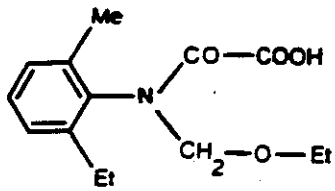
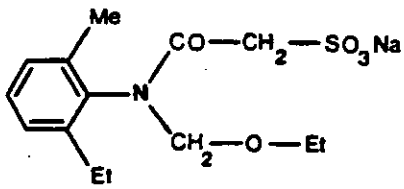
1. SCOPE

The analytical procedure described is suitable for the determination of 2-(ethoxymethyl)amino-N-(2-ethyl-6-methylphenyl)oxo-acetic acid [oxanilic acid (I)] and N-(ethoxymethyl-N-(2-ethyl-6-methyl phenyl)-3-oxoethane sulphonic acid as a sodium salt [sulphonic acid sodium salt (II)] in soil using external standardisation. The limit of determination of the method is 0·02 mg kg^{-1}

(I)



(II)



2. METHOD SUMMARY

A prepared soil sample is extracted by shaking with acetonitrile/water (1:1 v/v) containing 40 mM ammonium acetate for 30 minutes at room temperature. Centrifugation is performed and the aqueous extract then subjected to two successive Bond Elut C_{18} and C_8 column clean-up steps. The residue determination is by liquid chromatography using reversed phase ion pair mode with UV detection.

3. PROCEDURE

3.1 Sample Preparation

Soil samples received in zero contamination tubes should be allowed to thaw for approximately half to one hour prior to preparation.

The soil from the plastic tubes is either bulked or divided into the various depths required for analysis. The bulked sample is weighed and put through a 2 mm sieve to remove any stones, roots etc. before the sample is reweighed. The soil is then thoroughly mixed before taking a representative sample for analysis.

A moisture content should be evaluated on the sample. (A known weight of soil (approximately 20g) is placed in a petri dish and dried to constant weight by placing in an oven at 120°C maximum).

3.2 **Extraction**

a) Weigh representative samples of soil in duplicate (50g) into screw cap centrifuge tubes and spike at least three recovery samples with standard (dissolved in acetonitrile) as required. (See Section 6). One untreated control sample, one reagent blank and one spiked reagent blank should also be included. (See Section 5).

b) Add Acetonitrile : Water (1:1) containing 40 mM ammonium acetate (100 ml) to the centrifuge bottle and cap if firmly with a Teflon (PTFE) lined screw cap. Shake the samples vigorously on a mechanical shaker for 30 minutes, at room temperature.

c) Centrifuge the samples at 3000 rpm (approximately 2000 g) for 5 minutes.

3.3 **C_{18} Column Clean-up**

A C_{18} clean-up is necessary to remove nonpolar co-extractives from the sample.

3.3.1 **Column Conditioning**

a) Condition the C_{18} column with 2.5 ml of ethylacetate followed by 2.5 ml of methanol.

b) Wash the column with 2.5 ml of water.

3.3.2 Sample Application

Both of the analytes pass through the C_{18} column mainly unretained. Thus all of the application solution and the following washes/eluates should be collected in a suitable flask. e.g. 250 ml round bottom flask or 130 ml Winchester bottle.

a) Dilute out 40 ml of extract (equivalent of 20 g soil aliquot) with 60 ml of water and apply into the C_{18} column by using a 75 ml reservoir on the top of the cartridge. Filter through the solution at a speed not more than 10 ml/min by applying vacuum. Collect the eluate.

Note : The composition of the loading (application) solution should be kept at 20% acetonitrile in water.

b) Wash the column with 2 x 1 ml of water collect the wash to the application solution.

c) Elute the partly retained analytes (approx 10-20%) with 2 x 0.5 ml of acetonitrile + water (1:1 v/v).

Note : Leave each aliquot of 0.5 ml acetonitrile + water to act on C_{18} bed for 0.5-1 min. Collect the eluate to the application solution.

3.4 Evaporation

a) Transfer the sample from 3.3.2 (a-c) into a 250 ml round bottom flask if it hasn't been collected into that size of glassware and evaporate off the acetonitrile by concentrating the samples to approximately 10-20 ml volume using 65-70°C water bath.

Note : The analytes are not volatile therefore a warm water bath (65-70°C) can be used. Splashing and foaming can happen with acetonitrile + water mixture therefore proper care has to be taken and the splash-head has to be cleaned with water after each sample.

b) Transfer the sample quantitatively into 25 ml scintillation vial by using a suitable pipette and measure the volume. Use as much water for rinsing the round bottom flask to make up the volume to 20 ml. Combine the rinses to the sample.

3.5 pH Adjustment

a) Adjust the pH to pH = 1.85 - 2.0 by adding dropplets of 4M HCl under continuous stirring. When pH is close to the required value more dilute 1M HCl can be used.

Note : It is crucial to set the pH within the range of pH = 1.85-2.0 in order to make sure that the compounds will be retained on the following C_8 column clean-up. The pH adjustment should be carried out by using properly calibrated pH meter and glass electrode.

Once the pH is adjusted the following C_8 clean-up must be carried out immediately after, within the next half an hour.

3.6 C_8 Column Clean-up

3.6.1 Column Conditioning

a) Condition the column with 2.5 ml of ethylacetate followed by 2.5 ml of methanol.

b) Wash the column with 2.5 ml of water.

3.6.2 Sample Application

Apply the pH adjusted samples (pH = 1.85 - 2.0) onto the column with a slow application rate 1-2 ml/min using gentle vacuum.

Note : Under acidic conditions (pH = 1.85-2.0) the analytes will retained on the C_8 column. The C_8 clean-up must be carried out an immediately after pH adjustment.

3.6.3 Column Wash

Wash column with 2 x 1 ml of water. Drawn off the excess water by using vacuum or positive pressure.

3.6.4 Elution

a) Prepare the eluent acetonitrile + water (1:1 v/v) containing 10 mM low UV grade tetrabutyl-ammonium phosphate ion pair (PIC A low UV, Millipore Inc) reagent at a readjusted pH in the range of pH = 6.6-6.9. The pH adjustment should be carried out by adding ortho-phosphoric acid H_3PO_4.

Note : The tetrabutylammonium phosphate ion pair reagent is water soluble. The appropriate amount of PIC A, in order to make up 10 mM final solution, has to be dissolved in water first, before adding the acetonitrile, otherwise it may precipitate out from the acetonitrile + water mixture.

b) Elute the analytes into 3 ml HPLC vial with 1 x 0.5 ml followed by 2 x 0.4 ml eluent. Leave each aliquot of eluent on the column for approximately 1 min before applying the next. Draw off the retained solvent into the vial by using gentle vacuum or possitive pressure.

c) Measure the sample volume by using graduated pipette and make up the volume to the nearest precise value (normally 1.2 or 1.3 ml) with eluent. Calculate the sample concentration [g (soil extract)/ml].

3.7 Liquid Chromatography

The following conditions gave satisfactory results using a Waters 501 Pump coupled to 710B Waters Intelligent Sample Processor (WISP) with a Severn Analytical SA 6500 (SA 6510 or Beckman 166) UV detector and a CROCO-CIL™ HPLC column heater. An electronic data capture and processing unit eg. VG Instruments Multichrom Vax System was suitable for quantitation.

Column	: 25 cm x 4.6 mm i.d, stainless steel with 5 cm x 4.6 mm i.d guard column.
Column Packing	: Hypersil 5 μm SAS (C_1)
Column Temperature	: 95°C
Mobile Phase	: Acetonitrile:Water (30:70 v/v) + 10 mM PIC A low UV grade (tetrabutyl-ammonium-phosphate) ion pair. pH adjusted to 6.4-6.8 with H_3PO_4.
Flow Rate	: 1.2 ml/min
Injected Volume	: 50 μl of standard and sample
Wavelength	: 220 nm
HPLC run time	: 30-40 min depending whether there are long retention matrix impurities

Using these conditions the retention time of oxanilic acid and sulphonic acid are approximately 9.22 and 11.63 minutes, respectively. The sensitivity is such that 5.10^{-8} g standard give 50% full scale deflection (fsd) with electrometer absorbtion unit at 0.005 and potentiometric recorder set on 10 mV range. The electronic handling system or proper integrator is necessary in order to be able to quantify the results on peak area mode.

Typical chromatographic traces are included in Appendix 5.

3.8 **Calculation of Results**

i) The residues may be calculated in mg kg^{-1} for each sample extract using a mean standard response from injections bracketing the sample as follows:

$$\text{Residue} = \frac{\text{Pk area (Sample)}}{\text{Pk area (Std)}} \times \frac{\text{W (Std)}}{\text{I (Sample)}} \times \frac{\text{V(s)}}{\text{W(s)}} \quad \text{mg kg}^{-1}.$$

Where Pk area (Sample) = Peak area for sample (μVs)
Pk area (Std) = Average peak height area for bracketing standards (μVs)
W (Std) = Weight of analyte in reference standard (μg)
I (Sample) = Sample solution injection volume (ml)
V (s) = Solvent volume in sample extract (ml)
W (s) = Sample weight equivalent to sample extract (g)

ii) These sample residues should be further corrected using the average percentage recovery. (i.e. calculate each recovery sample as above, and express as a percentage of the fortification level. Then average all the recovery percentages for use in the calculation below).

$$\text{Corrected Residue (mg kg}^{-1}) = \frac{\text{Residue}}{\text{Average percentage recovery}} \times 100$$

iii) Results should finally be corrected for the soil moisture content using the following equation:

$$\text{Dry weight residue (mg kg}^{-1}) = \frac{\text{Wet Weight Residue} \times 100}{100 - \% \text{ Soil Moisure Content}}$$

Results should be corrected to two significant figures (S.F.) or one S.F. if the residue is <0.1 mg kg^{-1}

4. **LIMIT OF DETERMINATION**

The limit of determination has been defined as the smallest value of concentration of a compound in the analytical sample, that satisfies the three following requirements.

a) Is greater or equal to the limit of detection.
b) The recovery for the limit of determination is equal to or greater than 70%.
c) The coefficient of Variation (CV) at the limit of determination is equal to or smaller than 0.2 (equivalent to 20%).

It true assessment of the limit of determination of the method may be determined by fortification of untreated samples at the limit of determination levels with standards and subjecting them to the complete analytical procedure. The results should satisfy the three above mentioned criteria.

In these laboratories the limit of determination has been set at 0.02 mg kg^{-1}. 99

5 **CONTROLS REAGENT BLANKS/SPIKED REAGENT BLANKS**

The analyst must verify that the sample chromatograms are free from interferences orginating from either untreated soil samples or from the reagents, and that sample contamination with analytes has not occurred before or during the analysis. Therefore at least one control sample and one reagent blank (whole procedure followed in absence of soil) should be analysed alongside each set of samples. It is also useful to take through a spiked reagent blank to indicate whether anomalous results are due to the method, or adsorption/binding of the analyte by the matrix and to check that the spiking standard solution agrees with the standard used for GC analyses.

6 **RECOVERIES**

A minimum of two external recovery experiments should be run alongside each set of samples analysed; (that is untreated samples accurately fortified with a known amount of analytes prior to extraction), in the range 0.02 - 0.5 mg kg^{-1}.

For external standard recoveries, fortification levels should be based on the expected soil residue levels. When no residues are expected the recoveries should be fortified at low levels typically 0.1-0.3 mg kg^{-1} and include at least one fortified at the limit of determination. Provided the recovery values obtained are acceptable (mean values >70%; with a coefficient of variation CV <15%) they may be used to correct the determined residues, as in Section 3.8.

7 METHOD VALIDATION

a) Calibration graph for Standards

A series of recoveries were fortified at levels between 1.0 mg kg^{-1} and 0.02 mg kg^{-1}. These were then taken through the method. A series of standards in mobile phase were run and graphs plotted of peak area (μVs) versus analyte concentration ($\mu g\ ml^{-1}$) (See Appendix 6).

Linear relationships were observed for the standards.

b) A series of recoveries were fortified at analyte levels between 0.02 - 1.0 mg kg^{-1}. These were then taken through the method.

APPENDIX 1 : Apparatus

a) Equipment for the initial preparation of samples e.g. a Hobart Band Saw for cutting soil cores.

Flask shaker Model SF1 Stuart Scientific, UK.

c) Vacuum rotary evaporator with thermostatically controlled water bath, available from Buchi, Switzerland.

d) Complete Visiprep™ SPE twelve place vacuum manifold assembly with sample collector rack (for use with BOND ELUT™ disposable extraction columns). Supelco SA, Switzerland.

e) Liquid chromatograph fitted with sensitive UV detector e.g., Waters 501 pump coupled to a 710B Waters Intelligent Sample Processor (WISP), with a Severn Analytical Model SA6500, SA6510 UV detector or Beckmann Gold 166 UV detector, or equivalent system. The HPLC should be fitted with an appropriate integrator or data handling system e.g VG Instruments Multichrom Vax System.

f) HPLC column heater e.g CROCO-SIL™ rated up to 99±1°C available from Thames Chromatography, UK.

g) Laboratory centrifuge eg WIFUG Model 2000E WIFUG (A Division of Eltex of Sweden Ltd) Bradford, UK.

h) 25 cm x 4.6 mm i.d stainless steel HPLC column packed with Hypersil 5 µm SAS (C1). 5 cm x 4.6 mm i.d stainless steel guard column packed with the same packing (HICHROM UK).

APPENDIX 2 : Reagents

a) Solvents: glass distilled and HPLC Ror UV grade acetonitrile, (Code No. H049) ethylacetate and methanol, Romil Chemical UK.

b) Ultra pure water, Romil Chemicals UK.

c) C_{18} and C_8 BOND ELUT™ disposable extraction columns (3 ml) containing 500 mg bonded C_{18} and C_8. Supplier:- Jones Chromatography Ltd, UK (Part No 1210-2028 and No 1210-2029 respectively, manufactured by Analytichem International Inc, USA).

d) Tetrabutyl-ammonium phosphate ion pairing reagent PICA reagent Low UV grade (Part No 84189) Waters Chromatoraphy Division/ Millipore Corporation UK.

e) HCl, Analar, BDH, UK.

f) H_3PO_4, orthophosphoric acid (90% w/w) May & Baker Ltd, Dagenham, UK.

g) Oxanilic acid standard (ICIA5676/17) 2-(Ethoxymethyl)amino-N-(2-ethyl-6-methylphenyl)oxo-acetic acid (known purity, >97%).

h) Sulphonic acid sodium salt standard (ICIA5676/24) N-(Ethoxymethyl)-N-(2-ethyl-6-methylphenyl)-3-oxo ethane-sulphonic acid sodium salt (known purity, >95%).

APPENDIX 3 : Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in doubt, consult the appropriate safety manual (e.g. ICI Laboratory Safety Manual) containing recommendations and procedures for handling chemicals, and a monograph such as 'Hazards in the Chemical Laboratory' edited by L Bretherick, The Royal Society of Chemistry, London.

a) ACETONITRILE

Toxic by inhalation, in contact with skin
Highly flammable
Do not breath vapour
Avoid contact with eyes and skin
(RL 70 mg m^{-3})

b) ETHYLACETATE

Highly flammable
Avoid breathing vapour
Avoid contact with eyes
(RL 1400 mg m^{-3})

c) METHANOL

Highly flammable
Serious risk of poisoning by inhalation or swallowing
Avoid contact with skin and eyes
(CL 260 mg m^{-3})

d) 2-(Ethoxymethyl)amino-N-(2-ethyl-6-methylphenyl)oxoacetic-acid (oxanilic acid, ICIA5676/17)

Avoid contact with eyes, skin and clothing.
Avoid breathing vapours

e) N-(Ethoxymethyl)-N-(2-ethyl-6-methylphenyl)-3-oxoethane sulphonic acid sodium salt (Sulphonic acid ICIA5676/24).

Avoid contact with eyes, skin and clothing
Avoid breathing vapours.

f) HYDROCHLORIC ACID

Harmful Vapour
Causes burns, Irritating to respiretory system
Avoid breathing vapour
Prevent contact with eyes and skin
RL as HCl 7 mg m^{-3}

g) PHOSPHORIC ACID
Causes burns
Prevent contact with eyes and skin
RL 1 mg m^{-3}

APPENDIX 4 : Preparation of Analytical Standards

It is recommended that the following handling precautions should be taken when weighing the analytical standard material.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

Weigh out accurately, using a five figure balance, sufficient of oxanilic acid standard to allow dilution in acetonitrile to give a 1000 $\mu g\ ml^{-1}$ stock solution in volumetric flask. Weigh out accurately sufficient of sulphonic acid sodium salt to allow dilution in acetonitrile:water (9:1 v/v) solution to give a 1000 $\mu g\ ml^{-1}$ stock solution in volumeric flask. Make serial dilutions of these stock solution to give 100 $\mu g\ ml^{-1}$, 10 $\mu g\ ml^{-1}$ and 1.0 $\mu g\ ml^{-1}$ standard solutions in acetonitrile. These solutions should be used for the fortification of recovery samples.

Also prepare a dilution of 1 $\mu g\ ml^{-1}$ mixed standard solutions for the HPLC analysis from the stock solution in acetonitrile:water 4:6 v/v) containing 10 mM PICA a tetrabutyl ammonium phosphate ion pairing reagent and ortho-phosphoric acid to set the pH within the range of pH=6.5-6.8. When not in use, standard solutions should always be stored in a refrigerator at 4°C to prevent evaporation and concentration. Analytical standards should be replaced with freshly prepared standards after four months of use.