ANGLIA RUSKIN UNIVERSITY

THE DETECTION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN KERATINOUS MATRICES

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ANGLIA RUSKIN UNIVERSITY
ABSTRACT

FACULTY OF SCIENCE AND TECHNOLOGY

DOCTOR OF PHILOSOPHY

THE DETECTION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN KERATINOUS MATRICES

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The problems of non-steroidal anti-inflammatory drugs (NSAIDs) as environmental contaminants is an area of concern. NSAIDs are heavily relied upon to treat pain and inflammation. With such prevalence, these compounds are now entering the environment via many routes, such as water discharge and contaminated food. This results in subsequent exposure and effects on various animal species. One such example is diclofenac, which was associated with the extinction of Gyps vultures in Asia. The detection of diclofenac was based on post-mortem samples i.e. after a large decline in populations. In this research non-invasive samples i.e hairs and feathers are analysed pre-mortality as a preventive measure for early detection.

A simultaneous liquid chromatography-mass spectrometry (LC-MS) method for detection of eighteen compounds, either of known toxicological effects or future threat (NSAIDs - aceclofenac, carprofen, diclofenac, flunixin, ketoprofen, mefenamic acid, meloxicam, nimesulide, phenylbutazone, piroxicam and suxibuzone; metabolites - oxyphenylbutazone, 3-hydroxymethyl mefenamic acid, 4-hydroxydiclofenac, 4-hydroxynimesulide, 5-carboxymeloxicam, 5-hydroxyflunixin and 5-hydroxypiroxicam) has been developed and validated. A newly optimised sample preparation method was applied to hairs/feathers.

Precision of the analytical method was within 10% relative standard deviations for the majority of compounds. Recoveries averaged 83% and limits of detection (LOD) ranged 0.01 to 0.2µg/g. For diclofenac, flunixin, mefenamic acid, oxyphenylbutazone, piroxicam and 5-hydroxyflunixin, LODs were lower than previously reported. Various animal hairs/feathers were analysed (n=20) and in two samples piroxicam and phenylbutazone were individually detected, at 1.2µg/g ± 0.002 and 1.8µg/g ± 0.011 respectively.

The LC-MS method reported here has been validated for the first time using animal hair/feather samples. This range of NSAIDs and metabolites have never been reported before. LODs and LOQs of metabolites are reported for the first time. The detection of piroxicam and phenylbutazone in feathers highlights the viability of testing keratinous matrices.

Keywords: NSAIDs, diclofenac, liquid chromatography-mass spectrometry, feathers, non-invasive

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LIST OF ABBREVIATIONS

[M+]	Molecular ion
[M+HCO_2H]	Formic acid adduct ion
[M+ HCO_2-H]$^-$	Molecular ion plus formic acid minus one hydrogen
[M+H]-	Molecular ion plus one hydrogen
[M-2H]-	Molecular ion minus two hydrogens
[M-H]$^-$	Deprotonated molecular ion
[M-H-CO2]$^-$	Molecular ion minus one hydrogen and one carbon dioxide
£	British pounds
>	Greater than
<	Less than
≥	Greater than or equal to
≤	Less than or equal to
±	Plus or minus
=	Equal to
^{0}C	Degrees celcius
%	Percentage
∝	Selectivity
3HMA	3-hydroxymethyl mefenamic acid
4HN	4-hydroxynimesulide
4HD	4-hydroxydiclofenac
5CM	5-carboxymeloxicam
5HF	5-hydroxyflunixin
5HP	5-hydroxypiroxicam
A	Eddy diffusion
AC	Alternating current voltage
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
A_S	Asymmetry factor
B	Longitudinal diffusion
BNF	British National Formulary
BSE	Bovine spongiform encephalitis or mad cow disease in livestock
C	Mass transfer
C1	Confirmation ion 1
C2	Confirmation ion 2
CDER	Centre for Drug Evaluation and Research
cm	Centimeter

CNT	Coxib and traditional NSAID Trialists
CO_2	Carbon dioxide
COX	Cyclooxygenase enzyme
COX-1	Cyclooxygenase enzyme isoform 1
COX-2	Cyclooxygenase enzyme isoform 2
CYP450	Cytochrome P450 enzymes
Da	Dalton
DAD	Diode array detection
DC	Direct current
DL	Desolvation line
EIC	Extracted ion chromatogram
EMA	European Medicines Agency
EMEA	European Agency for the Evaluation of Medicinal Products
ESI	Electrospray ionisation
ESI^{-+}	Electrospray ionisation positive or negative mode
EU	European Union
FDA	Food and Drug Administration
G.	*Gyps*
GC	Gas chromatography
GC-MS	Gas chromatography coupled with mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
GI	Gastrointestinal tract
HETP	Height Equivalent to the Theoretical Plate
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography coupled with mass spectrometry
Ibuprofen-d_3	Deuterated ibuprofen
ICH	International Conference for Harmonisation
ID	Internal diameter
IUCN	International Union for Conservation of Nature
IUPAC	International Union of Pure and Applied Chemistry
k	Capacity factor
k_1	Capacity factor of less retained peak
k_2	Capacity factor of more retained peak
L/min	Litre per minute
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled with mass spectrometry

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limits of detection
Log C	Logarithm of Concentration
LOQ	Limits of quantification
M^+	Molecular ion
m/z	Mass-to-charge ratio
mg/ml	Milligram per millilitre
MHRA	Medicines and Healthcare Products Regulatory Agency
ml	Millilitre
mm	Millimeter
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
N	Plate number
NATA	National Association of Testing Authorities
NSAIDs	Non-steroidal anti-inflammatory drugs
ODS	Octadecylsilyl
OXY	Oxyphenylbutazone
PDA	Photo diode array
PAR	Peak area ratio
piroxicam-d_3	Deuterated piroxicam
pKa or	Acid dissociation constant values
pKb	Base dissociation constant values
Q	Quantification ion
Q-Q	Quantile-Quantile
Q/C1	Ratio of quantifier ion and first confirmation ion
Q/C2	Ratio of quantifier ion and second confirmation ion
R^2	Correlation coefficients
R/C	Mean relative response divided by concentration
RMM	Relative molecular mass
rpm	Revolutions per minute
RRF	Relative retention factor
RSD	Relative standard deviation
RSPB	The Royal Society for the Protection of Birds
RT	Retention times
R_s	Resolution
S/N	Signal to noise ratio

SAVE	Saving Asia's Vultures From Extinction
Scan	Scanning mode
SIM	Selected ion monitoring mode
SPE	Solid phase extraction
MP at T_0	98:2 % mix of water and acetonitrile
$t_{½}$	Half life
t_0	Dead time of column
t_r	Retention time
TIC	Total ion chromatogram
TMS	Trimethylsilyl
µg/g	Microgram per gramme
µg/L^{-1}	Microgram per litre
µg/ml	Microgram per millilitre
UHPLC	Ultra-high performance liquid chromatography
µl	Microlitre
µm	micrometer
UK	United Kingdom
UNODC	United Nations Office on Drugs and Crime
UPLC –MS	Ultra-performance coupled with mass spectrometry
USP	United States Pharmacopeia
UV	Ultraviolet
$W_{0.5}$	Peak at half width
WADA	World Anti-Doping Agency
WHO	World Health Organisation

LIST OF APPENDICES

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CHAPTER 1: NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND THEIR PRESENCE, DETECTION AND IMPACT IN THE ENVIRONMENT

1.1 RESEARCH RATIONALE

With newer pollutants, such as pharmaceuticals being detected in the environment and resulting in concerns of exposure to wildlife, conservation practices are now starting to utilise forensic analytical techniques. Wildlife forensics has a new challenge in dealing with the exposure of NSAIDs as endangered species are now at risk, namely the *Gyps* vulture species, birds of prey such as eagles, and water species such as the water vole, living in contaminated waterways. First demonstrated with the devastating population declines of three *Gyps* vulture species (discussed in section 1.2), these compounds are entering the environment via many routes including, treated water discharge or contaminated food sources. It is not surprising such compounds enter the environment owing to their prescription and availability over-the-counter. This is due to the situation that medical and veterinary professionals rely heavily on these pharmaceuticals to treat pain and inflammation of muscles, bones and joints. Further risks from exposure to such NSAIDs are now more apparent with growing research into the effects of NSAIDs in the environment (Arnold, et al., 2013; Cuthbert, et al., 2011; Hutchinson, et al., 2015; Mandal and Khadka, 2013; Zorilla, et al., 2014).

These compounds are already resulting in mass mortality (section 1.2) and continue to be of threat, yet remarkably, no methods have been published using alternative samples such as hairs and feathers, allowing for sampling pre-mortality. These alternative samples can be collected ethically without stress to the animal. This is especially important in species with already faltering numbers when conservation is paramount. Testing keratinous matrices pre-mortality will aid in early detection of NSAIDs, which could address the early signs of poisoning of protected species to prevent mass mortality and aid in the provision of treatment to the species effected by such compounds. Furthermore, it would help conservation efforts to remove exposure. Therefore this current project centres on the simultaneous detection NSAIDs in non-invasive samples (feathers and hairs).

This research includes a thorough literature review, providing essential background information on the known threats of NSAIDs, such as diclofenac, in the environment and the threats they pose to specific species, most notably vultures in India. Some understanding of the toxicity by relating the chemistry and the chemical process of NSAIDs, has allowed for an informed selection of metabolites, enhanced by a thorough

literature review, as included in this research. This enabled the research to centre on the NSAIDs and metabolites currently of concern and/or of future threat. Alongside considering current and future implications of exposure to NSAIDs to endangered species (Cuthbert, et al., 2011; Richards, et al., 2014; Sharma, et al., 2014; Zorilla, et al., 2014).

It has been documented, that other birds of prey and aquatic species, for example otters, are now at risk of NSAID exposure (Brodin, et al., 2013; Richards, et al., 2011; Sharma, et al*.,* 2014; Zorilla, et al., 2014). As reported in the vulture crisis (section 1.2.2) Oaks, et al. (2004) hypothesised that species are exposed to NSAIDs through the consumption of dead livestock that have been treated with these pharmaceuticals (section 1.2.2). In a recent publication by Sharma, et al. (2014), residues of diclofenac was detected in kidneys, liver, heart and spleen samples of two steppe eagles (*Aquila nepalensis*). From necropsy and histopathological studies, the authors concluded that the mortality was attributed to diclofenac toxicity. Furthermore, from these results the authors deduced that NSAIDs may be toxic to other birds of prey, such as eagles. Steppe eagles are closely related to the golden eagle (*Aquila chrysaetos*) in the UK and Europe and the vulnerable Spanish imperial eagle (*Aquila adalberti*). These birds are all at risk of renal failure (also known as kidney failure, in which kidneys fail to remove waste products from the blood) caused by diclofenac (Sharma, et al., 2014), (section 1.2.2) highlighting the need for diclofenac poisoning to be considered as a global problem and an early detection system to be made available.

Moreover, pharmaceutical compounds and their metabolites are entering waterways in the UK via human waste through treated and untreated municipal wastewaters. Conventional treatment facilities are not designed for the removal of these compounds and consequently, do not remove them, during waste water processing (Jelic, et al., 2012). Currently, there are more than 3000 pharmaceuticals licensed for use in the UK, of which NSAIDs make up twenty two (MHRA, 2010). Consequently it is not feasible to monitor such a large number of compounds (Jones, Voulvoulis and Lester, 2005). With the wide-ranging chemical and physical properties of these pharmaceuticals, water treatment facilities must have a selection process by which they identify those to test for (Jones, Voulvoulis and Lester, 2005). Hence, treatment facilities often only test for common compounds, such as acetaminophen and ibuprofen, due to their prevalence, and antibiotics, such as sulfamethoxazole (Jones, Voulvoulis and Lester, 2005; WHO, 2011).

There have been a few published studies documenting the presence of other pharmaceutical compounds. A study by Brodin, et al. (2013) showed how the benzodiazepine anxiolytic drug oxazepam altered the behaviour and feeding rate of wild European perch (*Perca fluviatilis*) at concentrations (1.8µg/L^{-1}) related to those detected in effluent surface waters. Authors suggested that the altered behaviour could be detrimental to the perch depending on whether they were at risk from predators. Therefore exposure risks to other aquatic species, beyond fish, are of concern. Waterways in the UK are home to otters and mink amongst others; the NSAIDs diclofenac and ibuprofen have already been detected in samples from otters (Richards, et al., 2011b). Richards (2011) reported the presence of diclofenac and ibuprofen in otter hair samples. Their findings showed external exposure within the aquatic environment, whilst suggesting that the presence of these two NSAIDs could have contributed to pathological lesions that were identified in the otter carcasses. These results highlight the potential exposure via digested material by the food chain or through drinking.

The detection of NSAIDs in waterways are especially concerning considering the potential exposure to protected aquatic species, such as the water vole (*Arvicola amphibious*), whose population declines have been reported in Britain (IUCN, 2014), thus putting the re-introduction at potential risk. If this were to be the case, the implementation of non-invasive samples is even more important where ethical considerations can restrict or forbid invasive sampling of protected species (as discussed in section 1.1.1).

This research has developed a new analytical method to analyse aceclofenac, acetylsalicylic acid, carprofen, diclofenac, flunixin, ibuprofen, ketoprofen, mefenamic acid, meloxicam, nimesulide, phenylbutazone, piroxicam and suxibuzone and seven major metabolites, oxyphenylbutazone, 3-hydroxymethyl mefenamic acid, 4-hydroxydiclofenac, 4-hydroxynimesulide, 5-carboxymeloxicam, 5-hydroxyflunixin and 5-hydroxypiroxicam. This research is novel in its approach to detecting not only this unique selection of NSAIDs, but also major metabolites of some of the parent compounds, which has never been addressed in this field before. In addition, the method also offers the detection of parent compounds (for example aceclofenac and piroxicam) that have not been previously analysed in one simultaneous method. Furthermore, it includes NSAIDs that have potential future threat and/or available on the market in areas of still large populations of vulture, such as in the Indian

subcontinent and have been neglected in current published methods (Taggart, et al., 2009).

1.1.1 Importance of developing new detection methods and alternative samples

In the field of wildlife forensics, current methods report the use of post mortem tissue samples for the detection of NSAIDs in endangered species (Mishra, et al., 2001; Shultz, et al., 2004). However, environmental conditions, such as extreme heat causing decay could impact on the quality and stability of analytes in these samples (Ng, et al., 2006; Mishra, et al., 2001; Richards, et al., 2014; Shultz, et al., 2004). The analysis of hair (section 1.4) in forensic and environmental investigations has gained increasing acceptance as a viable alternative to tissue and blood analysis. The analysis of alternative matrices, such as hair, is not only environmentally robust but durable too. Such analysis allows for a much wider window of detection, from months to even years, after administration or exposure (Baumgartner, et al., 1989; Jickells and Negrusz, 2008). Therefore the focus of this research is an investigation on the use of animal hair and feathers for the simultaneous detection of the NSAIDs.

The use of invasive samples can be restricted or even forbidden due to ethics. For example sampling blood from a live animal would cause undue stress. Richards, et al. (2014) states this is especially important in the case of protected populations with already faltering numbers. To avoid stress to animals during sample collection, non-invasive samples should be the preferred choice.

When analysing non-invasive samples, i.e. feathers, all of the sample can be utilised rather than the potential loss experienced in a degraded conventional tissue sample i.e. muscle, and enables analysis pre-mortality as well as post-mortality. Owing to low populations of endangered species and the continued threat from NSAIDs, there is an urgency to use non-invasive samples. It is therefore important that such methods are developed and validated, published and adopted for the protection of threatened species, such as eagles, vultures and water vole, this also aids in identifying other species at risk of exposure.

As such, this research developed and validated a simultaneous detection method that detects multiple compounds utilising liquid chromatography coupled with mass spectrometry (LC-MS). Furthermore, alternative samples, such as hair and feathers were used, as opposed to traditional blood and tissue samples. This research allows the continued improvement in wildlife and environmental monitoring, and wildlife

forensic investigations. Thus the newly validated method will not only aid in a timely response to wildlife mortality incidences, but could offer a means to monitor exposure of NSAIDs in other species, whilst enabling monitoring, ethically, in live animals. It is useful in alerting conservationists of possible future poisoning before another species is at the brink of the extinction. Hence, additionally giving an alternative method to post-mortem samples used currently. It also provides a new simultaneous method to identify such pollutants in the field of environmental and analytical sciences for other matrices. This project presents development in an emerging area of research which contributes to areas of environmental and wildlife monitoring. In turn it is essential in strengthening the worldwide wildlife and environmental monitoring effort further.

1.2 THE VULTURE CRISIS AND THE ROLE OF NSAIDS

The vulture crisis centred on the mass mortality of three *Gyps* vulture species, namely the Oriental white-backed vulture (*Gyps bengalensis),* long-billed and slender-billed vultures (*Gyps indicus* and *Gyps tenuirostris*, respectively), where populations rapidly declined to a loss of more than 95% (Prakash, 1999; Prakash, et al., 2003). Some researchers have even estimated a decline of 99.9% in some species and predict extinction in the wild in the near future (Markandya, et al., 2008; Oaks, et al., 2004; Prakash, 1999; Prakash, et al., 2003).

This section details, the early proposed causes of vulture demise and the difficulty in identifying the root cause of such mass mortality. It describes the break-through and consequently the role of diclofenac in the vulture crisis and ultimately, reflects on the phasing out of diclofenac. The ongoing conservation efforts and continued monitoring of diclofenac is also included.

1.2.1 Reported decline in vulture populations, early proposals

Decreases in vulture populations were first reported in 1999 in a publication by Prakash researching the status of vultures in Keoladeo National Park in Rajasthan (Prakash, 1999). The author recorded 95% decline in populations across three species of *Gyps* vultures; the oriental white-rumped vulture (*G.bengalensis*), the long-billed vulture (*G. indicus*) and Slender-billed Vulture (*G.tenuirostris*). At this early stage, most other populations were stable and therefore the study concluded that other declines were the result of a natural reduction in food availability and even persecution by humans and poisoning. At this stage *Gyps* vulture were not deemed to be at threat so no conservation effort was put in place.

In a follow up study by Prakash, et al. (2003), they explored different hypothesis for these declines in vultures. Spatial patterns were considered and evidence of regional decline was researched to see whether they differed between protected areas and outside. These hypothesis were assessed to see if food availability could have caused the decline. In this early major study, conclusions were drawn and in turn initial hypothesis were proven incorrect, as food source was readily available and no significant differences in regional declines were reported (Prakash, et al., 2003). The rapid decline was not seen in other birds of prey and unprecedented beyond previously reported cases. Declines proved independent of any spatial patterns, whether protected or nominal geographical area, and no pesticides or metals had been detected. Therefore, the authors concluded infectious disease was the most probable cause of the vulture decline due to the absence of chemical contamination causing the abnormal mass mortality.

Declines in populations were not restricted to India but in fact were mirrored in Pakistan (Gilbert, et al., 2006) and Nepal (Baral, et al., 2005, Chaudhary, et al., 2011). Some publications have since showed populations of the oriental white-rumped vulture declining on average at a rate of 43.9% per year in India (between 2000 and 2007) (Prakash, et al., 2007). Whilst in the Punjab province of India and in Pakistan the decline ranged between 11 and 61% (Murn, et al., 2002). Decline in Nepal was reported to be 14% from 2002 and 2011 (Chaudhary, et al., 2011). Whilst the rate of decline, today, are slower than those initially reported in the late 1990s, further declines in this protected species, with already low numbers, are of growing concern (Cuthbert, et al., 2011).

1.2.2 The breakthrough of the cause of vulture mass mortality

A major study carried out by Oaks, et al. (2004) to identify a probable cause of this case of mass mortality in *Gyps* vultures centred on post mortem analysis of 259 adult Oriental white-backed vultures, where authors reported urate (the salt of uric acid) deposits on the surface of internal organs. This lead to conclusions that the death of these vultures was caused by visceral gout, resulting in renal failure (Oaks, et al., 2004). To verify renal disease and to determine the cause, detailed diagnostic tests were carried out. In the post mortem studies by Mishra, et al. (2001), it was proposed that the presence of excess abdominal fat indicated that vultures were still consuming food at the time of death. These results again indicated a lack of food was not the primary cause of death (Shultz, et al., 2004) supporting Prakash, et al. (2003).

However, Shultz, et al. (2004) findings were in line with Oaks, et al. (2004), maintaining the same outcome of visceral gout in the dead vultures.

Considering the vultures food source, Oaks commented that domestic animals are likely to receive different veterinary care than wild animals, in such cases pharmaceuticals are often prescribed to treat livestock for common ailments thus, in turn increasing the risk of exposure. The authors investigated the food source of the Oriental white-backed species, showing it to be mostly dead domestic livestock and hypothesised that ingested veterinary pharmaceuticals via the consumption of contaminated carcasses might be responsible for the renal failure in the scavenging birds. Surveys were conducted in the region, covering veterinarians and pharmaceutical retailers, in the hope to identify pharmaceuticals known to have nephrotoxic properties, administered orally and what is prescribed routinely. The only drug identified to meet both criteria was the NSAID, diclofenac.

NSAIDs were first introduced in the 1970s, for human use as an anti-inflammatory and analgesic pharmaceutical (Smith, 2010). Diclofenac was initially registered for veterinary use in India in 1994 and in Pakistan in 1998 and was widely prescribed to treat lameness in domestic animals (Richards, 2010). However, it is well known to have a side effects causing hepatotoxicity and the increase in the concentration of uric acid in the kidneys resulting in renal failure (Mishra, et al., 2001; Shultz, et al., 2004). Research by Oaks, et al. (2004) revealed that the selling and use of diclofenac was prevalent on the Indian veterinary market at the time of the crisis; multiple companies were found to be selling it to treat livestock with an annual treatment estimate of approximately 10 million animals (Oaks, et al., 2004). Typically NSAIDs are used for the treatment of working animals to alleviate lameness and in turn increase their productive working life, this is especially important in a country where economy is relied on the working life of livestock (Richards, 2010).

Oaks, et al. (2004) detected residues of diclofenac, in the kidneys of Oriental white-backed vultures at concentrations of 0.051 to 0.643 µg/g after analysis using LC-MS (single compound analysis only). It was deduced that diclofenac was the most probable cause of mortality, and more specifically it was the exposure through consumption of contaminated carcasses with diclofenac, that ultimately led to renal failure and the demise of these vultures. Since initial research by Oaks, et al., (2004) proposing the actual cause of the mass mortality of *Gyps* vultures, many more papers have been published with similar findings (Green, et al., 2004; Shultz, et al., 2004). Shultz, et al.

(2004) concluded that vulture's corpses, without symptoms of visceral gout, contained no detectable residues of diclofenac. Therefore it was concluded that the occurrence of gout was related with the presence of diclofenac residues. How diclofenac causes visceral gout in vultures is still not fully understood (Cuthbert, et al., 2007b; Meteyer, et al., 2005) and is discussed in more detail in section 1.3.7. Studies have suggested the occurrence of diclofenac may initiate blood deficiency in the renal portal whereby valves open and close at random intervals redirecting the blood supply. This results in visceral gout after a build-up of uric acid in the bloodstream (Meteyer, et al., 2005).

1.2.3 Phasing out the use of diclofenac

With vultures at the brink of extinction it was necessary to set out conservation efforts and protection. Following the confirmation that diclofenac was the root cause of the mass mortality, the International Union for Conservation of Nature (IUCN) (IUCN, 2004) listed many species, including the *Gyps*, as critically endangered. However with such wide use and readily available sources of diclofenac on the Indian subcontinent, researchers called for conservation intervention and urgent action to be taken (Green, et al., 2004). This was to be of upper most importance in the main animal reserves that house endangered species for protection to prevent the continued exposure to contaminated carcasses (Oaks, et al., 2004; Green, et al., 2004; Shultz, et al., 2004). Green, et al. (2004) highlighted that the only probable way to achieve this urgent action was by setting out a ban on the diclofenac. Thus, the Indian government led a ban on diclofenac use, prohibiting both its manufacture and importation which came into force in May 2006. This was also followed with legislation being introduced in Nepal and Pakistan in the same year (Cuthbert, et al., 2011). Researchers, did however, note that the legislation banning the use of diclofenac would cause a likely increase in use of other NSAIDs, therefore further research to identify safer alternatives would be needed (Cuthbert, et al., 2007; Green, et al., 2004; Oaks, et al., 2004).

Until this point, only flunixin and ketoprofen had been reported to cause renal disease in chickens, cranes, quail and the African white-backed vultures (*Gyps africanus*) (Oaks, et al., 2004). Hence, it was vital to find alternative NSAIDs that would be relatively non-toxic and could offer a replacement for diclofenac use in the veterinary treatment of livestock. Prior to the ban in 2006, tests on the safety of the NSAID meloxicam were carried out on vultures after oral administration and feeding with tissues from meloxicam dosed cattle (Swan, 2006; Swarup, et al., 2007). It was found to be a suitable alternative to diclofenac owing to its low toxicity to vultures (Cuthbert,

et al., 2011, Naidoo, et al., 2007; Swan, et al., 2006; Swarup, et al., 2007). The mechanisms of how this pharmaceutical works is discussed further in section 1.3.7.

Despite the ban of diclofenac, there have since been discoveries of the pharmaceutical still being used; to conservationists this is of great concern (Cuthbert, et al., 2011). Therefore conservationists are frequently monitoring the use of diclofenac and steps are continually put into place to eradicate its use from agricultural farming systems in the Indian subcontinent. Cuthbert, et al. (2011) reported that diclofenac from human medication is being illegally used for veterinary purposes and summarises data collected from pharmacy visits from one and half to four years after the ban on diclofenac came into force. Across eleven Indian states it was apparent that diclofenac was still being sold on the market at considerably low cost compared to the safer alternative meloxicam. Mandal and Khadka (2013) reported that meloxicam was double the cost of diclofenac for the same size. For example, a 30ml vial costs 72 Indian Rupee respectively (£0.77) compared to 35 Rupee (£0.37) for diclofenac. With the high cost of the safer alternative it is no doubt that this explains, a slower use of meloxicam and the illegal use of diclofenac (Mandal and Khadka, 2013).

Another concern is, the majority of Nepalese and Indian pharmacists are not linking diclofenac with the vulture mortality, despite government awareness programs and training (Cuthbert, et al., 2011). It is also of interest to note that there are also calls for incentives to encourage the use of meloxicam, in order to attempt to break the heavy reliance on diclofenac (Cuthbert, et al., 2011; Mukherjee, et al., 2014).

In Cambodia, the use of diclofenac is entirely absent and studies have shown an increase in populations of *Gyps* vultures species since 2004 (Clements, et al., 2012). The noticeable outcome of recent studies into the availability of meloxicam is encouraging however, it is the sale of diclofenac and its illegal use that continue to be of concern. Conservationists are continually working on potential actions to eliminate the misuse and miss-selling of diclofenac. Measures to alter or restrict vial sizes and increase the price of diclofenac have been set out to make them less practical for veterinary use (Cuthbert, et al., 2011). The average cost of injectable diclofenac in 3ml vials is 2.1 - 4.1 Indian Rupee (£0.02 – 0.04) according to Cuthbert, et al. (2011). Veterinarians typically inject a 30ml vial of diclofenac when treating cattle thus by restricting vial sizes to 3ml, the cost of treatment with will raise to 62 -123 Indian Rupee (£0.62 – 1.24) acting as a deterrent to the misuse of diclofenac. Work to achieve the removal of diclofenac in excess of 3ml capacity is ongoing (Mukherjee, et al., 2014).

Recently, concerns of the use of diclofenac have spread to Europe after conservation foundations reported an Italian veterinary medicine company, FATRO, using loopholes in risk assessment protocols to have diclofenac approved for veterinary use in Italy and Spain (Richards, et al., 2014). Spain has the largest population of European vultures and if the presence of NSAIDs in livestock carcasses were in the food source of the vultures in the EU, the repercussions could be detrimental for the already faltering *Gyps* species (Richards, et al., 2014).

1.2.4 Ecological implications of vulture crisis

Ongoing threats extend beyond the use and ingestion of such pharmaceuticals; not only has the vulture demise led to considerable population decline, but as a result the ecological balance has quickly shifted. Vultures are remarkable scavengers and are key species in many ecosystems; they can safely digest a rotting carcass that may be lethal to other species and in the process they act as the cleaners of the environment removing potential toxic bacteria and infections (Prakash, et al., 2003; Stoyanova, Stefanov, and Schmutz, 2010). Concerns of the potential cultural and economic repercussion from the loss of these 'cleaners' extends to other ecosystems and their integrity (Markandya, et al., 2008). Therefore additional exposure of diclofenac could not only result in further mortality but could be damaging to the many other ecosystems which were never considered at risk before. With the loss of the vulture populations, there is a potential for increased occurrence of feral dogs and rats. Not only are these far less effective scavengers, than vultures, it could result in an increase of diseases attributed to dog and rat populations. Consequentially this could further kill other livestock and threaten human population (Markandya, et al., 2008).

There are ecological implications beyond the Indian subcontinent population of vultures too. Following the EU outbreak of Bovine Spongiform Encephalitis (BSE) or mad cow disease in livestock in the year 2000, carcasses and vulture feeding sites were controlled. The EU stipulated that carcasses must immediately be disposed of and authorities were to manage the food source given to vultures. There is a concern that this may change the feeding and foraging behaviour of vultures (Richards, et al., 2014). The safety of these sites has not yet been confirmed and carcasses that are given to the vultures may be intensively reared and hence could be from heavily medicated stock (Richards, et al., 2014). Thus, it is more important than ever to utilise screening methods, such as the employment of unconventional samples, before another mass mortality instance can occur in species at risk of exposure. Carcasses should be

continually monitored; conventional tissue samples can be utilised in the detection of NSAID residues however, the condition of these samples must be considered. Where conventional samples are decomposed, the use of unconventional samples are much more reliant and are not affected by environmental conditions. This research uses non-invasive samples which would aid current screening methods but also provides a viable alternative to traditional samples that may have degraded. The utilisation of a simultaneous detection method, as developed in this research, enables the detection of a variety of NSAIDs, including metabolites, in non-invasive samples.

1.3 AN OVERVIEW OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

Owing to the threat of NSAIDs on various species and the preceding literature, this section discusses the choice and selection of NSAIDs used in this research. It examines the chemical properties and structures of these NSAIDs and aims to relate the compounds to their potential toxicity. This section describes the use of NSAIDs (section 1.3.1), particularly in both human and veterinary medicine and pays attention to the mechanism of action in the body (section 1.3.6); how such compounds are released at the injury site are inhibited through specialist enzyme. It includes the modes of administration (section 1.3.5), mechanism of action and ultimately the adsorption, distribution and metabolism in the body (section 1.3.7). Particular interest is paid to human administration of NSAIDs due to the lack of literature on animal administration and mechanism of action, thus by understanding this, suggestions could be made on the little known information of NSAIDs action on animals.

1.3.1 The definition and uses of NSAIDs in human and veterinary medicine

There are more than fifty compounds that fall under the category of NSAIDs on the global market. These are pharmaceutical compounds that are used as everyday medicine for both human and veterinary treatment. NSAIDs include a range of compounds that are commonly used for pain relief and in the treatment of joint inflammation (Saraf, 2008) and are classed as non-steroidal to distinguish them from corticosteroid compounds which have similar actions, as discussed in section 1.3.7.

NSAIDs are not restricted to human use, as demonstrated by diclofenac as a veterinary pharmaceutical and its role in the vulture crisis (section 1.2). In veterinary treatment, NSAIDs are administered to livestock for pain relief and to manage conditions such as arthritis, joint lameness and laminitis, musculoskeletal discomfort, visceral pain and

post-operative pain (Mandal and Khadka, 2013). The use of NSAIDs in veterinary treatment is widespread due to their reliability, quick acting effects and low costs (Lees, et al., 2004; Suojala, Kaartinen and Pyorala., 2013) these are especially important in areas where conditions such as lameness can slow farming production. Animals are often used as working machines, vital to the livelihood of the farmer, thus the relied use of NSAIDs in working animals can increase their productive working life.

In human medicine NSAIDs are routinely implemented in the treatment of pain and stiffness resulting from inflammation and are most commonly prescribed for the treatment of rheumatic conditions such as arthritis (BNF, 2012). Whilst these compounds are prescribed, millions are continually bought over the counter making them among the most widely used drugs in the world (CNT, et al., 2013). These compounds have three main long lasting properties; anti-inflammatory, antipyretic and analgesic effects (Modi, et al., 2012) making them particularly useful for the treatment of continuous or regular pain as in rheumatic conditions. Differences in anti-inflammatory activity between NSAIDs are small; often there are considerable variations between users in both the response and tolerance (BNF, 2012). The British National Formulary, BNF (2012), states that up to 60% of patients respond to most common NSAIDs, such as, Ibuprofen or diclofenac. If patients do not respond to one of the main NSAIDS, they often respond well to an alternative. Therefore, alternatives to NSAIDs are not developed and reliance on NSAIDs is perpetuated. Consequently, this heavy reliance on these pharmaceuticals makes their prevalence in the environment a common occurrence.

1.3.2 Current NSAIDs known to be of threat

The research into NSAIDs and their exposure to endangered species, such as *Gyps* vulture, is limited, with safety testing carried out on only six NSAIDs, whereby five of which have proven toxic (Cuthbert, et al., 2015; Naidoo, et al., 2010; Oaks, et al., 2004; Swan, et al., 2006; Swarup, et al., 2007; Sharma, 2012; Zorilla, et al., 2014). Currently there is only one published analytical method representing the detection of NSAIDs in livestock tissue (Taggart, et al., 2009). Furthermore, there are only a handful of publications on the detection of NSAIDs in non-invasive samples such as animal hair (Richards, et al., 2011 and Richards, et al., 2011b). Conservationists and researchers have reported on the availability of a number of NSAIDs in areas of threat (Cuthbert, et al., 2011; Mandal and Kadka, 2013). This, in turn has provided an updated list of such

compounds that may pose a threat to endangered species as considered in this project.

Previous studies have also highlighted that other NSAIDs; carprofen, flunixin, ibuprofen, phenylbutazone and ketoprofen have been associated with mortality in chickens, cranes, quail and the African white-backed vultures (Cuthbert, et al., 2007b; Oaks, et al., 2004). Most recently, concerns have been expressed over the NSAID nimesulide (Figure 1.11) after its consumption by vultures has been associated with visceral gout and has similar toxic effects to diclofenac (Cuthbert, et al., 2015). Additionally, hepatotoxicity has been reported when used in human and veterinary medicine by Modi, et al. (2012). These studies have led to much debate on the safety of nimesulide, with the toxicity of still not understood despite its sale in pharmacies (Modi, et al., 2012), therefore, nimesulide has been included in this research (section 1.3.3, Table 1.1)

Another NSAID identified as a threat is ketoprofen; studies have found ketoprofen is toxic to vultures and has been found to be present in livestock carcasses in India (Naidoo, et al., 2010; Taggart, et al., 2009). Safety testing has been carried out to deduce symptoms of toxicity and clinical signs of necropsy or visceral gout and kidney damage related to ketoprofen mortality (Naidoo, et al., 2010). Research into the availability of ketoprofen on the market showed that 33% of Indian pharmacies stock six brands of ketoprofen, suggesting it has become widely used (Cuthbert, et al., 2011; Taggart, et al., 2009). Furthermore, ketoprofen and phenylbutazone have also been reported to be toxic to other avian species after studies in broiler chicken (Awan, et al., 2003). Post mortem studies on chickens showed necrosis at the injury site and liver (Awan, et al., 2003; Awan, et al., 2011). In addition, Mefenamic acid has been indicated in several cases of nephrotoxicity in rats including renal failure (Huq, 2007).

There are also reported concerns on the use of aceclofenac (Sharma, 2012). In humans diclofenac is a metabolite of aceclofenac (Bort, et al., 1996). As such, aceclofenac is likely to be toxic either independently or as a result of its metabolism in the body into diclofenac. More concerning are their major metabolites 4-hydroxyaceclofenac and 4-hydroxydiclofenac, will metabolise into each other. This metabolic pathway has also been documented in monkey, rat and human subjects (Bort, et al., 1996). As discussed in section 1.2.2, livestock are the primary food source of many birds of prey, especially carrion. Thus, given the documented metabolism of aceclofenac into diclofenac, in all mammal subjects tested to date (Sharma, 2012),

there is a real concern that this metabolism would occur in livestock and hence render this NSAID, especially in areas of endangered species, as toxic (Sharma, 2012). Therefore, the threat of these NSAIDs may extend beyond the parent compound itself and hence was included in this research (section 1.3.3, Table 1.1).

Alongside the concerns of the metabolic products of aceclofenac, likewise the NSAID suxibuzone metabolises to the known toxic phenylbutazone, with its links to gastrointestinal effects (Cuthbert, et al., 2007b). There is a noticeable lack of studies of metabolic pathways and possible subsequent toxicity in species that are at risk, such as vultures. Currently, and more importantly, there is a notable absence of the inclusion of metabolites in reported analysis methods and the analysis when their parent compounds have been implicated in toxicity. It is important to consider these metabolites as these compounds could also be toxic. Therefore a method that detects parent compounds alongside their metabolites, like the one developed and validated in this research, will offer a means of detecting these NSAIDs simultaneously. This is the first reporting of its kind in this field of research.

Despite findings from toxicity and safety studies, Cuthbert, et al. (2011) reported the NSAIDs; aceclofenac, diclofenac, flunixin meglumine, ibuprofen, ketoprofen, mefenamic acid, meloxicam, nimeuslide, phenylbutazone and piroxicam to be offered for sale on the Indian subcontinent by various pharmaceutical companies and veterinarians. Often these are sold in formulations with other NSAIDs as a secondary ingredient and in bolus and injectable forms. Surprisingly, with the exception of diclofenac, meloxicam and ketoprofen, little is known about the toxicity or safety of the other NSAIDs. Therefore the continued sale of these compounds is of serious concern and hence the development, and application thereafter, of this method is very important. This novel method offers a means to monitor these compounds and more conclusively identify them as potential threats in the event of recurring mass mortality.

1.3.3 NSAIDs used the in this study

Through a review of the literature, continued threat and exposure from a range of NSAIDs to protected species have been identified. This included species of an already declining population and those at potential risk. Furthermore, a review on NSAIDs availability, their toxicity or potentially toxic nature owing to similar chemistry, has informed a selection of NSAIDs to be included in this research (section 1.3.3 and 1.3.4). The NSAIDs investigated in this research were selected as they were either of known threat and toxicity or continued exposure, i.e. aceclofenac, carprofen,

diclofenac, flunixin, ibuprofen, ketoprofen, nimesulide and phenylbutazone (Cuthbert, et al., 2015; Naidoo, et al., 2010; Oaks, et al., 2004; Swan, et al., 2006; Swarup, et al., 2007; Sharma, 2012; Zorilla, et al., 2014) or available for sale in areas of protected species and/or may pose risk i.e. mefenamic acid, meloxicam and piroxicam (Cuthbert, et al., 2011).

In addition, it was identified that there was a lack of the inclusion of metabolites in analytical methods in this field. Specifically, there was only one published study looking at metabolites of aceclofenac (diclofenac and 4-hydroxydiclofenac) in cattle available to vultures (Galligan, et al., 2016). This current research ensures the inclusion of these metabolites alongside major metabolites of the known toxic parent compounds flunixin (5-hydroxyflunixin), nimesulide (4-hydroxynimesulide) and phenylbutazone (oxyphenylbutazone). It also includes the metabolite of meloxicam (5-carboxymeloxicam), currently the only safe alternative to diclofenac, and 5-hydroxypiroxicam, the major metabolite of piroxicam (Figure 1.12), considered to be related to meloxicam structurally (Figure 1.7). Lastly the metabolite 3-hydroxymethyl mefenamic acid is included as its parent compound mefenamic acid is found on sale in areas of protected species (Cuthbert, et al., 2011). The selection of these are discussed in more detail in section 1.3.7.1.

This research aims to provide a new analytical detection method. Thus, with a limited number of methods published in this research area, thirteen NSAIDs (Table 1.1) and seven metabolites (section 1.3.7.1, Table 1.2) have been included in initial investigations and preliminary method development and validated for eleven NSAIDs and seven metabolites, resulting in the first analytical method to analyse metabolites alongside parent compounds. This is also the first time that the NSAIDs selected to be investigated, in this field, have been analysed for their detection in feathers.

1.3.4 The chemical properties and structures of NSAIDs

NSAIDs are broadly structurally classified as enolic or carboxylic acids, which each have specific chemical grouping, such as oxicams and prazolones, and differing biological effects (Van Hoof, et al., 2004). These compounds have many functional groups in common. The structures of the NSAIDs investigated in this research are presented in the Figures 1.1 to 1.13 and their individual classification are presented in Table 1.1 Alongside the structures, relative molecular mass (RMM) and corresponding pK_a acid dissociation constants, are provided, which were later used in the development of an efficient sample extraction method and aid in the separation using

liquid chromatography. Table 1.1 shows each NSAID listed alongside their generic name and their corresponding International Union of Pure and Applied Chemistry (IUPAC) chemical name. It is necessary to consider that each NSAID will vary in their pharmacokinetics, pharmacodynamics and possible side effects, their modes of administration is discussed in detail in section 1.3.5.

The hepatotoxicity and nephrotoxicity side effects of NSAIDs are suggested to be linked to their chemical structure (Cuthbert, et al., 2007). The functional group considered to be toxic is the carboxylic acid group with a proximal amine group, as highlighted in the structure of diclofenac below by the red and blue rings respectively.

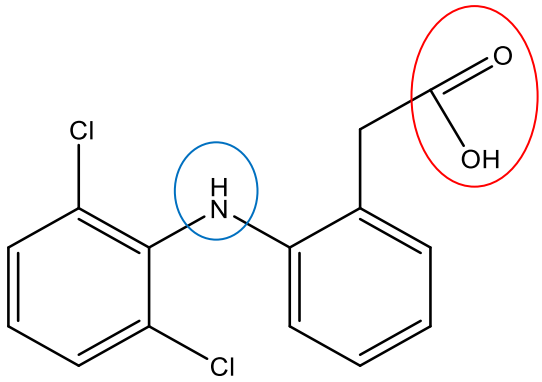


Figure 1.1 Structure of diclofenac

These functional groups are present in NSAIDs diclofenac, carprofen and flunixin that are known to be toxic (Figures 1.1, 1.2 and 1.3 respectively) and aceclofenac (Figure 1.4), that has been recently highlighted as a concern (Sharma, et al., 2012). However, this does not offer an explanation for ibuprofen and phenylbutazone (Figures 1.5 and 1.6) as both of these are found to cause nephrotoxicity in birds and lack a proximal amine group (Sharma, et al., 2012). Ibuprofen, however, does have a carboxylic acid group supporting conclusions drawn by Naidoo, et al. (2007). The proposed argument (Naidoo, et al., 2007) that meloxicam (Figure 1.7) is considered a safe alternative is on the premise that the structure does not contain a carboxylic acid, but does have an amine group.

Additionally, toxicity has also been linked with the metabolism these compounds (discussed further in section 1.3.7). During research on the pharmacokinetics of meloxicam, Naidoo, et al. (2007) found deficiencies in vulture metabolism, particularly in enzymes involved in the conversion of metabolites through a carboxy pathway (section 1.3.7). It is proposed meloxicam is a safe alternative (section 1.2.3) due to different enzymes used during its metabolism (section 1.3.7), however, in compounds

like diclofenac and ketoprofen, they suggested that these deficiencies can result in an accumulation of the parent compound and metabolite rendering these compounds toxic (Naidoo, et al., 2010b). However, no research has been published on this, thus it remains to be fully described and is beyond the scope of this research.

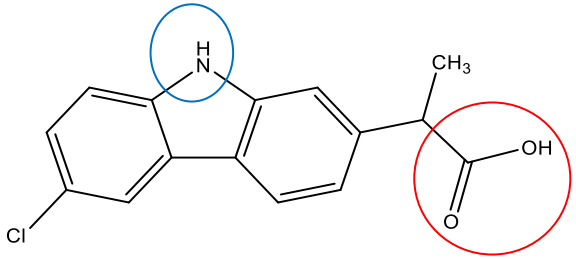


Figure 1.2 Structure of carprofen

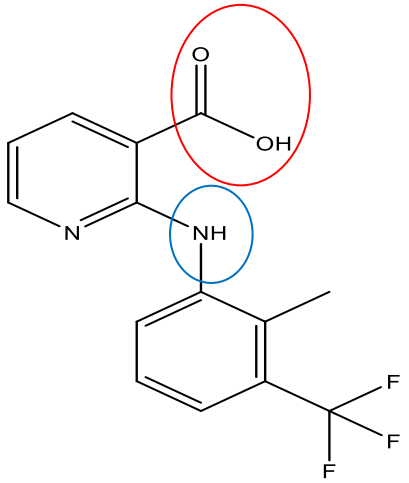


Figure 1.3 Structure of flunixin

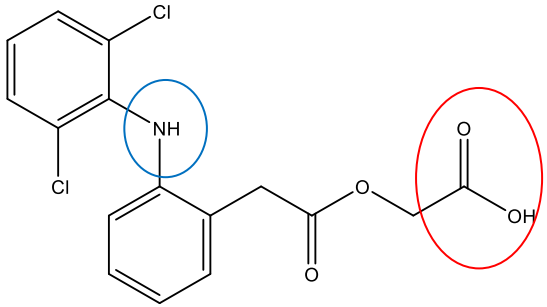


Figure 1.4 Structure of aceclofenac

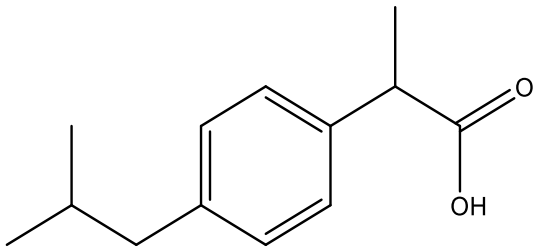


Figure 1.5 Structure of ibuprofen

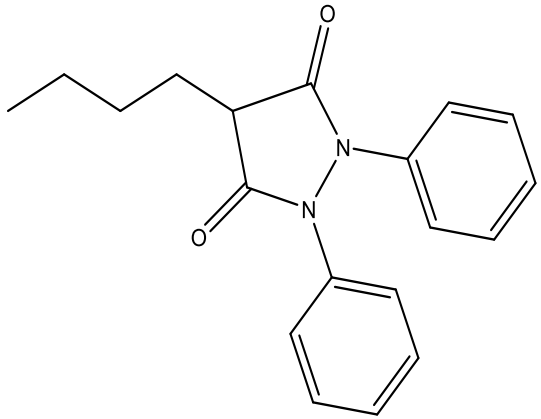


Figure 1.6 Structure of phenylbutazone

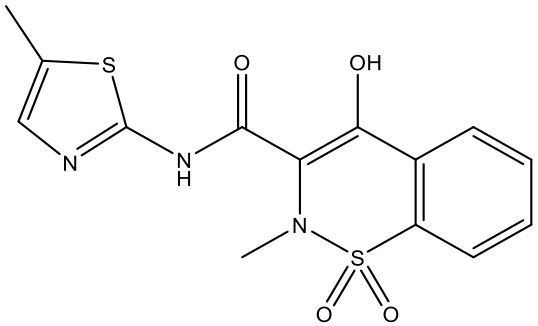


Figure 1.7 Structure of meloxicam

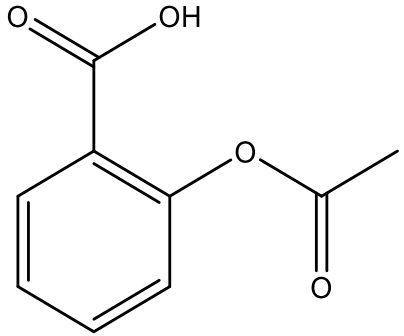


Figure 1.8 Structure of acetylsalicylic acid

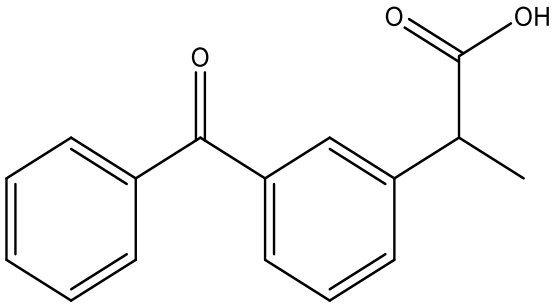


Figure 1.9 Structure of ketoprofen

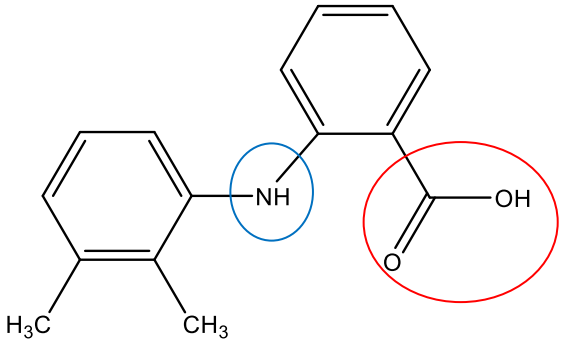


Figure 1.10 Structure of mefenamic acid

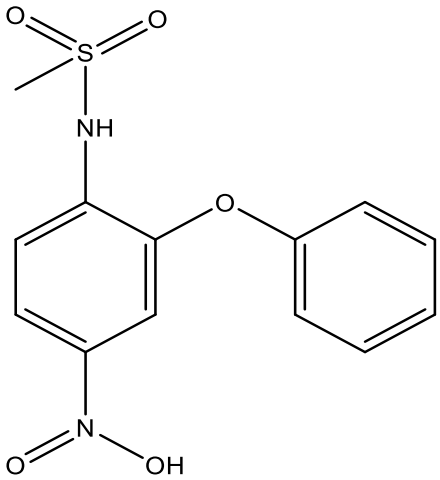


Figure 1.11 Structure of nimesulide

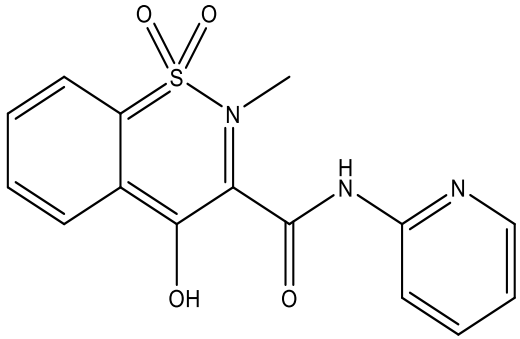


Figure 1.12 Structure of piroxicam

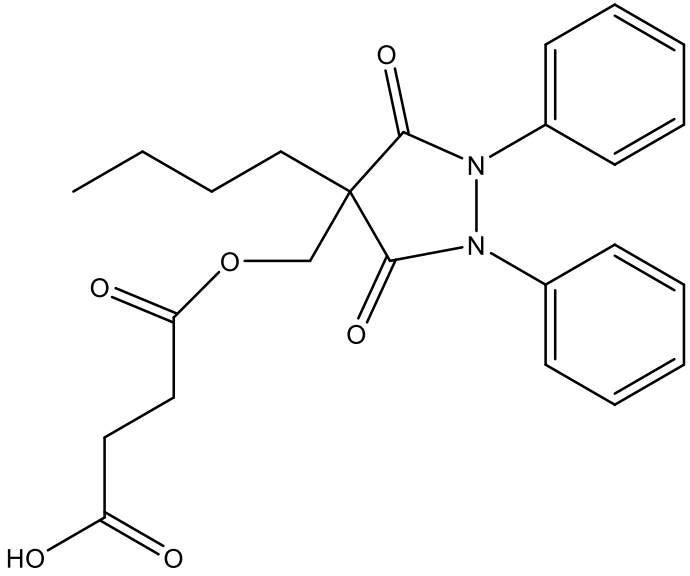


Figure 1.13 Structure of suxibuzone

Table 1.1 Classification of NSAIDs

NSAID	RMM	pKa	Classification	Chemical Group	IUPAC chemical name
aceclofenac	354.2	4.7	carboxylic acids	acetic acids	2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxyacetic acid
diclofenac	296.2	4.2			2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid
flunixin	491.5	5.8		aminonicotinic acid	2-[[2-Methyl-3-(trifluoromethyl)phenyl]amino]pyridine-3-carboxylic acid
nimesulide	308.1	6.5		anthralic acid	*N*-(4-Nitro-2-phenoxyphenyl)methanesulfonamide
mefenamic acid	241.3	4.2		fenamic acid	2-(2,3-dimethylphenyl)aminobenzoic acid
carprofen	273.7	4.4		propanoic acids	2-(6-Chloro-9*H*-carbazol-2-yl)propanoic acid
ketoprofen	254.3	4.5			2-(3-benzoylphenyl)propanoic acid
ibuprofen	206.3	4.4			2-(4-(2-methylpropyl)phenyl)propanoic acid
acetylsalicylic acid	180.2	3.5		salicylic acid	2-acetoxybenzoic acid
meloxicam	351.4	4.2	enolic acids	oxicams	4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide.
piroxicam	331.1	6.3			4-Hydroxy-2-methyl-N-(2-pyridinyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide
phenylbutazone	308.4	4.4		prazolones	4-butyl-1,2-diphenyl-3,5-pyrazolidinedione
suxibuzone	438	4.3			4-[[4-butyl-3,5-dioxo-1,2-di(phenyl)pyrazolidin-4-yl]methoxy]-4-oxobutanoic acid

Adapted from Richards, 2010; Van Hoof, et al., 2004

1.3.5 Modes of administration

The route of administration is an important factor in determining the onset, duration and intensity of the effects of the compound administered. The administration route will determine the length of time taken to reach the site of action (BNF, 2012; Jickells and Negrusz, 2008). This is also dependent on the individual, but ultimately it is the mode of administration that determines the time of action. All compounds in the body undergo four main stages; absorption, distribution, metabolism and excretion (Jickells and Negrusz, 2008).

In humans, absorption relates to the mode of administration and the resulting entry of the compound. NSAIDs are typically administered to humans orally in tablet form; however, they may be administered intravenously and intramuscularly, the latter referred to as parenteral, (which has the added benefit of reducing gastric irritation, rectally or topically. NSAIDs are short acting and have to be taken a number of times in one day (O'Shaughnessy, 2011). In oral administration, absorption is expected to be relatively slow compared to intravenous. None the less, taken orally, NSAIDs start to relieve pain within an hour after administration (BNF, 2012). The bioavailability of a compound is dependent on their solubility in the gastrointestinal tract (GI) and absorption within the body (Jickells and Negrusz, 2008).

Whilst parenteral modes of administration are preferred in veterinary medicine, oral administration is used as well (Merck, 2014). Cuthbert, et al. (2011) reported 83 oral and 80 injectable formulations of NSAIDs, which are available for sale on the Indian subcontinent as the primary formulations in veterinary treatment. European Medicines Agency (EMA) state a typical treatment via intramuscular injection at a dose of 2.5 µg/g per day for between one to three days treatment in cattle (EMA, 2003; EMA, 2009).

1.3.6 Mechanism of action in the body

NSAIDs, as defined in section 1.3.1, exhibit analgesic, antipyretic and anti-inflammatory properties. These compounds have a shared mechanism of action, namely the inhibition of the cyclooxygenase enzyme (COX) function responsible for the synthesis of the enzyme prostaglandin (Modi, et al., 2012). Prostaglandins are chemicals that maintain the inflammatory process; at the injury site prostaglandins are produced to express pain and fever in the body. This mechanism of action was discovered in ground-breaking research by Vane (1971) who first proposed inhibition of prostaglandin synthesis in acetylsalicylic acid. Vane's research subsequently helped

reveal that the therapeutic benefits and toxicity of NSAIDs are related to their affinity of COX inhibition mechanism (Lees, et al., 2004).

NSAIDs work by blocking arachidonic acid, a short chain fatty acid, from its binding site on the COX enzyme. Arachidonic acid serves as a substrate for inflammatory mediators which, through a series of cascade reactions, will produce eicosanoid mediators; prostaglandins and thromboxanes (Lees, et al., 2004; Modi, et al., 2012). In the presence of these mediators, an increased prostaglandin production occurs. Thus by means of NSAID administration, inhibition of these chemicals inhibits inflammation at the injury site.

Predominantly there are two main isoforms of the COX enzymes, namely COX-1 and COX-2. COX-1 generates prostaglandins that maintain organ function and are expressed in all tissues. COX-1 regulates many physiological processes including the protection of the GI tract and its mucosa. COX-2 is induced during the body's response to inflammation, prostaglandins in this case mediate pain and inflammation at the injury site (Lees, et al., 2004). As with most pharmaceuticals, NSAIDs are not without side effects and often cases of gastrointestinal and renal conditions are documented (BNF, 2012). NSAIDs are divided into two major groups; COX inhibitors and selective COX-2 inhibitors. NSAIDs that inhibit COX-1, such as first generation acetylsalicylic acid and ibuprofen, are associated with abdominal conditions like ulcers and bleeding of the gut due to the lowered prostaglandin levels (Lees et al. 2004; Vane & Botting 1995). Inhibition of these enzymes is often reversible, unlike in acetylsalicylic acid which is irreversible. For example, COX-1 is required for maintaining a thick stomach lining, by the irreversible blocking of such enzyme, long term use of acetylsalicylic acid could lead to thinning of the mucus that protects the stomach. In response new compounds have been developed to selectively inhibit COX-2 and as a result decrease the adverse GI side effects (Modi, et al., 2012).

The NSAID meloxicam is one of these newer NSAIDs, selectively inhibiting COX-2 and thus reducing risk of adverse effect on GI tract and renal function whilst offering the analgesic, anti-inflammatory and antipyretic properties. Whilst the main function of prostaglandins have been discussed, these enzymes have an important role in the regulation of blood circulation, and vascular permeability and kidney function (Lees, et al., 2004; Vane & Botting 1995). In animals, prostaglandins have a minor role in renal function, therefore, a possible consequence of continued treatment with NSAIDs can

ultimately lead to renal failure. Renal failure results in an accumulation of uric acid in the bloodstream and as a result causes visceral gout (Modi, et al., 2012).

1.3.7 NSAIDs metabolism

After the adsorption of an NSAID, metabolism occurs. Metabolism is a process by which the structure of the compound is changed to aid the removal of compounds from the body (Gibson and Skett, 2001). In principle, drug metabolism converts lipophilic ("fat-loving") compounds that cannot be efficiently eliminated by the kidneys into polar hydrophilic ("water loving") products that are easily excreted in urine. Metabolism is divided into two phases. Phase 1 reactions include 3 main types; oxidation, reduction, hydrolysis alongside N- and O-dealkylation and sulfoxide formation. Phase 1 reactions primarily involve introducing a chemically reactive group, for example a hydroxyl, a result of a hydrolysis reaction. These groups then serve as an attack site for the conjugating system of phase 2 reactions which involves the attachment of an ionised group to the drug. These include glucuronic acid, methyl or acetyl groups (Rang, et al., 2012). Phase 1 metabolism essentially prepares the compound to undergo phase 2 reactions. The attachment of an ionised group in phase 2 reactions increases the polarity of the compound and hence water solubility to enable its excretion and elimination from the body (Jickells and Negrusz, 2008).

Phase 1 reactions take place mainly in the liver although NSAIDs can undergo this metabolism phase in the lungs, kidneys and GI tract (Rang, et al., 2012). Metabolites produced in phase 1 reactions are often more chemically reactive, interfering with cellular function and reacting with certain types of cellular macromolecules. These reactions can result in hypersensitivity and necrosis as examples. Therefore, sometimes these products are more toxic than the parent compound itself. This is especially of concern when the parent compound are known to be toxic (section 1.3.2). Products from phase 2 reactions however, are, as mentioned, more polar and therefore cannot diffuse across membranes hence, their excretion as generally inactive metabolites (Lees, et al., 2004; Rang, et al., 2012).

Administered orally, NSAIDs may undergo first-pass metabolism where they travel through the liver so substantial metabolism of the parent compound can occur prior to entry into the circulation system. As a result the amount reaching the circulatory system is considerably less than the amount absorbed and hence their bioavailability is greatly reduced (O'Shaughnessy, 2011). Whilst first-pass metabolism will reduce the amount available for site of action of the parent compound, drugs that are metabolised into

active products will have a different profile of activity than a parent compound administered parenterally. As such, much larger dosages of drugs are needed when taken orally. However, often first-pass metabolism is important for many therapeutic compounds whereby a drug may only become pharmacologically active upon metabolism. As an example, acetylsalicylic acid has anti-inflammatory properties alongside inhibiting platelet function, it becomes hydrolysed to salicylic acid in phase 1 metabolism whereby it exhibits the desired anti-inflammatory properties (Moffat, Osselton and Widdop 2004; Rang, et al., 2012).

Metabolites are often more persistent in the body than their parent compounds, giving rise to longer clinical effects (Willis, et al., 1979). This is due to longer biological half-life ($t_{½}$). The $t_{½}$ is defined as the time required for drug concentrations in plasma to fall below 50% or one half (Jickells and Negrusz, 2008). An example is the main active metabolite of phenylbutazone; oxyphenylbutazone is excreted slowly in urine over twenty one days compared to two to five days of its parent compound (Dieterle, Faigle, Fruh and Mory, 1976). The disposition of compounds, including metabolites, in the body includes incorporation into hair, thus they are likely to be present in non-invasive keratinous samples, as discussed in more detail in section 1.4.1. These compounds diffuse from the blood supply at the base of the follicle into the hair itself. This incorporation is dependent on the chemistry of metabolites, such as their hydrophilic nature, owing to increased polarity, a result of metabolism. This exhibits lower plasma protein binding, and therefore this can result in metabolites being present in lower concentrations in blood, than their parent compound (Lees, et al., 2004; Modi, et al., 2012). This protein binding and the degree to which a compound will bind can affect its efficacy; with only the unbound components exhibiting the pharmacological effects. Protein binding describes the ability of proteins to form bonds with other compounds, hence only the unbound drug is free to interact with receptors, active in the body and able to be metabolised and excreted (Lees, et al., 2004; Modi, et al., 2012). For example, the major metabolite of meloxicam, 5-carboxymeloxicam, is 95% bound to protein meaning 5% is active in the system. This protein binding is presented in Table 1.2 for each metabolite.

The metabolism of each NSAID, in humans is well documented. However, in birds of prey, specifically vultures, and protected species such as water vole, there is a lack of knowledge in metabolic pathways and possible subsequent toxicity information. Currently the enzymes that control metabolism of NSAIDs are not described in *Gyps* Vulture. Moreover, the environmental fate and effects of these metabolites in veterinary

medicine is extremely limited (Crane, Boxall and Barrett, 2008). Despite this, research into the pharmacokinetics of meloxicam in humans enabled Naidoo, et al. (2008) to make some assumptions regarding NSAID metabolism in said species.

In mammals, tested to date i.e. monkey and rat, NSAIDs are metabolised by cytochrome P450 enzymes (CYP450), specifically meloxicam is metabolised by enzyme CYP2C9 during phase 1, and as a result the major metabolites 5-carboxymeloxicam and 5-hydroxymethyl meloxicam are produced. Naidoo, et al. (2008), using LC tandem mass spectrometry (LC-MS/MS), tentatively identified 5-hydroxymethyl meloxicam and one glucuronide conjugate metabolites and concluded that vultures are likely to use the same cytochrome P450 enzymes (CY450) and standard metabolic pathways as described in other animals. However, highlighted that the carboxy- metabolite was absent thus, as hydroxy- metabolites are converted into carboxy- metabolites, during phase 1 metabolism, by noncytochrome dependant pathway this would suggest the absence of the CYP450 pathways. Deficiencies in CYP450 may therefore explain the toxicity of NSAIDs, such as diclofenac and ketoprofen, as these deficiencies would allow the accumulation of parent compounds and their potentially toxic metabolites, which may interfere with intercellular function (section 1.3.2). As stated by Sharma (2012), whether the toxicity of parent compounds, such as diclofenac, is caused by themselves or by the metabolites remains unknown. Therefore, knowing phase 1 metabolites are often more chemically reactive than parent compounds and together with deficiencies in CYP450 enzymatic pathways, metabolites may pose a real threat to declining populations.

1.3.7.1 Metabolites to be detected in this research

There are two parts when considering metabolism of NSAIDs I) the metabolite in the food source i.e. ungulate and II) the metabolism within the species investigated i.e. bird of prey or the protected water vole. Currently there is a gap in the literature on the metabolism of NSAIDs in protected species, such as birds of prey. Hence, where this thesis discusses metabolism it has been primarily inferred through human metabolism, and covers metabolism in animals where possible and available. Whilst current literature is limited, research has suggested that mammals and birds of prey may use standard metabolism (cytochrome P450 enzyme system, as in man). Thus, with the uncertainty of the involvement of metabolites in toxicity and the lack of published analytical methods offering metabolite detection in this area, this research includes seven major metabolites alongside their parent compounds. This data has not been available previously.

The NSAIDs investigated in this research, as described in section 1.3.2 and 1.3.3, have been identified as either of known or potential threat through potential toxicity to endangered species. Further to this, a thorough literature review has identified the major metabolites (section 1.3.3) of some of these NSAIDs and their relation to hepatotoxicity and/or nephrotoxicity. As first introduced in section 1.3.3, the major metabolites included as an analyte of interest in the simultaneous detection method are; 4-hydroxydiclofenac, 3-hydroxymethyl mefenamic acid, oxyphenylbutazone, 5-hydroxyflunixin, 5-hydroxypiroxicam, 5-carboxymeloxicam and 4-hydroxynimesulide. These have been selected not only due to reported adverse effects (in humans and animals), but the threat they may pose. Their threats and metabolism are discussed in detail below. The structures of the metabolites discussed are presented in Table 1.2, alongside plasma protein binding percentages (section 1.3.7), and functional groups considered to be toxic, namely a carboxylic acid with a proximal amine, highlighted in red and blue rings respectively (section 1.3.4).

As discussed in previous section 1.3.6, parent compounds undergo metabolism and may produce active major metabolites, these in turn are often more chemically reactive. This is especially the case in the major metabolites (Table 1.2) of aceclofenac, diclofenac, mefenamic acid, phenylbutazone and suxibuzone, as discussed throughout this section.

As first introduced in section 1.3.2, the metabolic pathways of aceclofenac (Table 1.1) were reported by Bort, et al. (1996) in humans. The major metabolites of aceclofenac, 4-hydroxyaceclofenac (Table 1.2), is known to metabolise into diclofenac and its major metabolite 4-hydroxydiclofenac (Table 1.2), in several mammalian subjects. Thus, where the sale of aceclofenac is still legal on the sub-Indian continent in areas of endangered species, this could highlight another exposure route to diclofenac, even before the toxicity of aceclofenac itself has been confirmed. Sharma, et al. (2012) suggests that while metabolism has not been reported in livestock, given that aceclofenac metabolises into diclofenac in all mammalian species tested to date, and that livestock make up principal food source of scavengers, this highlights there may be a real threat from aceclofenac metabolising into diclofenac in the food source of protected species. For this reason the major metabolites of aceclofenac and diclofenac (diclofenac and 4-hydroxydiclofenac, respectively) (Table 1.2) have been included in this investigation.

In the case of 3-hyroxymethyl mefenamic acid (Table 1.2), the major metabolite of mefenamic acid, toxicological consequences remain unclear however, the metabolic pathway of this compound and its major metabolite employs the same noncytochrome dependant pathway that has been reported to be absent in birds of prey (section 1.3.6) (Naidoo, et al. 2008). Whether this would accumulate and render these compounds as toxic needs to be investigated. The fact remains that these compounds are closely related to diclofenac with very similar chemical structures (Figures 1.1, 1.11 and Table 1.2).

Whilst some NSAIDs produce toxic metabolites other NSAIDs may not, such as meloxicam, nimesulide. However, these are still of concern as there is a lack of knowledge surrounding the toxicity of the parent compounds and metabolic pathways, as is the case in meloxicam. Whilst currently the only safe alternative to diclofenac (section 1.2.3), meloxicam is reported to metabolise into 5-carboxymeloxicam (Table 1.2) in humans and mammals (Turck, Roth and Busch, 1996), the same group identified as potentially toxic by Cuthbert, et al. (2006) (section 1.3.4) and metabolised by the absent pathway in birds of prey, namely vultures (Naidoo, et al. 2008). Furthermore, with a relatively long half-life of twenty hours (Aberg, et al., 2009), if proven to be toxic it is highly likely that it would accumulate in the body and interfere with cellular function. For these reasons, 5-carboxymexloicam (Table 1.2) has been selected in this research as it is a carboxy metabolite

To date, there are only a few research papers on the detection of nimesulide and metabolites in human plasma and urine (Singla, Chawla and Singh, 2000). However, as recent publications are now suggesting nimesulide is toxic (Cuthbert, et al., 2015), the major metabolite 4-hydroxynimesulide is also included in this research. The pharmacokinetics and metabolism of nimesuide was studied in humans, whereby the main metabolite (4-hydroxynimesulide) (Table 1.2) provides anti-inflammatory and analgesic properties after phase 1 metabolism. It has been suggested that its toxicity is lower than that of the parent compound (Bernareggi, 2001). However, Bernareggi (2001) did note that nimesulide is almost exclusively eliminated by hepatic metabolism (metabolism in the liver) and thus, hepatic impairment can reduce the rate of elimination. If an accumulation was to occur this could cause adverse effects in protected species, but more research would need to be carried out to investigate this.

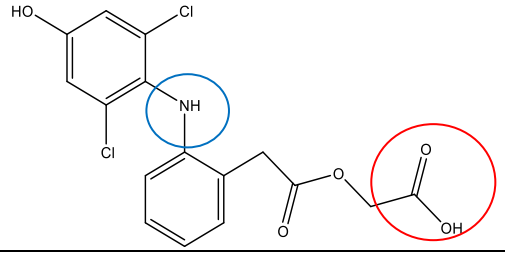
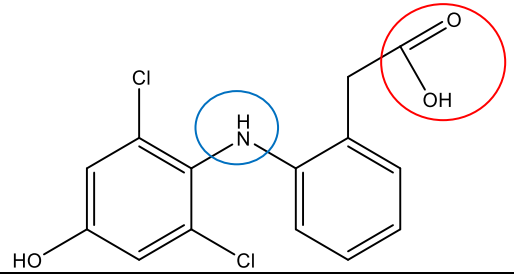
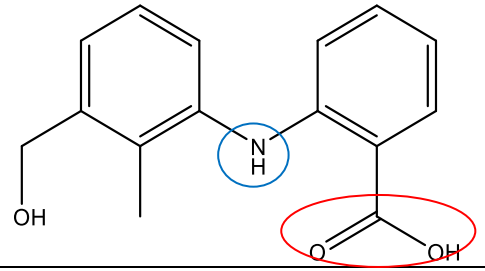
Other metabolites included in this research are oxyphenylbutazone (metabolite of phenylbutazone), 5-hydroxyflunixin (metabolite of flunixin) and 5-hydroxypiroxicam

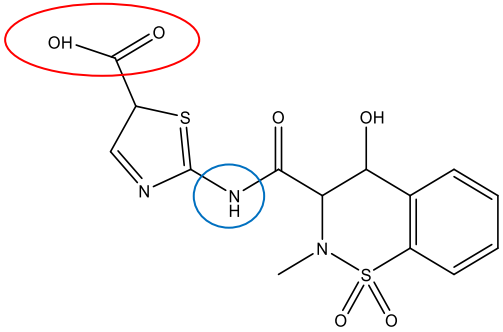
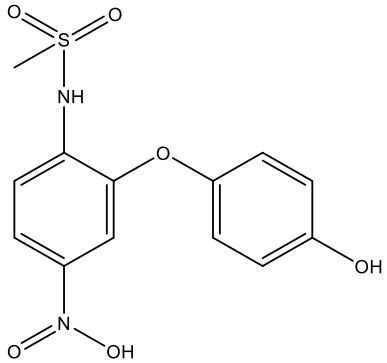
(metabolite of piroxicam) (Table 1.2). These metabolites have been selected as their parent compounds have been implemented in toxicity and have been reported on sale in areas of protected species (section 1.3.2 and 1.3.3). This is especially the case with oxyphenylbutazone, the major metabolite of phenylbutazone, and phenylbutazone itself (the major metabolite of suxibuzone, section 1.3.2, Table 1.2) are reported to have high incidences of GI adverse effects and blood deficiencies in humans and mammals (Woodhouse and Wynne, 1987). Thus as it seems most NSAID metabolites are, or have the potential of being toxic, this research has included not only those highlighted as a concern, like aceclofenac and diclofenac, but includes others that may be studied later showing them to be the same.

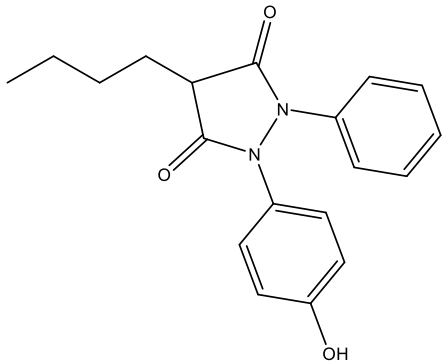
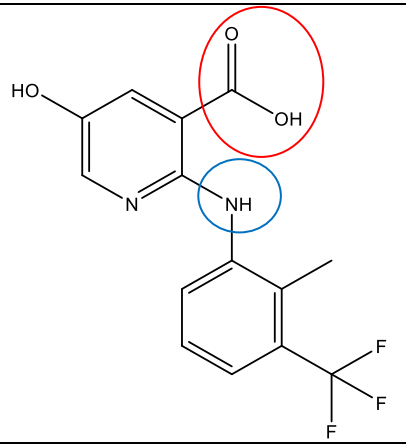
Some major metabolites of parent compounds (carprofen ester glucuronide - carprofen, salicylic acid - acetylsalicylic acid, 2-hydroxyibuprofen – ibuprofen and ketoprofen ester glucuronide – ketoprofen) (Table 1.2) have not been included in this research as, unlike those to be investigated, there has been no reporting's of toxicity or adverse effects in this field of research, often a result of efficient elimination from the body. As an example, carprofen metabolism has been reported to vary greatly between some animal species and humans but has shown not to accumulate and eliminate rapidly, its mechanism of action is still relatively unclear (Ray and Wade, 1982). Carprofen undergoes phase II metabolism to produce an ester glucuronide metabolite. As discussed in section 1.3.6, this would suggest this metabolite is polar and thus excreted with little activity in the body, this metabolite has not been implemented or documented in any toxicity.

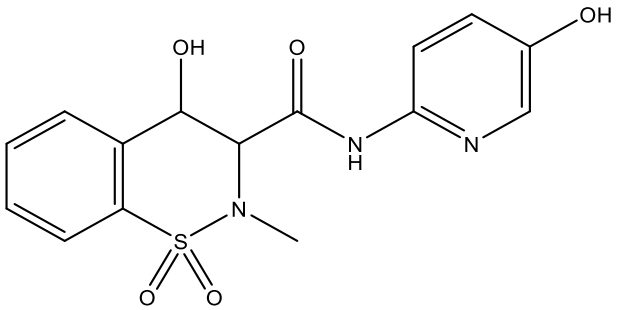
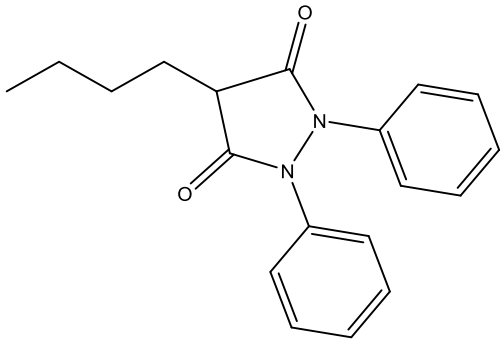
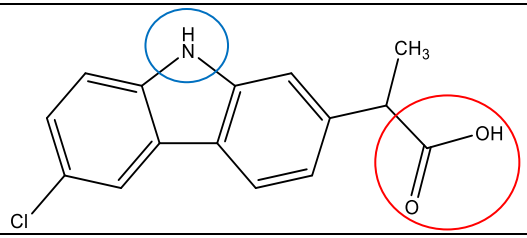
This research intends to simultaneously detect seven major metabolites of NSAIDs (Table 1.2) as identified that may be toxic to endangered species. It includes those where toxicity needs to be investigated further, due to the limited understanding of metabolic pathways and mechanism of action in birds of prey and mammals. This makes the analytical method presented in this current research novel, as this is the first reporting of its kind in this area.

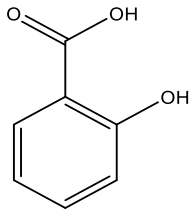
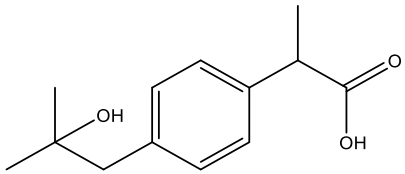
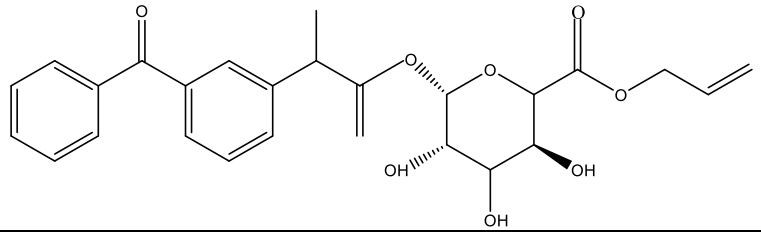
Table 1.2 Major metabolites and chemicals structures

Parent compound/NSAID	Major metabolite (% protein binding)	Chemical Structures	Metabolic phase and reaction	Reference
aceclofenac	4-hydroxyaceclofenac (74-79%)		Phase 1 hydroxylation	Bort, et al., 1996
diclofenac	4-hydroxydiclofenac (40%)		Phase 1 hydroxylation	Menasse, et al., 1978
mefenamic acid	3-hydroxymethyl mefenamic acid (52%)		Phase 1 hydroxylation	Huq, 2007

meloxicam	5-carboxymeloxicam (95%)		Phase 1 oxidation	Naidoo, et al., 2007
nimesulide	4-hydroxynimesulide (40%)		Phase 1 hydroxylation	Singla, Chawla, and Singh., 2000

phenylbutazone	oxyphenylbutazone (60%)		Phase 1 oxidation	Bakke, Draffan and Davies., 1974
flunixin	5-hydroxyflunixin (NA)*		Phase 1 hydroxylation	Wasfi, et al., 1998

piroxicam	5-hydroxypiroxicam (60%)		Phase 1 hydroxylation	Woodhouse and Wynne, 1987
suxibuzone	Phenylbutazone (NA)*		Phase 1 hydroxylation	Yasuda, et al., 1981
carprofen	carprofen ester glucuronide (50-60%)		Phase 2 conjugation	Rubio, et al., 1980

acetylsalicylic acid	salicylic acid (50-80%)		Phase 1 hydrolysis	Rang, et al., 2012
ibuprofen	2-hydroxyibuprofen (28%)		Phase 1 hydroxylation	Woodhouse and Wynne, 1987
ketoprofen	ketoprofen ester glucuronide (65%)		Phase 2 conjugation	Advenier, et al., 1983

*(NA) protein binding percentage not available

1.4 NON-INVASIVE SAMPLES

Previously in section 1.1.1, the emphasis on the importance of unconventional sample analysis was discussed in relation to non-invasive sampling from protected species. With continued threats to many species by the continued exposure to veterinary pharmaceuticals, particularly NSAIDs, there is an urgency for a method to enable the detection of such compounds using non-invasive samples such as hairs and feathers. As previously discussed, the use of primarily conventional samples, such as blood and tissue, could pose ethical issues, decomposition problems and conservation concerns when working with fragile populations. In this section; the concept of hair and feather analysis is considered, the incorporation of NSAIDs in hair is discussed including the use in the context of forensic and environmental sciences and advantages of promising environmentally robust samples selected to be analysed in this study.

1.4.1 The incorporation of NSAIDs and their metabolites in hair

Hair analysis works on the premise that as the hair grows, compounds are incorporated into the hair structure (Gaillard and Pépin, 1999). Essentially, hair, whether human or animal, is composed of keratin and is an outgrowth from a follicle (Harkey, 1993). Situated at the base of the follicle, the bulb generates the various layers of the hair shaft which includes the medulla (core layer), cortex and cuticle. There are three stages of hair growth, anagen, catagen and telogen respectively; during the anagen stage, compounds are incorporated into the hair from the bloodstream (Richards, 2010).

The main factors that influence the incorporation of compounds into the hair are melanin affinity, lipophilicity and basicity (pK_b) of the compound (Wenning, 2000). It is proposed that the incorporation of compounds in hair is through diffusion from the blood supply at the base of the hair follicle and thus, affected by the same parameters influencing the transport of the drug in the body i.e. adsorption, distribution and elimination (section 1.3.5 and 1.3.6) (Gaillard and Pépin, 1999). The compound must first cross the plasma membrane to permeate a cell, thus the chemistry of a compound plays an important role in its affinity to bind. As such, most veterinary pharmaceuticals are weak acids or bases and are present in the blood bonded to plasma protein or in the ionised form. It is only the fraction which is non-bonded i.e. non-ionised (section 1.3.7) and of suitable liposolubility that can undergo passive diffusion (Gratacos-Cubarsi, et al., 2006). Furthermore, plasma membrane exhibits low permeability to polar compounds thus basic and hydrophobic compounds have a stronger binding

capacity to hair (Moffat, Osselton and Widdop 2004). NSAIDs used in this project are generally weak acids and since they are often administered for long periods of time this will ensure a constant presence of parent compounds and metabolites, through their accumulation, in the body tissues and hair samples. As was the case in the dead carcasses of livestock on the Indian subcontinent.

It is proposed that after elimination of the parent compound from the body, metabolites are likely to be present and are often more persistent in the body due to longer $t_{½}$ (section 1.3.7) (Willis, et al., 1979). However, the parent compounds tend to be incorporated into the hair follicle more readily due to their lipophillic properties. For example, it was reported by Huq (2007) that the major metabolites of mefenamic acid (Table 1.2) are more soluble in water than parent compound thus incorporation into keratinous matrices may be lower than the parent compound. It is important to remember that in the food source parent compounds and metabolites are likely to be present therefore both compounds may pose a threat. As such, recent studies have suggested that the toxicity of diclofenac, to vultures specifically, is still unclear and whether it's the toxicity of diclofenac itself or in fact by its metabolites or a combination of both is unknown (Sharma, 2012). Therefore, metabolites have been included in this research.

The major advantage of hair analysis is the sampling of hair which is relatively non-invasive, as mentioned in section 1.1 and 1.1.1. The incorporation of compounds through the bloodstream and into a matrix such as hair can be applied to animals (section 1.4.2). Thus sampling, non-invasively, wildlife species that may be exposed to NSAIDs in the environment, may offer the detection of these compounds both pre or post mortality but could essentially offer an early detection method to identify exposure prior to mass mortality or loss of population.

1.4.2 History and advantages of hair analysis

The analysis of hair was first introduced in the 1960s and 1970s after the pioneering analysis to assess human exposure to heavy metals, such as lead or mercury, using atomic absorption spectroscopy (Baumgartner, et al., 1979). At this point examination of hair for organic chemicals was not possible as analytical methods were not sensitive enough (Kintz, 2004). However, ten years after the first investigations, Baumgartner, et al. (1979) reported differences in the concentrations of morphine along the hair shaft correlating to the time of heroin use. Reservations were still apparent in the scientific community and it wasn't until well after the 1990s, when numerous studies illustrated

hair to be a highly suitable matrix in the detection of drugs of abuse in forensic toxicology (Kintz, 2004). The detection of drugs of abuse in human hair and nails has since become a commonly used practice in forensic science, particularly the detection of illicit drugs (Englehart, et al., 1998; Henderson, 1993; Kintz, 2004; Ng, et al., 2006).

Hair analysis has gained increased acceptance as a viable alternative to the conventional analysis of blood and urine for drug testing in forensic toxicology (Nakahara, 1999). So far in forensic investigations, the analysis of feathers has primarily consisted of physical examinations in the identification of birds and external contaminates. However, in safety testing of food animals, poultry feathers are gaining recognition as matrices in which to monitor for residues of compounds that could be harmful to human health (Love, et al., 2012). In environmental investigations, the analysis of feathers, like hair, has been used since the 1960s as a viable matrix for monitoring heavy metals in birds (Burger, 1995; Haskins, Kelly and Weir, 2013; Pilastro, et al., 1993). Researchers have suggested birds will reduce their body burden by excreting metals into their feathers and it is thought this may be the case with other compounds too. Analysing keratinous matrices allows for a wide window of detection (months to years) of many compounds depending on the length and rate of hair growth (Baumgartner, et al., 1989). This is in contrast to the analysis of tissue samples where the detection window would have ceased, via excretion as discussed below and shown in Table 1.3.

In forensic toxicology, the screening test for drugs of abuse traditionally includes conventional matrices such as plasma, tissue, serum and blood. The analysis of these matrices is limited and such samples timeframe of detection falls far short in comparison to hair samples (Table 1.3). Furthermore, the exposure to environmental conditions and length of time contribute to the degradation of conventional matrices. As mentioned previously, it is clear that the longest window of detection is in hair (120 days, Table 1.3), with drugs of abuse detectable well after they would cease to be detected in the conventional samples, such as blood and urine.

Table 1.3 The timeframe of detection for drugs of abuse in corresponding matrices

Matrix	Detection timeframe / days
Saliva	1-36 (hours)
Blood / plasma	1-2
Urine	1-7
Sweat	1-14
Hair	120

Adapted from Richards, et al. (2014)

1.4.3 Feathers and the incorporation of analytes

In response to the limited knowledge of NSAID detection in alternative matrices, this research investigates the analysis of feather samples on the premise that, like hair, compounds will be incorporated and thus detectable via analytical instrumentation. Feathers are primarily made up of keratin and are formed in the same way as hair from follicles (Lucas and Stettenheim, 1972). The feather filament epidermis has three layers; the outermost layer is the feather sheath, the middle intermediate layer and the inner basal layer, the later forms the feather rachis and barb (Lucas and Stettenheim, 1972; Richards, 2010). Feathers grow rapidly until they reach their final size and remain attached to an inert follicle. The feather cycle, like in hair, is divided in a growth phase (anagen) which spans over several days to months or years, and a resting phase (telogen) which can span a few days to around fourteen months. All avian species undergo moulting to shed or replace feathers; most moults occur gradually but this varies for each species depending on breeding cycle habitat and migrations. Molts can also be either complete or partial; complete in which the bird replaces all feathers or partial where only some feather types are replaced (Lucas and Stettenheim, 1972; Richards, et al., 2014).

Once the feather is fully formed the blood vessels degenerate and thus the incorporation of any compound is stopped. A newly formed feather will have a direct blood supply hence NSAIDs are incorporated during the growth phase. If the concentrations in feathers accurately mirror the body burden during formation, feathers that are replaced should reflect the highest levels whilst feathers that are moulted last should accumulate the lowest concentrations (Haskins, Kelly and Weir, 2013; Richards, et al., 2014).

1.4.3.1 The selection of optimal feathers for analysis

There are five main types of feathers; contour or veined, the down, filoplume, semiplumes and bristles. Each of these types have a particular function, namely, the contours are the major flight feathers whilst the semiplumes act as insulation (Yu, et al., 2004). The contour feathers are laid in complex patterns of tracts over the body of the bird covering most of the surface and act as protection from the elements i.e. the sun and rain. The contour feathers are divided into those that cover the body and the flight feathers. The flight feathers of the wing are separated into three groups, the primaries, secondaries and tertiaries, collectively known as the remiges. The primaries are responsible for the forward thrust of the bird and are at the end of the wing, while the secondaries are located in the middle of the wing providing the lift. The tertiaries are the flight feather closest to the body, however, are not as important as primary and secondary feathers. The tail and coverts are the last flight feathers, of which the tail (retrices) act as the brakes and rudder controlling the flight while the coverts are at the very base of the flight feathers acting as a cover. The down, filoplumes, semiplumes and bristles, are thought to have sensory and offer insulation and protection. As there are many different types of feathers, the difficulty surrounds which are optimal for analysis (Proctor, 1993; Richards, et al., 2014), this is discussed below.

With the growth phase of some feathers taking months, this far exceeds the predicted 36 to 58 hour period between exposure to diclofenac and resulting mortality as presented by Oaks, et al. (2004). In a growing feather this lengthy timeframe should allow for the incorporation of the compounds of interest into the blood vessels in the feathers prior to death and allow for its detection thereafter. The difficulty is being able to identify the subsequent feathers whose growth coincides with the exposure. Given their length, primary and secondary flight feathers should be the optimal samples for analysis, however on ethical grounds and from an animal welfare prospective, the removal of these feathers in living birds is unacceptable, flight can be disrupted and can cause discomfort. Thus in living birds alone, body feathers, such as down, filoplume, semiplumes and the bristles, should be sampled, unless primaries or secondaries can be collected when moulted or lost naturally. These continuously grow and are relatively non-invasive, do not disrupt flight and can be collected from several locations of the body even external sites like identified nests may be advantageous. In this research, previously donated moulted feathers have been analysed (Chapter Five).

1.5 THE ANALYTICAL TECHNIQUES EMPLOYED IN THIS RESEARCH

This section includes the use of analytical techniques currently being used in the investigation of NSAIDs. There is specific emphasis on LC-MS as this is the technique used in this research. However, a comparison of LC-MS and LC coupled with tandem mass spectrometry (MS/MS) and gas chromatography coupled with mass spectrometry (GC-MS) is presented. GC-MS techniques are readily used and relied on in this field of research with LC-MS/MS being applied too, however, these involve expensive set up and running costs compared to LC-MS used in this research. This research has also highlighted the comparable, and in some cases, better sensitivity of LC-MS when compared to LC-MS/MS, as discussed in Chapter Four.

1.5.1 Review of use of analytical techniques

Current publications present analytical techniques for the detection of NSAIDs in tissue, serum, urine and wastewater, whether through singular or simultaneous analysis (Gallo, et al., 2006; Haj, et al., 1999; Ibanez, et al., 2009; Loffler and Ternes, 2003; Redderson and Heberer, 2003). In these publications the analytical methods employed varies between the traditional GC-MS and LC-MS, however, some have implemented tandem mass spectrometry (MS/MS), ultraviolet (UV) with diode array detection (DAD) (Hu, et al., 2012; Van Hoof, et al., 2004; Vinci, et al., 2006). Research conducted by Taggart, et al. (2009) is the first and only paper to describe the analysis of nine NSAIDs in ungulate liver tissues, collected from carcass dumps across seven Indian states, available to endangered vultures using LC-MS. This current project, implements the use of LC-MS to detect NSAIDs that previous methods do not include, such as aceclofenac and piroxicam (section 1.3.3), and also includes seven major metabolites. Results from Chapter Four shows the LC-MS technique employed to be more sensitive than published LC-MS and in some cases LC-MS/MS methods (section 4.4.5).

The importance of analytical detection methods was first discussed in the earlier section 1.1 with the limitations of the published method discussed throughout the chapter. It is apparent that overall, analytical techniques in this field are limited and so need to be developed. This research will provide this through the development of a new analytical method capable of detecting, simultaneously, NSAIDs and metabolites currently known to be of threat.

With the continued advances in chromatography and the applications of LC-MS gaining more and more interest, alongside the potential of analysing unconventional samples being both promising and growing, there is a gap in knowledge (SAVE, 2012) for a new method that will in turn fulfil the calls for an LC-MS method capable of the detection of more than ten NSAIDs (SAVE, 2012). Simultaneously employing LC-MS, this study aims to detect thirteen NSAIDs and seven major metabolites, including two internal standards flufenamic acid and piroxicam-d_3. The selection of internal standards is discussed in section 1.6.1 investigating their suitability within the method for development (section 3.1), and in Chapter Four for its validation.

1.5.2 LC-MS in comparison to other available techniques

LC-MS (also often referred to as high performance or ultra-performance liquid chromatography coupled with mass spectrometry; HPLC-MS or UPLC –MS respectively) is a fundamental separation technique in analytical laboratories (Shimadzu, 2012). It has been used for separating, analysing and purifying mixtures since the 1970s (McMaster, 2005). However it has not always been the preferred choice of technique; until the millennium, the favoured analytical technique was GC-MS (McMaster, 2005; Petrovic, et al., 2005). Unlike GC-MS, LC-MS has the capabilities of separating a variety of compounds varying from small molecular compounds and biological molecules and compounds, including those that are thermally liable and volatile (McMaster, 2005; Petrovic, et al., 2005). Furthermore, owing to the elevated polarity and weakly acidic nature of NSAIDs, employing LC avoids derivatisation steps that are required in GC-MS, especially in the analysis of acidic compounds (Barcelo and Hansen, et al., 2009). These factors are particularly important when analysing a wide range of environmental samples, from water to keratinous matrices, and where the analytical method is to be employed in countries with basic instruments where funding may be limited. This is especially important as LC-MS is often more cost effective than typically used LC-MS/MS (section 1.5.1).

In recent years it has been reported that LC-MS sales have nearly equalled to those of GC-MS and it is the advances in the technology, such as new interfacing techniques and more user friendly systems that have made LC-MS easier to implement (McMaster, 2005). Previously, interfaces that restricted or reduced gas flow into the mass spectrometer made combinations of GC-MS a widely used technique for years (Petrovic, et al., 2005; Shimadzu, 2012). In liquid chromatography, when vaporised, the solvent represents of volume 1000 times greater than that of carrier gas used in GC.

Developments have improved gas flow by using combinations of pumping and heating with an additional drying gas stream and ionisation at atmospheric pressure, this is discussed further 1.5.3.1. This is not without disadvantages, soft ionisation techniques can supress ion formation and in doing so will provide less structural information. However, it is important to remember LC-MS analysis will almost always yield a molecular ion (M^+) unlike GC-MS and therefore making it possible to limit the possible target analyte identity.

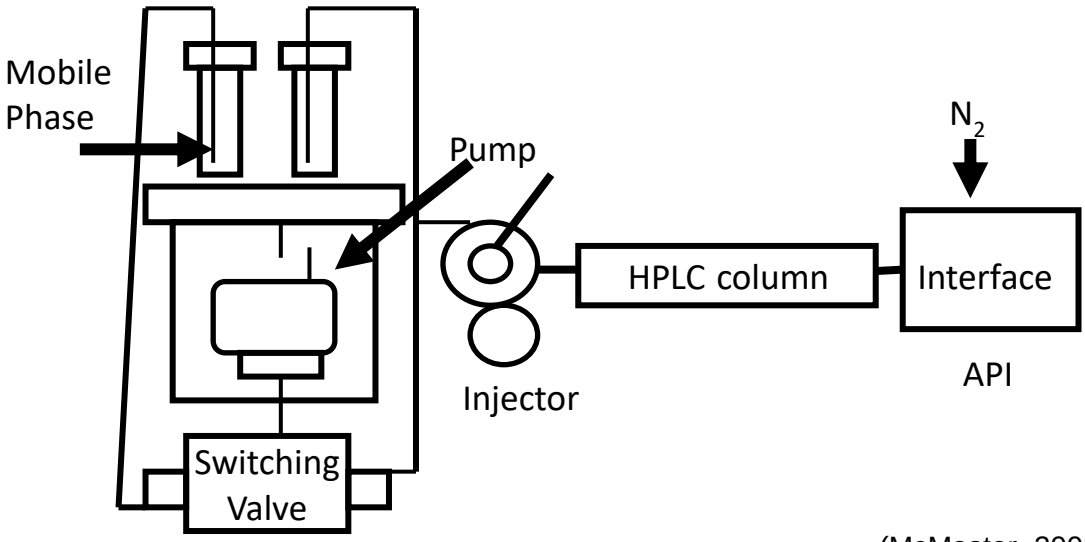
While both analytical methods require mobile and stationary phases the main difference between the two techniques are the mobile phases employed. GC-MS uses a gas phase typically an inert gas like helium, compared to solvents used in LC-MS. The latter is particularly disadvantageous due the large volumes used. LC-MS requires only ultra-pure solvents, meaning that the technique can become costly and less environmentally friendly than its GC-MS counterpart. Despite these disadvantages LC-MS still remains the most suited technique for this field of research and the matrix under analysis.

With these factors in mind, liquid chromatography has been selected as the technique of choice in this research as it provides a lower cost and simpler alternative to tandem mass spectrometry, whilst having many advantages over the use of GC-MS (Huber, 2007).

1.5.3 Liquid Chromatography with Mass Spectrometry

LC-MS uses HPLC as a means of sample separation, consisting of a solvent pump, a sample injector, column, detector and data collection component. A computer connects these components of the system together providing control to the LC, the flow of solvent and injection. Furthermore the computer is used for the data acquisition and processing post analysis; thus acquiring peak area, determine molecular weights of the components and fragmentation pattern (McMaster, 2005, Petrovic, et al., 2005).

The introduction of the analyte into the instrument begins with injection of the analyte into the mobile phase which carries the sample onto the column via a sample loop. When the liquid mobile phase reaches the injector the sample flows through the column by the flow of the liquid mobile phase where separation of analytes occurs. The analyte ultimately reaches the detector where it must pass through an interface when it enters the MS.

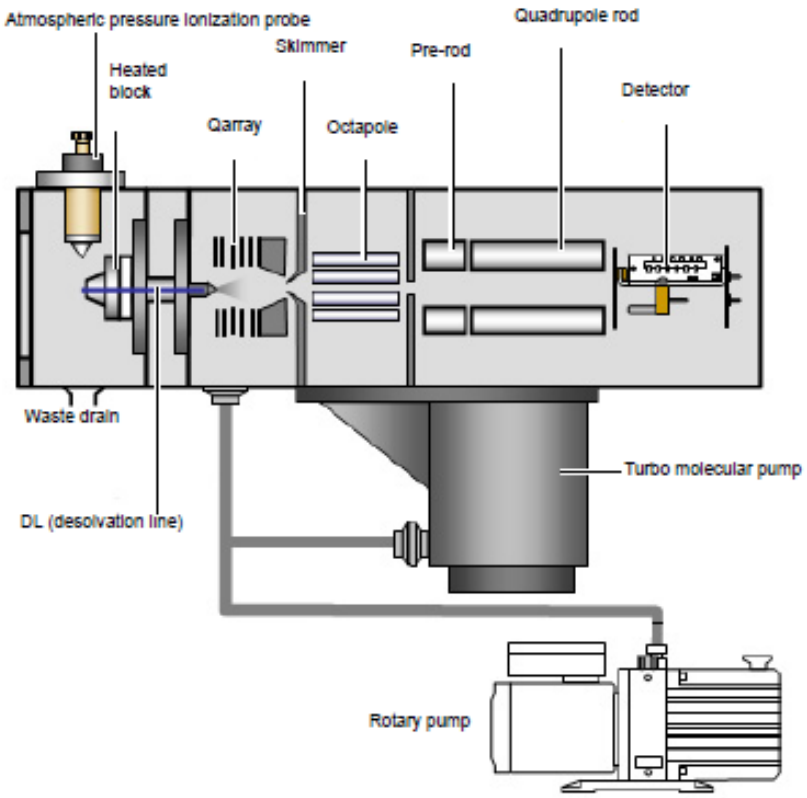


(McMaster, 2005)

Figure 1.14 HPLC configuration

The MS generates mass spectra to be interpreted in the identification of the compound. A more in-depth discussion on fragmentation, ions generated and choice of ions for detection is presented in Chapter Three. The MS consists of three main components; an ion source, which ionises analytes in a solution into a gas phase, a mass analyser which measures the mass of ions and the detector which measures the abundance. Critically, the analyte is introduced from the HPLC into the MS by being sprayed and ionised under atmospheric pressure by the atmospheric pressure ionization probe (e.g., Electrospray Ionisation (ESI) or Atmospheric Pressure Chemical Ionisation APCI probe). The ionised sample is introduced through the sample introduction unit, the desolvation line (DL). The charged droplets are heated by the DL thus, removing the solvent to introduce ions into the vacuum. The ions generated are focused and thereafter introduced into the quadrupole rods by the lens system comprising of the Qarray, skimmer, lens components and octapole (Figure 1.15). The Qarray and skimmer are responsible for focusing the scattering of the ions emitted from the DL. The Qarray main function is as an ion guide; where multi-stage high frequency ions are arranged. While the skimmer is the partition that separates the primary and secondary vacuum chambers. In the Octapole (a high-frequency ion guide located behind the skimmer), like the Qarray, high-frequency voltage is applied to eight plate electrodes to confine the ions and cause them to converge. Thereafter the resulting ions are separated in accordance to their mass-to-charge ratio (m/z) by the quadrupole mass analyser (section 1.5.3.2) (McMaster, 2005; Shimadzu, 2012). Figure 1.15 shows the configuration of the MS.

The final stage is the detector is made up of conversion dynode and electron multiplier whereby it detects positive and negative ions that have passed from the quadrupole rods. Ions are accelerated by the conversion dynode and collide. The collision of these rods releases ions and secondary electrons which travel to the electron multiplier. These secondary electrons are detected, amplified and sent to the data system.



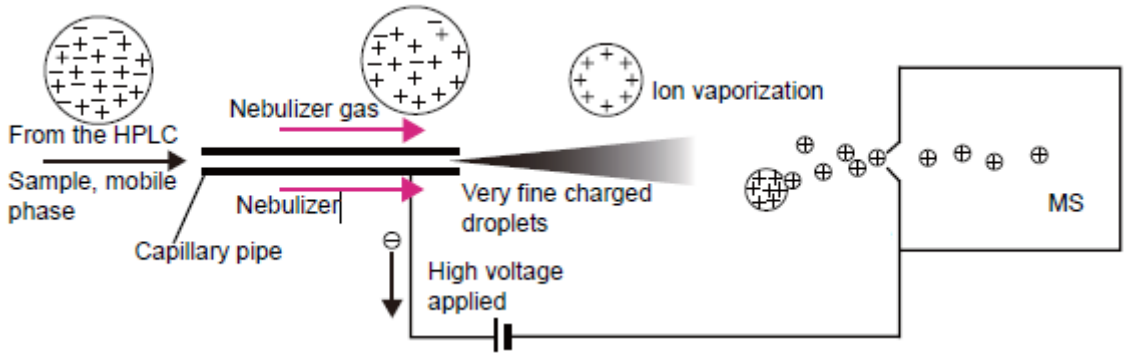
(Shimadzu, 2011)

Figure 1.15 Configuration of Mass spectrometer

1.5.3.1 Electrospray ionisation interface

Electrospray ionisation (ESI) belongs to the wider atmospheric pressure ionisation (API) techniques. In this ionisation technique, ions in the HPLC solution are transferred to the gas phase prior to sampling into the mass analyser. The interface between a liquid phase and a gas phase whilst maintaining a vacuum has posed difficulty for some time (McMaster, 2005), as introduced in section 1.6.1. With the advances in ESI this has changed (McMaster, 2005). ESI is a very soft ionisation technique therefore results in little fragmentation (section 3.2.5).

The process, as shown in Figure 1.16, involves the application of an electric field across the interface, a heated metal capillary pipe surrounded by nitrogen flow (nebulising gas), by which the eluent is passed. An electrospray is dispersed into fine aerosol of charged droplets where an electrostatic field causes further dissociation of analyte droplets (seen in Figure 1.17). The electrospray droplets possess positive or negative charges, dependant on the charge applied to the sampling cone. The drying gas then causes the droplets to vaporise as the charge concentration increases. Repulsive forces between ions with like charges exceed the cohesive forces to allow the resulting ions to be desorbed (ejected) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyser thus allowing separation of ions produced on the basis of mass to charge ratio (Watson and Sparkman, 2007).

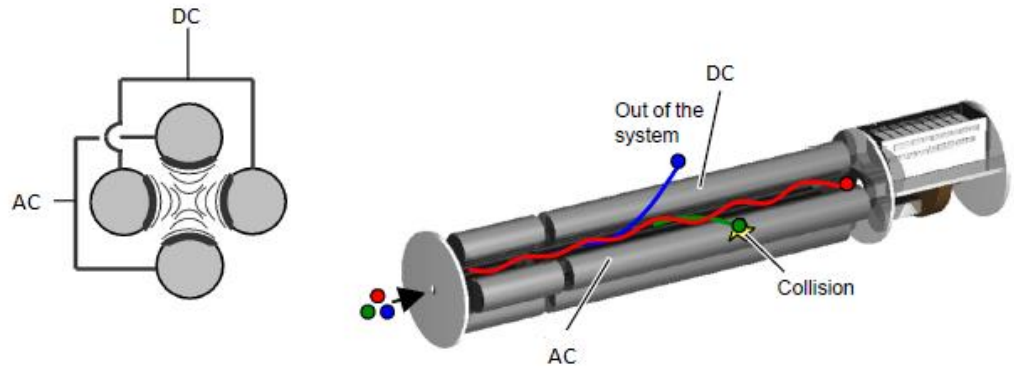


(Shimadzu, 2011)

Figure 1.16 Electrospray Ionisation

1.5.3.2 The quadrupole mass analyser

At the heart of the mass spectrometer is the analyser that measures the mass of an ion. The quadrupole mass analyser is the most widely used analyser due to its ease of use, mass range covered, good linear and dynamic range for quantitative work, resolution and quality of mass spectra (Watson and Sparkman, 2007). The analyser consists of four parallel rods arranged in a square with a void down the middle where the analytes are directed down, see Figure 1.17. The four rods are electrically connected to each other in opposite pairs, two with constant direct current (DC) voltage and two with alternating current (AC) voltage. The latter makes ions spiral as they pass down the quadrupole whilst the constant charge pulls the ions in a constant direction towards one pair of electrodes (Watson and Sparkman, 2007; McMaster, 2005; Shimadzu, 2012).



(Shimadzu, 2011)

Figure 1.17 Quadrupole mass analyser

The stability of an ion in the quadrupole is dependent on the size of the alternating and direct current. The stability refers to the analyte making it through the quadrupole rods rather than hitting the electrode where it is lost, depicted in Figure 1.17 in blue red and green. The conventional quadrupole works by scanning the voltages applied to the four parallel rods. During the majority of the scan the ion is unstable, however for a brief moment, scan lines past through the stable region and will result in the ion passing through to the detector at the other end and thus producing an emerging peak (Shimadzu, 2012).

The quadrupole can operate in two modes; scanning mode (scan) or selected ion monitoring mode (SIM). The latter is significantly more sensitive than scan mode, as it monitors only selected ions. Typically SIM mode is instigated for quantitation and monitoring of target compounds whilst scan mode is generally used for screening used for qualitative analysis and quantification when all analyte masses are unknown in advance. Sensitivity of scan mode is dependent on the number of ions scanned, scan speed and resolution. In LC-MS it is possible to run both positive and negative mode in order to analyse molecules that will ionize in positive and negative modes specifically. The NSAIDs in this research favour the latter (section 3.2.4) (McMaster, 2005; Watson and Sparkman, 2007).

1.6 DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD

This section introduces the preliminary investigations needed to be carried out to develop and validate an analytical method. Analytical methods employed in quantitative analysis are required to obtain reliable, repeatable and accurate data and as such, method development, and validation thereafter, is carried out to demonstrate that the

method of choice is suitable for its intended use (Ellison, Barwick and Farrant, 2009; Peters, Drummer and Mussholf, 2007).

A number of guidance documents regarding the development and validation of analytical methods have been published by various organisations (FDA, 2014; EMA, 1995; Huber, 2007). For example, IUPAC published “harmonized guidelines for single laboratory validation of method of analysis” whilst the United States Food and Drug Administration (FDA) have developed two guides; one for the validation of analytical methods and another for bioanalytical analysis (ICH, 2005; Huber, 2007). Investigations and guidelines set out by Commission Decision (2002), Centre for Drugs Evaluation and Research, International Conference on Harmonisation, and in Huber (2007) were followed throughout the analytical parts of this project.

1.6.1 Selection of NSAIDs and internal standards

In analytical chemistry the use of internal standards is often employed to aid in reproducible quantitative results. Internal standards are compounds that are known to the analysts and will produce a relative signal between the analyte and internal standard so that any changes in instrumental response or noise is counteracted (Nakahara, 1999). By calculating a ratio between the analytical response of an internal standard and that of the analyte, a calibration plot can be constructed. Therefore, on the addition of an internal standard to samples of unknown concentration this ratio can be calculated for quantification (Dolan, 2012). The first step of the method development was to choose a suitable internal standard for the simultaneous analysis of NSAIDs (section 3.1). It was also paramount to consider that the selected internal standards should not be present in samples, should form well-resolved peaks, elute around the analyte of interest and be stable. Additionally it is necessary for the internal standard to have a close chemical relationship to the analyte, i.e. of a similar structure, so that it has comparable chromatographic response namely; extraction characteristics, retention times, stability and detector response (Dolan, 2012).

Two internal standards were selected in this research, namely flufenamic acid and piroxicam-d_3, the former was selected owing to its common use by previous researchers on similar analysis (Kang and Kim, 2008, Niopas and Daftsios, 2002, Ou and Frawley, 1984). Piroxicam-d_3 was selected as a deuterated analogue having a similar structure and analytical response to target analyte (piroxicam under investigation in this case) and are unlikely to be found in environmental samples, such as feathers (Davison, Milan and Dutton, 2013, Owen and Keevil, 2012).

The suitability of the internal standards were verified during initial method development, recording retention time (RT) and relative retention factor (RRF) (section 3.1). The stability of these compounds (section 3.3) were tested prior to method validation thereafter. RT is the time taken for the compound to elute at maximum peak height from injection (Nakahara, 1999). RTs of compounds are dependent on many operational parameters of the instrument. Therefore the RRF (the ratio of retention time of analyte to retention time of internal standard) is calculated to correct the difference in analytical response i.e. peak area and noise, essentially providing a ratio that can be used for identification due to its independence from fluctuations in response.

1.6.2 Method optimisation

Method optimisation involves the development and modification of instrumental parameters such as the stationary phase, mobile phase, gradient elution programs (where mobile phase composition is changed during the analytical run), optimal solvent selection (section 1.6.2.8) and injection volume (section 1.6.2.9). As a result of these modifications, any that affect chromatographic profiles in a negative way are minimised, and those that improve performance are selected.

A good chromatographic profile is a peak, originating from Gaussian distribution, i.e. tall, sharp, narrow and symmetrical. The distinction between good chromatography and poor chromatography is influenced by several factors, for example, column efficiency (N) (section 1.6.2.1 and 1.6.2.2), capacity factor (k) (section 1.6.2.4), selectivity ($\propto$) (section 1.6.2.5), resolution (R_s) (section 1.6.2.6) and peak asymmetry (A_s) (section 1.6.2.7). Hence, these were studied to achieve an optimised method suitable for method validation (Chapter Four). During the development and modification of the gradient elution program, the chromatographic profiles of each compound were assessed until the most optimal method had been developed (section 3.2) (Huber, 2007; CDER, 1994). As part of this optimisation, the selection of a suitable column (section 1.6.2.3) was an important consideration alongside changes to the gradient elution program.

1.6.2.1 The Plate Theory of chromatography – column efficiency

Column efficiency is measured as theoretical plate number, an indirect measure of peak width and the ability of a column to produce narrow sharp peaks. The plate theory proposes that a chromatographic column is made up of a large number of separate layers, called theoretical plates and it is these plates that govern the shape of the

resulting peaks in the chromatogram (Braithwaite and Smith, 1996; Neue, 1997). In these plates, equilibration of the sample between the stationary and mobile phase occur. As the mobile phase moves through the column, this results in the transfer of mobile phase and analyte from one plate to the next (also known as mass transfer). In these plates, compounds with a greater affinity for the stationary phase will bond more strongly and thus, be retained in the column for longer. This results in the separation of compounds into bands and with the movement of the mobile phase the bands move down the column separating as they travel.

In terms of the resolving power to separate analytes in the column, it is said, the higher the plate numbers the more efficient the column, (Neue, 1997) and by reducing the particle size of the UHPLC column, efficiency (plate number) is increased (Neue, 1997). Hence, a column with a high plate number will produce a sharper (narrower) and more intense peak, showing normal distribution, i.e. Gaussian shape and overall results in better separation from adjacent peaks. Plate number or column efficiency can be calculated from the half-height method in the following equation 1.1, where t_r is the retention time of the peak of interest and $W_{0.5}$ is the peak width (units of time) at half-height (Dolan, 2016).

$$\mathbf{N = 5.54\left(\frac{t_r}{W_{0.5}}\right)^2} \qquad \text{(Equation 1.1)}$$

This half-height method is commonly used as it enables calculation of N if the peak is not fully separated from the neighbouring peak. This method assumes that the valley between the peaks is lower than half-height of the peak under investigation (Dolan, 2016).

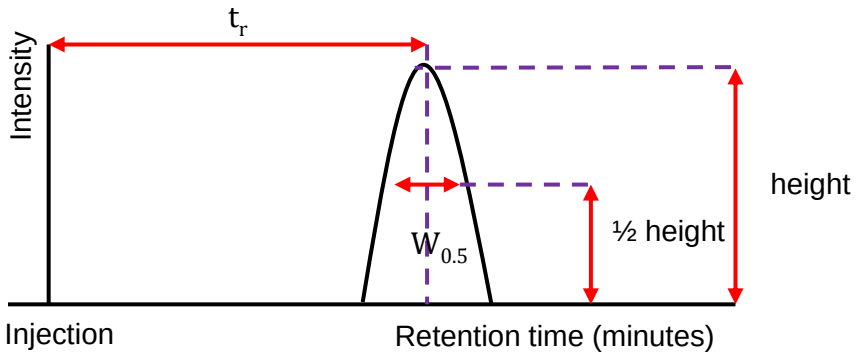


Figure 1.18 systematic of half height method for plate number

As an alternative to plate number (N) for column efficiency, plate height (H) is a measure of the length of column needed for the equilibrium process to proceed (the resolving power of the column). H is defined as the height equivalent of the theoretical plate (HETP) determined by plate number (as discuss above) and is given by the equation 1.2 (IUPAC, 2014). Where L is the length of column and N is the plate number (calculated in Equation 1.1).

$$\mathbf{HETP} = \mathbf{L}/\mathbf{N} \qquad \text{(Equation 1.2)}$$

1.6.2.2 The Rate Theory of chromatography

Whilst the plate theory helps to understand the processes inside the column, the rate theory of chromatography considers the time taken for the compound under analysis to equilibrate between the two phases. Thus, it considers the resulting band shape (band broadening) which is affected by kinetic variables such as, the rate of elution, diffusion (the net movement of molecules) and mass transfer of the analyte between the stationary and mobile phase (section 1.6.2.2) (Braithwaite and Smith, 1996; Neue, 1997).

Band broadening is a phenomenon that reduces the efficiency of separation in the analytical column, leading to poor chromatographic response, i.e. resolution (section 1.6.2.6), and peak asymmetry (section 1.6.2.7). For this reason the Van Deemter equation for height equivalent of the theoretical plate (HETP) (Equation 1.3) arises from rate theory to relate the resolving power of the column to the experimental variables that affect band broadening namely, Eddy diffusion (A), longitudinal diffusion (B) and resistance to mass transfer (C) (Neue, 1997). A graphical presentation on Equation 1.3 is presented in Figure 1.19.

$$\mathbf{HETP} = \mathbf{A} + \frac{\mathbf{B}}{\mathbf{u}} + \mathbf{Cu} \qquad \text{(Equation 1.3)}$$

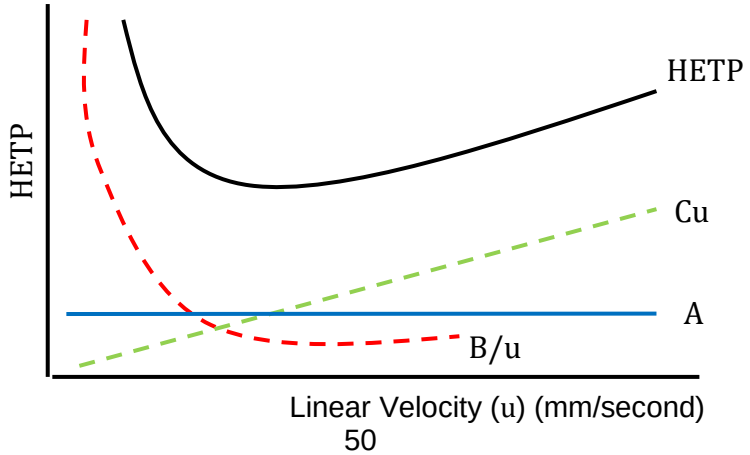


Figure 1.19 Graphical presentation of contributing factors to the Van Deemter equation

Eddy diffusion (A) (Figure 1.19) describes the movement of the analyte through the column, whereby the analyte will take different paths, at random, through the stationary phase. These paths occur due to small variations in particle size and lack of homogeneity of the stationary phase. This causes band broadening, a result of differences in path length, consequently resulting in a board peak shape (Braithwaite and Smith, 1996; Neue, 1997). As such, the effects of Eddy diffusion can be minimised by selecting a well packed column with smaller particle size, as was the case in this research by changing from a HPLC column with 5µm particle size to a UHPLC column of 1.7µm (section 1.6.2.3 and 3.2.1).

Meanwhile, an analyte will diffuse from the centre of a peak meaning the concentration of the analyte is at its highest in the centre and lowest at the edges of the peak as it diffuses, this process is called longitudinal diffusion (B) (Figure 1.19) (Neue, 1997). Longitudinal diffusion arises from the effects of linear velocity i.e. the flow rate. Band broadening will be worsened at low flow rates as the compounds under investigation will spend more time on the column resulting in a broader peak when compared to high linear velocity. To overcome this effect, a narrower column (decreasing the internal diameter) with a higher flow rate will ensure less time spent on the column resulting in tall, sharp and narrow peaks (section 1.6.2.3 and 3.2.1) (Neue, 1997).

Lastly, mass transfer (C) (introduced in section 1.6.2.1, Figure 1.19) refers to the time taken for the compound of interest to equilibrate between the stationary and mobile phase. The time spent in equilibration is thus dependent on the velocity of the mobile phase and the compounds affinity for the stationary phase. As such, if the analyte has a strong affinity to the stationary phase and the velocity of the mobile phase is high, then band broadening will occur as the mobile phase moves ahead of the analyte of interest. Unlike longitudinal diffusion, mass transfer is therefore worsened with higher linear velocity, however, effects can still be minimised in the same way i.e. the smaller the column diameter the less band boarding will result from mass transfer (Braithwaite and Smith, 1996). Mass transfer was an important consideration during the solvent selection (sections 1.6.2.8 and 3.3.2) and injection volumes (section 1.6.2.9 and 3.2.3).

It is important to understand these factors and their role in chromatographic response. It is apparent that these factors are therefore related to the column and the compounds

under investigation and therefore can be manipulated to optimise performance. As such, these factors were considered during method optimisation (section 3.2).

1.6.2.3 Column selection

The analytical method in this research was intially adapted from Baranowska and Kowalski's (2010) simultaneous detection HPLC method (section 2.2.1, Table 1.2). This was selected based on the inclusion of four NSAIDs covered in this research and its applicability to environmental samples, specifically waste and surface waters. The first stage in method development, was the selection of a column. A commonly used column for the type of analytes investigated in this research is the UHPLC kinetex column C18 with trimethylsilyl (TMS) with a particle size of 1.7 µm, internal diameter 2.1mm, 100mm in length.

The kinetex column is a reverse phase, meaning generally the stationary phase is relatively non-polar and the mobile phase is polar. Analytes dissolve in like for like, hence the most polar analytes are eluted from the column first followed by the other analytes in order of decreasing polarity (Synder and Dolan, 2007). This type of chromatography is commonly used in the analysis of NSAIDs and is particularly advantageous in the analysis of hydrophilic and hydrophobic compounds (Miao, Koenig and Metcalfe, 2002; Baranowska and Kowalski, 2010).

Additionally, improved peak symmetry (section 1.6.2.7) can be achieved at increased flow rates resulting in a shorter run time accompanied by the possible increase in resolution (section 1.6.2.6) (Guillarme and Veuthey, 2008; Neue, 1996). In a HPLC column, a larger internal diameter (ID), typically 4.6mm, requires higher volumes of mobile phase through high flow rates. Changing from a HPLC 4.6mm to a UHPLC 2.1mm ID column, as used in this research, can lower the flow rate, in turn lowering the solvent volume, thus achieving optimal linear velocity (the speed at which the solvent front travels the length of the column) without an increase in analysis time (section 1.6.2.2) (LGGC, 2015). Similarly a decrease in ID can increase sensitivity when injecting the same analyte mass. By changing the column diameter, the amount of stationary phase will reduce, in turn affecting the loading capacity, leading to an increased analyte concentration in the mobile phase (Neue, 1996). Therefore, the column selected in this research ensured a reduction in the solvent volume used without compromising the efficiency or selectivity of the analytical method (section 1.6.2.2). The analysis time was shortened, typically this is determined by the length in

column. In general the length is directly proportional to retention time amongst column efficiency and backpressure (Guillarme and Veuthey, 2008).

Whilst a reduction in analysis time and column length is acceptable, efficiency of chromatographic separation is paramount. Therefore, after the selection the UHPLC column, optimisation of the gradient method parameters were investigated (section 3.2.1), making changes to improve overall chromatographic separation and peak resolution (Huber, 2007; CDER, 1994).

1.6.2.4 Capacity factor

The first parameter to be evaluated was the capacity factor (k). k is equal to the ratio of retention time (t_r) of the peak under investigation to the retention time of the unretained peak or the dead time of the column (t_m), equivalent to the time where there is no affinity for the stationary phase, also referred to as dead time. This can be calculated in the following equation (CDER, 1994):

$$\boldsymbol{k} = \frac{\boldsymbol{t_r - t_0}}{\boldsymbol{t_0}}$$ (Equation 1.4)

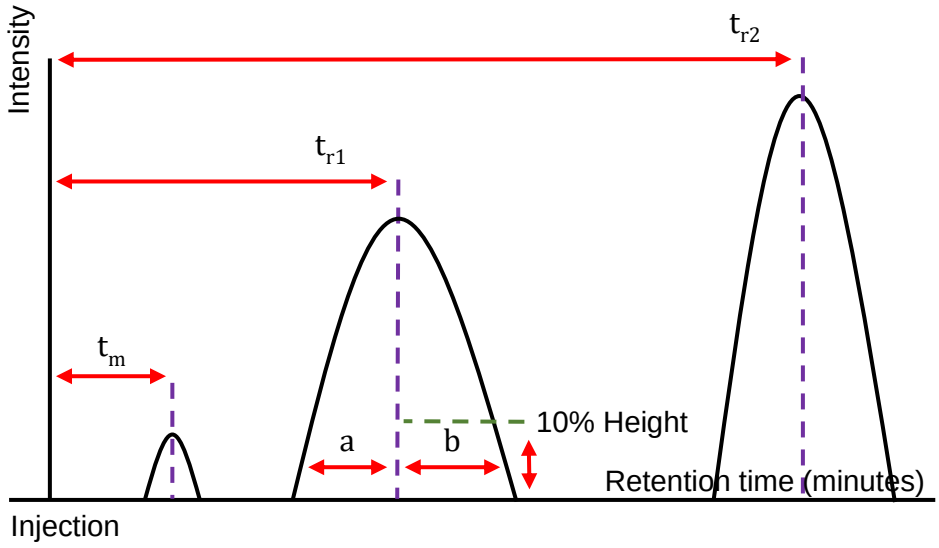


Figure 1.20 Systematic of peak separation use in the calculation of capacity factor, selectivity, resolution and peak asymmetry

An ideal k is between one and five, whereby if less than one it implies the analyte is not highly retained in the column and therefore, elution is quick compared to higher values (>5) signifying slow elution of anaytes from the column (IUPAC, 2014).

1.6.2.5 Selectivity

Selectivity ($\propto$), is defined as the ability to assess explicitly the analyte in the presence of other components expected to be present (Huber, 2007). It is a measure of the separating power of the column. It considers two peaks at any one time and measures the separation between the 2 compounds (the ratio capacity factors for both analytes). Selectivity of <1 suggests that compounds cannot be separated as they are more or less retained in one peak. When selectivity is >1 this shows the two analytes are separated from each other. Selectivity is calculated through the following equation:

$$\propto = \frac{k_1}{k_2} = \frac{t_{r2} - t_0}{t_{r1} - t_0} \qquad \text{(Equation 1.5)}$$

Where: k_1 is the capacity factor of the less retained peak (eluting first) and k_2 is the capacity factor of the more retained peak (eluting second). Calculated in values of time, as depicted in Figure 1.20.

1.6.2.6 Resolution

Where compounds are not fully separated, resolution (R_s), is the measure of peak overlap. To calculate R_s plate number (N), selectivity ($\propto$) and capacity factor (k) are incorporated into the equation. Selectivity and capacity factor are measures of retention in the column and therefore influence the retention of compounds. Consequently, increases in plate number and selectivity increase resolution, while an increase in capacity factor will decrease the resolution power (Dolan, 2016). It is paramount that all three factors are optimised to achieve optimal resolution overall. When peaks are fully resolved, R_s is said to be >2, however, mutual overlap is accepted for values between 1.0 and 1.5 (Huber, 2007; CDER, 1994). Resolution is calculated using the following equation, as depicted in (Figure 1.20):

$$\boldsymbol{R_s} = \frac{1}{4}\left(\frac{\propto - 1}{\propto}\right)\left(\frac{k}{k+1}\right)\sqrt{N} \qquad \text{(Equation 1.6)}$$

1.6.2.7 Peak asymmetry

The last parameter is peak asymmetry factor, a measure of peak tailing and fronting, describing peak shape. The ideal chromatographic profile was surmised earlier in the section (1.6.2.1), however, undesirable effects, in the form of peak tailing and fronting, can occur during method development. Tailing and fronting occur when the peak becomes distorted, typically fronting occurs when too much sample has been introduced and tailing can arise from effects of mass transfer, between equilibrium of the phases and the analyte in the plates (section 1.6.2.2). The following equation is employed to calculate overall peak asymmetry (CDER, 1994):

$$\boldsymbol{A_s = \frac{b}{a}} \qquad \text{(Equation 1.7)}$$

Where: A_s is the peak asymmetry factor, b is the distance from the point at peak midpoint to the trailing edge (measured at 10% of peak height) and a is the distance from the leading edge of peak to the midpoint (measured at 10% of peak height) (Figure 1.20). A peak asymmetry factor of <1 indicates fronting while >1 indicates tailing.

Peak asymmetry is important to consider in method development, as problems with asymmetrical peaks often present problems with resolution and quantification. They become more difficult to resolve and thus, integrate to provide a peak area resulting in quantification that is much less reproducible (Braithwaite and Smith, 1996).

After the investigation of these parameters, and optimisation of gradient elution program (section 3.2.1), retention times of all NSAIDs, and major metabolites were recorded, as shown in Table 3.3 and 3.4, alongside the RRF for identification purposes. These retention times were used throughout preliminary investigations, method validation (Chapter Four) and ultimately the identification process in the analysis of matrices (Chapter Five).

1.6.2.8 Solvent and mobile phase selection

Selecting solvents in liquid chromatography is an important step in achieving high sensitivity and selectivity, peak shape and chromatographic resolution (Ahuja and Rasmussen, 2007). Solvent selection can be just as important in method development as the instrumental parameters themselves, yet it is often overlooked (Ahuja and Rasmussen, 2007). The selection process must involve the selection of solvents that

analytical standards are prepared in, mobile phase constitution and use of additives. In LC-MS the use of additives is common practice. It involves the addition of chemicals to the mobile phase to supress unwanted ionisation in order to improve analyte response and enhance selectivity, this is especially advantageous in the analysis of complex matrices.

Formic acid, as used in this research, is a commonly used in a wide range of applications with LC-MS. The use of formic acid is to facilitate ionization by ensuring the analyte of interest is more basic than that of the solvent (Waters, 2015). For example, in the compounds investigated in this research, some are carboxylic acids having pK_a values ranging from 3.5 to 6.5 (Table 1.1) When the pH of the mobile phase is above this range, the acidic compounds are ionised. This results in poor chromatographic profiles due to shorter retention on the column. However, by lowering the pH below the pK_a, the compounds will become unionised and thus the ionisation of the carboxylic acids supressed, this increases the retention and results in better chromatography (Dolan, 2001; Wrezel and Pakula, 2005). Furthermore, the addition of formic acid suppresses the silanol activity of the stationary phase, essentially decreasing the unwanted interactions between basic molecules and the acidic silanol groups, thus, improving chromatographic separations resulting in better retention and peak shape of the analytes of interest (Dolan, 2001). These developments in solvent selection are discussed in detail in section 3.2.2.

The most obvious consideration for sample solvent and mobile phase selection is the solubility of the analyte. However, factors such as the ability to dissolve the analyte of interest, produce good chromatographic profiles (section 1.6.2.1), avoid degradation of the sample and increase selectivity should also be considered (Wrezel and Pakula, 2005). In the case of the latter, the solvent is an important consideration as upon injection and entry into the column thereafter, this is the place where the solvent and mobile phase are mixed for the first time. If incorrectly chosen, the disparity in solvent strength, between the solvent and mobile phase, can result in peak distortion, and thus analytical response (such as fronting and tailing). This distortion arises during the mass transfer of sample and mobile phase between the theoretical plates and equilibrium between the two phases (section 1.6.2.2). To negate the disparity in solvent strength, it is suggested, that an ideal sample solvent is the starting composition of the mobile phase (MP at T_0) (Wrezel and Pakula, 2005). MP at T_0, acetonitrile and methanol were investigated during method development, assessing the chromatographic profiles of compounds to find the optimal solvent (section 3.2.2).

1.6.2.9 Injection volumes

Chromatographic behaviour of a compound can be affected by many operational variables within the analytical method (section 1.6.2.2). This is especially relevant to injection volumes, whereby small changes can be made to obtain the best chromatographic results, giving rise to its investigation during method development (section 3.2.3). It is crucial that during development any changes or variables that can improve analytical response and chromatography are optimised (sections 1.6.2.1 to 1.6.2.8). Ideal chromatography involves achieving a peak profile of normal Gaussian distribution, one that is symmetrical, narrow and sharp. Whilst tailing, fronting (section 1.6.2.7) and band broadening (1.6.2.2) kept to a minimum. The latter, band broadening, is especially problematic as it can result in a loss of efficiency, poor resolution and poor chromatographic profiles of compounds investigated i.e. distorted peak shape and broad peak resolution. In this study, in addition to avoiding mass overloading, chromatographic profiles i.e. peak asymmetry (section 1.6.2.7) was investigated in the selection of the optimal injection volume, alongside RSD (section 3.2.3).

Overload in chromatography refers to the column condition where the sample size is so large and/or too concentrated, that performance is compromised (Synder and Dolan, 2007). This is described at two levels, either mass transfer or volume overload. The first considers the mass or concentration of the sample that is injected, whilst volume overload considers the injection volume, whether too large or too small. Band broadening effects arising with mass transfer (introduced in the rate theory, section 1.6.2.2, in the Van Deemter Equation (Equation 1.3)), will occur when too higher concentration saturates the column. As a result, the analyte will not equilibrate with the phases in the bands and will travel further down the column giving rise to broad peaks with drastically reduced resolution. Similarly in volume overload, effects of longitudinal diffusion will become apparent when high volumes of analyte are injected. Here peak broadening will be evident during the diffusion of the peak, if too much volume is injected then peaks will start to tail and retention times will increase (Hostettmann, Marston and Hostettmann, 1998).

To avoid any possible overload, the sample volume injected should be less than 10µl injection volume when using 100% strong solvent (Synder and Dolan, 2007). Following these guidelines injection volumes of 0.2µl, 2µl and 10µl were investigated (section 3.2.3) at corresponding concentrations of mixed NSAID standards prepared in 100% acetonitrile (section 2.3.3), with the starting composition of mobile phase and methanol.

1.6.3 Identification of NSAIDs and diagnostic ions

Compounds of interest can be identified through a series of parameters, whether qualitative i.e. comparison to standards, or quantitative i.e. determining the concentration of an unknown. In this research, compounds under investigation were initially identified by their RT and RRF. Solvent blanks were assessed by monitoring for any interference at RT of interest and mass spectra for each of the NSAIDs, in both the scan and selected ion monitoring (SIM) mode, were investigated (section 3.2.4). SIM (section 1.5.3.2) ensures a selective and sensitive method by distinguishing between detected compounds (Huber, 2007).

In the identification of NSAIDs, some researchers monitor only 2 diagnostic ions, however, the World Anti-Doping Agency or WADA (2003), recommend the analysis of at least three diagnostic ions, including one quantification ion and two confirmations ions, to ensure the correct identification of compounds of interest (WADA, 2003). In this thesis, the guidelines provided by the World Anti-Doping Agency are followed, ensuring the use of 3 ions for each of the NSAIDs, thus enabling a minimum of one ion ratio for the identification according to the Commission Decision (2002). The identification of NSAIDs and diagnostic ions is discussed in section 3.2.4 with the implementation of SIM in section 3.2.5.

1.6.4 Stability

Stability is one of the parameters investigated during method development and validation (section 3.3 and 4.2) as analytes can decompose prior to investigation whether during preparation, storage or analysis. To determine the stability of the chosen analytes during analysis time, method development should include an investigation into their short term stability (autosampler stability). Typical sample analysis time in this research ranged between 24 to 54 hours, with the majority of that time spent in vials in situ on the temperature controlled autosampler at 15^0C (section 3.3). These results offer a way of calculating, within certain degree of fluctuation, the allowed time span between sample preparation and analysis. In any analysis, products of degradation may exist and the presence of these can be monitored during stability testing (Huber, 2007). Furthermore, it is paramount to consider longer term stability (section 4.2), i.e. the maximum time from preparation to completion of all investigations and to account for standard storage in case of any unforeseen instrumental breakdown. With this in mind, a 6 day stability study was carried out in triplicate.

Stability considers samples to be stable if instrument response is within relative standard deviation (RSD) of 15% (Huber, 2007; Peters, 2007).

1.6.5 Precision and Accuracy

Precision is the close agreement between measurements, whilst accuracy is defined as the agreement between the true value and the experimental value. Precision is considered in intra-day repeatability and inter-day reproducibility studies (Huber, 2007). Repeatability refers to the precision of the analytical method achieved through replicate measurements made in a short time (section 3.4). Repeatability is often referred to as inter-assay precision. Intermediate precision expresses variations over days (section 4.2). Accuracy was considered in terms of % Recovery during the application of the method (section 5.2.2) (Miller and Miller, 2010).

Precision is determined and assessed by the relative standard deviation in percentage, often referred to as RSD. In terms of analytical methods acceptance criteria for precision is 15% RSD and if concentrations are nearer the limits of quantification (LOQ) then RSD of 20% are accepted (ICH, 2005; Peters, Drummer and Musshoff, 2007).

1.6.6 Linear range

The International Conference for Harmonisation (ICH) defines linearity as the capability of a method, within a range, to obtain results that are directly proportional to the concentration of the compound of interest in the sample. Linearity is investigated over a wide range of concentrations including at the very low and very high ends of concentrations. Ascertaining the linear range also provides information about the sensitivity of the method and the instrument, for example how low the method can detect at (Ellison, Barwick and Farrant, 2009). Results are presented as linear plots of instrumental response against concentration. A linear regression line is then fit and a linear regression equation calculated. Through the interpretation of linear regression plots, the linear range can be determined. The range of an analytical method is the region of upper to lower concentration levels that follow a linear trend within a degree of precision. Any data points that tail off and deviate at the low and high concentrations signify a change in non-linear behaviour in analytical response. When analysing linearity in this research the individual peak area ratios (peak area of the most abundant ion/most abundant ion of internal standard), were used for instrumental response. The resulting regression line of best fit is obtained from least squares which

minimises the sum of squared differences between the observed value and fitted values from the line (CDER, 1994; Ellison, Barwick and Farrant, 2009).

Analytical methods should be free from bias and thus random and of normal distribution (Peters, Drummer and Musshoff, 2007). Although standard deviation gives a measure of the spread about the mean, it does not indicate the shape of the distribution or the randomness of errors. Normality or rather normal distribution refers to the distribution of random errors; a random variable will result in normal distribution (Peters, Drummer and Musshoff, 2007). To test normality of these errors, within the investigation of linearity, the non-parametric Shapiro Wilk or goodness of fit test and Wald-Wolfowitz runs test have been applied (section 4.4.4) (Mehta and Patel, 2012). The assessed linear range has been discussed in section 4.4.

1.6.7 Limits of Detection (LOD) and Limits of Quantification (LOQ)

The LOD and LOQ are determined at the lower end of linear range, the LOD is defined as the lowest concentration that the analyte can be detected at but not necessarily quantified and similarly the LOQ is the lowest concentration that the analyte can be quantified with suitable precision and accuracy (Ellison, Barwick and Farrant, 2009; Peters, Drummer and Mussholf, 2007). Furthermore, LOD and LOQ assesses if instrumental response is distinguishable from a response of the blank. LOD and LOQ arecalculated as shown in equations 1.8 and 1.9 (IUPAC, 2014):

Limit of detection = $y_B + 3s_B$ (Equation 1.8)

Limit of quantification = $y_B + 10s_B$ (Equation 1.9)

Where $y_B$represents the response of the blank and $3s_B$ represents 3 times the standard deviation of the blank (10 times in LOQ $10s_B$).

1.7 RESEARCH AIMS

There is a considerable lack of research into NSAIDs detection and analysis in a wildlife context. A thorough literature review identified only one paper on the analysis of NSAIDs in such context, specifically the detection of nine NSAIDs in ungulate tissue available to endangered species using LC-MS (Taggart, et al., 2009). Furthermore, the method did not include NSAIDs since identified of concern nor any metabolites. This is especially important to consider, as some are more potent and persistent with slow excretion. From the initial literature reviews of this research it was identified there is a

need to address the lack of research surrounding the use of non-invasive samples in the detection of these compounds as currently in this field analysis is carried out on post-mortem traditionally used samples such as blood and tissue. Taggart, et al. (2009) analysed liver and kidney samples from livestock post-mortem. Other papers have only analysed for one NSAID, diclofenac and again only in post-mortem vulture samples (Oaks, et al, 2004). This research is novel in its approach to different samples (keratinous feathers and hairs) that could be sourced pre-mortality to quickly identify possible early exposure and some could be collected non-invasively through shedding. Additionally, feather samples are covered by this research but the method could be rolled out to samples (such as animal hair and furs) from other species that could be exposed to such pharmaceuticals.

Typically NSAIDs are analysed using LC-MS/MS in environmental samples such as wastewater, however, this project uses LC-MS which is a lower cost and simple alternative. This method could be used in countries with basic analytical instruments. This is the first reported research/detection to include the combination of these eleven NSAIDs, importantly it also includes seven metabolites never analysed before in a wildlife sample. Therefore the aims of this research are:

1) A critical review of literature to identify NSAIDs currently of environmental and toxic concern and to include other NSAIDs that pose a future environmental impact. To also include, metabolites that have been reported to be more potent and persistent in the body than their parent analyte.
2) To develop and validate a new method for these NSAIDs and metabolites using LC-MS.
3) To use the validated method on extracted keratinous samples and this extraction method will also become part of the validation.
4) Publish and collaborate findings to organisations to aid in wildlife conservation.

CHAPTER 2: METHODOLOGY

This chapter describes the LC-MS instrumental settings, methods and materials (section 2.1 and 2.2) used in this research. It is divided into three sections thereafter; (I) initial method development: preliminary development (section 2.3), (II) validation studies (section 2.4) and (III) sample preparation and application of the validated method (section 2.5). These sections highlight the various studies conducted in the development and validation of the analytical method and the application in feather samples. The LC-MS instrumental parameters have been discussed in the relevant section alongside the standards preparation and materials used.

2.1 CHEMICALS AND REAGENTS

Technical grade aceclofenac, acetylsalicylic acid, carprofen, diclofenac sodium, flufenamic acid, flunixin meglumine, ibuprofen, ketoprofen, mefenamic acid, meloxicam sodium, nimesulide, phenylbutazone, piroxicam, deuterated piroxicam (piroxicam-d_3) and suxibuzone (all in powder form) were purchased from Sigma-Aldrich, UK. Technical grade metabolites 4-hydroxydiclofenac, 5-hydroxyflunixin, 5-hydroxypiroxicam and oxyphenylbutazone (all in powder form) were purchased from Sigma-Aldrich, UK. Whilst 3-hydroxymethyl mefenamic acid, 4-hydroxynimesulide and 5-carboxymeloxicam (all in powder form) were obtained from Santa Cruz Biotechnology, Germany.

HPLC and LC-MS grade solvents acetonitrile, methanol and water alongside additives; formic acid, acetic acid and ammonium acetate were purchased through Fisher Scientific and Sigma-Aldrich, UK. Ultra-pure water suitable for HPLC use was purified using an Elga PURELAB Option, available at Anglia Ruskin University, Cambridge.

2.2 STANDARDS PREPARATION

2.2.1 Standards preparation: stock solution

Single and mixed NSAIDs stock solution (aceclofenac, acetylsalicylic acid, carprofen, diclofenac, flunixin, ibuprofen, ketoprofen, mefenamic acid, meloxicam, nimesulide, phenylbutazone, piroxicam and suxibuzone) of 1mg/ml were prepared in acetonitrile and methanol.

Stock solutions of 3-hydroxymethyl mefenamic acid, 4-hydroxydiclofenac, 4-hydroxylnimesulide, 5-carboxymeloxicam were prepared at 0.5mg/ml in acetonitrile and

prepared at 1mg/ml for 5-hydroxyflunixin, 5-hydroxypiroxicam and oxyphenylbutazone in acetonitrile. Internal standards (flufenamic acid and piroxicam-d_3) of 100µg/ml were prepared in acetonitrile.

2.2.2 Standards preparation: working solutions

Working solutions of single and mixed NSAIDs of 100µg/ml were prepared from the stock solution (1mg/ml) in acetonitrile and methanol (section 2.2.1). Working solutions of the metabolites were prepared at 50µg/ml and 100µg/ml from the single stock solutions (1mg/ml and 0.5mg/ml) in the presence of internal standard at 0.1µg/ml, in acetonitrile.

2.3 INITIAL METHOD DEVELOPMENT: PRELIMINARY INVESTIGATIONS

2.3.1 Optimisation of gradient elution program

The analytical method in this research was adapted from Baranowska and Kowalski's (2010) simultaneous detection HPLC method (Table 2.1). Optimisation was carried out using mixed NSAIDs standards as prepared in section 2.2.1.

Table 2.1 original HPLC method (Baranowska and Kowalski, 2010)

Original HPLC method	Time (minutes)	Solvent A % water/0.05% trifluoroacetic acid	Solvent B % methanol	Solvent C % acetonitrile
Gradient elution program	0	89	10	1
	10	50	30	20
	15	50	30	20
	17	30	20	50
	25	5	5	90
	30	89	10	1

A Shimadzu LCMS-2020 was employed during method development and validation (section 2.3.1) fitted with a kinetex C18 with trimethylsilyl (TMS) endcapping stationary phase column with a particle size of 1.7µm, internal diameter 2.1mm, 100mm in length. With a matching SecurityGuard 2.1mm guard column and cartridge. The column was purchased from Phenomenex. LABSolutions software was used for system control, data acquisition and data retrieval.

As Baranowska and Kowalski's (2010) method employed HPLC, initial changes in the method parameters were made to the gradient elution program time from 30 to 16 minutes to account for the change in column (from a HPLC Develosil RPAQUE-OUS-

AR-5 C30, 250mm in length, 4.6mm internal diameter and 5.8µm particle size), particularly its length, and technique being employed (LC-MS) (Table 2.2, section 3.2.1, Figure 3.3).

Table 2.2 Adapted gradient elution program analysis time, to account for change in column (method A)

Method A	Time (minutes)	Solvent A % water/0.1% formic acid	Solvent B % methanol/0.1% formic acid	Solvent C % acetonitrile/0.1% formic acid
Gradient elution program	5	50	30	20
	7.5	50	30	20
	8.5	30	20	50
	13	5	5	90
	16	89	10	1

Thereafter adaptions were made to the gradient elution program until the best separation was achieved (Table 2.3, section 3.2.1, Figure 3.4).

Table 2.3 Adapted gradient elution program (method B)

Method B	Time (minutes)	Solvent A % water/0.1% formic acid	Solvent B % methanol/0.1% formic acid	Solvent C % acetonitrile/0.1% formic acid
Gradient elution program	0.5	89	10	1
	1	60	25	15
	1.1			5
	5		30	
	8		20	
	10	5	5	90
	11 - 16	89	10	1

Further method development was carried out changing the mobile phase. Method C, involved the removal of methanol, leaving water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B) as two mobile phases (Table 2.4, section 3.2.1, Figure 3.5).

Table 2.4 Adapted gradient elution program, with two mobile phases (method C)

Method C	Time (minutes)	Solvent A % water/0.1% formic acid	Solvent B% acetonitrile/0.1% formic acid
Gradient elution program	0.5	98	2
	1	75	25
	5	65	35
	12.5	40	60
	15.5	0	100
	16.5	0	100
	17-18	99	1

Thereafter, adaptions were made at one minute, decreasing mobile phase B percentage to 12.5% B, and adjusting the percentage at five minutes to 25% B instead of 35% B. This method formed the final optimised gradient elution used in validation studies (section 2.3.1) (Table 2.5, section 3.2.1, Figure 3.6).

Table 2.5 Optimised LC gradient elution parameters (method D)

Optimised Method	Time (minutes)	Solvent A % water/0.1% formic acid	Solvent B% acetonitrile/0.1% formic acid
Final gradient elution program	0.5	98	2
	1.0	87.5	12.5
	5.0	75	25
	12.5	40	60
	15.5	0	100
	16.5	0	100
	17 - 20	99	1

2.3.2 Optimal solvent selection for standards

Mixed NSAID standards of 20µg/ml (section 2.2.1) were prepared in three solvents to test for analyte solubility and optimal solvent selection (section 3.2.2); 100% acetonitrile, 98:2 mix of water 0.1% formic acid and 2% acetonitrile 0.1% formic acid (to mimic the starting mobile phase constitution) and 100% methanol. Standards were analysed using parameters in Table 2.5 and 2.6.

2.3.3 Injection volumes

To investigate optimal injection volumes for ideal method performance (section 3.2.3), three injection volumes of 0.2µl, 2µl and 10µl were analysed at concentrations of 20µg/ml in acetonitrile, respectively using ibuprofen and mefenamic acid as examples. Based on initial results (section 3.2.3), mixed NSAID standards at 20µg/ml were prepared in 100% acetonitrile were injected in volumes of 0.2µl and 2µl.

2.3.4 Initial autosampler stability in an intra-day study over 54 hours

Mixed standards were analysed for 54 hour autosampler stability (15ºC). Stability (section 3.3) was investigated at three concentrations, 0.05, 0.5 and 5µg/ml respectively. These were prepared from the 100µg/ml mixed NSAID working solution in the presence of internal standard at 0.1µg/ml (section 2.2.2).

2.3.5 Intra-day repeatability precision studies

Intra-day repeatability precision (section 3.4) was tested at three concentrations, 0.05, 0.5 and 5µg/ml respectively. These standards were prepared from the 100µg/ml mixed NSAID working solution in the presence of internal standard at 0.01µg/ml (section 2.2.2).

2.4 VALIDATION STUDIES

2.4.1 LC-MS instrumental parameters

Validation was conducted on a Shimadzu LCMS-2020 was fitted with a kinetex C18 with trimethylsilyl (TMS) endcapping stationary phase column with a particle size of 1.7 µm, internal diameter 2.1mm, 100mm in length. With a matching SecurityGuard 2.1mm guard column and cartridge. The column was purchased from Phenomenex. LABSolutions software was used for system control, data acquisition and data retrieval.

Adapted from Baranowska and Kowalski's (2010) HPLC method (section 2.3.1, Table 2.2), the LC-MS parameters were developed (section 3.2.1, Figure 3.3 to 3.7) until a fully optimised method suitable for validation (Chapter Four) was achieved (Table 2.5 and 2.6).

The LC-MS analytical method employed gradient elution implementing two mobile phases; water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient elution was operated as described in section 2.2.1 and Table 2.5, at a flow rate of 0.5 ml/min. At the start and very end of the elution re-equilibration steps (0.5 minutes at the start and 3 minutes at the end) were included. At the end, the gradient was held at 99% A and 1% B.

The initial and optimised LC-MS parameters are shown in Table 2.6. Specifically, electrospray ionisation (ESI) was used in negative mode, and the MS was operated in SIM using ions from Table 3.6 between 4 and 14 minutes (section 3.2.4). The wash

solution used for the autosampler was 2-propanol and the optimal injection volume of each sample was 2µl (section 3.2.3).

Table 2.6 Optimised mass spectrometer settings

mass spectrometer parameters	Initial method development settings	Method validation settings
ionisation mode	Negative and positive ESI^{-+}	negative ESI^{-}
interface temperature	350^{o}C	
desolvation line (DL) temperature	held at 250^{0}C	
nitrogen nebulising gas flow rate	1.5L/min	
nitrogen drying gas	15L/min	10L/min
heating block	300^{0}C	
DL voltage	0	
Qarray voltage	0.0	
mass analyser mode	Scan m/z 50-400 between 0-20 minutes	SIM between 4-14 minutes
wash solution	2-propanol	
injection volume	0.2 µl	2µl
autosampler temperature	15^{0}C	
oven	50^{0}C	
photo diode array detector (PDA)	Scan between 190nm and 800nm	scan between 190nm and 400nm

2.4.2 Longer term stability in an inter-day study over six consecutive days

Longer term stability was considered for method validation over 6 days whereby samples were prepared and stored in a freezer in -20ºC conditions and thawed on the day of analysis.

Stability in method validation was investigated at three concentrations, as per section 2.3.4, in the presence of internal standard at 0.1µg/ml, in line with concentrations studied in the linearity (section 2.4.4).

2.4.3 Inter-day reproducibility precision studies

Inter-day reproducibility precision (section 4.3) was tested at three concentrations, as per section 2.3.5, in the presence of internal standard at 0.1µg/ml. The concentration of the internal standards was in line with that used throughout the validation.

2.4.4 Linearity and method detection limits

Linearity (section 4.4) was determined by investigating eleven different concentrations of the mixed NSAIDs standard in triplicate injections. Calibration standards (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25µg/ml) were prepared in acetonitrile from the 100µg/ml working solution in the presence of internal standards at 0.1µg/ml (section 2.2.2). LABSolutions software was used for data procurement while Microsoft and IBM SPSS Statistics was used for data interpretation (regression, residual analysis and statistical tests (Shapiro-Wilk normality test and Wald-Wolfowitz run test). Microsoft Excel was used to calculate the LOD and LOQ values.

2.5 SAMPLE PREPARATION AND APPLICATION OF VALIDATED METHOD

2.5.1 Feather sample pre-treatment

Feather samples were cut into 2-3 cm pieces (section 5.1.1, Figure 5.1). The samples were washed with warm water and sonicated for five minutes. This step was repeated until water was clear, the second and last washes were stored in vials and placed into the fridge for storage. Methanol was added and sonicated for a further five minutes, this wash was kept and refrigerated. Samples were left to dry in-between double layers of Whatman 542 ashless filter paper and once dry, transferred into sealable clear plastic sample bags (Figure 5.2). Which were frozen for two minutes in liquid nitrogen thereafter stored in a freezer until ready to be ground.

After being frozen, samples were cut into 2-3mm pieces (Figure 5.3) ground in an Agate jar and ground at 300 revolutions per minutes (rpm) for fifteen minutes in a Retsch PM100 (Figure 5.4). After grinding any remaining large pieces were cut with nail scissors until a powder like consistency was achieved (Figure 5.5).

2.5.2 Sample digestion and extraction

Aliquots of 50mg pulverised feathers were weighed in triplicate into 2ml Eppendorf tubes. Into each tube 100µl of each internal standard at a concentration of 1µg/ml was added followed by 1ml of methanol. In spiked samples, 100µl of a 0.1µg/ml mixed NSAIDs and metabolite standard was also added. These tubes were then placed into a Microtherm 56 shaker (CamLab Ltd) overnight, the temperature set at 40ºC for 17 hours.

After digestion, samples were filtered using dampened (with methanol) cotton wool. After the initial filtration, 1ml of methanol was added to the sample, and filtered once again followed by a further 1ml of methanol for rinsing.

To the filtrate, 1ml of hexane was added and mixed, the hexane layer was removed and kept for evaporation. Samples were evaporated to dryness using a miVac DNA sample condenser (Genevac Ltd) set at 40ºC and 1000 rpm. Dried samples were reconstituted in 100µl of acetonitrile. Where samples were not reconstituted immediately dried samples were stored in a freezer in -20ºC conditions and thawed on the day of analysis.

2.5.3 Analysis of spiked and unspiked feathers

Spiked and unspiked feather samples were prepared as per section 2.2 and analysed using the LC-MS settings in section 2.3.1. Results are presented in Chapter Five.

In recovery studies, samples were spiked (digested and filtered as per section 2.5.2) with 100µl of a 0.1µg/ml mixed NSAIDs and metabolites, alongside 100µl of each internal standard at a concentration of 1µg/ml. Standards were prepared in the same way (section 2.5.1 and 2.5.2) to mimic sample preparation for comparison in recovery studies.

CHAPTER 3: METHOD DEVELOPMENT RESULTS AND DISCUSSION

Method development encompasses the improvement of an analytical method through several sequential preliminary investigations. Preliminary investigations involve the modification of initial parameters and preparation techniques whereby, experimental improvements are incorporated to produce a method that is developed under optimal conditions (Huber, 2007). Whilst Chapter One explains the selection of HPLC and LC-MS techniques, and Chapter Two describing the preliminary investigations involved in the method development process, the results and discussion of those investigations are included in this chapter. It presents the results of LC-MS method optimisation prior to method validation (Chapter Four), including the assessment of chromatographic response, solvent selection and injection volume studies alongside initial stability and precision studies.

3.1 SELECTION OF INTERNAL STANDARDS

During initial method development the selected internal standards (IS) (piroxicam-d_3 and flufenamic acid) were studied in terms of chromatographic response. The characteristic ions used for their identification were taken from the literature specifically m/z 280, 341 and 250 for flufenamic acid and m/z 333, 341 and 250 for piroxicam-d_3 (Moffat, Osselton and Widdop, 2004). Retention times (RT) were recorded and used with diagnostic ions to establish correct identification. The RT and analysis of chromatographic profile for each IS are presented in Table 3.1. Figures 3.1 and 3.2 shows examples of the peak profile (total ion chromatogram, TIC) of each internal standard.

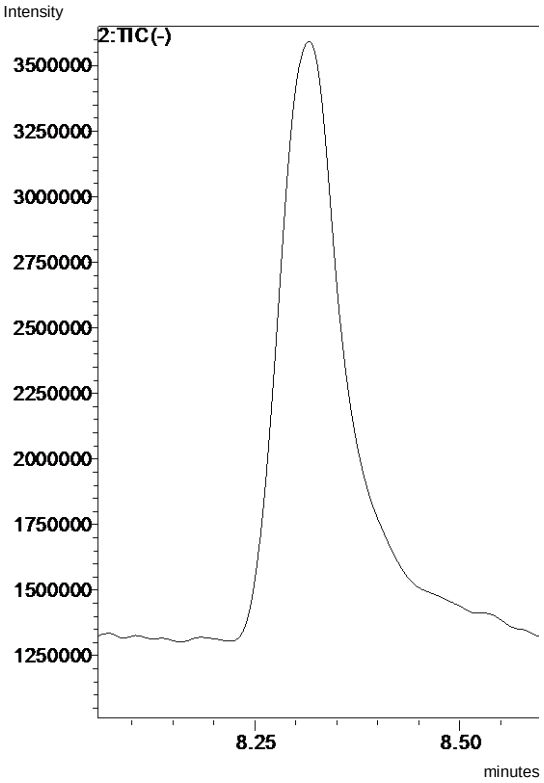


Figure 3.1 TIC peak profile of internal standard piroxicam-d_3

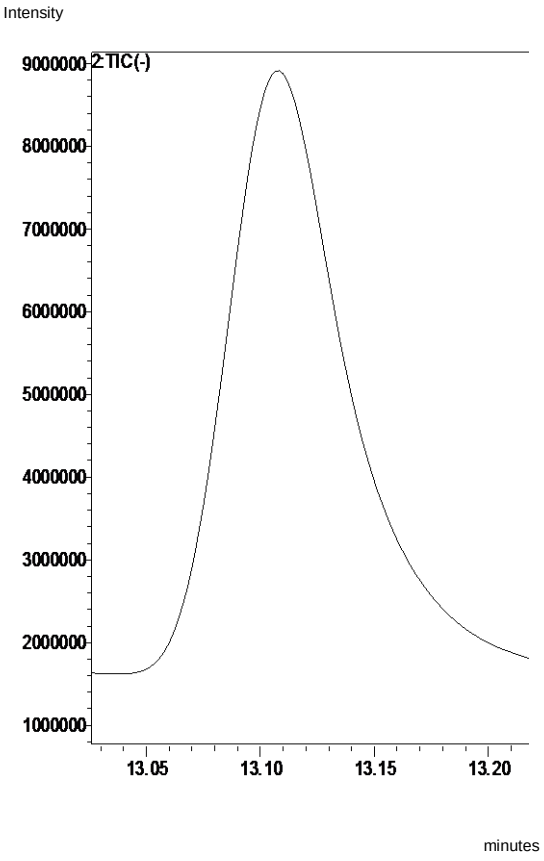


Figure 3.2 TIC peak profile of internal standard flufenamic acid

Table 3.1 Qualitative data for the chromatographic profile of internal standards piroxicam-d_3 and flufenamic acid

Internal Standard	RT[a]	k[b]	α[c]	R_s[d]	A_s[e]
Piroxicam-d_3	8.31	1.08	1.00	0.05	1.80
Flufenamic acid	13.11	2.27	1.56	0.60	2.00

[a]Mean retention time (minutes), [b]capacity factor, [c]selectivity
[d]resolution, [e]asymmetry

The peaks for both internal standards were well defined and sharp, selectivity was high for both compounds (>1) indicating they were both separated from neighbouring analytes. Both exhibited an ideal capacity factor of >1 (section 1.6.2.4) showing elution was neither too quick nor too slow. The resolution of both compounds was lower than the accepted >2, indicating mutual overlap with adjacent compounds, however, during SIM flufenamic acid was resolved from its neighbouring peak (mefenamic acid). In the case of Piroxcam-d_3, resolution was <1 due to its co-elution with its counterpart piroxicam. In LC-MS applications, deuterated standards are expected to exhibit this co-elution and therefore, do not required chromatographic resolution if, the MS is able to distinguish between the two compounds within a degree of certainty (Synder, Kirkland and Dolan, 2010). Both compounds had different mass spectral characteristics, hence, on the application of SIM (section 3.2.4 and 3.2.5), the method was proven to be selective enough to distinguish between the two.

The asymmetry (section 1.6.2.7) of each peak was also investigated. Both internal standards presented tailing with tailing factors calculated at 1.4 and 1.5 for piroxicam-d_3 and flufenamic acid respectively. This had an effect on the asymmetry factor which were both calculated over >1. Despite this the peaks still showed a good chromatographic profile (Gaussian distribution) and were repeatable throughout the method development (Figure 3.1 and 3.2, section 3.2.1). Furthermore, on initial investigations of mixed standards, in the presence of both NSAIDs and Internal standards, peak area ratios (PAR) were reasonable (0.75 to 3.3) (Table 3.2), this is especially important in terms of symmetry as any distortion in peak shape can make integration of peaks for quantification problematic (Synder, Kirkland and Dolan, 2010). The tailing factors did not cause any problems during the qualitative or quantitative stages of this research.

As mentioned, as part of the investigation of internal standards, single standards were analysed in the presence of these two compounds. Resolution between the internal

standards and NSAIDs was above accepted guidelines (>2) in all cases (with the exception of prioxicam-d_3 with piroxicam and flufenamic acid and mefenamic acid). From these early investigations RT and relative retention factor (RRF) were obtained (Table 3.2). Using RRF it was possible to determine which internal standard was used for which NSAID when calculating PAR for the entirety of the study.

A RRF closer to one indicates the closeness of analyte and internal standard, essentially providing a ratio between retention times. Using the RRF based on closeness in retention, and higher analytical response, the internal standard piroxicam-d_3 (Table 3.2, in blue) was used for NSAIDs acetylsalicylic acid, piroxicam, flunixin, ketoprofen, meloxicam, nimesulide, carprofen, diclofenac, suxibuzone and ibuprofen, and flufenamic acid (Table 3.2, in black) for NSAIDs aceclofenac, phenylbutazone and mefenamic acid. In this early developmental stage the metabolites (section 1.3.7.1) had not been procured, however, RRF value and RT are reported later in section 3.2.1.

Table 3.2 Retention times and RRF obtained through analysis of single NSAIDs standards

NSAID[a]	RT[b]	RRF[c]	PAR[d]
acectysalicylic acid	5.14 ± 0.009	1.62	0.75
piroxicam-d_3	8.31 ± 0.005	1.00	1.00
piroxicam	8.32 ± 0.004	1.00	1.75
flunixin	9.54 ± 0.007	0.87	1.31
ketoprofen	9.99 ± 0.00	0.83	1.09
meloxicam	10.18 ± 0.006	0.82	2.53
nimesulide	10.76 ± 0.007	0.77	3.33
carprofen	11.57 ± 0.002	0.72	1.40
diclofenac	11.99 ± 0.004	0.69	1.58
suxibuzone	12.04 ± 0.026	0.69	1.08
ibuprofen	12.13 ± 0.00	0.68	0.07
aceclofenac	12.13 ± 0.010	1.08	0.37
phenylbutazone	12.65 ± 0.009	1.04	0.65
mefenamic Acid	12.97 ± 0.00	1.01	0.29
flufenamic Acid	13.11 ± 0.005	1.00	1.00

.[a]100µg/ml, [b]retention time (n=3), [c]relative retention factor [d]mean peak area ratio (n=3), NSAIDs in blue= ratio with piroxicam-d_3, NSAIDs in black= ratio with flufenamic acid

As a result of these studies, the selection and suitability of both internal standards was proven. They were shown to have a good chromatographic profile, albeit a low tailing factor and good resolution, taking into account capacity factor and selectivity.

3.2 INITIAL METHOD DEVELOPMENT: PRELIMINARY INVESTIGATIONS

3.2.1 Optimised gradient elution program

In order to establish an optimised method for preliminary investigations and validation thereafter, changes in gradient elution program were investigated in terms of effects on chromatographic separation. The analytical method in this research was adapted from Baranowska and Kowalski (2010) (Table 2.1). Gradient elution programs were adapted as described in the methodology (section 2.3.1, method A (Table 2.2), method B (Table 2.3), method C (Table 2.4) and the optimised method D (Table 2.5)). TIC generated for each gradient elution program are presented in Figures 3.3 to 3.7 respectively. Figure 3.7 shows detection of more analytes than the other TIC as the metabolites (oxyphenylbutazone, 3-hydroxymethylmefenamic acid, 4-hydroxydiclofenac, 4-hydroxynimsulide, 5-carboxymeloxicam, 5-hydroxyflunixin and 5-hydroxyproixcam) had not been procured until this point.

Across all gradient elution programs there were similarities in the chromatographic profiles specifically, the general order of elution and retention times. The main differences between the methods was co-elution between compounds, the peak shape, resolution, and asymmetry factors. As such, it was recognized that chromatographic response was improved over the development stages, this is especially apparent on the comparison of Figure 3.3, when the first adaptions were made (Table 2.2), to Figure 3.6, the final method, ready for validation (Table 2.5). These improvements were brought about through initial changes in the gradient elution program (methods A and B), followed by an additional change in mobile phases (methods C and D).

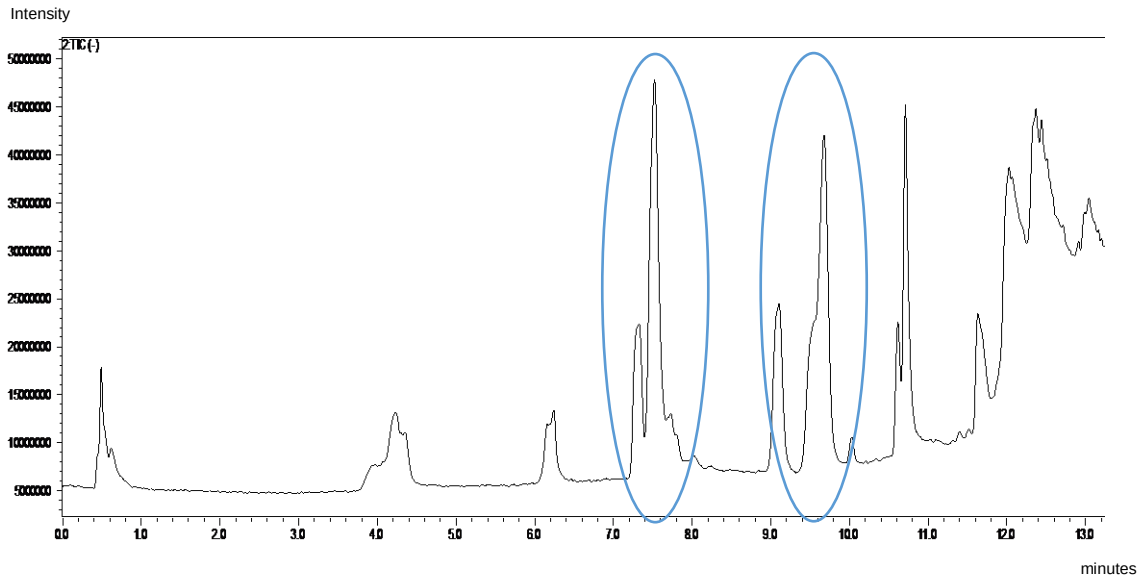


Figure 3.3 Chromatographic profiles (TIC) of mixed NSAID standards (100µg/ml) obtained with method A (Table 2.2)

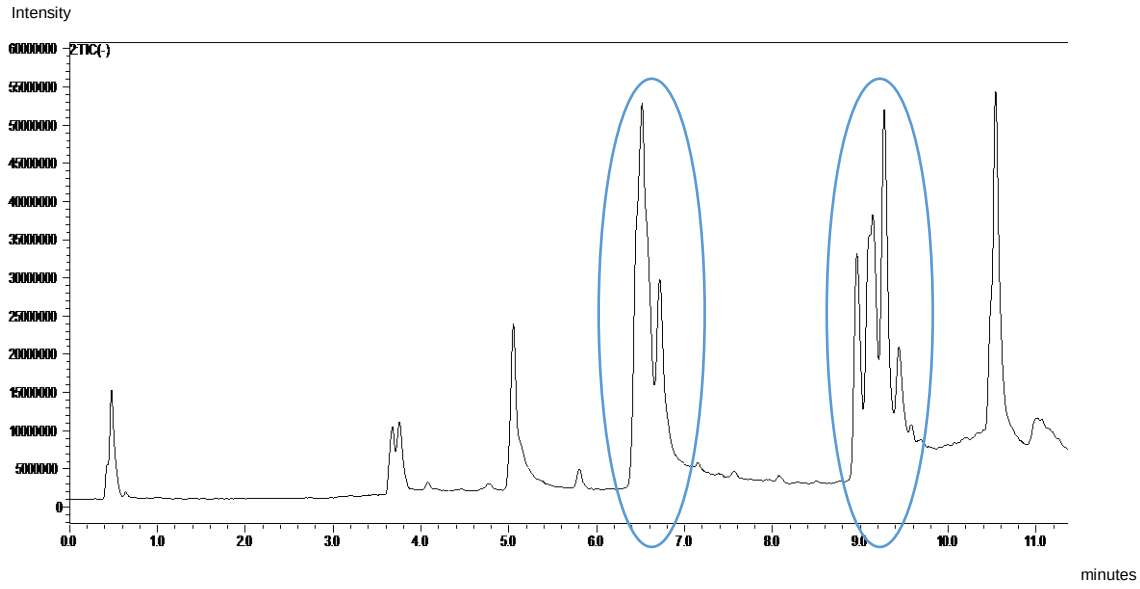


Figure 3.4 Chromatographic profiles (TIC) of mixed NSAID standards (100µg/ml) obtained with method B (Table 2.3)

Method optimisation first started with the implementation of method A (Table 2.1), which resulted in nineteen peaks eluting from the column. Chromatographic separation was poor with peak exhibiting shouldering, tailing, fronting and splitting. There was also poor separation between compounds (Figure 3.3); this analysis was carried out on a mixed standard of twelve NSAIDs and two internal standards. Therefore, using previous knowledge of RT obtained from the analysis of single NSAID standards (Table 3.1) it was possible to predict there were interference peaks with the possibility of co-elution between compounds. On interpretation of the results, it was indicated there was co-elution between meloxicam and ketoprofen around 7.5 minutes and nimesulide and

phenylbutazone around 9.5 minutes (as highlighted in Figure 3.3). With these results, changes in the gradient elution program were made. In method A (Table 2.2), the mobile phases increased by 20% within 2 minutes resulting in meloxicam, ketoprofen, nimesulide and phenylbutazone eluting within the same time. By concentrating on the retention area where the co-elution had occurred (7.5 to 9.5 minutes), the gradient was changed to slow the change in polarity and the increase in percentage change of methanol (0.1% formic acid). This would slow the elution of compounds resulting in further separation (Table 2.3).

Thereafter, similar results were achieved upon the application of method B (Figure 3.4), namely, flunixin, ketoprofen, meloxicam and nimesulide still co-eluted at 6.5 minutes. Undesirable chromatographic profiles were worsened in nimesulide and phenylbutazone with both compounds co-eluting with carprofen, diclofenac and aceclofenac at 9 minutes. Asymmetry factors were higher when comparisons were made between the two methods, for example carprofen asymmetry factor was 1.13 in method A compared to 2.97 in method B. Furthermore, where separation was present in the chromatogram, peaks did not exhibit base line separation, hence resolution and selectivity was low (<1).

The results showed significant improvement from method B to method C (Figure 3.4 and 3.5) with the removal of methanol from the mobile systems employed in method B (Table 2.3). All compounds eluted between 5 and 10 minutes whereby the percentage change of acetonitrile was from 5 to 90 % when this method (B) was employed. Acetonitrile has a higher elution strength than methanol which the compounds under investigation favoured. Therefore, in method C (Table 2.4), on the removal of methanol, the percentage change in acetonitrile was spread over a longer duration of time at a much slower rate, which resulted in the slower elution and better separation in compounds (Figure 3.5). Acetonitrile also has a lower viscosity than methanol and in turn reduces backpressure often resulting in better peak shape, as was the case across these two methods (Figure 3.4 and 3.5).

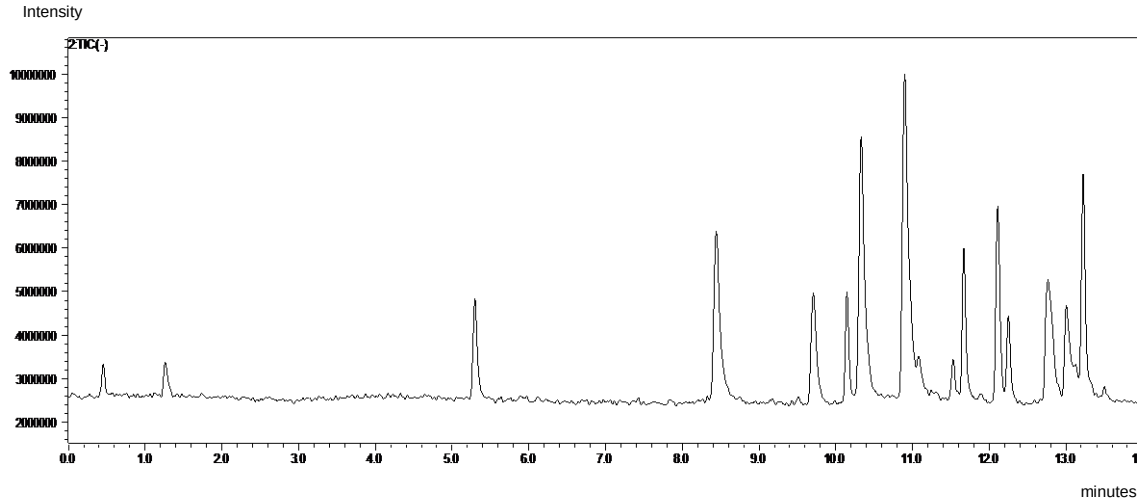


Figure 3.5 Chromatographic profiles (TIC) of mixed NSAID standards (100µg/ml) obtained with method C (Table 2.4)

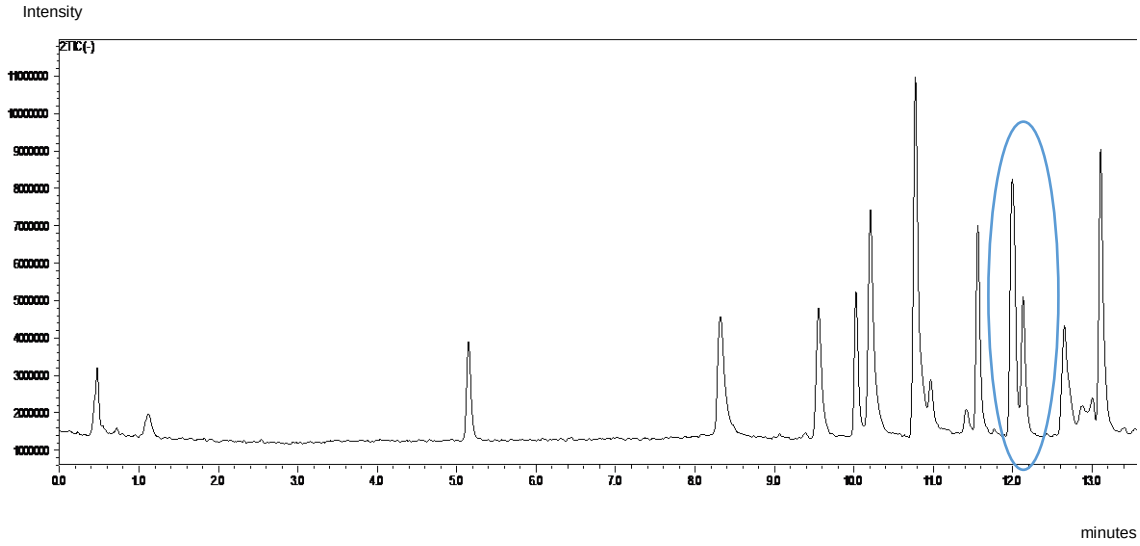


Figure 3.6 Chromatographic profiles (TIC) of mixed NSAID standards (100µg/ml) obtained with method D (Table 2.5)

The results showed further significant improvement between method C and D (Figures 3.5 and 3.6, Table 2.4 and 2.5), the peaks were Gaussian in shape and were tall and narrow. In addition resolution (section 1.6.2.6) and selectivity (>1) (section 1.6.2.5) were improved and overall asymmetry (<2) was good (Table 3.3). Resolution was considerably improved for compounds flunixin, ketoprofen, meloxicam, nimesulide, carprofen, aceclofenac and phenylbutazone, which was unachievable with previous methods (A and B). Compounds diclofenac, suxibuzone and ibuprofen still continued to co-elute in TIC (highlighted in Figure 3.6), however, peaks corresponding to each compound were distinguishable in SIM chromatograms, whereby, these compounds had an ideal capacity factor (=2) (section 1.6.2.4), selectivity indicated that the compounds could be separated (≥1) (Table 3.3). Therefore, all were deemed to yield a

good chromatographic profile.

This ideal chromatographic separation was brought by changes in the mobile phases employed. The method changes involved the removal of methanol and the increase in water and acetonitrile. Chromatographic separation is governed by the interaction between compounds and the mobile phase, the method employed was reverse phase chromatography (section 1.6.2). The compounds under investigation are weak acids in nature (section 1.4.1), hence an increase in the % organic in the mobile phase (acetonitrile in this case) increases the elution strength of the mobile phase. This worked to separate compounds that favoured this percentage change (Table 2.5) over a longer period. Hence, compounds would elute in order of decreasing polarity, with those of lower polarity having a higher affinity for the stationary phase and resulting in a higher capacity factor (Neue, 1997), as was the case for diclofenac, suxibuzone, ibuprofen, aceclofenac, phenylbutazone and mefenamic acid ($k \geq 2$). The main differences between methods C and D was the addition of a longer equilibrium stage at the end, this provided a longer time to re-equilibrate the column, whereby, there is a shift from 0% mobile phase A to 99% as required at the start of the next run. This stage also served to elute any strongly retained impurities from column prior to the next injection (Synder and Dolan, 2007).

The gradient elution program was optimised ensuring capacity factor, sensitivity, resolution, tailing factor and asymmetry were all within acceptable boundaries (section 1.6.2) (CDER, 1994). Initial development until this point had only investigated NSAIDs, hence, after the procurement of metabolites under investigation the method was then trialled. A mixed standard including both NSAIDs and metabolites was analysed under method D. Results were interpreted in the same manner as the mixed NSAID standard prior and are given in Figure 3.7 and Table 3.3.

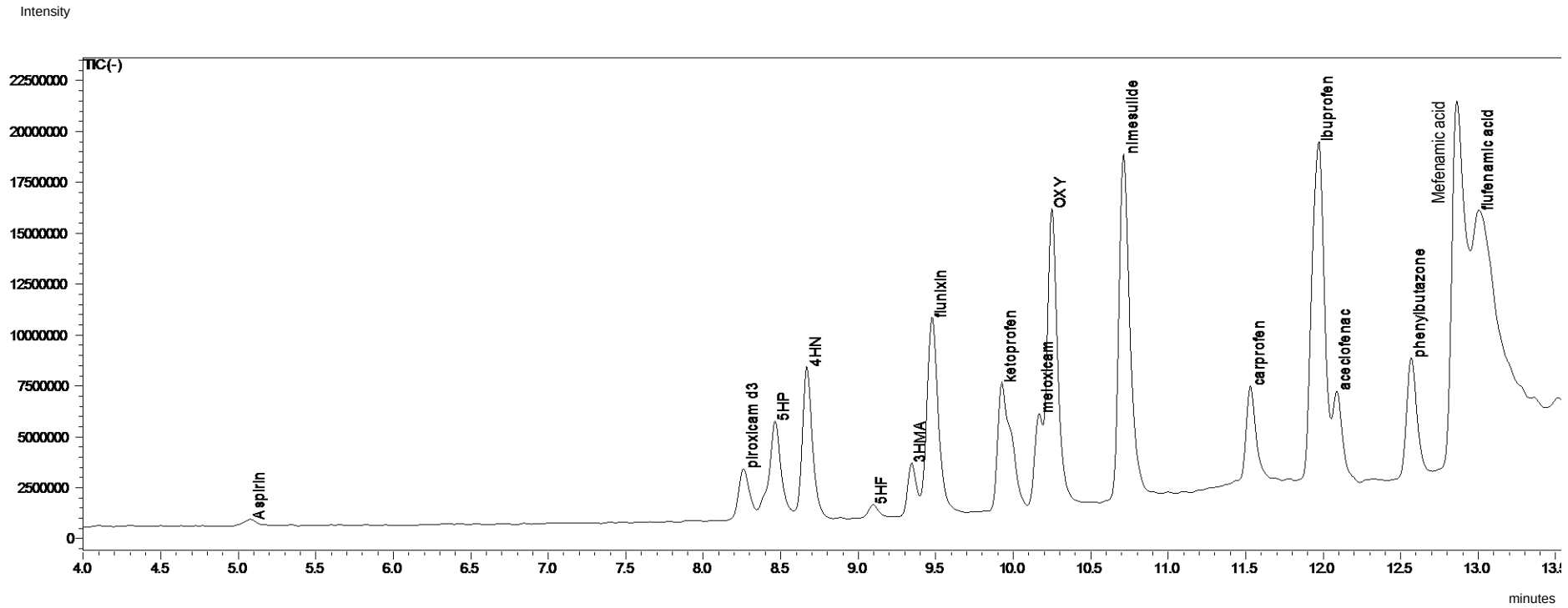


Figure 3.7 Chromatographic profiles (TIC) of mixed NSAID and metabolite standard (100µg/ml) obtained with method D (Table 2.5) (5HP= 5-hydroxypiroxicam, 4HN= 4-hydroxynimesulide, 5HF= 5-hydroxyflunixin, 3HMA= 3-hydroxymethyl mefenamic acid, OXY= oxyphenylbutazone)

Peak shapes were symmetrical, peaks were tall, sharp (narrow) and of Gaussian distribution. Resolution was high (>1) across all metabolites, with the exception of 5-hydroxypiroxicam and oxyphenbutazone. Selectivity was ideal (=1) and tailing factors were low ≤1.6. Metabolites were all eluted within the first half of the run, this was expected as metabolites are polar compounds (hydrophilic) and as such have a higher affinity for the mobile phase hence, eluting first (section 1.3.6) (Neue, 1997). Despite some co-elution between 5-carboxymeloxicam and 5-hydroxypiroxicam (8.5 minutes), ketoprofen and 4-hydroxydiclofenac (10.1 minutes) and meloxicam and oxyphenylbutazone not fully resolved at 10.2 minutes, on analysis of extracted ion chromatograms, with differing molecular ions, peaks were well separated.

Table 3.3 Qualitative data for the chromatographic profile of NSAIDs and metabolites, calculated during optimisation studies when employing method (D)

NSAID[a]	RT[b]	RRF[c]	k[d]	α[e]	R_s[f]	A_s[g]
acetylsalicylic acid	5.08 ± 0.03	0.62	0.27	0.79	4.70	1.61
piroxicam	8.27 ± 0.02	1.00	1.50	0.99	29.79	2.10
5-carboxymeloxicam	8.40 ± 0.02	0.93	1.10	0.98	1.27	1.63
5-hydroxypiroxicam	8.48 ± 0.02	1.02	1.12	0.99	0.60	2.10
4-hydroxynimesulide	8.68 ± 0.02	1.05	1.17	0.98	2.00	1.71
5-hydroxyflunixin	9.42 ± 0.03	1.14	1.27	0.95	4.37	1.60
3-hydroxymethyl mefenamic acid	9.35 ± 0.06	1.13	1.34	0.97	2.60	1.67
flunixin	9.50 ± 0.02	1.15	1.37	0.99	1.25	2.08
4-hydroxydiclofenac	9.94 ± 0.02	1.20	1.48	0.95	4.26	2.20
ketoprofen	10.01 ± 0.02	1.21	1.50	0.99	0.68	1.86
meloxicam	10.19 ± 0.02	1.21	1.54	0.98	1.75	2.95
oxyphenylbutazone	10.27 ± 0.02	1.24	1.56	0.99	0.80	1.97
nimesulide	10.73 ± 0.02	1.30	1.68	0.96	4.19	1.80
carprofen	11.55 ± 0.02	1.40	1.88	0.93	7.70	1.94
diclofenac	11.96 ± 0.02	1.45	1.98	0.97	4.22	2.22
suxibuzone	11.96 ± 0.02	1.45	1.99	1.00	0.02	1.87
Ibuprofen	11.97 ± 0.04	1.45	1.98	1.00	0.02	1.06
aceclofenac	12.11 ± 0.03	1.47	2.02	0.99	1.71	1.93
phenylbutazone	12.58 ± 0.02	0.96	2.14	0.96	0.25	1.49
mefenamic acid	12.97 ± 0.02	0.99	2.24	0.97	0.26	1.73

[a]100µg/ml, [b]retention time (mean RT ± SD) (n=30), [c] relative retention factor, [d]capacity factor, [e]sensitivity, [f]resolution, [g]asymmetry

From these studies method D proved to be the optimal gradient elution program and as such, upon the implementation of these conditions, the recorded RT and RRF for all NSAIDs and metabolites (Table 3.3 and 3.4) were used throughout preliminary

investigations, method validation (Chapter Four) and ultimately the identification process in the analysis of matrices (Chapter Five).

3.2.2 Optimal solvent selection for standards

The selection of solvents in liquid chromatography is an important and effective step in achieving optimal chromatographic resolution (section 1.6.2.6 and 1.6.2.8). In method development, the solvent the analytes of interest were dissolved in, were investigated to find which was optimal (as per section 2.3.2). To investigate the optimal solvent, three mixed NSAID standards at 20µg/ml were each made up in acetonitrile, methanol and the mobile phase mix at the start of the analysis (98% water 0.1% formic acid / 2% acetonitrile 0.1% formic acid, Table 2.5), for the purposes of the reader the latter will be called MP at T_0 (initial mobile phase (Table 2.5) at T_0) from here on. After analysing samples made up in acetonitrile, methanol and MP at T_0, Mean PAR were recorded (Table 3.4) and plot on comparison graphs alongside corresponding error bars of standard deviations (Figure 3.8).

Table 3.4 Mean peak area data obtained (n=3) during solvent selection investigations

NSAID[a]	Mean PAR recorded against corresponding mobile phase					
	acetonitrile	RSD%	methanol	RSD%	MP at T_0[b]	RSD%
acetylsalicylic acid	0.026	15.29	0.018	4.03	0.002	38.32
piroxicam	0.523	13.09	0.601	3.41	0.547	24.59
nimesulide	0.931	28.18	0.913	3.30	0.717	27.19
meloxicam	0.869	9.23	0.928	3.69	0.890	24.77
ketoprofen	0.157	7.23	0.185	0.46	0.121	36.18
flunixin	0.610	17.51	0.749	3.54	0.644	29.08
carprofen	0.238	4.36	0.273	0.83	0.115	39.71
suxibuzone	0.267	7.58	0.169	5.04	0.133	27.27
phenylbutazone	1.150	7.78	1.157	3.85	0.803	37.30
diclofenac	0.627	4.55	0.699	1.36	0.598	29.46
ibuprofen	0.007	7.34	0.013	8.37	0.003	70.29
mefenamic Acid	0.426	3.40	0.466	1.61	0.143	49.49

[a]20µg/ml, [b] MP at T_0 (98% water 0.1% formic acid / 2% acetonitrile 0.1% formic acid)

Results showed (Table 3.4, Figure 3.8) larger PAR when the analytes were prepared in acetonitrile and methanol whilst, analytical response using MP at T_0 were typically lower. Similarly, the RSD of the PARs were larger in MP at T_0 when compared to acetonitrile and methanol. Furthermore, standards prepared in MP at T_0 resulted in larger deviation between the triplicate data sets across all NSAIDs, when compared to

acetonitrile and methanol. In acetonitrile, RSD ranged from 3.40 to 28.18% compared to methanol ranging from 0.46 to 5.04%. However, despite higher RSDs of the PARs in acetonitrile, PAR were comparable with methanol and on occasion higher (i.e suxibuzone) (Table 3.4, Figure 3.8).

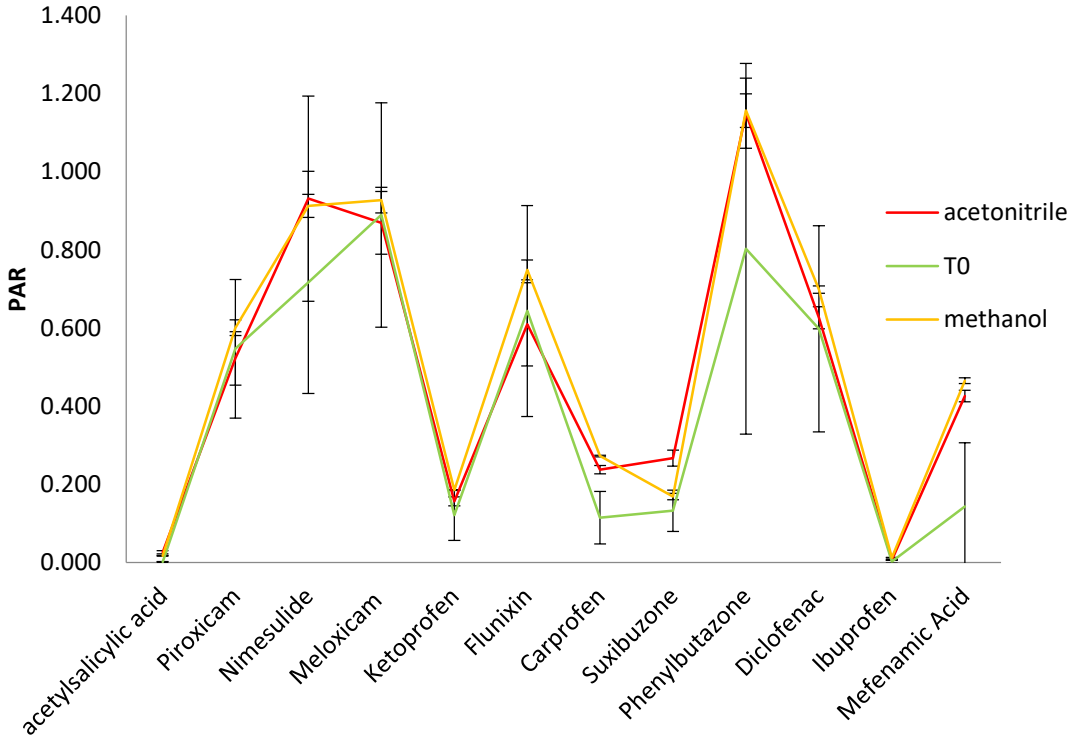


Figure 3.8 analytical response (mean PAR) for corresponding NSAIDs (1mg/ml) prepared in varying solvents

On the assessment of chromatographic profiles of each compound, some peak distortion and splitting was noted between the different solvents employed. It is well reported that solvent strength, in relation to the sample solvent, can result in peak distortion and splitting (Hawkins and Dolan, 2003; Wrezel, and Pakulathat, 2005), this was especially the case in acetylsalicylic acid. Figure 3.9 is an example of peak splitting in acetylsalicylic acid when it was prepared in acetonitrile.

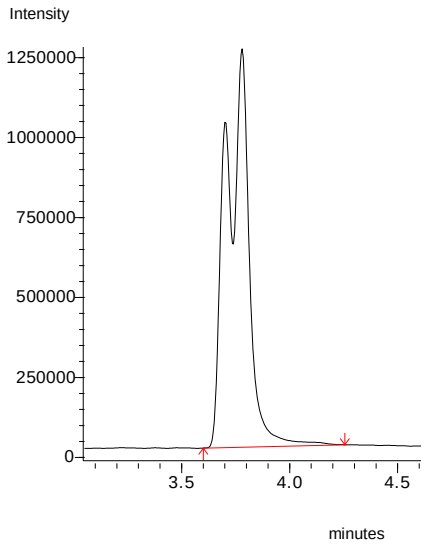


Figure 3.9 Peak splitting of acetylsalicylic acid made up in acetonitrile at 100µg/ml

This peak splitting was detected across all solvents in the case of acetylsalicylic acid. However, there was correlation between peak distortion and the use of MP at T_0, and this was especially the case in phenylbutazone and mefenamic acid where peak shape was most improved in acetonitrile. Acetylsalicylic acid was calculated to have a low capacity factor <1 (section 3.2.1) implying this compound was not highly retained by the column and thus elution was very fast. Perhaps, the peak splitting seen here is related to the disproportion in solvent strength between the sample solvent (100% acetonitrile) and the mobile phase increasing at the elution time (water 0.1% Formic acid at 65%) (section 1.6.2.2).

There were differences in peak shape across all solvents in majority of the NSAIDs investigated. A typical example is the NSAID suxibuzone (Figure 3.10), the overall peak appears symmetrical the main differences across the three solvents were at the base of the peak (shown using arrows). While acetonitrile was used as a solvent, slight tailing was present however, in methanol and MP at T_0 shouldering occurred alongside peak tailing, this was seen in all NSAIDs. Overall peak symmetry was improved in acetonitrile when compared with the other two solvents (Figure 3.10). As an exemplar, asymmetry factors (A_s) calculated in suxibuzone (as per section 1.6.2.7, Equation 1.7) resulted in the desired gaussian shaped peak (A_s = 1) (CDER, 1994) which was a tall, sharp and narrow. Meanwhile, in methanol exhibited tailing (A_s = 2). These affects were more prominent in MP at T_0 with a loss in symmetry and tailing present (A_s = 3.2).

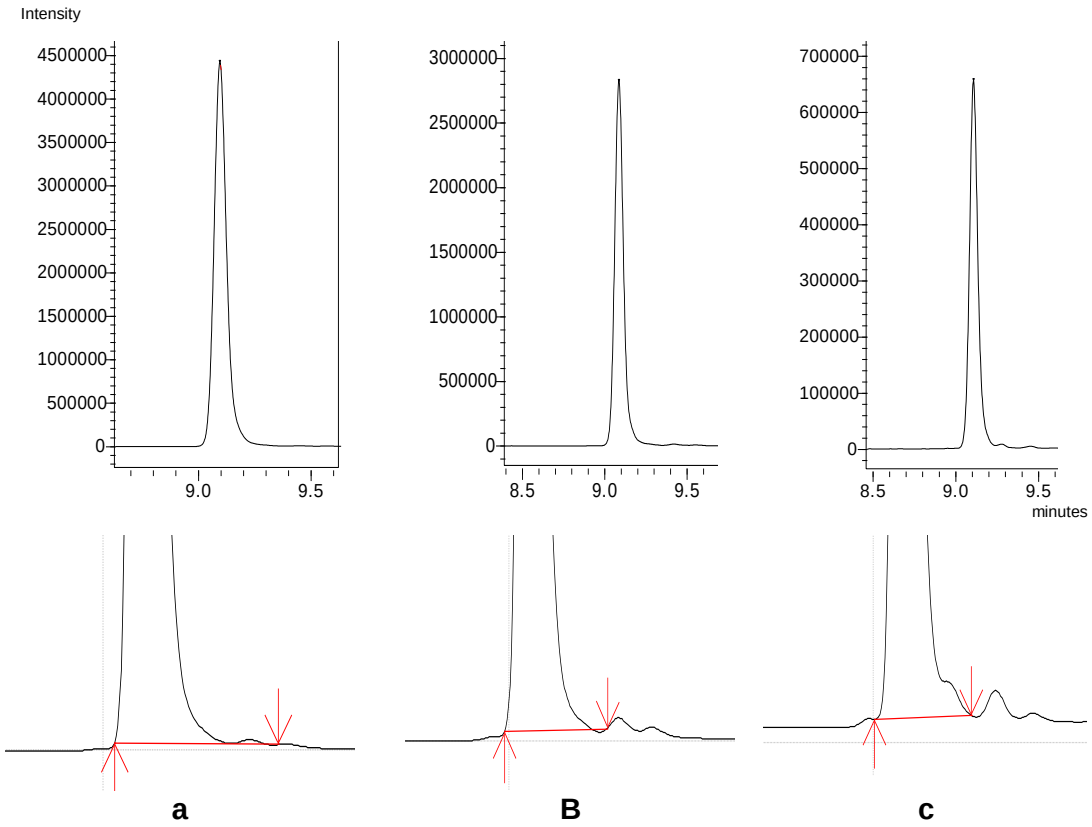


Figure 3.10 Peak shape in suxibuzone at 100µg/ml made up in acetonitrile (a), methanol (b) and MP at T_0 (c), alongside corresponding images of the peak base to show effects of different solvents and tailing factors

Based on the results obtained, most improved peak shape was obtained when acetonitrile was employed. Therefore, this was chosen as the solvent of choice. Thereafter, unless stated, all standards are prepared in 100% acetonitrile.

3.2.3 Injection volumes

Injection volumes were initially assessed in terms of the chromatographic profile i.e. peak asymmetry, using three volumes (0.2, 2 and 10 µl) (section 2.3.3). As previously highlighted (section 1.6.2.9), a good chromatographic peak should be Gaussian shaped. To determine optimal injection volumes, and consequently chromatographic response, tests were carried out on two NSAIDs, ibuprofen and mefenamic acid. These compounds were selected as ibuprofen had the lowest analytical response as an example (Table 3.6) of all NSAIDs (Table 3.2), and mefenamic acid was representative of all other NSAIDs (Table 3.6). Upon inspection of both samples injected at 10µl (20µg/ml), peak shapes were distorted, exhibiting poor peak symmetry as both compounds displayed band broadening, tailing and fronting (section 1.6.2.2, Figure

3.11 and 3.14). Tailing and fronting can occur when the peak shape becomes distorted, as was the case in Figure 3.11 and 3.14.

With this poor peak symmetry, injection volumes of 2 and 0.2 µl (20µg/ml), were investigated further. Throughout method development, an injection volume of 0.2µl had initially been used and until this method optimisation stage, 0.2µl had yielded satisfactory selectivity and sensitivity. On testing 0.2 and 2 µl injection volumes, peak shape was improved when compared to 10µl (Figures 3.11 to 3.14). Peaks were tall, sharp and narrow with only slight fronting at 0.2µl. Overall, only minor improvements could be seen between 0.2 and 2 µl, however, there was a significant difference noticed when comparison is made to the larger volume, on visual inspection and calculating peak asymmetry (Figure 3.12, 3.13, 3.15 and 3.16). As comparisons had only been made on ibuprofen and mefenamic acid, it was necessary to investigate all NSAIDs at 0.2 and 2 µl injection volumes to determine if one volume yielded a better analytical response than another.

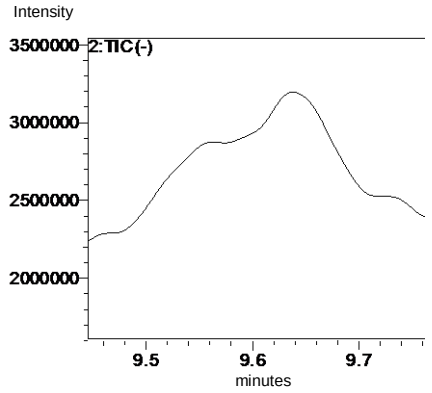


Figure 3.11 Chromatogram of ibuprofen with injection volume (10µl), resulting in volume overload (20µg/ml)

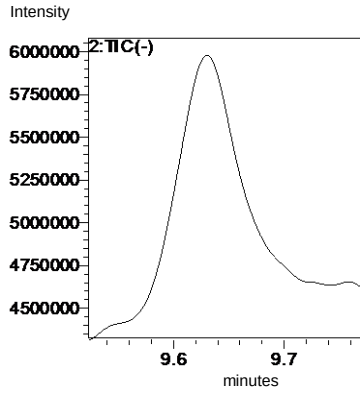


Figure 3.12 Chromatogram of ibuprofen with injection volume (2µl) (20µg/ml)

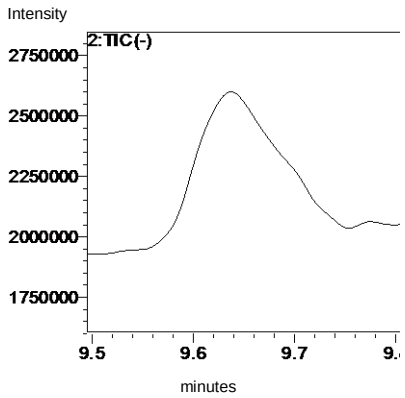


Figure 3.13 Chromatogram of ibuprofen with injection volume (0.02µl) (20µg/ml)

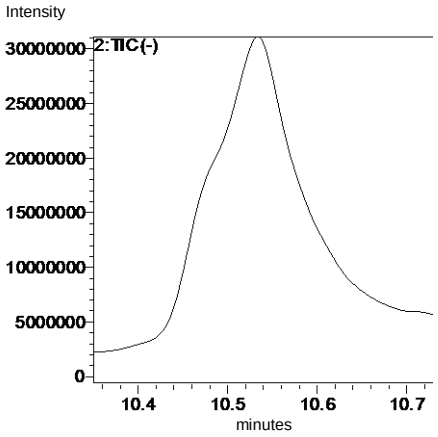


Figure 3.14 Chromatogram of mefenamic acid with injection volume (10µl), resulting in peak distortion (20µg/ml)

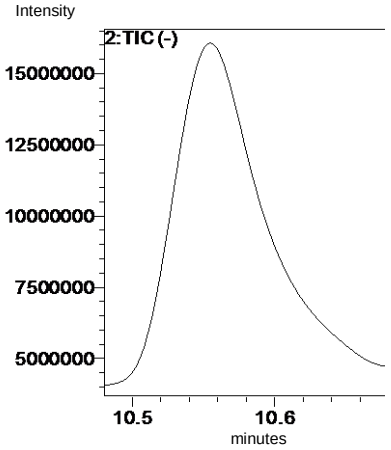


Figure 3.15 Chromatogram of mefenamic acid with injection volume (2µl) (20µg/ml)

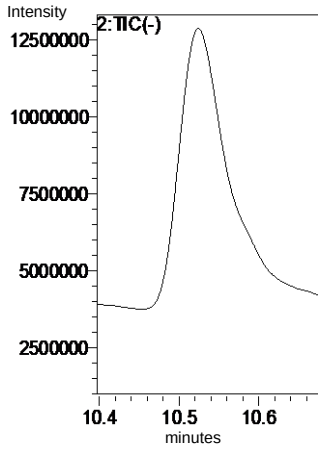


Figure 3.16 Chromatogram of mefenamic acid with injection volume (0.2µl) (20µg/ml)

After investigating all NSAIDs at 0.2µl and 2µl (20µg/ml), RSD of the PAR was calculated to assess the optimal injection volume. The RSD of PAR were higher for all NSAIDs at 0.2µl, (Table 3.5), compared to those recorded with the increased injection volume (2µl). RSD in PAR ranged between 3.4 to 28.18% in injection volumes of 0.2µl and 0.86 to 4.08% in 2µl injection volumes (Table 3.5). The only compound that did not conform to this was acetylsalicylic acid at 15.29% in 0.2µl injection and 39.39% in 2µl injection. The analytical response showed greater variation between triplicate analysis in the latter, leading to the increased RSD. As per the solvent investigations, acetylsalicylic acid, had lower analytical response compared to the other NSAIDs, therefore, this larger RSD is most likely to be associated with expected errors with low volumes. Upon injecting 0.2µl, the largest variations were seen in the analytical response for nimesulide with, 28.18% RSD in 0.2µl injection were compared to 1.09% RSD when injecting 2µl and with similar results recorded for flunixin (RSD ranging from 17.51% to 2.22% respectively). This data is presented in Table 3.5 and has been presented graphically in Figure 3.17.

Table 3.5 mixed NSAID analytical response (mean PAR, standard deviation and RSD%) recorded from injection volume studies at 0.2µl and 2µl in acetonitrile

NSAID	0.2µl Injection (0.02mg/ml)			2µl Injection (0.02mg/ml)		
	Mean PAR (n=3)	SD	RSD %	Mean PAR (n=3)	SD	RSD %
acetylsalicylic acid	0.026	0.004	15.29	0.082	0.032	39.39
piroxicam	0.523	0.068	13.09	0.517	0.010	1.92
nimesulide	0.931	0.262	28.18	1.016	0.011	1.09
meloxicam	0.869	0.080	9.23	0.811	0.021	2.57
ketoprofen	0.157	0.011	7.23	0.197	0.006	3.06
flunixin	0.610	0.107	17.51	0.580	0.013	2.22
carprofen	0.238	0.010	4.36	0.235	0.003	1.27
suxibuzone	0.267	0.020	7.58	0.248	0.010	3.90
phenylbutazone	1.150	0.089	7.78	1.080	0.024	2.25
diclofenac	0.627	0.028	4.55	0.655	0.006	0.86
ibuprofen	0.007	0.000	7.34	0.006	0.000	4.08
mefenamic Acid	0.426	0.014	3.40	0.452	0.012	2.63

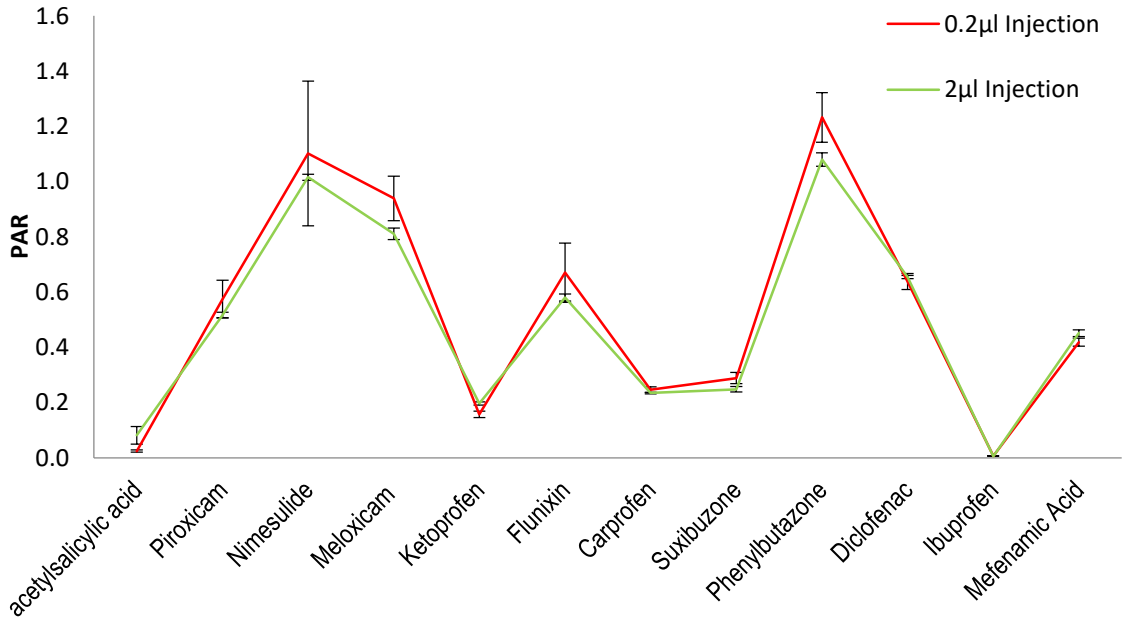


Figure 3.17 Comparison graph of 0.2 and 2µl injection volumes: mixed NSAIDs standard in acetonitrile against PAR

During the investigation of injection volumes, poor chromatographic profiles, including tailing and broadening, were noted at the highest volume of 10µl. This was likely to arise from the effects of mass transfer (section 1.6.2.2, in the Van Deemter Equation, Equation 1.3), where analytes may not have been able to equilibrate between the stationary phases and mobile phase leading to the band broadening. At the two lower injection volumes, peak distortion was likely linked with the effects of longitudinal diffusion, where slight distortion and higher RSD was reported when too lower volume (0.2µl) was injected. At this lowest volume higher RSD can be expected as results are less repeatable, with a tenfold increase to 2µl, the precision in analytical response was improved. Thus, as per the reported RSD, and chromatographic response discussed, when using an injection volume of 2µl instrumental response was higher with better precision across all NSAIDs.

3.2.4 Identification of NSAIDs and Diagnostic ions

To identify and confirm the presence of compounds in unknown samples, three identification reference points should be used in trace analysis, which this analytical method was ultimately applied (Commission Decision, 2002). Retention time (RT), relative retention factor (RRF), a quantification ion (Q), two confirmation ions (C1 and C2) and peak area ratios were used in this research (Migowska, 2012).

Retention times were previously recorded during the optimisation of the LC program, refer to Table 3.2 (section 3.2.1). Meanwhile, throughout method development, specifically during the identification of NSAIDs, using individual mass spectra for each compound (Table 3.2), possible fragmentation was predicted and ions were collated (Table 3.6). The collation of these ions allowed for the development of a SIM method. Following guidelines set out by World Anti-Doping Agency (WADA) (WADA, 2003), three diagnostic ions (one Q ion, two C ions) were used in the identification of NSAIDs and metabolites (section 1.6.3). In the case of metabolites oxyphenylbutazone, 4-hydroxynimesulide, 5-hydroxyflunixin and 5-hydroxyprioxicam only 2 diagnostic ions were present with relative abundancies above the accepted 10% (Commission Decision, 2002). This would still ensure at least one ion ratio could be reliably made. Primarily, ions were selected based on the three most abundant ions, as per the predicted fragmentation of the chemical structure.

Table 3.6 Compound table and their corresponding ions (scan negative ionisation mode). For each ion corresponding proposed fragmentation is stated.

NSAID	Retention time (minutes)	RMM	mass to charge ratio (m/z)		
			Quantification ion	Confirmation ion 1	Confirmation ion 2
acetylsalicylic acid	5.08	180.2	225 $[M-H+HCO_2H]^-$	179 $[M-H]^-$	151 $[M-CO-H]^-$
piroxicam d_3	8.26	331.4	333 $[M-H]^-$	341 $[M-H+HCO_2H]^-$	250 $[M-H-CHO_2]^-$
piroxicam	8.27	331.4	330 $[M-2H]^-$	323 $[M-8H]^-$	333 $[M+2H]^-$
5-carboxymeloxicam	8.40	381.4	380 $[M-H]^-$	379 $[M-2H]^-$	426 $[M-H+HCO_2H]^-$
5-hydroxyprioxicam	8.48	347.4	346 $[M-H]^-$	347$[M]^-$	
4-hydroxynimesulide	8.68	324.3	322 $[M-2H]^-$	323 $[M-H]^-$	
3-hydroxymethyl mefenamic acid	9.35	257.3	256 $[M-H]^-$	302 $[M-H+HCO_2H]^-$	257 $[M]^-$
5-hydroxyflunixin	9.42	312.2	311 $[M-H]^-$	379 $[M-H+HCO_2H]^-$	
flunixin	9.50	491.5	295 $[M-H]^-$	296 $[M+H]^-$	341 $[M-H+HCO_2H]^-$
4-hydroxydiclofenac	9.94	312.0	310 $[M-2H]^-$	311 $[M]^-$	356 $[M-H+HCO_2H]^-$
ketoprofen	10.01	254.3	299 $[M-H+HCO_2H]^-$	253 $[M-H]^-$	321 $[M+Na-H]^-$
meloxicam	10.19	351.4	350 $[M-H]^-$	352 $[M+H]^-$	396 $[M-H+HCO_2H]^-$
oxyphenylbutazone	10.27	324.4	323 $[M-H]^-$	391 $[M-H+HCO_2H]^-$	
nimesulide	10.73	308.1	307 $[M-H]^-$	308 $[M+H]^-$	375 $[M-H+HCO_2H]^-$
carprofen	11.55	273.7	272 $[M-H]^-$	318 $[M-H+HCO_2H]^-$	228 $[M-H-CHO_2]^-$
diclofenac	11.96	295.0	294 $[M-H]^-$	296 $[M+H]^-$	250 $[M-H-CHO_2]^-$
suxibuzone	11.96	438.0	307 $[M-C_5H_7O_4]^-$	483 $[M-H+HCO_2H]^-$	437 $[M-H]^-$
ibuprofen	11.97	206.3	251 $[M-H+HCO_2H]^-$	149 $[M-C_4H_9]^-$	131 $[M-C_3H_5O_2]^-$
aceclofenac	12.11	354.2	351 $[M-H]^-$	354	398 $[M-H+HCO_2H]^-$
phenylbutazone	12.58	308.4	307 $[M-H]^-$	308 $[M+H]^-$	375
mefenamic acid	12.97	241.3	240 $[M-H]^-$	307	265 $[M-H+HCO_2H]^-$
flufenamic acid	13.08	281.2	280 $[M-H]^-$	236 $[M-H-CHO_2]^-$	326 $[M-H+HCO_2H]^-$

Across all NSAIDs and metabolites, the most abundant ion was used as the quantification ion. In negative ionisation mode the deprotonated molecular ion $[M-H]^-$, where M depicts the whole molecule, was used as the quantification ion for all the major metabolites, parent compounds and the two internal standards, flufenamic acid and piroxicam-d_3, with the exception of 4-hydroxynimesulide, 4-hydroxydiclofenac, acetylsalicylic acid, ibuprofen, ketoprofen, piroxicam and suxibuzone respectively, as shown in Table 3.6 Similar approaches have been previously reported (Miksa, Cummings and Poppenga, 2005; Aberg, et al., 2009). As an example, Figure 3.18 shows the negative ionisation mass spectra of diclofenac. The most abundant ion is m/z 293, which is the deprotonated molecular ion $[M-H]^-$ (Table 3.6).

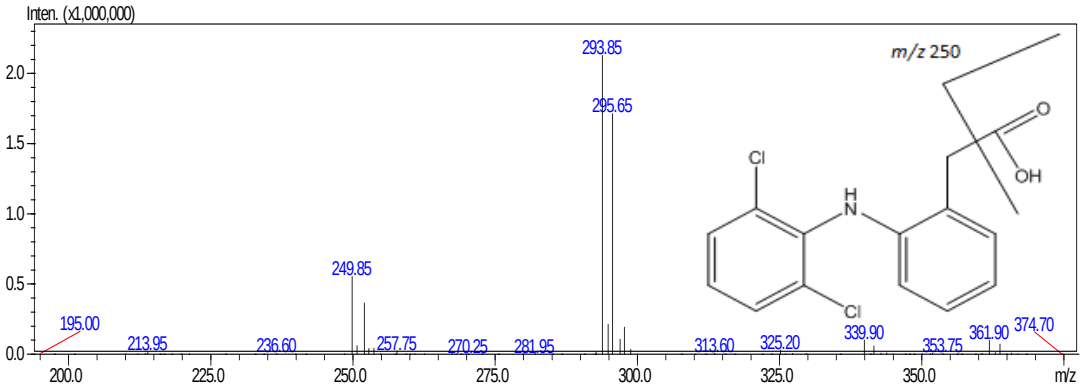


Figure 3.18 Negative ionisation mass spectra of diclofenac and corresponding proposed fragmentation (Moffat, Osselton and Widdop, 2004)

In NSAIDs acetylsalicylic acid, ibuprofen and ketoprofen the intensity of $[M-H]^-$ was not the most abundant ion, as shown in Figure 3.19, an example of ketoprofen. Instead the presence of ions representing a gain of 46Da indicated adduct formation with formic acid m/z 299 $[M-H+HCO_2H]^-$ from the mobile phase (Table 3.7), therefore this base ion was used for quantification of these compounds.

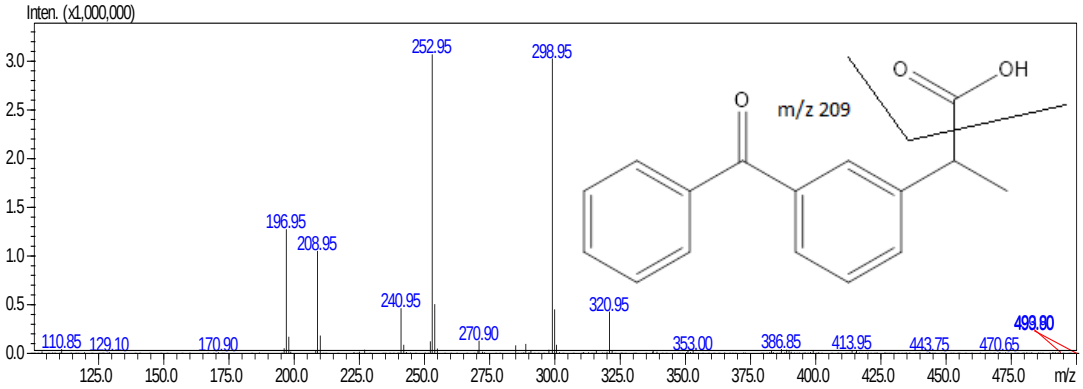


Figure 3.19 Negative ionisation mass spectra of ketoprofen.

Additionally, the base ion m/z 330 $[M\text{-}2H]^-$ was used for piroxicam and m/z 307 $[M\text{-}C_5H_7O_4]^-$ for suxibuzone (Miao, Koenig and Metcalfe, 2002). Meanwhile the deprotonated $[M\text{-}2H]^-$ ion was identified as the base ion for 4-hydroxydiclofenac and 4-hydroxynimesulide, these findings are in line with those reported by Kang and Kim (2008).

Alongside the selection of quantification ions, the next abundant ions were selected for confirmation (Table 3.6), in majority of the compounds these were either deprotonated molecular ions or formic acid adducts where they had not previously been selected, often the loss of a CO_2 group was used as the third confirmatory ion (Petrovic, et al., 2005). In doing this, possible fragmentation can be predicted based on the chemical structure. For example, in the fragmentation of diclofenac (Figure 3.18), as an example, the molecular ion is identified at m/z 293 $[M\text{-}H]^-$, the confirmatory ion, the next abundant, is selected at m/z 295 indicates a gain of 1 Da $[M+H]^-$, thereafter m/z 250 $[M\text{-}H\text{-}CO_2]^-$ indicates a loss of 45 Da represents the loss of the CO_2 group. The presence of ion m/z 339 $[M\text{-}H+HCO_2H]^-$ represents a gain of 46Da, the adduct formation of formic acid.

In the example of metabolites, the proposed fragmentation of the major metabolite of diclofenac; 4-hydroxydiclofenac (Figure 3.20), shows the molecular ion $[M^-]$ identified at m/z 312, whilst the base ion is recorded at m/z 310, indicating a loss of i.e. 2 Da [M-2H]. A further confirmatory ion, the next abundant after the molecular ion, is selected at m/z 355 representing a gain of 46Da, the adduct formation of formic acid $[M+HCO_2H]^-$.

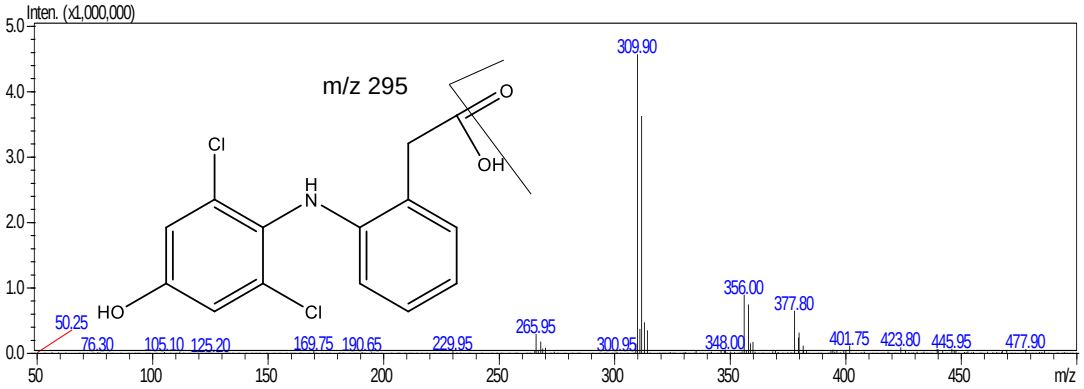


Figure 3.20 Negative ionisation mass spectra of 4-hydroxy diclofenac and corresponding proposed fragmentation

All other fragment ions have been listed in Table 3.6, with chemical structures originally presented in section 1.3.4.

3.2.5 Implementation of SIM for selectivity

Due to the co-elution of piroxicam, piroxicam-d_3 and 5-carboxymeloxicam at 8.26 minutes, diclofenac, ibuprofen and suxibuzone co-eluting at 11.92 minutes and 4-hydroxydiclofenac and ketoprofen (Figure 3.21) and the metabolite oxyphenylbutazone with meloxicam at 9.88 and 10.15 minutes respectively, SIM was used.

With different molecular and confirmation ions, as depicted in Table 3.6, selective ion monitoring mode (SIM), was employed. By only scanning ions of interest, SIM allows for the discrimination between co-eluting compounds thus confirming SIM is the far more selective (Figure 3.21 and 3.22). Figure 3.21 shows the SIM chromatogram of diclofenac at 11.92 minutes and suxibuzone at 11.95 minutes, ibuprofen elutes at 11.90 minutes, however, due to low intensity can only be seen in extracted ion mode. Whilst Figure 3.22 shows the SIM chromatogram of 4-hydroxydiclofenac at 9.85 minutes and ketoprofen 9.92 minutes. Both are examples of how SIM has separated one peak eluted in TIC into two separate peaks.

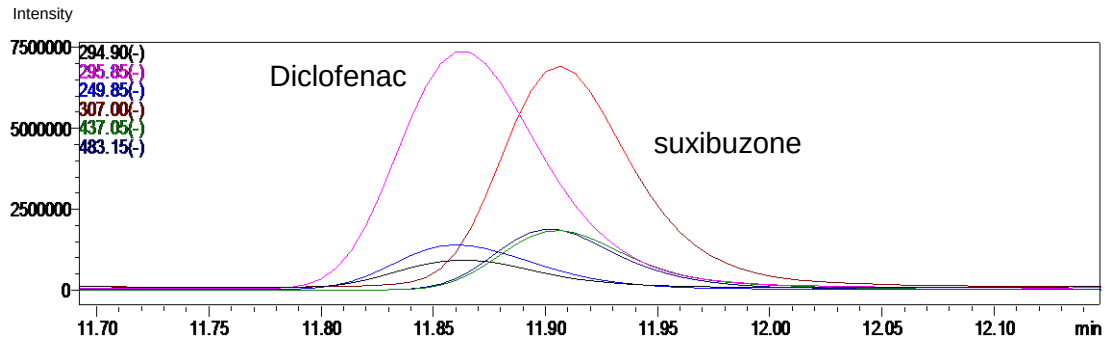


Figure 3.21 The selected ion chromatogram of diclofenac (m/z 295), suxibuzone (m/z 307) (0.125µg/ml)

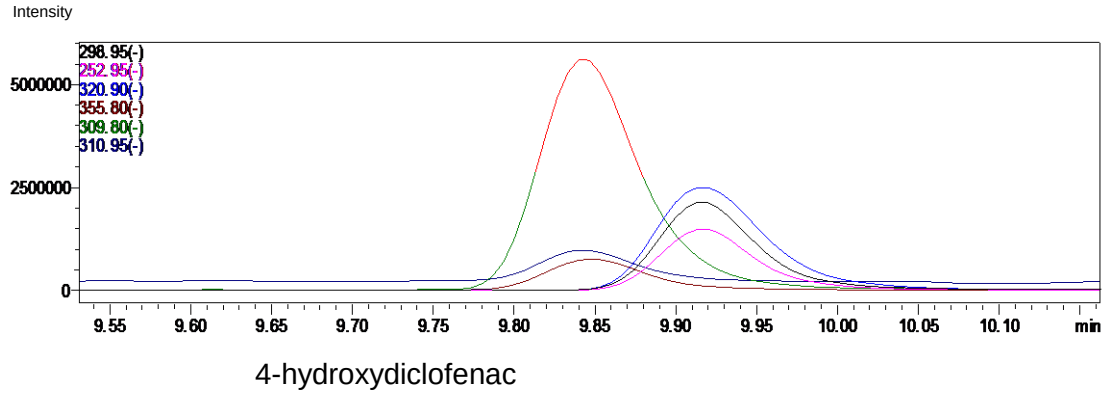


Figure 3.22 The selected ion chromatogram of 4-hydroxydiclofenac (m/z 310) and ketoprofen (m/z 299) (10µg/ml)

Similarly, problems with co-elution between oxyphenylbutazone and meloxicam were negated by the use of SIM as shown in Figure 3.23. By implementing the selected ions m/z 323, 391 for the metabolite oxyphenylbutazone and m/z 350, 352 and 396 for meloxicam, these compounds were identified at 10.19 and 10.11 respectively.

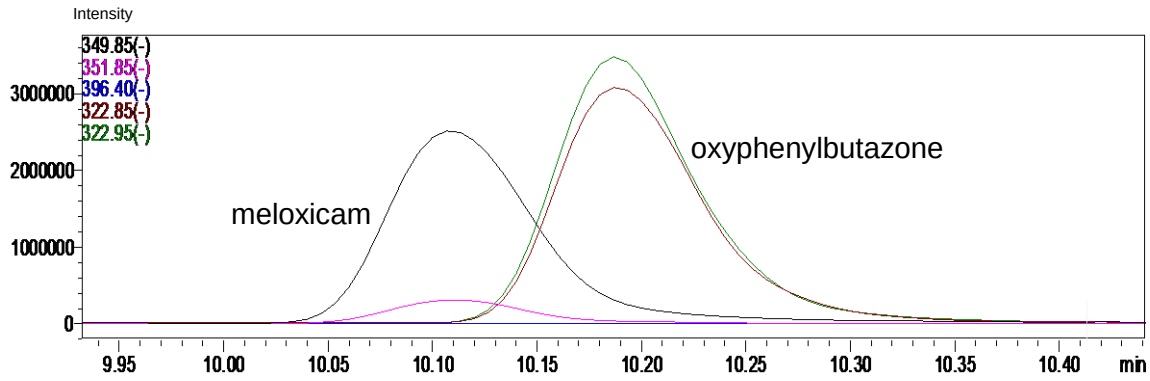


Figure 3.23 The selected ion chromatogram of the NSAID meloxicam (m/z 350) and the metabolite oxyphenylbutazone (m/z 323) (10µg/ml)

Co-elution among three compounds; 5-carboxymeloxicam, piroxicam and piroxicam-d_3, was negated through the use of SIM mode and selected ions m/z 380, 379 and 426 for 5-carboxymeloxicam, m/z 330, 323 and 333 for piroxicam and m/z 333 for piroxicam-d_3 (as discussed in section 3.2.4) the compounds were separately identified at 8.23 and 8.21 minutes respectively. The co-elution present in scan mode of piroxicam and its deuterated analog (piroxicam-d_3) is to be expected (section 3.1), as the latter is the isotopically labelled version of piroxicam resulting in chemically similar structures just differed by 3 atomic mass units and thus same chromatographic retention times (Synder, Kirkland and Dolan, 2010).

By employing SIM, compounds exhibiting co-elution can be identified and quantified (section 5.2.1). Based on the selectivity of the analytical method in SIM mode, results for the method validation presented in Chapter Four are obtained from analysis in SIM mode.

3.3 INITIAL AUTOSAMPLER STABILITY IN AN INTRA-DAY STUDY OVER 54 HOURS

During initial method development, autosampler stability was investigated. This short term stability study was carried out to determine that the compounds investigated were stable in a mixed standard, made up in acetonitrile, for the duration of 54 hours. The time was selected based on the average analysis length during preliminary investigations to ascertain the optimal length on which mixed standards could be stored directly on the autosampler. During method validation, stability was reassessed (section 4.2) to allow for longer term analysis and investigated the stability of a mixed standard spread over a number of days with storage between analysis in -20ºC conditions. Stability was investigated as per method described in Chapter Two section 2.3.4.

3.3.1 Initial autosampler stability of internal standards

It is necessary to consider the stability of both internal standards, piroxicam-d_3 and flufenamic acid and, as quantification involves the ratio of analytical response between the analyte of interest and the internal standard.

Analytical response were obtained through SIM analysis and mean relative response factor (R_c) is calculated using m/z 333 for piroxicam-d_3 and m/z 280 for flufenamic acid (section 3.2.5, Table 3.6). Figure 3.24 and 3.25, shows the mean peak area values presented alongside 15% RSD (depicted by the horizontal lines). Results show the

deviation is random throughout the runs, without a pattern and within the recommended RSD of ±15% (Peters, Drummer, Musshoff, 2007). Therefore, over the 54 hour stability studies both internal standards (0.01µg/ml) are stable with small fluctuations (RSD) within hours, denoted by the error bars at each corresponding hour, (Figure 3.24 and 3.25). Both internal standards are within the acceptable limits throughout the 54 hour duration.

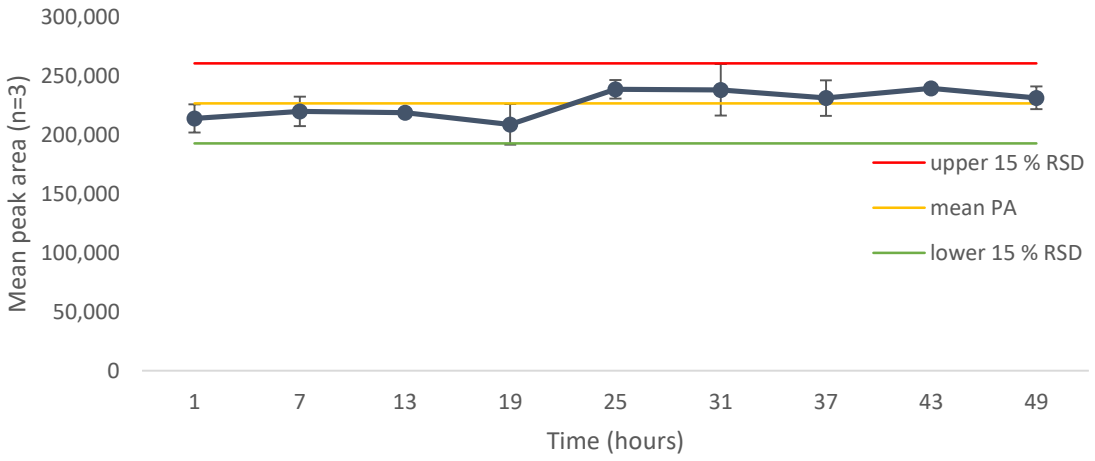


Figure 3.24 54 hour stability results (mean peak area against time) for internal standard piroxicam-d3 (m/z 332)

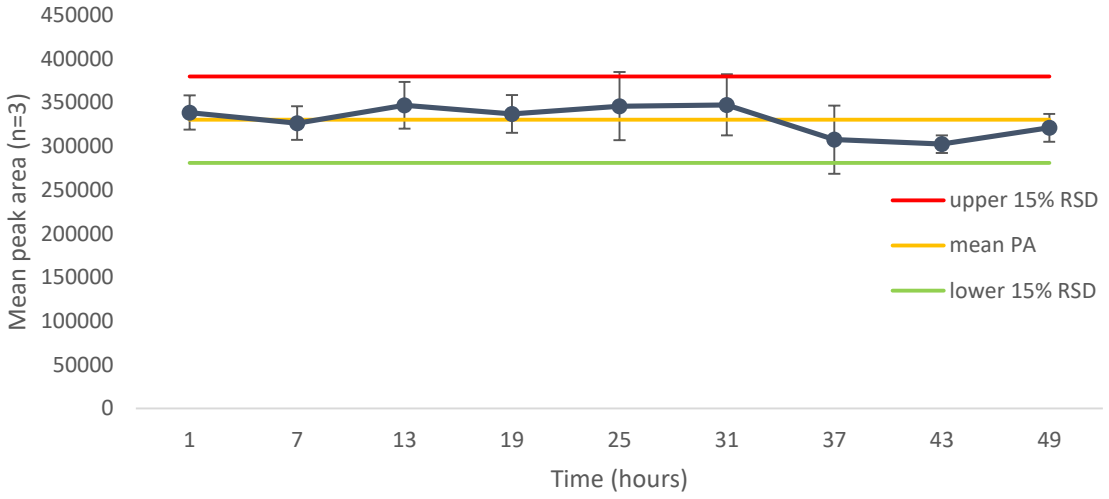


Figure 3.25 54 hour stability results (mean peak area against time) for internal standard flufenamic acid (m/z 279)

This short term study investigated the stability of both internal standards, present in a mixed standard at a concentration of 0.01µg/ml, when stored directly on the

autosampler during a 54 hour study. Following the interpretation of the data collected for the internal standards, both were proven stable for storage on the autosampler for the duration of the average analysis time (Figure 3.24 and 3.25).

These results show the stability of flufenamic acid and piroxicam-d_3 thus, supporting the selection and suitability as internal standards for this method of simultaneous detection of NSAIDs using LC-MS.

3.3.2 Initial autosampler stability of mixed NSAIDs

This method has determined stability for NSAIDs in a mixed standard prepared in acetonitrile. In short term stability studies, mixed NSAIDs standards were investigated at three different concentration levels (0.05, 0.5, 5µg/ml) (section 2.3.4). The results of the stability studies indicate compounds were stable (within acceptance criteria of ±15% RSD, section 1.6.4) during analysis on the temperature controlled autosampler (15ºC) for the duration on 54 hours, albeit acetylsalicylic acid and ibuprofen (Figure 3.27 and Appendix I).

Figure 3.26 shows an example of the NSAID aceclofenac during the 54 hour study at all concentrations. For all NSAIDs (Appendix I to III), analytical response (mean PAR) were plot against time, with horizontal lines depicting the ±15% RSD and the mean PAR over 54 hours. Aceclofenac, like all NSAIDs investigated, apart from acetylsalicylic acid and ibuprofen, was within ±15% RSD throughout the 54 hours across all concentrations, with low RSD overall.

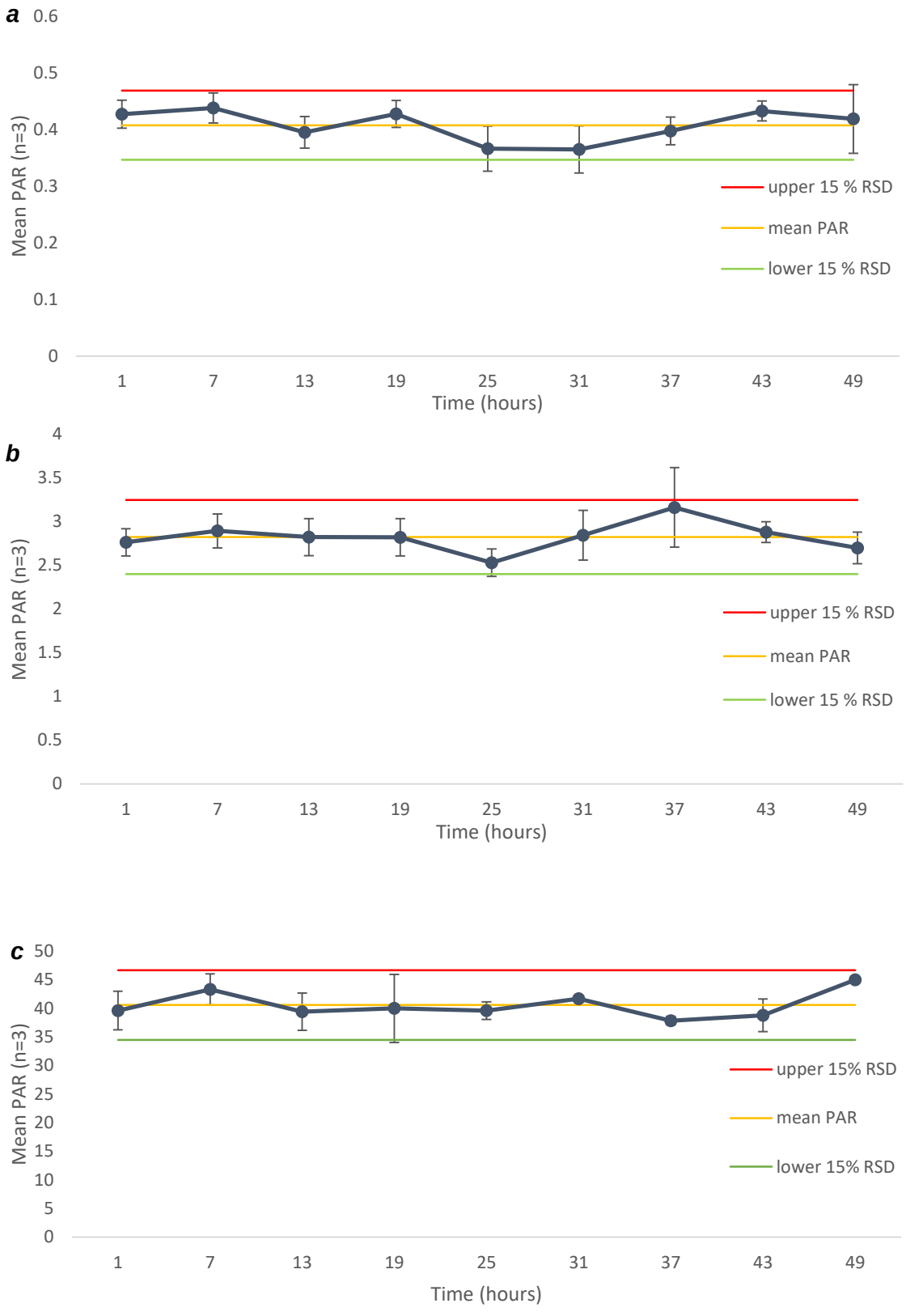


Figure 3.26 Graph of mean PAR (n=3) over 54 hours for aceclofenac stability at, (a) 0.05µg/ml, (b) 0.5µg/ml, (c) 5µg/ml.

In the case of acetylsalicylic acid and ibuprofen the analyte response fluctuated and resulted in larger standard deviations, especially at the lowest concentration (0.05 µg/ml) with low PARs (Figure 3.27, Appendix I to III). This was also the case, for these compounds, throughout the method development and particularly the repeatability study in section 3.4.1. It is inherent that as the concentration lowers so did analytical response and the resulting ratio, thus errors and larger variance will increase (Saar, et al., 2012), but they should still be within the acceptance criteria Ibuprofen is stable at higher concentrations (0.5 and 50µg/ml) and Figure 3.27 shows the instability of this compound with larger standard deviations at the lower concentration.

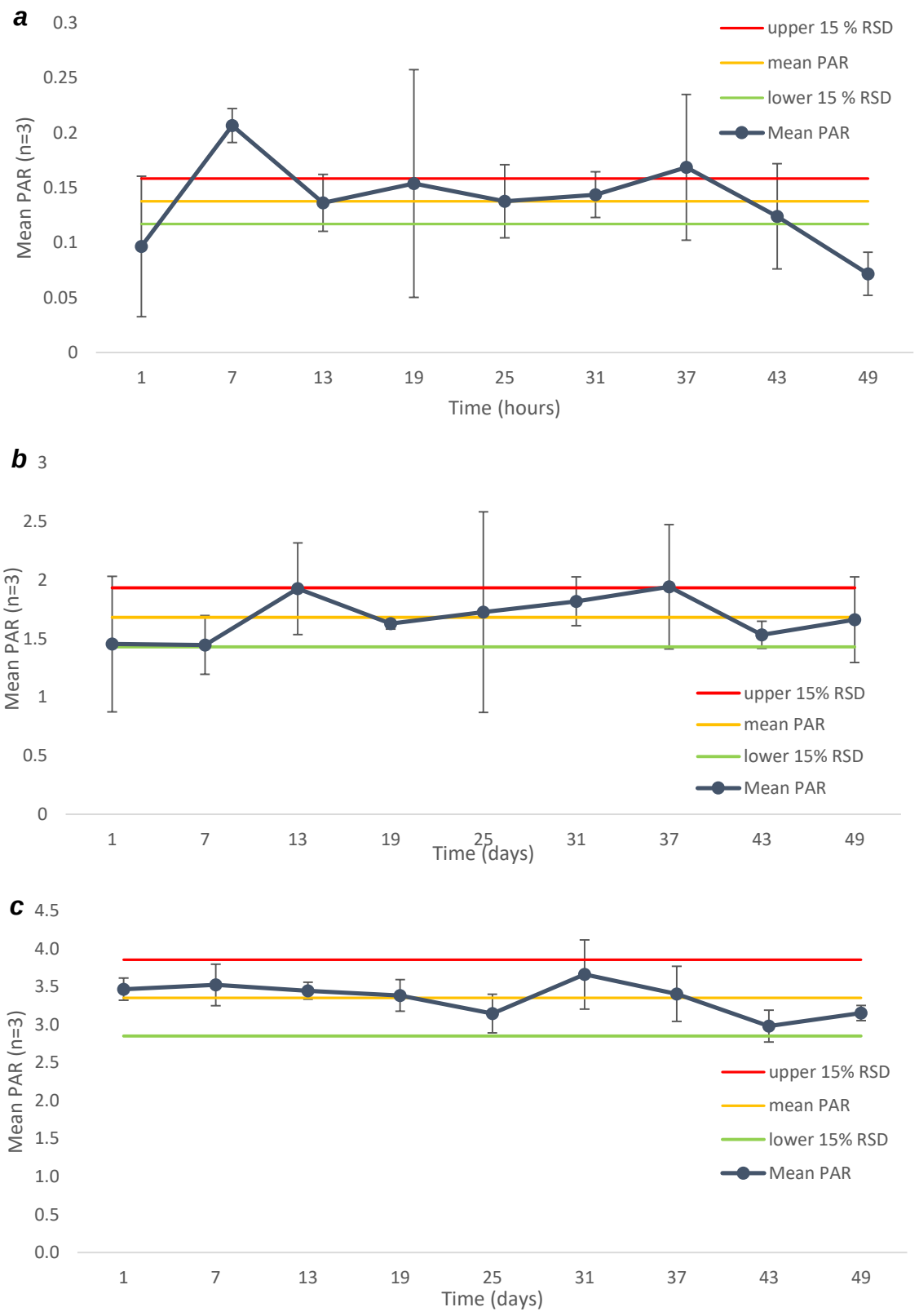


Figure 3.27 Graph of mean peak area ratio (n=3) over 54 hours for ibuprofen stability at (a) 0.05µg/ml, (b) 0.5µg/ml, (c) 5µg/ml.

The stability over 54 hours of each NSAID in acetonitrile has been determined and are stable across 0.05, 0.5 and 5µg/ml concentrations for the full duration of 54 hours with the exception of acetylsalicylic acid that showed instability across all concentrations and ibuprofen at the mid and higher concentration. With regards to the metabolites, at the highest concentration (5 µg/ml) 5-carboxymeloxicam and 5-hydroxyflunixin were beyond ±15% RSD. With 5-carboxymeloxicam and 5-hydroxyflunixin, there were notable decreases in analytical response between 43 and 54 hours and this could suggest that these metabolites are only stable up to 43 hours when stored in a mixed standard directly on the autosampler (Appendix I to III). In the case of ibuprofen, instability was apparent at the lowest concentration, this could be linked with expected fluctuation near its limits of detection. The instability of ibuprofen is contrary to the published findings; analysing ibuprofen and its deuterated counterpart, ibuprofen-d_3, with LC-MS/MS, Grenier, et al. (2011) reported ibuprofen to be stable for a period of up to 24 hours at concentrations of 0.03 and 0.7µg/ml in ambient temperature. The mix of compounds and the choice of technique is different between this research and the study by Grenier, et al. (2011), thus the stability of ibuprofen in mixed standards should be studied further.

3.4 PRELIMINARY INVESTIGATION OF PRECISION

This study assessed the repeatability of samples, prior to method validation. Intra-day precision was investigated over 54 hours as per methods section 2.3.5.

3.4.1 Intra-day repeatability study

This study was investigated using low, mid and high concentrations within the linear range (section 4.4) at 0.05, 0.5 and 5µg/ml. Each concentration was investigated with nine replications over a 54 hour period, each analysed in triplicate. The PAR (analyte: IS) and corresponding standard deviation (n=3) were recorded for the duration of 54 hours. Corresponding relative standard deviation were calculated. In Table 3.7 the data is presented for mixed standards (containing NSAIDs and metabolites) at the concentrations, and includes their corresponding PAR.

Table 3.7 Intra-day repeatability results of peak area ratio (analyte:IS) and their respective SD and RSD of three concentrations of mixed NSAID standards.

NSAID	Intra-assay PAR[a] repeatability					
	0.05µg/ml	RSD[b] %	0.5µg/ml	RSD[b] %	5µg/ml	RSD[b] %
aceclofenac	0.41 ± 0.03	6.86	2.82 ± 0.17	5.98	40.56 ± 1.56	3.85
acetylsalicylic acid	0.14 ± 0.04	26.99	0.77 ± 0.19	24.47	2.51 ± 0.31	12.41
carprofen	2.02 ± 0.11	5.56	16.33 ± 1.49	9.12	46.72 ± 4.28	9.17
diclofenac	2.93 ± 0.20	6.76	30.06 ± 2.32	7.72	91.75 ± 5.87	6.40
flunixin	9.35 ± 0.67	7.17	80.06 ± 7.99	9.98	202.68 ± 9.81	4.84
Ibuprofen	0.14 ± 0.04	28.43	1.68 ± 0.19	11.16	3.35 ± 0.17	5.10
ketoprofen	2.05 ± 0.15	7.13	13.55 ± 1.01	7.48	39.38 ± 2.70	6.87
mefenamic acid	1.20 ± 0.10	8.32	5.83 ± 0.31	5.32	75.05 ± 3.29	4.39
meloxicam	4.79 ± 0.24	5.42	37.12 ± 3.49	9.41	138.57 ± 10.78	7.78
nimesulide	17.44 ± 1.05	6.00	114.10 ± 9.23	8.09	220.30 ± 14.17	6.43
oxyphenylbutazone	9.05 ± 0.66	7.33	89.39 ± 7.24	8.09	196.32 ± 10.03	5.11
phenylbutazone	0.45 ± 0.04	9.58	3.71 ± 0.47	12.53	49.82 ± 3.39	6.80
piroxicam	4.23 ± 0.30	7.16	30.15 ± 1.98	6.58	84.77 ± 6.64	7.84
suxibuzone	4.40 ± 0.35	8.04	39.48 ± 3.71	9.41	120.83 ± 8.61	7.13
3-hydroxymethylmefenamic acid	2.29 ± 0.24	10.28	25.74 ± 2.11	8.21	82.25 ± 8.55	10.39
4-hydroxydiclofenac	3.88 ± 0.28	7.18	38.10 ± 2.75	7.21	112.92 ± 6.90	6.11
4-hydroxynimesulide	4.21 ± 0.28	6.71	45.44 ± 4.43	9.76	141.88 ± 9.38	6.61
5-carboxymeloxicam	1.05 ± 0.09	8.14	8.29 ± 0.68	8.21	37.70 ± 13.68	36.27
5-hydroxyflunixin	0.73 ± 0.10	13.51	3.59 ± 0.37	10.38	66.01 ± 10.78	16.33
5-hydroxypiroxicam	5.78 ± 0.32	5.62	43.61 ± 3.92	7.90	162.62 ± 8.79	5.41

[a]peak area ratio of analyte peak area/ internal standard peak area (n=3) ± SD [b]Relative Standard Deviation (RSD)

At 0.05µg/ml, all NSAIDs, with the exception of acetylsalicylic acid and ibuprofen are within the accepted ±15% RSD, with precision actually closer to 10% RSD for all NSAIDs. In acetylsalicylic acid and ibuprofen, shown graphically in Appendix I, these compounds showed high variability (>20% RSD) in results at this lowest concentration across the duration of the study (26.99% and 28.43% respectively, Table 3.7).

At the two lower concentrations (0.05 and 0.5µg/ml) the analytical responses are low in comparison to those recorded across the other compounds analysed. This in turn resulted in low PARs, as an example, acetylsalicylic acid, at 0.05 µg/ml, PARs ranged between 0.02 and 0.38 thus producing a RSD of 63.5% overall. At this concentration both compounds exhibited poor peak symmetry (tailing factor >1), with similar results

achieved at 0.5µg/ml, in line with findings during the stability investigations in section 3.3.2.

At 0.5µg/ml all NSAIDs were within 15% RSD with most compounds actually nearer 10% RSD. The exception to this was acetylsalicylic acid with a recorded RSD at 24.47%. At this mid-range concentration, precision was improved in acetylsalicylic acid, recorded at 24.47%, and ibuprofen within 15% RSD (Table 3.7). In acetylsalicylic acid analytical response was also much closer to that of the internal standards when compared to 0.05µg/ml. However, at these concentration the internal standard, piroxicam-d_3, is almost tenfold less than the concentration of acetylsalicylic acid being detected, this indicates that the sensitivity of this compound was much less than other compounds. This would mean a concentration that would otherwise yield a repeatable analytical response, in the case of acetylsalicylic acid sensitivity is much lower.

At the high (5µg/ml) concentration investigated the majority of compounds are within 15% RSD. However, larger variations were recorded in metabolites 5-carboxymeloxicam and 5-hydroxyflunixin. In the case of 5-carboxymeloxicam and 5-hydroxyflunixin, RSD was recorded at 36.27% and 16.33% respectively (Table 3.7). In both metabolites there was a notable decrease in analytical response between 43 and 54 hours with large deviations between the triplicate data set, for example in PARs recorded for 5-hydroxyflunixin at 48 hours 53% RSD was recorded. This could be due a loss in stability of these compounds, whereby there are a number of factors are involved (section 3.3.2). After 43 hours, the compound may have started to degrade leading to a drop in concentration and ultimately a fall in analytical response, additionally 5-carboxymeloxicam was identified as a compound that co-eluted with piroxicam and piroxicam-d_3 (section 3.2.5) whereby the latter compounds are more responsive at higher concentrations. On inspection of the chromatography of these metabolites both showed peak distortion, possibly exhibiting overloading, this was investigated further through the assessment of linear range (section 4.4).

Overall, the results show that all the compounds were repeatable, all are within 10-15% RSD at the three concentrations studied, with the exception of the discussed acetylsalicylic acid and ibuprofen and metabolites 5-carboxymeloxicam and 5-hydroxyflunixin beyond 43 hours. Furthermore, as the concentration increases so does the method's precision. This was especially the case for ibuprofen and acetylsalicylic acid; at the lowest concentration it is apparent that the RSD is beyond the accepted 15% for both compounds and more precisely 0.5µg/ml is beyond the calculated LOQ

for acetylsalicylic acid. At the highest concentration acetylsalicylic acid is within 15% RSD.

3.5 SUMMARY

The analytical method developed in this project was optimised through several preliminary investigations. These involved modifications in initial parameters to achieve an optimal analytical method, as has been presented in this chapter.

Initial investigations were carried out to first access the chromatographic profile of both internal standards employed in this research. Peaks were tall and sharp and within recommended criteria. With differing mass spectral data, both compounds were separated from neighbouring peaks and therefore, proven to be selective enough. Meanwhile, in the case of flufenamic acid (the second internal standard) mutual overlap with neighbouring mefenamic acid was recorded, however resolution was within acceptable resolution limits (=1). Both internal standards were repeatable and reproducible throughout qualitative and quantitative studies, therefore, supported the selection of these compounds.

Substantial changes were made to during the optimisation of the gradient elution program including, the mobile phases and column employed. Changes in polarity of the mobile phases resulted in shifts in elution order and the resolution of analytes. Peak shapes across NSAIDs and metabolites were Gaussian in shape and tailing factor was low (≤1.5). The final optimised method ensured resolution and selectivity (>1) was improved and overall asymmetry (<2) was ideal.

Optimal solvent selection was also considered in the development stages; formic acid was used as an additive to improve chromatographic separations and resulted in better retention and peak shape. The optimal solvent was found to be acetonitrile, based on reproducible results, larger peak areas, better shaped peaks and improved base line resolution.

Optimal injection volumes were investigated whereby notable peak distortion occurred at 10 µl, whilst 0.2 and 2µl resulted in symmetrical peak shape. Despite this, at the two lowest volumes only slight differences were noted. However, on analysis of RSD, at injection volumes of 2µl RSD were improved, examples were provided in the NSAID nimesulide at 0.2 µl RSD was recorded at 21.72% compared to 1.63% at 2µl. These

reductions in deviations were noted across majority of NSAIDs. Hence, based on this data, injection volumes at 2µl were found to be optimal.

Selectivity was assessed and base line resolution was achieved in scan mode for all NSAIDs studied except for aceclofenac, diclofenac, ibuprofen, piroxicam, piroxicam-d_3, oxyphenylbutazone, suxibuzone, 4-hydroxydiclofenac and 5-carboxymeloxicam. However, all compounds were resolved using SIM. From these results, it is concluded, that SIM mode is the far more selective method. Using SIM is advantageous when analysing feather samples as there may be ions present in the matrix of the sample that could possibly interfere. Blanks were analysed between samples to ensure no interferences were present that would inhibit the detection of analytes of interest. No cross contamination or interferences were observed, this further ensures that method developed here is selective.

During method development initial investigations into the stability of mixed standards (in acetonitrile) were carried out to determine the stability of compounds when stored directly on the autosampler. The analysis time (54 hours) ensured the investigation of the average time a mixed standard spent on the temperature controlled autosampler (15ºC) during method development. The results from the study determined the short term stability of 5-carboxymeloxicam and 5-hydroxyflunixin is up to 43 hours when stored in a mixed standard directly on the autosampler. Whilst for all other compounds, with the exception of acetylsalicylic acid and ibuprofen, were stable for the duration of the 54 hour investigation. Stability studies also included 54 hour investigations of both internal standards in a mixed standard. Both were proven stable and within the required RSD of ±15% for 54 hours storage on the autosampler.

Alongside initial autosampler stability, supporting precision studies were carried out to provide an initial indication of the methods precision, piror to validation (Chapter Four). From these precision studies, as expected, when the concentration increased so did the methods precision. Where the method was not deemed precise, this fell in line with the linear ranges as statistically analysed throughout section 4.3 and the LOD and LOQs (section 4.4.5) calculated thereafter. This was especially evident in the metabolites; 5-carboxymeloxicam and 5-hydroxyflunixin.

Whilst method development resulted in improvements in chromatographic separations of most NSAIDs, compounds acetylsalicylic acid and ibuprofen consistently showed low analytical responses and variation throughout. The compounds showed inconsistencies in precision and stability studies and therefore could not reliably be

detected by this analytical method (falling outside the criteria set out by the Commission Decision). It was therefore not plausible to include these two compounds in the validated method (Chapter Four) nor apply them to the application in real samples (Chapter Five). This is an area for further investigation in future research (section 6.3).

Upon optimisation and meeting the required guidelines (Huber, 2007; CDER, 1994), the method was ready for validation. Following this, Chapter Four presents the investigations and supporting data that were carried out and collated to validate the analytical method.

CHAPTER 4: METHOD VALIDATION RESULTS AND DISCUSSION

The results from method validation (section 2.4) are included in this chapter subsequent to its development as outlined in Chapter Three. The LC-MS method has been developed in Chapter Three for the simultaneous detection of eleven NSAIDs and seven major metabolites (section 1.3.3). There are four aspects of this research that make this analytical method novel; (i) the range of NSAIDs selected, (ii) the inclusion of metabolites, (iii) the simultaneous detection of twenty compounds and two internal standards and (iv) the sample preparation involved, the latter is detailed in Chapter Five.

The analytical method presented was validated through a series of parameters and laboratory tests set out by Commission Decision (2002) and in Huber (2007) in accordance with the Centre for Drugs Evaluation and Research, and International Conference on Harmonisation. These studies establish whether the results met requirements for the intended analytical application. The studies carried out during the validation were selectivity (section 4.1), longer term, six day stability (section 2.4.2 and 4.2), inter-assay precision (section 2.4.3 and 4.3), linear range (section 2.4.4 and 4.4) and limits of detection and quantification (section 2.4.4 and 4.4.5). The assessment of linear range included linear regression of which there was visual examinations of plots, analysis of residuals and statistical tests Shapiro-Wilk test for normality and Wald-Wolfowitz runs test for randomness. These tests would ensure the data is free from bias and any variations from linearity are insignificant and random.

4.1 RESULTS OF SELECTIVITY

Selectivity was investigated during method development stages (section 3.2.1) and through the implementation of the SIM method. Diagnostic ions were collated for all analytes of interest and were used to resolve any co-elution between NSAIDs and their metabolites (section 3.2.4 and 3.2.5). As discussed in the preceding chapter, compounds found to overlap had different quantification and confirmation m/z ions (Table 3.7), this allowed for discrimination between compounds and thereafter enables reliable quantification. Based on the selectivity of the analytical method in SIM mode, this method was validated and results are presented in this chapter.

4.2 LONGER TERM STABILITY IN AN INTER-DAY STUDY

Autosampler stability time was investigated during initial method development (section 3.3). In the method validation to allow for longer term analysis, i.e. if analysis was

spread over a number of days, stability of compounds over 6 days, with a freezing and thawing stage between analysis, was studied.

4.2.1 Longer term stability of internal standards

Like initial autosampler stability studies (section 3.3), the stability of both internal standards was also investigated over a longer term stability study as discussed in Chapter Two, section 2.4.3.

Over the period of 6 days both flufenamic acid and piroxicam-d_3 peak areas recorded are within acceptance criteria of ±15% RSD with no specific pattern. From the data presented (Figure 4.1 and 4.2) both internal standards are stable across the duration of 6 days. This includes the time from sample preparation and the time spent in storage in freezer conditions.

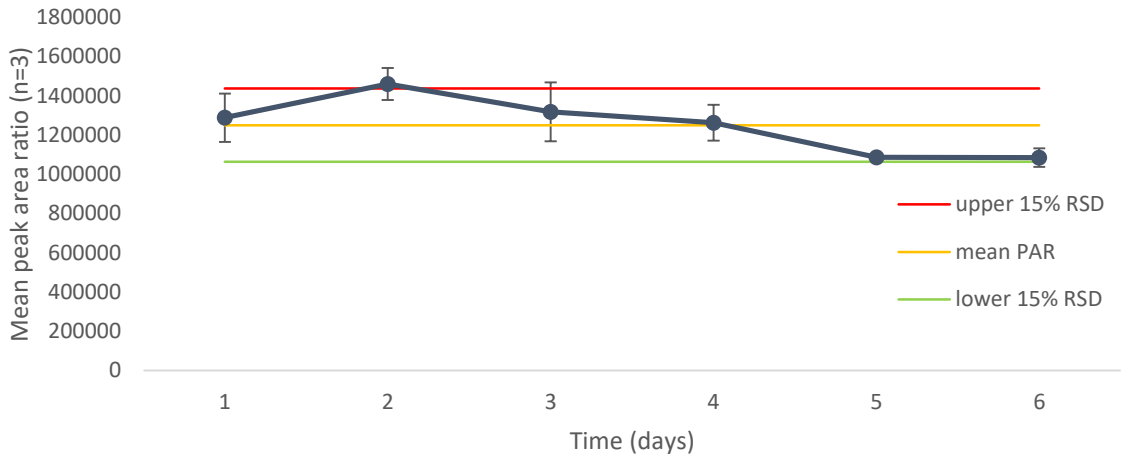


Figure 4.1 Six day stability results for internal standard piroxicam-d3 (m/z 332); mean peak area (0.1µg/ml).

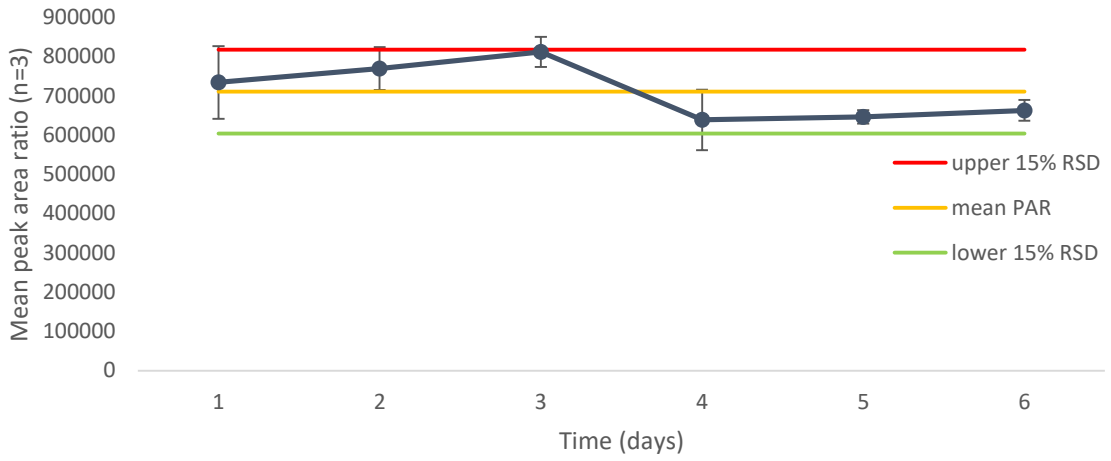


Figure 4.2 Six day stability results for internal standard flufenamic acid (m/z 279); mean peak area (0.1µg/ml).

On the second day there was slight increase in analytical response in piroxicam-d_3 this has resulted in the mean peak area being close to the upper 15%. The analytical responses recorded on this day had an RSD of 5.6%, showing a small variation in peak areas, denoted by the error bars. This increase in response could be down to instrumental fluctuations i.e. indeterminate error, this is especially relevant as only peak area was used thus the results are not relative to any such fluctuations.

During six day stability studies, the concentration of both internal standards was the same (0.1µg/ml), selected based on the linear range this research investigated (section 4.4). It was however apparent, that whilst the concentration is the same not all analytes yield the same analytical response, as was the case in these two compounds.

Together with the initial investigations (section 3.3), these results show the stability of flufenamic acid and piroxicam-d_3 are also stable over the duration of six days. This, supports their selection and suitability as internal standards for this method of simultaneous detection of NSAIDs using LC-MS.

4.2.2 Longer term stability of mixed NSAID standards in an inter-day study over six consecutive days

Investigations were carried out over three concentrations (0.05, 0.5 and 5µg/ml) and at the lowest concentration of 0.05µg/ml the majority of NSAIDs and metabolites fell within ±15% RSD over the six day study, with some falling within ±10% RSD, showing high precision and stability in compounds (Figure 4.3 and Appendix IV, V and VI). As with initial autosampler investigations (section 3.3), similar results were noted in the

metabolites 5-carboxymeloxicam and 5-hydroxyflunixin, both having RSD larger than ±15%. It was apparent that at this concentration (0.05µg/ml), 5-carboxymeloxicam was close to its calculated LOD (0.03µg/ml, Table 4.5) and beyond the LOQ at 0.12µg/ml (section 4.4.5, Table 4.5), where larger fluctuations in analytical response are expected, as discussed in section 3.3.2 (Peters, Drummer and Musshoff, 2007). However, RSD was within the lower acceptance range of ±20% RSD throughout the duration of the study (section 3.4). In the case of 5-hydroxyflunixin, the analytical response on day 3 and 4 was outside of these lower accepted ranges indicating a drop in analytical response. On inspection of the chromatogram, poor peak symmetry was noted and peaks were less intense. This is most likely to relate to fluctuations in instrumental response and indeterminate errors that cannot be explained.

At the 0.5µg/ml all NSAIDs were within the accepted ±15% RSD with NSAIDs aceclofenac (Figure 4.3), meloxicam and piroxicam even lower (Appendix IV). At 5µg/ml all compounds were within acceptance values (refer to Appendix VI and VII). These results indicate that the compounds under investigation are most stable at concentrations above LOD.

Figure 4.3 shows an example of the NSAID aceclofenac during the 6 day stability study at all concentrations. Horizontal lines depict ±15% RSD for each concentration (0.05, 0.5 and 5µg/ml), and the mean PAR over the duration of six days. Figure 4.3 shows that aceclofenac is stable over 6 days in a mixed NSAID standard in acetonitrile when stored as per storage conditions in section 2.4.2.

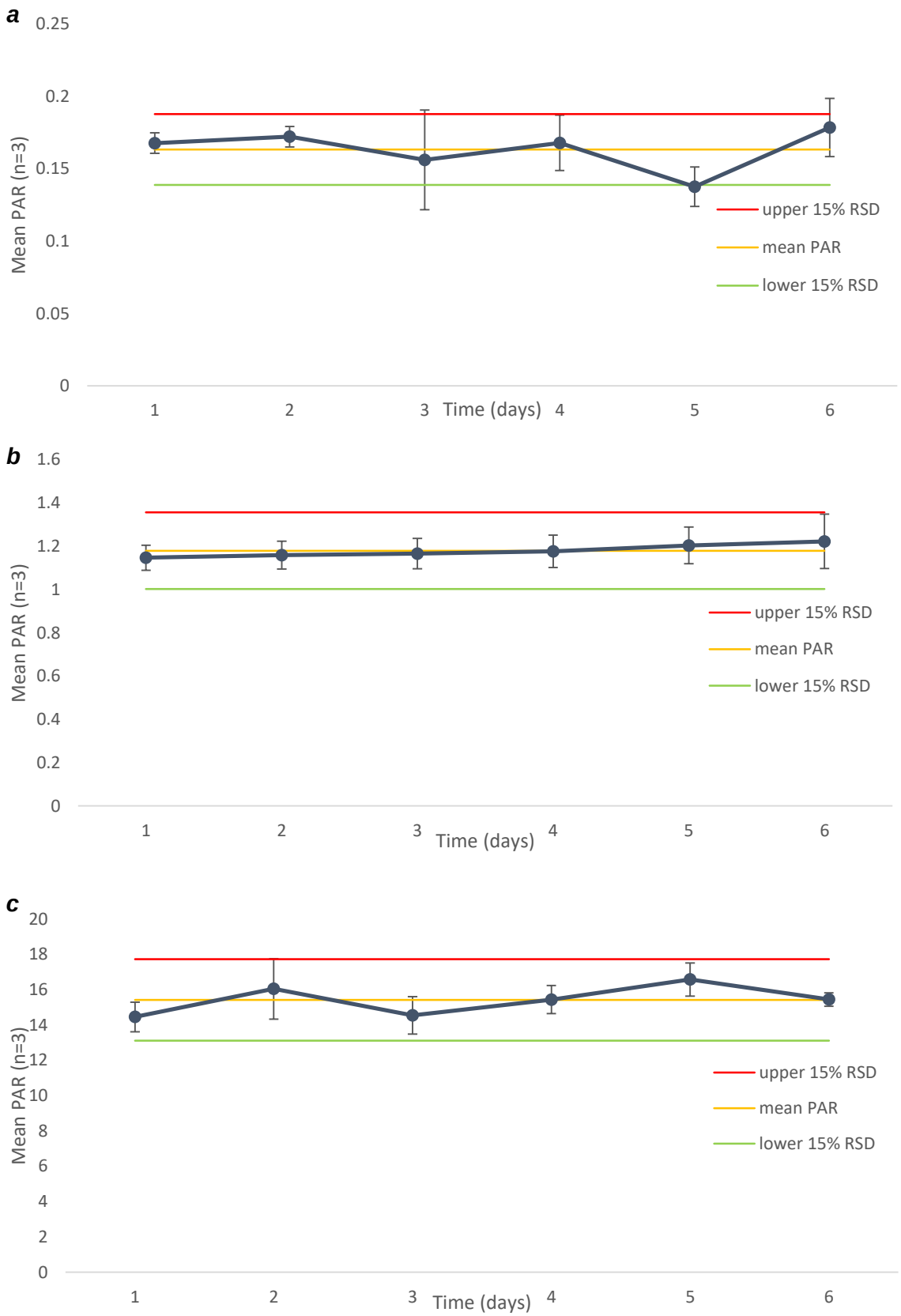


Figure 4.3 Graph of Mean Peak Area Ratio (n=3) over 6 days for aceclofenac stability trial, (a) 0.05µg/ml, (b) 0.5µg/ml, (c) 5µg/ml.

From the 6 days stability study, all NSAIDs, except 5-carboxymeloxicam and 5-hydroxyflunixin, were within acceptance criteria of ±15% RSD (Appendix IV, V and VI), thus, are stable for the duration of the study. In the case of the mentioned metabolites, 5-carboxymeloxicam (IV-e) and 5-hydroxyflunixin (IV-f) appear stable at higher concentrations (0.5 and 5 µg/ml) with an RSD for each within ±15% for the duration of six days. However, at the lowest concentration (0.05µg/ml), i.e. near LOD, stable up to 3 days.

4.3 PRECISION OF THE ANALYTICAL METHOD

Precision of the analytical method was initially investigated during the development of the analytical method. This section presents an inter-assay reproducibility precision study based over six days in triplicate analysis as per protocols in section 2.4.3.

4.3.1 Inter-day reproducibility precision study

At the lowest concentration, 0.05µg/ml, all NSAIDs results were precise and within 15% RSD. However, the metabolites 5-carboxymeloxicam and 5-hydroxyflunixin had RSD over 15% (16.46% and 22.59% respectively); the results are shown in Table 4.1. Like intra-day repeatability studies (section 3.4), data collected for these compounds had larger RSD at the lowest concentrations only. Hence, as the concentration increased so did corresponding precision. This indicates, in the case of these compounds, the analytical method is the most precise at higher concentrations.

Table 4.1 Inter-day repeatability results of three concentrations of mixed NSAID standards

NSAID	Inter-assay PAR[a] repeatability					
	0.05µg/ml	RSD[b] %	0.5µg/ml	RSD [b] %	5µg/ml	RSD[b] %
aceclofenac	0.16 ± 0.01	8.93	1.18 ± 0.03	2.43	15.41 ± 0.83	5.36
carprofen	0.20 ± 0.02	7.69	1.29 ± 0.12	9.93	7.46 ± 0.38	5.13
diclofenac	0.52 ± 0.05	9.62	2.90 ± 0.28	9.51	17.51 ± 0.86	4.90
flunixin	2.60 ± 0.27	10.25	17.55 ± 1.70	9.67	16.47 ± 0.96	2.06
ketoprofen	0.18 ± 0.03	14.77	0.81 ± 0.05	6.79	3.84 ± 0.14	3.61
mefenamic acid	0.37 ± 0.05	13.95	2.28 ± 0.20	8.63	27.00 ± 2.38	8.82
meloxicam	12.04 ± 0.78	6.44	60.44 ± 2.60	4.30	73.77 ± 1.72	2.34
nimesulide	21.82 ± 1.68	7.69	73.33 ± 5.65	7.71	77.17 ± 2.03	2.64
oxyphenylbutazone	5.94 ± 0.44	7.47	43.98 ± 3.27	7.43	56.48 ± 1.11	1.97
phenylbutazone	0.60 ± 0.06	10.02	3.42 ±0.19	5.50	38.76 ± 2.18	5.62
piroxicam	5.21 ± 0.40	7.75	30.35 ± 0.98	3.24	62.42 ± 0.92	1.47
suxibuzone	0.80 ± 0.03	3.76	4.02 ± 0.39	9.62	23.38 ± 0.96	4.10
3-hydroxymethyl mefenamic acid	0.48 ± 0.04	8.90	3.55 ± 0.32	8.89	19.61 ± 0.81	4.11
4-hydroxydiclofenac	0.36 ± 0.02	6.49	2.69 ± 0.26	9.65	16.18 ± 0.88	5.46
4-hydroxynimesulide	9.33 ± 0.83	8.88	50.86 ± 3.48	6.84	61.97 ± 1.27	2.05
5-carboxymeloxicam	0.05 ± 0.01	16.46	0.53 ± 0.03	6.50	4.13 ± 0.20	4.81
5-hydroxyflunixin	0.12 ± 0.03	22.59	0.21 ± 0.02	11.24	1.26 ± 0.06	4.87
5-hydroxypiroxicam	0.75 ± 0.05	6.75	7.18 ± 0.56	7.86	33.47 ± 0.72	2.14

[a]peak area ratio of analyte peak area/ internal standard peak area (n=3) ± SD [b]relative standard deviation (RSD)

On inter-day repeatability study results, it was apparent that 5-carboxymeloxicam and 5-hydroxyflunixin had larger RSD (>15%) (Table 4.1) at the lowest concentration, however it was still within 15% in mid-range and high concentrations. In 5-carboxymeloxicam and 5-hydroxyflunixin, at the lowest concentration, when investigating the RSD of triplicate analysis on day 3 and 4 there were large deviations and lower analytical responses than those recorded across the other four days. However, as these were in mixed NSAID standards, and no other compounds exhibited the same drop in analytical response, these fluctuations were compound dependent and thus, in section 4.2.2, linked to indeterminate errors. Meanwhile, in section 3.3.2 (autosampler stability) and section 3.4.1 (intra-day precision) high RSD and similar drops in analytical response were recorded around 43 hours suggesting that on the autosampler that 5-carboxymeloxicam and 5-hydroxyflunixin were unstable beyond this time in a mixed standard. The data presented in Table 4.1 is supported by these earlier

findings and suggests that the analytical method cannot yield precise data, for these compounds, beyond this point at low concentrations. RSD within day three was 26.13% and on day 4, analytical response was lower (peak area ratio 0.09) than those recorded across the six days (mean peak area ratio 0.14), similar variations were observed in 5-carboxymeloxicam, (refer to Appendix IV). This could be the result the previously discussed fluctuations at the lower concentration of the working concentration range, where more fluctuation is expected when closer to LOQ (Peters, Drummer and Musshoff, 2007). LOQ for 5-carboxymeloxicam (0.123µg/ml) and 5-hydroxyflunixin (0.070µg/ml) is reported in section 4.4.5, Table 4.5)

At 0.5µg/ml all NSAIDs were within ±15% RSD, therefore analysis at this concentration appears precise (≤7.54%) across intra-day studies. At 5µg/ml all NSAIDs, are within a RSD of ±15%, ranging from 1.47% for piroxicam to 4.90% for diclofenac.

Overall, the inter-assay reproducibility study has shown the analytical method to be precise and within acceptance criteria (Huber, 2007; Peters, Drummer and Musshoff, 2007) and often lower, within 5-10% RSD. The precision of the method also increased as the concentration increased.

4.4 THE ASSESSED LINEAR RANGE

The linear range (section 1.6.6) of the analytical method presented in this thesis was accessed experimentally as per the method described in section 2.4.4. Results from this study are presented and discussed in sections 4.4.1 to 4.4.5. The linear range of each NSAID and selected metabolites was investigated through (I) linear regression (section 4.4.1), (II) relative response (section 4.4.2), (III) analysis of residuals (section 4.4.3) and (IV) statistical tests (section 4.4.4). Statistical tests such as Shapiro-Wilk and Wald-Wolfowitz were employed to test the normality of results and randomness of errors in each data set (first described in section 1.6.6). Thereafter, the limits of detection (LOD) and limits of quantification (LOQ), together with signal to noise ratio, of each compound was determined from linear regression and compared to literature where possible (section 4.4.5).

4.4.1 Linear regression

In the first assessment of the linear range, regression analysis was carried out. Linear graphs were plotted of PAR versus concentration (µg/ml) for each NSAID and metabolite. Through this analysis, it is expected that at very low and very high concentrations there are points that tail off and plateau where instrumental response is

non-linear, these concentrations are thus beyond the linear range of the compound and furthermore, beyond the working range of the detector (Huber, 2007). The latter is especially obvious at higher concentrations where it is possible to see peaks that exhibit overloading, refer to section 1.6.2.2 and 1.6.2.9. Over the whole concentration range investigated (0.01 to 25 µg/ml), results were non-linear at the highest concentrations of 5, 10 and 25 µg/ml. All NSAIDs and metabolites showed MS detector saturation at these concentrations (5, 10 and 25 µg/ml) and in some cases at 1 and 2.5 µg/ml (i.e. nimesulide, oxyphenylbutazone and 3-hydroxymethyl mefenamic acid). Figure 4.4 is the linear graph of nimesulide plotted over the whole concentration range, it shows the highest concentrations that have plateaued and resulted in a non-linear response.

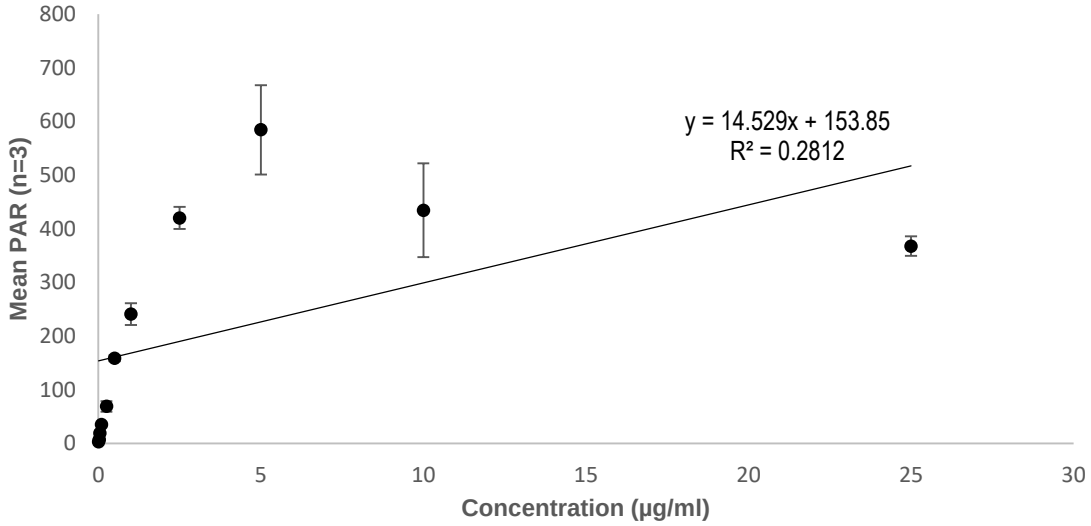


Figure 4.4 Linearity plot of nimesulide (0.01 to 25µg/ml)

The ICH defines the linear range as the interval between the upper and lower concentration whereby the analytical response exhibits linearity. To investigate further, these non-linear concentrations were removed from each of the linear regression graphs and a further calibration graph of PAR versus concentration was plot. As an exemplar shown in Figure 4.5 of nimesulide, each NSAID resulted in a linear relationship (Appendix VII).

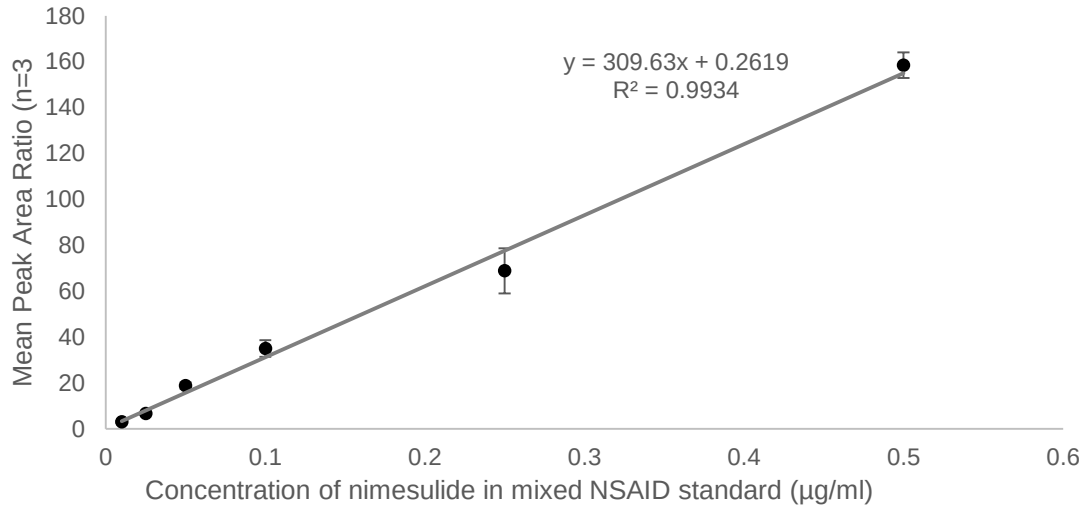


Figure 4.5 linear range plot of nimesulide (0.01 to 0.5µg/ml)

Correlation coefficients (R^2) value were calculated and recorded at 0.993 ± 0.002 (n=22) across all compounds. R^2 is a measure of the degree of linear association between x and y residuals. Ranging from 0.0 to 1.0, the larger the R^2 value, the closer the correlation between all the data points and the line of best fit, an R^2 value of 1 represents a perfect fit. It is said, a 10% relative uncertainty in this linear range would require R^2 values of 0.99 (Ellison, Barwick and Farrant, 2009). On comparison to the R^2 values obtained in the linearity study, all NSAIDs conformed to 10% relative uncertainties (Apendix VII, Table 4.2). It is important to remember that R^2 cannot generally be taken as a sole measure of linearity. Whilst it is an indication of good linearity when data is spread evenly, around the line of best fit, and without anomalies, high R^2 values can be obtained from a large spread of data that would otherwise produce a non-linear graph (Miller and Miller, 2010). Therefore, the linear range of each compound was investigated statistically through a series of tests including, the analysis of residuals, goodness of fit and runs test.

4.4.2 Relative response

Linearity data was further analysed by plotting a graph of relative response (mean PAR divided by concentration) against the concentration, on a log scale (Figure 4.6 and 4.7 and Appendix VIII). Deviations from linearity can be difficult to detect so by analysing plots of relative response, if linearity is achieved then the resulting plot should be within ±5% (95 to 105%) of the mean relative response factor (R_c) (Huber, 2007). It is possible to predict the linear range as the method is deemed linear until the point where the relative response falls outside the accepted RSD. Deviations should

therefore, be equally distributed between positive and negative values, however, areas of high and low concentration will typically yield a negative deviation a result of their non-linear response. R_c designates the line of constant response (Figure 4.6, 4.7 and Appendix VIII).

As an example, Figure 4.6 shows the plot for nimesulide where data points beyond the linear range were identified at the highest concentrations (5, 10 and 25 µg/ml respectively), as originally highlighted in Figure 4.4 and in Figure 4.6. Including these data points cause bias in the intercept by shifting the regression line hence the resulting correlation coefficient falls outside the accepted 0.99 (Figure 4.4, R^2 = 0.28). On the removal of these data points a further concentration point (2.5 µg/ml, highlighted in Figure 4.6) was identified outside ±5% and hence was removed. The data points were plot again (Figure 4.7) and became spread around the mean, within ±5%, indicating, until this point and through this interpretation, the linear range of the compound was 0.01 to 1µg/ml.

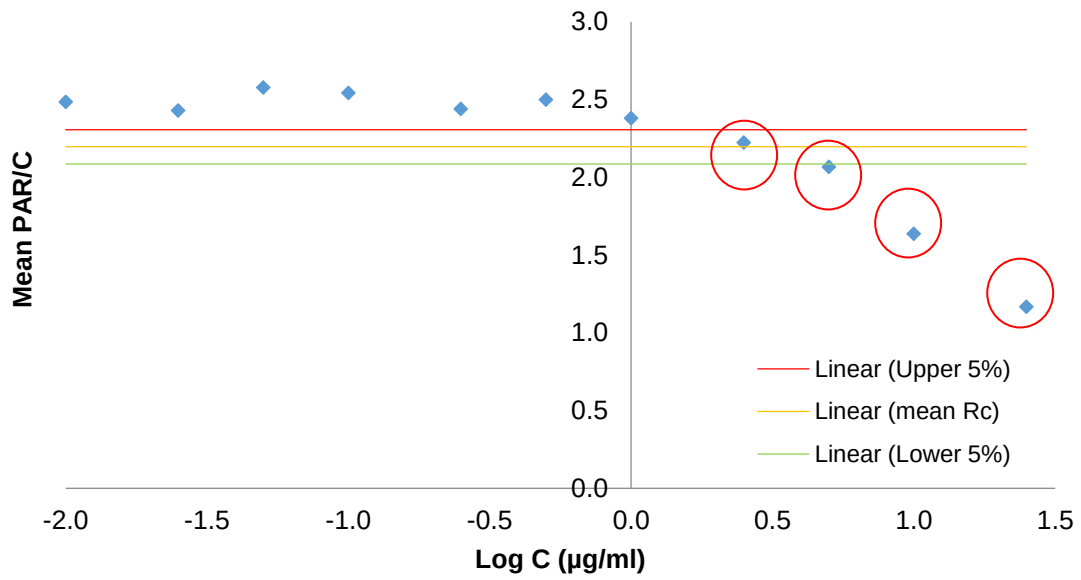


Figure 4.6 Relative response (mean PAR/C) of nimesulide versus log concentration

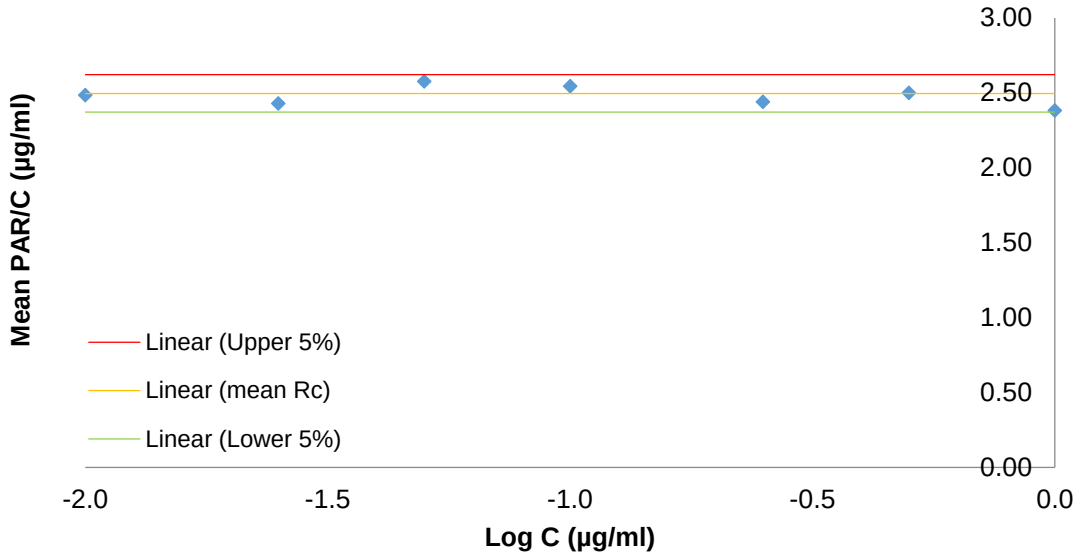


Figure 4.7 Relative response (mean PAR/C) of nimesulide versus log concentration (0.01 to 1µg/ml)

Upon examination of the relative response graphs for each compound (Appendix VIII) and correlating linear graphs a predicted linear range was obtained (Table 4.2). Thereafter, another method was used on the concentration range investigated to confirm the linear range of the compounds, namely the analysis of residuals, the calculated difference between the observed value and the predicted value obtained from the linear regression (section 4.4.3).

4.4.3 Analysis of residuals

The analysis of residuals can aid further in the identification of problems with poor or incorrect curve fitting (Figure 4.8) (Ellison, Barwick and Farrant, 2009). If the data is of good fit, the residuals are expected to be randomly distributed around zero (the ideal result indicating there is no deviation from the observed value) (Figure 4.9). Deviations from this random distribution can indicate problems within the data set, for example, a curve indicates the line of best fit has been fitted through the data set that follows a non-linear trend, this was initially seen in Figure 4.4 (section 4.4.1) at the highest concentrations for nimesulide (5, 10 and 25 µg/ml) where MS detector saturation was recorded.

On analysis of the residuals, residual plots were produced (residuals against concentration). Uneven distribution of values was apparent at lower and upper

concentrations (Figure 4.8), as previously discussed in the interpretation of Figure 4.5. This resulted in residual plots not randomly distributed around zero and therefore represented a loss of linearity, this can be seen in nimesulide, Figure 4.8, which resulted in a curve. Subsequently, the analytical responses that were non-linear, at the high and low end of the concentration range investigated (section 4.4.1, Table 1.2), were removed for all compounds and residual plots were obtained (Appendix IX). Figure 4.9 shows an ideal response in nimesulide; the residuals are randomly distributed around zero in linear range 0.01 to 0.5µg/ml.

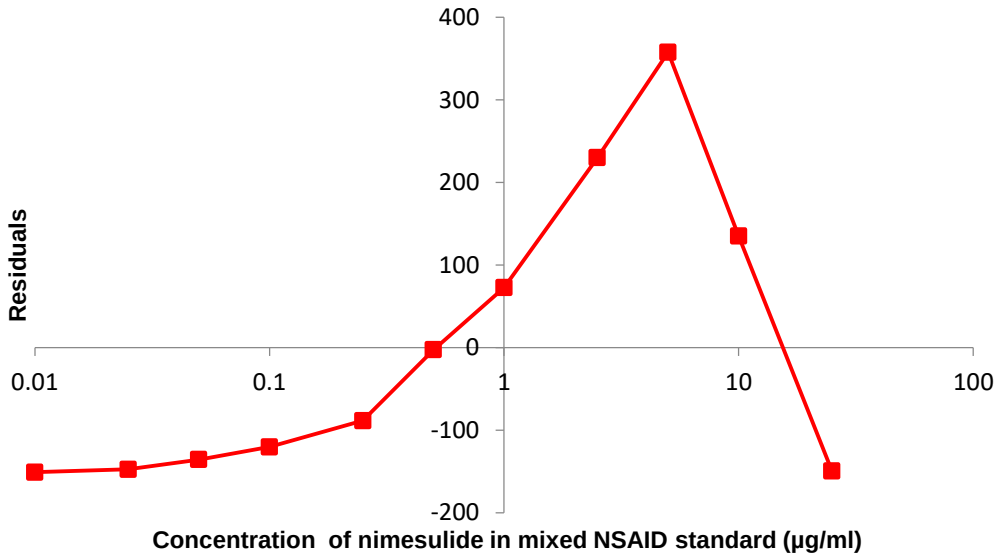


Figure 4.8 Biased residual plot of nimesulide (0.01 to 25 µg/ml)

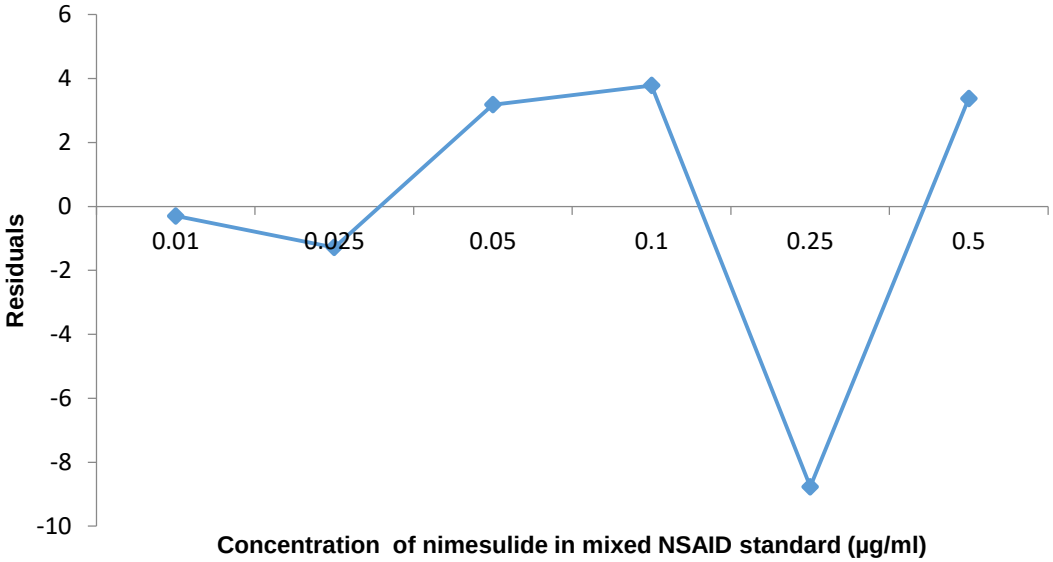


Figure 4.9 Unbiased residual plot of nimesulide (0.01 to 0.5 µg/ml)

Using regression and residual analysis (Microsoft Excel) (Appendix IX), concentration ranges were assessed and the resulting linear range are given in Table 4.2.

Table 4.2 linear range and R^2 values after regression and residual analysis

NSAID	linear range µg/ml	R^{2a}
aceclofenac	0.010-2.5	0.996
carprofen	0.025-1.0	0.991
diclofenac	0.010-5.0	0.995
flunixin	0.010-1.0	0.996
ketoprofen	0.025-5.0	0.993
mefenamic acid	0.010-10	0.991
meloxicam	0.010-2.5	0.996
nimesulide	0.010-0.5	0.993
oxyphenylbutazone	0.010-0.5	0.994
phenylbutazone	0.050-2.5	0.996
piroxicam	0.010-2.5	0.990
suxibuzone	0.025-2.5	0.990
3-hydroxymethyl mefenamic acid	0.010-0.5	0.993
4-hydroxydiclofenac	0.010-1.0	0.998
4-hydroxynimesulide	0.010-5.0	0.995
5-carboxymeloxicam	0.025-1.0	0.995
5-hydroxyflunixin	0.025-5.0	0.998
5-hydroxyprioxicam	0.010-2.5	0.998

[a]mean correlation coefficient (R^2) (n=3) data from linear regression

4.4.4 Statistical analysis

For further analysis of the residual plots and to confirm the normality of the results for the linear range (Table 4.2), the statistical package IBM SPSS Statistics (SPSS) was employed to perform the Shapiro-Wilk test (Shapiro and Wilk, 1965). Normality of results can be assessed by two main methods: graphically or numerically. Suitable for small samples sizes of less than 50 samples, this statistical test involves the numerical assessment of normality through the comparison of analytical response, PAR, with the predicted analytical response obtained from the residual analysis (linear range as per Table 4.2). The difference between the two is calculated and residuals obtained and analysed (Miller and Miller, 2010). Thereafter, the output of normal quantile-quantile (Q-Q) plots are assessed graphically for normality (Figure 4.10).

The Shapiro-Wilk test will return a mean, its standard deviation and a p-value. P-values represent the probability of observing a value greater than or equal to the critical value

(0.05), representing 95% confidence interval, if the null hypothesis is retained. If the distribution of errors is normal, the statistical test retains a null hypothesis (≥0.05) indicating the data is of normal distribution. If the p-value is lower than the significance level of 0.05, the null hypothesis must be rejected as it indicates a result significantly different from non-normal distribution. In the statistics used throughout the linearity study the confidence limit used has been 95% thus, the significance level used was p = 0.05.

For graphical assessment, in the case of the Q-Q plots (Figure 4.10), if the data is normally distributed then the data points will be close to the regression line. If the data appears in a non-linear manner then the data is not normally distributed. Normality is depicted graphically by Q-Q plots and an exemplar is shown in Figure 4.10 of nimesulide.

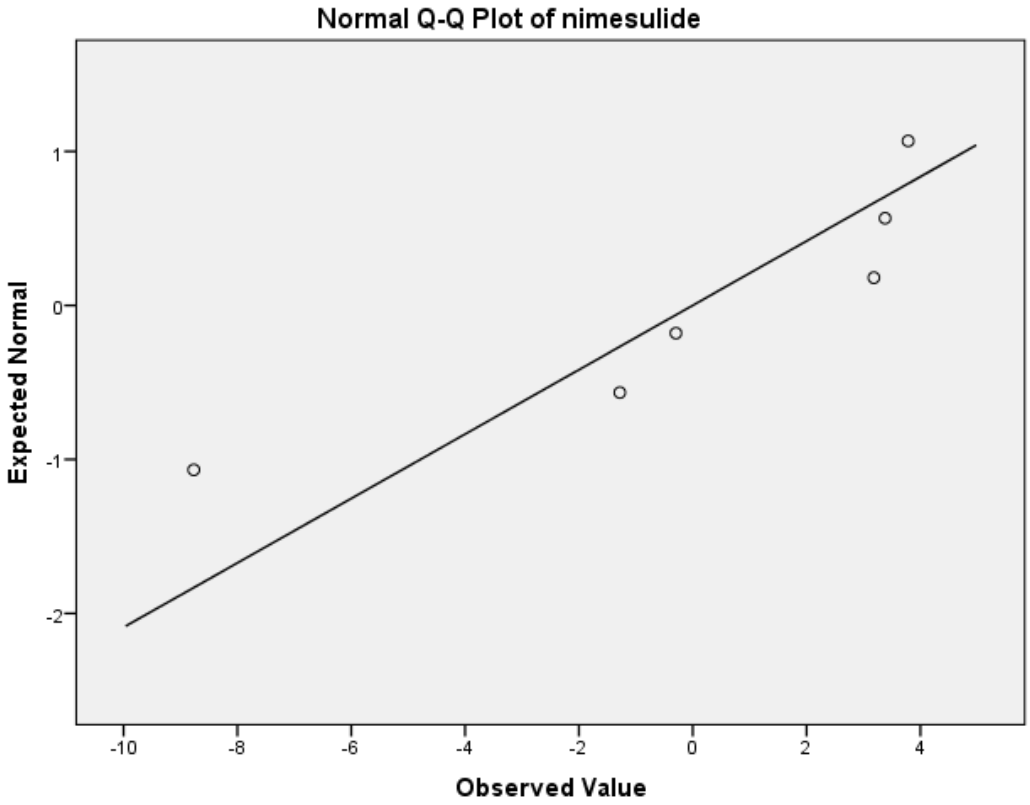


Figure 4.10 One-Sample Shapiro Wilk Test; Q-Q plot for nimesulide

On visual analysis, the linearity of the data points (Figure 4.10 and Appendix X) for all NSAIDs and metabolites are close to the regression line thus showing normal distribution. Employing the Shapiro Wilk test on all compounds to numerically test for normality resulted in the null hypothesis being retained (≥ 0.05) (Table 4.3). Significant

values ranged from 0.052 to 0.818, above the required 95% significance limit (≥ 0.05), and in agreement with the Q-Q plots, indicating the data points were normally distributed and thus linear.

Table 4.3 Shapiro Wilk test results; null hypothesis of normal distribution and corresponding significance value (tested on predicted linear range in Table 4.2)

NSAID	P value[a]	Retain null hypothesis
aceclofenac	0.240	✓
carprofen	0.306	✓
diclofenac	0.093	✓
flunixin	0.052	✓
ketoprofen	0.236	✓
mefenamic acid	0.550	✓
meloxicam	0.322	✓
nimesulide	0.088	✓
oxyphenylbutazone	0.067	✓
phenylbutazone	0.124	✓
piroxicam	0.298	✓
suxibuzone	0.096	✓
3-hydroxymethyl mefenamic acid	0.126	✓
4-hydroxydiclofenac	0.064	✓
4-hydroxynimesulide	0.143	✓
5-carboxymeloxicam	0.298	✓
5-hydroxyflunixin	0.818	✓
5-hydroxyprioxicam	0.130	✓

[a]≥ 0.05= retain, <0.05= reject ✓ = retained

After the Shapiro Wilk test, it was necessary to consider the randomness in the data set; therefore, the Wald-Wolfowitz runs test was employed (Wapole, 2002). In linearity it is expected that there will be a slight negative or positive residual from the line of best fit and these will occur at random. However, by including points that tail off and plateau at the very low and high concentrations, attempting to fit the line of best fit through a set of data points actually on a curve may yield a sequence of non-random negative and positive residuals. These residuals are a sequence of increasing and decreasing events called runs. By testing these residuals, the test makes no assumptions on the normality or distribution of the data. However, by utilising the runs test on the same principle of the Shapiro Wilk test, this can be used to evaluate the linearity by verifying

the distribution of the residuals. This test considers whether the number of runs is small enough for the null hypothesis, to be retained or rejected. (Epshtein, 2004). As such, if the distribution of residuals is of random distribution, the statistical test retains a null hypothesis ($p \geq 0.05$). If the sample has too many or too few runs then the test suggests that the residuals are not randomly distributed confidence limits are still the same and work on 95% probability level (Epshtein, 2004).

Figure 4.11 is an example of the graphical presentation of Runs test for the NSAID nimesulide. If the Runs are of normal distribution and mutually independent errors then the p-value will increase and in doing so the number of Runs gets closer to the centre of Gaussian distribution curve. If the p-value falls below the significance interval of 95% (<0.05) then the number of runs falls within one of the ends of the curve, indicating too few or too many runs. According to Miller and Miller (2010) too few or too many runs indicates a departure from randomness, hence, falling below the significance level. In Figure 4.11, as depicted by the red line and overlapping description, the number of observed runs is 4, with a p-value of 1 this indicates that the null hypothesis is retained and the distribution of residuals is randomly distributed.

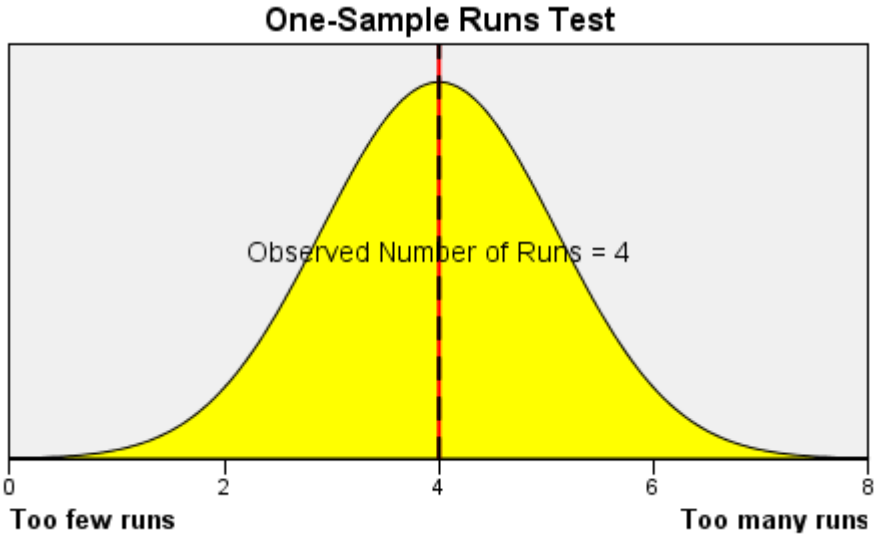


Figure 4.11 One-Sample Runs Test, Gaussian distribution curve for nimesulide (data based residuals)

As expected the null hypothesis is retained for all NSAIDs with p-values above the significance level of ≥ 0.05 ranging from 0.160 to 1.000 (Table 4.4). This shows that the distribution of data is normal and results are of random distribution.

Table 4.4 Wald-Wolfowitz hypothesised runs test results; null hypothesis (normal distribution of error) and p-values

NSAID	p-value[a]	Retain null hypothesis
aceclofenac	0.648	✓
carprofen	0.648	✓
diclofenac	0.160	✓
flunixin	0.648	✓
ketoprofen	0.648	✓
mefenamic acid	1.000	✓
meloxicam	1.000	✓
nimesulide	1.000	✓
oxyphenylbutazone	0.648	✓
phenylbutazone	0.648	✓
piroxicam	0.252	✓
suxibuzone	0.431	✓
3-hydroxymethyl mefenamic acid	1.000	✓
4-hydroxydiclofenac	0.913	✓
4-hydroxynimesulide	0.648	✓
5-carboxymeloxicam	0.648	✓
5-hydroxyflunixin	1.000	✓
5-hydroxyprioxicam	0.952	✓

[a]≥ 0.05= retain, <0.05= reject ✓ = retain

The Shapiro-Wilk and Wald-Wolfowitz statistical tests have been successfully applied to residual analysis and have verified results determined from regression analysis. Both tests have confirmed the analytical data, from the linear range (Table 4.2), is of normal distribution and residuals are randomly distributed.

On comparison to the literature, the linear ranges reported here are comparable and in some cases span a wider concentration range including linearity at lower concentrations. For example, Abdel-Hamil, Novotny and Hamza (2001) used a LC-MS method and determined the linear range of diclofenac between 0.05-0.3µg/ml and 0.1-0.5µg/ml for ketoprofen compared to the wide range of 0.01-5.0 µg/ml and 0.025-5.0µg/ml (Table 4.2) obtained in this research respectively. Furthermore, research by Hu, et al. (2012), reported linear ranges for compounds flunixin and meloxicam (0.008 - 0.786 µg/ml, for both compounds), whilst the lowest concentration in this range was lower than reported in this research (0.010-5.0µg/ml for flunixin and 0.010-2.5µg/ml for meloxicam), research by Hu, et al. (2012), implemented LC-MS/MS. Therefore, it could

be deemed that these lower concentrations are a result of the increased sensitivity obtained using tandem MS, but the lower limits found by this research only using LC-MS are very close. This current research offered the same degree of linearity over a wider working range suitable for application in real samples.

In the context of the research area these linear ranges fall in line with the concentrations that have been detected in wildlife samples, such as diclofenac residues detected at 0.05-0.643 µg/g (Oaks, et al., 2004). Thus, the linear range reported in this method spans concentrations that could be detected in a real sample setting.

4.4.5 Limits of detection and quantification

The IUPAC method (Equation 1.8 and 1.9) was used to calculate the LOD and LOQs for each NSAID and comparisons are made to the literature and presented in Table 4.5. LOD values range from 0.010 to 0.196µg/g and LOQ range from 0.033 to 0.576µg/g for nimesulide and ketoprofen respectively. Expressed as units of mass on column, these values were checked alongside the signal to noise ratio (S/N) as another means of estimating the LOD and LOQ. The lowest concentration at which the signal to noise ratio is 3:1 is considered as LOD and 10:1 is considered as LOQ (Table 4.5).

Table 4.5 Comparison of calculated and reported Limits of detection and quantification

NSAID	LOD[a] (from this research)		S/N[b]		LOD (from literature)		LOQ[c] (from this research)		S/N[d]		LOQ (from literature)		Analytical technique and matrix
	µg/ml	µg/g	µg/ml	µg/g	µg/ml	µg/g	µg/ml	µg/g	µg/ml	µg/g	µg/ml	µg/g	
aceclofenac	0.044	0.056	0.050	0.064	0.002	0.003	0.139	0.177	0.250	0.318	NR	NR[e]	LC-MS/MS, rat plasma[f]
carprofen	0.042	0.053	0.050	0.064	0.002	0.003	0.131	0.167	0.100	0.127	0.004	0.005	LC-MS/MS, bovine milk[g]
diclofenac	0.057	0.073	0.050	0.064	1.000	1.094	0.189	0.240	0.100	0.109	1.000	1.094	LC-MS, bovine serum[h]
flunixin	0.02	0.025	0.025	0.032	0.100	0.109	0.061	0.078	0.050	0.055	0.100	0.109	LC-MS, bovine serum[h]
ketoprofen	0.154	0.196	0.050	0.064	0.002	0.002	0.453	0.576	0.250	0.255	0.003	0.003	LC-MS/MS, bovine milk[g]
mefenamic acid	0.088	0.112	0.025	0.032	0.500	0.547	0.309	0.393	0.050	0.055	0.500	0.547	LC-MS, bovine serum[h]
meloxicam	0.043	0.055	0.025	0.032	0.018	0.018	0.146	0.186	0.100	0.102	0.020	0.020	LC-MS/MS, bovine milk[g]
nimesulide	0.008	0.010	0.025	0.032	0.002	0.003	0.026	0.033	0.250	0.318	0.005	0.006	LC-MS/MS, Swine muscle[i]
oxyphenylbutazone	0.022	0.028	0.025	0.032	1.000	1.094	0.048	0.061	0.100	0.109	1.000	1.094	LC-MS, bovine serum[h]
phenylbutazone	0.095	0.121	0.050	0.064	0.050	0.055	0.293	0.373	0.100	0.109	0.050	0.055	LC-MS, bovine serum[h]
piroxicam	0.038	0.048	0.050	0.064	0.100	0.109	0.127	0.162	0.100	0.109	0.500	0.547	LC-MS, bovine serum[h]
suxibuzone	0.036	0.046	0.025	0.032	0.003	0.003	0.123	0.156	0.100	0.109	0.005	0.005	LC-MS/MS, bovine milk[g]
3-hydroxymethyl mefenamic acid	0.023	0.029	0.025	0.032	NR	NR	0.071	0.090	0.100	0.102	NR	NR	NR
4-hydroxydiclofenac	0.031	0.039	0.025	0.032	0.0002	0.0002	0.094	0.120	0.100	0.127	NR	NR	LC-MS/MS, rat plasma[f]
4-hydroxynimesulide	0.063	0.080	0.100	0.127	NR	NR	0.218	0.277	0.100	0.102	NR	NR	NR
5-carboxymeloxicam	0.034	0.043	0.050	0.064	NR	NR	0.123	0.156	0.500	0.511	NR	NR	NR
5-hydroxyflunixin	0.022	0.028	0.025	0.032	0.056	0.057	0.07	0.089	0.100	0.102	0.072	0.074	LC-MS/MS, bovine milk[g]
5-hydroxyprioxicam	0.028	0.036	0.025	0.032	NR	NR	0.084	0.107	0.100	0.102	NR	NR	NR

[a]LOD= $y_B + 3s_B$ [b]signal to noise ratio 3:1 [c]LOQ= $y_B + 10s_B$ [d]signal to noise ration 10:1 [e]Not Reported, [f]Kim, et al., 2012, [g]Dowling, et al., 2009, [h]Miksa, Cummings and Poppenga, 2005, [i]Hu, et al., 2012,

The LODs from this research are lower than compared to the literature (Table 4.5) in the case of diclofenac, flunixin, mefenamic acid, oxyphenylbutazone, piroxicam and 5-hydroxyflunixin. Out of these compounds, of particular interest are the limits calculated for diclofenac. Not only do these falls below reported LOD and LOQ data previously published, at an LOD of 0.073µg/g this is in line with the previously detected residues of diclofenac at concentrations between 0.05 – 0.643µg/g (Oaks, et al., 2004). Additionally, Taggart, et al. (2009) detected residues in liver samples ranging from 0.16 to 5.60µg/g for ketoprofen and 0.01 to 1.65µg/g for meloxicam. As seen in linearity studies, not only do these fall within the working linear range (0.01 – 3.18 µg/g, Table 4.2) of this method, for ketoprofen the highest concentration detected is below the calculated LOD (0.196µg/g) and for meloxicam, at an LOD of 0.055µg/g, the lowest concentration is well within the detection limits of this method. Thus, the LODs and LOQs as presented here, fall in line with concentrations that could be present in a real sample which is paramount to this research area (Oaks, et al., 2004; Taggart, et al., 2009). If similar levels are detected in feather samples, which have not be studied until now, this method is fit for purpose with regards to the linear range, LODs and LOQs found.

As shown in Table 4.5, the LOD for flunixin, calculated by this research, is 0.025µg/g compared to the reported 0.1µg/g (Miksa, Cummings and Poppenga, 2005). This was the same for mefenamic acid reported at 0.547 µg/g compared to the calculated LOD from this research at 0.112µg/g (Miksa, Cummings and Poppenga, 2005; Dowling, et al., 2009). The authors have employed LC-MS/MS, which is considered a more sensitive analytical technique. However, this LC-MS method has proven more sensitive than previously published (Table 4.5). Furthermore, in nimesulide the calculated LOD is below its calculated linear range (Table 4.2) at 0.01µg/g and for metabolites 3-hydroxymethyl mefenamic acid, 4-hydroxynimesulide, 5-hydroxyflunixin calculated LODs (0.029, 0.080, 0.028µg/g respectively) are very close to the lower end of the linear range. This is important when quantifying as the linear range is effectively measuring the sensitivity, whilst the LOD is the lowest concentration detected within a degree of statistical certainty (Bernal, 2014). The principal benefit of determining these limits is the methods capability of detecting and determining trace concentrations, thus, it is important to develop methods that offer lower LODs capable of detecting trace levels in a real sample setting (Miller and Miller, 2010).

Where LODs and LOQs were higher than those reported in the literature (e.g. meloxicam at 0.055µg/g and nimesulide at 0.010µg/g (Dowling, et al., 2009; Hu, et al.,

2012), LC-MS/MS was used (Table 4.5). LC-MS/MS is considered more sensitive than LC–MS used in this research and as such, lower limits would be expected. However, it is important to remember the limits of detection between techniques only differs very little when LC-MS/MS is supposed to offer greater sensitivity.

To the author's knowledge, oxyphenylbutazone, 4-hydroxydiclofenac and 5-hydroxyflunixin are the only metabolities, in this research, that LOD and LOQ has been previously reported (Miksa, Cummings and Poppenga, 2005; Kim, et al., 2012; Dowling, et al., 2009). No data is currently available for 3-hydroxymethyl mefenamic acid, 4-hydroxynimesulide, 5-carboxymeloxicam and 5-hydroxyprioxicam. This is the first time in which these metabolites have been included in method validation and in a simultaneous detection method. Therefore, these LODs and LOQs for metabolites 3-hyrdoxymethyl mefenamic acid, 4-hydroxynimesulide, 5-carboxymeloxicam and 5-hydroxypiroxicam are revealed for the first time.

4.5 OVERALL DISCUSSION AND OUTCOMES OF METHOD VALIDATION

Method validation parameters were selected as per the guidelines set out in Huber and CDER, incorporating parameters set out by Commission Decision, ICH, USP and WADA. The LC-MS method was validated in selected ion monitoring (SIM) mode.

In the intra-day precision studies, similar results were obtained to initial intra-day studies (section 3.3). At the lowest concentration (0.05µg/ml) large RSD were recorded for 5-carboxymeloxicam and 5-hydroxyflunixin. On closer inspection of the individual data sets, variation was particularly high on day 3 and 4 of analysis. Lower analytical responses of the analytes than those of the internal standards resulted in low analytical ratios thus introduced larger variation. High RSD at the lowest concentrations were in line with the working linear range and expected variabilities at the lower end. Overall analytical responses recorded for all other NSAIDs were precise and within the accepted RSD.

For the linearity study, each NSAID was tested at a wide concentration range (0.01 to 25µg/ml). Linearity was assessed using the following methods; i) visual examination, ii) linear regression, iii) relative response graphs (R/C vs log C) iv) analysis of residuals, vi) Shapiro Wilk test for normality and Wald-Wolfowitz runs-test of randomness to determine the working range, linear range and most importantly the LOD and LOQ for each NSAID.

Results showed that not all NSAIDs followed a linear trend especially at the lower and higher concentrations thus indicating these concentrations were beyond the linear range. Through visual analysis of the correlation coefficient predictions on the linear range were made but through statistical tests, for example plotting graphs of relative response, looking at the residuals and the test of normality and randomness, outliers were identified and eliminated to predict the linear range. The resulting correlation coefficients resulted in near true linear fit for all NSAIDs. The Shapiro Wilk test and Runs test were applied and showed normal distribution and randomness in data for all NSAIDs. Therefore, from the interpretation of these statistical tests, the results are valid to use.

Using the data from linear range LODs and LOQs were calculated. The LODs for diclofenac, flunixin, mefenamic acid, oxyphenylbutazone, piroxicam and 5-hydroxyflunixin are lower than compared to the literature and very close to reported LODs for meloxicam, nimesulide and phenylbutazone, suggesting that this method is comparable, if not more sensitive, than some currently published methods. The LOD for diclofenac is below the reported 0.098 and 0.225 µg/g lethal dose to vultures and in line with previously detected residues at concentrations between 0.05 – 0.643µg/g. Meanwhile, for metabolites 3-hydroxymethyl mefenamic acid, 4-hydroxynimesulide, 5-carboxymeloxicam and 5-hydroxyprioxicam this is the first reporting of LODs and LOQs. The LODs and LOQs should be published hereafter. The limits across the compounds were found to be in line with the linear range as documented in Table 4.2.

Therefore the validation of a novel analytical method employing LC-MS for the simultaneous detection of eleven NSAIDs and seven metabolites has been reported. From the interpretation and critique of the results the data presented is within acceptance criteria. The method has been validated to allow for identification in negative ionisation SIM with high precision and accuracy, whilst also being sensitive and selective to detect at concentrations that are known to be toxic in this research area.

CHAPTER 5: SAMPLE EXTRACTION METHOD DEVELOPMENT AND ANALYSIS, THE APPLICATION OF THE VALIDATED METHOD

This chapter presents the results and discussion obtained from the analysis of donated animal hairs and feathers. It includes the development of an optimal sample preparation/extraction method and the application of the validated method (Chapter Four). The focus of this chapter is the requirement for a time efficient and cost effective method for potential application in laboratories with low budgets for analytical testing and sample testing. Selectivity and recovery (section 5.2) of NSAIDs in spiked feather samples were studied in order to take into account any matrix affect that may prove problematic to the trace analysis of NSAIDs, are described. Feather samples (red kite and parrot feather) (n=15) and cat hair (n=2) were analysed (section 5.3) and two NSAIDs detected, quantified.

5.1 OPTIMAL SAMPLE PREPARATION METHOD DEVELOPMENT

5.1.1 Sample pre-treatment development

During the development of the sample preparation/extraction three sample preparation methods were trialled. They consisted of i) manual cutting with nail scissors, ii) a mechanical homogenizer (MP Bio FastPrep), using a lysing matrix to grind the sample in individual tubes and iii) a PM100 Planetary Ball Mill, using a specialist grinding jar and liquid nitrogen as a dry freezing additional preparation step. During sample preparation development, time optimisation (from whole sample to desired powdered sample ready for analysis), alongside the homogenisation were important. Very early in the sample preparation method development, manual cutting was disregarded due to inconsistency in overall homogenisation (the tough nature of the barb and rachis were problematic and resisted fine cutting with nail scissors), the time involved (2-3 hours per sample, Table 5.1) and loss of sample (small particles of the sample were lost on the surface of the nail scissors, the wall of the container and to the external environment). Whilst this method has the lowest set up costs (approximately £2 to £15, Table 5.1) of all three methods, the time to process the sample (Table 5.1) was longer than other methods.

The second method investigated was the use of a mechanical homogenizer. This method made grinding the vane of the feather possible, thus, was advantageous over manual cutting, particularly in terms of the consistency in homogenisation of the sample (1 - 2mm sized particles, powder like consistency), that was previously

unachievable. Despite this advantage, like manual cutting, mechanical means failed to grind the rachis, which were only broken down into smaller fragments. Therefore, this method was disregarded as overall homogenisation of the sample could not be achieved, with some parts of the feather still intact. Additionally, this technique is particularly costly compared to the other methods investigated (Table 5.1). When using the homogeniser, each sample is ground in small tubes containing a specialist lysing matrix (small grinding particles used to break down the sample) so these tubes can only be used once. The costs of each tube is £20 (MP Biomedicals, 2016), thus is obviously a disadvantage with regards to a low cost method. Therefore, it would prove costly when used in the field with large numbers of samples.

The last method employed in this research was the PM100 Planetary Ball Mill. This method was particularly advantageous over the previous two methods investigated. All parts of the feather were broken down; the vanes pulverised into a powder and grinding of the barbs and rachis partially achieved, which had not been possible until this point using the other two methods. Furthermore, the grinding jar and balls are re-useable meaning there is only one set up cost involved. Whilst this method had achieved improved homogenisation of the all parts of the sample, the brittle yet tough nature of the rachis resisted complete pulverisation. After carrying out a review of the literature into feather grinding methods, there was a variety of methods used for sample pre-treatment, however, there was no single method suitable for all samples. Often authors employed an additional preparation stage to the samples, to make them more malleable, for example acid digestions, dry ashing and freezing, with the latter of particular interest (Barone and Schmidt, 2006; Chen, 2015; Dauwe, et al., 2004; Gochfield, 1991).

The use of dry-freezing in the preparation of chicken feathers used in feather meal was recently reported by Chen (2015). Samples were dry-frozen with liquid nitrogen prior to any grinding, which resulted in making the sample, particularly the troublesome rachis, more brittle-like, thus easier to grind. Whilst improvements had been made in the homogenisation of the rachis, until this point no method achieved a powder like consistency of this part of the sample. Therefore, liquid nitrogen as an additional prepared stage was employed (Figure 5.1 and 5.2). Samples were pre-cut (Figure 5.1) and placed into sample bags and frozen (Figure 5.2). After freezing the sample for 2 minutes, 2-3 cm pieces were placed into the grinding jar (Figure 5.3) and pulverised for 30 minutes in two 15 minute intervals using the ball mill (Figure 5.4). This additional preparation step made a notable difference to the rachis, by splitting into smaller (0.5 –

1 cm) pieces, making them much easier, but still included, an additional hand cutting step thereafter (Figure 5.5). However, considering the disadvantages of the hand cutting method, particularly the time involved, development to improve this method further continued.

Figure 5.1 Feathers roughly cut

Figure 5.2 Feathers frozen in liquid nitrogen for 2 minutes

Improvements started with greatly reducing the initial size of feather samples from 2 - 3 cm to 2 - 3 mm pieces, of which were ground for 15 minutes initially to see if there were any improved visible results. By cutting into smaller pieces the rachis were ground to powder in half the time of the larger size pieces whilst eliminating and improving the need for manual cutting (Figure 5.3 to 5.5).

Figure 5.3 Samples are cut into 2-3mm pieces into a grinding jar

Figure 5.4 Samples are ground for 15 minutes in PM100 Planetary Ball Mill

Figure 5.5 large remaining pieces are cut until a powder like consistency is achieved

Variation in sample structure type and quantity, created differences in the end product after the grinding process. Some feathers were soft down type, others were stronger contour and veined feathers with hard rachis (see section 1.4.3.1 for feather types with respect to optimal feather selection). Hence, on occasions, large pieces were left after grinding. However, these were brittle at this stage therefore an additional manual cutting step was manageable with nail scissors. As all parts of the sample were ground into a powder, with the satisfactory results, this sample pre-treatment method was employed, using the PM100 Planetary Ball Mill to prepare all samples. A comparative view of all three sample preparation methods trialled is presented in Table 5.1.

Table 5.1 Sample preparation methods against time optimisation, associated cost, quality of sample, advantages and disadvantages of the method used

Method	Approximate time to process one sample	Set-up costs (£)	Cost per sample (£)	Homogenisation of sample	Advantages	Disadvantages
Hand cutting with nail scissors	2-3 hours	2 -15	none	Different sized pieces, some larger pieces of barbs present	Low set up costs	Risk of minor injuries to user, loss of sample and very time consuming
MP Bio FastPreo Mechanical homogeniser	30 minutes – 1 hour	4100[a]	20.0[b]	Vane is ground, Barb in intact	Shorter time Consistency in homogenisation of vane	Sample is left very static And barbs are left whole
PM100 Planetary Ball Mill	30 minutes	990[c]	0.27[d]	All parts of the sample are ground	Shortest time of sample grinding Consistency in homogenisation of vane and barb	Some large pieces of barbs require hand cutting

[a]MP Biomedicals, 2016, [b]cost of lysing matrix tube per sample, MP Biomedicals (2016), [c]Retsch, [d]cost of liquid nitrogen per litre, Retsch (2016)

5.1.2 The selection of Liquid-Liquid Extraction

Biological matrices, such as hair and feathers, contain proteins, salts and other organic compounds that could be chemically similar to the analyte of interest (i.e. pharmaceutical compounds). As such, some of these can be extracted and hence can cause interference. Therefore, it is often necessary to employ an extraction step that will remove unwanted interferences (Majors, 2013), in turn improving chromatographic interpretation (section 1.6.2.6 and 1.6.2.7), and enhance the detection of analytes of interest whilst minimising background noise (Kataoka 2003).

During a study by Richards (2010), the use of SPE in keratinous matrices was investigated and resulted in unsatisfactory results, particularly in the detection of NSAIDs, as no detectable peaks were produced. This included those expected for the internal standards. In this research, alternative sample clean-up process was investigated. Liquid-Liquid extraction (LLE) involved initial extraction with hexane to remove the majority of the interferences, i.e. non-polar compounds, fats and proteins, and methanol to extract NSAIDs (section 2.5.2). This hypothesis was assumed as that the NSAIDs are freely soluble in methanol but this is not the case in hexane. Furthermore, methanol is commonly used in the extraction of organic compounds from keratinous matrices, such as hair (Gratacos-Cubarsi, et al., 2006). After the employment of LLE the chromatography was cleaner and interference peaks were reduced (Figure 5.6).

There are both advantages and disadvantages to the LLE process; the advantages include it is lower in costs in comparison to SPE, has shorter method development time and is easy to perform. The disadvantages are the use of large volumes of solvents, difficulty in automation, and expensive. The advantages outweigh the disadvantages and this is why LLE was selected as the sample clean-up method in this research.

The LLE method employed in this research is described in section 2.5.2, where samples were extracted with hexane and methanol in triplicate. The methanol layer was dried and reconstituted in acetonitrile (100µl), the optimal solvent investigated during method development (section 3.2.2).

5.2 ANALYSIS OF SPIKED SAMPLES

Blank feathers and feather samples spiked with mixed NSAID and metabolite standard (0.1µg/ml) in the presence of internal standards at 1µg/ml (section 2.5.3) were

analysed using the validated method (Chapter Four, section 2.4.1). This section examines any possible matrix effects post extraction and therefore doesn't compromise the selectivity of the validated method (Huber, 2007) and calculates the recovery of extraction method. While working with complex matrices and LC-MS, it is important to consider matrix effects. These effects alter the ionization efficiency when molecules in the matrix co-elutes with the analytes of interest (Taylor, 2005). To remove such effects sample extraction method LLE (section 5.1.2) was used.

5.2.1 Selectivity

Upon the application of the validated SIM method (section 3.2.5), it was paramount to first analyse a blank feather sample (without spiking, Figure 5.6) to document any possible interferences from the matrix, which may have remained after the sample clean-up process. Furthermore, a solvent blank was analysed in between each sample (Figure 5.7). The results showed that, there were no interference peaks which overlapped the retention times of interest, and furthermore, no ions of interest in SIM were detected relating to the blanks.

Selectivity was also investigated by analysing spiked feather samples. As such, these samples were initially spiked with 0.1µg/ml mixed NSAID and metabolites standard. The selection of this concentration was based on the findings from the literature, as described in section 4.4, whereby Taggart, et al. (2009). The samples were spiked at concentrations within the working linear range of the NSAIDs under investigation (Table 4.2) and above the corresponding LOD and LOQ (Table 4.5). Peaks other than those of compounds of interest were eluted within the sample run (Figure 5.6) however, they did not interfere and could have been typical of proteins and other organic compounds associated, and to be expected, with keratinous matrices (as first introduced in section 5.1.2).

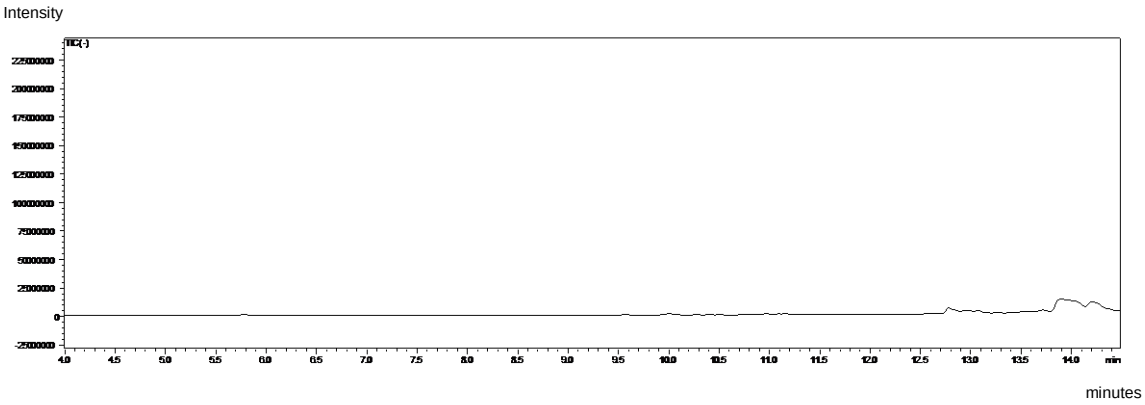


Figure 5.6 Chromatogram of a blank feather sample used as a negative control

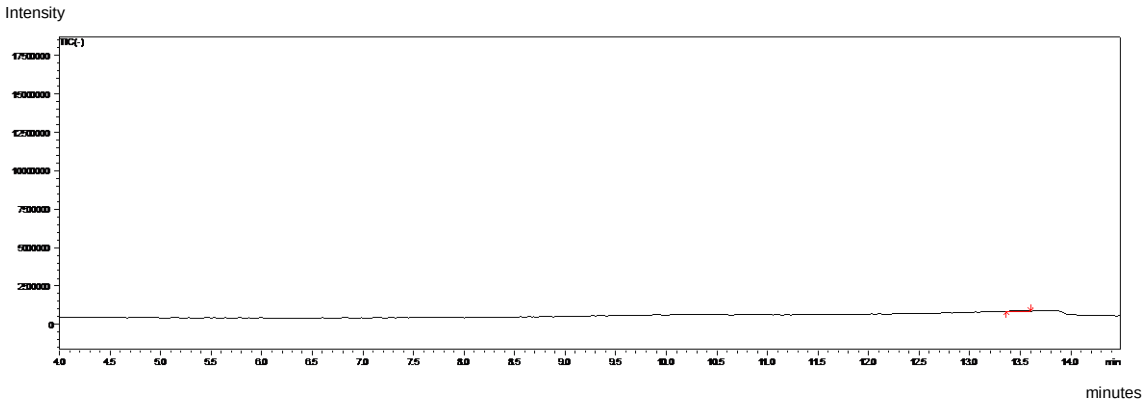


Figure 5.7 Typical chromatogram of a mid-run blank

On the analysis of the spiked feather samples (0.1µg/ml), all compounds, with the exception of 5-carboxymeloxicam and 5-hydroxyflunixin, were detected (fully resolved, Figure 5.8 and 5.9) and retention times recorded (Table 5.2). Throughout analysis, deviation in retention times was low ranging from ± 0.02 to 0.11. RRF were calculated and varied ±0.02 from those recorded in Table 3.2 and 3.3. Low variation in retention times were recorded between compounds detected in spiked samples compared to retention times of standards (Table 3.2 and 3.3). Variation in retention time between standards and spiked samples ranged from 0.00 to 0.22 minutes.

In compounds 5-carboxymeloxicam and 5-hydroxyflunixin, the analytical response was affected by a more intense peak eluting at the end of the analysis time (12.5 minutes, Figure 4.6). This larger peak in turn reduced the sensitivity and detection limit. Despite this, in method validation results (Chapter Four) these compounds were not only detectable at the spiked concentration (0.1µg/ml) (section 4.4), the PAR was greater (Table 4.1) and above the calculated LODs and LOQs (Table 4.5). This suggests, these compounds experienced ion suppression most likely related to other organic compounds in the feather under analysis.

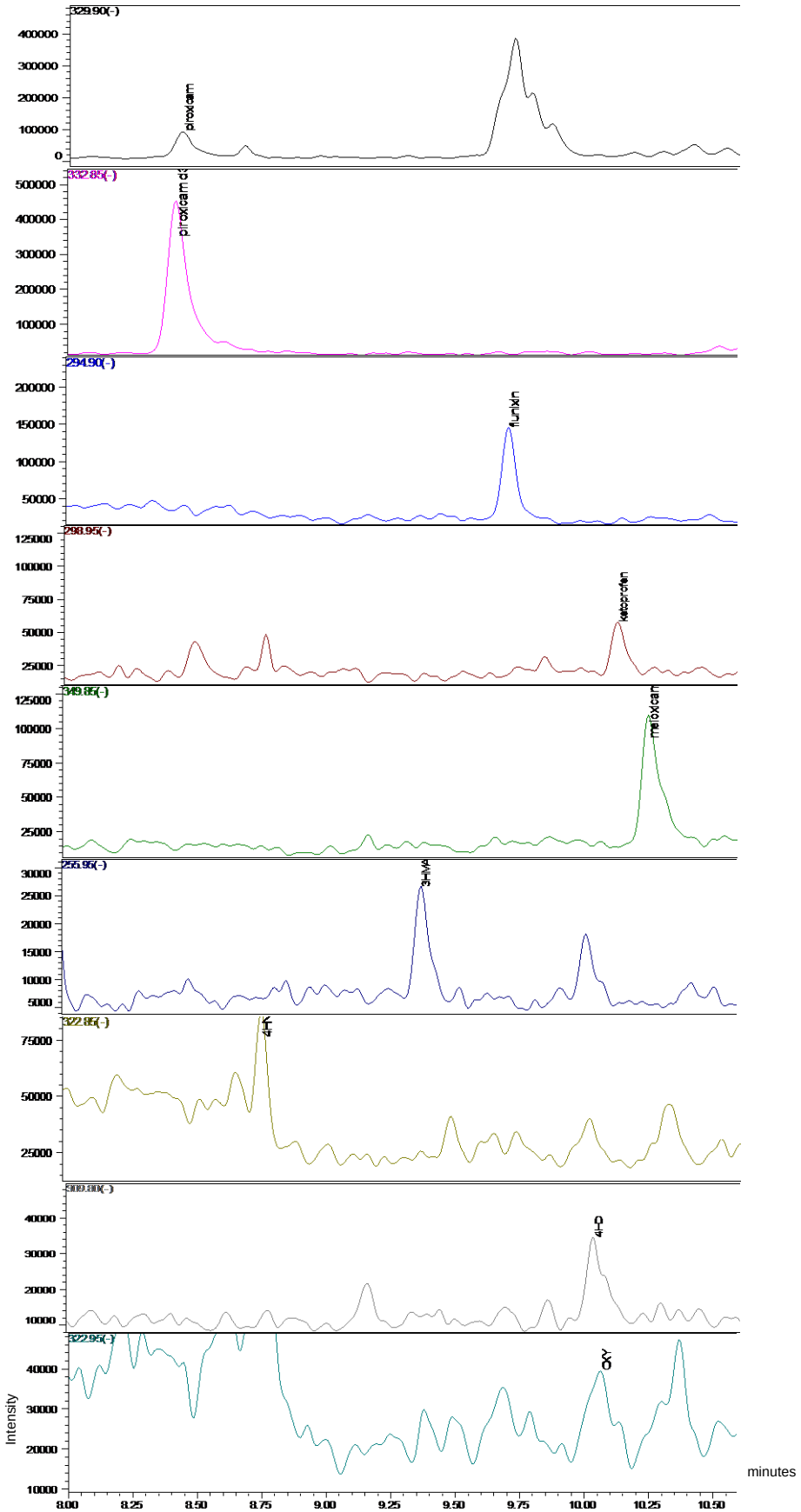


Figure 5.8 SIM chromatogram of mixed NSAIDs in a spiked (0.1µg/ml) feather sample (8.00 to 10.30 minutes)

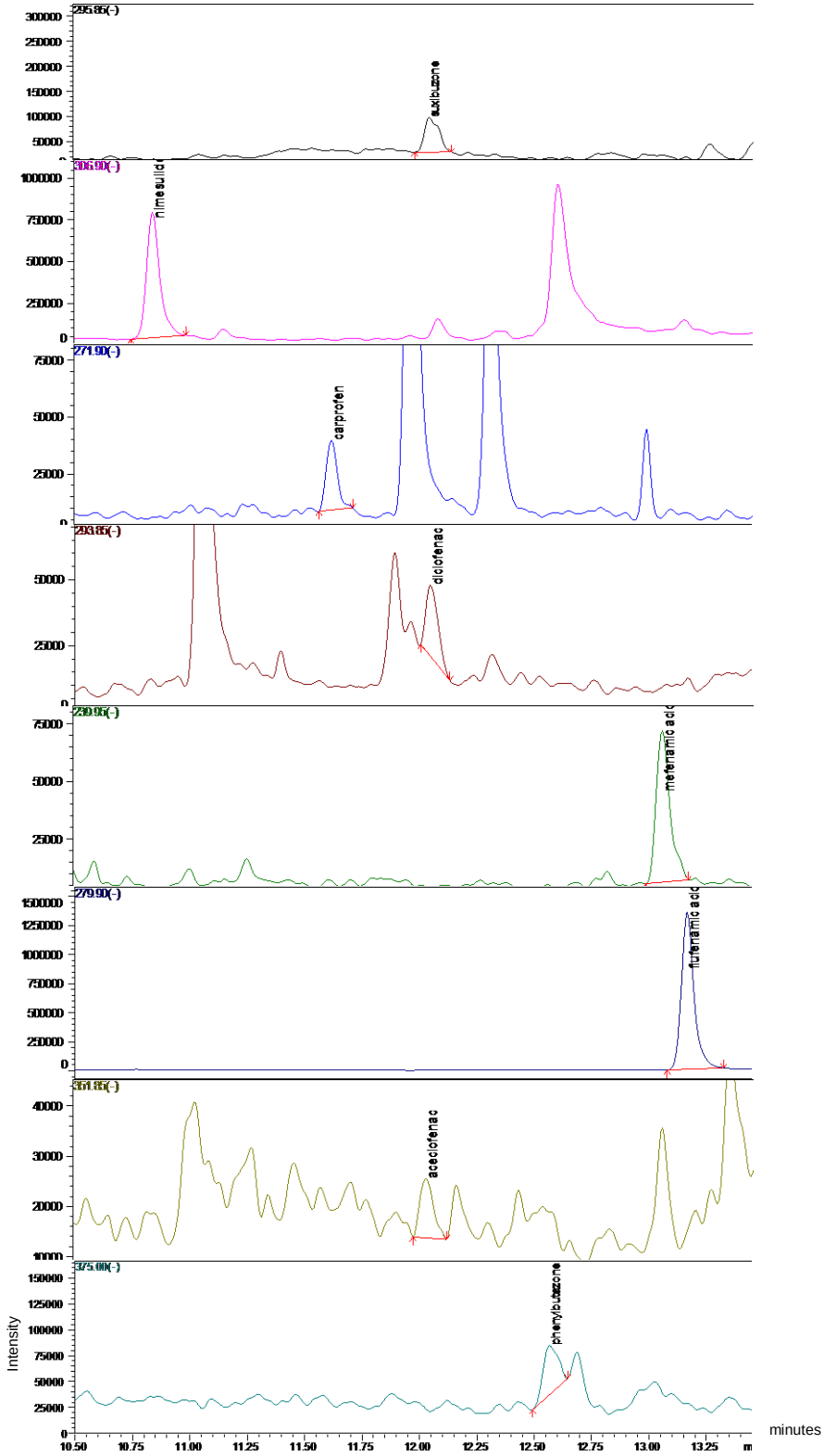


Figure 5.9 SIM chromatogram of mixed NSAIDs in a spiked (0.1µg/ml) feather sample (10.50 to 13.25 minutes)

Table 5.2 Retention times and RRF of NSAIDs and metabolites in spiked feather samples at 0.1µg/ml

NSAID	RT[a] (minutes)	RRF
piroxicam	8.31 ± 0.23	0.99
piroxicam-d_3 (internal standard)	8.28 ± 0.04	1.00
5-carboxymeloxicam	Not detected	
5-hydroxypiroxicam	8.55 ± 0.03	1.02
4-hydroxynimesulide	8.75 ± 0.03	1.05
3-hydroxymethyl mefenamic acid	9.42 ± 0.03	1.13
5-hydroxyflunixin	Not detected	
flunixin	9.60 ± 0.03	1.15
4-hydroxydiclofenac	10.00 ± 0.03	1.19
ketoprofen	10.08 ± 0.03	1.20
meloxicam	10.27 ± 0.03	1.23
oxyphenylbutazone	10.05 ± 0.02	1.20
nimesulide	10.82 ± 0.03	1.29
carprofen	11.60 ± 0.02	1.39
diclofenac	12.02 ± 0.02	1.44
suxibuzone	12.03 ± 0.02	1.44
aceclofenac	12.09 ± 0.07	1.44
phenylbutazone	12.60 ± 0.13	0.96
mefenamic acid	13.04 ± 0.02	0.99
flufenamic acid (internal standard)	13.08 ± 0.02	1.00

[a]RT ± SD (n=3)

Throughout the method development, three diagnostic ions (one quantifying ion, two confirmation ions) were selected for each of the analytes of interest, with exception of the metabolites, 5-hydroxypiroxicam, 4-hydroxynimesulide, 5-hydroxyflunixin and oxyphenylbutazone, as discussed in section 3.2.4 (Table 3.6). The use of three diagnostic ions is paramount in the identification and confirmation of target analytes in samples (section 5.3). In this analysis, alongside identification, the use of SIM negated any issues with co-elution between compounds as discussed in section 3.2.5, in particular, parent compounds aceclofenac, diclofenac, piroxicam, piroxicam-d_3, suxibuzone and metabolites; 4-hydroxydiclofenac and oxyphenylbutazone

As previously discussed, interferences from complex matrices can have effects on the detection of compounds that are present at trace levels. As such, these interferences can affect the detection of the analytes. Whilst SIM, together with LLE can help such issues, it is important to remember that complete removal of background peaks is not possible (LGGC, 2010). This was particularly the case, as highlighted previously, in

compounds 5-carboxymeloxicam and 5-hydroxyflunixin, whereby the analytical response was affected by a more intense peak related to the matrix (Figure 4.6). As a result, it is expected that complex matrices may share particular m/z with analytes of interest and hence cause problems when identifying compounds. Through the implementation of three diagnostic ions, this research meets the requirements set out by various agencies, such as the American Society for Mass Spectrometry (ASMS), Commission Decision (2002), United Nations Office on Drugs and Crime (UNODC) (2009) and WADA (2003), whilst allowing for the confirmation of compounds of interest in spiked and unspiked samples. For all compounds investigated, diagnostic and quantification ions were always distinguishable from interferences as were the ion ratios of the internal standards.

From the results obtained during the analysis of spiked samples, the validated method was proven capable of detecting all NSAIDs of interest in the complex feather and furthermore, their identification thereafter is made with both reliability and confidence.

5.2.2 Recovery

After the preparation of the sample, including the extraction of the analytes of interest from the matrix, the percentage recovery was determined as a measure of the effectiveness of sample preparation (accuracy). In doing so the percentage recovery can be used to correct the final results accordingly for the compounds of interest that may or may not be detected in the unspiked samples (section 5.3). To calculate the percentage recovery, a comparison is made of mean PAR of the extracted samples with those recorded from standards prepared in the same way (Equation 5.1) (UNODC, 2009).

$$\% \text{ Recovery} = \left(\frac{mean\ PAR\ of\ extracted\ samples}{mean\ PAR\ of\ extracted\ standards}\right) \times 100 \qquad \text{(Equation 5.1)}$$

The RSD was calculated alongside % recovery in both the extracted spiked samples and standards as a means of testing the precision of the extraction method.

The percentage recoveries were recorded for each compound and are presented in Table 5.3. Similar to selectivity studies (section 5.2.1) 5-carboxymeloxicam and 5-hydroxyflunixin were not detected as the analytical signal was low and could not be distinguished from a larger peak eluting at the end of the analytical run (section 5.2.1, Figure 5.6). Over the compounds investigated % recoveries were high ranging between 62.3% for 3-hydroxymethyl mefenamic acid to 99.6% for flunixin. Low recoveries were

recorded in 4-hydroxynimesulide, oxyphenylbutazone and 5-hydroxypiroxicam at 7.2%, 11.4% and 14.2% respectively. These low recoveries can be attributed to signal suppression due to matrix effects or the extraction process itself, as highlighted in section 5.2.1. When comparisons are made between PAR during method validation (section 4.3) and those in Table 5.3, the loss in signal is apparent as analytical response is reduced, supporting this hypothesis. Furthermore, whilst this research has resulted in a low percentage recovery for oxyphenylbutazone at 11.4%, Grippa, et al. (2000), proposed that degradation of the compound can cause low % recovery. The authors hypothesised that oxyphenylbutazone, and its parent compound phenylbutazone, start to degrade at acidic pH. However, both compounds had not shown degradation during stability studies previously (sections 3.3.2 and 4.2.2). Thus, the low % recoveries are most likely related to matrix effects, particularly signal suppression and areas of large noise affecting detection limits.

Additionally, in this study % recoveries were higher than previously reported for ketoprofen, diclofenac and piroxicam. In recovery studies in wastewater analysis ketoprofen had a reported recovery of 61%, compared to 70.6% reported in this method, and 78% compared to 88.9% for diclofenac in the same study (Gros, Petrovic and Barcelo, 2006). These compounds were extracted from wastewaters using SPE and LC-MS/MS. Furthermore, in aceclofenac studies, a % recovery of 85% was reported by Kim, et al. (2012) in rat plasma using LC-MS/MS. This is only 5% difference to the 80.5% reported by this research. This method has therefore shown that improved and comparable accuracies can be achieved with the cheaper alternative LLE in combination with LC-MS, supporting the aims of this experiment (section 1.7).

Table 5.3 A table of % recoveries, including corresponding peak area and %RSD for extracted spiked samples and extracted standards.

NSAID	Extracted Spiked Sample		Extracted Standard		Recovery %
	Mean PAR (n=3)	RSD %	mean PAR (n=3)	RSD %	
aceclofenac	0.007	17.3	0.008	17.6	80.5
carprofen	0.062	10.2	0.065	9.3	95.1
diclofenac	0.106	13.0	0.119	13.2	88.9
flunixin	0.282	7.7	0.284	7.0	99.6
ketoprofen	0.101	6.4	0.143	18.3	70.6
mefenamic acid	0.031	12.2	0.034	15.1	88.2
meloxicam	0.500	14.9	0.712	11.9	70.3
nimesulide	0.689	11.0	0.798	13.3	86.4
oxyphenylbutazone	0.058	3.7	0.516	20.0	11.4
phenylbutazone	0.037	7.7	0.043	18.5	87.1
piroxicam	0.268	13.2	0.290	7.7	92.5
suxibuzone	0.135	10.9	0.154	13.8	87.8
3-hydroxymethyl mefenamic acid	0.045	11.7	0.071	17.8	62.3
4-hydroxydiclofenac	0.076	11.2	0.105	16.2	72.0
4-hydroxynimesulide	0.132	12.4	1.844	14.6	7.2
5-carboxymeloxicam	Not detected		0.007	13.9	
5-hydroxyflunixin	Not detected		0.046	15.4	
5-hydroxypiroxicam	0.101	17.0	0.717	19.0	14.2

As a measure of precision, RSD were recorded for extracted spiked samples. All compounds RSD were recorded within ≤20% acceptance criteria (FDA, 2001) ranging from 7.0% for flunixin to 20% for oxyphenylbutazone (Table 5.3). Overall, this would indicate that the extraction method precision varies between compounds and offers low to high precision.

In the extracted standards, there was a combination of low to high precision, with RSD ranging from 3.7% for oxyphenylbutazone to 17% for 5-hydroxypiroxicam. These higher RSD values can be attributed to errors in sample preparation and during the extraction process, such as filtration. Where 5-carboxymeloxicam and 5-hydroxyflunixin were detected in extracted standards, this suggests these compounds are more than likely to have experienced ion suppression from matrix effects, hence not being detected in the spiked feather samples. Comparison between RSD of extracted samples and extracted spiked samples were on average comparable, differing by

±2.6%, with the exception of ketoprofen, oxyphenylbutazone and phenylbutazone with larger deviations of 13%.

The FDA (2001) states that the recovery of an analyte need not be 100%, but should be consistent, reproducible and precise (the latter of which, RSD were recorded within ≤20% for all compounds). This method ensured the best representation of percentage recoveries by using comparisons between extracted spiked samples and standards prepared under the same conditions, essentially, ensuring both samples would be exposed to the same solvent and sample preparation and thus, allow for more realistic results and percentage recoveries. This avoided unrealistic comparisons to reference standards which effectively represent 100% recovery and thus result in unusually high recoveries. The recovery values and supporting RSDs achieved in this study show the suitability of the method in hand and its reproducibility.

5.3 ANALYSIS OF UNSPIKED SAMPLES

A number (n=17) of unspiked feather and animal fur samples were analysed (in triplicate) using the validated method, these included wild bird feathers (from Red Kite (*Milvus milvus*), Buzzard (*Buteo buteo*) and Raven (*Corvus*)) (n=13), donated parrot feathers (n=2) and cat hair (n=2). These samples were prepared as per the methods detailed in section 2.4.4, and were analysed in the presence of both internal standards, piroxicam-d_3 and flufenamic acid, at a concentration of 1µg/ml. This section considers the systematic approach undertaken to identify target analytes in unspiked samples. For each suspected target analyte in an unspiked sample, the mass spectrum was compared to that of the spiked sample to allow for an authentic comparison. In particular it presents the analysis of two real samples, in which piroxicam and phenylbutazone have been detected in feather samples.

Guidelines, as set out by the Commission Decision (2002) and UNODC (2009), were followed in the identification process in the analysis of unspiked samples. Data collected in the form of mass spectra were analysed in conjunction with retention time (RT) and relative retention factor (RRF) for identification purposes (Table 5.4).

5.3.1 Feather analysis and detection of NSAIDs

On the analysis of unspiked feather and animal fur samples (n=17) (Table 5.4), two peaks, corresponding to both internal standards, were positively identified. At retention times of 8.28 ± 0.04 minutes for piroxicam-d_3 and 13.08 ± 0.02 minutes for flufenamic acid (Figure 5.10), all three diagnostic ions characteristic to each internal standard

(piroxicam-d_3 m/z 333, 341, 250 and flufenamic acid m/z 280, 236, 326) were present and in the same ratio. Figure 5.10 shows the analysis carried out in SIM, implementing diagnostic ions to establish correct identification of piroxicam-d_3 and flufenamic acid alongside corresponding mass spectra.

Peaks other than those of interest, were detected at the end of the analytical run (Figure 5.6). These were disregarded, through the interpretation of mass spectra, namely diagnostic ions, as no identification matches were made to compounds of interest. Due to the complex nature of keratinous matrices (section 1.4), these peaks are most likely to relate to components within the matrix (as discussed in section 5.2) (Figure 5.6).

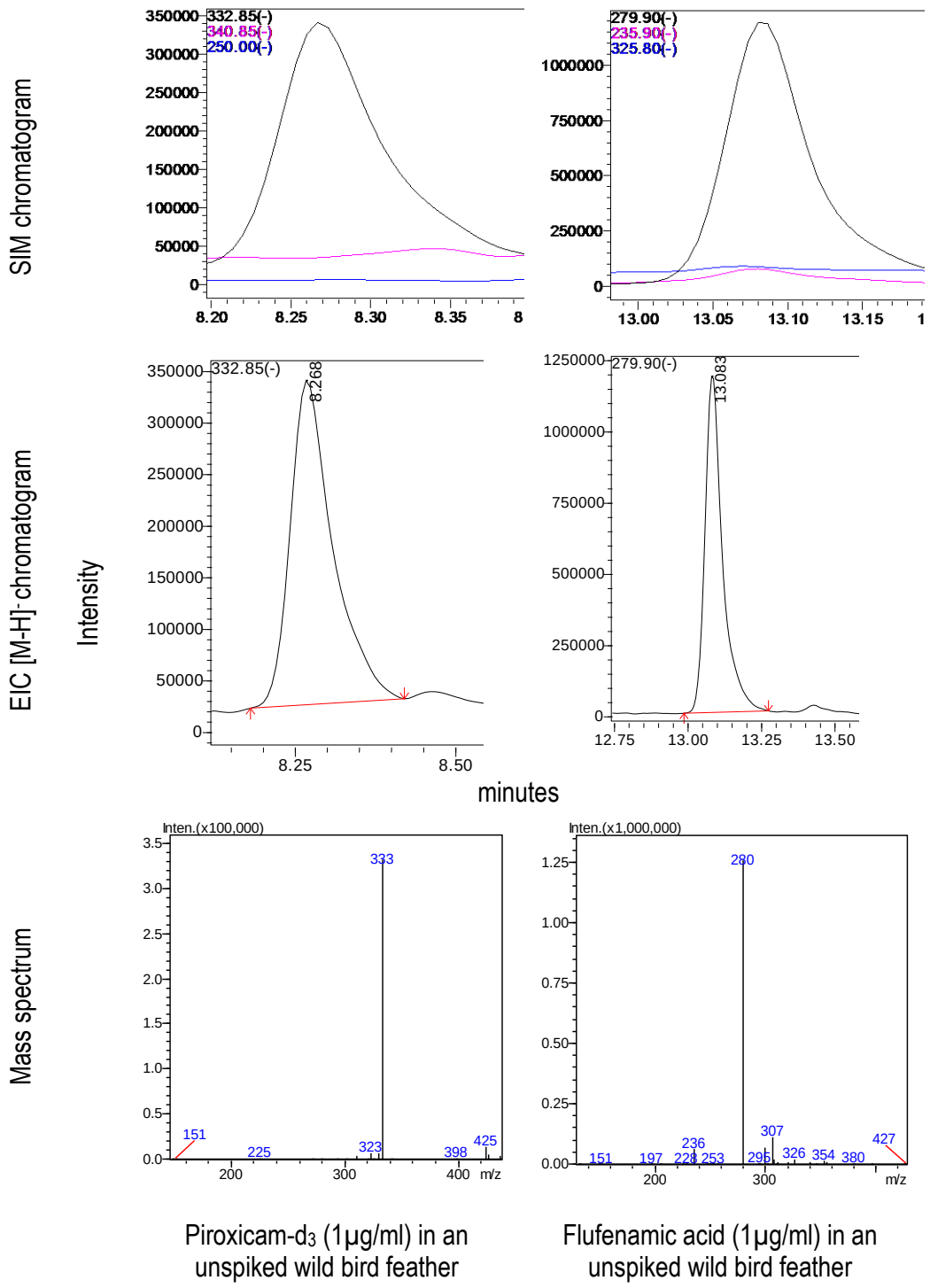


Figure 5.10 Detected peaks of internal standards piroxicam-d_3 (8.28 ± 0.04 minutes) and flufenamic acid (13.08 ± 0.02 minutes) in an unspiked feather sample at concentrations of 1µg/ml

Upon the analysis of the various samples (Table 5.4), retention times and mass spectra were compared to the positive controls and spiked samples. From two samples, Red Kite feather (S1, Table 5.4) and parrot feathers (S14, Table 5.4) analysed, two additional peaks at retention times consistent with the NSAIDs piroxicam (7.59 minutes) and phenylbutazone (12.87 minutes) were detected using the validated SIM method (section 3.2.4, 3.2.5 and Chapter Four), in two individual samples (Figures 5.11 and 5.12). Thereafter, corresponding retention times were compared to standards and verified not to be present in the blank. No interference peaks were consistent with the retention times for the identified target analytes consequently, supporting their identification. Figures 5.11 and 5.12 show SIM and extracted Ion chromatogram (EIC) chromatograms of piroxicam and phenylbutazone as detected in a spiked feather sample (0.1µg/ml). Alongside standards (positive controls) at 0.1µg/ml compared to that of the in unspiked feather samples for qualitative identification. EIC chromatograms show only one peak relating to the quantification ion, this allows for the accurate quantification of each compound.

Table 5.4 Keratinous matrices analysed (n=3) during the analysis of unspiked samples

Sample number	Animal	Sample Type	Traceability code	**Compound(s) detected**
S1	Red Kite	Moulted primaries and downy feathers	(nest code 02.029)	Piroxicam, internal standards- piroxicam-d_3 and flufenamic acid
S2	Red Kite	Moulted primaries and downy feathers	(nest code 02.083)	internal standards- piroxicam-d_3 and flufenamic acid
S3	Red Kite	Moulted primaries and downy feathers	(nest code 01.026)	
S4	Red Kite	Moulted primaries and downy feathers	(nest code 07.004)	
S5	Red Kite	Moulted primaries and downy feathers	(nest code 05.076)	
S6	Red Kite	Moulted primaries and downy feathers	(nest code 02.064)	
S7	Red Kite	Moulted primaries and downy feathers	(nest code 02.062)	
S8	Red Kite	Moulted primaries and downy feathers	(nest code 02.041)	
S9	Red Kite	Moulted primaries and downy feathers	(nest code 02.037)	
S10	Red Kite	Moulted primaries and downy feathers	(nest code 02.003)	
S11	Red Kite	Moulted primaries and downy feathers	(nest code 05.22)	
S12	Red Kite	Moulted primaries and downy feathers	(nest code 03.92)	
S13	Red Kite	Moulted primaries and downy feathers	(nest code 05.032)	
S14	Crimson Rosella Parrot	moulted primaries, downy and filoplume feathers	026	Phenylbutazone and internal standards- piroxicam-d_3 and flufenamic acid
S15	Crimson Rosella Parrot	moulted primaries, downy and filoplume feathers	028	internal standards- piroxicam-d_3 and flufenamic acid
S16	Cat	Domesticated cat hair, collected during brushing	024	internal standards- piroxicam-d_3 and flufenamic acid
S17	Cat	Domesticated cat hair, collected during brushing	025	internal standards- piroxicam-d_3 and flufenamic acid

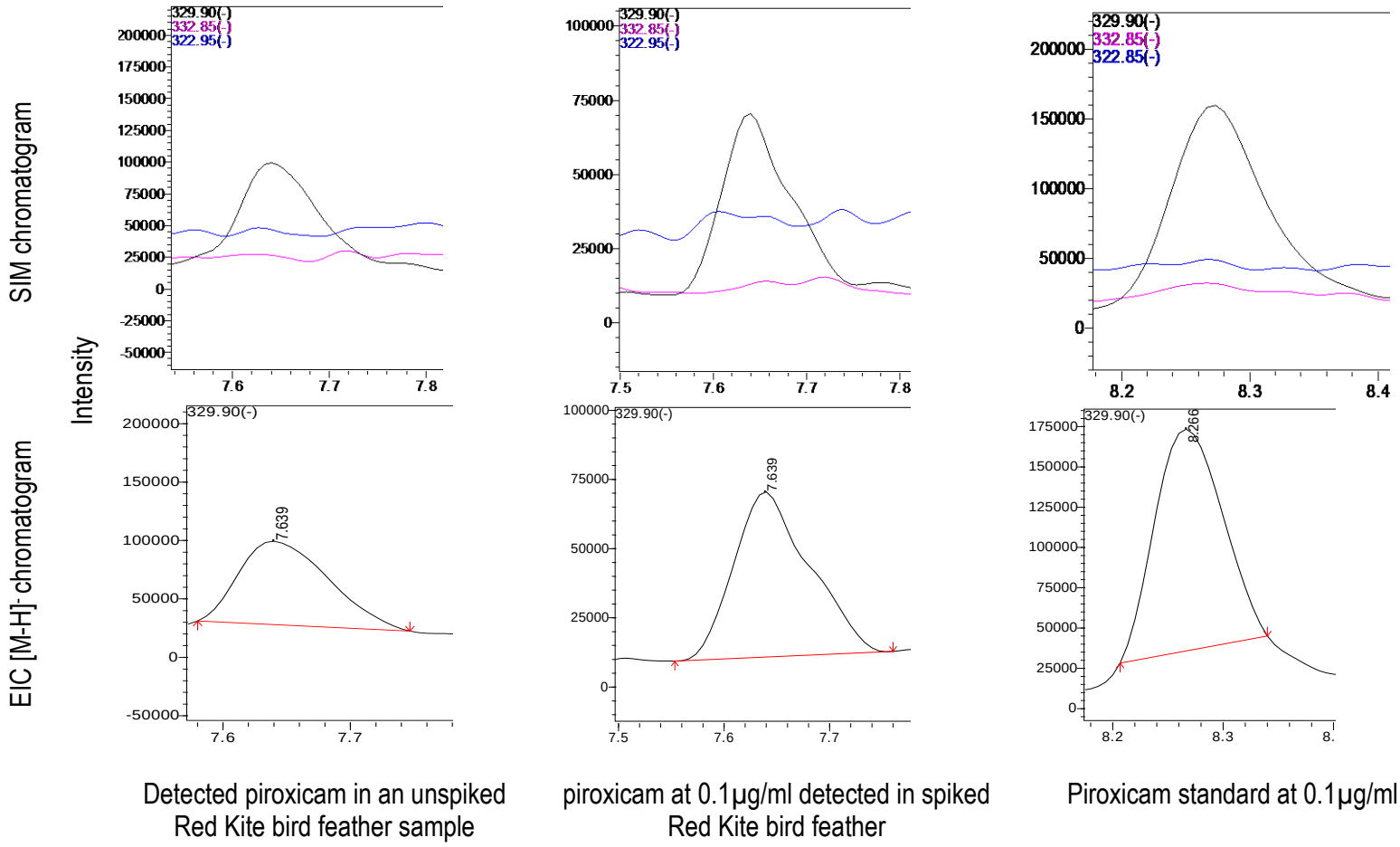


Figure 5.11 SIM and EIC chromatograms of piroxicam in a Red Kite feather, spiked feather sample and 0.1µg/ml standard

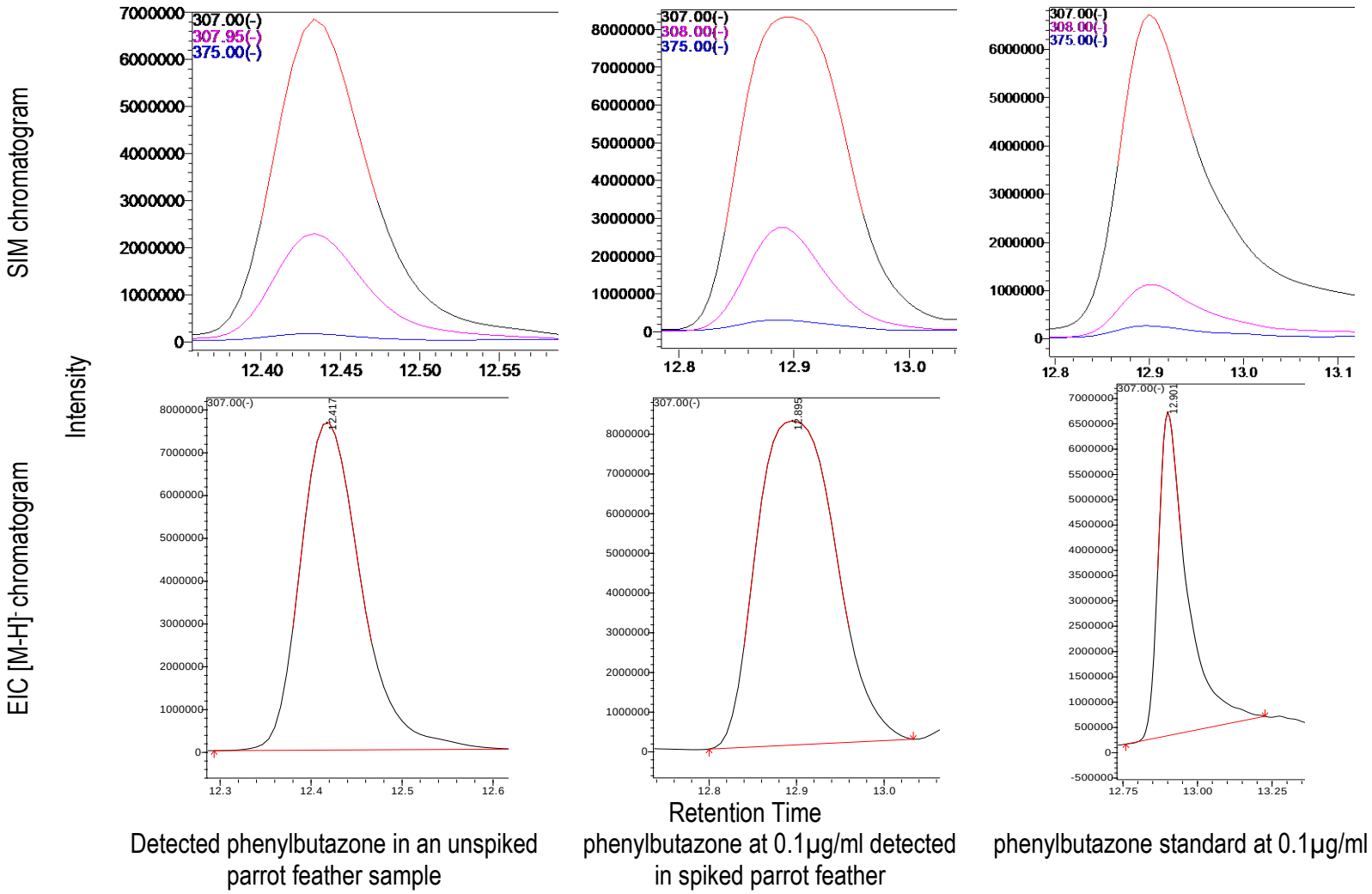


Figure 5.12 SIM and EIC chromatograms of phenylbutazone in a parrot feather, spiked feather sample and 0.1µg/ml standard

Table 5.5 details the detection of piroxicam and phenylbutazone in terms of comparing retention times and ions present and ion intensities correlating to that of their presence in spiked samples and standards. Figure 5.13 is a comparison of mass spectra to spiked samples and standards. The differences in retention times (Table 5.5) ranged from 0.04 minutes in detected piroxicam and 0.44 minutes in phenylbutazone. This meant the retention times, when compared with those recorded from spiked samples and standards, were proven to fall within the 0.5 and 7.6% for piroxicam and 3.3 and 3.6% in phenylbutazone respectively. Difference in retention time for piroxicam was within ±2% acceptance criteria set by the UNODC (2009) when comparisons were made between the unspiked and spiked feather samples and thus support its positive identification. Despite the retention time of detected phenylbutazone being over the ±2% acceptance criteria, calculated RRF were within the tolerance value of 2.5% for LC (Commission Decision, 2002). Differences in RRF (Table 5.5) were recorded at 1.03% for phenylbutazone. It was therefore possible to make a positive identification based on retention time for piroxicam and RRF for phenylbutazone.

The UNODC (2009) state that the mass spectrum should have a good visual match hence, thereafter, comparisons were made to the mass spectra of the detected NSAIDs in the spiked samples and the presence of diagnostic ions (Figure 5.13). Three diagnostic ions were used in the identification of each compound as per section 3.2.4 (m/z 330, 323 and 332 in piroxicam and m/z 307, 308 and 375 for phenylbutazone) (Table 3.6). All diagnostic ions were present and within the same ratio and abundancies further confirming the positive match (Table 5.6).

Table 5.5 and Figure 5.13 show the ratio of diagnostic ions for each detected NSAID. The abundancies of these ions were recorded and are listed in Table 5.5. Ion abundancies differed by ±3.4 to 3.6% in piroxicam ±0.8 to 2.3% in phenylbutazone (between ions in unspiked and spiked feather samples and unspiked feather samples and standards respectively). For the purpose of identification, these relative abundancies are within the ±20% allowable error as set out by the Commission Decision (2002) and UNODC (2009).

Table 5.5 Comparison table of NSAIDs detected in standards, unspiked and spiked feather samples including relative ion abundancy

Sample	NSAID detected	Detection in Unspiked samples			RRF	Detection in Spiked samples			RRF	Detection in Standards			RRF
		RT (min)[a]	Ions present (m/z)[b]	Ion abundancy %		RT (min)[a]	Ions present (m/z)[b]	Ion abundancy %		RT (min)[a]	Ions present (m/z)[b]	Ion abundancy %	
S1- Red Kite feather	piroxicam	7.63	Q 330	100	0.92	7.59	Q 330	100	0.99	8.26	Q 330	100	0.99
			C1 323	10			C1 323	14			C1 323	7	
			C2 332	4			C2 332	4			C2 332	3	
S14- parrot Feather	phenylbutazone	12.43	Q 307	100	0.97	12.87	Q 307	100	0.96	12.90	Q 307	100	0.96
			C1 308	29			C1 308	28			C1 308	25	
			C2 375	2			C2 375	2			C2 375	3	

[a]minute (n=3), [b]mass to charge, Q quantification ion, C confirmatory ion

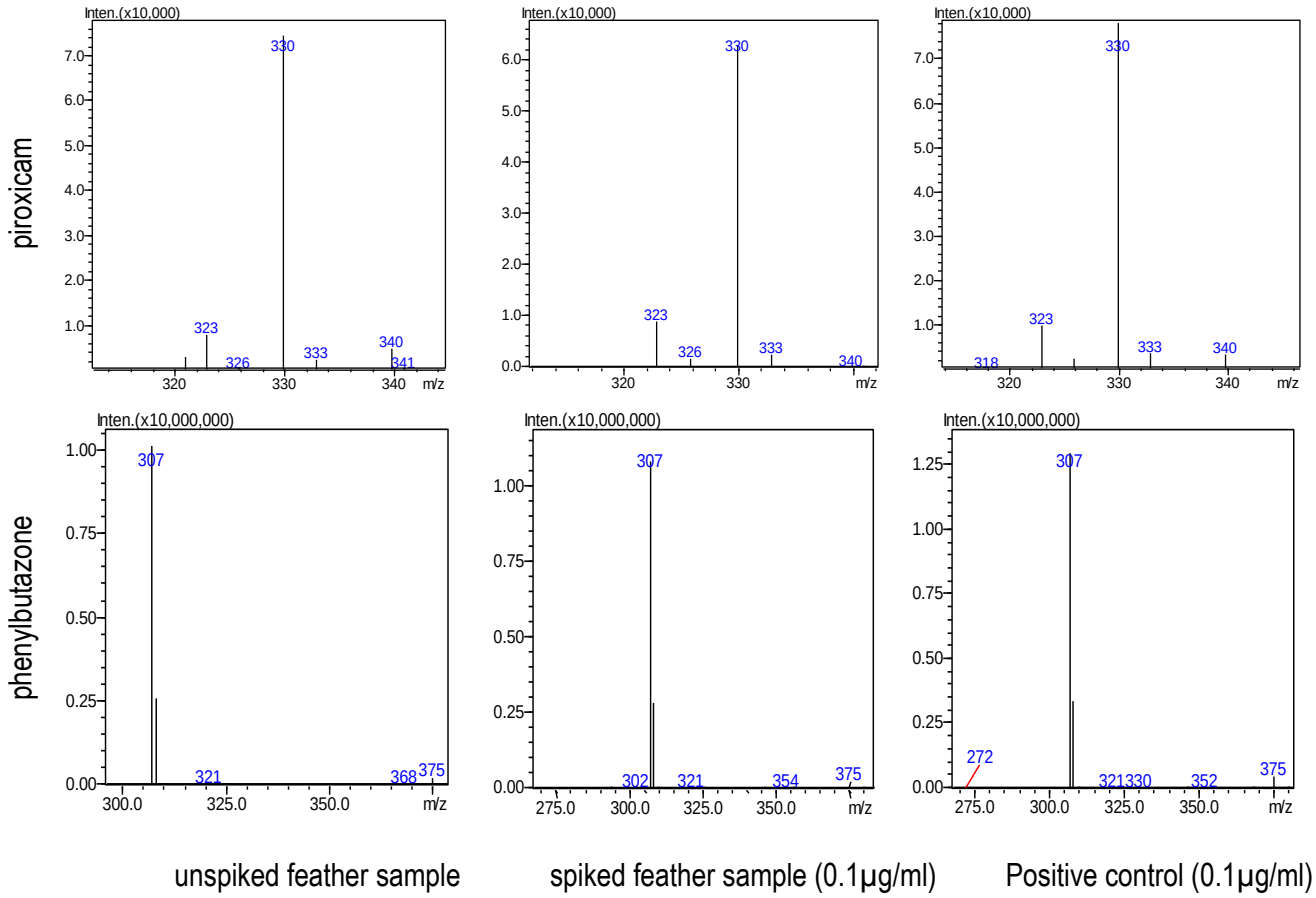


Figure 5.13 Mass spectra comparison of piroxicam in a Red Kite feather and phenylbutazone in a parrot feather, spiked feather sample and 0.1µg/ml standard

Ion ratios were calculated between the quantification ion and each confirmatory ion thereafter. Table 5.6 presents this data which includes the peak areas and ion ratios (Q/C1 and Q/C2) as calculated for piroxicam and phenylbutazone alongside their corresponding diagnostic ions.

The Commission Decision (2002) state that one ion ratio, within ±20%, can be used as an identification point. Typically calculated between Q/C1, as this research implemented three diagnostic ions, two ion ratios were calculated in the identification of piroxicam and phenylbutazone (Q/C1 and Q/C2). From the data shown (Table 5.6) at least one ion ratio of both compounds is within the accepted threshold thus, meeting the minimum requirements for a further identification point. Cooper (2010) states that for assays in LC-MS, ratios may be more dependent on concentration and time than GC-MS. Therefore, proposes a more appropriate range to use with LC-MS would be acceptable up to ±30%. In spite of this upper threshold this method has shown to be comparable to GC-MS lower acceptance criteria.

Table 5.6 Ion ratios and % differences of piroxicam and phenylbutazone from feather sample. Data shown includes diagnostic ions and corresponding peak areas

Ion	Peak Area			Ion ratios						% difference between ion ratios (unspiked and spiked sample)		% difference between ion ratios (unspiked and standard)	
	Unspiked feather sample	Spiked Feather sample	Standard (positive control)	Unspiked feather sample		Spiked Feather sample		Standard (positive control)					
				Q/C1	Q/C2	Q/C1	Q/C2	Q/C1	Q/C2	Q/C1	Q/C2	Q/C1	Q/C2
piroxicam													
Q-330	63078	74595	130245	7.15	25.15	9.33	29.07	13.20	35.22	30.6	15.6	23.3	17.5
C1-323	8827	7992	9865										
C2-332	2508	2566	3698										
Phenybutazone													
Q-307	10278814	15495746	12908935	3.61	51.04	3.88	52.93	3.91	29.25	7.51	3.70	1.9	82.8
C1-308	2651521	4297337	3301523.7										
C2-375	194196	303580	441270										

Q = quantification ion, C = confirmatory ion

This research implemented four identification points per analyte detected, as recognised by the Commission Decision (2002) (section 3.2.5). There has been little research published on the detection of NSAIDs in keratinous matrices, especially their detection using LC-MS. Therefore, following the criteria as set out by the Commission Decision (2002), has ensured any positive identification exceeds those in currently available guidelines.

Therefore, at this stage all identification points have been fulfilled and within the accepted limits as set out by guidelines in Commission Decision (2002), Cooper (2010) and the UNODC (2009). Comparisons were made to piroxicam and phenylbutazone as detected in a spiked feather sample, to account for the matrix effect, and to positive control standards, where highlighted these also met acceptance limits; thus, confirming the presence of the two aforementioned NSAIDs. Additionally, as there were no interferences at the retention times for piroxicam and phenylbutazone this indicated the presence of such NSAIDs was not a result of contamination nor carryover, as not found in the blank, from the spiked samples. Following this confirmation, it was necessary to follow steps to quantify these compounds.

5.3.2 Quantification of detected NSAIDs in feather samples

Using linear regression, the resulting peak area ratios were used to calculate the concentrations of piroxicam and phenylbutazone, after their positive identification. The concentrations (µg/ml), once calculated, were converted into µg/g for comparisons. As a result both compounds were detected (n=3) at concentrations of 1.2 ± 0.002µg/g for piroxicam (S1, Red Kite feather) and 1.8 ± 0.011µg/g for phenylbutazone (S14, Crimson Rosella Parrot).

Both piroxicam and phenylbutazone were detected at concentrations above their LOD (0.048 and 0.121µg/g, respectively) and LOQs (0.162 and 0.373µg/g, respectively) (Table 4.5). The national association of testing authorities (NATA) defines these limits as the smallest concentration at which analytical response is readily distinguishable from the noise (NATA, 2013) and, the lowest point at which compounds can be positively identified according to predetermined criteria, threshold limits and levels of confidence. This was the case in the detection of these compounds in the unspiked feather samples. Both compounds were easily distinguishable from the noise and, at concentrations of 1.2µg/g and 1.8µg/g (piroxicam and phenylbutazone respectively), were within the linear range validated for this method used in this research (Table 4.2), which were determined as 0.01-2.5µg/ml and 0.05-5 µg/ml, respectively.

Bernal (2014) stated that this lowest concentration should be determined within acceptable precision and accuracy (Bernal, 2014). Thus, throughout method development and validation (section 3.4 and 4.3) the precision of the method was proven to be within acceptable limits. Therefore, these factors support the quantification of these compounds. Additionally, these compounds (piroxicam and phenylbutazone) have been positively identified through four separate identification points all within the thresholds.

5.3.3 Linking findings with the literature

The Red Kite feathers analysed in this research were collected from the nests of birds of prey and monitored feeding sites in association with the Welsh Kite Trust. Whilst the health background of the wild birds is unknown, as these samples were collected at feeding sites, where the meat given to the birds is supplied through local abattoirs. There is a real possibility that such meat may have been contaminated with compounds from veterinary prescriptions, such as NSAIDs (first highlighted in section 1.2.4). Furthermore, indications to the possible identity of the species sampled were provided. These included the Red Kite (*Milvus milvus*), Buzzard (*Buteo buteo*) and Raven (*Corvus*). In the case of the donated parrot feathers, these were collected from a crimson rosella parrot *Platycercus elegans*. With this background information and owing to the positive identification of piroxicam and phenylbutazone, it is possible to link with consumption, exposure route and threat they may pose.

Originally confined to Wales, the Red Kite, has been through a reintroduction scheme run by the Royal Society for the Protection of Birds (RSPB) in 1989. This programme has seen their populations increase and now spread across England and Wales. Despite increased sightings and populations, the IUCN still have these birds of prey registered as near threatened status, stating this species are currently experiencing moderately rapid population decline. It is suggested, that their population declines are now typically from poisoning caused by pesticides and persecution, such as illegal poisoning to kill predators of livestock e.g. foxes and wolves. Additionally, the Red Kite suffered intense human persecution until the mid-1950s where, rabbit myxomatosis devastated food supply. Since then, poor breeding success in the 1960s, caused by effects of organochlorine pesticides have resulted in further population decline.

A recent study by Orros and Fellowes (2014) suggested that the feeding habits of these red kite species are changing. Birds have been reported in the city of Reading feeding in residential gardens. The authors documented the red kites are not fussy

eaters, stating that they feed on leftovers, processed meat and small carcasses. This is not without concern as exposure to this meat may be contaminated with potentially toxic by-products, whether this be veterinary pharmaceuticals or pesticides that would effectively be uncontrolled. It is important to remember that veterinarians are, legally, allowed to make their own decisions when prescribing approved compounds not normally used in the species being treated (RCVS, 2015). However, it is more likely that the meat used as the feeding stations was contaminated with these pharmaceuticals. Naidoo, et al., (2010) stated that there are several NSAIDs whose safety to birds of prey, such as the protected vultures, has not been tested and therefore this highlights the ongoing concern of such practices. Often alternative cheaper NSAIDs are frequently used in the place of the more expensive prescribed compounds (Cuthbert, et al., 2011). This was and still is the case with the use of diclofenac after its ban in 2006 on the Indian subcontinent instead of the recommended meloxicam, the cost of diclofenac reported at £0.37 compared to £0.77 for a vial of meloxicam, as first discussed in section 1.2.3.

Unlike the detection of piroxicam in the wild bird feathers, detection of phenylbutazone in the parrot feather is more than likely to relate to birds direct treatment with the veterinary compound. Commonly a pharmaceutical used in equine medicine, phenylbutazone is a widely used NSAID that has been reported in avian medicine including Psittaciformes (parrots) and raptors (Ritchie, et al., 1994). In veterinary medicine, piroxicam and phenylbutazone are typically used to treat arthritic pain and occasionally, piroxicam is prescribed in cases of cancers in treatment for cats, dogs and horses (Bullman-Fleming, Turner and Rosenberg, 2010; Iwabe, Ramírez-López and Juárez-Sánchez, 2009; Knapp, et al., 1994). Although, currently, there is no published research on the treatment of birds of prey with piroxicam, like phenylbutazone, its use has been reported in avian species, such as parrots. This is supported by necropsy findings and biomedical analysis carried out by Awan, et al. (2012) whereby the authors found piroxicam to be safe in avian species at concentrations of 2µg/g. It was suggested piroxicam's good pharmaceutical effects in human medicine may lend itself useful in veterinary medicine. Research carried out by Dama (2014), proposed piroxicam is likely to be safe to endangered species, such as vultures, due to the similarity to the safer alternative meloxicam. Dama (2014) also reported the increased use of oxicam derivatives (Table 1.1) is more than likely to occur and urged that the proposed safety of piroxicam is first validated experimentally. Increased incidents of oxicam derivatives is supported by Cuthbert, et al*,* (2011), study which states piroxicam is available for sale in areas of protected species.

The toxicity of phenylbutazone in avian species has been tested in broiler chickens (Awan, et al, 2003). In line with a study by Embert (1986), Awan, et al. (2003) concluded that phenylbutazone is hepatotoxic in avian species at 50µg/g body weight after increased serum levels were recorded indicating cellular degeneration in the liver muscles. Despite this, no nephrotoxicity and no increase in uric acid levels were recorded to further suggest toxicity, as was previously found in line with mortality from diclofenac (Oaks, et al., 2004; Taggart, et al., 2009). Furthermore, the reported toxic dose is more than double the dose recommended in raptors (Ritchie, et al., 1994), and thus should be used with some degree of caution. However, there have been a number of authors reporting instances of mortality with renal disease and gout in protected species of vultures after treatment with phenylbutazone thus supporting such findings (Cuthbert, et al., 2007; Naidoo, et al., 2010; Pain, et al., 2008; Ragni, 2014). Particularly a study by Fourie (2014) into South African Cape vultures (*Gyps coprotheres*) found these birds were toxic to phenylbutazone at concentrations of 1.7µg/g, stating those levels are double that found in ungulate tissue.

As first introduced in Chapter One (section 1.3.7), if the use of piroxicam and, especially the toxic phenylbutazone, is to increase, it should be executed with some degree of caution as both NSAIDs are inhibitors of the COX-1 enzyme and are associated with adverse abdominal conditions like ulcers of the gut (Lees, et al. 2004). In response, the development and introduction of newer, more favourable COX-2 inhibiting NSAIDs, such as the NSAID meloxicam, have seen an increase in use. Selectively inhibiting COX-2, meloxicam reduces the risk of adverse effect on renal function whilst offering the analgesic, anti-inflammatory and antipyretic properties.

If piroxicam use is deemed to be safe, its use should be encouraged in areas of protected species or under continued reintroduction programs. Research into the indications suggesting phenylbutazone is toxic advocate that its continued use through conservation programmes should be discouraged. Animals treated with the latter should be controlled as to avoid ending up at approved feeding sites, this in turn would limit the possible exposure routes and would ultimately safeguard protected species from potentially devastating population declines.

The detection of these compounds highlights the exposure routes that birds of prey and other species are at risk to. More studies need to be carried out on the safety of common veterinary pharmaceuticals that are readily available in areas of protected species, including cheap and safe alternatives that can be continually implemented.

Whilst only piroxicam and phenylbutazone were detected in the bird feathers, this is not to say that other NSAIDs were not present in the matrix. Other compounds could well have been in trace amounts lower than the limit of detection of the method developed/validated in this research. As this method allows detection of eleven parent compounds and five metabolites, it could be employed as an initial screening method as a means of testing, quickly, efficiently and most importantly pre-mortality, species that show signs of poisoning or ill health prior to more expensive confirmatory methods.

5.4 OVERALL SUMMARY

Method development of an optimal sample preparation method involved the trial of various means of grinding feathers, from manual cutting to using a mechanical ball mill. As expected, mechanical means reduced processing time and resulted in better homogenisation of the sample. The use of a mechanical PM100 Planetary Ball Mill, together with the use of liquid nitrogen as proposed in this research to help dry freeze, shortened preparation time drastically to achieve optimal results in 30 minutes compared to the hours spent manual cutting. LLE was employed for extraction of analytes and an additional clean-up stage was introduced to avoid interferences.

Results, during selectivity studies (section 5.2.1) showed the diagnostic ions to be distinguishable from any interferences present. There were no changes in RT and RRF of parent NSAIDs and metabolites recorded. The results obtained in the selectivity investigation have proven the capability of the validated method, particularly the ability of detecting all NSAIDs, albeit 5-carboxymeloxicam and 5-hyrdoxyflunixin, of interest in the complex matrix.

Recovery studies showed majority of NSAIDs with recoveries between 62.3 to 99.6%, with the exception of 4-hydroxynimesulide, oxyphenylbutazone and 5-hydroxypiroxicam. Where low recoveries were recorded this was linked with errors during sample extraction method. Throughout the study, precision in the extraction method was always within ≤20% RSD acceptance criteria. Results were comparable and in some cases better than the literature showing the extraction methods suitability, repeatability and reliability.

In feather analysis, two compounds of interest (piroxicam and phenylbutazone) were detected in unspiked wild bird and parrot feathers. These were identified using RT and RRF, of which were proven to fall within acceptance criteria and tolerance values. The diagnostic ions were also important in the identification process of these NSAIDs. Good

visual match and ions within the same ratio and abundancies resulted in both compounds within the ±20% allowable error. After confirming the presence of piroxicam and phenylbutazone, both compounds were quantified. Piroxicam was detected at a concentration of 1.2µg/g and 1.8µg/g for phenylbutazone. These were within the linear range and above the LOD.

The LD_{50} (lethal for 50% of the population) data available for piroxicam and phenylbutazone in birds of prey species is limited with discrepancies (section 5.3.3). The concentration of phenylbutazone detected in this research, falls below the published toxic levels and in line with the recommended dosages as discussed in section 5.3.3. Meanwhile detected at 1.2µg/g, piroxicam is recommended to be safe in avian species due to its similarity to the safer alternative meloxicam and after toxicity studies revealed no hepatotoxicity or renal abnormalities at concentrations of 2.2µg/g.

The safety of piroxicam should be investigated scientifically like meloxicam, before it is use is encouraged. Research into the toxicity of phenylbutazone advocate that its use should be discouraged, if implemented in toxicity and mass mortality like diclofenac, a ban on the use of phenylbutazone in veterinary treatment should follow. Hence, more studies need to be carried out on the safety of common veterinary pharmaceuticals that are readily available in areas of protected species.

This, to the author's knowledge, is the first instance that this type of pharmaceuticals have been detected in feathers. The method ensures a low-cost simple alternative to currently used expensive post-mortem tissue samples when analysing the exposure to NSAIDs that may pose a real threat to populations of endangered species.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

This chapter concludes the findings from this research and provides recommendations for areas of further research. The chapter summarises the rationale behind the research, whilst providing an overall conclusion to the analytical findings, relating results to the research aims throughout, as discussed throughout Chapter Three (method development), Four (method validation) and Five (application of the method). At the close of the conclusion the opportunities for collaboration are discussed in terms of conservation and recommendations of the research.

6.1 SUMMARY OF THE STUDY RATIONALE

Endangered species and their exposure to compounds, such as NSAIDs, in the environment has become an increasing challenge in wildlife forensics. First documented as the cause of the 95% population decline of the *Gyps* vulture species, NSAIDs, namely diclofenac have a toxic effect on protected populations. Research, as introduced in section 1.1.1, showed diclofenac to be toxic to eagles and detection of ibuprofen on the external surface of otter hair. Thus, concerns regarding possible exposure to other protected species, such as the water vole and red kite, both of which have seen population declines in the UK over recent years, are of continued concern. Despite research increasingly looking into the effects and risks that such compounds pose in the environment, surprisingly, there are no analytical methods employing hair testing to allow for testing pre-mortality in wildlife forensic cases. Current methods rely on post-mortem tissue samples (section 1.4), despite the knowledge that these can be affected by environmental conditions. Hair samples are not only environmentally robust but allow for a much wider window of detection. Thus, this gap in knowledge highlights the importance for hair/feather sample analysis which enables pre-mortality detection of target compounds.

Therefore, the primary focus of this research was to develop, and validate, a simultaneous detection method capable of detecting nineteen compounds and two internal standards metabolites with LC-MS as the chosen technique. Thereafter, feathers from red kite and parrots and cat hair samples were analysed and positive identifications were made (Chapter Four). Additionally, it was important to ensure the analytical method was not only current but the most relevant to the research field. Hence, an important aim of this study was to identify the NSAIDs of concern, and to include these in the detection method alongside NSAIDs that could pose a future

impact and their metabolites, for example aceclofenac, highlighted as future threat and metabolises into the toxic diclofenac.

Since the vulture crisis, many conservationists and researchers have reported on the toxicity and availability of NSAIDs in areas of protected species, particularly vultures (section 1.3.2). For example in diclofenac and ketoprofen, whilst highlighting the lack of safety testing in other NSAIDs which are readily available, but have been proven to be toxic in other mammalian species, for example nimesulide and mefenamic acid. Metabolites were not included in previous studies. As such, thirteen NSAIDs and seven metabolites were included in this research for the development of simultaneous detection method as introduced in section 1.3.3 and 1.3.7.1.

6.2 ANALYTICAL STUDIES

6.2.1 Method development

A series of preliminary investigations were carried out in the method development stages to ensure the analytical method developed is robust, sensitive, specific and fit for purpose. Investigations included LC-MS method optimisation, of which included solvent selection, injection volumes, stability and selectivity investigations.

Early in the method development, necessary changes to the LC program were required to optimise the most suitable conditions to allow for the simultaneous detection of thirteen NSAIDs, seven metabolites and two internal standards. Alterations were made to the gradient elution settings and mobile phases used. The weakly acidic nature of NSAIDs favoured (98-0%) a decrease in water and an increase in acetonitrile (2-100%). The elution strength of acetonitrile proved much better than that of methanol thus, the latter was removed from the mobile phases employed. Ultimately, separation of all NSAIDs, albeit diclofenac, ibuprofen and suxibuzone, was achieved using total ion chromatogram.

Thereafter, solvents for standard preparation; 100% acetonitrile, 100% methanol and the starting mix of mobile phase, and injection volumes of 0.2, 2 and 10 µl were investigated. Injection volumes of 2µl in 100% acetonitrile yielded the best analytical response and high repeatability (10.5% RSD (n=12)) of results for all NSAIDs studied.

Initial analysis were carried out individually and then as mixed standards in scan mode. For final method validation and sample analysis, SIM was used. With three diagnostic ions per analyte between 4-14 minutes, separation of eleven NSAIDs, seven

metabolites and two internal standards, in one analytical method was achieved. Due to low ion intensities (above the accepted 10% abundance) for oxyphenylbutazone, 4-hydroxynimesulide, 5-hydroxyflunixin and 5-hydroxyprioxicam, only two ions were used.

Initial autosampler stability studies (54 hours) were conducted to determine the suitable duration from preparation to storage of compounds of interest. Both internal standards were proven to be stable over the duration of the study. Meanwhile, the NSAIDs and metabolites were stable across 0.05, 0.5 and 5µg/ml concentrations for the duration of 54 hours with the exception of acetylsalicylic acid, ibuprofen and metabolites 5-carboxymeloxicam and 5-hydroxyflunixin. In the case of the latter two compounds (5-carboxymeloxicam and 5-hydroxyflunixin), were proven to be stable for up to 43 hours with storage directly on the autosampler. In precision studies, at the three concentrations investigated, analytical responses were repeatable and within 10-15% RSD. Where the acceptance criteria was not met, namely in the compounds above, the concentrations at which this investigation was conducted fell, on occasions, beyond the working range of the compounds mentioned, therefore, this should be investigated further.

6.2.2 Method validation conclusions

Subsequent to the method development, longer term stability (six consecutive days) and corresponding precision for validation of the analytical method was conducted. Stability studies indicated that mixed standards were stable for the full six days however, for metabolites 5-carboxymeloxicam and 5-hydroxyflunixin for up to 3 days. This assay was based on a mixed standard that was stored in the freezer between analysis. Like initial studies, the method was deemed precise and within 10-15% RSD, with the exception of 5-carboxymeloxicam and 5-hydroxyflunixin. Good linearity (R^2 >0.99) was obtained. The data was tested for normality and randomness of errors, of which both statistical tests retained the null hypothesis, thus data was deemed to be of normal distribution.

The employment of the cheaper LC-MS has proven to be an effective alternative to the often employed LC-MS/MS and GC-MS. This has been demonstrated through comparable and in some cases lower LODs and LOQs than those reported in the literature. In instances of higher LODs, comparisons were made to the more superior LC-MS/MS, as such, LODs fell within previously reported concentration ranges investigated (section 4.4.5) despite sensitivity expected to be lower in LC-MS.

Furthermore, on comparison to the reported detection of NSAIDs in tissues (Taggart, et al., 2008), diclofenac was detected at concentrations of 0.05 to 0.0643µg/g. As such, the method developed in this research is capable of detecting NSAIDs at these concentrations (Table 4.5) in a real sample setting and therefore fit for purpose. No comparisons could be made for the metabolites as no such data is available, to the author's knowledge. This is an area for further research nonetheless. As it stands, would mean this research is the first instance whereby such analytes have been included in the simultaneous detection reported.

With the successful validation of the analytical method (Chapter Four), to the author's knowledge, this is the first LC-MS method that is not only capable of detecting simultaneously the selection of NSAIDs included in this study, but furthermore, facilitates the detection of seven major metabolites alongside parent compounds. This may be the first reporting of LODs of metabolites.

6.2.3 Application of the analytical method; feather analysis

The LC–MS method developed and validated in this research was applied to real sample analysis, particularly the testing of feathers. In its first stages, an optimal sample preparation method was developed and optimised. Throughout this development, time and cost implications were of upmost importance. The method was developed to the use of liquid nitrogen, whereby overall homogenisation was improved and shorter preparation time achieved. The optimal preparation method involved the use of a PM100 planetary ball mill. Unlike other cutting methods, mechanical means resulted in all parts of the feather ground to a powder like consistency suitable for analysis and in only 30 minutes. To ensure matrix effects were minimal, liquid liquid extraction was optimised. LLE was selected due to its user friendly procedure over the costly and complex nature of solid-phase extraction.

Feather samples were spiked at concentrations in line with the literature and analysed in selectivity and recovery studies. This tested the capabilities of the method proving it was selective and sensitive enough for the application in complex matrices. Such studies considered the efficiency of the sample clean-up and how much of the analyte of interest was recovered. All compounds, with the exception of 5-carboxymeloxicam and 5-hydroxyflunixin, were detected in spiked samples and resolved with no interferences at the retention times of interest. The method was capable of simultaneously detecting eleven NSAIDs and five major metabolites in complex feather samples. In recovery studies RSD was within acceptance criteria for majority of

NSAIDs with overall recovery ranging from 62.3 to 99.6%. In precision studies, results were precise and within acceptance criteria throughout which showed the suitability and reliability of the extraction method.

In unspiked feathers two NSAIDs were detected above their LOD, piroxicam in a red kite feather and phenylbutazone in a parrot feather. Both compounds were positively identified according to four separate identification points (retention time, relative retention factors, relative ion intensities and a minimum of one ion ratio). Piroxicam was detected at a concentration of 1.2µg/g and phenylbutazone at 1.8µg/g.

The successful application of this method fulfils the research question; can NSAIDs be detected in alternative keratinous matrices, such as hair and feathers? This has been possible with the detection of piroxicam and phenylbutazone from two individual bird feathers. The analytical method, as presented in this thesis, thus provides a means of testing eleven NSAIDs and five metabolites in environmentally robust samples, which has not been published before. Whilst within high precision and acceptance criteria as set out by various recognised organisations (Commission Decision, 2002; FDA, 2014; EMA, 1995; Huber, 2007; UNODC, 2009).

Therefore, the analytical method presented in this thesis has proven to be both sensitive and selective for detection and quantification of compounds of interest in complex matrices. It is hoped that findings from this research can be circulated to conservationists and organisations currently relying on conventional tissue analysis, as an alternative method. As well as being a means of detecting NSAIDs, the use of feathers and hairs provides a way of screening protected species pre-mortality. The testing of hair and feathers for this reason is essential, whether it is applied to testing the food source directly or in the initial signs of poisoning.

6.3 SUGGESTIONS FOR FURTHER RESEARCH

The lack of precision and instability, as reported throughout method development, in compounds acetylsalicylic acid and ibuprofen were problematic and ultimately led to their exclusion in the validated method. Whilst this method offers a means of detecting compounds identified as a threat and those that may pose a threat, further work should be carried out to include acetylsalicylic acid and ibuprofen in future methods.

During stability studies this research considered the typical analysis time encountered by the analyst. It would be beneficial to consider the longer term stability of mixed NSAID standards so that not only can it provide researchers and conservationists alike

with an accurate time permitted for sample analysis, but would account for any instrument breakdowns which could span for several days or weeks.

Recovery studies proved the extraction offered precision between samples however, overall recovery could be considered further. Where RSD was high and recoveries were low, this indicates potential areas of error. Loss in sample may be experienced during the extraction, filtration and evaporation stage and hence could be an area suggested for further research. This is especially important if research into detection of compounds in keratinous matrices continues, as recovery studies are absent in current publications.

The testing of keratinous matrices should continue and be encouraged with longer term sample analysis. This research has provided a relatively short sample preparation time of eighteen hours from sample collection to analysis, including the timely grinding, filtration and digestion stages. It is important to remember that the time mentioned is not per sample but would be allocated for >5 samples prepared and analysed concurrently. The continuation of this research and development to include many other commonly prescribed compounds would strengthen this novel method further. It would provide conservationists and researchers with an alternative means of detecting potentially toxic by-products in wildlife forensic cases.

Calls for further donations of keratinous matrices should continue especially in cases of suspected NSAID toxicity or suspicious mortality. Whether these samples come from the UK or other areas of protected species such as Europe and the Indian subcontinent would be dependent on collaborators but should be encouraged. One species that could be used to investigate the possible exposure and hence presence of NSAIDs could be the American mink (*Neovison vision*). Hair samples could be collected for analysis of NSAIDs during mink culling programmes or during bio-monitoring efforts. As a result the detection of NSAIDs, in these cases, would indicate their presence in the water ways of protected species.

Though LC-MS has been selected based on cost and comparable sensitivity to more expensive techniques, other techniques could be employed to provide a relatively novel method implementing a variety of instrumentation. In-house method transfer would be possible and would implement GC-MS. This would be valuable research to see if results achieved using LC-MS were comparable to GC-MS. However, method transfer should be carried out with some degree of caution; as discussed in Chapter One,

derivatisation steps are required in the analysis of NSAIDs in GC-MS so would require further method optimisation.

Further research into the detection of metabolites in keratinous matrices should be considered in greater detail. The analytical method presented in this research was a first in terms of detecting parent compounds alongside seven major metabolites. However, there is support for further research into their presence in keratinous matrices. As discussed in Chapter One, metabolites are often more persistent in the body and given that NSAIDs are taken under repeat administration, these potentially toxic compounds are likely to accumulate in the body. Thus, making their detection in keratinous samples possible. However, as metabolites were not detected in a real sample setting this still remains to be confirmed or refuted.

There is also a lack of research into the metabolic pathways of common veterinary pharmaceuticals in birds of prey and livestock. Pharmacokinetic and pharmacodynamic studies are far beyond the remit of this research and would involve ethical considerations throughout however, should be considered in future toxicity studies. Such results would aid in the identification of safer alternatives to toxic compounds.

6.4 RECOMMENDATIONS OF THE RESEARCH

Fundamentally, the testing of keratinous matrices should continue and most importantly be adopted in cases of wildlife forensics and conservationists working with protected and endangered species. Whether this is in the UK, EU or Indian subcontinent, this analytical method offers a means of monitoring protected species pre-mortality, which is currently not available. With reports of NSAID toxicity as recently as 2015, for example nimesulide, international collaboration is needed. In areas of protected species the adoption of the testing of keratinous matrices is most important, this includes conservation groups monitoring endangered and protected species in the UK, such as the red kite and water vole. If the adoption of this method has a slow uptake then it could be used as a supplementary method. This would be especially advantageous in biomonitoring efforts of protected species prior to potential instances of mass mortality.

The need of safety testing, in birds of prey especially, is paramount. Currently only meloxicam has been scientifically tested and deemed safe to avian species, more NSAIDs need to be studied. Control of potentially toxic NSAIDs in veterinary medicines need to be considered too, and where possible, at the point of treatment. When clinical

decisions made regarding treatment of an animal, the consequential exposure to other populations needs to be considered.

It is the hope of the author that this intriguing and interesting area of research is continued, whether that be through the further research discussed in section 6.3 or the recommendations made above. It is anticipated that together with currently published research and knowledge gained, NSAIDs in the environment will not result in such a devastating population decline as was documented in the vultures in the 1990s.

REFERENCES

Aberg, A., Olsson, C., Bondesson, U. and Hedeland, M., 2009. A mass spectrometric study on meloxicam metabolism in horses and the fungus *Cunninghamella elegans*, and the relevance of this microbial system as a model of drug metabolism in the horse. *Journal of Mass Spectrometry*, 44, pp.1026-1037.

Advenier, C., Roux, A., Gobert, C., Massias, P., Varoquaux, O. and Flouvat, B., 1983. Pharmacokinetics of ketoprofen in the elderly. *British Journal of Clinical Pharmacology*, 16, pp.65-70.

Ahuja, S. and Rasmussen, H. eds., 2007. *HPLC Method Development for Pharmaceuticals*. London: Elsevier.

Arnold, K., Boxall, A., Brown, R., Cuthbert, R., Gaw, S., Hutchinson, T., Jobling, S., Madden, J., Metcalfe, C., Naidoo, V., Shore, R., Smits, J., Taggart, M. and Thompson, H. 2013. Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems. *Biology Letters*, 9 (20130492) pp.1-4

Awan, A., Ashraf, M., Malik, A., Akram, M. and Murtaza, G., 2012. Toxicity Study of Piroxicam in Broilers. *Latin American Journal of Pharmacy*, 31(3), pp. 456-460.

Awan, A., Nazir, T., Ashraf, M., Umer, O. and Rehman, H.U., 2011. Studies of ketoprofen Toxicity in Avian Species. *Journal of Basic and Applied Sciences*, 7(2), pp.127-132.

Awan, A., Nazir., T., Ashraf, M., Umer, O. and Rehman, H., 2003. Study of phenylbutazone Toxicity in Avian Species. *Pakistan Journal of Pharmacy*, 16(1), pp.25-28.

Bakke, O., Draffan, G. and Davies, D., 1974. The Metabolism of Phenylbutazone in the Rat. *Xenobiotica*, 4(4), pp.237-254.

Baral, N., Gautam, R. and Tamang, B., 2005. Population status and breeding ecology of White-rumped Vulture *Gyps bengalensis* in Rampur Valley, Nepal. *Forktail*, 21(1), pp.87-91.

Baranowska, I. and Kowalski, B., 2010. Using HPLC Method with DAD Detection for the Simultaneous Determination of 15 Drugs in Surface Water and Wastewater. *Polish Journal of Environmental Studies*, 20(1), pp.21-28.

Barcelo, D. and Hansen, P. ed., 2009. *Biosensors for the Environmental Monitoring of Aquatic Systems*. New York: Springer.

Barone, J. and Schmidt, W. The United States Of America As Represented By The Secretary Of Agriculture. 2006. *Compositions and films comprised of avian feather keratin*. Pat. US7066995 B1.

Baumgartner, A., Jones, P., Baumgartner, W. and Black, C., 1979. Radioimmunoassay of Hair for Determining Opiate-Abuse Histories. *Journal of Nuclear Medicine*, 20, pp.748-752.

Baumgartner, W., Hill, V. and Blahd, W., 1989. Hair analysis for drugs of abuse. *Journal of Forensic Sciences*, 34(6), pp.1433-1453.

Bernal, E., 2014. Limit of Detection and Limit of Quantification Determination in Gas Chromatography. In: Guo, X, ed. 2014 Advances in Gas Chromatography. InTech. pp. 57-81.

Bernareggi, A., 2001. Clinical pharmacokinetics and metabolism of nimesulide. *Inflammopharmacology*, 9 (1,2), pp.81-89.

Bonesi, L. and Palazon, S., 2007. The American mink in Europe: Status, impacts, and control. *Biological Conservation*, 134, pp.470-483.

Bort, R., Ponsoda, X., Carrasco, E., Gomes-Lechon, M. and Castell, J., 1996. Metabolism of aceclofenac in humans. *Drug Metabolism and Disposition*, 24(8), pp.834-841.

Braithwaite, A. and Smith, F., 1996. *Chromatographic methods*. 5th ed. Dordrecht: Kluwer Academic Publishers.

British National Formulary, BNF., 2012. *BNF 64*. Basingstoke: Royal Pharmaceutical Science. 655.

Brodin, T., Fick, J., Jonsson, M. and Klaminder, J., 2013. Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science*, 339(6121), pp.814-815.

Bulman-Fleming, J., Turner, T. and Rosenberg, M., Evaluation of adverse events in cats receiving long-term piroxicam therapy for various neoplasms. Journal of Feline Medicine and Surgery, 12, pp. 262-268.

Burger, J. and Gochfield, M., 1995. Biomonitoring of heavy metals in the pacific basin using avian feathers. *Environmental Toxicology and Chemistry*, 14(7), pp.1233-1239.

Centre for Drug Evaluation and Research (CDER)., 1994. Validation of Chromatographic Methods. *Reviewer Guidance*, 3(1), pp.1-10.

Chaudhary, M., Subedi, T., Giri, J., Baral, H., Bidari, B., Subedi, H., Chaudary, B., Chaudary, I., Paudel, K. and Cuthbert, R., 2011. Population trends of Critically Endangered *Gyps* vultures in the lowlands of Nepal. *Bird Conservation International*, 22(3), pp.270-278.

Chen, Z., 2015. *Further assessment of PPCPs in feed grade chicken feather meal including potential sources*. Ph.D. Clemson University. Available at:

<http://tigerprints.clemson.edu/cgi/viewcontent.cgi?article=3098&context=all_theses> [Accessed 21.10.2015].

Clements, T., Gilbert, M., Rainey, H., Cuthbert, R., Eames, J., Bunnat, P., Teak, S., Chansocheat, S. and Setha, T., 2012. Vultures in Cambodia: population, threats and conservation. *Bird Conservation International*, 1(1), pp.1-18.

Commission Decision 96/43/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

Cooper, G., Paterson, S., Osselton, M., 2010. The United Kingdom and Ireland Association of Forensic Toxicologists: forensic toxicology laboratory guidelines (2010). *Science & Justice*, 50(4), pp. 166-176.

Coxib and traditional NSAID Trialists' (CNT) Collaboration, 2013. *Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials.* [pdf] The Lancet: Elsevier. Available at:
<http://download.thelancet.com/pdfs/journals/lancet/PIIS0140673613609009.pdf?id=eaaKc4Dg_itmm9DUzq5Ou> [Accessed 11 December 2014].

Crane, M., Boxall, A. and Barrett, K. 2008. *Veterinary Medicines in the Environment*. New York: CRC Press.

Crawford Scientific., 2014. *Sample Diluent Effects in HPLC.* [online] Crawford Scientifc. Available at: <http://www.crawfordscientific.com/Chromatography-Technical-Tips-Sample-Diluent-Effects-in-HPLC.html> [Accessed 20 October 2015].

Cuthbert, R., Dave, R., Chakraborty, S., Kumar, S., Prakash, S., Ranade, S. and Prakash, V., 2011. Assessing the ongoing threat from veterinary non-steroidal anti-inflammatory drugs to Critically Endangered *Gyps* vultures in India. *Oryx*, 45(3), p.420-426.

Cuthbert, R., Pain, D., Green, R., Swan, G. and Swarup, D., 2007. Comparative toxicity studies of NSAIDs in birds: a criticism of Reddy et al. *Environmental Toxicology and Pharmacology*, 23, pp.254–255.

Cuthbert, R., Parry-Jones, J., Green, R. and Pain, D., 2007b. NSAIDs and scavenging birds: potential impacts beyond Asia's critically endangered vultures. *Biology Letters*, 3, pp.91–94.

Cuthbert, R., Taggart, M., Saini, M., Sharma, A., Das, A., Kulkarni, M., Deori, P., Ranade, S., Shringarpure, R., Galligan, T. and Green, R., 2015. Continuing mortality of vultures in India associated with illegal veterinary use of diclofenac and a potential threat from nimesulide. *Oryx*, pp. 1-9.

Dama, M., 2014. The diclofenac ban is helping vulture conservation; what further Pharmaceutical threats loom ahead?. *Current Science*, 107(4), pp. 564-565.

Dauwe, T., Janssens, E., Bervoets, L., Blust, R. and Eens, M., 2004. Relationships between metal concentrations in great tit nestlings and their environment and food. *Environmental Pollution*, 131(3), pp. 373-380.

Davison, A., Milan, A. and Dutton, J., 2013. Potential problems with using deuterated internal standards for liquid chromatography-tandem mass spectrometry. *Annals Clinical Biochemistry*, 50(3), pp. 274.

Dieterle, W., Faigle, J., Fruh, F., Mory, H., Theobald, W., Alt, K. and Richter, W., 1976. Metabolism of phenylbutazone in man. *Drug Research*, 26(4), pp.572-577.

Dolan, J., 2001. Retaining Polar Compounds. *LGGC*, 19(11), pp.1132-1135.

Dolan, J., 2012. When should an Internal Standard be Used?. *LGGC North America*, [online] Available at:<http://www.chromatographyonline.com/when-should-internal-standard-be-used-0?id=&sk=&date=&pageID=4> [Accessed 10 November 2015].

Dolan, J., 2016. Column Plate Number and System Suitability. *LGGC Europe*, 29(3), pp. 130-134.

Dowling, G., Malone, E., Haribson, T. and Martin, S., 2009. Analytical strategy for the determination of non-steroidal anti-inflammatory drugs in plasma and improved analytical strategy for the determination of authorised and non-authorised non-steroidal anti-inflammatory drugs in milk by LC–MS/MS. *Food Additives & Contaminants: Part A,* 27(7).

Dulaurent, S., Gaulier, J., Baudel, J., Fardet, L., Maury, E. and Lachatre, G., 2008. Hair analysis to document non-fatal pesticide intoxication cases. *Forensic Science International*, 176, pp.72–75.

Ellison, S, Barwick, V and Farrant, T (2009). *Practical Statistics for the Analytical Scientist*. 2nd ed. Cambridge: RSC Publishing.

EMEA, 2003. *Diclofenac summary report*. London: The European Agency for the Evaluation of Medicinal Products.

EMEA, 2009. *European public MRL Assessment Report (EPMAR)*. London: The European Agency for the Evaluation of Medicinal Products.

Englehart, D., Lavins, E. and Sutheimer, C., 1998. Detection of drugs of abuse in nails. *Journal of Analytical Toxicology*, 22. pp.314-318.

Epshtein, N., 2004. Structure of chemical compounds, methods of analysis and process control: Validation of HPLC techniques for pharmaceutical analysis. *Pharmaceutical Chemistry Journal*, 38(4), pp.40-56.

Eurachem., 1998. *The fitness for Purpose of Analytical Methods. A laboratory Guide to Method Validation and Related Topics.* Middlesex: EURACHEM Working Group.

European Medicines Agency (EMA)., 1995. *Validation of Analytical Procedures: Text and Methodology. Note for guidance on validation of analytical procedures: Text and Methodology*. London: European Medicines Agency.

Food and Drug Administration (FDA). 2001. *Guidance for Industry Bioanalytical Method Validation.* [pdf] FDA. Available at :< http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf> [Acessed 21 January 2016].

Food and Drug Administration (FDA). 2014. Guidance for Industry Analytical Procedures and Methods Validation for Drugs and Biologics. *Analytical Procedures and Methods Validation for Drugs and Biologics*. Rockvillie: U.S. Department of Health and Human Services Food and Drug Administration

Fourie, T., 2014. The safety of carprofen, flunixin and phenylbutazone in the Cape Vulture (*Gyps coprotheres*) – a pilot study. MVSc. University of Pretoria. Available at: < http://repository.up.ac.za/bitstream/handle/2263/46035/Fourie_Safety_2015.pdf?sequence=1&isAllowed=y> [Accessed 6 November 2015]

Gaillard, Y. and Pépin, G., 1999. Testing hair for pharmaceuticals. *Journal of Chromatography B*, 733. pp.231–246.

Gallo, P., Fabbrocino, S., Dowling, G., Salini, M., Fiori, M., Perretta, G. and Serpe, L., 2010. Confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A*, 1217, pp. 2832-2839.

Gallo, P., Fabbrocino, S., Vinci, F., Danese, V., Nasi, A., and Serpe, L., 2006. Multi-Residue Determination of Non-Steroidal Anti-Inflammatory Drug Residues in Animal Serum and Plasma by HPLC and Photo-Diode Array Detection. *Journal of Chromatographic Science*, 44(10), pp.585-900.

Gibson, G. and Skett, P. *Introduction to Drug Metabolism*. Cheltenhan: Nelson Thornes.

Gilbert, M., Watson, R., Virani, M., Oaks, J., Ahmed, S., Chaudhry, M., Arshad, M., Mahmood, S., Ali, A. and Khan, A., 2006. Rapid population declines and mortality clusters in three Oriental white-backed vulture Gyps bengalensis colonies in Pakistan due to diclofenac poisoning. *Oryx*, 40(4), pp.388-399.

Gochfield, M., Saliva, J., Lesser, F., Shukla, T., Bertrand, D., Burger, J., 1991. Effects of color on cadmium and lead levels in avian contour feathers. *Archives of Environmental Contamination and Toxicology*, 20(4), pp. 523-526.

Gratacos-Cubarsi, M., Castellari, M., Valero, A. and Garcia-Regueiro, J., 2006. Hair analysis for veterinary drug monitoring in livestock production. *Journal of Chromatography B*, 834, pp. 14-25.

Green, R., Newton, I., Schultz, S., Cunningham, A., Gilbert, M., Pain, D. and Prakash, V., 2004. Diclofenac poisoning as a cause of vulture declines across the Indian subcontinent *Journal of Applied Ecology*, 41, pp.793-800.

Grenier, A., Andrade, L., Awad, A. and Pekol, T., 2011. *Validation of LC–MS-MS Methods for the Determination of Ibuprofen in Miniature Swine Plasma and Synovial Fluid.* Available: http://www.chromatographyonline.com/lcgc/article/articleDetail.jsp?id=719051&pageID=2. Last accessed 20 Sept 2014.

Gros, M., Petrovic, M. and Barcelo., D., 2006. Development of a multi-residue analytical methodology based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters.*Talanta*, 70, pp.678-690.

Guillarme, D. and Veuthey, J-L., 2008. *Guidelines for the use of UHPLC Instruments*. [pdf] University of Geneva. Available at: http://www.unige.ch/sciences/pharm/fanal/lcap/Guidelines%20for%20the%20use%20of%20UHPLC%20instruments%20-%20site%20WEB%20labo.pdf [Accessed 6 November 2015]

Haj, B., Ainri, A., Hassan, M., Khadem, R. and Marzouq, M., 1999. The GC/MS analysis of some commonly used non-steroidal anti-inflammatory drugs (NSAIDs) in pharmaceutical dosage forms and in urine. *Forensic Science International*, 105(1), pp.141-153.

Haskins, S., Kelly, D. and Weir, R., 2013. Trace element analysis of turkey vulture (*Cathartes aura*) feathers. *Journal of Radioanalytical and Nuclear Chemistry,* 295(2), p.1331.

Hawkins, C. and Dolan, J., 2003. Understanding Split Peaks. *LGGC North America*, 21(12), pp. 1134-1138.

Henderson, G., 1993. Mechanisms of drug incorporation into hair. *Forensic Science International*, 63, pp.19-29.

Hilton, M. and Thomas, K., 2003. Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography–electrospray tandem mass spectrometry. *Journal of Chromatography A*, 1015(1), pp.129-141.

Hostettmann, K., Marston, A. and Hostettmann, M., 1998. *Preparative Chromatography Techniques*. Springer: New York.

Hu, T., Peng, T., XJ, L., Chen, D., Dai, H., Deng, X., Yue, Z., Wang, G., Shen, J., Xia, X., Ding, S., Zhou, Y., Zhu, A. and Jiang, H., 2012. Simultaneous determination of thirty non-steroidal anti-inflammatory drug residues in swine muscle by ultra-high-performance liquid chromatography with tandem mass spectrometry. *Journal of Chromatography A*, 1219(1), pp.104-113.

Huber, L., 2007. *Validation of Analytical Methods*. New York: Informa Healthcare. I-65.

Huq, F., 2007. Molecular modelling analysis of the metabolism of mefenamic acid. *Journal of Pharmacology and Toxicology*, 2(1), pp.72-79.

Hutchinson, T., Madden, J., Naidoo, V. and Walker, C., 2015.Comparative metabolism as a key driver of wildlife species sensitivity to human and veterinary pharmaceuticals*. Philosophical Transactions B*, 370 (1664).

Ibanez, M., Guerrero, C., Sancho, J. and Hernandex, F., 2009. Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. *Journal of Chromatography A*, 1216(1), pp.2529-2539.

ICH., 2005. Validation of Analytical Procedures: Text and Methodology. *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use,* 4(1), pp.1-13.

ICUN, 2014. *The IUCN Red list of threatened Species.* [online] Available at: <http:// The IUCN Red list of threatened Species>[Accessed 04 February 2015].

IUCN, 2004. *Red List - Gyps indicus.* [online] Available at: <http://www.iucnredlist.org/details/22729731/0> [Accessed 26 Aug 2014].

IUPAC., 2014. *Compendium of Chemical Terminology, 2nd ed. (the "Gold Book").* [online] INTERNATIONAL Union of Pure and Appiled Chemistry. Available at: < http://goldbook.iupac.org/index.html> [Accessed 6 November 2015].

Iwabe, S., Ramírez-López, L. and Juárez-Sánchez, M., 2009. The use of piroxicam as an adjuntive treatment for squamous cell carcinoma in the third eyelid of a horse. Veterinaria México, 40(4), pp. 389-395.

Jedziniak, P., Szprengier-Juszkiewicz, T., Pietruk, K., Sledzinska, E. and Zmudzki, J., 2012. Determination of non-steroidal anti-inflammatory drugs and their metabolites in milk by liquid chromatography–tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 403(10), pp.2955-2963.

Jelic, A., Gros, M., Petrovic, M., Ginebreda, A. and Barcelo., 2012. Bringing Science into River Management Plans. In: H. Guasch, A. Ginebreda, A and A. Geiscinger, eds. 2012. *The Handbook of Environmental Chemistry*. Berlin: Springer. pp.1-23.

Jickells, S. and Negrusz, A., 2008. *Clarke's Analytical Forensic Toxicology*. London: Pharmaceutical Press.

Jones, O., Voulvoulis, N., Lester, J., 2005. Human Pharmaceuticals in Wastewater Treatment Processes. *Critical Reviews in Environmental Science and Technology*, 35, pp.401-427

Kang, W. and Kim., 2008. Simultaneous determination of aceclofenac and its three metabolites in plasma using liquid chromatography–tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 46(3), pp.587-591.

Kataoka, H. 2003. New trends in sample preparation for clinical and pharmaceutical analysis. *Trends in Analytical Chemistry*, 22, p. 232 – 243.

Kim, E., Ahn, B., Noh, K., Kang, W. and Gwak, H., 2012. Quantitative determination of aceclofenac and its three major metabolites in rat plasma by HPLC-MS/MS. *Journal of separation science*, 35(17), pp.2219-2222.

Kintz, P., 1996. *Drug Testing in Hair*. Florida: CRC Press.

Kintz, P., 2004. Value of hair analysis in postmortem toxicology. *Forensic Science International*, 142, p. 127-134.

Knapp, D., Richardson, R., Chan, T., Botooms, G., Widmer, W., DeNicola, D., Teclaw, R., Bonney, P. and Kuczek, T., 1994. Piroxicam therapy in 34 dogs with transitional cell carcinoma of the urinary bladder. Journal of Veterinary Internal Medicine, 8(4), pp. 273-278.

Lees, P., Landoni, M., Giraudel, J. and Toutain, P., 2004. Pharmacodynamics and pharmacokinetics of non-steroidal anti-inflammatory drugs in species of veterinary interest. *Journal of Veterinary Pharmacology and Therapeutics*, 27, pp.479-490.

LGGC, 2010. *Sample Preparation.* [pdf] LCGC's CHROMacademy. Available at: *http://www.chromacademy.com/lms/sco59/Sample_Preparation_Liquid-Liquid_Extraction_Techniques.pdf* [Accessed 14 April 2016].

Li, W., Zhang, J. and Tse, F., 2013. *Handbook of LC-MS Bioanalysis: Best Practices, Experimental Protocols, and regulations*. New Jersey: Wiley.

Loffler, D. and Ternes, T., 2003. Determination of acidic pharmaceuticals, antibiotics and ivermectin in river sediment using liquid chromatography–tandem mass spectrometry. *Journal of Chromatograpy A*, 1021(1), pp.133-144.

Love. D., Halden, U., Davis, M. and Nachman, K., 2012. Feather meal: A previously unrecognized route for re-entry into the food supply of multiple pharmaceuticals and personal care products (PPCPs). *Environmental Science and Technology*, 46, pp.3795-3802.

Lucas, A. and Stettenheim, P., 1972. *Avain Anatomy Integument Part 1*. US: U.S. Dept. of Agriculture.

Majors, R, 2013. *Sample Preparation Fundamentals for Chromatography.* [pdf] Agilent Technologies. Available at: <https://www.agilent.com/cs/library/primers/Public/5991-3326EN_SPHB.pdf> {Accessed 22 January 2015].

Mandal, R. and Khadka, K., 2013. Is diclofenac still a threat to Gyps Vulture? A case from Eastern Nepal and adjoining areas in India. *International Journal of Pure and Applied Zoology*, 1(3), pp.205-208.

Markandya, A., Taylor, T., Longo, A., Murty, M., Murty, S. and Dhavala, K., 2008. Counting the cost of vulture decline-an appraisal of the human health and other benefits of vultures in India. *Ecological Economics*, 67, pp.194-204.

McMaster, M., 2005. *LC/MS: A Practical User's Guide*. [e-book] New Jersey: Wiley. Available through: Google Books < http://books.google.co.uk> [Accessed 10 December 2014]

Mehta, C.R., and Patel, N.R., 2012. Exact Tests. Chicago: SPSS Inc.

Mennasse, R., Hedwall, P., Kraetz, J., Pericin, C., Riesterer, L., Sallmann, A., Ziel., R. and Jaques., R. 1978. Pharmacological properties of diclofenac sodium and its metabolites. *Scandinavian Journal of Rheumatology*, 22, pp.5-16.

Merck, 2014. Nonsteriodal Anti-inflammatory Drugs. [online] Available at: http://www.merckmanuals.com/vet/pharmacology/anti-inflammatory_agents/nonsteroidal_anti-inflammatory_drugs.html [Accessed 5 Feburary 2015]

Meteyer, C., Rideout, B., Gilbert, M., Shivaprasad, H. and Oaks, J., 2005. Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed oriental white-backed vultures (*Gyps bengalensis*). *Journal of Wildlife Diseases*, 41, pp.707-716.

Method to Assess Chronic Exposure to Diazinon in Rats. *Journal of Analytical Toxicology*, 29, pp. 805-809.

MHRA, 2010. MHRA Public Assessment Report: *Non-steroidal anti-inflammatory drugs and cardiovascular risks in the general population*. [pdf] London: Medicine and Healthcare Regulatory Agency. Available at: < http://www.mhra.gov.uk/home/groups/s-par/documents/websiteresources/con068576.pdf> [Accessed 10 December 2015]

Miao, X., Koenig, B. and Metcalfe, C., 2002. Analysis of acidic drugs in the effluents of sewage treatment plants using liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Chromatography A*, 952, pp.139-147.

Migowska, N., Caban, M., Stepnowski, P. and Kumirska, J., 2012. Simultaneous analysis of non-steroidal anti-inflammatory drugs and estrogenic hormones in water and wastewater samples using gas chromatography–mass spectrometry and gas chromatography with electron capture detection. *Science of the Total Environment*. 441, pp. 77-78.

Miksa, I., Cummings, M. and Poppenga, R., 2005. Multi-Residue Determination of Non-Steroidal Anti-Inflammatory Drug Residues in Sera by Liquid Chromatography-Mass Spectrometry. *Journal of Analytical Toxicology*. 29 (1), pp.94-104.

Miller, J. and Miller, J., 2010. *Statistics and Chemometrics for Analytical Chemistry*. 6th ed. Harlow: Prentice Hall. pp. 110-154.

Mishra, S., Prasad, G., Minakshi, M., Malik, Y., Kmahajan, N. and Praksh, V., 2001. Vulture mortality: Pathology and microbiological Investigations. *Indian journal of Animal Sciences*, 72(4), pp.283-286

Modi, C., Mody, S., Patel, H., Dudhatra, G., Kumar, A. and Avale, M., 2012. Toxicopathological overview of analgesic and anti-inflammatory drugs in animals. *Journal of applied Pharmaceutical Science, 2*(1), pp.149-157.

Moffatt, A., Osselton, M. and Widdop, B., 2004. *Clarke's. Analysis of drugs and poisons in pharmaceuticals, body fluids and post-mortem material.* 3rd ed. London: Pharmaceutical Press.

MP Biomedical, 2016. *MP Biomedicals.* [online] Available at: <http://www.mpbio.com> [Accessed 12 April 2016].

Muarer, H., Tauvel, F. and Kraemer, T., 2001. Screening Procedure for Detection of Non-Steroidal Anti-inflammatory Drugs and their Metabolites in Urine as Part of a Systematic Toxicological Analysis Procedure for Acidic Drugs and Poisons by Gas Chromatography- Mass Spectrometry after Extractive Methylation. *Journal of Analytical toxicology*, 25(4), pp.237-244.

Mukherjee, A., Galligan, T., Prakash, V., Paudel, V., Khan, U., Prakash, S., Ranade, S., Shastri, K., Dave, R., Donald, P., Bowden, C., 2014. Vulture Safe Zones to save Gyps Vultures in South Asia. *Mistnet*, 15(3), pp.4-21.

Murn, C., Anderson, M. and Anthony, A., 2002. Aerial survey of African white-backed vulture colonies around Kimberley, Northern Cape and Free State provinces, South Africa. *South African Journal of Wildlife Research,* 32(2), pp.45-152.

Mwangala, F., Sarna, L., Galloway, T. and Webster, G., 1993. Distribution of fenvalerate and permethrin residues on cattle hair following variable application rates of

impregnated ear tags. Pesticide Science, [e-journal] 39(3). Abstract only. Available through: Wiley Online Library Website < http://onlinelibrary.wiley.com> [Accessed 9 November 2015].

Naidoo, V., Wolter, K., Cromarty, A., Bartels, P., Bekker, L., McGaw., Taggart, M., Cuthbert, R. and Swan., G., 2008. The pharmacokinetics of meloxicam in vultures. *Journal of Veterinary Pharmacology and Therapeutics*, 31(2), pp.128-134.

Naidoo, V., Wolter, K., Cromarty, Diekmann, M., Duncan, N., Meharg, A., Taggart, M., Venter, L. and Cuthbert, R., 2010. Toxicity of non-steroidal anti-inflammatory drugs to Gyps vultures: a new threat from ketoprofen. *Biology Letters*, 6(3), pp.339-341.

Naidoo, V., Venter, L., Wolter, K., Taggart, M. and Cuthbert, R., 2010b. The Toxicokinetics of ketoprofen in *Gyps coprotheres*: toxicity due to zero-order metabolism. *Archives of Toxicology*, 84, pp.761–766.

Nakahara, Y., 1999. Hair analysis for abused and therapeutic drugs. *Journal of Chromatography B*, 733. 161-180.

Neue, U., 1997. *HPLC Columns, Theory, Technology and Practice.* New Yok: Wiley-VCH.

Ng, L., Vincent, A., Halliwell, B. and Wong, K., 2006. Action of diclofenac on kidney mitochondria and cells. *Biochemical and Biophysical Research Communication,* 348, pp.494-500

Niopas, I. and Daftsios, A., 2002. A validated high-performance liquid chromatographic method for the determination of glibenclamide in human plasma and its application to pharmacokinetic studies. *Journal of pharmaceutical and Biomedical Analysis*, 28(3-4), pp. 653-657.

Oaks, J., Gilbert, M., Virani, M., Watson, R., Meteyer, C., Rideout, B., Shivaprasad, H., Ahmed, S., Chaudhry, M., Arshad, M., Mahmood, S., Ali, A. and Khan, A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, 427, pp.630-633.

Orros, M. and Fellowes, M., 2014. Supplementary feeding of the reintroduced Red Kite (*Milvus milvus*) in UK gardens. *Bird Study*, 1, pp. 1-4.

O'Shaughnessy, K., 2011. *New Guide to Medicines and Drugs*. 8th ed. London: Dorling Kindersley.

Ou, C. and Frawley, V., 1984. Liquid-chromatographic determination of indomethacin in blood from newborns with patent ductus arteriosus. *Clinical Chemistry*, 30(6), pp. 898-901.

Owen, L. and Keevil, B., 2012. Testosterone measurement by liquid chromatography tandem mass spectrometry: the importance of internal standard choice. *Annals of clinical Biochemistry*, 49, pp. 600-602.

Pain, D., Bowden, C., Cunningham, A., Cuthbert, R., Das, D., Gilbert, M., Jakati, R., Jhala, J., Khan, A., Naidoo, V., Oaks, J., Parry-Jones, J., Prakash, V., Rahmani, A., Ranade, S., Baral, H., Senacha, K., Saravanan, S., Shah, N., Swan, G., Swarup, D., Taggart, M., Watson, R., Virani, M., Wolter, K. and Green, R., 2008. The race to prevent the extinction of South Asian vultures. *Bird Conservation International*, 18, pp.s30-s48.

Peters, F., 2007. Stability of analytes in biosamples—an important issue in clinical and forensic toxicology?. *Analytical and Bioanalytical Chemistry*, 388, pp.1505-1519.

Peters, F., Drummer, O. and Musshoff, F., 2007. Validation of new methods. *Forensic Science International*, 165, pp.216-224.

Petrovic, M., Hernando, M., Diaz-Cruz, S. and Barcelo, D., 2005. Liquid chromatography–tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. *Journal of Chromatography A*, 1067(1-2), pp.1-14.

Pilastro, A., Congiu, L., Tallandini, L. and Turchetto., 1993. The use of bird feathers for the monitoring of Cadmium pollution. *Archives of Environmental Contamination and Toxicology*, 24, pp.355-358.

Prakash, V., 1999. Status of vultures in Keoladeo National Park, Bharatpur, Rajasthan, with special reference to population crash in *Gyps* species. *Journal of the Bombay Natural History Society,* 96, pp.365–378.

Prakash, V., Green, R., Pain, D., Ranade, S., Saravanan, S., Prakash, N., Venkitchalam, R., Cuthbert, R., Rahmani, A. and Cunningham, A., 2007. Recent Changes in Populations of Resident *Gyps* Vultures in India. *Journal of the Bombay Natural History Society*, 104(2), pp. 129-135.

Prakash, V., Pain, D., Cunningham, A., Donald, P., Prakash, N., Verma, A., Gargi, R., Sivakumar, S. and Rahmani, A., 2003. Catastrophic collapse of Indian white-backed *Gyps bengalensis* and long-billed *Gyps indicus* vulture populations. *Biological Conservation*, 109, pp.381-390.

Proctor, N., 1993. *Manual of ornithology: avian structure & function*. Yale: Yale University

Ragni, 2012. *Vulture culture at threat from diclofenac*. Available at: < http://www.vettimes.co.uk/article/vulture-culture-at-threat-from-diclofenac/> [Accessed 9 November 2015].

Rang, H., Dale, M., Ritter, J., Flower, R. and Henderson, G., 2012. *Rang and Dale's Pharmacology*. 7th ed. Edinburgh: Elsevier Churchill Livingstone.

Ray, J. and Wade, D., 1982. The pharmacokinetics and metabolism of ^{14}C-carprofen in man. *Biopharmaceutics and Drug Disposition*, 3, pp.29-38

Redderson, K. and Heberer, T., 2003. Multi-compound methods for the detection of pharmaceutical residues in various waters applying solid phase extraction (SPE) and gas chromatography with mass spectrometric (GC-MS) detection. *Journal of Separation Science*, 26(1), pp.1443-1450.

Retsch, 2016. *Planetary Ball Mill PM100*. [online] Available at: <http://www.retsch.com/products/miling/ball-mills/planetary-ball-mill-pm-100/> [Accessed 17 May 2016].

Richards, 2010. Detection of Non-steroidal anti-inflammatory drugs in hair, nails and feathers using GC-MS with emphasis on diclofenac: a forensic tool for wildlife conservation. PhD thesis. Anglia Ruskin University, Cambridge, UK.

Richards, N., Cook, G., Simpson, V., Hall, S., Harrison, N. and Scott, K., 2011b. Qualitative detection of the NSAIDs diclofenac and ibuprofen in the hair of Eurasian otters (Lutra lutra) occupying UK waterways with GC–MS. *European Journal of Wildlife Research*, 57(1), pp.1107-1114.

Richards, N., Hall, S., Harrison, N., Gautam, L., Scott, K., Dowling, G., Zorilla, I. and Fajardo, I., 2014. Merging Wildlife and Environmental Monitoring Approaches with Forensic Principles: Application of Unconventional and Non-Invasive Sampling in Eco-Pharmacovigilance. *Journal of Forensic Research*, 5(3), pp.1-16.

Richards, N., Hall, S., Scoot, K. and Harrison, N., 2011. First detection of an NSAID, flunixin, in sheep's wool using GC-MS. *Environmental Pollution*, 159, pp.1446-1450.

Ritchie, B. and Harrison, G., 1994. Formulary. In:. In: B. Ritchie, G. Harrison. and L. Harrison, ed. 1994. *Avain Medicine: Principles and Application.* Florida: Wingers Publishing. pp.457-482.

Rothe M., Pragst, F., Thor, S. & Hunger, J., 1997. Effect of pigmentation on the drug deposition in hair of grey-haired subjects. *Forensic Science International*, 84, pp.53 – 60.

Royal College of Veterinary Surgeons (RCVS), 2015. *Code of Profesional Conduct for Veterinary Surgeons: 4. Veterinary medicines.* [online] Available at: <https://www.rcvs.org.uk/advice-and-guidance/code-of-professional-conduct-for-veterinary-surgeons/supporting-guidance/veterinary-medicines/ > [Accessed 9 November 2015].

Rubio, F., Seawall, S., Pocelinko, R., DeBarbieri, B., Benz, W., Berger, L., Morgan, L., Pao, J., Williams, T. and Koechlin, B., 1980. Metabolism of Craprofen, a Nonsteriodal

Anti-Inflammatory Agent, in Rats, Dogsand Humans. *Journal of Pharmaceutical Sciences*, 69(11), pp.1245-1253.

Saar, E., Gerostamoulos, d., Drimmer, O. and Beyer, J., 2012. Assessment of the stability of 30 antipsychotic drugs in stored blood specimens. *Forensic Science International*, 215(1-3), pp.152-158.

Saraf, S., 2008. N*SAIDs Non-Steroidal Anti-Inflammatory Drugs: An Overview*. PharmaMed Press: Hyderabas.

Saving Asia's Vultures From Extinction (SAVE), 2012. *Report from the 2nd meeting.* [online] Available at: <http://www.save-vultures.org/save_latestnews.html.> [Accessed 26 Aug 2014].

Shaprio, S. and Wilk, M., 1965. An Analysis of Variance Test for Normality (Complete Samples). *Biometrika*. 52(3), pp. 591-611.

Sharma, A., Saini, M., Singh, S., Prakash, V., Das, A., Dasan, R., Pandey, S., Bohara, D., Galligan, T., Greens, R., Knopp, D. and Cuthbert, R., 2014. Diclofenac is toxic to the Steppe Eagle Aquila nipalensis: widening the diversity of raptors threatened by NSAID misuse in South Asia. *Bird Conservation International*, 1(1), pp.1-5.

Sharma, P., 2012. Aceclofenac as a potential threat to critically endangered vultures in India: a review. *Journal of Raptor Research*, 46(3), pp.314-318

Shimadzu., 2011. *High Performance Liquid Chromatograph Mass Spectrometer LCMS-2020 Instruction Manual*. Japan: Shimadzu.

Shimadzu., 2012. Handbook for LC/MS. *Handbook for LC/MS*. 2(1), pp.1-39.

Shultz S., Baral H., Charman, H., Cunningham, A., Das, D., Ghalsasi, G., Goudar, M., Green, R., Jones, A., Nighot, P., Pain, D., and Prakash, V., 2004. Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Proceedings of the Royal Society of London B Biological Sciences (Suppl.),* 271, pp.458-460.

Singla, A., Chawla, M. and Singh, A., 2000. Nimesulide: Some pharmaceutical and pharmacological aspects – An update. *Journal of Pharmacy and Pharmacology*, 52, pp.467-486.

Smith, D., 2010. *Metabolism, Pharmacokinetics and Toxicity of Functional Groups: Impact of Chemical Building Blocks on ADMET*. Cambridge: Royal Society of Chemistry.

Stoyanova, Y., Stefanov, N. and Schmutz, J., 2010. Twig used as a tool by the Egyptian Vulture (Neophron percnopterus). Journal of Raptor Research, 44(2), pp.154-156

Suojala, L., Karrtinen, L. and Pyorala, S., 2013. Treatment for bovine Escherichia coli mastitis – an evidence-based approach. *Journal of Veterinary Pharmacology and Therapeutics*, 36 (6), pp.521-531.

Swan, G., Naidoo, V., Cuthbert, R., Green, R., Pain, D., Swarup, D., Prakash, V., Taggart, M., Bekker, L., Das, D., Diekmann, J., Diekmann, M., Killian, E., Meharg, A., Patra, R., Saini, M. and Wolter, K., 2006. Removing the Threat of Diclofenac to Critically Endangered Asian Vultures. *PLOS biology*, 40(3), pp.395-402.

Swartz, M., 2010. *Seeing is Believing: Detectors for HPLC.* [online] Waters. Available at: <http://www.chromatographyonline.com/seeing-believing-detectors-hplc?id=&sk=&date=&pageID=3> [Accessed 6 November 2015].

Swarup, D., Patra, R., Prakash, V., Cuthbert, R., Das, D., Avari, P., Pain, D., Green, R., Sharma, A., Saini, M., Das, D. and Taggart, M., 2007. Safety of meloxicam to critically endangered Gyps vultures and other scavenging birds in India. *Animal Conservation*, 10(2), pp.192-198.

Synder, L. and Dolan, J., 2007. *High-Performance Gardient Elution: The Practical Application of the Linear-Solvent-Strength Model.* New Jersey: Wiley and Sons.

Synder, L., Kirkland, J. and Dolan, J., 2010. *Introduction to Modern Liquid Chromatography*. 3rd ed. New Jersey: Wiley and Sons.

Taggart, M., Senacha , K., Green, R., Cuthbert, R., Jhala, Y., Meharg, A., Mateo, R. and Pain, D., 2009. Analysis of Nine NSAIDs in Ungulate Tissues Available to Critically Endangered Vultures in India. *Environmental Science and Technology*, 43(12), pp.4561-4566.

Taylor, P., 2005. Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. *Clinical Biochemistry*, 38, pp. 328– 334.

Tsatsakis, A., Tutudaki, M., Tzatzarakis, M., Psaroudakis, K., Dolapskis, G. and Michalodimitrakis, M., 1998. Pesticide deposition in hair: preliminary results of a model study of methomyl incorporation into rabbit hair. *Veterinary and Human Toxicology*, 40(4), pp. 200-203.

Turck, D., Roth, W. and Busch, U., 1996. A review of the clinical pharmacokinetics of meloxicam. *British Journal of Rheumatology*, 35(1), pp.13-16.

Tutudaki, M. and Tsatsakis, A., 2005. Pesticide Hair Analysis: Development of a GC-NCI-MS

UNODC, 2009. United Nations Office on Drugs and Crime. *Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens*. [Online] Available at: http://www.unodc.org/documents/scientific/validation_E.pdf [Accessed 6 November 2015].

Van Hoof, N., De Wasch, K., Poelmans, S., Noppe, H. and De Brabander, H., 2004. Multi-residue liquid chromatography/tandem mass spectrometry method for the detection of non-steroidal anti-inflammatory drugs in bovine muscle: optimisation of ion trap parameters. *Rapid Communications in Mass Spectrometry*, 18(1), pp.2823-2829.

Vane, J.R., & Botting, R.M., 1995. New insights into the mode of action of anti-inflammatory drugs. *Inflammation Research*, 44, p. 1 – 10.

Vane, J., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biology*, 231, pp.232-239

Vinci, F., Fabbrocino, S., Flori, M., Serpe, L. and Gallo, P., 2006. Determination of fourteen non-steroidal anti-inflammatory drugs in animal serum and plasma by liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry*, 20(22), pp.3412-3420.

WADA, 2003. World anti-doping agency. *Identification criteria for qualitative assays incorporating chromatography and mass spectrometry*. [pdf] [Online] Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf [Accessed 06 August 2015].

Wapole, R., 2002. *Probability & Statistics for Engineers & Scientists*. 8th ed. New Jersey: Prentice and Hall.

Wasfi, I., Boni, N., Abdel Hadi, A., Elghazali, M., Zorob, O., Alkatheeri, N. and Barezaiq, I., 1998. Pharmacokinetics, metabolism and urinary detection time of funixin after intravenous administration in camels. *Journal of Veterinary Pharmacology and Therapeutics*, 21, pp.203-208.

Waters., 2015. *Solvents and Caveats for LC/MS.* [online] Waters. Available at: <http://www.waters.com/waters/en_GB/Solvents-and-Caveats-for-LC-MS/nav.htm?cid=10091173&locale=en_GB> [Accessed 20 October 2015].

Watson, J and Sparkman., 2007. *Introduction to Mass Spectrometry*. 4th ed. West Sussex: Wiley.

Wenning, R., 2000. Potential problems with the interpretation of hair analysis results. *Forensic Science International*, 107, p. 5-12.

Willis, J., Kendall, M., Flinn, R., Thornhill, D. and Welling, P., 1979. The pharmacokinetics of diclofenac sodium following intravenous and oral administration. *European Journal of Clinical Pharmacology*, 16, pp.405-410.

Woodhouse, K. and Wynne, H., 1987.The pharmacokinetics of Non-Steroidal Anti-Inflammatory Drugs in the Elderly. *Clinical pharmacokinetics*, 12, pp.111-122.

World Health Organization (WHO)., 2011. *Pharmaceuticals in Drinking-water.* Switzerland: WHO Press.

Wresel, P. and Pakula, C., 2005. *Key Factors in Sample Diluent Selection for HPLC Assays of Active Pharmaceutical Ingredients.* [online] LCGC. Available at: <http://www.chromatographyonline.com/key-factors-sample-diluent-selection-hplc-assays-active-pharmaceutical-ingredients?id=&sk=&date=&pageID=2> [Accessed 6 November 2015].

Yasuda, Y., Shindo, T., Mitani, N., Ishida, N., Oono, F. and Kageyama, T., 1981. Comparison of the Absorption, Excretion and Metabolism of Suxibuzone and Phenylbutazone in Humans. *Journal of Pharmacutical Sciences*, 71(5), pp.565-572.

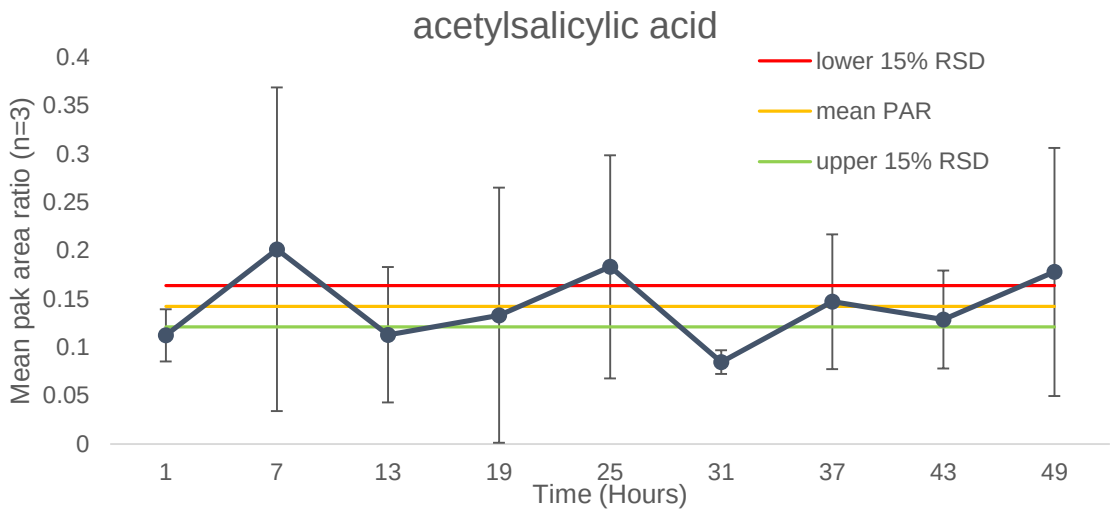
Yritia, M., Parra, P., Fernanez, J. and Barbanoj, J., 1999. Piroxicam quantitation in human plasma by high-performance liquid chromatography with on- and off-line solid-phase extraction. Journal of Chromatography A, 846(1-2), pp.199-205.

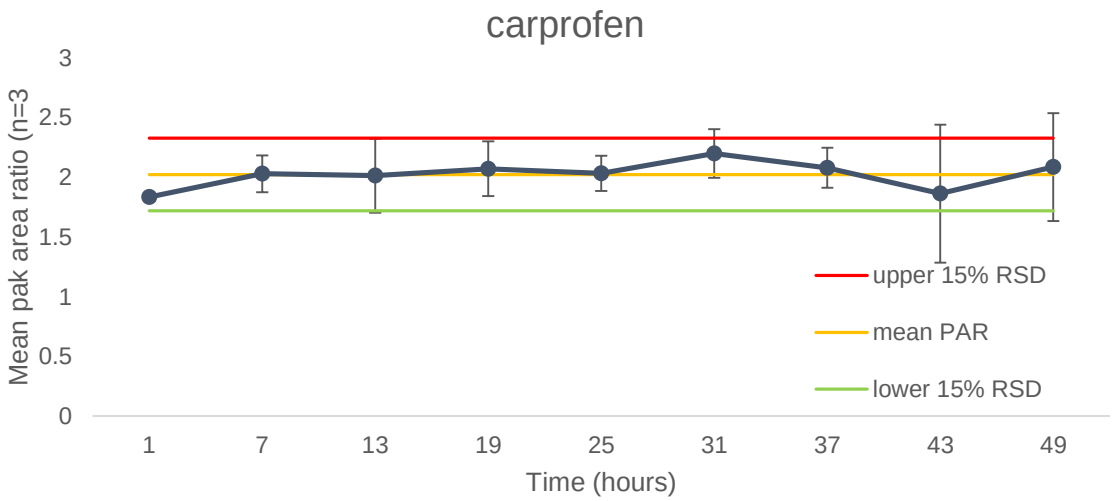
Yu, M., Yue, Z., Wu, P., Wu, D. and Mayer J., 2004. The developmental biology of feather follicles. *International Journal of Developing Biology*, 48 pp.181-191.

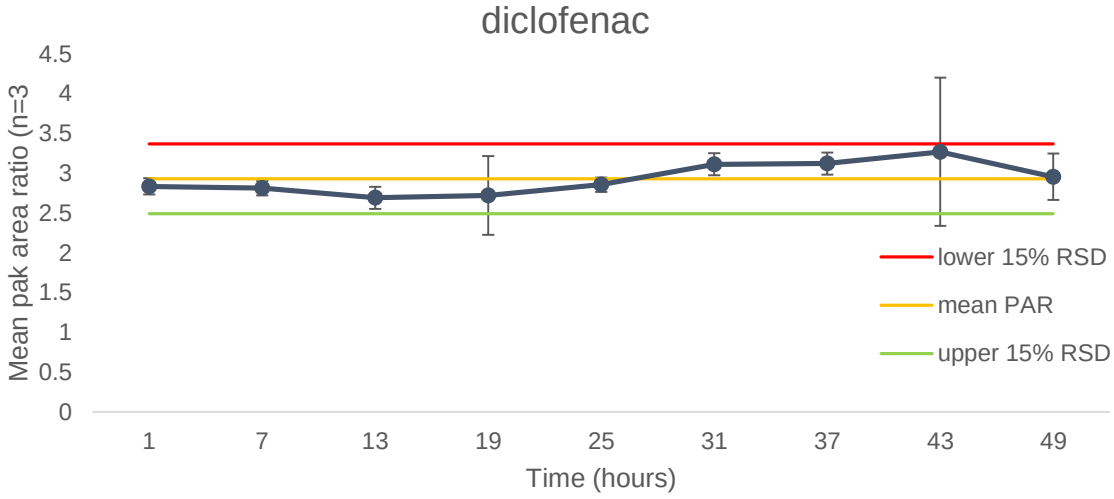
Zorilla, I., Martinez, R., Taggart, M. And Richards, N., 2014. Suspected Flunixin Poisoning of a Wild Eurasian Griffon Vulture from Spain. Conservation Biology, 00 (0) pp.1-6

APPENDICES

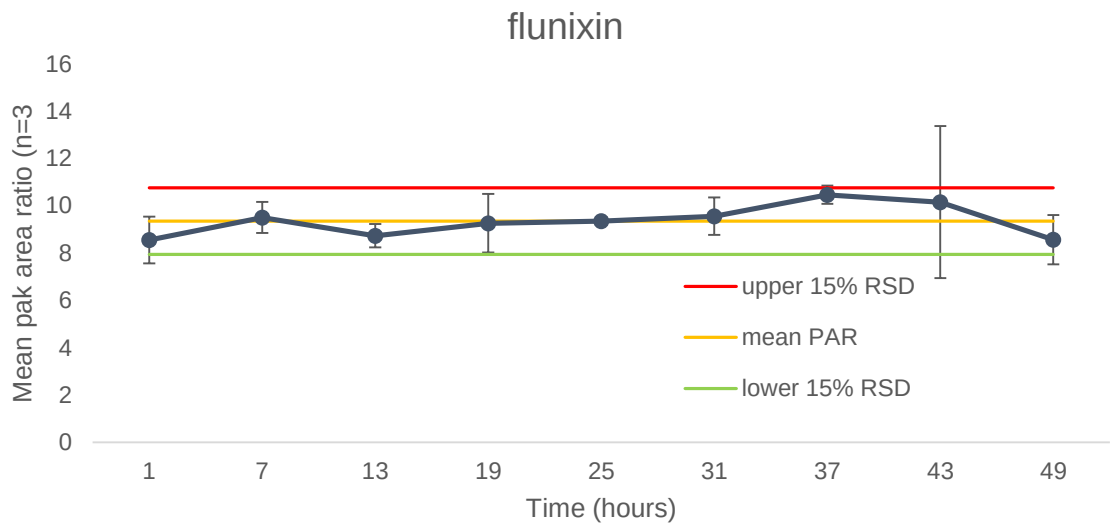
Appendix I-a Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.05µg/ml

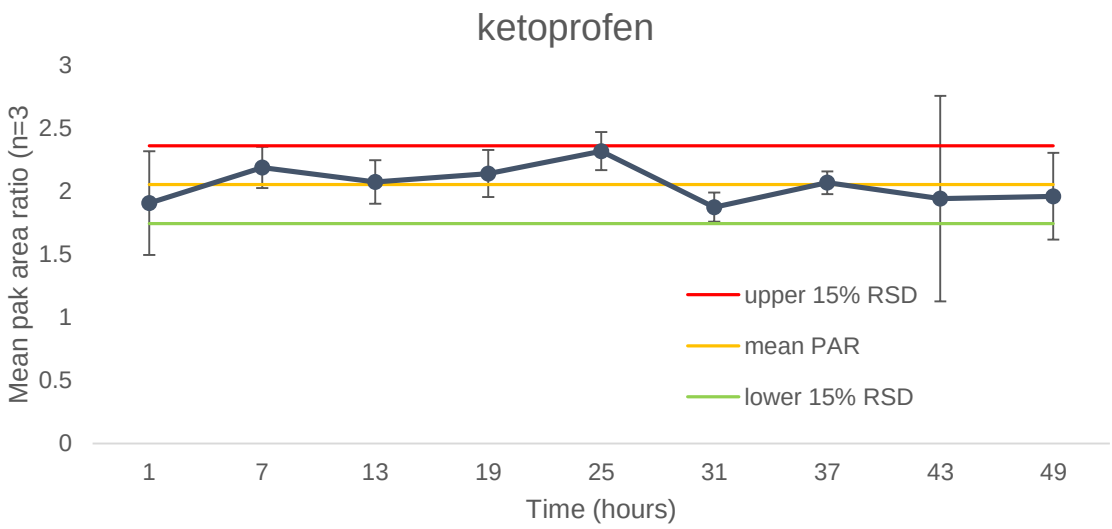


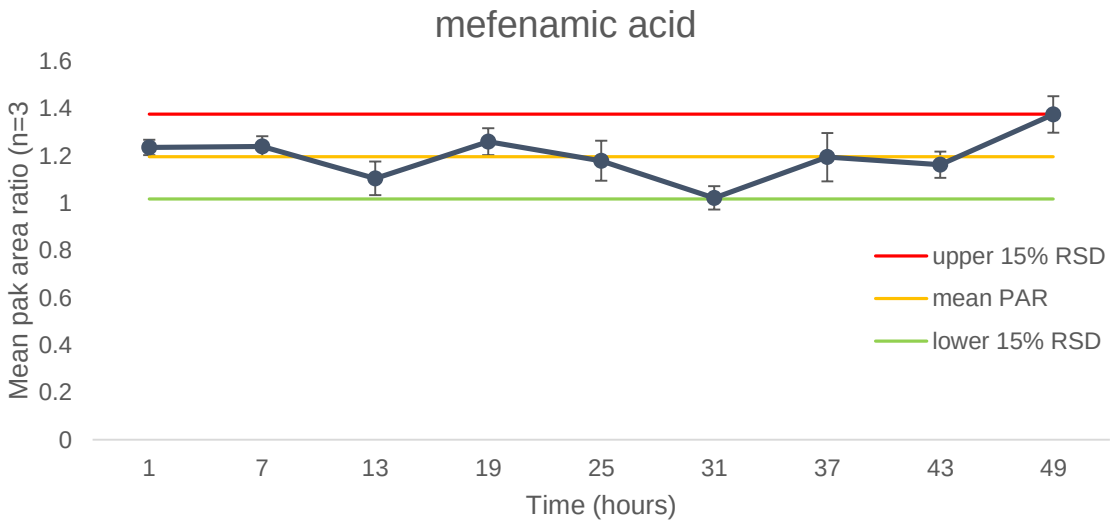




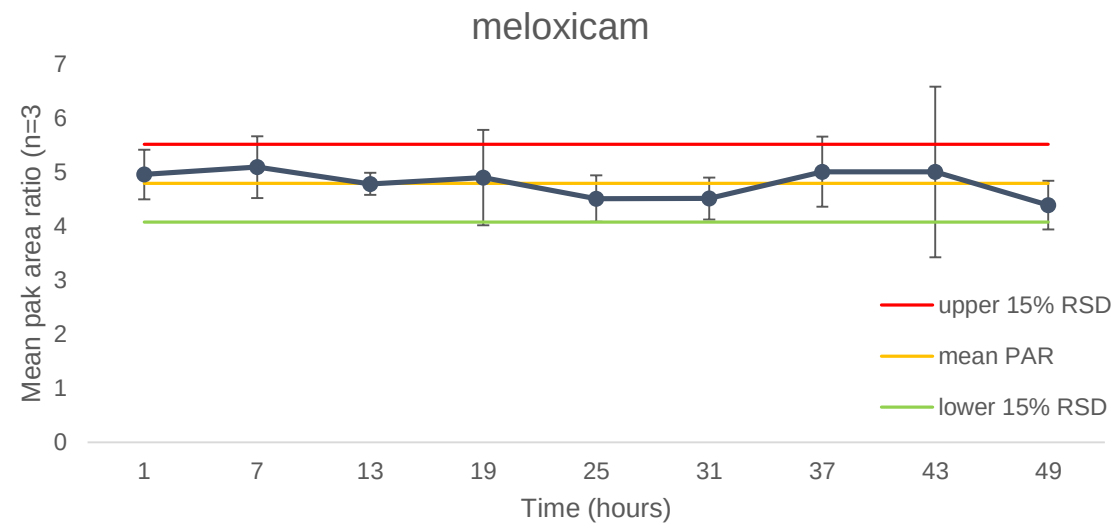
Appendix I-b Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.05µg/ml

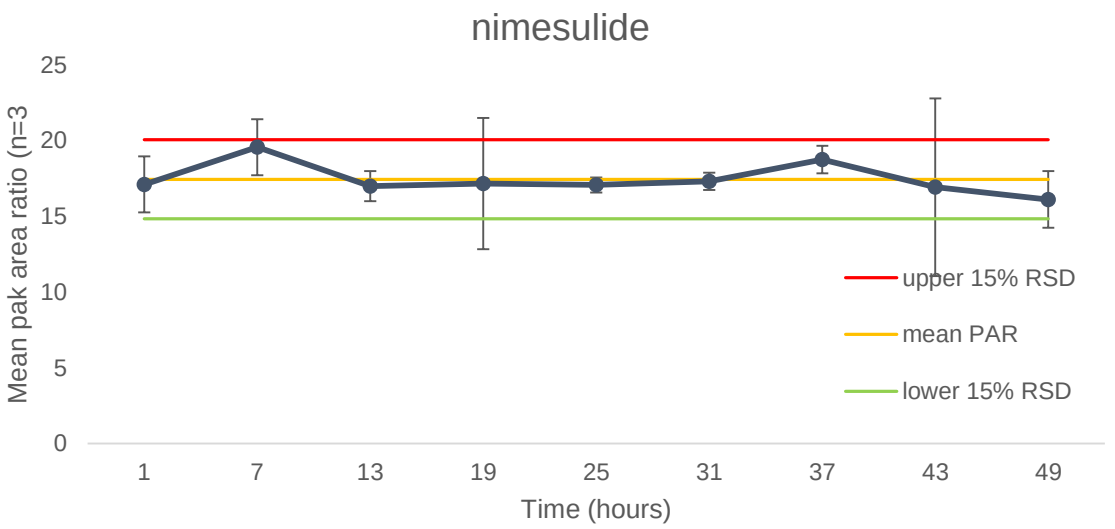


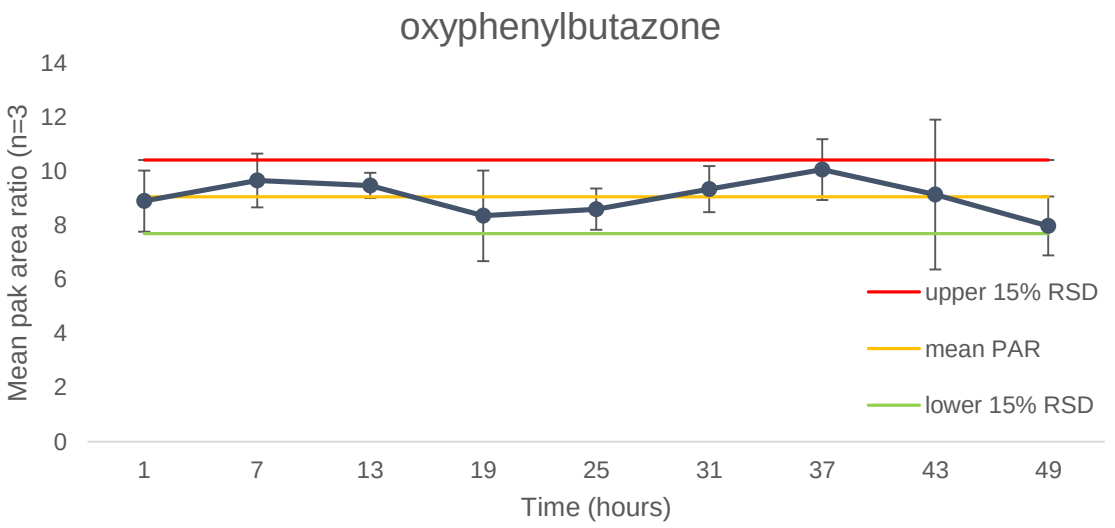




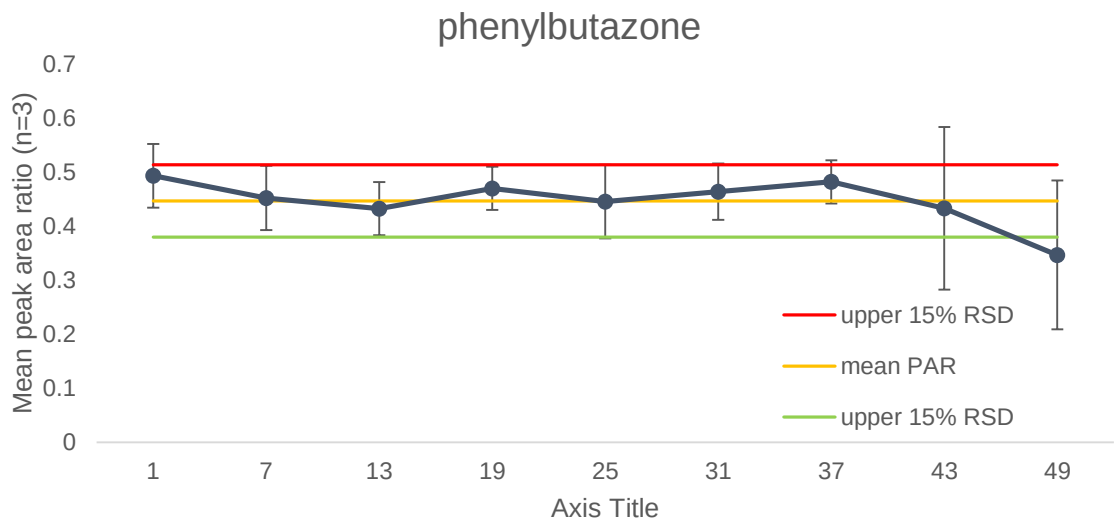
Appendix I-c Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.05µg/ml

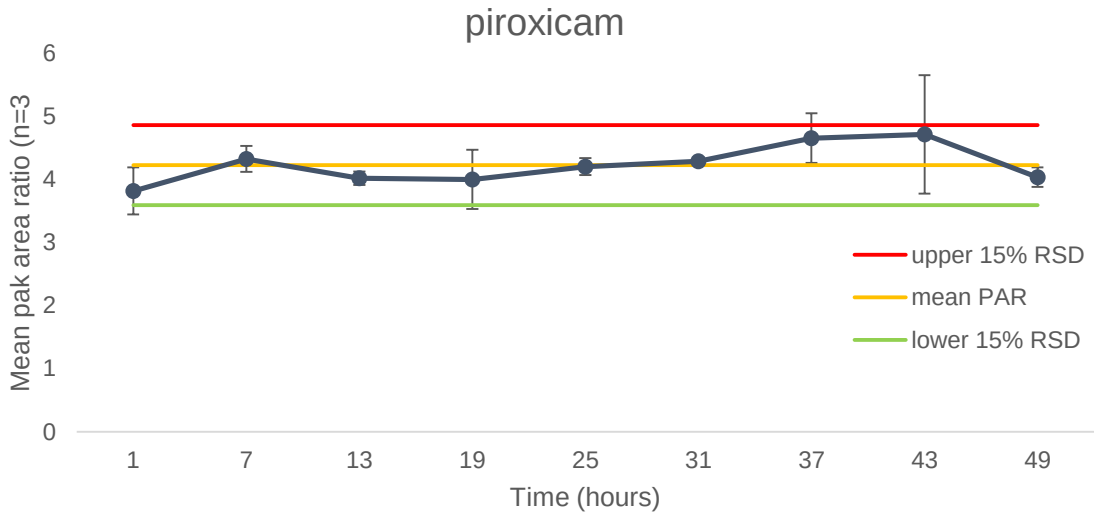


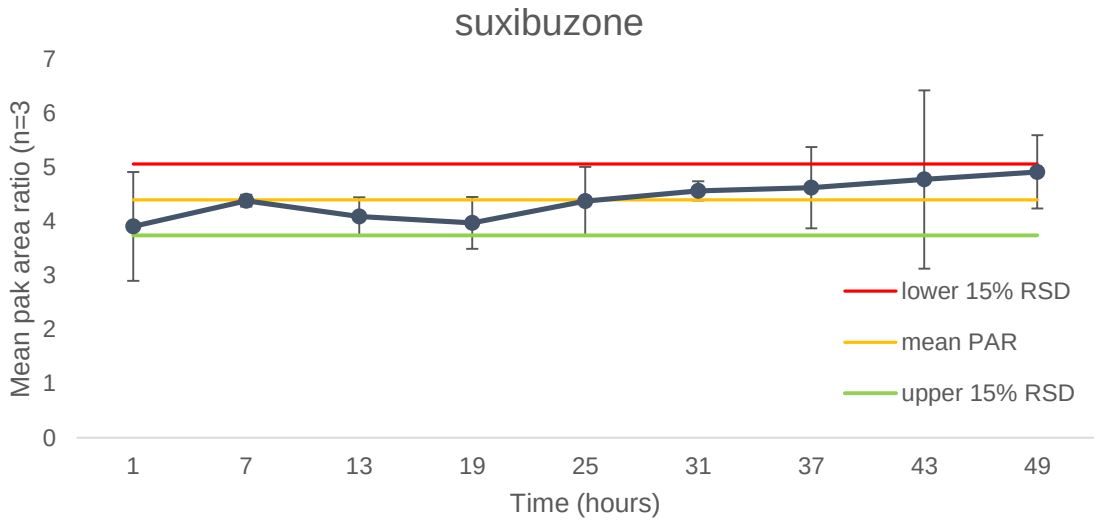




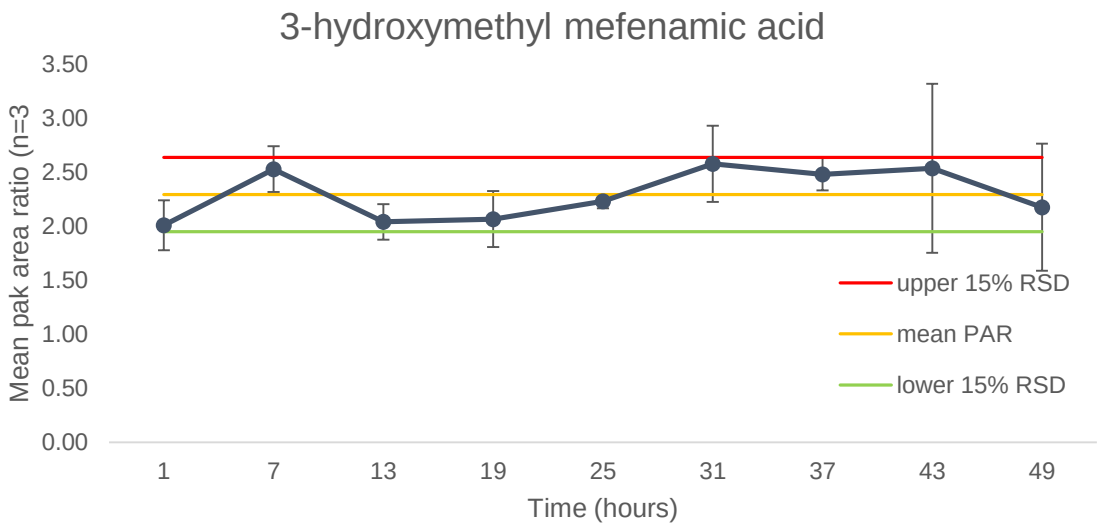
Appendix I-d Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.05µg/ml

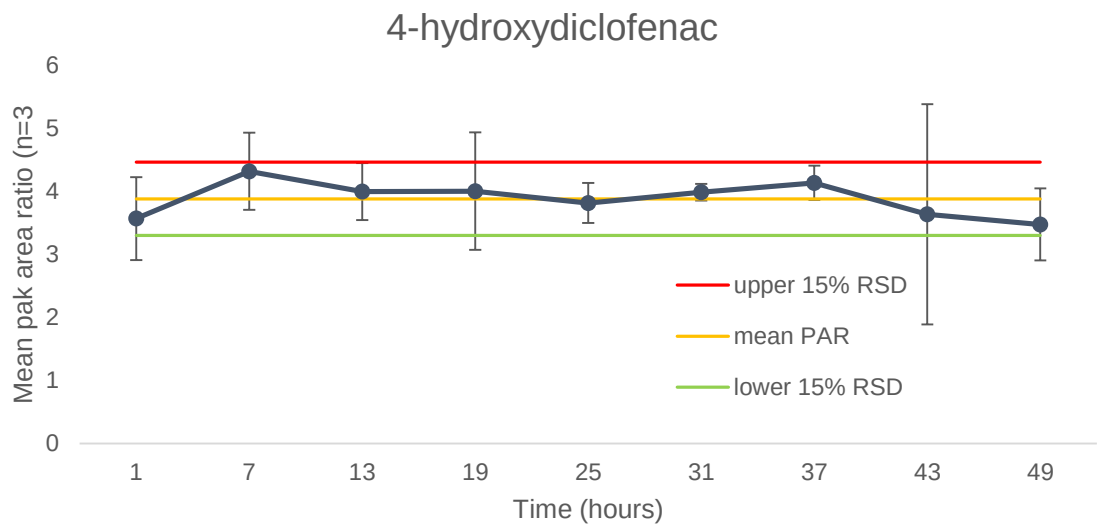


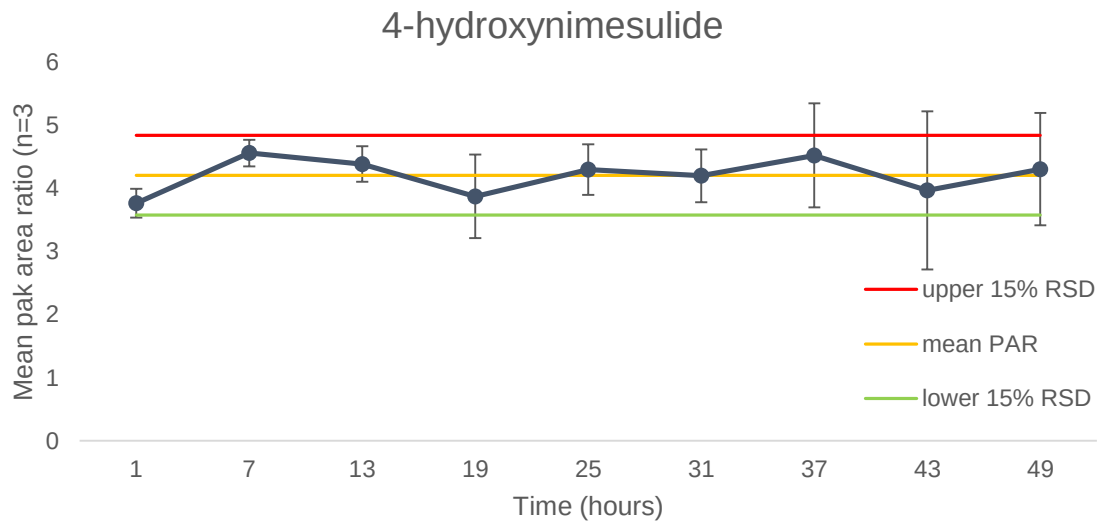




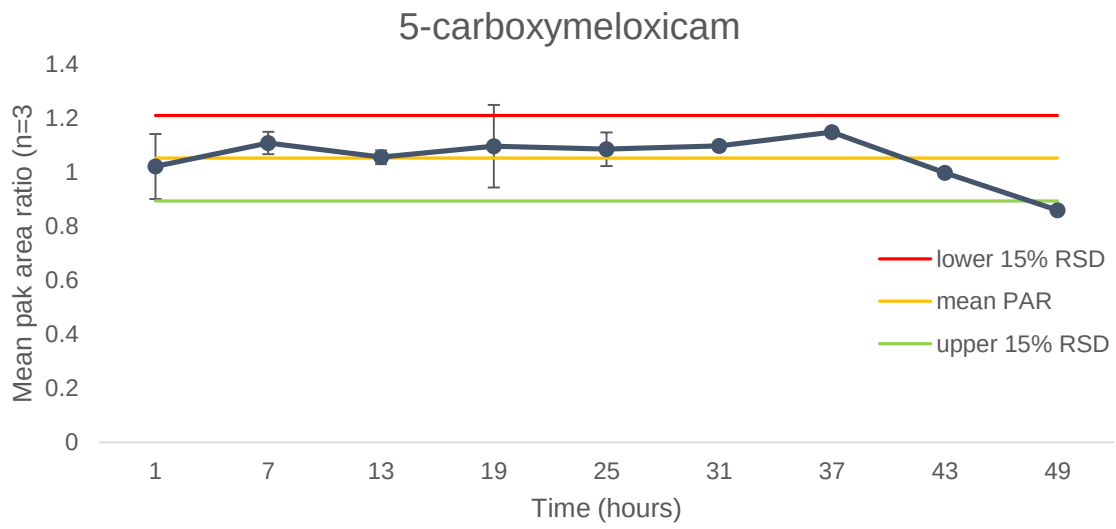
Appendix I-e Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.05µg/ml

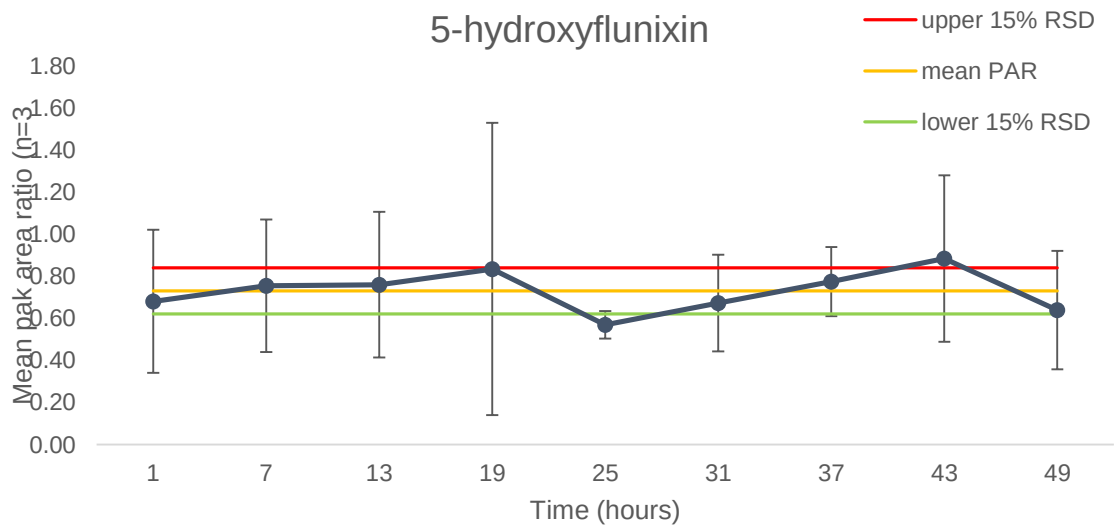


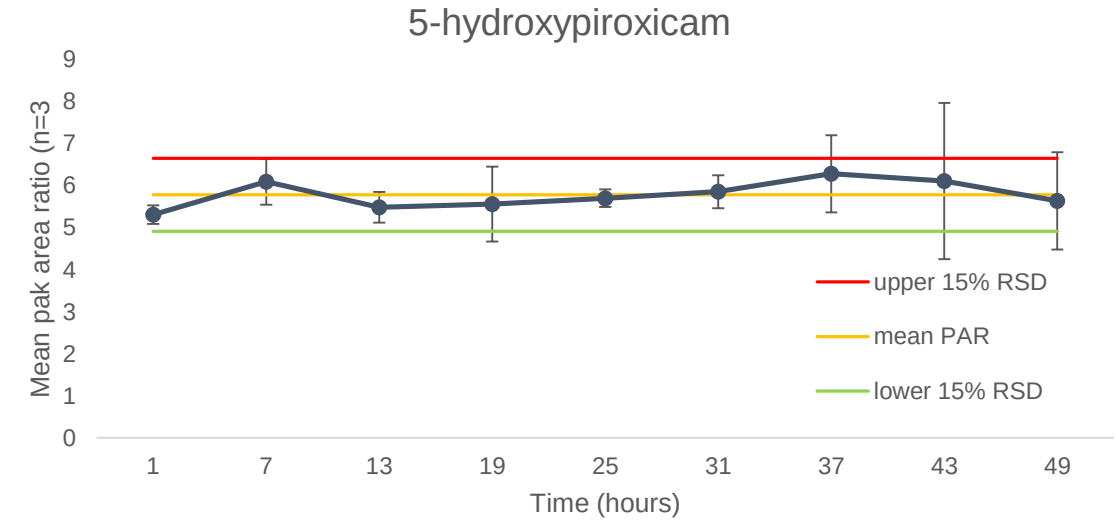




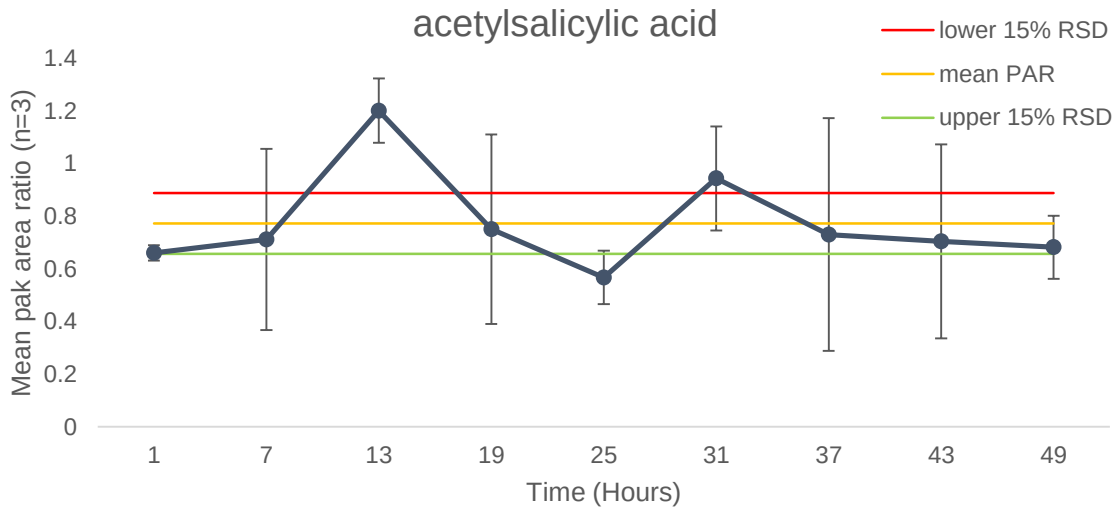
Appendix I-f Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.05µg/ml

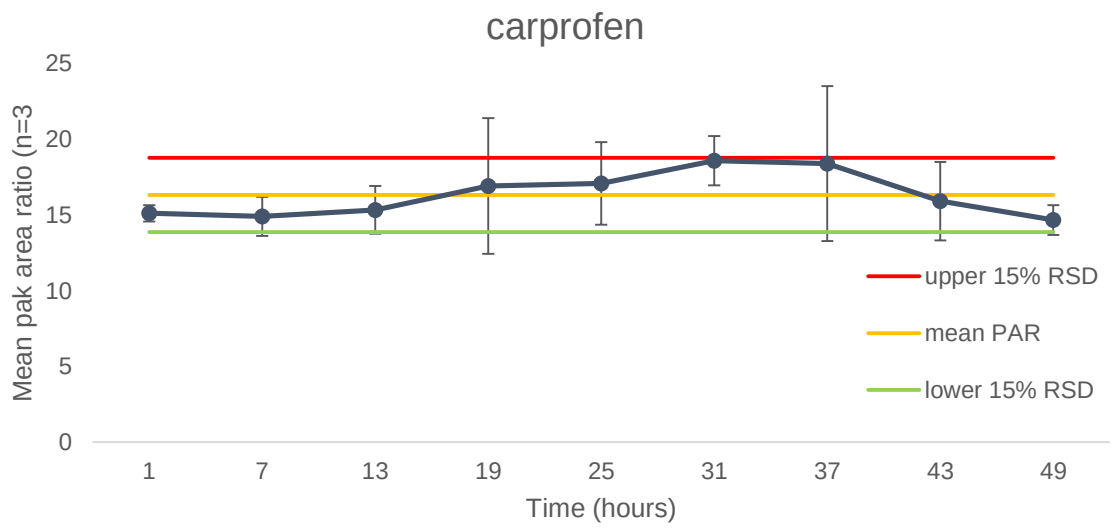


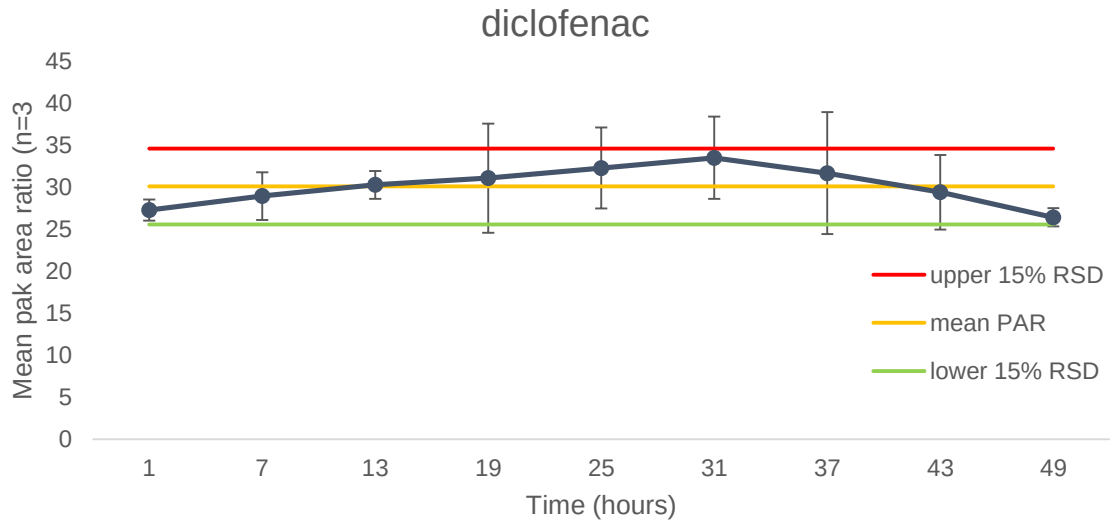




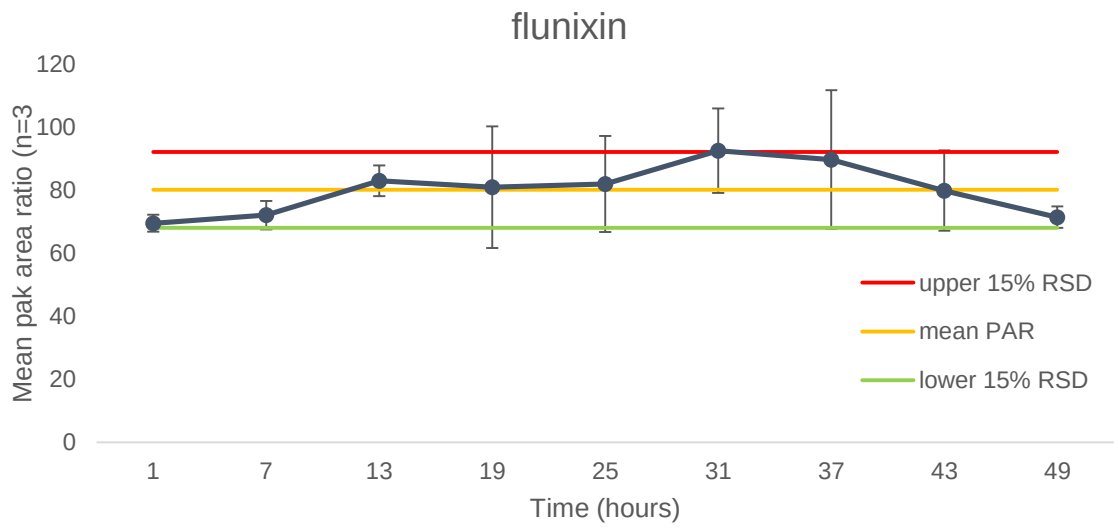
Appendix II-a Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.5µg/ml

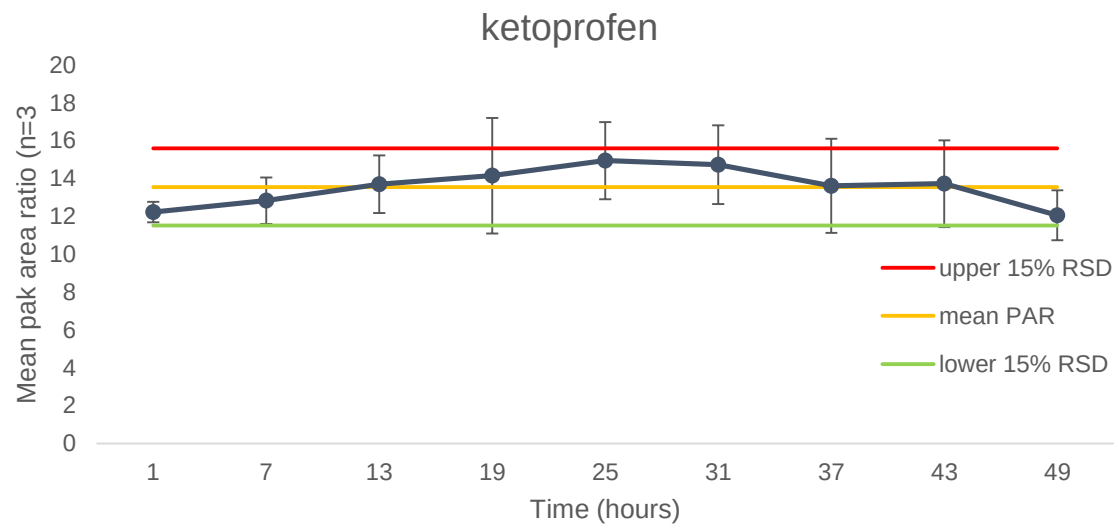


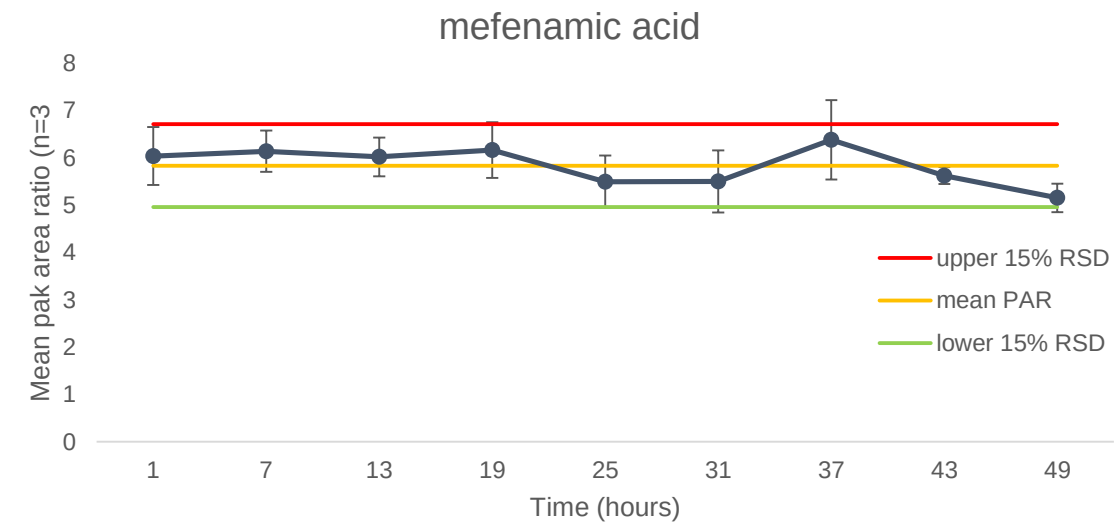




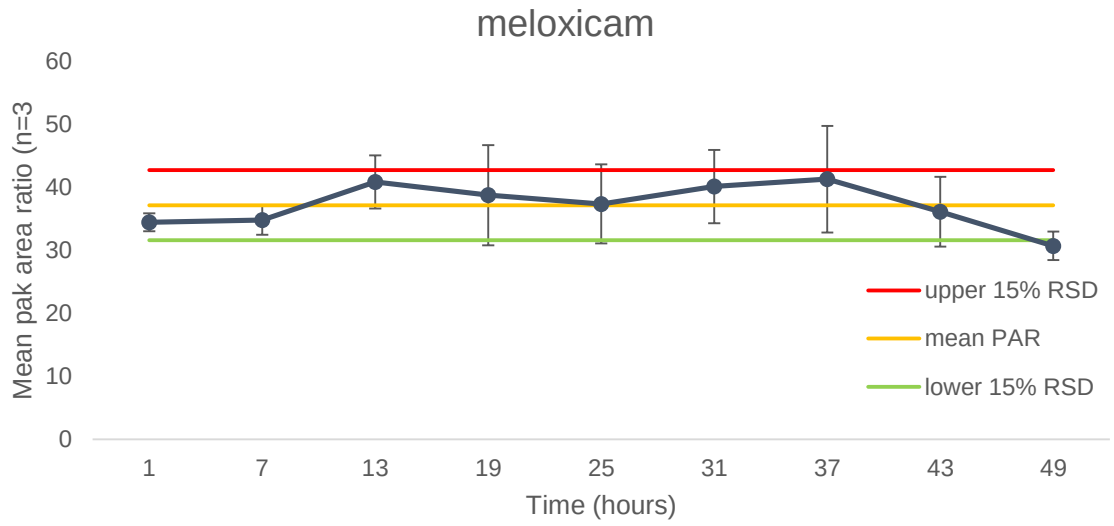
Appendix II-b Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.5µg/ml

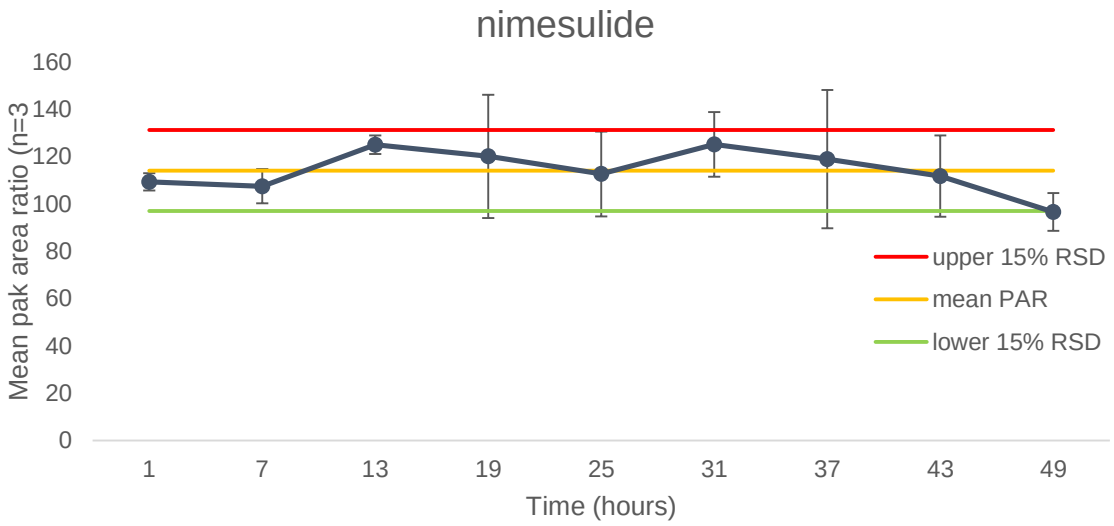


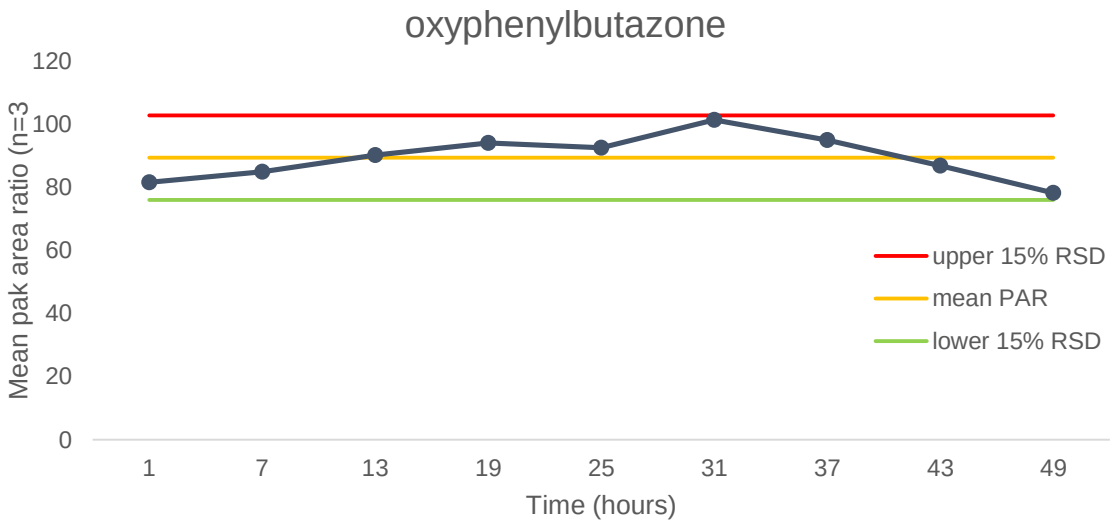




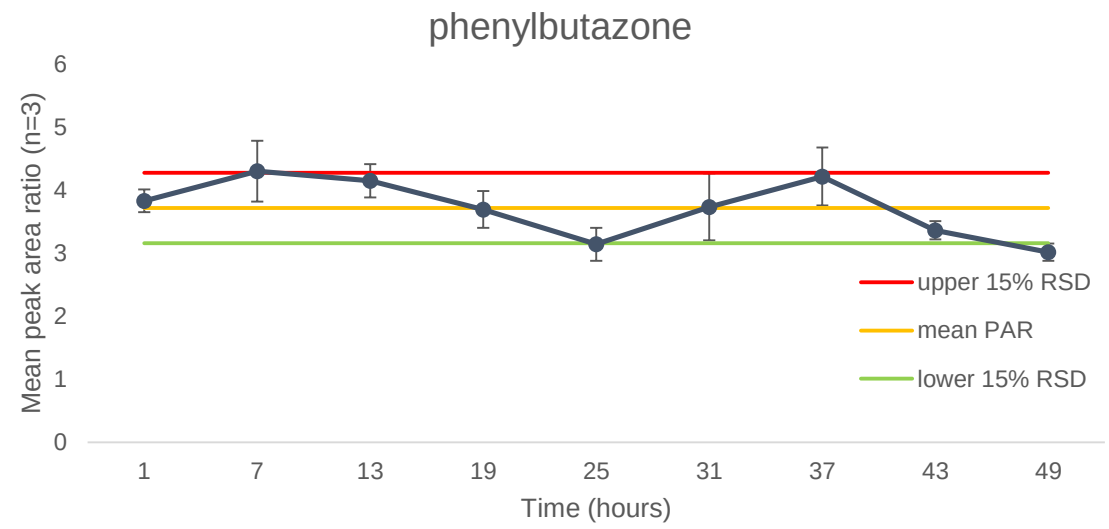
Appendix II-c Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.5µg/ml

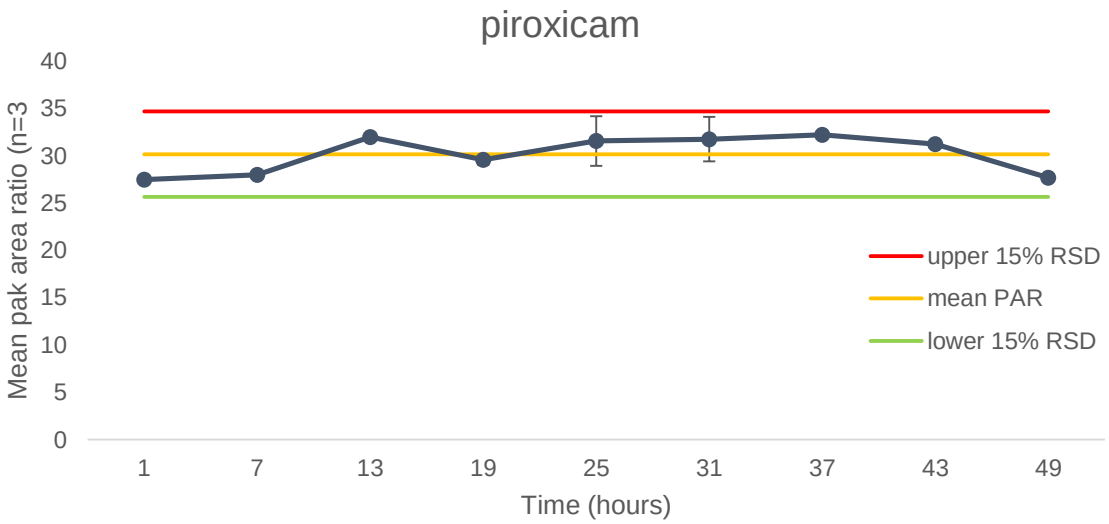


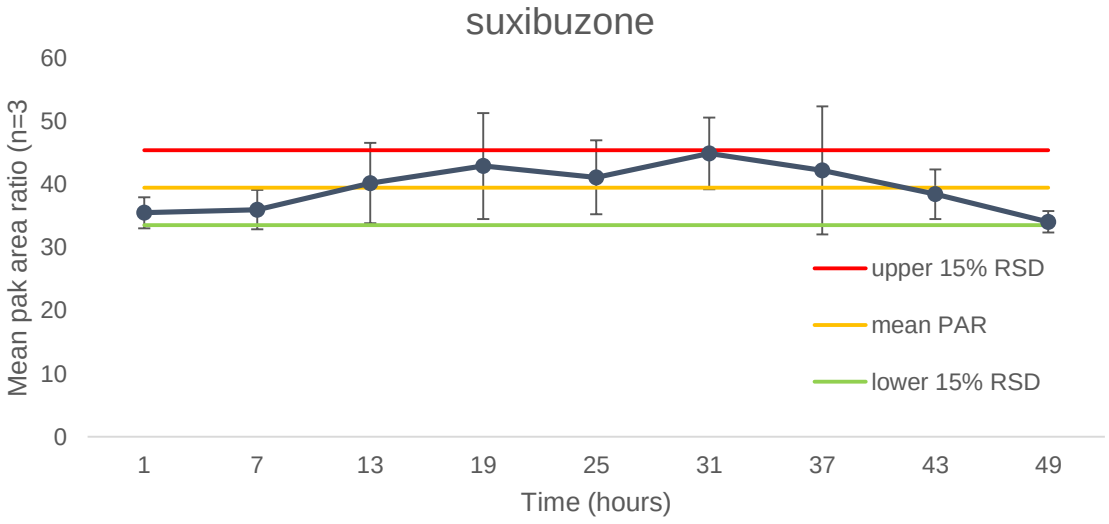




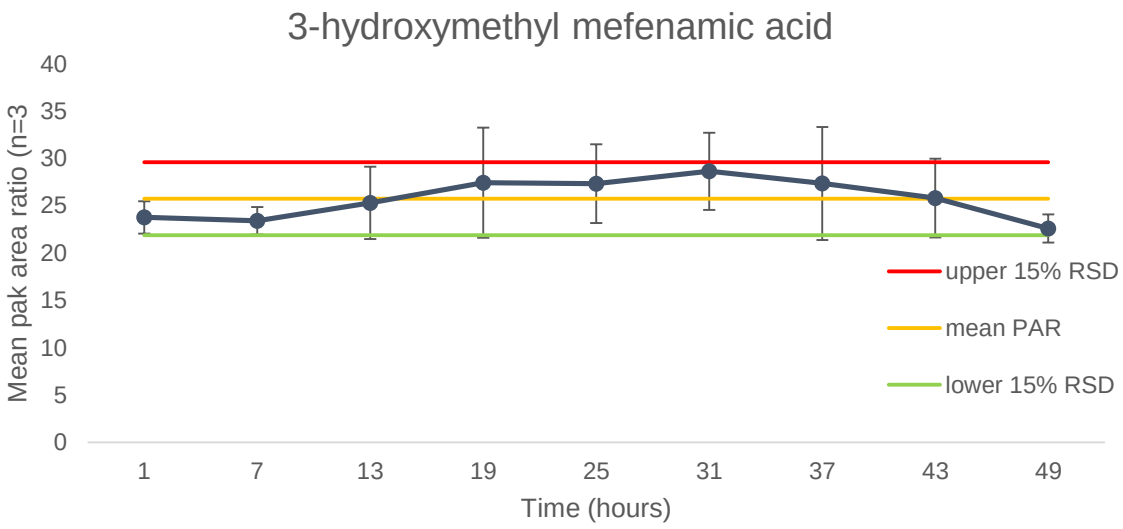
Appendix II-d Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.5µg/ml

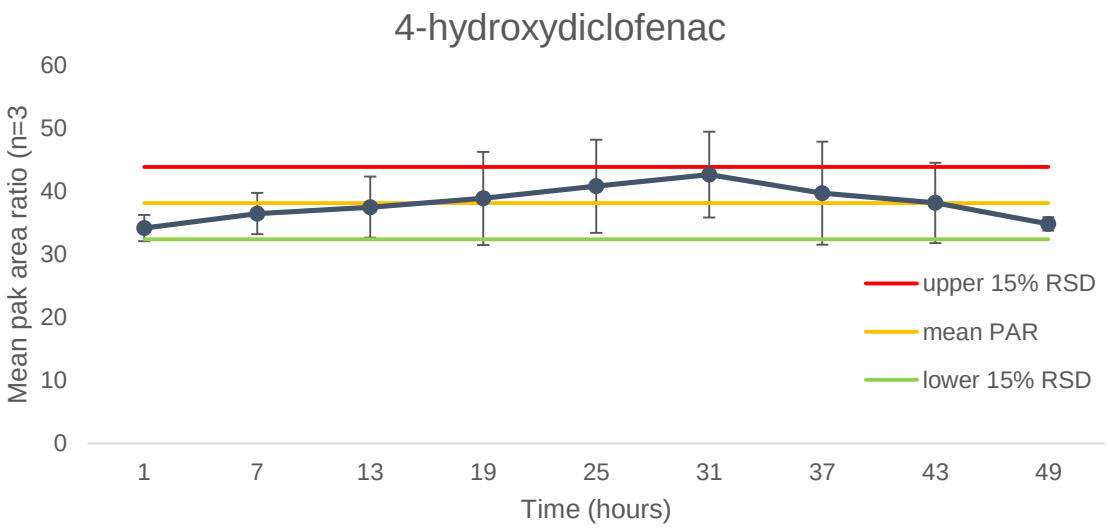


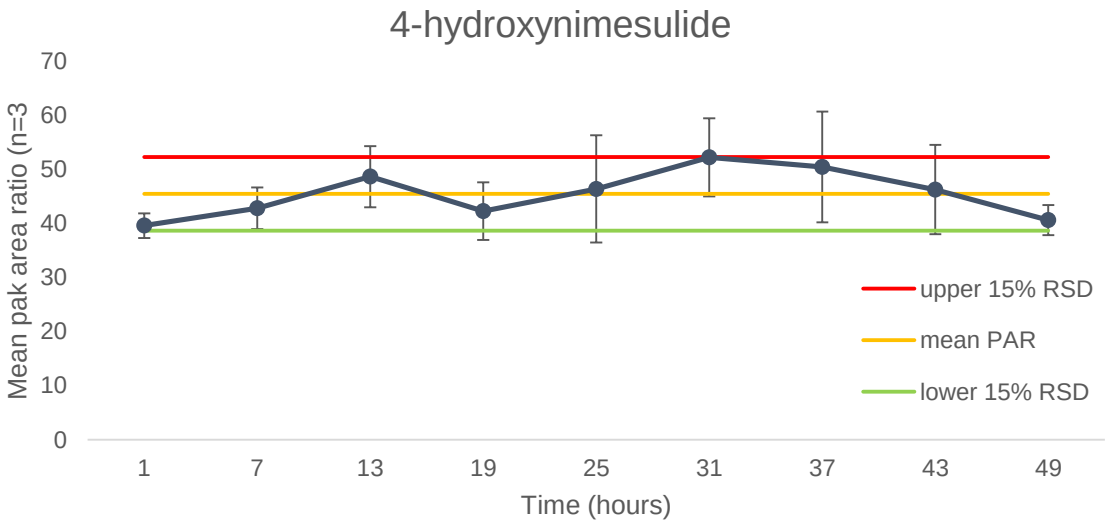




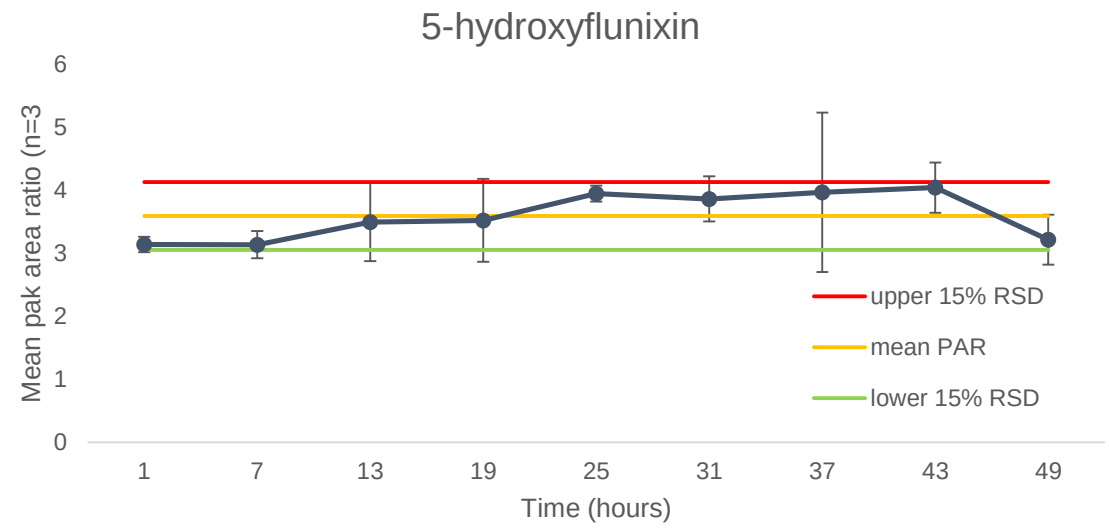
Appendix II-e Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.5µg/ml

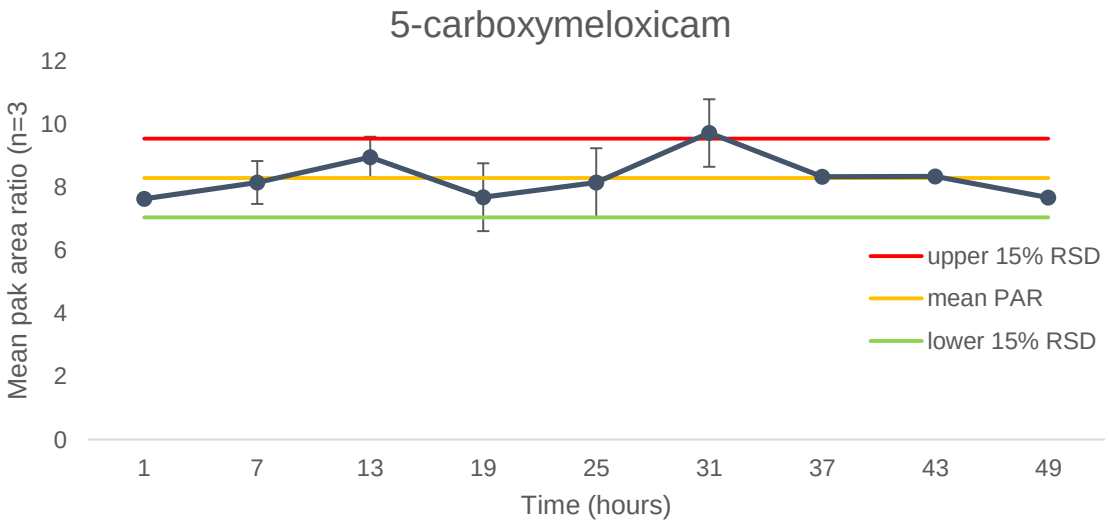


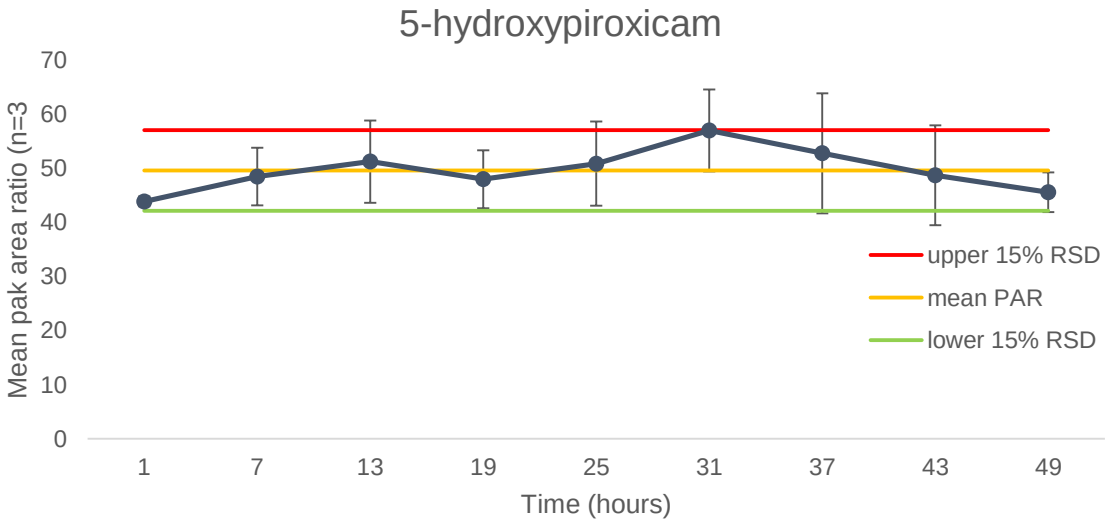




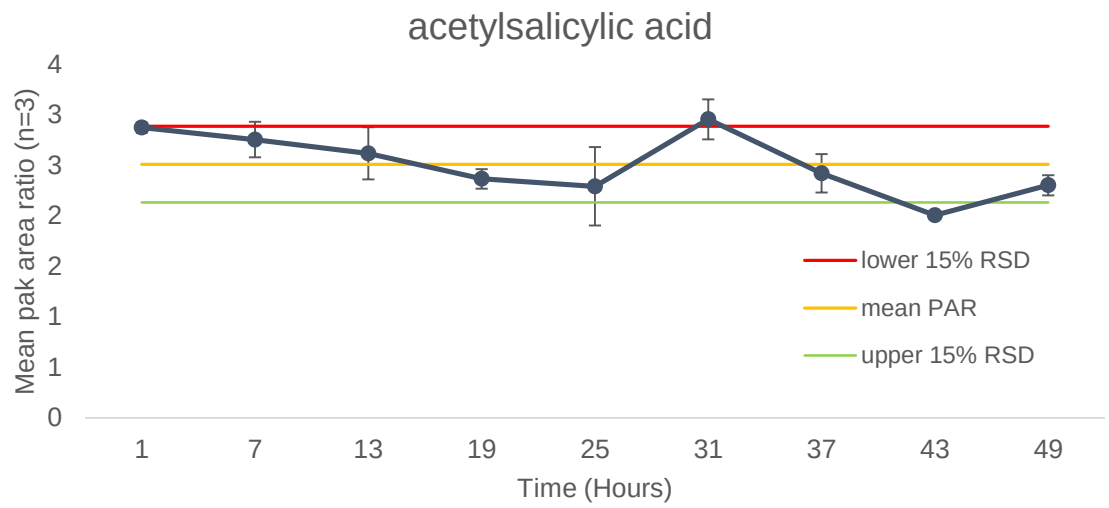
Appendix II-f Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 0.5µg/ml

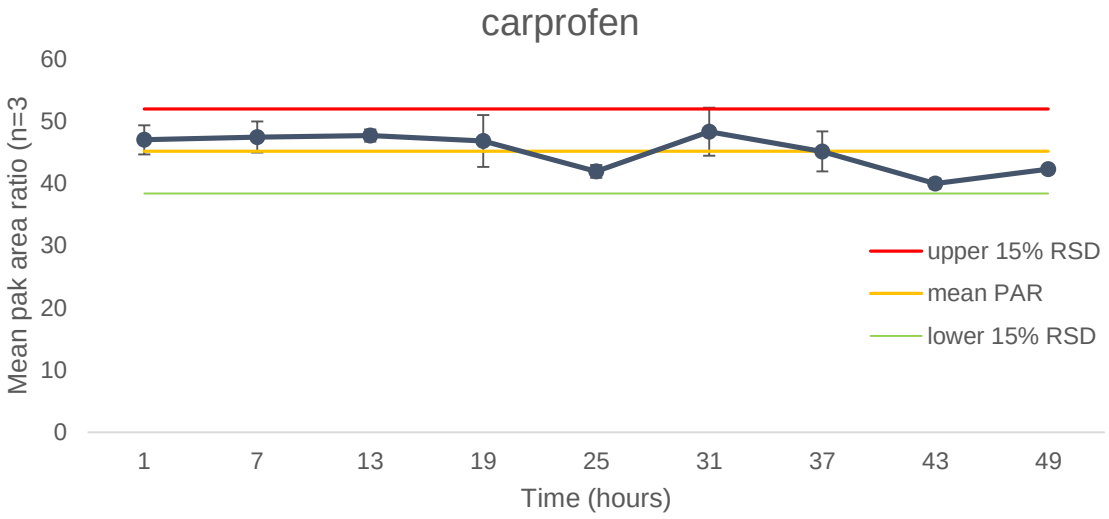


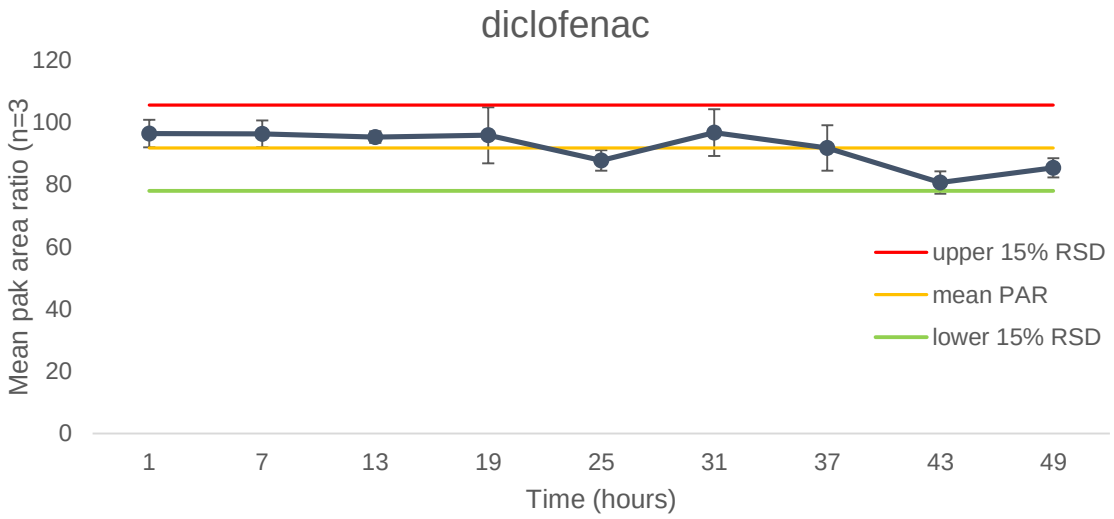




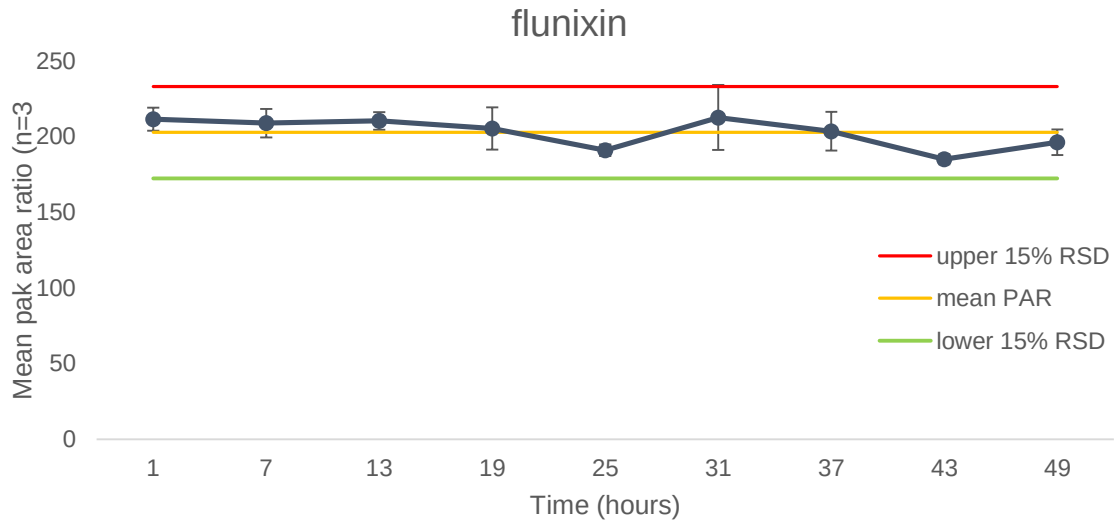
Appendix III-a Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 5µg/ml

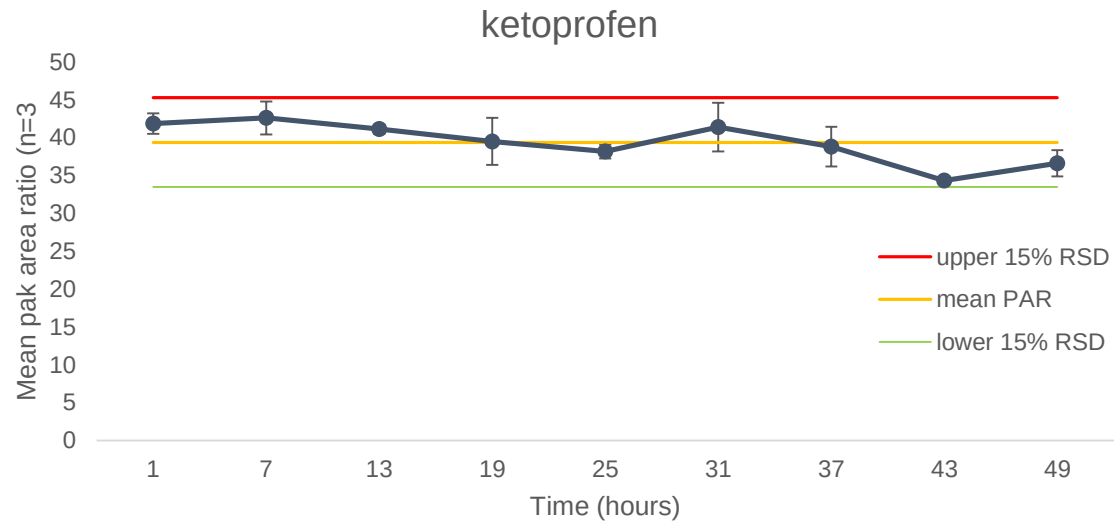


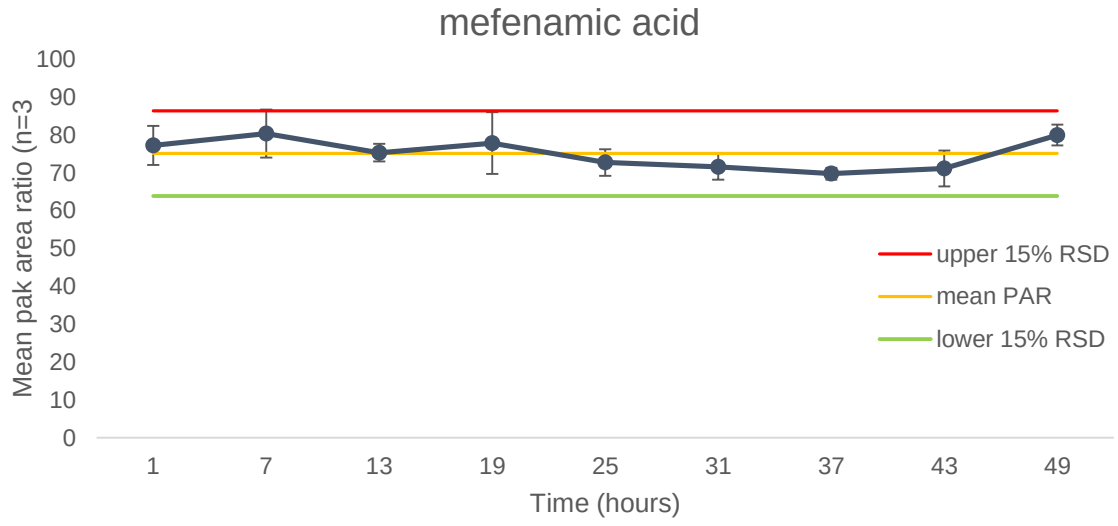




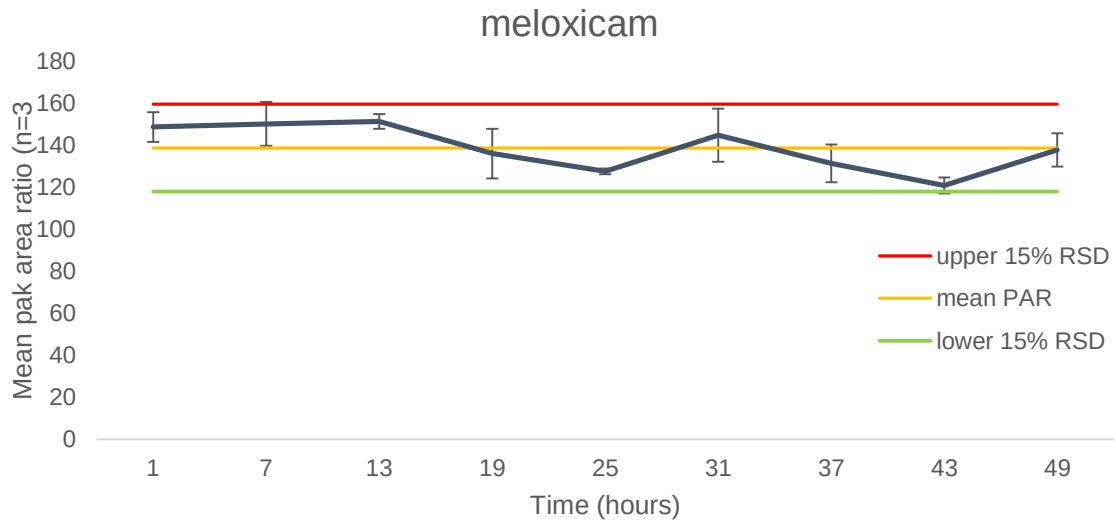
Appendix III-b Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 5µg/ml

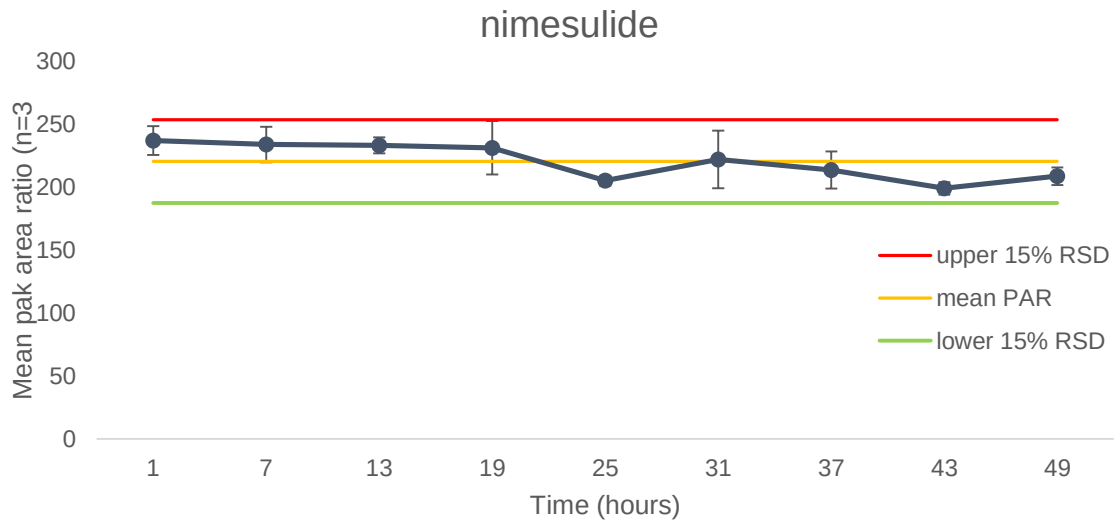


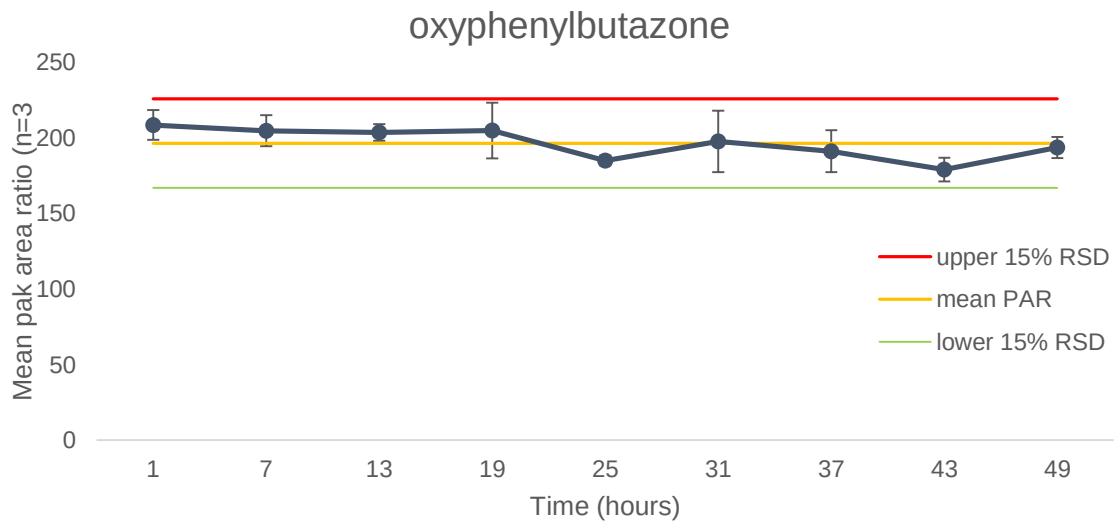




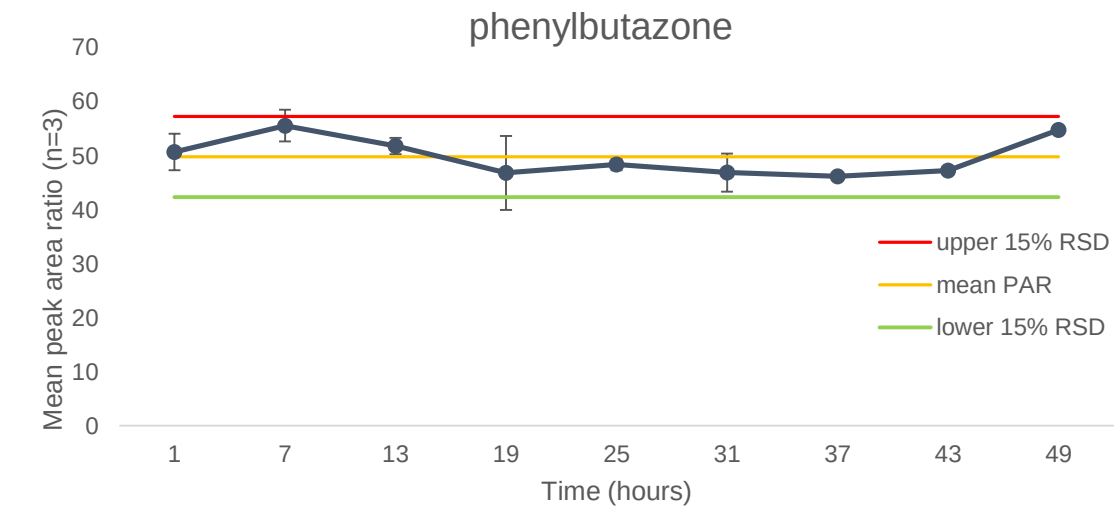
Appendix III-c Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 5µg/ml

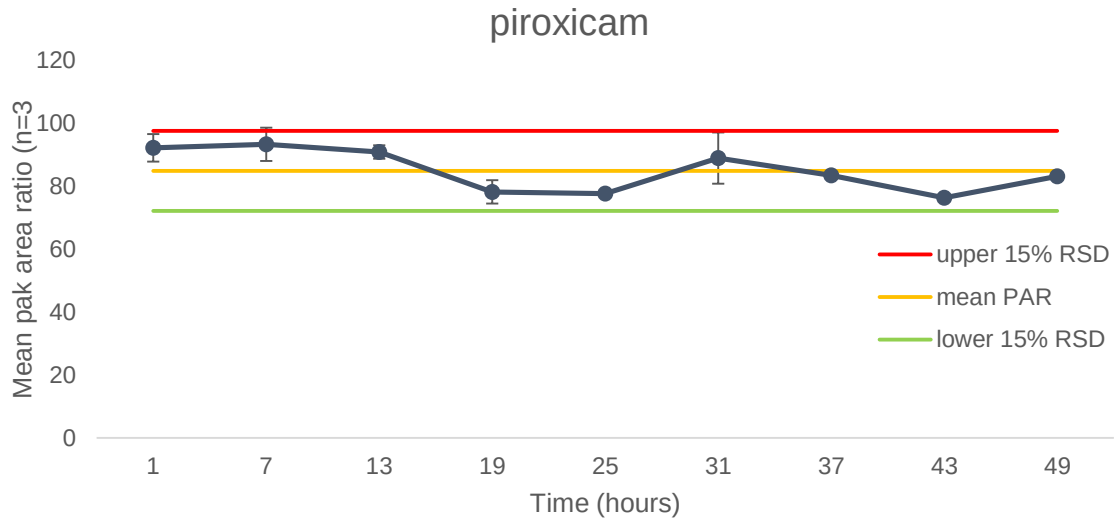


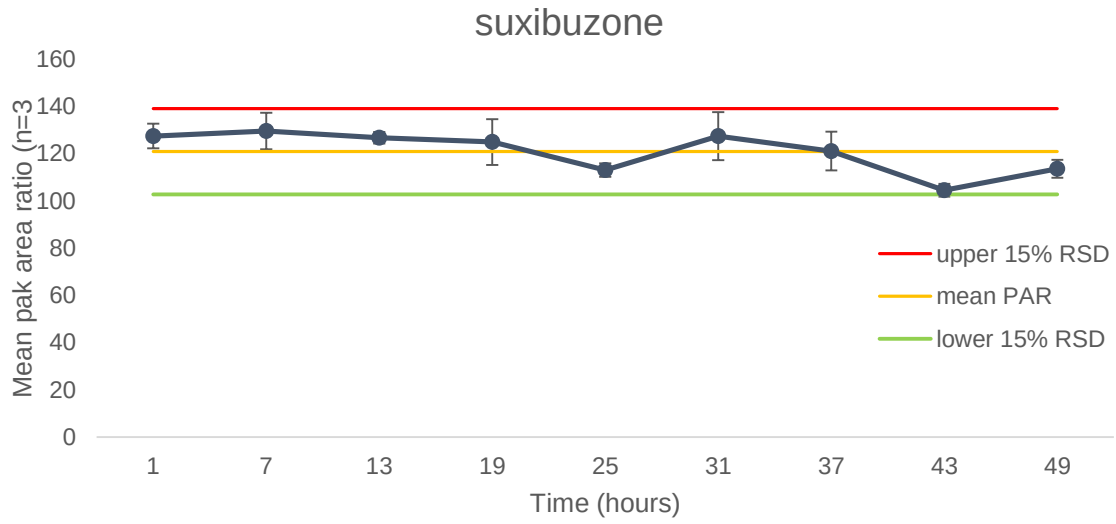




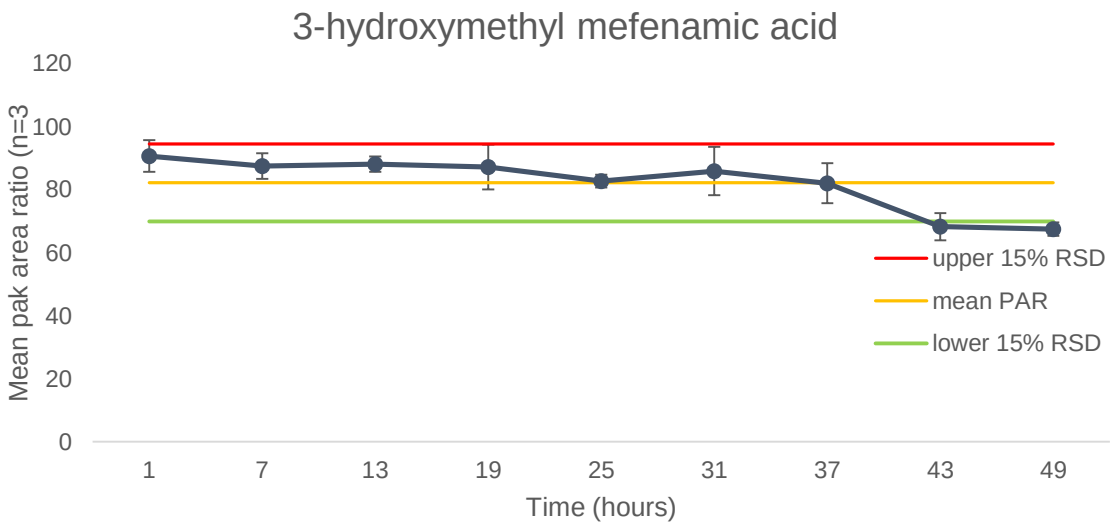
Appendix III-d Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 5µg/ml

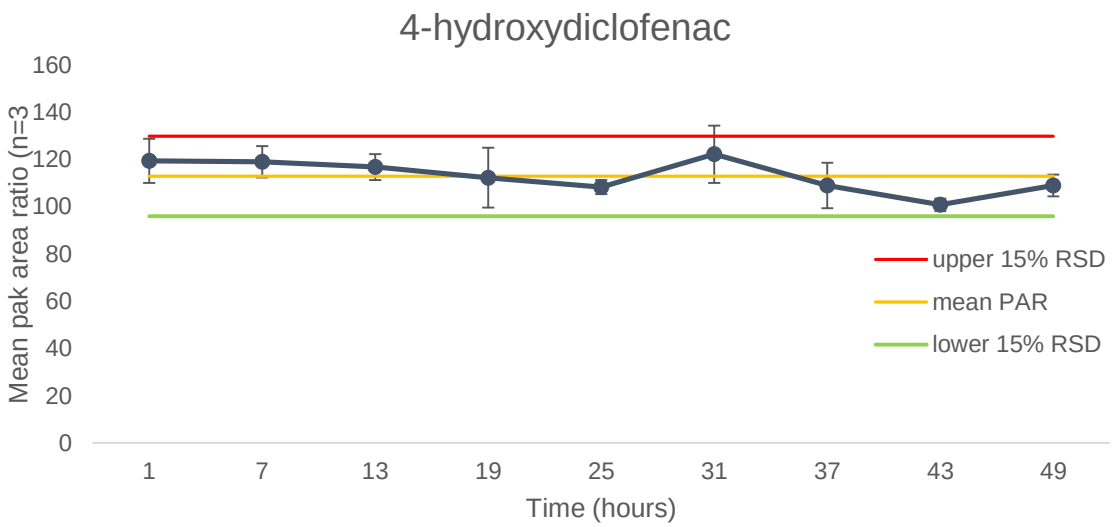


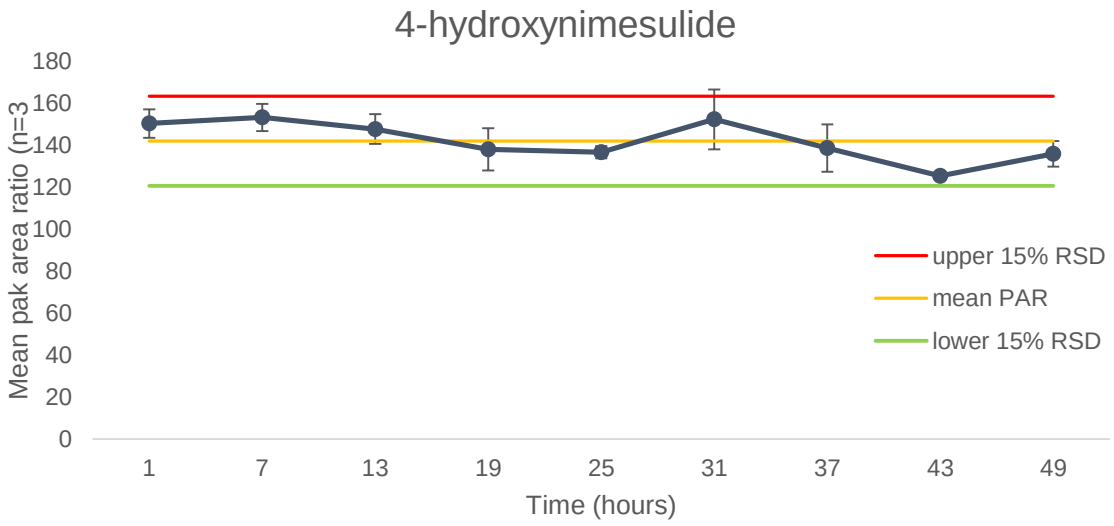




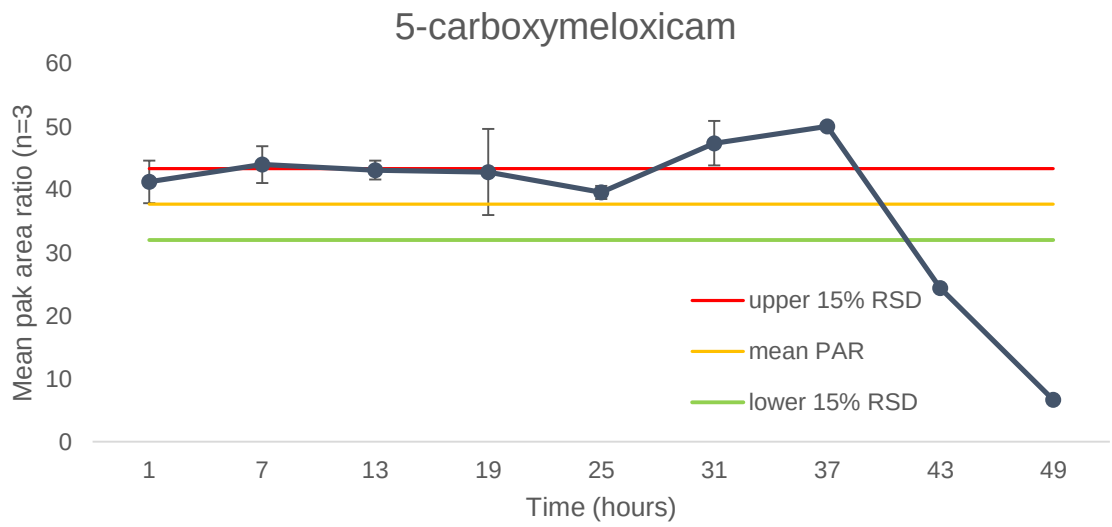
Appendix III-e Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 5µg/ml

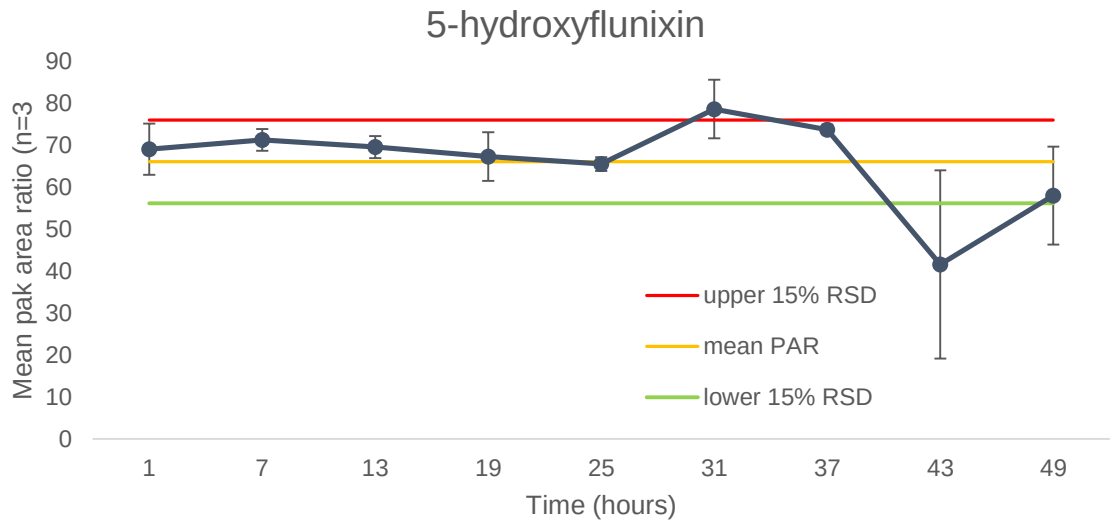


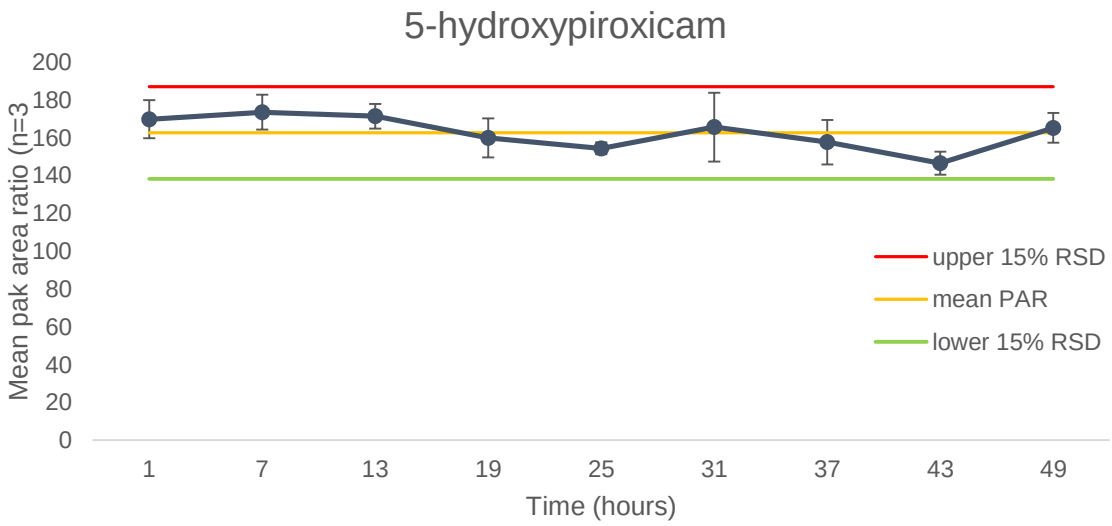




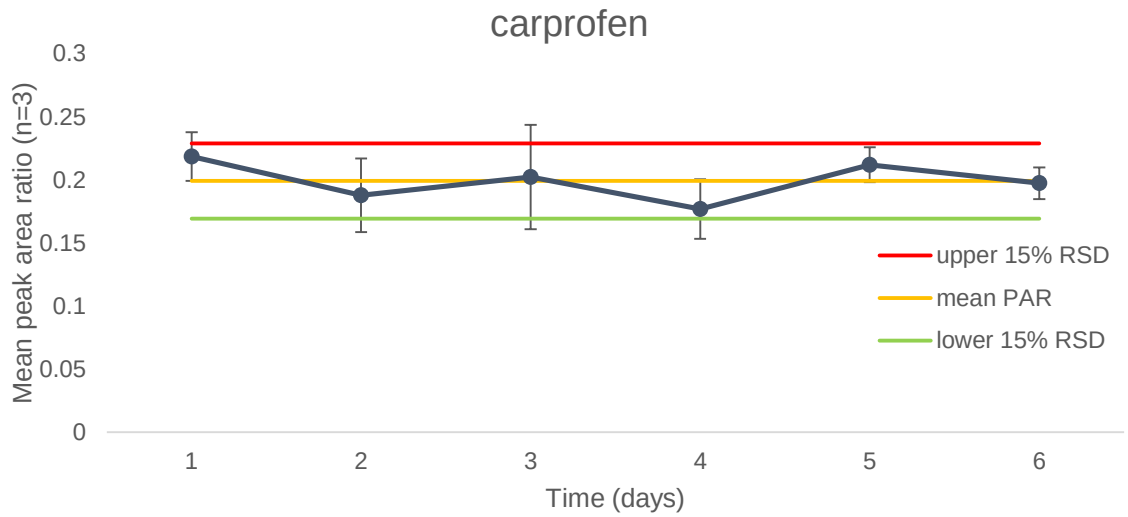
Appendix III-f Graph of mean peak area ratio (n=3) over 54 hours stability trial studied at 5µg/ml

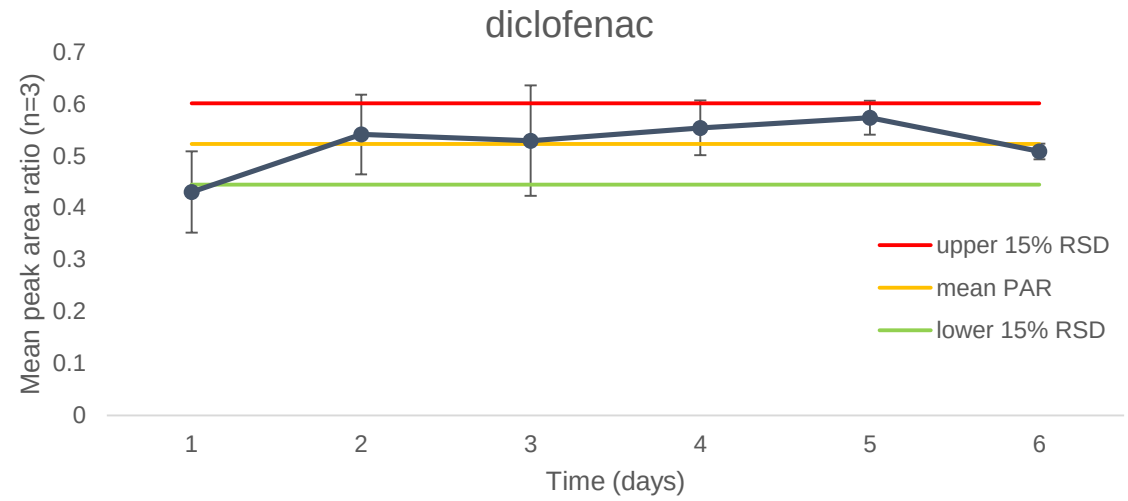


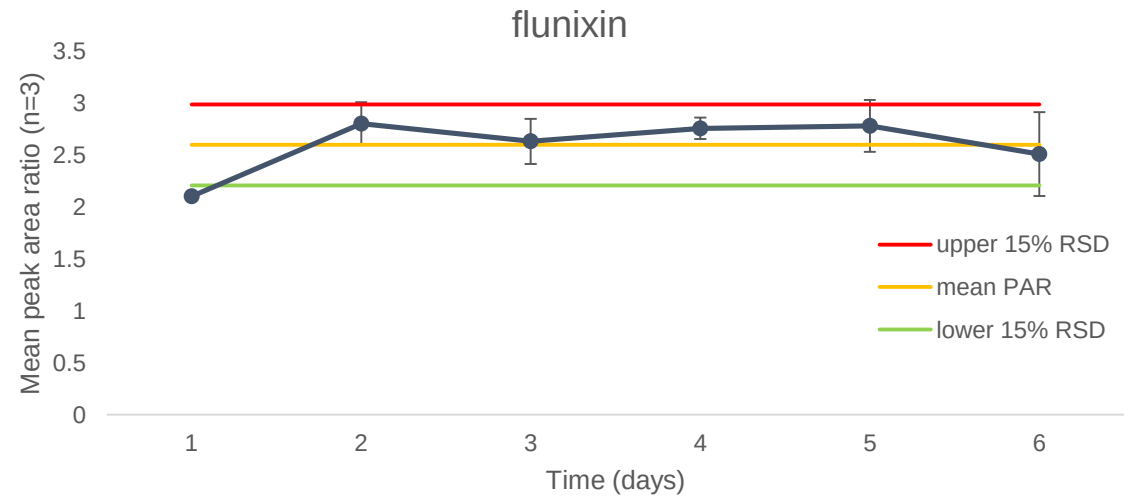




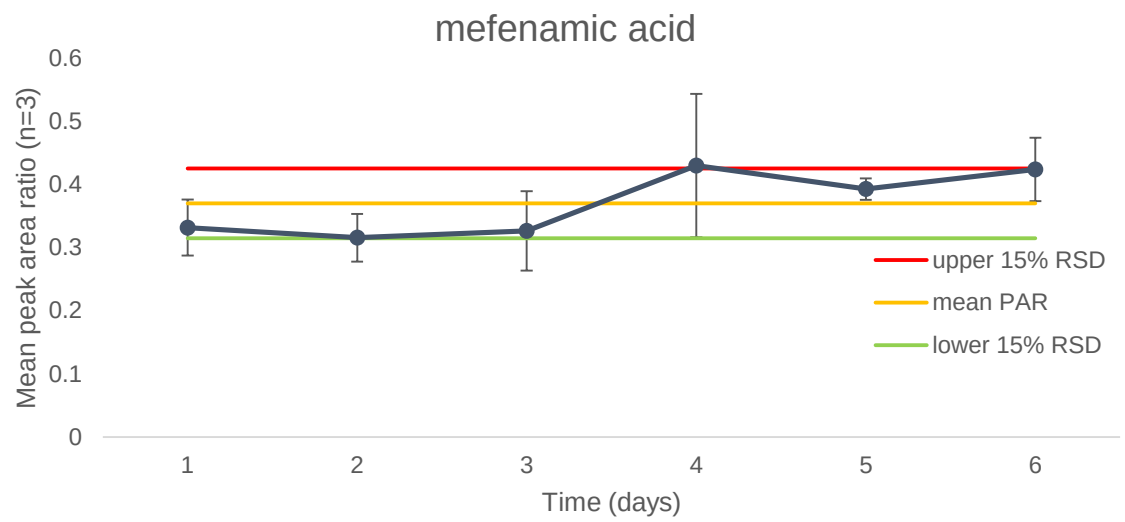
Appendix IV-a Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.05µg/ml

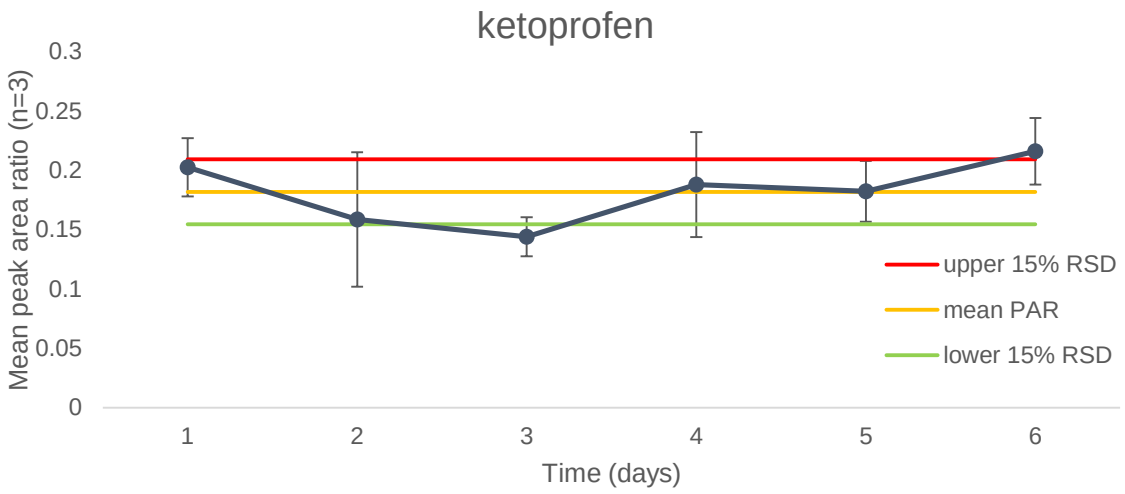


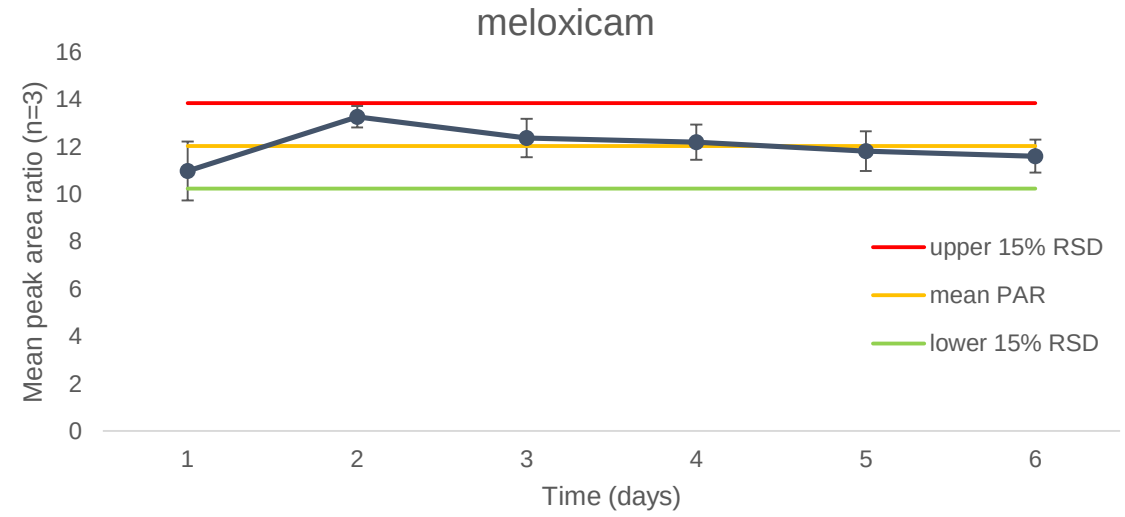




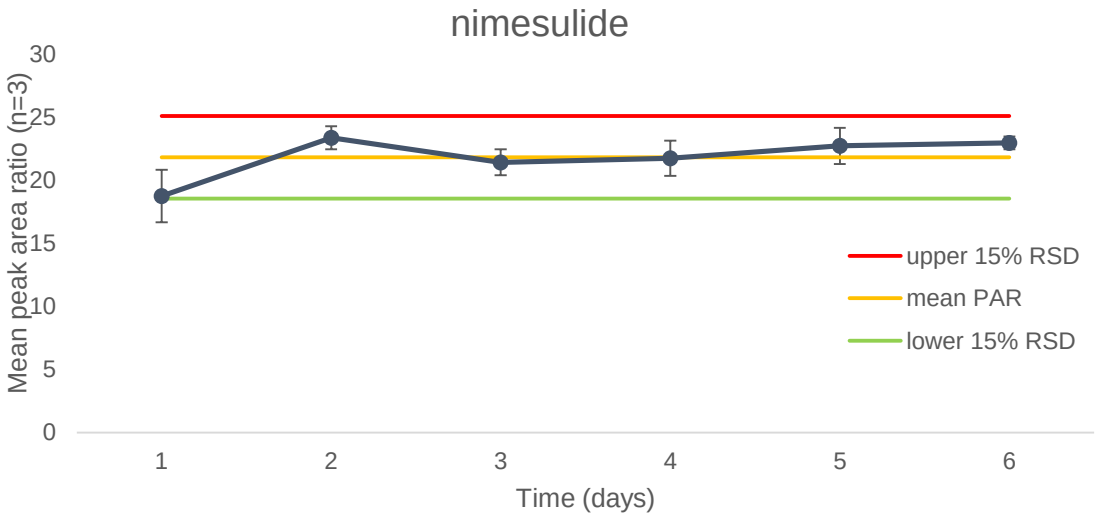
Appendix IV-b Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.05µg/ml

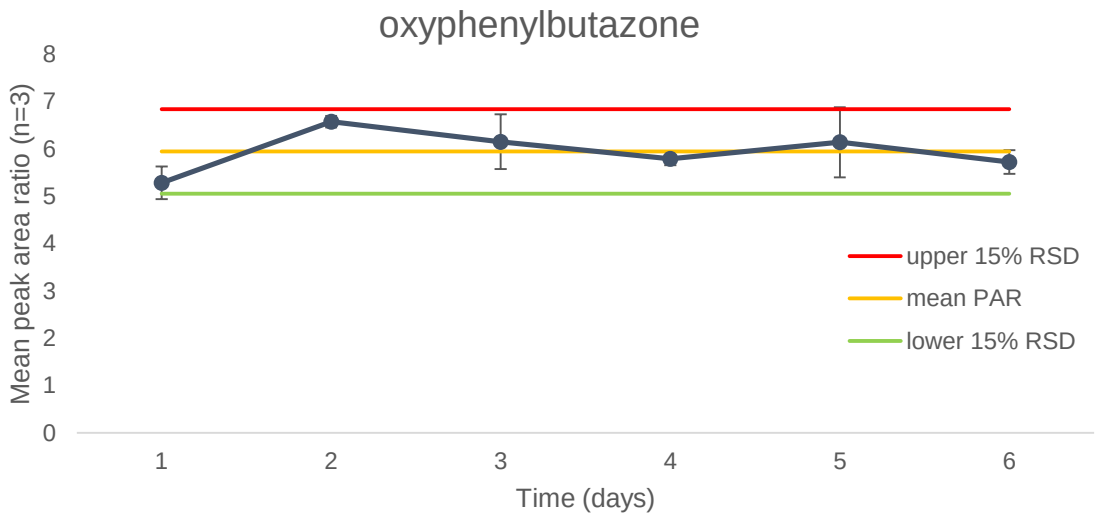


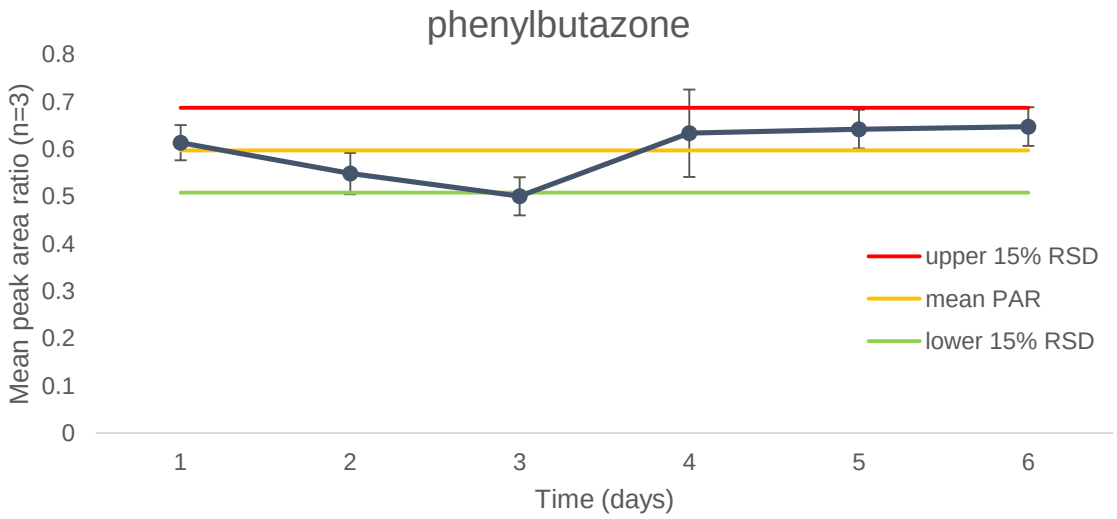




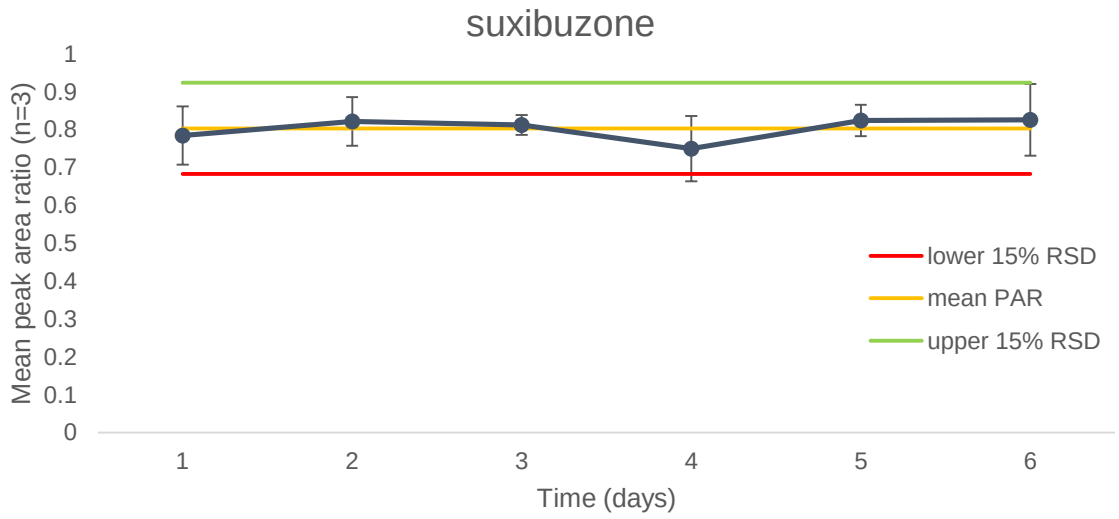
Appendix IV-c Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.05µg/ml

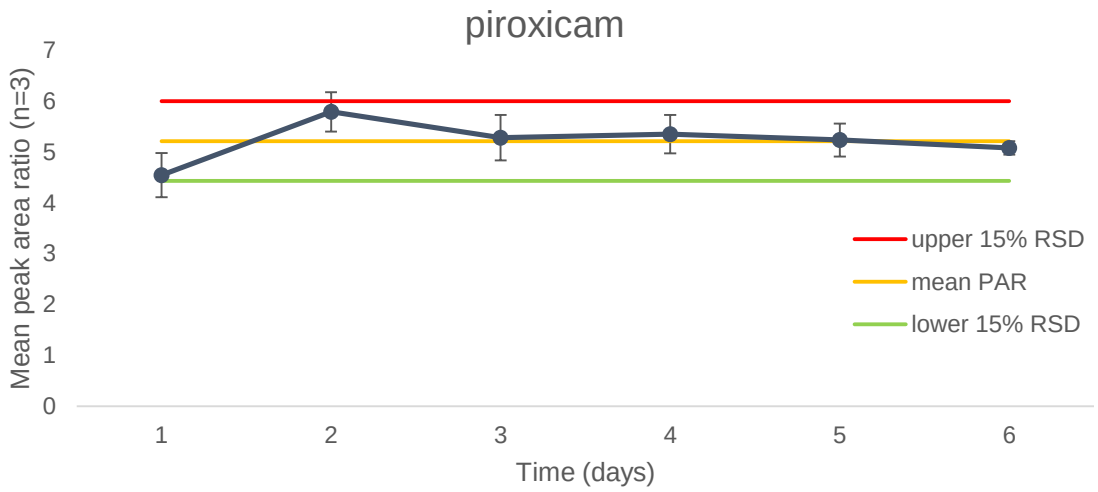


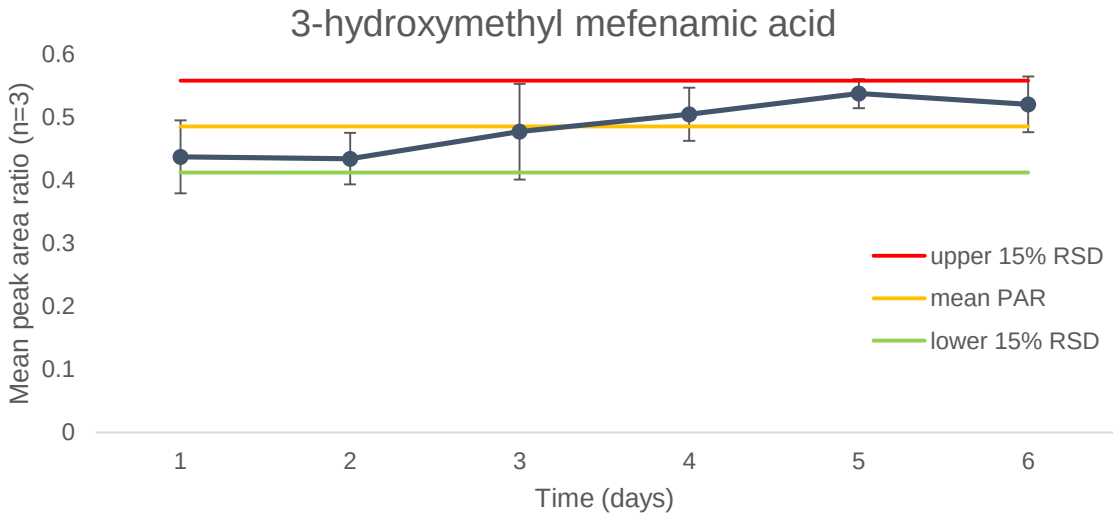




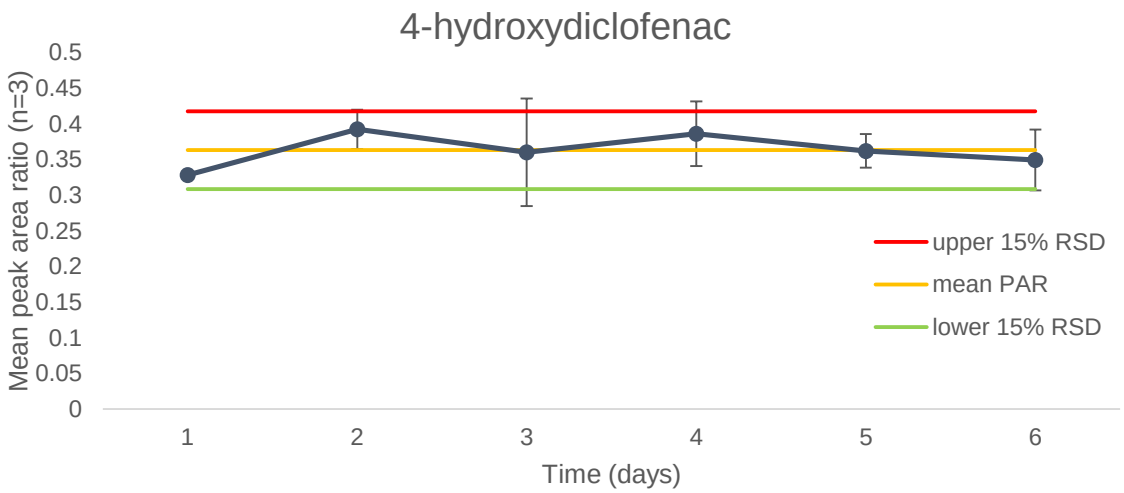
Appendix IV-d Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.05µg/ml

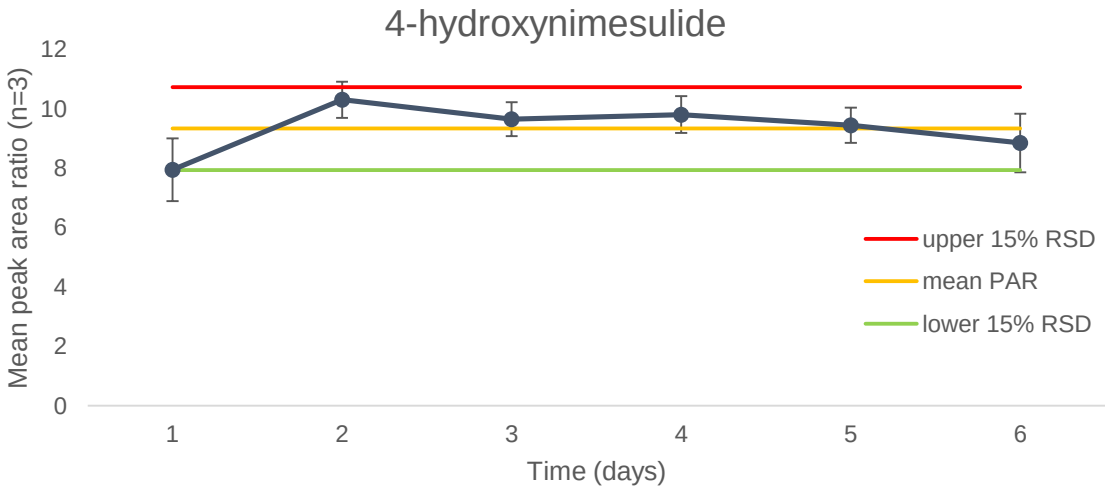


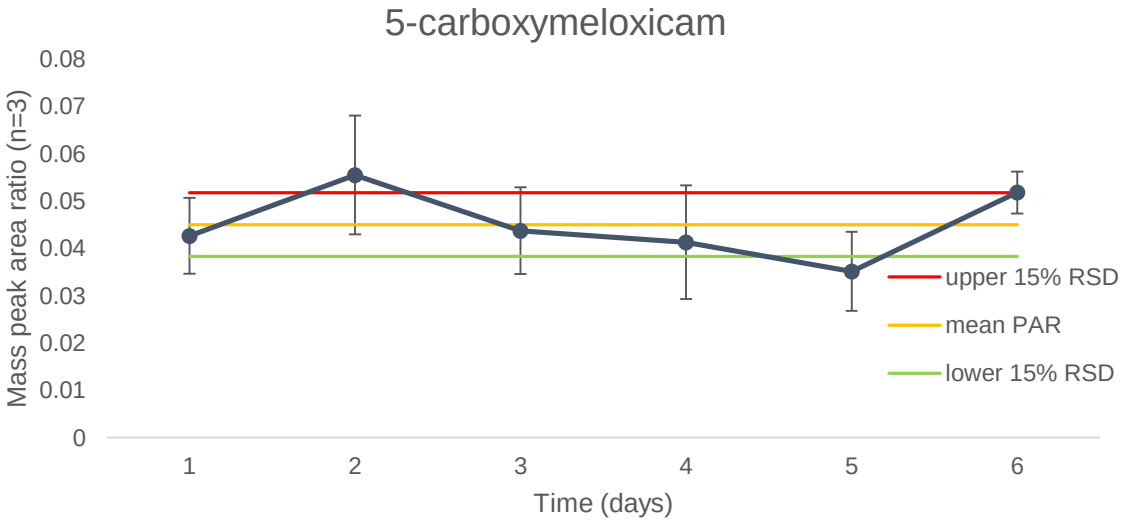




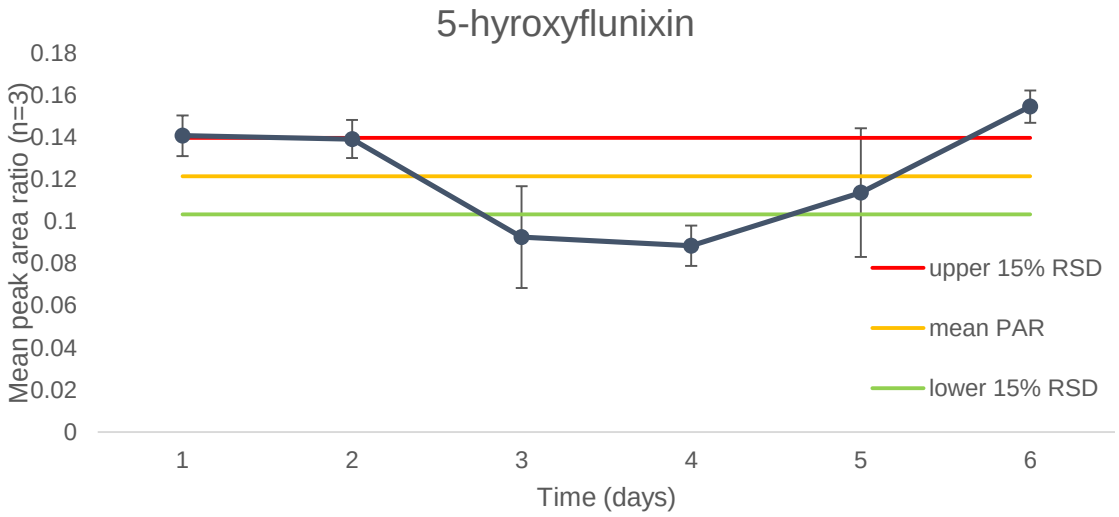
Appendix IV-e Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.05µg/ml

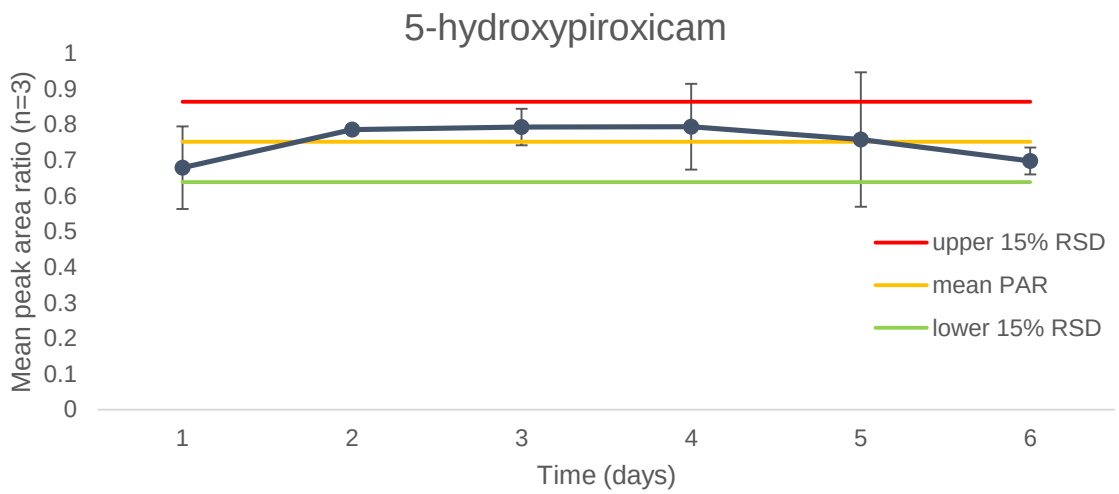




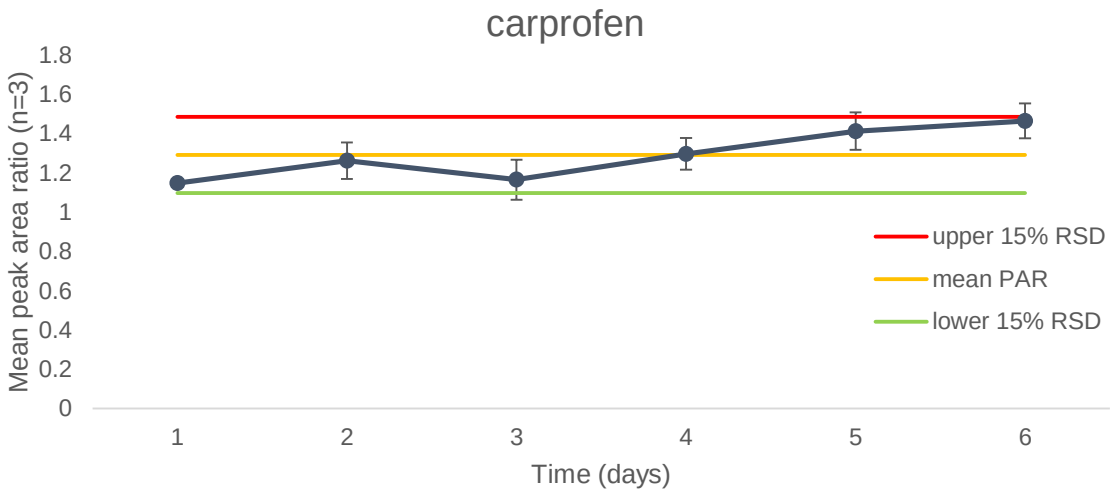


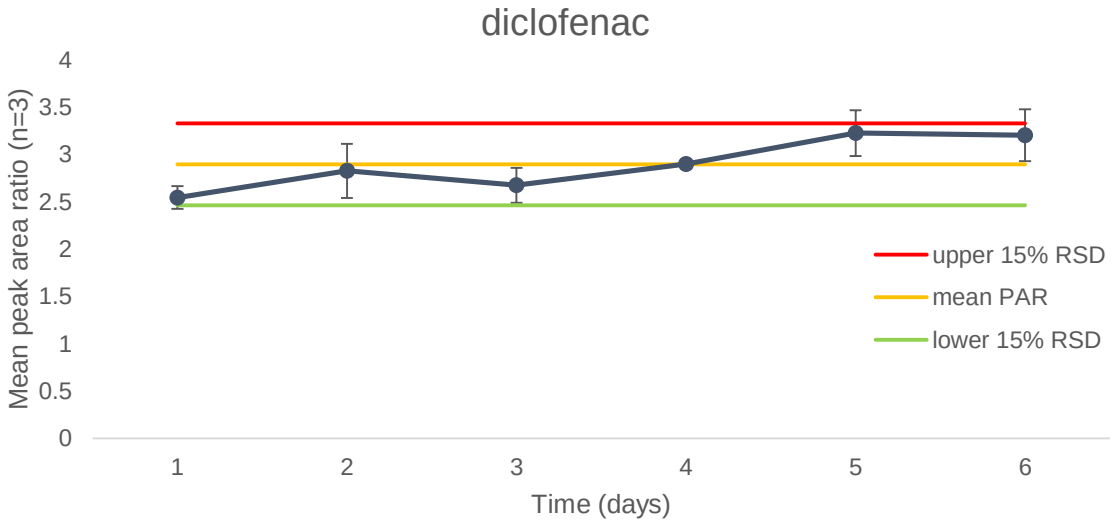
Appendix IV-f Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.05µg/ml

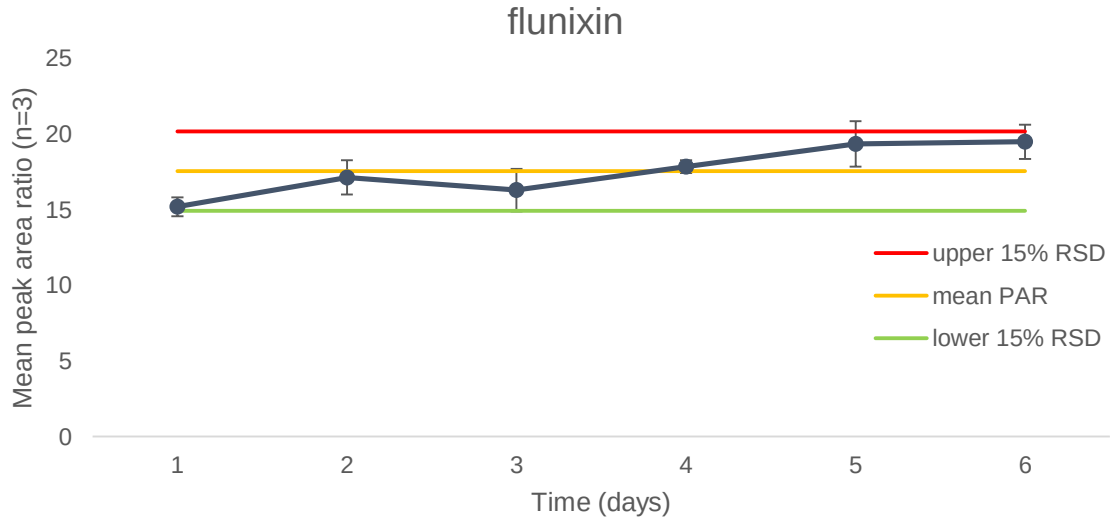




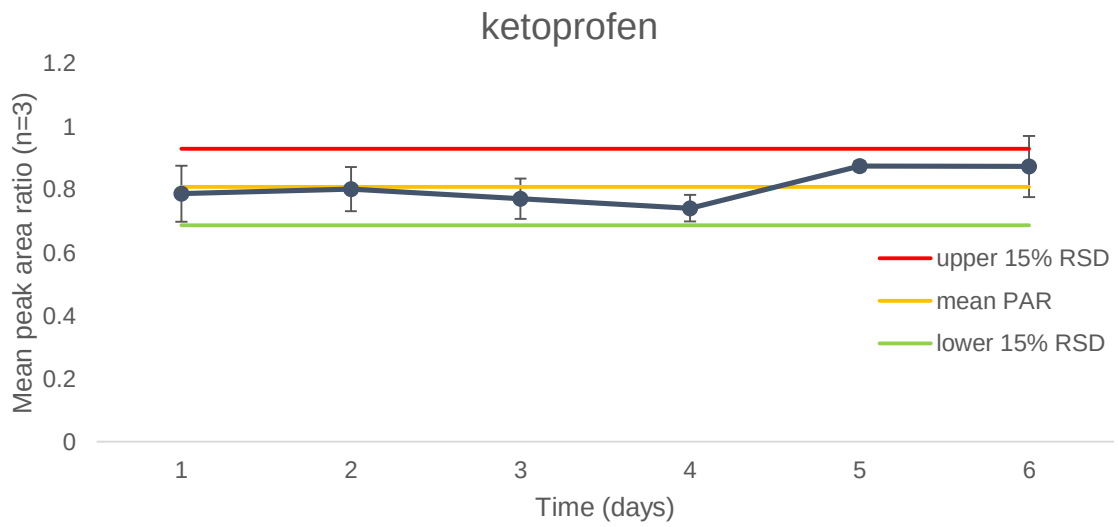
Appendix V-a Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.5µg/ml

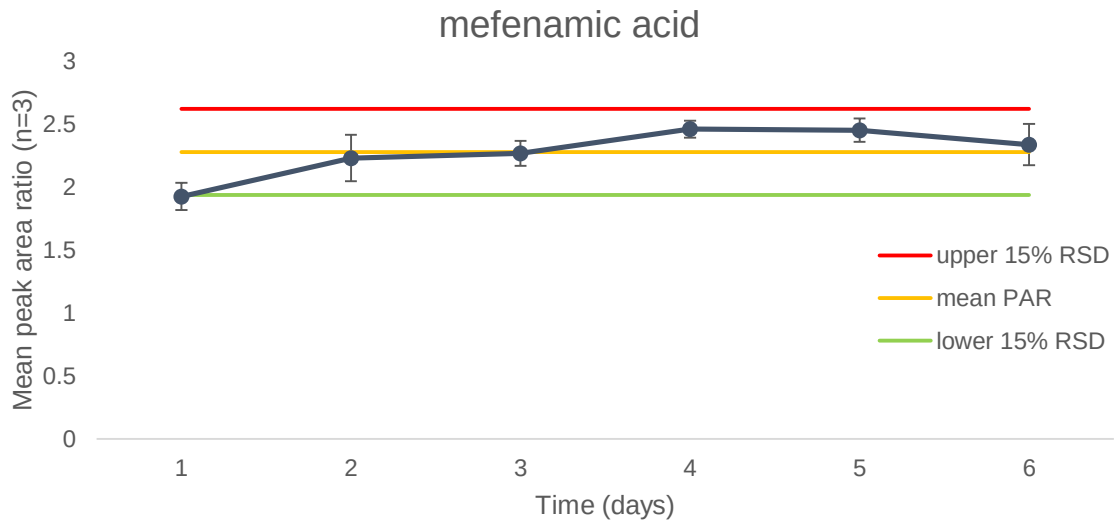


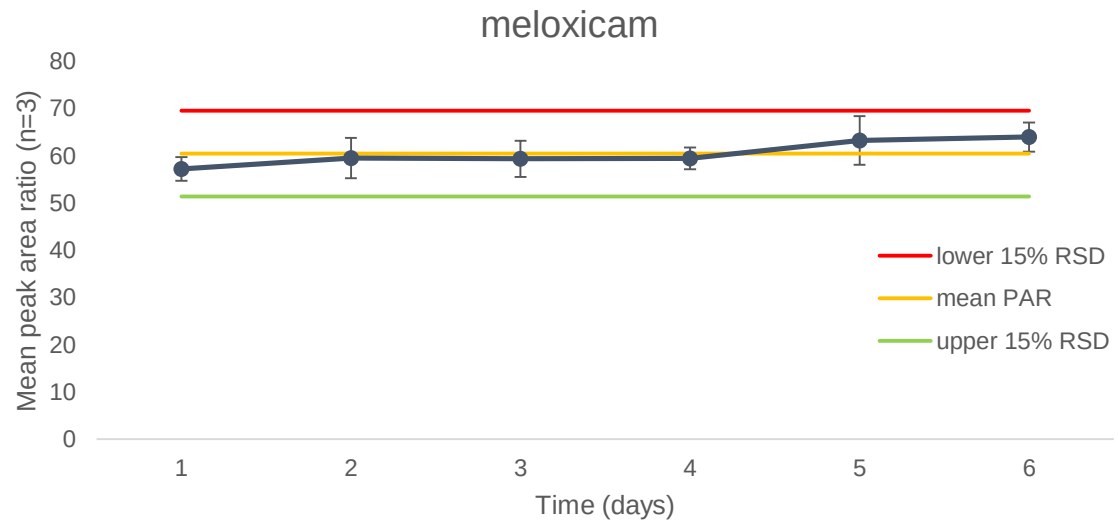




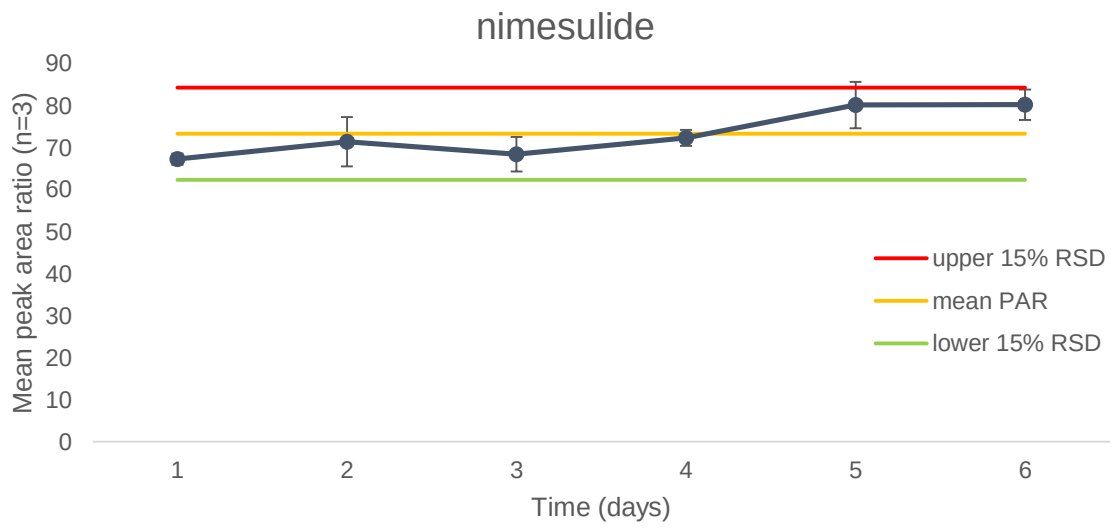
Appendix V-b Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.5µg/ml

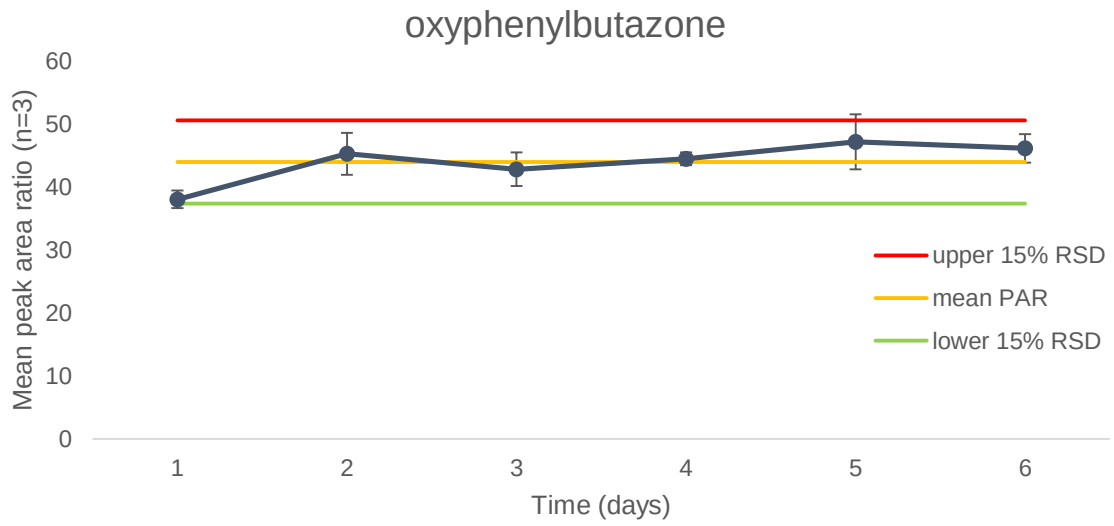


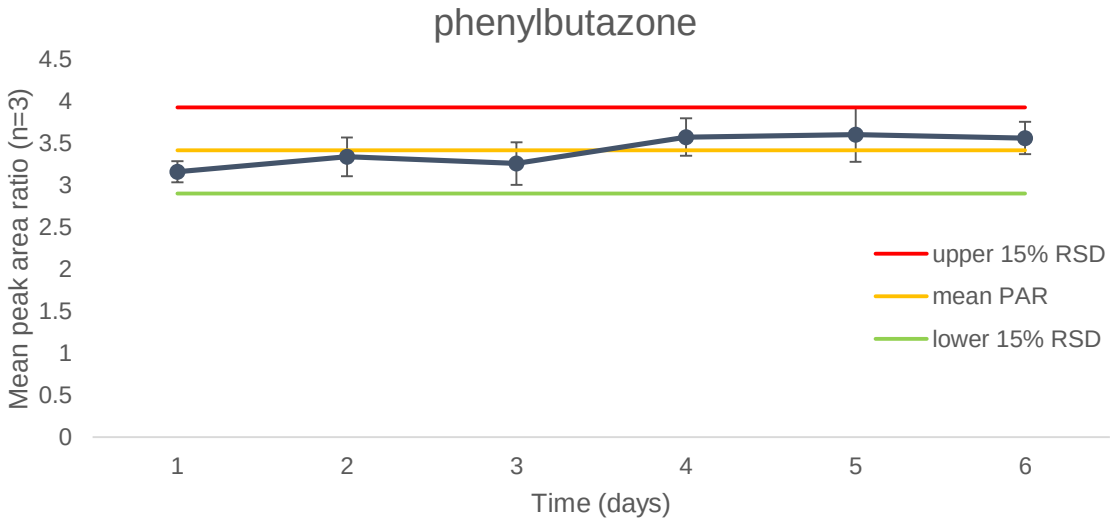




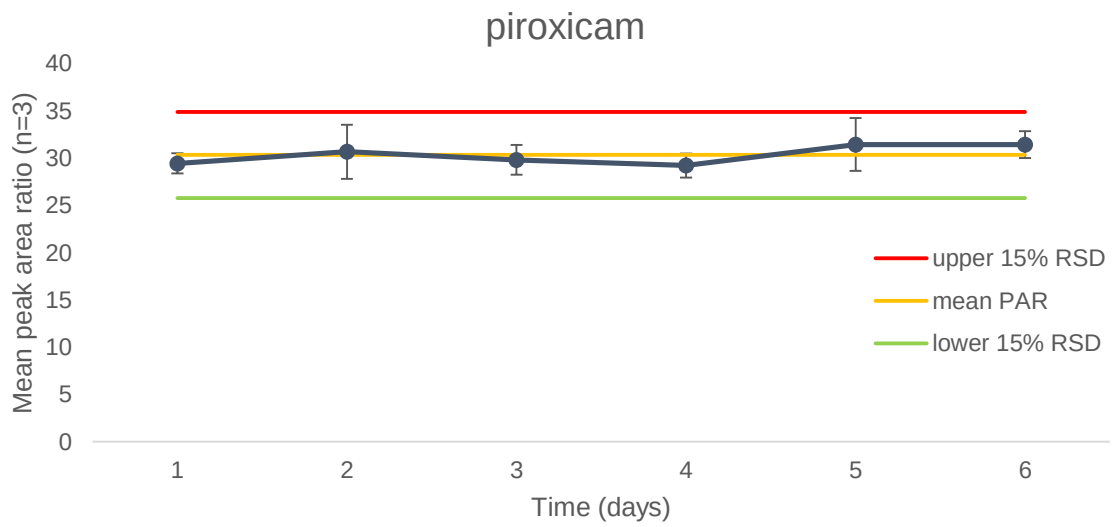
Appendix V-c Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.5µg/ml

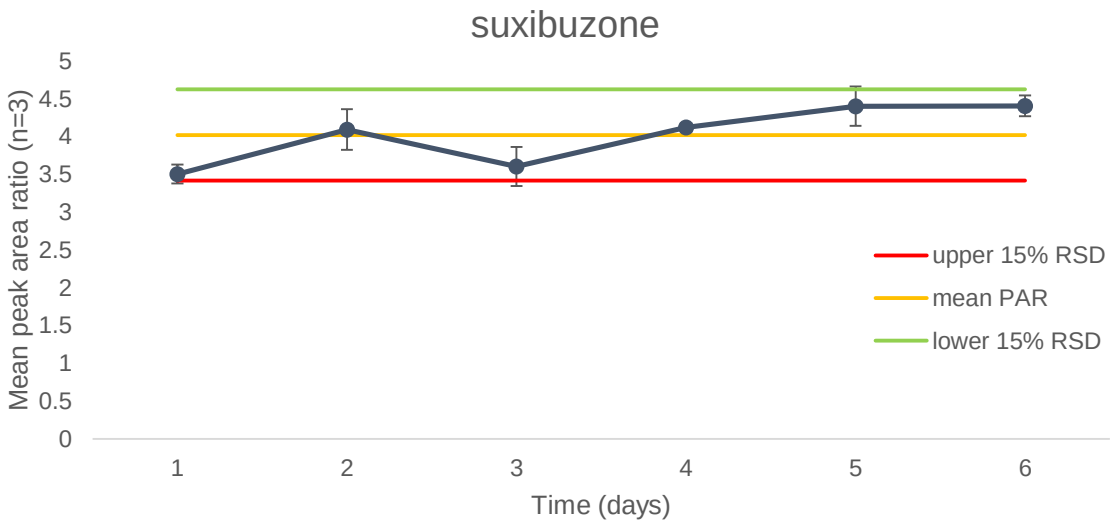


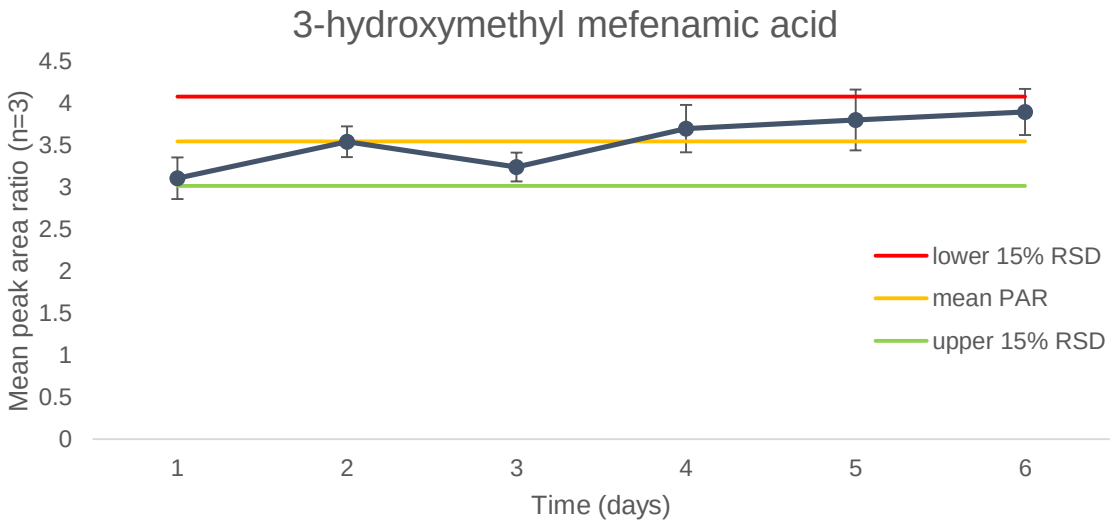




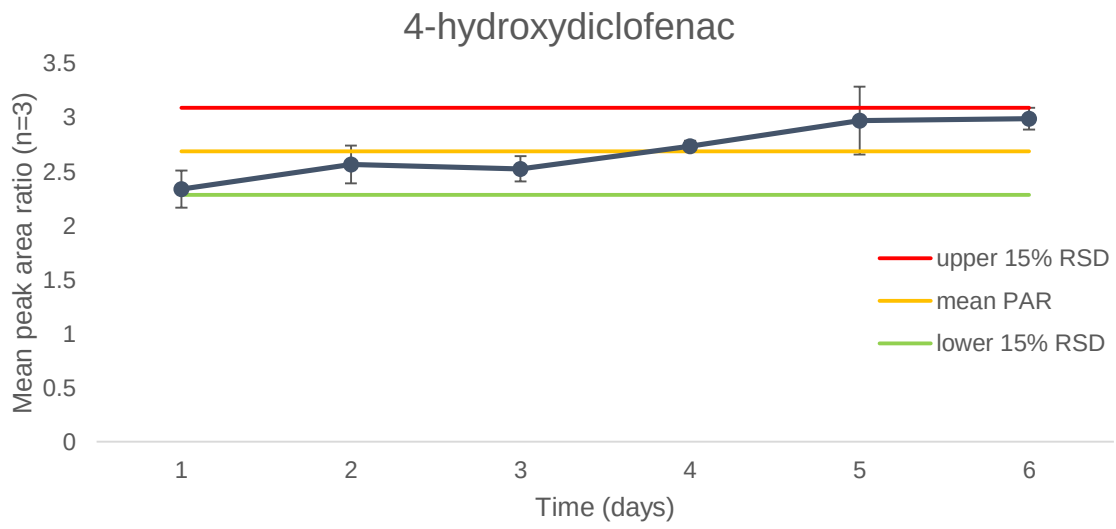
Appendix V-d Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.5µg/ml

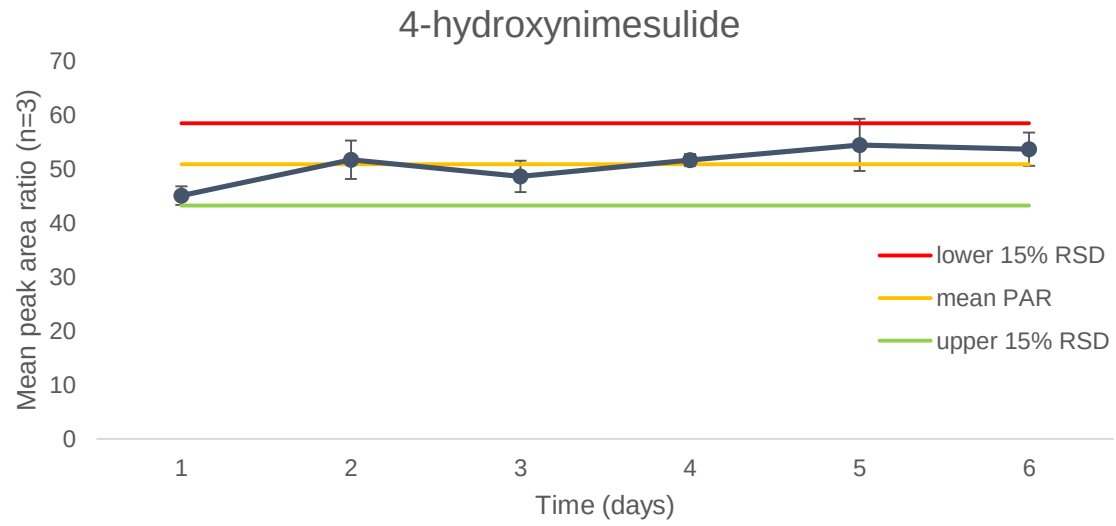


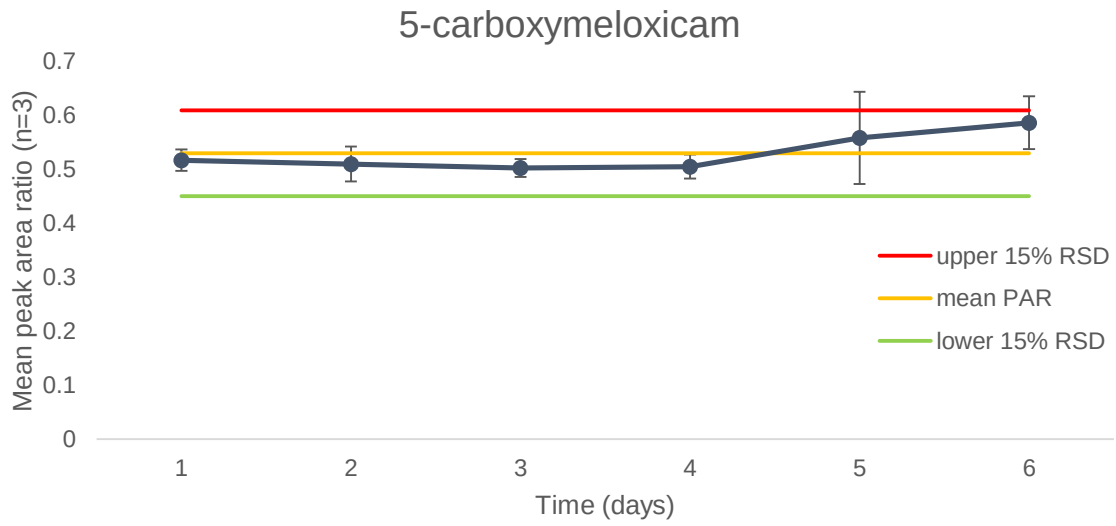




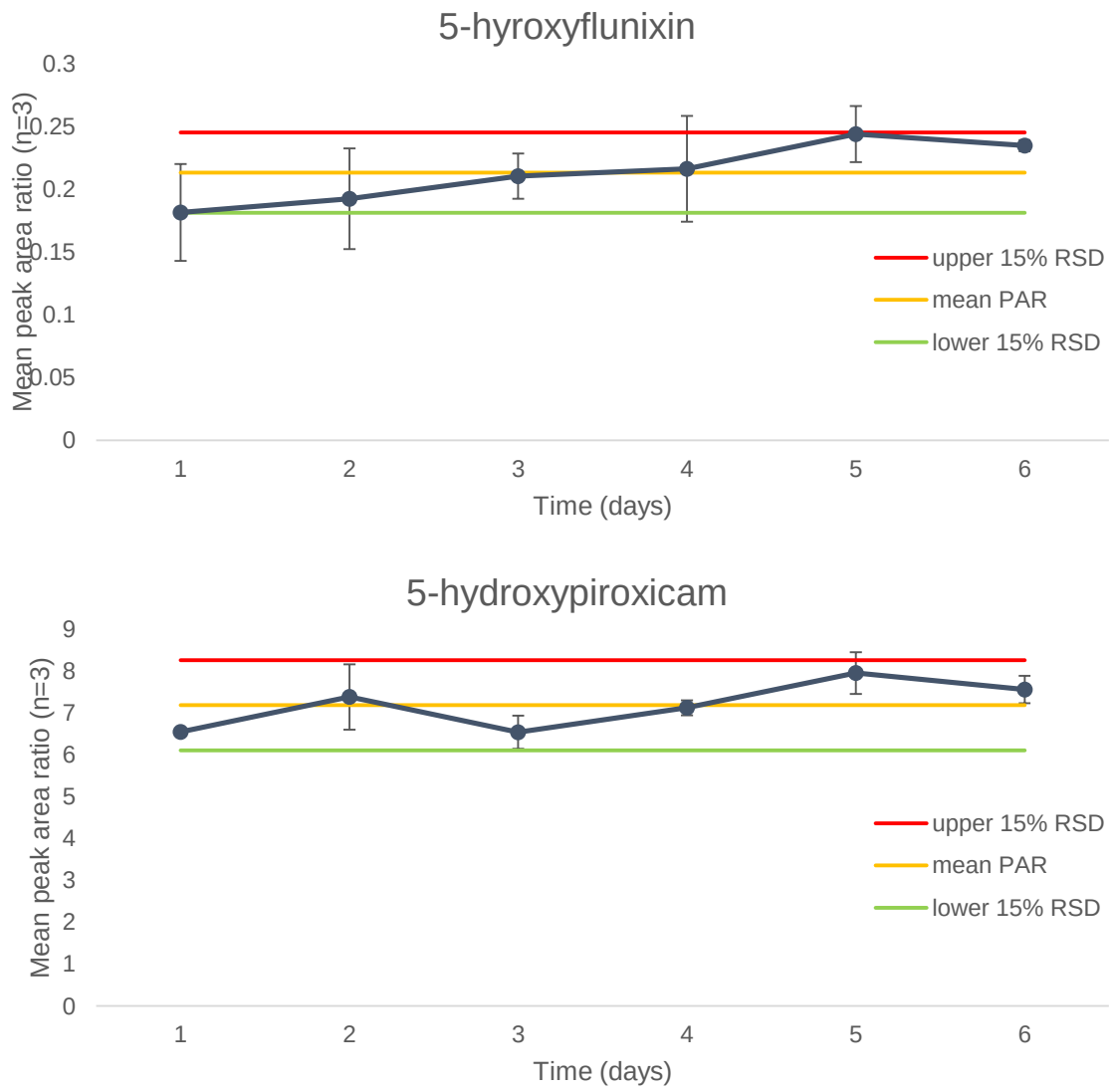
Appendix V-e Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.5µg/ml



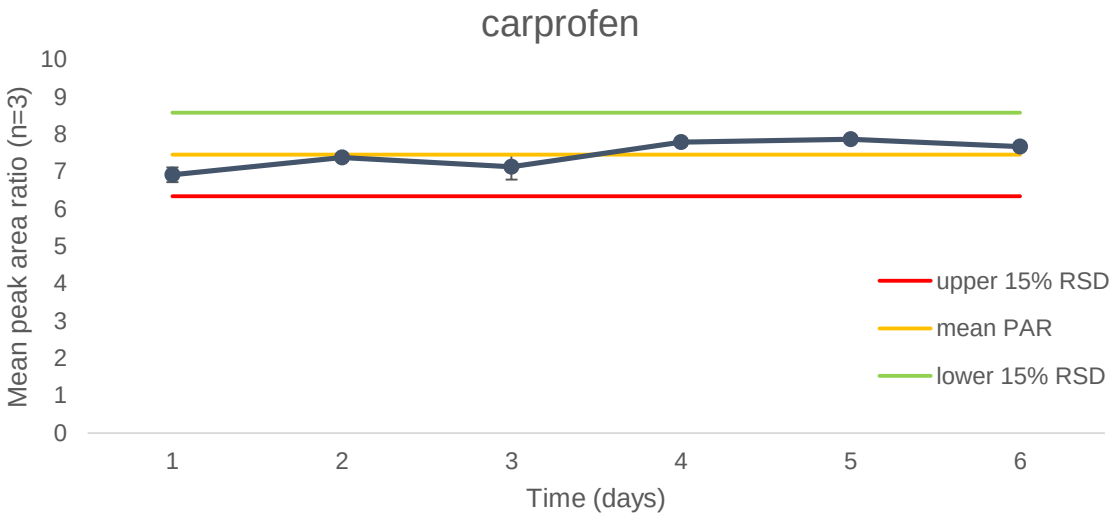


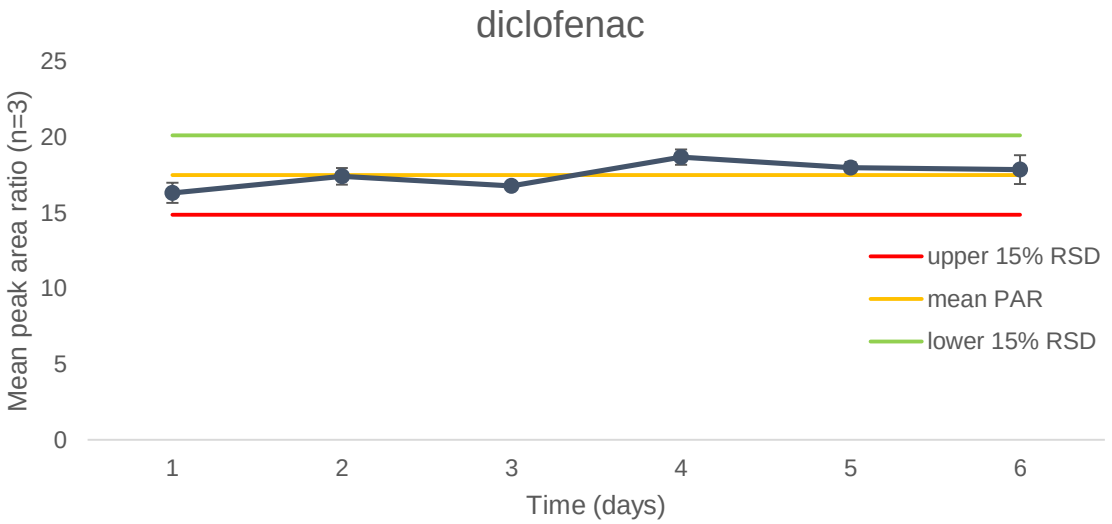


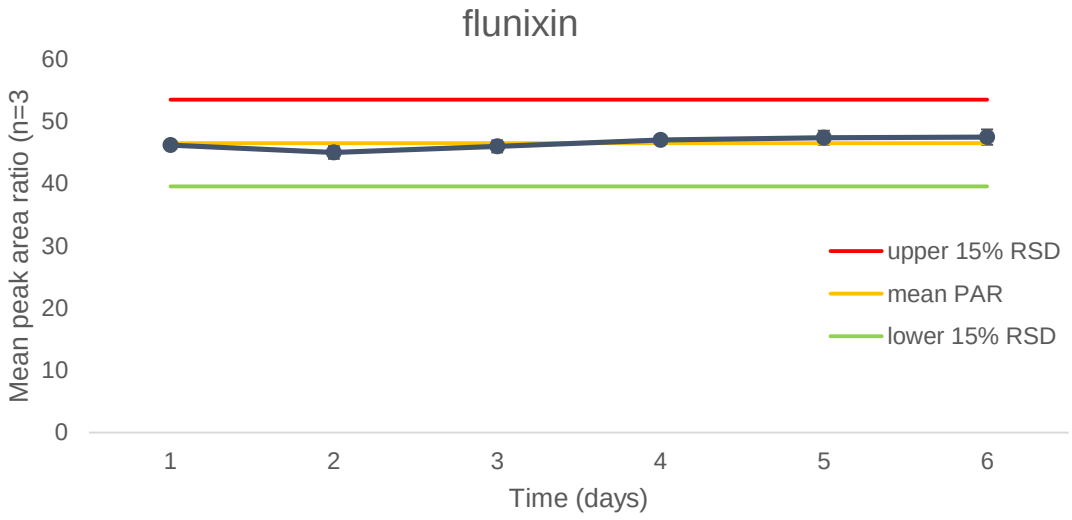
Appendix V-f Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 0.5µg/ml



Appendix VI-a Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 5µg/ml

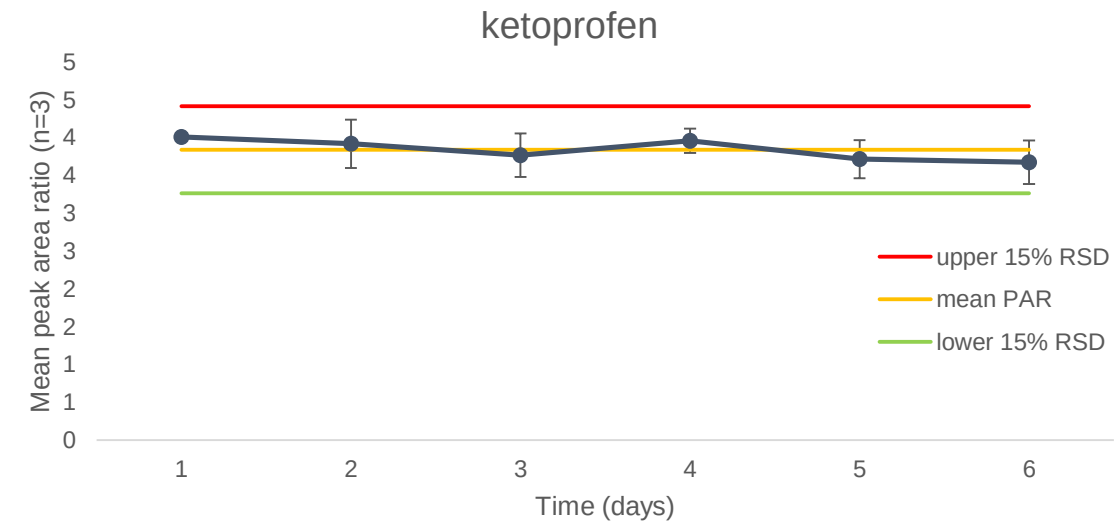


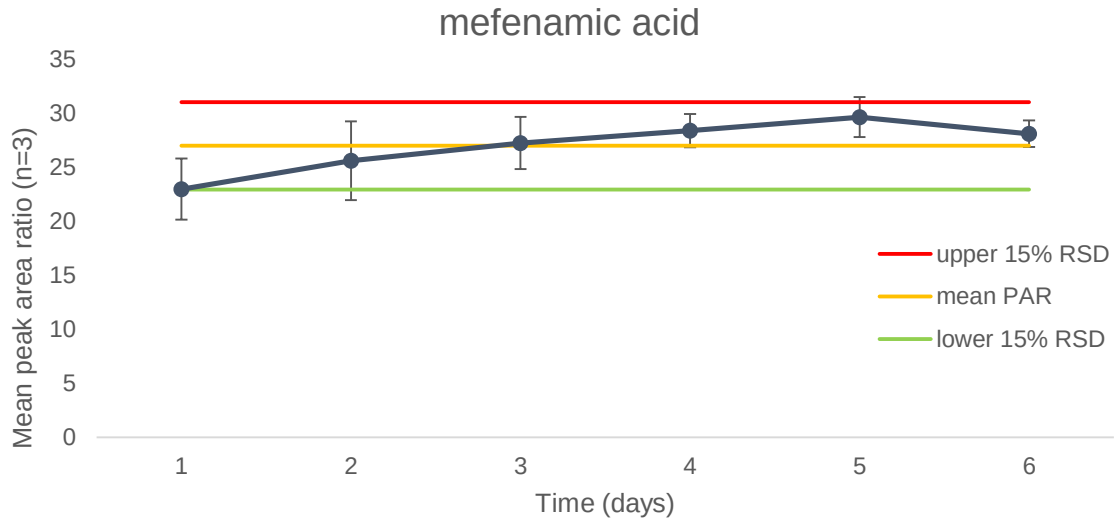


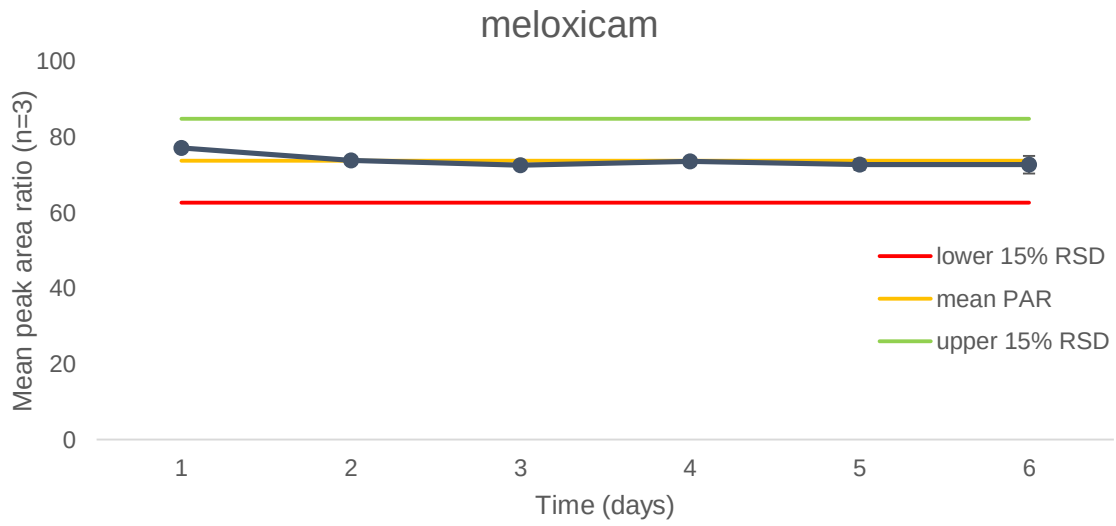


Appendix VI-b Graph of mean peak area ratio (n=3) over 6 days stability trial

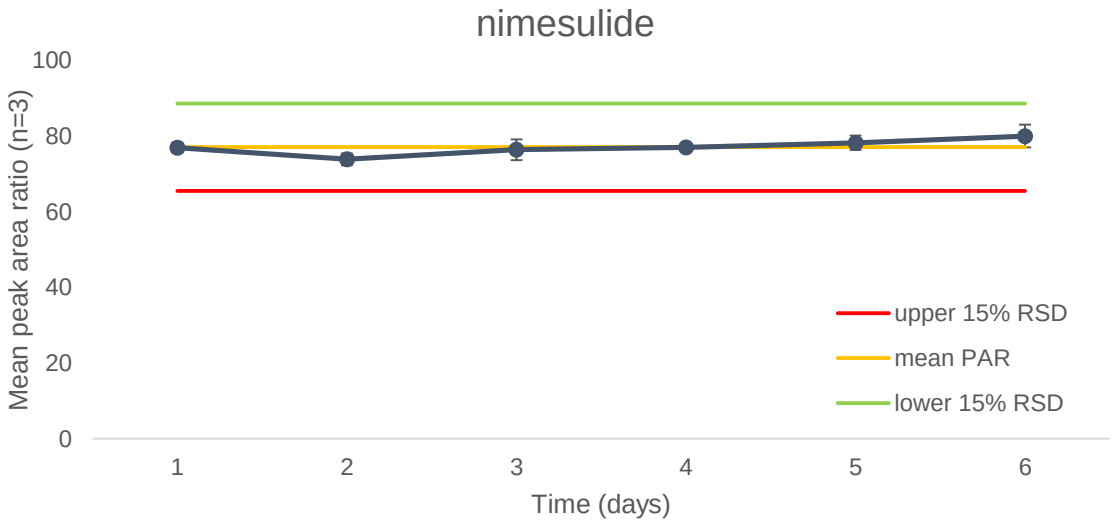
studied at 5µg/ml

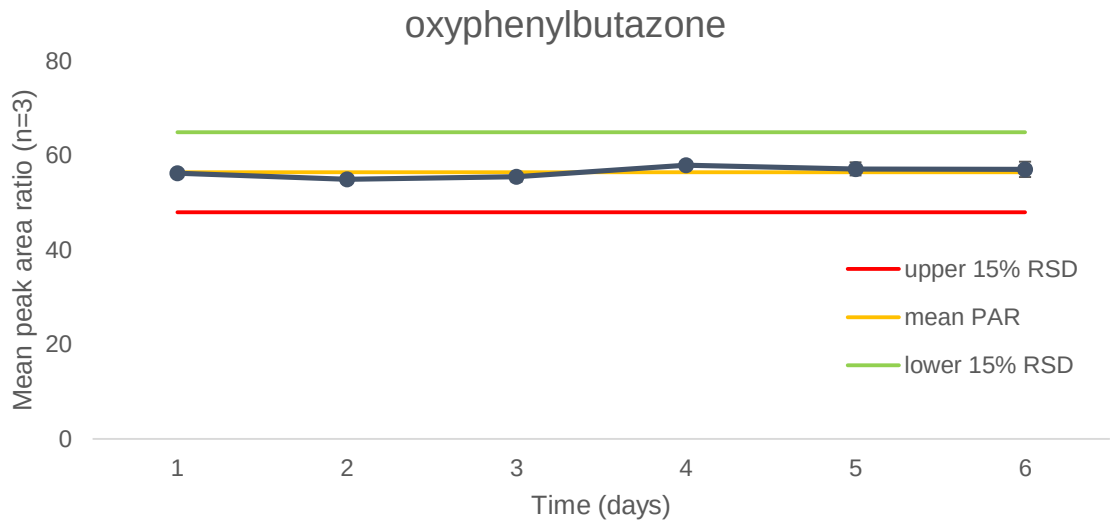


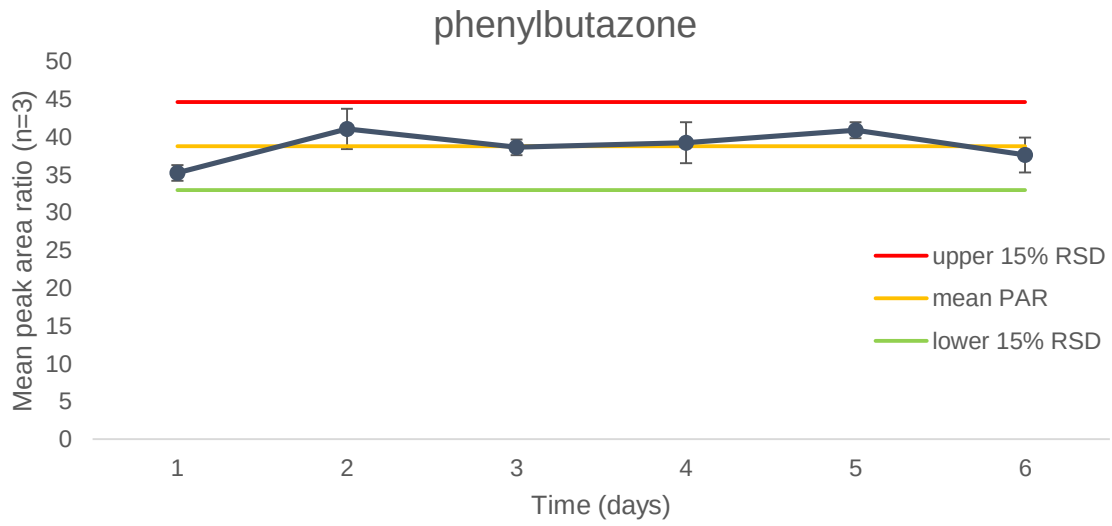




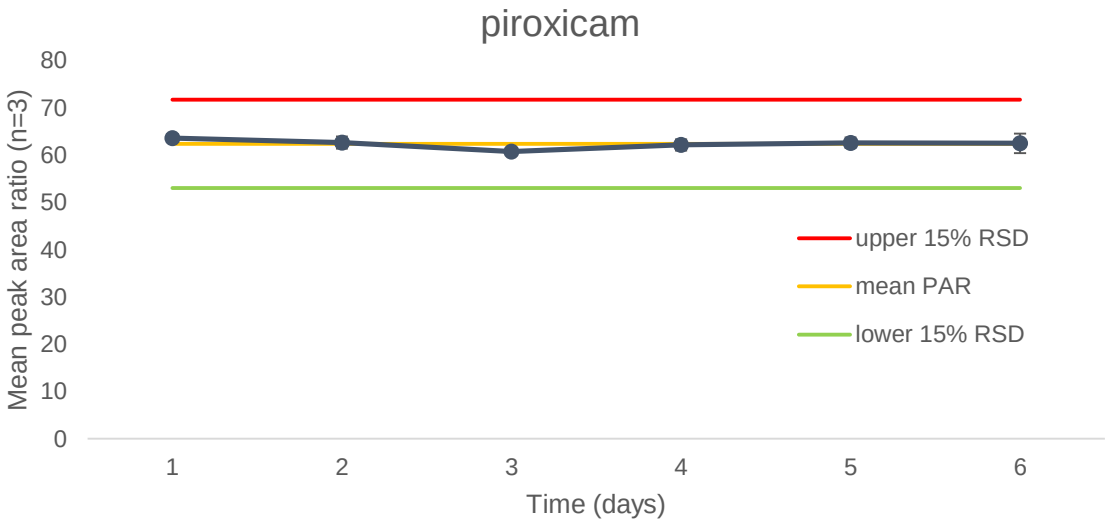
Appendix VI-c Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 5µg/ml

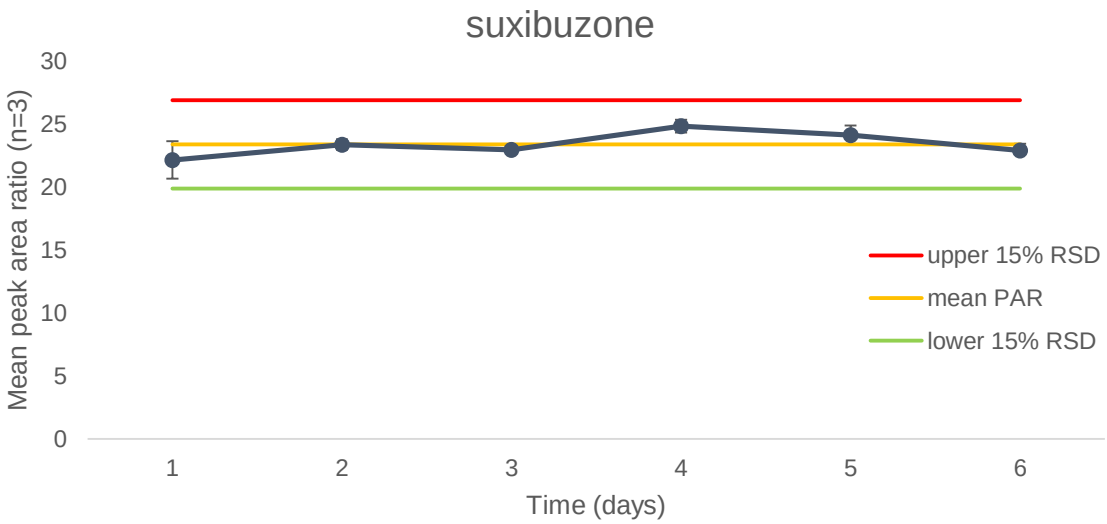


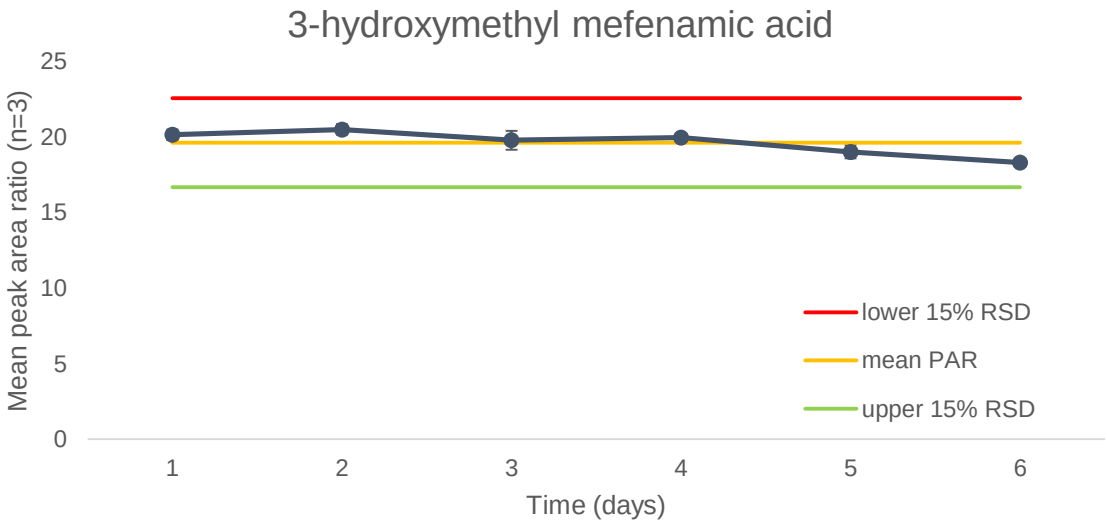




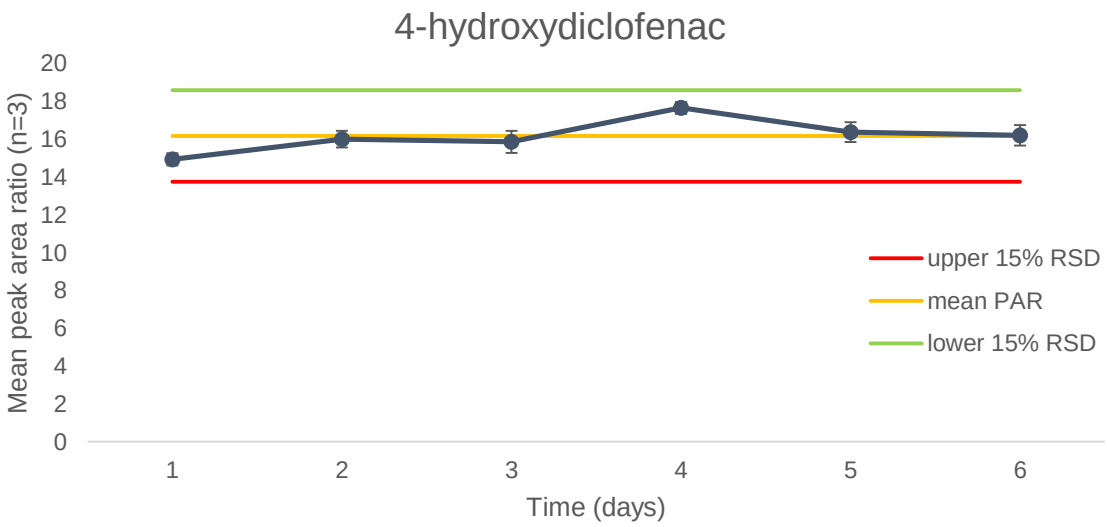
Appendix VI -d Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 5µg/ml

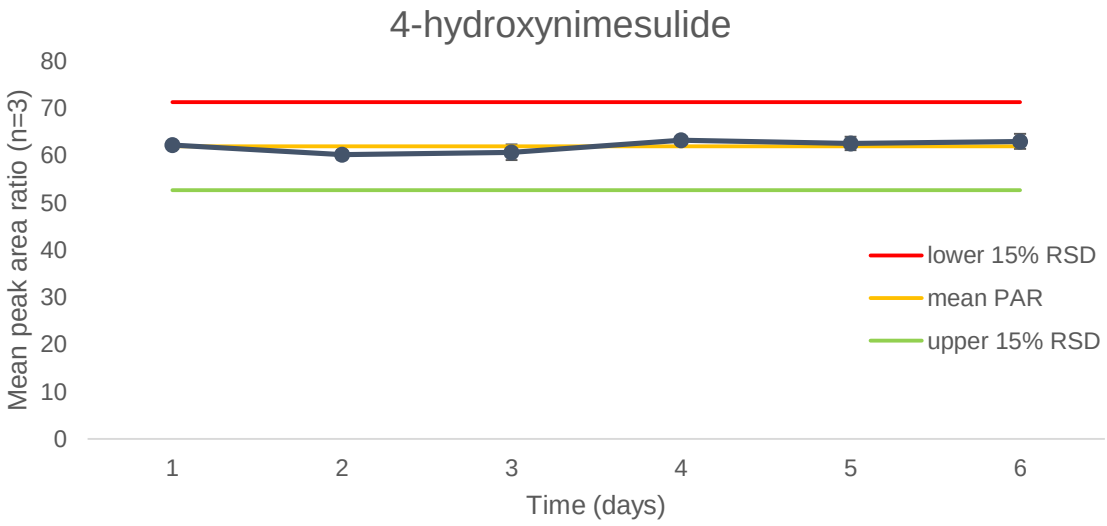


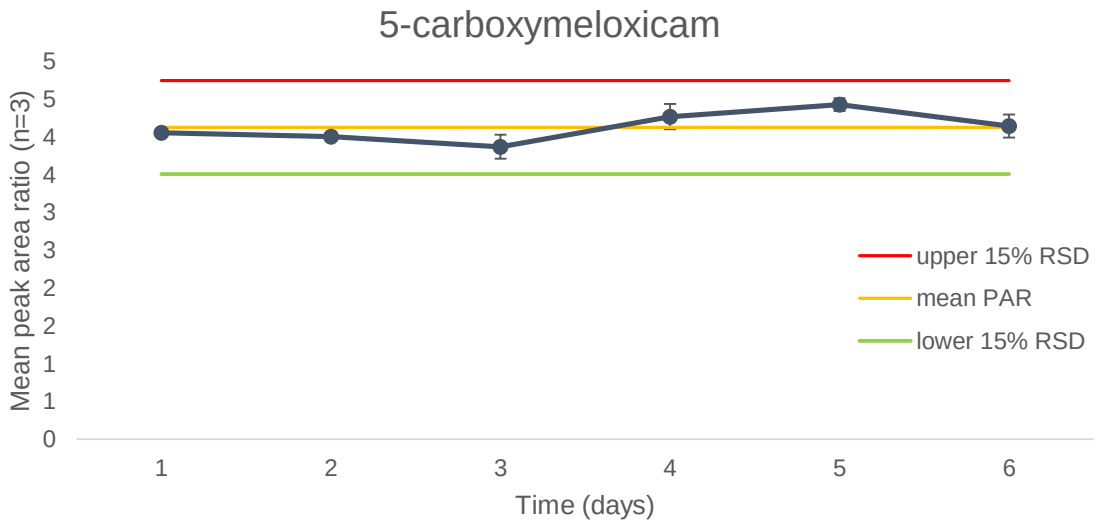




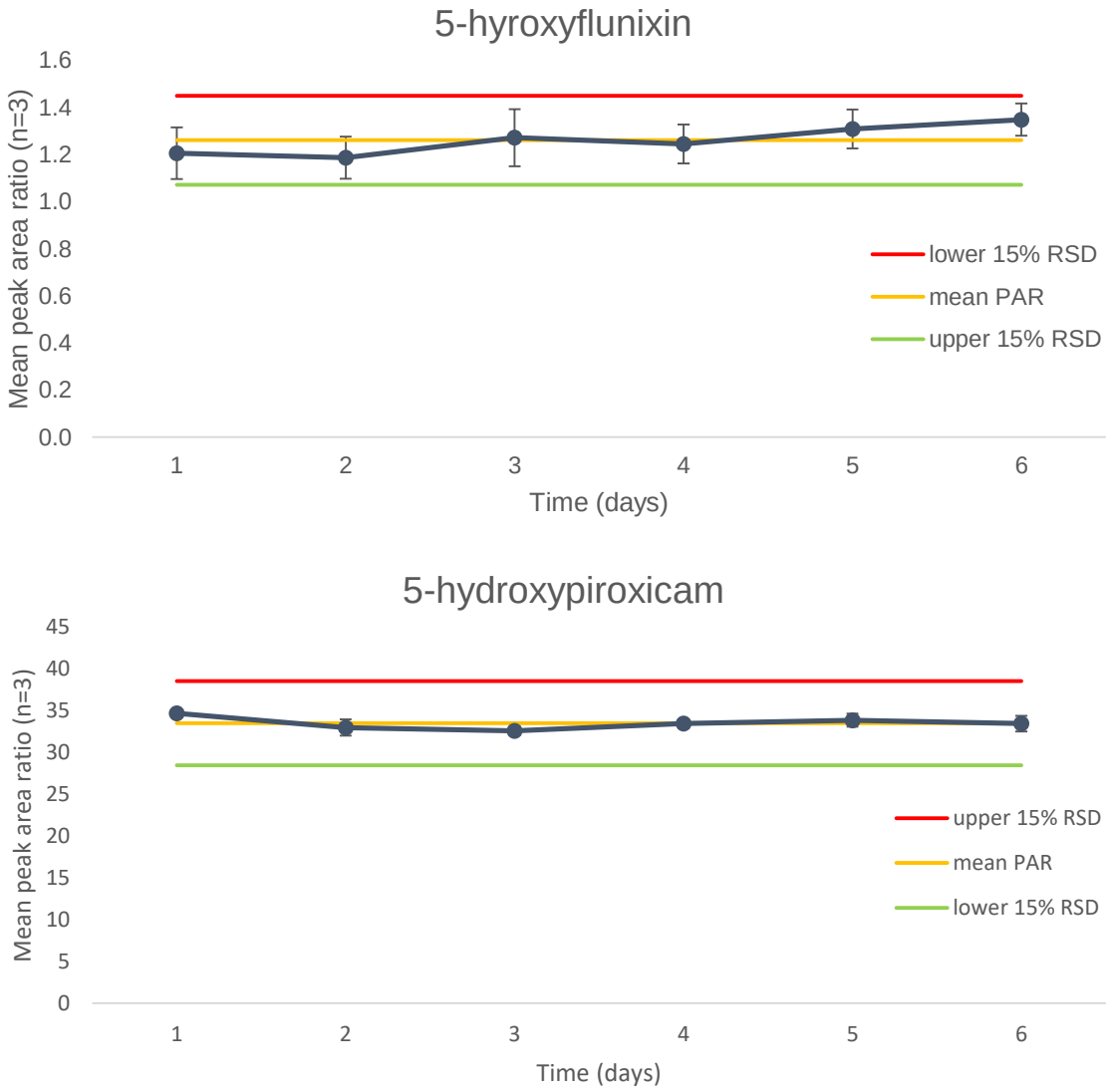
Appendix VI -e Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 5µg/ml



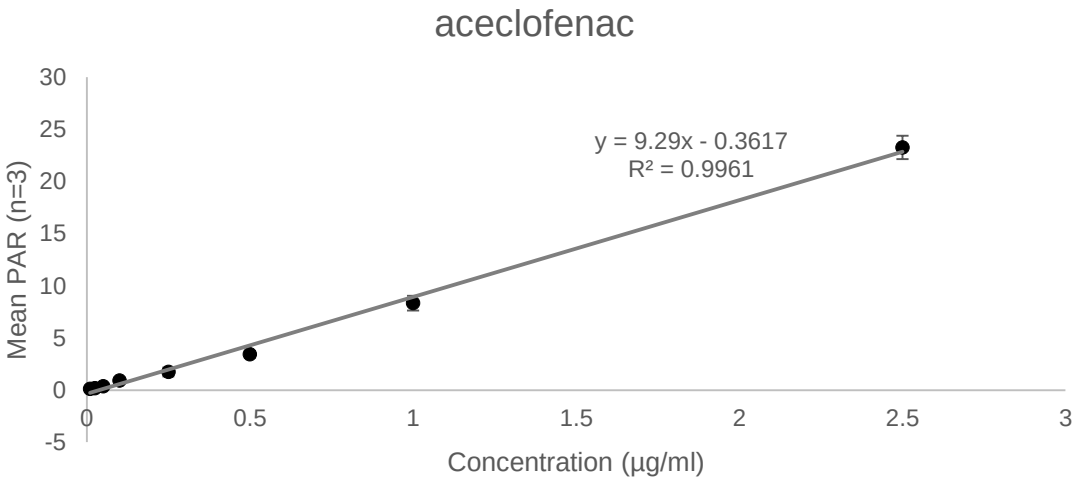


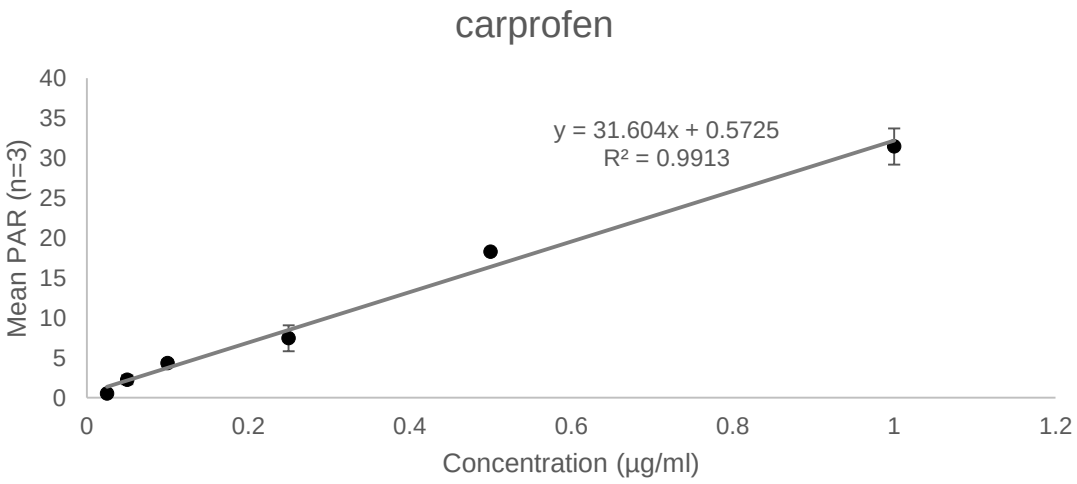


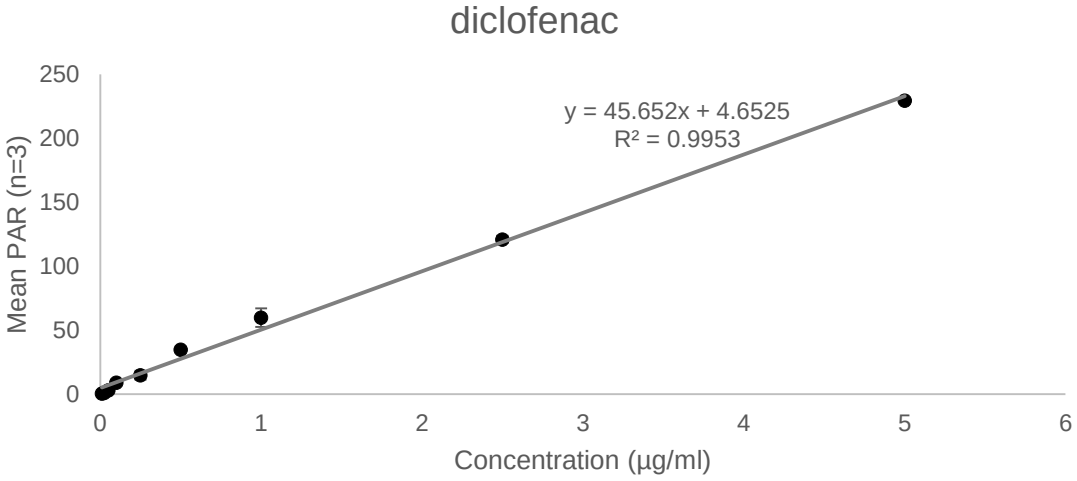
Appendix VI -f Graph of mean peak area ratio (n=3) over 6 days stability trial studied at 5µg/ml



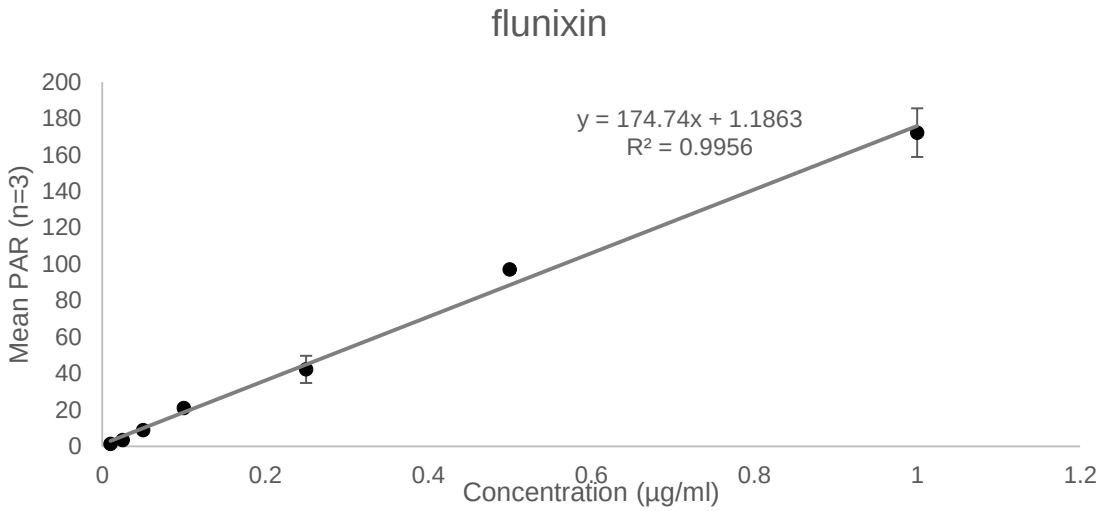
Appendix VII-a linearity plots of NSAIDs concentration against mean peak area ratio (n=3)

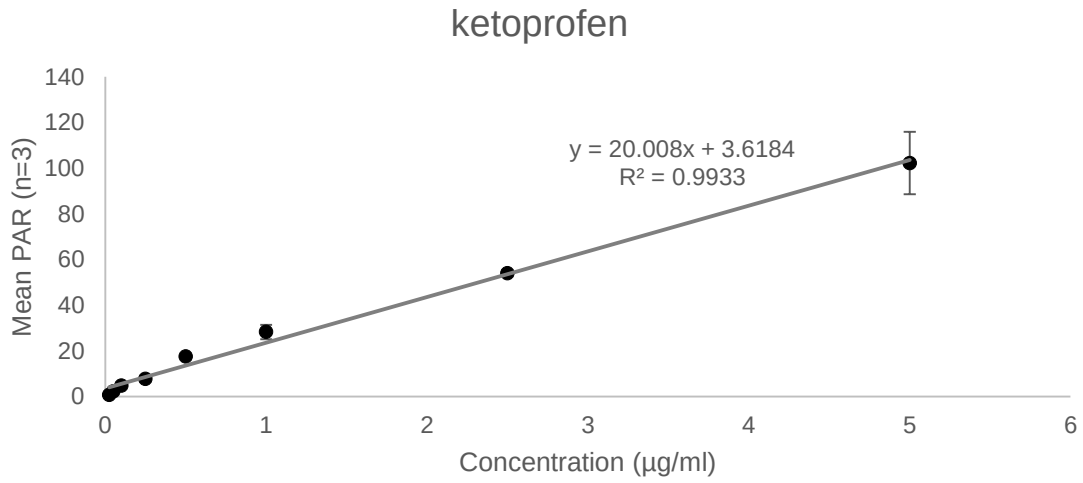


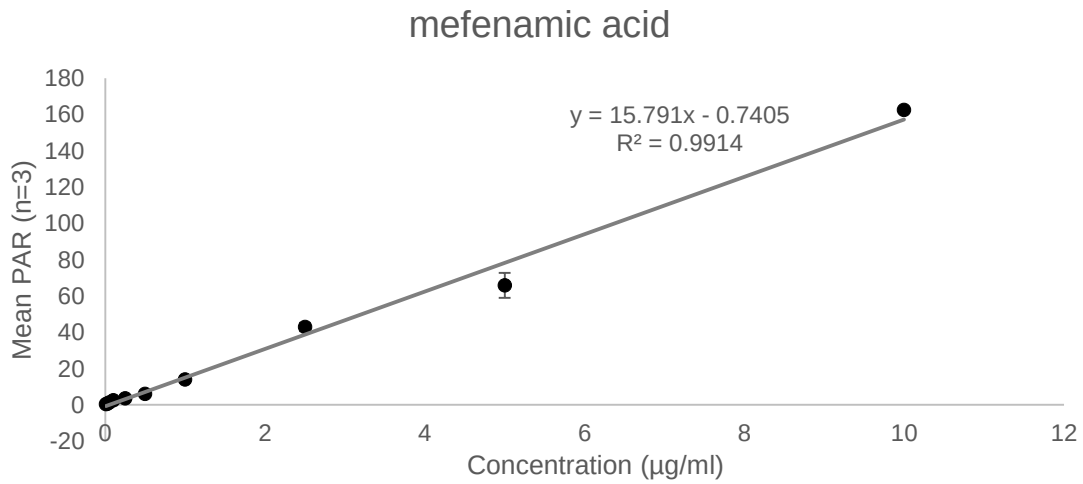




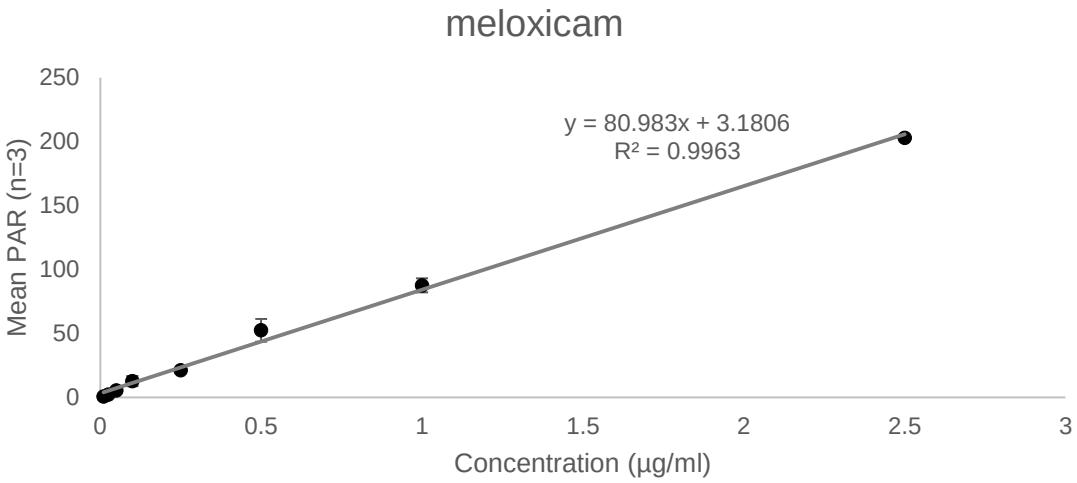
Appendix VII-b linearity plots of NSAIDs concentration against mean peak area ratio (n=3)

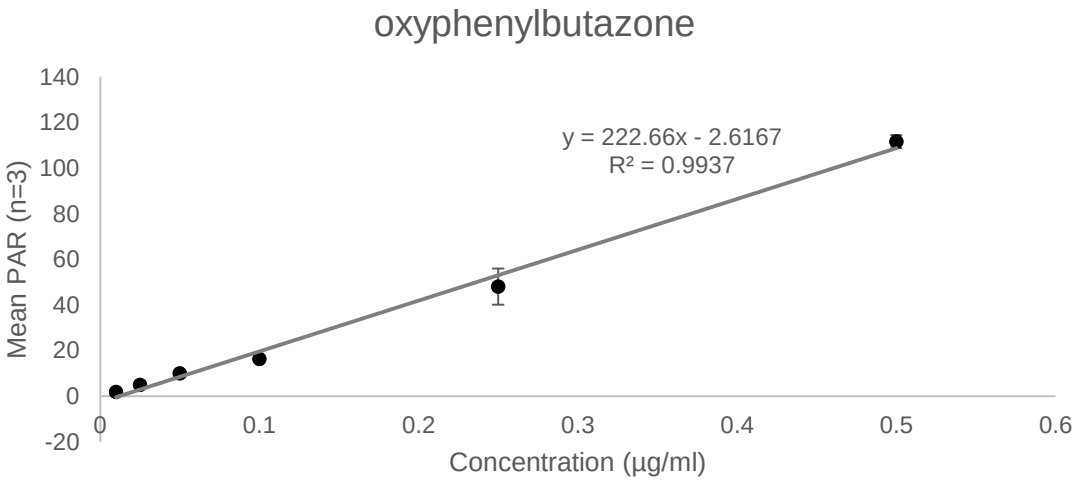


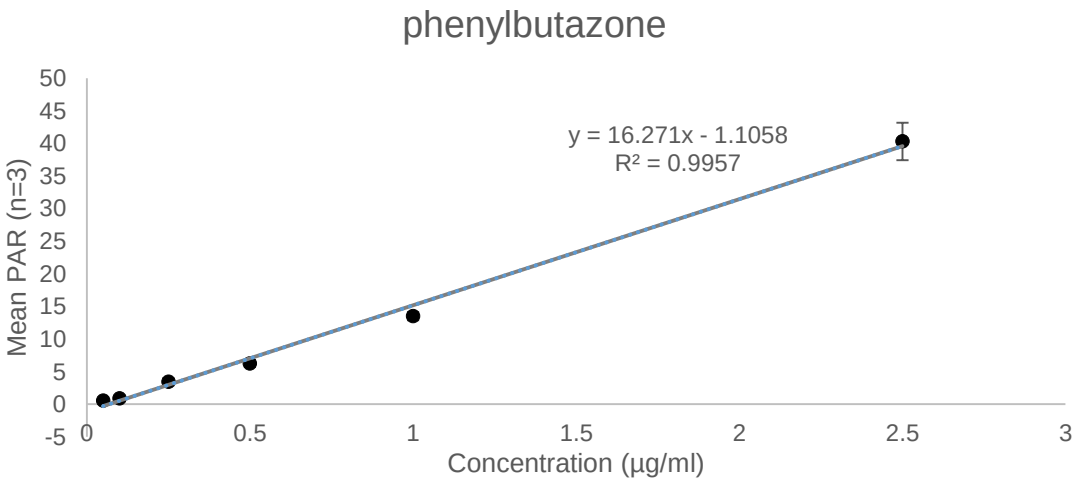




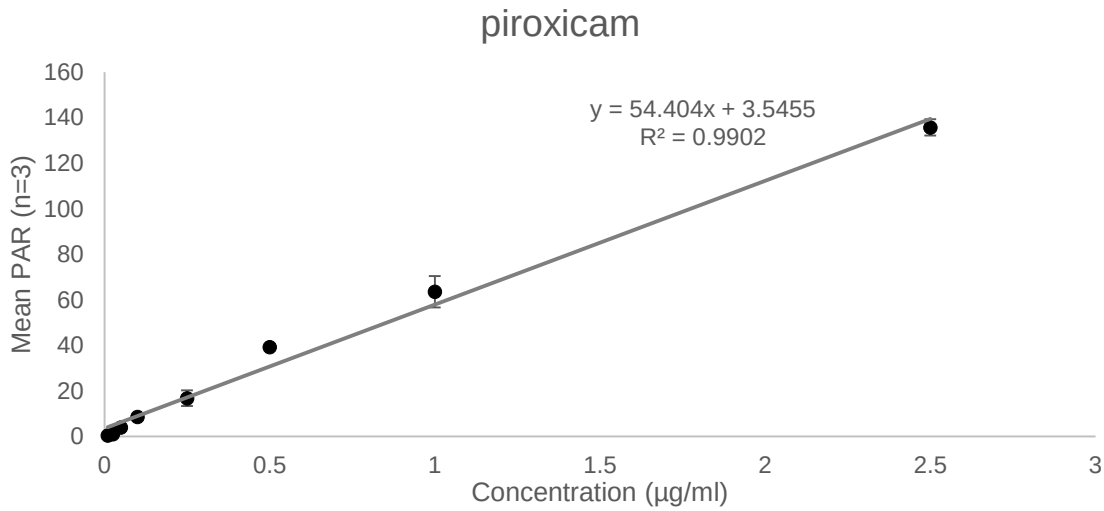
Appendix VII-c linearity plots of NSAIDs concentration against mean peak area ratio (n=3)

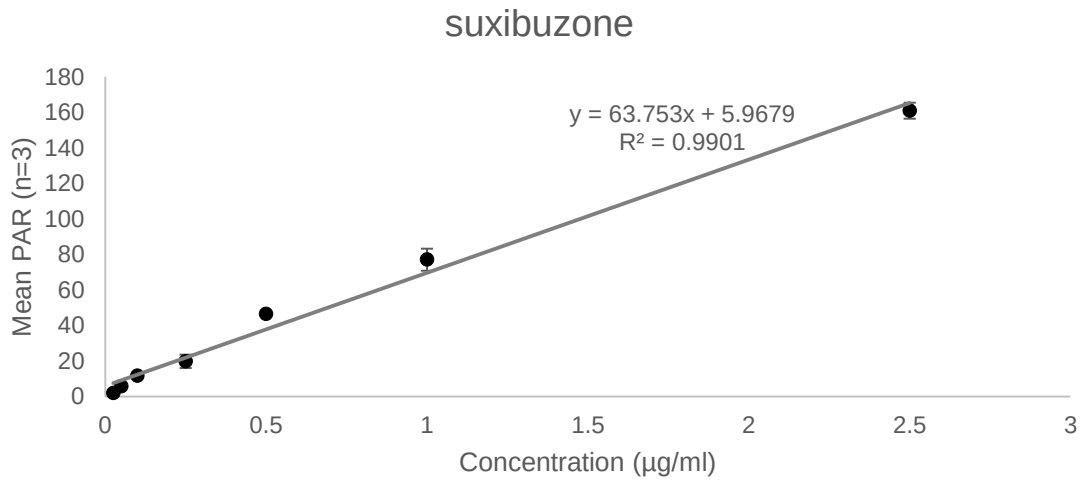


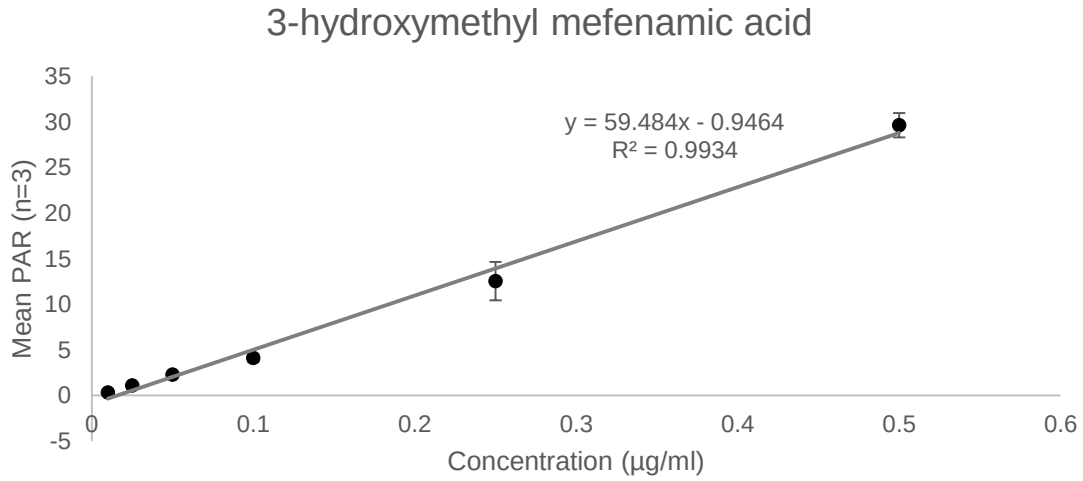




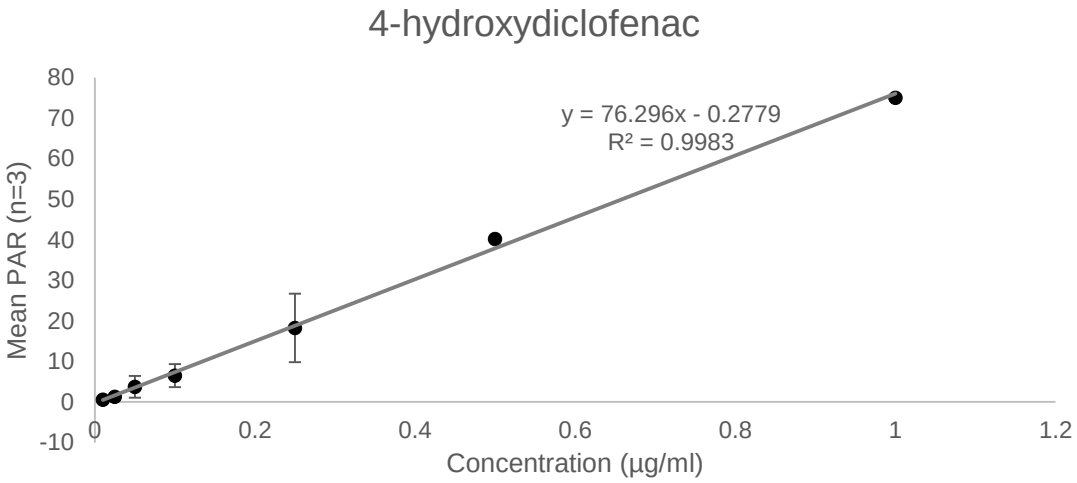
Appendix VII-d linearity plots of NSAIDs concentration against mean peak area ratio (n=3)

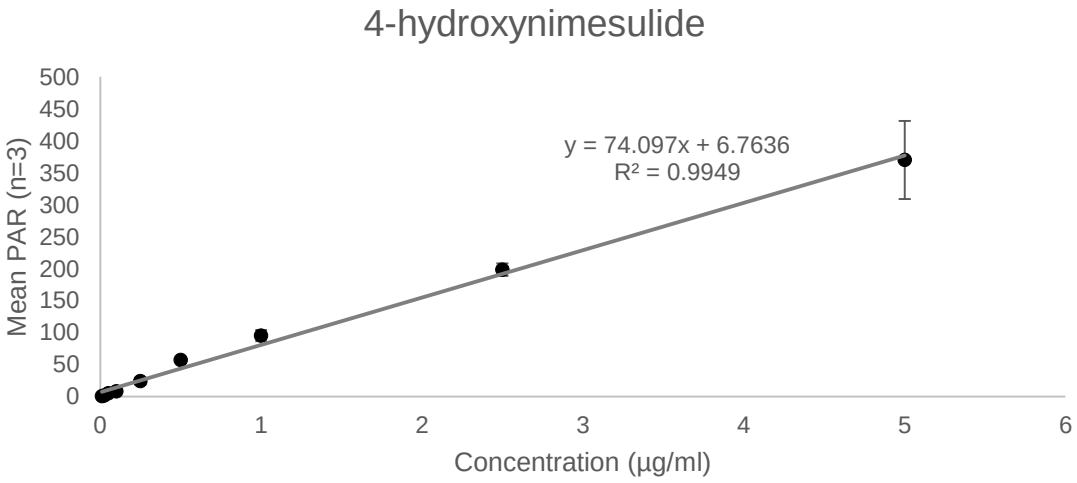


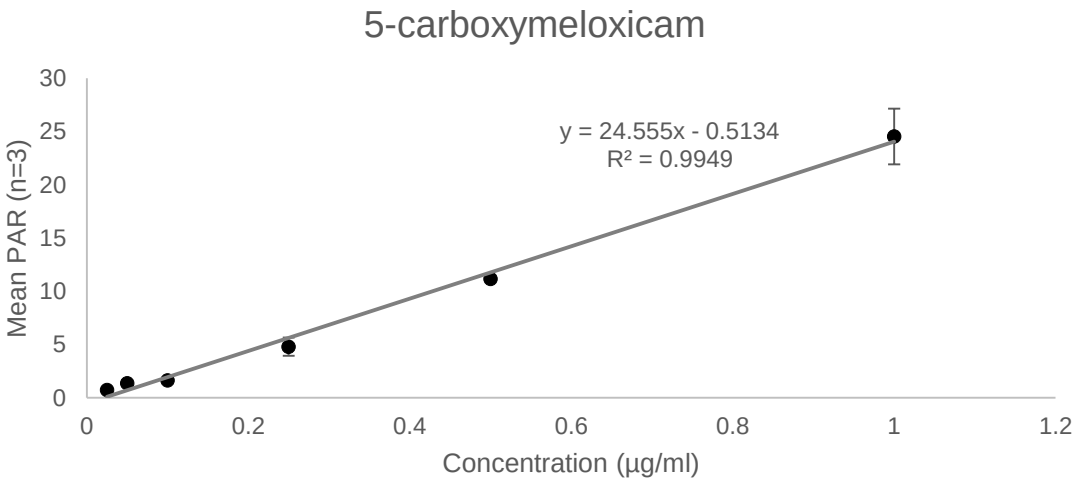




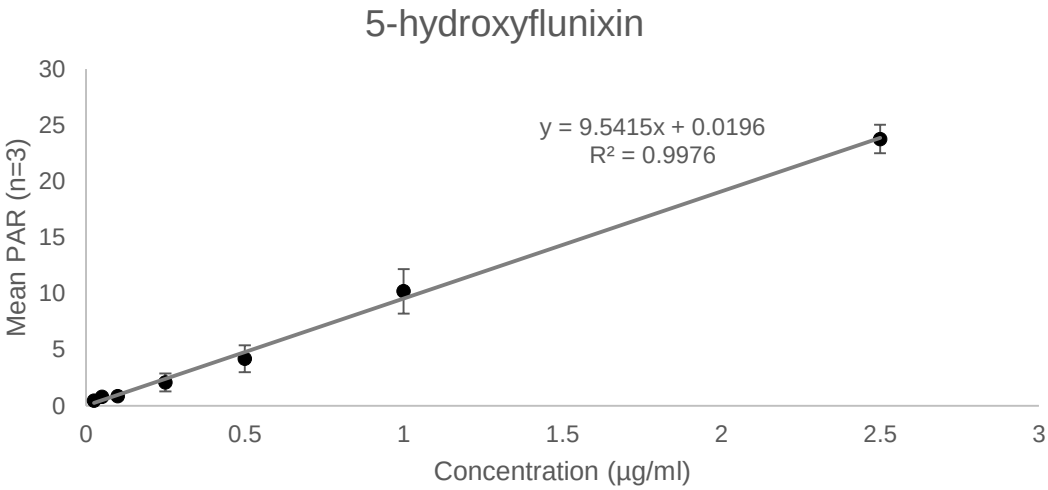
Appendix VII-e linearity plots of NSAIDs concentration against mean peak area ratio (n=3)

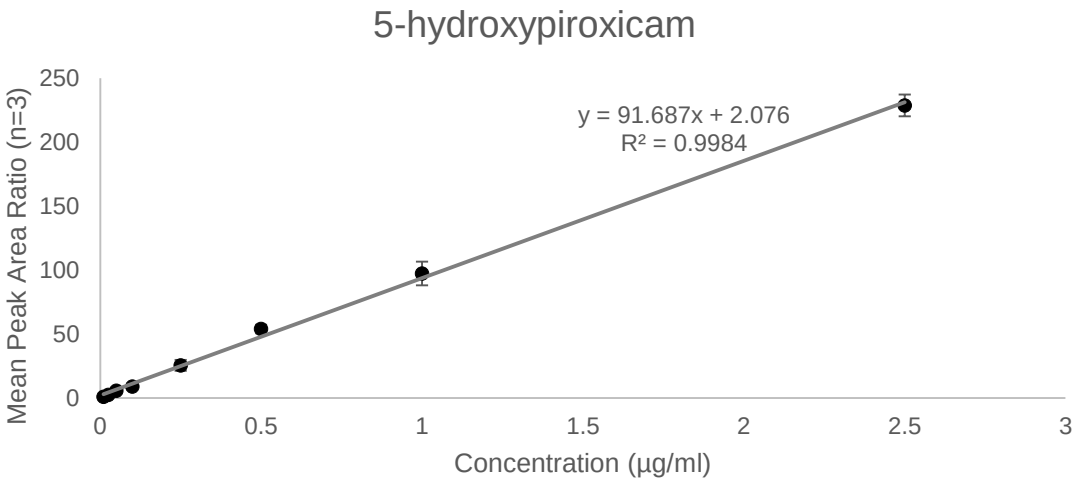




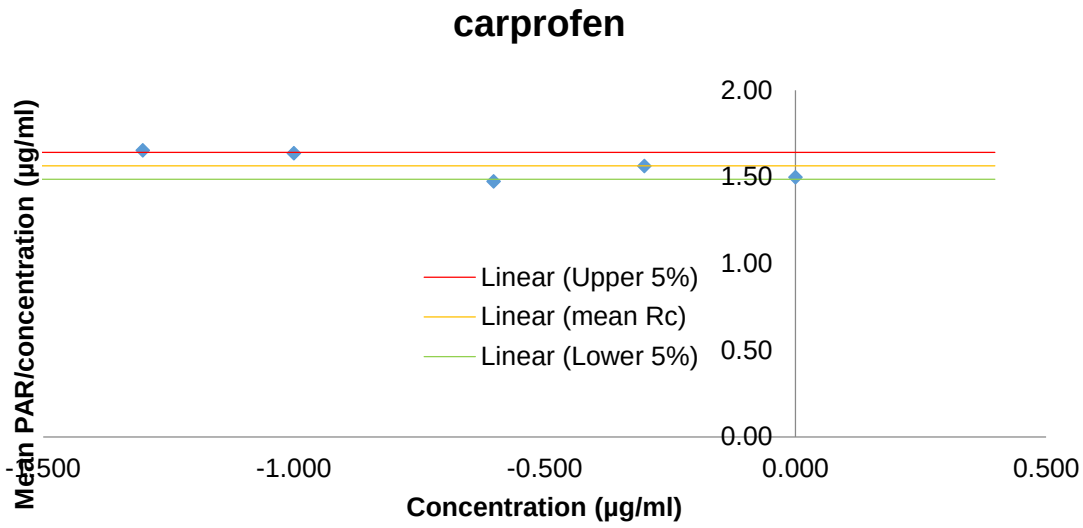


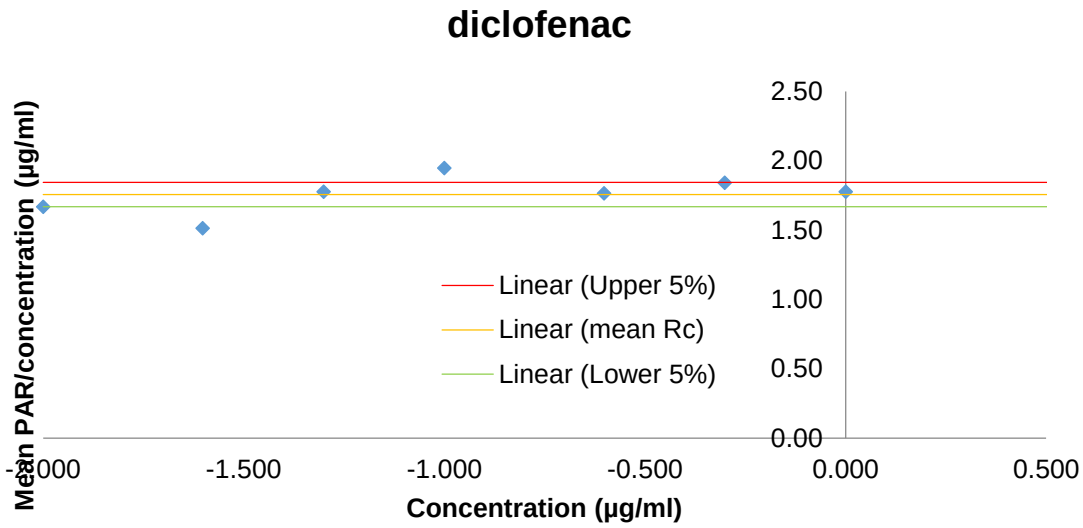
Appendix VII-f linearity plots of NSAIDs concentration against mean peak area ratio (n=3)

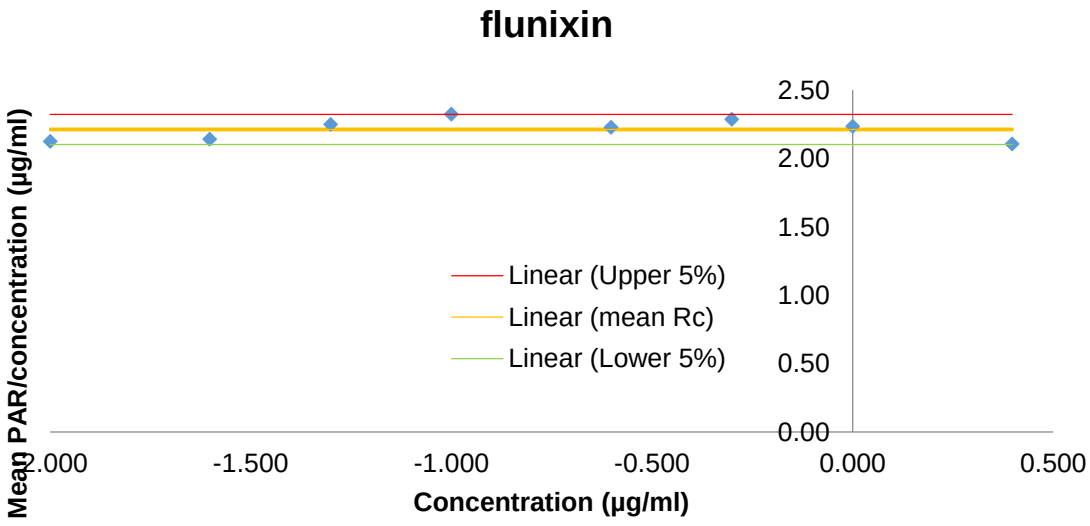




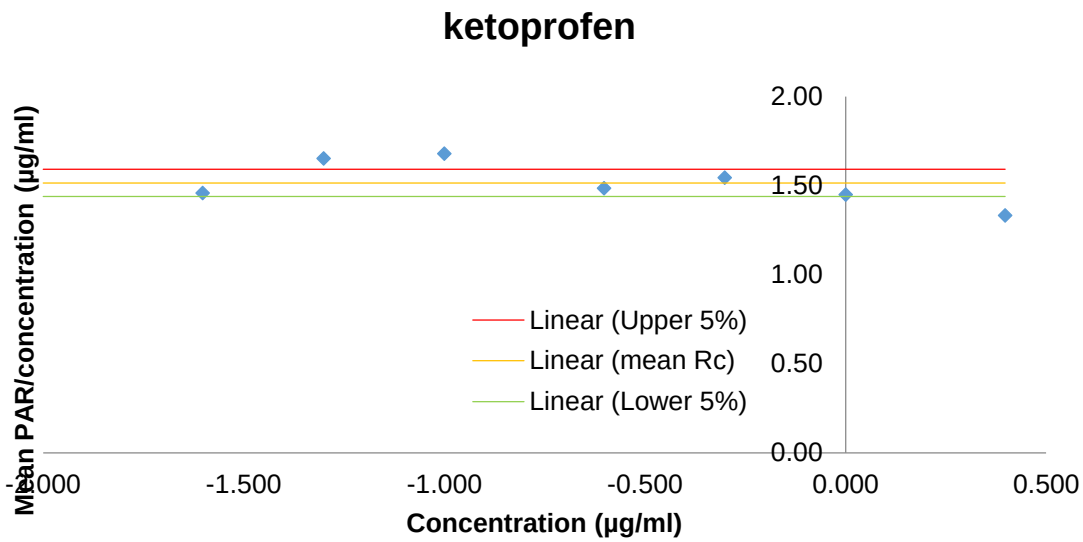
Appendix VIII-a Mean peak area ratio/concentration vs log concentration plots for all NSAIDs

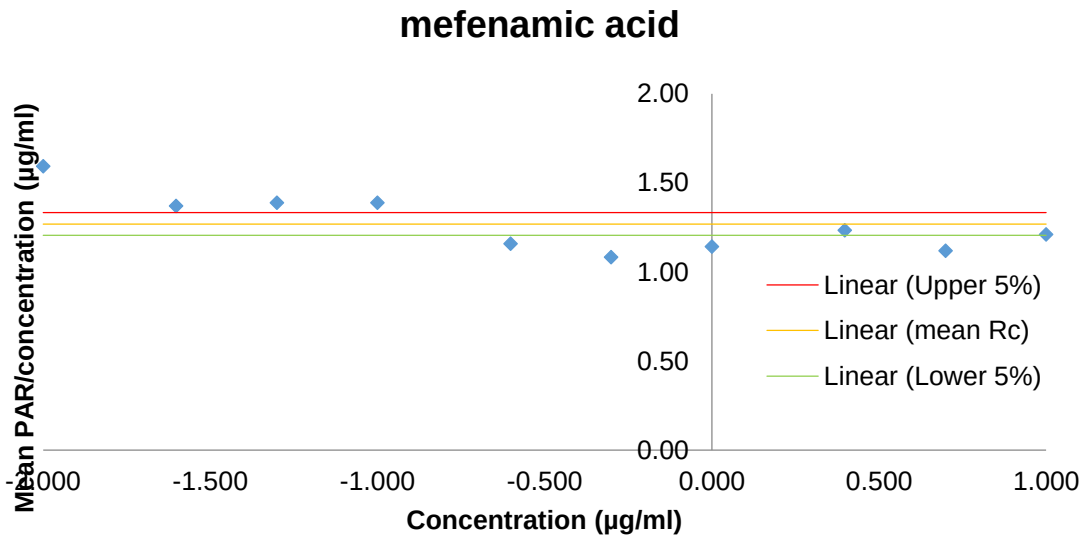


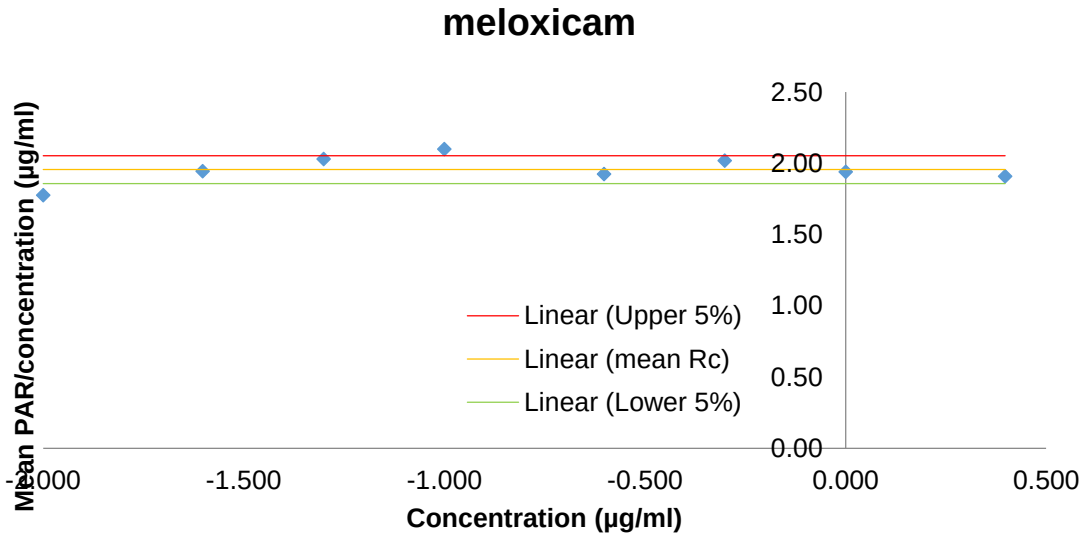




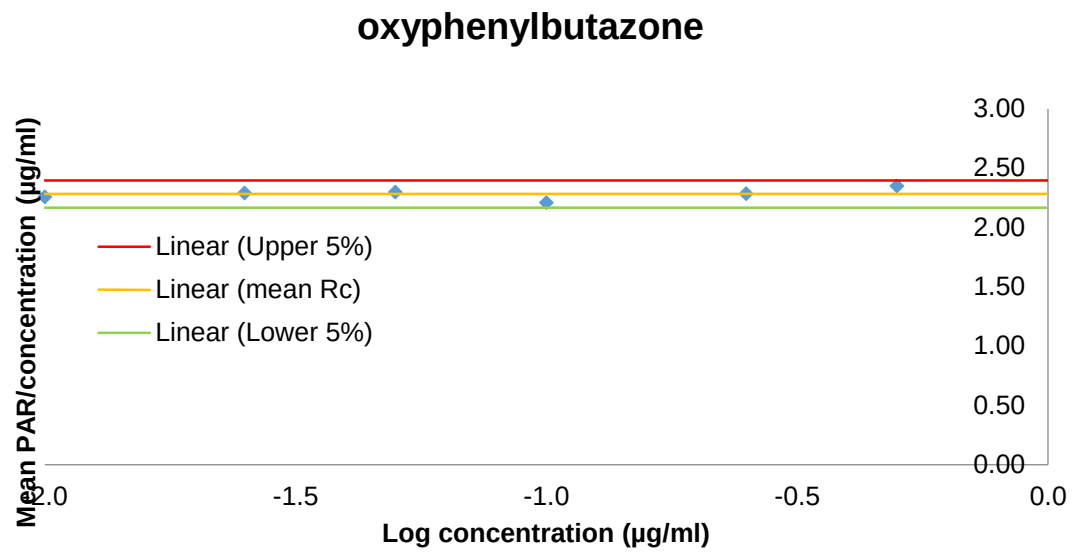
Appendix VIII-b Mean peak area ratio/concentration vs log concentration plots for all NSAIDs

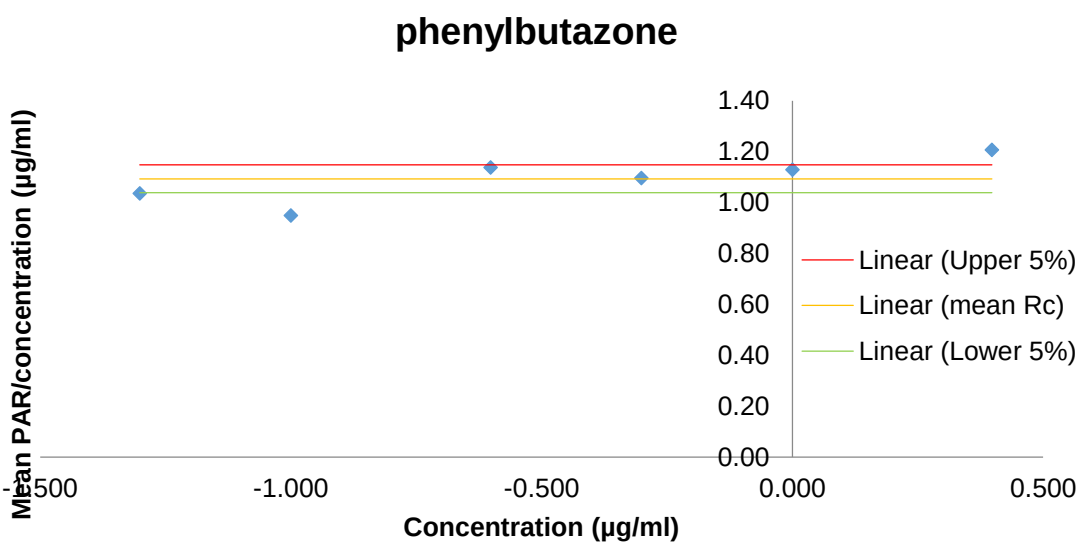


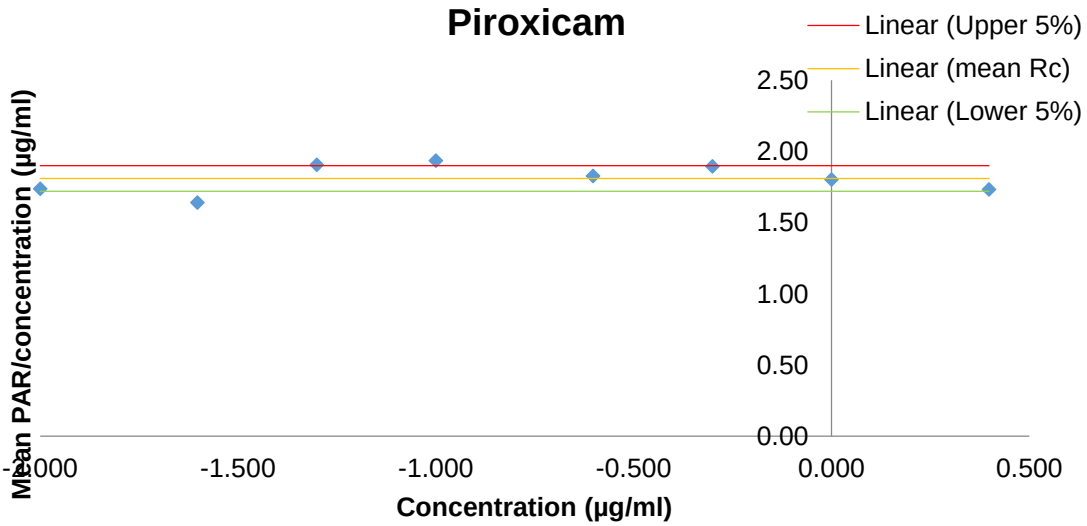




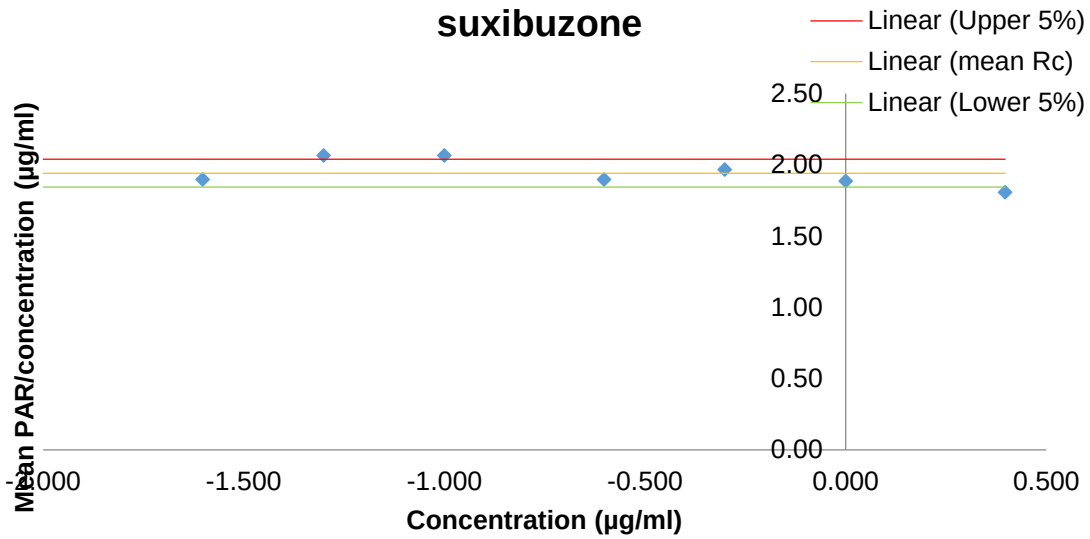
Appendix VIII-c Mean peak area ratio/concentration vs log concentration plots for all NSAIDs

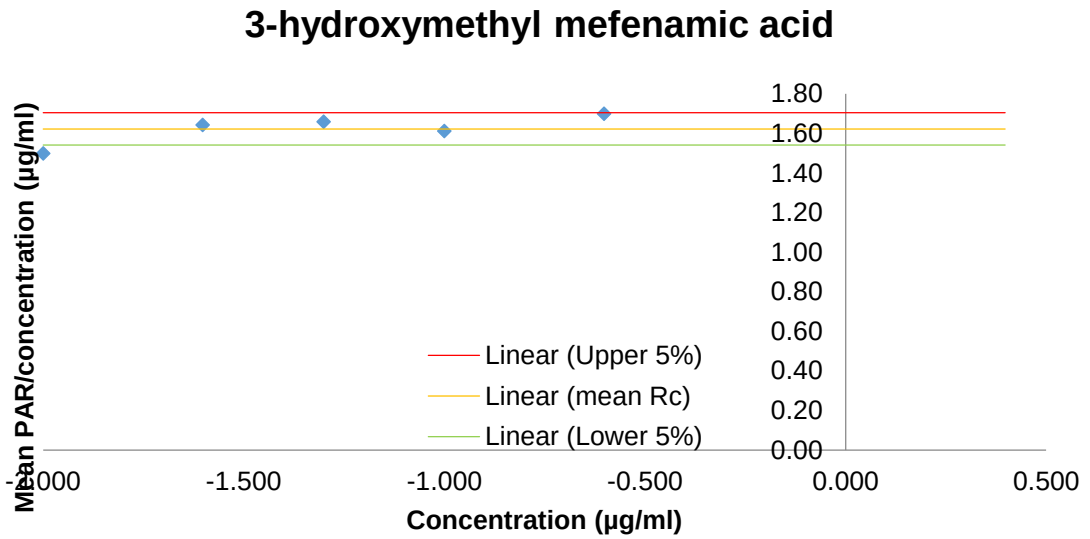


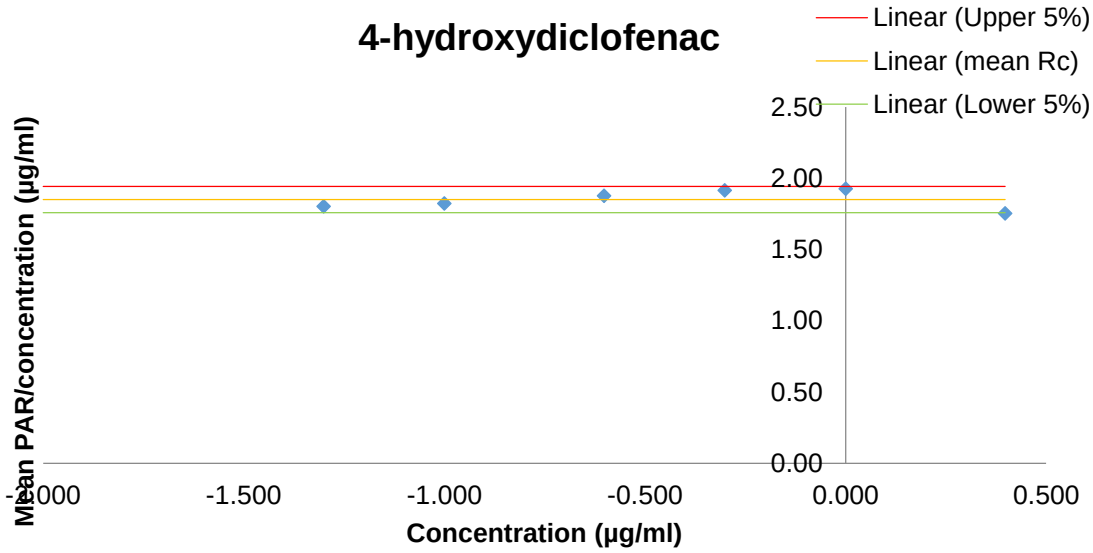




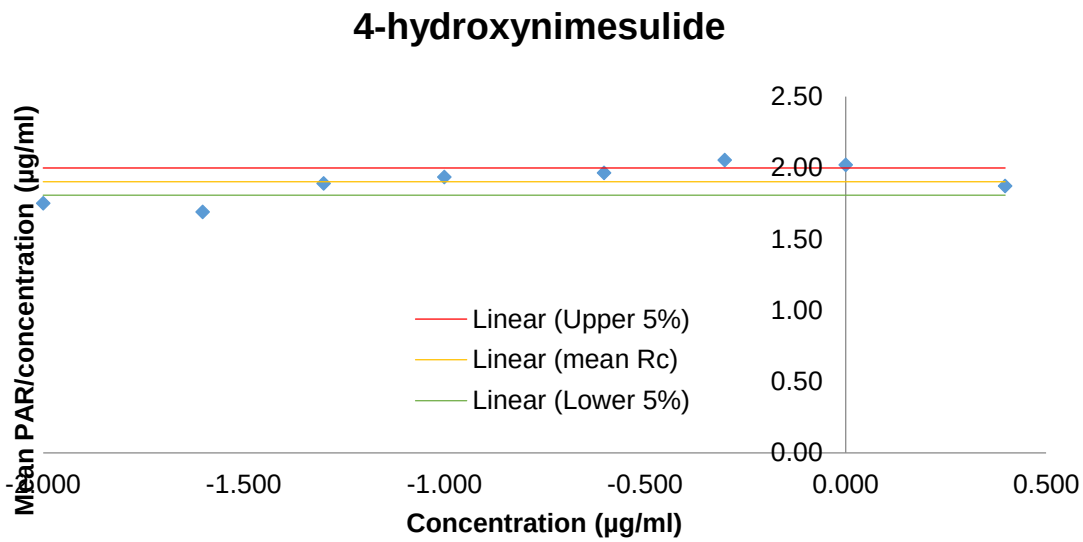
Appendix VIII-d Mean peak area ratio/concentration vs log concentration plots for all NSAIDs

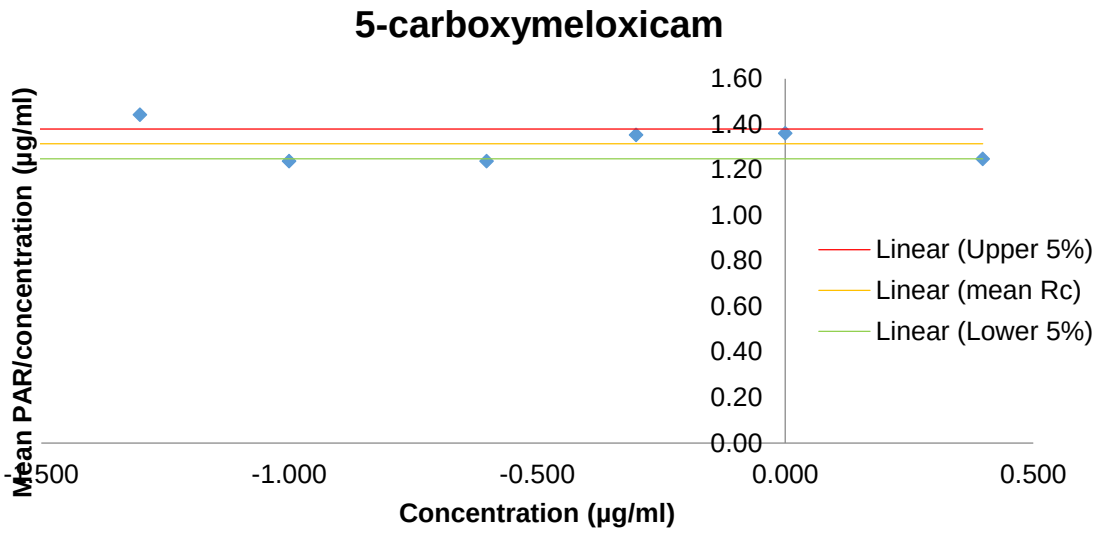


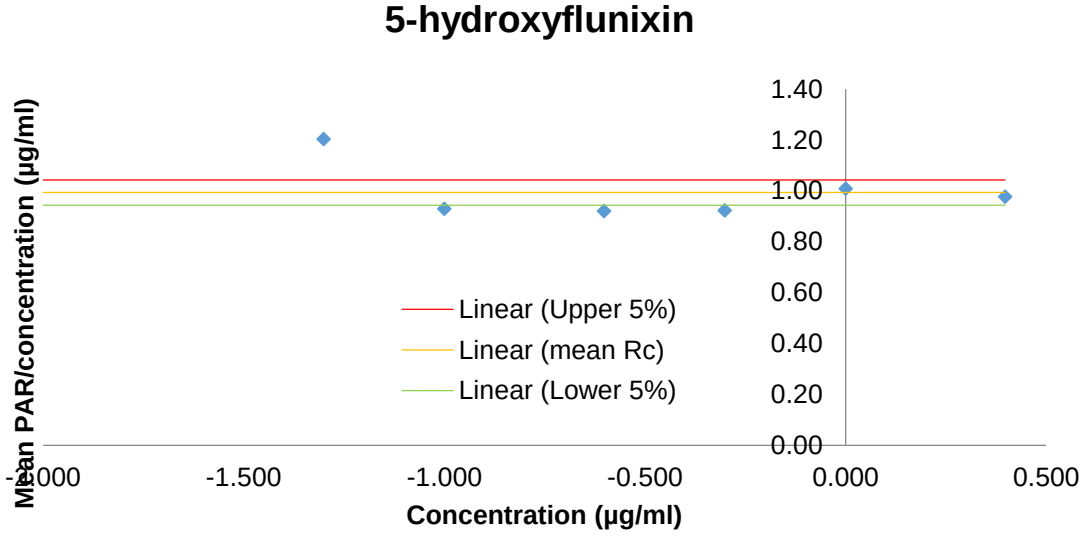




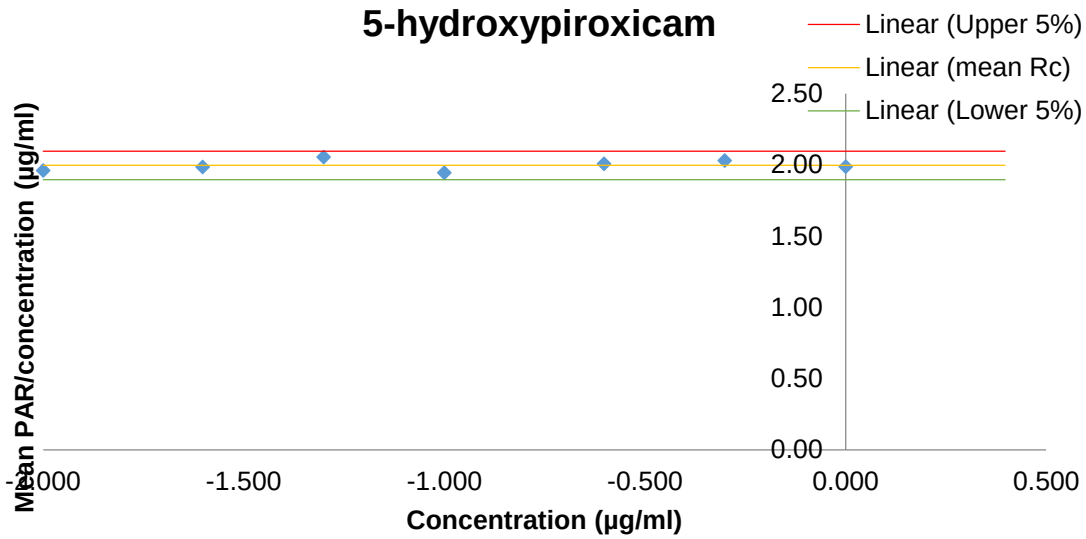
Appendix VIII-e Mean peak area ratio/concentration vs log concentration plots for all NSAIDs



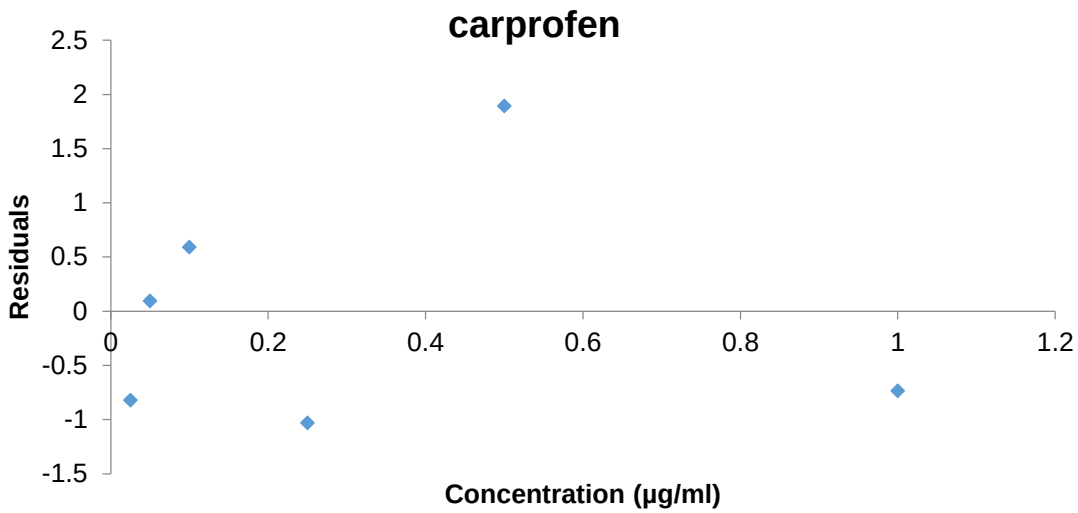


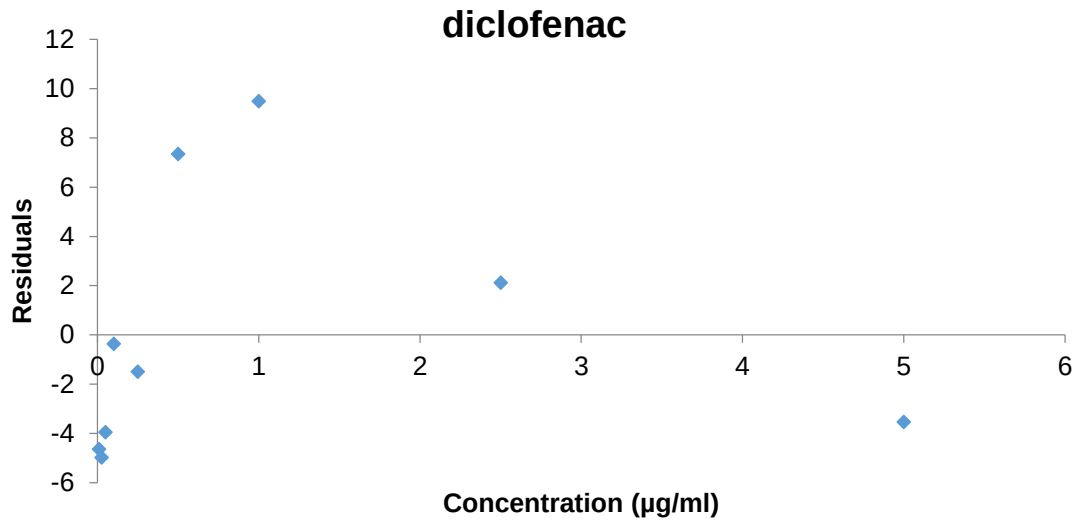


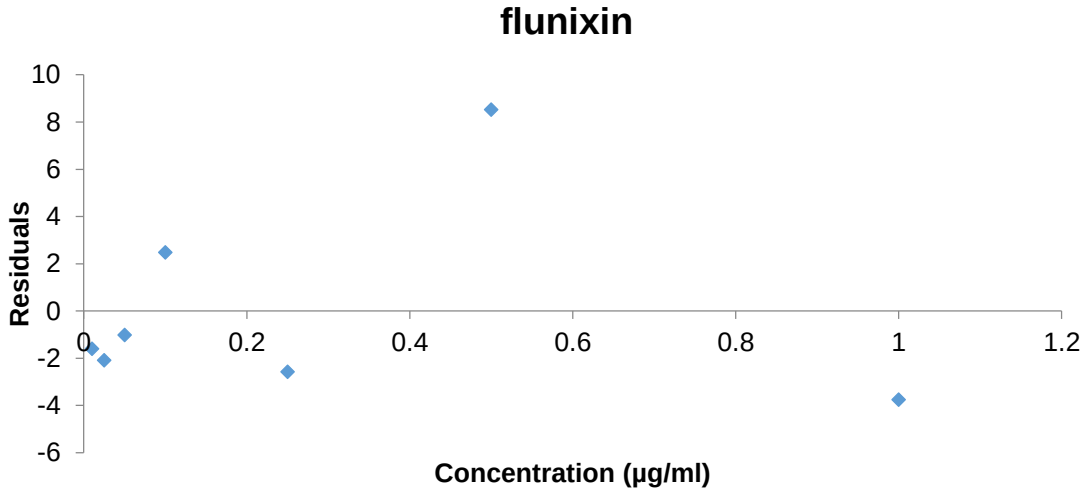
Appendix VIII-f Mean peak area ratio/concentration vs log concentration plots for all NSAIDs



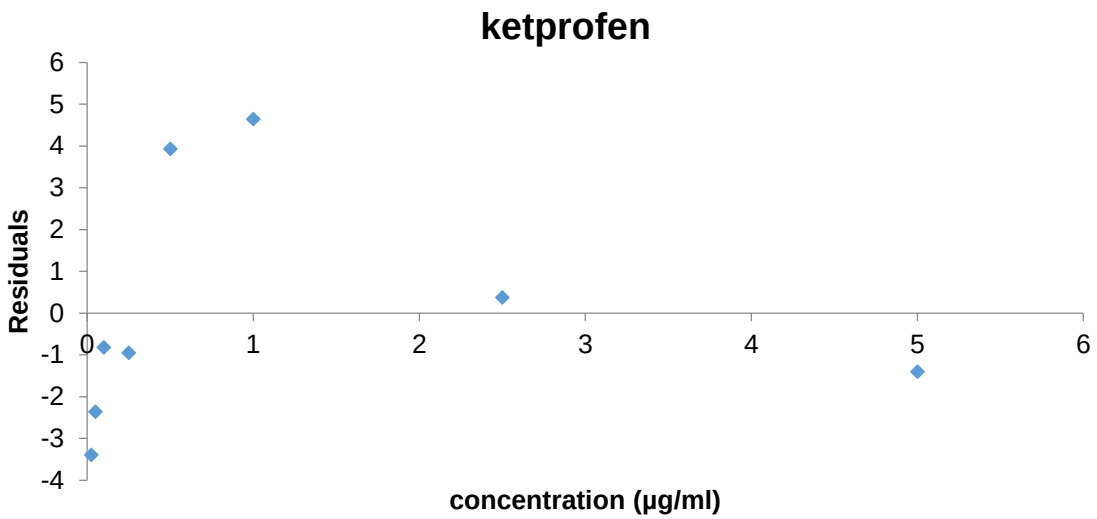
Appendix IX-a Residual plots of NSAIDs over working linear range (µg/ml)

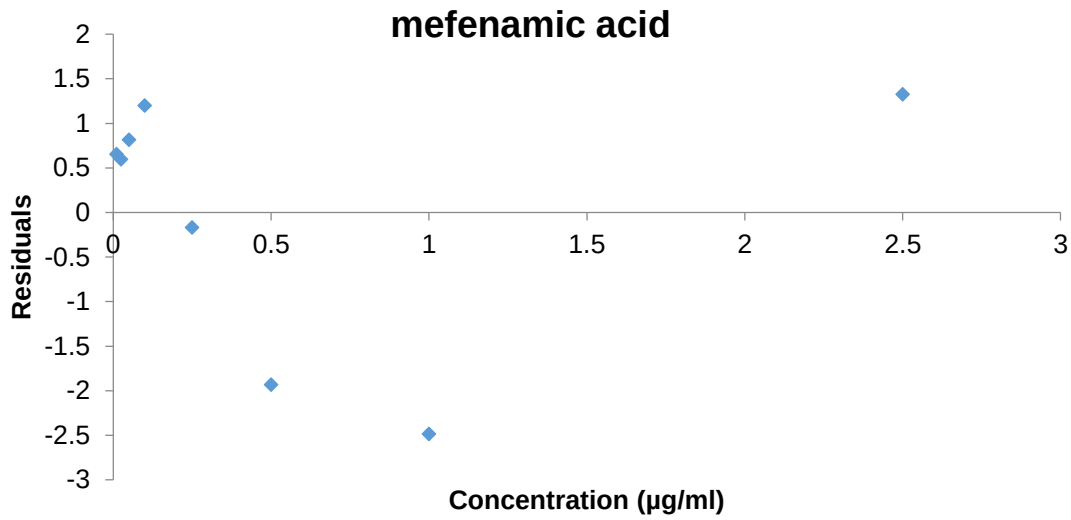


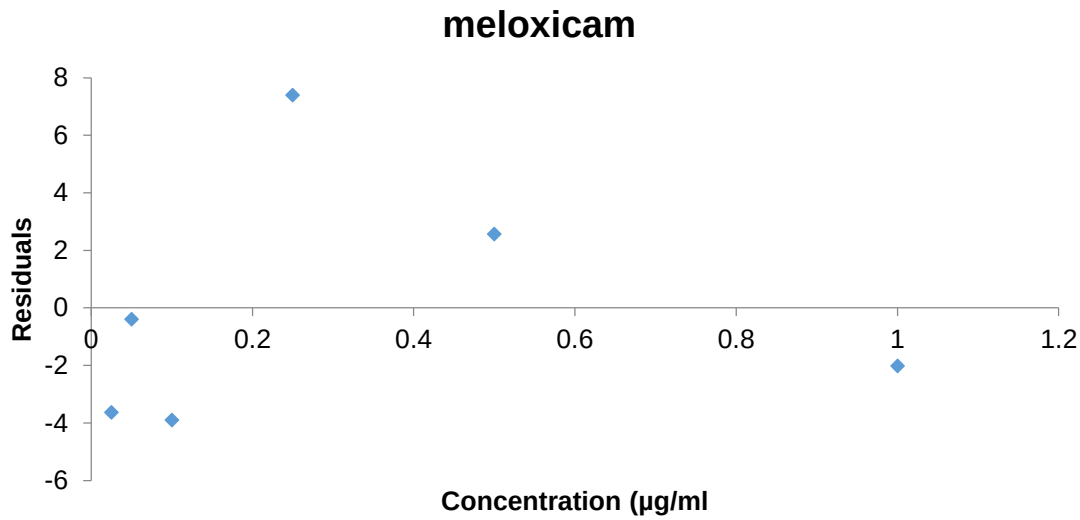




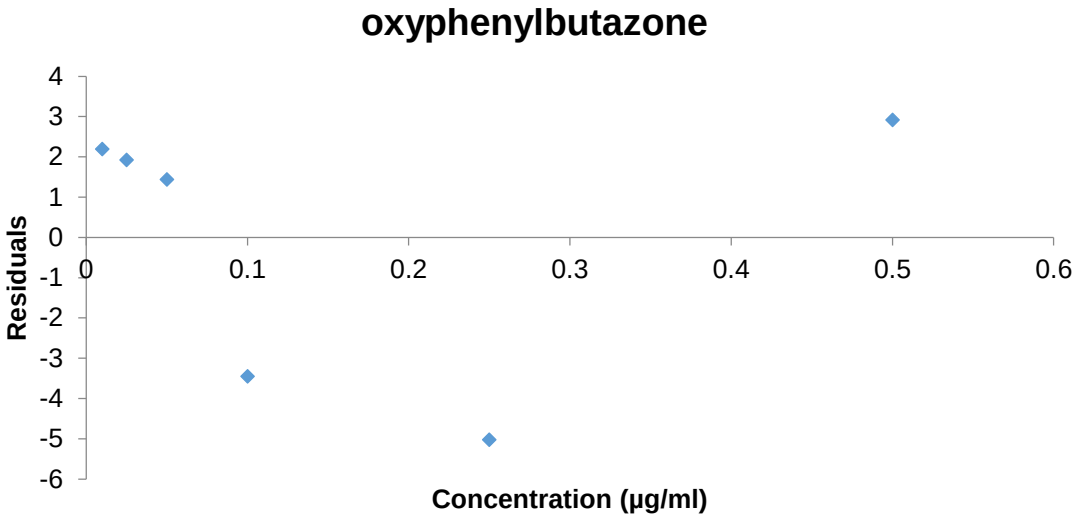
Appendix IX-b Residual plots of NSAIDs over working linear range (µg/ml)

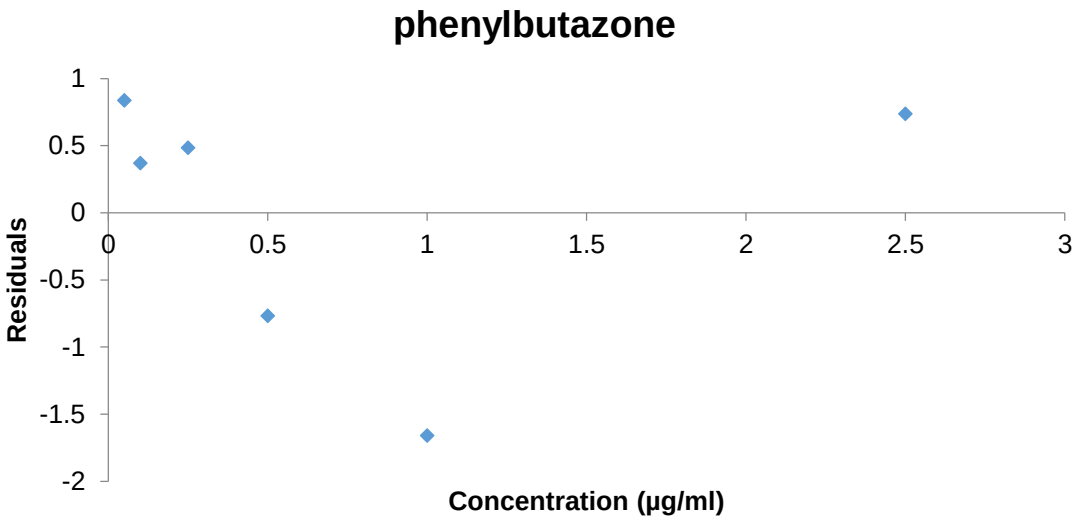


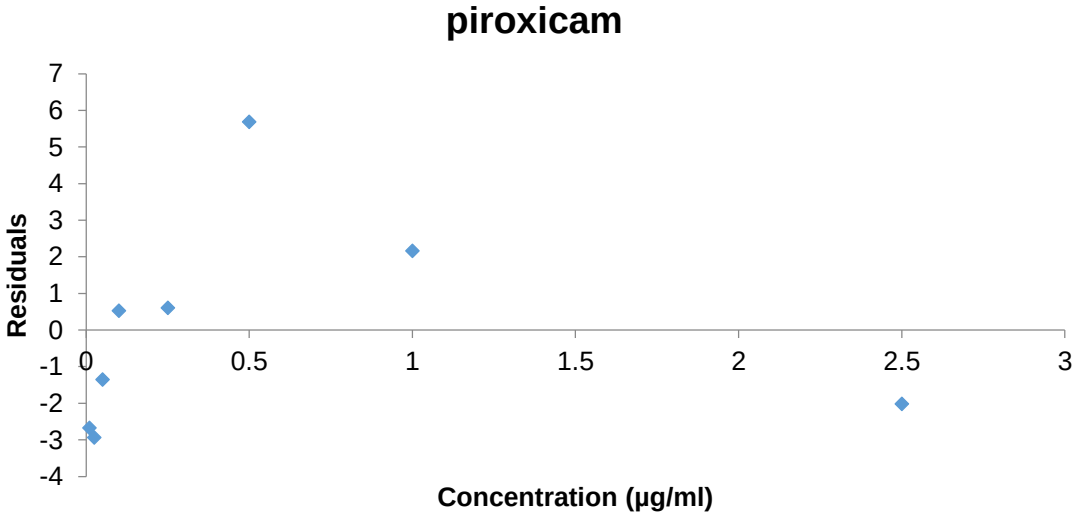




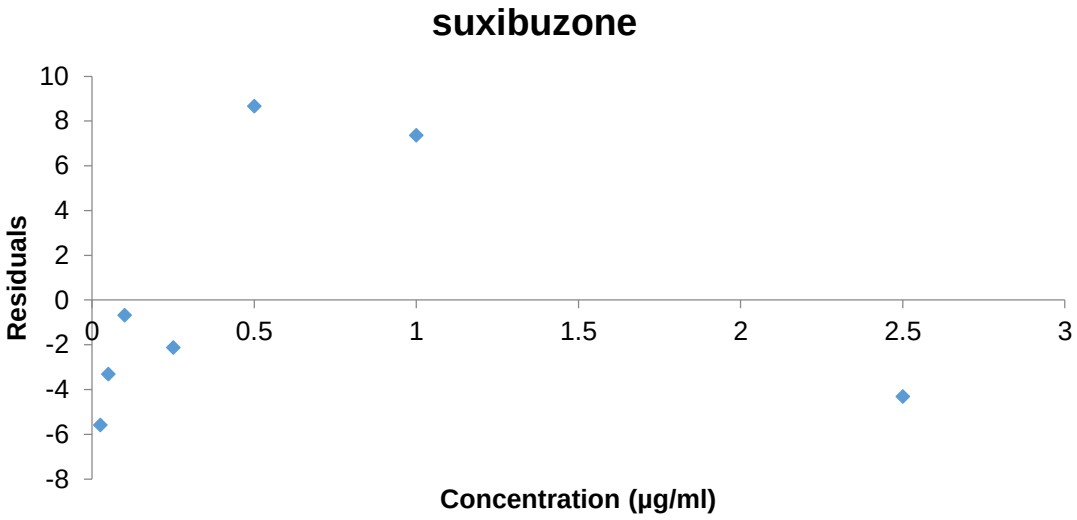
Appendix IX-c Residual plots of NSAIDs over working linear range (µg/ml)

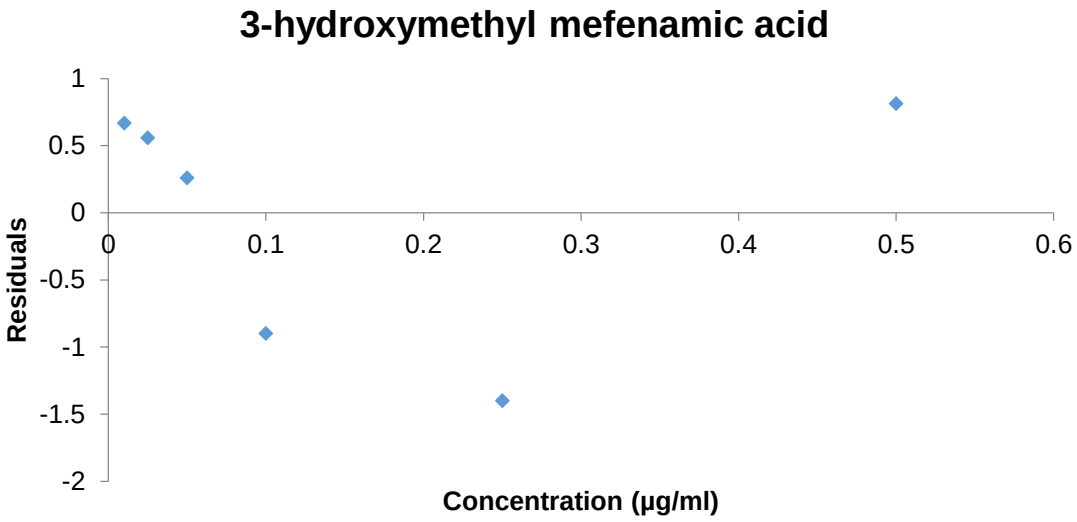


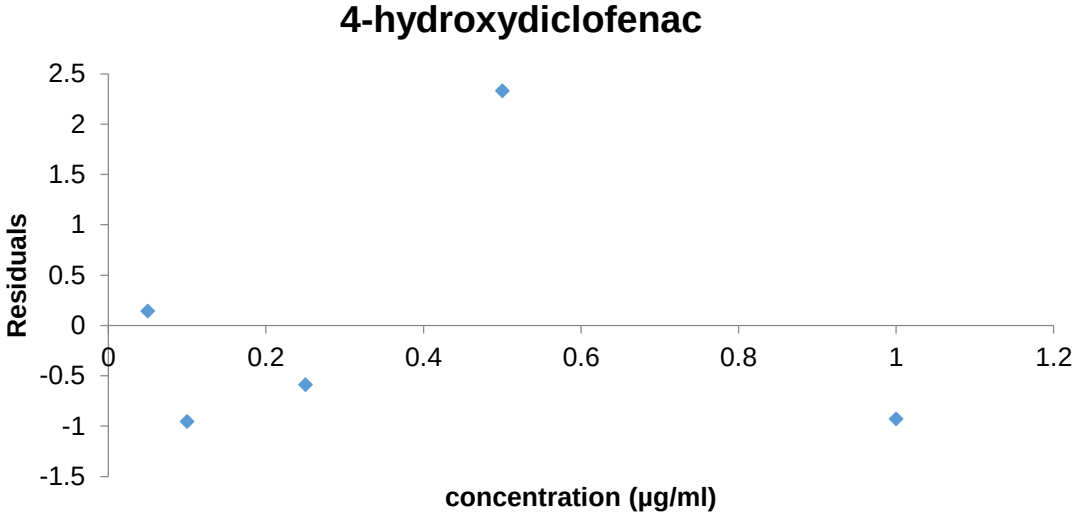




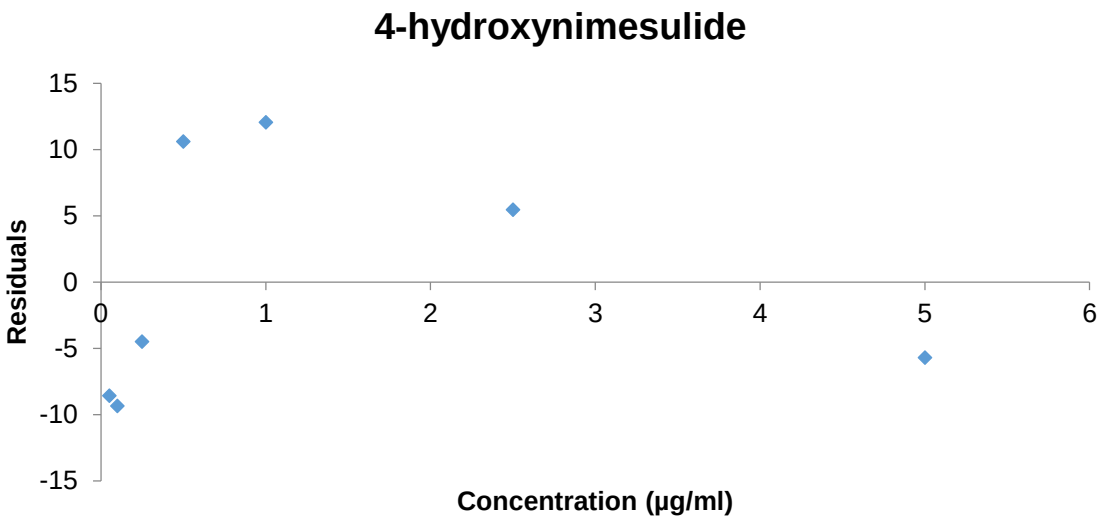
Appendix IX-d Residual plots of NSAIDs over working linear range (µg/ml)

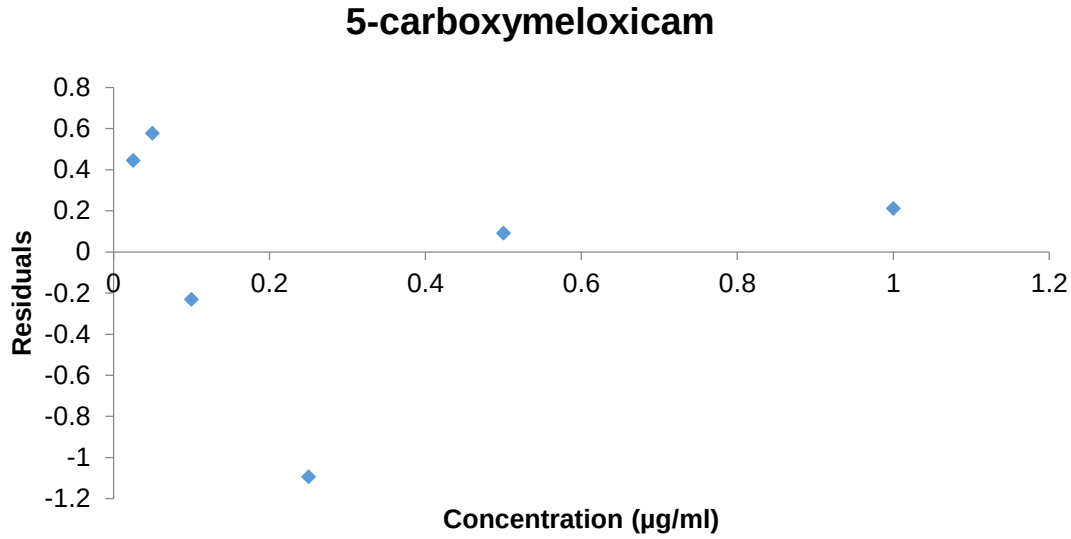


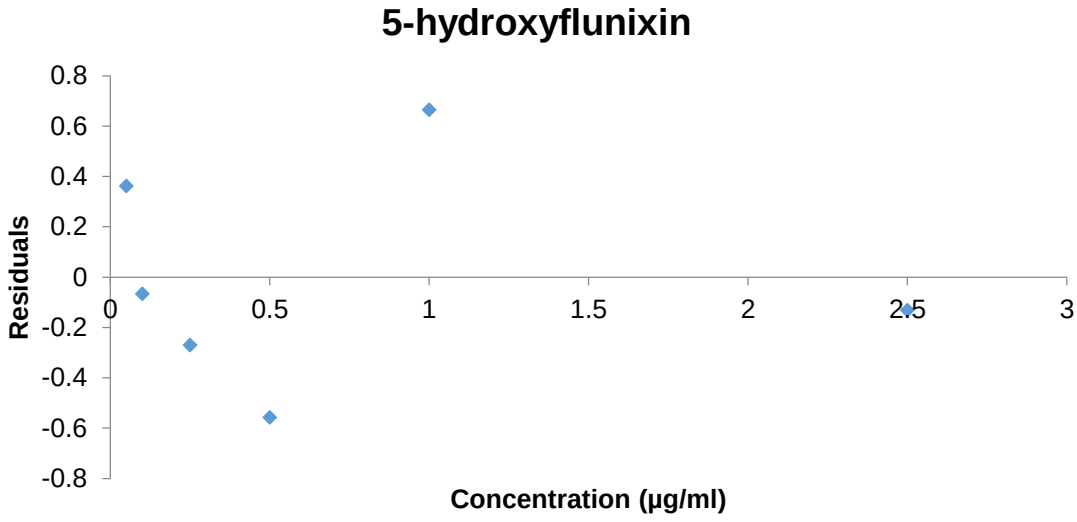




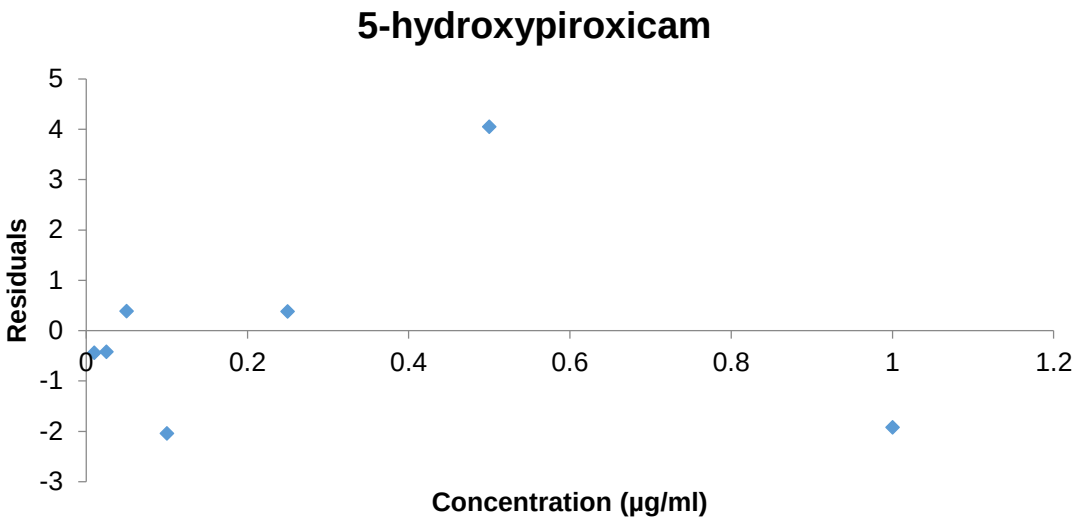
Appendix IX-e Residual plots of NSAIDs over working linear range (µg/ml)



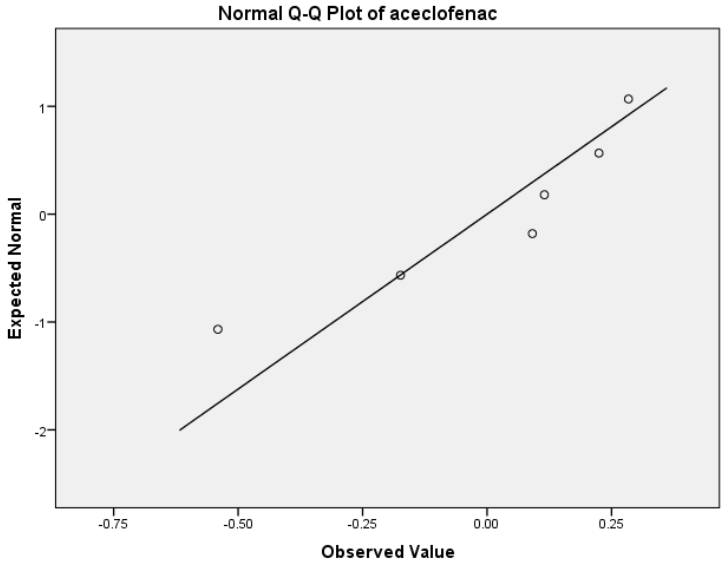


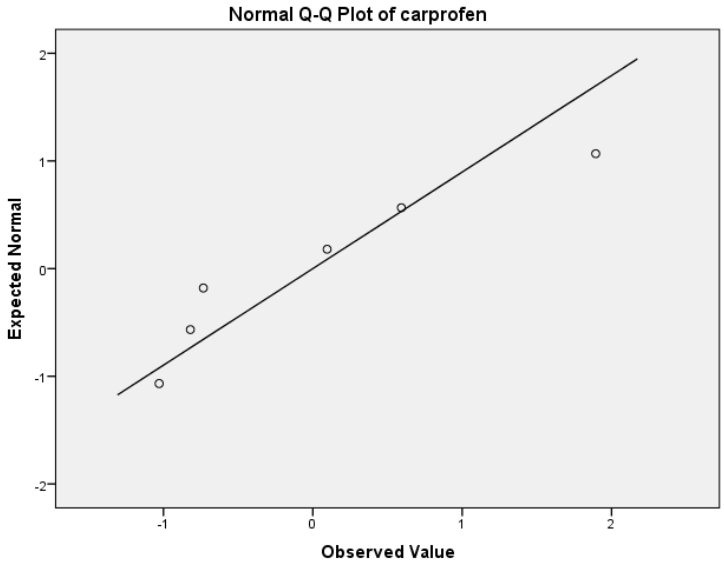


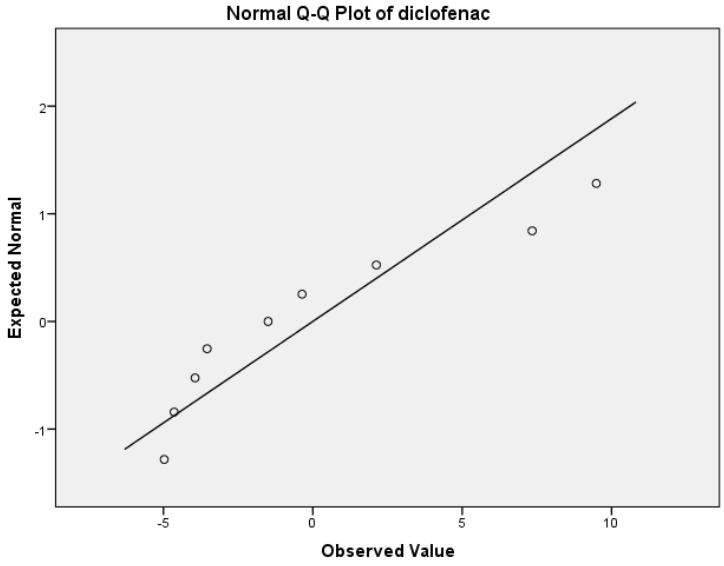
Appendix IX-f Residual plots of NSAIDs over working linear range (µg/ml)



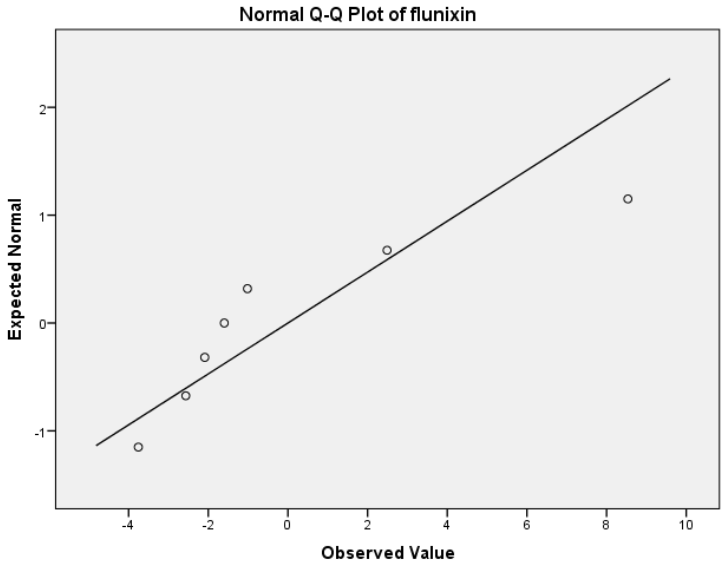
Appendix X-a Q-Q plots for one-sample Shapiro Wilk test

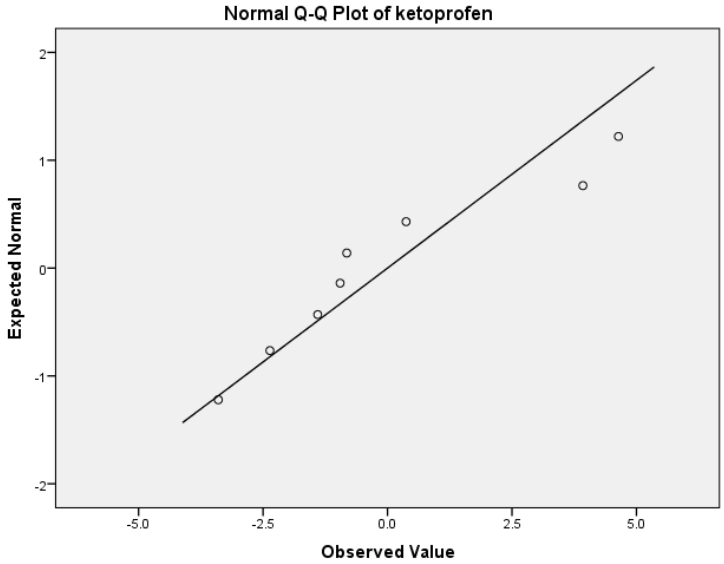


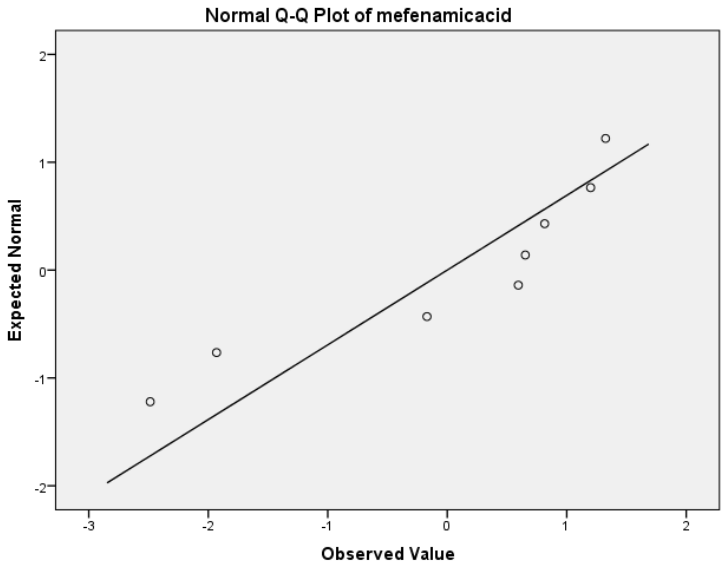




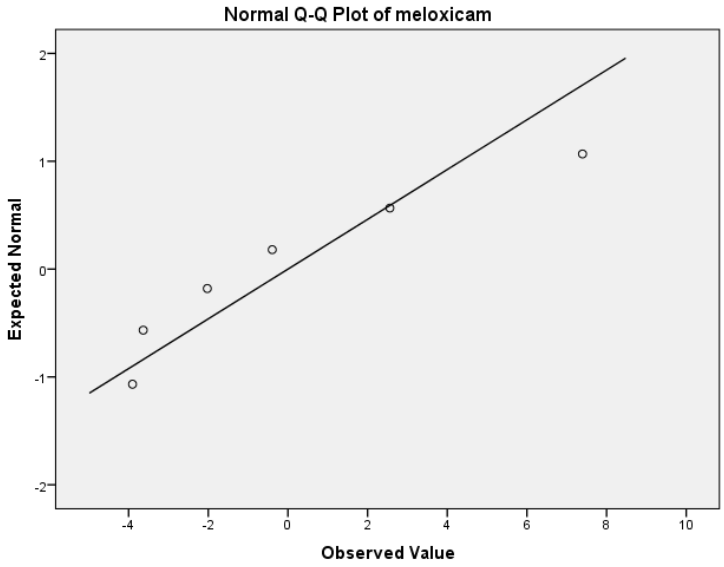
Appendix X-b Q-Q plots for one-sample Shapiro Wilk test

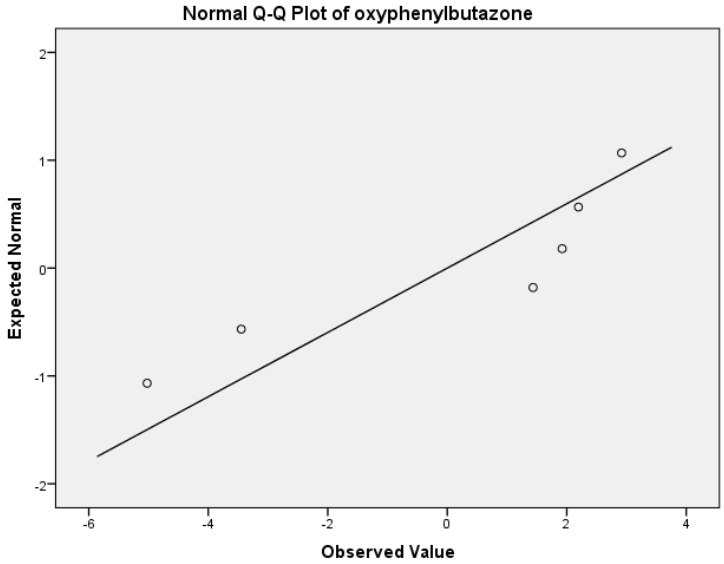


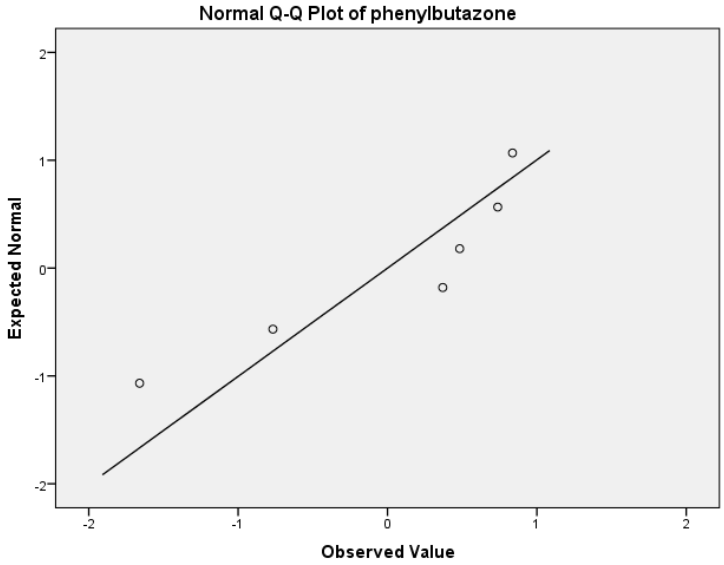




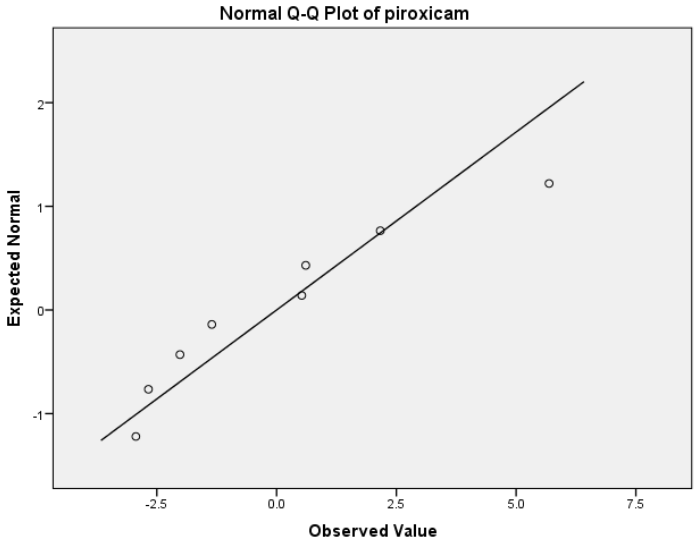
Appendix X-c Q-Q plots for one-sample Shapiro Wilk test

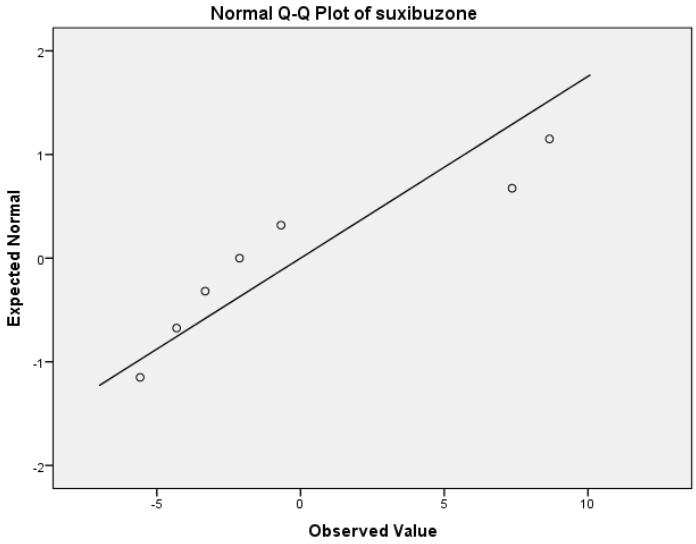


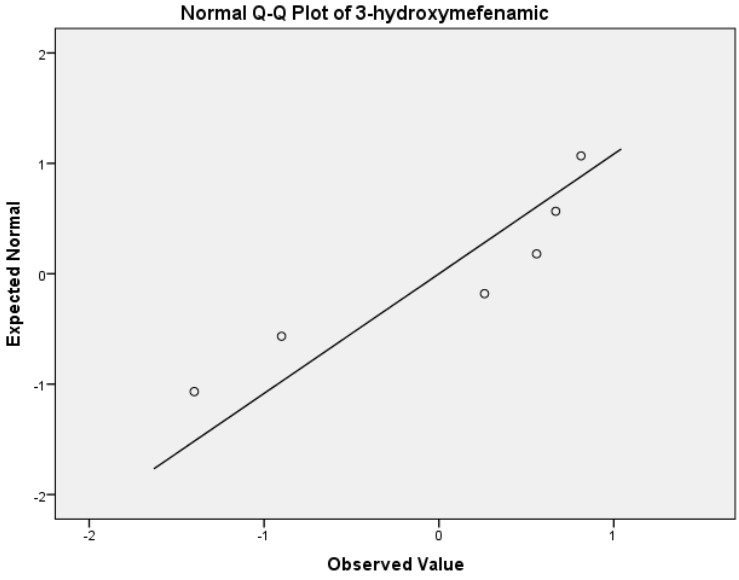




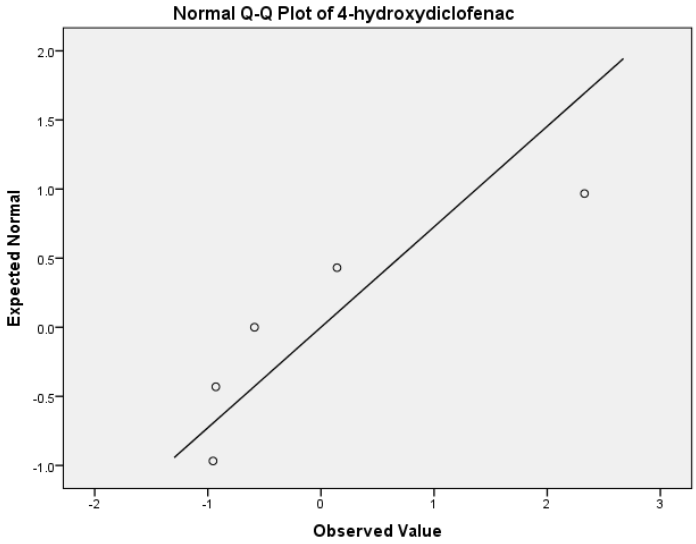
Appendix X-d Q-Q plots for one-sample Shapiro Wilk test

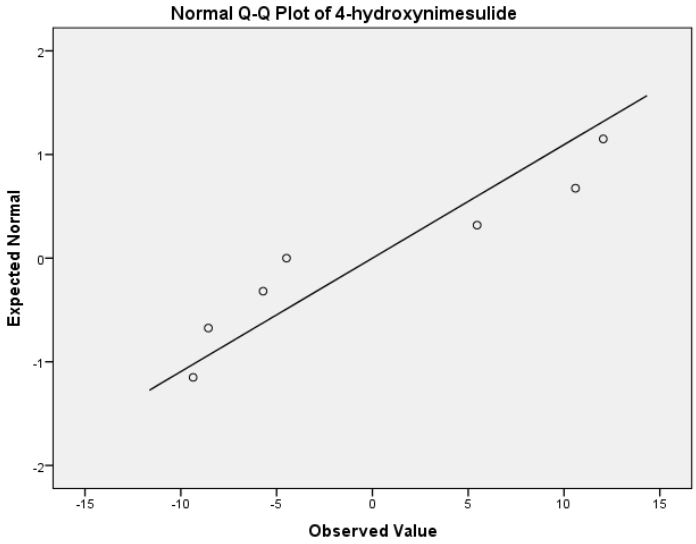


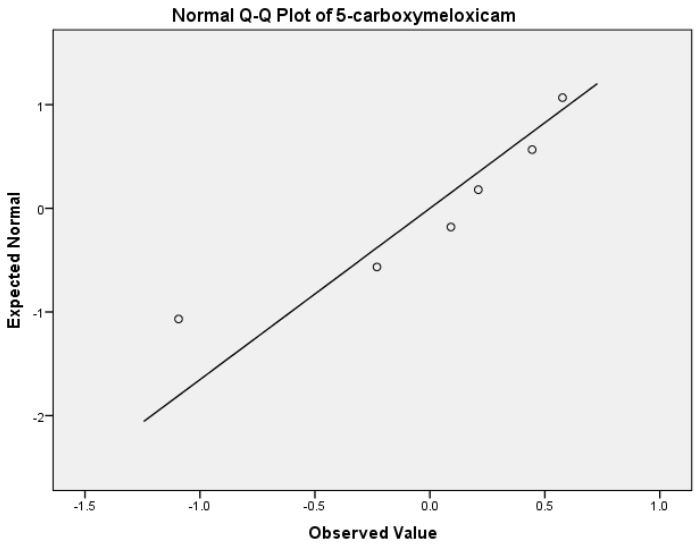




Appendix X-e Q-Q plots for one-sample Shapiro Wilk test







Appendix X-b Q-Q plots for one-sample Shapiro Wilk test