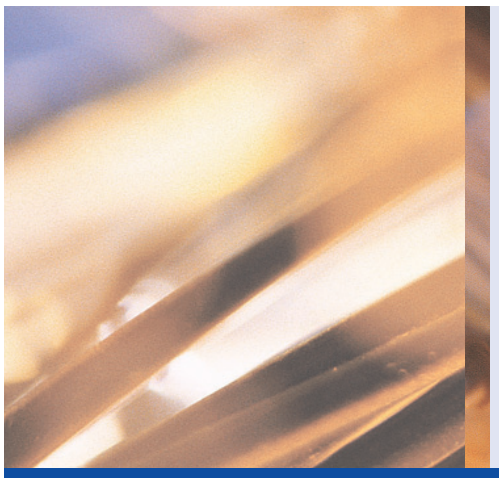


Separation Science Focus

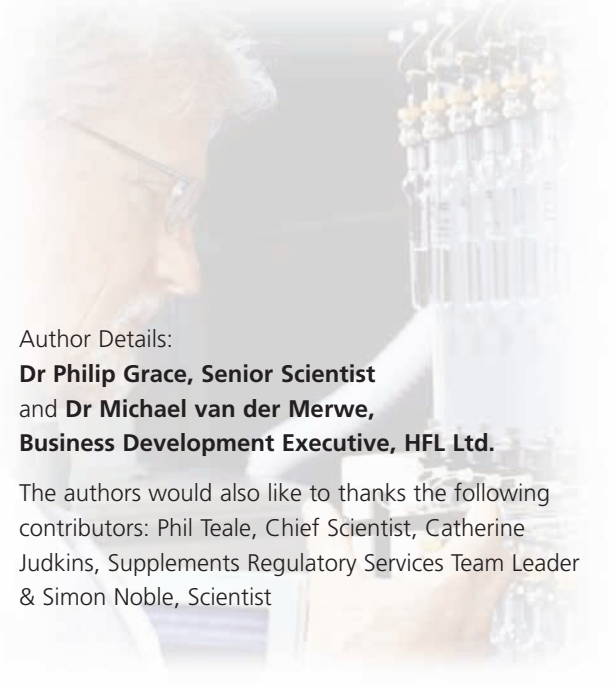
THE USE OF LC-MS/MS AND GC-MS/MS IN THE SPORTS, HEALTHCARE AND PHARMACEUTICALS SECTORS

Dr Philip Grace and Dr Michael van der Merwe

Established more than 40 years ago, HFL is the only laboratory in the world engaged in both contract research and drug surveillance screening to protect the integrity of human and animal sports. This unique background in high-integrity analysis adds an extra dimension to HFL's services supporting pharmaceutical and biopharmaceutical development.

Operating from state-of-the-art facilities near Cambridge, UK, HFL provides laboratory services supporting four major sectors; pharmaceutical, sports, healthcare and government. With a unique blend of core expertise and capabilities in proteomics, immunochemistry, chromatography and mass spectrometry, HFL has particular strengths in high-sensitivity, quantitative analysis of small chemical entities and large biological molecules in biological matrices. This paper describes four case examples applying LC-MS/MS, GC/MS/MS and uHPLC technologies to support our activities in these sectors.

"THE USE OF NUTRITIONAL SUPPLEMENTS TO ENHANCE ATHLETIC PERFORMANCE IS NOW WELL ESTABLISHED"



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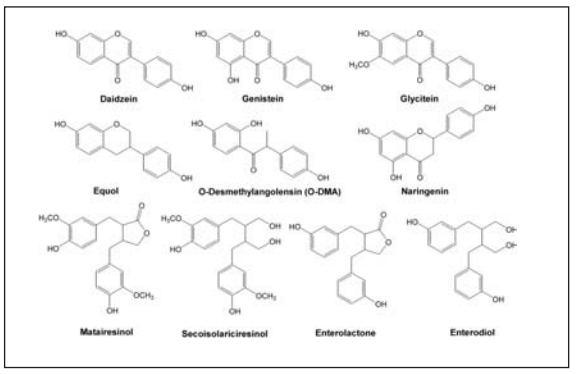
HEALTHCARE

Phytoestrogens are diphenolic compounds that naturally occur in a variety of plants that can form part of the human diet. Interest in phytoestrogens stems from the belief that they may offer a protective effect against a number of human conditions including cardiovascular disease, certain forms of cancer, and menopausal symptoms. Investigation of these potential health benefits relies to a large part on the use of analytical methods that are capable of accurately quantifying the levels of phytoestrogens in biological fluids.



The main types of phytoestrogen of interest are the isoflavones and the lignans. Isoflavones occur in foods such as legumes and in particularly high levels in soy products where they are present mainly as glycosides.

The most abundant isoflavones are genistein and daidzein. Upon entering the human body, the glycosides are hydrolysed by intestinal bacteria to form aglycones. These aglycones may be further metabolised, for example daidzein can be transformed toO-desmethylangolensin (O-DMA) or to equol or they can be converted in the liver to beta-glucuronide or sulfate conjugates which circulate in the blood or are excreted in urine and faeces. Lignans occur naturally as aglycones or as mono-and diglycosides. The variation in the type and extent of conjugation means that there are potentially a great number of possible lignan conjugates. Lignans are found in a much greater variety of foods than the isoflavones, being present in grains, seeds, and in particularly high concentrations in linseed. However, despite being so widespread, there is very little data available on their biological effects, or on the amounts present in foods and biological fluids.

Analysis of phytoestrogens in biological fluids has been accomplished by time resolved fluorescence immunoassays, however, due to the large number of potential metabolites and their close structural homology the use of chromatographic methodologies such as GC-MS or LC-MS/MS is desirable. The high separation power of capillary GC is a potential advantage in this instance but in terms of reliability and quality of quantification we have found LC-MS/MS to be the method of choice. Following addition of triply-^{13}C labelled analogues, to act as internal standards, samples were enzyme hydrolysed to cleave glucuronic acid conjugates, extraction from the biological matrix was accomplished using a polymeric phase extraction cartridge with subsequent analysis in negative ion electrospray ionisation mode. Due to the requirement to separate the twelve analytes from each other and other endogenous materials a relatively long run time of 14 minutes was required. Clearly when utilising expensive equipment such as LC-MS/MS the run time is an important consideration.

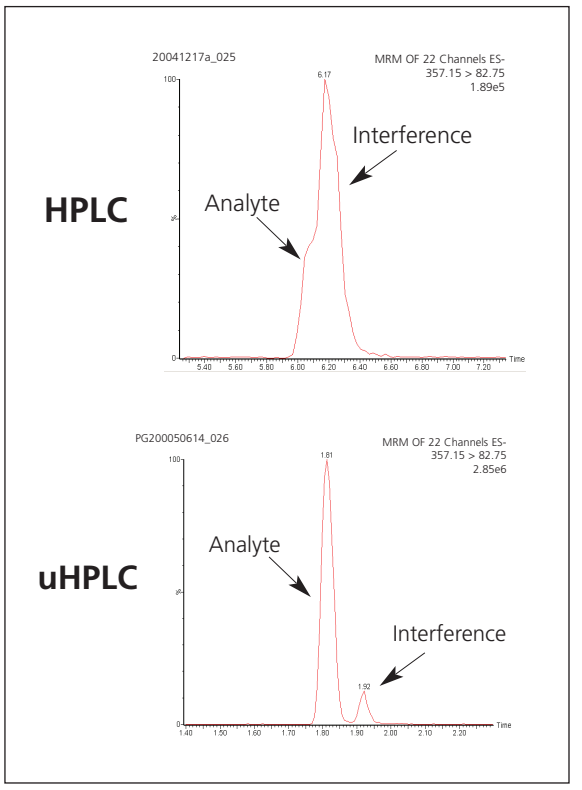


Figure 1. Resolution of a matrix interference using uHPLC

One solution that we successfully applied was to make use of a column switching arrangement with two analytical columns being employed. This allowed the run time to be decreased to 10 minutes, a significant and worthwhile improvement in throughput. A second highly attractive approach is to make use of ultra HPLC.

This provided a significant increase in throughput with an analytical run time of 5.5 minutes, in addition a significant improvement in sensitivity, between 4 and 8 times dependant upon the analyte, was observed. Even more impressive was the fact that even with the decreased analytical run time a matrix interference which coeluted with one of the analytes was clearly resolved using u-HPLC *(Figure 1)*.

THE USE OF LCMS TO DETECT SEDATIVES IN PLASMA (SPORTS)

Traditionally, urine has been the matrix of choice in sports testing. There are several reasons for this including the non-invasive nature of urine collection, the fact that many drugs and metabolites are concentrated in urine and that for many drugs, particularly the anabolic steroids, urine provides an extended detection period. However, blood has many attractions as a matrix of choice, particularly in the equine where urine samples cannot be produced 'on demand'.

In effect, urine is well suited to post race sampling but is inappropriate for alternative sampling strategies, such as pre race, at the training yard, etc. in addition urine is inappropriate as a matrix for the detection of the abuse of a number of the newer protein based therapeutic agents.

For these reasons, there is an increasing requirement to utilise blood serum or plasma for equine sports testing. However, the traditional approach of using full scan GCMS to detect a wide range of potential analytes is inappropriate for the analysis of some potent drugs and drug classes, a good example of this is the sedatives.

The use of sedatives at the time of racing must be monitored as there exists potential abuse to calm an excitable animal or, arguably more sinister, to 'stop' a horse for the purpose of gambling. The sedatives also represent an interesting challenge to the analyst due to the high analytical sensitivity required and the diverse structures of the various sedative types.

Despite the fact that the sedatives are structurally diverse, the majority are basic in nature, this offers the possibility of developing generic extraction procedures. Similarly, basic drugs are amenable to generic atmospheric pressure ionisation conditions, the use of LC-MS/MS also offers the advantage of eliminating the requirement for derivatisation and the use of multiple reaction monitoring to simultaneously look for several analytes at high sensitivity.

Modern MS/MS systems employ collision cell configurations (for example the SCIEX Linac™ cell) designed to allow rapid switching between MRM events allowing the analysis of dozens of analytes without time windowing. Utilising a simple liquid / liquid extraction followed by API LC-MS/MS on a SCIEX3000 system we have developed a screen capable of detecting in excess of twenty sedative drugs, most with low $pg.ml^{-1}$ sensitivity. The generic nature of the method allows the ready addition of other basic drugs to the screen.

THE USE OF GCMS TO DETECT STEROIDS IN SUPPLEMENTS (SPORTS AND HEALTHCARE)

The use of nutritional supplements to enhance athletic performance is now well established with a range of products being available. The use of supplements, although frowned upon in some quarters, is allowed in all sports provided they are free of performance enhancing substances such as anabolic steroids. A number of high profile cases have arisen where it as been claimed that detection of the use of an anabolic steroid resulted from a nutritional supplement contaminated with an anabolic steroid or one of many steroid precursors used in the supplement industry.

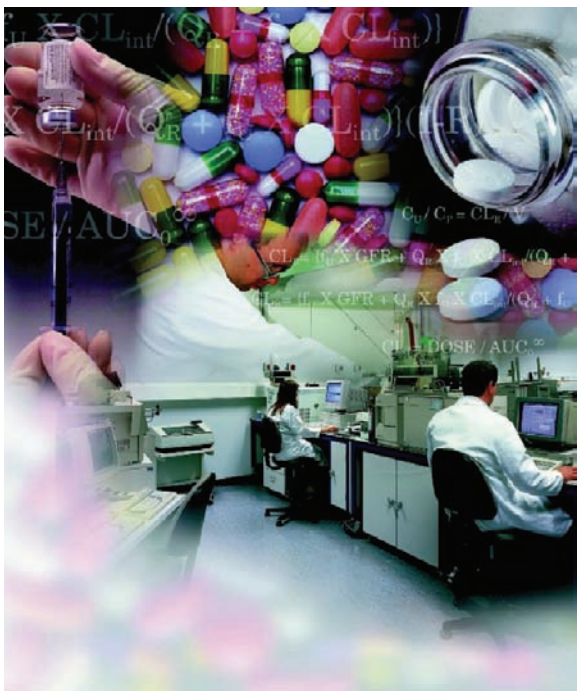
Due to the potency and potential long period of action of the anabolic steroids highly sensitive techniques are used to detect their abuse, as a result similarly sensitive methods are required to analyse nutritional supplements for contamination at trace levels. This requirement is further complicated by the diverse nature of the supplements and ingredients requiring testing, from simple whey powders to mixtures of herbs, flavourings and colourants. HFL has developed a generic methodology capable of detecting a wide range of steroids in supplements.

GCMS remains the most appropriate methodology for detection of androgenic and anabolic steroids due to the separation power offered by capillary columns and the high sensitivity of modern instrumentation. However, when dealing with complex matrices high sensitivity and separation power are not in themselves the complete answer, appropriate sample preparation and derivatisation procedures are equally important.

In this case initial extraction from the solid matrix was accomplished using methanol, following dilution with buffer the sample was concentrated using a polymeric phase SPE and purified using a silica SPE cartridge.

The majority of potential steroid contaminants in sports supplements are precursors of the endogenous steroids nadrolone / testosterone or synthetic 17·-alklylated analogues. Judicious choice of the derivatisation procedure is required to ensure diagnostic high mass ions are generated, for absolute sensitivity and certainty we employ GC-MS/MS, again choice of the derivative formed is important. For this reason we choose to form t-butyldimethylsilyl derivatives, this provides an assay capable of low part per billion sensitivity for a diverse range of matrix types.

uHPLC APPLIED TO THE QUANTIFICATION OF DRUGS IN PLASMA (PHARMACEUTICAL)



API LC-MS/MS is now well established as the technique of choice for bioanalysis in the pharmaceutical industry. But, with ever more potent drugs being developed and strict requirements for registration needing to be observed, increasingly sensitive and expensive instrumentation is required. The relatively high cost of instrumentation, increased sample numbers and need for rapid production of results has long been a driver pushing the analyst to develop faster and faster methods.

However, there are limits to which this is possible, ion suppression is a very real problem with API sources, especially when analysing complex matrices such as plasma. In addition the speed of solvent gradients and the performance of the stationary phase is limited by a number of factors not least the particle size. The advent of viable uHPLC systems promises to revolutionise this situation, an example of an early investigation of the potential of uHPLC to bioanalysis is the analysis of ciprofloxacin.

Ciprofloxacin is an antibiotic used to treat a variety of complaints including anthrax. Previously we developed an LC-MS/MS assay for this drug in plasma. The methodology provided a run time of 4 minutes with good sensitivity and linearity. Transfer of the methodology to uHPLC proved extremely rapid, less than 1 hour, and analysis of standards confirmed that the expected sensitivity gains, due to lessened peak width, could be attained. But how would the system perform with real samples?

In practice using uHPLC the run time could be decreased from 4 minutes to 0.8 minutes allowing 8 batches of samples to be analysed in 9 hours rather than the 46 hours required using traditional technology. In addition, the assay attained twice the sensitivity despite the fact that 3 times less volume was injected. These significant advantages were attained without compromising quality, comparison of 20 samples analysed using the two techniques provided a 0.998 correlation and a gradient of 1.012 indicating excellent agreement.