Development and evaluation of an LC-ESI-MS method for the simultaneous detection of five major opium alkaloids

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Development and evaluation of an LC-ESI-MS method for the simultaneous detection of five major opium alkaloids

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Abstract

The aim of this work was to establish an analytical method for the simultaneous detection of five major opium alkaloids in poppy seeds by liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS). Once opium alkaloids were detected in poppy seeds, toxicological studies were carried out to establish if these compounds were detected in oral fluid (OF) of participants who ingested muffins containing poppy seeds.

It is known that the ingestion of poppy seeds has caused positive opiate drug test results and much work has been reported in the scientific literature in the last 20 years. Researchers in the field have investigated alternatives to differentiate between heroin administration and that of other opiate drugs versus poppy seed ingestion. Most of the work which has been carried out relates to establishing illicit heroin use by examining biological matrices for the presence of acetylcodeine, thebaine, papaverine, noscapine and their associated metabolites.

The research methodology consisted of establishing an LC-ESI-MS method for the simultaneous detection of five major opium alkaloids (morphine, codeine, thebaine, papaverine and noscapine). A deuterated internal standard (morphine-d3) was used for the quantitation of alkaloids in harvested poppy seeds and oral fluid samples. Due to technical difficulties, 3 LC-MS instruments were employed in this work. Electrospray ionisation was employed in all mass spectrometers but the analysers included an ion trap with octopole, a triple quadrupole and a hybrid quadrupole Orbitrap.

Suitable extraction procedures were determined and harvested seeds purchased from a number of supermarkets were analysed for the presence of five alkaloid compounds using the LC-MS method. A small scale pilot study with 6 participants was carried out to establish if it was possible to fail an OF drug test for opiates after consuming poppy seed muffins. OF samples were collected post ingestion using Quantisal™ kits and the level of each of the opiates was monitored.

The findings were that an LC-ESI-MS method was established for the simultaneous detection and quantitation of five major alkaloids. However, the method development process involved finding a solution to co-elution of morphine and codeine. The process also included resolving the issue of thebaine producing two peaks with identical mass spectra and separated by a difference of 6 minutes in retention time.

Varying levels of alkaloids were identified in harvested poppy seeds: levels of these compounds differed considerably within and between batches of poppy seeds. These findings could be attributed to a number of factors, for example, where and how the plants were grown and methods of harvesting.

Two poppy seed muffins were consumed as part of a toxicology study. Morphine was detected in the 5 minute sample in 5 out of the 6 participants with concentrations in OF of 0.5-0.8 ng mL^{-1}; codeine was detected in 2 of the 6 participants at 1.5 and 2.6 ng mL^{-1}. Thebaine, noscapine and papaverine were also detected in OF of a number of participants, which has not been previously reported in the literature. However, it should be noted that the values calculated are only estimated since the peak area ratios obtained were found to be less than the lowest concentration (10 ng mL^{-1}) in the linear calibration range.

In conclusion, an LC-ESI-MS method for the simultaneous detection and quantitation of five major opium alkaloids has been established and has been used to detect alkaloids in harvested poppy seeds and oral fluid samples. From a small pilot toxicology study, oral fluid results indicate that levels of morphine and codeine do not exceed the SAMSHA 40 ng mL^{-1} cut-off after ingestion of a realistic amount of poppy seeds contained within bakery products.

List of Contents

Abstract.....i
List of Contents.....iii
List of Figures.....vi
List of Tables.....x
List of Abbreviations.....xii
Acknowledgements.....xiii
Declaration.....xiv

1. OPIUM AND OPIATES.....1
   1.1. History of opium poppies.....1
      1.1.1. In the beginning.....1
      1.1.2. 10^{th} – 12^{th} centuries.....1
      1.1.3. Paracelsus.....2
      1.1.4. The (very) political years.....3
      1.1.5. The chemical years.....5
      1.1.6. The Opium Wars (1839-1842 and 1856-60).....6
      1.1.7. The discovery of diacetylmorphine.....6
      1.1.8. Modern uses of the opium poppy.....9
   1.2. *Papaver somniferum* L.....9
      1.2.1. Opium, opiates and their uses.....9
      1.2.2. Pharmaceutical industry.....12
      1.2.3. Food industry.....13
      1.2.4. Opiates and abuse.....15
   1.3. Opium and opiates.....16
   1.4. Alkaloid biosynthesis in *Papaver somniferum* L.....18
      1.4.1. Biosynthesis of major opium alkaloids.....18
   1.5. Toxicology.....23
      1.5.1. Toxicology of the opium alkaloids.....23
      1.5.2. Poppy seed ingestion versus heroin use.....33
2. OPIATES IN ORAL FLUID.....35
   2.1. Biological matrices and drug testing.....35

2.1.1. Saliva vs oral fluid 36
2.1.2. Pharmacokinetics of drugs in oral fluid 37
2.1.3. Challenges of using oral fluid in drug testing 43
2.1.4. Extraction of drugs 44
2.1.5. Interpretation of oral fluid results 45
2.2. GC-MS versus LC-MS 46
2.3. Research aims 47
3. LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC-MS) 48
3.1. LC-MS 48
3.1.1. Liquid chromatography 48
3.1.2. Mass spectrometry 50
3.1.3. Mass spectrometry analysers 53
3.2. Analytical method development and validation 55
4. ESTABLISHING AN LC-MS TESTING METHOD FOR THE SIMULTANEOUS DETECTION OF THE FIVE MAJOR OPIUM ALKALOIDS 58
4.1. Experimental 58
4.1.1. Chemicals and reagents 58
4.1.2. Instrumentation 58
4.1.3. Analysis of results 59
4.2. Method development – LC-ESI-MS 60
4.2.1. Selectivity 67
4.2.2. Linearity 68
4.2.3. Precision 69
4.2.4. Other validation parameters 72
4.3. NMR analyses of thebaine 72
4.4. Further method development 76
4.5. Validation of the final LC-ESI-MS method 78
4.5.1. Selectivity 78
4.5.2. Linearity 78
4.5.3. Precision 79
4.5.4. Solvent effects 80
4.6. Comparison with LC-ESI-MS (triple quadrupole) 93
5. ANALYSIS OF POPPY SEEDS AND POPPY SEED CONTAINING FOOD PRODUCTS 103
5.1. Experimental 103

5.1.1. Chemicals, reagents and poppy seeds 103
5.1.2. LC-MS instrument 103
5.1.3. Calibration graphs and calculations 104
5.1.4. Preparation of poppy seeds and poppy seed containing products 104
5.2. Results and Discussion 105
5.2.1. Extraction of alkaloids from poppy seeds 105
5.2.2. Alkaloids in poppy seeds and poppy seed products 107
6. TOXICOLOGY OF POPPY SEED ALKALOIDS IN ORAL FLUID 114
6.1. Experimental 114
6.1.1. Reference material and intermediate stock solutions 114
6.1.2. LC-MS instrument and analytical method 114
6.1.3. Solid phase extraction procedure 116
6.1.4. Preparation of poppy seed containing products – mini muffins 116
6.2. Toxicology study 117
6.2.1. Matrix-matched calibration solutions 119
6.3. Results and discussion 121
6.3.1. Selectivity 121
6.3.2. Linearity 123
6.3.3. Precision 124
6.3.4. Matrix Effects 126
6.3.5. Toxicology study 127
7. CONCLUSION AND FURTHER WORK 139
7.1. Conclusion 139
7.2. Further work 141
8. References 144

List of Figures

Figure 1.1 (a) Antique bottle of Sydenham's laudanum [26] and (b) antique bottle of Dover's Powders [27] 3
Figure 1.2 Basic synthetic route from opium to diacetylmorphine 7
Figure 1.3 (a) Antique bottle of Heroin from Bayer Pharmaceutical Company [44] and (b) Label from a product from Bayer Pharmaceutical Company containing aspirin and heroin [45] 8
Figure 1.4 A lanced poppy with a flower in Myanmar [56] 9
Figure 1.5 Map showing the Golden Triangle: Myanmar, Lao People's Democratic Republic (Laos) and Thailand 10
Figure 1.6 Poppy seeds inside a mature poppy pod [61] 11
Figure 1.7 General process from field to API used by the pharmaceutical industry 12
Figure 1.8 Latex being collected from incised pods and opium collected [56] 16
Figure 1.9 General phenanthrene structure 17
Figure 1.10 General isoquinoline structure 17
Figure 1.11 Chemical structures of the major opium alkaloids 17
Figure 1.12 Chemical structures of shikimic, chorismic and prephenic acids 18
Figure 1.13 Biosynthetic route from prephenic acid to phenylalanine 19
Figure 1.14 Biosynthesis from L-dopa to (S)-scoulerine and (R)-reticuline in alkaloid production in *Papaver somniferum*. Enzyme abbreviations are as follows, BBE: berberine bridge enzyme, CNMT: coclaurine N-methyltransferases, CYP80B3: N-methylcoclaurine 3'-hydroxylase, DRR: 1,2-dehydroreticuline reductase, DRS: 1,2-dehydroreticuline synthase, NCS: norcoclaurine synthase, 4'-OMT: 3'-hydroxy-N-methyltransferase 4'-O-methyltransferase, 6OMT: 6-O-methyltransferase, TYDC: tyrosine decarboxylase. Adapted from [99-101] 20
Figure 1.15 Biosynthetic interconversion from (R)-reticuline to form thebaine, codeine and morphine in *Papaver somniferum*. Enzyme abbreviations are as follows, COR: codeinone reductase, CYP719B1: salutaridine synthase, SaIAT: salutaridinol 7-O-acetyltransferase, SaIR: salutaridine:NADH 7-oxidoreductase. Adapted from [99-101] 21
Figure 1.16 Biosynthetic interconversion from (S)-scoularine to form noscapine in *Papaver somniferum*. Adapted from [99-101] Enzyme abbreviations are as follows: CYP719A21: (S)-canadine synthase, CYP82X2: secoberbine intermediates 3-hydroxylase, PSCXE1: papaveroxine carboxylestaerase, PSMT1: (S)-scoulerine-9-O-methyltransferase, PSMT2:

papaveroxine intermediate 4'-O-methyltransferase, PSSDR1: noscapine synthase, TNMT: tetrahydroprotoberbine cis-N-methyltransferase. 22
Figure 1.17 Central and peripheral nervous systems[107] 24
Figure 1.18 Structural differences between morphine, codeine and thebaine 26
Figure 1.19 Chemical structure of papaverine 28
Figure 1.20 Main metabolic pathways for morphine and codeine. "Glu" denotes a glucuronide group 30
Figure 1.21 Chemical structure of noscapine and its metabolites 31
Figure 1.22 Chemical structure of diacetylmorphine 32
Figure 2.1 Major saliva producing glands where (1) represents the parotid glands, (2) represents the submandibular glands and (3) represents the sublingual glands (Figure from Aps, 2005[163]) 36
Figure 2.2 Ionisation of a weak base of pKa 8.5 with varying pH 40
Figure 2.3 (a) Quantisal™, (b) OraSure® and (c) Intercept® oral fluid collection kits 44
Figure 3.1 Diagram representing silicon backbone of a C18 stationary phase with –Si-OH groups. Adapted from Restek HPLC column selection guide[214] 50
Figure 3.2 Block diagram of the processes involved in mass spectrometry after HPLC separation 50
Figure 3.3 Comparison of ionisation methods in relation to analyte polarity and molecular weight 51
Figure 3.4 Ion formation in an ESI source. Adapted from Bayne & Carlin (2010)[172] 52
Figure 3.5 APCI ion source. Adapted from Bayne & Carlin (2010)[172] 52
Figure 3.6 Trapping system of an ion trap analyser. Adapted from Bayne & Carlin (2010)[172] 53
Figure 3.7 Image of a triple quadrupole[233] with block diagram outlining ion formation and separation 54
Figure 3.8 Diagram of a linear trap quadrupole orbitrap mass spectrometer[236] 55
Figure 4.1 Chromatogram and associated mass spectra for morphine injection for (a) peak 1 at 1.33 minutes and (b) peak 2 at 10.53 minutes 61
Figure 4.2 Chromatogram and associated mass spectra for codeine injection for (a) peak 1 at 1.33 minutes (b) peak 2 at 2.86 minutes and (c) peak 3 at 11.14 minutes 62
Figure 4.3 Chromatogram and associated mass spectra for thebaine injection (a) peak 1 at 1.38 minutes (b) peak 2 at 7.26 minutes and (c) peak 3 at 9.81 minutes 63

Figure 4.4 Chromatogram and associated mass spectra for noscapine injection (a) peak 1 at 8.28 minutes and (b) peak 2 at 9.66 minutes 64
Figure 4.5 Chromatogram and associated mass spectra for papaverine injection (a) peak 1 at 8.54 minutes and (b) peak 2 at 10.32 minutes 65
Figure 4.6 An example of the resulting chromatogram and associated mass spectrum from a blank injection 65
Figure 4.7 Single peaks for (a) morphine (b) codeine (c) papaverine (d) noscapine and (e) two peaks for thebaine 67
Figure 4.8 Data and associated calibration graph for morphine 70
Figure 4.9 Data and associated calibration graph for codeine 70
Figure 4.10 Data and associated calibration graph for papaverine 71
Figure 4.11 Data and associated calibration graph for noscapine 71
Figure 4.12 Chromatogram for thebaine showing two peaks: (a) mass spectrum for peak at 1.31 minutes and (b) mass spectrum for peak at 8.62 minutes 72
Figure 4.13 ^{1}H-NMR spectra for thebaine in (a) D_2O:CD_3CN ratio 80:20 (v/v), (b) D_2O:CD_3CN ratio 50:50 (v/v), (c) D_2O:CD_3CN ratio 20:80. All solutions contain 1% CD_3COOD 73
Figure 4.14 ^{1}H-NMR spectrum of thebaine in 100% D_2O (+ 1% CD_3COOD) 74
Figure 4.15 Formation of thebaine-D^+ complex ions, HA^+ and HB^+ from epimers A and B[254] 75
Figure 4.16 Extracted chromatograms from a mixed injection of (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine, (e) papaverine and (f) noscapine 78
Figure 4.17 Proposed fragments formed from morphine during ionisation in (A) LC-ESI-MS and (B) LC-MS^n instruments[263-265] 95
Figure 4.18 Proposed fragments formed from codeine during ionisation in (A) LC-ESI-MS, (B) LC-MS^n and (C) both instruments[263-265] 96
Figure 4.19 Proposed fragments formed from thebaine during ionisation in (A) LC-ESI-MS, (B) LC-MS^n instruments[232] 96
Figure 4.20 Proposed fragments formed from papaverine during ionisation in (A) LC-ESI-MS, (B) LC-MS^n and (C) both instruments[266] 97
Figure 4.21 Proposed fragments formed from noscapine during ionisation in both LC-ESI and LC-MS^n instruments[266] 97
Figure 4.22 Chromatogram showing all five alkaloids separated using PFPP column and LC-MS^n tandem mass spectrometer. The quantitation ion highlighted in Table 4.20 was used (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine), (e) papaverine, (f) noscapine ... 98

Figure 4.23 Chromatograms for (a) morphine, (b) codeine, (c) thebaine, (d) papaverine, (e) noscapine, (f) morphine-d3 in triplicate 100
Figure 6.1 Chromatograms for (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine, (e) papaverine, (f) noscapine 121
Figure 6.2 Extracted chromatograms for (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine, (e) papaverine, (f) noscapine 122
Figure 6.3 (a) Extracted chromatogram using *m/z* 414 for a known injection of noscapine, (b) mass spectrum of noscapine, (c) extracted chromatogram using *m/z* 414 for blank oral fluid spiked with morphine-d3, (d) mass spectrum of peak of retention time 8.00-8.24 minutes from chromatogram (c) 123
Figure 6.4 Combined participant time-concentration profile for morphine 128
Figure 6.5 Combined participant time-concentration profile for morphine (reduced time points on x-axis) 128
Figure 6.6 Combined participant time-concentration profile for morphine obtained from the repeat study 129
Figure 6.7 Combined participant time-concentration profile for morphine obtained from the repeat study (reduced time points on x-axis) 130
Figure 6.8 Combined participant time-concentration profile for codeine 131
Figure 6.9 Combined participant time-concentration profile for codeine obtained from the repeat study for participant 1 132
Figure 6.10 Combined participant time-concentration profile for codeine obtained from the repeat study for participants 2 and 3 (reduced time points on x-axis) 132
Figure 6.11 Combined participant time-concentration profile for thebaine 133
Figure 6.12 Combined participant time-concentration profile for noscapine 134
Figure 6.13 Combined participant time-concentration profile for noscapine (reduced time points on x-axis) and participant 1 removed 135
Figure 6.14 Combined participant time-concentration profile for papaverine obtained from the repeat study 136

List of Tables

Table 1.1 Alkaloid content by % weight of opium in *Papaver setigerum* by electrophoresis [58] and *Papaver somniferum* by HPLC [59] 10

Table 1.2 Countries (and amount in tons) producing opiate raw materials rich in morphine and thebaine. 13

Table 1.3 Legislation/treaties relating to control of opium 15

Table 4.1 Initial LC-MS conditions used for attempted detection of opium alkaloids 60

Table 4.2 Optimised mass spectrometer settings for initial MS/MS experiments 66

Table 4.3 Linear equations and associated R^2 values for morphine, codeine, papaverine and noscapine 68

Table 4.4 Analyte specific parameters for LCQ Advantage mass spectrometer from HPLC (* - ion used for quantitation) 76

Table 4.5 Mobile phase composition and gradient program for the final LC-ESI-MS method on the PFPP column 77

Table 4.6 Linear equations and associated R^2 values for morphine, codeine, thebaine, papaverine and noscapine 79

Table 4.7 %CV for morphine, codeine, thebaine, papaverine and noscapine with varying concentration 80

Table 4.8 Effect on reproducibility of individual alkaloid compounds in acetonitrile (ACN) at 50 ng mL^{-1} 81

Table 4.9 Effect on reproducibility of individual alkaloid compounds in ACN at 300 ng mL^{-1} 82

Table 4.10 Effect on reproducibility of individual alkaloid compounds in ACN at 1000 ng mL^{-1} 83

Table 4.11 Methanol:water (25:75, v/v) at 50 ng mL^{-1} 84

Table 4.12 Methanol:water (25:75, v/v) at 300 ng mL^{-1} 85

Table 4.13 Methanol:water (25:75, v/v) at 1000 ng mL^{-1} 86

Table 4.14 Methanol:water (50:50, v/v) at 50 ng mL^{-1} 87

Table 4.15 Methanol:water (50:50, v/v) at 300 ng mL^{-1} 88

Table 4.16 Methanol:water (50:50, v/v) at 1000 ng mL^{-1} 89

Table 4.17 Methanol:water (75:25, v/v) at 50 ng mL^{-1} 90

Table 4.18 Methanol:water (75:25, v/v) at 300 ng mL^{-1} 91

Table 4.19 Methanol:water (75:25, v/v) at 1000 ng mL^{-1} 92

Table 4.20 Analyte specific parameters for 4500 QTRAP tandem mass spectrometer. DP-declustering potential; EP – entrance potential; CXP – collision cell exit potential. (* - ion used for quantitation) 94
Table 4.21 Peak area ratios for morphine, codeine, thebaine, papaverine and noscapine 101
Table 4.22 Mean (x), standard deviation (σx) and co-efficient of variation (CV%) for cumulative data for each of the five alkaloids at 20 ng mL^{-1} and 200 ng mL^{-1} 102
Table 4.23 Linear equations and associated R^2 values for the five major opium alkaloids. 102
Table 5.1 Poppy seed brand and country of origin 103
Table 5.2 Extraction solvents and associated polarity index values 106
Table 5.3 Range and mean weight of morphine (ng) per gram of poppy seeds 108
Table 5.4 Range and mean weight of codeine (ng) per gram of poppy seeds 108
Table 5.5 Range and mean weight of thebaine (ng) per gram of poppy seeds 109
Table 5.6 Range and mean weight of noscapine (ng) per gram of poppy seeds 110
Table 5.7 Comparison of levels of alkaloids identified in harvested poppy seeds, seeds from the surface of bread rolls and seeds heated with no matrix 111
Table 5.8 Comparison of alkaloids identified in poppy seeds 112
Table 6.1 Mobile phase composition and gradient program 115
Table 6.2 Analyte specific parameters for Q-Exactive™ mass spectrometer. CID – in-source collision induced dissociation energy. *-ion used for quantitation 115
Table 6.3 Weights of muffins provided in each of the kits for the toxicology study 117
Table 6.4 Final concentrations for intermediate stock solutions of morphine, codeine, thebaine, papaverine and noscapine 119
Table 6.5 Concentration of alkaloids present in calibration solutions 120
Table 6.6 Calibration range, linear equation and R^2 values for morphine, codeine, thebaine, papaverine and noscapine. 124
Table 6.7 Mean (x), standard deviation (σx) and co-efficient of variation (%CV) for 10 injections of mixed alkaloid oral fluid samples of 200 ng mL^{-1}, containing 100 ng mL^{-1} morphine-d3 125
Table 6.8 Peak area ratios for morphine codeine, thebaine, papaverine and noscapine, showing mean (x), standard deviation (σx) and co-efficient of variation (%CV) for 10 QC samples analysed a period of four days 126

List of Abbreviations

APCI	**Atmospheric pressure chemical ionisation**
GC-MS	**Gas chromatography-mass spectrometry**
LC-ESI-MS	**Liquid chromatography-electrospray ionisation-mass spectrometry**
LC/MS/MS	**Liquid chromatography tandem mass spectrometry**
LLE	**Liquid-liquid extraction**
NMR	**Nuclear magnetic resonance spectroscopy**
PFPP	**Pentafluorophenyl phase with propyl spacer**
RP-LC	**Reversed phase liquid chromatography**
SPE	**Solid phase extraction**

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the Faculty Ethics Committee on 28th October 2013.

I declare that the word count of this thesis is 36,225 words.

Name: Michelle Carlin

Signature:

Date: 16th March 2016

1. OPIUM AND OPIATES

1.1. History of opium poppies

1.1.1. In the beginning…

Poppies have been grown and used in daily culture for many thousands of years but the exact date or place of origin has not been established: it is however proposed by a number of scholars that *Papaver somniferum* L., the opium poppy originated in Sumer, a region in ancient Mesopotamia (modern day Iraq and Kuwait) around 5000 BC.[1] Much has been written with respect to the opium poppy over the many years since this time and the writings relate not only to the cultivation and harvest of this crop but to the investigations of the chemistry of the plant and its medicinal uses and to the wars that have been fought.[1-11] Opium, a latex based product of the opium poppy has been the basis for the two Opium wars (1839-1842 and 1856-1860) fought between Great Britain and China but also many a personal war has been fought against the addiction to opium and the alkaloid compounds extracted and further synthesised from it.[5,12]

Although other writings have been published before this time, Hippocrates (450-380 BC) a Greek physician, was one of the first people to highlight and document the importance of the poppy in its many forms for medical purposes [2,3,13] and as such, the use of the poppy for anaesthesia was well documented in the *Corpus Hippocraticum*.[14] This is a collection of 60 documents detailing medical practices and procedures, not all written by Hippocrates himself, but as a collection form the basis of some modern medical practices.[13] Poppies were also recommended as treatment where pain was recorded as one of the patient's symptoms in a number of passages from these documents.[14] However from history, the opium poppy disappeared for almost 1000 years with no real reason or explanation as to why.

1.1.2. 10^{th} – 12^{th} centuries

In between the 10^{th} and 12^{th} centuries it has been reported that the opium poppy was cultivated in the Abbasid Caliphate (what now covers Iraq, Afghanistan, Turkey and India): most of what was produced in this region however was predominantly used in "home-use" as poppy cake (this is now used as livestock feed) or was taken to local markets and sold. Arab traders however commenced the first "trafficking" of opium to other parts of the

world, such as, to Indonesia, China and along the North African coast to Moorish Spain. Since opium was not a common product at this time in many parts of the world, some people were sceptical about its use, but the Arab traders continued with their plight and persevered and eventually opium became welcomed rather than feared.[15]

The routes that were taken by these Arab traders crossed many a desert or other arid landscape and they also encountered bandits: in order to make sure their product arrived at its destination safely, these traders would choose routes that were not on maps of the time and thus made their trek even more inhospitable. These routes are still used in what is now modern day Afghanistan and are still used to traffic opium and its subsequent products through to other parts of the world.[16]

Opium became widely known and used as an analgesic in areas where the opium was harvested and trafficked. It was not only found to be of use as an analgesic but was also found to be useful in managing many other ailments. At this point, Egypt and Mesopotamia were the leaders in the opium growing areas of the world and this eventually greatly aided the international trade in opium.[17]

The Crusades (1095-1291) were military campaigns that were initially sanctioned by the Roman Catholic Church to ensure access for Christians to the Holy Lands of Jerusalem.[18] With the West involved in fighting with the East, it was possible for opium to make its way back to Europe with the return of those who had been fighting in the Holy Lands and it has been reported that the Arab traders pushed this process forward. Due to the nature of the voyage, the opium that eventually reached Europe was not found to be in the best condition but was opium nonetheless: at this point in history, the demand for opium greatly increased.[15,19]

1.1.3. Paracelsus

Philippus Aureolus Theophrastus Bombastus von Hohenheim (1493-1541 AD), a Swiss-German alchemist and physician also known as Paracelsus, has the title of founding father of toxicology attributed to him.[20] Amongst his many contributions to toxicology and medicine, he is reported to have introduced laudanum, at this time a solid form of opium latex derived from poppies. He studied the effects of laudanum on many people, including himself which he characterised as causing sleep: he is reported to have said that “an ideal medicine puts the disease to sleep without killing the patient”.[15] He is also widely reported

to have prescribed the opium product as a treatment for many forms of pain throughout the body. He recorded many of his prescriptions and had a number of apprentices. After the death of Paracelsus, many scholars and followers of Paracelsus started to disseminate his teachings throughout parts of Europe.[21]

1.1.4. The (very) political years

By the early 17th century, laudanum became a reference to a liquid preparation of opium and alcohol, also known as a tincture of opium.[22] Thomas Sydenham (1618-1683), a British physician whose aim was to relieve suffering, produced the opium tincture that was easily prepared and would in the end, be easy to administer to all patient types. Sydenham made this tincture of opium a proprietary medication sold as Sydenham's Laudanum with this preparation sold as a "cure all".[23] One of the apprentices of Sydenham, Thomas Dover (1660-1742) later went on to produce an opium based powder and between Sydenham's Laudanum and Dover's Powder (Figure 1.1 showing both preparations), opium took its place firmly in the legitimate pharmacies of the time.[24,25] Whether the ready availability and affordability of opium aided the popularity of the two preparations or the preparations aided the availability of the opium in the UK is not clear, however what is clear is that from this point onwards in history, the relationship of the West with opium spirals out to include drug addiction and war on grand scales which have continued to the present day.

a. b.

Figure 1.1 (a) Antique bottle of Sydenham's laudanum [26] and (b) antique bottle of Dover's Powders [27]

For many hundreds of years, the opium poppy had been cultivated and harvested in India: as previously mentioned as was the case in Abassid Cailphate, the opium grown in India was a product used by families as a remedy for all things. After the Battle of Plassey (a

battle between the British and the Nawal of Bengal and his French allies which resulted in significant control over the Indian subcontinent by colonial powers) in 1757 the Honourable East India Company of England was established and this association of businessmen and aristocrats ensured that trade in products such as cotton, silk, tea, and most importantly, opium was successful.[28,29] English Governors in India were introduced, with one of the roles being to oversee the cultivation and harvest of the opium crop.

There were numerous challenges faced by the English governors, the local Indian farmers and both governments, but by the 1780's it is estimated that the area of land used in the cultivation and harvest of opium poppy was approximately 250,000 acres.[15] The expanse and size of this kind of operation resulted in a change in the harvesting methods in order to standardise the process and make the product more stable to transport and eventually to sell at auction in Calcutta. From reports of the time it is estimated that the annual income from opium was thought to be in excess of £2 million with the largest customer of India being China in the East leaving the Anatolian Plateau, or more specifically Turkey, to become the main supplier of opium to the West.[30]

In Turkey around the 1780's and probably before, all parts of the plant were utilised, from the poppy seed oil used in cooking and lighting to the poppy cake being used by poorer families to bulk out their bread. It has been reported[31] that at this time, Turkish opium was far more potent than the Indian variety, with the plants growing 6-8 feet in height (in comparison to Indian plants which would typically grow 3-6 feet) and containing around 10-13% by weight morphine in comparison to 4-8% in the Indian variety: this was determined by analysis of the product years later.[32] This Turkish opium was transported from Smyrna to predominantly the British ports of Dover, Bristol, Liverpool but mostly to London.[15]

British people at the time did not wish to purchase opium from Turkey and preferred that their opium was "home" grown, whether that be from the UK or from one of the other countries within the British Empire. Prizes were offered for studies on the growing of medicinal plants and for opium, with a number of eminent British scientists proving that it was possible to grow opium on British land, including the cold Scottish countryside.[33] However what was problematic were the yields that could be produced: these yields would not be as profitable as imported opium from other countries since the cost of producing the opium would be much higher in the UK. The reasons for this were the yield was lower and the cost of labour was higher, even if women and children were employed to carry out

the laborious task of harvesting.[34] It was also found that if appropriate harvesting techniques were not used, some of the crop would be lost, further reducing the yield. Some of the home-grown opium was used and Britain continued to import opium from Turkey but by the year 1805, the British had begun to export approximately a quarter of this opium to the United States of America.[35]

1.1.5. The chemical years

It was also in 1805 when German pharmacist Freidrich Sertürner (1783-1841) reported his findings of his isolation of the alkaloid morphine from opium.[1,11] He had seen the use of opium with his sister who had died of consumption (pulmonary tuberculosis) but he had also noted that the opium had eventually lost its effectiveness. He decided to investigate this phenomenon by carrying out many experiments to identify the extracts of the opium. Sertürner published a paper outlining his findings from opium extracts and showed that his "poppy acid" showed different chemical reactions than with opium alone and stated that the "cause was something non-acidic but biologically active". In terms of scientific deductions this is a significant one as at this time in history; it was assumed that all pharmacologically active compounds were acidic in nature with the acidity of the compound being directly related to the strength of the biological activity.[36] It took a number of years and much devotion from Sertürner before he could convince the scientific community to accept his findings, however he eventually proved that he had isolated a compound, which he called *principum somniferum* (later called morphine) by carrying out a chlorate precipitation. He also proved that by weight, the product was more than ten times more active than the same weight of opium. These results were determined by experimenting with the morphine and the opium on himself and others and although he was probably addicted to the alkaloid compounds, he established that morphine could have many practical uses in treating pain and beyond.[37] It eventually took the efforts of Joseph-Louis Gay-Lussac, a renowned chemist, to help Sertürner to be acknowledged for the work but that would not happen for a further 10 years when his patience was rewarded.[38]

Morphine was not fully appreciated pharmaceutically until the invention of the hypodermic needle in 1853, long after the death of Sertürner.[39,40] Morphine was initially used to cure addictions to alcohol, opium and laudanum amongst other preparations but it was not known at this time that morphine itself was highly addictive.[23,33]

The work of Sertürner began a renewed interest in organic chemistry over the following years, which resulted in other alkaloid compounds being isolated from opium: the most notable of these being narcotine (noscapine) in 1817 and codeine in 1832, both by French chemist Pierre Jean Robiquet (who had been a student under the tutelage of Gay-Lussac) [41], thebaine (in 1832 by Pierre-Joseph Pelletier and Thibournery who was the manager of his factory [19]), and papaverine in 1849 (by Georg Merck [42], son of Emanuel Merck founder of the Merck Pharmaceutical and Chemical Company).

1.1.6. The Opium Wars (1839-1842 and 1856-60)

In the early 19th century, many chemical advancements were made with regards to understanding opium alkaloids but also at this time the two Opium Wars were being fought between Britain and China. By the 1830's, China was almost being destroyed by opium as it was heavily used as a recreational drug and a high proportion of the population were addicted.[23] The Emperor asked Queen Victoria for assistance to stop the opium being brought in to China but this request was ignored and action was taken by the Emperor to ban the importation and use of opium. However opium was a very lucrative business for all and because imports of opium by British merchants (as well as other countries) to China were banned therefore losing money, the British decided to try to rectify the situation: they used the fact that the Chinese wished British merchants to comply with "barbaric Chinese law" as the reason for citing a war.[43] Britain won the war resulting in the legalisation of opium in China but the situation also began changes to world politics and international trade.[19] The second opium war was waged against China by France and Britain and resulted in the continued legalisation of opium and a large payment to both France and Britain.

1.1.7. The discovery of diacetylmorphine

Diacetylmorphine, the major pharmacological active compound of heroin, was synthesised from morphine in 1874 by Charles Romley Alder Wright, a British scientist who was investigating a non-addictive alternative to morphine.[4,6] The general synthetic process for the synthesis of diacetylmorphine from opium is shown in Figure 1.2.

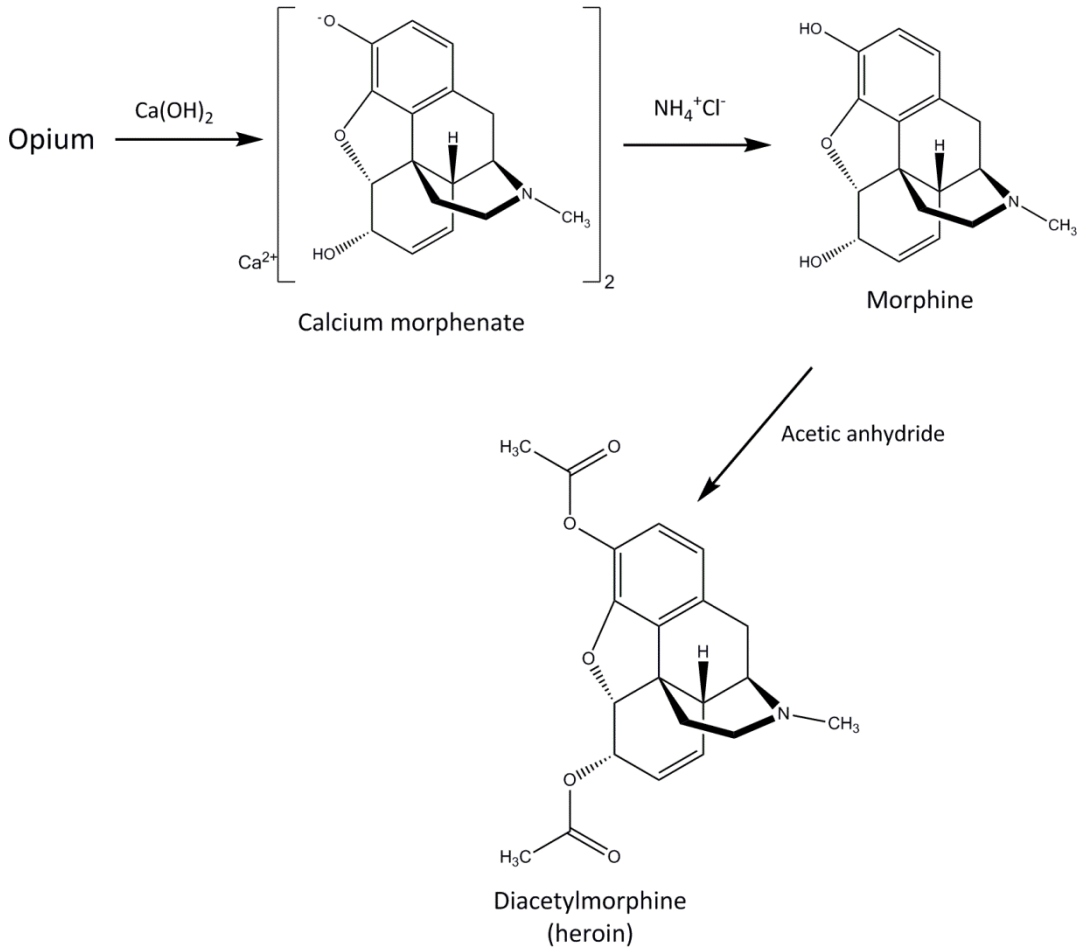


Figure 1.2 Basic synthetic route from opium to diacetylmorphine

In 1898, the German chemical company of Bayer introduced diacetylmorphine to the market as Heroin (Figure 1.3a). This product was hailed as a wonder drug and one that did not have the same side effects as shown by morphine. It also appeared not to show the same addictive properties as morphine and was used in the treatment of morphine addiction (known as demorphinisation).[4] It was later found that patients treated with heroin did not desist in their use of the preparation once their 'treatment' phase had concluded but continued to self-administer the drug.[19] Bayer Company went on to use diacetylmorphine in other preparations alongside their other new drug aspirin (Figure 1.3b).

a. 

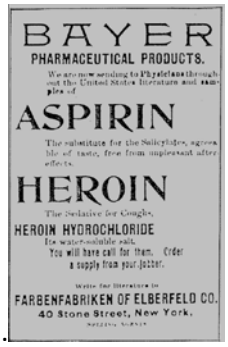
b. 

Figure 1.3 (a) Antique bottle of Heroin from Bayer Pharmaceutical Company [44] and (b) Label from a product from Bayer Pharmaceutical Company containing aspirin and heroin [45]

It wasn't until 1899 that physician Horatio C. Wood (1841-1921) found that in order to maintain the same level of effect on an individual over a lengthy time, the dose of heroin had to be increased. He also proposed that there was not enough experimental data to make an accurate conclusion that heroin was not an addictive compound.[46]

It was proven over the years that heroin was far more addictive than morphine but that its properties were of far more use than morphine. It was proposed that to reduce the possibility of addiction that heroin should not be used in the treatment of minor ailments such as coughs, but to be prescribed only in severe acute pain (not in cases of chronic pain). However, heroin was found to be a most effective drug and physicians and pharmacists were at odds about using it since no other drug of the time equalled the pharmacological effects of heroin: they therefore continued to use it.[47] Many other heroin preparations became commercially available, including mixtures with other drugs such as cocaine, cannabis and the less problematic ipecacuanha (an expectorant).[48]

Over a number of years, physicians realised the devastating effect of heroin and its addictive properties. However, smugglers and other criminals of the time took very little time in comparison to realise just how much of a commodity heroin was.[33] They recognised heroin's pharmacological effectiveness, in comparison to morphine, but also the power of exploitation they had with heroin. Around 1924, the legitimate manufacture of heroin by the pharmaceutical companies ceased and most of the heroin was provided by smugglers.[49] Public perception of the drug changed from one extreme to the other and over time, heroin produced worldwide criminal organisations of such magnitude that modern day society is still dealing with it today.

1.1.8. Modern uses of the opium poppy

In the 21st century, the two main legitimate uses of the opium poppy are as a source of alkaloid compounds for the pharmaceutical industry and as a source of poppy seeds for the food industry.[50] In addition, this plant is used illicitly in the manufacture of diacetylmorphine.[51]

1.2. *Papaver somniferum* L.

Of the plant family Papaveraceae (common name poppy) the genus *Papaver* has two species containing morphine, codeine, thebaine, noscapine (also called narcotine) and papaverine: *Papaver somniferum* L. (Figure 1.4) and *Papaver setigerum D.C.* [52-54] Thebaine has been reported in *Papaver orientale* L. *and Papaver bracteatum* Lindl. but no biosynthetic interconversion to codeine and morphine has been found in these species.[55]

Figure 1.4 A lanced poppy with a flower in Myanmar [56]

It is a continual debate as to whether *Papaver setigerum* D.C. is a subspecies of *Papaver somniferum* L. or whether *Papaver setigerum* D.C. is a separate species altogether as it is thought that *Papaver somniferum* L. is the product of many years of optimized breeding but for clarity, in this work it is assumed that they are separate species.[52,53,55,57]

1.2.1. Opium, opiates and their uses

Although it is known that alkaloid compounds can be found in both *Papaver somniferum* L. and *Papaver setigerum* D.C., *Papaver somniferum L.* has considerably higher levels of the

five major alkaloids by percent weight of opium than that present in *setigerum*, as can be seen in Table 1.1.

Table 1.1 Alkaloid content by % weight of opium in *Papaver setigerum* by electrophoresis [58] and *Papaver somniferum* by HPLC [59]

	***Papaver* subspecies**	
Alkaloid	*Setigerum*	*Somniferum*
Morphine	2.3 %	7.65 - 25.15 %
Codeine	2.6 %	1.21 - 6.37 %
Thebaine	Detected but not quantified	0.97 - 6.38 %
Papaverine	4.7 %	0.51 - 5.33 %
Noscapine	10.2 %	4.03 - 15.22 %

Papaver somniferum L., is an annual crop cultivated worldwide but is legitimately grown by the pharmaceutical and food industries in France, Spain, Turkey, Holland, Hungary, Poland, Romania, Czechoslavakia, Yugoslavia, India, Central and Southern America, Canada, Australia and Iran.[2,60] The world's illicit crop of opium originates predominantly in an area well known for the production of opium and heroin known as the "Golden Triangle" (see Figure 1.5) and Afghanistan. These areas are also well known for drug smuggling.

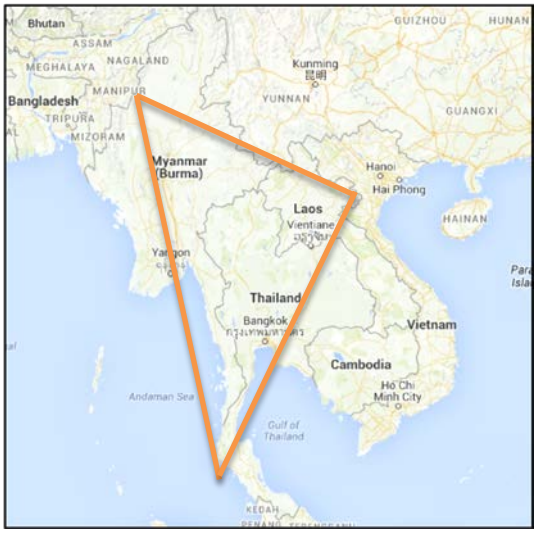


Figure 1.5 Map showing the Golden Triangle: Myanmar, Lao People's Democratic Republic (Laos) and Thailand

Papaver somniferum L. is an herbaceous plant that when grown in the southern hemisphere is generally sown in July, with the crop managed between August and December, and harvested between January and April. This final phase includes harvesting, assessment of crop quality and payment. In the northern hemisphere the crop is generally sown in late autumn/early winter.[11] The plants emerge in the spring when flowering occurs, subsequently forming ripe capsules in May/June. In this growth process, the capsules will change from blue/green to yellow. There is a limited window for harvesting of the latex: this is 5-10 days after the flowering petals fall from the plant. The dried latex product is called opium from which morphine and other alkaloid compounds can be extracted. If left to fully mature, the plant will form poppy seeds (Figure 1.6) within the capsule which can be mechanically harvested and collected by a sieving process.[3,11]

Figure 1.6 Poppy seeds inside a mature poppy pod [61]

Harvesting methods have been shown to greatly affect the level of alkaloids in the final opium product. If the opium is harvested too early in the process the product is found to be watery and too late in harvesting produces opium with significantly lower levels of alkaloids.[62] Poor practices in collection also reduce the size and quality of the crop: for example if the incision made in the pod is too deep, the pod is punctured and the latex lost, ultimately resulting in loss of opium. When carried out skilfully, most of the pods will be incised more than once during the harvesting of an opium field. The whole process will typically last several days to weeks, depending upon the size of the field.[51]

1.2.2. Pharmaceutical industry

In modern day society, opium continues to have many uses; in particular it is used as a source of narcotic raw material (NRM) from which the pharmaceutical industry synthesises active pharmaceutical ingredients (API). See Figure 1.7 for the general pharmaceutical process from field to API.

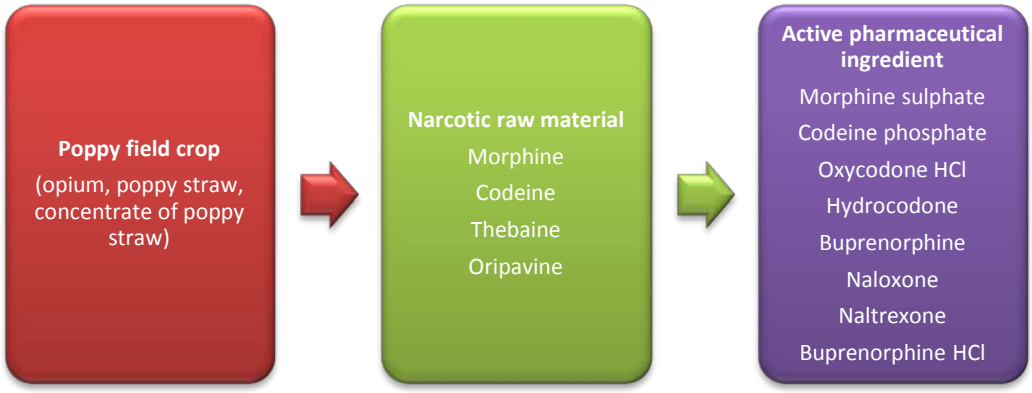


Figure 1.7 General process from field to API used by the pharmaceutical industry

Papaver somniferum L. with higher levels of morphine and codeine (but predominantly morphine) were historically grown by the pharmaceutical industries however, products from crop (opium and poppy straw) of this strain were open to exploitation by less than salubrious individuals/groups who could produce heroin (diacetylmorphine). Strains of *Papaver somniferum* L. were bred to contain higher levels of thebaine and also oripavine: these compounds are controlled by the International Narcotics Control Board (INCB) because of their convertibility to opioids subject to abuse (as are morphine and codeine due to their potential for abuse).[60,63] The INCB is an independent body monitoring the implementation of The United Nations International Drug Control Conventions (Single Convention on Narcotic Drugs of 1961 as amended by 1972 Protocol, Convention on Psychotropic Substances 1971 and the United Nations Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances 1988).[64,65]

The main countries licitly producing *Papaver somniferum* L. crops for the pharmaceutical industry are shown in Table 1.2. Most countries will grow crops higher in either morphine or thebaine content but some countries produce both. The annual crop in tons is also provided in Table 1.2 showing the values for 2013 but also the values estimated for 2014.[60]

Table 1.2 Countries (and amount in tons) producing opiate raw materials rich in morphine and thebaine.

	Crop rich in morphine		Crop rich in thebaine	
Country of origin	**2013**	**2014***	**2013**	**2014***
Australia	184	220	268	311
France	131	145	15	29
Hungary	27	20	3	10
India	32	51	3	5
Spain	116	143	58	61
Turkey	65	108	n/a	n/a
Other	38	22	1	1

*these values have been estimated by the INCB; n/a – not applicable

Most of the morphine and codeine (83% and 91%, respectively) produced worldwide by the pharmaceutical industries is obtained from poppy straw with the remaining portions extracted from opium. Poppy straw comprises all parts of the *Papaver somniferum* L. crop after mowing but it does not include the seeds. Poppy straw can be obtained from all of the countries listed in Table 1.2 but India is the only country permitted to supply opium to the world's pharmaceutical market.[66]

1.2.3. Food industry

It is not clear at what point poppy seeds were included in food preparation but archaeological artefacts found in an area of Lucerne, Switzerland dating from approximately 6000 years ago (the Neolithic period) identified evidence of poppy cultivation.[19] The artefacts included poppy cake and poppy heads.[67,68] Other archaeological artefacts have been identified from other sites that include items with poppy head symbols but the Swiss find is the first evidence of the use in food preparation.[7,69] It is possible to surmise that if our ancestors were growing a crop that they would have used as much of the product of their labours as was possible and so the use of seeds in cooking would have been a natural evolution.

Papaver somniferum L. is cultivated for the pharmaceutical industry but a by-product of the process of harvesting poppy straw is poppy seeds.[60] This source of poppy seeds is used by the food industry and are included in cakes, on bread products and sold to supermarkets and specialist shops for use in cooking/baking recipes. It was initially thought that the

seeds and any products derived from them would not contain any alkaloid compounds due to the fact that the seeds develop after the latex.[10,11] In the late 1970's, it was noted that poppy seeds contained alkaloids found in opium. However it was not until the 1980's that research in this field escalated and in 1998 a paper was published by Meadway*, et al.*[70] which highlighted that it was possible to fail a urine drug test after the consumption of a bread product containing poppy seeds.[52,70-77]

Over the last 10-15 years it has become increasingly apparent that the presence of alkaloids in the food chain is a problem and can potentially lead to serious repercussions.[52,70-72,76-87] In Germany in particular, where poppy seeds are used and readily incorporated into food products, measures have been considered for some time to try to reduce the amount of morphine present in poppy seeds intended for the food chain. It has been reported that poppy seeds used for decorative purposes can contain up to 100 mg/kg of morphine however German authorities have recommended a limit of 20 mg/kg.[75]

In 2011 the European Food Safety Authority (EFSA) published information relating to alkaloids in food products and provided a risk assessment with respect to public health.[88] The information was gathered from a call from EFSA regarding any data relating to levels of morphine, codeine, thebaine, noscapine and papaverine from poppy seeds destined for the food market. From this research, it was noted that poppy seed use in food preparation varies considerably in European countries, and they are culturally and traditionally used in Central-Eastern Europe in many food products such as bread, desserts and fine bakery wares (bakery products containing sugar and fat at more than 5% by dry weight of fat). In comparison, poppy seeds are not used as extensively in other parts of Europe, such as in the United Kingdom. The panel assessing the data evaluated the potential dietary exposure to morphine as well as the morphine levels that were found in biological material post-consumption of the poppy seed products: they considered morphine only due to the higher pharmacological activity of morphine in comparison to the other four opium alkaloids.

A number of significant points were highlighted from this research; the first being that the estimated dietary morphine exposure. Based on information provided from three European countries where there is a higher consumption of poppy seed products and from data on the occurrence of morphine in poppy seeds, it was estimated that daily intake of morphine could be from 3 – 90 μg/kg body weight per day. It was also hypothesised, using the same data that portions of food items having high poppy seed content could provide morphine exposure from approximately 38 – 200 μg/kg body weight per portion for adults. From

evaluation of extensive scientific literature sources, the EFSA panel concluded that it was possible for an individual to suffer effects from ingestion of poppy seed products but this was concluded from a number of cases reported from Germany only.[88]

It has also been shown that washing and other pre-treatments of the seeds can reduce morphine levels by up to 90% as it is believed that the alkaloid content found when analysing poppy seeds is due to external contamination from the pod previously containing the latex and not from the inside of the seeds.[76]

1.2.4. Opiates and abuse

Currently, the control of opium falls under the responsibility of the United Nations under the Single Convention on Narcotic Drugs 1961[89] and is monitored by the International Narcotics Control Board (INCB). There are a number of pieces of legislation that have been written pertaining to the control of drugs which make reference to opium, or are specific to opium and are shown in Table 1.3.

As previously mentioned (section 1.3.1), there are a wide range of countries cultivating *Papaver somniferum L.* legitimately for the pharmaceutical and food industries with the illicit crops being grown in the Golden Triangle and Afghanistan. The crops destined for the pharmaceutical and food markets are tightly controlled since farmers cultivating, harvesting and selling the plant and its products must possess a licence.[64] On the other hand, monitoring and controlling the illicit crops is a far more difficult task.[51]

Table 1.3 Legislation/treaties relating to control of opium

Act	Year
Pharmacy Act [90]	1868
Poisons & Pharmacy Act [91]	1908
International Opium Convention [92]	1912
Dangerous Drugs Act [93]	1920
Single Convention on Narcotic Drugs [89]	1961 (as amended in 1971)
Misuse of Drugs Act [94]	1971
United Nations Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances [95]	1988

The UNODC World Drug Report provides an annual review of international drug markets and in the latest report, opiates and opioids continue to be the principal drugs causing the most concern and drug-related deaths globally. It has been highlighted that Afghanistan is the major producer of illicit opium crops and this has continued to be the case for the last three years where the area under cultivation is estimated to have been 209,000 ha in 2013. In comparison, the global area under cultivation for the illicit market was estimated to be 296,700 ha. From this area in Afghanistan, it is estimated in the report that the total amount of the opium product is 5,500 tons with the potential to produce 560 tons of heroin of unknown purity.[51]

1.3. **Opium and opiates**

Opium is the raw material that is formed from a milky exudate obtained by incising the unripe capsules of *Papaver Somniferum* L. when it is air dried (Figure 1.8).[11]

Figure 1.8 Latex being collected from incised pods and opium collected [56]

More than 100 alkaloids have been identified from opium, with five of them accounting for almost all of the quantitative alkaloid content.[50,96] These alkaloid compounds can very broadly be classed as phenanthrene or isoquinoline alkaloids, with general structures shown in Figures 1.9 and 1.10, respectively.

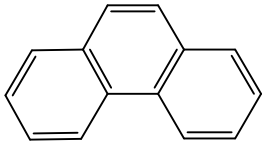


Figure 1.9 General phenanthrene structure

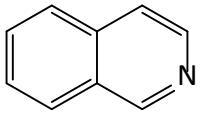


Figure 1.10 General isoquinoline structure

Of the five major alkaloids, morphine, codeine and thebaine are classed as phenanthrene alkaloids; the other two (papaverine and noscapine) fall into the benzoisoquinoline category.[97] Structures of all of these alkaloids are shown in Figure 1.11. The other minor alkaloids exist in one of the two categories but have been reported in trace levels only.[11]

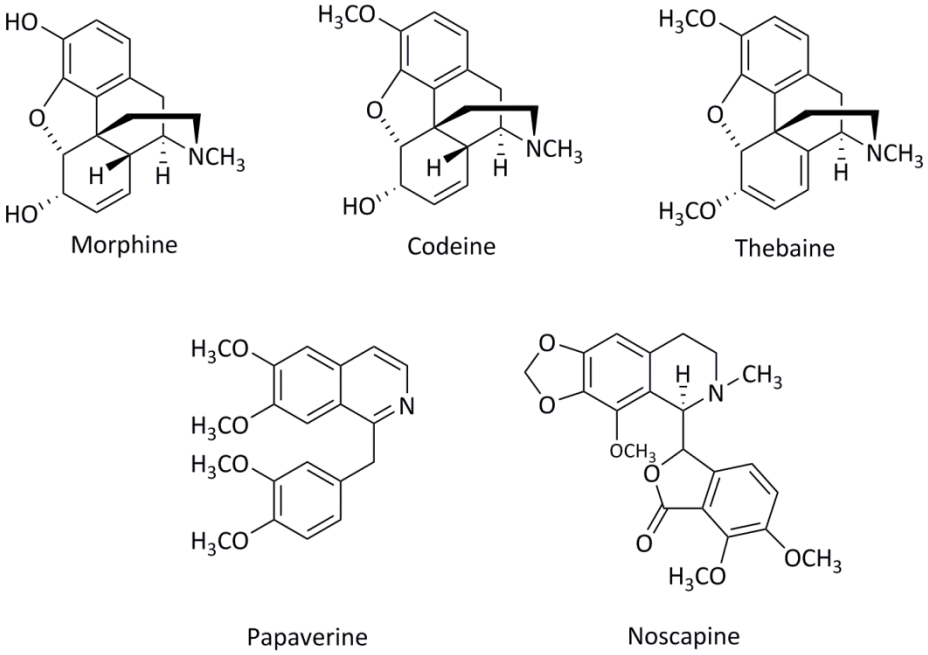


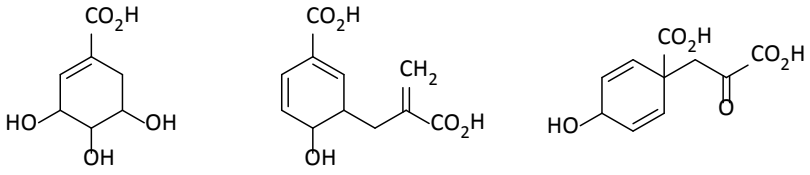
Figure 1.11 Chemical structures of the major opium alkaloids

1.4. **Alkaloid biosynthesis in *Papaver somniferum* L.**

Of the many alkaloid compounds present in *Papaver somniferum* L. the five major alkaloids of interest in this work are morphine, codeine, thebaine, papaverine and noscapine. These compounds comprise the largest percent, by weight, of dried opium as shown in Table 1.1.[58,59] These compounds are the most pharmacologically active compounds, some more than others, and have throughout the years found uses in the pharmaceutical industry (see section 1.3.2). The biosynthesis of the morphine, codeine, thebaine, papaverine and noscapine are important in this context for this research and will be further discussed.

1.4.1. Biosynthesis of major opium alkaloids

Morphine, codeine, thebaine, papaverine and noscapine are derived from amino acids such as L-phenylalanine and L-tyrosine and share a heterocyclic ring with nitrogen. The biosynthesis of alkaloid compounds in general, begins from a number of biosynthetic pathways in plants which can start from photosynthesis which the alkaloid syntheses occurring via the acetate, mevalonate, deoxyxylulose or shikimate pathways. For the opium alkaloids of interest here, the shikimate pathway is the significant one. Shikimic, chorismic and prephenic acids (Figure 1.12) are the precursors to many natural products which contain an aromatic benzene ring. [98]



Shikimic acid Chorismic acid Prephenic acid

Figure 1.12 Chemical structures of shikimic, chorismic and prephenic acids

The initial alkaloid synthesis within the *Papaver somniferum* L. starts from shikimic acid which is biosynthesised to produce chorismic acid then prephenic acid. From prephenic acid, the amino acid, phenylalanine is produced by the mechanisms shown in Figure 1.13.[98]

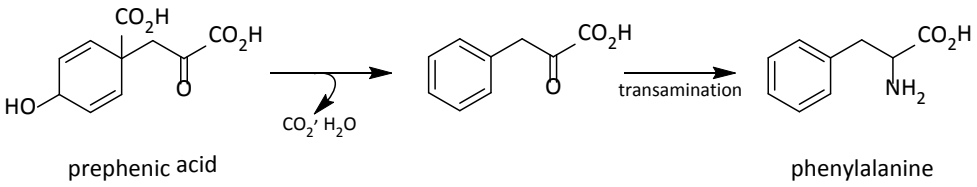


Figure 1.13 Biosynthetic route from prephenic acid to phenylalanine

Phenylalanine is the amino acid from which the opium alkaloids are further biosynthesised. From L-phenylalanine, L-tyrosine is formed via phenylalanine hydroxylase (PAH) which is further synthesised to L-dopa, also known as (R)-3,4-dihydroxyphenylalanine.[99] L-Dopa is then further biosynthesised by enzymatic metabolism to produce papaverine with the biosynthetic pathway shown in Figure 1.14. Figure 1.14 also shows the pathway from (S)-coclaurine to (S)-reticuline, which is the central biosynthetic pathway intermediate through which thebaine, morphine and codeine, via (R)-reticuline is shown in Figure 1.15. The synthesis of noscapine, formed via (S)-scoularine is shown in Figure 1.16.

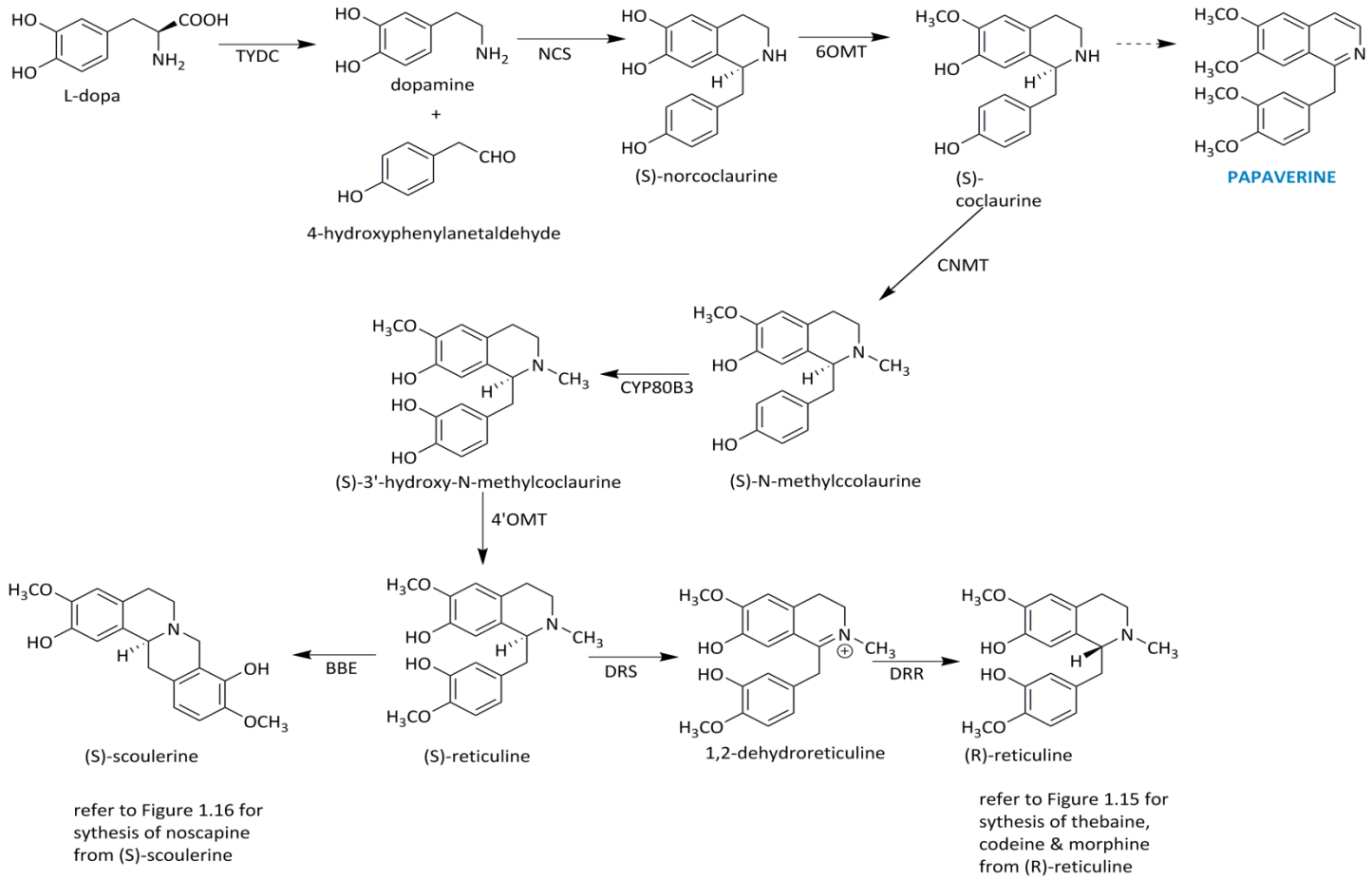


Figure 1.14 Biosynthesis from L-dopa to (S)-scoulerine and (R)-reticuline in alkaloid production in *Papaver somniferum*. Enzyme abbreviations are as follows, BBE: berberine bridge enzyme, CNMT: coclaurine N-methyltransferases, CYP80B3: N-methylcoclaurine 3'-hydroxylase, DRR: 1,2-dehydroreticuline reductase, DRS: 1,2-dehydroreticuline synthase, NCS: norcoclaurine synthase, 4'-OMT: 3'-hydroxy-N-methyltransferase 4'-O-methyltransferase, 6OMT: 6-O-methyltransferase, TYDC: tyrosine decarboxylase. Adapted from [99-101]

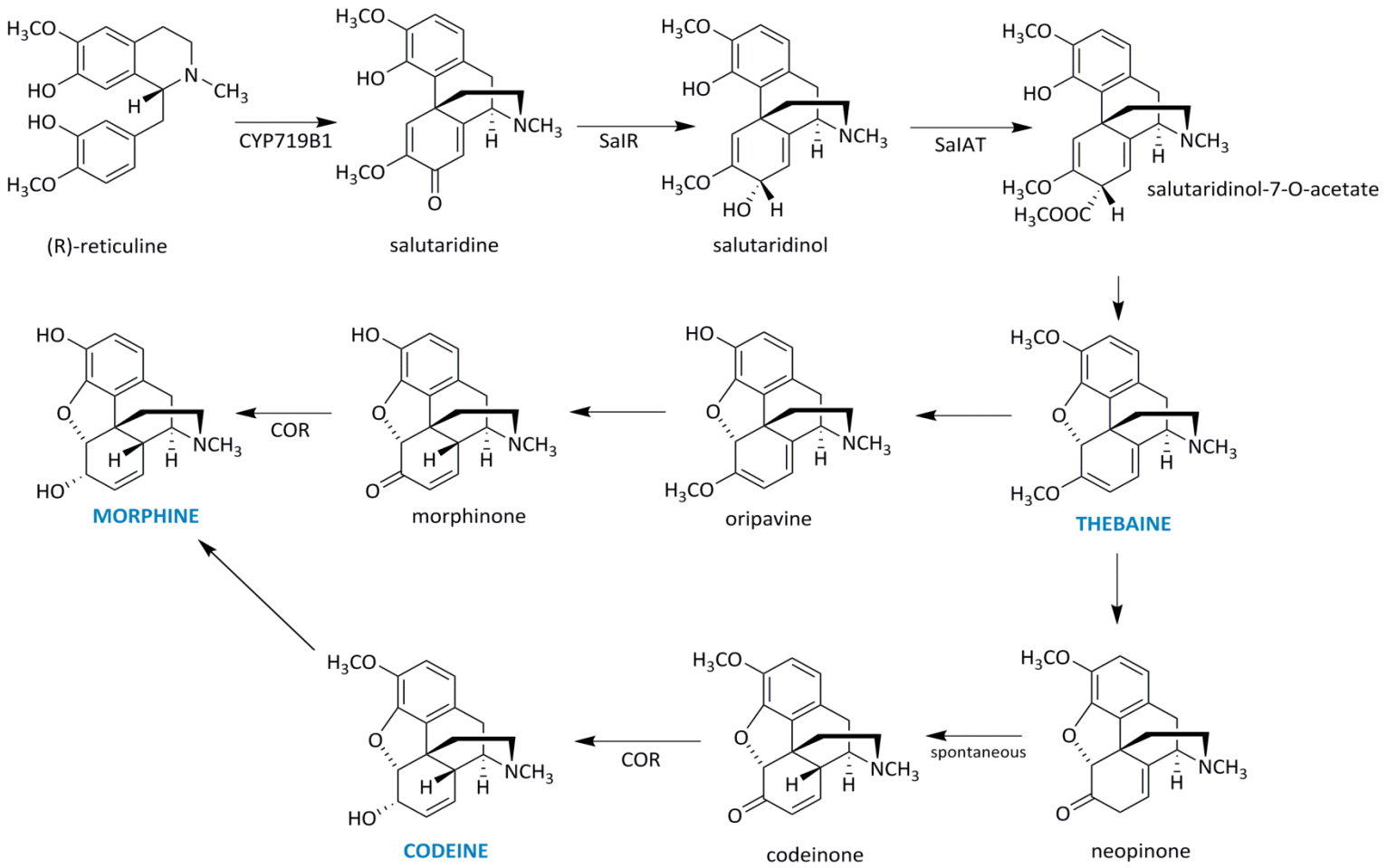


Figure 1.15 Biosynthetic interconversion from (R)-reticuline to form thebaine, codeine and morphine in *Papaver somniferum*. Enzyme abbreviations are as follows, COR: codeinone reductase, CYP719B1: salutaridine synthase, SalAT: salutaridinol 7-O-acetyltransferase, SalR: salutaridine:NADH 7-oxidoreductase. Adapted from [99-101]

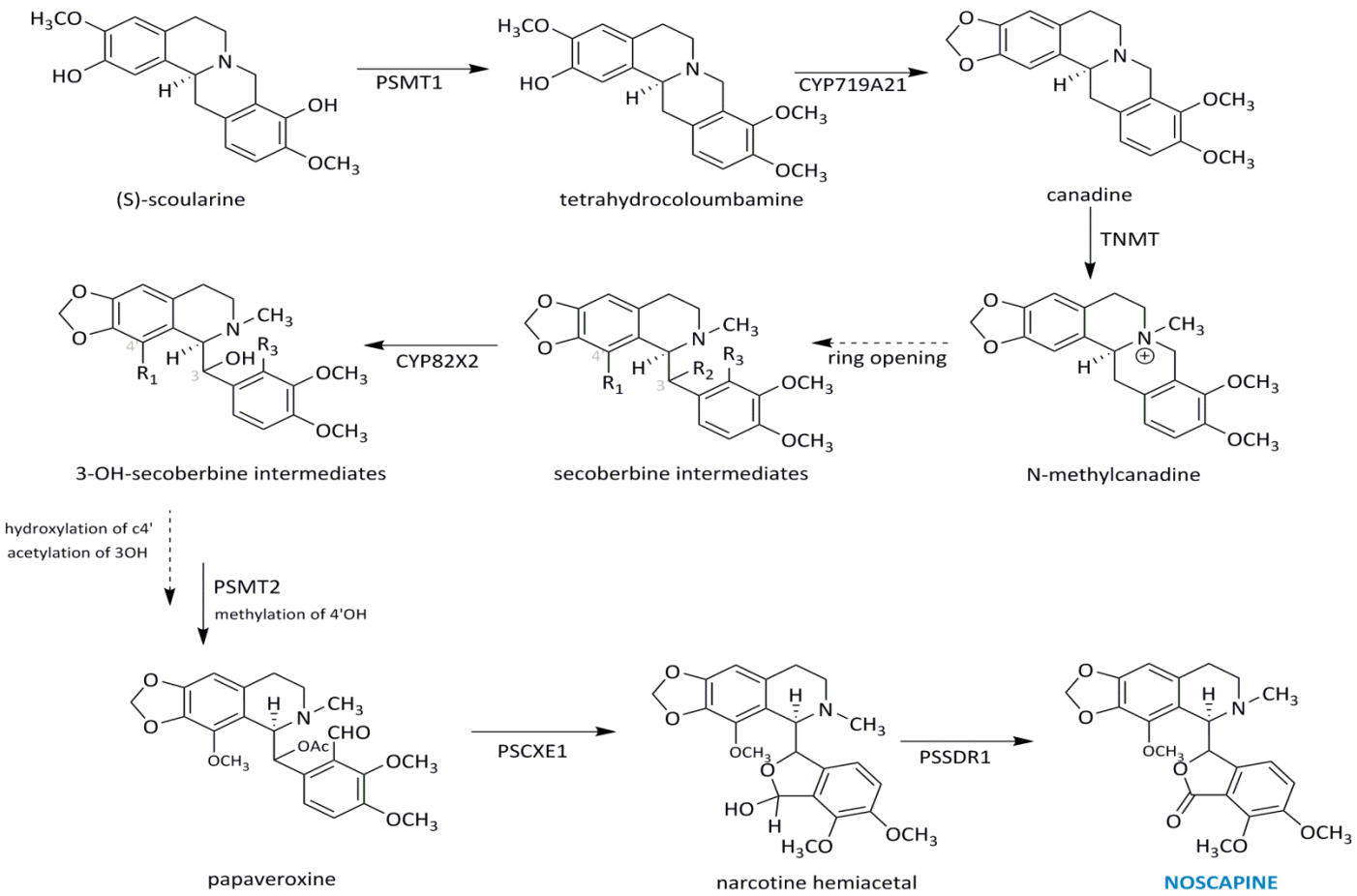


Figure 1.16 Biosynthetic interconversion from (S)-scoularine to form noscapine in *Papaver somniferum*. Adapted from [99-101] Enzyme abbreviations are as follows: CYP719A21: (S)-canadine synthase, CYP82X2: secoberbine intermediates 3-hydroxylase, PSCXE1: papaveroxine carboxylestaerase, PSMT1: (S)-scoulerine-9-O-methyltransferase, PSMT2: papaveroxine intermediate 4'-O-methyltransferase, PSSDR1: noscapine synthase, TNMT: tetrahydroprotoberbine cis-N-methyltransferase.

1.5. **Toxicology**

Within the field of toxicology, the processes of absorption, distribution, metabolism and elimination (ADME) of substances are studied. The route of administration, or the way in which a substance enters the body will greatly affect the bioavailability of the compounds present. In order for a compound to have an effect on an individual the compound and/or its metabolites must enter the flow of blood around the body. The blood then carries the compound(s) to sites of action where the resultant effect is produced.[102,103]

The main routes of administration are intravenously (I.V.), inhalation, orally, subcutaneously, intramuscularly and dermal sorption although other routes of administration are possible. A process known as first-pass metabolism can be seen for some compounds when administered orally. This process is one where a portion, sometimes large, of the original dose of the compound will be lost before it reaches the stomach and is seen in the oral administration of some opiate drugs. This loss occurs where the drug compound is transported from the gastrointestinal tract via the hepatic portal vein to the liver where it is metabolised.[104]

1.5.1. Toxicology of the opium alkaloids

Morphine, codeine, thebaine, papaverine and noscapine are the five major opium alkaloids of interest in this work and their physical and structural properties as well as their associated toxicology, where appropriate, are discussed below. As a group of drugs having originated from opium, they are classed as **opiates**; drugs synthetically derived from these opiate compounds, for example diacetylmorphine, are called **opioids**. The term opioid is used generally to include all drug compounds that work to relieve pain at the opioid receptors in the body.[105]

1.5.1.1. Pain and analgesia

Pain can be the response to external stimuli, such as, from an injury, from a fall or a burn and is typically associated with the peripheral nervous system (see Figure 1.17). This type of pain is generally termed **nociceptive pain** and the pain is generally associated with tissue damage. Nociceptive pain can be managed or treated with paracetamol, non-steroidal anti-

inflammatory drugs (NSAIDS) such as ibuprofen and aspirin or codeine or morphine (or other opioid analgesics), depending upon the severity of the pain.[104,106]

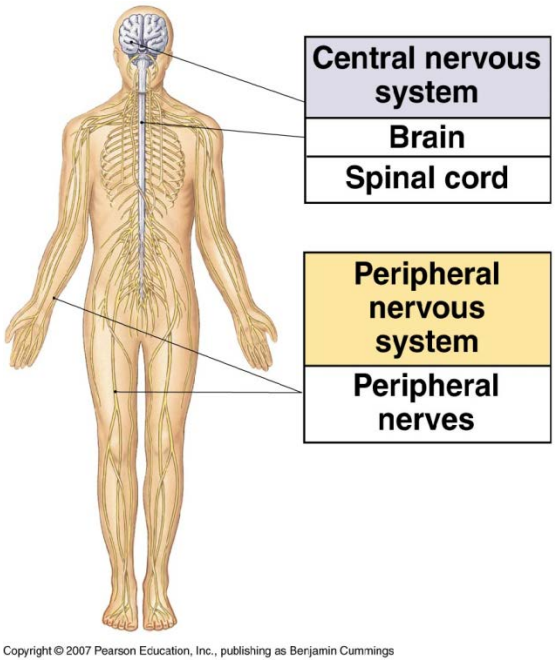


Figure 1.17 Central and peripheral nervous systems[107]

On the other hand, severe chronic pain can occur as a result of damage to the brain or nervous system, such as is found in cases of patients suffering a stroke. This type of pain is generally termed **neuropathic pain** and is typically managed with tricyclic antidepressants or a combination of other drugs since conventional analgesics have not proved useful in treating this type of pain.[106]

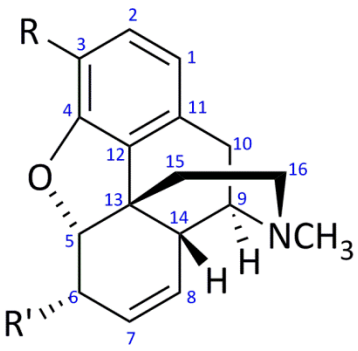
The effects that opioid analgesics have on the body have been identified as being due to the interactions of these compounds with the endogenous opioid system. There are three classical opioid receptors within the central nervous system (CNS) as well as in the peripheral tissues however most of the analgesia produced occurs by inhibiting the nociceptive transmission in the CNS. The opioid receptors in the CNS are classified as delta (δ), kappa (κ) and mu (μ) with the μ-receptors thought to provide most of the analgesic effects.[105] All of the receptors are responsible for different aspects of analgesia and sedation, although the effects of the δ-receptors are not fully understood.[108]

Opioids can be categorised with respect to their effect at the opiate receptors: they are classified as an agonist, a partial agonist or an antagonist. Agonists interact with a receptor to produce an optimum response from that specific receptor, partial agonists bind to the specific receptors but cause only a partial functional response, irrespective of the amount of drug administered and, antagonists bind to specific receptors but produce no functional response. At the same time, antagonists prevent an agonist from binding to that specific receptor.[105,108]

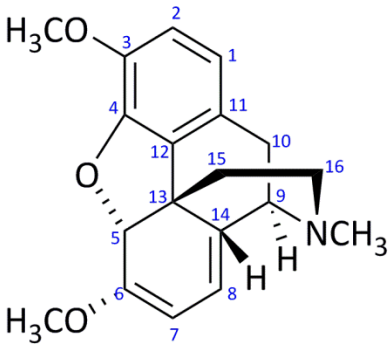
All opioids are metabolised predominantly by the cytochrome P450 (CYP450) enzyme system but the UDP-glucuronosyltransferases (UGTs) also play a minor role, in particular the formation of glucuronides by UGT2B7.[109]

1.5.1.2. Morphine

Morphine is the classic opioid analgesic and the one to which all other painkillers are compared.[105] It is a μ-opioid agonist and is well known that of the phenanthrene opium derived alkaloids, morphine is the most potent in activity.[104,110,111] Morphine is used in the treatment of moderate to severe pain and is used in palliative care of cancer patients, for pain management. It is well known that repeated morphine administration may cause tolerance and dependence in individuals therefore tends not to be prescribed for long periods of time; however this is not considered such a problem when used in palliative care. [108,112,113] The chemical structure of morphine is shown in Figure 1.18 and it can be seen that morphine has two planar and two aliphatic ring structures that are approximately perpendicular to the rest of the molecule. It has a phenolic hydroxyl group and an alcohol group in position 6, with the phenolic functional group contributing to the analgesic properties of morphine.[11]



Compound	R	R'
Morphine	-OH	-OH
Codeine	$-OCH_3$	-OH



Thebaine

Figure 1.18 Structural differences between morphine, codeine and thebaine

Routes of administration for morphine can vary and can include oral, transdermal and parenterally although with all drugs, route of administration can greatly affect the bioavailability. When administered parenterally, morphine is rapidly absorbed but orally administered morphine is prone to first-pass metabolism[103,104] with approximately 40-50% of the original dose reaching the CNS within 30 minutes for immediate release and within 90 minutes for extended release forms.[108] Morphine is distributed around the body into the kidneys, liver, lungs and because morphine crosses the blood-brain barrier, it is also found in the brain.[103]

90% of the morphine dose is metabolised by phase II conjugation to form morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). M6G has been shown to have potent analgesic properties and is itself a μ-opioid agonist but the same is not true for M3G.[104,112,114] Other minor metabolites of morphine are formed and are shown in Figure 1.20.

After parenteral administration, up to 90% of the dose of morphine is eliminated in the urine in 24 hours with approximately 10% as free morphine and 65-70% as conjugated morphine. The rest is composed of other minor metabolites.[103]

1.5.1.3. Codeine

Codeine is used in the treatment of mild to moderate pain but the side effects caused by long-term use of the drug, as well as dependency and tolerance, means that codeine is not prescribed for long periods of time.

Like morphine, codeine is a μ-opioid agonist but to a lesser extent.[111] The main difference in chemical structure between the two is that codeine is the methyl ether form of morphine and is structurally a modification of an hydroxyl (-OH) group on the C3 of the phenanthrene to a methoxy ($-OCH_3$) group. This methoxy group reduces the overall pharmacological activity of codeine (in comparison to morphine) but because 10% of the dose of codeine administered is metabolised to morphine, it is an effective analgesic.[115] Codeine has been shown to have approximately half of the pharmacological potency of morphine.[108]

The main route for administration for codeine is orally and via this route it is well absorbed.[103] Approximately 5% of the codeine dose is metabolised to the active metabolite morphine by CYP2D6 by O-demethylation[104,111] with approximately 80% of the codeine dose being metabolised to form codeine-6-glucuronide (C6G) by UGT2B7.[115] Due to the similarities in chemical structure to M6G, C6G also has pharmacological properties, although again to a lesser extent. [116] Between 10-15% of the codeine administered is converted to normorphine via CYP3A4 which is in turn glucuronidated to form normorphine-6-glucuronide (N6G). The main metabolic pathways for codeine are shown in Figure 1.20.

Around 86% of the orally administered dose of codeine is eliminated in the urine in 24 hours with 40-70% of this as free or conjugated codeine; 5-15% as free or conjugated morphine and 10-20% as free or conjugated normorphine.[103]

1.5.1.4. Thebaine

Thebaine has no therapeutic uses but is used by the pharmaceutical industry as a starting material from which other opioid drugs are synthesised.[63] The main compounds synthesised from thebaine are morphine, codeine, oxycodone, hydrocodone and buprenorphine.[117]

From codeine to thebaine, the difference in chemical structure is the modification from an hydroxyl group on the C3 position of the phenanthrene to a methoxy group. There is also a change of position of double bond from the C7-C8 position to the C8-C14 position, shown in Figure 1.18. The structural differences at the phenolic hydroxyl group and the C3 position are known to greatly to affect the biological activity and subsequent metabolism of the opioids and in the case of thebaine, the changes produce a compound with no therapeutic properties.[63]

1.5.1.5. Papaverine

Papaverine is an antispasmodic which induces smooth muscle relaxation and vasodilation and has been used in the past in the treatment of erectile dysfunction[118] and treating heart arrhythmias.[119] It has few uses nowadays although there have been a few publications highlighting the use of papaverine in some medical cases particularly in cases of stroke and as an antispasmodic.[120-124]

Papaverine is structurally different to the phenanthrene alkaloids such as morphine. It falls into the benzylisoquinoline group of alkaloids and has methoxy groups in the C6, C7, C3' and C4' positions. The chemical structure of papaverine is shown in Figure 1.19.

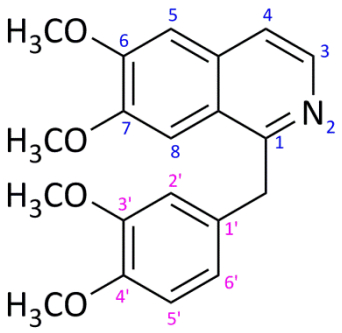


Figure 1.19 Chemical structure of papaverine

There is little information with respect to the structure activity relationship (SAR) of papaverine although it is known that papaverine works by inhibiting cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) phosphodiasterases present in smooth muscle. It has also been identified that papaverine blocks calcium ion

channels, inhibiting the release of calcium; this along with the inhibition of cAMP and cGMP induces vasodilation and relaxation in the smooth muscle.[120]

Papaverine can be administered orally but also by intracavernosal injection for treating erectile dysfunction.[125] It is well absorbed after oral administration but undergoes extensive first-pass metabolism. The main metabolites produced are 4'-hydroxypapaverine and 6-hydroxypapaverine with 50-80% of the original dose eliminated in the urine within 48 hours and the 6-hydroxy form accounting for more than 30%.[103,126]

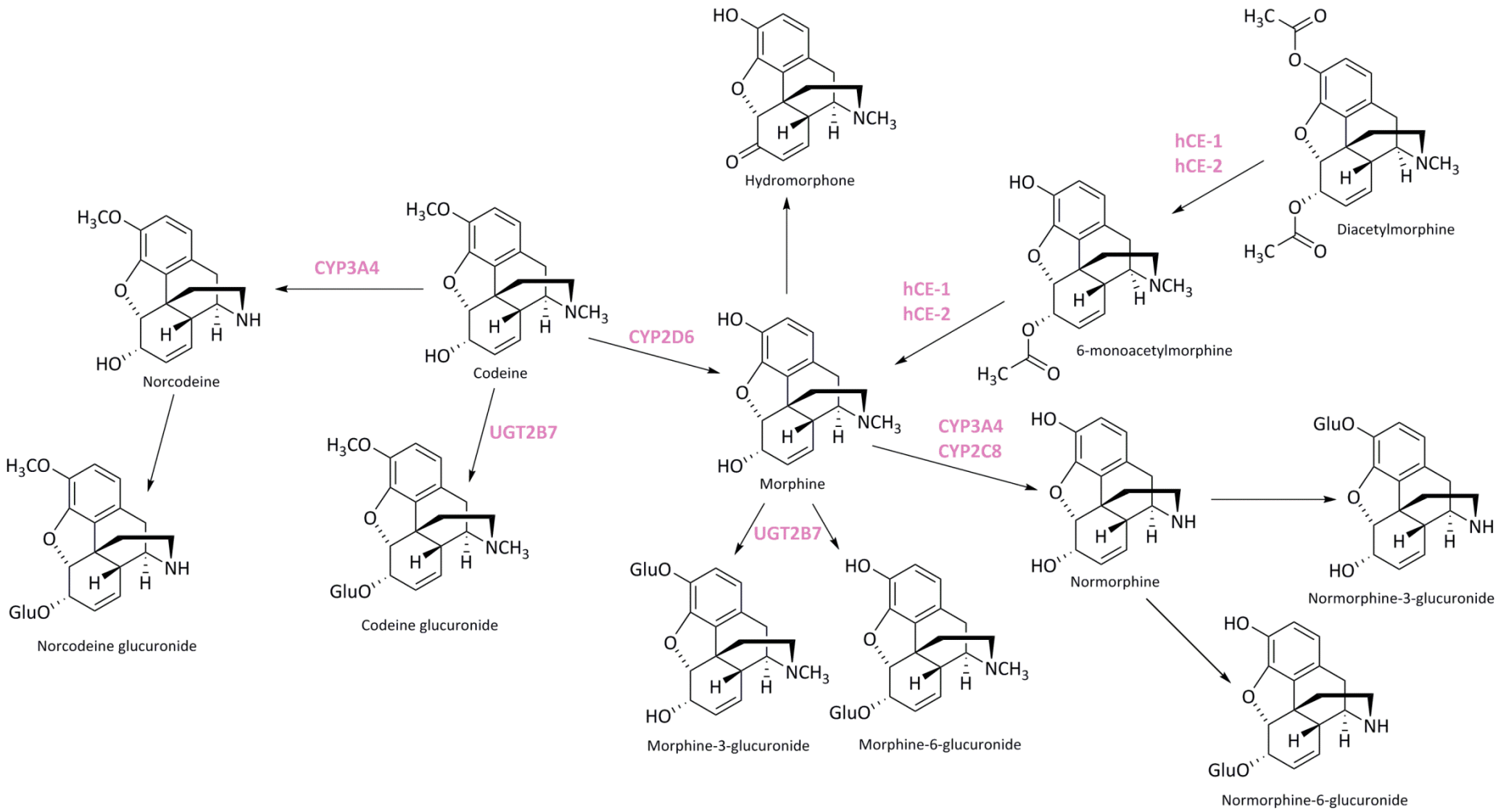


Figure 1.20 Main metabolic pathways for morphine and codeine. "Glu" denotes a glucuronide group

1.5.1.6. Noscapine

Noscapine (Figure 1.21) has been used as an antitussive and recently has been studied as a treatment for some cancers.[127,128] Noscapine can be orally administered although the mechanism of action is not fully understood.[129] The main metabolites of noscapine have been identified as cotarnine, meconine, narcotoline and nornoscapine[130,131], also shown in Figure 1.21.

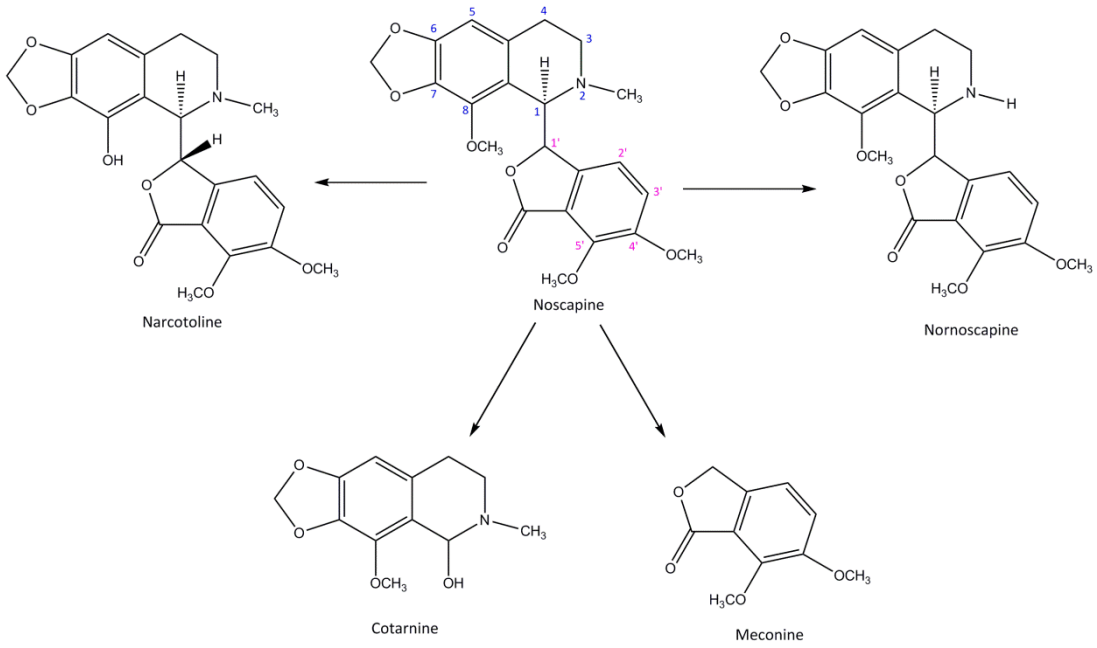


Figure 1.21 Chemical structure of noscapine and its metabolites

The main routes of elimination for noscapine and its metabolites are not well understood and little is available in the literature, possibly due to the limited applications, at the current time. However it has been highlighted recently that in *in vitro* tests and when applied to mice, the main enzymes involved in the metabolism of noscapine were found to be CYP3A4/5 for the formation of narcotoline but many phase I isoenzymes were involved in the production of cotarnine and meconine.[128]

1.5.1.7. Diacetylmorphine

Diacetylmorphine is a powerful opioid agonist that is used legitimately to aid in pain management in palliative care but due to the severe tolerance and dependency properties, has few other uses medically.[132] It is considered a pro-drug as it has a short half-life and is quickly metabolised to 6-monoacetylmorphine and then onto morphine: it is rarely found in biological matrices.[133] Diacetylmorphine is the active compound present in street heroin samples however these "street" samples of heroin more often than not contain other analgesics, other alkaloids extracted from opium, caffeine, sugars and other non-active compounds to dilute the heroin and make it go further.[134]

The chemical structure of diacetylmorphine, shown in Figure 1.22, differs from the morphine structure (see Figure 1.18) in that the phenolic hydroxyl group of morphine in the C3 position and the hydroxyl group in the C6 position are replaced by acetyl functional groups.

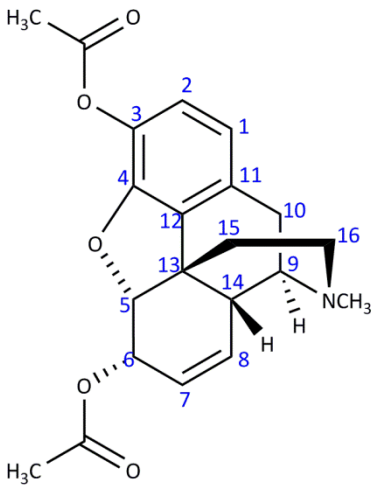


Figure 1.22 Chemical structure of diacetylmorphine

The main metabolic pathways for diacetylmorphine are shown in Figure 1.20. The main metabolite used to confirm the ingestion of diacetylmorphine is 6-monoacetylmorphine (6-MAM).[135,136] 6-MAM has a relatively short half-life of 6-25 minutes[103] and is generally found in blood for between 30 minutes to 1 hour after administration which means that there is a short window of detection for positively identifying heroin administration.[137,138]

1.5.2. Poppy seed ingestion versus heroin use

Poppy seeds are used in many foodstuffs, predominantly bread products but are also incorporated into desserts and other cake recipes, worldwide.[139] In parts of Eastern Europe, poppy seeds are also used in pasta and noodle dishes and are incorporated to add a nutty flavour to a dish.

It is known that the ingestion of poppy seeds has caused positive opiate drug test results and much work has been reported in the scientific literature in the last 20 years.[70-72,74,77,78,80,85-87,140-142] The positive opiate results have been found in urine, blood and oral fluid but when confirming opiate drugs in biological matrices, codeine and morphine presence are identified and quantified. When it is necessary to confirm administration of heroin (diacetylmorphine as the pharmacological active), 6-monoacetylmorphine presence will be investigated in the biological matrix but it is not always possible to detect this compound. Researchers in the field have investigated alternatives to differentiate between heroin administration and that of other opiate drugs versus poppy seed ingestion. Most of the work which has been carried out relates to establishing illicit heroin use by examining biological matrices for the presence of acetylcodeine (an impurity of illicit heroin), thebaine, papaverine, noscapine and their associated metabolites.[143] A study published by Hill, *et al.*[141] in 2005 highlighted the possibility of differentiation of heroin use and poppy seed ingestion using hair and urine as the matrices. It was suggested that 6-MAM could be used as the marker for heroin administration since it was detected in hair of heroin users versus not in the urine of individuals ingesting poppy seeds. However, the comparison of data in two different matrices is unusual and it is not really possible to draw any meaningful interpretation from these findings: it would have been better if a direct comparison of the results between heroin users and individuals ingesting poppy seeds in the same matrix was carried out.

Thebaine has also been investigated as a potential marker for poppy seed ingestion due to the fact that it has been found in many poppy seeds analysed but not in street heroin samples.[140] To complicate the matter, ElSohly *et al.*[140] published a paper in 1988 highlighting that it was not possible to detect thebaine in urine of individuals ingesting poppy seeds however Cassella *et al.*[78] subsequently published a paper in 1997 reporting that it was possible. This discrepancy could be due to an increased sensitivity of the gas chromatograph – mass spectrometer (GC-MS) used in the more recent of the papers.

It has been suggested that the use of morphine and codeine concentration ratios in biological matrices may also be used to indicate the difference between heroin and poppy seeds: it was noted in a short communication published in Forensic Toxicology in 2011[139] that much lower concentrations of morphine and codeine were found in urine in comparison to those of heroin users. It was noted in this communication that further investigation was required before any meaningful interpretation could be deduced from these findings. However, many parameters could have affected the findings that were not discussed. It is neither possible to establish a "standard" dose of diacetylmorphine present in street heroin nor is it possible to ascertain an exact dose of morphine and codeine provided from poppy seeds therefore the findings reported cannot be considered completely reliable. No further publication reporting any advancement in the study has been published, to date.

At this current time, it is not possible to differentiate between heroin administration and poppy seed ingestion other than by the detection of 6-MAM. Although other biological markers have been investigated, no other compound is a serious candidate when 6-MAM is no longer detectable.

2. OPIATES IN ORAL FLUID

2.1. **Biological matrices and drug testing**

For toxicology testing, blood is by far the best matrix as it can provide information on what compounds are present in the body at a given time: this makes it easier to interpret symptoms or effects that may be experienced by the individual.[144] However, blood is not an easy matrix to collect either at the roadside (e.g. for the assessment of driving under the influence of drink or drugs) or in workplaces where random or organised drug screening takes place. Blood collection requires the services of a trained individual, such as a phlebotomist who can take the blood samples which is something that is not very practical at the roadside. The use of blood as a biological matrix also brings with it many potential health and safety issues particularly relating to pathogens that may be present in blood and the subsequent endangering of the collector. As a result, alternative matrices have been considered for both roadside and workplace testing.[82]

For workplace drug testing, urine was always the preferred specimen collected; however this matrix has a number of negative factors associated with interpretation: urine is a predominantly water based product eliminated from the body over a period of time and can therefore only provide information within the last 24 hours.[145] Urine was the matrix of choice for roadside drug testing in some countries (e.g. Belgium and France)[146] but an alternative non-invasive, easy to collect specimen and sampling method with little or no embarrassment on the part of the collector or individual providing the specimen is preferred. For these and many other reasons, alternative matrices have been sought for both workplace and roadside drug testing.

When considering alternative matrices, urine can be eliminated as a potential matrix of choice as it poses problems with collection, particularly at the roadside. It is also difficult to control collection to reduce adulteration without severe embarrassment for both collector and individual providing the specimen.[147] Hair has been shown to be useful in providing information on drug use in a window of between a few weeks and a few months, depending upon the length of hair of the individual. However, since for both workplace and roadside drug testing it is necessary to establish if an individual has recently taken drugs or has drugs in their system at the time of testing, hair is also not an appropriate matrix of choice.[82,145] Sweat has been investigated by a number of researchers[145,148-151] as an alternative matrix for current drug use. PharmChek® drugs of abuse sweat patch and

AlcoPro sweat patch drug test are two commercially available patches which can be returned to the company for analysis by GC-MS however, like hair, sweat patches tend to be used to provide drug information over a longer window of detection than is required for roadside and workplace drug testing.[152]

Oral fluid, as an alternative matrix for drug testing has been investigated over a number of years by many researchers and is proving to be an excellent choice with respect to being able to provide information about current drug use.[74,79,83,84,86,153-157] Oral fluid is also easier to collect than blood and does not require a trained practitioner as is required for blood collection.[158]

2.1.1. Saliva vs oral fluid

Saliva contains 99% water with the rest comprising proteins (largely α-amylase which aids in the preliminary digestion of food in the oral cavity), mucins and electrolytes dissolved in the aqueous phase (most importantly Ca^{2+}, Cl^-, HCO_3^-, K^+, Mg^{2+}, Na^+ and NH_3/NH_4^+).[159,160] Saliva originates from three major salivary glands called the parotid, submandibular and sublingual glands (see Figure 2.1) and a number of minor glands collectively called the Von Ebner glands which are found in the connective tissue below the circumvallate papillae (the small raised bumps on the tongue towards the back of the mouth).[161] The submandibular and sublingual glands are seromucous glands whereas the parotid and Von Ebner glands are completely serous glands.[162] In unstimulated saliva, 65% of the secretions originates from the submandibular glands, 23% from the parotid glands, 8% from the Von Ebner glands and 4% from the sublingual glands.[161]

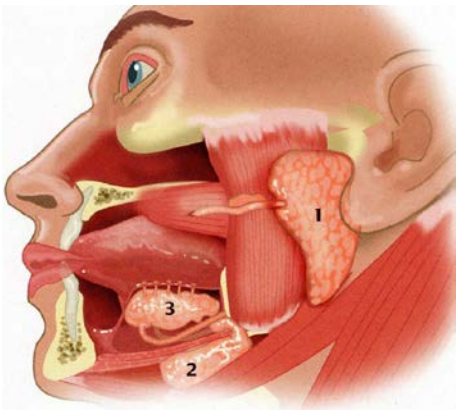


Figure 2.1 Major saliva producing glands where (1) represents the parotid glands, (2) represents the submandibular glands and (3) represents the sublingual glands (Figure from Aps, 2005[163])

The combination of salivary glands will produce between 0.5 and 1.5 L of saliva in a day; however, the production of saliva is affected by the circadian rhythm of salivation and can also be affected by disease and administration of prescription and illicit drugs.[158,163,164]

For clarification, the term "oral fluid" will be used referring to the fluid that is collected from the mouth. This is because "saliva" refers only to the liquid that is secreted from the salivary glands in the mucous membranes of the mouth. Oral fluid (OF) refers to the liquid collected from the oral cavity that will contain the secretions from the salivary glands (saliva) but also other components such as cellular material, gingival crevicular fluid and blood. It was decided at the 1993 New York Academy of Sciences meeting on saliva testing that the term "saliva" referred to the fluid specifically collected from the salivary glands and that "oral fluid" referred to the whole sample collected from the mouth when adsorbents are placed into the oral cavity or when the sample is collected by expectoration (the process of allowing the saliva to collect in the oral cavity and allowing the fluid to drain from the mouth into a container).[165]

2.1.2. Pharmacokinetics of drugs in oral fluid

Over the years, a number of researchers have investigated the mechanism of transfer of drugs (and their metabolites, where appropriate) into oral fluid.[86,153,155,156,158,164,166,167] From the majority of these studies, it has been shown that for the most part, oral fluid contains the parent drug as opposed to metabolite(s).[164] It has also been highlighted that drugs can transfer from the blood plasma to the saliva as salivary glands are supplied by arterial blood permitting a relatively rapid transfer.[83]

There are however, a number of factors which should be taken into account when considering the transfer of drugs from plasma to oral fluid: these include the level of health of the individual, other drugs being administered and also the chemistry of the drug being transferred. It was reported by Aps in 2005[163] that disease such as multiple sclerosis, cystic fibrosis, diabetes mellitus, HIV, individuals with kidney dysfunction and alcoholic liver cirrhosis would have a difference in composition of saliva in comparison to a non-affected individual. For example, for individuals who have diabetes mellitus, a reduction in the flow of saliva is noted whereas individuals with a kidney dysfunction, an increase in salivary urea levels results in saliva with a higher pH than is normally expected.[163] Unstimulated saliva

has a pH range of 6.0 - 7.2 but a salivary pH of 8.0 has been found in saliva which has been collected via stimulation.[102]

There are a number of methods by which the transfer of drugs from plasma to saliva is facilitated: passive transcellular diffusion, ultra-filtration and active transport.[168] Passive transcellular diffusion is the primary method of transfer of drugs from blood to saliva and depends upon factors such as the chemical properties of the drug, the pH of the saliva , concentration of un-ionized drug (since the ionized form of the drug will not passively diffuse across cellular membranes), drug-protein binding (only free-fraction can diffuse), and characteristics of the membrane.[169] Most drugs are transferred by this mechanism, which transports the drug across cellular membranes down a concentration gradient without utilising energy.[169] The mechanism of ultra-filtration allows small molecules of typically less than 300 Da to pass into the saliva, however molecules of up to 150 Da have been found to transfer minimally. Active transport is the main transport mechanism for many electrolytes and some proteins, but not drugs.[83,169,170]

It is important to establish if drug compounds will cross the cell membranes from the plasma to the oral fluid. The pK_a of a drug is the acid-base dissociation constant and is used to establish whether a drug molecule will be found in the ionised or unionised form at a particular pH. Unionised drugs cross cell membranes and the Henderson-Hasselbalch equation is used to determine the ionisation of a drug with a particular pK_a value, at particular saliva pH values.[171]

The equation for the ionisation, or dissociation, of an acid at equilibrium in aqueous solution is provided below (equation 2.1), where HA is the conjugate base, H^+ represents the hydroxonium ions (H_3O^+) in aqueous solution and A^- represents the weak acid in aqueous solution[172]:

$$HA_{(aq)} \rightleftharpoons H^+_{(aq)} + A^-_{(aq)} \qquad \textbf{(2.1)}$$

From this the general Henderson-Hasselbalch[173] equation can be derived (equation 2.2)

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \qquad \textbf{(2.2)}$$

Where K_a = dissociation constant of the weak acid

$pK_a = -\log K_a$

$pH = -\log[H^+]$

$[A^-]$ = molarity of the weak acid

[HA] = molarity of the conjugate base

It is possible to apply this acid-base theory to acidic and basic drugs which can transfer from the plasma to saliva and a value known as the saliva:plasma ratio (S/P) can be determined using the following equations.

For basic drug compounds the following equation can be used:

$$S/P = \frac{1+10^{(pK_d - pH_s)} x\, f_p}{1+ 10^{(pK_d - pH_p)}\, x\, f_s} \qquad \textbf{(2.3)}$$

For acidic drug compounds the following equation can be used:

$$S/P = \frac{1+10^{(pH_s - pK_d)} x\, f_p}{1+ 10^{(pH_p - pK_d)}\, x\, f_s} \qquad \textbf{(2.4)}$$

Where S/P = concentration of drug in saliva to the concentration of drug in plasma

pH_s = pH of saliva

pH_p = pH of plasma

pK_d = pK_a value of the drug

f_p = fraction of drug unbound in plasma

f_s = fraction of drug unbound in saliva

Morphine is an amphoteric basic molecule that possesses both an amine and a phenol group which are ionisable.[174] In relation to the amine group, morphine has a pK_a constant of 8.0 but with respect to the phenol group, morphine has a pK_a constant of 9.9.[103] The saliva:plasma (S/P) ratio for morphine is approximately 1.2, although this is an average value reported (range 0.3 – 34.3).[175-177]

Codeine has the presence of the amine group, as is the case with morphine, however codeine does not include an ionisable phenol group. Codeine has a methoxy (-OCH_3) group in position 3 in place of the hydroxyl group present in morphine. The absence of phenolic group, or any other readily ionisable group, on codeine means that it is not amphoteric and has only one pK_a constant of 8.2.[173] For codeine, the S/P is approximately 3.3[170] however a value of 3.7[178] has also been reported.

Thebaine, like both morphine and codeine has an amine group but has two methoxy groups in position 3 and 6 and has a pK_a constant of 8.2.[103] In comparison, diacetylmorphine has a pK_a constant of 7.6[103] and papaverine and noscapine have pK_a constants of 6.4 and 6.2, respectively.[103] The S/P ratios of thebaine, noscapine or papaverine have not been reported in the scientific literature, however the S/P ratio for diacetylmorphine has been reported as 2.13[176] and a value of 6 for 6-MAM.[170,176,179]

The physiological pH of saliva is typically pH 6.0 – 7.2[102,167] and pH 7.4[180] for plasma: when the pH value is lower than the pK_a constant of a basic drug, the compound will become positively charged and therefore will be in an ionised state (Figure 2.2).[172]

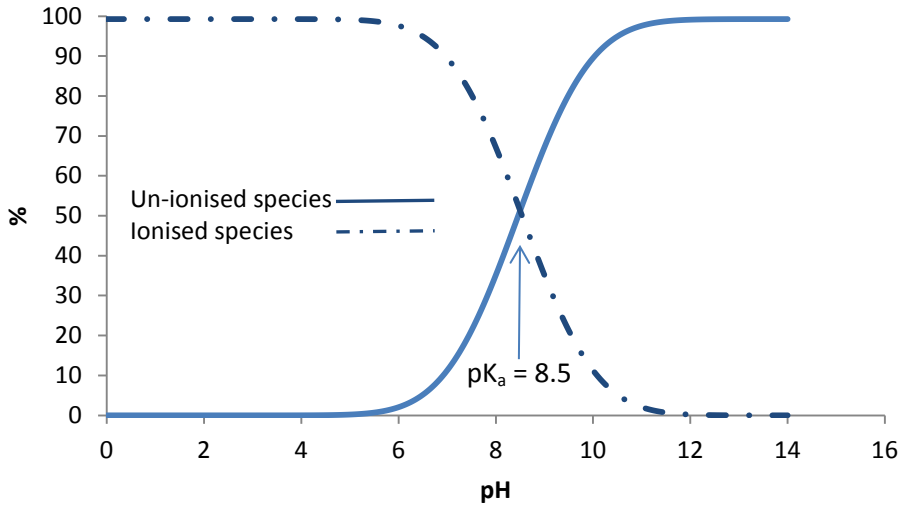


Figure 2.2 Ionisation of a weak base of pKa 8.5 with varying pH

For morphine, codeine and thebaine where pK_a constants are close to the physiological pH of saliva and plasma, they will exist in both the ionised and unionised form. Only the

unionised portion of these three compounds will readily transfer by passive diffusion from the plasma to the saliva.

In the case of papaverine and noscapine where the pK_a constants are 6.4 and 6.2, respectively, the pK_a constants are below the pH values of saliva and plasma. This should mean that both of these compounds will be predominantly found in the unionised form and will, therefore more readily transfer from plasma to saliva by passive diffusion.

2.1.2.1. Pharmacokinetics of opiates in oral fluid

The fate of the major opium alkaloids found on entering the human body has been explained in section 1.6.1, however the explanation predominantly relates to the legitimate administration of the opiates from the perspective of pain management. The main route of administration of poppy seeds, and poppy seed containing food products, is oral and this method of administration brings with it some considerations when interpreting the results of the analyses of oral fluid samples. It is well established that when drugs are administered orally, they contaminate the oral cavity and lead to elevated S/P ratios immediately post consumption. [83,158,166]

More specifically, in relation to detecting opiates in oral fluid, the current scientific literature relates only to the presence of morphine and codeine and no, or very limited, data exist in relation to levels of thebaine, papaverine or noscapine in this biological matrix. Some publications have reported the findings in relation to the intra-venous administration and smoking of heroin but again, only morphine, codeine and associated metabolite presence have been reported.[164,166,179]

With respect to the compounds observed in oral fluid, the parent compound is most often reported.[164] In 1990, Cone published a study, where 20 mg and 10 mg of morphine sulphate were administered intra-muscularly to a participant and oral fluid was stimulated by sour candy, and found that morphine levels were at a peak in oral fluid 30 minutes post administration of the dose. Using GC-MS, it was established that after a 20 mg dose, peak oral fluid morphine level was 37.8 ng mL^{-1}, 10.8 ng mL^{-1} after a 10 mg dose and thereafter declined in both cases. As part of the same study, a 120 mg and a 60 mg of an intramuscular dose of codeine phosphate was administered to an individual and peak oral fluid levels were obtained between 30 – 45 minutes post administration. The peak levels

for codeine were found to be 307.6 ng mL^{-1} and 183.9 ng mL^{-1}, respectively: as was the case with morphine, the codeine levels rapidly declined.[175] This study was fairly limited as it only included two participants with a known history of heroin abuse and was not repeated but, levels of morphine, codeine and their associated metabolites present in other biological matrices were included for comparison.

Other more recent studies, involving the oral administration of codeine sulphate (120 mg/70 kg and 60 mg/70 kg doses) with a larger number of participants did not find morphine in any of the oral fluid samples but found codeine in approximately 26% of the samples analysed: the codeine was found in a concentration range of 2.5 – 3961 ng mL^{-1} using GC-MS.[178,181]

In 2003, a study carried out by Rohrig and Moore[74] investigated the implications of ingestion of both bakery products containing poppy seeds and raw poppy seeds. Two sites were involved in the study: at site 1, 4 participants consumed 3 commercially available poppy seed bagels within 1 hour. At site 2, 3 participants consumed 1 poppy seed bagel plus as many raw poppy seeds as was possible (14.82 g, 9.82 g and 20.82 g were consumed by the participants). The study revealed that at site 1, morphine and codeine were not found in any of the oral fluid samples analysed; it should however be noted that the first specimen was collected 1 hour post ingestion of the poppy seed bagel. At site 2, codeine was not found in the oral fluid samples but morphine was found at a peak level 15 minutes post consumption for all 3 participants. The highest concentration obtained for one of the participants at this site was 205 ng mL^{-1}.

In a more recent publication by Newmeyer, *et al.* [177] in 2015 a controlled administration of two quantities of 45 g of raw poppy seeds was carried out and the levels of morphine and codeine in oral fluid were monitored with an immunoassay Dräger Drug Test® 5000 and LC-MS. The authors reported that each of the 45 g quantities of poppy seeds contained 15.7 mg of morphine and 3.1 mg of codeine and were administered 8 hours apart to 17 participants. The findings of this study were that all oral fluid samples were deemed positive by falling above the 20 ng mL^{-1} analytical cut-off of the Dräger Drug Test 5000 for opiates. The peak oral fluid levels for morphine was obtained between 30 minutes and 1 hour post administration and found to be 177 ng mL^{-1}; for codeine the peak oral fluid level was obtained between 30 minutes and 2.5 hours post administration and was found to be 32.6 ng mL^{-1}.

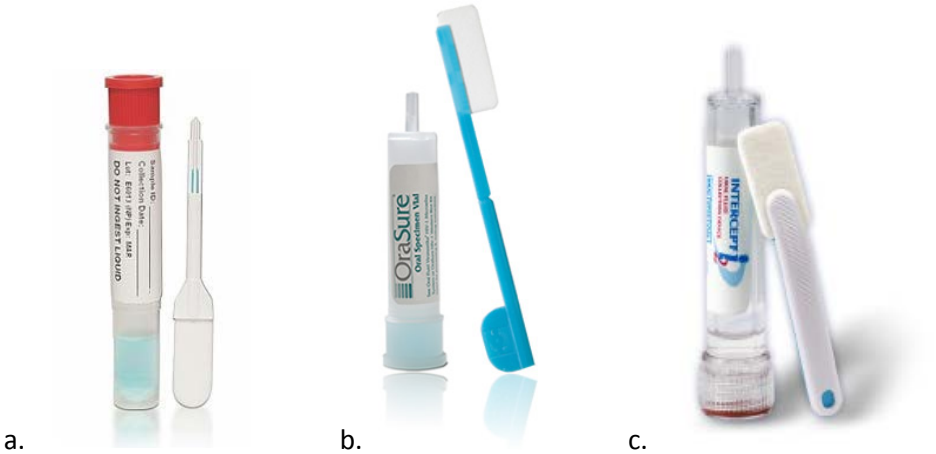
For comparison, diacetylmorphine and 6-monoacetylmorphine (6-MAM) have been detected in oral fluid after 2 minutes by GC-MS following administration by intra-venous injection or smoking of heroin.[176] Higher levels of diacetylmorphine were observed where the heroin was smoked (3534 – 20,580 ng mL^{-1} at peak level), in comparison to intravenous injection (6-30 ng mL^{-1} at peak level). The authors of this study suggest the reason for this observation being that the oral cavity was contaminated in the smoking process. 6-MAM (640 – 3577 ng mL^{-1} at peak levels) and morphine (6 – 142 ng mL^{-1} at peak levels) were also observed in the oral fluid samples by both routes of administration however it was reported that the levels of each of the compounds decreased very quickly after the peak levels were obtained.

2.1.3. Challenges of using oral fluid in drug testing

Although more practical than urine for workplace and roadside testing, oral fluid has its disadvantages: as with many biological materials, this matrix poses some pathological problems with the risk of infection if not handled appropriately. Oral fluid contains mucins which make the matrix more viscous to handle in comparison to urine and for some individuals, the production of saliva is a problem (Section 2.1.2).

The process of collecting the oral fluid sample has also been shown to greatly affect the drugs detected. In a review carried out by Crouch[158] in 2005, it was highlighted, for example, that codeine concentrations detected in non-stimulated versus stimulated oral fluid, were 3.6 times higher in non-stimulated oral fluid. The main methods of collection of oral fluid are expectoration, stimulated expectoration (usually using citric acid), mechanical stimulation (involving the chewing of gum or other inert material), and using commercially available adsorbent collection pads.[182] Over the last few years, commercially available collection kits have overtaken the other methods of collection; some of the gums used in mechanical stimulation were shown to adsorb some of the drug compounds, citric acid was shown to increase the rate of production of saliva therefore reducing the overall concentration of drug in oral fluid (impacting on interpretation of the S/P ratios of the drugs) and expectoration (which would involve allowing oral fluid to collect in the oral cavity and allowing the fluid to drain unaided into a collection pot) was found to be an unpopular method of collection.[183,184]

There are a number of commercially available oral fluid collection devices currently offered on the market. These kits tend to include an adsorbent pad which is used to collect the oral fluid and a buffer or preservative solution within a container for ease of transportation to the laboratory. [185] Three of these kits are shown in Figure 2.4 and include the Quantisal™, OraSure® and Intercept® collection kits.



a. b. c.

Figure 2.3 (a) Quantisal™, (b) OraSure® and (c) Intercept® oral fluid collection kits

2.1.4. Extraction of drugs

Extraction is a technique used to remove potentially interfering compounds from a matrix and to allow the concentration of target drug analytes.[186,187] The two main methods of extraction of drugs from oral fluid, as with other biological matrices, are liquid-liquid extraction (LLE) and solid phase extraction (SPE).[188] LLE is a technique employing an immiscible organic solvent of known polarity and typically involves altering the pH of the biological matrix such that either acidic/neutral analytes or basic analytes will be extracted into an appropriate organic solvent.[102,189] LLE was traditionally employed in toxicology laboratories but due to the improvement of adsorbent bed chemistries, increased understanding of adsorption mechanisms, commercial availability and ease of use, SPE has become very popular.[186,188,190-192]

Commonly employed chemistries involved in solid phase extractions are normal phase (NP), reversed phase (RP), ion exchange and mixed mode.[188] Normal phase SPE employs a polar packing material, typically modified silica (for example with aminopropyl or cyanopropyl functionalization) in the extraction of polar analytes. The matrix should be less polar than the analytes of interest in order to effect the separation and a solvent, of an

appropriate polarity to disrupt the binding mechanism of the analytes from the sorbent bed.[189]

Reversed phase SPE uses the opposite mechanism to that employed in normal phase SPE: a non-polar packing material (for example, silica modified with alkyl or aryl functional groups) is used to extract non-polar analytes. Again, a solvent of appropriate polarity will be used to desorb the analytes from the sorbent bed.[172]

Electrostatic interactions between charged groups of the analyte(s) and the adsorbent packing material are employed in ion exchange SPE to effect a separation: the cartridges contain either adsorbent for anionic or cationic exchanges. Packing material composed of sulphonic acid groups is an example of a strong cation exchanger and quaternary amine groups as an example of a strong anion exchanger.[193]

Mixed mode SPE employs multiple retention mechanisms and is suited when analytes are to be extracted from a complex matrix.[189] Only analytes which have the properties to interact with both of the adsorbent mechanisms will be retained and subsequently desorbed and all other components will pass through the cartridge.[194]

2.1.5. Interpretation of oral fluid results

The European Workplace Drug Testing Society (EWDTS) has provided guidelines[195] for the use, analysis, detection and detection of drugs in oral fluid: the document covers points for consideration when carrying out this type of work and includes acceptable collection methods and guidelines for the interpretation of oral fluid tests. The EWDTS guidelines have included screening and confirmation cut-offs for the detection of opiates (morphine) in neat oral fluid and have incorporated the 40 ng mL^{-1} analytical cut-off set down by the Substance Abuse and Mental Health Services Administration (SAMHSA) in the USA.[196] However, due to the increased sensitivity of most analytical instruments employed in toxicology laboratories, 40 ng mL^{-1} is recommended as a screening.[195]

If an oral fluid sample is found to be positive for only morphine, it can be interpreted as being due to the administration of morphine, use of heroin (diacetylmorphine) or because of the ingestion of poppy seeds. If the specimen is found positive for codeine alone, it can be interpreted as being due to codeine administration only.[178] Where a combination of

morphine and codeine are present in oral fluid it can be interpreted in a number of ways: if the level of morphine is greater than that of codeine, it is assumed that this is as a result of the use of heroin or the administration of morphine.[83] It has also been suggested that this finding could also be as a results of recent ingestion of poppy seed products.[177] On the other hand, if the codeine level is greater than the morphine, it is proposed that this is due to administration of codeine.[167]

By comparison, in order to establish if an individual has used heroin, it is necessary to identify either a combination of diacetylmorphine, 6-MAM and morphine, a combination of 6-MAM and morphine or 6-MAM only.[176,197] It has been reported that 6-MAM is found oral fluid samples when heroin has been used. However, it is also widely known that 6-MAM has a short half-life and so may not always be detected.[179]

2.2. **GC-MS versus LC-MS**

In the field of forensic toxicology, gas chromatography-mass spectrometry (GC-MS) was the predominant analytical technique used in systematic toxicological analysis for many years.[198] The theoretical and practical aspects of the separation of drugs and/or their metabolites and the subsequent fragmentation patterns produced by the hyphenated GC-MS technique are well understood. The instruments and methods for the detection and quantitation of these analytes have been proven to be robust and reproducible and many commercially available databases were, and still are, readily available. These instruments became the work horses of many, if not all, forensic toxicology and drug analysis laboratories but in 1968, the work of a research group lead by Victor Tal'rose reported the first attempt to hyphenate a liquid chromatograph with a mass spectrometer.[199] It was to take until 1980 before the first commercial instrument was built by Finnigan (now incorporated into the company Thermo Scientific) and until 1989 when Sciex (now incorporated into the company AB Sciex) introduced a more user-friendly model that LC-MS finally started to take a place alongside GC-MS instruments.[200]

It has taken many years for LC-MS, in its many forms, to finally be accepted in working forensic toxicology laboratories but the technique is now widely used in the field and has many applications.[201-204] Although the reproducibility of this technique between systems has made the introduction of databases relevant to toxicological screening a challenge,

commercially available instruments and their software have made the transition from GC-MS to LC-MS less of a chore. LC-MS appears now to be a well-established technique in many working laboratories and its applications in forensic toxicology are much reported in the scientific literature.[172,202,204-208] It is for these reasons that LC-MS was employed in this work: the theory of LC-MS will be discussed in Chapter 3.

2.3. **Research aims**

The main aim of this research is to establish an analytical method for the simultaneous detection of the five major opium alkaloids in poppy seeds by liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS). If opium alkaloids are detected in poppy seeds, a further aim is to carry out toxicological studies to establish if these compounds can be detected in oral fluid of participants who ingest poppy seeds products, and whether or not it is possible to differentiate between poppy seed ingestion and administration of other diacetylmorphine/morphine/codeine products.

3. LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC-MS)

3.1. **LC-MS**

Liquid chromatography hyphenated to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS^n) are analytical techniques that have found favour and have been implemented in many analytical laboratories over the last 20 years or so. [79,201-203,205-209] LC-MS is a hyphenated technique coupling a liquid chromatography separation technique with detection of the separated analytes by mass spectrometry, providing not only retention time data for each analyte but information on the masses of molecular ion(s) or fragments formed in reactions within the mass spectrometer.

There are many options or parameter choices that can differ in LC-MS however the basic principles of LC-MS are constant through each form of instrumentation. The theoretical descriptions of the main components of typical instruments follow.

3.1.1. Liquid chromatography

High performance liquid chromatography (HPLC) is a well-known chromatographic separation technique with a wide range of applications in many different fields of analytical science. It is used to separate mixtures of compounds into their individual components by means of an interaction between the compound, a liquid mobile phase and a stationary phase.

The commonly used modes in HPLC are reversed phase, normal phase, and size exclusion, with reversed phase being widely used in both forensic drug and toxicological analyses.[72,135,137,141,209,210]

In reversed phase liquid chromatography (RP-LC), separation of a mixture of compounds takes place as an interaction between the stationary phase packed within the column and a mobile phase. These RP-LC systems employ a non-polar stationary phase and a polar mobile phase. Typically the packing materials within the reversed phase columns are composed of spherical silica particles which are coated with hydrophobic alkyl chains of varying length. A longer chain length provides a more non-polar *stationary phase* than a shorter chain length. Other column packing materials can include phenyl groups. These RP-

LC columns will be used with mobile phases containing mixtures of aqueous/organic solvents in order to effect an appropriate separation of compounds in a mixture.

3.1.1.1. Column chemistry

When using reversed phase chromatography, compounds are separated on the basis of their hydrophobic nature: each compound will exhibit an adsorption phenomenon in which it is adsorbed onto the stationary phase depending upon packing material and analyte chemistries. When the solvent strength of the mobile phase is sufficient to compete with the hydrophobic forces keeping the compound adsorbed to the stationary phase, it will be eluted from the column and subsequently detected.[211] The most commonly used column chemistry used in RP-LC is a C8 or a C18 column. Researchers employing LC-MS for the detection of opiates in a variety of matrices have also employed C18 columns in their analyses.[50,57,209,210]

Reversed phase column chemistry is based on columns packed with spherical silica particles coated with hydrophobic alkyl (-CH_2-) groups of varying length however in this case, the particles are coated with a dodecyl (C18) chain. Chromatographic separations occur predominantly due to Van der Waals interactions between alkyl groups of the compound and the functional groups of the stationary phase however, the actual separations are far more complex: multiple retention mechanisms may be taking place at the same time. [212]

The spherical particles are coated with the C18 chain but unreacted silanol groups (-Si-OH) are also present, as shown in Figure 3.1. These groups are present as they should serve as a support for the C18 alkyl groups but, due to factors such as steric hindrance, silanol groups are found unreacted: they are weakly acidic, hydrophilic in nature and are considered to be strong adsorption sites. These unreacted silanol groups can influence greatly the chromatographic properties which in turn can lead to poor resolution of peaks.[213]

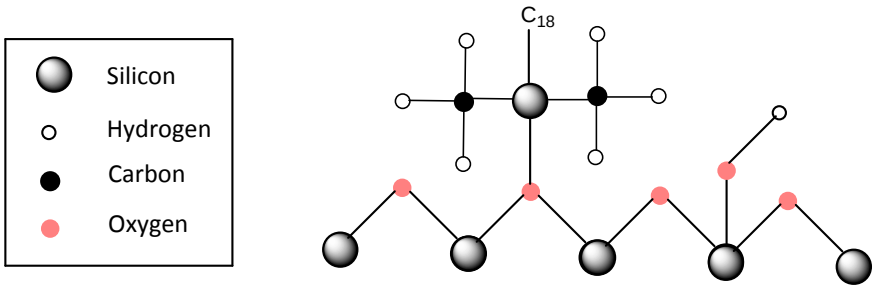


Figure 3.1 Diagram representing silicon backbone of a C18 stationary phase with –Si-OH groups. Adapted from Restek HPLC column selection guide[214]

3.1.2. Mass spectrometry

Currently, many different types of mass spectrometers are commercially available which can be hyphenated to a liquid chromatograph instrument: each of the separated compounds to the MS instrument directly from the LC instrument. A basic block diagram of an MS instrument is shown in Figure 3.2.

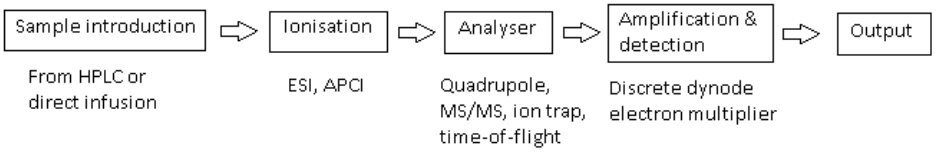


Figure 3.2 Block diagram of the processes involved in mass spectrometry after HPLC separation

Depending upon the application, the commercial supplier and capital available to spend on an LC-MS instrument, many choices are possible with respect to the method of ionisation (ion source) and the analyser. The discussion of the many different types of LC-MS instruments available are out with the scope of this work however, a number of the commonly encountered instrument setups will be considered.

3.1.2.1. Ionisation

Ions can be formed by a number of mechanisms in LC-MS; the most widely available and therefore most commonly employed ionisation techniques are electrospray ionisation (ESI)

and atmospheric pressure chemical ionisation (APCI). Each of the ionisation mechanisms is useful depending upon the physicochemical properties of the compound(s) being analysed such as the molecular weight, and polarity but also instrumental parameters used in the initial separation using HPLC.[215] For example, relatively low flow rates of mobile phase are required when using ESI in comparison to APCI where higher flow rates may be employed. Figure 3.3 shows the use of ESI and APCI depending upon polarity and molecular weight of the analytes.

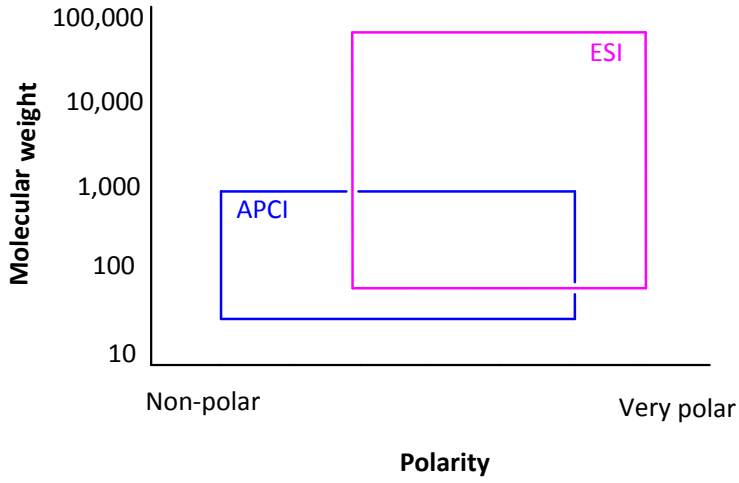


Figure 3.3 Comparison of ionisation methods in relation to analyte polarity and molecular weight

3.1.2.2. Electrospray ionisation (ESI)

The ion sources used on all LC-MS instruments employed in this work use the principle of electrospray ionization to generate gas phase analyte ions from the liquid phase introduced from the HPLC instrument.[216] ESI is a soft-ionisation technique whereby quasi-molecular ions are formed: as positively or negatively charged ions depending upon the mode employed.[217,218]

There has been much discussion over the years on the mechanism of formation of charged ions using ESI sources however it is thought that the charged ions are formed by the analyte(s) in the liquid phase being sprayed into a chamber maintained at atmospheric pressure.[217-220] The liquid is introduced into the source in the presence of a heated drying gas, usually nitrogen, and a strong electrostatic field. The electrostatic field produced

causes dissociation of the analyte molecules, with the drying gas causing the solvent to evaporate. On evaporation of the solvent, the charge concentration of the droplets increases which in turn leads to the forces between ions of the same charge ejecting ions into the gas phase: this process is known as desolvation.[221,222] This process finally gives rise to very small droplets containing single charged analyte molecules.[217,219] A diagram representing the droplet formation in ESI is shown in Figure 3.4. Once the ions are formed, they then pass through a capillary chamber into the mass analyser.

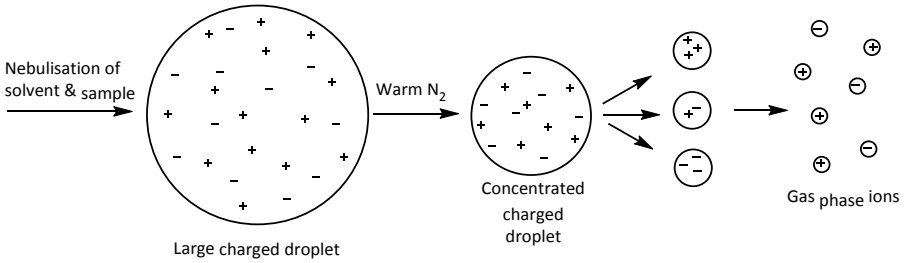


Figure 3.4 Ion formation in an ESI source. Adapted from Bayne & Carlin (2010)[172]

3.1.2.3. Atmospheric pressure chemical ionisation (APCI)

APCI exploits similar ionisation methods as is used in ESI however in APCI the evaporation of the solvent and the formation of ions are two separate processes. With APCI, the ion source is set up in a similar way to ESI but a corona needle (Figure 3.5) is used to ionise the gas and solvent molecules.

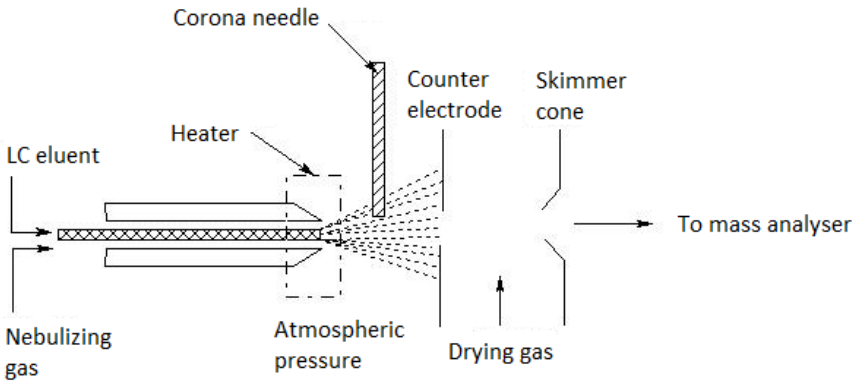


Figure 3.5 APCI ion source. Adapted from Bayne & Carlin (2010)[172]

These charged gas and solvent ions then interact with the analyte and cause ionisation through charge transfer.[223] LC-MS with APCI ionisation has found application in drug and toxicological analyses.[224-227] APCI is typically used with analytes that are not readily ionised with ESI and are small and thermally stable.[223]

3.1.3. Mass spectrometry analysers

The analyser in a mass spectrometer is essentially used to filter ions of a chosen mass-to-charge (m/z) or m/z range before being detected. The filtering process occurs on the basis of the behaviour of the ions in either a magnetic or electric field. Mass analysers include, for example, single quadrupole, ion trap, and triple quadrupole.[172,194]

3.1.3.1. Ion trap

The ion trap analyser is an electric field instrument used to "trap" ions by applying a charge from the appropriate electrode of the analyser. Ion trap analysers consists of a doughnut shaped electrode alongside two other hyperbolic electrodes called end-cap electrodes, (Figure 3.6).

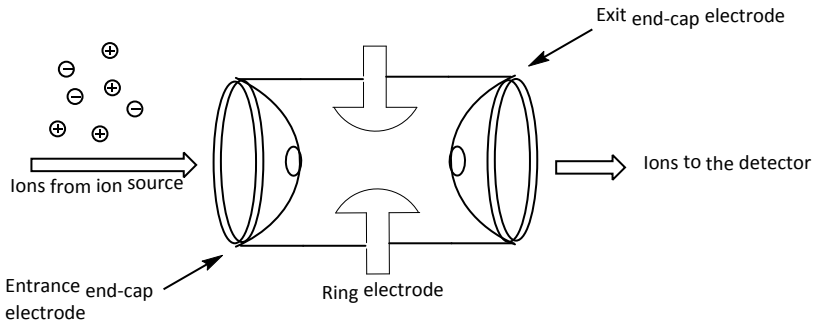


Figure 3.6 Trapping system of an ion trap analyser. Adapted from Bayne & Carlin (2010)[172]

The ions are trapped in the centre of the ring electrodes by applying radio frequency (RF): the ion trap is filled with a damping gas, typically helium, which is used to collide with the ions thus reducing their kinetic energies. Ions are hence trapped and ejected to the

detector depending on their mass to charge ratio by selectively altering the RF voltage of the trap.[228]

Ion trap analysers can be susceptible to space-charge repulsion effects as a result of too many ions being present in the trap generating a distortion of the electrical field. Consequently this can lead to a reduction in the overall performance of the analyser.[229] Ion trap instruments have however been successfully utilised in forensic and analytical applications for many years.[207,230-232]

3.1.3.2. Triple quadrupole

Triple quadrupole analysers employs three quadrupoles in tandem (Q1, Q2 and Q3 in Figure 3.7) to separate and filter the ions of interest. Q1 is used to filter ions of a particular m/z value by altering the RF and DC voltages; Q2 is then used as a collision cell where the ion or ions from Q1 are focussed and can interact with a collision gas causing charged fragments to form. These fragment ions will then be filtered in Q3 in the same way as in Q1.[231]

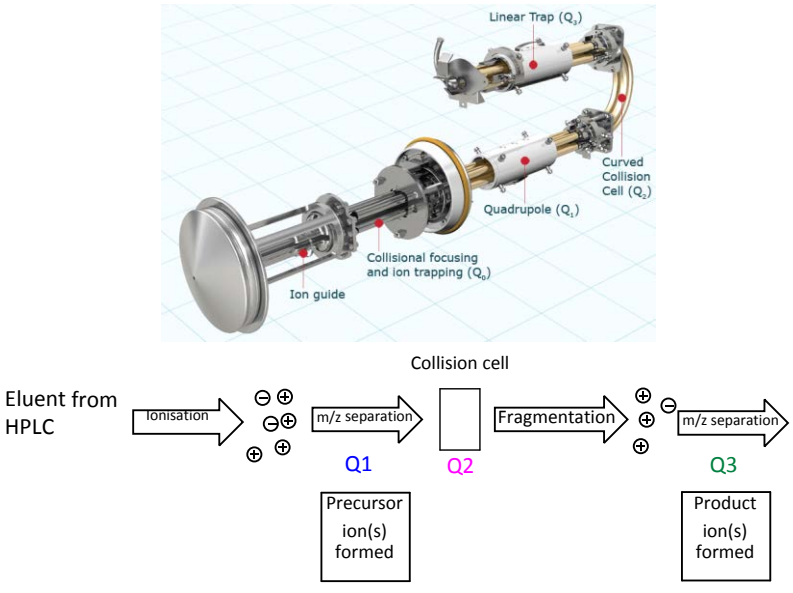


Figure 3.7 Image of a triple quadrupole[233] with block diagram outlining ion formation and separation

3.1.3.3. Orbitrap

A relatively new type of analyser to commercial LC-MS instruments is an Orbitrap.[234] This instrument admits ions from the ESI source into a linear ion trap. Here, ions of a particular mass range are "trapped" before reaching a curved quadrupole (C-trap) which is surrounded by two flat lenses with apertures that allow ion transport.[235]

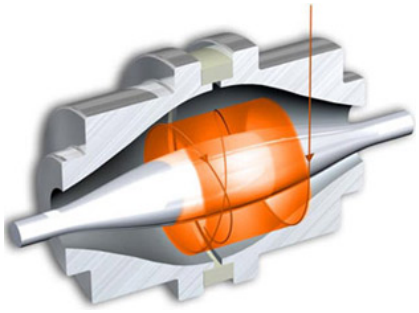


Figure 3.8 Diagram of a linear trap quadrupole Orbitrap mass spectrometer[236]

The Orbitrap is a small electrostatic device with a single spindle-shaped electrode (Figure 3.8). When the ions are injected into the Orbitrap, they are trapped by orbiting around a central electrode.[237] The ions are detected when a stable electrostatic field is achieved.[235]

3.2. **Analytical method development and validation**

One of the aims of this research is to establish an analytical method for the simultaneous detection of the five major opium alkaloids morphine, codeine, thebaine, papaverine and noscapine. When faced with a new analyte or mixture of analytes, it is necessary to develop an analytical method for the detection and, in this case, quantitation of these analytes.

Method development in LC-MS involves the consideration of all aspects of analysis from sample preparation techniques to instrumental parameter settings to achieve an optimum output and data which can be analysed to provide meaningful interpretation. [238] Once a suitable sample preparation and analytical method for the detection and quantitation of a

method has been obtained, validation of that process is required to prove that method performs in the manner for which it is intended and that it performs consistently well.

Peters *et al.*[239] published a paper in 2007 outlining essential parameters required when validating a new analytical method with particular reference to methods in analytical toxicology. It was proposed by the authors that selectivity, linearity, accuracy, precision, limit of detection (LOD), lower limit of quantitation (LLOQ), recovery and robustness should be established and where appropriate, matrix effects should also be included when validating an analytical method. These validation parameters still stand today and are accepted in the field of analytical toxicology and drug analysis: they are briefly defined below.

- **Selectivity (specificity):** the ability to unambiguously detect the analyte of interest in the presence of other components which may be expected to be present in the sample being analysed.

- **Calibration model:** a relationship between analyte concentration in a sample and detector response must be established. This is predominantly linear however other models, such as quadratic models may be used.

- **Accuracy (bias):** this is a measure of the closeness of agreement between an accepted reference value and an actual sample result.

- **Precision:** this is the closeness of agreement between a series of measurements obtained from analysis of the same homogeneous sample under the prescribed conditions.

- **Limit of detection (LOD):** this is the lowest analyte concentration in a sample which can be detected but not necessarily quantitated as an exact value. It is generally accepted that when a signal-to-noise (S/N) ratio is of ≥ 3:1 is achieved, LOD is established.

- **Lower limit of quantitation (LLOQ):** this is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

It is generally accepted that when a S/N ratio is of ≥ 10:1 is achieved, LLOQ is established.

- **Recovery:** this is determined as a percentage of analyte concentration detected after extraction from a matrix in comparison to the concentration intentionally added to blank spiked matrix.

- **Robustness:** this is a measure of the capacity of the method to remain unaffected by small but deliberate variations in the analytical parameters.

- **Matrix effects:** these effects can either increase the ionisation (enhance) or decrease the ionisation (supress) of certain analytes. A number of factors such as sample preparation technique, matrix type, mobile phase additives and ionisation type can produce matrix effects.[239]

4. ESTABLISHING AN LC-MS TESTING METHOD FOR THE SIMULTANEOUS DETECTION OF THE FIVE MAJOR OPIUM ALKALOIDS

4.1. Experimental

4.1.1. Chemicals and reagents

4.1.1.1. LC-MS

Organic solvents (methanol, acetonitrile, chloroform, isopropyl alcohol) of HPLC grade were purchased from Sigma-Aldrich (Poole, Dorset) as was acetic acid. Drug standards of codeine (1 mg mL^{-1}) in methanol and morphine (1 mg mL^{-1}) in methanol were purchased from LGC Standards (Teddington, Middlesex) and powder form drug standards of codeine, morphine sulphate salt monohydrate, thebaine, noscapine hydrochloride hydrate and papaverine hydrochloride were purchased from Sigma-Aldrich (Poole, Dorset). Deuterated morphine (100 µg mL^{-1}) in methanol was purchased from Sigma Aldrich (Poole, Dorset) and used as an internal standard.

4.1.1.2. NMR

Only thebaine was analysed using NMR and standards were prepared by dissolving approximately 10 mg of the powdered standard into a number of solvent ratios of deuterated water (D_2O) and acetonitrile-d3 (CD_3CN) incorporating 1% of deutero-acetic acid (CD_3COOD) to mimic the mobile phase conditions of the liquid chromatograph. Deuterated solvents were purchased from Goss Scientific (Crewe, Cheshire). LC-grade water was obtained from a Millipore purification system.

4.1.2. Instrumentation

4.1.2.1. LC-ESI-MS (ion trap)

HPLC was performed using an LC Surveyor system (Thermo Finnigan, Hemel Hempsted, UK) which was equipped with a pump, auto-sampler and column heater.

A Gemini 3 µm C18, 100 x 2.0 mm column fitted with a Gemini C18, 4.0 x 2.0 mm guard column (Phenomenex, Cheshire, UK) was used and was thermostated at 30^{o}C.

Mass spectrometry was performed using an LCQ Advantage (Thermo Finnigan, Hemel Hempsted, UK) ion trap mass spectrometer. The mass spectrometer was operated in a positive electrospray ionisation mode. Selection and tuning of the mass spectrometer settings for each of the drug analytes were performed using direct infusion, involving the direct introduction of each of the analytes in methanol.

4.1.2.2. LC-ESI-MS (triple quadrupole)

A comparative method was used in collaboration with the toxicology laboratory of the Institut de Recherche Criminelle de la Gendarmerie Nationale (IRCGN), Paris. The instrument used was a 1260 HPLC (Agilent Technologies, Paris, France) coupled to a 4500 QTRAP tandem mass spectrometer (AB Sciex, Les Ulis, France). The mass spectrometer was operated in a positive electrospray ionisation mode. The column used was an Allure™ pentafluorophenyl phase with a propyl spacer (PFPP) column, 5 µm, 50 x 2.1 mm fitted with an Allure™, PFPP 10.0 x 2.1 mm guard column (Restek, Lisses, France) thermostated to 40 ^{o}C. The auto-sampler tray was maintained at 8 ^{o}C.

4.1.2.3. Nuclear magnetic resonance spectroscopy

All ^{1}H-NMR spectra were obtained using a JOEL ECS 400 NMR spectrometer operating at 399.8 MHz.

4.1.3. Analysis of results

Data analysis was carried out using XCaliber 2.0 software package supplied with the ThermoFinnigan LC system and LCQ Advantage ion trap mass spectrometer.

Analysis of data obtained using the Agilent HPLC 1260 hyphenated to the AB Sciex 4500 mass spectrometer was analysed using Analyst 1.6 software package.

All ^{1}H-NMR spectra were analysed using the Delta 5.03 software package.

4.2. **Method development – LC-ESI-MS**

A paper published in 2006 by Sproll *et al.*[76], reported the use of an LC/MS/MS method for analysing the presence of morphine and codeine in poppy seeds and carrying out a risk analysis of what happens to those alkaloids when they enter the food chain. Bucelli *et al.*[230] in 2009 also published a paper reporting the successful analysis of hair samples for the detection of drugs of abuse using LC-ESI-MS; using both of these published methods, instrumental parameters were chosen as the starting point for the initial method development although the detection of thebaine, papaverine and noscapine were not included in these articles. The initial LC method used is shown in Table 4.1 where the column used was a Gemini 3 µm C18, 100 x 2.00 mm column fitted with a Gemini C18, 4.0 x 2.0 mm guard column; solvent A was acetonitrile + 1% acetic acid (pH 1.5); solvent B was water + 5% acetonitrile + 1% acetic acid (pH 2.5) and the mass spectrometer was run in positive mode electrospray ionisation using a capillary temperature of 230 ^{o}C, a spray voltage of 6 kV and capillary voltage of 36 V.

Table 4.1 Initial LC-MS conditions used for attempted detection of opium alkaloids

Mobile phase composition **Solvent A:** Acetonitrile + 1% acetic acid **Solvent B:** Water + 5% acetonitrile + 1% acetic acid			
Time (minutes)	**%A**	**%B**	**Flow rate (µL min^{-1})**
0.00	0	100	200
10.00	65	35	200
12.00	65	35	200
14.00	0	100	300
20.00	0	100	300

At this point in the work, deuterated morphine was not available but solutions of morphine, codeine, thebaine, papaverine and noscapine were prepared at a concentration of 2000 ng mL^{-1} in methanol and water (50:50, v/v). All drug standards were prepared from powdered material as described in section 4.1.1. The mass spectrometer was set to perform analysis over a 12 minute run time and was set for selected ion monitoring (SIM) for morphine with a quasi-molecular ion, [M-H^{+}], of *m/z* 286 ± 2.0; codeine with a quasi-

molecular ion of *m/z* 300 ± 2.0; thebaine with a quasi-molecular ion of *m/z* 312 ± 2.0; papaverine with a quasi-molecular ion of *m/z* 340 ± 2.0 and finally noscapine with a quasi-molecular ion of *m/z* 414 ± 2.0.

The solution of morphine was introduced to the LC-MS as described above and the resulting chromatogram and associated mass spectrum is shown in Figure 4.1. Under these conditions, morphine was found to have a retention time of 1.33 minutes which shows that it is almost unretained by the column and has a high affinity for the mobile phase. The associated mass spectrum (Figure 4.1.a.) shows the [M-H$^+$] at *m/z* 286.05 however a second peak also appears in the chromatogram with a retention time of 10.53 minutes and a [M-H$^+$] at *m/z* 340.24. It was assumed at this point, that this peak was due to carryover from previous injections when the mass spectrometry method was being established.

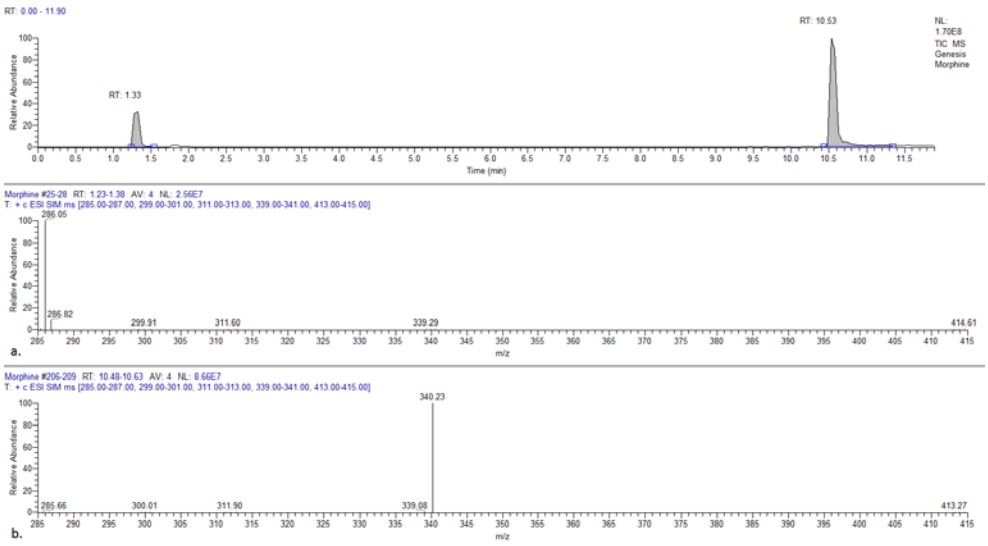


Figure 4.1 Chromatogram and associated mass spectra for morphine injection for (a) peak 1 at 1.33 minutes and (b) peak 2 at 10.53 minutes

When codeine was introduced (chromatogram and associated spectra shown in Figure 4.2), it was noted that there was a peak found with a retention time of 1.33 minutes with the associated mass spectrum showing the codeine [M-H$^+$] at *m/z* 300.06. The same or very similar retention time to morphine is not completely unexpected due to the similarity in chemical structures of the two compounds: the only difference is the replacement of an hydroxyl group on the 3-position with a methoxy group.

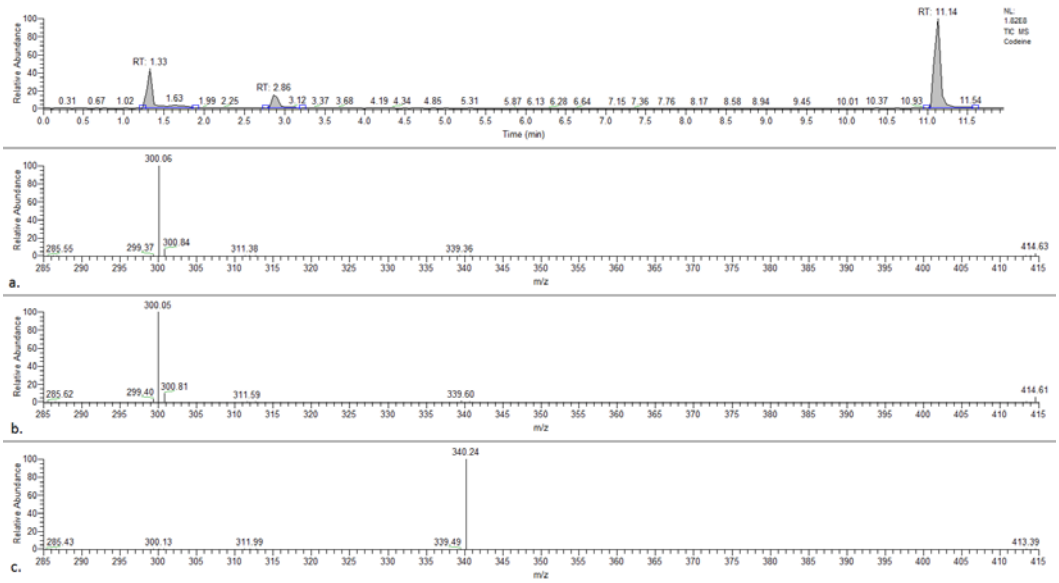


Figure 4.2 Chromatogram and associated mass spectra for codeine injection for (a) peak 1 at 1.33 minutes (b) peak 2 at 2.86 minutes and (c) peak 3 at 11.14 minutes

There is also a peak with a retention time of 11.14 minutes with a mass spectrum that appears very similar to that of papaverine. In this chromatogram, there is also a third peak with a retention time of 2.86 minutes with a corresponding mass spectrum with the most abundant peak found with *m/z* 300.05.

Possible explanations for the presence of two peaks with the same mass spectral output was that the solution was too concentrated resulting in more than one peak for one compound or that the powdered drug standard which had been purchased was not pure. It is well known that in the synthesis and preparation of drug standards, impurities, albeit in small proportions, will be present in the final product.[240,241] In order to asses which, if any of these was true, the concentration of the drug standard was reduced and the resulting chromatogram was the same as shown in Figure 4.2 but with smaller peak areas. In order to assess this, a pure drug standard, purchased as a 1 mg mL^{-1} pre-prepared standard in methanol in a glass ampoule, was purchased from LGC Standards (Teddington, Middlesex) and from this, a 1000 ng mL^{-1} codeine standard was prepared and injected again. The resulting chromatogram produced only one peak for codeine with a retention time of 1.33 minutes, proving that the presence of the second peak was due to an impurity in the standard.

When the thebaine drug standard was injected into the instrument, the resulting chromatogram, shown in Figure 4.3, was of a poor quality: the peak thought to be papaverine was still present but there were two peaks present (1.38 minutes and 7.26 minutes) which showed the same ion with *m/z* 311.94. Due to the fact this this issue had also been seen with the injection of the codeine standard, it was initially thought that sample impurities explained the presence of multiple peaks. However, it was not possible to obtain a pure drug standard for thebaine and the fact that there was a large difference in retention time between the two peaks (which was not seen in the case of codeine) warranted further investigation (Section 4.3).

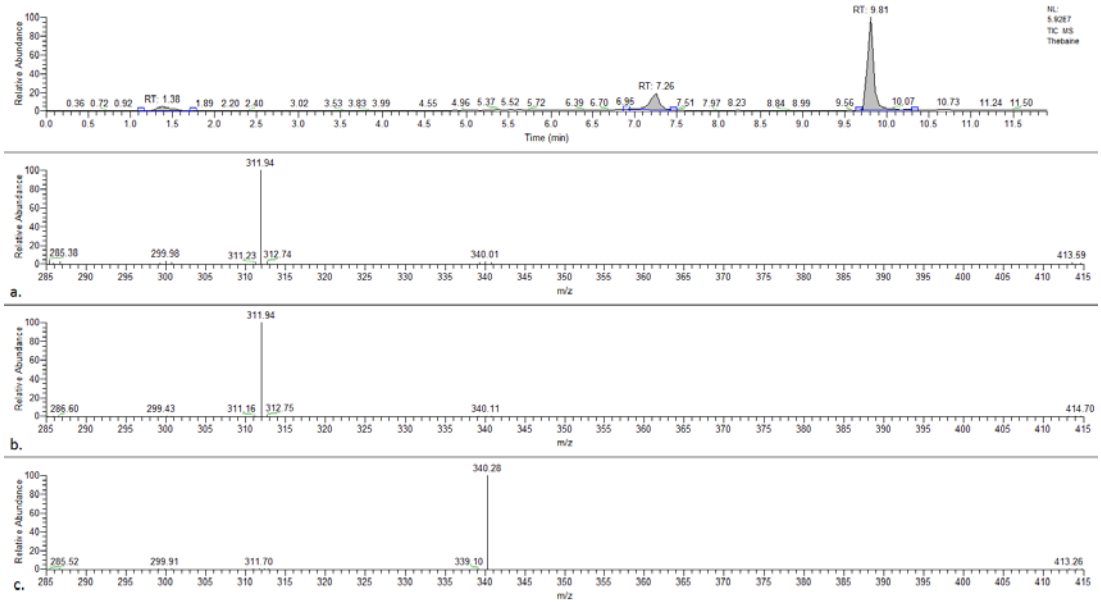


Figure 4.3 Chromatogram and associated mass spectra for thebaine injection (a) peak 1 at 1.38 minutes (b) peak 2 at 7.26 minutes and (c) peak 3 at 9.81 minutes

Noscapine was then introduced to the system and again two peaks were noted (Figure 4.4): one peak was found at a retention time of 8.28 minutes with associated mass spectrum showing the most abundant ion *m/z* of 414 and, as with the previously injected alkaloids, an extra peak consistent with the quasi-molecular ion *m/z* of 340 for papaverine.

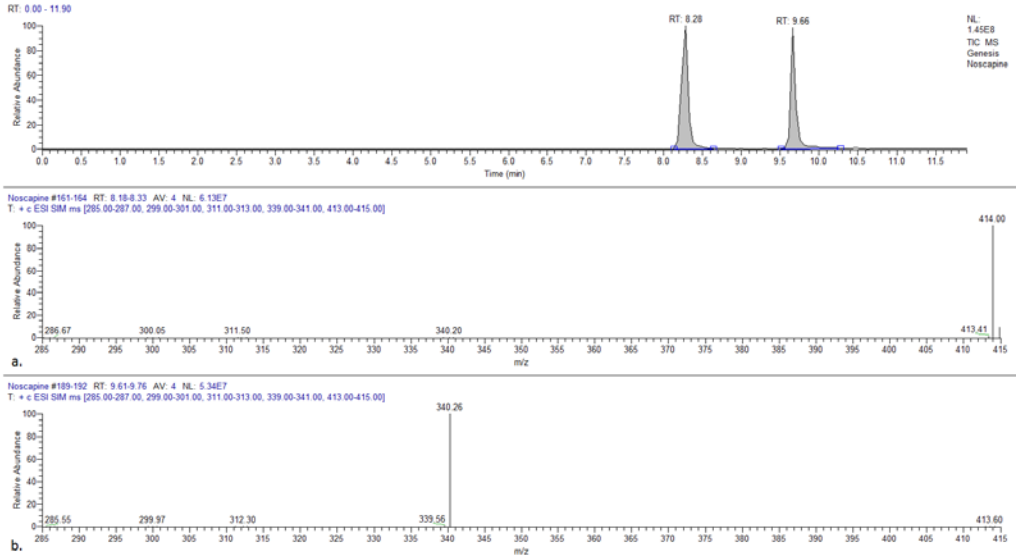


Figure 4.4 Chromatogram and associated mass spectra for noscapine injection (a) peak 1 at 8.28 minutes and (b) peak 2 at 9.66 minutes

Papaverine was injected into the instrument and the resulting chromatogram and associated mass spectra are shown in Figure 4.5. It was initially thought that the extra peak found in the chromatograms for morphine, codeine, thebaine and noscapine with *m/z* of 340 was due to carryover from early injections of papaverine which were used to establish the mass spectrometry method. However, two peaks were found again when papaverine itself was injected.

In order to establish if both peaks were due to papaverine (one from column/system overload and the other from injection) it was necessary to consider alternative hypotheses. Although it was possible that column/system overload had taken place, which can be seen from the high peak area values in most of the previously noted chromatograms, it was also possible that since the instrument was also used many other researchers in different fields, that the peak could be due to a compound that was not papaverine.

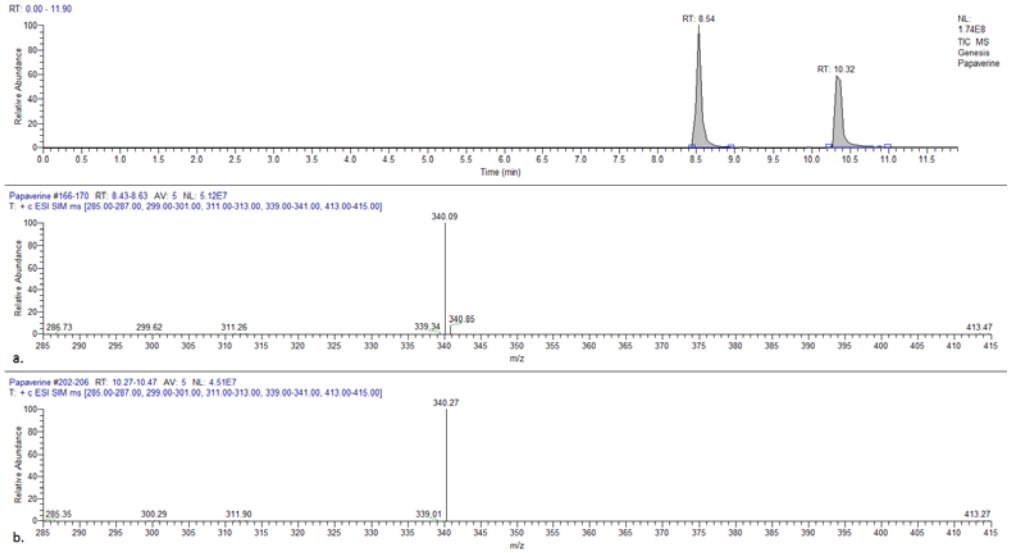


Figure 4.5 Chromatogram and associated mass spectra for papaverine injection (a) peak 1 at 8.54 minutes and (b) peak 2 at 10.32 minutes

Blank injections of the solvent used to prepare the drug standards were injected into the LC-MS at the beginning of the sequence and between each of the standards; as can be seen from one of the blank injections shown in Figure 4.6, the extra peak was persistent. This is true for all of the blank injections therefore with this information, it was decided to reduce the concentration of the drug standards by half in order to avoid overloading the column but also to reduce or eliminate carry over in subsequent injections. This did not resolve the matter, as the extra peak was still present, even after column flushing.

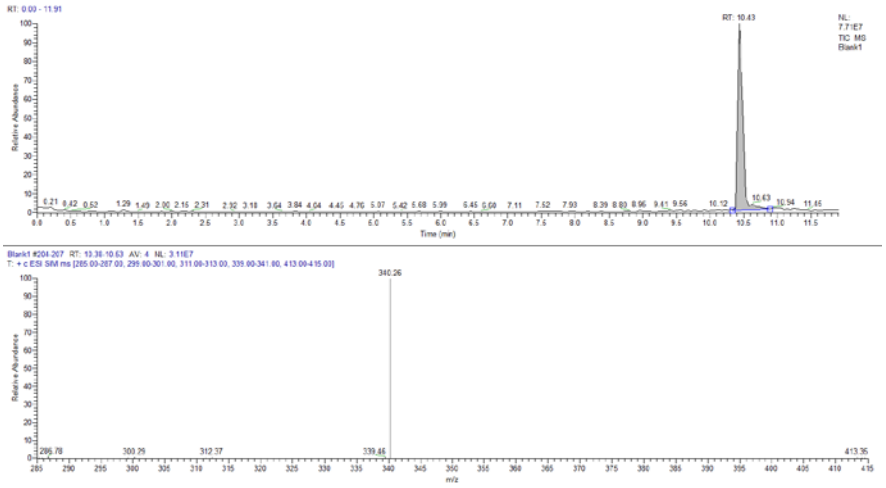


Figure 4.6 An example of the resulting chromatogram and associated mass spectrum from a blank injection

Since it was possible that the peak was carryover on the column, another C18 column was connected to the HPLC and morphine and codeine were injected under the same LC-MS method as previous alkaloid injections. It was found that the extra peak was still present which allowed the conclusion to be drawn that column carryover was not the reason for the presence of the extra peak.

After establishing that column carryover was not the reason for the extra peak, a decision was made to carry out mass spectrometry/mass spectrometry (MS/MS) experiments. Using selective ion monitoring (SIM) and searching only for the quasi-molecular ion for each of the alkaloids did not provide sufficient discriminating power. Therefore, transitions from precursor ion (quasi-molecular ion) to product ions (fragment ions) were monitored.

To optimize the mass spectrometer settings for each of the alkaloid compounds, a 100 ng mL^{-1} standards of morphine, codeine (both from 1 mg mL^{-1} drug standards in methanol), thebaine, papaverine and noscapine (from powder) were prepared and introduced to the mass spectrometer using positive electrospray ionisation. Each alkaloid solution was introduced using direct infusion: once the quasi-molecular ion (precursor ion) for each of the alkaloids was found, the collision energy of the mass spectrometer was increased until the peak for the quasi-molecular ion halved in abundance and other stable peaks were present (product ions). The optimised settings are provided in Table 4.2.

Table 4.2 Optimised mass spectrometer settings for initial MS/MS experiments

Compound	Quasi-molecular ion (*m/z*)	Monitored transition mass (*m/z*)	Collision Energy (eV)
Morphine	286	286 → 268	33
Codeine	300	300 → 215, 243	34
Papaverine	340	340 → 202	38
Noscapine	414	414→ 220	27

The chromatogram in Figure 4.7 shows that using this method it was possible to detect one peak for morphine, codeine, noscapine and papaverine but two peaks for thebaine. It

should be noted that in this mass spectrometry method, thebaine was monitored by SIM only which was an error.

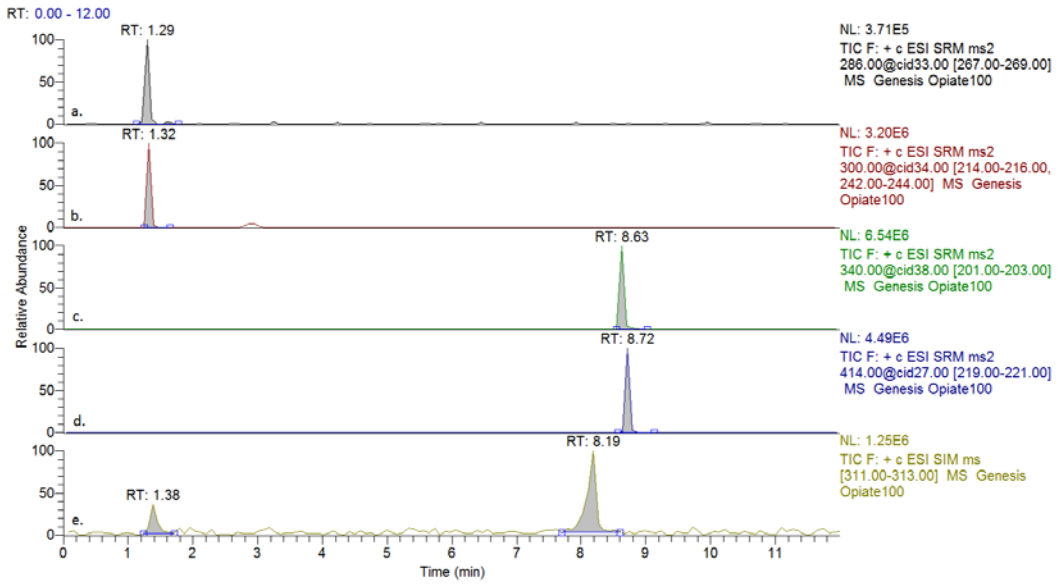


Figure 4.7 Single peaks for (a) morphine (b) codeine (c) papaverine (d) noscapine and (e) two peaks for thebaine

The MS/MS transition for thebaine was subsequently included in the method but still the presence of the two peaks persisted. At this point, it was decided that the issue of the presence of two peaks for thebaine required further investigations but these will be discussed in section 4.4.

For the validation of an analytical method it is necessary to ensure, at the very minimum, that selectivity, linearity, accuracy, precision, and limits of detection/quantitation should be considered.[239] These properties were hence considered in relation to this analyte mixture.

4.2.1. Selectivity

Section 3.2 defines this validation parameter as being able to prove that the analyte, or analytes, of interest should be unambiguously detected in the presence of other components from the sample matrix. In this case, the matrix was aqueous mobile phase. In

order to assess this parameter, multiple blank samples were injected before, between and at the end of the sample sequences and all blanks showed the absence of peaks for the alkaloid compounds being analysed by this method. When morphine, codeine, papaverine and noscapine were injected individually, only one peak was found (Figure 4.7) therefore showing that selectivity was established.

4.2.2. Linearity

In order to assess if linearity was possible for each of the compounds using this analytical method, a series of calibration solutions were prepared in the range 0 – 1000 ng mL^{-1}. Similar ranges had been used in published studies in the scientific literature in the analysis of opiates in poppy seeds and other biological matrices.[70,140,177,242] Calibration graphs were constructed using concentration and peak area. The resulting calibration graphs were constructed using a minimum of five data points, including a blank as is suggested in the literature.[239,243] The equation of the line and coefficient of determination (R^2) for morphine, codeine, papaverine and noscapine are provided in Table 4.3.

Table 4.3 Linear equations and associated R^2 values for morphine, codeine, papaverine and noscapine

Compound	Linear equation	R^2	Concentration Range (ng mL^{-1})
Morphine	y = 14942x + 60372	0.9897	0 – 200
Codeine	y = 38087x + 0.9996	0.9999	0 – 1000
Papaverine	y = 95953x + $1x10^6$	0.9989	0 – 1000
Noscapine	y = 182484x + 523763	0.9923	0 – 200

It is generally accepted in method validation, that a minimum R^2 value of 0.99 is expected to demonstrate linearity.[244] Codeine, papaverine and noscapine calibration graphs meet these criteria and are therefore linear, however morphine fell slightly lower than the desired value. It should be noted that these graphs were constructed using only one data set therefore in order to determine if linearity was reproducible, precision should be determined.

4.2.3. Precision

Precision has been defined in section 3.2 as the closeness of agreement between a series of measurements. Precision in chromatography methods is generally determined by repeating injections at different points along the linear range of concentrations and assessing the coefficient of variation (%CV) or relative standard deviation.[244,245]

For morphine, codeine, papaverine and noscapine, mixed calibration solutions were prepared at 1, 10, 50, 100, 500 and 1000 ng mL^{-1}; at each concentration four repeat injections were carried out. A blank was run at the beginning and end of the sequence and between each of the four injections of the same concentration. From the peak area for each of the alkaloids, the mean ($\bar{x}$) peak area for the four injections at each concentration point, the standard deviation (σ) and the percent coefficient of variation (%CV) was calculated: from this, a calibration graph was constructed.

For morphine (Figure 4.8), it was not possible to detect morphine at the lower end of the concentration range. Other validated LC-MS methods have been published which included morphine: Coles *et al.*[242] in 2007 reported LOD of 0.05 ng mL^{-1} and LOQ of 2 ng mL^{-1} and Taylor and Elliott[209] in 2009 reported an LOD of 0.34 ng mL^{-1} and an LOQ of 10 ng mL^{-1}. By comparison, the lowest detected concentration for morphine using this LC-MS method was 50 ng mL^{-1}: this is considerably higher than the values reported in the literature. The coefficient of determination (R^2) value for this data is also poor: a value of 0.7758 is much lower than the expected value of 0.99 indicating substantial error associated with using the equation from this data. It should also be noted that the calibration graph from the available data has been constructed with only 4 data points plus the blank: 5 points plus the blank is the minimum requirement[246,247] therefore it was necessary to increase the number of points in the range. It is expected that the %CV values should be 15% or less and 20% or less[239,244,245] at the lowest concentration points however, as can be seen from the tabulated data in Figure 4.8, only the repeat injections of morphine at 1000 ng mL^{-1} would meet this criteria.

Concentration (ng mL^{-1})	$\bar{x}$ peak area	σ	% CV
0	0	0	0
1	0	0	0
10	0	0	0
50	255705	299579	117
100	1217997	255554	24
500	1489118	536633	36
1000	2075914	314665	15

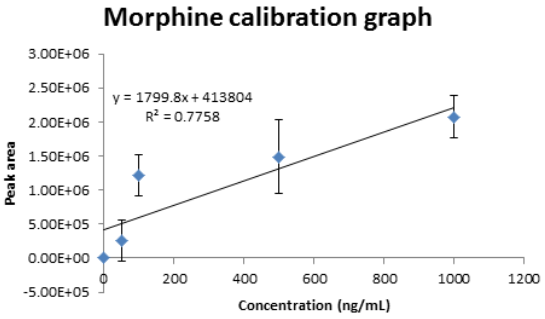


Figure 4.8 Data and associated calibration graph for morphine

When this data set was analysed for codeine concentration (Figure 4.9), it was possible to detect 10 ng mL^{-1}, which is lower than that found for morphine. The published literature has several reports describing the detection and quantitation of codeine; Coles *et al.*[242] in 2007 reported LOD of 0.05 ng mL^{-1} and LOQ of 2 ng mL^{-1} and Newmeyer *et al.*[177] reported an LOD of 0.5 ng mL^{-1} and LOQ of 1 ng mL^{-1}. As was the case with morphine, the lowest detectable concentration for codeine from this work is considerably higher than those stated in the published literature. The R^2 value of 0.9973, obtained when the average peak area values were plotted against concentration is greater than the minimum value of 0.99. However, when considering the coefficient of variation for each of the concentration points, the values far exceed the 15% or 20% threshold desired.

Concentration (ng mL^{-1})	$\bar{x}$ peak area	σ	% CV
0	0	0	0
1	0	0	0
10	39953	25018	63
50	136539	39701	29
100	333697	82627	25
500	3097998	742972	24
1000	6678602	1511142	23

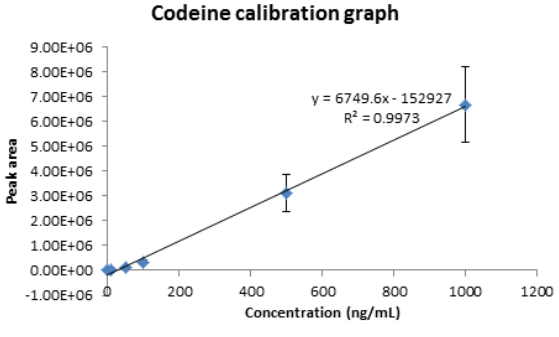


Figure 4.9 Data and associated calibration graph for codeine

Papaverine injections provided linear data (Figure 4.10) with an acceptable R^2 value of 0.9996 with 6 data points and a blank but the %CV values at all the data points except at 500 and 1000 ng mL^{-1}, exceed the 15% (or 20% for lowest concentration) limit previously described. It was also possible to detect papaverine at a concentration of 1 ng mL^{-1}. There are currently no comparable LC-MS quantitative methods for papaverine with available for LOD and LOQ data. Although the data obtained for papaverine is much better than that obtained for morphine and codeine, it still falls short of acceptable limits required to validate a method.

Concentration (ng mL^{-1})	$\bar{x}$ peak area	σ	% CV
0	0	0	0
1	371691	124395	33
10	2411971	813266	34
50	14712091	3707716	25
100	27057093	6548087	24
500	122224987	10867228	9
1000	236071917	31936835	14

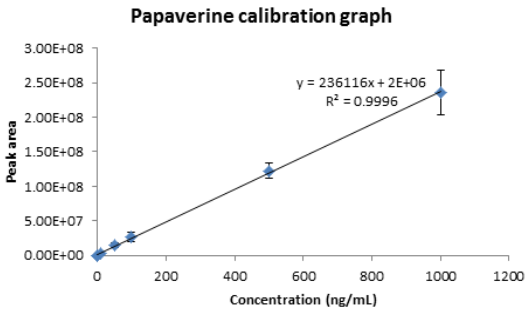


Figure 4.10 Data and associated calibration graph for papaverine

It was possible to detect noscapine at 1 ng mL^{-1} and to construct a linear calibration graph but with an R^2 value of 0.985, it was with some error. Notably, if the 1000 ng mL^{-1} point was removed, an R^2 value of 0.9977 was obtained. However, when the %CV values are considered, only the 500 and 1000 ng mL^{-1} data points meet the acceptable criteria with both having values less than 15%.

Concentration (ng mL^{-1})	$\bar{x}$ peak area	σ	% CV
0	0	0	0
1	172239	60812	35
10	2220854	516	23
50	6730359	1403278	21
100	20020260	7212539	36
500	84188085	10643872	13
1000	133254813	23744589	18

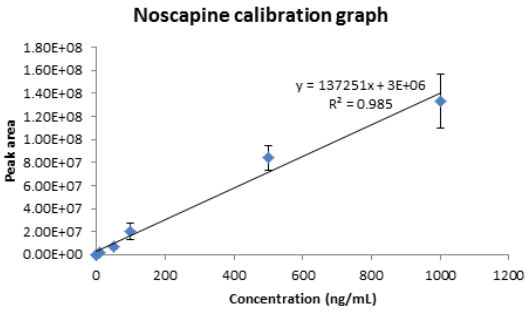


Figure 4.11 Data and associated calibration graph for noscapine

4.2.4. Other validation parameters

It is clear, that for morphine, codeine, papaverine and noscapine that the data, and therefore the method, did not meet the prescribed requirements for method validation. Since the validation parameters discussed above are not acceptable, it was decided not to continue with determining the other appropriate validation parameters but to assess where the method could be adapted in order to achieve a better outcome. With this in mind, it was decided to try to resolve the issue of two peaks with very similar mass spectral data appearing in the chromatogram for thebaine. If this issue could be resolved, the development of an analytical method for the simultaneous detection of all five alkaloid compounds might be achieved.

4.3. **NMR analyses of thebaine**

It was noted that using the initial LC-ESI-MS method that all compounds could be detected but that there were two peaks present in the chromatogram for thebaine (Figure 4.12) with both having almost identical mass spectral data. There was also no linear correlation produced for either of the peaks when attempting to construct a calibration graph. A series of experiments were carried out using nuclear magnetic resonance (NMR) to investigate this finding.

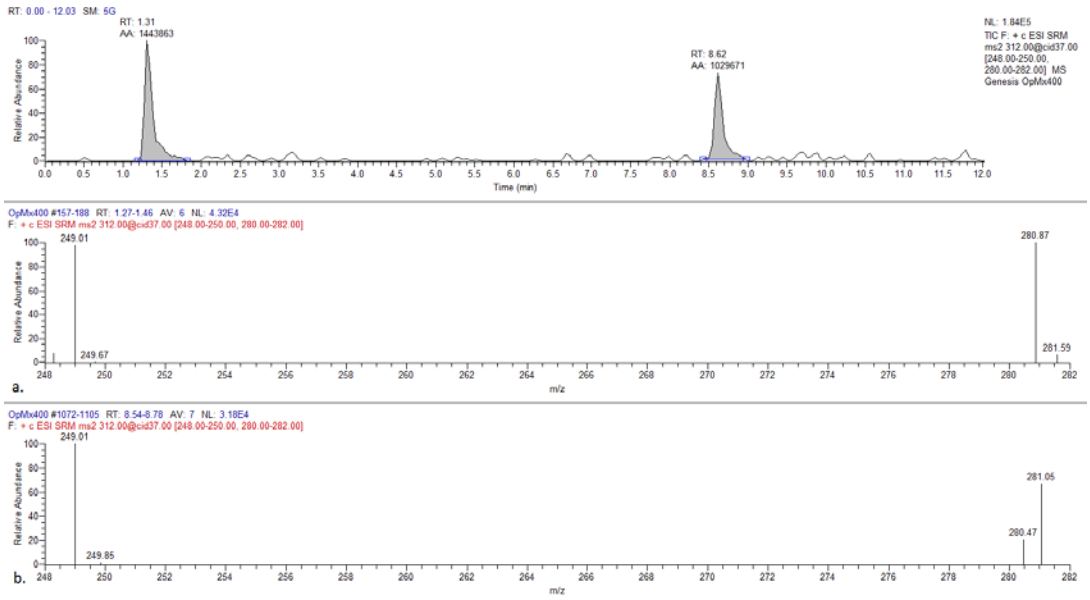


Figure 4.12 Chromatogram for thebaine showing two peaks: (a) mass spectrum for peak at 1.31 minutes and (b) mass spectrum for peak at 8.62 minutes

NMR has been used to elucidate structures of molecules for many years[248] but has been used more specifically to attempt to elucidate the structure of alkaloids since the 1970s. In 1975, Terui *et al.*[249] reported the ^{13}C spectra for morphine alkaloids thebaine and sinomenine; Theuns *et al.*[250,251] reported the structure of thebaine using both ^{13}C and ^{1}H NMR with the most recent article in this field published by Caldwell *et al.*[252] who reported the configurational analysis of thebaine and codeine using ^{13}C and ^{1}H NMR in 1996.

In the HPLC method described above, two separate mobile phases were used: acetonitrile + 1% acetic acid and water + 5% acetonitrile + 1% acetic acid. It was decided to establish if there was any difference in the chemical structure of thebaine with varying proportions of the solvent acetonitrile to aqueous component and also to alter the amount of acid present in the solvent. It was hoped this would help explain why the chromatography showed two peaks. Solutions were prepared for ^{1}H NMR in varying ratios of deuterated water (D_2O):deuterated acetonitrile (CD_3CN) + 1% deutero-acetic acid (CD_3COOD) to investigate the effect of the mobile phase composition on the structure of thebaine. Figure 4.13 shows resulting ^{1}H-NMR spectra with varying mobile phase composition.

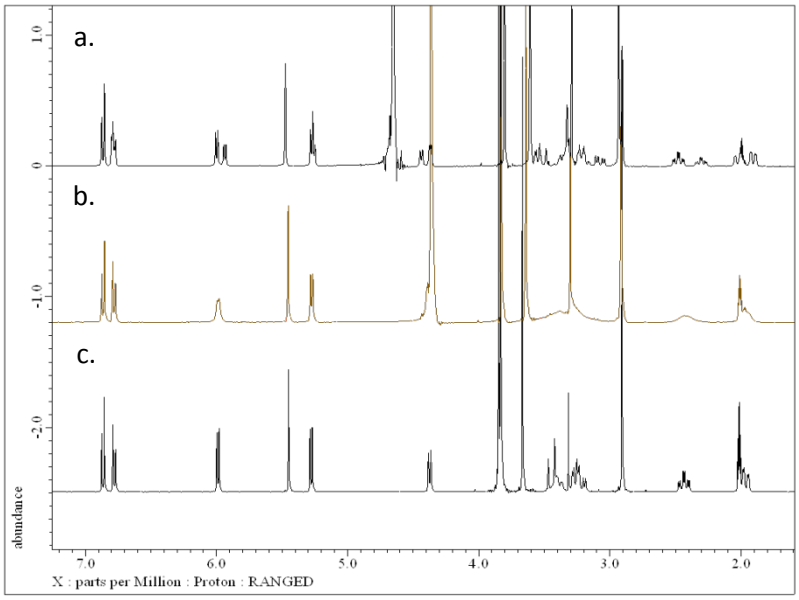


Figure 4.13 ^{1}H-NMR spectra for thebaine in (a) D_2O:CD_3CN ratio 80:20 (v/v), (b) D_2O:CD_3CN ratio 50:50 (v/v), (c) D_2O:CD_3CN ratio 20:80. All solutions contain 1% CD_3COOD

It is proposed that in the presence of deutero-acetic acid the nitrogen atom of the thebaine is deuterated and thus promotes solubility in the polar mobile phase at the beginning of

the LC-MS method. The ^{1}H-NMR spectrum shown in Figure 4.13 (a.) indicates the presence of two isomeric forms (epimers) of the thebaine-D^{+} complex ion in an approximate ratio of 55:45. These ratios were determined from the integration of signals obtained from the spectra. The spectrum in Figure 4.13 (c.), in which the solvent is predominantly deuterated acetonitrile, is less complicated showing either the presence of one form of the thebaine-D^{+} complex ion or the presence of the two forms exchanging so quickly that the ^{1}H-NMR cannot differentiate between the two and so and averaged spectrum is recorded. The spectrum shown in (b.) of Figure 4.13 shows a spectrum with broad signals obtained from a 50:50 water:acetonitrile solvent indicative of a slow exchange between the two forms. When the acetic acid content was increased from 1% to 2%, no difference was noted between the spectra therefore concluding that the exchange was based on the solvent composition, rather than pH, which is consistent with the findings of Caldwell *et al*.[252]

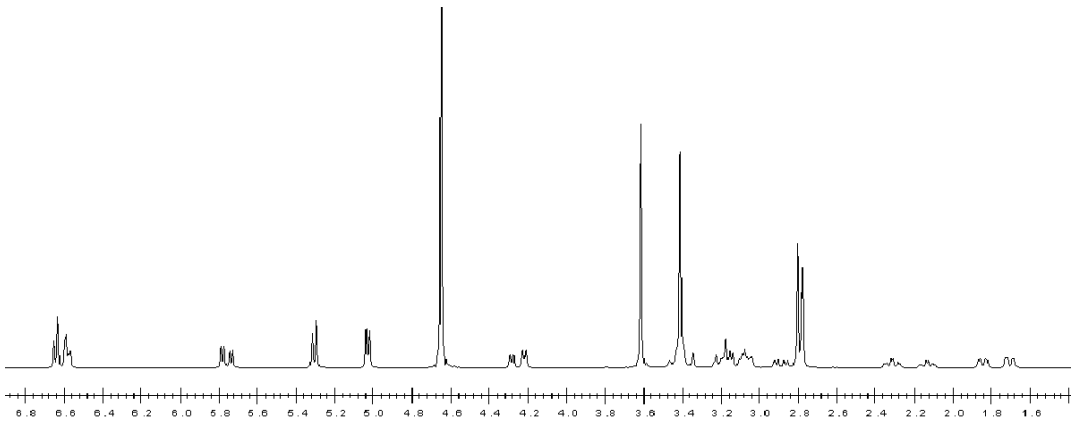


Figure 4.14 ^{1}H-NMR spectrum of thebaine in 100% D_2O (+ 1% CD_3COOD)

For comparison, a spectrum was obtained for thebaine in deuterated water with 1% deutero-acetic acid (Figure 4.14): again two epimers are formed in the same ratio as found above. It is suggested that thebaine can exist in two epimeric forms shown in Figure 4.15 (epimer A and B): in the presence of the acetic acid, it is thought that the epimers are protonated (HA^{+} and HB^{+}). The presence of the lone pair on the nitrogen atom of the unionised form enables nitrogen inversion. Nitrogen inversion is the process by which the lone pair of electrons moves from one side of the molecule to the other via quantum mechanical tunnelling.[253] In the process of inversion, the nitrogen starts off in the sp^3 hybridised form (tetrahedral); as the lone pair moves through the nucleus the nitrogen

becomes sp^2 hybridised (trigonal planar) and then finally returns to the sp^3 hybridised form when the lone pair makes it to the other side of the nitrogen to where it started.

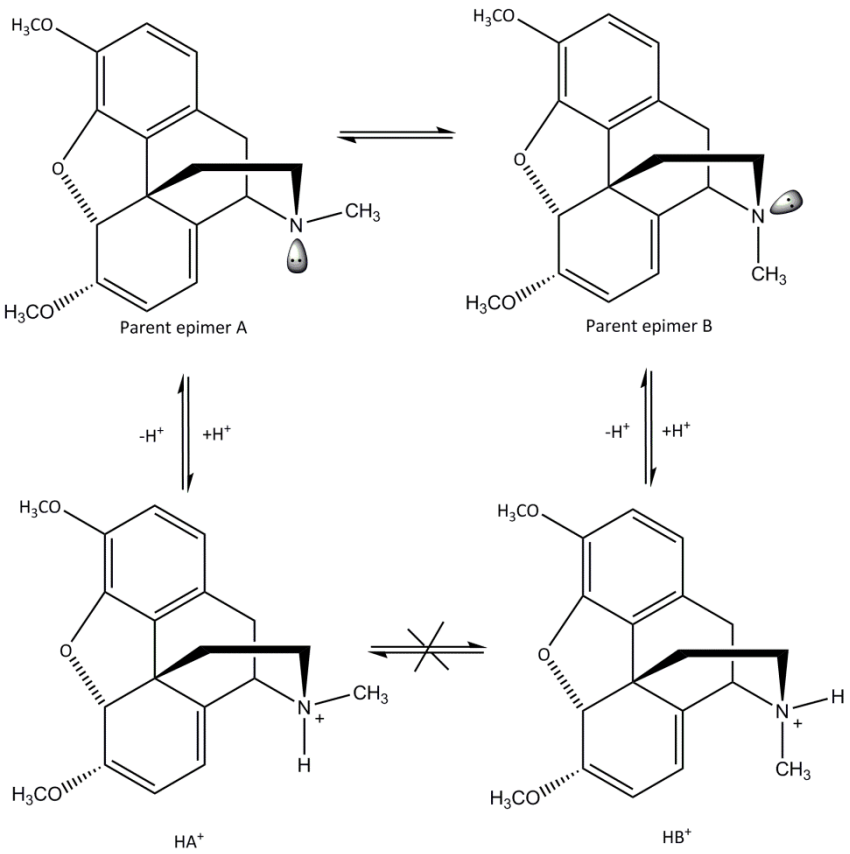


Figure 4.15 Formation of thebaine-D^+ complex ions, HA^+ and HB^+ from epimers A and B[254]

In order to establish if the conclusion drawn from this NMR study was replicated if the same sample conditions were analysed by the LC-MS, standards of thebaine of concentration 1000 ng mL^{-1} in acetonitrile with percent of acetic acid of 1, 2, 4, 6 and 8%. These standards were then analysed by the LC-MS method outlined in the experimental section. It was noted that the optimum solution for preparation of thebaine standards was acetonitrile with 1% acetic acid. All other acetonitrile/acetic acid combinations produced poor peak shape for both thebaine peaks. It was also noted that it was not possible to eliminate the second peak for thebaine from the chromatogram. It was possible to produce a stable form of the thebaine to construct a calibration graph in the concentration range of 0-1000 ng mL^{-1} (5 points and a blank) with an R^2 value of 0.9987. It was however decided

this was not acceptable and that a definitive identification and quantitation of one form of thebaine would be achieved by the elimination of the second peak.

4.4. **Further method development**

An instrument failure occurred once this suite of experiments was complete, leading to a pause in experimentation. On resumption, a related but slightly different method using deuterated morphine (morphine-d3) as internal standard was investigated.

As previously carried out and explained in Section 4.2, to establish the optimum mass spectrometer parameters for each of the compounds of interest, a solution of each alkaloid and the deuterated morphine were prepared in methanol at a concentration of 100 ng mL^{-1}; the solutions were then introduced to the mass spectrometer directly with the aid of an external syringe driver. The parameters were optimised automatically by the mass spectrometer for each of the compound masses and the settings were stored; these parameters are shown in Table 4.4.

Table 4.4 Analyte specific parameters for LCQ Advantage mass spectrometer from HPLC (* - ion used for quantitation)

Compound	*m/z*	Monitored transition mass (*m/z*)	Collision Energy (eV)
Morphine	286	286 → 201*, 229	33
Codeine	300	300 → 215*, 243, 282	32
Thebaine	312	312 → 183, 249, 281*	28
Papaverine	340	340 → 202* 202 → 171	36 32
Noscapine	414	414→ 220*, 353	29
Morphine-d_3	289	289 → 201*, 229	30

With the findings from the NMR study (reported in section 4.3) showing that the solvent choice for the sample preparation has a significant impact on the form that the thebaine

was found in, it was decided to include the use of a buffered mobile phase. Based on the experience of the author, and in discussion with colleagues from the department of toxicology of the French Institut de Recherche Criminelle de la Gendarmerie Nationale (IRCGN), Olivier Roussel[255] and Sophie Salle[256], a mobile phase composition of A: water + 2 mM ammonium formate + 0.2% formic acid and B: acetonitrile + 2 mM ammonium formate + 0.2 % formic acid was used with the details of the HPLC method shown in Table 4.5. The auto-sampler tray was held at 8 ^{o}C to reduce any effects that temperature may have on the thebaine epimer formation and the final standard opiate mix solutions (containing morphine, codeine, thebaine, papaverine, noscapine and morphine-d3) were prepared in methanol:water (50:50, v/v), as had been used previously. When the method was run on the C18 column, single peaks were observed for all of the alkaloid compounds, including thebaine however the peak shape was poor, with tailing and fronting observed. An alternative to the C18 column is the Allure™ pentafluorophenyl phase with a propyl spacer (PFPP) column 5 µm, 50 x 2.1 mm fitted with an Allure™ PFPP 10.0 x 2.1 mm guard column (Restek, Buckinghamshire, UK). This column had a proven successful separation of morphine and codeine in the toxicology laboratory of the IRCGN, and had provided good peak shape.[255,256] The HPLC gradient method is shown in Table 4.5 and the column was thermostated to 40 ^{o}C. The same mass spectrometer settings were used as shown in Table 4.4.

Table 4.5 Mobile phase composition and gradient program for the final LC-ESI-MS method on the PFPP column

Mobile phase composition **Solvent A:** Water + 2 mM ammonium formate + 0.2% formic acid, pH 2.4 **Solvent B:** Acetonitrile + 2 mM ammonium formate + 0.2 % formic acid, pH 4.8			
Time (minutes)	**%A**	**%B**	**Flow rate (µL/min)**
0.00	90	10	350
2.00	90	10	350
10.00	10	90	350
11.00	10	90	350
12.00	90	10	350
14.00	90	10	350

Using this method and the new column, it was again found that single peaks were observed for all of the alkaloid compounds, including thebaine and that the quality of the peaks obtained was far better and that it was possible to achieve different retentions times for morphine and codeine, i.e. no co-elution as was found using the Gemini column. (Figure 4.16).

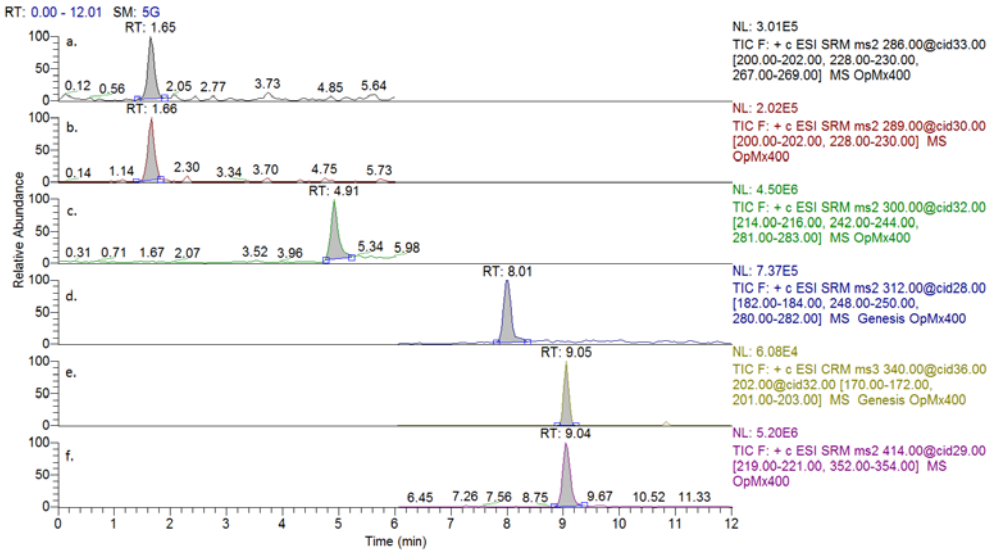


Figure 4.16 Extracted chromatograms from a mixed injection of (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine, (e) papaverine and (f) noscapine

4.5. Validation of the final LC-ESI-MS method

4.5.1. Selectivity

As discussed in section 4.2.1, selectivity was established by running blanks at the beginning and end of the sequence and in between each set of standards in the sequence. Since no peaks were found at the same retention time as any of the alkaloid compounds or the internal standard in any of the blank solutions, selectivity of the method had been established.

4.5.2. Linearity

Linearity of the method was established by preparing mixed calibration standards of morphine, codeine, thebaine, papaverine, noscapine and morphine-d3 at 10 points across

the concentration range 10 – 500 ng mL^{-1}. Calibration standards were prepared in triplicate and each calibration point was injected in triplicate resulting in three separate data sets.

Using the ions highlighted in Table 4.4 for quantitation, average peak area ratios (drug peak area/internal standard peak area) for each of the alkaloids were used to construct calibration graphs; the linear equations, R^2 values and concentration range are provided in Table 4.6. The calibrations graphs were constructed with a minimum of 6 data points plus a blank.

Table 4.6 Linear equations and associated R^2 values for morphine, codeine, thebaine, papaverine and noscapine

Compound	Linear equation	R^2	Concentration range (ng mL^{-1})
Morphine	y = 0.0047x – 0.0100	0.9957	0 – 200
Codeine	y = 0.014x – 0.0675	0.9976	0 – 200
Thebaine	y = 0.0214x – 0.2152	0.9915	0 – 200
Papaverine	y = 0.019x – 0.1795	0.9952	0 – 400
Noscapine	y = 0.0735x – 0.6081	0.9985	0 – 500

The coefficient of determination (R^2) value for all of the alkaloid compounds is greater than 0.99, as described in section 4.2.2, therefore this method can be described as linear for morphine, codeine, thebaine, papaverine and noscapine.

4.5.3. Precision

Precision was established as described in Section 4.2.3, by carrying out repeat injections at a number of points along the concentration range: from these repeat 9 injections, the percent coefficient of variation (%CV) was determined and the final results are shown in Table 4.7.

Table 4.7 %CV for morphine, codeine, thebaine, papaverine and noscapine with varying concentration

Concentration (ng mL^{-1})	Coefficient of variation (%)				
	Morphine	Codeine	Thebaine	Papaverine	Noscapine
0	0	0	0	0	0
10	86	106	56	72	48
15	84	69	45	117	37
20	73	40	42	49	46
30	47	31	44	46	33
40	39	43	23	46	34
50	32	47	37	36	29
100	58	29	37	67	23
200	30	52	52	64	122
300	24	20	27	35	31
400	15	47	64	57	24
500	56	54	72	84	51

From this data, it can be seen that the values are very high and in some instances, the coefficient of variation exceeds 100%. In validation of an analytical method, the acceptable coefficient of variation is 15% with 20% accepted at the lowest concentration values. In this case, all values, at all concentration points exceed the recommended limits.

4.5.4. Solvent effects

Due to the challenge of obtaining reproducibility of the method, a series of experiments were designed to establish if the solvent used for preparation of the calibration standards had any subsequent effect on the reproducibility of the method. Since it had already been found that solvent choice had significant effects on resulting chromatography in the NMR study (section 4.3), it was decided to vary the composition of the solvent and monitor the subsequent output from the instrument. Since it had already been established that the HPLC method produced five individual peaks for the alkaloids plus the morphine-d3 and that the response from a series of calibration standards was linear, it was the hypothesis that that the problem of variation was as a result of an effect of the mass spectrometer. In

order to test this hypothesis, the HPLC was excluded from this study and instead direct infusion to the mass spectrometer was employed.

Mixed opiate solutions were prepared in acetonitrile, methanol: water (25:75, 50:50 and 75:25, v/v) at concentrations of 50, 300 and 1000 ng mL^{-1}. Each solution was introduced by direct infusion to the mass spectrometer and the output monitored for 2.00 minutes: each compound was monitored 20 times over the 2.00 minute time frame. The data for each compound was then used to calculate the mean ion intensity, standard deviation and the % coefficient of variation. Each solvent is discussed individually in the following sections.

4.5.4.1. Acetonitrile

Acetonitrile was used as the solvent and each %CV values for each of the individual alkaloids at 50, 300 and 1000 ng mL^{-1} are provided in Table 4.8, 4.9 and 4.10, respectively.

Table 4.8 Effect on reproducibility of individual alkaloid compounds in acetonitrile (ACN) at 50 ng mL^{-1}.

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	67	-	-	-	-	-
Codeine (C)	-	16	-	-	-	-
Thebaine (T)	-	-	22	-	-	-
Papaverine (P)	-	-	-	12	-	-
Noscapine (N)	-	-	-	-	12	-
M + C	99	117	-	-	-	-
M + d3	47	39	-	-	-	7
M + T	36	-	33	-	-	-
M + N	66	-	-	-	15	-
M + P	80	-	-	10	-	-
M + C + N	88	62	-	-	14	-
M + C + P	59	29	-	16	-	-
M + C + T	72	30	19	-	-	-
M + T + N	60	-	20	-	19	-
M + T + P	69	-	32	18	-	-
M + C + T + d3	42	22	42	-	-	26

Table 4.9 Effect on reproducibility of individual alkaloid compounds in ACN at 300 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	45	-	-	-	-	-
Codeine (C)	-	16	-	-	-	-
Thebaine (T)	-	-	16	-	-	-
Papaverine (P)	-	-	-	10	-	-
Noscapine (N)	-	-	-	-	15	-
M + C	36	23	-	-	-	-
M + d3	30	-	-	-	-	16
M + T	50	-	13	-	-	-
M + N	53	-	-	-	11	-
M + P	67	-	-	12	-	-
M + C + N	42	18	-	-	16	-
M + C + P	21	22	-	15	-	-
M + C + T	23	15	16	-	-	-
M + T + N	14	-	19	-	13	-
M + T + P	52	-	29	10	-	-
M + C + T + d3	29	23	42	-	-	7

Table 4.10 Effect on reproducibility of individual alkaloid compounds in ACN at 1000 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	42	-	-	-	-	-
Codeine (C)	-	27	-	-	-	-
Thebaine (T)	-	-	15	-	-	-
Papaverine (P)	-	-	-	15	-	-
Noscapine (N)	-	-	-	-	19	-
M + C	135	34	-	-	-	-
M + d3	21	-	-	-	-	12
M + T	24	-	14	-	-	-
M + N	86	-	-	-	16	-
M + P	68	-	-	79	-	-
M + C + N	29	18	-	-	20	-
M + C + P	54	26	-	26	-	-
M + C + T	58	11	30	-	-	-
M + T + N	44	-	17	-	13	-
M + T + P	46	-	46	18	-	-
M + C + T + d3	23	17	39	-	-	16

In acetonitrile, it appears from the data obtained that there is no correlation between the concentration of alkaloid and the variation observed.

4.5.4.2. Methanol:water (25:75, v/v)

Methanol:water (25:75, v/v) was used as the solvent and each %CV values for each of the individual alkaloids at 50, 300 and 1000 ng mL^{-1} are provided in Table 4.11, 4.12 and 4.13, respectively.

Table 4.11 Methanol:water (25:75, v/v) at 50 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	24	-	-	-	-	-
Codeine (C)	-	18	-	-	-	-
Thebaine (T)	-	-	19	-	-	-
Papaverine (P)	-	-	-	17	-	-
Noscapine (N)	-	-	-	-	21	-
M + C	26	25	-	-	-	-
M + d3	23	-	-	-	-	20
M + T	21	-	40	-	-	-
M + N	26	-	-	-	29	-
M + P	21	-	-	18	-	-
M + C + N	16	18	-	-	18	-
M + C + P	16	26	-	20	-	-
M + C + T	26	31	39	-	-	-
M + T + N	17	-	40	-	44	-
M + T + P	26	-	49	19	-	-
M + C + T + d3	28	-	39	-	-	18

Table 4.12 Methanol:water (25:75, v/v) at 300 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	11	-	-	-	-	-
Codeine (C)	-	16	-	-	-	-
Thebaine (T)	-	-	19	-	-	-
Papaverine (P)	-	-	-	22	-	-
Noscapine (N)	-	-	-	-	22	-
M + C	15	14	-	-	-	-
M + d3	18	-	-	-	-	14
M + T	17	-	23	-	-	-
M + N	25	-	-	-	13	-
M + P	21	-	-	13	-	-
M + C + N	17	19	-	-	14	-
M + C + P	20	21	-	14	-	-
M + C + T	22	28	32	-	-	-
M + T + N	24	-	25	-	11	-
M + T + P	21	-	36	11	-	-
M + C + T + d3	24	26	41	-	-	22

Table 4.13 Methanol:water (25:75, v/v) at 1000 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	15	-	-	-	-	-
Codeine (C)	-	18	-	-	-	-
Thebaine (T)	-	-	21	-	-	-
Papaverine (P)	-	-	-	11	-	-
Noscapine (N)	-	-	-	-	18	-
M + C	20	24	-	-	-	-
M + d3	19	-	-	-	-	16
M + T	19	-	29	-	-	-
M + N	16	-	-	-	18	-
M + P	28	-	-	22	-	-
M + C + N	26	26	-	-	24	-
M + C + P	20	20	-	16	-	-
M + C + T	22	29	47	-	-	-
M + T + N	21	-	21	-	14	-
M + T + P	15	-	25	17	-	-
M + C + T + d3	24	22	32	-	-	24

When methanol:water (75:25, v/v) was used, it appears that the reproducibility of this solvent is better when compared to the values obtained when acetonitrile was used as the solvent. At the lowest concentration of 50 ng mL^{-1}, the %CV values are generally higher for individual alkaloids and combinations of alkaloids than were found at the highest concentration of 100 ng mL^{-1}. It should be noted that the data shows that when individual alkaloids are introduced in this solvent, the %CV values are lower than when the compound is injected in a mixture with other alkaloids.

4.5.4.3. Methanol:water (50:50, v/v)

Methanol:water (50:50, v/v) was used as the solvent and each %CV values for each of the individual alkaloids at 50, 300 and 1000 ng mL^{-1} are provided in Table 4.14, 4.15 and 4.16, respectively.

Table 4.14 Methanol:water (50:50, v/v) at 50 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	7	-	-	-	-	-
Codeine (C)	-	6	-	-	-	-
Thebaine (T)	-	-	11	-	-	-
Papaverine (P)	-	-	-	3	-	-
Noscapine (N)	-	-	-	-	4	-
M + C	16	13	-	-	-	-
M + d3	12	-	-	-	-	7
M + T	14	-	30	-	-	-
M + N	12	-	-	-	15	-
M + P	19	-	-	9	-	-
M + C + N	10	9	-	-	13	-
M + C + P	14	8	-	9	-	-
M + C + T	14	13	21	-	-	-
M + T + N	10	-	16	-	8	-
M + T + P	15	-	23	9	-	-
M + C + T + d3	17	19	11	-	-	14

Table 4.15 Methanol:water (50:50, v/v) at 300 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	7	-	-	-	-	-
Codeine (C)	-	3	-	-	-	-
Thebaine (T)	-	-	5	-	-	-
Papaverine (P)	-	-	-	3	-	-
Noscapine (N)	-	-	-	-	4	-
M + C	18	23	-	-	-	6
M + d3	6	-	-	-	-	-
M + T	7	-	13	-	-	-
M + N	8	-	-	-	5	-
M + P	7	-	-	7	-	-
M + C + N	14	9	-	-	8	-
M + C + P	8	5	-	5	-	-
M + C + T	8	9	10	-	-	-
M + T + N	4	-	5	-	4	-
M + T + P	10	-	12	9	-	-
M + C + T + d3	7	6	6	-	-	5

Table 4.16 Methanol:water (50:50, v/v) at 1000 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	4	-	-	-	-	-
Codeine (C)	-	2	-	-	-	-
Thebaine (T)	-	-	4	-	-	-
Papaverine (P)	-	-	-	2	-	-
Noscapine (N)	-	-	-	-	3	-
M + C	2	6	-	-	-	-
M + d3	3	-	-	-	-	4
M + T	4	-	5	-	-	-
M + N	4	-	-	-	4	-
M + P	4	-	-	3	-	-
M + C + N	5	6	-	-	4	-
M + C + P	4	5	-	4	-	-
M + C + T	4	4	6	-	-	-
M + T + N	5	-	6	-	4	-
M + T + P	5	-	7	2	-	-
M + C + T + d3	4	3	6	-	-	3

From the data obtained for methanol:water (50:50, v/v) as the solvent, this is the best solvent to use as in comparison to the other two solvents discussed, this solvent has provided the lowest variation values at all concentrations. The %CV values found for individual alkaloids and for all combinations of alkaloids at 1000 ng mL^{-1} is very good (Table 4.16). These results show that, in comparison to the %CV values obtained for other solvents, the solvent composition can have a significant effect on the reproducibility of the final method employed in the mass spectrometer.

4.5.4.4. Methanol:water (75:25, v/v)

The final solvent used in this study was methanol:water (25:75, v/v) which was used in the same way as the previous solvents were. Each %CV values for each of the individual alkaloids at 50, 300 and 1000 ng mL^{-1} are provided in Table 4.17, 4.18 and 4.19, respectively.

Table 4.17 Methanol:water (75:25, v/v) at 50 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	48	-	-	-	-	-
Codeine (C)	-	29	-	-	-	-
Thebaine (T)	-	-	48	-	-	-
Papaverine (P)	-	-	-	16	-	-
Noscapine (N)	-	-	-	-	30	-
M + C	24	29	-	-	-	-
M + d3	27	-	-	-	-	27
M + T	29	-	40	-	-	-
M + N	29	-	-	-	22	-
M + P	34	-	-	22	-	-
M + C + N	44	24	-	-	24	-
M + C + P	30	45	-	21	-	-
M + C + T	41	43	52	-	-	-
M + T + N	37	-	38	-	18	-
M + T + P	25	-	54	18	-	-
M + C + T + d3	36	40	51	-	-	25

Table 4.18 Methanol:water (75:25, v/v) at 300 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	31	-	-	-	-	-
Codeine (C)	-	26	-	-	-	-
Thebaine (T)	-	-	38	-	-	-
Papaverine (P)	-	-	-	16	-	-
Noscapine (N)	-	-	-	-	20	-
M + C	23	24	-	-	-	-
M + d3	39	-	-	-	-	23
M + T	22	-	33	-	-	-
M + N	24	-	-	-	27	-
M + P	27	-	-	14	-	-
M + C + N	42	26	-	-	27	-
M + C + P	30	24	-	17	-	-
M + C + T	17	33	36	-	-	-
M + T + N	32	-	26	-	15	-
M + T + P	21	-	43	13	-	-
M + C + T + d3	57	23	36	-	-	193

Table 4.19 Methanol:water (75:25, v/v) at 1000 ng mL^{-1}

	Coefficient of variation (%)					
Compound(s)	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**	**Morphine-d3**
Morphine (M)	-	-	-	-	-	-
Codeine (C)	-	14	-	-	-	-
Thebaine (T)	-	-	42	-	-	-
Papaverine (P)	-	-	-	13	-	-
Noscapine (N)	-	-	-	-	21	-
M + C	37	33	-	-	-	-
M + d3	26	-	-	-	-	27
M + T	31	-	30	-	-	-
M + N	27	-	-	-	11	-
M + P	27	-	-	16	-	-
M + C + N	44	20	-	-	21	-
M + C + P	25	19	-	14	-	-
M + C + T	24	27	42	-	-	-
M + T + N	30	-	24	-	18	-
M + T + P	25	-	34	16	-	-
M + C + T + d3	27	16	32	-	-	20

When the proportion of methanol was increased to 75% (25% water), the variation also increases: even at the highest concentration of 1000 ng mL^{-1}, high %CV values were obtained for individual alkaloids. Again, as is seen with acetonitrile, there appears to be no correlation between the concentration and the combination of alkaloids in solution.

When considering all of the data for all of the solvents in this study, the variation of %CV values when other alkaloids are present may be due to a phenomenon known as matrix effects or competitive ionisation, which has been reported to occur in the ionisation step LC-MS methods.[238,257-259] Matrix effects can be defined as the variation of ionisation efficiency due to the presence of co-eluting or other interfering substances from the matrix.[257] In 2004, Tang *et al*.[260] reported that it was possible to obtain linearity with a mixture of compounds (reserpine, caffeine and MRFA) even though competitive ionisation using ESI-MS was found. It was found in this study that it was possible to prove that the ion

signals for caffeine and MRFA were suppressed in the presence of caffeine in a solvent system of methanol:water (50:50, v/v) + 1% acetic acid: the authors suggested that reducing the flow rate of mobile phase greatly increases the sensitivity of the ESI-MS system. The suggested flow rates were found to be in the nano range (10 – 100 nL min^{-1}). In the same article, it was stated that the competition between the compounds will be affected by the number of ions in solution. At this current time, the exact mechanism of action of competition between ions remains unknown.[257]

It was not possible to adapt the LC-MS method being employed in this work to nano range flow rates due to the limitations of the instrument. However, in 2002 it was reported by Matz and Hill[261] that the solvent can significantly affect the ionisation in ESI-MS systems. In an experiment where methanol:water (50:50, v/v) was used in the analysis of a mixture of amphetamine compounds, it was found that at high concentration, competitive ionisation occurred in the presence of six amphetamine compounds in a mixture. It was found that the solvent decreased the competitive ionisation but also reduced the sensitivity of the method. However, by adding either 5% acetic acid or 1% formic acid, it was also shown that the competition between ions was greater. Since the established LC-MS method in this work required the formic acid in order to maintain the thebaine (and the other alkaloids) in an appropriate form to obtain one peak and a linear relationship between concentration and peak area ratio, it was concluded that the removal of the modifier (formic acid) was not appropriate.

From this point on, even though it had not been included in the solvent study, the mobile phase A (water + 2 mM ammonium formate + 0.2% formic acid) was used to prepare the standards and any samples/quality control (QC) solutions. It is recommended that samples/standards are prepared in the mobile phase used at the beginning of the HPLC method in order to avoid potential problems with mixing of different solvents which ultimately have an impact on ionisation.[262]

4.6. **Comparison with LC-ESI-MS (triple quadrupole)**

In order to determine if the results obtained from the method used on the LC-ESI-MS method were due to limitations of the particular instrument or were in fact the true results,

the method was transferred to a LC-ESI-MS triple quadrupole instrument in the toxicology department of the Institut de Recherche Criminelle de la Gendarmerie Nationale (IRCGN).

Instrument specific information is provided in section 4.1.2.2. In order to establish the method using the triple quadrupole instrument, it was necessary to prepare stock solutions of each of the five opium alkaloids in methanol at a concentration of 100 ng mL^{-1}. Each individual standard was directly infused to the mass spectrometer, including the internal standard (morphine-d3) and the instrument set to optimise the system for each compound as had been carried out in the development of the LC-ESI-MS method recently discussed. The Q1 and Q3 masses obtained for each of the five alkaloids are shown in Table 4.20. Each of the Q3 ions were extracted, the signal and peak shapes produced were examined and a decision made on which ions were best to use for identification and subsequent quantitation. These decisions were based on the quality of the peak produced and the strength of the signal for each ion.

Table 4.20 Analyte specific parameters for 4500 QTRAP tandem mass spectrometer. DP- declustering potential; EP – entrance potential; CXP – collision cell exit potential. (* - ion used for quantitation)

Compound	Q1 mass	Q3 Mass transitions (*m/z*)	Collision Energy (eV)	DP (V)	EP (V)	CXP (V)
Morphine	286	286 → 153	49	90	10	10
		286 →165*	47	90	10	6
Codeine	300	300 → 215*	33	91	10	6
		300 →165	43	91	10	6
Thebaine	312	312 → 266*	21	50	10	8
		312 →152	29	50	10	6
Papaverine	340	340 → 171*	45	81	10	14
		340 →324	39	81	10	10
Noscapine	414	414→ 353*	29	90	10	10
		414→220	27	90	10	6
Morphine-d_3	289	289 →152	47	90	10	12
		289 →165*	47	90	10	4

As described in Section 3.1.2.1, the method of ionisation can vary in mass spectrometry depending upon the type of instrument used. In this work, both instruments employed electrospray ionisation (ESI) but the energy used to cause the ionisation varies. The energy required to cause fragmentation is specific to the instrument and to the analytes. In the case of the LC-ESI-MS instrument, the specific energies required to cause fragmentation are shown in Table 4.4 and the energies employed with the LC-ESI-tandem mass spectrometer are provided in Table 4.20. Depending upon the energy used, different product ions (fragment ions) can be formed: the suggested fragment ions formed in both instruments are provided for morphine (Figure 4.17), codeine (Figure 4.18), thebaine (Figure 4.19), papaverine (Figure 4.20) and noscapine (Figure 4.21).

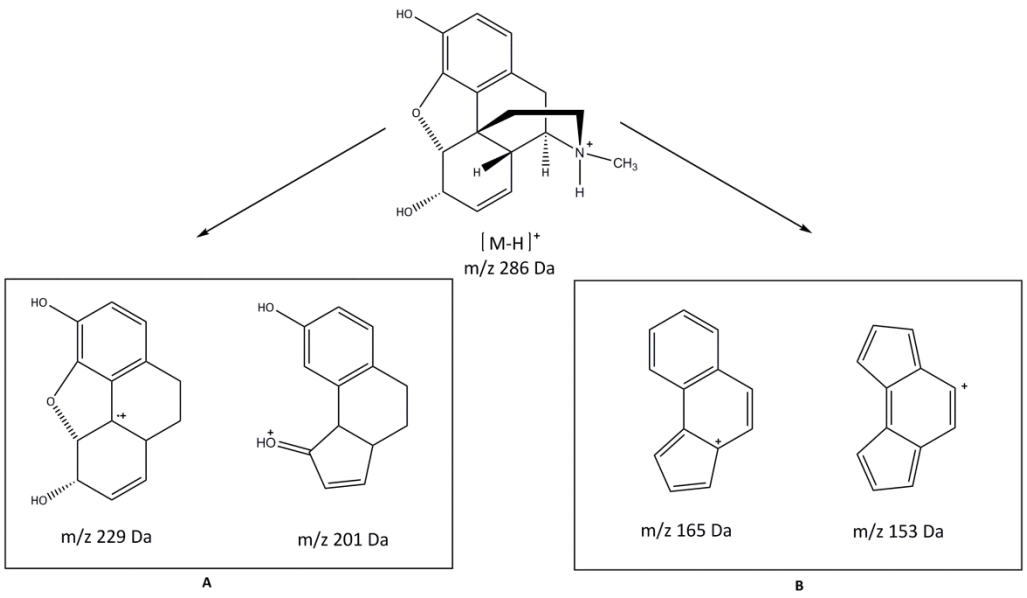


Figure 4.17 Proposed fragments formed from morphine during ionisation in (A) LC-ESI-MS and (B) LC-MS^n instruments[263-265]

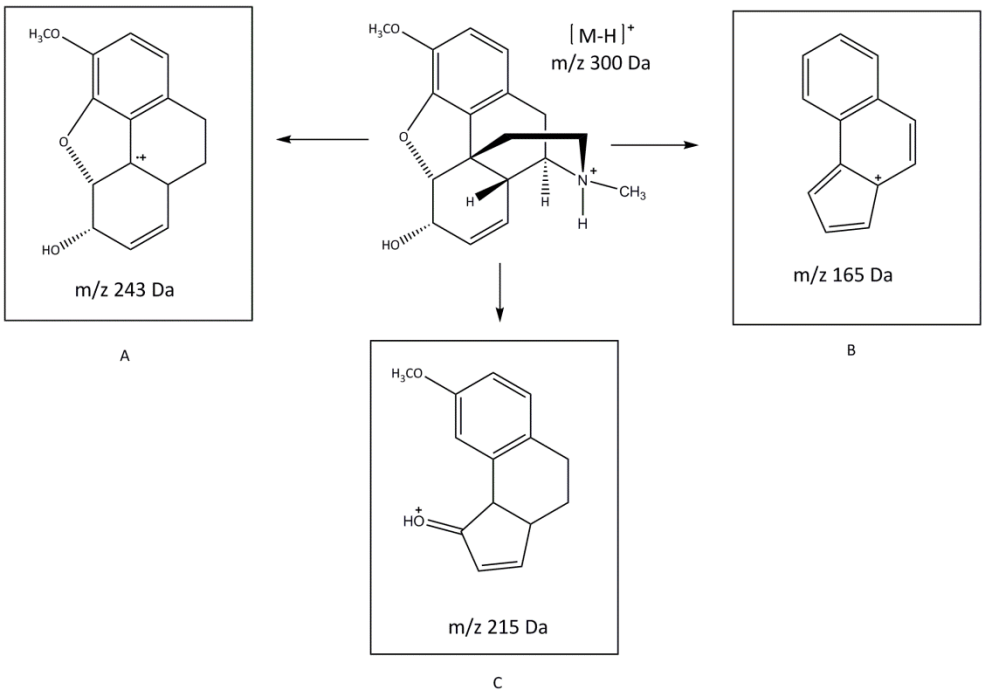


Figure 4.18 Proposed fragments formed from codeine during ionisation in (A) LC-ESI-MS, (B) LC-MS^n and (C) both instruments[263-265]

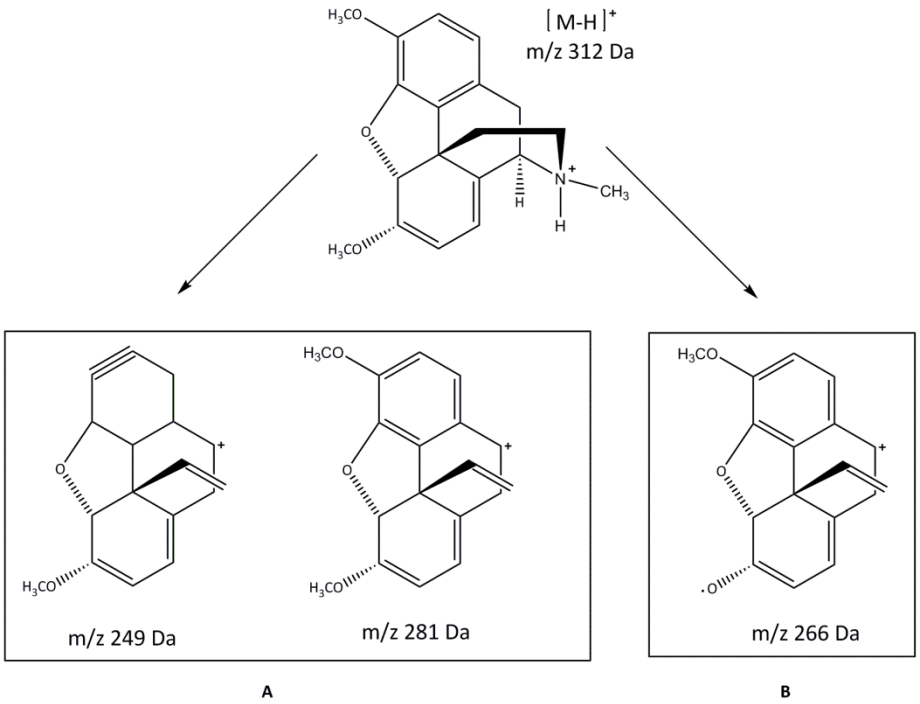


Figure 4.19 Proposed fragments formed from thebaine during ionisation in (A) LC-ESI-MS, (B) LC-MS^n instruments[232]

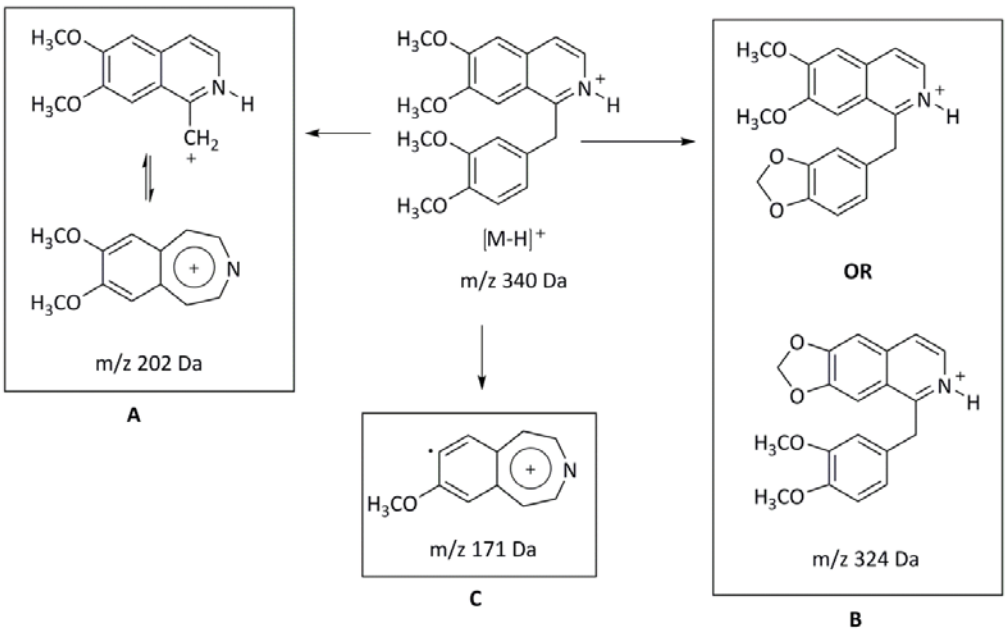


Figure 4.20 Proposed fragments formed from papaverine during ionisation in (A) LC-ESI-MS, (B) LC-MS^n and (C) both instruments[266]

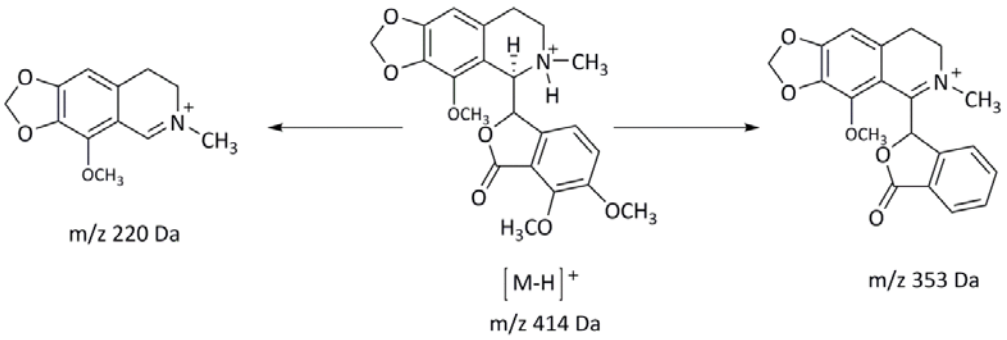


Figure 4.21 Proposed fragments formed from noscapine during ionisation in both LC-ESI and LC-MS^n instruments[266]

Once the optimum settings for the mass spectrometer were established the instrument was set up with the HPLC mobile phase gradient method, and the Allure™ pentafluorophenyl phase with a propyl spacer (PFPP) column as used previously in this work. An initial injection of a mixture of morphine, codeine, thebaine, papaverine, noscapine and morphine-d3 was introduced in order to establish if the method still provided detection of all alkaloids and the internal standard. Resulting chromatograms for all compounds are shown in Figure 4.22 proving that the method successfully detected the alkaloids of interest.

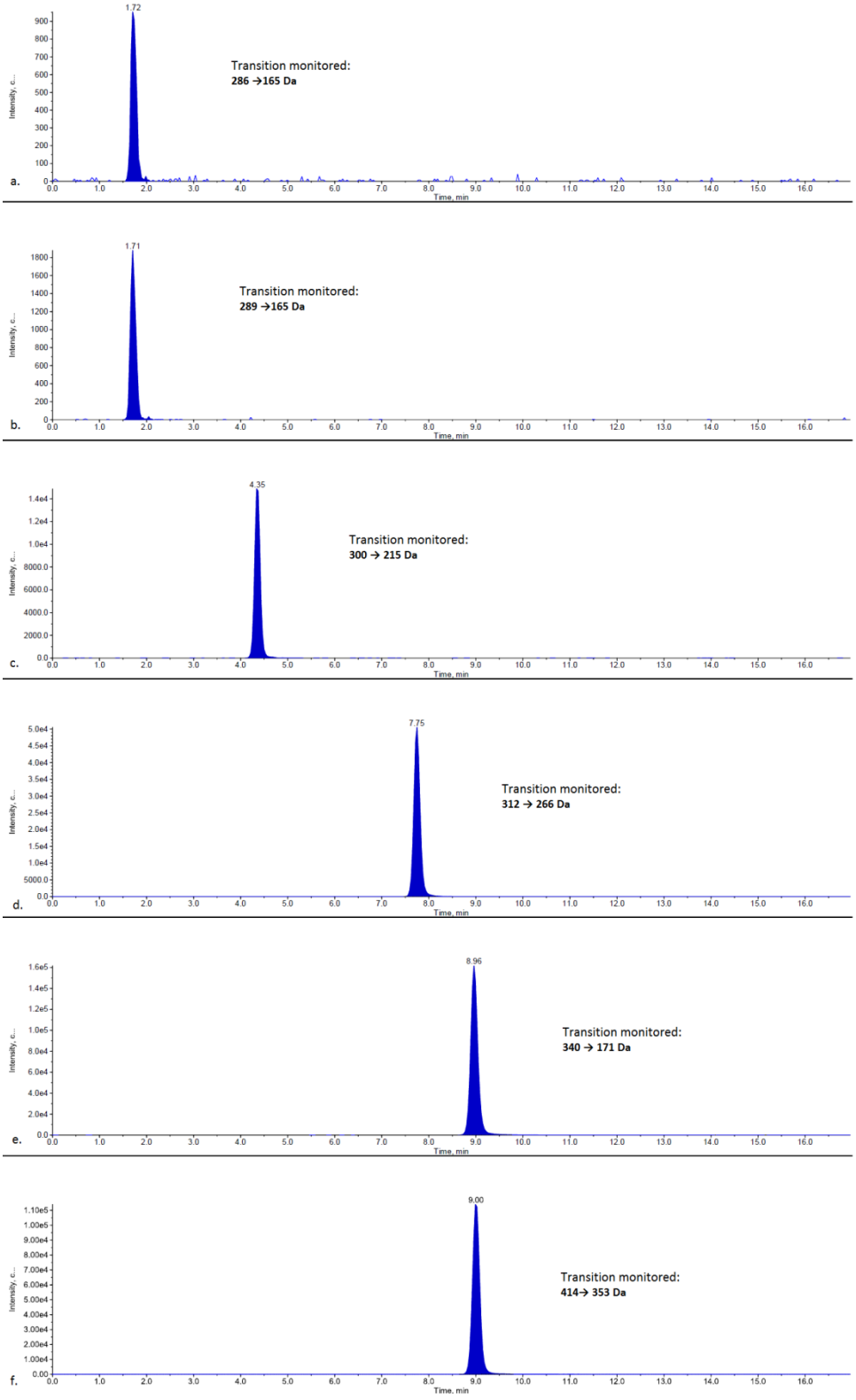


Figure 4.22 Chromatogram showing all five alkaloids separated using PFPP column and LC-MS^n tandem mass spectrometer. The quantitation ion highlighted in Table 4.20 was used (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine), (e) papaverine, (f) noscapine

To establish if linearity was achieved using this method on the LC-MS^n instrument, three mixed calibration solutions containing morphine, codeine, thebaine, papaverine and noscapine were prepared in the aqueous mobile phase at 20 ng mL^{-1} and at 200 ng mL^{-1} and each solution was analysed in triplicate. The resulting chromatograms are shown in Figure 4.23. Blanks of aqueous mobile phase were run at the beginning and end of the sequence and in between solution run in triplicate. To the mixed solutions, deuterated morphine (morphine-d3) as an internal standard was added at a concentration of 100 ng mL^{-1}; these are the same conditions as had been used in the LC-ESI-MS (ion trap) method. Only the lowest concentration and highest concentration values were used to verify that linearity could be established and to determine the relative standard deviation for comparison with the current data set. This limited data acquisition was due to constraints of instrument time.

As can be seen in the chromatograms shown in Figure 4.23, the repeat injections for morphine, codeine, thebaine, papaverine, noscapine and morphine-d3 all show good peak shape and reproducible retention times using this instrument. Even though the peaks for papaverine and noscapine have similar retention times (8.99 and 9.01 minutes, respectively) because different ion transitions are being monitored it is possible to be certain that each peak relates to a separate compound.

In order to assess precision of the method and thus compare the findings to the original LC-ESI-MS method, a 20 ng mL^{-1} and a 200 ng mL^{-1} mixed solution was prepared three times and each was injected in triplicate. Blank aqueous mobile phase was injected at the beginning and end of the sequence, as well as between each data set. The resulting peak area for each of the alkaloids and peak area for morphine-d3 were used to obtain peak area ratios with the final data provided in Table 4.21.

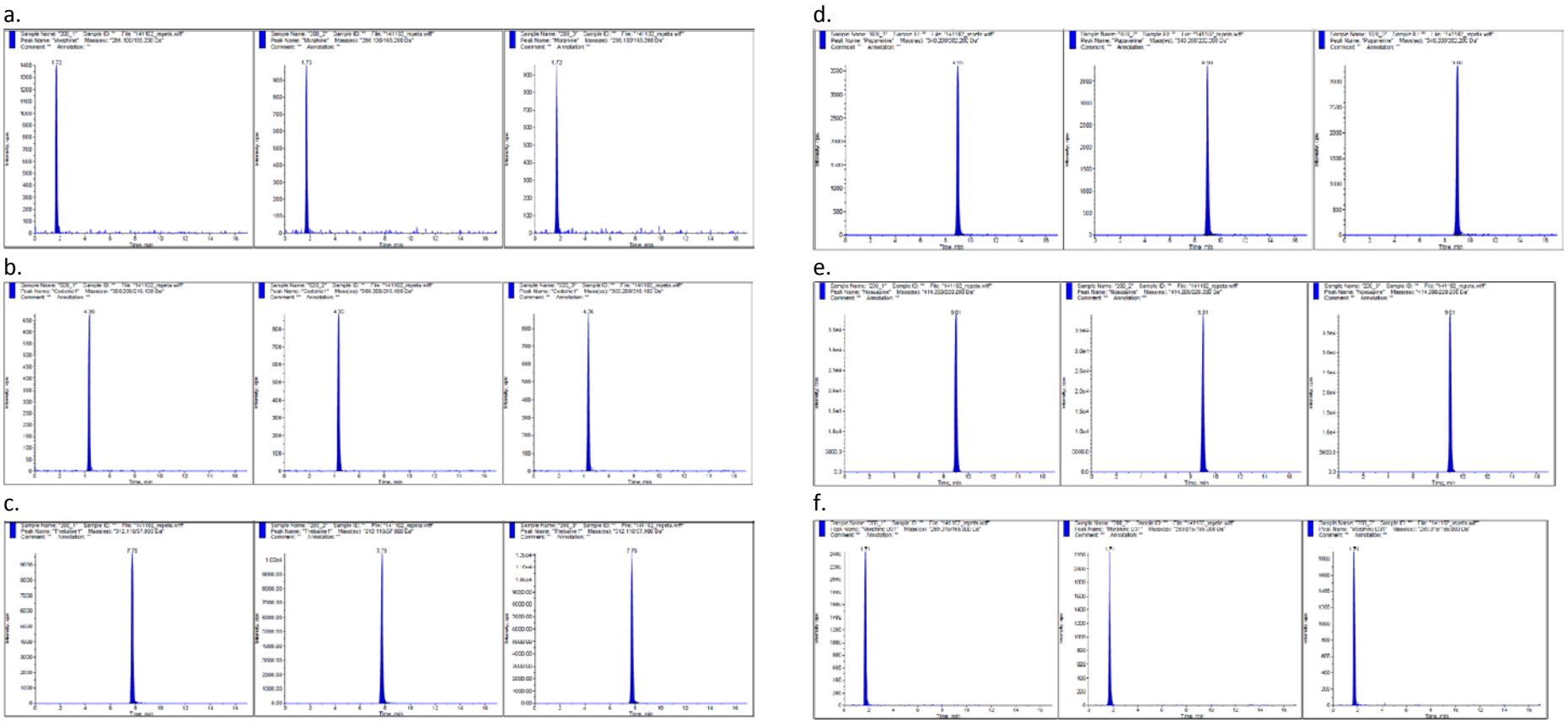


Figure 4.23 Chromatograms for (a) morphine, (b) codeine, (c) thebaine, (d) papaverine, (e) noscapine, (f) morphine-d3 in triplicate

Table 4.21 Peak area ratios for morphine, codeine, thebaine, papaverine and noscapine

	Coefficient of variation (%)					
Data set	**Concentration (ng mL^{-1})**	**Morphine**	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**
1	20	22	18	71	97	105
	200	6	47	5	8	7
2	20	21	14	11	9	10
	200	25	27	12	11	13
3	20	46	16	8	10	8
	200	2	6	9	7	5

It can be noted that for data set 1 the coefficient of variation values for morphine, thebaine, papaverine and noscapine do not meet the ≤ 20% accepted response for the value close to the LOQ of 20 ng mL^{-1}. Only codeine has a value of 18% which is just under this 20% limit. In comparison, codeine is the only compound of the 5 alkaloids to be in excess of the 15% limit whereas the others fall considerably below, showing acceptable results for the solutions at a concentration of 200 ng mL^{-1}. For data set 2, morphine has a %CV value of 21%, just over the 20% limit but all other alkaloids are below 15% at 20 ng mL^{-1}; in comparison, at 200 ng mL^{-1} morphine and codeine are considerably higher (25% and 27%, respectively) than the 15% limit expected at this concentration. On the other hand, thebaine, papaverine and noscapine all have %CV values lower than 15%.

Finally, for data set 3, morphine shows a large %CV value of 46% at 20 ng mL^{-1} however codeine, thebaine, papaverine and noscapine all fall considerably below the acceptable limit of 20%. For the alkaloids at 200 ng mL^{-1}, all were found to be considerably lower than the 15% limit. It appears that there are limited data consistencies between the three data sets. For example, if all of the %CV values for all of the alkaloids at 20 ng mL^{-1} were consistently higher than 20% it might be considered that 20 ng mL^{-1} was below the acceptable lowest limit of quantitation however, this is not the case. It may also be expected that at a higher concentration of 200 ng mL^{-1} that there may be less variation due to the fact that peak area are larger however, again, this is not seen to be the case in this instance. A comparison of the combined data for all 20 ng mL^{-1} injections and all 200 ng mL^{-1} injections was carried out and the results provided in Table 4.22.

Table 4.22 Mean ($\bar{x}$), standard deviation (σ_x) and co-efficient of variation (%CV) for cumulative data for each of the five alkaloids at 20 ng mL^{-1} and 200 ng mL^{-1}

Compound		Morphine	Codeine	Thebaine	Papaverine	Noscapine
Concentration (ng mL^{-1})	Parameter					
20	$\bar{x}$	0.06	0.97	2.05	5.24	6.58
	σ_x	0.02	0.15	0.70	2.19	2.89
	%CV	**29**	**16**	**34**	**42**	**44**
200	$\bar{x}$	0.62	10.64	22.84	74.83	75.35
	σ_x	0.12	3.14	5.72	24.31	7.06
	%CV	**19**	**29**	**25**	**32**	**9**

Using this combined data, calibration graphs were constructed in order to establish if the data obtained using this instrument was linear. Although using a two-point calibration , including a blank is not best practice for method validation, it was employed only to determine if the relationship between concentration and peak area ratio and the values for coefficient of determination were acceptable; the linear equation and R^2 values for each of the alkaloids is provided in Table 4.23.

Table 4.23 Linear equations and associated R^2 values for the five major opium alkaloids

Compound	Linear equation	R^2
Morphine	y = 0.0031x + 0.0009	1
Codeine	y = 0.0534x – 0.0461	0.9999
Thebaine	y = 0.1147x – 0.1152	0.9999
Papaverine	y = 0.3791x – 1.106	0.9992
Noscapine	y = 0.3789x – 0.4734	0.9999

As was found with the LC-ESI-MS method, linearity can be achieved using the LC-ESI-MS^n method however an unresolved issue with reproducibility remains.

5. ANALYSIS OF POPPY SEEDS AND POPPY SEED CONTAINING FOOD PRODUCTS

5.1. Experimental

5.1.1. Chemicals, reagents and poppy seeds

Organic solvents (methanol, acetonitrile, chloroform, isopropyl alcohol) of HPLC grade were purchased from Sigma-Aldrich (Poole, Dorset). Deuterated morphine (100 µg mL^{-1}) in methanol was purchased from Sigma Aldrich (Poole, Dorset). Liquid nitrogen was obtained from BOC Industrial Gases (Manchester). Poppy seeds were purchased from a number of supermarkets in the UK with the country of origin, where available, provided in Table 5.1: the supermarket name has not been included but each brand has been assigned a number for reference.

Table 5.1 Poppy seed brand and country of origin

Poppy seed source reference	Country of origin
#1	China
#2	Unknown
#3	Turkey
#4	Unknown
#5	Holland
#6	Netherlands
#7	Unknown
#8	Netherlands

5.1.2. LC-MS instrument

HPLC was performed using an LC Surveyor system (Thermo Finnigan, Hemel Hempsted, UK) which was equipped with a pump, auto-sampler and column heater. Mass spectrometry was performed using an LCQ advantage (Thermo Finnigan, Hemel Hempsted, UK) ion trap mass spectrometer. The mass spectrometer was operated in a positive electrospray ionisation mode using the method established through the studies reported in chapter 4.

5.1.3. Calibration graphs and calculations

Calibration solutions were prepared in mobile phase on the day of analysis of each of the poppy seed extractions. The resulting equations from the calibration were used to determine the concentration of the solution of poppy seeds and the final alkaloid levels in ng g^{-1} were determined by taking into account the dilution factor from the solvent and the original weight of the poppy seeds used. The method for the extraction of alkaloids from poppy seeds included the addition of deuterated internal standard prior to extraction (see section 5.2.1).

5.1.4. Preparation of poppy seeds and poppy seed containing products

5.1.4.1. Poppy seed muffins

Mini poppy seed muffins were prepared by mixing together 175 g of self-raising flour, 112 g of caster sugar, 50 g of poppy seeds, ½ teaspoon of bicarbonate of soda, 70 g of melted butter, two small eggs, the zest of one lemon and 175 mL of skimmed milk. The ingredients were mixed into a batter and added to each of the dimples of mini-muffin trays purchased from Lakeland (Cumbria). The trays were then placed into an electric oven set to 180 ^{O}C and left to cook for 15 minutes. The final weight of poppy seeds in each mini-muffin was approximately 1.8 g. They were then left to cool to room temperature. Each poppy seed muffin weighed approximately 20 g. The poppy seed muffins were immersed in liquid nitrogen, crushed using a mortar and pestle and transferred to a spice blender for homogenisation prior to extraction and analysis by LC-MS using the method detailed below.

5.1.4.2. Poppy seed topped rolls/buns

A comparative study was carried out to establish if there was a difference between alkaloid levels resulting from poppy seeds incorporated into the matrix of the muffin to those resulting from poppy seeds coated onto a bread roll. The dough for the rolls was prepared using 280 g of strong white bread flour, 1 ½ tablespoons of sugar, 1 teaspoon of salt, ¾ teaspoon of fast action yeast, 2 tablespoons of skimmed milk powder, 150 mL of water and 2 tablespoons of oil. The dough was mixed in a commercially available Morphy Richards

compact bread maker (using the “dough” program) made available in the food technology laboratory. The dough was then split into 4 equal portions and each one was pressed into poppy seeds. The rolls were then placed into an oven at 190 ^{O}C and left to cook for 25 minutes, according to the recipe of the bread mix. The rolls were left to cool to room temperature and the poppy seeds were scraped from the surface using a metal spatula and homogenised in a spice blender prior to extraction and analysis by LC-MS using the method detailed below.

5.1.4.3. Thermally processed poppy seeds

In order to assess if the muffin matrix had any effect on the level of alkaloids found in the poppy seeds, raw poppy seeds from different suppliers were placed on a baking tray and heating at 180 ^{O}C for 15 minutes: they were then left to cool to room temperature, homogenised in a spice blender prior to extraction and analysis by LC-MS using the method detailed below.

5.2. Results and Discussion

5.2.1. Extraction of alkaloids from poppy seeds

Meadway, *et al*.[70] reported a method for the extraction of alkaloid compounds from poppy seeds which included the use of an aliquot of a solution of saturated ammonium chloride with concentrated ammonium solution at a pH of 9.0. The alkaloids were then extracted using chloroform:isopropanol (90:10, v/v) and back extracted into 0.5 M sulphuric acid. When this method was attempted in the laboratory, it was found that the final extracted product contained mostly oily residue, which was not readily miscible with the aqueous mobile phase used as the solvent for reconstitution. Since it is known that poppy seed oil can be extracted from poppy seeds[8,267,268], it was concluded that the extraction method was not as selective as desired and an alternative extraction method should be identified.

Seven solvents, which covered a broad range of polarity index values, and the chloroform:isopropanol (90:10, v/v) solvent used in the above-mentioned study were chosen for comparison. A list of the solvents and associated polarity index values are

provided in Table 5.2, where a lower polarity index value indicates a solvent of lower polarity.

Table 5.2 Extraction solvents and associated polarity index values

Solvent	Polarity index (Snyder)[269]
Diethyl ether	2.8
Dichloromethane	3.1
Chloroform	4.1
Isopropanol	4.3
Acetonitrile	5.8
Methanol	6.6
Water	9
Chloroform/isopropanol (90:10, v/v)	Unknown

For each solvent, extractions were carried out at pH 3.5, pH 5.0, pH 7.0 and pH 9.0 in order to obtain the optimum extraction conditions for the alkaloids from the poppy seeds. In order to avoid contamination by plastic residues from pipette tips by the solvents, glass pipettes were employed.

Poppy seeds from a batch of the #1 seeds were homogenised using a spice blender and approximately 200 mg of the seeds were weighed into glass tubes (4 tubes for each of the solvents). For each solvent, pH was altered to produce a solution of poppy seeds and solvent (1 mL) at pH 3.5, pH 5.0, pH 7.0 and pH 9.0. To each solution, deuterated internal standard (morphine-d3) was added. The tubes were then capped and placed into an ultrasonic bath for 10 minutes, centrifuged at 4000 rpm for 10 minutes. The appropriate layer was transferred to a clean glass tube, where it was dried down under nitrogen at 40^{o}C and reconstituted in 100 µL of aqueous mobile phase: extracts were filtered using a 0.22 µm nylon syringe filter before being transferred to a glass insert held in an auto-sampler vial for the LC-MS instrument.

The resulting chromatograms were obtained for each of the alkaloid compounds and were compared with respect to the presence of alkaloid and internal standard peaks, peak shape, and interferences from the matrix/solvent combination. It was found that at the extremes of the polarity scale (diethyl ether, dichloromethane and water), the chromatograms

produced were very complex and peak shape of alkaloids was very poor, independent of pH.

In contrast, the best result, in terms of alkaloid presence and peak shape, was obtained using chloroform:isopropanol (90:10, v/v) but at pH 3.5. For this reason, this extraction solvent was used for the extraction of alkaloids from harvested (raw), and all thermally processed poppy seeds.

5.2.2. Alkaloids in poppy seeds and poppy seed products

5.2.2.1. Harvested poppy seeds

Raw/harvested poppy seeds were purchased from different supermarkets: the source reference and country of origin for each of the poppy seed batches is provided in Table 5.1. No information was available from the supermarkets on processing that may have occurred prior to packaging and distribution of the poppy seeds and no information was available on the exact sub-species of *Papaver somniferum* L. plant harvested for the supply of the poppy seeds to the supermarkets. Seeds were extracted using the method described in section 5.2.1 and analysed by LC-MS using the method described in section 4.4. From each batch of poppy seeds, a minimum of 6 different portions weighing approximately 200 mg were extracted and analysed.

For each source, the mean weight of each of the alkaloids in poppy seeds was calculated and the range was identified. When the levels of morphine in poppy seeds from each of the different sources was compared (Table 5.3), it was found that there was much variation within batch but also between sources of poppy seeds. For example, no morphine was identified in any of the 15 randomly selected portions of seeds from source #4 however from source #2, when 15 randomly selected portions of these seeds were analysed, the levels of morphine ranged from 2,638 – 63,994 ng g^{-1}. The country of origin for both of these sources is unknown.

Table 5.3 Range and mean weight of morphine (ng) per gram of poppy seeds

Poppy seed source reference	Mean weight of morphine (ng g^{-1})	Range (ng g^{-1})
#1	1233	233 – 3,197
#2	29,652	2,638 – 63,994
#3	121	0 – 769
#4	ND	ND
#5	5,840	864 – 10,837
#6	1,620	141 – 4,223
#7	1,059	0 – 4,754
#8	62	0 – 312

ND – not detected

When the same comparison was carried out for codeine (Table 5.4) it was also found that there was much variation within batch and between sources of poppy seeds as was the case with morphine. Source #2, which was found to have a level of morphine much higher than the other sources, was also found to have a higher level of codeine. No other similarities can be drawn from the data.

Table 5.4 Range and mean weight of codeine (ng) per gram of poppy seeds

Poppy seed source reference	Mean weight of codeine (ng g^{-1})	Range (ng g^{-1})
#1	2,308	1,426 – 4,520
#2	8,507	474 – 23,307
#3	157	0 – 651
#4	72	52 – 106
#5	2,610	0 – 5,441
#6	153	61 – 349
#7	5,688	236 – 14,607
#8	117	94 – 157

When this same comparison was carried out for thebaine (Table 5.5) it was found that of the poppy seeds analysed, 50% of the sources seeds did not contain thebaine. It was found that the same source with the highest levels of morphine and codeine also exhibited highest levels of thebaine.

Table 5.5 Range and mean weight of thebaine (ng) per gram of poppy seeds

Poppy seed source reference	Mean weight of thebaine (ng g^{-1})	Range (ng g^{-1})
#1	1,251	285 – 2,480
#2	42,950	1,977 – 133,493
#3	ND	ND
#4	ND	ND
#5	6,363	841 – 12,561
#6	112	0 – 343
#7	ND	ND
#8	ND	ND

ND – not detected

Noscapine was identified in only 2 of the 8 sources of poppy seeds (Table 5.6). It was found that the seeds from source #7 contained the highest levels of noscapine of the two sources where noscapine was identified. When the levels of the other alkaloids present in source #7 seeds, it was found that morphine (0 – 4,754 ng g^{-1}) and codeine (2,361 – 14,607 ng g^{-1}) were also identified at levels in the higher range with the respect to the other sources.

Papaverine was detected in some of the analysed seeds but peaks were so small that it was not possible to quantify them.

Source #2 was found to contain morphine (2638 – 63,994 ng g^{-1}), codeine (474 – 23,307 ng g^{-1}) and thebaine (1,977 – 133,493 ng g^{-1}) at levels higher in comparison to other sources. It has been identified that sub-varieties of *Papaver somniferum* L. will have different alkaloid content and compositions.[57,88] However, this taxonomic information was not available from the suppliers of the seeds.

Table 5.6 Range and mean weight of noscapine (ng) per gram of poppy seeds

Poppy seed source reference	Mean weight of noscapine (ng g^{-1})	Range (ng g^{-1})
#1	ND	ND
#2	ND	ND
#3	ND	ND
#4	ND	ND
#5	534	0 – 2,970
#6	ND	ND
#7	2,224	291 – 10,700
#8	ND	ND

ND – not detected

It has been known since 1920[270] that factors, such as, the season in which the plants are grown, weather conditions and quality and type of fertiliser used can greatly affect the levels of alkaloids biosynthesised by *Papaver somniferum* L. In turn, the levels of alkaloids found in opium latex will also be affected. No data currently exist that compares levels of alkaloids in opium latex and alkaloids from the same plant but it is assumed that the levels would correlate. On this basis, the country of origin, where the plant was grown in the field (e.g. in the shade or direct sunlight), the quality of the soil can all affect the levels of alkaloids in the poppy seeds obtained from the plant.[76,87] This means that if a batch of poppy seeds are harvested from one field, naturally there will be variation in the levels of alkaloids from each of the plants. It is also possible that a batch of poppy seeds is the combination of multiple fields in one country. These factors may explain why there is such variation within batch and between sources of poppy seeds as discussed above.

5.2.2.2. Harvested versus thermally processed poppy seeds

A comparison was carried out to establish if there was a difference in the levels of alkaloids identified between harvested poppy seeds, as described above, poppy seeds which were baked on top of a bread roll, poppy seeds incorporated into a muffin matrix, and poppy seeds heated in an oven in the absence of bread/muffin matrix.

Harvested poppy seeds were prepared for analysis as described in Section 5.2.2.1. Thermally processed poppy seeds were prepared for analysis as described in Section 5.1.3.3. Poppy seed muffins and poppy seed topped rolls were prepared for analysis as described in Sections 5.1.3.1 and 5.1.3.2, respectively. For each source of harvested poppy seeds, poppy seeds heated without matrix, and poppy seeds scraped from the bread roll, 15 randomly selected portions weighing approximately 200 mg were extracted and analysed.

For the poppy seed muffins, approximately 400 mg of homogenised poppy seed/muffin mixture was extracted. However, the muffin matrix greatly interfered with the extraction process. When the chromatograms were analysed for the extracted muffins, no alkaloids were identified therefore results have not been included in this discussion.

The seeds from 3 randomly selected sources were extracted and analysed with the results shown in Table 5.7. Again, as was established with extractions of harvested poppy seeds (Section 5.2.2.1) there was much variation in the alkaloids identified and also in the levels of those alkaloids present.

Table 5.7 Comparison of levels of alkaloids identified in harvested poppy seeds, seeds from the surface of bread rolls and seeds heated with no matrix

		Harvested		**Seeds on bread roll**		**Heated (no matrix)**	
Source reference	**Alkaloid**	Mean (ng g^{-1})	Range (ng g^{-1})	Mean (ng g^{-1})	Range (ng g^{-1})	Mean (ng g^{-1})	Range (ng g^{-1})
#1	Morphine	545	8 – 1,888	11	0 – 33	63	0 – 304
	Codeine	82	0 – 284	3	0 – 27	5	0 – 54
#6	Morphine	217	0 – 431	1	0 – 11	ND	ND
	Codeine	175	0 – 418	3	0 – 21	ND	ND
	Papaverine	11	0 – 64	ND	ND	ND	ND
	Noscapine	34	0 – 80	ND	ND	ND	ND
#8	Morphine	25	0 – 96	ND	ND	15	0 – 49
	Codeine	30	0 – 81	5	0 - 19	11	0 – 52

ND – not detected

What was identified from this data was that whether the seeds were heated on the surface of the bread roll or were heated with no bread matrix, the levels of alkaloids (if detected)

were considerably lower than in the harvested seeds. Koleva *et al*.[271] reported that morphine content could be reduced by 10-50% in the process of baking and Sproll *et al.*[75] reported that the process of grinding and baking could reduce the morphine content of poppy seeds by up to 84%.

When comparing the results from the current work to levels published in the literature (Table 5.8) it can be seen that these findings are in-keeping with those published by Sproll *et al*.[76], published in 2006. This group of researchers employed an LC/MS/MS method for the detection of morphine, codeine, papaverine and noscapine in poppy seeds. The work published by Grove *et al*.[272] from 1976 appears to show much lower levels for morphine and codeine. However, this could have been due to the sensitivity of the GC-MS instrument employed.

Table 5.8 Comparison of alkaloids identified in poppy seeds

	Current work	**Grove *et al*.[272] (1976)**	**Sproll *et al*.[76] (2006)**
Compound	Range (ng g^{-1})		
Morphine	0 – 63,994	600 – 2,300	1,000 – 270,000
Codeine	0 – 23,307	100 – 500	0 – 56,000
Thebaine	0 – 133,493	NI	NI
Papaverine	ND	NI	ND
Noscapine	0 – 10,700	NI	0 – 21,000

ND – not detected; NI – not included in the study

The levels of alkaloids identified in the current work are generally lower than those found by Sproll *et al*.[76] but this could be due to the same factors that may influence the levels of alkaloids in poppy seeds (highlighted in section 5.2.2.1).

It was clear from the data obtained in this current work, and from other studies published in the literature, that there is much variation in the levels of alkaloids identified in poppy seeds. This variation can be attributed to a variety of natural parameters, such as weather and soil conditions, but also in the way that the seeds are harvested.[72] Processing methods prior to packaging and even the baking process has been shown to greatly affect the level of alkaloids.[75] The findings of this study correlate with the studies published in the literature.

When poppy seeds are consumed on a bun or roll, it has been estimated that each roll contains 1 – 4 g of poppy seeds.[72] Due to the reduction of the levels of alkaloids in the baking process, it has been estimated that if the poppy seeds contained 100,000 ng g^{-1} of morphine, the amount of seeds ingested would not cause any significant effect on an individual.[76] The implications of the ingestion of 1-4 g of baked poppy seeds with respect to workplace and roadside drug testing will be discussed in Chapter 6.

6. TOXICOLOGY OF POPPY SEED ALKALOIDS IN ORAL FLUID

6.1. Experimental

6.1.1. Reference material and intermediate stock solutions

Morphine, codeine and morphine-d3 were all prepared from reference standards in 1 mL ampoules purchased from Cerilliant (LGC Promochem). These morphine and codeine (1 mg mL^{-1}) standards were transferred into 10 mL volumetric flasks and filled to volume with methanol producing 100 µg mL^{-1} intermediate stocks.

Thebaine, papaverine hydrochloride and noscapine were all purchased in powder form from Sigma Aldrich and stock solutions of approximately 1 mg mL^{-1} in methanol were prepared. The final concentrations of free opiate for thebaine, papaverine and noscapine were 1.15 mg mL^{-1}, 1.26 mg mL^{-1} and 1.21 mg mL^{-1}, respectively. A mixed intermediate stock solution in methanol of approximately 10 µg mL^{-1} was prepared for thebaine, papaverine and noscapine (11.5, 12.6 and 12.1 µg mL^{-1}, respectively).

Phosphate buffer was prepared by dissolving 6.8 g of potassium dihydrogen phosphate in 400 mL of deionized water, the pH of which had been adjusted to 6.0 using 1 M potassium hydroxide solution. This was then made up to 500 mL in a volumetric flask with deionized water.

Morphine-d3 was used as the internal standard and was purchased from LGC Promochem as a 100 µg mL^{-1} reference standard in methanol. This was transferred to a 10 mL volumetric flask and filled to volume with methanol, producing a 10 µg mL^{-1} intermediate stock. From this, a 1000 ng mL^{-1} working solution of the morphine-d3 was prepared in methanol.

6.1.2. LC-MS instrument and analytical method

Due to instrument failure of the Thermo LC-ESI-MS and it being decommissioned, it was necessary to transfer the original method to another LC-MS instrument in the Faculty. The instrument made available was a Dionex™ UltiMate™ 3000 liquid chromatograph hyphenated to a Q-Exactive™ hybrid quadrupole orbitrap mass spectrometer (Thermo Finnigan, Hemel Hempsted, UK) and was employed for the toxicology study. The column used was the same Allure™ pentafluorophenyl phase with a propyl spacer (PFPP) column, 5

µm, 50 x 2.1 mm fitted with a guard column (Thames Restek, Buckinghamshire, UK) thermostated to 40^{o}C, which had been employed previously. The HPLC method used is shown in Table 6.1.

Table 6.1 Mobile phase composition and gradient program

Mobile phase composition **Solvent A:** Water + 2 mM ammonium formate + 0.2% formic acid **Solvent B:** Acetonitrile + 2 mM ammonium formate + 0.2% formic acid			
Time (minutes)	**%A**	**%B**	**Flow rate (µL min^{-1})**
0.00	90	10	350
2.00	90	10	350
10.00	10	90	350
11.00	10	90	350
12.00	90	10	350
14.00	90	10	350

The mass spectrometer was operated in a positive electrospray ionisation mode with the analyte specific settings shown in Table 6.2. As with all other mass spectrometers employed in this research, the mass spectrometer settings were established by directly infusing a 100 ng mL^{-1} solution of each of the five alkaloids and the deuterated internal standard (morphine-d3).

Table 6.2 Analyte specific parameters for Q-Exactive™ mass spectrometer. CID – in-source collision induced dissociation energy. *-ion used for quantitation

Compound	**[M-H^{+}] mass (Da)**	**Monitored transition mass (m/z)**	**CID (eV)**
Morphine	286*	286 → 201, 229	55
Codeine	300*	300 → 215, 243	52
Thebaine	312*	312 → 58, 249, 281	20
Papaverine	340*	340 → 202, 324	40
Noscapine	414*	414 → 220, 353	25
Morphine-d3	289*	289 → 201, 229	55

Data obtained from the Q-Exactive™ instrument was analysed using XCaliber 3.0 software package supplied with the ThermoFinnigan instrument.

6.1.3. Solid phase extraction procedure

Bond Elut Certify (130 mg bed mass, 3 mL volume) mixed mode cartridges were used. The cartridges employ both a non-polar sorbent (C8) and a strong cation exchanger and these types of mixed mode SPE cartridges have been successfully employed in the extraction of opiates from biological matrices.[79,191,273-276] The SPE method was designed using a combination of a number of scientific papers, SPE guides from SPE column manufacturers and personal experience.[74,79,154,185,277]

The cartridge was conditioned with 2 mL of methanol followed by 2 mL of phosphate buffer (0.1 M, pH 6.0). The oral fluid sample was loaded and allowed to drip through under gravity. The cartridges were then washed with 1 mL of water, followed by 500 µL of 0.01 M formic acid. The cartridge was then air dried for 10 minutes under full vacuum, then 50 µL of methanol was added and the cartridge was dried for a further 2 minutes.

The basic opiate drugs were eluted using 2 x 1 mL of methanol: ammonia (98:2, v/v) which was prepared on the day the extractions were carried out. The final extracts were evaporated under nitrogen at 40^{o}C and reconstituted in 100 µL aqueous mobile phase (A). Each reconstituted extract was subsequently filtered through a 0.22 µm nylon syringe filter before being transferred to a glass insert held in an auto-sampler vial for the LC-MS instrument.

6.1.4. Preparation of poppy seed containing products – mini muffins

Mini poppy seed muffins were prepared by mixing together 175 g of self-raising flour, 112 g of caster sugar, 50 g of poppy seeds, ½ teaspoon of bicarbonate of soda, 70 g of melted butter, two small eggs, the zest of one lemon and 175 mL of skimmed milk. The ingredients were mixed into a batter and this was added in equal amounts to each of the dimples of mini-muffin trays purchased from Lakeland (Cumbria). Twenty-eight mini muffins were prepared which equates to approximately 1.8 g of poppy seeds per muffin.

The trays were then placed into an electric oven set to 180 ^{O}C and left to cook for 15 minutes. They were left to cool to room temperature and then 2 muffins were weighed and placed into zip lock bags for the toxicology study (kits 1-6). Two sets of mini poppy seed muffins were also prepared, weighed, frozen for one week, defrosted at room temperature then used in the toxicology study (kit A and B). The weights of the mini-muffins are provided in Table 6.3.

Table 6.3 Weights of muffins provided in each of the kits for the toxicology study

Kit number	Weight (g) of muffin 1	Weight (g) of muffin 2
1	19.9	19.6
2	20.1	19.5
3	21.7	22.1
4	21.6	21.0
5	18.6	18.5
6	19.2	19.3
A	19.6	19.5
B	18.5	18.3

6.2. **Toxicology study**

This small scale pilot study was designed to establish if eating two mini poppy seed muffins made it possible to exceed the 40 ng mL^{-1} analytical cut-off established by SAMHSA for opiates in oral fluid, as discussed in Section 2.1.5, was carried out.

Six participants were provided with an information sheet outlining the steps to be followed in the collection of the oral fluid samples for the toxicology study. They were also provided with seven Quantisal™ oral fluid collection kits, 2 mini poppy seed muffins, a collection bag, a pen and paperwork from ethical approval (which included consent form, medical questionnaire and research proposal). Participants were provided with training on how to use the Quantisal™ collection kits prior to commencing the study. The mini poppy seed muffins were prepared in the food technology laboratory of Northumbria University as outlined in Section 6.1.4 in the same week that the toxicology study was carried out. Each

of the participants carried out their sampling on a different day of the same week. All of the oral fluid samples, with the adsorbent pad removed, were frozen at -20 ^{o}C until the following week when defrosting at room temperature, extraction and analysis by LC-MS took place.

Two of the participants (numbers 4 and 6) also agreed to repeat the study the following week: four mini poppy seed muffins from the original batch were frozen, defrosted and the toxicology study repeated. These oral fluid samples were refrigerated overnight at 4 ^{o}C, after which extraction and analysis by LC-MS took place. These samples were collected and stored overnight in order to compare the results with the oral fluid samples which were frozen to establish if freezing the oral fluid had a detrimental effect on the results obtained.

For the collection, participants were asked to rinse their mouth out with water, wait ten minutes and collect the first oral fluid sample which was to be labelled CONTROL. Next, the two mini poppy seed muffins were to be consumed and 5 minutes should be allowed to pass before the next oral fluid sample was collected: this was to be labelled SAMPLE 1. Oral fluid samples 2 – 6 were then to be collected at 20 minutes, 1 hour, 2 hours, 4 hours and 8 hours post-ingestion of the muffins. These collection times were in-keeping with other toxicology studies investigating the detection of opiates in oral fluid after controlled opiate or poppy seed ingestion.[74,85,177,278] All oral fluid collection was carried out by all six participants in the same week. The oral fluid samples of the two participants who repeated the study where the oral fluid samples were refrigerated (not frozen), were labelled A (originally participant 6) and B (originally participant 4).

Finally, two working solutions containing morphine, codeine, thebaine, papaverine and noscapine at concentrations of 200 ng mL^{-1} and 1000 ng mL^{-1} were prepared in methanol using the intermediate stock solutions prepared above. The final concentrations for each of the compounds are provided in Table 6.4.

Table 6.4 Final concentrations for intermediate stock solutions of morphine, codeine, thebaine, papaverine and noscapine

Compound	Final concentration in 1000 ng mL^{-1} intermediate stock	Final concentration in 200 ng mL^{-1} intermediate stock
Morphine	1000 ng mL^{-1}	200 ng mL^{-1}
Codeine	1000 ng mL^{-1}	200 ng mL^{-1}
Thebaine	1150 ng mL^{-1}	230 ng mL^{-1}
Papaverine	1260 ng mL^{-1}	252 ng mL^{-1}
Noscapine	1210 ng mL^{-1}	242 ng mL^{-1}

Using the 1000 ng mL^{-1} and 200 ng mL^{-1} working stocks, calibration solutions at concentrations of 10, 20, 30, 40, 50, 100 and 200 ng mL^{-1} were prepared in 1 mL of blank oral fluid. The blank oral fluid was pooled oral fluid collected by expectoration by the researcher over the period of two days. The oral fluid was refrigerated prior to preparation of the calibration solutions. Each of the calibration solutions was then added to 3 mL of buffer solution in a Quantisal™ oral fluid collection kit and frozen alongside the oral fluid samples provided by the participants from the toxicology study. Calibration solutions were prepared in triplicate.

Five separate quality control (QC) samples were prepared in 1 mL of oral fluid, resulting in approximately 100 ng mL^{-1} of morphine, codeine, thebaine, papaverine, noscapine and morphine-d3. For the 100 ng mL^{-1} QCs the concentration of morphine, codeine and morphine-d3 was 100 ng mL^{-1}, 115 ng mL^{-1} for thebaine, 126 ng mL^{-1} for papaverine and 121 ng mL^{-1} for noscapine.

6.2.1. Matrix-matched calibration solutions

On the day of analysis, calibration solutions were defrosted at room temperature and to each of the calibration oral fluid solutions, 100 µL of the 1000 ng mL^{-1} working solution of morphine-d3 was added, resulting in a final concentration of morphine-d3 of 100 ng mL^{-1}. The concentration of each of the alkaloids present in each of the calibration solutions is provided in Table 6.5. The calibration solutions were extracted by SPE and prepared for analysis, as described in Section 6.1.3.

Table 6.5 Concentration of alkaloids present in calibration solutions

Calibration concentration (ng mL^{-1})	Compound	Actual concentration (ng mL^{-1})
10	Morphine & codeine	10
	Thebaine	12
	Papaverine	13
	Noscapine	12
20	Morphine & codeine	20
	Thebaine	23
	Papaverine	25
	Noscapine	24
30	Morphine & codeine	30
	Thebaine	35
	Papaverine	38
	Noscapine	36
40	Morphine & codeine	40
	Thebaine	46
	Papaverine	50
	Noscapine	48
50	Morphine & codeine	50
	Thebaine	58
	Papaverine	63
	Noscapine	61
100	Morphine & codeine	100
	Thebaine	115
	Papaverine	126
	Noscapine	121
200	Morphine & codeine	200
	Thebaine	230
	Papaverine	252
	Noscapine	242

6.3. **Results and discussion**

6.3.1. Selectivity

When selectivity was established in Section 4.5.1, mixed alkaloid solutions of known concentrations were prepared and compared to a blank matrix. For the method development phase (Chapter 4) the matrix was blank mobile phase A, which was composed of water + 2 mM ammonium formate + 0.2% formic acid. For this toxicology study, the matrix used was oral fluid therefore, a mixed alkaloid solution containing all five alkaloids plus the internal standard was prepared in oral fluid: extraction was then carried out using the SPE method (Section 6.1.3) and the extract analysed. A mixed alkaloid extract with approximately 200 ng mL^{-1} of each of the five alkaloids (+ 100 ng mL^{-1} morphine-d3) was analysed using the instrument methods (Section 6.1.2).

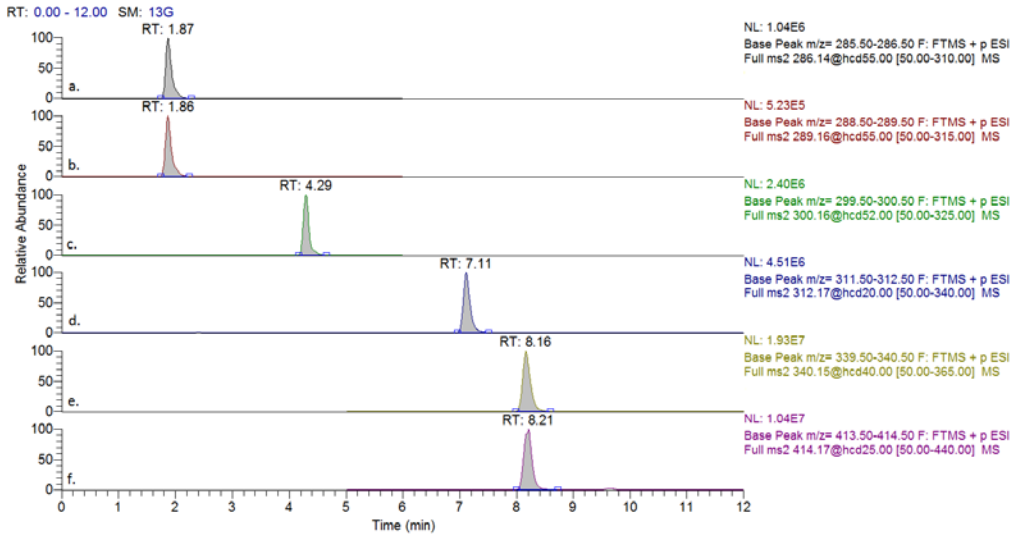


Figure 6.1 Chromatograms for (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine, (e) papaverine, (f) noscapine

All chromatograms (Figure 6.1) are the result of extracting the quasi-molecular ion for each alkaloid: as can be seen, there appears to be no interferences from the oral fluid matrix. An aqueous mobile phase blank was injected into the LC-MS instrument and no interferences for the alkaloids were found. However, in order to assess the selectivity in the case of a biological matrix, oral fluid spiked only with morphine-d3 (100 ng mL^{-1}) was prepared in the same way as described for the known mixed alkaloid extract. The quasi-molecular ion was

extracted for each of the alkaloids and the internal standard and the subsequent chromatograms are shown in Figure 6.2.

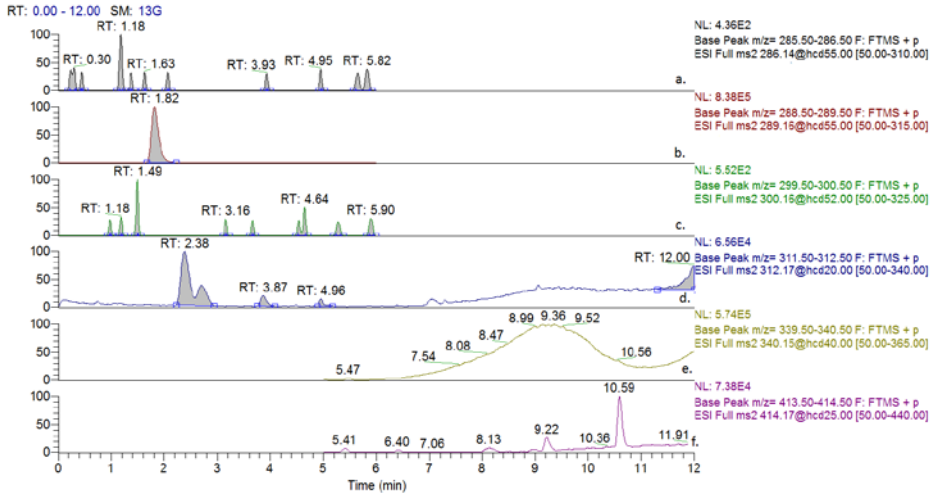


Figure 6.2 Extracted chromatograms for (a) morphine, (b) morphine-d3, (c) codeine, (d) thebaine, (e) papaverine, (f) noscapine

When the chromatograms were examined from the blank oral fluid spiked with morphine-d3, it can be seen that, as expected, there is a large peak observed for the internal standard (Figure 6.2.b). For morphine, codeine and thebaine there were no matrix peaks which caused interference at the expected retention times for each of these alkaloids (1.87, 4.29 and 7.11 minutes, respectively); the chromatogram for papaverine shows baseline fluctuation which could be due to increased pressure in the system due to changes in the mobile phase gradient. Other blank oral fluid samples were injected (post SPE extraction but no morphine-d3) and also showed a similar baseline but when the mass spectra were analysed, the expected ions indicative of the presence of papaverine were not found.

In the chromatogram for noscapine (Figure 6.2.f), a peak was found between 8.00 – 8.24 minutes which has a retention time similar to that expected for noscapine (8.21 minutes): the mass spectrum of this peak was compared to the mass spectrum from a known injection of noscapine (Figure 6.3.). As can be seen, all of the expected ions for noscapine (*m/z* 220, 353 and 414) are not found in the mass spectrum for the peak of similar retention time in the oral fluid blank (Figure 6.3.d) therefore this component of the matrix would not affect the interpretation of the presence of noscapine in a sample.

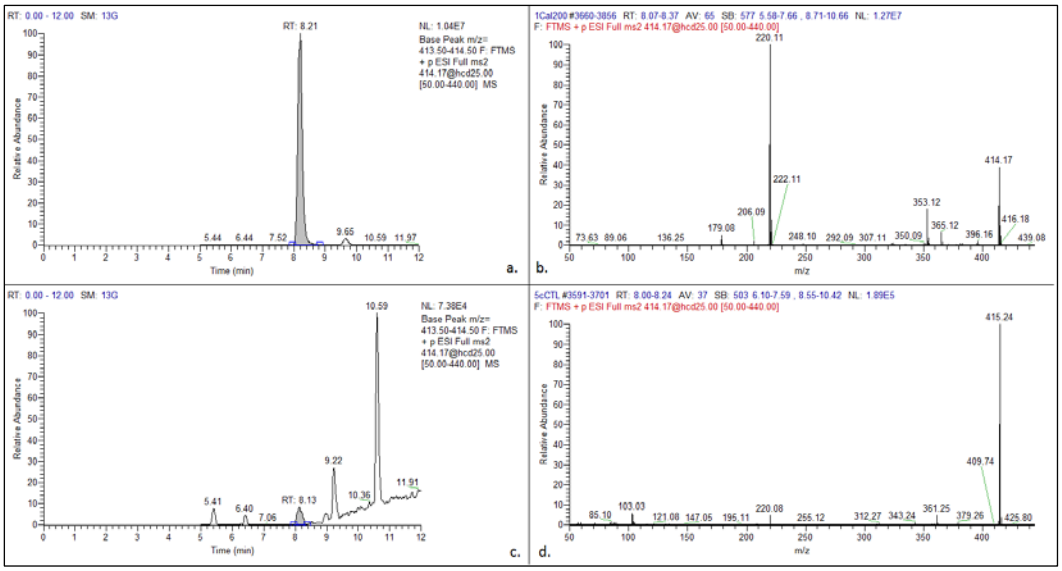


Figure 6.3 (a) Extracted chromatogram using *m/z* 414 for a known injection of noscapine, (b) mass spectrum of noscapine, (c) extracted chromatogram using *m/z* 414 for blank oral fluid spiked with morphine-d3, (d) mass spectrum of peak of retention time 8.00-8.24 minutes from chromatogram (c)

Since it was possible to show that blank oral fluid spiked with only morphine-d3 internal standard showed no interferences for the five alkaloids it can be assumed that the method is selective based upon the recommendations for analytical method validation published in 2007 by Peters[239], and explained in section 4.5.1.

6.3.2. Linearity

Linearity was established using the extracted calibration samples, prepared as in section 6.2.1. In practical method validation, a minimum of 5 data points should be used to prepare a calibration graph and each point should be prepared a minimum of three times.[243] Therefore in this study, three separate calibration sets were prepared in oral fluid, extracted using SPE and the data produced was used to calculate the peak area ratio (opiate drug/internal standard), mean and standard deviation for each of the concentration points. The ratio of the peak area produced for the drug compound divided by the peak area obtained for an appropriate internal standard is recommended for reliable quantitation in analytical methods applied to biological matrices and has been used in the previous chapters.[239]

The data obtained was used to construct calibration graphs: a minimum of five concentration points plus a blank were used. The concentration range, linear equation

from the line of best-fit for the calibration data and the co-efficient of determination (R^2) obtained for each of the opiates are provided in Table 6.6.

Table 6.6 Calibration range, linear equation and R^2 values for morphine, codeine, thebaine, papaverine and noscapine.

Compound	Concentration range (ng mL^{-1})	Linear equation	R^2
Morphine	0 – 200	y = 0.0092x – 0.0059	0.9994
Codeine	0 – 200	y = 0.0194x + 0.0055	0.9975
Thebaine	0 – 200	y = 0.0437x + 0.0682	0.9985
Papaverine	0 – 200	y = 0.2066x + 0.6244	0.9983
Noscapine	0 – 200	y = 0.1095x + 0.7234	0.9954

Since the R^2 value for each of the five alkaloids is greater than 0.99, it can be concluded that the data is linear.

6.3.3. Precision

In order to assess the quality of the data produced using this method, pooled oral fluid was used to prepare 10 separate spiked samples containing approximately 200 ng mL^{-1} of each of the five alkaloids plus internal standard (100 ng mL^{-1}), extracted using the SPE method outlined in Section 6.1.3 and analysed by the LC-MS method described in Section 6.1.2.

The peak area ratios for each of the alkaloids for the 10 injections, mean values, standard deviation and co-efficient of variation values are provided in Table 6.7.

Table 6.7 Mean ($\bar{x}$), standard deviation (σ_x) and co-efficient of variation (%CV) for 10 injections of mixed alkaloid oral fluid samples of 200 ng mL^{-1}, containing 100 ng mL^{-1} morphine-d3

Sample number	Peak area ratios (opiate/morphine-d3)				
	Morphine	**Codeine**	**Thebaine**	**Papaverine**	**Noscapine**
1	1.7820	2.9968	9.0411	30.4039	17.8829
2	1.7987	3.2099	9.1101	31.6306	20.1764
3	1.7504	3.1078	8.8784	29.8126	19.2470
4	1.7467	3.1437	9.0107	30.7348	19.7190
5	1.7702	3.2083	9.2064	31.3650	19.6585
6	1.7298	3.0515	8.9459	30.7253	19.0242
7	1.7689	3.1582	9.1664	31.1406	19.3667
8	1.7737	3.0598	9.0140	30.7827	18.6688
9	1.7685	3.0225	9.1758	31.2501	18.8078
10	1.7693	2.9875	8.7978	30.3220	18.4352
$\bar{x}$	**1.7658**	**3.0946**	**9.0347**	**30.8168**	**19.0986**
σ_x	**0.0193**	**0.0831**	**0.1341**	**0.5473**	**0.6788**
%CV	**1.09**	**2.69**	**1.48**	**1.78**	**3.55**

When assessing the precision, the recommended acceptance criteria is obtaining coefficient of variation values of 15% or less.[246] As can be seen in Table 6.7, all five alkaloid compounds have a %CV of less than 4% which is much lower than the 15% limit therefore showing that the method is precise, or more specifically, repeatable. Accuracy was determined to be less than 15% variation for all alkaloids except papaverine: for this compound the variation was found to be 26% which exceeds the recommended limit.[239]

6.3.3.1. Repeatability

As part of the validation process, quality control (QC) mixed opiate samples were prepared at concentrations of 100 ng mL^{-1}, including morphine-d3 at 100 ng mL^{-1}. These QC different QC samples were prepared, extracted and analysed in the sample way as calibration and participant samples. The QC samples were bracketed between participant samples and blanks. Table 6.8 shows the repeatability of the combined QC data.

Table 6.8 Peak area ratios for morphine codeine, thebaine, papaverine and noscapine, showing mean ($\bar{x}$), **standard deviation (σ_x) and co-efficient of variation (%CV) for 10 QC samples analysed a period of four days**

Parameter	Morphine	Codeine	Thebaine	Papaverine	Noscapine
QC1	0.9612	1.5742	1.4634	6.2556	4.5583
QC2	1.0447	1.7298	1.6943	7.1971	5.1888
QC3	1.0006	1.6423	1.6555	6.9561	5.0065
QC4	1.0272	1.7944	1.6525	6.9377	4.8596
QC5	0.9758	1.8000	1.6281	6.6821	4.8853
QC6	1.0283	1.7208	1.6356	7.0490	5.0805
QC7	1.0099	1.7108	1.6939	7.3957	5.1580
QC8	1.0377	1.8387	1.7176	7.6651	5.3115
QC9	0.9891	1.7646	1.6284	7.1578	4.8177
QC10	0.9612	1.5742	1.4634	6.2556	4.5583
$\bar{x}$	**1.0083**	**1.7306**	**1.6410**	**7.0329**	**4.9851**
σ_x	**0.0288**	**0.0825**	**0.0739**	**0.4057**	**0.2301**
%CV	**2.86**	**4.77**	**4.51**	**5.77**	**4.61**

All co-efficient of variation values are less than 6% and since it acceptable to have values of less than 15%[246], these findings show that the method is repeatable.

6.3.4. Matrix Effects

It is recommended in bio-analytical method development that matrix effects should be evaluated and that this can be assessed by preparing five samples of the biological matrix and spiking with the analytes of interest for subsequent extraction and analysis.[257,258,279] The data obtained can then be used to determine if the analytical method is affected by matrix effects. When precision of the method was assessed (section 6.3.3), 10 separate aliquots of oral fluid were spiked, extracted and analysed by LC-MS: this data was used to assess the precision of the method but it can also be used to evaluate potential matrix effects. In 2006, Matuszewski, *et al.*[280] recommended that the co-efficient of variation should not exceed 3-4% in order to consider the method practically free from matrix effects and reliable: since the precision data obtained for each of the alkaloids (Table 6.7) shows

that the %CV values are all less than 4%, it can be concluded that any matrix effects from the use of oral fluid are minimal and do not greatly affect the analytical method.

6.3.5. Toxicology study

As described in section 6.2, control oral fluid samples were collected by participants prior to consuming the poppy seed muffins, followed by further samples collected at 5 minutes, 20 minutes, 1 hour, 2 hours, 4 hours and 8 hours post-consumption. Six participants were involved in the study with two participants repeating the study one week apart and all samples were treated in the same way as the calibration standards, which were matrix matched (section 6.2.1). Using the peak area ratio, the concentration of any of the alkaloid compounds identified in the oral fluid samples was calculated. It should be highlighted that all of the peak area values obtained for the alkaloids identified in the oral fluid samples were below the 10 ng mL^{-1} peak area ratio values. The linear equations were still used to calculate an approximate concentration in order to assess if any correlation between participants could be identified. The same six participants were recruited to repeat the toxicology study: the only difference from the previous study was that samples were stored overnight in the refrigerator (not frozen and defrosted). The two poppy seed muffins consumed were prepared in the same way as described in section 6.1.4 and the poppy seeds in each muffin weighed approximately 1.8 g (~3.6 g in total).

6.3.5.1. Morphine

Morphine was identified in the first 1 or 2 oral fluid samples (collected at 5 and 20 minutes) of all participants, except participant A (Figure 6.4). Participant A and participant 6 are the same person but samples were collected one week apart. It should be noted that although the morphine concentration appears to "peak" in the 5 minute and 20 minute sample, the y-axis scale does not exceed 1.2 ng mL^{-1}, which is considerably lower than the 40 ng mL^{-1} analytical cut-off recommended by SAMHSA[196] in the United States of America and by the EWDTS[195] in Europe. SAMHSA have recently drafted a proposal for Federal workplace testing programs to use a screening cut-off of 30 ng mL^{-1} for opiates with a 15 ng mL^{-1} confirmation cut-off[281]: even if these cut-off values were used in this case, the concentrations found would not exceed the proposed concentration limits.

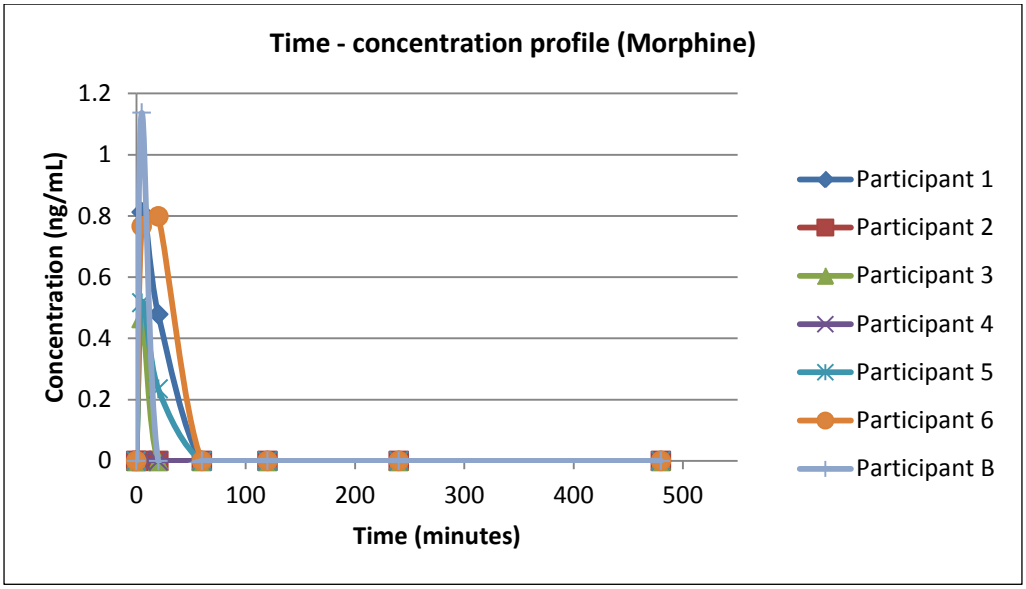


Figure 6.4 Combined participant time-concentration profile for morphine

After the 1 hour post ingestion sample, there is no change in the concentration, i.e. the concentration of morphine remains at zero. In order to compare the participant differences in the first hour, the x-axis of the graph in Figure 6.4 was amended to remove the later time points (Figure 6.5).

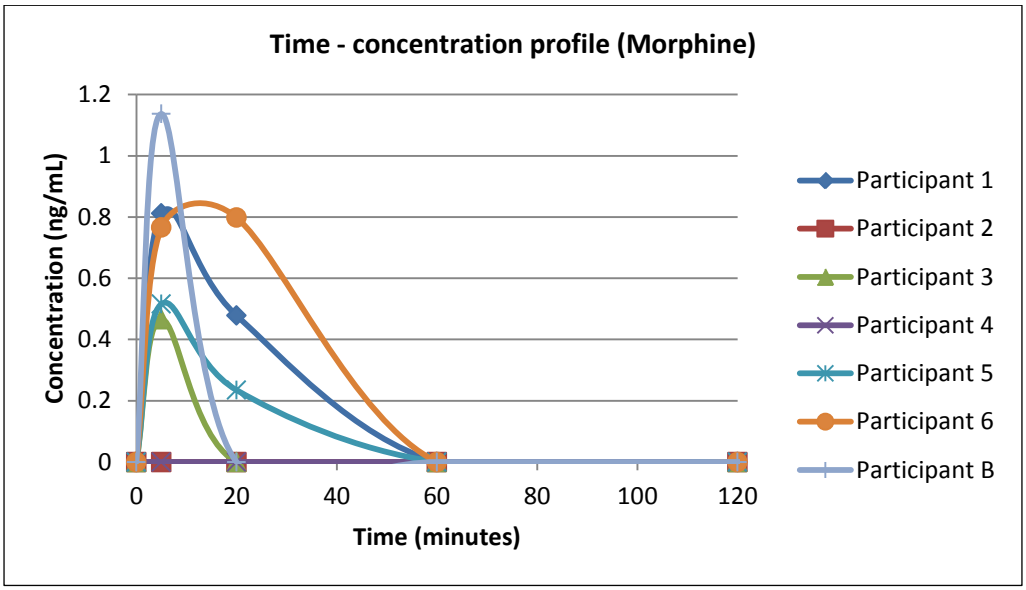


Figure 6.5 Combined participant time-concentration profile for morphine (reduced time points on x-axis)

When the study was repeated and the oral fluid samples were refrigerated rather than frozen, the findings (Figure 6.6) were different to those previously found. For participants 3, 5 and 6, morphine was identified in the 4 hour post ingestion sample and in participant 5, morphine was even noted in the 8 hour post ingestion samples. As mentioned above, it should be noted that the values are very low and below the lowest concentration point of the calibration solutions.

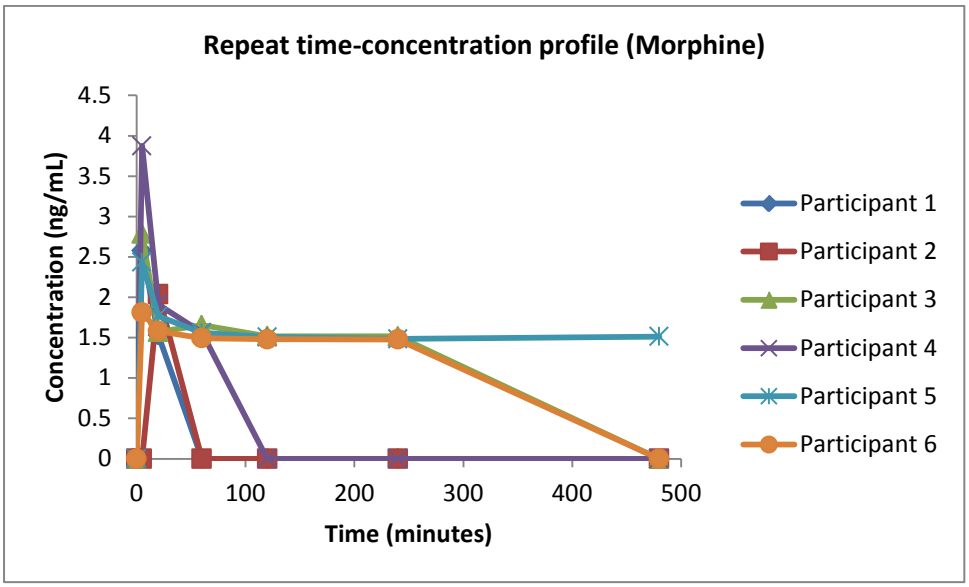


Figure 6.6 Combined participant time-concentration profile for morphine obtained from the repeat study

All participant numbers allocated in the original study were allocated to the same person in the repeat study, to avoid confusion. As can be seen in Figure 6.6, morphine was identified in the oral fluid of all participants in at least one of the collected oral fluid samples. If the x-axis of the graph is modified to make the initial data easier to view (Figure 6.7) it is possible to see that in the 5 minute and 20 minutes post ingestion samples, morphine was detected in all six participants.

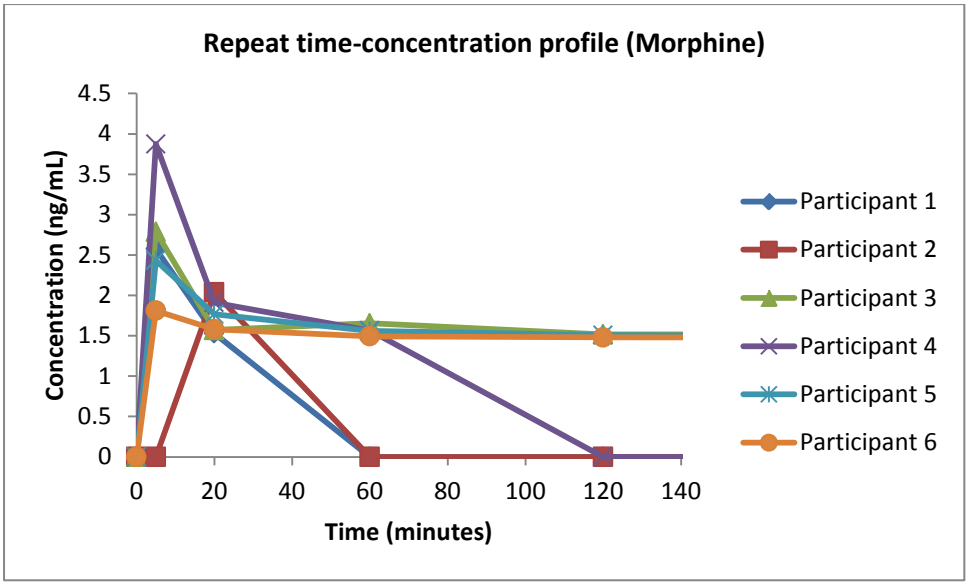


Figure 6.7 Combined participant time-concentration profile for morphine obtained from the repeat study (reduced time points on x-axis)

6.3.5.2. Codeine

Codeine was identified in the 5 minute oral fluid samples of only 2 of the 6 participants (Figure 6). As was found in the case of morphine, the graph appears to show a "peak" in the first sample but again, the y-axis scale should be noted: the highest value is less than 3 ng mL^{-1}, which is also much less than the SAMSHA analytical cut-off of 40 ng mL^{-1} and the proposed cut-off of 15 ng mL^{-1} as was also the case with morphine.

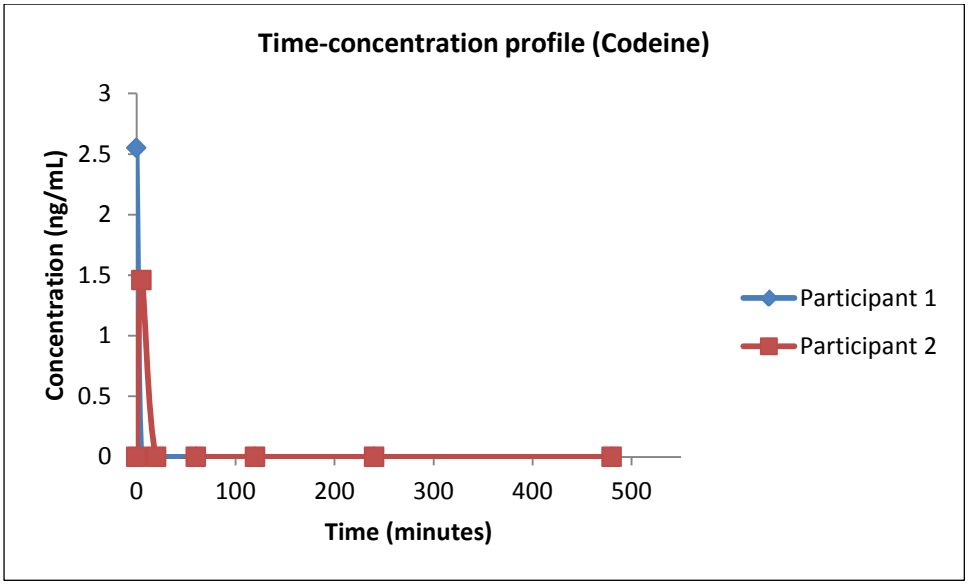


Figure 6.8 Combined participant time-concentration profile for codeine

In the repeat study, codeine was identified in the oral fluid samples of participants 1, 2 and 3 (identified in participants 1 and 2 in the original study). When the data was analysed, the results from participant 1 (Figure 6.9) show that there is an initial detection of codeine in the 5 minute post ingestion sample which then decreases until the 1 hour post ingestion samples was collected. However in the 2 hour post ingestion sample, the level of codeine increases to approximately 28 ng mL^{-1}. If the 40 ng mL^{-1} SAMSHA analytical cut-off is employed, this sample would still be classed as a negative however, if the suggested 15 ng mL^{-1} cut-off was used, this sample would be classed as positive. This result was only seen for participant 1 and is the only one sample to come close to the 40 ng mL^{-1} SAMSHA analytical cut-off.

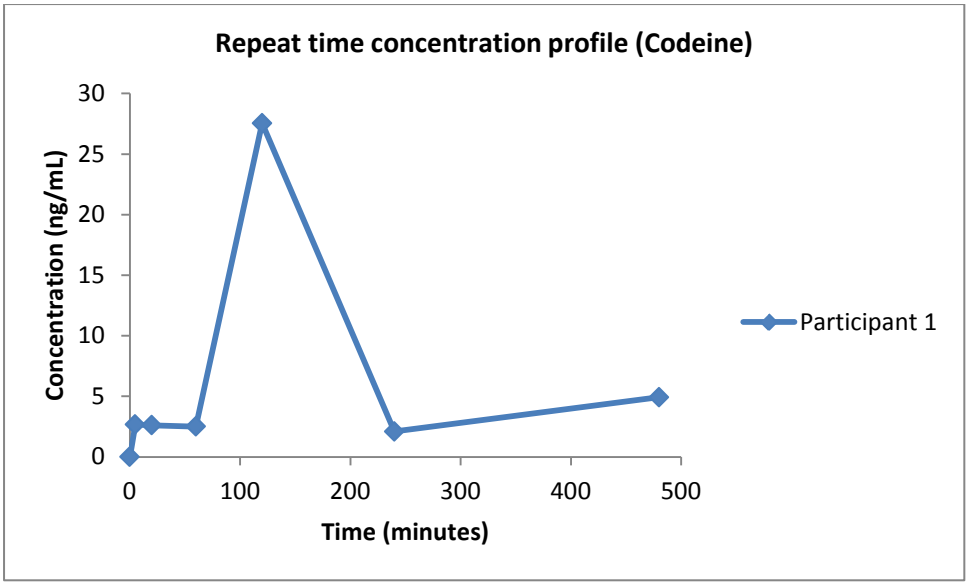


Figure 6.9 Combined participant time-concentration profile for codeine obtained from the repeat study for participant 1

Codeine was also identified in the oral fluid sample of participants 2 and 3 (Figure 6.10) but the extrapolated concentration values were more similar to those found in the original study (Figure 6.8).

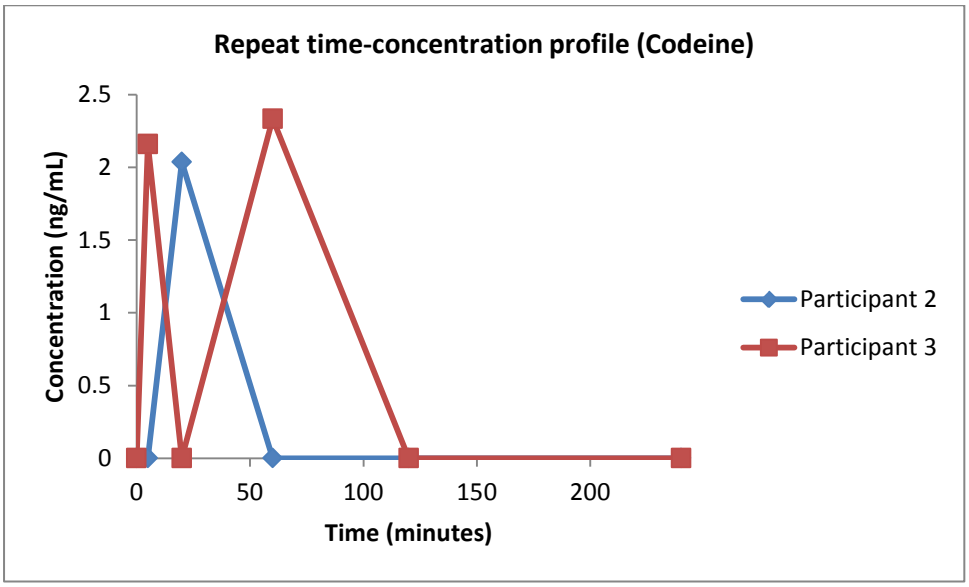


Figure 6.10 Combined participant time-concentration profile for codeine obtained from the repeat study for participants 2 and 3 (reduced time points on x-axis)

6.3.5.3. Thebaine

Currently, the guidelines for the presence of opiates in oral fluid are confirmed by the presence of morphine and codeine only: if heroin administration is suspected, this is usually confirmed by the presence of 6-MAM (see Section 2.1.5). Thebaine, was proposed by Cassella, *et al.*[78] in 1997 as a potential marker in urine for poppy seed consumption. In 2007, El-Haj, *et al.*[282] showed that thebaine was a useful marker, also in urine, for opium use (this included poppy seeds). In the latter study, users of illicit heroin were included in the study and no thebaine was detected. However urine is a different matrix to oral fluid. As yet, there are no guidelines to provide interpretation of thebaine in oral fluid, or in biological matrices in general.

When the oral fluid from all participants was analysed for the presence of thebaine, it was identified in the samples 4 of the 6 participants (Figure 6.11).

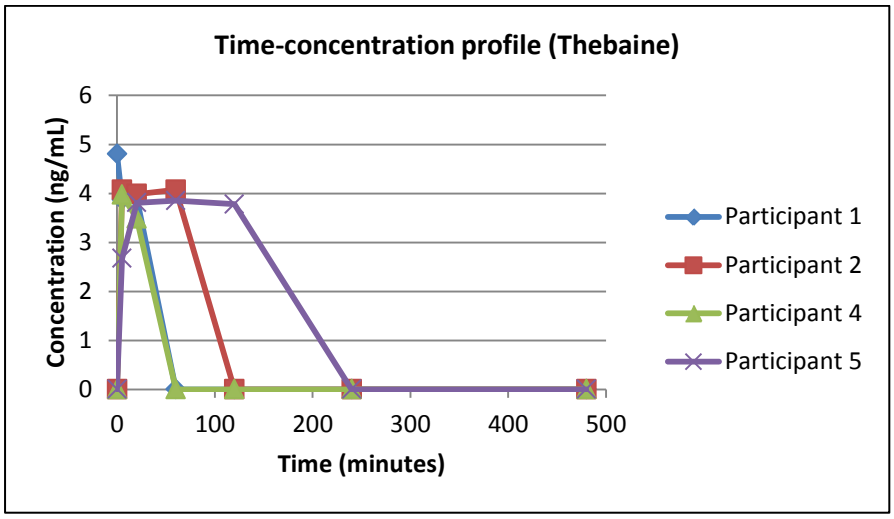


Figure 6.11 Combined participant time-concentration profile for thebaine

As with morphine and codeine, the y-axis scale is small which makes the results appear more pronounced than in reality. However, for participants 1 and 4, thebaine was found in the 5 and 20 minute samples, at concentrations less than 5 ng mL^{-1} but thebaine was detected for up to 1 hour for participant 2 and 2 hours for participant 5.

Thebaine was not detected in any of the oral fluid samples of any of the six participants in the repeat toxicology study. Thebaine has not been reported in oral fluid in the scientific

literature to date. Whether these findings are significant warrants further investigation as it is necessary to establish if thebaine can be detected in the oral fluid of individuals who have consumed diacetylmorphine (in the form of heroin), morphine or codeine preparations.

6.3.5.4. Noscapine

No data currently exists in the published literature with respect to the interpretation of noscapine presence in oral fluid. Noscapine was identified in the oral fluid of 4 of the 6 participants (Figure 6.12).

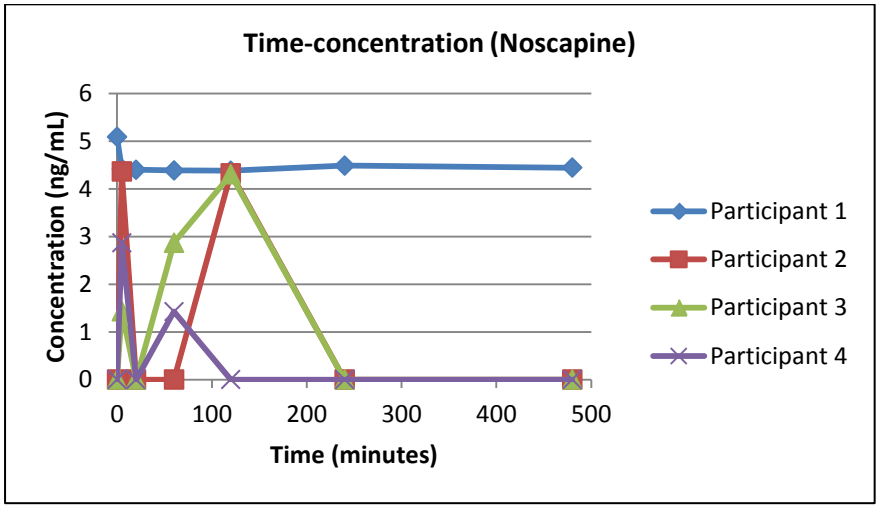


Figure 6.12 Combined participant time-concentration profile for noscapine

For participants 2-4 noscapine was identified in the 5 minute sample (all with less than 6 ng mL^{-1}) but when the 20 minute sample was analysed, no noscapine was identified: when the 1 hour sample was analysed, it was found that noscapine was again identified in the oral fluid of participants 3 and 4, albeit at less than 3 ng mL^{-1} for both. Noscapine was not found in the 1 hour sample of participant 2, but was then identified in the 2 hour sample: no noscapine was identified in the 4 hour sample. In the case of participant 2, no noscapine was identified in the 20 minute or 1 hour sample but was identified in the 2 hour sample.

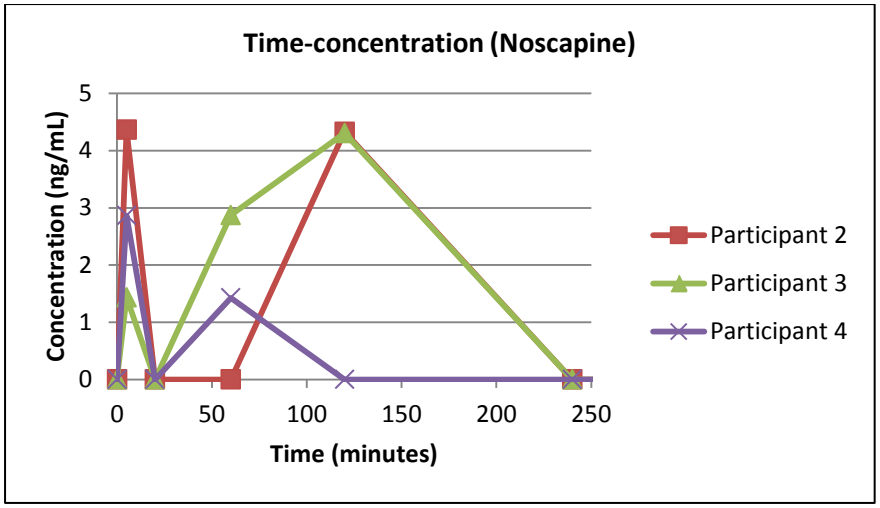


Figure 6.13 Combined participant time-concentration profile for noscapine (reduced time points on x-axis) and participant 1 removed

The results obtained from the oral fluid samples of participant 1 were different to all of the other participants: it was found in this case that the noscapine level increases to a point in the 5 minute sample and then continued to be identified until the final 4 hour sample. It is possible that the oral cavity of the participant was contaminated with poppy seeds for the duration of the study. However, if this was the case, it would be expected that similar results for this participant would also be seen for the other alkaloids. Since this was not the case, the reasons for this are unknown and since no literature in relation to the pharmacokinetics of papaverine in oral fluid exists, it can be deemed an anomaly. It should however be taken into account that these values are so small, and below the lowest calibration standard, therefore the findings may be as a result of the limitations of the method used.

Noscapine has not been reported in oral fluid in the scientific literature to date. As was highlighted in the case of thebaine (Section 6.3.5.3), whether these findings are significant warrants further investigation to establish if noscapine can be detected in the oral fluid of individuals who have consumed diacetylmorphine (in the form of heroin), morphine or codeine preparations.

6.3.5.5. Papaverine

Papaverine was only detected in oral fluid samples in the repeat study (Figure 6.14) and was detected in 3 of the 6 participants. Again, it should be noted that in all samples, papaverine was found in concentrations lower than the lowest concentration point of the calibration solutions. Papaverine was also identified in the control sample for participant 1, which is unexpected. However, the same participant also had noscapine identified in the control sample and continued to be identified throughout the full sample collection time in the original study. On further investigation, the participant collected the samples as outlined in the instruction sheet and as per the training provided and had not consumed any other pharmaceutical/over-the-counter preparations or used any topical treatments therefore this remains an anomaly, currently unexplained.

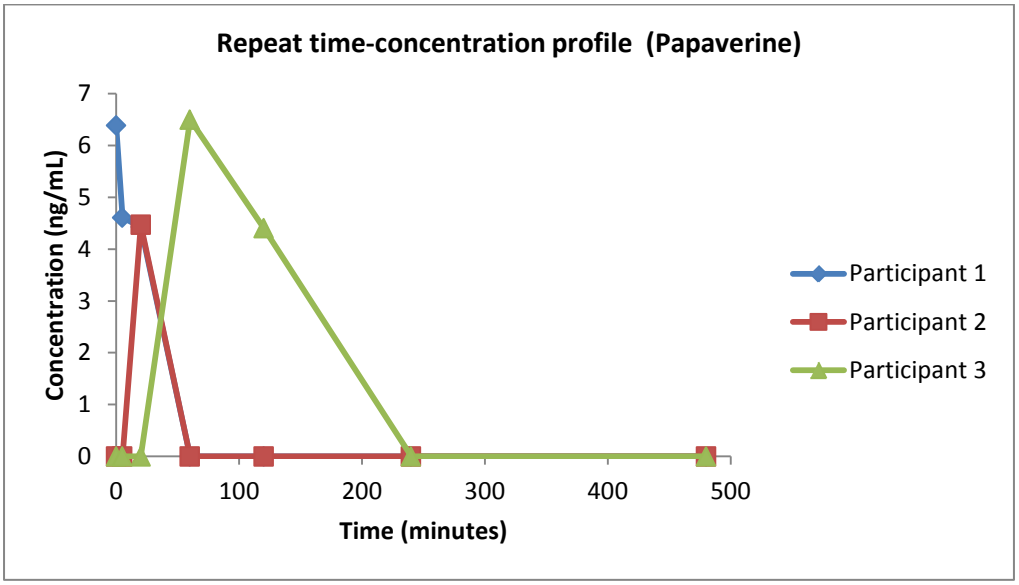


Figure 6.14 Combined participant time-concentration profile for papaverine obtained from the repeat study

Papaverine has not been reported in oral fluid in the scientific literature to date. As was highlighted in the case of thebaine (Section 6.3.5.3) and noscapine (Section 6.3.5.4).

6.3.5.6. Interpretation of oral fluid results

In 2003, Rohrig and Moore published their findings in relation to the presence of morphine and codeine in oral fluid of participants who consumed poppy seeds.[74] As discussed in section 2.1.2.1, the researchers found that it was possible to exceed the 40 ng mL^{-1} cut-off with the presence of morphine in oral fluid post-consumption of poppy seed bagels and loose poppy seeds. Since 2014, a group of researchers from the National Institute on Drug Abuse (NIDA), in the United States of America have published a number of papers on the interpretation of the concentration of morphine and codeine in oral fluid and urine of participants who consumed poppy seeds.[74,85,177,278] These studies report the findings of what appears to be one larger study where two quantities of 45 g of raw poppy seeds were consumed 8 hours apart by participants and oral fluid was collected (Section 2.1.2.1 for more information on the studies). From these publications, it was reported that it was possible to exceed the 40 ng mL^{-1} cut-off by the presence of morphine up to 1 hour post-consumption of the seeds. However, 90 g of raw poppy seeds does seem unrealistic, even if over an 8 hour period. As discussed in Section 5.2.2.2, the average poppy seed bread roll contains 1 – 4 g poppy seeds.[72] In this study, approximately 3.6 g of poppy seeds were ingested since two mini-muffins were consumed: none of the participants exceeded, or even came close to the 40 ng mL^{-1} cut-off recommended by SAMHSA and EWDTS. There are however a number of factors that should be considered in the overall interpretation of these findings.

It has been reported that the circadian rhythms of salivation in human biological systems can have an effect on the transfer of drugs from blood into saliva.[163] It is also possible that in the process of cooking the poppy seeds in the muffin matrix, the alkaloid content of the poppy seeds was reduced as it has been shown by a number of researchers that the cooking and processing of poppy seeds can greatly affect the alkaloid content: in 1998, Meadway, *et al.*[70] demonstrated that a difference in the opiate content of cooked, sieved and raw poppy seeds and concluded that the processing method prior to consumption would affect the alkaloid content ingested. Other studies showed that washing, grinding and baking could reduce the alkaloid content by up to 70%, depending upon the combinations of parameters used.[75] Since the poppy seeds used in this study were purchased from a supermarket and limited information was available on the treatment of the seeds, it is not possible to determine at this point if the processing of the seeds had any effect on the results obtained. A number of researchers[72,278] have also reported that the

variety of poppy seeds and the geographical origin can have an effect on the alkaloid content: again, this information was not available for the poppy seeds used in this study therefore it is possible that these parameters could have had an influence on the findings of this study.

Factors such as disease of an individual can greatly affect the production of saliva and the quality of the saliva, thus affect the transfer of drugs (Section 2.1.2): all participants in this study were healthy, drug-free individuals with no known sensitivity to opiates. The findings of this limited toxicology study are interesting since it has been reported by other researchers that it is possible to exceed the 40 ng mL^{-1} cut-off recommended by SAMHSA and EWDTS. However it is the opinion of this researcher that based on the reduction of alkaloids as a result of the baking process (Section 5.2.2.2) and the average of 1 – 4 g of poppy seeds consumed, that a negative roadside or workplace drug testing result in oral fluid would be found. In order to establish if these findings are representative, the study should be increased to include more participants, collection at different times of the day and to have more control of the amount of poppy seeds (and level of alkaloids) consumed by individuals.

7. CONCLUSION AND FURTHER WORK

7.1. **Conclusion**

The aim of this work was to establish and evaluate an LC-ESI-MS method for the detection and quantitation of morphine, codeine, thebaine, papaverine, and noscapine: the five major opium alkaloids.

The LC-ESI-MS method was initially developed using a reversed phase LC column and an ion trap octopole mass spectrometer: there were many challenges to overcome to achieve a method for the simultaneous detection of the five alkaloid compounds. The biggest challenge was solving the problem of the appearance of two peaks in the chromatogram for thebaine; both of which had very similar mass spectral data. Experiments were carried out using NMR, which established that two epimers of thebaine were being formed in the solvent chosen for sample preparation. When this solvent was changed to the aqueous mobile phase used in the LC method, one peak in the chromatogram was eventually achieved for thebaine.

The method was finally modified to include the use of a pentafluorophenyl phase with propyl spacer (PFPP) column and finally all five compounds could be detected and quantified. The established method included the use of water + 2mM ammonium formate + 0.2% formic acid mobile phase (A) and acetonitrile + 2mM ammonium formate + 0.2% formic acid mobile phase (B). The gradient method commenced with 90% mobile phase A, which was held for 2 minutes and then altered to 10% A over 8 minutes and held for 1 minute. The instrument was then re-equilibrated to 90% over 3 minutes. The ion transitions monitored were *m/z* 286→201 for morphine, *m/z* 300→215 for codeine, *m/z* 312→281 for thebaine, *m/z* 340→202 for papaverine, *m/z* 414→220 for noscapine and *m/z* 289→201 for morphine-d3, although at least two product ions were used to qualify each compound. This method was then transferred to an LC instrument hyphenated to a triple quadrupole mass spectrometer in the IRCGN toxicology laboratory in Paris for comparison and the results correlated to those found with the original method.

For evaluation, this method was applied to the analysis of poppy seeds for the detection of alkaloids: a comparison between harvested (raw) seeds, seeds which were baked on the surface of a bread roll and seeds thermally processed without bread matrix. The extraction

method employed chloroform:isopropanol (90:10, v/v) at pH 3.5 with ultrasonication for 10 minutes, followed by 10 minutes of centrifugation at 4000 rpm.

The extract was transferred to a clean glass tube, where it was dried down under nitrogen at 40 ^{o}C and reconstituted in 100 µL of aqueous mobile phase and filtered using a 0.22 µm nylon syringe filter.

Morphine, codeine, thebaine and noscapine were identified in poppy seeds from a number of supermarkets in the UK. There was much variation in the levels of alkaloids identified in poppy seeds. This variation can be attributed to a variety of natural parameters, such as weather and soil conditions, but also in the way that the seeds are harvested. Processing methods prior to packaging and even the baking process has been shown to greatly affect the level of alkaloids. When these results were compared to those of thermally processed poppy seeds, whether from the surface of a bread roll or were heated with no bread matrix, the levels of alkaloids (if detected) were considerably lower than in the harvested seeds. These findings correlate with the studies published in the literature.

Due to technical issues, the method was modified for use with an LC instrument hyphenated to a hybrid linear orbitrap for a toxicology study. For this study, two poppy seed muffins were consumed (~ 3.6 g of poppy seeds) and oral fluid was collected using Quantisal™ oral fluid collection kits at 5 minutes, 20 minutes, 1 hour, 2 hours, 4 hours and 8 hours post-consumption. Oral fluid was used as the biological matrix as it has been shown that opiate drugs will transfer from blood to saliva and thus to oral fluid; it can therefore provide information on recent drug use, and is easily collected and stored.

Extraction was carried out using Bond Elut Certify (130 mg bed mass, 3 mL volume) mixed mode cartridges; the method of extraction included conditioning of the cartridges with methanol and phosphate buffer (0.1 M, pH 6.0). The oral fluid sample was then applied and allowed to drip through under gravity. The cartridges were then washed with water and 0.01 M formic acid before being air dried for 10 minutes under full vacuum. 50 µL of methanol was added and the cartridge was dried for a further 2 minutes and the alkaloids were eluted using methanol: ammonia (98:2, v/v).The final extracts were then evaporated under nitrogen at 40 ^{o}C and reconstituted in 100 µL mobile phase A with each extract being filtered through a 0.22 µm nylon syringe filter.

Morphine was detected in oral fluid in the 5 minute sample in 5 out of the 6 participants; codeine was identified in the 5 minute oral fluid samples of only 2 of the 6 participants; thebaine was identified in 5 out of the 6 participants for up to 2 hours post-consumption; noscapine was identified in the 5 minute sample for 3 participants then decreased to zero before being identified in the oral fluid of 2 of the participants in the 1 hour sample. The noscapine level then decreased to zero at 2 hours and was not detected in any of the later samples collected. Papaverine was detected in the oral fluid of 3 of the 6 particiapnts but was not identified in the 4 hours post ingestion sample or in the subsequent sample collected. For all of the opium alkaloids identified, they were found in concentrations less than 10 ng mL^{-1}, apart from codeine identified the oral fluid of participant 1 (27 ng mL^{-1} at the 2 hour post ingestion time point).

In conclusion, a simultaneous LC-MS method for the detection and quantitation of five major opium alkaloids has been established and has been used to detect alkaloids in harvested poppy seeds and oral fluid samples. The method has been shown to be selective, precise and linear. From a small pilot toxicology study, oral fluid results indicate that levels of morphine and codeine do not exceed the SAMSHA and EWDT 40 ng mL^{-1} cut-off after ingestion of a realistic amount of poppy seeds contained within bakery products. Thebaine, papaverine and noscapine were also identified in the oral fluid samples for many of the participants and since this has been previously unreported in the scientific literature, this is a significant finding. Further investigation is however required to establish if these findings could add to the debate on whether it is possible to differentiate between poppy seed ingestion and administration of other opiate preparations, including heroin.

7.2. **Further work**

Although a sensitive and linear method was established for the detection and quantitation of five major opium alkaloids, the coefficient of variation values in repeatability studies for morphine, codeine, thebaine, papaverine and noscapine exceeded the 15% limit expected. In order to account for this factor, calibration graphs were produced on every occasion that poppy seeds or oral fluid extractions were analysed. Quality control samples were included at various points throughout the entire run to ensure the calibration graph produced was fit for purpose to determine concentrations of alkaloids in the samples. Other researchers

have published reproducible LC-MS methods for the detection of multiple alkaloids in poppy seeds and biological matrices. Newmeyer *et al.*[177] reported coefficient of variation values of less than 9% for morphine and codeine in both blood and oral fluid. Sproll *et al.*[76] also reported values of 9% or less for an LC-MS method where morphine, codeine, papaverine and noscapine were detected and quantified. Whether the inclusion of thebaine in the method has affected the overall repeatability of the method in this work should be investigated.

Since this method includes five alkaloids and morphine-d3 as an internal standard it is possible that competitive ionisation is taking place in the process of electrospray ionisation. Competitive ionisation can take place in ESI methods where multiple analytes are present in a mixture: ions are formed however factors such as the chemical structure of the analytes, the concentration of analytes in solution and the mobile phase used to introduce the analytes to the mass spectrometer have all been shown to have an effect in these cases.[217,222,238,259,260,283] In order to attempt to achieve repeatability, competitive ionisation could be investigated.

It was shown in this work that there is much variation in the levels of alkaloids present in poppy seeds and that this can be explained by the fact that they are a natural product which can be affected by external environmental factors that cannot be controlled. However, the method of extraction should be further investigated to establish overall extraction recoveries. In this work, a known amount of deuterated internal standard was added to the poppy seeds prior to extraction however, it should be considered that this is not a true representation of the "extracted" alkaloids. Most researchers in this field assume that most of the alkaloids identified from poppy seeds are as a result of external contamination of the surface of the seeds[72,75,76] but it is possible that alkaloids are present in the matrix of the seeds, even if it is a small amount. When the poppy seeds incorporated into a muffin matrix were analysed, the resulting chromatograms were very complex and it was concluded that the extraction method was not selective enough when the muffin matrix was present. Further work is required to modify the extraction and sample preparation method to eliminate the muffin matrix from chromatograms. An alternative method for the separation of the poppy seeds from the matrix could be identified as the results from poppy seeds in this matrix would add to the findings of this study.

In relation to the toxicology experiments carried out, it is proposed that the sample size of participants should be increased to determine if the findings are representative. Only 6 participants were included in this pilot study however using this data and from other publications, a power calculation should be carried out to establish the number of participants required for a full toxicology study.[284,285] In this work, it was found that based on a realistic ingestion of poppy seeds, no alkaloids were detected in oral fluid above the SAMSHA and EWDT 40 ng mL^{-1} cut-off. To extend this study, other biological matrices could be included. Blood is the best matrix as it can provide information on what compounds are present in the body at a given time: this makes it easier to interpret symptoms or effects that may be experienced by the individual.[144] Urine can provide a longer window of detection of since it is an elimination product of the body: drugs and/or metabolites can be detected in urine for up to 24 hours post-administration.[145,286] On this basis, it would advantageous to repeat the toxicology study to include the collection of oral fluid, blood and urine for the best conclusion to be drawn. Obtaining blood as part of the toxicology study would also allow for the determination of the saliva or oral fluid:plasma ratios of opium alkaloids. A number of articles published in the literature report the saliva:plasma ratios for morphine and codeine[83,167,170] but no reports currently exist with respect to thebaine, noscapine or papaverine. Establishing the saliva:plasma ratios for these compounds could make a good addition to knowledge on the subject.

At this current time, there is no published literature in this field which includes the detection of thebaine, papaverine or noscapine in oral fluid. When the toxicology study is continued, further work should include the investigation of the presence of these compounds in oral fluid. It would be beneficial to establish if these compounds can be identified in the oral fluid of individuals who use illicit heroin and in the oral fluid of individuals who administer legitimate opiate containing preparations. If thebaine, papaverine and noscapine are not detected in the oral fluid of these individuals, it may be possible to differentiate between the ingestion of poppy seeds/poppy seed products and other opiate containing drugs (both licit and illicit). However, much work will be required to establish if this is the case.

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