, 1588.

(84) Daughton, C. G. In *Reviews of Environmental Contamination and Toxicology Volume 210;* Springer: 2011, 59.

(85) Thompson, T. In *The Observer*; 10 November ed.; Guardian News and Media Limited: UK, 2002; <http://www.theguardian.com/>

(86) Hudson, J. *Can. Soc. Forensic Sci. J.* **1989**, *22*, 203.

(87) Shrivastava, P.; McLean, C. J.; Aberl, F.; Bonenberger, J.; Berg, R.-P.; Zimmermann, R. In *Enabling Technologies for Law Enforcement and Security;* International Society for Optics and Photonics: 1997, 183.

(88) Frederick, K. A.; Pertaub, R.; Shi Kam, N. W. *Spectrosc. Lett.* **2004**, *37*, 301.

(89) Ebejer, K. A.; Lloyd, G. R.; Brereton, R. G.; Carter, J. F.; Sleeman, R. *Forensic Sci. Int.* **2007**, *171*, 165.

(90) Wilson, A.; Aitken, C.; Sleeman, R.; Carter, J. *Forensic Sci. Int.* **2013**, *236,* 67.

(91) Keller, T.; Schneider, A.; Tutsch-Bauer, E.; Jaspers, J.; Aderjan, R.; Skopp, G. *Int. J. Ion Mobility Spectrom.* **1999**, *2*, 22.

CHAPTER 2

Experimental

This chapter provides details of the materials and methods used to perform the experiments described in this thesis.

2.1 Materials and instruments

2.1.1 Starting materials and solvents

All reagents were of analytical grade, purchased from Sigma-Aldrich (UK) and used as received unless otherwise stated. This included gold(III) chloride trihydrate, with a molecular mass of 393.83 g mol^{-1}, and Protein A/G with a molecular weight of 66 kDa. Analytical grade deionised water (dH_2O), methanol (MeOH), hydrochloric acid 32 % (HCl (aq)), sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4), sodium hydrogen carbonate ($NaHCO_3$), sodium chloride (NaCl), glycerol, dimethyl sulfoxide (DMSO), tri-sodium citrate ($Na_3H_5C_6O_7$), 50 mL centrifuge tubes, 25G 0.5 mm sterile needles, Swann-Morton disposable scalpels and Eppendorf microcentrifuge tubes (1.5 and 2 mL) were all purchased from Fisher Scientific (UK). Dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) were purchased from Pharmacia Biotech (UK). Rabbit polyclonal anti-cocaine antibody was purchased in 1 mL units of serum from Europa Bioproducts (UK) and purified as reported in Section 2.2.1. Sheep polyclonal anti-cocaine antibody and horseradish peroxidase (HRP)-labelled mouse monoclonal anti-sheep IgG were purchased from Abcam (UK) and Jackson ImmunoResearch laboratories (USA), respectively. Cocaine-horseradish peroxidase (cocaine-HRP) and cocaine-bovine serum albumin (cocaine-BSA) haptens were purchased from Randox (UK) and Europa Bioproducts (UK), respectively.

Sephadex PD-10 desalting columns were purchased from GE Life Sciences (UK). 'Slide-A-Lyzer' mini dialysis units (10,000 MWCO), 'NAb Protein A plus' spin columns, Coomassie brilliant blue R250, bromophenol blue sodium salt, ammonium persulfate (APS), 10 MWCO dialysis tubing, sulfo-N-hydroxysuccinimide sodium salt (sulfo-NHS), ethylene dichloride (EDC), *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), 96-well Nunc C8 Maxisorp microtiter plates, 3,3',5,5' tetramethylbenzidine (TMB) substrate solution, and horseradish peroxidase (HRP) were all purchased from Thermo Scientific (UK). α-thio-ω-carboxy polyethylene glycol (PEGCOOH, MW 3317 Da) and α-Mercapto-ω-amino polyethylene glycol hydrochloride ($PEGNH_2$, MW 3163 Da) were purchased from Iris Biotech GMBH (Germany). Ultrapure agarose, Novex Sharp unstained protein standard (molecular weight marker), Gibco rat tail collagen I, Novex enhanced chemiluminscence (ECL) substrate reagent and stable 3,3´-diaminobenzidine (DAB) were all purchased from Invitrogen (UK). 18 x 16 cm glass plates, and 16 cm x 8 cm x 1.5 mm gel spacers were purchased from Web Scientific (UK).

N,N,N'-tetramethylethylenediamine (TEMED) was purchased from Bio Rad (UK). Cerilliant Cocaine standard (1 mg mL^{-1} in acetonitrile) was purchased from LGC Standards (UK). Nitrocellulose membrane and Millex GP syringe driven filter units (0.22 μm) were purchased from Millipore (UK and USA, respectively). 15 mL centrifuge tubes were purchased from Corning B. V. Life Sciences (The Netherlands). Vivaspin20 (30,000 MWCO) and Vivaspin500 (100,000 MWCO) centrifuge tubes were purchased from Sartorius Stedim Biotech (UK). Holey carbon film 300 mesh copper grids were purchased from Agar Scientific (UK). Everyday Value dried skimmed milk powder was purchased from a local grocery store (Tesco, UK), and Dow Corning food grade RTV silicon was purchased from MB Fibreglass (UK). Fabriano Roma 100 % cotton ivory illustration paper was purchased from Atlantis Art, London (UK).

Benzoylecgonine standard (Cerilliant; 1 mg mL^{-1} in MeOH) was kindly donated by Simon Hudson of HFL Sports Science, LGC Health Sciences (Fordham, UK).

Scottish banknotes of both £10 and £20 denominations from general circulation were kindly provided by the Scottish Crime and Drug Enforcement Agency (SCDEA). Ten fingerprint samples on glass microscope slides obtained from five volunteers at a methadone treatment clinic were kindly donated by Intelligent Fingerprinting Ltd. (Norwich, UK). Five newly minted £20 GBP banknotes, along with five £20 and ten £10 GBP banknotes from general circulation were kindly provided by Kevin Wills on behalf of the Bank of England (Notes Division, Banking Services; Loughton, UK). Approximately 1 g of seized 'street' cocaine (of unknown purity) was kindly provided by Steven Hamilton of the Norfolk Constabulary (Serious and Organised Crime Directorate, Wymondham, UK).

2.1.2 Gel electrophoresis buffers and solutions

The analysis of proteins and the coating of banknote samples were performed using polyacrylamide gels and gel electrophoresis. The buffers and gel solutions applied for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), non-denaturing polyacrylamide gel electrophoresis (native-PAGE) and agarose gel electrophoresis are described in this chapter. The components of the buffers used for SDS-PAGE and native-PAGE, and of the SDS-PAGE gels are listed in **Tables 2.1** and **2.2**, respectively. 1 L of protein stain for SDS-PAGE was prepared by firstly dissolving 1.0 g Coomassie brilliant blue R250 in 500 mL MeOH and 400 mL dH_2O, then adding 100 mL glacial acetic acid. 1 L of de-stain for SDS-PAGE was prepared by adding 70 mL glacial acetic acid to 100 mL of MeOH, and making the volume up to 1 L with dH_2O. For native-PAGE, the protein stain was prepared by firstly dissolving 0.25 g Coomassie brilliant blue R250 in 125 mL methanol and 100 mL dH_2O, and then adding 25 mL glacial acetic acid. The de-stain solution used for native-PAGE was the same as that used for SDS PAGE.

Table 2.1. The components of the buffers used for SDS PAGE and native-PAGE in this study, prepared based on the original Laemmli buffer system.[1]

	Sample buffer	Electrophoresis buffer
SDS PAGE	5 mL 0.6 M Tris buffer, pH 6.8 0.5 g SDS 5.0 g sucrose 250 µL β-mercaptoethanol 5 mL 0.5 % w/v bromophenol blue (made up to 50 mL with dH_2O)	6.0 g Tris 28.8 g glycine 1.0 g SDS (made up to 1 L with dH_2O)
Native-PAGE	15.5 mL 1 M Tris buffer, pH 6.8 2.5 mL 1 % w/v bromophenol blue 7 mL dH_2O 25 mL glycerol (diluted 1:5 before use)	3.0 g Tris 14.4 g glycine (made up to 1 L with dH_2O, adjusted pH to 8.3)

Table 2.2. The components used for the preparation of a 15 % polyacrylamide gel for SDS PAGE.[2]

Resolving gel	Stacking gel
2 mL 1.875 M Tris buffer (pH 8.8)	1 mL 0.6 M Tris buffer (pH 6.8)
2.85 mL dH_2O	7.5 mL dH_2O
5 mL 30 % acrylamide/bis-acrylamide	1.35 mL 30 % acrylamide/bis-acrylamide
100 µL 10 % w/v SDS	100 µL 10 % w/v SDS
50 µL 10 % w/v APS	50 µL 10 % w/v APS
25 µL TEMED	25 µL TEMED

1 L of 5 x Tris-borate EDTA (TBE) buffer stock was prepared for agarose gel electrophoresis by dissolving 54 g of Tris and 27.5 g of boric acid with 20 mL of 0.5 M EDTA (pH 8.0). The solution was then made up to a final volume 1 L with dH_2O. 0.5 x TBE electrophoresis buffer was prepared by diluting the 5 x TBE buffer stock to 1:10 in dH_2O. 200 mL 0.2 % w/v agarose gel mix was prepared by combining 0.4 g of ultrapure agarose with 100 mL of dH_2O and heating to 85 °C to

melt the agarose. The mixture was cooled to 60 °C and 100 mL of 1 x TBE buffer (pH 8.0) was added. The solution was immediately poured into a 15 x 15 cm gel tray and set at room temperature (RT).

2.1.3 Instrumental techniques

Centrifugation of large (20 - 50 mL) tubes was performed using an Avanti J-25 centrifuge. Centrifugation of small (1.5 – 2.0 mL) tubes and Protein A spin columns were performed using a Beckman Coulter Allegra X-22R and an MSE Micro Centaur Pluse centrifuge, respectively. Samples for SDS PAGE analysis were heated to 100 °C using a Grant-Bio PHMT Thermoshaker (at 0 rpm). 8 x 10 cm^2 SDS-PAGE and native-PAGE gels were cast in a Hoefer S245 dual gel cassette, and performed using a Hoefer Mighty Small II vertical electrophoresis system. 16 x 18 cm^2 native-PAGE gels were cast using a Hoefer SE600 dual cassette. 15 x 15 cm^2 agarose gels were prepared and run using a Scie-Plas HU-15 standard horizontal electrophoresis gel unit. Both SDS-PAGE and agarose gel electrophoresis were performed using an Amersham Pharmacia Biotech EPS 601 power supply. Colorimetric imaging of SDS-PAGE and native-PAGE gels was performed using an HP photosmart C4580 scanner. Chemiluminescent imaging was performed using a Fuji LAS-3000 imaging system; images were obtained at 30 s intervals in chemiluminescent increment mode and at high sensitivity, for a total exposure time of 5 min. Photographic images of gels were made using a Canon IXUS 115HS handheld digital camera.

UV-Vis absorption spectra were recorded on a Hitachi U-3000 spectrophotometer at RT. Quartz cuvettes with a 1 cm path length were used. Microtiter plate absorbance intensity readings were recorded using a Perkin Elmer Wallac Envision 2103 multilabel microplate reader. HRP-labelled anti-cocaine antibody was separated from unconjugated HRP by gel filtration with a 350 mL of GE Health Life Sciences Superdex 200 column using an Amersham Pharmacia Biotech AKTA Explorer. 20 mM phosphate buffered saline with 150 mM NaCl (pH 7.2) was used as a running buffer. Absorbance intensity of the fractions was measured at 280 nm.

Transmission electron microscope (TEM) images were obtained using a Joel 2000EX TEM operating at 200 kV. Imaging was performed by Dr Colin McDonald (School of Chemistry, University of East Anglia, Norwich, UK). LC-MS confirmation analysis of cocaine in extracted fingerprint samples was performed using a Thermo LTQ Orbitrap Discovery high resolution accurate mass LC-MS running in full scan positive ion electrospray mode. The orbitrap was operating at a mass resolution of 30,000 FWHM at *m/z* 400. The analysis was performed by Simon Hudson at HFL Sports Science, LGC Health Sciences, Fordham, UK. Confirmation analysis of cocaine in oral fluid samples was performed using a GC-MS^2 by Michalakis Michael at LGC Ltd. (Teddington, UK).

2.2 Developing a competitive enzyme-immunoassay (cEIA) for the quantitation of cocaine on banknotes

2.2.1 Anti-cocaine antibody purification

Polyclonal anti-cocaine antibody was isolated from the supplied rabbit serum samples by immunoprecipitation using ammonium sulfate,[3] followed by Protein A chromatography. A saturated solution of ammonium sulfate in dH_2O was prepared by adding 190 g of $(NH_4)_2SO_4$ to 200 mL of dH_2O and stirring overnight at RT. The solution was made 24 h in advance and stored at 4 °C before use. The 1 mL aliquots of purchased serum were centrifuged at 10,000 xg for 30 min at 4 °C. The resulting supernatant was removed by pipette and the pellet discarded. The serum was then cooled to 4 °C in an iced waterbath. 400 µL of the pre-prepared saturated ammonium sulphate solution was added dropwise for every 1 mL of serum. The solution was then gently stirred for 3 h at 4 °C before being transferred to 1.5 mL Eppendorf tubes. Centrifugation was performed at 4000 xg for 10 min at 4 °C, after which the supernatant was discarded and the pellet drained by inverting the sample tube gently over tissue paper. The pelleted material was resuspended in 200 µL of 10 mM phosphate buffer (pH 7.4; PB) by drawing repeatedly into a wide-gauge Pasteur pipette. The final volume of the IgG solution was then made up to 500 µL with PB,

and the IgG solution transferred in aliquots of 200 µL into mini dialysis units. Dialysis was performed against 2 L of 10 mM PB (pH 7.4) for 2 h at 4 °C, after which the solution was transferred to 1.5 mL Eppendorf tubes and stored at 4 °C overnight.

Immunochromatography was performed using Protein A spin columns, with a sample capacity of ≤ 500 µL, in accordance with the manufacturer's instructions. Three columns were used to accommodate the 900 µL of solution to be purified. Briefly, the columns were equilibrated before use by washing twice with 400 µL of 10 mM PBS (150 mM NaCl, pH 7.2). Wash solutions were removed from the columns by centrifugation for 1 min at 4,000 xg. The solution containing the antibody was added at 300 µL per column, and the columns were incubated at room temperature on a rotary mixer for 10 min. The columns containing bound antibody were then washed three times with 400 µL of 10 mM PBS (pH 7.2). The antibody was eluted from the columns using three 400 µL additions of 0.1 M glycine (pH 2.0). Each of the resulting three fractions was neutralised with 40 µL of 1.0 M Tris buffer (pH 8.5). The presence of antibody in the first and second fractions was determined by recording the absorbance intensity at 280 nm, measured using a UV-Vis spectrophotometer as described in **Section 2.1.3**. Confirmation was performed by SDS-PAGE.[2] For this, 10 µL of each fraction was diluted in an equal volume of sample buffer and heated to 100 °C for 5 min. 15 µL of each sample was then loaded onto a 15 % SDS-polyacrylamide gel alongside a molecular weight marker and run at a constant voltage of 180 V for 60 min. The Laemmli sample and electrophoresis buffers, gel mixes and staining solutions used were all prepared as described in **Section 2.1.2**. The resulting gels were imaged by scanning as described in **Section 2.1.3**. After the presence of antibody was confirmed, the two fractions were combined and dialysed in mini-dialysis units for 2 h against 2 L of 10 mM PB (pH 7.4). The solutions were then aliquoted and stored at -20 °C for up to six months.

2.2.2 Extraction of cocaine from banknote and latent fingerprint samples

Cocaine was extracted from banknote samples using a modified version of the method described by Esteve-Turrillas *et al.*.[4] Each banknote was rolled and placed in a 20 mL glass test tube with 15 mL of MeOH. The tube was sealed and vortex-mixed for 5 min. The banknote was then removed from the tube and rinsed with a further 5 mL of methanol. The two extract volumes (15 and 5 mL) were combined to make up the 20 mL final volume. The solvent was evaporated under reduced pressure at 40 °C and the extract reconstituted in 0.5 mL of MeOH. The reconstituted extract was centrifuged at 14,000 rpm for 7 min at 4 °C to remove any solids, and the supernatant retained. Each extract was then divided into two halves; one half for cEIA and the other half for confirmation analysis by LC-MS. Extract portions set aside for LC-MS confirmation were kept in MeOH and stored at 4 °C. For the immunoassay, 50 µL of the extract was taken and the solvent was removed by drying under a gentle stream of argon. The extracts were then reconstituted in 50 µL of 10 mM PB (pH 7.4) containing 2 % v/v MeOH and stored at 4 °C overnight until analysed.

Fingerprint samples obtained from volunteer subjects known to be taking illicit drugs were provided by Intelligent Fingerprinting Ltd. The samples were taken by deposition of a fingerprint onto a clean glass microscope slide. Oral fluid samples were collected from the volunteers at the same time and analysed by GC-MS[2], as described in **Section 2.1.3**. Fingerprints from drug-free volunteers were taken in the same manner as for the test subjects and used as negative controls. Cocaine was extracted from each fingerprint separately using a similar method as described previously for the banknote samples. Specifically, the microscope slide with the fingerprint was inserted into a 50 mL centrifuge tube and 20 mL of MeOH was added. The tube was sealed and vortex-mixed for 5 min. The solvent was evaporated under reduced pressure at 40 °C and the extract reconstituted in 0.5 mL of 10 mM PB (pH 7.4) containing 2 % v/v MeOH. The samples were stored at 4 °C overnight until analysed.

2.2.3 Preparation of standard and sample solutions

A cocaine standard stock solution (1 mg mL^{-1}) was prepared in advance of analysis as follows. A 50 µL aliquot of cocaine standard was taken and the acetonitrile removed by evaporation under a gentle stream of argon. The standard was then reconstituted in 50 µL of 10 mM PB (pH 7.4) containing 2 % v/v acetonitrile. A standard curve was prepared on the day of analysis by diluting the stock solution to known concentrations in 10 mM PB (pH 7.4). The concentrations of cocaine for each curve was as follows; 0.78, 1.56, 3.125, 6.25 12.5, 25, 50 and 100 ng mL^{-1} for the analysis of banknote extracts, and 0.195, 0.39, 0.78, 1.56, 3.125, 6.25 12.5, and 25 ng mL^{-1} for the analysis of fingerprint extracts.

Banknote extracts were diluted to 1:500 by adding 1.0 µL of sample to 499 µL of 10 mM PB (pH 7.4), or to 1:1000 by adding 1.0 µL of sample to 999 µL of PB immediately prior to analysis. Fingerprint extracts were not diluted prior to analysis.

2.2.4 Anti-cocaine cEIA method

A schematic overview of the developed cEIA method is shown in **Figure 2.1**. The assay involved the adsorption of the purified anti-cocaine antibody (1 µg mL^{-1}) to a microtiter plate at 100 µL/well in 100 mM carbonate buffer, pH 9.5 (optimised as described in **Section 2.2.5**). The plate was incubated overnight (18 h) at 4 °C and then washed three times with 10 mM PBS containing 0.05 % v/v Tween-20 (150 mM NaCl, pH 7.4; PBS/T). Blocking buffer, PBS containing 1 % w/v bovine serum albumin (PBS/BSA), was added at 200 µL/well and incubated at RT for 2 h. The plate was then washed a further three times with PBS/T before the addition of pre-diluted standards and banknote extracts (in PB) at 50 µL/well. The plate was incubated for a further 2 h at RT after which 50 µL of the cocaine-HRP conjugate (1:20 in sample buffer (blocking buffer with 0.05 % v/v Tween-20), optimised as described in **Section 2.2.5**), was added to each well. The solutions were mixed by gently tapping the side of the plate several times and then incubated for 2 h at room temperature. The plate was then washed four times with PBS/T. TMB substrate solution was added at

100 µL/well followed by a 20 min incubation at RT in the absence of light. Sulfuric acid (0.5 M in dH_2O) was added at 100 µL/well to stop the enzymatic reaction, after which the absorbance of each well was measured at A_{450} using a multi-label plate reader as described in **Section 2.1.3**. Wells without antibody were used as a blank. The intensity of the absorbance measured was inversely proportional to the quantity of cocaine in the banknote extract.

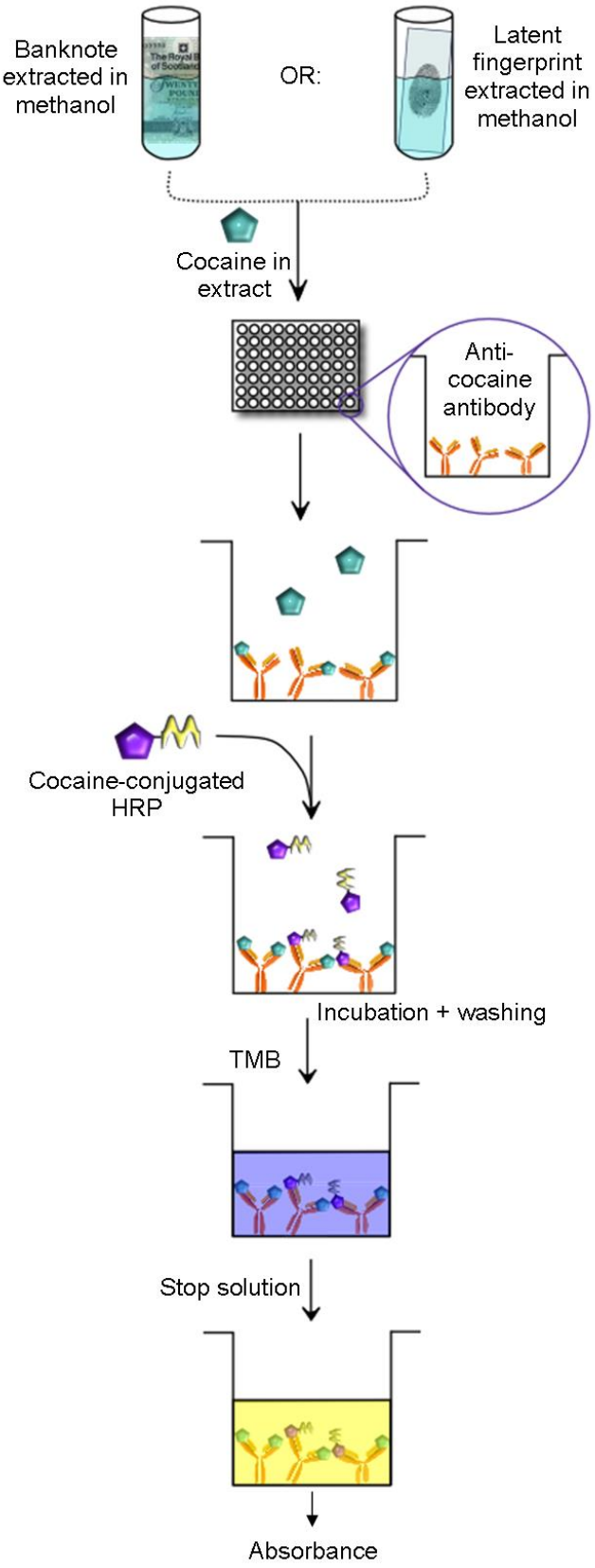

Figure 2.1. A schematic representation of the cEIA for the detection of cocaine in extracts taken from banknotes or latent fingerprints.

Absorbance intensities were corrected for cocaine-HRP binding in the absence of cocaine as shown in **Equation 2.1**. The standard curve was generated by plotting the cocaine concentration (on a logarithmic scale) against the logit of the corrected absorbance intensity (Logit (B/B_0)).[5] For this, Equation 2.2 was applied.[6]

$$Corrected\ A_{450} = \left(\frac{B}{B_0}\right) \qquad (2.1)$$

$$Logit\left(\frac{B}{B_0}\right) = In\left(\frac{\frac{B}{B_0}}{1-\frac{B}{B_0}}\right) \qquad (2.2)$$

In Equations 2.1 and 2.2: B is the absorbance intensity of the sample or standard solution, and B_0 is the absorbance intensity produced by the 0 ng mL^{-1} cocaine standard solution.

2.2.5 cEIA method optimisation

Anti-cocaine antibody (1 µg mL^{-1}, 100 µL/well) was adsorbed onto a micro-titer plate by overnight incubation (18 h) at 4 °C as described in **Section 2.2.4**. Initially, the antibody was diluted in either 100 mM carbonate buffer (pH 9.5), or 10 mM PB (pH 7.4) prior to addition to the wells. The resulting relative concentration of antibody immobilised onto the plate was then estimated using an enzyme-labelled molecule of antigen, in this case HRP-labelled cocaine (cocaine-HRP), as follows. 50 µL of cocaine-HRP (1:20 in blocking buffer) was applied to each well. Unbound hapten was removed by washing three times with PBS/T. TMB substrate solution was added at 100 µL/well followed by a 20 min incubation at RT in the absence of light. Sulfuric acid (0.5 M in dH_2O) was added at 100 µL/well to stop the enzymatic reaction, after which the absorbance of each well was measured at A_{450} using a multi-label plate reader as described in **Section 2.1.3**. Wells without antibody were used as a blank.

The concentration of cocaine-HRP bound, as indicated by the intensity of the coloured product, was proportional to the amount of antibody bound to the plate.

The optimal dilution factor for the cocaine-HRP was similarly determined based on the coloured product produced by the bound enzyme. The cocaine-HRP was subjected to a series of dilution factors from 1:20 – 1:160 in blocking buffer, and applied to a microtiter plate containing anti-cocaine antibody bound at a concentration of 1 µg mL^{-1} as described previously. Wells with BSA bound at 100 ng mL^{-1} in place of the antibody were used as negative controls. Unbound hapten was removed by washing three times with PBS/T, and the presence of enzyme detected using TMB substrate solution as described for the labelling of bound antibody in the previous paragraph.

The addition of Tween-20 to the blocking buffer as a means of preventing non-specific binding of cocaine-HRP was investigated using a preliminary set of cocaine standards. Wells containing anti-cocaine antibody, and negative control wells with BSA were prepared as previously described. Blocking buffer was then added at 200 µL/well and incubated at RT for 2 h. The plate was washed three times with PBS/T before the addition of four pre-diluted cocaine standards (in PB) at 50 µL/well. The cocaine standards, at the concentrations 0, 100, 500 and 1000 ng mL^{-1}, were prepared as described in **Section 2.2.3**. Cocaine-HRP, diluted to 1:20 in sample buffer, was then added at 50 µL/well. The solutions were mixed by gently tapping the side of the plate several times and then incubated for 2 h at RT. The plate was washed four times with PBS/T, and enzyme substrate added and measured as described as described for the labelling of bound antibody at the beginning of this section.

2.2.6 Limit of detection (LOD), precision and specificity

The detection limit of the cEIA was calculated from the average absorbance values from six replicate analyses of the blank minus three times the standard deviation of

the average. The resulting absorbance value was then extrapolated from the log-logit standard curve.

The precision of the assay was estimated based on repeat measurements of calibration standard samples prepared in assay buffer (10 mM PB, pH 7.4). Standards at three different cocaine concentrations (6.25, 25 and 100 ng mL^{-1}) were used. The intra-assay precision was determined using four replicates of each standard in a single analysis. Inter-assay precision was similarly assessed by analysing the same sample, as four repetitions, across three separate analyses undertaken on different days over a period of 30 days.

The analytical specificity, or cross-reactivity, of the assay was tested for benzoylecgonine (BE), the cocaine analogue and main metabolite of cocaine found in urine. Three BE standard solutions (6.25, 25.0 and 100.0 ng mL^{-1}) were prepared in assay buffer (10 mM PB, pH 7.4) using the same method applied for the preparation of cocaine standards in **Section 2.2.3**. The standards were analysed as four repeat measurements by cEIA as described in **Section 2.2.4**, alongside a complete set of cocaine standards. The % cross-reactivity of BE was calculated relative to the cocaine standard curve,[7] as shown in Equation 2.3.

$$Cross\ reactivity\ (\%) = \frac{[Apparent\ cocaine]/ng\,mL^{-1}}{[Added\ BE]/ng\,mL^{-1}} \times 100 \qquad (2.3)$$

In Equation 2.3: [Apparent cocaine] is the concentration of cocaine calculated from the standard curve based on the absorbance intensity, and [Added BE] indicates the actual concentration of BE from which the absorbance intensity was measured.

2.3 Location-specific immunodetection of cocaine on banknotes

2.3.1 Comparing gel matrices for cocaine immobilisation

In this study, a number of different gel matrices were initially tested in order to gauge their suitability for application onto banknotes. A summary of the gel types and the methods used to prepare them is provided in Table 2.3. The mechanical properties of the gels were established by preparing gels set to a *ca*. 2 mm thickness around small squares (*ca*. 2 x 3 cm^2) of cotton paper. This was carried out by pouring the pre-prepared gel mixes into custom moulds (*ca*. 5 x 7 x 0.5 cm^3, cast out of food grade silicon in a mould prepared using small interlocking plastic building blocks) containing the cotton paper. The paper was submerged into the gel mix using tweezers, and the gels set as per the conditions specified in Table 2.3.

Table 2.3. A summary of the different types of gel matrices compared in this study. The methods used to make the gels are provided alongside each gel type.

Medium	Content	Method(s) used
Gelatin	3, 5 and 7 % w/v	30 mL of 3, 5 or 7 % w/v porcine skin gelatin was prepared in dH_2O. The solution was heated to 55 °C in a glycerol bath for 30 min,[8] then poured into the mould and set at RT or 4 °C.
Cross-linked gelatin	7 % w/v	7 % w/v gelatin gels were prepared and set as described above. For cross-linking, the gels were soaked for 2 h in 4 % PFA* in 0.1 M PBS (50 mM NaCl, pH 7.4) at RT.[9]
Casein	7 % w/v	20 mL of 7 % w/v bovine casein was prepared in dH_2O. The pH was adjusted to 10 by adding 1.0 M NaOH. The solution was then heated to 65 °C for 45 min in a glycerol bath with continuous stirring. The solution was filtered through one layer of muslin 'butter cloth' and cooled to RT before being poured into the mould and set at RT.
Collagen	1.0 mg mL^{-1}	Collagen (3 mg mL^{-1}) in 0.2 % acetic acid (pH 3-4) was diluted 1:2 in 2 mM PBS (100 mM NaCl, pH 7.4) and stored on ice to prevent gelation. The pH of the solution was adjusted to 7.4 with 1 M NaOH, before being warmed to RT for *ca*. 10 min. The solution was poured into moulds and set for 1-2 h at 37 °C.[10]
Cross-linked collagen	1.8 mg mL^{-1}	2.5 mL 2 mM PBS, 0.1 mL 1 M NaOH and 4 mL collagen (3 mg mL^{-1}, as above) were placed on ice. The collagen was added dropwise to the PBS and the pH adjusted to 7.6 using the NaOH. The solution was warmed to RT, poured into moulds and set for *ca*. 40 min at RT.[10] The gels were removed from the mould, soaked in 4 % PFA* for 30 min at RT and then washed in PBS with 0.05 % v/v Tween-20 for 10 min at RT.
Agarose	0.2 % w/v	A 0.2 % w/v solution of agarose was prepared by adding 12 mg of ultrapure agarose to 6 mL of 10 mM PB (pH 7.4). The solution was heated to 95 °C until the agarose dissolved. It was poured into the mould and set for *ca*. 1 h at RT.[11]
LMP† agarose	0.2, 0.5 and 1.0 % w/v	0.2, 0.5 and 1.0 % w/v agarose solutions were prepared by adding 0.04, 0.1 and 0.2 g of agarose to 20 mL of dH_2O, respectively. The gels were further prepared and set as described above.

*PFA = paraformaldehyde and †LMP = low melting point.

Permeability testing was performed using gels cast to a *ca*. 5 mm thickness on top of the glass wool at the base of a chromatography column. The glass wool was first lined with filter paper before the gel solutions (7 % w/v gelatin, 0.2 % w/v agarose, and 2 mg mL^{-1} collagen) were prepared and cast as described in **Table 2.3**. The set gels were washed by applying 20 mL of PBS/T (10 mM PBS/0.05 % Tween-20) above the gel layer. The solution was then left to flow through the gel for *ca*. 1.5 h at 4 °C with the column tap set to 'open', after which the eluent was discarded. 20 mL of primary antibody solution (1:100 in PBS/T) was then applied on top of the gel and left to permeate for a further 1.5 h at 4 °C. The absorbance intensity of the antibody solution was measured at 280 nm as described in **Section 2.1.3** before and after gel permeation.

Cross-linking of the gel matrices was performed using a pre-prepared 4 % solution of paraformaldehyde (PFA) in 50 mM PBS (0.137 M NaCl, pH 7.4). 500 mL of this solution was prepared by first heating 400 mL of the PBS to 60 °C and stirring gently throughout. 20 g of PFA was then added. 1 M NaOH was added dropwise until the solution became clear. Once clear, the solution was cooled to RT and sterile-filtered using a 0.22 µm filter unit. The volume was made up to 500 mL with PBS, before being divided into 20 mL aliquots and stored at -20 °C.

2.3.2 In-gel immunodetection method development for polyacrylamide gels

Samples of cocaine-bovine serum albumin (cocaine-BSA) hapten, and control samples containing primary anti-cocaine or secondary HRP-labelled antibody were imbedded into a polyacrylamide gel using a modified version of the native-polyacrylamide gel electrophoresis (native-PAGE) method described by Walker.[12] The gels were cast as homogenous Tris-HCl slab-gels, using a single separating gel mix rather than a layered stacking- and separating- gel approach. An 8 x 10 cm^2 gel cassette was prepared using 0.75 or 1.5 mm spacers. Gel mixes were made using different acrylamide concentrations and cross-linker ratios, as listed in **Table 2.4**. The pre-prepared gel mix was added slowly to the cassette and allowed to set at RT.

10 µL of the molecular weight marker was loaded into the first well of the gel. 15 µL of sample, diluted 1:1 in 1x native-PAGE sample buffer (prepared as described in **Section 2.1.2**), was then added to each remaining well. Electrophoresis was performed at 120 V constant voltage for approximately 3 h in native-PAGE electrophoresis buffer, prepared as reported in **Section 2.1.2**. Gels which were not required for in-gel immunodetection were stained using the native-PAGE protein stain, prepared as described in **Section 2.1.2**. De-staining was performed using the SDS-PAGE de-stain solution described in the **Section 2.1.2**.

Table 2.4. Solutions for preparing Tris-HCL separating gels for native-PAGE; the different acrylamide concentrations and cross-linker ratios used in this study.

	% Acrylamide				
Components (in mL):	5.0 %	7.5 %	8.0 %	8.5 %	10.0 %
30 % acrylamide/bis-acrylamide	2.5	3.75	4.0	4.25	5.0
dH_2O	8.4	3.75	6.9	6.65	5.9
Tris Buffer (1.5 M, pH 8.8)	3.8	7.425	3.8	3.8	3.8
10 % APS	0.15	0.075	0.15	0.15	0.15
TEMED	0.008	0.009	0.009	0.009	0.006

In-gel immunodetection was performed on unstained gels using a modified version of the method described by Desai *et al.*.[13] Following electrophoresis, the gel was removed from the cassette and transferred to a gel box. The gel was then submerged in approximately 50 mL of 50 % isopropanol/water for 15 min at RT. This was followed by a 15 min wash step in approximately 50 mL of dH_2O. The gel was then incubated for between 1 and 3 h (as listed in **Table 2.5**) at RT in a 20 mL solution of anti-cocaine primary antibody diluted in sample buffer. Two types of primary anti-cocaine antibody were used in this study; rabbit antibody (purified as reported in **Section 2.2.1)**, and sheep antibody, which was diluted directly from the purchased stock solution. The conditions used for each of the antibody types are as

shown in **Table 2.5**. Following incubation with the primary antibody, the gel was washed three times for 10 min in approximately 50 mL of wash buffer (either PBS/0.05 v/v Tween-20 (PBS/Tween-20), or 1x Tris-buffered saline (TBS)/0.05 v/v Tween-20 (TBS/Tween-20), depending on the type of sample buffer used). 20 mL of an appropriately diluted solution of HRP-labelled secondary antibody in sample buffer was then added to the gel and incubated for 1 h at RT. **Table 2.5** lists the dilutions of secondary antibody alongside the type of sample buffer used. The gel was then washed a further three times as previously described.

Table 2.5. Method development for the in-gel immunodetection using polyacrylamide gels; a summary of the different conditions tested during this study. The combination of conditions for each experiment (numbered 1 – 10) is shown.

Experiment:	Gel (% acrylamide)	1° antibody dilution	1° antibody incubation time (h)	2° antibody dilution	2° antibody incubation time (h)	Sample buffer used	HRP substrate
1	10	1:100	1	1:5,000	1	5.0 % w/v BSA/PBS/Tween-20[a]	DAB
2	10	1:100	1	1:5,000	1	1.0 % w/v BSA/PBS/Tween-20[a]	DAB
3	7.5[b]	1:50	1	1:1,000	1	1.0 % w/v BSA/PBS/Tween-20[a]	DAB
4	5	1:50	1	1:1,000	1	1.0 % w/v BSA/PBS/Tween-20[a]	DAB
5	7.5[b]	1:50	1	1:1,000	1	1.0 % w/v BSA/PBS/Tween-20[a]	DAB
6	7.5[b]	1:50	1	1:400	1	0.5 % w/v BSA/PBS/Tween-20[a]	DAB
7	7.5[b]	1:50	2	1:200	1	0.5 % w/v BSA/PBS/Tween-20[a]	ECL
8	7.5[b]	1:25	2	1:100	1	0.5 % w/v BSA/TBS/Tween-20[c]	ECL
9[d]	7.5	1:2,500	3	1:10,000	1	0.5 % w/v BSA/TBS/Tween-20[c]	ECL
10[d]	7.5	-	-	1:1,000	1.5	0.5 % w/v BSA/TBS/Tween-20[c]	ECL

[a]'PBS' is 10 mM PBS with 100 mM NaCl (pH 7.4). 0.05 % v/v Tween-20 was used.

[b]TEMED volume adjusted to 7.5 µL (experiment 3) and 6.0 µL (experiments 5 - 7).

[c]1x TBS and 0.05 % v/v Tween-20 were used.

[d]Experiments 9 and 10 were performed using sheep polyclonal anti-cocaine antibody (also used in **Section 2.3.4**) with a corresponding HRP-labelled anti-sheep IgG secondary antibody.

The colorimetric substrate DAB was initially applied for the detection of HRP within the gels for experiments 1 – 5, as listed in **Table 2.5**. 20 mL of DAB substrate solution, pre-equilibrated to RT, was added to the gel after the final wash step. The gel was incubated in the DAB solution for 1 – 2 min, or until the coloured product became visible. The gel was then rinsed four times with wash buffer before being placed between acetate sheets and imaged. An enhanced chemiluminescence (ECL) substrate solution was also applied for the detection of HRP in gels, as shown for experiments 7 – 10 in **Table 2.5**. For this, 10 mL of the ECL substrate solution was prepared by mixing 5 mL of reagent A (stable peroxidase buffer) with 5 mL of reagent B (luminol enhancer). This was added to the gel immediately after the final wash step, and was followed by a 5 min incubation in the dark at RT. The ECL solution was removed after incubation, and the gel was briefly rinsed (for around 15 s) in dH_2O to remove any excess. The gel was then placed between acetate sheets and imaged at 30 s intervals for a total exposure time of 5 min, as detailed in **Section 2.1.3**.

2.3.3 Detection of antibody interaction by 'dot blot'-style assay

Interaction between the primary and labelled secondary antibody was detected using a modified protocol based on the original method of Hawkes *et al.*.[14] A 1 mg mL^{-1} solution of primary antibody in 10 mM PB (pH 7.4) was prepared, along with a 1 mg mL^{-1} solution of BSA in PB as a negative control. A rectangle of nitrocellulose membrane was cut and pre-labelled with pencil in a grid pattern. 2 µL of anti-cocaine antibody stock solution (as purchased), or 2 µL of a control solution of BSA (5 mg mL^{-1} in PB) was slowly spotted onto the circle drawn in the centre of each grid using a micropipette and allowed to dry at RT. Once dry, the membrane was transferred to a 10 cm plastic Petri dish and submerged in approximately 20 mL of blocking buffer (5 % w/v BSA/TBS/0.05 % v/v Tween-20) for 45 min at RT. The membrane was then incubated for 30 min in 10 mL of secondary antibody solution (1:1000 in TBS/0.05 % v/vTween-20, TBS/T) at RT. The membrane was washed once in TBS/T for 15 min at RT, followed by two times in TBS/T for 5 min at RT, and

a final time for 5 min in TBS at RT. 10 mL of ECL substrate solution, prepared by mixing 5 mL of reagent A with 5 mL of reagent B, was added to the membrane and incubated for 1 min in the dark and at RT. The membrane was then rinsed for approximately 15 s in dH_2O, placed between acetate sheets and imaged at 30 s intervals for a total exposure time of 3 min, as detailed in **Section 2.1.3.**

2.3.4 HRP labelling of sheep polyclonal anti-cocaine antibody

Anti-cocaine IgG was labelled with HRP using the periodate-mediated method of conjugation as reported by Tijssen and Kurstak,[15] and Wisdom.[16] 2 mg of HRP was dissolved in 500 µL of sterile-filtered dH_2O. 100 µL of freshly prepared 0.1 M sodium periodate was added to the HRP solution and stirred for 20 min at RT, during which a colour change of orange to green was observed. The resulting solution was dialysed in mini-dialysis units overnight at 4 °C against 2 L of 1 mM sodium acetate buffer (pH 4.4). 50 µL of the anti-cocaine antibody was made up to 500 µL in 10 mM sodium carbonate buffer (pH 9.5). The pH of the dialysed enzyme was adjusted to 9.0 – 9.5 by adding 10 µL of 0.2 M sodium carbonate buffer (pH 9.5), after which the solution containing the antibody was immediately added. The mixture was then stirred for 2 h at RT. 50 µL of freshly prepared sodium borohydride solution (4 mg mL^{-1} in dH_2O) was added and the mixture was stirred for a further 2 h at RT. The antibody-HRP hapten was purified using a gel filtration column as described in **Section 2.1.3**. The fractions from the first peak at A_{280} were selected, and the presence of the hapten was confirmed by SDS-PAGE, performed as described in **Section 2.2.1**, and by UV-Vis spectrophotometry at A_{280} and A_{403}. Fractions found to be containing the hapten were then concentrated to a 1 mL volume by centrifugation in a Vivaspin20 tube at 2,500 xg for 40 min at 4 °C. The purity of the labelled antibody, as indicated by the Rz value, was determined using the ratio of the absorbance intensity at A_{275}:A_{403}.[17] The concentration of the labelled antibody was estimated based on the absorption of HRP at 403 nm using the extinction co-efficient 102 mM^{-1} cm^{-1}.[18] 5 mg of BSA was added to the labelled antibody to make a final

BSA concentration of 5 mg mL^{-1}. The solution was divided into 1 mL aliquots and stored at -20 °C.

2.3.5 Preparation of spiked cotton paper, partial banknote and whole banknote samples

Cotton paper was cut into rectangular-shaped pieces of approximately 5 x 7 cm^2 and pre-labelled with pencil in a grid pattern. Small circles (*ca*. 3 mm) were drawn in the centre of each grid to indicate the location of the spiked sample. The cocaine standard solution (1 mg mL^{-1} in MeOH), and a cocaine-BSA hapten at various concentrations in PB were used for spiking. BSA (5 mg mL^{-1} in PB), anti-cocaine antibody and HRP-labelled secondary antibody solutions were used as control samples. 2 - 10 µL of each standard or control solution was slowly spotted onto the centre of each circle using a micropipette. The paper was then allowed to dry for 2 - 3 h at RT before analysis by in-gel immunodetection. For the partial banknote samples, £5 GBP notes were cut width-wise into *ca*. 6 cm wide strips and then spiked in the same manner as the cotton paper samples.

Whole or partial banknote samples were spiked with 'street' cocaine using the approach described by Ebejer *et al.*.[19] For the partial banknote samples, £5 GBP notes were first cut width-wise into *ca*. 4 cm wide strips before spiking. Newly minted £20 GBP notes were used as whole banknote samples. A flat glass plate was dusted with 25 mg of cocaine to represent a 'contaminated surface.' The banknote was then rubbed over the surface at random, in order to achieve sporadic but overall coverage. The same process was performed for both sides of the note. The contaminated glass plate was weighed before and after spiking to enable the quantity of cocaine adhered to the note to be estimated.

2.3.6 In-gel immunodetection of cocaine on spiked cotton paper, partial banknote and whole banknote samples

In-gel immunodetection was performed using a method based on that developed for PAGE gels in Section 2.3.2. Figure 2.2 shows a schematic overview of the developed method for immunodetection on whole banknotes.

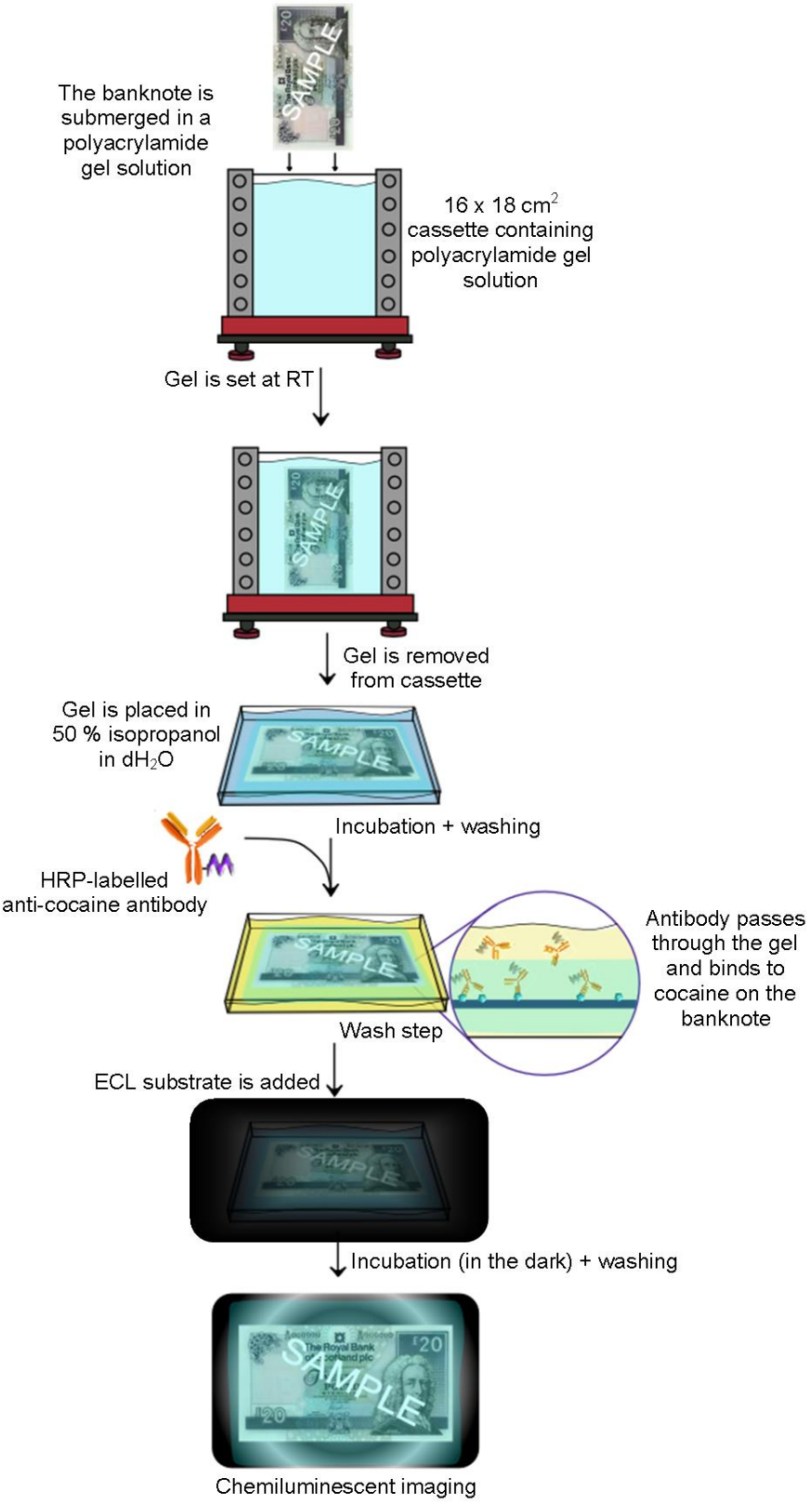

Figure 2.2. A schematic representation of the in-gel immunodetection method for the detection of cocaine on banknotes.

An appropriately sized gel cassette, as indicated in **Table 2.6**, was assembled using either 0.75 or 1.5 mm spacers. A gel mix containing 7.5 % acrylamide was then prepared using the components listed in **Table 2.4**. The spiked cotton paper, partial banknote samples or whole banknote samples were prepared as described in **Section 2.3.5**. The cotton paper samples were initially coated in the pre-prepared gel mix using one of two different approaches. For the first method, the sample was placed inside the cassette and the gel mix slowly poured around it; in the second, the gel mix was added slowly to the cassette beforehand and the sample was slowly lowered into the solution using tweezers until completely immersed. All banknote samples were gel-coated using the second approach. For whole banknotes, the sample was gently lowered length-ways into the gel mix and pushed down with tweezers until completely immersed. Air bubbles in the gel mix were removed by gently tapping the cassette, or by sucking out the air through a narrow pipette tip. A thin layer of ethanol (*ca*. 3 mm) was floated on top of the gel before it was left to set for *ca.* 40 min at RT.

Once set, the gel containing the sample was gently removed from the cassette and transferred to a gel box. For the whole banknote samples, excess gel (beyond *ca*. 1.5 cm of the edge of the banknote) was removed using a scalpel prior to transfer to the gel box. Once transferred, the gel was then submerged in 50 % isopropanol in dH_2O (using the volumes listed in **Table 2.6**) for 15 min at RT. This was followed by a 15 min wash step in dH_2O. The gel was then incubated for 2 h at RT in a solution of either rabbit or sheep polyclonal anti-cocaine antibody (diluted in 0.5 % w/v BSA/1x TBS/0.05 % v/v Tween-20 (pH 7.5, sample buffer)), as indicated in **Table 2.6**. HRP-labelled sheep anti-cocaine antibody, prepared as described in **Section 2.3.4**, was used an alternative to the HRP-labelled secondary antibody in later experiments (**Table 2.6**). After incubation with the primary antibody, the gel was then washed three times for 10 min in TBS/T (1x TBS/0.05 v/v Tween-20, pH 7.5) using the volumes listed in **Table 2.6**. HRP-labelled goat anti-rabbit IgG, or HRP-labelled anti-sheep IgG secondary antibody in sample buffer was then added to the gel and incubated for 1 h at RT. The gel was washed a further three times as described

previously. For gels treated with HRP-labelled primary antibody, the previous two steps involving the addition of the HRP-labelled secondary antibody and subsequent wash step were omitted. The ECL substrate solution, at the volumes indicated in **Table 2.6**, was prepared by performing a 1:1 dilution of reagent A with reagent B, as described in **Section 2.3.2**. The ECL solution was added to the gel and incubated for 5 min at RT in the absence of light. The solution was then removed and the gel was briefly rinsed (around 15 s) in dH_2O, before being placed between acetate sheets and imaged at 30 s intervals for a total exposure time of 5 min, as detailed in **Section 2.1.3**.

Table 2.6. The in-gel immunodetection of cocaine using a polyacrylamide gel matrix; a summary of the different conditions used for the analysis of spiked cotton paper, partial banknote (BN) and whole BN samples during this study.

Sample type:	Gel size (cm^2)	Gel volume (mL)	50 % isopropanol volume (mL)[a]	1° antibody type	1° antibody volume (mL)	1° antibody dilution	2° antibody volume (mL)	Wash buffer volume (mL)	ECL substrate volume (mL)
Cotton paper	8 x 10[b]	15	50	Rabbit	20	1:25	20	50	10
Partial BN	8 x 10[d]	15	50	Rabbit[c]	20	1:25	20	50	10
Whole BN	18 x 16[d]	45	100	HRP-labelled sheep	40	1:200	-	100	20

[a]An equal volume of dH_2O was used in the wash step thereafter.

[b]0.75 mm gel spacers were used.

[c]HRP-labelled sheep anti-cocaine antibody (at a 1:200 dilution) was also used.

[d]1.5 mm gel spacers and 1.5 M Tris-buffer at pH 7.5 rather than pH 8.5 was used for these gels.

2.4 Anti-cocaine antibody functionalisation of gold nanoparticles: a comparison between different techniques

2.4.1 Synthesis of 16 nm citrate-stabilised gold nanoparticles

16 nm citrate-stabilised gold nanoparticles (cAuNP) were synthesised according to the method of Turkevich *et al.*.[20] A 1.3 mM solution of aqueous tri-sodium citrate was prepared by dissolving 50 mg of tri-sodium citrate in 50 mL of dH_2O. A 0.2 mM aqueous solution of gold (III) chloride trihydrate was similarly prepared by dissolving 12.5 mg of the gold (III) chloride trihydrate in 100 mL of dH_2O. Both solutions were heated to 60 °C, combined and immediately heated further to 85 °C while being stirred. The combined solution was then stirred for a further 2.5 h at 85 °C. The resulting burgundy-coloured solution was cooled to RT, filtered using a 0.22 µm filter and stored at 4 °C. The concentration of AuNP was calculated using the absorption at 520 nm and the extinction co-efficient 2.4×10^8 M^{-1} cm^{-1}.[21]

2.4.2 Synthesis of 40 nm cysteamine-stabilised gold nanoparticles

Cysteamine stabilised AuNP were synthesised using a modified version of the method described by Niidome *et al.*.[22] The particles were prepared by sodium borohydride ($NaBH_4$) reduction of gold (III) chloride trihydrate in the presence of the cystamine stabiliser, using a molar ratio of 56 Au:0.1 $NaBH_4$:85 cysteamine. 400 µL of 213 mM cysteamine hydrochloride was added to 40 mL of 1.42 mM gold (III) chloride trihydrate and stirred for 20 min at RT. 10 µL of 10 mM sodium borohydride was added and the solution stirred vigorously in the dark and at RT. After 10 min, the speed of stirring was reduced and left overnight. The presence of AuNP was observed by the formation of a deep red colouration. The particles were purified by dialysis for 24 h against 4.5 L of dH_2O at RT using 10 MWCO dialysis tubing. The concentration of AuNP was calculated using the absorption at 528 nm and the extinction co-efficient 4.7×10^9 M^{-1} cm^{-1}.[23]

2.4.3 Gold nanoparticle characterisation by transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to characterise the size and morphology of the AuNP. 10 µL of the AuNP in solution was placed onto a holey carbon film 300 mesh copper grid. Excess solvent was removed from the sample by touching the side of the grid with absorbent tissue paper. The grid was then allowed to dry overnight at RT before imaging. Imaging was performed as described in Section 2.1.3.

2.4.4 Antibody functionalisation of 16 nm AuNP using a PEG-carboxyl linker

Antibody-functionalised AuNP were prepared using a modified version of the protocol described by Mason *et al.*, whereby the PEG-carboxyl linker was self-assembled onto cAuNP, followed by the addition of the anti-cocaine antibody *via* the formation of an amide bond.[24] A w/v ratio of 1 PEG:1 AuNP was self-assembled by adding 20 mg of α-thio-ω-carboxy polyethylene glycol (3,317 Da, PEGCOOH) to 20 mL of cAuNP prepared as described in Section 2.4.1. The mixture was then stirred overnight at RT. Unbound PEG was removed by centrifugation filtration in a Vivaspin20 tube at 2,500 xg for 15 min at 4 °C. Following centrifugation, the AuNP-PEGCOOH were resuspended in 10 mL of 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) and stored at 4 °C.

Addition of the anti-cocaine antibody to the terminal -COOH functional group on the PEG was performed using sulfo-NHS/EDC chemistry, in a similar manner to that described by Won and Sim.[25] 10 µL of an EDC/sulfo-NHS solution (0.1 M EDC and 0.1 M sulfo-NHS in dH_2O) was added to 1 mL of AuNP-PEGCOOH. Immediately, 100 µL of anti-cocaine antibody (diluted to 10 µg mL^{-1} in 10 mM PB, pH 7.4) was added. The solution was then mixed thoroughly by drawing repeatedly into a wide-mouthed pipette, before being stirred gently for 2 h at RT. Unbound antibody was

removed by centrifugation at 14,000 rpm for 15 min at 4 °C, after which the AuNP were resuspended in 1.0 mL of 10 mM PB (pH 7.4). The centrifugation step was repeated for a total of three times, yielding 1.0 mL of purified AuNP-PEGCOOH-antibody. The antibody-functionalised particles were stored at 4 °C until needed.

2.4.5 Antibody functionalisation of 16 nm AuNP using a PEG-amine linker

cAuNP with a PEG-amine ($PEGNH_2$) monolayer were synthesised in a similar manner to the AuNP-PEGCOOH described in **Section 2.4.4**. Briefly, a w/v ratio of 1 PEG:1 AuNP was produced by adding 20 mg of α-mercapto-ω-amino poly(ethylene glycol) hydrochloride (MW 3163 Da) to 20 mL of cAuNP. The PEG was self-assembled onto the gold surface by stirring overnight at RT. Unbound $PEGNH_2$ was removed by centrifugation in a Vivaspin20 tube at 2,500 xg for 15 min at 4 °C. Following centrifugation, the AuNP-$PEGNH_2$ were resuspended in 10 mL of 10 mM PB (pH 8.0) and stored at 4 °C until used the same day.

Functionalisation of the AuNP-$PEGNH_2$ was performed using the method reported by Puertas *et al.* for the addition of antibody to aminated magnetic particles.[26] Anti-cocaine antibody (purified as stated in **Section 2.2.1**) was diluted to 0.5 mg mL^{-1} in 10 mM PB (pH 7.0). 50 µL of sodium periodate (100 mM in dH_2O) was added to 1.0 mL of the antibody solution and incubated for 2 h at 4 °C in the dark. Excess sodium periodate was removed using a PD-10 desalting column equilibrated by washing four times with 8.0 mL 10 mM PB (pH 8.0), as per the manufacturer's instructions. The 1.0 mL of oxidised antibody was applied to the column, allowed to enter the packed column bed completely, and then made up to 2.5 mL with 1.5 mL of 10 mM PB (pH 8.0). The column flow-through was discarded before the antibody was eluted from the column using 3.5 mL of 10 mM PB (pH 8.0). The 3.5 mL of oxidised antibody solution was added to 10 mL of AuNP-$PEGNH_2$. The mixture was stirred gently for 1 h at RT, after which 2.5 mL of milk buffer (1 % w/v skimmed milk powder in 10 mM PB, pH 7.4) was added. The AuNP were then stirred for a further 1 h at RT.

The antibody-functionalised AuNP-PEGNH_2 were purified by glycerol gradient using a modified version of the method described by Karthik *et al.*.[27] 900 µL of 80 % v/v glycerol in dH_2O (pH 7.8) was added to a 15 mL centrifuge tube. 1.3 mL of 30 % v/v glycerol in dH_2O (pH 7.8) was layered on top. 5 mL of the AuNP-PEGNH_2-antibody was then floated on top of the glycerol solutions, followed by centrifugation at 2,500 xg for 2 h at 30 °C. After centrifugation, the AuNP-PEGNH_2-antibody had moved from the top buffer layer to the 30 % glycerol layer lower down in the tube. The AuNP were extracted from this 30 % glycerol layer by piercing the side of the tube with a 25G needle and collecting the AuNP in a 10 mL syringe. The extracted volume (*ca.* 1.5 mL) was made up to 5 mL with 10 mM MES (pH 6.0). The glycerol was removed by centrifugation in a Vivaspin20 tube (30,000 MWCO), at 2,000 xg for 15 min (4 °C), after which the AuNP-PEGNH_2-antibody were resuspended in 5 mL of 10 mM MES (pH 6.0). Centrifugation was repeated for a total of four wash steps. The AuNP-PEGNH_2-antibody were resuspended in a final volume 2 mL of 10 mM MES (pH 6.0) after the last wash step. 2 mL of 0.25 M sodium cyanoborohydride in 10 mM PB (pH 8.0) was added to the 2 mL of AuNP-PEGNH_2-antibody and stirred for 30 min at RT. Excess reducing agent was removed by centrifugation in a Vivaspin20 tube at 2,000 xg for 15 min at 4 °C. Centrifugation was repeated a total of three times. The AuNP-PEGHN_2-antibody were resuspended in 4 mL of 10 mM PB (pH 7.4) after each centrifugation. Once purified, modification of the AuNP surface was measured by UV-Vis spectrophotometry at 520 nm. The functionalised AuNP were stored at 4 °C.

2.4.6 Antibody functionalisation of 16 nm AuNP using a Protein-A/G intermediate

Antibody immobilisation onto the surface of cAuNP *via* an SPDP-Protein A/G intermediate was performed using a modified version of the method described by Leggett *et al.*.[28] The Protein A/G was first modified by addition of the SPDP bifunctional linker. 60 µL of SPDP (10 mM in DMSO) was added to 2.5 mL of Protein A/G (5 µM in 100 mM PB, pH 7.8). The solution was stirred at RT for 30 min.

Unbound SPDP was removed using a Sephadex PD-10 desalting column equilibrated with 10 mM PB (pH 7.4) as per the manufacturer's instructions. Briefly, 2.5 mL of the SPDP-Protein A/G solution was applied to the column, and the flow-through discarded. The column was washed four times with a total of 8 mL of 10 mM PB (pH 7.4) and the SPDP-Protein A/G eluted in a further 3.5 mL of 10 mM PB (pH 7.4). The presence of SPDP in the elution was determined by adding 25 µL of DTT (100 mM in dH_2O) to 1 mL of the SPDP-Protein A/G eluent. The mixture was then stirred for 30 min at RT. The presence of 2-thiopyridine in the resulting solution was measured by UV-Vis spectrophotometry at 343 nm, and its concentration subsequently determined based on the recorded absorption using the extinction co-efficient 8.08 x 10^3 M^{-1} cm^{-1}.[29,30] This concentration was in turn used as the overall concentration of SPDP-Protein A/G, based on the finding that separated 2-thiopyridine is equivalent to the 2-pyridyl disulfide content of the modified protein as reported by Stuchbury *et al.*.[29]

The SPDP-Protein A/G was adsorbed onto the surface of the AuNP by combining the two at a molar ratio of 100 SPDP-Protein A/G:1 AuNP, and stirring overnight at RT. Purification of the AuNP-Protein A/G was performed by centrifugation in Vivaspin500 tubes at 2,500 xg for 12 min at 4 °C. 10 of the Vivaspin500 tubes were used to accommodate 5 mL of the AuNP in 500 µL aliquots. Each aliquot was resuspended in 500 µL of 10 mM PB (pH 7.4) following centrifugation. The wash step was repeated for a total of three times. The shift in absorption maximum that followed the addition of Protein A/G to the particle surface was measured by UV-Vis spectrophotometry at around 520 nm.

Anti-cocaine antibody, purified as described in **Section 2.2.1**, was added to 10 mL of AuNP-Protein A/G to make a final concentration of 10 µg mL^{-1}. The solution was stirred gently for 1 h at RT. After incubation, 2.5 mL of milk buffer (1 % w/v in 10 mM PB) was added and the AuNP stirred for a further 1 h at RT. The solution was divided into ten 1 mL aliquots and transferred to 1.5 mL Eppendorf tubes. Unbound antibody was removed by centrifugation at 8,000 rpm for 30 min at 4 °C, after which the AuNP

were resuspended in 1.0 mL of 10 mM PB (pH 7.4). The centrifugation step was repeated for a total of three times. After the final centrifugation, the AuNP were resuspended in 250 µL of 10 mM PB (pH 7.4) per aliquot. The aliquots were combined to make up a total of 2.5 mL of purified AuNP-Protein A/G-antibody. The functionalised particles were stored at 4 °C until needed.

2.4.7 Antibody functionalisation of 40 nm cysteamine-stabilised AuNP

Antibody functionalisation of the 40 nm cysteamine-stabilised AuNP, synthesised as stated in **Section 2.4.2**, was performed using the method of functionalisation described in **Section 2.4.5**.

2.4.8 Detection of bound antibody by SDS PAGE

A modified version of the SDS-PAGE method reported by Puertas *et al*. was used for the detection of antibody bound to the surface of the functionalised AuNP.[26] 500 µL of the functionalised AuNP were first concentrated by centrifugation in a Vivaspin500 tube at 2,500 xg for 15 min at 4 °C. Following centrifugation, 10 µL of concentrated AuNP was taken from above the membrane and transferred to a 1.5 mL Eppendorf tube. 10 µL of a 40 % w/v SDS and 20 % v/v mercaptoethanol solution in dH_2O was added and the solution incubated at 100 °C for 10 min. The aggregated AuNP cores were removed by centrifugation at 8,800 xg for 10 min, producing a small black pellet. The supernatant was transferred to a fresh tube and the pellet discarded. In a separate tube, 10 µL of the supernatant was added to 10 µL of SDS-PAGE sample buffer (prepared as described in **Section 2.1.2**) and incubated for 5 min at 100 °C. The solution was cooled for approximately 5 min before 10 µL was loaded onto a 15 % SDS-polyacrylamide gel, prepared as described in **Section 2.1.2**. SDS-PAGE was performed as detailed in **Section 2.2.1**.

2.4.9 Visualisation of AuNP surface modification and size by agarose gel electrophoresis

In this study, the method reported by Hanauer *et al.* was applied for the separation of AuNP on the basis of size.[31] Antibody-functionalised AuNP were concentrated by centrifugation using a Vivaspin500 tube at 2,500 xg for 15 min at 4 °C. 20 µL of concentrated AuNP solution was then taken from above the membrane and loaded directly onto a 0.2 % w/v agarose gel prepared in 1 x TBE buffer (pH 8.0). The gels were run with a 15 cm electrode spacing at a constant voltage of 150 V for 60 min, using a 0.5 x TBE electrophoresis buffer (pH 8.0). The gel and buffer solutions were prepared as described in Section 2.1.2. Photographic images of the gels were obtained immediately after electrophoresis, as described in Section 2.1.3.

2.4.10 Estimation of bound antibody concentration using enzyme labelled antigen

In this study, HRP labelled cocaine (cocaine-HRP) was used as an indication of the presence of antibody on the surface of functionalised AuNP. An attempt was made to estimate the number of antibodies bound to each AuNP based on the intensity of the enzyme product generated by the bound labelled antigen. A schematic version of the developed method is shown in Figure 2.3.

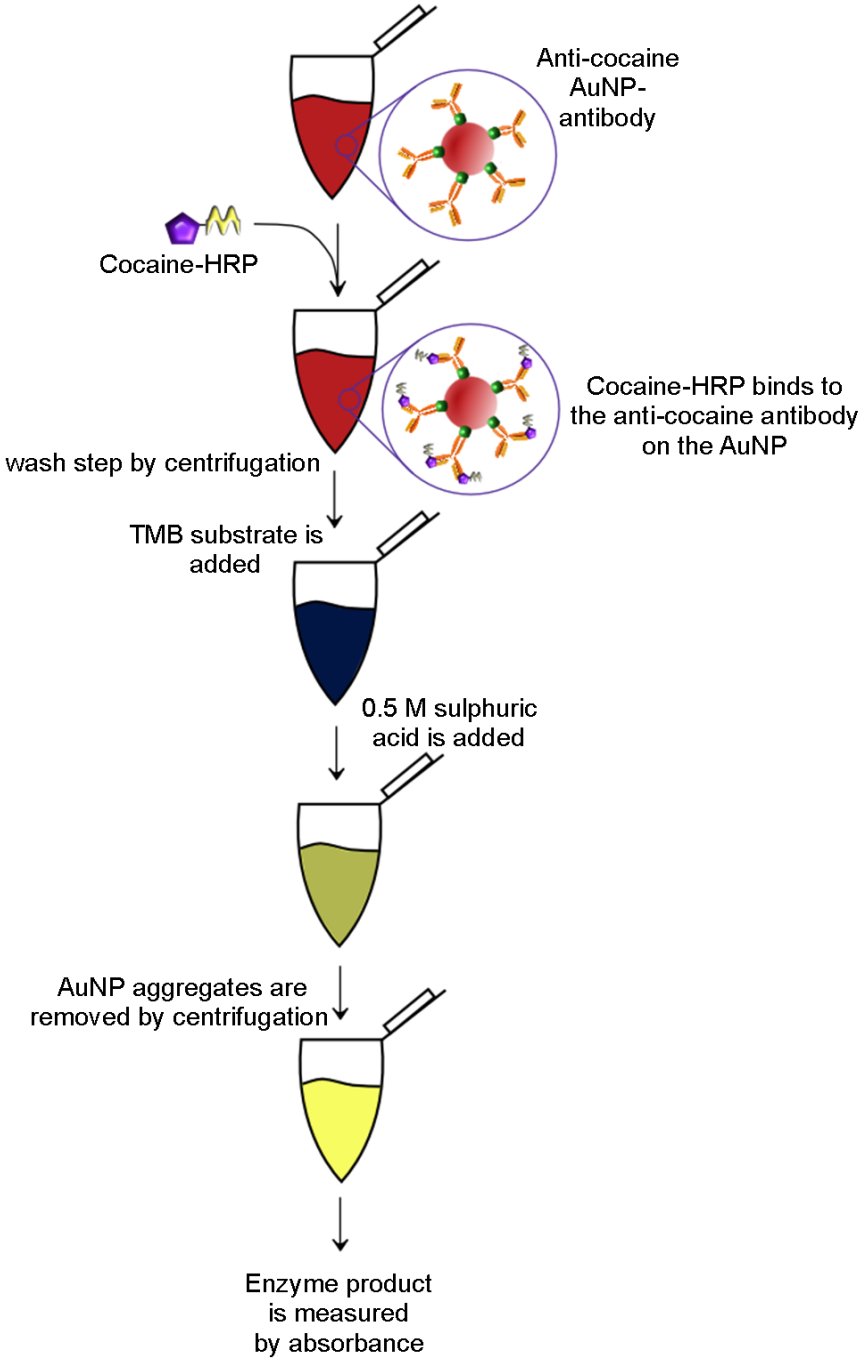

Figure 2.3. A schematic diagram of the detection of anti-cocaine antibody on the surface of functionalised AuNP using the HRP-labelled cocaine hapten.

The concentration of the cocaine-HRP hapten was first calculated based on the absorption of HRP at 403 nm, as described in **Section 2.3.4**. A 1.0 μM cocaine-HRP stock solution was then prepared. Six 500 μL standard solutions of cocaine-HRP,

ranging in concentration from 0 to 100 nM, were prepared by diluting the 1.0 μM stock in 10 mM PB (pH 7.4). 200 μL of TMB substrate solution was added to each standard followed by a 20 min incubation in the dark at RT. 200 μL of sulfuric acid (0.5 M in dH_2O) was added. A 1:2 dilution of the resulting enzyme product solution was made in 10 mM PB (pH 7.4), before the absorbance intensity was measured at 450 nm.

For the detection of antibody bound to AuNP, a 1:20 dilution of the cocaine-HRP was made in 10 mM PB (pH 7.4). 2.0 μL of this solution was added to 1.0 mL of functionalised AuNP and stirred at room temperature for 30 min. Two 500 μL aliquots of the solution were placed into two Vivaspin500 tubes. Unbound cocaine-HRP was removed by centrifugation at 2,500 xg for 12 min at 4 °C, and the solutions resuspended in 500 μL of 10 mM PB (pH 7.4) per aliquot. Centrifugation was repeated for a total of four wash steps, or until the supernatant below the membrane remained colourless when TMB substrate solution was added. The concentration of AuNP was then determined by measuring the extinction at 520 nm and using the extinction co-efficient 2.4 x 10^8 M^{-1} cm^{-1}.[21] Each 500 μL aliquot was then transferred to a 1.5 mL Eppendorf tube, and the presence of HRP determined using the TMB substrate solution in the same way as for the cocaine-HRP standard solutions. The resulting solutions were centrifuged at 10,000 xg for 10 min at 4 °C to remove the aggregated AuNP, producing a small black pellet. 500 μL of the supernatant was taken and the remaining solution and pellet discarded. A 1:1 dilution of the supernatant was made in 10 mM PB (pH 7.4) and the absorbance intensity measured by UV-Vis spectrophotometry at 450 nm.

The HRP concentration in the sample was determined based on the absorbance intensity of the sample at 450 nm, using a linear regression of the standard curve. A 1:1 ratio of bound cocaine-HRP:antibody was assumed. The average molar ratio of AuNP:antibody was calculated for each type of functionalised particle based on the concentration of AuNP after the final wash step (measured as stated). The molar ratio was expressed as the number of antibody per particle.

2.4.11 Determination of antigen binding efficacy using a microtiter plate-based assay

The efficacy with which the antibody-functionalised AuNP were able to bind cocaine was determined using a cocaine-BSA hapten, which was adsorbed onto micro-titer plates to form the basis of a simple immunoassay. A schematic overview of this method is shown in **Figure 2.4**. Cocaine-BSA (1 mg mL^{-1} in 10 mM PB, pH 7.4) was first diluted to 100 µg mL^{-1} in 100 mM carbonate buffer (pH 9.5). 100 µL of this cocaine-BSA solution was then added to each well of the plate (**Figure 2.4A**). BSA (100 µg mL^{-1} in 100 mM carbonate buffer, pH 9.5) was added to an equal number of wells on the plate and used as a negative control (**Figure 2.4B**). The plate was incubated overnight at 4 °C, then washed three times with PBS/T (10 mM PBS with 100 mM NaCl/ 0.05 % v/v Tween-20, pH 7.4). 200 µL of blocking buffer (1.0 % w/v BSA/PBS) was added to each well and incubated for 2 h at RT. The plate was then washed a further three times with PBS/T before the addition of either antibody-functionalised AuNP, or unbound antibody (100 µg mL^{-1} in 10 mM PB, pH 7.4) at 100 µL/well. This was followed by 2 h of incubation at RT, after which the plate was washed three times with PBS/T. 100 µL of the HRP-labelled secondary antibody solution (1:5,000 in 10 mM PB, pH 7.4) was then added to each well, and the plate was incubated for a further 2 h at RT. The plate was washed a final four times with PBS/T. TMB substrate solution was added at 100 µL/well followed by a 20 min incubation at RT in the absence of light. Sulfuric acid (0.5 M in dH_2O) was added at 100 µL/well to stop the enzymatic reaction, after which the absorption of each well was measured at 450 nm using the multi-label plate reader as described in **Section 2.1.3**. Wells without protein bound were used as control blanks. The intensity of the absorbance measured was proportional to the concentration of the antibody, or the functionalised AuNP bound to the adsorbed cocaine-BSA.

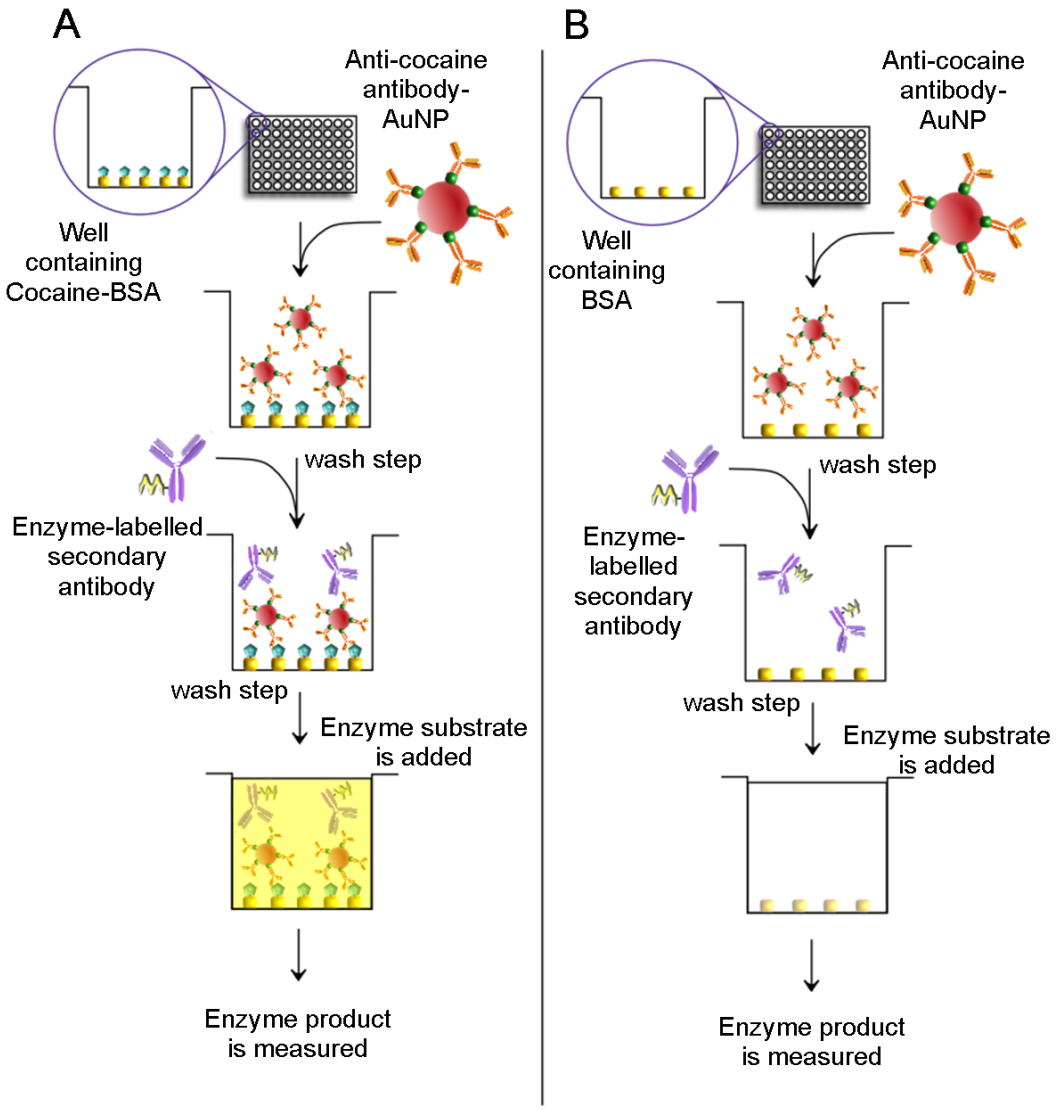

Figure 2.4. A schematic diagram of the immunoassay-based method developed for determining the binding characteristics of the anti-cocaine AuNP-antibody in the (A) presence and (B) absence of a cocaine-BSA hapten bound to a microtiter plate. Detection of the antibody-functionalised AuNP was performed using an HRP-labelled secondary antibody, as shown.

2.5 References

(1) Laemmli, U. *Nature* **1970**, *227*, 680.

(2) Walker, J. M. In *The Protein Protocols Handbook, 3rd ed.*; Walker, J. M., Ed.; Springer: New York, 2009, 177.

(3) Page, M.; Thorpe, R. In *The Protein Protocols Handbook, 2nd ed.*; Walker, J. M., Ed.; Springer: 1996, 721.

(4) Esteve-Turrillas, F. A.; Armenta, S.; Moros, J.; Garrigues, S.; Pastor, A.; de la Guardia, M. *J. Chromatogr. A* **2005**, *1065*, 321.

(5) Kidwell, D. A.; Athanaselis, S. A. In *Handbook of forensic drug analysis;* Smith, F. P., Ed.; Elsevier Academic Press: San Diego, 2005, 235.

(6) Wild, D. *The immunoassay handbook;* 4th ed.; Elsevier Science: Oxford, 2013.

(7) Jickells, S.; Negrusz, A. *Clarke's Analytical Forensic Toxicology;* Pharmaceutical Press: London, 2008.

(8) Chambi, H.; Grosso, C. *Food Res. Int.* **2006**, *39*, 458.

(9) Griffioen, H. A.; Van der Beek, E.; Boer, G. J. *J. Neurosci. Meth.* **1992**, *43*, 43.

(10) O'Connor, S. M.; Stenger, D. A.; Shaffer, K. M.; Maric, D.; Barker, J. L.; Ma, W. *J. Neurosci. Meth.* **2000**, *102*, 187.

(11) Wester, K.; Andersson, A. C.; Ranefall, F.; Bengtsson, E.; Malmstrom, P. U.; Busch, C. *J. Pathol.* **2000**, *190*, 503.

(12) Walker, J. M. In *The Protein Protocols Handbook;* 3rd ed.; Walker, J. M., Ed.; Springer: New York, 2009, 171.

(13) Desai, S.; Dworecki, B.; Cichon, E. *Anal. Biochem.* **2001**, *297*, 94.

(14) Hawkes, R.; Niday, E.; Gordon, J. *Anal. Biochem.* **1982**, *119*, 142.

(15) Tijssen, P.; Kurstak, E. *Anal. Biochem.* **1984**, *136*, 451.

(16) Wisdom, G. B. In *The Protein Protocols Handbook;* 3rd ed.; Walker, J. M., Ed.; Humana Press: New York, 2009.

(17) Avrameas, S. *Histochem. J.* **1972**, *4*, 321.

(18) Schonbaum, G. R.; Lo, S. *J. Biol. Chem.* **1972**, *247*, 3353.

(19) Ebejer, K. A.; Winn, J.; Carter, J. F.; Sleeman, R.; Parker, J.; Körber, F. *Forensic Sci. Int.* **2007**, *167*, 94.

(20) Turkevich, J. S.; Stevenson, P. C.; Hillier, J. *Discuss. Farady Soc.* **1951**, *11*, 55.

(21) Storhoff, J. J.; Lazarides, A. A.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L.; Schatz, G. C. *J. Am. Chem. Soc.* **2000**, *122*, 4640.

(22) Niidome, T.; Nakashima, K.; Takahashi, H.; Niidome, Y. *Chem. Commun.* **2004**, 1978.

(23) Niemeyer, C. M.; Ceyhand, B.; Gao, S.; Chi, L.; Peschel, S.; Simon, U. *Colloid. Polym. Sci.* **2001**, *279*, 68.

(24) Mason, M. D.; Eck, W.; Craig, G.; Sigdel, A.; Ritter, G.; Old, L. J.; Tang, L.; Brennan, M. F.; Allen, P. J. *Acs Nano* **2008**, *2*, 2263.

(25) Won, S. H.; Sim, S. J. *Analyst* **2012**, *137*, 1241.

(26) Puertas, S.; Moros, M.; Fernandez-Pacheco, R.; Ibarra, M. R.; Grazu, V.; de la Fuente, J. M. *J. Phys. D Appl. Phys.* **2010**, *4,* 474012.

(27) Karthik, K., Singh, P., Das, P. *Small Ruminant Res.* **2011**, *99*, 7.

(28) Leggett, R.; Lee-Smith, E. E.; Jickells, S. M.; Russell, D. A. *Angew. Chem., Int. Edit.* **2007**, *46*, 4100.

(29) Stuchbury, T.; Shipton, M.; Norris, R.; Malthouse, J.; Brocklehurst, K.; Herbert, J.; Suschitzky, H. *Biochem. J.* **1975**, *151*, 417.

(30) Carlsson, J.; Drevin, H.; Axen, R. *Biochem. J.* **1978**, *173*, 723.

(31) Hanauer, M.; Pierrat, S.; Zins, I.; Lotz, A.; Sonnichsen, C. *Nano Lett.* **2007**, *7*, 2881.

CHAPTER 3

Development of a competitive enzyme immunoassay for the detection of cocaine on banknotes and in latent fingerprints

This chapter describes the development of a competitive enzyme immunoassay (cEIA) method for the quantification of cocaine in forensic samples, and its application for the detection of cocaine extracted from banknotes and latent fingerprints.

3.1 Introduction

3.1.1 Antibodies

Antibodies, also known as immunoglobulins (Ig), are large immunological proteins that exhibit unique individual specificity for a broad range of target molecules.[1] Antibodies are synthesised by B lymphocytes and are used by the mammalian immune system to identify and neutralise invading foreign bodies.[1-3] Initially, antibodies are present in a membrane-bound form on the surface of a B-cell and are referred to as B-cell receptors (BCR). The interaction of a BCR with its specific antigen facilitates activation of the B cell and subsequent differentiation into cells responsible for the synthesis of large quantities of antibody in a soluble, extracellular form.[1] Exploitation of this immunisation process has enabled the production of specifically-targeted antibodies for a wide range of analytical applications. Antibodies can be produced *via* the direct immunisation of a mammalian host, such as a mouse, rabbit or sheep, with the target molecule.[3] The resulting polyclonal antibodies obtained from the serum of the animal consist of a mixture of molecules of different epitope specificity and affinity.[1-3] Purification of polyclonal antibodies is typically

performed in a series of steps based on antibody class and antigen binding affinity. Alternatively, monoclonal antibodies can be generated from a single activated B cell, which is fused with an immortalised cell line to form a hybridoma. These hybridoma cells produce antibodies with single antigen epitope specificity, and can be selected and cloned to produce antibodies with optimal binding affinity.[2]

All monomeric antibodies are “Y”-shaped molecules constructed of paired heavy and light polypeptide chains tethered together by disulfide bonds.[1-3] A typical structure is shown in **Figure 3.1**. At the N-terminal end the four chains combine to form two antigen binding fragments (FAb), the structure of which are variable and depend on the target antigen.[1,4] At the C-terminal end the constant region (Fc), made up of the two heavy chains, is responsible for B-cell activation and exhibits little structural variation within each antibody class.[1] Immunoglobulins have been divided into five different classes according to the structure of the Fc region; IgG, IgM, IgA, IgD and IgE.[1,3] Of these, IgG is produced in the highest yield in response to immunisation and is used almost exclusively in immunoassays.[3] Furthermore, IgG binds to its epitope with the highest affinity, remains stable throughout purification procedures and features several functional groups which enable chemical coupling with only minimal interference to antigen binding, making it highly suited to immunoassay-based applications.[3]

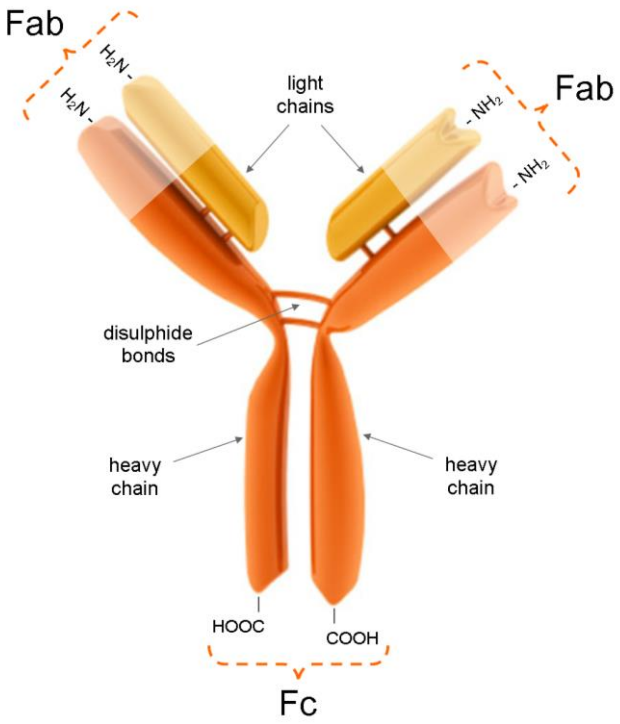

Figure 3.1. A schematic representation of the structure of an antibody.[1] The molecule is made up of paired heavy and light chains joined together by disulfide bonds. The antibody binding sites (Fab) are situated at the N-terminus, and the constant fragment (Fc) at the C-terminus.

Small target molecules, such as drugs, are not large enough to produce an immune response in an animal host.[3] The molecule must therefore be conjugated to a carrier protein to form a hapten prior to immunisation.[5] Proteins such as bovine serum albumin (BSA) and keyhole limpit hemocyanin (KLH) are typically used for this purpose.[3] Although this enables the production of antibodies for drugs like cocaine, it can also increase the ability of the antibody to detect compounds other than the antigen, a phenomenon known as cross-reactivity. Polyclonal antibodies produced using a hapten immunogen can be specific for the carrier protein rather than the compound, a risk which can be lowered significantly by employing an immunoaffinitiy purification step.[3]

3.1.2 Enzyme Immunoassays

The role of antibodies within the immune system enables them to be targeted towards a vast array of antigens, from cells and biomolecules right through to synthetic chemicals.[1] This flexibility has enabled the development of immunoassays targeting a wide range of different analytes. Immunoassays are based around the capture of a target analyte, even at minute concentrations, by a corresponding primary antibody within an often complex sample matrix.[3] This process is afforded by the unique capability of antibodies to strongly bind antigens with a high level of specificity.

Capture of the analyte by the antibody is followed by the addition of a secondary reagent containing the signal-generating component of the assay, commonly known as the tracer, which enables subsequent measurement.[3] Generally speaking, the tracer is made up of two main parts; the first of these facilitates specific binding to a target such as the primary antibody or analyte, and the second is a label compound or molecule responsible for emitting a detectable signal.[3] The original radioimmunoassay (RIA), developed by Berson and Yalow in 1959, used a radioactive iodine (^{131}I) label for this purpose.[6] A wide range of different RIAs were produced in the decades that followed, a number of which are still in use today.[3,7]

Concerns over the safety and handling of radioactive material have led to the development of a variety of alternative labelling methods ranging from proteins to fluorophores and metal colloids.[7-9] Of these, enzyme labels are currently the most widely used and will be focused on within this thesis. The presence of an enzyme can be detected at low concentrations by using its catalytic properties to generate a coloured, fluorescent, luminescent, or electrochemically active product from a 'neutral' substrate.[3] The rate at which the catalytic conversion of substrate to product occurs can be as high as 10^7 molecules per minute from a single enzyme.[3] This signal amplification process enhances the sensitivity of the assay significantly when compared with labels generating just one signal event.[9] The activity of enzymes commonly used in enzyme immunoassays are more stable than that of the ^{125}I RIA

label, however the conditions under which the catalytic conversion reaction of substrate to product occurs is susceptible to interferences and therefore close monitoring of assay conditions at this stage is required.[3,9]

Horseradish peroxidase (HRP) is one of the most commonly used enzymes in immunoassays.[3] HRP is a 44 kDa glycosylated metalloprotein with an iron(III) protoporphyrin IX heme group, two calcium atoms and six lysine residues available for conjugation without any loss of enzyme activity.[10] Its catalytic activity as an oxidoreductase enables the reduction of hydrogen peroxidase in the presence of a number of different hydrogen donors, and it can be used to generate coloured, luminescent or fluorescent derivatives in combination with various substrates.[3,10] A typical chromogenic substrate for HRP employed in EIAs is 3,3',5,5'-tetramethylbenzidine (TMB), which generates a blue coloured product with absorbance maxima at 370 nm and 652 nm, and which can be stabilised with the addition of sulfuric or phosphoric acid to yield a yellow solution with a maximum absorbance at 450 nm.[11] The absorbance of colorimetric substrates of this kind is measured by spectrophotometry.

Labelled conjugates made up of HRP covalently bound to an antibody or antigen can be formed using a number of chemical linking strategies. One of the simplest methods for coupling HRP to protein uses glutaraldehyde, a homobifunctional aldehyde which reacts with amine groups that are present on most proteins.[12] Although easy to perform, the reaction is prone to the formation of enzyme or protein polymers and is therefore less favourable than other coupling strategies.[3] A more popular method for coupling HRP exploits the glycoprotein nature of the enzyme.[13] In this method, the carbohydrate residues on HRP are oxidised by sodium periodate to produce aldehyde groups. These in turn react with amine groups on the protein to form a stable conjugate.[13,14] Competitive immunoassays can require the coupling of HRP to a small, non-protein molecule which is almost always the analyte of interest.[3] In this case the coupling technique employed will depend on the functional groups available on the molecule. Carboxyl-based approaches, such as the reaction of a

carboxylic acid with a carbodiimide, are often used although other coupling reactions can be appropriate.[3,15]

Many different formats of multi-step enzyme immunoassays (EIAs) exist, and these can be broadly categorised as homogeneous or heterogeneous depending on whether separation of bound from unbound enzyme is required prior to addition of the substrate.[3,8,15,16] In a homogenous system, separation of the unbound enzyme is not necessary, as it can be distinguished from the bound enzyme via either activation or de-activation of its catalytic activity.[16] The simple, one-step nature of this type of assay format makes it the fastest and easiest type of EIA. The most widely used of these is an EIA first reported by Rubenstein *et al.* in 1972 for the analysis of morphine, introduced commercially as the 'enzyme multiplied immunoassay technique' (EMIT) for the analysis of drugs of abuse in urine.[3,17] In this competition-based assay system, the tracer is an analyte molecule labelled with the enzyme glucose-6-phosphate dehydrogenase (PDH).[17] In the absence of free analyte, the PDH-labelled analyte is bound to the antibody and this inhibits the activity of the enzyme. Free analyte in a sample will compete for antibody binding sites with the labelled analyte, resulting in the presence of unbound, catalytically active PDH from which a signal can be measured.[15,17]

In a heterogeneous EIA format, the bound and free forms of the enzyme tracer are indistinguishable and therefore a separation step is required prior to measurement.[3,9] The separation step gives this type of assay two distinct advantages over a homogeneous EIA. Firstly, removal of potential endogenous interference from the sample during the wash step effectively acts to increase the sensitivity of this type of assay.[3] Secondly, the presence of the wash step also removes potential matrix interferents, thereby negating the need for a preliminary extraction step and making this type of assay particularly useful for complex sample types.[3,18] Although many variations of heterogeneous EIA exist, three main assay designs are the most commonly used; the indirect enzyme-linked immunosorbent assay (ELISA), the 'sandwich' ELISA, and the competitive EIA (also known as the 'competitive ELISA',

or simply ‘EIA’).[3] All three involve the initial adsorption of an antigen or capture antibody onto a solid phase in the form of a 96-well microtiter plate.[3]

The ELISA assay design is based around the use of an enzyme-labelled antibody as a means of detection.[19,20] **Figure 3.2** shows a schematic overview of two commonly used assays. In an indirect ELISA (**Figure 3.2A**), the sample is added directly to the microtiter plate and the analyte of interest adheres to its surface.[3,9,15,16] A primary antibody is then added, followed by a secondary antibody specific for the type of primary antibody (for example, rabbit IgG). The secondary antibody is labelled with an enzyme to enable detection upon addition of the substrate. The two antibody or ‘sandwich’ ELISA (**Figure 3.2B**) differs from the indirect ELISA in that an antibody, known as the capture antibody, is bound to the microtiter plate prior to addition of the sample. A second primary antibody, targeting a different epitope on the antigen to the capture antibody, is then added and the excess removed by washing. Detection is facilitated by an enzyme-labelled secondary antibody as for the indirect ELISA. In ELISA assays a direct labelling approach is employed, producing a signal that is proportional to the amount of antigen present in the sample,[3,9,15,16] as is illustrated in **Figure 3.2**.

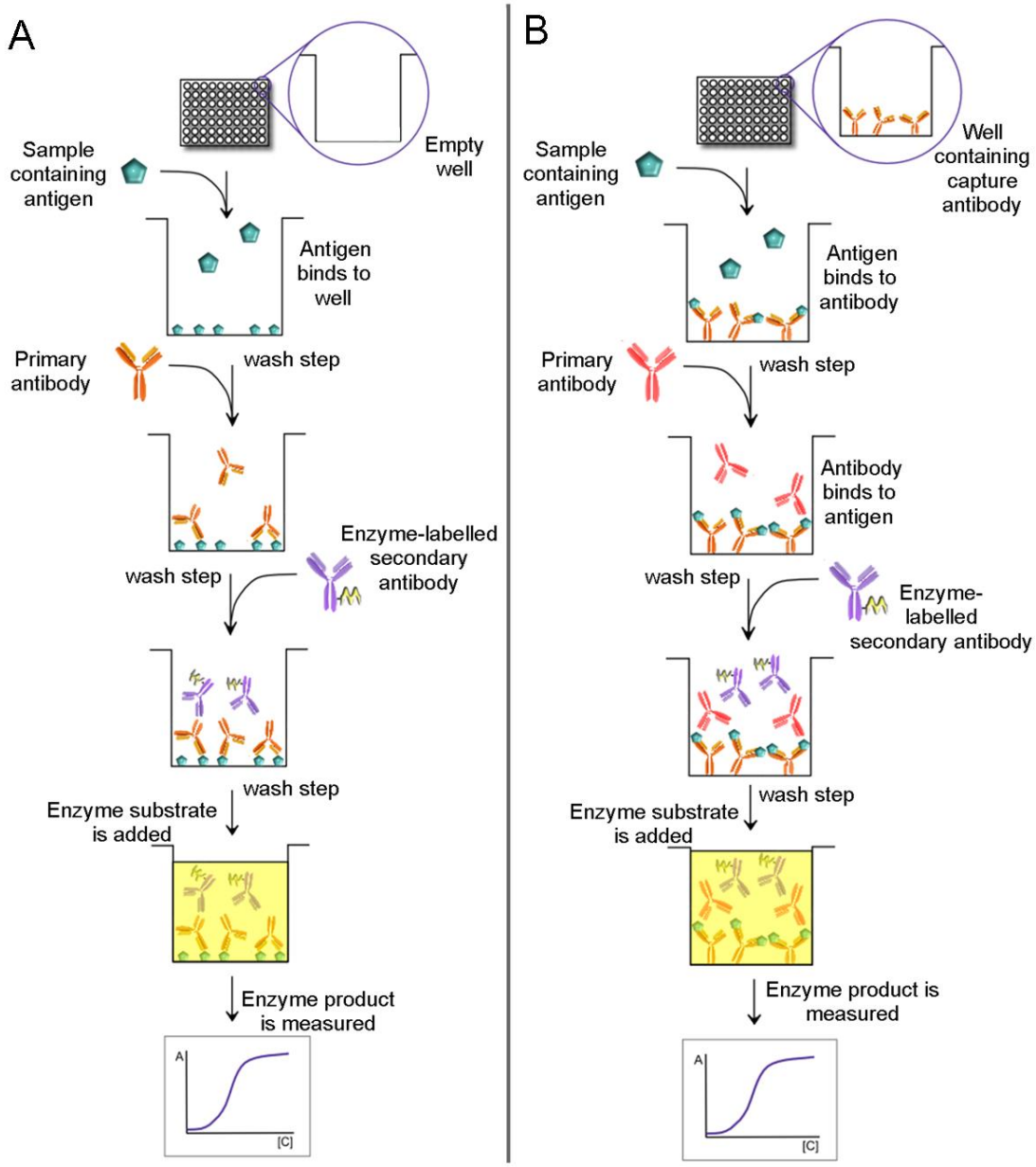

Figure 3.2. Schematic representations of ELISA methods. The formats for indirect ELISAs (A) and 'sandwich' ELISAs (B) are shown.

Competitive EIAs are particularly suited for the detection of low-level concentrations of small compounds such as drugs.[21] They differ from the ELISA assay format in that they involve an indirect method of generating a signal, involving an enzyme conjugated to the target molecule rather than a secondary antibody.[22] In this type of competitive assay format (shown in **Figure 3.3**), capture antibody is first immobilised

onto the surface of a microtiter plate. Enzyme-labelled analyte and the sample of interest are then incubated together before excess enzyme and sample are removed by washing. During the incubation, the enzyme-labelled antigen competes for antibody binding sites with free analyte in the sample.[22] The higher the concentration of analyte in the sample, the lower the concentration of bound enzyme; as a result the signal generated is inversely proportional to the amount of analyte in the sample as the calibration curve in **Figure 3.3** illustrates.[3,9,22]

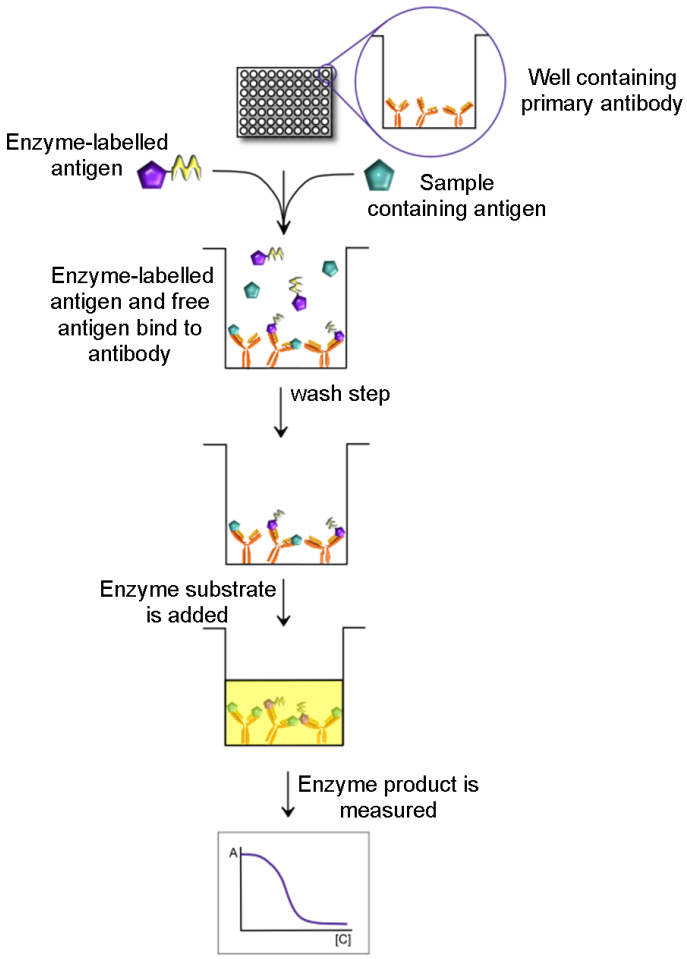

Figure 3.3. A schematic representation of a competitive-style EIA method.

3.1.3 Enzyme immunoassays for the detection of cocaine

Immunoassays were originally introduced to forensic toxicology laboratories for the preliminary screening of drugs of abuse in urine samples.[23] The commercial EMIT

'drugs of abuse urine' assay (EMIT DAU), released in the 1970s, was the first type of EIA employed in this way.[24] The manufactured immunoassay kits provided a means of testing for a variety of drug and drug metabolite compounds in urine samples that is fast, accurate, inexpensive and easy to perform.[23,25] Since then, the variety of EIAs available for the screening of drugs of abuse in urine has expanded rapidly to include a range of different formats such as competitive EIA, ELISA and the homogeneous cloned enzyme donor immunoassay (CEDIA).[18,23,25] Advances in technology have meant that EIAs can now be easily automated, enabling more accurate analysis of a larger number of samples and making them all the more suitable for screening purposes, as discussed in **Chapter 1**.[26]

While most commercial immunoassays have been developed for a urine matrix, they have also been applied by forensic toxicologists to other matrices including blood, tissue homogenates, hair extracts, oral fluid, sweat and meconium.[26-28] The use of non-urine matrices poses two main challenges; firstly, the concentration of the target compound is often lower in such samples, and so the sensitivity of the immunoassay may not be appropriate. Secondly, non-urine matrices, particularly postmortem specimens, are usually more complex in their composition and this can increase the potential for interference.[28]

The cross-reactivity of an EIA is determined by its primary or capture antibody, and is therefore source (*i.e.* manufacturer) dependent.[28] The ability of an immunoassay to detect cross-reactive species can be a useful characteristic or, depending on the specificity required for analysis, a nuisance and a manner in which false-positive results can be generated.[3] Like other commercial EIAs for drugs of abuse, assays for cocaine detection are designed for urine analysis and therefore target benzoylecgonine as the primary metabolite.[3,18,21] Cross-reactivity of some types of assays, marketed as 'cocaine and metabolite' assays, can be high for cocaethylene and cocaine (> 100 %) in particular.[25,26,29]

Some researchers have exploited this cross-reactivity by applying these types of competitive EIAs for the detection of cocaine, rather than benzoylecgonine, in non-urine samples that may be of value in a forensic investigation. The use of commercial competitive EIA assays, such as the STC Cocaine Metabolite Micro-Plate EIA, has been reported for the detection of cocaine in postmortem blood and tissue,[26] oral fluid,[29] sweat,[30] meconium,[31] and hair extracts.[32] In these types of sample cocaine is present at concentrations that are much lower than in urine, for example in oral fluid where the concentration can be up to thirty times less.[29] Despite this, the assays evaluated were capable of identifying the presence or absence of cocaine to a degree of accuracy comparable to confirmatory analyses performed using RIA,[26] biochip microarray,[31] and GC-MS.[29,30] As outlined in **Chapter 1**, the definition of cocaine presence or absence was based on whether the concentration was above or below a cut-off concentration, respectively. The cut-off concentrations were as low as 20 ng g^{-1} and 10 ng mL^{-1} for some sample types,[29,31] so quantitative analysis by means of competitive EIA with a high level of sensitivity was therefore required. These studies illustrate the levels of sensitivity that can be achieved using competitive EIAs, and their suitability for the analysis of even complex sample matrices such as postmortem blood or tissue homogenate. At present, a commercial enzyme immunoassay specifically targeting cocaine rather than cocaine and metabolites is not available. The development of a cocaine-specific competitive enzyme immunoassay for the analysis of cocaine in a forensic setting is therefore presented in this chapter.

3.1.4 Aim of the research presented in this chapter

The high proportion of paper currency in general circulation contaminated with cocaine has been well documented.[33] The methods of cocaine contamination, through trade or use of the drug, and the spread of contamination to other notes *via* bank sorting machines has been described in detail in **Chapter 1**. As transfer of cocaine to other banknotes is a diluent process, the concentration of cocaine on a note contaminated in this way would be lower than if the note had contacted cocaine

directly. As such, a concentration of cocaine on a note that is beyond that of the general circulation could be associated with drug use or trade and thus provide incriminating evidence. Circumstantial evidence of this nature can be crucial to law enforcement in combating cocaine trafficking and trade.

Interest has also grown in recent years in gaining information from latent fingerprints beyond suspect identification. Additional information in a forensic context, such as the use by or recent exposure of an individual to illicit drugs, could provide a greater link between a fingerprint and a suspect. As already highlighted in **Chapter 1**, the successful detection of cocaine and other drug compounds in latent fingerprints is well-documented using a number of different analytical approaches, particularly for prints artificially doped with the compound of interest. Rowell *et al*. reported the detection of isolated patches of cocaine contamination in a fingerprint obtained from an individual being treated at a drug addiction centre.[34] The non-homogenous pattern of cocaine contamination in the print was likely the result of the subject's fingertip coming into contact with the drug prior to the time of sampling, and thereby indicative of recent drug exposure. Analytical methods for the detection of these types of cocaine residues in latent fingerprints could be used in a forensic context, as their presence would provide further evidence of a suspect's involvement in drug use or trade.

The methods used in previous studies for the detection of cocaine on banknotes and in latent fingerprints have been described in detail in **Chapter 1**. As mentioned, chromatography techniques coupled with MS are the most commonly used, although alternative approaches such as SALDI-TOF-MS and Raman spectroscopy have also been reported.[34,35] The application of immunological methods of detection is limited to only a few studies. Examples of methods include; antibody-functionalised magnetic and gold nanoparticles with fluorescent labelling which were successfully applied for the detection benzoylecgonine in latent fingerprints,[36-38] and the application of a strip-based urine immunoassay for the detection of cocaine extracted

from banknotes.[39] These studies illustrate the potential of immunological methods for the detection of cocaine in such complex sample matrices.

From the examples given in the introduction of this chapter, it is clear that competitive EIAs offer an attractive alternative to the current methods available for the quantitative detection of cocaine in forensic samples such as banknotes and latent fingerprints. Assays of this nature offer excellent sensitivity and specificity in a simple to use and cost effective manner, making them highly suited for forensic analysis without requiring specialised chemicals or advanced instrumentation.

This chapter describes the development of a competitive EIA for the detection of cocaine in latent fingerprints and paper currency. The preliminary efficacy of the assay was assessed based on the analysis of ten Bank of Scotland (UK) banknotes obtained from general circulation, the results of which were compared with a parallel analysis performed by LC-MS on the same sample extracts. The competitive EIA was also used for the detection of cocaine in ten latent fingerprints obtained from five test subjects being treated for the habitual use of drugs, including cocaine, at a methadone treatment clinic. The concentrations determined in the extracted fingerprint samples were compared with the cocaine concentrations analysed in corresponding oral fluid samples by GC-MS. The application of a competitive EIA of for the detection of cocaine in forensic samples of this nature has not been previously reported.

3.2 Results and discussion

3.2.1 Anti-cocaine antibody purification

Rabbit polyclonal anti-cocaine antibody was purified from neat serum by ammonium sulfate precipitation followed by an immunochromatography step performed using Protein A spin columns, as described in Chapter 2 (Section 2.1). The amount of antibody in the three fractions obtained from the spin columns was determined by

measurement of the absorbance at 280 nm. **Figure 3.4A** shows a typical elution profile from one of the columns, where the highest concentration of antibody is found in the first two of the eluted fractions. This was consistently the case over the course of three separate purifications where six columns were used at a time.

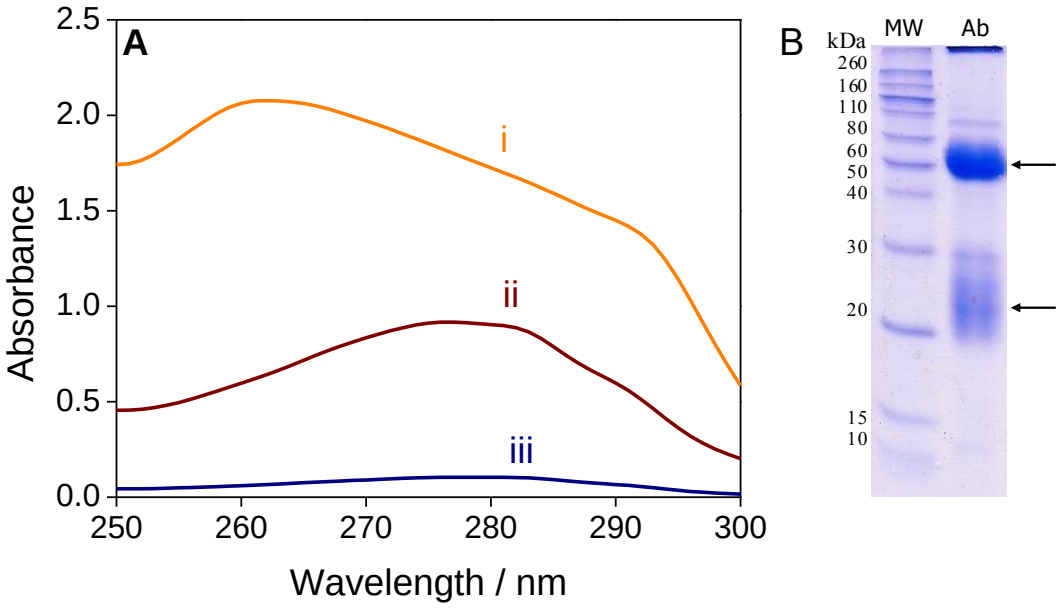

Figure 3.4. Confirmation of the purification of anti-cocaine antibody from serum. Typical UV-visible absorption spectra of the three Protein A spin column fractions (i-iii) are shown in (A). The corresponding SDS PAGE result shows the purified antibody ('Ab') alongside a molecular weight marker ('MW') in (B). The heavy and light chains of the antibody are indicated by the top and bottom arrows in (B), respectively.

Analysis by SDS PAGE of the first two fractions obtained from the spin columns was performed in order to identify the presence of purified IgG antibody. A typical result is shown alongside the molecular weight marker in **Figure 3.4B**. As mentioned in **Chapter 5** (**Section 5.2.4**), IgG antibody is made up of two fragments, a heavy chain and a light chain, which will separate in the presence of SDS, resulting in two characteristic bands (at around 20-30 and 50-60 kDa) in an SDS PAGE gel. Two

fragments of the correct molecular weights (shown by the arrows in **Figure 3.4B**) were present in the gel, confirming the presence of IgG. The absence of other major bands in the gel indicates that the polyclonal antibody is sufficiently purified for application in an immunoassay-based method.

3.2.2 Competitive EIA method development

The initial phase of EIA development involved the adsorption of the purified anti-cocaine antibody onto the solid phase, in this case the polystyrene surface of a 96-well Nunc Maxisorp microtiter plate. Adsorption occurs *via* the interaction of the carbohydrate moiety on the Fc region of the antibody with hydrophilic binding sites on the surface of the plate.[40] Previously reported methods have noted that the optimum adsorption of antibody occurred at alkaline conditions.[41] In order to confirm this, a comparison was made between the adsorption of antibody in 100 mM carbonate buffer (pH 9.5), and in 10 mM phosphate buffer (PB, pH 7.4). The resulting relative concentration of antibody immobilised onto the plate was then estimated using cocaine-HRP based detection, as described in **Chapter 2** (**Section 2.2.5**). The concentration of cocaine-HRP bound, as indicated by the intensity of the coloured product, was considered proportional to the amount of antibody bound to the plate. The results shown in **Figure 3.5** indicate that a more intense signal, and hence a higher concentration of adsorbed antibody was produced by dilution of the antibody in the alkaline carbonate buffer rather than PB.

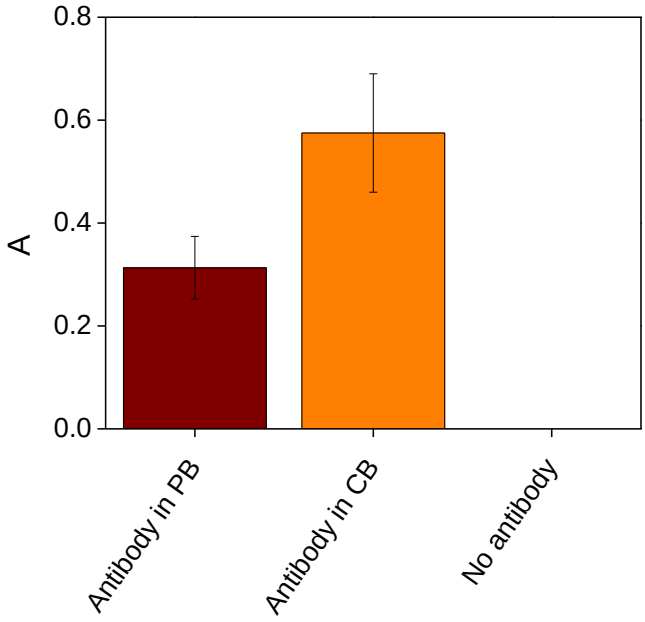

Figure 3.5. The absorbance intensity at 450 nm from cocaine-HRP bound to immobilised anti-cocaine antibody; a comparison between antibody adsorption buffers. PB represents 10 mM phosphate buffer (pH 7.4), and CB represents 100 mM carbonate buffer (pH 9.5). Results were obtained using a 1:20 dilution of cocaine-HRP in PBS/BSA. The anti-cocaine antibody was immobilised for 18 h as a 1 μg mL^{-1} solution, and the incubation time after addition of the cocaine-HRP was 2 h. Each absorbance value represents the mean ± SD of at least four measurements.

The optimal dilution factor for the cocaine-HRP was similarly determined based on the coloured product produced by the bound enzyme. The cocaine-HRP was subjected to a series of dilution factors from 1:20 – 1:160 in PBS/BSA, and applied to a micro-titer plate containing anti-cocaine antibody bound at a concentration of 1 μg mL^{-1}. Wells with BSA bound at 100 ng mL^{-1} in place of the antibody were used as controls. As shown in **Figure 3.6**, the greatest absorbance intensity signal and hence the greatest concentration of bound cocaine-HRP was generated using the 1:20 dilution factor. However, the control wells also showed that non-specific binding of cocaine-HRP occurred at dilution factors of 1:80 and greater (**Figure 3.6**).

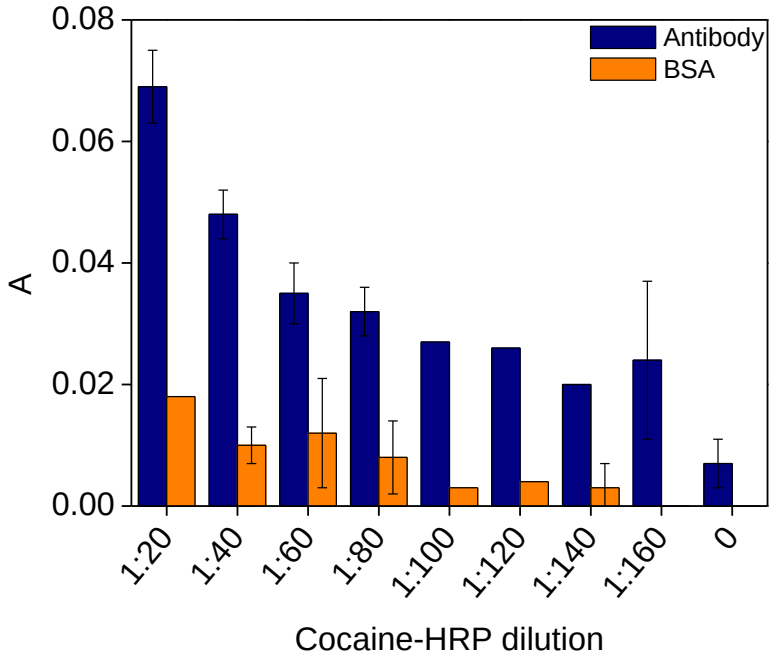

Figure 3.6. The absorbance intensity at 450 nm from cocaine-HRP bound to immobilised antibody; the application of cocaine-HRP at different dilution factors using wells with BSA bound in place of antibody as negative controls. Each absorbance value represents the mean ± SD of at least four measurements.

Detergents such as Tween-20 are often used in immunoassays as an additional blocking agent, alongside proteins such as casein or BSA.[42] Tween-20 was in this case added to the cocaine-HRP dilution buffer, and its effect on non-specific binding evaluated using a similar method to the one described in the previous paragraph. Four cocaine standards, at concentrations ranging from 0 – 1000 ng mL^{-1}, were initially used to test the response of the assay. These were added to a series of wells containing immobilised antibody. Wells containing BSA were used as negative controls as described in the previous paragraph. Cocaine-HRP, at the determined optimal dilution factor of 1:20 in sample buffer (blocking buffer with 0.05 % v/v Tween-20), was then added to each well and the enzyme product measured as detailed in **Chapter 2** (**Section 2.2.5**). As shown in **Figure 3.7A**, dilution of the cocaine-HRP to a factor 1:20 in buffer containing Tween-20 resulted in a significant

reduction in non-specific binding in wells without antibody. The addition of the four cocaine standards also led to a reduced absorbance signal in response to an increase in cocaine concentration, as expected for this type of assay. This is illustrated by the preliminary standard curve shown in **Figure 3.7B**, which has the beginnings of the characteristic sigmoidal shape of a calibration curve produced by this type of competitive assay.[3]

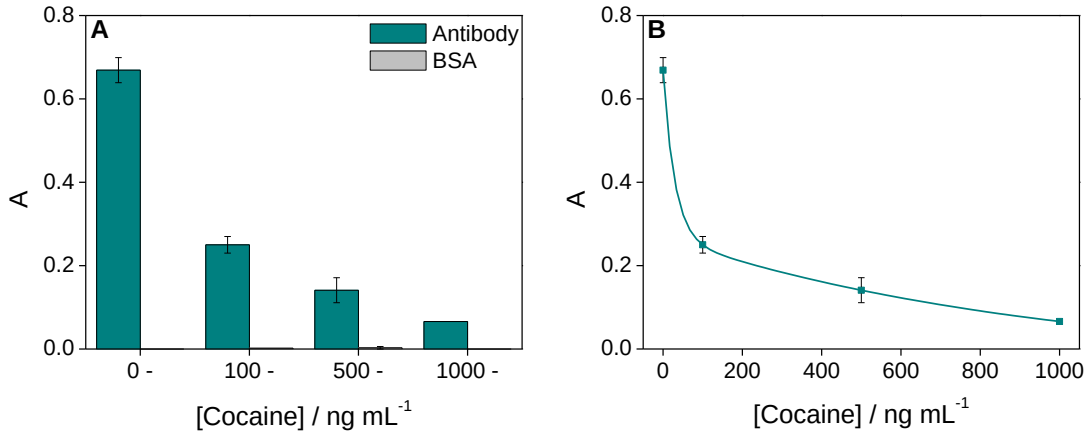

Figure 3.7. The absorbance intensity at 450 nm produced by cEIA using four cocaine standards and cocaine-HRP diluted to 1:20 in blocking buffer containing 0.05 % v/v Tween-20. In (A), the signal produced by wells containing antibody is shown alongside the signal from negative control wells (with BSA bound in place of antibody). A preliminary standard curve generated from four cocaine standards with concentrations ranging from 0 – 1000 ng mL^{-1} is shown in (B). Each point represents the mean ± SD of at least four measurements.

Overall, it was established during development of the competitive EIA (cEIA) that the optimal conditions for antibody adsorption onto the microtiter plate occurred with antibody diluted to 1 µg mL^{-1} in 100 mM carbonate buffer (pH 9.5). In addition, the greatest degree of cocaine-HRP binding was achieved using a dilution factor of 1:20 for the conjugate. Non-specific binding of the cocaine-HRP to the microtiter plate at

this dilution factor was prevented by dilution in sample buffer containing 0.05 % v/v Tween-20. All further analyses were performed using these optimised conditions.

3.2.3 Calibration, LOD, specificity and precision

Cocaine standards diluted in buffer were used to generate calibration curves for the analysis of cocaine extracted from banknotes and latent fingerprints. A series of standard concentrations were initially analysed to determine the linear range of the assay. Once this was established, the calibration curve was run alongside two extracted banknote samples to ensure that the absorbance signal of the samples fell within the range of the curve. The banknote samples used were two Scottish £20 notes obtained from general circulation and extracted as described in **Chapter 2** (**Section 2.2.2**). The serial dilution range, from 1:500 and 1:4000, was based on a previous experiment which indicated that a sample extract diluted to 1:16 was too concentrated to produce a signal. As shown in **Figure 3.8**, the extracted samples were in this case sufficiently diluted to produce a signal, and the signals produced fell well within the range of the standard curve. Of the dilution factors applied, it was concluded that 1:500 and 1:1000 were sufficient for the generation of an absorbance signal which was suitable for the quantitation of cocaine using the standard curve concentrations shown in **Figure 3.8**. The results confirmed the suitability of the assay for the detection of cocaine even at ng mL^{-1} extract concentrations.

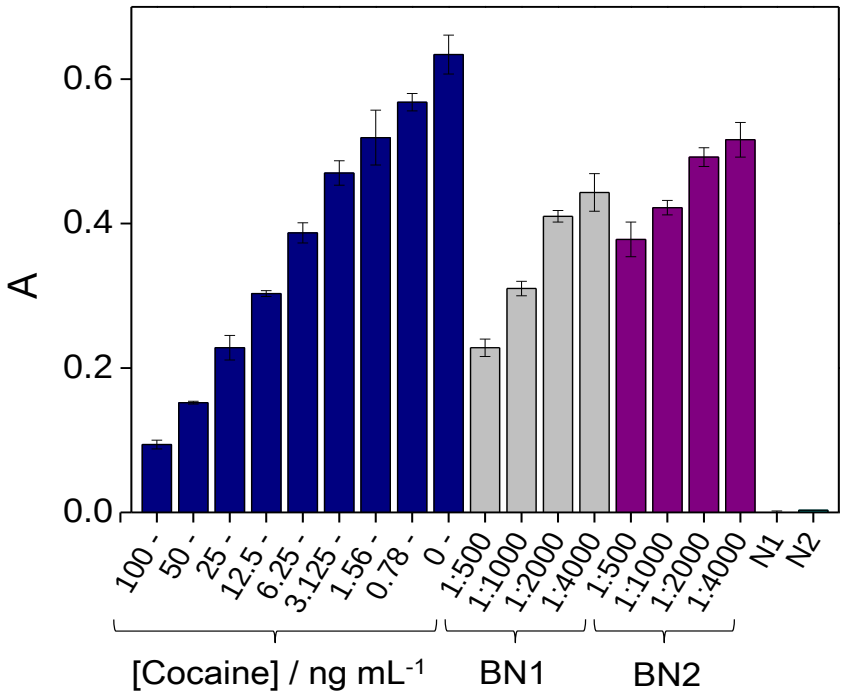

Figure 3.8. The absorbance intensity at 450 nm obtained using the cEIA by a series of cocaine standard solutions (shown in blue), and from two extracted banknote samples (shown in grey and purple, respectively). The two banknote samples were diluted from 1:500 to 1:4000, as indicated. N1 and N2 are negative controls performed using wells without the anti-cocaine primary antibody. Each absorbance value represents the mean ± SD of at least four measurements.

A further set of standard concentrations ranging from 0.78 to 100.00 ng mL^{-1}, and from 0.195 to 25.00 ng mL^{-1} was used for the analysis of extracts from banknotes and latent fingerprints, respectively. Lower concentrations of standards were used for the analysis of fingerprint extracts to account for the lower concentrations of cocaine present in the samples. Examples of typical calibration curves for the analysis of banknotes and fingerprints are shown in **Figures 3.9A** and **3.10B**. The curves were linearised by plotting the logarithm of the concentration against the logit of the signal,[43] calculated as described in **Chapter 2** (**Section 2.2.4**). The linear data showed good correlation coefficients (r^2 = 0.999) on the logit-log plots using a linear regression for both calibration curves (**Figures 3.9B** and **3.10B**).

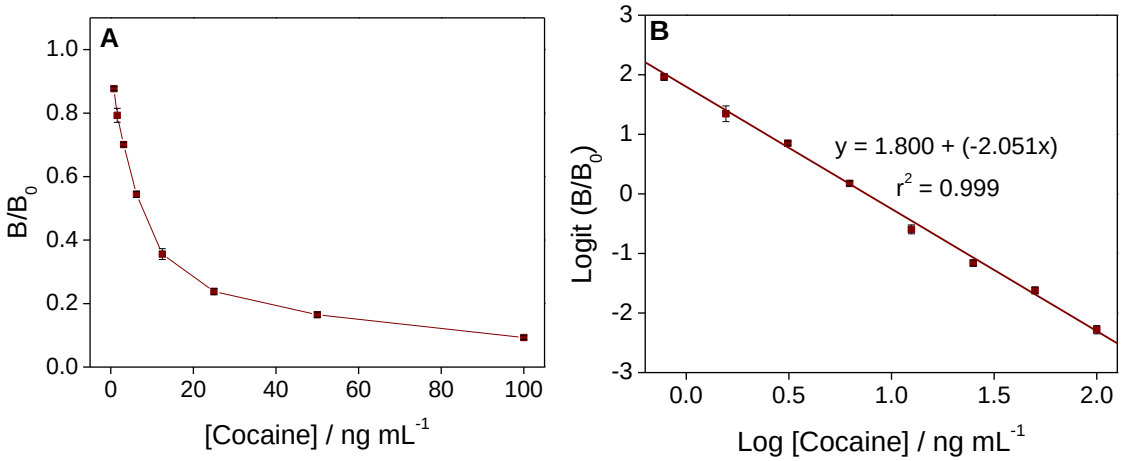

Figure 3.9. A typical standard curve developed for the analysis of extracted banknote samples. (A) The corrected absorbance response (B/B_0) is shown as a function of cocaine concentration in the range of 0.78 to 100.00 ng mL^{-1}. Each point represents the mean ± SD of at least three measurements. The corresponding logit-log linear calibration curve is shown in (B).

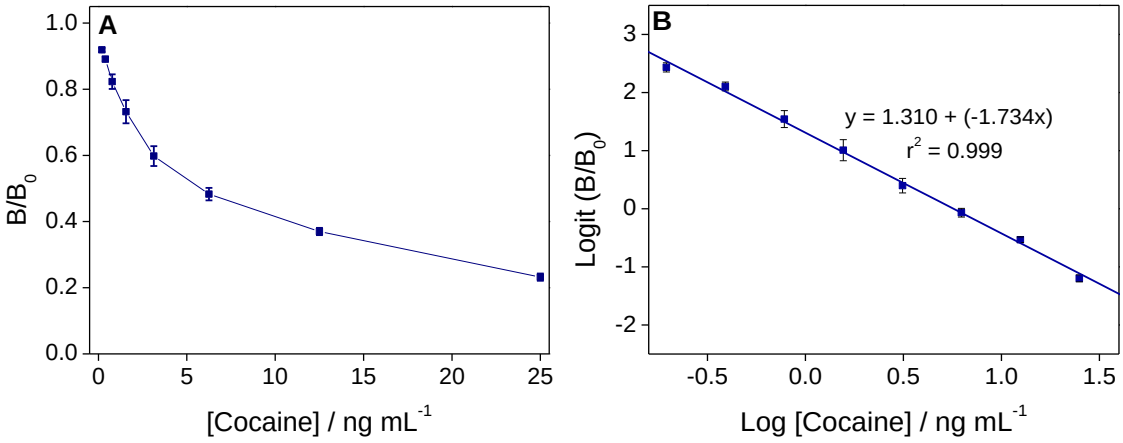

Figure 3.10. A typical standard curve developed for the analysis of extracts from latent fingerprints. (A) The corrected absorbance response (B/B_0) is shown as a function of cocaine concentration in the range of 0.195 to 25.00 ng mL^{-1}. Each point represents the mean ± SD of at least three measurements. The corresponding logit-log linear calibration curve is shown in (B).

The detection limit of the cEIA was calculated from the average absorbance values from six replicate analyses of the blank minus three times the standard deviation of the average. The resulting absorbance value was then extrapolated from the log-logit standard curve. The minimum detectable concentration of cocaine was determined as being 0.162 ng mL^{-1}. This detection limit compares favourably to the 0.95 ng mL^{-1} limit reported for the detection of cocaine and metabolites in oral fluid using a commercial micro-plate EIA,[29] and to the 1.0 ng mL^{-1} achieved using GC-MS with solid phase extraction for the detection of cocaine from banknote samples.[44]

As discussed in **Section 3.1.3**, the cross-reactivity of an EIA depends on the specificity of the primary antibody, and is therefore manufacturer specific. In this study, the cross-reacting species of the primary antibody used were not specified by the manufacturer. As the antibody is polyclonal, it was assumed that some degree of cross-reactivity would be present. The analytical specificity or cross-reactivity of the cEIA was therefore determined for benzoylecgonine (BE), the primary cocaine metabolite in urine and an analogue of cocaine. The BE standard was diluted to three different concentrations in assay buffer (PB) rather than the recommended blank sample matrix, as this was not available for the analysis of banknotes, the reasons for which are discussed in more detail in **Section 3.2.4**.

The relative cross-reactivity was calculated from a cocaine standard curve as described in **Chapter 2** (**Section 2.2.6**). The results, expressed in absorbance intensity, are shown alongside the corresponding cocaine standards in **Figure 3.11**. Relative to 100 % for the cocaine standards, the cross-reactivity for BE was 71, 63 and 41 % at 6.25, 25.0 and 100.0 ng mL^{-1}, respectively. This shows that cross-reactivity is present in the assay, and is particularly high at the low cocaine concentrations expected on banknotes and in latent fingerprint samples. Although this result may overestimate of the level of cross-reactivity possible in actual samples, it nevertheless indicates that the antibody inherently cross-reacts with metabolites such as BE, something which should be noted when interpreting the

results of the assay. The indication of cross-reactivity also stresses the need for confirmation of assay results using a different method of analysis.

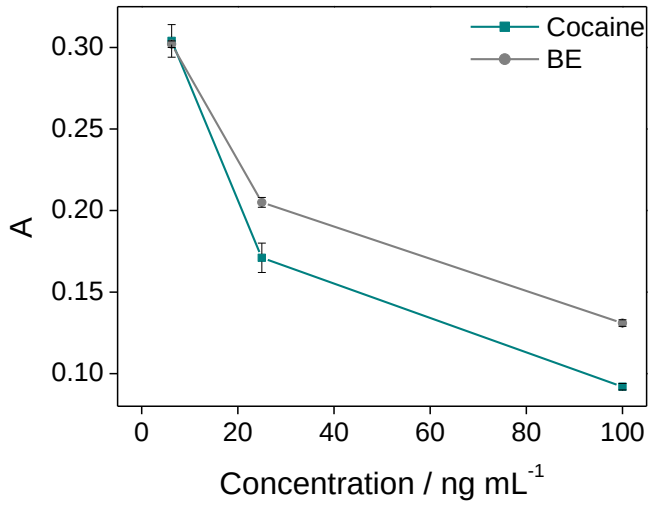

Figure 3.11. The cross-reactivity of the cEIA for benzoylecgonine (BE), shown alongside the corresponding cocaine standards. Each point represents the mean ± SD of four measurements.

The precision of the assay was determined based on repeat measurements of calibration standard samples prepared in assay buffer (10 mM PB, pH 7.4). Standards at three different cocaine concentrations (6.25, 25 and 100 ng mL^{-1}) were used. The intra-assay precision was determined using four replicates of each standard in a single analysis (n = 4). Inter-assay precision was similarly assessed by analysing the same sample, as four repetitions, across three separate tests performed on different days over a period of 30 days (n = 12). The results shown in **Table 3.1** indicate that the developed cEIA shows an acceptable level of precision for cocaine quantification, with confidence variables of 1.58-6.54 % and 4.38-6.69 % for intra- and inter-assay precision, respectively.

Table 3.1. Precision of the cEIA for the quantification of cocaine.

	Intra-assay (n = 4)		Inter-assay (n = 12)	
Cocaine (ng mL^{-1})	Mean ± SD (ng mL^{-1})	CV[a] (%)	Mean ± SD (ng mL^{-1})	CV[a] (%)
6.25	6.100 ± 0.097	1.58	6.363 ± 0.279	4.38
25.0	25.119 ± 0.894	3.56	25.849 ± 1.207	4.67
100.0	98.723 ± 6.456	6.54	98.416 ± 6.587	6.69

[a]CV is the coefficient of variation.

3.2.4 Analysis of ten UK banknote samples

UK banknotes comprise of cotton and linen paper.[45] As described in **Chapter 1**, the way in which paper currency is handled and transferred means that a number of contaminants for example; dust, soil, food particles or cosmetics, can all be found on the fibres of a banknote. At a trace level, predominantly caffeine, nicotine, paracetamol and other pharmaceutical compounds have been reported on banknotes.[46] However, drugs such as cocaine and its metabolite benzoylecgonine, tetrahydrocannabinol (THC), heroin, amphetamines, and ketamine are also often present.[33]

Typically, cocaine is extracted from banknotes using an organic solvent. For chromatographic analyses, this is followed by a form of sample clean-up such as centrifugation or filtration.[47] Such steps can be avoided with immunochemical analysis simply by diluting the sample in buffer prior to analysis.[15] Optimising immunoassays for pre-diluted samples lessens possible matrix effects by reducing the overall concentration of potential interferents in the sample, while maintaining an analyte concentration that is within the working range of the assay.

As highlighted in **Chapter 1**, extracts from banknotes could contain a number of potential immunoassay interferents. Consequently, the influence of the 'matrix effect' on the cEIA was determined. General practise to test matrix effects involves spiking

blank sample matrices with an appropriate standard at a known concentration and thus determining the accuracy of the results. As the majority of banknotes in general circulation are known to be contaminated with cocaine such notes could not be used for spiking. Previously uncirculated banknotes were also avoided as they lack the appropriate 'handling and transfer' contaminants to properly represent the sample matrix. Instead, cocaine was extracted from ten UK banknote samples using a modified version of the method described by Esteve-Turrillas *et al.*, as detailed in **Chapter 2** (**Section 2.2.2**).[48] Esteve-Turrillas *et al.* reported recoveries of 101 ± 2 and 98 ± 3 % in spiked banknote samples extracted using their method followed by GC-MS^2 analysis.

The extracts from the banknote samples were each subjected to the two dilution factors (1:500 and 1:1000), as established in **Section 3.2.3**, and analysed using the cEIA method described in **Chapter 2** (**Section 2.2.4**). The cocaine concentration in each sample was estimated using the standard curve, corrected for the dilution factor and compared to determine what effect doubling the optimised concentration of raw extract would have on the accuracy of the assay. Results showing the change in absorbance relative to cocaine concentration in the standard solutions, and the absorbance response to the doubled extract concentration are shown in **Figures 3.12A** and **3.12B**, respectively.

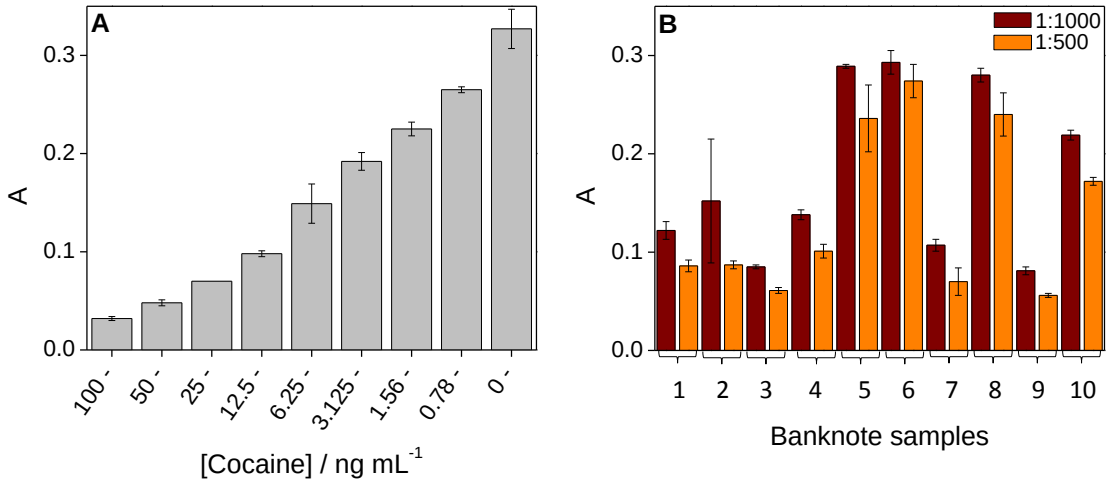

Figure 3.12. (A) The absorbance intensity at 450 nm obtained using the cEIA by a series of cocaine standard solutions, and (B) from ten extracted banknote samples. The ten banknote samples were diluted to a factor of 1:1000 and 1:500. The results from each dilution are shown in the order of lowest extract concentration to highest as indicated by the figure legend. Each absorbance value represents the mean ± SD of at least four measurements.

The absorbance values shown in **Figure 3.12A** were used to generate the standard curve. The absorbance values given in **Figure 3.12B** were then used to calculate the cocaine concentrations from this in **Figure 3.12A**, the results of which are shown in **Table 3.2**. The average difference in cocaine concentration between the two extract dilutions across all of the ten banknote samples was ± 0.810 µg mL^{-1}. The differences ranged from ± 0.037 to 1.915 µg mL^{-1}. The variation in concentration between the two sample dilutions showed that the matrix does affect the response of the assay, although the variation between the two dilutions was minimal and did not exceed the standard deviation obtained from the repeat measurements (**Table 3.2**). As described by Wild, a more dilute sample will often yield an improved immunoassay response given that the concentration of the analyte is still within the detection limits of the assay, as applying the dilution decreases the overall quantity of potential interferents present in each sample.[3] As shown in **Figure 3.12B**, an overall increase in the absorbance signal was obtained using the more dilute (1:1000) rather than the more concentrated (1:500) samples, suggesting that the 1:1000 dilution provides a more appropriate matrix for the cEIA. Based on this, the quantitative

results obtained using the 1:1000 sample dilution were applied for the parallel analysis with LC-MS hereafter.

Table 3.2. Results of the quantitative analysis of cocaine extracted from ten UK banknote samples undertaken using the cEIA. The average cocaine concentrations obtained using two different sample dilutions (1:500 and 1:1000), and the variation between them are shown.

	[Cocaine] / μg mL^{-1}		Variation
Banknote sample	1:500 dilution[a]	1:1000 dilution[a]	[cocaine] / μg mL^{-1}
1	9.416 ± 1.182	9.322 ± 1.655	0.094
2	9.232 ± 0.913	7.317 ± 4.233	1.915
3	17.592 ± 1.294	19.131 ± 1.031	1.539
4	6.884 ± 0.978	6.921 ± 0.553	0.037
5	0.742 ± 0.532	0.385 ± 0.025	0.357
6	0.292 ± 0.143	0.297 ± 0.143	0.005
7	13.800 ± 5.686	11.945 ± 1.320	1.855
8	0.656 ± 0.294	0.467 ± 0.107	0.189
9	17.999 ± 1.567	19.950 ± 1.567	1.951
10	2.048 ± 0.118	1.893 ± 0.177	0.155

[a]Each concentration represents the mean ± SD of at least three measurements.

3.2.5 Method comparison with LC-MS

The results obtained from the cEIA for the banknote extracts were compared with those obtained by a conventional LC-MS method. The ten UK banknote samples, extracted and analysed as described in the previous section (**Section 3.2.4**), were also analysed by LC-MS using the same cocaine standard as a reference. The LC-MS analysis was performed by Simon Hudson at HFL Sports Science using the method described by Hudson *et al.*.[49] **Table 3.3** shows the concentration values obtained using the cEIA and the LC-MS methods. Using a paired t-test it was

determined that the concentrations obtained using the two techniques did not differ significantly from one another at P = 0.1.

Table 3.3 Results of the quantitative analysis of cocaine extracted from ten UK banknote samples undertaken using the cEIA[a] and LC-MS methods.

Banknote sample	cEIA / μg mL^{-1}	LC-MS / μg mL^{-1}
1	9.322 ± 1.655	4.181
2	7.317 ± 4.233	10.026
3	19.131 ± 1.031	13.044
4	6.921 ± 0.553	4.699
5	0.385 ± 0.025	0.448
6	0.297 ± 0.143	0.245
7	11.945 ± 1.320	8.583
8	0.467 ± 0.107	0.529
9	19.950 ± 1.567	13.780
10	1.893 ± 0.177	2.205

[a]Each cEIA value represents the mean ± SD of at least three measurements.

A direct comparison of the two sets of quantitative data is shown in **Figure 3.13**. With the exception of two outliers, the linear relationship indicates a high degree of similarity between the detection of cocaine using the two methods. A possible explanation for the discrepancies in the data are the effect of interferents in the sample extract on the assay, particularly as some of the extracts analysed were observed as being cloudy and discoloured. However, it was also noted that overall the concentrations determined using the cEIA were slightly lower than those detected by LC-MS, the opposite of what would be expected if the assay were affected by either matrix interference or cross-reactivity.

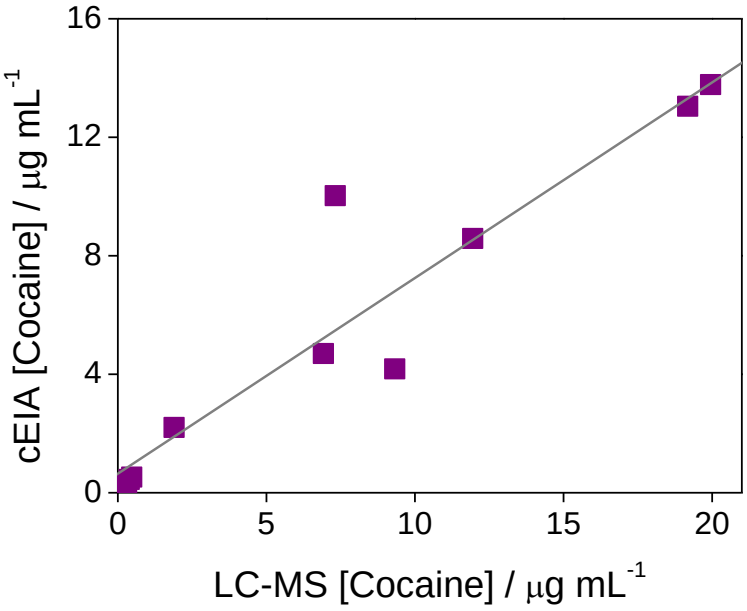

Figure 3.13. A comparison between the concentrations of cocaine extracted from ten UK banknote samples, determined by cEIA and LC-MS. Each cEIA result represents the mean ± SD of at least three measurements.

BE and other analogous compounds were also detected by the LC-MS in the extracted samples (results not shown). BE in particular was detected at concentrations similar to cocaine in a number of samples. Despite the inherent cross-reactivity of the assay for BE indicated by the results in **Section 3.2.3**, the assay was not affected by cross-reactivity when applied to the analysis of cocaine in real banknote samples due to the similarity between the results of the assay compared to those of the confirmatory LC-MS analysis. Overall, the results show that the developed cEIA therefore enables the quantitation of cocaine in complex banknote extracts to a degree of sensitivity that is comparable to the current chromatographic-mass spectrometry technique.

3.2.6 Analysis of twelve latent fingerprint samples

The two main mechanisms by which latent fingerprints can become contaminated by cocaine have been discussed in **Chapter 1**. The first is through contact transfer,

known to occur when the fingertip of an individual comes into contact with the crystalline drug powder. The crystals of cocaine stick to the sweaty skin of the fingertip for a short time, and *via* the sweat are transferred to surfaces that the skin comes into contact with. The second route for contamination of fingerprints is as a result of the individual taking the drug itself. When a drug enters the body it is transported *via* the circulation to the liver, where it is metabolised, transported to the kidneys and then excreted in the urine. During this process, metabolites can be secreted onto the skin surface from the circulation through the action of sweating.[50] Hazarika *et al.* showed that drug metabolites such as BE were present at a detectable level in the fingerprints of cocaine users.[37,38] In addition, the use of SALDI-TOF-MS enabled the detection of EDDP, a major metabolite of methadone, alongside its parent drug in the same fingerprint from an individual taking methadone.[34] As methadone is taken as an oral dose, it was concluded that the presence of the parent drug in the fingerprint arises from its secretion from the blood in the same manner as for metabolites. The presence of cocaine at a detectable level in the sweat of a fingerprint from a known cocaine user has not previously been studied.

In this study, two fingerprint samples were taken from five individuals attending a methadone treatment clinic, for a total of ten fingerprint samples. Two additional fingerprints were obtained from two separate drug-free volunteers (one print per volunteer) to serve as negative control samples. The fingerprints were extracted and analysed by cEIA as described in **Chapter 2** (**Sections 2.2.2** and **2.2.4**, respectively). The absorbance intensity produced by the extracted fingerprint samples are shown in **Figure 3.14B**, alongside the standard curve in **Figure 3.14A** (developed as outlined in **Section 3.2.3**). The signals produced by the extracted sample fell within range of the standard curve, indicating that the sensitivity of the cEIA was suitable for the analysis of this type of sample matrix. For the samples positive for cocaine, such as in P2(a) and (b), the signal was in the lower range of the calibration curve and therefore nearing the detection limit of the assay. This showed that cocaine was present at low concentrations but nonetheless detectable levels, as to be expected from low-analyte

samples of this nature. The low cocaine concentrations present in the fingerprint samples analysed during this study indicate that for detection using the cEIA in a forensic setting a suitable cut-off concentration would first need to be established.

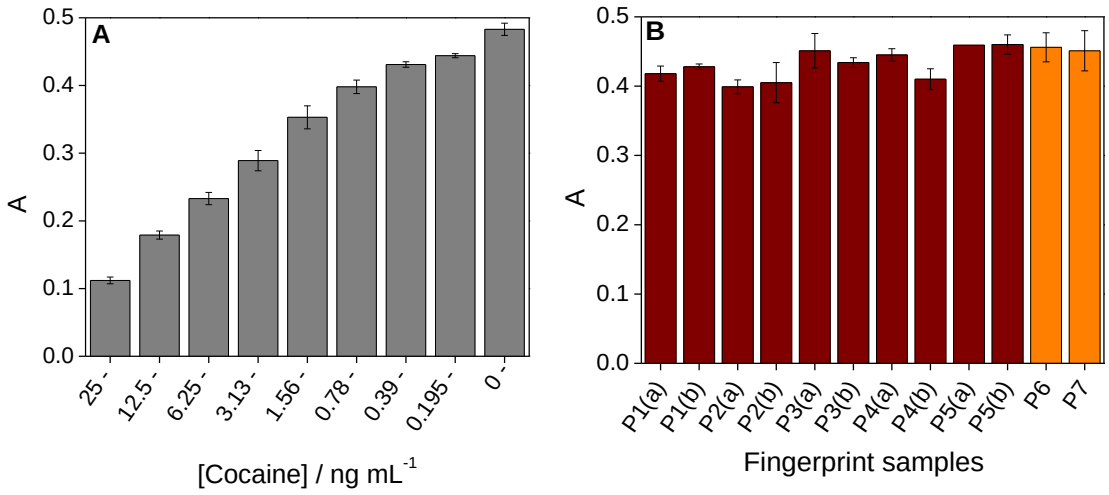

Figure 3.14. (A) The absorbance intensity at 450 nm produced using the cEIA by a series of cocaine standard solutions, and (B) from twelve extracted latent fingerprint samples. The first ten fingerprints, P1-P5, were collected from five volunteers at the methadone treatment clinic. The prints were collected two at a time as indicated by (a) and (b). P6 and P7 were single prints obtained from two separate drug-free individuals as negative controls. Each absorbance value represents the mean ± SD of at least three measurements.

3.2.7 Comparison with GC-MS analysis of corresponding oral fluid samples

As described in **Chapter 1**, studies have shown a strong correlation between cocaine concentration in oral fluid and in plasma, proving oral fluid to be an appropriate sample matrix for the detection of drug use.[51,52] Due to its rapid elimination, with a half-life of only 3 h, the detection of cocaine in oral fluid is a suitable indicator of recent drug use close to the time of sampling.[53] In this study, oral fluid samples were taken from five individuals attending a methadone treatment clinic at the same time

as providing the latent fingerprint samples used for the cocaine analysis. The fingerprint samples were extracted and analysed using the cEIA method as reported in **Section 3.2.6.** The results were compared with the results of a GC-MS analysis performed by LGC Forensics of the corresponding oral fluid samples.

The concentrations of cocaine determined in the oral fluid samples by GC-MS, and the extracted fingerprints by cEIA are shown in **Figure 3.15A** and **B**, respectively. Cocaine was detected in oral fluid samples from three of the five test subjects, with the sample from subject P2 containing the highest concentration (19 ng mL^{-1}). This corresponds to the highest concentration of cocaine determined using the cEIA in both fingerprints from subject P2 at 0.728 and 0.879 ng mL^{-1} for samples (a) and (b), respectively. The oral fluid samples from subjects P1 and P3 also tested positive for cocaine. These positive cocaine results correspond to positive fingerprint samples, although the relative concentrations are slightly different. The fingerprints from subject P4 gave cocaine results where one print was significantly higher than the other print. Since the oral fluid results from volunteer P4 were negative, it is possible that contamination by contact transfer could account for this result. The fingerprint samples P4(a), both prints from P5 and the negative control samples (P6 and P7) did not contain cocaine at detectable concentrations based on the sensitivity of the assay.

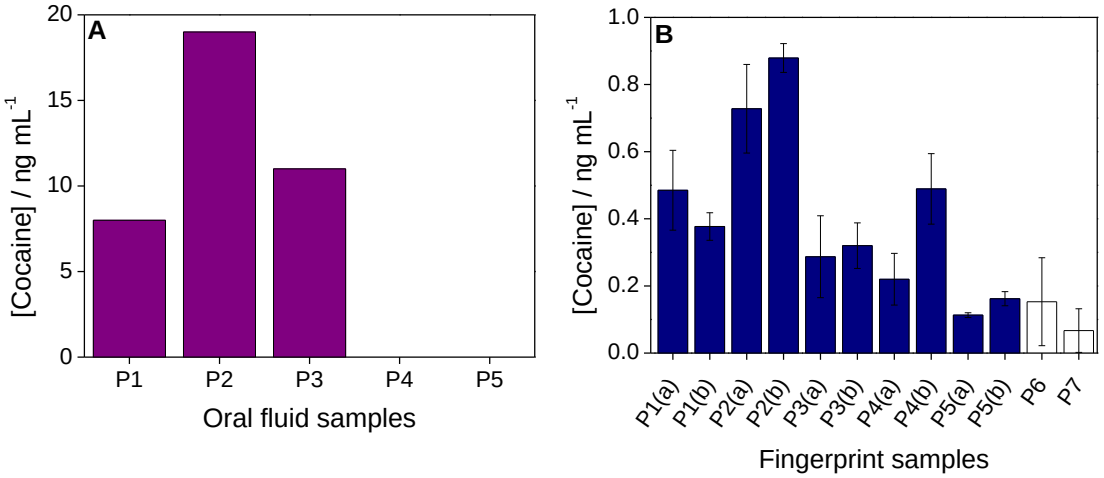

Figure 3.15. (A) Results of a quantitative analysis of cocaine by GC-MS on oral fluid samples taken from five individuals (numbered P1 – P5) from a methadone clinic, and (B) results of the analysis of cocaine extracted from twelve fingerprint samples by cEIA. The first ten fingerprints, P1-P5, correspond to the individuals from which oral fluid was also taken. P6 and P7 were single prints obtained from two separate drug-free individuals as negative controls. Each cEIA value represents the mean ± SD of at least three measurements.

As indicated in **Figure 3.11** (**Section 3.2.3**), the cEIA showed a degree of cross-reactivity for BE, particularly at low analyte concentrations. As mentioned in **Section 3.1.4**, the detection of BE has been previously reported in latent fingerprints from known cocaine users.[37,38] It is important that the potential cross-reactivity of the assay be taken into account when interpreting the results for this type of analysis.

The overall trend of the data shows that the cocaine present in the latent fingerprint samples as determined by the cEIA reflects that of the oral fluid concentrations, particularly as the concentrations detected were similar in both of the fingerprint samples from the same subject. The developed cEIA method proved to be readily applicable for the analysis of these types of samples, even at cocaine concentrations within the fingerprint extract of less than 1 ng mL^{-1}. As the presence of the drug in oral fluid is a recognised indicator of recent cocaine use, the results obtained

suggest, for the first time, that the detection of cocaine in the sweat of a latent fingerprint can be similarly linked to consumption.

3.3 Conclusions

In this chapter, a non-destructive and sensitive competitive enzyme immunoassay (cEIA) has been developed for the detection of low concentrations of cocaine in complex forensic samples such as extracts from banknotes and latent fingerprints. The assay was developed using a polyclonal anti-cocaine antibody successfully purified from serum. The precision and sensitivity achieved by the assay, with a LOD of 0.162 ng mL^{-1}, is suitable for the quantitation of cocaine in samples of forensic significance, and compares favourably to the detection limits of both a GC-MS-based method and a commercial micro-plate EIA.

The quantification of cocaine extracted from ten UK banknote samples was performed using the cEIA and validated by parallel LC-MS analysis. A statistically significant agreement was found between the results from both methods, indicating that the cEIA has the required specificity for the analysis of complex, heavily contaminated banknote extracts. Despite showing cross-reactivity for BE during specificity testing, the results of the assay were not affected by the presence of BE and other potential interferents in the samples. Trace levels of cocaine were detected on all ten banknotes sampled using the assay. Although no dataset currently exists for the concentrations of cocaine present on UK banknotes, the concentrations detected were in agreement with the background levels of cocaine detected on Irish Euro banknotes in general circulation.[54]

The cEIA was similarly applied to the quantification of cocaine extracted from latent fingerprints donated by five individuals attending a methadone treatment clinic. The results were compared to the cocaine concentrations determined by GC-MS in oral fluid samples obtained from the test subjects at the time the fingerprints were taken. The overall trend of the data showed, for the first time, that the cocaine detected at

similar concentrations in more than one latent fingerprint from the same individual could be linked with those in oral fluid, and therefore also be associated with consumption.

Overall, the developed cEIA offers a straight forward, cost-effective alternative to chromatography-MS techniques for the quantitation of cocaine in forensic samples, without the need for specialised instrumentation. The simplicity of the developed cEIA provides a method that would be readily applicable for everyday analysis in a forensic laboratory.

3.4 References

(1) Murphy, K.; Travers, P.; Walport, M. *Janeway's Immunobiology;* 7th ed.; Garland Science: New York, 2008.

(2) Voet, D.; Voet, J. G. *Biochemistry;* 4th ed.; John Wiley & Sons: Hoboken, NJ, 2011.

(3) Wild, D. *The immunoassay handbook;* 4th ed.; Elsevier Science: Oxford, 2013.

(4) Lu, B.; Smyth, M. R.; O'Kennedy, R. *Analyst* **1996**, *121*, 29R.

(5) Spector, S.; Parker, C. W. *Science* **1970**, *168*, 1347.

(6) Yalow, R. S.; Berson, S. A. *Nature* **1959**, *184*, 1648.

(7) Lequin, R. M. *Clin. Chem.* **2005**, *51*, 2415.

(8) Wu, A. H. *Clin. Chim. Acta* **2006**, *369*, 119.

(9) Voller, A.; Bartlett, A.; Bidwell, D. *J. Clin. Pathol.* **1978**, *31*, 507.

(10) Veitch, N. C. *Phytochemistry* **2004**, *65*, 249.

(11) Josephy, P. D.; Eling, T.; Mason, R. P. *J. Biol. Chem.* **1982**, *257*, 3669.

(12) Avrameas, S. *Histochem. J.* **1972**, *4*, 321.

(13) Tijssen, P.; Kurstak, E. *Anal. Biochem.* **1984**, *136*, 451.

(14) Wisdom, G. B. In *The Protein Protocols Handbook;* 3rd ed.; Walker, J. M., Ed.; Humana Press: New York, 2009.

(15) Wisdom, G. B. *Clin. Chem.* **1976**, *22*, 1243.

(16) Voller, A.; Bidwell, D.; Bartlett, A. *B. World Health Organ.* **1976**, *53*, 55.

(17) Rubenstein, K. E.; Schneider, R. S.; Ullman, E. F. *Biochem. Biophys. Res. Co.* **1972**, *47*, 846.

(18) Jickells, S.; Negrusz, A. *Clarke's Analytical Forensic Toxicology;* Pharmaceutical Press: London, 2008.

(19) Engvall, E.; Perlmann, P. *Immunochemistry* **1971**, *8*, 871.

(20) Voller, A.; Bidwell, D.; Huldt, G.; Engvall, E. *B. World Health Organ.* **1974**, *51*, 209.

(21) Carney, S.; Wolf, C. E.; Tarnai-Moak, L.; Poklis, A. *J. Clin. Lab. Anal.* **2012**, *26*, 130.

(22) Van Weeman, B.; Schuurs, A. *FEBS Letter* **1971**, *15*, 232.

(23) Langman, L. J.; Kapur, B. M. *Clin. Biochem.* **2006**, *39*, 498.

(24) Allen, L. V.; Lou Stiles, M. *Clin. Toxicol.* **1981**, *18*, 1043.

(25) Kerrigan, S.; Phillips Jr, W. H. *Clin. Chem.* **2001**, *47*, 540.

(26) Moore, K. A.; Werner, C.; Zannelli, R. M.; Levine, B.; Smith, M. L. *Forensic Sci. Int.* **1999**, *106*, 93.

(27) Mali, N.; Karpe, M.; Kadam, V. *J. Appl. Pharm. Sci.* **2011**, *1*, 58.

(28) Moody, D. E. In *Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation, Volume 7;* Meyers, R. A., Wiley: New York, **2000**.

(29) Niedbala, R. S.; Kardos, K.; Fries, T.; Cannon, A.; Davis, A. *J. Anal. Toxicol.* **2001**, *25*, 62.

(30) Spiehler, V.; Fay, J.; Fogerson, R.; Schoendorfer, D.; Niedbala, R. S. *Clin. Chem.* **1996**, *42*, 34.

(31) Marin, S. J.; Merrell, M.; McMillin, G. A. *J. Anal. Toxicol.* **2011**, *35*, 40.

(32) Musshoff, F.; Kirschbaum, K.; Graumann, K.; Herzfeld, C.; Sachs, H.; Madea, B. *Forensic Sci. Int.* **2012**, *215*, 60.

(33) Sleeman, R.; Burton, F.; Carter, J.; Roberts, D.; Hulmston, P. *Anal. Chem.* **2000**, *72*, 397A.

(34) Rowell, F.; Hudson, K.; Seviour, J. *Analyst* **2009**, *134*, 701.

(35) Frederick, K. A.; Pertaub, R.; Shi Kam, N. W. *Spectrosc. Lett.* **2004**, *37*, 301.

(36) Hazarika, P.; Russell, D. A. *Angew. Chem., Int. Edit.* **2012**, *51*, 3524.

(37) Hazarika, P.; Jickells, S. M.; Wolff, K.; Russell, D. A. *Angew. Chem., Int. Edit.* **2008**, *47*, 10167.

(38) Hazarika, P.; Jickells, S. M.; Wolff, K.; Russell, D. A. *Anal. Chem.* **2010**, *82*, 9150.

(39) Shrivastava, P.; McLean, C. J.; Aberl, F.; Bonenberger, J.; Berg, R.-P.; Zimmermann, R. In *Enabling Technologies for Law Enforcement and Security;* International Society for Optics and Photonics: 1997, 183.

(40) Esser, P. *Thermo Scientific Nunc Bulletin* **1988**, 1.

(41) Cantarero, L.; Butler, J.; Osborne, J. *Anal Biochem* **1980**, *105*, 375.

(42) Esser, P. *Thermo Scientific Nunc Bulletin No. 8* **1990**, 1.

(43) Kidwell, D. A.; Athanaselis, S. A. In *Handbook of forensic drug analysis;* Smith, F. P., Ed.; Elsevier Academic Press: San Diego, 2005, 235

(44) Jenkins, A. J. *Forensic Sci Int.* **2001**, *121*, 189.

(45) Ebejer, K. A.; Winn, J.; Carter, J. F.; Sleeman, R.; Parker, J.; Körber, F. *Forensic Sci. Int.* **2007**, *167*, 94.

(46) Carter, J. F.; Sleeman, R.; Parry, J. *Forensic Sci. Int.* **2003**, *132*, 106.

(47) Dixon, S. J.; Brereton, R. G.; Carter, J. F.; Sleeman, R. *Anal. Chim. Acta* **2006**, *559*, 54.

(48) Esteve-Turrillas, F. A.; Armenta, S.; Moros, J.; Garrigues, S.; Pastor, A.; de la Guardia, M. *J. Chromatogr. A* **2005**, *1065*, 321.

(49) Hudson, S. M., S.; Timbers, S. In *Proceedings of the 18th International Conference of Racing Analysts and Veterinarians, New Zealand;* Dunmore Publishing Ltd: Auckland, 2010, 133.

(50) Kidwell, D. A.; Holland, J. C.; Athanaselis, S. *J. Chromatogr. B* **1998**, *713*, 111.

(51) Vindenes, V.; Lund, H.; Andresen, W.; Gjerde, H.; Ikdahl, S.; Christophersen, A.; Øiestad, E. *Forensic Sci. Int.* **2012**, *219*, 165.

(52) Bosker, W. M.; Huestis, M. A. *Clin. Chem.* **2009**, *55*, 1910.

(53) Scheidweiler, K. B.; Spargo, E. A. K.; Kelly, T. L.; Cone, E. J.; Barnes, A. J.; Huestis, M. A. *Ther. Drug Monit.* **2010**, *32*, 628.

(54) Bones, J.; Macka, M.; Paull, B. *Analyst* **2007**, *132*, 208.

CHAPTER 4

Location-specific immunodetection of cocaine on banknotes

This chapter describes the development of an immunodetection method for the immobilisation and subsequent localised staining of cocaine on banknotes using an enzyme-labelled anti-cocaine antibody and an acrylamide gel matrix.

4.1 Introduction

4.1.1 Methods of immunodetection in biology

Immunohistochemistry (IHC) was the first technique developed for the immunostaining of antigen in a solid, semi-permeable matrix, commonly a cellular component within frozen or embedded tissue.[1] The principle began with a publication by Coons *et al.* in 1941 reporting the application of a fluorescently-labelled antibody for the detection of cellular antigen in frozen tissue sections.[2,3] Since then, the technique has evolved to overcome initial limitations in sensitivity by the introduction of steps to prevent non-specific staining, as well as by the incorporation of different detection systems.[1,3,4] The successful formation of HRP-labelled antibody by Avrameas *et al.* was particularly important in the development of modern methods of IHC, as it enabled the colorimetric localisation of antigen which can be directly observed *via* light microscopy.[5,6]

Three main steps are involved in the preparation of non-frozen tissue samples for IHC staining. In the first of these, preservation of the tissue matrix is commonly achieved by treatment with a fixative such as formaldehyde.[1] Treatment with formaldehyde or a similar cross-linking agent generates the formation of irreversible, covalent cross-links by the establishment of methylene bridges between the amide or

amine end-groups of proteins in the tissue section.[7,8] The cross-links act to maintain the structural and antigenic integrity of the tissue, and thereby limit extraction, diffusion or displacement of antigen during subsequent processing.[8] During the fixation process, previously soluble proteins become anchored to structural proteins in the tissue and are rendered insoluble; other cellular components such as nucleic acids, lipids and carbohydrates are not fixed directly but become entrapped within the network of insoluble cross-links.[7] A gel-like proteinaceous network is generated within the cells of the tissue section, forming a semi-permeable solid phase in which location-specific immunostaining can be performed.[1] Although the overall purpose of fixation is to render the cells in the tissue sample into a condition of stasis that is as close to *in vivo* as possible, it can also improve antibody penetration into the tissue section.[9] The gel-like, porous nature of the network formed by protein cross-links facilitates the entry of antibodies, and the degree of this porosity can be controlled *via* the use of different types of fixatives.[9]

After fixation, the preserved tissue is dehydrated, embedded, thin-sectioned and treated with a blocking agent to prevent endogenous staining prior to immunodetection.[10] Addition of the primary antibody targeted to the cellular component of interest is facilitated by a simple incubation step in a solution of diluted antibody or anti-serum.[4] During this incubation the antibody diffuses into the tissue section and binds to the antigenic material which has been rendered immobile *via* the process of fixation.[8] As the antibody molecule cannot be seen using a light microscope, a labelling mechanism is required to enable visualisation of the bound antibody and thereby the localisation of the target antigen.[1] A variety of labels ranging from fluorescent compounds to active enzymes have been used for this purpose.[11] When using active enzymes as a label, it is crucial that the enzyme substrate remains situated at the source of the antigen rather than diffusing across the specimen. For this reason, the use of specialised enzyme substrates which yield a product in the form of a coloured precipitate is common.[11]

Horseradish peroxidase (HRP) is one of the most popular enzyme labels for IHC, particularly as its use enables visualisation *via* a wide range of different colorimetric substrates.[4,11] HRP can be readily coupled either directly onto the primary antibody, or applied indirectly by means of a labelled secondary antibody as described in Chapter 3.1.2 for use in ELISA-style immunoassays. Although signal amplification is inherently achieved by the activity of the enzyme, the sensitivity of an HRP label can be further enhanced by the application of more than one enzyme per antibody, using techniques such as enzyme bridging or the peroxidase antiperoxidase (PAP) method.[1] Substrates such as tetramethyl benzidine (TMB) or diaminobenzidine (DAB), which yield a blue or brown precipitate in the presence of the enzyme respectively, are commonly used.[4]

IHC of cellular antigens performed on cultured cells, also known as immunocytochemistry (ICC), is based on the same methodology as that used for the immunostaining of tissue specimens.[1] Only the medium in which the staining occurs differs between the two techniques. In 2D and 3D cell culture, cells are grown either as a monolayer or as a 3D network within a cross-linked, gel-like matrix designed to mimic interstitial tissue structures as closely as possible.[12] Polymer networks capable of absorbing many times their weight in water, known as hydrogels, are commonly used as matrices for this type of cell culture.[13] The swelling characteristic of hydrogels renders them semi-permeable, in that free water molecules can move within the matrix.[13] This in turn allows cells to be mobile within the matrix in a manner that more closely resembles their behaviour *in vivo* than if the cells are grown as a monolayer.[12,14] In addition to being biocompatible, hydrogels are inexpensive, non-toxic and can densify over several orders of magnitude with only modest increases in concentration.[15] Both natural and synthetic hydrogels have been successfully applied for cell culture applications.[12]

Preparation of 2D or 3D cell cultures for immunostaining is performed in a similar manner as for the immunostaining of tissue specimens. The cytological specimen is first treated with a fixative such as PFA, followed by the application of a blocking

agent and subsequent immunostaining in the same manner as for the IHC detection in tissue specimens.[16,17] The cells remain within the culture matrix throughout this process, and particularly for natural hydrogels this can lead to the cross-linking of protein or polymers in both the intra- and extracellular environment.[18] However, as shown by the successful immunostaining of cellular antigen reported by Ma *et al.* and O'Conner *et al.* within natural hydrogel matrices fixed with PFA, the degree of polymerisation of the hydrogel matrix does not prevent antibodies from permeating the gel.[16,17]

In addition to 3D cell culture matrices, unfixed hydrogels are also known to be permeable to antibodies. Prior to the advent of enzyme immunoassays, gels such as agar-agar, agarose, gelatin and polyacrylamide were used to visualise antibody-antigen interaction.[19,20] Known as gel diffusion analysis, the technique was first described by Oudin in 1946 who reported that the addition of an antiserum to an antigen-containing agar gel resulted in the formation of discrete lines of precipitate.[21] The method was furthered by Ouchterlony who developed a two-dimensional or 'double-diffusion' version by showing that antigen and antibody in adjacent wells within a thin agar gel would diffuse towards one another in the medium, resulting in thin lines of immune precipitate where the reactants meet.[22] Although the method of Ouchterlony double immunodiffusion was initially developed using agar, this gel medium has a strong negative charge and has therefore more recently been replaced by agarose gels, which possess little charge and therefore minimise gel-reactant interaction.[23] The technique illustrates the permeability of the aforementioned gel matrices to antibodies, and indicates their suitability as a matrix for immunodetection.

4.1.2 Types of hydrogels used in this study

As described in Section 4.1.1, agarose gels are commonly used for the analysis of antibody-antigen interaction by Ouchterlony immunodiffusion and have therefore been shown to be permeable to antibodies. Agarose is a galactopyranose-based

linear polysaccharide purified from agar.[24] Gelation of a homogeneous agarose solution occurs according to a coil-helix transition that takes place when the gel is cooled from 99 °C to a temperature below the ordering temperature, which is typically around 35 °C for normal agarose.[15] The gel is formed when an infinite three-dimensional network of bundled helical fibres of agarose connected by flexible chains develops, the process of which is shown in **Figure 4.1**.[25,26] The pore size between these fibres varies from 100 – 300 nm and is inversely proportional to the concentration of agarose used to make the gel.[27,28] Agarose gels have been used as a matrix for 3D cell culture and subjected to immunostaining as described for IHC specimens in **Section 4.1.1**. Wester *et al.*, for example, transferred fibroblasts originally cultured in a monolayer into a matrix of 6 % w/v agarose prior to IHC processing.[29] The resulting 'cell-gels' were successfully applied as control samples for the staining of tissue sections, showing homogeneous immunostaining of cells even in slices of different thicknesses.[29]

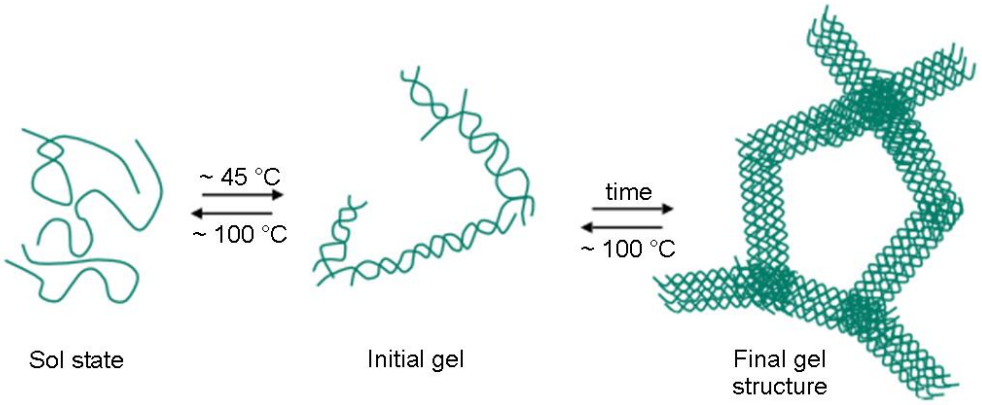

Figure 4.1. The gel structure of agarose.[26]

Collagen and gelatin are naturally derived hydrogels which, like agarose, are popular as matrices for 2D and 3D cell culture.[30] Collagen is a structural protein of a helical conformation made up of three polypeptide strands. These helices in turn aggregate to form elongated fibrils, the arrangement of which gives the collagen its unique

strengthening properties.[31] Collagen is naturally present in abundance in almost all connective tissues in mammals including tendons, ligaments and skin. Insoluble collagen fibrils make up the greater part of the extracellular matrix (ECM), a proteinaceous network designed to arrange and provide mechanical support to cells, thereby maintaining tissue strength and structure.[16,32] Due to their mechanical strength, ease of gelation and abundance in mammalian tissue, collagen hydrogels are now extensively used as scaffolds for the encapsulation of cells in culture.[15,17] The gelation of collagen occurs at physiological pH and temperature, and begins with the entropy-driven aggregation of triple-helical collagen monomers.[31] The resulting small aggregates self-assemble into thin filaments, which in turn cross-link into larger collagen fibres.[32] Non-covalent entanglement of these fibres yields the irregular three-dimensional matrix which makes up the gel, as illustrated in **Figure 4.2**.[15] Collagen gels exhibit large pore sizes, between 5 and 10 μM, the size of which can be fine-tuned *via* modest changes in temperature, pH, ionic strength and collagen concentration.[30] The inherent porosity of collagen gels readily facilitates the staining of antigenic material in 3D cell cultures by way of immunodetection. Indeed, the use of ICC as a method of detection is well-described for cells embedded in collagen gel matrices,[16,17,33] as well as for mixed gel matrices made up of collagen in combination with another hydrogel such as agarose.[15,34]

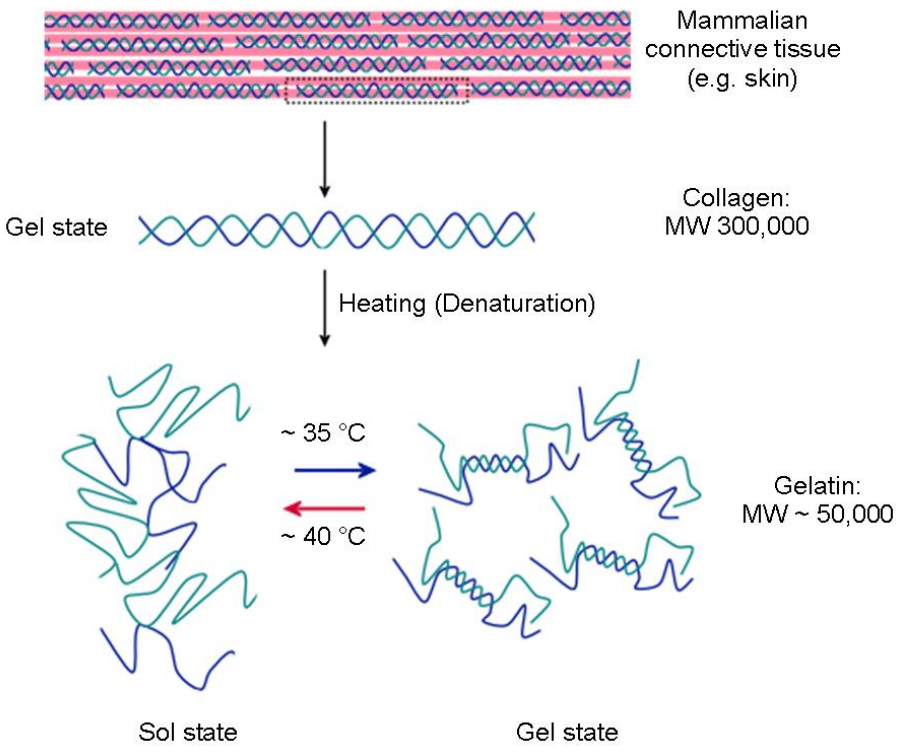

Figure 4.2. The gel structures of collagen and gelatin.[35,36]

The irreversible hydrolysis of collagen, *via* thermal denaturation or chemical degradation, breaks down its triple helical structure to give a heterogeneous mixture of single or multi-stranded polypeptides known as gelatin.[37] An aqueous solution of gelatin will enter the sol state at around 40 °C and forms a thermoreversible gel upon cooling.[38] During gelation the peptide strands in gelatin undergo coil-helix transition followed by aggregation of the helices, leading to the reformation of a collagen-like triple-helical structure.[37] The formation and gelation of gelatin is shown alongside that of collagen in **Figure 4.2**. Like collagen, gelatin hydrogels are of biological origin and therefore popular for use in cell culture matrices, often in combination with other strengthening materials such as chitosan and PEG.[39,40] Its application as a cell culture matrix followed by ICC staining has also been reported.[41] In addition, gelatin hydrogels and films are widely used in the biomedical, pharmaceutical, and food

industries for applications ranging from tissue engineering, through to the formation of drug capsules and as a sealant for preserving food.[38,42,43]

Casein hydrogels are, like gelatin, a mainstay of the food industry. They are commonly used as sealants for the preservation of moisture or gas-sensitive food products such as fresh produce.[44,45] Caseins are phosphoproteins principally found in cow's milk, where they are present in clustered formations, known as micelles, for the binding of calcium and phosphorous.[46] Caseins are readily purified from milk *via* a number of different methods, the most common of which involves acid precipitation which produces caseins as an insoluble solid.[47] The gelation of casein in an aqueous solution occurs *via* the formation of covalent protein cross-linkages, which yields a network of linear and globular aggregates.[46] Polymerisation of caseins can be induced by increases in temperature or pH, or by the introduction of an enzyme such as transglutaminase.[44,47,48] This well-described gelation process has traditionally been harnessed for the production of dairy products such as cheese and yoghurt, as well as for application in casein-based paints and natural adhesives.[47] More recently, the various advantages of casein hydrogels, including biocompatibility and non-toxicity, has led to increased interest in their use for biomedical applications such as drug delivery vehicles and soft tissue adhesives.[49]

Acrylamide gels are synthetic hydrogels which are widely used in biological research for the size-dependent separation of macromolecules, including whole proteins, protein fragments or nucleic acids, by gel electrophoresis.[50,51] Formation of the gel occurs *via* the polymerisation of acrylamide into long chains, which in turn are covalently cross-linked by a bifunctional compound such as bis-acrylamide to form a porous meshwork the structure of which is shown in **Figure 4.3**.[52] Polymerisation is initiated by the addition of ammonium persulfate (APS), from which tetramethylethylenediamine (TEMED) catalyses the formation of free radicals to facilitate and accelerate polymerisation.[52] The rate of polymerisation therefore depends on the concentrations of APS and TEMED used, as well as temperature, pH and the absence of oxygen, which acts as an inhibitor.[52] The strength and

permeability of the gel can be controlled by adjusting the total acrylamide concentration and weight percentage of the bis-acrylamide cross-linker, each of which is inversely proportional to the effective pore size of the gel.[51,53]

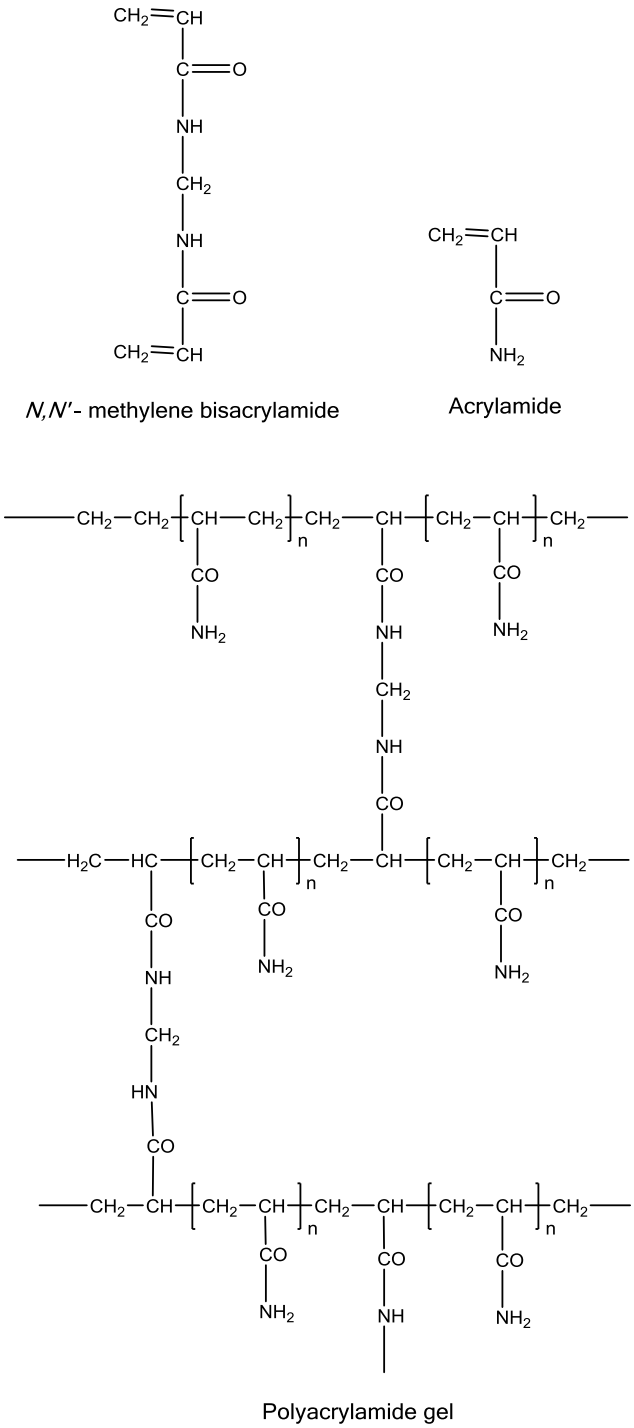

Figure 4.3. The structure of acrylamide, bis-acrylamide and polyacrylamide gel.[52]

The immunodetection of antigenic proteins in acrylamide gels is most commonly performed *via* indirect methods such as western blotting, where proteins within the gel are first transferred to a membrane prior to immunostaining. The direct immunoprobing of material within the gel has historically been avoided due to the common belief that acrylamide gels are impermeable to large proteins such as antibodies.[54] However, the limitations of western blotting led to the development of a method for the in-gel immunodetection of antigenic material, first reported by Burridge and Olden and Yamada following separation by SDS polyacrylamide gel electrophoresis.[50,55] The technique involves fixation of the protein fragments within the gel and simultaneously removing the SDS *via* an incubation step in trichloroacetic acid or isopropanol with acetic acid.[50] It is thought that the action of the fixative may also act to improve the permeability of the gel to antibodies.[50,54] Immunodetection is then facilitated by the direct incubation of the gel containing the antigenic material in solutions of primary and then HRP-labelled secondary antibody.[50] Since its inception, in-gel immunodetection has been adapted to include different visualisation techniques such as radioactive and fluorescent labelling, and has been applied for the detection of several different cellular antigens.[56,57] Due to the time involved in performing the analysis, several days in total, western blotting has remained the more widely used method for immunodetection in acrylamide gels. However, a recent improvement of the in-gel immunodetection method by Desai *et al.*, involving a short incubation step in 50 % isopropanol in water in place of the previously complicated fixation process, now enables results to be obtained within hours rather than days, significantly improving its practicality.[54]

4.1.3 Aim of the research presented in this chapter

The presence of background levels of cocaine on paper currency in general circulation is known. As described in Chapters 1 and 3.1.4, it is of forensic relevance to establish the concentrations of cocaine present on individual banknotes, as this may enable banknotes to be linked to direct avenues of contamination through criminal activities. At present, the analysis of cocaine on banknotes performed by law

enforcement agencies in the UK is focussed on the use of ion-mobility spectrometry (IMS) as a means of quantification. The method involves taking a representative sample of cocaine along two strips on the side of the sample note by vacuum sampling as detailed in **Chapter 1**.[58] Recently, concerns have been raised that the area sampled may not provide an adequate representation of the cocaine concentration present on the note in its entirety. For example, the primary location of cocaine on banknotes could be around the edges of the note rather than spread uniformly across it, in which case the cocaine present would be greater than indicated using the present method of sampling. An analytical method which enables the areas on banknotes where cocaine contamination most frequently occurs is therefore necessary to affirm the authenticity of the results obtained using the current means of analysis. It is of interest that the analytical method is developed to enable both the presence and location of the cocaine on banknotes to be determined.

As described in **Section 4.1.1**, biological methods of immunodetection such as IHC and ICC enable the location-specific detection of cellular antigens in specimens such as fixed tissue sections. Immunodetection of this nature is useful for biologists as it enables the context of the antigenic material to be established in relation to other structural components in the sample. The specific and sensitive nature of immunological detection methods facilitated by the antigen recognition ability of antibodies makes them highly suited for the detection of molecules at low concentrations in complex sample matrices, as discussed in **Chapter 3.1.1**. The successful quantitative immunodetection of cocaine extracted from banknotes using a plate-based enzyme immunoassay was reported in **Chapter 3**. As discussed in **Chapter 3.3**, this study proved the efficacy of an antibody-based method for the detection of cocaine at the low concentrations present on banknotes, and in the presence of possible interferents present in the sample matrix.

The present chapter describes the development of a novel immunostaining method for the location-specific detection of cocaine on the surface of banknotes, based on the use of techniques previously applied to biological samples for the visualisation of

cellular antigens. The application of IHC methods for the detection of antigenic material has been previously described by Miller *et al.*, who reported the successful immunostaining of brain cells on cotton fabric using IHC-based methodology.[59] The stability of cocaine in a proteinaceous tissue network formed by fixation using formaldehyde has also been studied by Viel *et al.* and Cingolani *et al.*, who separately report the successful extraction of cocaine and benzoylecgonine from liver specimens up to seven days after fixation, indicating the suitability of a cross-linked hydrogel matrix for cocaine detection.[60,61] In this study, cocaine was affixed onto the surface of the banknote *via* the application of a hydrogel matrix in a similar manner as for the ICC-based detection of antigenic materials within a 3D cell culture matrix. Different hydrogels of varying properties, as detailed in **Section 4.1**, were compared in terms of strength, stability and permeability to antibody to determine the most effective type of matrix for this purpose.

Immunostaining of the immobilised cocaine on the banknote surface was subsequently performed using an anti-cocaine primary antibody, either pre-labelled with HRP or in conjunction with an HRP-labelled secondary antibody. Visualisation was enabled by the application of a chemiluminescent substrate, producing a luminescent signal on the areas of the banknote in which cocaine was present. As detailed in **Chapter 1**, UK banknotes are printed on paper consisting of a mixture of cotton and linen, with cotton being the major constituent. In the first instance, paper made from 100 % cotton was therefore used as a representation of a banknote. Cotton paper without cocaine added, and cotton paper spiked with a cocaine standard were used as negative and positive representative samples respectively throughout the study.

The developed method was applied for the detection of cocaine firstly on partial banknote samples spiked with cocaine standard. Whole banknote samples obtained from general circulation were then analysed alongside whole banknote samples on which half of the note had been spiked with ‘street’ cocaine. Newly minted banknotes with or without cocaine were used as positive and negative controls, respectively.

The results obtained, for the first time, demonstrate the successful location-specific immunostaining of cocaine on banknotes within an acrylamide hydrogel matrix.

4.2 Results and discussion

4.2.2 A comparison of gel matrices for cocaine immobilisation

Hydrogel matrices were investigated in this study as a potential means of affixing cocaine onto the surface of banknotes. The application of a matrix was required to prevent the movement or diffusion of cocaine during subsequent immunostaining. The properties of hydrogels, including their mechanical strength, biocompatibility, large pore size and high level of transparency make them highly suited for immunostaining-based applications. This is illustrated by their frequent use as 2D and 3D cell culture matrices for ICC staining. The properties of the different types of gel matrices of biological origin used in this study, namely agarose, casein, collagen, and gelatin, are described in detail in Section 4.1.2. Hydrogels made from natural polymers were primarily selected over synthetic polymers for this study as they are readily available, cost-effective, non-toxic, biodegradable and easily manipulated. Synthesis of the different gel types was performed as described in Chapter 2.3.1 (Table 2.3).

The gels were compared in terms of mechanical strength and adhesive properties based on the observed level of binding to cotton paper as a representation of a banknote. The strength of the gel was based on consistency; high was defined as a solid gel which retained its shape when light pressure was applied, medium as a semi-solid gel which yielded to pressure and low as a gel which was too weak to maintain its structure. The adhesion of the gel was similarly based on the level of adherence to the cotton paper; high was well attached even after incubation in buffer, medium was initially attached but adherence did not last for the duration of the buffer incubation, and low was not attached. The permeability of the gels to antibody was then determined using a gel column as described in Chapter 2.3.1.

The results of the hydrogel comparison are summarised in **Table 4.1** in terms of the mechanical strength, level of adhesion and permeability of each gel type. As shown, the mechanical strength of both the collagen and casein gels was insufficient to properly adhere to the cotton paper, thereby rendering them ineffective as a matrix for immunostaining. Agarose and gelatin conversely provided the greatest degree of mechanical strength and proved sufficiently able to adhere to the paper. However, both the agarose and the gelatin hydrogels proved impermeable to antibodies when prepared using the concentrations of polymer required for a stable barrier around the paper. For this reason, synthetic polymers were selected in favour of biologically derived hydrogels as a possible matrix for the immobilisation of cocaine.

Table 4.1. A summary of the different types of hydrogel matrices compared in this study; the results are summarised in terms of mechanical strength, adhesive properties and permeability to antibodies.

Medium	Content	Strength	Adhesion	Permeability
Gelatin	3, 5 and 7 % w/v	Medium	High	Impermeable
Cross-linked Gelatin	7 % w/v	High	High	Impermeable
Casein	7 % w/v	Low	Low	Permeable
Collagen	1.0 mg mL^{-1}	Low	Low	Permeable
Cross-linked Collagen	1.8 mg mL^{-1}	Medium	Low	Permeable
Agarose and LMP Agarose	0.2 % w/v	Medium	Low	Permeable
LMP† Agarose	0.5 and 1.0 % w/v	High	Medium	Impermeable

*PFA = paraformaldehyde and †LMP = low melting point.

4.2.3 In-gel immunodetection method development for polyacrylamide gels using rabbit anti-cocaine antibody

Acrylamide gels, commonly used for the separation of biomolecules by gel electrophoresis, were chosen as a potential alternative to a natural hydrogel as a matrix for the immobilisation of cocaine on banknotes. The properties of polyacrylamide gels and their previous application for in-gel immunostaining is

described in **Section 4.1.2**. As the mechanical strength of covalently cross-linked acrylamide gels is known, the permeability of the gels to antibody after treatment with 50 % isopropanol in water was first investigated. The immunostaining of acrylamide gels was performed using the method of Desai *et al.* following separation by non-denaturing PAGE as described in **Chapter 2.3.2**.[54] The gels were run with a sample of cocaine-BSA hapten applied as the antigenic target, a BSA sample as the negative control and a sample of anti-cocaine primary antibody as a positive control of secondary antibody binding. Immunostaining was performed as detailed in **Chapter 2.3.2** (**Table 2.5**). The 3,3'-diaminobenzidine (DAB) enzyme substrate that was used forms a visible red-brown precipitate within a gel when HRP is present. The results of the in-gel immunodetection are shown in **Figure 4.5**. The results obtained from the initial immunostaining of 10 % w/v PAGE gels, prepared as described in **Chapter 2.3.2** (**Table 2.4**, experiments 1 and 2), are shown in **Figure 4.5B** and **C**. A gel containing the equivalent set of standards and samples was also separated by non-denaturing PAGE but stained for all protein using Coomassie brilliant blue R250 rather than immunostaining; the results are shown in **Figure 4.5A.**

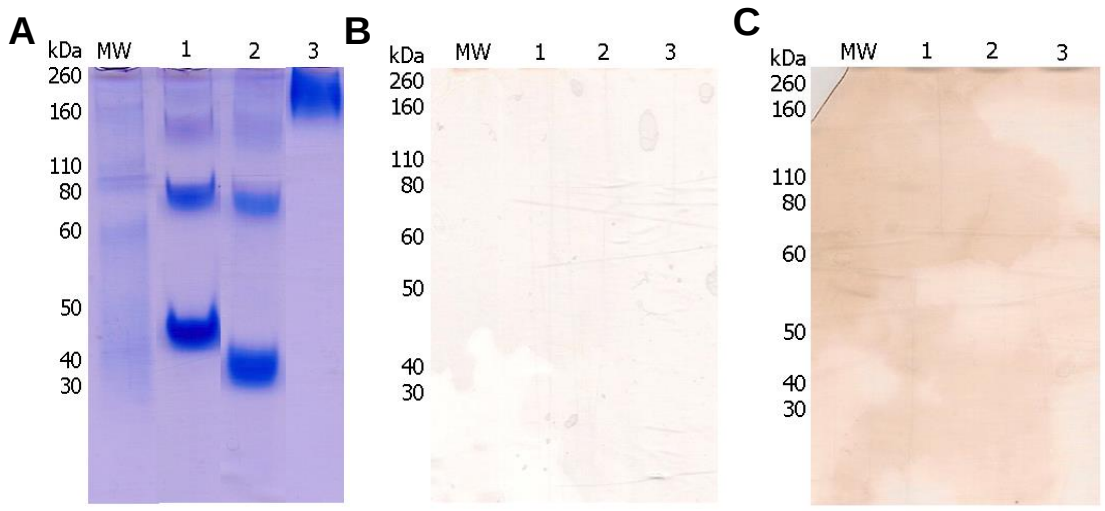

Figure 4.5. The in-gel immunodetection of cocaine-BSA in a 0.75 mm thick, 10 % w/v acrylamide PAGE gel stained using DAB as an enzyme substrate. The total protein in the gel, stained by Coomassie brilliant blue R250, is shown in (A). The corresponding immunostained gels treated with a 5 % and a 1 % w/v BSA antibody diluent buffer are shown in (B) and (C), respectively. After the molecular weight marker (MW), the samples in each gel are labelled as follows; (1) BSA, (2) cocaine-BSA and (3) anti-cocaine primary antibody. All samples were pre-diluted to 1 mg mL^{-1} in PB prior to analysis.

Overall, the results in **Figure 4.5** show that the immunodetection procedure had not been successful when applied to 10 % w/v acrylamide PAGE gels. Anti-cocaine primary antibody, applied to lane 3 of the gels shown in **Figure 4.5**, was included in the analysis as a positive control which should always be stained after application of the HRP-labelled secondary antibody. It was therefore expected that brown-red staining would be present in lane 3 of the immunostained gels (**Figure 4.5B** and **C**) at the same position as the protein-stained band in lane 3 of **Figure 4.5A.** As this was not the case, the results suggest that the secondary antibody was not penetrating the gels to facilitate staining; this may have been caused by too high a concentration of BSA in the blocking buffer, or by a too high concentration of acrylamide used to prepare the gels. In order to determine if the blocking buffer was preventing specific binding of the secondary antibody, blocking buffer containing two concentrations of BSA (5 % or 1 % w/v) were compared on identical 10 % w/v acrylamide gels. The

results are shown in **Figure 4.5B** and **C** for 5 % or 1 % w/v BSA blocking buffer, respectively. As shown in **Figure 4.5C**, no specific staining was observed as a result of the reduced BSA blocking buffer, indicating that it was likely the concentration of acrylamide in the gel was preventing immunodetection rather than the blocking buffer.

As detailed in **Section 4.1.2**, the concentration of acrylamide in a gel is inversely proportional to its effective pore size.[51] The effect of decreasing the concentration of polyacrylamide in the gels as a means of enabling specific immunostaining was therefore investigated based on the results described in the previous paragraph. Gels with acrylamide concentrations of 7.5 % w/v and 5 % w/v were prepared as described in **Chapter 2.3.2** (**Table 2.4**), and immunostained using the conditions defined in **Table 2.5** (experiments 4 and 5). A sample containing HRP-labelled secondary antibody was run as a substrate control together with the other control samples included in the 10 % w/v gel. A molecular weight marker was not included, as the location of the bands was determined based on their position in the identical but protein stained gel rather than respective to the molecular weight standards.

As shown in lane 4 of **Figure 4.6**, staining in the lane containing the secondary antibody substrate control showed that the substrate was generating a good level of staining. Otherwise, no specific staining was observed in either of the gels with lower acrylamide concentrations including in the lane containing the primary antibody, where it was expected that staining would occur as a result of binding of the HRP labelled antibody. Based on the results obtained, it was thought that the DAB enzyme substrate used may not provide the level of sensitivity required to enable visualisation of the cocaine at the low concentrations likely to be present on banknotes. It was also noted that the 5 % w/v gel was prone to frequent tearing, and at times proved too fragile to remain wholly intact throughout the immunostaining procedure (results not shown).

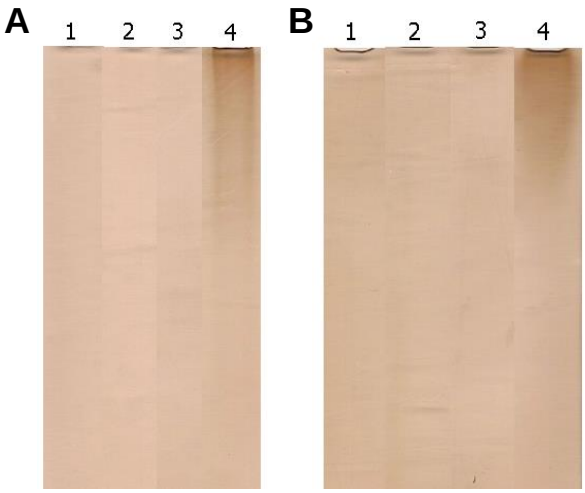

Figure 4.6. The in-gel immunodetection of cocaine-BSA in 0.75 mm thick, 5 % w/v and 7.5 % w/v acrylamide PAGE gels stained using DAB as an enzyme substrate are shown in (A) and (B), respectively. The samples in each gel are labelled as follows; (1) BSA, (2) cocaine-BSA, (3) anti-cocaine primary antibody and (4) HRP-labelled secondary antibody. All samples were pre-diluted to 1 mg mL^{-1} in PB prior to analysis.

Enhanced chemiluminescent (ECL) substrates are commonly applied for the detection of antigenic material in immunoblotting procedures, such as western blots, as an alternative to colorimetric staining.[62] Chemiluminescence can be simply defined as a term describing chemical reactions which emit light.[63] Reactions of this type require compounds which exhibit chemiluminescence, such as luminol and its derivatives.[63] The oxidation of luminol in solution produces a dianion intermediate, the decomposition of which leads to the production of an excited state and the emission of light in the region of 425 nm.[63] This reaction can be catalysed by HRP in the presence of hydrogen peroxide, the rate of which in turn can be improved up to 1000-fold by the addition of an enhancer such as *p*-iodophenol (PIP).[64] ECL reactions of this type provide more prolonged, intense and stable light emission, and are therefore preferentially used for the immunostaining of biomolecules present at low concentrations.[64] An ECL substrate was therefore applied instead of DAB for in-gel immunodetection in the next set of experiments, to determine whether the increased level of sensitivity offered by this type of HRP substrate would facilitate

visualisation of cocaine-BSA within the gels. The presence of the luminescent staining of the gel was facilitated using a cooled-CCD camera as part of the Fujifilm LAS-3000 imaging system, which converts photons into digital signals. The resulting gel image shows a negative version of the stained gel, in which the luminescent signal produced by the presence of HRP is converted into 'staining' of a grey or black appearance on a white background.

Electrophoresis of a 7.5 % w/v polyacrylamide gel was performed with the same set of samples analysed by 5 % w/v and 7.5 % w/v gels. Immunostaining of the 5 % w/v and 7.5 % w/v gels was performed as described in **Table 2.5** of **Chapter 2.3.2** (experiments 7 and 8). The results of the analysis are shown in **Figure 4.7**. The gel shown in **Figure 4.7A** was stained for protein using Coomassie brilliant blue R250 and run using the same method as for the immunostained gels. The results showed that the increased sensitivity offered by the ECL substrate led to the increased specific staining in both gels shown in **Figure 4.7B** and **C**, particularly in lane 4 where a bright band of staining was observed at the same location where HRP-labelled secondary antibody was shown to be present by the protein-stained band in **Figure 4.7A**. The anti-cocaine antibody was included in lane 3 as a positive control in all of the gels shown in **Figure 4.7**. Staining was expected to occur in lane 3 of the immunostained gels (**Figure 4.7B** and **C**) at the same position as the protein stained band visible at the top of lane 3 in **Figure 4.7A**, as a result of interaction between the primary antibody in the gel and the secondary antibody applied to the gel during staining. This staining can be seen to be present in both **Figure 4.7B** and **C**, and is indicated by the dark grey coloration present in Lane 3 which is not observed in the BSA negative control lane (lane 1) of either gel.

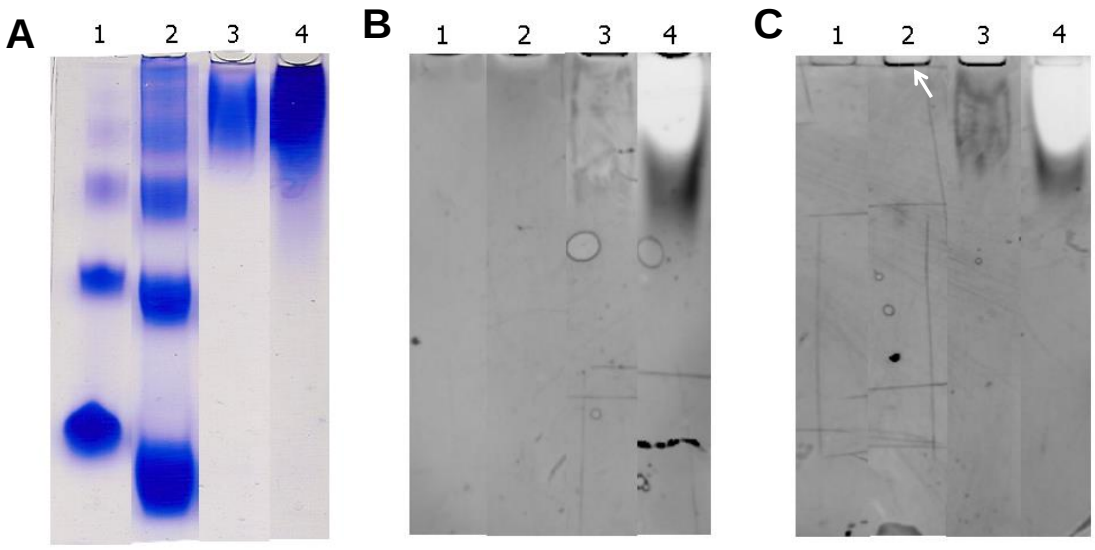

Figure 4.7. The in-gel immunodetection of cocaine-BSA in a 0.75 mm thick, 7.5 % w/v acrylamide PAGE gel stained with an ECL enzyme substrate. The total protein in the gel, stained by Coomassie brilliant blue R250, is shown in (A). Immunostaining was performed on two gels identical to the one shown in (A) using two different types of buffer (PBS and TBS); the results of the gels analysed using PBS and TBS are shown in (B) and (C), respectively. An exposure time of 3.5 min was used for both gels. The samples in each gel are labelled as follows; (1) BSA, (2) cocaine-BSA, (3) anti-cocaine primary antibody and (4) HRP-labelled secondary antibody. All samples were pre-diluted to 1 mg mL^{-1} in PB prior to analysis. The possible immunostaining of cocaine-BSA is indicated by the white arrow in lane 2 of (C).

As described by Miller, the efficacy of both primary and secondary antibody binding can depend on the type of buffer (PBS or TBS) used during immunostaining, the preference of which can vary between different types of antibodies.[65] A comparison was therefore made between immunostaining performed using PBS and TBS as the antibody diluent and wash buffers using the same protocol in order to optimise antibody binding. The resulting PBS and TBS-treated gels are shown in **Figure 4.7B** and **C**, respectively. Improved staining was observed in **Figure 4.7C**, as indicated by the darker staining in the anti-cocaine primary antibody control lane (lane 3) of **Figure 4.7C** compared to the lighter staining in **Figure 4.7B**. This showed that immunodetection performed using TBS buffers led to improved interaction between

the primary and secondary antibody, thereby resulting in improved staining in the gel. TBS buffer was therefore applied for all of the in-gel immunostaining performed hereafter. Positive immunostaining of cocaine-BSA, present in lane 2, was not observed in the gel treated with PBS buffers (**Figure 4.7B**). However, a small band of staining present at the base of the sample well in lane 2 (indicated by the white arrow in **Figure 4.7C**), which was not present in lane 1 containing BSA as a negative control, may have resulted from the detection of cocaine in this area. This presence of protein at this location in lane 2 of the gel stained for protein (**Figure 4.7A**) increased the likelihood of the staining being indicative of the location of the cocaine-BSA conjugate in the gel, although these results alone were not considered conclusive.

4.2.4 In-gel immunodetection of cocaine on spiked cotton paper and partial banknote samples using rabbit anti-cocaine antibody

The developed in-gel immunodetection method, applied using the optimised conditions described in **Section 4.2.3**, was used for the detection of cocaine standard on 100 % cotton paper samples as a representation of a banknote matrix. Spiking of the cotton paper samples with a solution of cocaine standard as well as cocaine-BSA, a BSA negative control, and primary and secondary antibody positive controls was performed as described in **Chapter 2.3.5**. The paper was spiked by applying 2 µL of each solution to each side of the paper sample in the grid pattern shown below in the images of **Figure 4.8**. The circles indicate the exact location of the spiked solutions within the grid. In-gel immunodetection was performed using a 1:25 dilution of rabbit anti-cocaine antibody as described in **Chapter 2.3.6**.

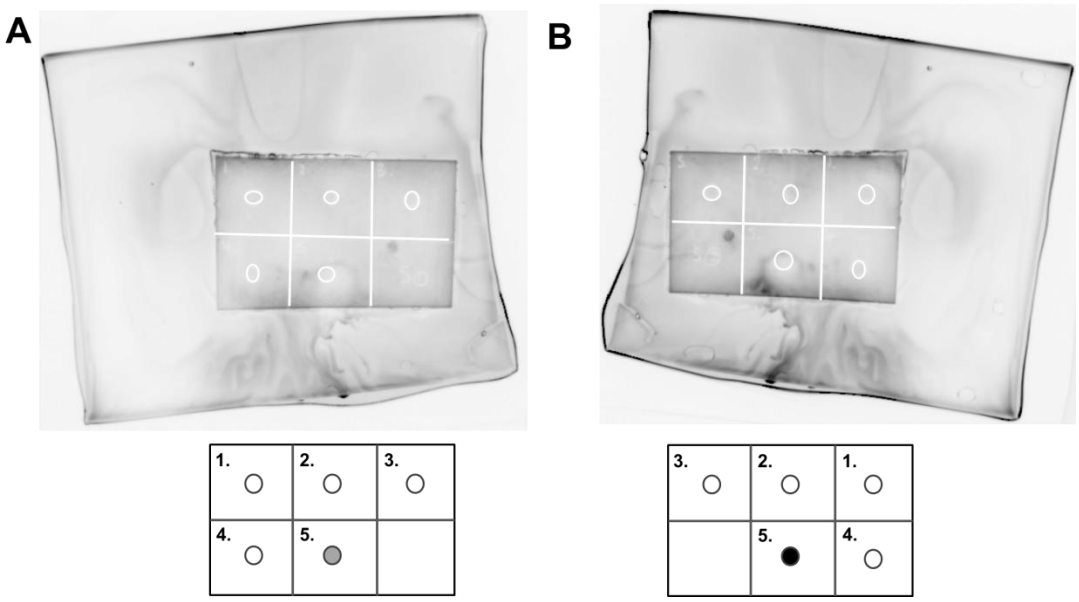

Figure 4.8. The immunostaining of a spiked cotton paper sample within a 1.5 mm thick, 7.5 % w/v acrylamide gel. (A) and (B) show each side of the paper. Standard and control samples for spiking were applied to both sides of the paper using the grid layout shown; each circle indicates the exact location of the spiked substance within the grid. The stained areas on the gel are indicated by black shading in the diagram. The solutions for spiking were as follows; (1) BSA, (2) cocaine-BSA, (3) anti-cocaine primary antibody, (4) cocaine standard (1 mg mL^{-1} in MeOH) and (5) HRP-labelled secondary antibody. All samples, except the cocaine standard, were pre-diluted to 1 mg mL^{-1} in PB prior to applications. Imaging was performed using an exposure time of 5 min.

Both sides of a typical gel after immunostaining are shown in **Figure 4.8**. The presence of HRP-labelled secondary antibody is indicated by areas of dark (black/grey) staining within the gel. HRP-labelled secondary antibody, applied directly onto the surface of the paper as a staining control in grid 5, was indicated by the presence of dark staining around and underneath the circled area. The staining for this control appeared to migrate or 'bleed' away from the spiking location, indicating the possible movement of the secondary antibody within the gel. This was expected based on the solubility of the protein in the aqueous gel solution immediately after application and before the gel had set. The observed pattern of staining could not be

attributed to the location of spiked cocaine or cocaine-BSA on the surface of the cotton paper, therefore indicating that immunostaining of cocaine had in this case not been successful.

In-gel immunodetection was performed on partial banknote samples in the same way as previously applied for the staining of the cotton paper sample shown in **Figure 4.8**. The partial banknote sample in **Figure 4.9A** was spiked with a higher volume of cocaine standard and cocaine-BSA (5 µL for each) than the 2 µL volume used in the previous experiment. The spiking volume was further increased to 10 µL for the partial banknote shown in **Figure 4.9B**. In-gel immunodetection was performed using a 1:25 dilution of rabbit anti-cocaine antibody as described in **Chapter 2.3.6** for both samples. A similar result was obtained for the partial banknote shown in **Figure 4.9A** as for that of the cotton paper sample shown in **Figure 4.8**, with the greatest intensity of staining occurring in grid 5 around the region of the note spiked with the HRP-labelled secondary antibody staining control. The migration or 'bleeding' of the stain, noted previously on the cotton paper sample, was once again evident. No further localised staining was observed, indicating the immunodetection of cocaine was unsuccessful.

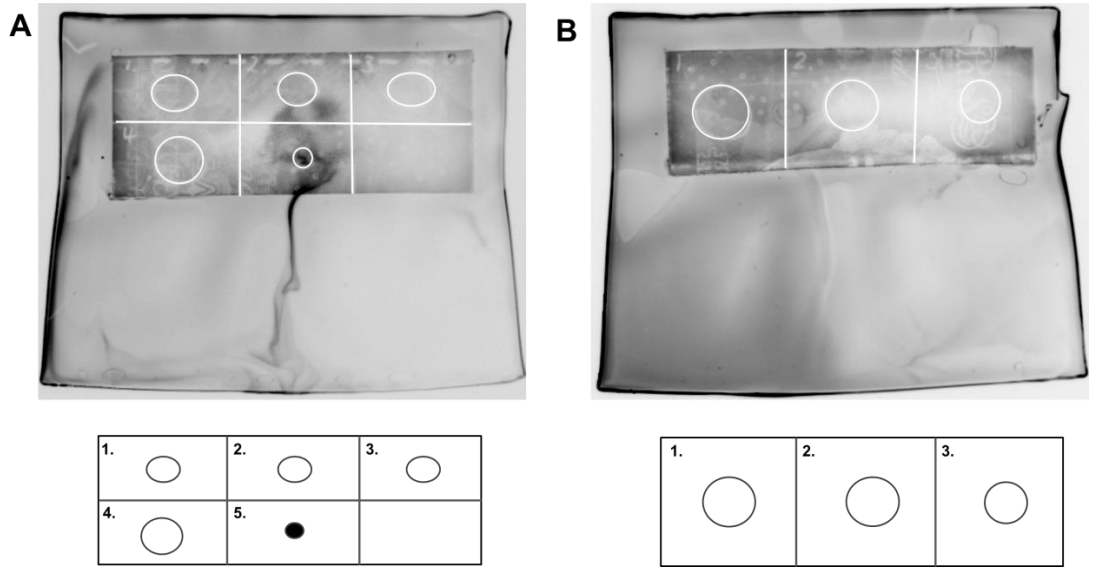

Figure 4.9. The results of the immunostaining of two parts of a £5 banknote sample obtained from general circulation within 1.5 mm thick, 7.5 % w/v acrylamide gels are shown in (A) and (B). Standard and control samples for spiking were applied to the notes using the grid layouts shown; the circle indicates the exact location of the spiked substance within the grid. The stained areas on the gel are indicated by black shading in the diagram. The solutions for spiking were as follows; (1) BSA, (2) anti-cocaine primary antibody, (3) cocaine-BSA, (4) cocaine standard (1 mg mL^{-1} in MeOH) and (5) HRP-labelled secondary antibody in (A), and (1) BSA, (2) cocaine standard (1 mg mL^{-1} in MeOH) and (3) anti-cocaine primary antibody in (B). All samples, except the cocaine standard, were pre-diluted to 1 mg mL^{-1} in PB prior to applications. The images were obtained using 2 and 3 min exposure times for (A) and (B), respectively. The contrast of (A) was adjusted by +1.

The application of an increased volume of cocaine-BSA and cocaine standard onto the partial note shown in **Figure 4.9B** was used to increase the antigenic concentration on the sample in the next experiment, and thereby improving the chance of successful immunostaining. Both the primary antibody and secondary antibody controls were omitted from this sample so as not to detract from any cocaine-specific staining. As shown in **Figure 4.9B**, a region of white staining was observed around the circled areas in grids 2 and 3 spiked with cocaine-BSA and

cocaine standard, respectively. The presence of white areas of staining in gels or immunoblots stained using an ECL substrate, known as hollow or 'ghost' bands, is a commonly reported phenomenon.[66] These ghost bands are thought to result from the presence of too much antigenic material on the gel or membrane, although their actual origin is not well understood.[66] It was thought that the white regions of staining on the gel in **Figure 4.9B** may also be the result of excess cocaine being present due to the high spiking volumes used. A repeat of the procedure on a partial banknote sample spiked with lower volumes cocaine-BSA and the cocaine standard solution, such as 7.5 µL, would be necessary for this to be determined.

4.2.5 In-gel immunodetection method development for polyacrylamide gels using sheep anti-cocaine antibody

At this point in the study, the manufacturer of the polyclonal rabbit anti-cocaine antibody discontinued the supply of this antibody. The immunodetection method was therefore re-optimised using a new sheep polyclonal anti-cocaine antibody with a corresponding HRP-labelled anti-sheep IgG secondary antibody. Immunodetection was initially performed on a gel containing cocaine-BSA, a BSA negative control, and primary and secondary antibody controls after separation by non-denaturing PAGE, as described for the rabbit primary antibody in **Section 4.2.3**.

Immunostaining of the 7.5 % acrylamide gels was performed after PAGE using a 1:2,500 dilution of primary antibody, and a 1:10,000 dilution of secondary antibody as described in **Chapter 2.3.2**. The results of the analysis are shown in **Figure 4.10**. The gel shown in **Figure 4.10A** was stained for all protein by Coomassie brilliant blue, run using the same method as for the immunostained gel in **Figure 4.10B**. The only staining present in the gel shown in **Figure 4.10B** was obtained from the HRP-labelled secondary antibody staining control in lane 3. As expected, no staining was observed in lane 1 containing the BSA negative control. However, there was also an absence of staining in lane 2 where the anti-cocaine primary antibody control was present. This indicated that HRP labelling of the primary antibody *via* secondary

antibody binding had not been successful. The experiment was repeated using a higher concentration of secondary antibody (a 1:5,000 dilution) from which the same result was obtained (results not shown). Overall these results suggested that the primary and secondary antibodies were failing to interact with one another within the gel. A dot-blot style assay was therefore performed in order to confirm that the secondary antibody was able to bind to the primary in the absence of the gel.

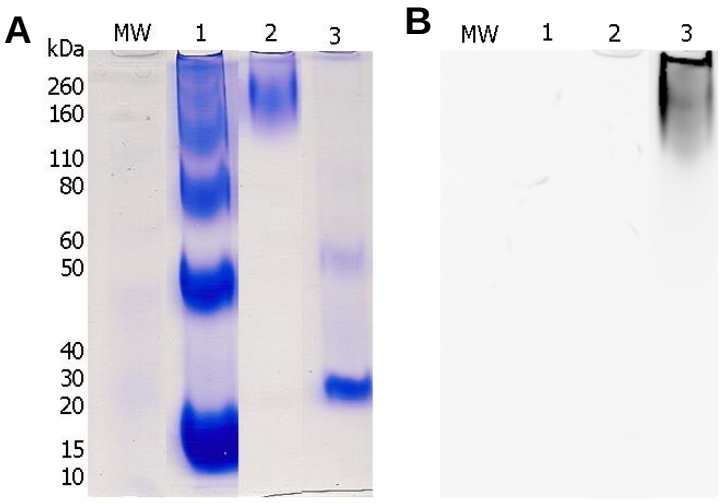

Figure 4.10. The in-gel immunodetection of cocaine-BSA in a 0.75 mm thick, 7.5 % w/v acrylamide PAGE gel stained with an ECL enzyme substrate. The total protein in the gel, stained by Coomassie brilliant blue R250, is shown in (A). The corresponding immunostained gel imaged using an exposure time of 3.5 min is shown in (B). After the molecular weight marker (MW), the samples in the gel are labelled as follows; (1) BSA, (2) anti-cocaine primary antibody and (3) HRP-labelled secondary antibody. All samples were pre-diluted to 1 mg mL^{-1} in PB prior to analysis.

4.2.6 In-gel immunodetection of cocaine on nitrocellulose membrane samples using sheep anti-cocaine antibody

A dot-blot style assay on nitrocellulose membrane was used as a means of testing for interaction between the anti-cocaine primary antibody and the HRP-labelled

secondary antibody. The assay was performed as described in **Chapter 2.3.3**. Primary antibody and BSA spotted onto the nitrocellulose membrane were used as the test and negative control, respectively. As indicated by the results in **Figure 4.11**, the primary antibody (circle 1) was successfully visualised using the HRP-labelled secondary antibody. No staining was present on the area with BSA bound in (circle 2). This proved that a specific interaction was occurring between the two antibodies, indicating that perhaps the presence of the gel was interfering with secondary antibody binding.

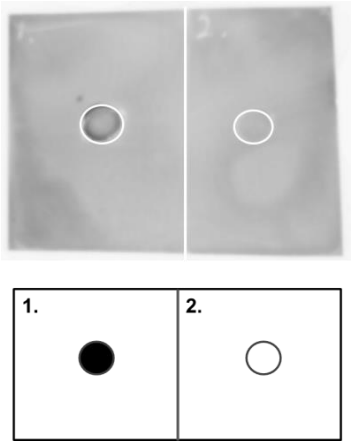

Figure 4.11. The results of a 'dot-blot'-style assay for the detection of antibody interaction, performed on nitrocellulose membrane spotted with anti-cocaine primary antibody and BSA, within the circles labelled (1) and (2), respectively. Spotting of the solutions was performed at a concentration of 1 mg mL^{-1} for both proteins. Imaging was performed using an exposure time of 1 min.

In order to determine whether the presence of the acrylamide gel was interfering with the interaction between the primary and secondary antibodies, a nitrocellulose membrane was prepared in the same way as for the dot blot, but with an additional HRP-secondary antibody staining control also included. In-gel immunodetection was then performed on the membrane as described in **Chapter 2.3.6**, in a similar manner as for the cotton paper samples. A 1:100 dilution of HRP-labelled secondary antibody was used. As shown in **Figure 4.12**, the resulting gel was only stained in the area

where the secondary antibody control was present on the membrane (circle 3). No staining was observed where the primary antibody (circle 1) or the BSA negative control (circle 2) had been spotted. This indicated that the presence of the gel did indeed interfere with the interaction between the primary and secondary antibody, and confirmed that an alternative means of enzyme labelling would be necessary to use this type of antibody for in-gel immunodetection using the developed method.

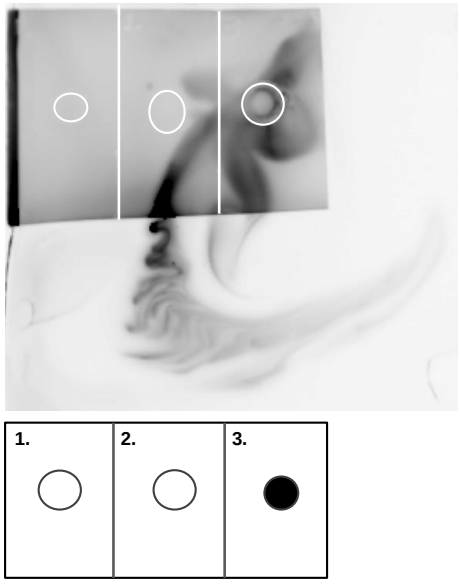

Figure 4.12. The results of a 'dot-blot'-style in-gel immunoassay for the detection of antibody interaction, performed on nitrocellulose membrane within a 1.5 mm thick, 7.5 % w/v acrylamide PAGE gel. The membrane was spotted with anti-cocaine primary antibody, BSA and HRP-labelled secondary antibody within the circles labelled (1), (2) and (3), respectively. Spotting of the solutions was performed at a concentration of 1 mg mL^{-1} for both proteins. Imaging was performed using an exposure time of 3 min.

4.2.7 HRP labelling of sheep anti-cocaine antibody

The results in **Section 4.2.6** showed that the sheep anti-cocaine primary antibody could not be labelled using the HRP-labelled secondary antibody due to the interference with secondary antibody binding caused by the presence of the gel. The direct labelling of the primary anti-cocaine antibody with HRP to form an antibody-

HRP conjugate was therefore performed to overcome this problem. HRP labelling was achieved using the well-described periodate-mediated method of conjugation as detailed in **Chapter 2.3.4**. This method of conjugation is performed as a two-step process; in the first, monosaccharide residues on the HRP are oxidised with periodate to produce aldehyde groups. In the second step, the aldehyde groups are allowed to react with amine groups on the antibody, leading to the formation of Schiff bases which are reduced to give a stable conjugate.[67]

After conjugation, the resulting HRP-labelled antibody was separated from unbound HRP by gel filtration. The presence of protein in the eluted fractions from the column was monitored by absorbance at 280 nm as shown in **Figure 4.13A**. Two peaks were present in the elution spectrum, the first of which was thought to contain the labelled antibody based on its larger size compared with the free HRP in the second peak. The abrupt change in absorbance at 280 nm mid-way through the first peak was the result of an air bubble in the column. The presence of HRP-labelled antibody was confirmed in the fractions of the first peak by SDS PAGE, the results of which are shown in **Figure 4.13B**. The presence of antibody in the fractions B10 through to C2 is indicated by the two bands in the gel of molecular weights corresponding to those of the HRP-modified heavy and light chains of the antibody at around 116–126 kDa and 30-40 kDa, respectively. The presence of free HRP in fraction C6 is also indicated by a single band corresponding to the molecular weight of the enzyme (**Figure 4.13B**).

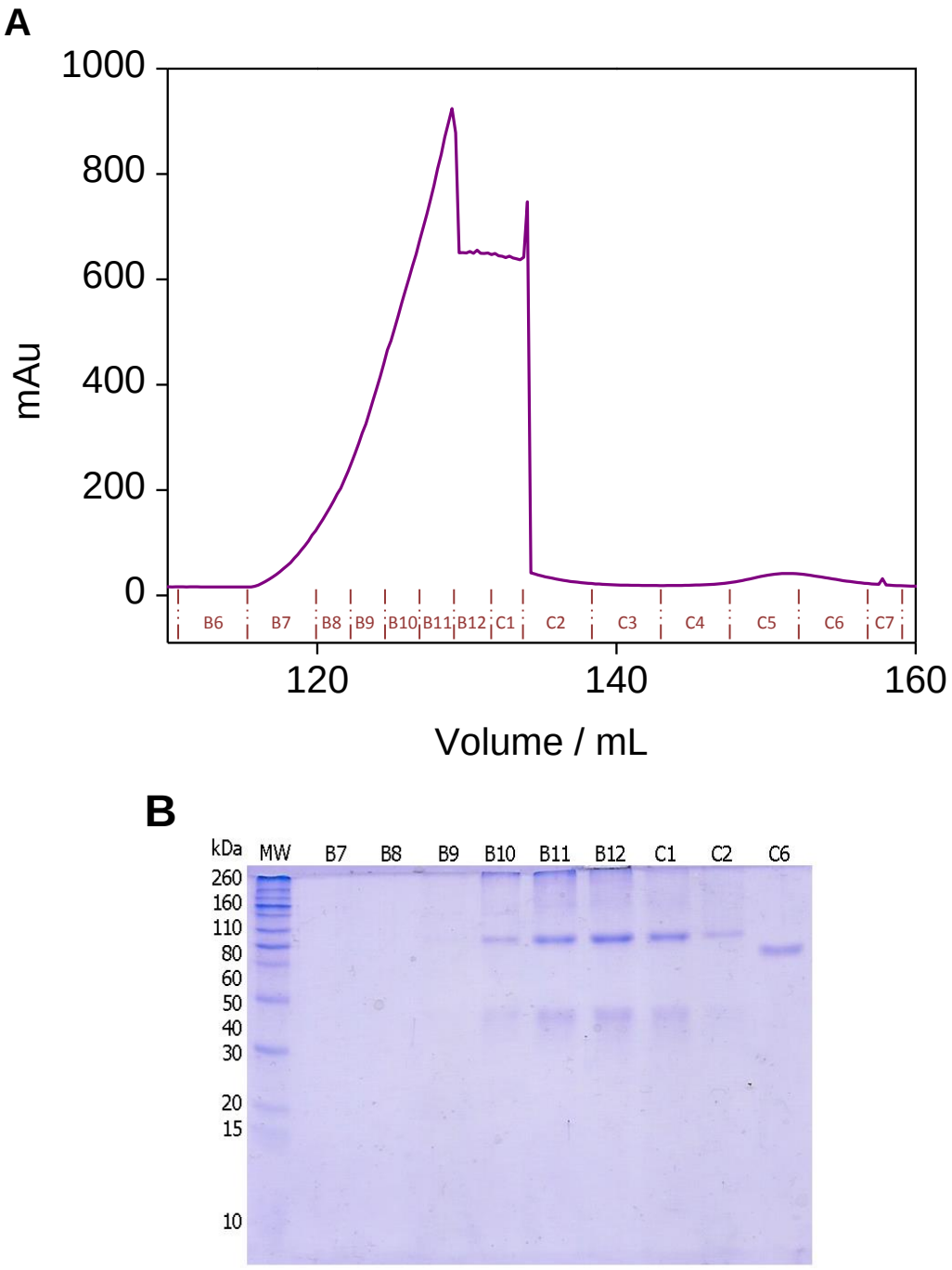

Figure 4.13. A typical UV-visible absorption trace at 280 nm obtained from the separation of HRP-labelled antibody from unbound HRP by gel filtration is shown in (A). The volumes of fractions B6 through C7 are as indicated below the spectrum. SDS PAGE confirmation of the fractions from the first peak (B7 through B12, C1 and C2) and from the second peak (C6) is shown in (B) alongside a molecular weight marker ('MW').

The fractions from the first peak containing the labelled antibody as determined by SDS PAGE were combined, and the solution concentrated to a final volume of 1 mL as described in **Chapter 2.3.4**. The presence of the HRP-antibody hapten was then confirmed by UV-Vis absorption spectrophotometry. A typical example of the absorption spectrum obtained from HRP-labelled antibody is shown in **Figure 4.14**. The two characteristic absorption bands present at 280 and 403 nm relate to the total protein in the solution and the haem group of HRP, respectively. The Rz value of the labelled antibody was determined by the ratio of the absorbance at 280 and 403 nm; this value provides a measure of the haem content of the preparation and can be used as an indicator of the purity of the conjugate.[67] The concentration of the conjugate was also calculated based on the absorbance of the haem group of HRP at 403 nm as described in **Chapter 2.3.4**. A typical Rz value of 0.3 and a concentration of 2 μM of labelled hapten was obtained.

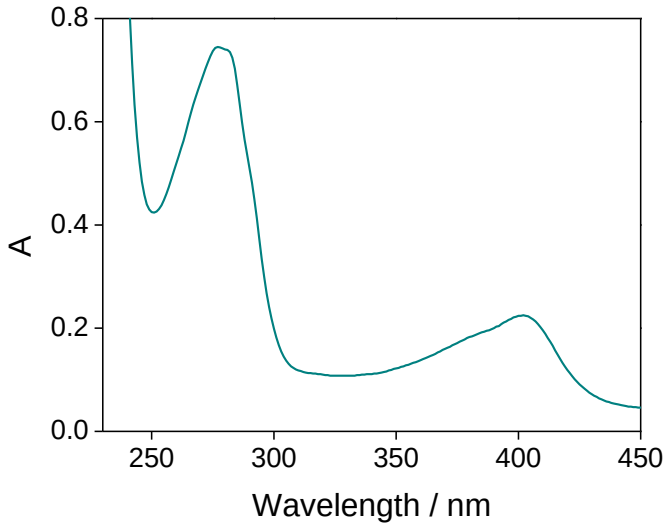

Figure 4.14. A typical UV-visible absorption spectrum of HRP-labelled anti-cocaine antibody. The hapten shown had an Rz value of 0.3 and a concentration of 2.2 μM.

4.2.8 In-gel immunodetection of cocaine on partial and whole banknote samples using HRP-labelled sheep anti-cocaine antibody

In-gel immunodetection was performed on partial banknote samples using the HRP-labelled anti-cocaine antibody as described in Chapter 2.3.6. The method used was based on that previously applied for the immunostaining of partial banknote samples using a rabbit primary antibody with a corresponding HRP-labelled secondary antibody in Section 4.2.4. The same spiking volume of 10 µL per solution for the gel shown in Figure 4.9B was applied. Cocaine standard was initially used as a test sample, alongside solutions of BSA and the HRP-labelled primary antibody applied as negative and positive control samples, respectively. In-gel immunodetection was performed using a 1:200 dilution of the HRP-labelled anti-cocaine antibody. The results are shown in Figure 4.15A. As expected, localised staining was observed at the location where the HRP-labelled antibody positive control had been applied to the note in grid 3. Significant movement or 'bleeding' of the stain, as previously shown in the gel spiked with HRP-labelled secondary antibody shown in Figure 4.8, was not present. Localised staining was not seen in the area spiked with cocaine standard, indicating that the immunodetection of cocaine had not been successful (circle in grid 1). Staining was also not present in the area where the BSA negative control had been applied (white circle in grid 2), as expected. Further staining in the gel occurred in an area where neither cocaine nor BSA had been applied to the banknote, in the position indicated by the blackened circle in the diagram (grid 2) below the gel shown in Figure 4.15A. The cause of this staining, whether it was specifically stained cocaine standard which had become mobile within the gel or another non-specifically stained element, is not known.

Overall, the results showed that the cocaine standard could not be detected using the immunodetection method. It was thought that this may have been the result of the nature of the cocaine standard used for spiking, which was in solution rather than as a crystalline powder. Previous studies have shown that cocaine contamination of banknotes occurs by crystals of cocaine becoming embedded in the weave of the

fibres that make up the note,[68] a mechanism which is likely to rely on the granular nature of the cocaine to occur. In order to better mimic this adhesion process, crystalline cocaine rather than cocaine as a solvent solution was applied for the spiking of the banknotes hereafter.

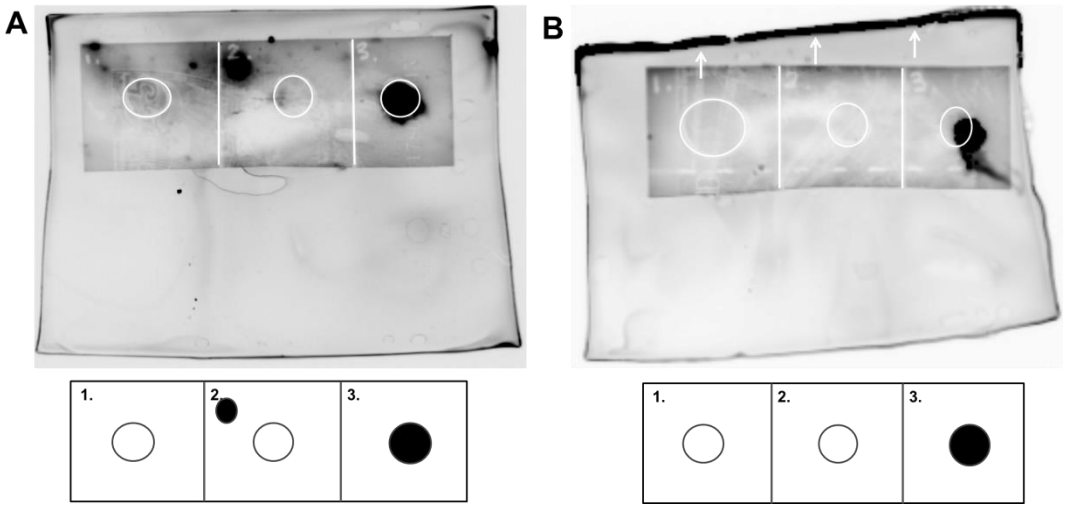

Figure 4.15. The results of the immunostaining of two partial banknote samples within a 1.5 mm thick, 7.5 % w/v acrylamide gels are shown in (A) and (B). Standard and control samples for spiking were applied to the notes using the grid layouts shown; the circle indicates the exact location of the spiked substance within the grid. The solutions for spiking were as follows; (1) cocaine standard (1 mg mL^{-1} in MeOH), (2) BSA and (3) HRP-labelled anti-cocaine primary antibody. All samples, except the cocaine standard, were pre-diluted to 1 mg mL^{-1} in PB prior to applications. The images were obtained using 2 and 3 min exposure times for (A) and (B), respectively. The contrast of (A) was adjusted by +2. Intense staining is indicated by the white arrow in (B).

The procedure was repeated for another part of the same banknote sample spiked with 'street' cocaine rather than cocaine standard in solution. The street cocaine, which was received as a crystalline powder, was used as a more realistic representation of the form and purity of cocaine likely to be present on a banknote,

rather than pure cocaine standard applied as a solution in solvent. It was also considered that the cocaine crystalline powder could behave differently within the gel in terms of solubility and mobility prior to gelation. Spiking was performed by rubbing both sides of the labelled area of the note onto a surface covered in crystalline cocaine as described in Chapter 2.3.5. It was noted that spiking in this way proved difficult and inaccurate, as the powder was prone to adhere to other parts of the banknote sample due to the small size of the area chosen for spiking. Immunostaining was performed on the spiked note in the same way as for the gel shown in Figure 4.15A. The resulting gel is shown in Figure 4.15B. As expected, the greatest intensity of the staining occurred on the part of the banknote in grid 3 where the HRP-labelled antibody positive control had been applied. No further localised staining was observed on the surface of the partial banknote in either grid 1, where the cocaine sample had been applied, or in grid 2 where the BSA negative control was present. This showed that the specific immunodetection of cocaine on the spiked banknote sample had not been successful. However, a dark band of staining was present at the top of the gel away from the banknote sample, as indicated by the white arrows in Figure 4.15B. Staining in this region was not present in the note that had been spiked with the cocaine standard shown in Figure 4.15A. This result suggested that this band could relate to the presence of cocaine within the gel, as the cocaine may have migrated to the top of the gel prior to gelation and been immunostained during subsequent analysis. Further analysis was necessary in order to confirm this.

The immunodetection procedure was therefore repeated in order to determine whether the staining at the top of the banknote shown in Figure 4.15B was the result of movement of the spiked cocaine within the gel prior to gelation, or an artefact of the detection method. Due to the difficulty of accurately applying the crystalline cocaine to only one small area of a partial banknote as experienced in the previous experiment, two separate partial banknotes, one with and one without cocaine, were instead analysed. This enabled the presence of cocaine to be determined in the absence of possible artefacts introduced by the method of spiking. A £5 note

obtained from general circulation was pre-washed with methanol to remove any potential background cocaine and cut into quarters, two of which were used for the analysis. Cocaine was then applied to both sides of the positive partial banknote using a spiking method similar to that described by Ebejer *et al.*,[69] which involved spreading cocaine over a flat surface before rubbing the note over the contaminated surface as detailed in **Chapter 2.3.5**. Immunostaining of the two samples was then performed as described in **Chapter 2.3.6**.

The results of the immunostaining are shown in **Figure 4.16**. The two sides of the negative and positive banknotes are shown in **A** and **B**, and then **C** and **D**, respectively. A clear difference was observed in the intensity of the staining between the positive and negative notes. Significantly darker staining was seen on the positive note in **Figure 4.16C** and **D** than on the negative note in **Figure 4.16A** and **B**. The areas of staining in **Figure 4.16C** and **D** occurred both in the gel around the note as well as on the note itself, and the patterns of staining at the bottom of the gels (indicated by the white arrows in **Figure 4.16**) were similar to those previously observed where positive control samples had spread or 'bled' into the surrounding gel area. The results show that the staining of cocaine was successful based on the difference between the positive and negative samples; however, the results also indicated that the spiked cocaine is not static within the gel and instead appears to move around in the gel mix possibly prior to gelation.

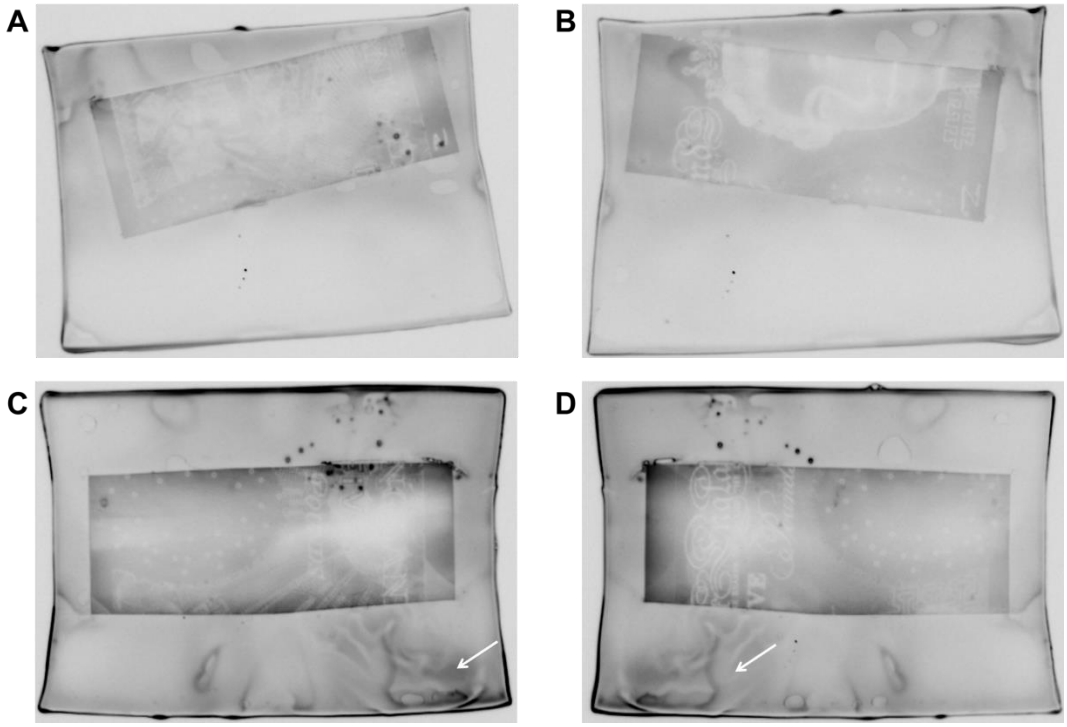

Figure 4.16. The immunostaining of partial banknote samples within 1.5 mm thick, 7.5 % w/v acrylamide gels. (A) and (B) show each side of part of a £5 banknote obtained from general circulation and pre-washed with MeOH. (C) and (D) show each side of a different part of the same banknote spiked with 5 mg of street cocaine. Imaging was performed using a 5 min exposure time. All images were contrast adjusted by +2. Areas where the staining has migrated or 'bled' are indicated by the white arrows in (C) and (D).

It was reported by Bohannon that the washing of banknotes with solvents such as methanol may affect the structure of the fibres within the note, and thereby also affect the adherent properties of the fibres to cocaine crystals entrapped within them.[70] In order to eliminate the possibility of the methanol wash-step affecting the results obtained in the previous experiment, the immunostaining procedure was repeated on a £5 banknote obtained from general circulation which was not pre-washed prior to analysis. The results of the analysis are shown in **Figure 4.17**, with the negative note shown in **A** and **B** and the positive note in **C** and **D**. The positive and negative notes are readily distinguishable based on the intensity of the dark staining as observed in the previous experiment. Overall, the staining on the positive

note is the darkest on the note itself rather than the surrounding gel, suggesting improved adherence of the spiked cocaine to the note. The more intense of the staining observed in **Figure 4.17C**, and to a lesser extent **Figure 4.17D** was situated around the edge of the banknote sample. The location of this staining could have resulted from the migration of cocaine towards the edges of the banknote during gelation, or may alternatively be the area on the banknote where the majority of the spiked cocaine is present. A higher intensity of staining was also observed in the negative in **Figure 4.17A** and **B** than was previously observed in **Figure 4.16A** and **B**. The higher intensity possibly resulting from background cocaine contamination already present on the note obtained from general circulation.

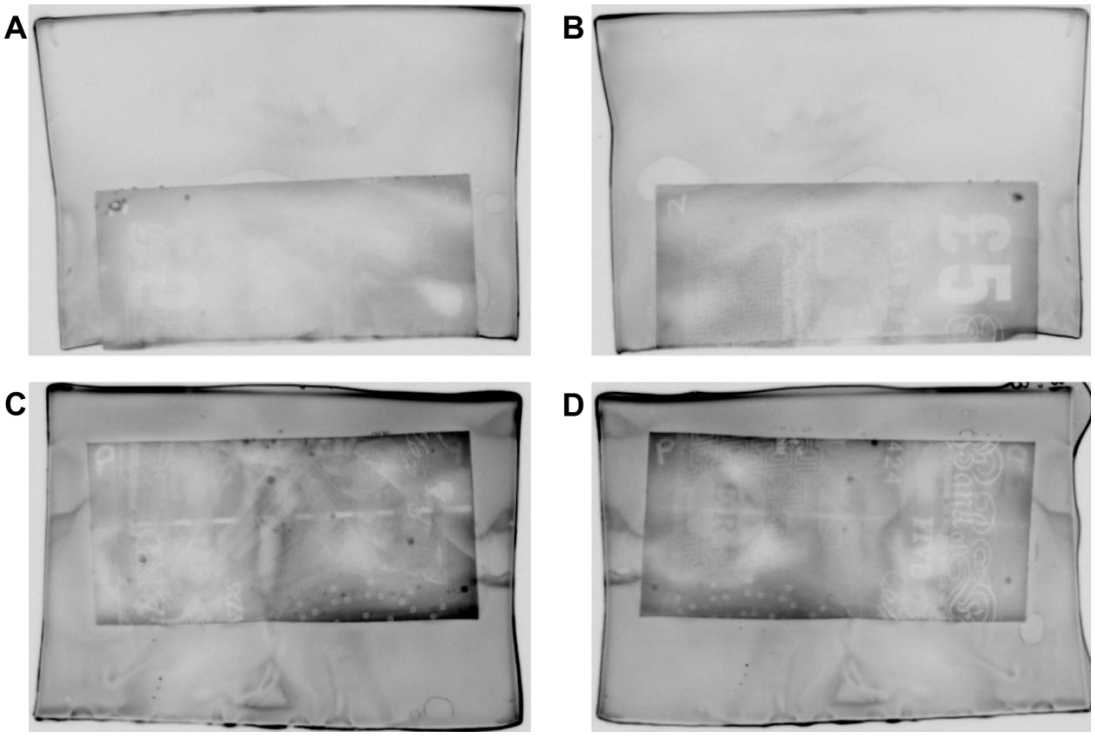

Figure 4.17. The immunostaining of partial banknote samples within 1.5 mm thick, 7.5 % w/v acrylamide gels. (A) and (B) show each side of part of a banknote obtained from general circulation. (C) and (D) show each side of a different part of the same banknote spiked with 5 mg of street cocaine. Imaging was performed using a 5 min exposure time. All images were contrast adjusted by +2.

Furthering the successful immunodetection of cocaine on banknotes, the method was then adapted and applied to whole banknote samples. Immunodetection was performed as described in Chapter 2.3.6. The different steps in the method were performed as demonstrated by the images shown in Figure 4.18.

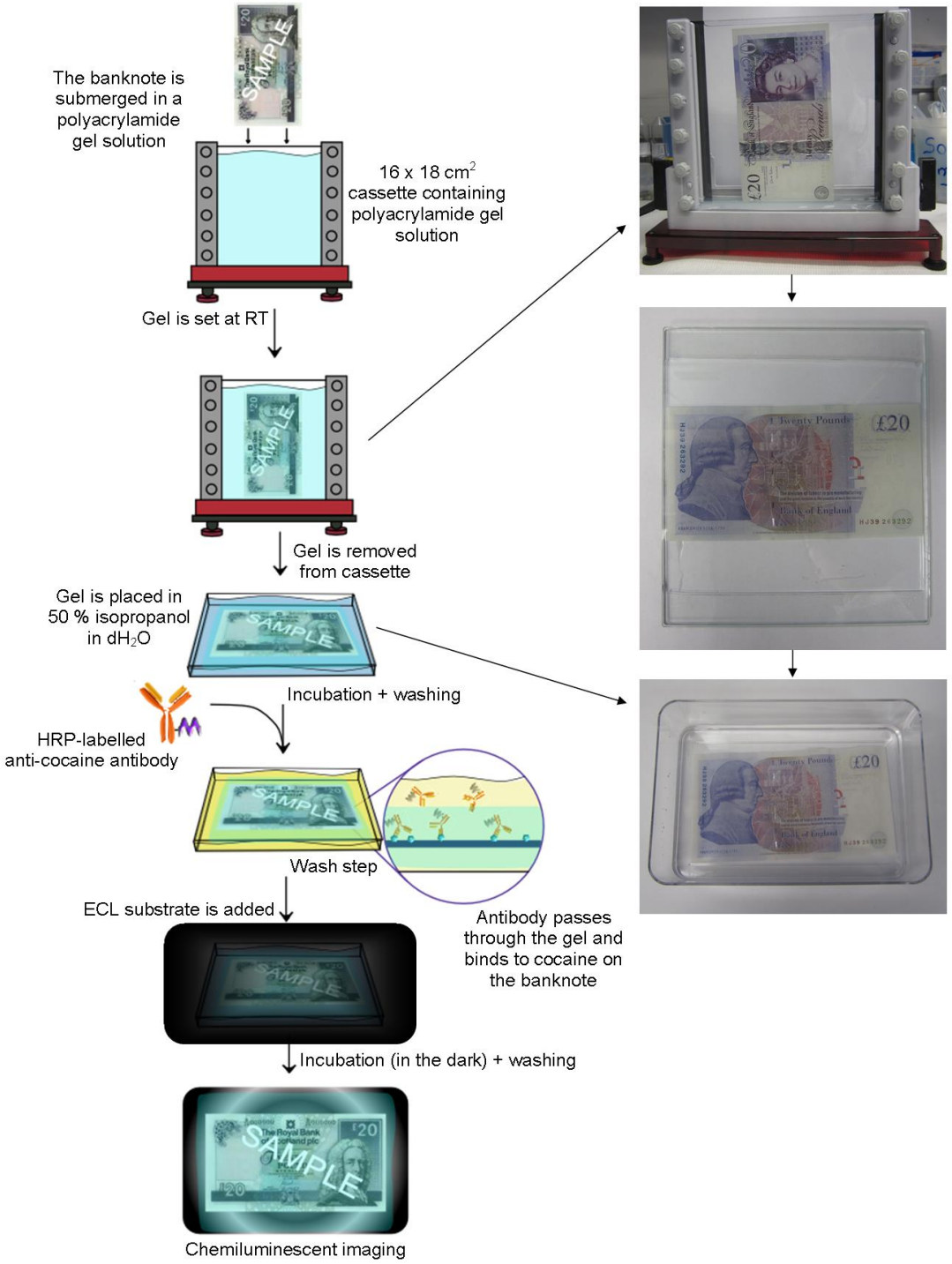

Figure 4.18. A schematic representation showing the steps performed during the in-gel immunodetection for the detection of cocaine on banknotes. Images on the right of the figure are photographs taken during the immunodetection procedure.

As banknotes from general circulation are known to contain background levels of cocaine contamination, newly minted notes (obtained directly from the Bank of England) were instead used as a truly negative sample matrix for the initial testing of the method. A typical result obtained from the immunostaining of a newly minted banknote is shown in **Figure 4.19A** and **B**. A background level of light, grey-coloured staining was observed on the note particularly in areas where air bubbles had developed in the gel during gelation, which have been labelled by white circles in the figure. The results of the negative control were compared with the immunostaining obtained from a newly minted banknote spiked with cocaine over the whole of the note. A typical example of this is shown in **Figure 4.19C** and **D**. The staining present on the note spiked with cocaine was darker and more intense than that observed on the negative control note, although not the same degree of difference as observed in the partial banknote samples shown in **Figures 4.16** and **4.17**. The increase in the intensity of the staining was thought to relate to the level of wear of the banknotes, as the partial banknotes had been obtained from general circulation and was therefore more aged and worn than the newly minted notes. This association was based on two studies which showed that the wear or ageing of banknotes leads to loosening and spreading of the tight weave of fibres that form the material of the note.[71,72] This in turn leads to greater porosity within the weave, in which cocaine crystals can more readily become entrapped and retained by the loosened fibres.

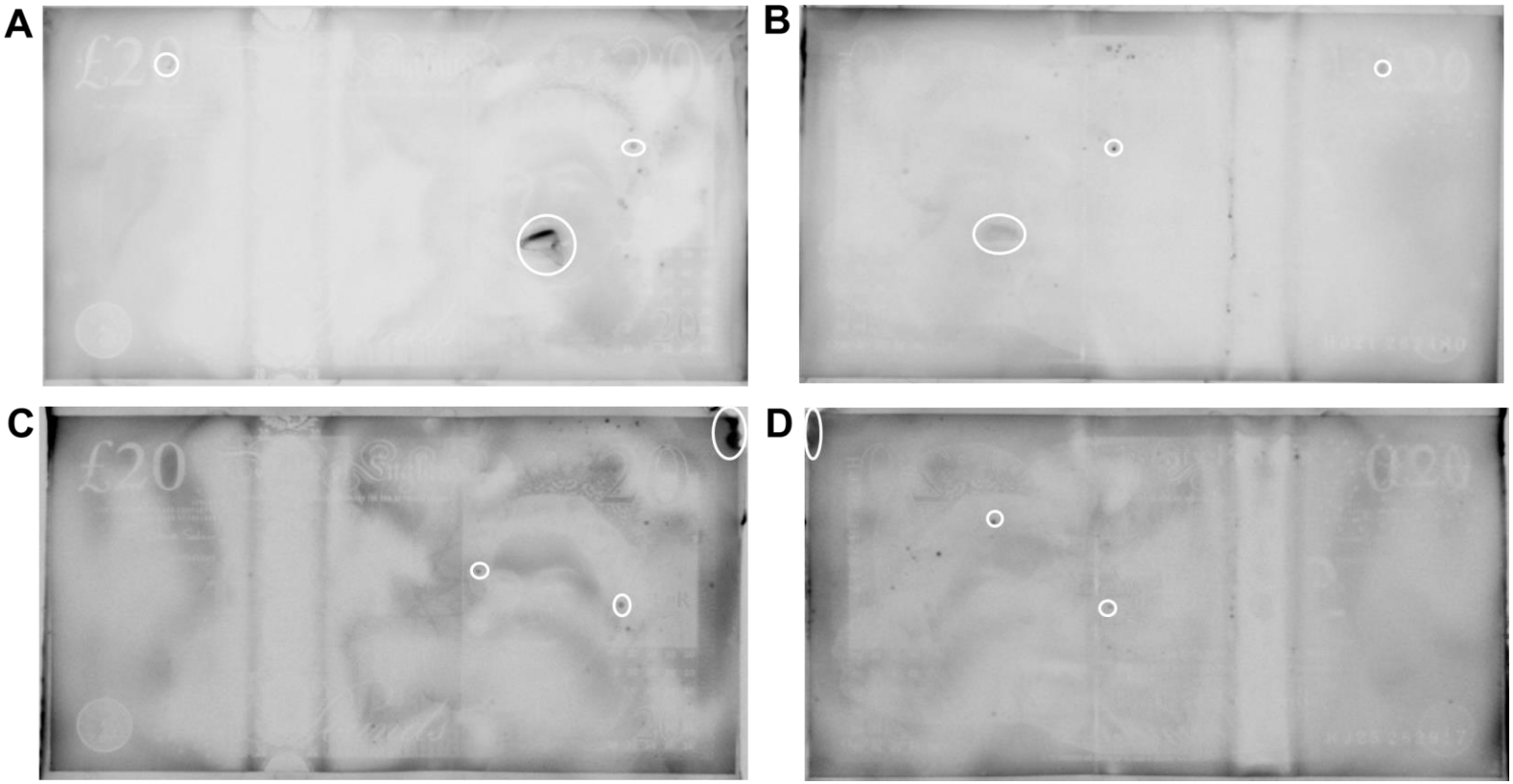

Figure 4.19. The immunostaining of newly minted £20 banknotes within 7.5 % w/v acrylamide gels. (A) and (B) show each side of part of one banknote. (C) and (D) show each side of a second newly minted banknote spiked with 10 mg of street cocaine over the whole of the note. Imaging was performed using a 5 min exposure time. All images were contrast adjusted by +2. The white circles indicate where air bubbles are present in the gels.

Although a clear difference was observed between the immunostained negative and positive banknotes, whether the staining occurred at the location where the cocaine had been applied to the banknote could not be confirmed using banknotes where cocaine had been applied to the whole of the note. In order to test the location-specificity of the staining, newly minted banknotes where half of the note had been spiked with cocaine and the other left absent were used. The typical results of the half-spiked note are shown alongside another negative newly-minted note in **Figure 4.20**. The division between the positive cocaine-spiked side of the note (labelled 'P') and the non-spiked negative side of the note (labelled 'N') is indicated by the white line in **Figure 4.20C** and **D**. Darker and more intense staining was observed on both sides of the positive banknote in agreement with the previous experiments (**Figure 4.19**). The area where the darkest staining occurred was at the edge of the banknote, rather than over the whole of the positive side of the note. This result suggested that the cocaine had migrated towards the edge of the note during gelation instead of remaining in its original spiked location.

The migration of the cocaine was thought to result from both the vertical orientation and the size of the cassette used for the application of the gel to the surface of the banknote. The results shown in **Figure 4.20** illustrate the need for the development of bespoke equipment which would improve both the practicality and the results produced using the in-gel immunodetection method. For example, a horizontal rather than vertical gel cassette slightly larger than the dimensions of the banknote would reduce both the movement of the note during application, as well as the volume of gel solution needed. This could lead to a reduction in the mobility of the cocaine during gelation and therefore improve the repeatability of the method. The development of a gel incubator colander of the appropriate dimensions would also enable the gel to be transferred more easily between solutions, thereby preventing damage to the fragile edges of the gel (like the damage indicated by the white circle at the edge of the banknote shown in **Figure 4.20C** and **D**).

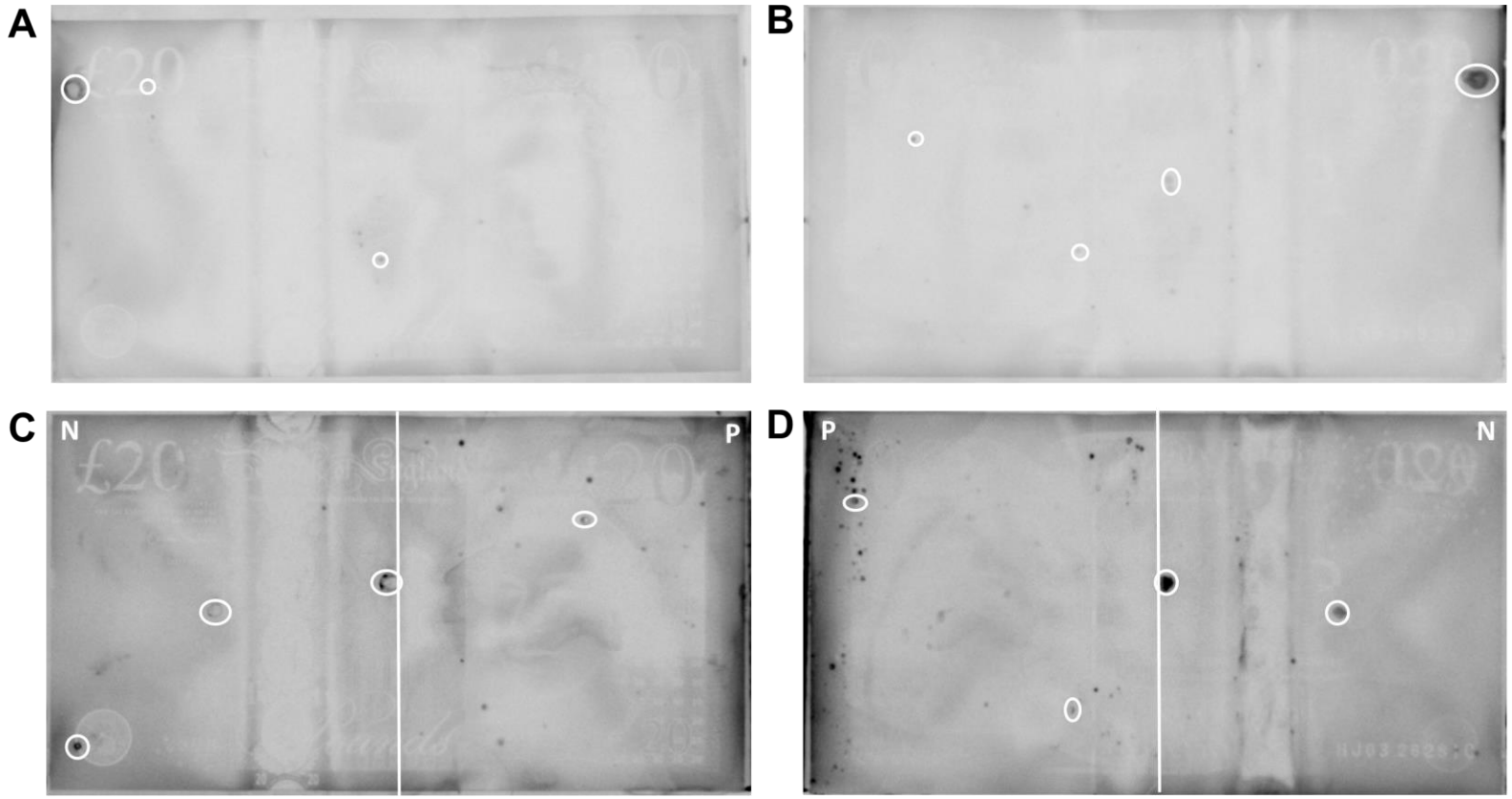

Figure 4.20. The immunostaining of newly minted £20 banknotes within 7.5 % w/v acrylamide gels. (A) and (B) show each side of part of one banknote. (C) and (D) show each side of a second banknote, where half of the note is spiked with 10 mg of street cocaine. The side of the note spiked with cocaine is labelled 'P', and the side without cocaine is labelled 'N'. Imaging was performed using a 5 min exposure time. All images were contrast adjusted by +2. The white circles indicate where air bubbles are present in the gels.

The entrapment and adherence of cocaine crystals within the fibres of banknotes was suggested by Moss and by Sleeman *et al.* as relating to the age and therefore the structural integrity of the weave of the banknote.[71,72] The immunodetection method was therefore applied for the analysis of six aged banknotes obtained from general circulation in order to confirm whether this suggestion affected the results obtained. The also enabled the analysis of actual samples with unknown levels of cocaine contamination to be performed. All of the notes analysed were of a worn and yellowed appearance, and were more pliable compared to the newly minted notes used in the previous experiments. The results of the immunostaining of two of the notes obtained from general circulation are shown in **Figure 4.21**. Sides 1 and 2 of the first note are shown in **Figure 4.21A** and **B**, and side 1 and 2 of the second are shown in **Figure 4.21C** and **D**. The nature of the staining observed on both the first (**Figure 4.21A** and **B**) and the second (**Figure 4.21C** and **D**) from general circulation differed from that of the newly minted notes shown in **Figures 4.19** and **4.20**. For instance, small circular dots of intense dark grey staining were present on the older notes in **Figure 4.21**, as opposed to the overall grey hue seen on the newly minted notes. These localised spots of intense staining were thought to result from the detection of the levels of cocaine contamination present on banknotes in general circulation. The areas where the darkest staining occurred were on the right and left hand edge of the sides of the banknote shown in **Figure 4.21C** and **D**, respectively. This staining may have resulted from the migration of cocaine towards that edge of the banknote during gelation. However, due to the unknown nature of the sample and therefore the undetermined levels of cocaine contamination and location, the staining may also be indicative of the presence of a large quantity of cocaine on that edge of the banknote. The difference between the type of staining between the newly minted and the older banknotes was thought to be due to the loosening of the banknote fibres that allows the entrapped cocaine crystals to remain in place even during application and subsequent gelation of the acrylamide gel. However, due to the unknown levels of cocaine present on the banknotes obtained from general circulation, the genuine immunostaining of cocaine could not be confirmed based on these results alone.

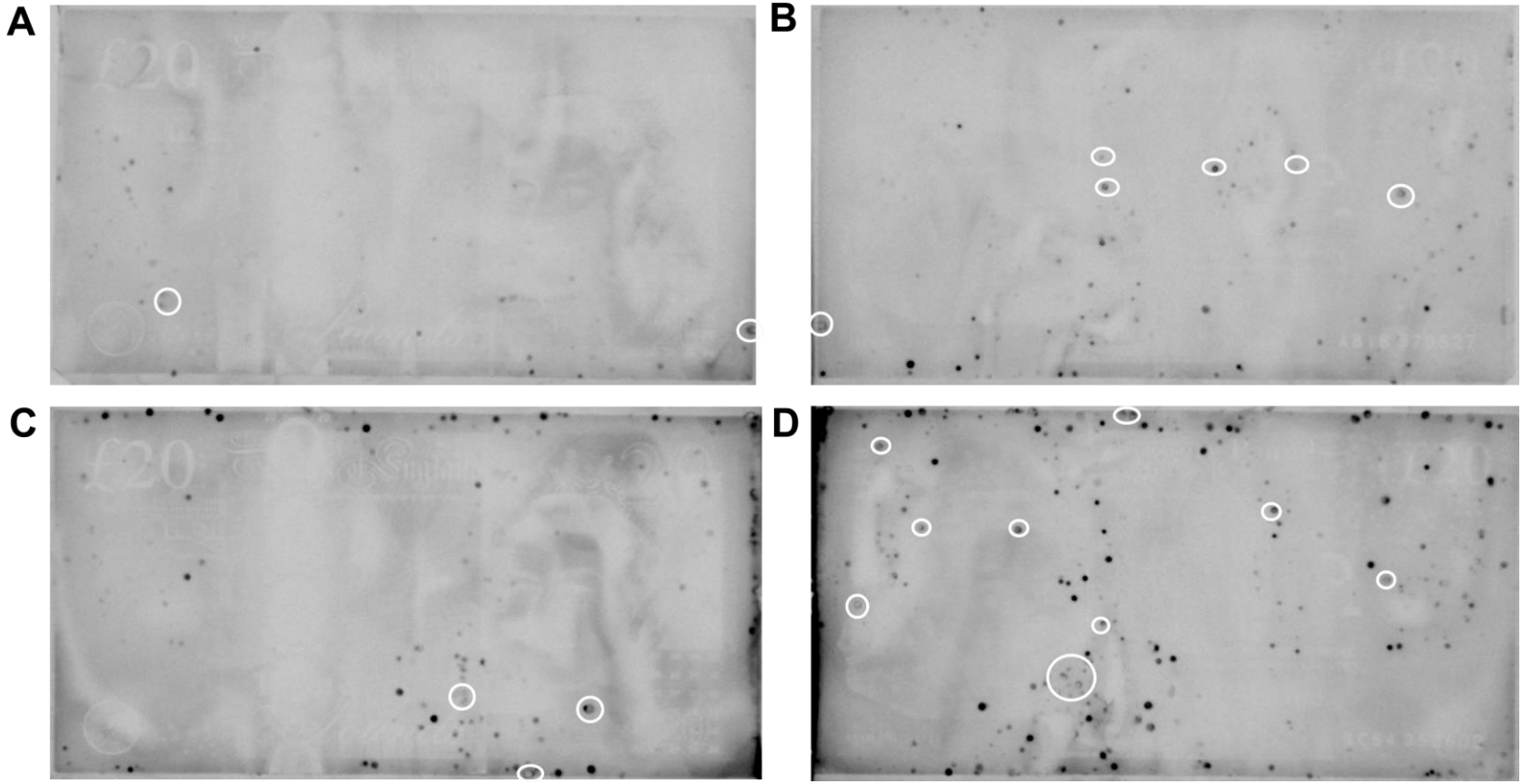

Figure 4.21. The immunostaining of two worn £20 banknotes obtained from general circulation within 7.5 % w/v acrylamide gels. The two sides of the first banknote are shown (A) and (B), and each side of the second banknote in (C) and (D). Imaging was performed using a 5 min exposure time. All images were contrast adjusted by +2. The white circles indicate where air bubbles are present in the gels.

In order to confirm whether the small, intense spots of immunostaining observed on the banknotes obtained from general circulation were the result of genuine immunodetection of cocaine on the surface of the banknote, the procedure was repeated on a set of half-spiked banknotes similar to that previously performed on the newly minted notes. A typical result is shown in **Figure 4.22** alongside that of another non-spiked banknote from general circulation. Sides 1 and 2 of the non-spiked note are shown in **Figure 4.22A** and **B**, and side 1 and 2 of the half-spiked are shown in **Figure 4.22C** and **D**. Overall, a greater number of stained areas were observed on the half-spiked banknote in **Figure 4.22C** and **D** as compared to the non-spiked note in **Figure 4.22A** and **B**. A greater number of stained areas were also present on the cocaine-spiked half of the positive banknote (labelled 'P') than the non-spiked half of the banknote (labelled 'N'). In particular, the side of the banknote shown in **Figure 4.22D** featured a greater number of dark spots of staining on the positively spiked half. The areas where the darkest staining occurred were on the left and right hand edges of the 'P' sides of the banknote shown in **Figure 4.22C** and **D**, respectively. The staining at the edge of the banknote in **Figure 4.22C** and **D** was less intense than that observed on the edge of the banknote shown in **Figure 4.21C** and **D**. The nature of the staining also differed between the two banknotes; for example, the staining in **Figure 4.22C** and **D** appeared as circular spots, whereas the staining in **Figure 4.21C** and **D** was present as one band of dark colouration. The less intense and spotted staining in **Figure 4.22C** and **D** on the sides of the banknote where cocaine had been applied suggests that this result may relate to the presence of cocaine on the banknote, rather than the migration of cocaine towards the edge of the banknote during gelation. It was noted that although the spiking performed using an excess (*ca.* 10 mg) of crystalline 'street' cocaine proved more successful than spiking with the cocaine standard in solution, the overall amount of cocaine adhered to the banknotes spiked in this manner is unknown. The spiked banknotes analysed in this study may not realistically reflect the quantities of cocaine likely to be present on banknotes in general circulation (as shown in **Table 1.2**). The production of partial or whole banknote 'standard' samples containing known quantities of cocaine which

more accurately reflect those previously reported in general circulation would be beneficial for the validation of the in-gel immunodetection.

Overall, the staining present on the spiked note was of similar appearance in terms of size and shape as the staining present on the non-spiked notes from general circulation rather than the newly minted notes. This indicated that the nature of the staining could indeed relate to the age and structural integrity of the banknote as originally suggested. The results suggest that using the developed immunodetection method enables the location-specific visualisation of cocaine on the surface of banknotes, thereby fulfilling the original aim of the study.

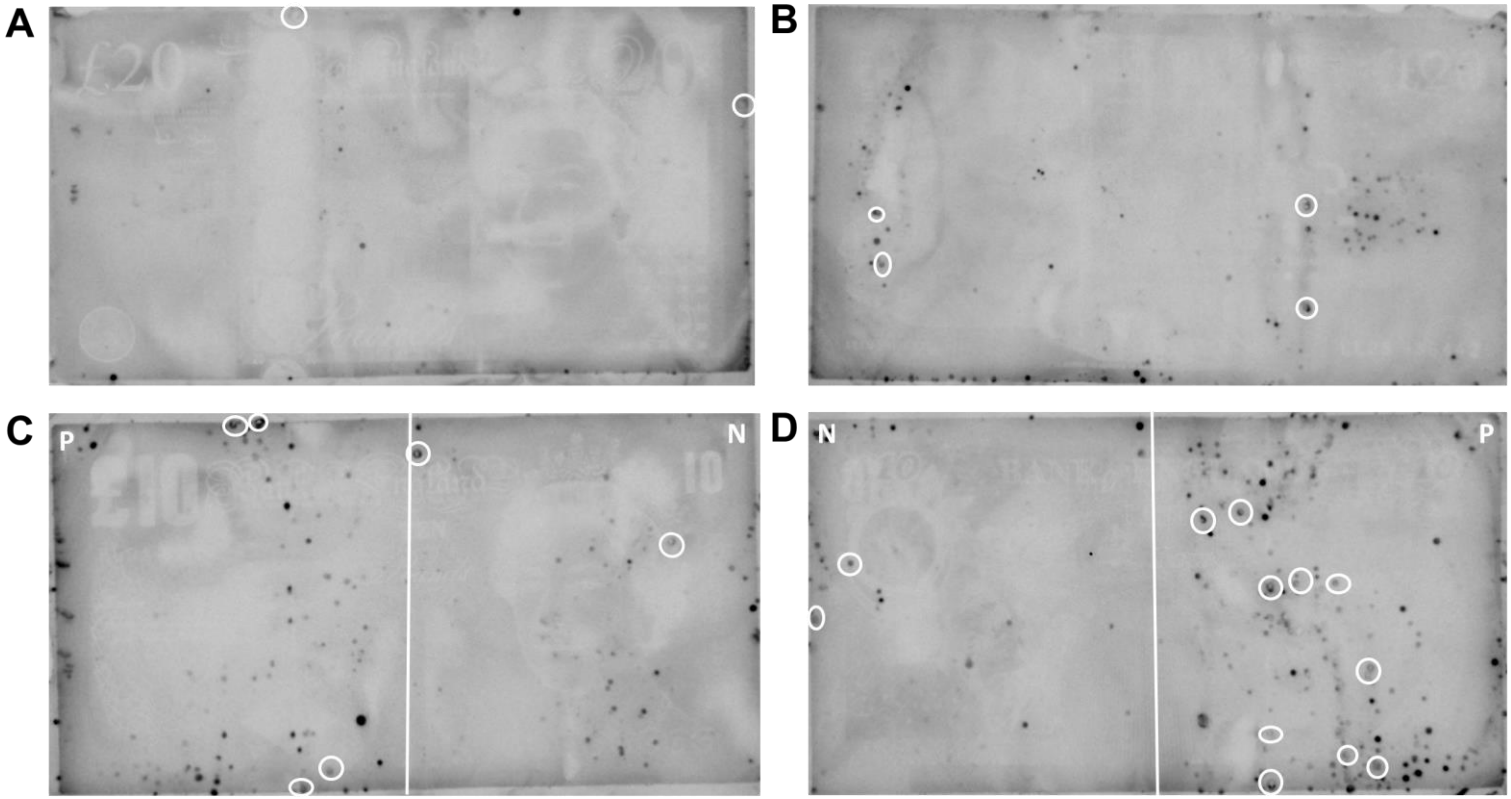

Figure 4.22. The immunostaining of one worn £20 banknote, and one worn £10 banknote obtained from general circulation within 7.5 % w/v acrylamide gels. (A) and (B) show each side of the £20 banknote. (C) and (D) show each side of the £10 banknote, where half of the note is spiked with 10 mg of street cocaine. The side of the note spiked with cocaine is labelled 'P', and the side without cocaine is labelled 'N'. Imaging was performed using a 5 min exposure time. All images were contrast adjusted by +2. The white circles indicate where air bubbles are present in the gels.

4.3 Conclusions

The aim of the work presented in this chapter was to develop a method for the location-specific immunodetection of cocaine on banknotes in order to determine the efficacy of the IMS sampling method currently used by law enforcement in the UK for the detection of cocaine on banknotes.[58] Immunodetection was selected as a means of detecting cocaine on the notes based on the previous application of immunological methods for the location-specific staining of cellular antigens in solid or semi-solid matrices such as tissue specimens. An acrylamide hydrogel was used as a means of affixing the cocaine on the banknotes to facilitate immunostaining. Hydrogels are commonly applied as scaffolding material in 2D and 3D cell culture which enables subsequent staining for cellular antigen by ICC methods. The porosity, biocompatibility and mechanical strength of hydrogels make them well suited for these types of applications. As well as being widely used for the separation of biomolecules by gel electrophoresis, hydrogels such as agarose have also been used for the testing of antibody-antigenic interactions by diffusion analysis. This shows that hydrogels are suitably permeable to allow the passive diffusion of large proteins such as antibodies.

In the present study, the mechanical strength and antibody permeability of a number of different biologically derived hydrogels, namely collagen, gelatin, casein and agarose were determined in order to judge their suitability as a matrix for the immobilisation of cocaine on banknotes. Hydrogels of biological origin were initially chosen as they are non-toxic, biodegradable, easily manipulated, cost-effective and widely available. The results obtained after applying the hydrogels to cotton paper as a representation of a banknote showed that the gels possessed too little mechanical strength to properly adhere to the surface of the note at the low concentrations of polymer required to enable antibody permeation. Based on these results, the use of biologically derived hydrogels as a matrix for cocaine detection on banknotes was discontinued in favour of a synthetic polymeric hydrogel matrix.

An acrylamide hydrogel was selected as an alternative to a biological hydrogel due to its previous application for the in-gel immunodetection of proteinaceous antigen after separation by gel electrophoresis. The method involved treatment of the gel with 50 % isopropanol in water as a fixative, the application of which enables the immobilisation of antigenic material within the gel and reportedly also increases its permeability to antibodies.[54] In-gel immunodetection of cocaine-BSA as an antigenic target was initially performed in acrylamide gels after separation by non-denaturing PAGE. Immunostaining was achieved using a rabbit anti-cocaine primary antibody with a corresponding HRP-labelled secondary antibody, after which visualisation of bound HRP was enabled *via* incubation in DAB as an enzyme substrate. Inclusion of primary and secondary antibody controls in the PAGE gels led to optimisation of the method, during which the enzyme substrate was changed from a colorimetric to an ECL substrate. This enhanced the sensitivity of the staining method and resulted in the positive staining of the primary antibody control by the labelled secondary antibody.

During the study, the manufacturer discontinued production of the original rabbit polyclonal anti-cocaine antibody. The remainder of the study was therefore continued with a sheep polyclonal anti-cocaine antibody, the cocaine-binding efficiency of which could not be compared with that of the original antibody due to variation introduced by the different labelling approaches used for each antibody type. The sheep polyclonal antibody was applied for in-gel detection on PAGE gels using a corresponding HRP-labelled secondary antibody. Labelling of the primary antibody *via* the HRP-labelled secondary antibody proved unsuccessful using this approach, despite the successful interaction between the primary and secondary antibodies established by a dot-blot style assay. Repetition of the dot-blot assay within an acrylamide gel proved that the presence of the gel interfered with the interaction between the primary and secondary antibodies. Direct labelling of the primary antibody with HRP was therefore undertaken to eliminate the need for a secondary-antibody based method of detection. Labelling of the primary antibody with HRP was performed using the well-described periodate-mediated method of conjugation, in

which addition of HRP binds to the Fc region of IgG and therefore does not affect the antigen-binding capacity of the antibody.[67] The successful formation of the antibody-HRP hapten was confirmed by SDS PAGE and UV-Vis absorption spectrophotometry at 280 and 403 nm, yielding a conjugate of suitable concentration and purity.

The HRP-labelled anti-cocaine antibody was initially unsuccessful at the in-gel immunodetection of cocaine standard, which was applied to small areas of partial banknote samples in a solvent solution. However, the detection of cocaine on partial banknote samples spiked with a sample of crystalline 'street' cocaine as a more realistic substance for spiking proved successful using the developed method. The presence of cocaine on the partial banknotes was indicated by darker regions of staining on the notes which had been spiked with cocaine, compared to the lighter background levels of staining present on non-spiked partial banknotes. The method yielded different levels of staining for partial notes obtained from general circulation after pre-washing with methanol as compared with those which had not been washed prior to spiking and analysis. The results confirmed the reports of Bohannon suggesting that pre-washing of banknotes in methanol affects the structure of the fibrous network that makes up the note, thereby affecting that way in which cocaine becomes embedded and retained by those fibres.[70]

Based on the successful immunodetection of cocaine on partial banknotes, the method was adapted for application to whole banknote samples. Detection was initially performed on newly minted banknotes as a true negative sample matrix without any possible cocaine contamination which could be obtained from general circulation. Immunodetection of the newly minted notes showed a clear difference in the intensity of staining when newly minted negative control samples were compared with newly minted banknotes which had been spiked with cocaine over the whole of the note. The staining observed on the newly minted notes was uniformly grey in colour over the whole of the note, the hue of which was darker on the notes which were positive for cocaine. The results suggested that the cocaine was successfully

being detected using the developed method. As the location of the cocaine on the note could not be confirmed based on these results alone, further analysis was performed in order to achieve the overall aim of the detection of cocaine on the banknote *in situ*. Newly minted banknotes where half of the note had been spiked with street cocaine and the other half left blank were used as a means of determining whether the location of the cocaine on the note could be labelled using the developed method of staining. The results showed that the overall degree of staining was darker for the half-spiked note than for the negative control. An area of intense staining was also present but only at the edge of the spiked half of the note, rather than over its entirety. This suggested that the spiked cocaine had become mobile within the gel mix during application and prior to gelation, and had thereby migrated to areas of the note on which it had not originally been applied.

The findings of previous publications suggest that the age and wear of a banknote may affect its ability to entrap and retain cocaine within its fibres.[71,72] The fibres of a banknote loosen with age, and the weave becomes more porous and this reportedly facilitates increased entry and retention of cocaine crystals within the fibrous network. Based on this concept, the retention of cocaine on newly minted notes would be less than the used notes in general circulation. As such, six banknotes from general circulation were analysed by immunostaining to determine whether the nature and intensity of the staining differed from that obtained from the newly minted banknotes. The analysis of the notes also represented the detection of cocaine on a genuine sample matrix on which unknown concentrations of cocaine could be present. The appearance of the notes was described as worn and aged as compared to the newly minted control sample. The results of the immunodetection of cocaine showed that the staining appeared as dark grey or black spots on the surface of the banknote, rather than the overall dark grey stain present on the newly minted notes. Immunodetection of cocaine was also performed on banknotes from general circulation half-spiked with ‘street’ cocaine in the same way as for the newly minted notes. A greater number of dark, stained spots were observed on the spiked half of the banknote when compared with the unspiked half. This suggested that the

staining was related to the presence and location of cocaine on the surface of the banknote.

Overall, the development of a novel in-gel immunodetection method for the visualisation of cocaine on the surface of banknotes is reported. The results shown in this chapter suggest that using the developed method enables the direct immunostaining of the cocaine at the location in which it is present on the banknote. The use of specialised equipment, such as a bespoke gel casting cassette for the gelation of the banknotes, would provide a more consistent matrix and decrease mobility of the cocaine prior to fixation and would thereby improve the developed technique. The production of a set of 'standard' banknote samples spiked with cocaine at concentrations reflecting those likely to be present on banknotes in general circulation would also be greatly beneficial for the further development and validation of the in-gel immunodetection method presented in this study. The possible application of antibody-functionalised nanoparticles to enhance the sensitivity of the developed method is also suggested to enable the visualisation of cocaine at even lower concentrations that presently detectable. The formation of anti-cocaine antibody-functionalised gold nanoparticles is described in Chapter 5.

Overall, the results presented in this chapter suggest that cocaine can be present at variable locations across the whole of the banknote. Based on the banknote samples analysed during this study it is therefore possible that the current IMS sampling method used by UK law enforcement provides estimates which either over- or understate the concentrations of cocaine that are actually present on the whole of the banknote. A method in which the whole of the banknote, rather than a representative area is sampled would provide more accurate indication of the cocaine contamination on a banknote.

4.4 References

(1) Dabbs, D. J. *Diagnostic Immunohistochemistry;* 2nd ed.; Elsevier: Philadelphia, 2006.

(2) Coons, A. H.; Creech, H. J.; Jones, R. N. *Exp. Biol. M.* **1941**, *47*, 200.

(3) Coons, A. H. *Ann. NY Acad. Sci.* **1971**, *177*, 5.

(4) Tubbs, R. R.; Sheibani, K.; Deodhar, S. D.; Hawk, W. A. *Cleveland Clin. Q.* **1981**, *48*, 245.

(5) Avrameas, S. *Histochem. J.* **1972**, *4*, 321.

(6) Murphy, K.; Travers, P.; Walport, M. *Janeway's Immunobiology*, 7th ed.; Garland Science: New York, 2008.

(7) Gubisne-Haberle, D.; Hill, W.; Kazachkov, M.; Richardson, J. S.; Peter, H. Y. *J. Pharmacol. Exp. Ther.* **2004**, *310*, 1125.

(8) Montero, C. *J. Histochem. Cytochem.* **2003**, *51*, 1.

(9) Renshaw, S. *Immunohistochemistry;* Scion Publishing: Oxfordshire, 2007.

(10) Glauert, A. M. *Fixation, Dehydration and Embedding of Biological Specimens;* Elsevier North-Holland: Amsterdam, 1975.

(11) Ramos-Vara, J. *Vet. Pathol.* **2005**, *42*, 405.

(12) Tibbitt, M. W.; Anseth, K. S. *Biotechnol. Bioeng.* **2009**, *103*, 655.

(13) Hoffman, A. S. *Adv. Drug Deliver. Rev.* **2012**, *64*, 18.

(14) Smalley, K. S.; Lioni, M.; Herlyn, M. *In Vitro Cell. Dev.-An.* **2006**, *42*, 242.

(15) Ulrich, T. A.; Jain, A.; Tanner, K.; MacKay, J. L.; Kumar, S. *Biomaterials* **2010**, *31*, 1875.

(16) Ma, W.; Fitzgerald, W.; Liu, Q. Y.; O'Shaughnessy, T. J.; Maric, D.; Lin, H. J.; Alkon, D. L.; Barker, J. L. *Exp. Neurol.* **2004**, *190*, 276.

(17) O'Connor, S. M.; Stenger, D. A.; Shaffer, K. M.; Maric, D.; Barker, J. L.; Ma, W. *J. Neurosci. Meth.* **2000**, *102*, 187.

(18) Raub, C. B.; Suresh, V.; Krasieva, T.; Lyubovitsky, J.; Mih, J. D.; Putnam, A. J.; Tromberg, B. J.; George, S. C. *Biophys. J.* **2007**, *92*, 2212.

(19) Oudin, J. *Method. Enzymol.* **1980**, *70*, 166.

(20) Silverstein, A. M. *A History of Immunology;* Elsevier: New York, 2009.

(21) Oudin, J. *CR Acad. Sci. Paris* **1946**, *222*, 115.

(22) Ouchterlony, O. *Acta Pathol. Mic. SC* **1949**, *26*, 507.

(23) Bailey, G. S. In *The Protein Protocols Handbook;* Springer: 1996, p 749.

(24) Armisen, R.; Galatas, F. *FAO Fisheries Technical Paper,* **1987**.

(25) Normand, V.; Lootens, D. L.; Amici, E.; Plucknett, K. P.; Aymard, P. *Biomacromolecules* **2000**, *1*, 730.

(26) Rees, D. A.; Welsh, E. J. *Angew. Chem., Int. Ed.* **1977**, *16*, 214.

(27) Johnson, E. M.; Berk, D. A.; Jain, R. K.; Deen, W. M. *Biophys. J.* **1996**, *70*, 1017.

(28) Pluen, A.; Netti, P. A.; Jain, R. K.; Berk, D. A. *Biophys. J.* **1999**, *77*, 542.

(29) Wester, K.; Andersson, A. C.; Ranefall, F.; Bengtsson, E.; Malmstrom, P. U.; Busch, C. *J. Pathol.* **2000**, *190*, 503.

(30) Pedersen, J. A.; Swartz, M. A. *Ann. Biom. Eng.* **2005**, *33*, 1469.

(31) Jenkins, C. L.; Raines, R. T. *Nat. Prod. Rep.* **2002**, *19*, 49.

(32) Shoulders, M. D.; Raines, R. T. *Annu. Rev. Biochem.* **2008**, *78*, 929.

(33) O'Connor, S. M.; Andreadis, J. D.; Shaffer, K. M.; Ma, W.; Pancrazio, J. J.; Stenger, D. A. *Biosens. Bioelectron.* **2000**, *14*, 871.

(34) O'Connor, S. M.; Stenger, D. A.; Shaffer, K. M.; Ma, W. *Neurosci. Lett.* **2001**, *304*, 189.

(35) O'Leary, L. E.; Fallas, J. A.; Bakota, E. L.; Kang, M. K.; Hartgerink, J. D. *Nat. Chem.* **2011**, *3*, 821.

(36) Roussenova, M.; Enrione, J.; Diaz-Calderon, P.; Taylor, A.; Ubbink, J.; Alam, M. *New J. Phys.* **2012**, *14*, 035016.

(37) Veis, A.; Cohen, J. *Nature* **1960**, *186,* 720.

(38) Bigi, A.; Cojazzi, G.; Panzavolta, S.; Rubini, K.; Roveri, N. *Biomaterials* **2001**, *22*, 763.

(39) Zhao, F.; Grayson, W. L.; Ma, T.; Bunnell, B.; Lu, W. W. *Biomaterials* **2006**, *27*, 1859.

(40) Fu, Y.; Xu, K.; Zheng, X.; Giacomin, A. J.; Mix, A. W.; Kao, W. J. *Biomaterials* **2012**, *33*, 48.

(41) Awad, H. A.; Quinn Wickham, M.; Leddy, H. A.; Gimble, J. M.; Guilak, F. *Biomaterials* **2004**, *25*, 3211.

(42) Matsuda, S.; Iwata, H.; Se, N.; Ikada, Y. *J. Biomed. Mater. Res.* **1999**, *45*, 20.

(43) Hafidz, R. M.; Yaakob, C.; Amin, I.; Noorfaizan, A. *Int. Food Res. J.* **2011**, *18*, 787.

(44) Chick, J.; Ustunol, Z. *J. Food Sci.* **1998**, *63*, 1024.

(45) Avena-Bustillos, R. J.; Cisneros-Zevallos, L. A.; Krochta, J. M.; Saltveit Jr, M. E. *Postharvest Biol. Tech.* **1994**, *4*, 319.

(46) Horne, D. S. *Curr. Opin. Colloid In.* **2002**, *7*, 456.

(47) Lucey, J.; Singh, H. *Food Res. Int.* **1997**, *30*, 529.

(48) Zhong, Q.; Daubert, C. R.; Velev, O. D. *Langmuir* **2004**, *20*, 7406.

(49) Song, F.; Zhang, L.-M.; Shi, J.-F.; Li, N.-N. *Colloid. Surface. B* **2010**, *79*, 142.

(50) Olden, K.; Yamada, K. M. *Anal. Biochem.* **1977**, *78*, 483.

(51) Chrambach, A.; Rodbard, D. *Science* **1971**, *172*, 440.

(52) Rickwood, D. a. H., B.D. *Gel Electrophoresis of Proteins: A Practical Approach;* IRL Press: Oxford, 1981.

(53) Grattoni, C. A.; Al-Sharji, H. H.; Yang, C.; Muggeridge, A. H.; Zimmerman, R. W. *J Colloid. Interf. Sci.* **2001**, *240*, 601.

(54) Desai, S.; Dworecki, B.; Cichon, E. *Anal. Biochem.* **2001**, *297*, 94.

(55) Burridge, K. *P. Natl Acad. Sci.* **1976**, *73*, 4457.

(56) Adair, W. S.; Jurivich, D.; Goodenough, U. W. *J. Cell Biol.* **1978**, *79*, 281.

(57) Anderson, R. G.; Vasile, E.; Mello, R. J.; Brown, M. S.; Goldstein, J. L. *Cell* **1978**, *15*, 919.

(58) Dussy, F. E.; Berchtold, C.; Briellmann, T. A.; Lang, C.; Steiger, R.; Bovens, M. *Forensic Sci. Int.* **2008**, *177*, 105.

(59) Miller, R. T.; Grantham, R.; Lockett, B.; Temple-Camp, C.; Pang, J. *Am. J. Foren. Med. Path.* **2002**, *23*, 321.

(60) Viel, G.; Nalesso, A.; Cecchetto, G.; Montisci, M.; Ferrara, S. D. *Forensic Sci. Int.* **2009**, *193*, 79.

(61) Cingolani, M.; Cippitelli, M.; Froldi, R.; Gambaro, V.; Tassoni, G. *J. Anal. Toxicol.* **2004**, *28*, 16.

(62) Baeyens, W.; Schulman, S.; Calokerinos, A.; Zhao, Y.; Garćia Campaña, A. M.; Nakashima, K.; De Keukeleire, D. *J. Pharmaceut. Biomed.* **1998**, *17*, 941.

(63) Dodeigne, C.; Thunus, L.; Lejeune, R. *Talanta* **2000**, *51*, 415.

(64) Xiong, X.; Ouyang, J.; Xiong, X.; Zhai, S. *TrAC Trend. Anal. Chem.* **2009**, *28*, 961.

(65) Miller, R. T. In *Society for Applied Immunohistochemistry, 2001 Annual Meeting.* <http://www. ihcworld. com/_books/Technical-IHC. Pdf>

(66) Alegria-Schaffer, A.; Lodge, A.; Vattem, K. *Method. Enzymol.* **2009**, *463*, 573.

(67) Wisdom, G. B. In *The Protein Protocols Handbook;* 3rd ed.; Walker, J. M., Ed.; Humana Press: New York, 2009.

(68) Ebejer, K. A.; Lloyd, G. R.; Brereton, R. G.; Carter, J. F.; Sleeman, R. *Forensic Sci. Int.* **2007**, *171*, 165.

(69) Ebejer, K. A.; Winn, J.; Carter, J. F.; Sleeman, R.; Parker, J.; Körber, F. *Forensic Sci. Int.* **2007**, *167*, 94.

(70) Bohannon, J. *Science* **2007**, *316*, 3.

(71) Moss, J., M. Sc. Thesis, University of Bristol, UK, 1997.

(72) Sleeman, R.; Burton, F.; Carter, J.; Roberts, D.; Hulmston, P. *Anal. Chem.* **2000**, *72*, 397A.

CHAPTER 5

A comparison of four different approaches for the functionalisation of gold nanoparticles with anti-cocaine antibody

This chapter describes a comparative study of four different approaches for the functionalisation of gold nanoparticles with anti-cocaine antibody *via* ligand or protein intermediates.

5.1 Introduction

5.1.1 Gold nanoparticles

Gold nanoparticles (AuNPs) have been known to mankind for many centuries. The technical use of colloidal gold as a pigment in glass dates back to as early as the 4th century AD.[1,2] The modern scientific understanding of colloidal gold began with the work of Michael Faraday in the 1850s. Faraday's studies of the reduction of gold chloride using phosphorous produced the first concoction of gold nanoparticles, and led to the initial recognition that the colour of the solution resulted from the minute size of the particles.[1,3,4] Since then, simplification of the routes of synthesis has enabled the production of gold particles in a range of different shapes and sizes in the nanometre range (1-100 nm) with a high degree of precision and accuracy.[5]

The synthesis of AuNP in both aqueous and organic solvents typically involves addition of a reducing agent to a solution of gold salt (such as gold chloride), which leads to the nucleation of Au ions to nanoparticles.[6] The presence of a stabilising agent, which is either absorbed or chemically bound to the surface of the AuNP, is required to lend an overall charge to the particle. This charge causes the AuNP to

repel one another and thereby remain colloidally stable in solution.[6] For the most common route of synthesis in aqueous solution, citric acid serves as both a reducing agent by triggering the nucleation of gold chloride, and a stabiliser by then absorbing onto the particles to provide a net negative charge.[7,8] Similar routes of synthesis can be used to produce particles of different geometries such as rods[9] and stars,[10] or a different combination of reducing and stabilising agents can be applied to enable synthesis of the particles in organic solvents.[11]

The defining feature of all nanoparticles is their small size, and for inorganic nanoparticles this leads them to possess properties that are not found in bulk samples of the same material.[12] One of the most prominent features of gold particles in the nm size range is their capacity to strongly absorb and scatter visual light as a result of their characteristic surface plasmon resonance (SPR). SPR is the collective oscillation of free electrons on the surface of AuNP in response to excitation caused by the absorption of energy from light.[13] Change in the shape or size of the nanoparticle causes a shift in the electric field density on its surface, and this in turn leads to a change in the frequency of the oscillation.[14] As a result, AuNP of different shapes and sizes can be differentiated based on their optical properties, such as absorbance at higher or lower wavelengths for larger or smaller particles, respectively.[6,14] This phenomenon is responsible for the characteristic brightly coloured solutions of AuNP which makes them so attractive for labelling purposes.

Certain properties of AuNP can be readily modified by replacing the stabiliser molecule present on the surface of the particle after synthesis with a ligand of the desired specificities.[6] As thiol moieties bind with high affinity to gold surfaces *via* the formation of Au-sulfur bonds, thiol-modified ligands are most commonly used for such ligand-exchange reactions.[6,13] The choice of ligand offers flexibility in terms of the environment in which the AuNP can be applied. For example, the addition of a ligand with either a hydrophilic or hydrophobic terminal group would enable the particles to be soluble in either aqueous or organic solvents, respectively. Ligands of

this type can also be used to provide a scaffold onto which additional molecules, such as proteins, can be bound.[5]

AuNPs have become widely used in the field of analytical chemistry.[15] Certain properties of AuNPs make them particularly attractive for analytical applications. The bright and photo-stable colouration of AuNP, ranging from blue-green to a deep red in the visible spectrum, that results from their unique SPR can be exploited for the purpose of detection.[16] Resistance of AuNP to oxide formation lends stability under ambient conditions, thereby providing an inert metal scaffold onto which organic molecules can be bound.[6] Controlled and straightforward immobilisation of organic molecules directly onto the gold surface is possible either by employing gold-sulfur bonds, or *via* the use of a ligand as mentioned in the previous paragraph.[17] This enables AuNP to be readily functionalised for a wide range of different purposes.

5.1.2 Antibody functionalisation of gold nanoparticles

The advantageous characteristics of AuNPs are commonly exploited in the form of bio-functionalised particles, which can be assembled by encapsulating the AuNP in a coating of biological material such as DNA,[18] protein[19] or lectin.[17] The addition of such biomolecules enables AuNPs to be specifically directed to targets of interest *via* molecular recognition.[3] The earliest examples of such surface functionalised AuNP were the AuNP-antibody conjugates developed in the 1980s for immunostaining in electron microscopy.[3] The immunological specificity of the antibody in combination with the electron density of the AuNP made antibody functionalised AuNP particularly attractive for such applications.[15] Since then, a wide variety of different methods ranging from immunostaining within cells through to immunoassays have been developed which similarly harnass the molecular recognition of the antibody and combine this with the unique visual properties of the AuNP for detection.[6,13,20] In recent developments, antibody-functionalised AuNP have been applied for a variety of applications including biological staining, disease diagnosis and cancer therapy.[16]

Although the most commonly exploited feature of AuNP for application in immunodetection is their unique colorimetric properties, another applicable characteristic which is true for all metal nanoparticles is their high surface to volume ratio.[21] A large surface area is advantageous for the purpose of analytical detection. The binding of an antibody-functionalised AuNP to its target analyte, followed by the addition of a fluorescent marker or enzyme label yields a signal that is 10-fold greater than when only a single labelling molecule is bound.[22] This signal amplification property makes functionalised AuNP particularly useful for the detection of small compounds, afforded by the molecular specificity of the antibody, in samples containing low concentrations of analyte.[20] In combination with the other advantages of using AuNP, this makes antibody-functionalised AuNP particularly useful for the detection of small compounds, such as drugs, at the low concentrations present in samples of forensic interest. Antibody-functionalised AuNP are of particular interest in this study due to their previous application by Leggett *et al.* for the purpose of detecting nanogram quantities of cotinine, the metabolite of the drug nicotine, in fingerprints.[23] Hazarika *et al.* reported a similar technique involving antibody-functionalised magnetic particles for the detection of metabolites from various illicit substances, notably including that of cocaine.[24] It is based on this principle that this study aims to produce a successful variety of antibody-functionalised gold nanoparticles for the purpose of detecting cocaine.

The functionalisation of AuNP with antibody can be performed using a number of different approaches.[5] The simplest of these involves the non-specific physical absorption of the antibody directly onto the gold surface *via* hydrophobic interactions, as described by Natan *et al.*.[25] Such particles have been used for many different applications such as for the staining of macrophage cells using a specific targeting antibody,[17] as a labelled reporter antibody in a chemiluminescence-based immunoassay system,[19] and as the solid phase in a similar immunoassay-based method.[18] However, it has been reported that addition of the antibody *via* direct absorption could compromise the target specificity offered by the functionalised AuNP due to variations in the integrity of the antibody and orientation of the

binding.[26] Instead, other methods have been developed based on the encapsulation of the AuNP in a monolayer of protein or functionalised ligand onto which the antibody can be covalently bound. This not only introduces potential for more oriented and reliable binding of the antibody, but can also enhance particle solubility and stability depending on the nature of the protein or ligand used.[26-29]

5.1.3 Aim of the research presented in this chapter

A number of strategies have been reported for the covalent binding of antibodies onto a gold surface by way of a ligand intermediate.[3,26] The most robust form of anchorage onto gold was reported by Giersig and Mulvany as that provided by thiolated ligands, which exploit the strong gold-sulfur bond formed between a soft acid (gold) and an equally soft thiolate base.[30] Because of this, the use of a heterobifunctional ligand with a thiol group at one terminus and a functional group for binding of the antibody at the other has proven advantageous for this purpose. In this study, four different approaches involving heterobifunctional ligands were used for the covalent binding of anti-cocaine antibodies onto the surface of AuNP.

In the first of these approaches, a thiolated PEG ligand with terminal carboxyl functionality (PEGCOOH) was employed for the addition of antibody to the surface of AuNP. As described by Mason *et al.*, PEGCOOH can easily be self-assembled onto citrate-stabilised AuNP and in doing so provides increased solubility in aqueous solvents.[31] Activation of the -COOH functional group using the well-documented EDC/NHS method enables covalent binding of the antibody *via* any one of a number of exposed amine residues,[32] as illustrated in **Figure 5.1A**. AuNP functionalised with antibody using this type of approach are highly stable in aqueous buffers and relatively straightforward to assemble. Their synthesis has been reported for a number of different applications including as part of an immunoassay for the signal enhancement of HRP,[33] and for the detection of fingerprints by the targeting of specific amino acids.[34] Due to the number of available binding sites on the antibody, functionalisation using this approach results in randomly bound antibody in a variety

of different orientations. As described by Puertas *et al.*, this can lead to an antibody positioned in a manner in which the FAb region of the antibody is not available for antigen binding.[35] In this study, a comparison was made between this approach and more directed methods of functionalisation to determine the effect of the antibody orientation on the efficacy of the functionalised AuNP.

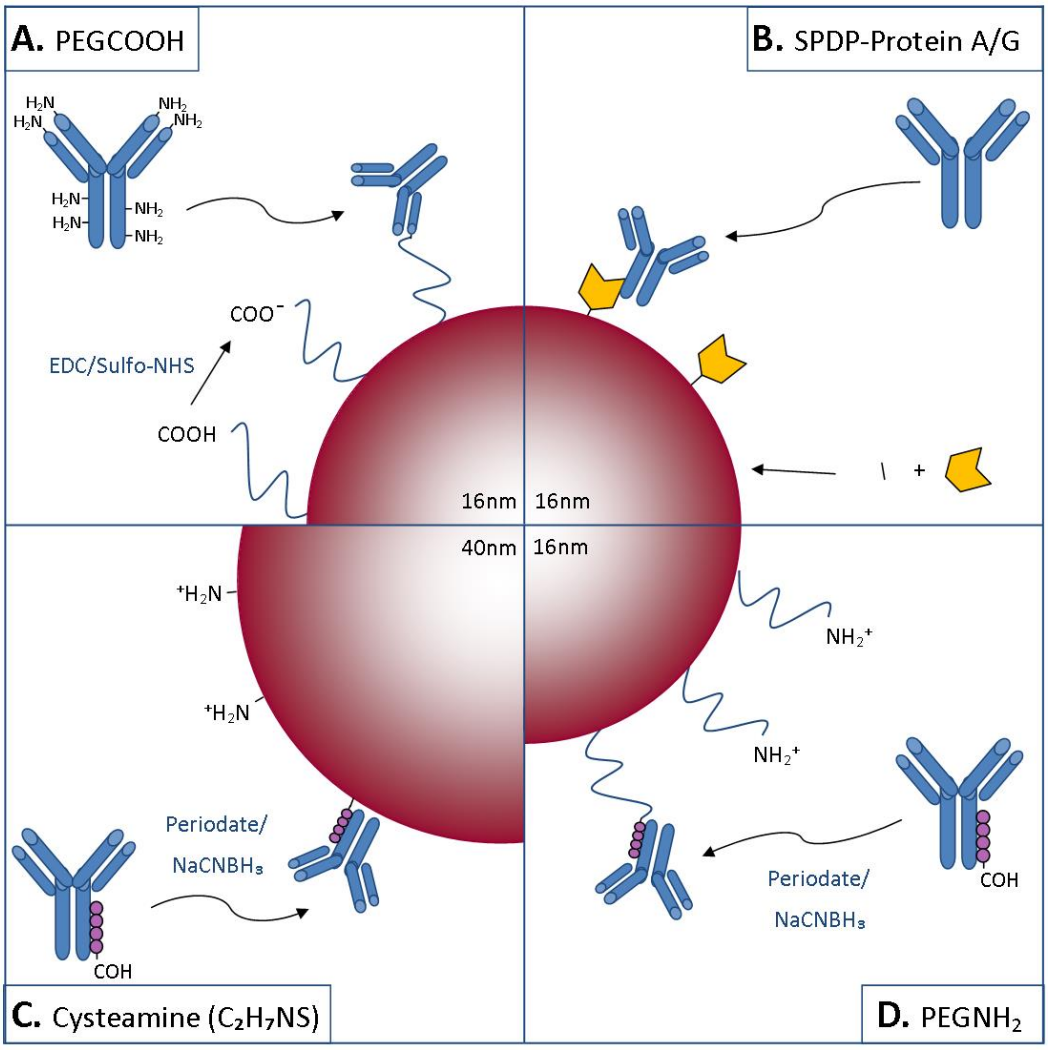

Figure 5.1. The four different strategies for immobilising antibodies onto AuNP used in this study. The use of a PEGCOOH linker is shown in (A), Protein A/G modified with an SPDP linker in (B), a short cysteamine linker in (C) and a $PEGNH_2$ linker in (D). The antibody is represented in blue and the Protein A/G in yellow.

In the second approach used for the antibody-functionalisation of AuNP, oriented binding of the antibody was achieved *via* a protein intermediate covalently coupled to the surface of the AuNP. Bacterial IgG-binding proteins, such as Protein A and Protein G, are commonly employed for the oriented binding of antibody, as their preferential binding of the Fc region leads to presentation of the FAb region for optimal antigen binding.[26,36] As mentioned, Leggett *et al.* successfully detected drug metabolites in latent fingerprints using antibody-functionalised AuNPs with an SPDP modified Protein A intermediate.[23] In this study, the chimeric Protein A/G, a fusion protein which offers four IgG binding sites with the combined binding specificities of both Proteins A and G, was used.[36] The Protein A/G was immobilised onto the surface of the AuNP by way of a heterobifunctional SPDP ligand as described by Leggett *et al.*, the structure of which is shown in **Figure 5.2**. The addition of SPDP to Protein A/G was facilitated by the lysine groups of the protein in combination with the NHS ester on the ligand. Once conjugated to the Protein A/G, the disulfide group of the SPDP was then immobilised onto the gold. By exploiting the interaction between Protein A/G and the Fc region of the antibody, this approach to functionalisation orients the antibody so that the antigen-binding region is directed away from the gold. This directed binding of the anti-cocaine antibody *via* Protein A/G is illustrated in **Figure 5.1B**.

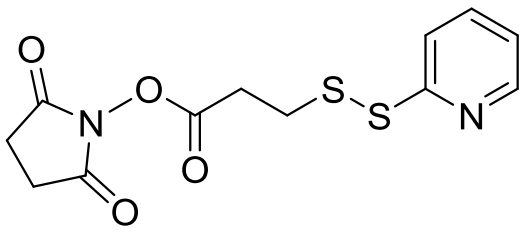

Figure 5.2. The structure of the *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) bi-functional linker.

Directed antibody binding can also be performed using a ligand with an amine functional group, as described by Puertas *et al.*.[35] In this method binding is achieved *via* the polysaccharide moiety located on the Fc region of the antibody, resulting in presentation of the FAb region for antigen binding in a similar manner as obtained using Protein A/G. In this method the aldehyde on the polysaccharide moiety is first oxidised before being added to the amine-modified particles. The oxidised antibody forms a Schiff base intermediate in the presence of the aminated ligand on the particle which, when reduced, forms a strong bond between antibody and ligand. AuNP functionalised with antibody using an aminated ligand have previously been synthesised by Kumar *et al.* for the imaging of intracellular biomarkers.[37] Niidome *et al.* report the functionalisation of 40 nm aminated AuNP, synthesised by sodium borohydride reduction using a short cysteamine linker, with DNA using a similar method.[38] In the third method for functionalisation used in this study, the synthesis of cysteamine-stabilised AuNP, as described by Niidome *et al.*, was combined with the antibody functionalisation method of Puertas *et al.* for the functionalisation of AuNP with anti-cocaine antibody.[35,38] A schematic of this approach is shown in **Figure 5.1C**.

As 40 nm cysteamine-AuNP are charge-stabilised by only a short linker, it was thought that the length of the cysteamine may generate AuNP of insufficient solubility in aqueous solutes and at the conditions required for antibody attachment. Because of this, in addition to antibody functionalisation of 40 nm cysteamine-AuNP using the method shown in **Figure 5.1C**, 16 nm citrate-stabilised AuNP (cAuNP) with an aminated PEG linker ($PEGNH_2$) were also synthesised and functionalised *via* the polysaccharide moiety of the antibody as a means of comparison. This fourth approach to functionalisation is described in **Figure 5.1D**.

In this chapter, AuNP functionalised with anti-cocaine antibody using the four different approaches described were compared in terms of antibody orientation, particle stability and solubility. UV-Vis spectrophotometry and transmission electron microscopy (TEM) were first used to characterise the AuNPs after synthesis. After functionalisation of the AuNP, the presence of anti-cocaine antibody on the particle

surface, and the overall cocaine-binding efficiency of the functionalised AuNP were determined by means of gel electrophoresis, enzyme-labelled antigen and application in a plate-based immunoassay. Based on the characterisation of each type of functionalised AuNP, the most effective functionalisation approach was selected for the production of anti-cocaine antibody-functionalised AuNP. The anti-cocaine particles were produced for eventual use for the detection of cocaine on forensic samples such as banknotes.

5.2 Results and discussion

5.2.2 Synthesis of gold nanoparticles

16 nm cAuNP were synthesised according to the method of Turkevich *et al.*,[7] as described in **Section 2.4.1**. The particles, typically a deep red colour, absorb light in the visible spectrum with a maximum at *ca.* 520 nm, yielding an absorption spectrum as shown in **Figure 5.3**. The concentration of the newly synthesised cAuNP shown in **Figure 5.3** was determined to be 3.35 nM using the extinction coefficient 2.4×10^8 M^{-1} cm^{-1}.[39] AuNP of 40 nm diameter were synthesised by sodium borohydride reduction in the presence of a cysteamine stabiliser using the method reported by Niidome *et al.*,[38] as detailed in **Section 2.4.2.** A similar absorption spectrum was produced as for the citrate-stabilised AuNP, but with an absorption maximum at around 528 nm. The concentration of the particles shown in **Figure 5.3** was found to be 0.24 nM using the extinction co-efficient 4.7×10^9 M^{-1} cm^{-1}.[40]

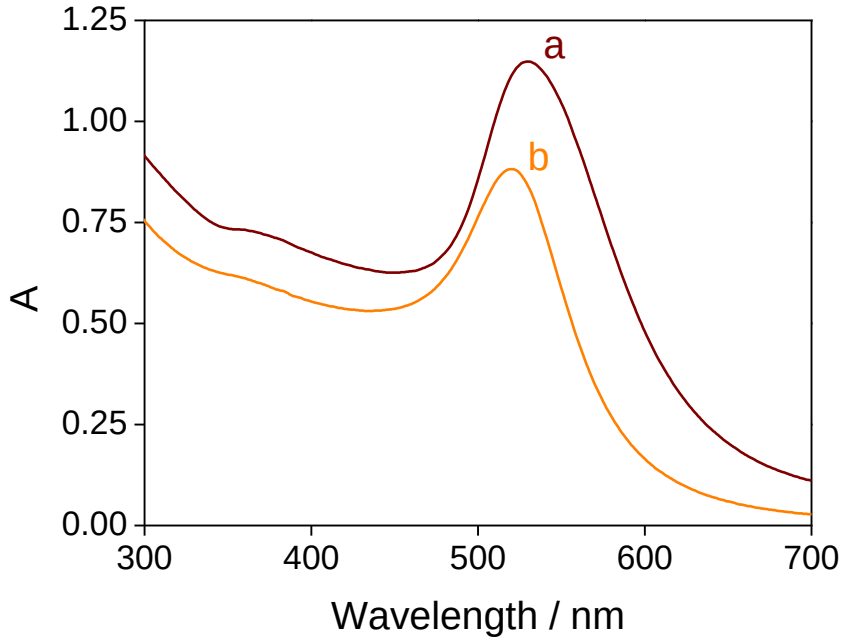

Figure 5.3. UV-visible absorption spectra of 40 nm cysteamine-stabilised AuNP (a) and 16 nm citrate-stabilised AuNP (b).

TEM was used in order to determine the size of the gold nanoparticles. The average diameter of 101 citrate-stabilised AuNP and 100 cysteamine-stabilised AuNP were measured from the TEM images shown in **Figure 5.4**. The size distribution for each particle type is shown in **Figure 5.5**. The calculated average diameter of the cAuNP and cysteamine AuNP was 15.6 ± 2.1 nm and 37.4 ± 4.1 nm, respectively. Of the two particle types, the larger cysteamine AuNP showed the greatest size variation when compared with the smaller cAuNP.

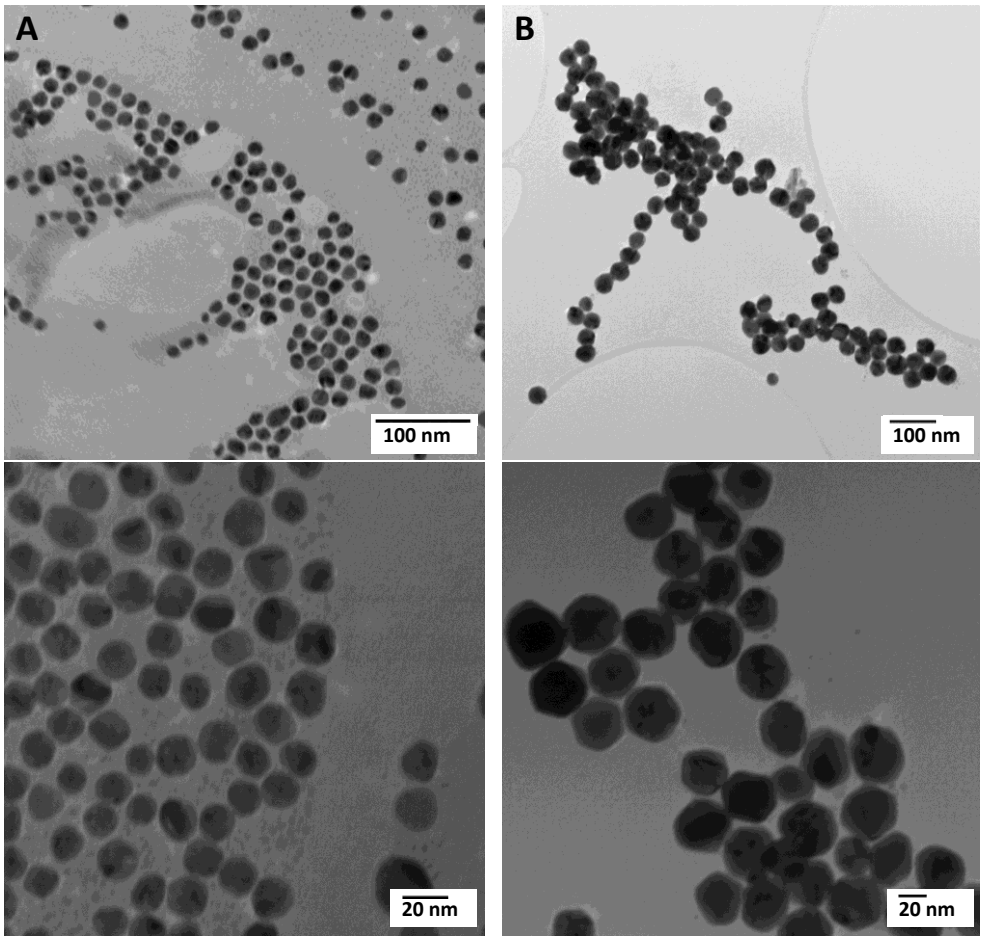

Figure 5.4. TEM images of the citrate-stabilised AuNP (A) and cysteamine-stabilised AuNP (B), taken at 40,000x (top images) and 120,000x (bottom images) magnification.

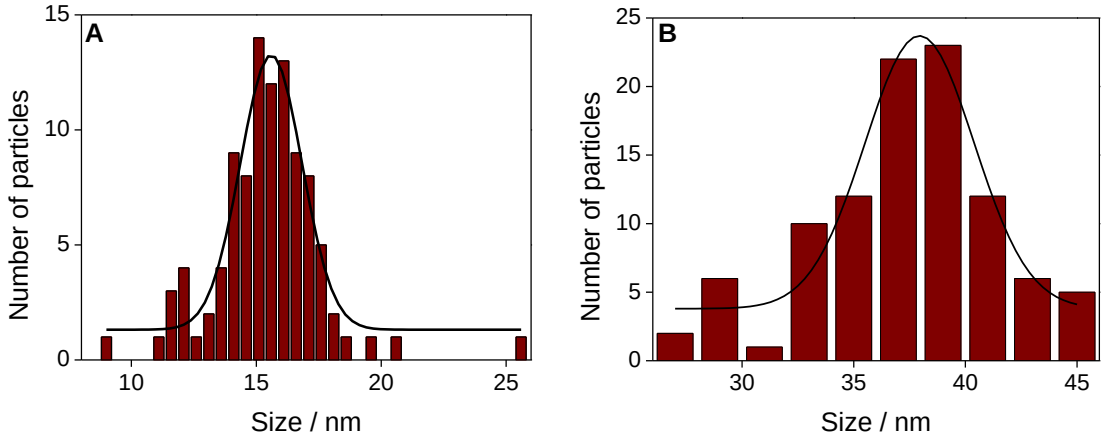

Figure 5.5. The size distribution of 101 citrate-stabilised AuNP (A) and 100 cysteamine-stabilised AuNP (B).

5.2.3 Antibody functionalisation of the AuNPs

The addition of antibody onto the surface of AuNPs was performed in this study using the four different approaches shown in Figure 5.1. For the first of these, antibody functionalised cAuNP with a PEGCOOH linker were prepared using a modified version of the protocol described by Mason *et al.*.[31] The self-assembly of a PEGCOOH monolayer around the particles was facilitated by the formation of gold-sulfur bonds between the surface of the cAuNP and the thiol terminal group of the ligand. This was followed by the EDC/NHS activation of -COOH functional groups on the PEG, which in turn enabled the addition of antibody *via* the formation of amide bonds. Purification of the AuNP-PEGCOOH-antibody was performed by centrifugation. The modification of the surface of the cAuNP surface, in this case by the addition of PEGCOOH and antibody, was indicated by a red-shift in the absorption maximum by approximately 2 nm per addition (from 520 to 522 nm) of PEG or protein. The increase in wavelength of the extinction maximum is indicated in the UV-Vis absorption spectra of the AuNP, and enables each step in the functionalisation process to be monitored. Figure 5.6 shows typical examples of absorption spectra for the step-wise addition of PEGCOOH and antibody to the surface of the cAuNP. AuNP functionalised using PEGCOOH proved to be highly stable in aqueous buffered solutions, and generated a good yield of antibody-functionalised AuNP as illustrated in Figure 5.6.

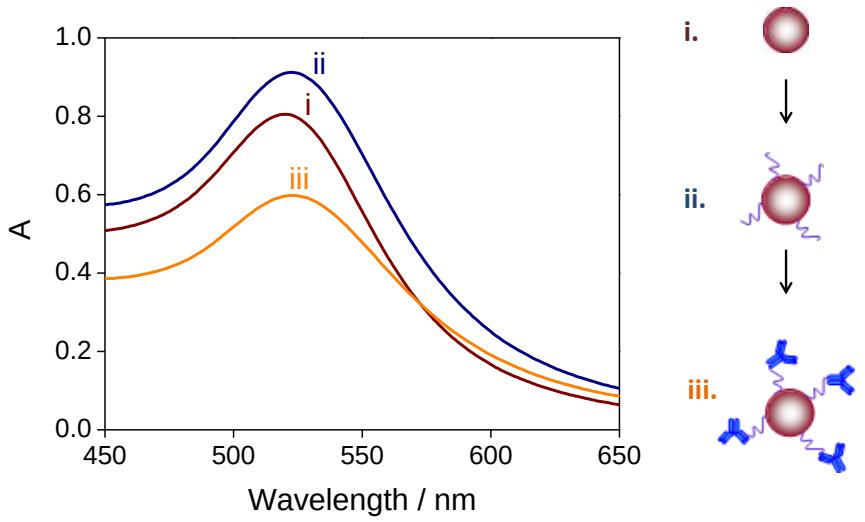

Figure 5.6. UV-VIS absorption spectra of the stages of antibody functionalisation of 16 nm AuNP *via* a PEGCOOH linker, as indicated by the numbers i-iii in the schematic shown. The different particle types and their absorption maxima at around 520 nm are as follows: (i) Citrate AuNP (520 nm), (ii) AuNP-PEGCOOH (2x concentration; 522 nm) and (iii) AuNP-PEGCOOH-antibody (524 nm).

For the second approach used in this study, 16 nm cAuNP were functionalised with antibody by way of an aminated PEG ligand. The method used for the addition of an antibody to an amine-functionalised ligand was based on that applied by Puertas *et al.* to aminated magnetic particles.[35] In this approach, binding of the antibody to the ligand occurs *via* an aldehyde on the polysaccharide moiety of the Fc region of the antibody, as detailed in **Section 5.1.3**. Antibody functionalisation was performed as described in **Chapter 2.4.5**. A $PEGNH_2$ monolayer was first self-assembled onto the surface of the cAuNP. The aldehyde group on the polysaccharide region of the antibody was then oxidised by sodium periodate before being applied to a solution of the AuNP-$PEGNH_2$. The resulting antibody-functionalised AuNP-$PEGNH_2$ were purified by glycerol gradient using a modified version of the approach described by Karthik *et al.*.[41] The reaction between the oxidised aldehyde of the antibody and the amine of the AuNP-PEG resulted in the formation of a Schiff base intermediate. This intermediate was reduced by incubation with sodium cyanoborohydride to form a

strong covalent bond between linker and antibody, after which the resulting AuNP-$PEGNH_2$-antibody were purified once again by glycerol gradient as previously described.

The increase in the absorption maxima following the addition of both the $PEGNH_2$ and the antibody are indicated by the UV-Vis absorption spectra shown in **Figure 5.7**. As shown, the extinction maximum typically increased by 2 – 3 nm following each step in the functionalisation process, indicating the successful modification of the AuNP surface. The newly synthesised antibody-functionalised AuNP-$PEGNH_2$ showed relatively good stability in aqueous buffered solutions. The concentration of the antibody-functionalised AuNP obtained was lower than that of the AuNP-PEGCOOH-antibody. This was indicated by the absorption maximum of 0.468 obtained for the AuNP-$PEGNH_2$-antibody (shown in **Figure 5.7**(iii)), which was lower than the absorption maximum of 0.631 obtained for the AuNP-PEGCOOH-antibody shown in **Figure 5.6** (iii). The AuNP-$PEGNH_2$-antibody were prone to adhering to both glass and plastic sample vials, although this was lessened with the addition of milk buffer as a stabiliser in the final stage of functionalisation, as described in **Chapter 2.4.5**.

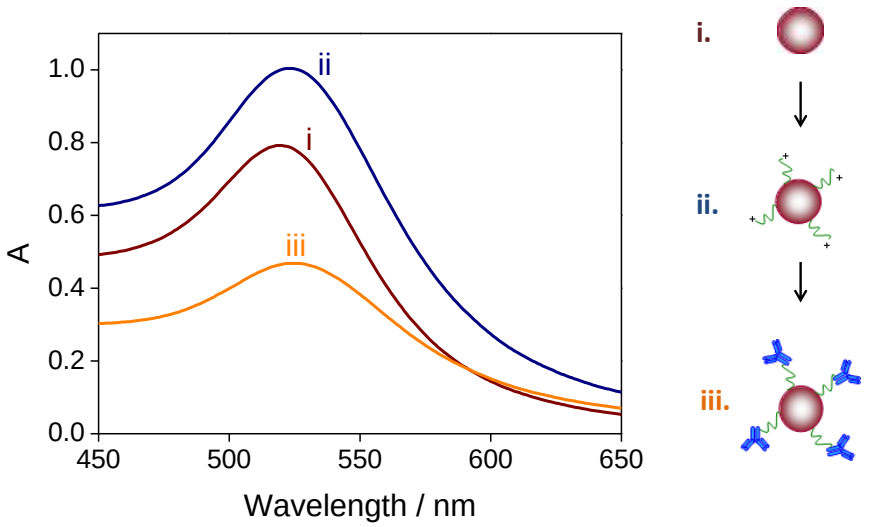

Figure 5.7. UV-VIS absorption spectra of the stages of antibody functionalisation of 16 nm AuNP *via* a $PEGNH_2$ linker, as indicated by the numbers i-iii in the schematic shown. The different particle types and their absorption maxima at around 520 nm are as follows: (i) Citrate AuNP (520 nm), (ii) AuNP-$PEGNH_2$ (2x concentration; 523 nm) and (iii) AuNP-$PEGNH_2$-antibody (525 nm).

In the third approach used for the immobilisation of antibody onto the surface of 16 nm cAuNP in this study, a modified Protein A/G intermediate was employed as a means of facilitating antibody binding (**Figure 5.1**). Functionalisation was performed using a modified version of the Protein A-based method reported by Leggett *et al.*,[23] as described in **Chapter 2.4.6**. Protein A/G was first modified to include a disulfide group *via* the addition of an SPDP bi-functional linker. The succinimidyl ester portion of the SPDP chemically reacts with the primary amine groups present on Protein A/G when the two are mixed. The SPDP-Protein A/G was purified by gel filtration, after which the presence of the SPDP was determined in the column eluent by the addition of a DTT solution as described by Carlsson *et al.*.[42] DTT was used to facilitate the release of 2-thiopyridine from the 2-pyridyl disulfide groups present on the SPDP-modified proteins. The release of 2-thiopyridine was confirmed by the appearance of a characteristic absorption band at 343 nm which is not seen in the absorption spectrum of the thiolated protein, as shown in **Figure 5.8**.

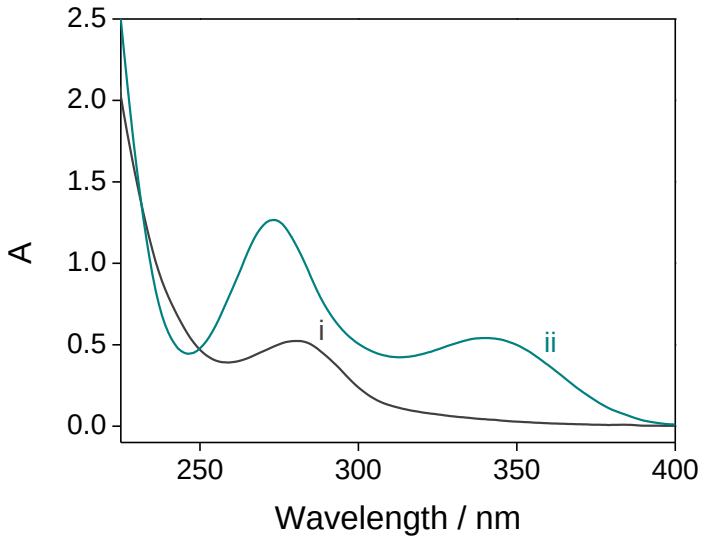

Figure 5.8. The UV-Vis absorption spectra of a solution of SPDP-Protein A/G before (i) and after (ii) treatment with DTT. The characteristic absorption band of 2-thiopyridine is present at 343 nm in (ii) only.

The concentration of the SPDP modified Protein A/G in the eluent was not determined by the absorbance intensity at 280 nm due to the inherent absorption of the 2-pyridyl disulfide groups of the SPDP at that wavelength.[42] Instead, the concentration was determined as that of the released pyridine-2-thione, based on the 1:1 ratio of separated 2-thiopyridine to 2-pyridyl disulfide present in the thiolated protein reported by Stuchbury *et al.*.[43] For this, the extinction coefficient 8.08 x 10^3 M^{-1} cm^{-1} and the absorbance intensity at 343 nm were applied.[42,43] Based on the absorbance intensity of 0.538 at 343 nm shown in **Figure 5.8**, a typical SPDP modified Protein A/G concentration of 66.58 µM was obtained.

The modified Protein A/G was self-assembled onto the surface of 16 nm cAuNP in the same manner as for the AuNP-PEGCOOH and AuNP-$PEGNH_2$, as described in **Chapter 2.4.6**. The AuNP-Protein A/G were then incubated with a solution of antibody, during which the antibody was immobilised onto the AuNP surface by the preferential binding of the Protein A/G to the Fc region of the antibody. The increase

in the absorption maximum following the addition of both the SPDP-Protein A/G and the antibody is indicated by the UV-Vis absorption spectra shown in **Figure 5.9**. Successful modification of the AuNP surface was indicated by a typical increase in the extinction maximum of around 3 nm following each successive addition of protein. The AuNP-Protein A/G proved stable in aqueous buffered solutions both before and after addition of the antibody, and yielded a higher concentration of particles than that produced using the PEGCOOH approach to functionalisation. This was indicated by the absorption maximum of 0.760 obtained for the AuNP-Protein A/G-antibody (shown in **Figure 5.9** (iii)), which was higher than the absorption maximum of 0.631 obtained for the AuNP-PEGCOOH-antibody shown in **Figure 5.6** (iii).

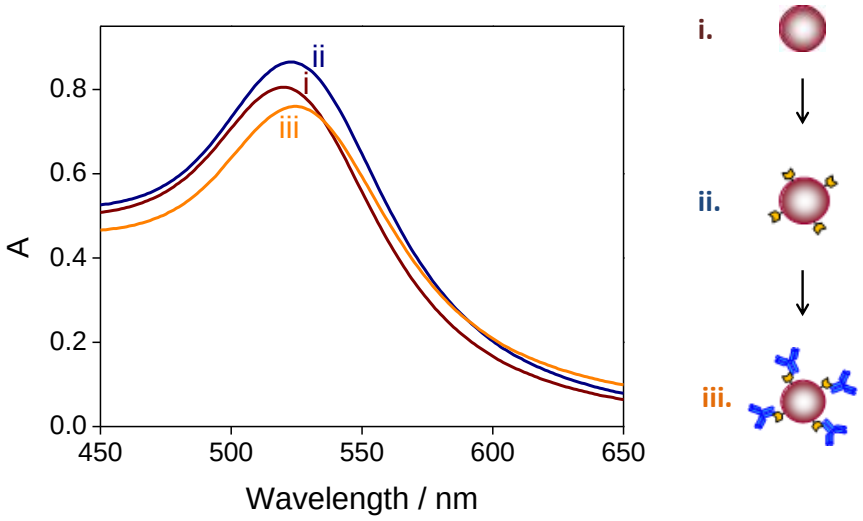

Figure 5.9. UV-VIS absorption spectra of the stages of antibody functionalisation of 16 nm AuNP *via* an SPDP modified Protein A/G intermediate, as indicated by the numbers i-iii in the schematic shown. The different particle types and their absorption maxima at around 520 nm are as follows: (i) Citrate AuNP (520 nm), (ii) AuNP-Protein A/G (1.5x concentration; 523 nm) and (iii) AuNP-Protein A/G-antibody (526 nm).

The final approach for the addition of antibody to the surface of AuNP used in this study was performed using 40 nm AuNP stabilised by a cysteamine ligand. The functionalisation of these AuNPs occurred through the binding *via* the polysaccharide moiety of the Fc region of the antibody to the amine functional group of the ligand, as used for the fuctionalisation of AuNP-PEGNH_2 (described in **Chapter 2.4.5**). The resulting AuNP-antibody were purified using a modified approach to the one reported by Karthik *et al.*[41]. Prior to purification it was observed that the colour of the AuNP solution changed from deep red to purple, an indication that the particles had become aggregated. During purification by glycerol gradient (as described in **Chapter 2.4.5**) the majority of the AuNP became insoluble and adhered to the wall of the centrifuge tube. Aggregation of the particles was confirmed by the UV-Vis absorption spectra shown in **Figure 5.10**. Similar results were obtained during repeated experiments. Because of the instability of the 40 nm cysteamine-AuNP upon addition of the antibody, the application of this type of AuNP was discontinued at this point. Nanoparticle characterisation was continued for the three remaining methods of functionalisation.

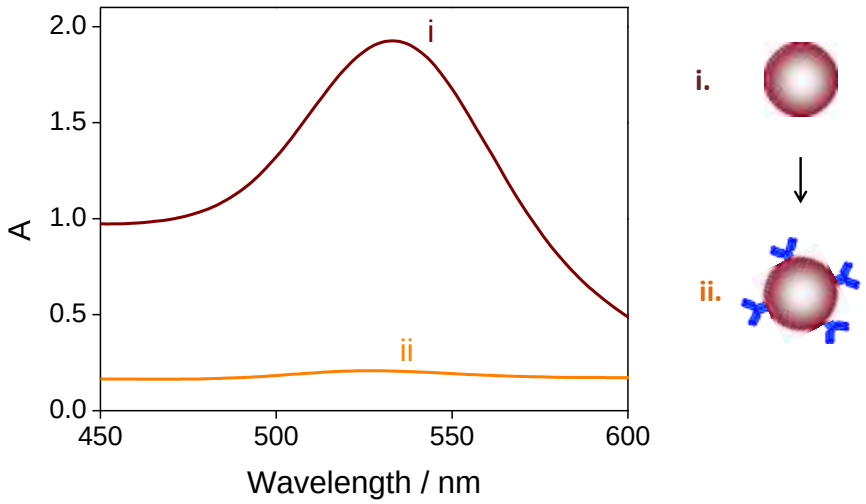

Figure 5.10. UV-VIS absorption spectra of the stages of antibody functionalisation of 40 nm cysteamine-stabilised AuNP, as indicated by numbers i and ii in the schematic shown. The particle types and their absorption maxima at around 520 nm are as follows: (i) Cysteamine AuNP (528 nm), and (ii) Cysteamine AuNP-antibody (aggregated).

5.2.4 Detection of bound antibody by SDS PAGE

As described in **Section 5.1.3.**, Puertas *et al.* reported the successful application of SDS-PAGE for determining the presence of antibody on the surface of functionalised magnetic particles.[35] A solution of SDS and mercaptoethanol was used to fragment antibodies present on the surface of the particles, and the resulting fragments were separated and visualised by SDS-PAGE as described in **Chapter 2.4.8**. The findings of Puertas *et al.* indicated that the fragmentation pattern of the antibody is dependent upon the orientation of the antibodies when bound to the particle.[35] A solution of free antibody treated with SDS and mercaptoethanol will fragment into two parts, a light chain and a heavy chain, which yield characteristic bands in an SDS-PAGE gel at around 20-30 and 40-60 kDa, respectively. A schematic of this fragmentation pattern is shown in **Figure 5.11A**. According to the research of Puertas *et al.*, although the fragmentation pattern of antibody does not change, the components of the antibody that are covalently bound to a particle will remain covalently bound and thus will not be observed in a gel.[35] For example, an antibody that is bound to the particle *via* its heavy chain will result in a gel with only the band from the light chain being present. This approach was applied to the three types of antibody-functionalised AuNPs used in this study. A schematic of the expected results of this study is shown in **Figure 5.11B**.

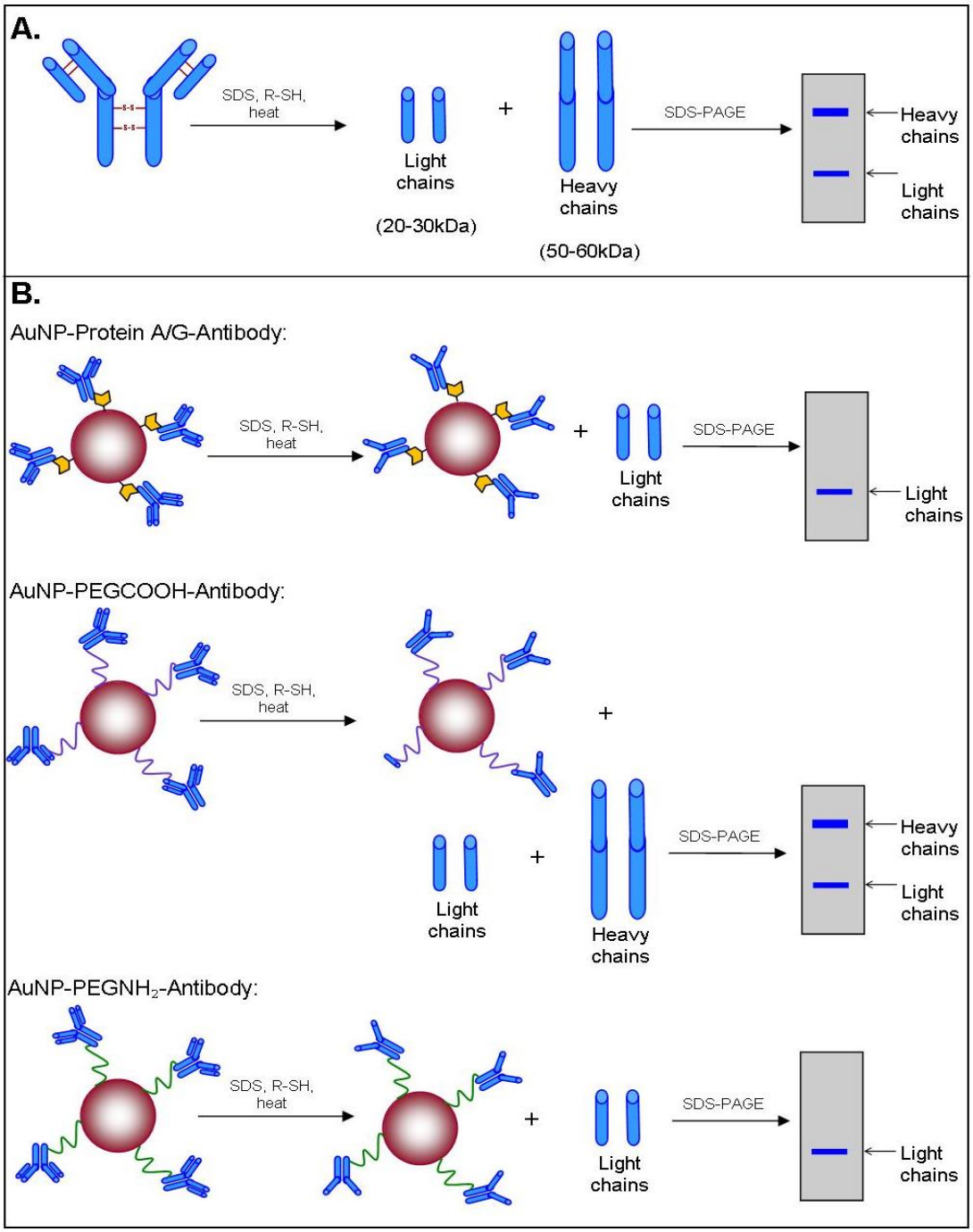

Figure 5.11. The characteristic fragmentation pattern of free antibody in an SDS-PAGE gel following treatment with SDS and mercaptoethanol is shown in (A). The fragmentation patterns of antibody in SDS-PAGE gels expected when the same SDS and mercaptoethanol treatment is applied to antibody immobilised onto the surface of AuNP using the three approaches are shown in (B). The expected results are based on those reported by Puertas *et al.*.[35]

A typical example of an SDS-PAGE gel of fragmented antibody from the surface of antibody-functionalised AuNP-PEGCOOH, prepared as described in **Chapter 2.4.4**, is shown in **Figure 5.12**. Free antibody in the lane B of the gel shown in **Figure 5.12** generated the characteristic two-band profile as predicted by the expected results in **Figure 5.11A**. The two characteristic bands of the antibody, at around 20-30 and 40-60 kDa respectively, are indicated by the arrows in **Figure 5.12**. The antibody fragments obtained from the AuNP-PEGCOOH-antibody (**Figure 5.12**, lane C) yielded two bands of approximately the same molecular weights as for the free antibody. No visible protein bands were present in the lane containing the AuNP-PEGCOOH negative control (**Figure 5.12**, lane A). The results obtained support the prediction illustrated in **Figure 5.11B** that, due to the random binding of the PEGCOOH to one of several available amine groups on the antibody, the orientation of the bound antibody is variable and thus both the heavy and the light chain fragments can be present in the gel. The bottom of the two characteristic antibody fragments relates to the light chain of the antibody and was seen to be weaker in the fragments obtained from the AuNP-PEGCOOH-antibody (**Figure 5.12**, lane C) than in the fragments obtained from the free antibody (**Figure 5.12**, lane B). This result suggests that binding of the antibody to the AuNP occurs preferentially *via* the light chain of the antibody, as this would result in a greater number fragments and therefore more intense staining of the band relating to the heavy chain fragments in the gel as indicated in **Figure 5.11B**. Overall, the presence of the characteristic bands of the antibody confirms the presence of antibody on the surface of the AuNP, thus proving the successful functionalisation of the AuNP.

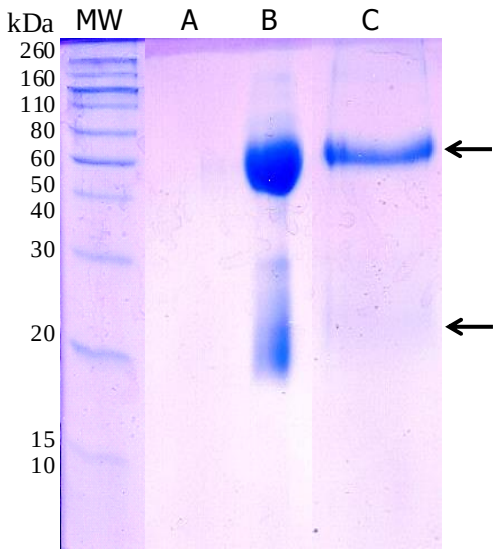

Figure 5.12. A typical 15 % acrylamide SDS-PAGE gel showing the protein fragments obtained from antibody-functionalised AuNP-PEGCOOH following treatment with SDS and mercaptoethanol (lane C). AuNP-PEGCOOH without antibody were run as a control in lane A alongside the molecular weight marker (MW), and a stock solution of free anti-cocaine antibody was run in lane B.

A typical SDS-PAGE analysis of fragmented antibody from AuNP-Protein A/G-antibody (prepared as described in **Chapter 2.4.6**) is shown in **Figure 5.13**. The characteristic fragmentation bands at 20-30 and 40-60 kDa of free antibody in lane A are indicated by two arrows in **Figure 5.13**. A single band at *ca*. 50 kDa was produced by the antibody fragmented from the AuNP-Protein A/G, a band which corresponds to the MW of the heavy chain of the antibody (**Figure 5.13**, lane C). The band does not relate to that of the AuNP-Protein A/G control in lane B of **Figure 5.13**, the MW of which (*ca*. 66 kDa) instead corresponds to that of free Protein A/G and is thought to have been the result of insufficient purification of the AuNP-Protein A/G.[36]

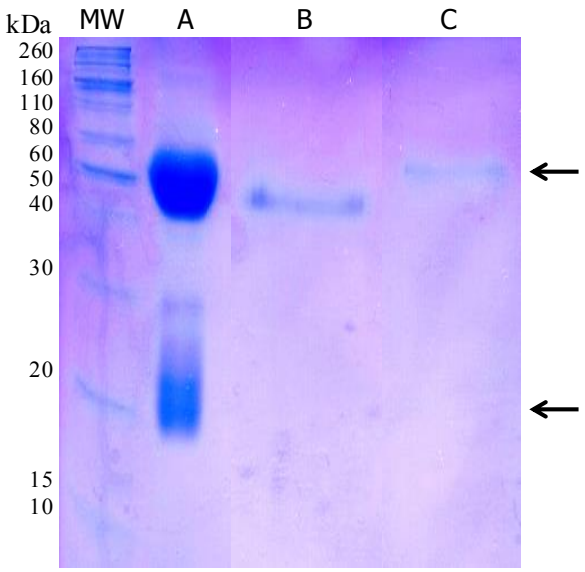

Figure 5.13. A typical 15 % acrylamide SDS-PAGE gel showing the protein fragments obtained from antibody-functionalised AuNP-ProteinA/G following treatment with SDS and mercaptoethanol (lane C). A stock solution of free anti-cocaine antibody was run in lane A alongside the molecular weight marker (MW), and AuNP-ProteinA/G were run as a control in lane B.

Based on the results shown in **Figure 5.13**, it was concluded that predominantly heavy chain antibody fragments had been dissociated from the AuNP-Protein A/G-antibody rather than light chains as predicted in **Figure 5.11B**. The presence of heavy chains in the gel requires immobilisation of the antibody to have occurred *via* the FAb fragment, instead of the Fc region required for optimal orientation of antibody onto the particle surface. This suggests that although Protein A/G binds preferentially to the Fc region of the antibody, other mechanisms of binding involving the FAb fragment of the antibody can occur. This phenomenon has been previously reported by Clarizia *et al.,* who showed that up to 50 % of Protein A/G interactions result in misalignment of the antibody.[44] An additional possibility is that light chain fragments in the gel resulting from antibody bound to Protein A/G *via* the Fc region were present, but at concentrations insufficient for staining to occur. Overall, the results were considered indicative of the presence of antibody bound to the surface of the

AuNP-Protein A/G, but the exact orientation of the antibody would require confirmation by additional methods of analysis.

Stabilisation of functionalised AuNP-PEGNH_2 with 1 % w/v milk buffer after addition of oxidised antibody (as described in **Chapter 2.4.5**) initially generated SDS-PAGE results from which the presence of antibody fragments could not be distinguished from the bands of the milk proteins. Functionalisation without milk buffer was attempted, but the resulting AuNP-PEGNH_2-antibody particles proved unstable and aggregated during purification (results not shown). SDS PAGE was subsequently performed using antibody-functionalised AuNP-PEGNH_2 following an additional wash step to remove excess milk buffer. The resulting SDS PAGE is shown in **Figure 5.14**, the lanes of which contain as follows; fragments from free antibody in lane A, a AuNP-PEGNH2 negative control in lane B, antibody fragments obtained from the AuNP-PEGNH_2-antibody in lane C and protein fragments obtained from milk buffer in lane D. The antibody fragments obtained from the AuNP-PEGNH_2-antibody (**Figure 5.14**, lane C) yielded an additional band which is not present in either the AuNP-PEGNH_2 or the milk buffer control in lanes B and D, respectively. The MW of the band (*ca*. 60 kDa) corresponds to the heavy chain fragment of the free antibody solution in lane A, as indicated by the top arrow in **Figure 5.14**, rather than the light chain fragment predicted in **Figure 5.11B**. The low concentration at which the heavy chain fragment is present in relation to the milk proteins makes it difficult to confirm the presence or absence of antibody functionalisation using this approach.

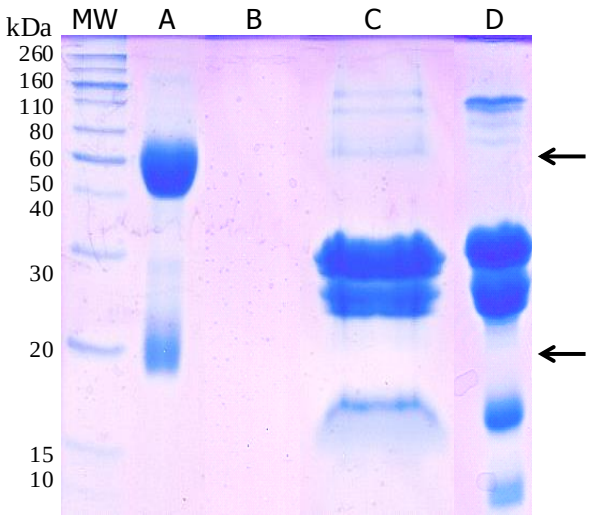

Figure 5.14. A typical 15 % acrylamide SDS-PAGE gel showing the protein fragments obtained from antibody-functionalised AuNP-PEGNH_2 following treatment with SDS and mercaptoethanol (lane C). A stock solution of free anti-cocaine antibody was run in lane A alongside the molecular weight marker (MW). AuNP-PEGNH_2 and a 0.5 % w/v solution of milk buffer in PB were run as controls in lanes B and D, respectively.

5.2.5 Visualisation of AuNP surface modification and size by agarose gel electrophoresis

In this study, cAuNP modified by the addition of proteins and ligands were separated from one another on the basis of size using 0.2 % w/v agarose gels as described in **Chapter 2.4.9**. The method used has been previously applied by Hanauer *et al.* for the separation of gold and silver nanoparticles on the basis of their size and shape.[45] Haneuer *et al.* noted that modification of the particle surface *via* the addition of a PEG monolayer led to increased particle stability and therefore improved band resolution in the gels.[45] This observation was exploited in this study as a means of visualising modification of the AuNP surface following the addition of either protein or ligand at each stage of the functionalisation process. The use of this approach was based on the prediction that each successive addition would incrementally increase the size of the particle, and thereby slow its migration through the gel. The predicted movement of the different types of modified and functionalised cAuNP is

schematically illustrated in **Figure 5.15**. Overall, it was expected that with each successive addition of ligand or protein the resulting band would be at a position further up the gel than the un-modified cAuNP. AuNP-$PEGNH_2$ were the exception to this rule, as the positive charge of the amine functional group under alkaline conditions would cause the particles to migrate upwards in the gel towards the negative rather than the positive electrode. Addition of antibody to the AuNP-$PEGNH_2$ would reverse the charge of the AuNP to an overall negative, resulting in a band in the gel at a similar position to the AuNP-PEGCOOH-antibody due to the equivalent size of the PEG ligand.

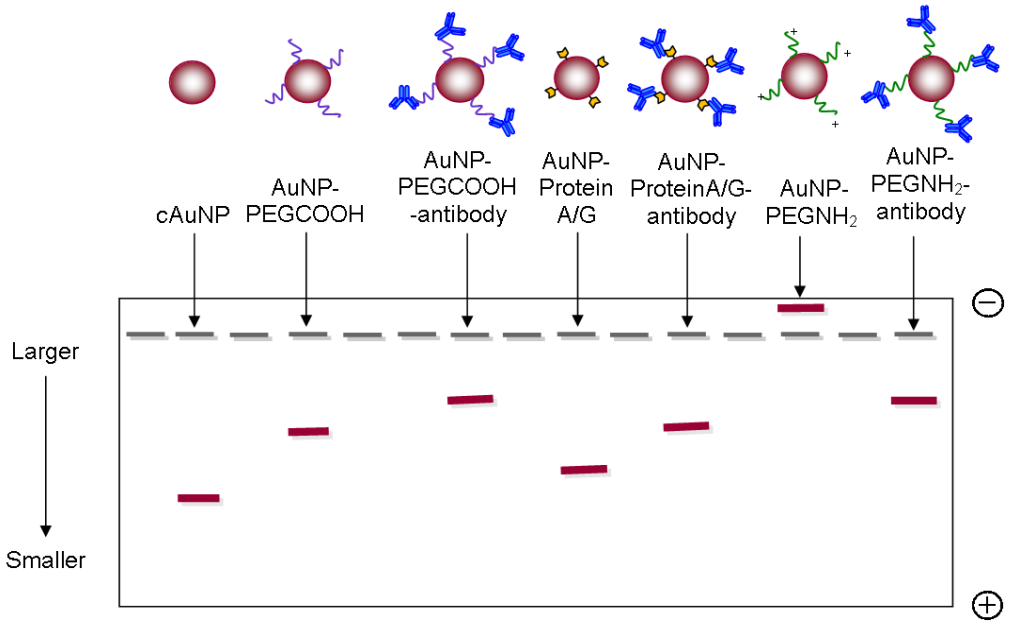

Figure 5.15. The expected separation of cAuNP and functionalised cAuNP by agarose gel electrophoresis, on the basis of increased size resulting from the addition of protein or ligand.

As illustrated by the typical agarose gel results shown in **Figure 5.16**, the band position of the AuNP following each successive addition of protein or ligand corresponded to the predicted migration in the gel shown in **Figure 5.15**. For each addition of protein or ligand, the overall size of the particle increased and its

migration through the gel was slowed sufficiently for the different stages in the functionalisation process to be separated. The characteristic burgundy colour of the AuNP, resulting from their surface plasmon resonance as described in **Section 5.1.1**, enabled visual recognition of the bands without the need for additional staining.

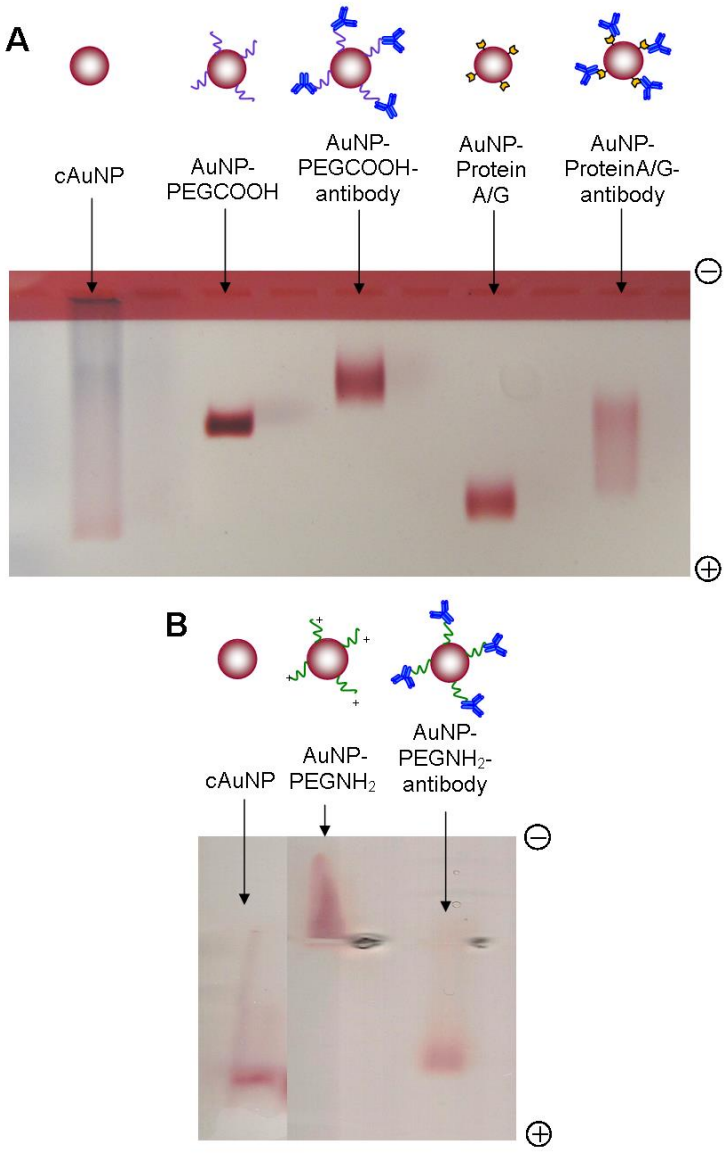

Figure 5.16. True colour photographs of 0.2 % w/v agarose gels of cAuNP at each stage of the functionalisation process. The step-wise addition of PEGCOOH, modified Protein A/G and anti-cocaine antibody to the surface of cAuNP are shown in (A), and the addition of $PEGNH_2$ and subsequently anti-cocaine antibody are shown in (B).

The results in **Figure 5.16A** in particular confirm the observation of Hanauer *et al.* that the addition of a ligand or protein to the cAuNP considerably improves the stability of the AuNP,[45] allowing them to migrate as a band through the gel rather than a streak. Separation on the basis of charge is also illustrated in **Figure 5.16B**, where negatively charged cAuNP can be distinguished from positively charged AuNP-PEGNH_2 by their migration towards different electrodes. The positive charge of the PEGNH_2 was reversed after the successful addition of antibody, causing the AuNP-PEGNH_2-antibody to migrate downwards in the gel towards the positive electrode as predicted in **Figure 5.15**. Overall, separation of AuNP based on the successive addition of protein or ligand to the surface of the particles by agarose gel electrophoresis proved successful in this study. The successful step-wise functionalisation of AuNP was visually confirmed using this method.

5.2.6 Estimating the number of bound antibody per AuNP using enzyme-labelled antigen

Enzyme labelled antigen (cocaine-HRP) was used to simultaneously label and quantify anti-cocaine antibody on the surface of functionalised AuNP. The advantage of using enzyme-labelled antigen over indirect antibody labelling (by way of secondary antibody, for example) is that it enables not only detection of the antibody, but also the specificity and efficacy of antigen binding by the functionalised AuNP to be measured. This was particularly important in this study due to the undefined cocaine-binding ability of the polyclonal antibody.

As described in **Chapter 2.4.10**, cocaine-HRP was applied to the antibody-functionalised AuNP, after which addition of enzyme substrate enabled the presence of bound HRP to be determined. Negative controls in the form of AuNP without antibody, in the case of AuNP-Protein A/G, or AuNP functionalised with BSA in place of antibody were used to distinguish any element of non-specific binding. The presence of cocaine-HRP bound to the antibody-functionalised AuNP was determined by the addition of enzyme substrate, and the resulting visible colour

change in the presence of HRP was measured by absorbance as described in **Chapter 2.4.10**. A standard curve of cocaine-HRP at concentrations ranging from 10 - 75 nM was used to determine the amount of the labelled antigen bound to the AuNP. From this, the relative number of antibody per AuNP was estimated based on an assumed 1:1 ratio of bound cocaine-HRP:antibody. Although a 2:1 ratio of cocaine-HRP:antibody is possible based on the two available antigen-binding sites on each antibody, the 1:1 ratio was used in this study in order to provide an estimate of the maximum possible number of antibodies present.

A typical standard curve used for determining the concentration of cocaine-HRP bound to the antibody-functionalised AuNP is shown in **Figure 5.17**. The absorbance spectra of the enzyme product obtained from the standard solutions are shown in **Figure 5.17A**, alongside the corresponding linear regression of the curve based on absorbance intensity at 450 nm in **Figure 5.17B**.

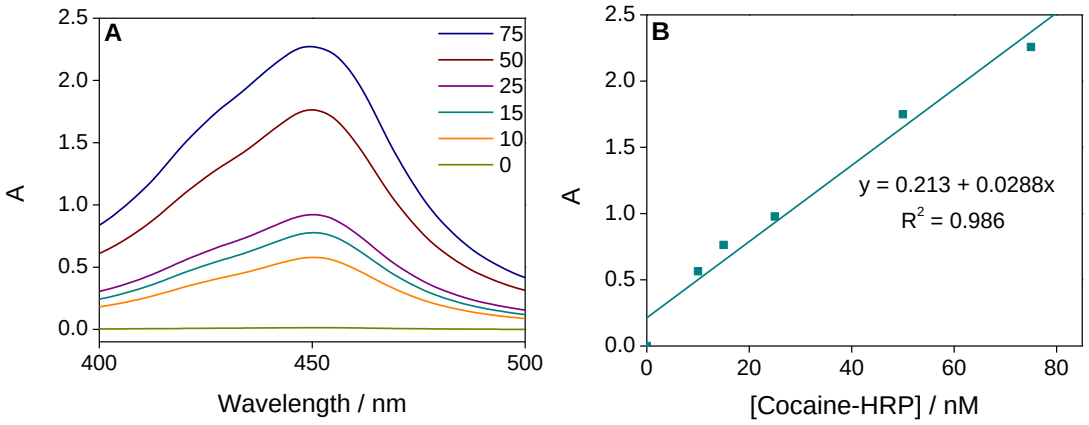

Figure 5.17. A typical standard curve of cocaine-HRP for the quantitation of anti-cocaine antibody on the surface of functionalised AuNP. (A) The absorption spectra of the enzyme product produced by cocaine-HRP standard solutions at concentrations ranging from 10 to 75 nM. Each spectrum represents the mean of at least two measurements. A corresponding linear calibration curve of absorbance intensity at 450 nm is shown in (B).

The absorption spectra of the enzyme product obtained from solutions of antibody-functionalised AuNP treated with cocaine-HRP alongside their respective negative controls are shown in **Figure 5.18**. As indicated in **Figure 5.18A** and **B**, the absorbance intensity at 450 nm was significantly higher for the AuNP-PEGCOOH and AuNP-Protein A/G with antibody bound than those without. These results showed the successful identification of bound antibody on the surface of the functionalised AuNP using the enzyme-labelled antigen. It also confirmed that binding of cocaine-HRP occurred in a specific fashion, as indicated by the lack of enzyme product where no antibody was present. An absorbance intensity considerably lower than that of the AuNP-PEGCOOH-antibody and AuNP-Protein A/G-antibody was obtained from antibody-functionalised AuNP-$PEGNH_2$ following treatment with cocaine-HRP (**Figure 5.18C**). As for the other types of AuNP, AuNP-$PEGNH_2$ with and without antibody could be distinguished from one another based on an increase in absorbance intensity in the presence of the antibody. However, the difference in absorbance intensity between the AuNP-$PEGNH_2$-antibody and the negative control was significantly less than that of the other two types of functionalised AuNP. Based on this result it was concluded that the considerably fewer antibodies had been successfully bound to the AuNP surface using this PEG-NH_2 based approach for AuNP functionalisation.

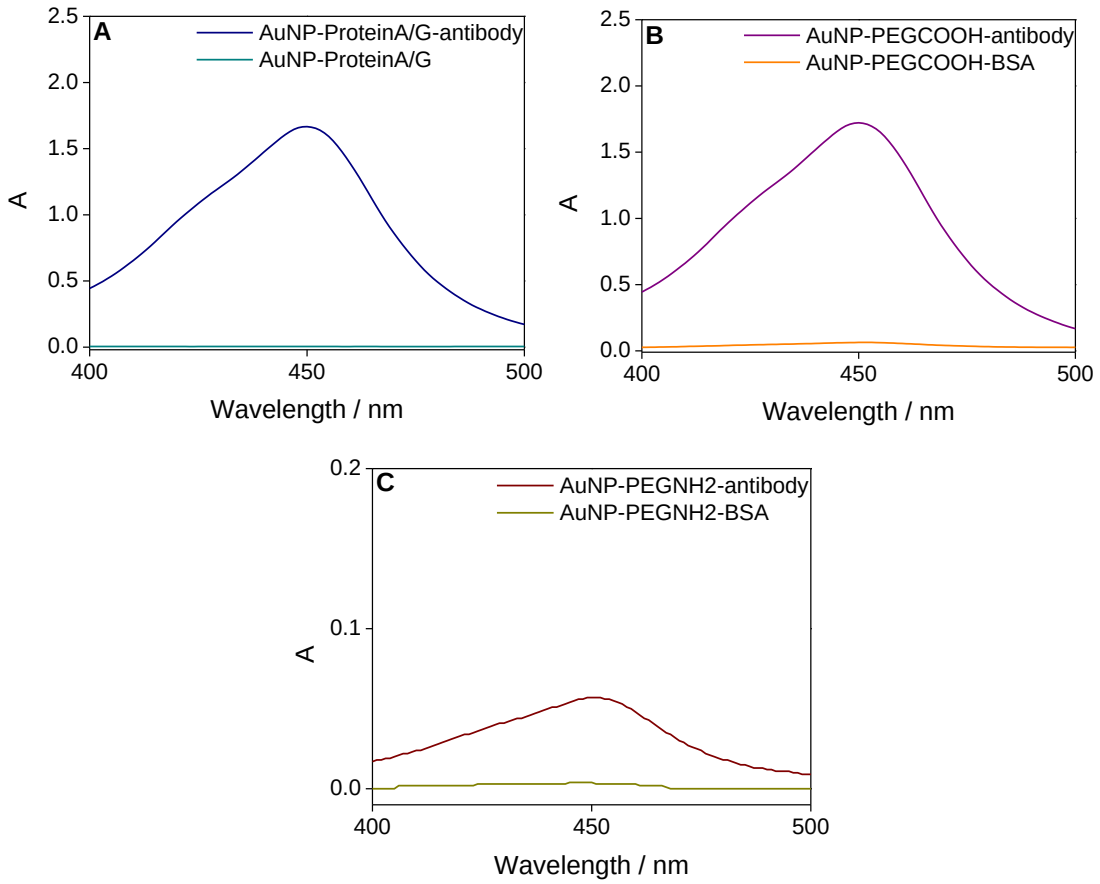

Figure 5.18. The absorption spectra of the enzyme product obtained from cocaine-HRP-treated anti-cocaine antibody-functionalised cAuNP measured at around 450 nm. The types of functionalised AuNP shown are as follows: (A) AuNP-Protein A/G-antibody with un-functionalised AuNP-Protein A/G used as a negative control, (B) AuNP-PEGCOOH-antibody with BSA-functionalised AuNP-PEGCOOH as a negative control, and (C) AuNP-PEGNH_2-antibody with BSA functionalised AuNP-PEGNH_2 as a negative control. Each spectrum represents the mean of at least two measurements.

As described in **Chapter 2.4.10**, the concentration of cocaine-HRP was determined based on the absorbance intensity of the enzyme product at 450 nm and a standard curve as shown in **Figure 5.17**. The number of antibody on the surface of the functionalised AuNP was in turn estimated based on the number of active cocaine binding sites present, using a 1:1 concentration ratio of cocaine-HRP:antibody as described previously. The estimated results for the number of antibody per particle

for each type of functionalised AuNP are shown in **Table 5.1**. As indicated, the highest number of antibodies per particle was achieved using the AuNP-Protein A/G method of functionalisation. As discussed in **Section 5.1.3**, the preferential binding of Protein A/G to the Fc region yields antibody positioned with the FAb region correctly oriented for optimal binding of the antigen. Conversely, binding by way of a –COOH functional group can occur *via* a number of available amine groups and therefore produces a variety of different antibody orientations. The lower number of antibody per AuNP estimated for the AuNP-PEGCOOH-antibody compared to the AuNP-Protein A/G-antibody using the labelled antigen approach could therefore result from this random orientation, as antibody positioned without the FAb region exposed would not be detected using this method. The low number of antibody per AuNP-$PEGNH_2$ fell outside the range of the cocaine-HRP standard curve for accurate quantification, and was considered indicative of poor binding of antibody to the AuNP when using this approach. Overall, the AuNP-Protein A/G directed approach to antibody functionalisation proved the most successful based on the results shown.

Table 5.1. The estimated number of antibody per AuNP, based on the number of active cocaine binding sites, shown alongside the concentrations of both the antibody and AuNP for each of the antibody functionalisation approaches used.

	A_{450}	[Antibody] (nM)	[AuNP] (nM)	Antibody / AuNP
AuNP-PEGCOOH-antibody	1.722	52.40	2.75	19.1
AuNP-ProteinA/G-antibody	1.666	50.45	1.43	35.2
AuNP-$PEGNH_2$-antibody	0.057	0.07	0.48	1.2

5.2.7 Determination of antigen binding efficacy using a microtiter plate-based assay

The binding efficacy of the antibody-functionalised AuNP was determined using a cocaine-BSA hapten immobilised onto a microtiter plate. As binding of the cocaine-

BSA to the microtiter plate occurs *via* interaction with BSA only, the cocaine component of the hapten would be presented for binding in a manner that could mimic the position of cocaine on the surface of forensic samples such as banknotes. The cocaine-BSA was applied to each well of the microtiter plate in excess and under the same conditions to ensure that each of the wells was consistently coated with the maximum possible number of haptens. The presence of functionalised AuNP bound to the immobilised cocaine-BSA was determined using a HRP-labelled secondary antibody targeted to the primary antibody on the surface of the AuNP. Addition of the enzyme substrate enabled the presence of bound AuNP-antibody to be measured by absorbance intensity at 450 nm as explained in **Chapter 2.4.11**. Wells with BSA bound in place of cocaine-BSA were used as negative controls to distinguish any element of non-specific binding by the functionalised AuNP. The addition of free anti-cocaine antibody instead of the functionalised AuNP enabled any signal amplification generated by the particles to be measured. The anti-cocaine antibody functionalised AuNP and the free anti-cocaine antibody were applied to the wells in excess in order for the maximum number of bound AuNP or antibody to be measured. A comparison was made between the signal obtained from the free antibody, and that produced by the antibody-functionalised AuNP bound to the cocaine-BSA in each of the wells. As the free antibody and antibody-functionalised AuNP were applied in excess, the results were compared based on the assumption that the maximum possible number of each would be bound to the wells.

The results of the cocaine-BSA plate assay, expressed as the absorbance intensity of the enzyme product, are shown in **Figure 5.19** for the application of free antibody, AuNP-Protein A/G-antibody and AuNP-PEGCOOH-antibody, respectively. As indicated, the greatest absorbance intensity was obtained when antibody functionalised AuNP-Protein A/G was added to wells containing cocaine-BSA. This signal was greater than that generated following the addition of both the free antibody, and of the antibody functionalised AuNP-PEGCOOH. The signal produced by the AuNP-PEGCOOH-antibody was lower than that of the free antibody, indicating that AuNP functionalised using this approach were ineffective at producing an

amplified signal. A representative negative control, obtained using AuNP-Protein A/G-antibody applied to wells with only BSA bound, is also shown in **Figure 5.19**. As indicated by the absence of signal, non-specific binding was not observed during the assay. Overall, the results indicate that the AuNP-Protein A/G-antibody were the most effective means of labelling cocaine on a solid surface, yielding a signal that was greater than that produced by the antibody alone.

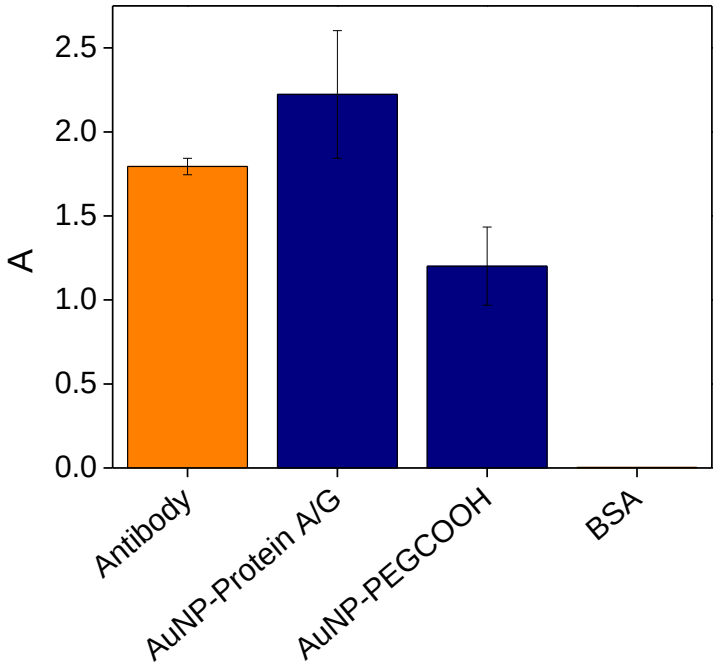

Figure 5.19. The absorbance intensity at 450 nm from HRP-labelled primary antibody, or anti-cocaine antibody-functionalised AuNP bound to cocaine-BSA immobilised onto a microtiter plate. The results obtained after the application of antibody, AuNP-Protein A/G-antibody, and AuNP-PEGCOOH-antibody are shown from left to right. A representative result obtained from a BSA control well is shown on the far right. Each absorbance intensity value represents the mean ± SD of at least three measurements.

5.3 Conclusions

Four different approaches for the functionalisation of AuNPs with anti-cocaine antibody using different ligand or protein intermediates were compared in this chapter. The AuNP were successfully functionalised with antibody using all but one of the approaches selected for the study. The successful modification of the gold surface *via* the addition of antibodies onto the gold surface was performed using a PEG linker with a –COOH terminal functional group, an aminated PEG ligand and a modified Protein A/G intermediate. The direct functionalisation of 40 nm cysteamine-stabilised AuNP *via* the amine group on the cysteamine proved unsuccessful, as the linker was insufficient in length to provide stability under the aqueous conditions required for antibody addition.

Modification of the particle surface following the addition of protein or ligand, and the subsequent attachment of the antibody was monitored by measuring the absorption maximum of the AuNP by UV-Vis spectrophotometry at approximately 520 nm. The adsorption of protein or ligand onto the gold surface affects the SPR of the AuNP, and this effect is expressed as a shift in the absorption maximum of the particles. During the functionalisation of AuNP by the addition of PEGCOOH, $PEGNH_2$ or SPDP-Protein A/G, a shift in the maximum of 2 – 3 nm was recorded after each successive modification. This proved that the modification of the AuNP surface had been successful.

The addition of protein or ligand to the particle surface was also characterised by visual separation using agarose gel electrophoresis. Using this technique, AuNP modified *via* the addition of the different ligands, and the subsequent attachment of antibody could be differentiated from one another on the basis of charge and size. Using this technique, it was shown that the modified particles travelled more slowly and showed increased stability within the gel when compared to the un-modified AuNP. Functionalisation using the $PEGNH_2$ ligand was illustrated by the positively charged aminated ligand, which caused the AuNP to travel towards the negative rather than the positive electrode in the gel, and the subsequent reversal of this

charge following the addition of the antibody. Overall it was shown that the successful functionalisation of AuNP with antibody was performed using the three different approaches used in this study, and that these could be visualised by the position of different self-staining bands in an agarose gel.

SDS-PAGE was used for the detection of antibody fragmented from the surface of the different types of functionalised AuNP. It was expected that the orientation of the immobilised antibodies on the surface of the AuNP would be demonstrated based on the presence of heavy or light antibody fragments in the gel. This in turn would provide a means of differentiating antibody bound at variable orientations, such as those immobilised *via* a PEGCOOH ligand, with the directed Fc-mediated attachment achieved using Protein A/G or $PEGNH_2$ intermediates. Of the three different functionalised AuNP used in this study, only the AuNP-PEGCOOH-antibody yielded results consistent with a positive identification of variably-bound anti-cocaine antibody. Interference from blocking buffer protein used to stabilise the AuNP-$PEGNH_2$, and the presence of only one of the two characteristic antibody fragments derived from the AuNP-Protein A/G yielded inconclusive results for these particles.

Enzyme-labelled antigen (cocaine-HRP) was found to bind to the antibody-functionalised AuNP at significantly higher concentrations than for AuNP without antibody bound. Using this method, all three types of functionalised AuNP were confirmed as being specifically able to bind cocaine. The number of antibody bound to each AuNP was estimated based on the concentration of antigen bound. A ratio of antibody to AuNP of 19:1 and 35:1 were estimated for the AuNP-PEGCOOH and AuNP-Protein A/G, respectively. The AuNP-$PEGNH_2$ was found to have too low a signal for the concentration of antibody to be accurately determined using this method. The results indicate that the optimal binding efficacy of the functionalised AuNP to cocaine-HRP was obtained when using the modified Protein A/G method of functionalisation, which may be due to the favourable orientation of the antibody when bound using this approach. AuNP functionalised with antibody by way of a

$PEGNH_2$ did not result in sufficient binding of the labelled antigen to be used for the detection of cocaine.

A plate-based assay using a cocaine-BSA hapten was used to determine the binding efficacy of the antibody functionalised AuNP in a manner that might mimic the presentation of cocaine on the surface of a forensic sample such as a banknote. Using a HRP-labelled secondary antibody, the binding of the functionalised AuNP was detected by measuring the absorbance intensity following addition of an enzyme substrate. By using wells with BSA bound in place of the cocaine hapten, it was shown that the functionalised AuNP bound specifically in the presence and not the absence of immobilised cocaine. Furthermore, the intensity of the signal obtained after the application of antibody functionalised AuNP-Protein A/G was greater than that of both the AuNP-PEGCOOH and the free antibody. This showed that the AuNP-Protein A/G-antibody could be successfully used for the detection of cocaine at low concentrations by producing an amplified signal when compared with that produced by antibody alone. In order for the binding efficacy of the AuNP to be more comprehensively compared to that of the free antibody, a similar assay where a gradient of different concentrations of cocaine-BSA immobilised onto wells of the microtiter plate could be applied. In this way, a response curve could be generated which would indicate whether the amplified signal produced by the presence of the AuNP-Protein A/G-antibody is present even at the low concentrations of analyte likely to be present on samples of forensic interest.

Of the four approaches to functionalisation compared in this study, the PEGCOOH-based approach was the simplest to perform and yielded the most consistently stable antibody-functionalised AuNP. However, the cocaine-binding efficacy of the antibody-functionalised AuNP-COOH was lessened due to the random rather than oriented binding of the anti-cocaine antibody onto the PEGCOOH on the AuNP surface. The antibody-functionalised AuNP-Protein A/G on the other hand provided enhanced cocaine-binding due to the oriented nature of the bound antibody on the Protein A/G intermediate. Based on the extensive characterisation of each type of antibody

functionalised AuNP, it was therefore determined the SPDP modified Protein A/G-based approach proved to be the most effective method of generating antibody-functionalised particles for the application of cocaine detection in forensic samples such as banknotes.

5.4 References

(1) Zsigmondy, R. A. In *Nobel Lectures, Chemistry 1922-1941,* Elsevier Publishing Company: Amsterdam, **1966**.

(2) Freestone, I.; Meeks, N.; Sax, M.; Higgitt, C. *Gold Bull.* **2007**, *40*, 270.

(3) Wilson, R. *Chem. Soc. Rev.* **2008**, *37*, 2028.

(4) Thompson, D. *Gold Bull.* **2007**, *40*, 267.

(5) Reddy, V. R. *Synlett.* **2006**, *2006*, 1791.

(6) Parak, W. J.; Sperling, R. A.; Rivera gil, P.; Zhang, F.; Zanella, M. *Chem. Soc. Rev.* **2008**, *37*, 1896.

(7) Turkevich, J. S.; Stevenson, P. C.; Hillier, J. *Discuss. Farady Soc.* **1951**, *11*, 55.

(8) Frens, G. *Nature* **1973**, *241*, 20.

(9) Pérez-Juste, J.; Pastoriza-Santos, I.; Liz-Marzán, L. M.; Mulvaney, P. *Coordin. Chem. Rev.* **2005**, *249*, 1870.

(10) Yamamoto, M.; Kashiwagi, Y.; Sakata, T.; Mori, H.; Nakamoto, M. *Chem. Mater.* **2005**, *17*, 5391.

(11) Brust, M.; Walker, M.; Bethell, D.; Schiffrin, D. J.; Whyman, R. *J. Chem. Soc., Chem. Commun.* **1994**, 801.

(12) Auffan, M.; Rose, J.; Bottero, J. Y.; Lowry, G. V.; Jolivet, J. P.; Wiesner, M. R. *Nat. Nanotechnol.* **2009**, *4*, 634.

(13) Boisselier, E.; Astruc, D. *Chem. Soc. Rev.* **2009**, *38*, 1759.

(14) Eustis, S.; El-Sayed, M. A. *Chem. Soc. Rev.* **2006**, *35*, 209.

(15) Omidfar, K.; Khorsand, F.; Darziani Azizi, M. *Biosens. Bioelectron.* **2013**, *43*, 336.

(16) Boisselier, E.; Astruc, D. *Chem. Soc. Rev.* **2009**, *38*, 1759.

(17) Cortie, M. B.; Pissuwan, D.; Cortie, C. H.; Valenzuela, S. M. *Gold Bull.* **2007**, *40*, 121.

(18) Jia, C. P.; Liu, M. Y.; Huang, Y. Y.; Lou, X. H.; Yao, S. H.; Jin, Q. H.; Zhao, J. L.; Xiang, J. Q. *Analyst* **2010**, *135*, 327.

(19) Li, Z. P.; Wang, Y. C.; Liu, C. H.; Li, Y. K. *Anal. Chim. Acta* **2005**, *551*, 85.

(20) Aveyard, J.; Nolan, P.; Wilson, R. *Anal. Chem.* **2008**, *80*, 6001.

(21) Dreaden, E. C.; Alkilany, A. M.; Huang, X.; Murphy, C. J.; El-Sayed, M. A. *Chem. Soc. Rev.* **2012**, *41*, 2740.

(22) Nagl, S.; Schaeferling, M.; Wolfbeis, O. S. *Microchim. Acta* **2005**, *151*, 1.

(23) Leggett, R.; Lee-Smith, E. E.; Jickells, S. M.; Russell, D. A. *Angew. Chem., Int. Edit.* **2007**, *46*, 4100.

(24) Hazarika, P.; Jickells, S. M.; Wolff, K.; Russell, D. A. *Angew. Chem., Int. Edit.* **2008**, *47*, 10167.

(25) Natan, M. J.; Lyon, L. A.; Musick, M. D. *Anal. Chem.* **1998**, *70*, 5177.

(26) Trilling, A. K.; Beekwilder, J.; Zuilhof, H. *Analyst* **2013**, *138*, 1619.

(27) Yoshimoto, K.; Hoshino, Y.; Ishii, T.; Nagasaki, Y. *Chem. Commun. (Camb)* **2008**, 5369.

(28) Veronese, F. M.; Pasut, G. *Drug Discov. Today* **2005**, *10*, 1451.

(29) Sakura, T.; Takahashi, T.; Kataoka, K.; Nagasaki, Y. *Colloid. Polym. Sci.* **2005**, *284*, 97.

(30) Giersig, M.; Mulvaney, P. *Langmuir* **1993**, *9*, 3408.

(31) Mason, M. D.; Eck, W.; Craig, G.; Sigdel, A.; Ritter, G.; Old, L. J.; Tang, L.; Brennan, M. F.; Allen, P. J. *Acs Nano* **2008**, *2*, 2263.

(32) Grabarek, Z.; Gergely, J. *Anal. Biochem.* **1990**, *185*, 131.

(33) Cao, C.; Sim, S. J. *Biosens. Bioelectron.* **2007**, *22*, 1874.

(34) Spindler, X.; Hofstetter, O.; McDonagh, A. M.; Roux, C.; Lennard, C. *Chem. Commun.* **2011**, *47*, 5602.

(35) Puertas, S.; Moros, M.; Fernandez-Pacheco, R.; Ibarra, M. R.; Grazu, V.; de la Fuente, J. M. *J. Phys. D. Appl. Phys.* **2010**, *43*, 474012.

(36) Eliasson, M.; Olsson, A.; Palmcrantz, E.; Wiberg, K.; Inganäs, M.; Guss, B.; Lindberg, M.; Uhlen, M. *J. Biol. Chem.* **1988**, *263*, 4323.

(37) Kumar, S.; Aaron, J.; Sokolov, K. *Nat. Protoc.* **2008**, *3*, 314.

(38) Niidome, T.; Nakashima, K.; Takahashi, H.; Niidome, Y. *Chem. Commun.* **2004**, 1978.

(39) Storhoff, J. J.; Lazarides, A. A.; Mucic, R. C.; Mirkin, C. A.; Letsinger, R. L.; Schatz, G. C. *J. Am. Chem. Soc.* **2000**, *122*, 4640.

(40) Niemeyer, C. M.; Ceyhan, B.; Gao, S.; Chi, L.; Peschel, S.; Simon, U. *Colloid. Polym. Sci.* **2001**, *279*, 68.

(41) Karthik, K., Singh, P., Das, P. *Small Ruminant Res.* **2011**, *99*, 7.

(42) Carlsson, J.; Drevin, H.; Axen, R. *Biochem. J.* **1978**, *173*, 723.

(43) Stuchbury, T.; Shipton, M.; Norris, R.; Malthouse, J.; Brocklehurst, K.; Herbert, J.; Suschitzky, H. *Biochem. J.* **1975**, *151*, 417.

(44) Clarizia, L.-J. A.; Sok, D.; Wei, M.; Mead, J.; Barry, C.; McDonald, M. J. *Anal. Bioanal. Chem.* **2009**, *393*, 1531.

(45) Hanauer, M.; Pierrat, S.; Zins, I.; Lotz, A.; Sonnichsen, C. *Nano Lett.* **2007**, *7*, 2881.

CHAPTER 6

Conclusions and Future Work

This thesis describes the development of a competitive immunoassay for the quantitation of cocaine extracted from banknotes, and a novel method for the location-specific immunodetection of cocaine on the banknote surface. The conclusions of the developed methods and their application for the detection of cocaine both on and extracted from banknotes are summarised in this chapter. The application of the competitive immunoassay for the detection of cocaine extracted from latent fingerprints, and a comparison of different functionalisation techniques for the synthesis of antibody-gold nanoparticles for cocaine detection are also described. Suggestions for future work in relation to the current study are highlighted.

6.1 The quantitation of cocaine on banknotes by cEIA

Chapter 3 described the development of a non-destructive and sensitive competitive enzyme immunoassay (cEIA) for the detection of low concentrations (*ca.* 1 – 20 ng mL^{-1}) of cocaine in diluted extracts from banknote samples. The application of the cEIA for the quantitation of cocaine in complex forensic samples such as extracts from banknotes and latent fingerprints was also outlined. A polyclonal anti-cocaine antibody was successfully purified from rabbit serum to form the basis of the assay. The precision and sensitivity of the cEIA were found to be suitable for the quantitation of cocaine in samples of forensic significance, as indicated by the LOD of 0.162 ng mL^{-1} which compares favourably to the detection limits of both a GC-MS method and a commercial micro-plate EIA.[1,2]

The developed method was applied for the quantitation of cocaine extracted from ten UK banknotes from general circulation as a representation of a genuine sample matrix. Trace levels of cocaine ranging from 0.3 to 20.0 µg mL^{-1} were detected on all

of the samples analysed, at concentrations that were similar to the levels of cocaine detected on Irish Euro banknotes from general circulation.[3] The quantitative assay results were validated *via* a parallel LC-MS^2 analysis of the same samples, and a statistically significant agreement was found between the two sets of results. This indicated that the assay performed to the desired level of specificity required for the analysis of cocaine in complex, heavily contaminated banknote extracts. The primary cocaine metabolite benzoylecgonine as well as other potential interferents were detected by LC-MS^2 in the extracted samples, but despite showing cross-reactivity for benzoylecgonine the quantitative results of the assay were unaffected.

The versatility of the cEIA was further demonstrated *via* its application for the quantitation of cocaine extracted from ten latent fingerprints. The prints were donated by five individual volunteers, known to be taking cocaine, who were attending a methadone treatment clinic. The concentrations of cocaine detected in the fingerprint extracts using the cEIA ranged from 0 to 19.0 ng mL^{-1}. The results from the cEIA showed similar results for multiple fingerprints obtained from one volunteer; for example, the cocaine concentrations 0.728 and 0.879 ng mL^{-1} were detected in two fingerprint samples from the same individual. The results of the cEIA were compared with the quantitative analysis of cocaine by GC-MS performed on oral fluid samples obtained from the five volunteer subjects at the time of fingerprinting. Cocaine was detected by GC-MS in the oral fluid samples from three of the five individuals tested. The results showed, for the first time, that the concentrations of cocaine detected by cEIA in latent fingerprints followed the same trend as those of the oral fluid samples. The detection of cocaine in both oral fluid and the extracts of latent fingerprints could therefore be combined as an indicator of recent consumption.

Overall, the developed cEIA presents a user-friendly, economical alternative to the more commonly used chromatography-MS techniques for the quantitation of cocaine in forensic samples such as banknotes, without the need for sophisticated instrumentation. Enzyme immunoassays such as the cEIA presented in this study only use aqueous solvents, do not require specialist training to perform and can be

easily automated, making them readily applicable for everyday analysis in a forensic laboratory.

6.2 Direct immunostaining of cocaine on banknotes

A novel immunological method developed for the location-specific staining of cocaine on the surface of a banknote is presented in Chapter 4. The method was developed as a future means for determining a pattern for the location of cocaine contamination on a banknote, with a view to establishing the efficacy of the method currently used by UK law enforcement agencies for the detection of cocaine on banknotes by IMS.[4]

Immunological methods are widely used for the location-specific staining of cellular antigens in fixed tissue specimens.[5] Hydrogel matrices, due to their strength and biocompatibility, are also widely used for gel electrophoresis or as 2D or 3D cell culture matrices, among other biological applications.[6] The subsequent immunodetection of target molecules within these matrices is facilitated by the porous nature of hydrogels, which are known to be permeable to large proteins such as antibodies.[7,8] In the present study, the mechanical strength and antibody permeability of the biologically derived hydrogels collagen, gelatin, casein and agarose were determined in order to judge their suitability as a means of affixing cocaine onto the surface of banknotes for subsequent immunostaining. The results obtained after applying the hydrogels to cotton paper as a representation of a banknote showed that the gels possessed too little mechanical strength to properly adhere to the surface of the note at the low concentrations of polymer required to enable antibody permeation.

A synthetic acrylamide hydrogel was instead used based on its previous application for the in-gel immunodetection of proteinaceous antigen after separation by gel electrophoresis.[9] Treatment of the acrylamide gel with 50 % isopropanol in water as a fixative enables the immobilisation of antigenic material within the gel and reportedly also increases its permeability to antibodies.[9] The in-gel immunodetection of cocaine-BSA as an antigenic target was initially performed in acrylamide gels after

separation by non-denaturing PAGE. Immunostaining of the gels using a rabbit anti-cocaine primary antibody, a corresponding HRP-labelled secondary antibody and 3,3'-diaminobenzidine (DAB) as an enzyme substrate initially proved unsuccessful. However, further optimisation of the method and the introduction of an enhanced chemiluminescence (ECL) substrate for increased sensitivity led to the positive staining of the anti-cocaine primary antibody control by the HRP-labelled secondary antibody.

Due to the discontinuation of the manufacturing of the original rabbit polyclonal anti-cocaine antibody, a sheep polyclonal anti-cocaine antibody was instead selected and used for the remainder of the study. The cocaine-binding efficacy of the two antibody types was not compared due to variations introduced by the different means of HRP-labelling used for each. The in-gel immunodetection of cocaine-BSA as an antigenic target in acrylamide gels after separation by non-denaturing PAGE was repeated. The sheep anti-cocaine antibody using a corresponding HRP-labelled secondary antibody was applied with an ECL enzyme substrate, however detection using this method proved unsuccessful. Staining in the control lane containing the primary anti-cocaine antibody as a positive control was not observed, which led to the conclusion that interaction between the primary and the secondary antibodies was not occurring. This was investigated using a dot-blot style assay, which showed that interaction between the antibodies was successful, however not in the presence of the acrylamide gel.

The anti-cocaine primary antibody was subsequently directly labelled with HRP in order to remove the need for secondary antibody-mediated detection. Labelling was achieved using the well-described periodate-mediated method of conjugation, in which addition of HRP occurs *via* the Fc region of IgG and therefore does not affect the antigen-binding capacity of the antibody.[10] The successful formation of the antibody-HRP hapten was confirmed by SDS PAGE and UV-Vis absorption spectrophotometry, yielding a conjugate of suitable concentration and purity. The in-gel immunodetection of cocaine standard (applied to small areas of partial banknote

samples in a solvent solution) using the HRP-labelled anti-cocaine antibody initially proved unsuccessful. It was thought that the effectiveness of the in-gel immunodetection may have been compromised by the solute rather than powdered nature of the standard used for spiking.

A partial banknote sample spiked with a sample of 'street' cocaine as a more realistic substance for spiking was successfully immunostained for cocaine using the developed method. The presence of cocaine was indicated by darker regions of staining on the notes which had been spiked with cocaine, compared to the lighter background levels of staining present on non-spiked partial banknotes. The method yielded different levels of staining for partial notes obtained from general circulation after pre-washing with methanol as compared with those which had not been washed prior to spiking and analysis. The results confirmed the reports of Bohannon suggesting that pre-washing of banknotes in methanol affects the structure of the fibrous network that makes up the note, thereby affecting that way in which cocaine becomes embedded and retained by those fibres.[11]

The in-gel immunodetection method was subsequently adapted and applied to the analysis of whole banknote samples. Initially, newly minted notes were analysed as true negative samples; a clear difference in the intensity of staining was observed between newly minted negative control notes and those which had been spiked with cocaine over the whole of the note. These initial results suggested that the cocaine was successfully being detected using the developed method. In order to confirm whether the staining was occurring at the location where cocaine was applied to the note, the analysis was repeated using newly minted banknotes where half of the note had been spiked with street cocaine and the other half left blank as a negative control area. The results showed an area of intense staining at the edge of the spiked half of the note rather than over its entirety. The staining observed was also more intense over the whole of the gel when compared with negative control notes rather than just in the spiked area. The results indicated that the spiked cocaine had

become mobile within the gel mix prior to gelation and had thereby migrated to areas of the note on which it had not originally been applied.

The developed method was applied to the analysis of six banknotes from general circulation as a representation of a genuine sample matrix on which unknown concentrations of cocaine could be present, and to confirm whether the nature and intensity of the staining differed from that obtained from the newly minted banknotes. The appearance of the notes was described as worn and aged as compared to the newly minted control samples, which based on the findings of previous studies leads to an increased ability to entrap and retain cocaine within the banknote fibres.[12,13] The result of the analysis showed staining in the form of dark grey or black spots on the surface of the banknote, rather than the overall dark grey stain from the cocaine spiked on the newly minted notes. Analysis was also performed on banknotes from general circulation half-spiked with 'street' cocaine in the same way as for the newly minted notes yielding greater number of dark, stained spots on the spiked half of the banknote when compared with the unspiked half. The results suggested that the staining related to the presence and location of cocaine on the surface of the banknote. The problem of the cocaine migrating after application to the banknotes described in the previous paragraph was therefore partially overcome by applying cocaine to used rather than newly minted banknotes. The used banknotes provided improved adherence of the cocaine as described in previous studies.[14,15]

Overall, the development of a novel in-gel immunodetection method for the visualisation of cocaine on the surface of banknotes was reported. The results obtained suggested that using the developed method enables the direct immunostaining of the cocaine at the location in which it is present on the banknote. The results obtained from the banknote samples analysed during this study suggest that cocaine can be present at variable locations across the whole of the banknote. Based on the sporadic levels of contamination on the banknotes sampled, it is possible that the current IMS sampling method used by UK law enforcement either over- or underestimates the concentration of cocaine that are actually present on the

whole of the banknote. A method in which the entire banknote, rather than a representative area is sampled would therefore provide more accurate indication of the cocaine contamination on banknotes.

6.3 Method comparison for the antibody-functionalisation of AuNP

Four different approaches for the functionalisation of AuNPs with anti-cocaine antibodies using different ligand or protein intermediates were investigated and the results compared in Chapter 5. The AuNP were successfully functionalised with antibody using all but one of the approaches selected for the study. The addition of antibodies onto the AuNP was performed using a PEG linker with a –COOH terminal functional group, an aminated PEG ligand and an SPDP-Protein A/G intermediate. Of the four approaches used only the direct functionalisation of 40 nm cysteamine-stabilised AuNP *via* the amine group on the cysteamine proved unsuccessful. This was due to the instability of the particles under aqueous conditions resulting from the insufficient length of the stabilising ligand.

The successful functionalisation of the AuNP was demonstrated by a shift in the absorption maximum of the AuNP by 2 - 3 nm after each subsequent addition of protein or ligand. The increase in the size of the AuNP following the addition of ligand or protein was also visualised by agarose gel electrophoresis; this showed that the modified AuNP travelled more slowly within the gel when compared with un-modified AuNP, and that the position of the self-staining bands formed in the gel reflected the size or charge difference between each addition. Differentiation between the three different modified AuNP at each stage of the functionalisation process was possible using this technique.

SDS-PAGE was applied for the detection of antibodies fragmented from the surface of the different types of functionalised AuNP. It was expected that the orientation of the immobilised antibody on the surface of the AuNP would be demonstrated based

on the presence of heavy or light antibody fragments in the gel. Of the three different functionalised AuNP used in this study, only the AuNP-PEGCOOH-antibody yielded results consistent with a positive identification of variably-bound anti-cocaine antibodies. However, at least one of the two characteristic antibody fragments was identified in the lanes of both the AuNP-Protein A/G and the AuNP-$PEGNH_2$. This showed that although SDS PAGE proved ineffective as a means of determining the position of the antibody on the surface of the functionalised AuNP, the presence of antibody was successfully demonstrated using this technique.

Enzyme-labelled antigen (cocaine-HRP) was applied to the functionalised AuNP as a means of labelling bound antibodies on the AuNP surface. Using this method, all three types of functionalised AuNP were confirmed as being specifically able to bind cocaine. The number of antibody bound to each AuNP was estimated based on the concentration of bound cocaine-HRP. A ratio of antibody to AuNP of 19:1 and 35:1 were estimated for the AuNP-PEGCOOH and AuNP-Protein A/G, respectively. The AuNP-$PEGNH_2$ was found to have too low a signal for the concentration of antibody to be accurately determined using this method. The results indicated that the optimal binding efficacy of the functionalised AuNP to cocaine-HRP was obtained when using the Protein A/G method of functionalisation. This optimal binding is likely to have occurred due to the favourable orientation of the antibody obtained *via* the Protein A/G intermediate. AuNP functionalised with antibody by way of a $PEGNH_2$ did not result in sufficient binding of the labelled antigen to be used for the detection of cocaine.

A plate-based assay was developed using a cocaine-BSA hapten as an antigen. The assay was used to determine the binding efficiency of the antibody-functionalised AuNP in a manner that might mimic the presentation of cocaine on the surface of a forensic sample such as a banknote. It was shown that the functionalised AuNP bound specifically in the presence of the immobilised cocaine-BSA hapten. The results also demonstrated that the functionalised AuNP would not bind in the absence of the cocaine-BSA hapten. Furthermore, the intensity of the signal obtained

after the application of antibody functionalised AuNP-Protein A/G was greater than that of both the AuNP-PEGCOOH and the free antibody. This showed that the AuNP-Protein A/G-antibody could be successfully used for the detection of cocaine at low concentrations by producing an amplified signal when compared with that produced by antibody alone.

Based on the extensive characterisation of each type of antibody functionalised AuNP, it was demonstrated that of the four approaches of nanoparticle functionalisation that were compared in this study, the SPDP-Protein A/G-based approach proved to be the most effective method for the formation of anti-cocaine antibody-AuNP conjugates for cocaine detection in forensic samples such as banknotes.

6.4 Future work

In this study, a cEIA was developed for the detection of cocaine in samples of forensic interest. The versatility and robustness of the assay was demonstrated by applying the method to the analysis of cocaine extracted from 'real' banknote samples and fingerprints. Further comparison between cocaine detected in oral fluid samples and corresponding fingerprint extracts could be of interest in this area, particularly in relation to the timing of peak drug concentrations in oral fluid and blood, and between the peak concentrations in blood and sweat after consumption of the drug.[16,17] The peak cocaine concentration in oral fluid, for example, occurs sooner after the drug is taken than the peak concentration in the sweat of a fingerprint, which is likely to occur later. Establishing a relationship between the two peaks in concentration may enable the approximate window of time during which the cocaine was taken to be estimated based on the combined analysis of oral fluids and of fingerprints. Furthermore, the adaptation of the assay for the detection of alternative metabolites of cocaine other than benzoylecgonine, such as ecgonine methyl ester or cocaethylene, is recommended. The detection of metabolites such as ecgonine methyl ester in particular, which derives from enzymatic action in the body rather

than spontaneous hydrolysis, would enable the critical differentiation between recent cocaine consumption and possible environmental cocaine contamination beyond that currently possible using enzyme immunoassay-style analytical methods. The peak concentration of ecgonine methyl ester following the consumption of cocaine could provide further information as to the window of time during which the drug was taken. Validation of the cEIA should be performed to enable this simple method to be introduced into a forensic laboratory setting. Particularly the application of the assay for the analysis of cocaine extracted from fingerprints would require a much more extensive study during which a suitable cut-off concentration for the differentiation between 'positive' and 'negative' samples would need to be established.

In order to improve the developed method for the location-specific immunodetection of cocaine on banknotes, specialised equipment such as a bespoke gel caster for the more consistent application of the acrylamide gel matrix to the banknotes should be used. This would decrease mobility of the cocaine prior to fixation as was observed on some of the banknote samples during this study. The application of the in-gel immunodetection method to a greater number of cocaine-spiked and negative control banknotes is also recommended to ensure the repeatability of the staining method. The development of 'standard' banknote samples harbouring known quantities of cocaine at concentrations reflecting those likely to be found in general circulation would be beneficial for this process. The analysis of a larger cohort of banknotes from general circulation should then be performed. This would establish with certainty whether cocaine contamination occurs over the whole or specific parts of banknotes in general circulation, thereby completing the overall aim of the present study. The results from this sample survey could then be related to the effectiveness of the sampling method currently used by UK law enforcement agencies for the detection of cocaine, in terms of whether the area of the banknote presently sampled is genuinely representative of the quantity of cocaine present on the whole of the note.[4]

The application of the antibody-functionalised nanoparticles described in Chapter 5 to enhance the sensitivity of the developed immunostaining method for the detection of cocaine on banknotes is also suggested. The use of the functionalised AuNP would enable the visualisation of cocaine at even lower concentrations than presently detectable due to the signal enhancing properties of the functionalised nanoparticles which were demonstrated in this study. Permeability studies to determine whether the acrylamide gel is sufficiently porous to allow passive diffusion of the particles would first be required.

Overall, the results shown in this thesis have demonstrated the suitability of immunological methods for the detection of illicit substances such as cocaine in complex sample matrices of forensic importance.

6.5 References

(1) Jenkins, A. J. *Forensic Sci. Int.* **2001**, *121*, 189.

(2) Niedbala, R. S.; Kardos, K.; Fries, T.; Cannon, A.; Davis, A. *J. Anal. Toxicol.* **2001**, *25*, 62.

(3) Bones, J.; Macka, M.; Paull, B. *Analyst* **2007**, *132*, 208.

(4) Dussy, F. E.; Berchtold, C.; Briellmann, T. A.; Lang, C.; Steiger, R.; Bovens, M. *Forensic Sci. Int.* **2008**, *177*, 105.

(5) Dabbs, D. J. *Diagnostic Immunohistochemistry;* 2nd ed.; Elsevier: Philadelphia, 2006.

(6) Tibbitt, M. W.; Anseth, K. S. *Biotechnol. Bioeng.* **2009**, *103*, 655.

(7) Ulrich, T. A.; Jain, A.; Tanner, K.; MacKay, J. L.; Kumar, S. *Biomaterials* **2010**, *31*, 1875.

(8) Oudin, J. *Method. Enzymol.* **1980**, *70*, 166.

(9) Desai, S.; Dworecki, B.; Cichon, E. *Anal. Biochem.* **2001**, *297*, 94.

(10) Wisdom, G. B. In *The Protein Protocols Handbook;* 3rd ed.; Walker, J. M., Ed.; Humana Press: New York, 2009.

(11) Bohannon, J. *Science* **2007**, *316*, 3.

(12) Moss, J., M. Sc. Thesis, University of Bristol, UK, 1997.

(13) Sleeman, R.; Burton, F.; Carter, J.; Roberts, D.; Hulmston, P. *Anal. Chem.* **2000**, *72*, 397A.

(14) Ebejer, K. A.; Winn, J.; Carter, J. F.; Sleeman, R.; Parker, J.; Körber, F. *Forensic Sci. Int.* **2007**, *167*, 94.

(15) Roberts, D.; Carter, J.; Sleeman, R.; Burton, I. *Spectrosc. Eur.* **1997**, *9*, 16.

(16) Samyn, N.; De Boeck, G.; Verstraete, A. G. *J Forensic Sci.* **2002**, *47*, 1380.

(17) Wille, S. M.; Raes, E.; Lillsunde, P.; Gunnar, T.; Laloup, M.; Samyn, N.; Christophersen, A. S.; Moeller, M. R.; Hammer, K. P.; Verstraete, A. G. *Ther. Drug Monit.* **2009**, *31*, 511.

Publications and Presentations

Publications

van der Heide S, Russell DA. 2014 'Optimisation of immuno-gold nanoparticle complexes for antigen detection.' *In preparation.*

van der Heide S, Hardwick S, Cunningham A, Russell DA. 2014 'Location-specific immunodetection of cocaine on banknotes.' *In preparation.*

van der Heide S, Hardwick S, Cunningham A, Hudson S, Russell DA. 2014 'A competitive enzyme immunoassay for the quantitation of cocaine on banknotes.' *In preparation.*

Oral Presentations

'Nanoparticles: Small things for big applications.' van der Heide, S. Royal Society of Chemistry sponsored Norwich Science Café, the Maddermarket Theatre Norwich, March 2014.

'A competitive enzyme immunoassay for the quantitation of cocaine on banknotes.' van der Heide S, Cunningham A, Hardwick S, Russell DA. Royal Society of Chemistry Analytical Research Forum 2013, GlaxoSmithKline and University of Hertfordshire, July 2013.

Poster Presentations

'Detective Jack Drake and the case of the drug money.' van der Heide S, Cunningham A, Hardwick S, Russell DA. University of East Anglia Showcase of Postgraduate Research 2013, The Forum, Norwich, June 2013.

'Find the drug metabolites: Follow the gold.' van der Heide S, Russell DA. University of East Anglia Showcase of Postgraduate Research 2012, The Forum, Norwich, June 2012 (Awarded 'Best Overall Entry').

'A comparison of antibody-gold nanoparticle conjugates for the detection of cocaine.' van der Heide S, Russell DA. School of Chemistry Research Colloquium, University of East Anglia, July 2012.

'Antibody-functionalised gold nanoparticles for cocaine detection.' van der Heide S, Russell DA. Royal Society of Chemistry Analytical Research Forum 2012, Durham University, July 2012.

'Antibody-nanoparticles conjugates for the detection of cytokines in latent fingerprints.' van der Heide S, Russell DA. Royal Society of Chemistry Analytical Research Forum 2011, University of Manchester, July 2011.

'Antibody-nanoparticle conjugates for the detection of immune markers in latent fingerprints.' van der Heide S, Russell DA. . School of Chemistry Research Colloquium, University of East Anglia, July 2011.