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Robust Extraction and Clean-up Method for Analysis of Organophosphates, Synthetic Pyrethroids, Organochlorines, Diflubenzuron and Triflumuron on Raw Wool.

By

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SUMMARY

This report describes a robust method for the extraction of hydrophobic pesticides in raw wool and for clean-up of the extract prior to analysis. The pesticides covered by this method include organophosphates (OPs), synthetic pyrethroids (SPs), organochlorines (OCs) and the hydrophobic insect growth regulators (IGRs), diflubenzuron and triflumuron. In keeping with the original intention of Draft TM 59, the method described is not intended to be prescriptive; it simply seeks to provide guidance for the newer laboratories that are having problems with isolation of pesticides from the difficult wool wax matrix.

The procedures described remove the high molecular weight and polar materials that are encountered on raw wool, and produce relatively clean pesticide-containing fractions that can be chromatographed reliably and reproducibly. The methods can be performed manually and do not require specialised equipment. It is intended that laboratories will substantially modify the procedures to suit the equipment and supplies that they have on hand, and the analytes that they need to determine. As an example, quantitation of the analytes (with the exception of diflubenzuron and triflumuron) in this procedure is by gas chromatography (GC) using dual electron capture detector (ECD) and a phosphorous-specific thermionic specific detector (TSD). It is likely that most modern laboratories will already be using more selective mass spectrometry for the detection of most pesticides, however the tuning, set-up, selectivity and optimisation of these detectors is highly dependent on the brand and model of the detectors (and in some cases, different units), and is beyond the scope of this paper. It is possible that laboratories can operate both mass-selective detectors and TSD/ECD in parallel while they develop their own robust procedures.

The method described uses a mixture of hexane and ether to extract lipid material and hydrophobic pesticides from raw wool. The bulk of the high molecular mass wool wax is removed from the extraction solution using gel permeation chromatography (GPC), and a small silica gel chromatography column is used to isolate pesticides of different polarities into two different fractions. The more polar pesticides (the organophosphates) are analysed separately using suitable GC conditions with a specific TSD detector. The wool wax fraction containing the less polar pesticides is relatively free of interferences and can be analysed by GC with ECD, however a confirmatory analysis using a different GC column is strongly advised if the relatively non-specific ECD is used.

The more polar IGRs are thermally unstable under GC conditions, and they are analysed separately using a simpler solid phase extraction (SPE) clean-up with quantitation using high performance liquid chromatography (HPLC).

It is important to note that the extraction procedure used in this paper is only suitable for greasy wool samples. For extraction of scoured wools, it is essential to use polar solvents and more aggressive extraction conditions that swell the fibre.

INTRODUCTION:

The determination of pesticide residues in greasy wool is a complex analysis requiring a number of operations. The steps include extraction of the pesticides from the raw wool, separation of the pesticides from the bulk of the wool wax matrix, followed by analytical determination and quantitation of the various classes of chemicals. The pesticides discussed in this submission are all lipophilic (fat soluble) and tend to associate with the wool wax present on the surface of the wool fibre. Extraction conditions that will remove the wool wax from the wool will also remove these pesticides.

Wool wax is an extremely difficult, complex and variable analytical matrix, being composed primarily of wool wax esters, but it also contains free fatty acids, free fatty alcohols and sterols, and their breakdown products from oxidation. Many of these wool wax components have similar polarity and molecular weights to the pesticides, and they can potentially cause interferences in the analytical procedure. The high molecular weight waxes and high polarity components on the raw wool can also contribute to degradation of the chromatography columns and equipment employed in the procedure, and this can lead to changing retention times, matrix effects on injection, and general non-reproducibility, and this has been reported by some of the newer laboratories. This paper provides simple, low cost, manual procedures for extraction of pesticides from raw wool, and for preparation of pesticide-containing solutions that can be analysed reproducibly and consistently.

The paper provides general guidance on the specific analysis and quantitation steps, however these will vary from laboratory to laboratory, and will depend on the equipment already purchased, and the specific analytes being sought. The quantitation methods outlined in this paper use either liquid chromatography with a diode array detector, or gas chromatography with low cost and widely available electron capture and phosphorus specific detectors.

However, for the gas chromatographic separations, many laboratories will already own mass-selective detectors that will vary from ion-trap to multi-quadrupole models, and that might operate in a variety of modes from total ion to MS-MS and beyond. The tuning and optimisation of these units must be done on a machine-by machine basis, and is beyond the scope of this paper. Our intention is to provide clean-up conditions for raw wool extracts that will allow stable chromatographic conditions; these are an essential requirement to allow further optimisation and tuning of conditions to occur.

LITERATURE METHODS FOR ANALYSIS OF WOOL WAX MATERIALS

A number of approaches have been utilised for isolation of the pesticides from the wool wax. These methods have largely been developed to test for pesticide residues in lanolin used in cosmetic and pharmaceutical preparations. The traditional method for analysis involved solvent partitioning [1] but was very time intensive and problems were often encountered with the formation of emulsions. Diserens [2] used a two stage SPE procedure, first removing most of the lipid material on a diatomaceous earth column, followed by a secondary reversed phase cleanup using a silica-based C-18 adsorbent. Quantitation was by gas chromatography (GC) using both electron capture and flame photometric detection. This method was demonstrated as being suitable for a range of OP and OC pesticides.

The Official Monographs for Lanolin and Modified Lanolin by the United States Pharmacopoeial (USP) Convention [3] recommend a gel permeation chromatography (GPC) cleanup procedure, followed by GC determination with both electron capture and flame-photometric detection. This method is cited as being suitable for a range of OP and OC pesticides. This approach using GPC was also employed by Heikes [4] for the cleanup and analysis of lanolin for OP and OC pesticides, using a combination of electron capture, flame photometric and mass spectrometric techniques after gas chromatographic separation.

The European Pharmacopoeia method for pesticides in lanolin [5] also uses gel permeation chromatography, followed by solid phase extraction with magnesium silicate as a second cleanup step to remove the small amount of lanolin co-eluting with the pesticide fraction. This is followed by gas chromatographic separation with either electron capture or thermionic detectors. The method is cited for a range of OP, SP and OC pesticides.

Jones [6] modified the GPC parameters of the USP method to allow analysis of SP pesticides, followed by a secondary alumina solid phase extraction step to produce a robust cleanup procedure suitable for

OP, SP and OC pesticides. He used GC with quantitation by electron capture and thermionic specific detectors.

The above authors have demonstrated that silica-based GPC cleanup followed by a solid phase extraction can be used to isolate wool wax fractions that can then be analysed using relatively simple GC methods using low cost detectors. The current CSIRO method is based on this previous work, but addresses several of the problems that were encountered.

A common problem with the published GPC methods is that they are based on traditional GPC procedures that use large volume columns and high solvent flow rates. The solvent volumes involved are large and this adds significant cost and time to the clean-up and analysis. The current CSIRO method uses a modern commercial GPC column that allows use of lower injection sizes, lower flow rates and lower overall solvent volumes.

An additional problem is that electron capture detectors are not specific only to compounds that contain halogens. A signal can be obtained from halogen-free compounds if they are present in larger concentrations. This means that an analyst cannot be confident that a chromatographic response was obtained from a trace concentration of a pesticide, or from a larger quantity of a non-pesticidal material with a similar elution time. It also means that there may be a great deal of baseline noise that may make quantitation difficult. It is essential that results obtained from electron capture detectors are confirmed using different chromatographic conditions, or mass-selective detectors must be used before the presence of an analyte can be reported.

DESCRIPTION OF CSIRO ANALYSIS METHOD

The analytical test specimen, prepared by blending, conditioning and sub-sampling, is extracted with 5% diethyl ether-hexane solvent, either in a soxhlet apparatus or overnight at ambient temperature, to remove the wool wax and associated pesticides. It is important to note that this procedure is only suitable for greasy wool samples as the mild extraction procedures will only recover pesticides from the external wool wax layer. Solvents that swell the fibre (such as hot methanol) are needed to extract pesticides associated with internal lipids from scoured wool.

An aliquot of the extraction solvent is taken to dryness. The addition of a non-volatile “keeper” is recommended to minimise any pesticide losses during the drying steps. If the dried sample is not processed immediately, it may be stored (in a refrigerator) until required for analysis. The wax residue is warmed and dissolved in a suitable solvent in preparation for the following GPC/solid phase extraction (SPE) cleanup procedure for analysis of organophosphates, organochlorines and synthetic pyrethroids, or for the SPE clean-up of diflubenzuron and triflumuron. Appropriate internal standards may be added to the extraction solvent.

For analysis of organophosphates, organochlorines and synthetic pyrethroids, the reconstituted hexane solution is passed through a commercial high resolution GPC column. Columns may also be prepared as detailed in references 3 - 6, however higher volumes of solvent may be needed. The dimensions of the column will dictate the flow rates required, and the sample volume injected onto the column. When the wool wax solution is injected onto the column, the larger molecular weight wool wax esters will be eluted first, and this volume is discarded. The fraction containing the pesticides is retained, and later eluting materials (lower molecular weight) are discarded. The column must be characterised using a solution of wool wax, together with solutions containing the pesticides of interest to determine the precise volumes to be collected.

The solvent used to perform the GPC elution is carefully selected so that the pesticide-containing fraction from the GPC column can be transferred directly to a preconditioned silica SPE column, where the pesticides will be retained. A 30% solution of dichloromethane in n-hexane has been found to be suitable for the GPC/SPE columns used at CSIRO. The cartridge is then eluted with a slightly stronger solvent to ensure removal of all of the SP and OC pesticides (Fraction A). The silica cartridge is then eluted with a second solvent to remove the more polar OPs, and collected separately as Fraction B. The solvent is removed from the two fractions, and the residues are reconstituted in solvent containing an appropriate internal standard before each is analysed separately by GC, using electron capture and thermionic detection.

Fraction A is a very narrow polarity fraction that contains the less polar SP and OC pesticides. This fraction contains relatively little of the wool wax sterols, fatty acids, alcohols, or their degradation products, and it produces a relatively flat GC baseline with few interferences in the ECD chromatograms.

Even so, because the ECD is non-specific, the identity of peaks that appear in the retention windows for organochlorine and synthetic pyrethroid pesticides should be checked by analysis on a different GC column or with a mass-selective detector before the presence of an analyte can be reported.

Fraction B collected from the secondary solid phase clean-up column contains the more polar OPs which are analysed using a thermionic specific detector (TSD) tuned to provide maximum sensitivity for compounds that contain phosphorous. The fraction containing the OPs has a greater concentration of impurities, but because the detector is tuned to be sensitive to phosphorous, the interferences are not seen in the chromatogram.

Internal standards may be added at a number of steps in the analysis to check on the efficiency of the procedure. Appropriate internal standards will be of the same classes of chemicals, but be pesticides that are not used on sheep and are not expected to be encountered during analysis of wool samples.

The lipophilic insect growth regulators (IGR), diflubenzuron and triflumuron are analysed using a separate aliquot from the original wool extract. The reconstituted wool extract is simply transferred to a solid phase clean-up cartridge, and the bulk of the non-polar wool wax is eluted with a less polar solvent. The IGRs are then eluted from the clean-up column with a selective solvent, and analysed by HPLC using diode array detection. The purity of the eluted peaks at the retention time corresponding to diflubenzuron and triflumuron is checked by comparing the UV spectra of the eluted peak with the UV spectra of the pure pesticides. Again, internal standards are used, chosen from related benzoylureas that are not used on sheep

The reported procedures were used by CSIRO in the IWTO Interlaboratory Proficiency Program from 1999 to 2001, with satisfactory results [7, 8, 9, 10].

EXPERIMENTAL

Wool extraction and preparation of sample for cleanup:

The analytical test specimen (10g) is extracted to remove the pesticides using 5% diethyl ether in hexane (100 mL) as the solvent, with gentle agitation overnight. After centrifuging a 10 mL aliquot is taken, keeper (1-decanol) is added and the sample is taken to dryness in a Dri-Bath at a temperature of 45 – 55 ^{o}C using a gentle stream of nitrogen. Care must be taken to remove the sample as soon as it is dry, otherwise loss of volatile pesticides may occur.

For analysis of organophosphates, organochlorines and synthetic pyrethroids, the residues are reconstituted in 1 mL of hexane containing two internal standards (one OP and one SP).

For analysis of diflubenzuron or triflumuron, a separate aliquot of extraction solvent is taken to dryness and reconstituted in 8 mL of 30% dichloromethane in n-hexane, and an appropriate internal standard.

Cleanup procedure and analysis for OP, SP and OC pesticides:

The method involves a GPC separation, followed by SPE clean-up using a silica column. Two different fractions are collected for analysis. Fraction ‘A’ will contain the SP and OC pesticides, while the OPs are eluted in Fraction ‘B’.

The cleanup procedure uses a Styragel HR 0.5 column (Waters), fed with 30% dichloromethane in n-hexane at 1 mL/minute, with an injection volume of 0.4 mL. Under these conditions, most of the wool wax elutes in the first 9 minutes and is discarded. The pesticides elute from the GPC column in the following 15 minutes and this volume is collected.

The pesticide fraction is passed immediately through an SPE column (for example Bond Elut silica, 500 mg and 2.8 mL size (Varian)) that has been preconditioned with 30% dichloromethane in hexane. The eluent is collected. A solution of 40% dichloromethane in n-hexane (9 mL) is immediately passed through the SPE column and also collected. The two volumes are combined as Fraction A. A solution of 5% methyl tert-butyl ether (MTBE) in dichloromethane (5 mL) is then immediately passed through the SPE column and collected as Fraction B. It is important that the adsorbent in the SPE column is not allowed to dry between these operations. After solvent removal using a gentle stream of nitrogen, the residues are reconstituted in 1 mL hexane containing the appropriate GC internal standard (SP/OC for Fraction A, OP for Fraction B). Again, particular care must be taken with the drying step to ensure that volatile pesticides are not lost.

GC conditions:

A Varian 3400 gas chromatograph, fitted with an electron capture detector and a phosphorus specific detector (TSD) is used. The GC is operated to the manufacturer's recommendations. Chromatographic separation is achieved on a relatively non-polar capillary column, for example, fused silica capillary column, BP5: 5% phenyl dimethyl siloxane (SGE), or equivalent. OCs and SPs are determined in Fraction A using a column connected to the ECD, by comparison with known standards. The OPs are similarly determined in Fraction B using the TSD. The column oven is programmed with a temperature gradient up to 300 °C. It is necessary to heat the columns to near their maximum recommended temperatures to remove any wool wax components that have passed through the clean-up procedures.

Cleanup procedure and analysis for diflubenzuron and triflumuron:

The extraction of the wool and preparation of the sample for cleanup is detailed above. It should be noted that diflubenzuron and triflumuron are poorly soluble in the normal ether-hexane solvent used for extraction of the other hydrophobic pesticides, and if high concentrations of these pesticides are expected (greater than 100 mg/kg on the wool), dichloromethane must be used as extraction solvent. If diflubenzuron or triflumuron are found at concentrations near 100 mg/kg when ether-hexane has been used as solvent, the wool sample must be re-extracted with dichloromethane and the extracts added to the previous solution to ensure that the extraction is complete.

An aliquot of the extraction solution is dried, then dissolved in 8 mL of 30% dichloromethane in n-hexane. This is passed through an SPE column (for example, Bond Elut silica, 500 mg and 2.8 mL size (Varian)) that has been preconditioned with the same solvent. The eluent is discarded. The column is then immediately eluted with 9 mL of 40% dichloromethane in n-hexane which is also discarded. The pesticides are eluted from the column with 5 mL of 5% MTBE in dichloromethane. This eluent is collected, dried in a gentle stream of nitrogen, and reconstituted in an appropriate injection solvent for analysis by normal phase HPLC.

The HPLC separation uses a diol column with a dichloromethane - heptane mixture as the mobile phase. Other columns and separation conditions may be used, and it important to adjust solvent conditions to ensure that possible interfering components are selectively moved away from the main analyte and internal standard peaks to ensure optimum quantitation.

Routine quantitation of diflubenzuron or triflumuron is conducted at 280 nm. The purity of peaks eluting at the retention time corresponding to diflubenzuron and triflumuron is checked by comparing the UV spectra of the eluted peak (at front, mid and trailing edge) with the UV spectra of the pure pesticides. Again, internal standards are chosen from related benzoylureas that are not used on sheep.

REFERENCES

1. *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, sec. 29.014.

2. Diserens, H. (1989) *Simplified Extraction and Cleanup for Multiresidue Determination of Pesticides in Lanolin*. J. Assoc. Off. Anal. Chem. Vol. 72, No. 6. pp991-993.

3. USP, Official Lanolin and Modified Lanolin Monographs (1994). In USP 23; The United States Pharmacopoeial Convention: Washington, DC, pp 869 - 872.

4. Heikes, D.L and Craun, J.C (1992) *Rapid Multiresidue Procedure for the Determination of Pesticides in Anhydrous Laonlin and Lanolin-Containing Pharmaceutical Preparations Utilizing Gel Permeation Chromatography Cleanup with Gas Chromatographic and Mass Spectrometric Techniques.*J. Agric. Food Chem. *40,* pp 1586 – 1590.

5. European Pharmacopoeia (2004) Wool Fat: Adeps Lanae. Fifth Edition, Supplement 5.2. 07/2005: 0134.

6. Jones, F.W. (1996) *Multiresidue Analysis of Pesticides in Wool Wax and Lanolin Using Gel Permeation and Gas Chromatography.* J. Agric. Food Chem. *44,* pp 3197 – 3201.

7. Australian National Residue Survey. (1999) *First NRS Laboratory Performance Evaluation, March – June 1999.*

8. Australian National Residue Survey. (1999) *Second NRS Laboratory Performance Evaluation, October 1999.*

9. Australian National Residue Survey. (2000) *Third NRS Laboratory Performance Evaluation, March 2000.*

10. Australian National Residue Survey. (2000) *Fourth NRS Laboratory Performance Evaluation, October 2000.*