"Traditionally, livestock carcasses on the Indian subcontinent have been left out by the millions for scavengers, particularly vultures, to consume. Over the last decade several species of the vultures that were the primary consumers of these carcasses face extinction, with diclofenac residues in the livestock carcasses implicated in the cause"

The Importance of Reliable and Efficient Sample Preparation in the Development of a Novel Forensic Method to Detect Diclofenac Residues in Vultures and Livestock Animals

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INTRODUCTION

Diclofenac (*Figure 1*) is a non-steroidal anti-inflammatory drug (NSAID) that is extensively used to treat pain and reduce inflammation in humans and animals. Other commonly encountered NSAIDs include ibuprofen and aspirin. First introduced as an analgesic and anti-inflammatory agent for humans in the 1970s, it was in widespread use in veterinary medicine by the 1980s [1]. Diclofenac was registered for veterinary use on the Indian subcontinent in the mid to late 1990s [2,3]. Here, livestock animals are often used as working machines, and diclofenac was regarded by veterinarians and farmers as a 'wonder drug' because of its evident benefits very quickly after dosage. The drug's effectiveness, however, arises from its abatement of the symptoms rather than the root cause of the impairment. Multiple administration is often necessary, especially in elderly ailing animals, where nature eventually takes its course.

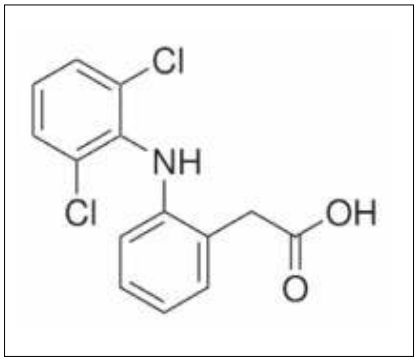

Figure 1. Structure of diclofenac

Traditionally, livestock carcasses on the Indian subcontinent have been left out by the millions for scavengers, particularly vultures, to consume. Over the last decade several species of the vultures that were the primary consumers of these carcasses face extinction, with diclofenac residues in the livestock carcasses implicated in the cause [4]. Despite the introduction of various regulatory measures to prevent further manufacture of diclofenac within and import into the Indian subcontinent, the concern remains that stockpiles exist and formulations are still available from nearby countries such as China and Tibet where the manufacture of veterinary diclofenac is legal [5]. Although clinical trials identified the NSAID meloxicam as an effective and safer alternative to diclofenac [6,7], it remains more expensive and is therefore not as popular.

The discovery that diclofenac was available for veterinary use in some parts of Africa in 2007 raised concern for those with an interest in the conservation of Africa's already imperilled vultures and other susceptible avian species. These worries are not confined to diclofenac only. According to a survey of captive facilities; exposure to carprofen, flunixin, ibuprofen, ketoprofen and phenylbutazone may also adversely affect avian species [8]. Given that NSAIDs are registered worldwide for administration to livestock animals, it is critical to be able to monitor for their presence in the environment, in the carcasses of animals available to any vulnerable species, and in the latter themselves.

Most conventional methods of diclofenac detection still require extraction of the drug residues from the tissues of the dead bird or livestock animal. These samples must be in sufficiently good condition for analysis, therefore retrieval as soon as possible after death is critical. However, carcasses, particularly those in remote locations, may not be found for days, weeks or months, during which time they can be subjected to extreme environmental conditions. Given these factors, a method that could detect residues in the more long-lived keratinous matrices was deemed useful for long-term monitoring and conservation work.

To address this, an alternate technique was developed to identify diclofenac residues in vultures and livestock animals by GC-MS (published elsewhere). A preliminary multiscreen method for the simultaneous detection of carprofen, diclofenac, flunixin, ibuprofen, ketoprofen and phenylbutazone was also developed to address the dearth of residue detection methods for the other NSAIDs of concern. The diclofenac detection method was tested using vulture feathers, human hair and nails for eventual application to the analysis of talons, beaks, hooves and bones.

The method was developed principally for dissemination to African laboratories working with Foundation for Analytical Science & Technology in Africa (FASTA) and particularly to the chemistry laboratory of the Jomo Kenyatta University of Agriculture & Technology (JKUAT) in Nairobi, Kenya. The intention is that the method be routinely used in wildlife forensic investigations both to ascertain the cause of death of African vultures and to evaluate presence or absence of diclofenac and NSAIDs of concern in the agricultural environment. FASTA is a charitable company that was established to support scientific education, analytical research and the preservation of the environment in Africa via capacity-building and technology transfer.

This paper reviews the development of this methodology, the technical challenges faced, with emphasis on sample preparation, and the role of the miVac sample condenser in increasing the overall efficiency and reliability of the method and its validation.

DEVELOPMENT OF A METHOD TO DETECT DICLOFENAC IN HAIR, NAIL AND FEATHER SAMPLES

The development of the diclofenac detection method required extensive stepwise preliminary trials, namely: assessing its solubility and stability in various solvents, selecting a derivatising agent, optimising derivatisation, establishing instrument sensitivity and testing the extraction process. All samples were dried down at 40°C and reconstituted with derivatising agent before being run on the GC-MS. The method validation comprised extracting samples of hair, nails and feathers in methanol overnight, drying down the extracts and derivatising with N,O-Bis(trimethylsilyl)trifluoroacetamide with 1.0% trimethylchlorosilane (BSTFA 1.0% TMCS) and ethyl acetate prior to analysis (Richards 2009, unpublished data).

In the preliminary stages of the research, samples were evaporated to dryness at 40°C under a steady stream of nitrogen in a Techne Dri-block heater. However, this method was time-consuming, inconvenient and results lacked uniformity. Fitting samples beneath the needles prior to drying could take 10 – 15 minutes. Samples prepared from methanolic solution (1.0 – 2.0ml) took at least 45 minutes

to dry down, frequently over an hour. Extracted samples often took several hours and even then were not completely dry. The system also required close monitoring and samples dried down at different rates within the heater. Reconstitution of samples containing a small residue of methanol resulted in incomplete derivatisation or reaction inversion. Work was occasionally delayed if a shipment of nitrogen was late, and the system was limited to 30 samples at a time.

Sample preparation is the cornerstone of method development. As such it should be easy to carry out, efficient and cost-effective, needing a minimum of consumables, particularly if it will be used in developing countries. Each step must be repeatable and reproducible prior to validation. Rapid and reliable preparation is especially important for an application such as this, where mitigating measures and monitoring strategies depend on obtaining accurate results in a timely manner. All samples must be free from artefact so that any incongruous results need not be attributed to sample preparation. Due to the problems outlined above, an alternate method of sample evaporation was required. The miVac DNA concentrator from Genevac Ltd (UK) was selected, not only because it has been successfully used in other similar applications [9], but because of the ease and convenience of usage, specifically: the comparative speed of drying, increased number of samples that can be dried down simultaneously, and uniformity of the result. Samples prepared from solution (1.0 and 2.0 ml) required up to 15 minutes only to completely dry down while extracted samples took up to one hour. No residual methanol was detected. Up to 44 samples, prepared in either 2.0 or and 4.0ml vials could be dried down simultaneously. Vial trays can be custom-made to accommodate the preferred vial volume(s). While the tray used for this work was designed to accommodate both 2.0 and 4.0ml vials, if only 2.0ml vials had been used then up to 78 samples could have been dried down at one time. Complete dry down was achieved in all case with confidence.

Figure 2. miVac DNA concentrator.

The miVac (*Figure 2*) dries by boiling the samples under vacuum. As the pressure in the system drops, so does the boiling point and therefore the temperature of the samples. Samples containing methanol will routinely boil at -20°C until they dry when they will warm up to the temperature of the system, typically not more than +40°C. The sample vials are spun in a centrifuge rotor during evaporation to prevent samples boiling over and any resultant sample loss and/or cross contamination. Vial holders can be custom made to accommodate the favoured vial size.

Using the miVac DNA concentrator with solid aluminium rotor, up to 44 samples could be dried down in approximately 15 minutes, a great improvement on the nitrogen blow down system. There were no incidents of partial or incomplete drying, eliminating anomalous results due to poor or incomplete derivatisation. In addition, the miVac runs free from operator attention and requires no consumables, rendering suitable for use in areas were the supply chain of scientific materials may be weak.

CONCLUSION

At least 15,000 samples were dried down over the course of the method development and subsequent validation. Though this may represent a standard weekly run for a commercial laboratory, it is nonetheless a large number for a single PhD researcher to prepare and process. The sample preparation process and method validation were substantially improved by replacing the nitrogen blow down device with the miVac DNA concentrator. The analysis of diclofenac in feathers, hair and nails was facilitated and results could be interpreted with confidence. The concentrator purchased for this research will be shipped to the JKUAT laboratory in Nairobi so that it can be used for this application and in follow-up research.

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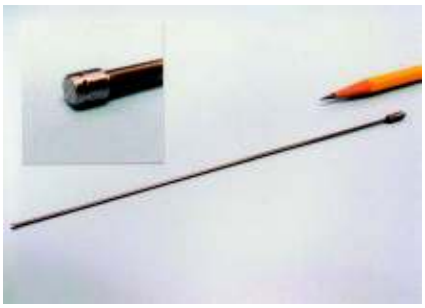
Micro Nozzles for Delicate Wash Down Applications



A new range of micro wash down nozzles for washing cuvettes, test tubes, 96 well plates and other similar laboratory receptacles as well as purging/drying gas chambers, has been introduced by miniature component specialists **Lee Products**. They are constructed entirely from 303 and 304 stainless steel. Lee nozzles are only 6" long, feature a 0.062 Minstac fitting at one end and have a total nozzle restriction of 1800 Lohms.

Two versions are available, one with ten 0.010" diameter holes and one with twenty 0.007" diameter holes to ensure an ultra fine spray, which is ideal for delicate, yet effective wash down in critical areas. However, it is intended to produce a version with forty 0.005" diameter holes in the future. Various lengths are available on request, as are specials to suit specific applications.

Circle no. 80

Expanding Gene Expression Assay Portfolio

Integrated DNA Technologies (IDT) has expanded its PrimeTime qPCR portfolio to include new assay scales that enable more affordable gene expression studies. The PrimeTime Mini (100 reactions), Standard (500 reactions) and XL (2500 reactions) Assay scales better reflect customer needs for validation and screening studies.

Stephen Gunstream, IDT's OEM Business Unit Leader, commented: "Our new qPCR scales ensure that all scientists, including those who only require limited reactions, can have access to the most precise quantitative PCR chemistry available." The new assays can be ordered through IDT's RealTime PCR Design Tool. The tool allows for ultimate design flexibility and includes the option to select specific exon spanning sites, as well as review all primer and probe sequences prior to purchase.

Circle no. 81