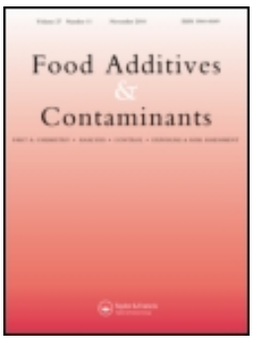

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Determination of ethylenethiourea (ETU) and propylenethiourea (PTU) in foods by high performance liquid chromatography–atmospheric pressure chemical ionisation–medium-resolution mass spectrometry

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Abstract
A robust and sensitive method for the determination of ethylenethiourea (ETU) and iso-propylenethiourea (i-PTU) in foods is reported. ETU and i-PTU were extracted by blending with dichloromethane (DCM) in the presence of sodium sulphate, sodium carbonate, thiourea and ascorbic acid. $^{2}H_4$-ETU and *n*-PTU were used as internal standards. After filtration the DCM was removed by rotary evaporation and the extract re-dissolved in water before analysis by reversed-phase liquid chromatography with detection by atmospheric pressure chemical ionization-mass spectrometry using a double focusing mass spectrometer at a resolution of 5000. Mean recoveries of ETU and i-PTU from fruit-based, cereal-based and meat-based infant foods, potato chips and tinned potatos at 0.01 mg kg^{-1} and from pizza and yoghurt at 0.02–0.1 mg kg^{-1} were 95% and 97% respectively. Precision, including both repeatability and internal reproducibility, was in the range of 3.1–13.1%.

Keywords: *ethylenethiourea (ETU), propylenethiourea (PTU), liquid chromatography-mass spectrometry (LC/MS), processed foods*

Introduction

Ethylenethiourea (ETU; imidazolidine-2-thione), and iso-propylenethiourea (i-PTU; 4-methylimidazolidine-2-thione) are, respectively, degradation products of the ethylenebisdithiocarbamate (EBDC) and propylenebisdithiocarbamate (PBDC) fungicides, collectively called alkylenebisdithiocarbamates (ABDCs), which include some of the most widely used fungicides in agriculture and horticulture. The EBDC fungicides include mancozeb, maneb, zineb and metiram, while propineb is the main example of a PBDC. The fungicides are considered to have low toxicity, but ETU and i-PTU are of much greater toxicological concern (Lentza-Rizos 1990). ETU has been found to produce thyroid disorders, birth defects and cancers in laboratory animals (WHO 1988) and to produce genotoxic effects (Dearfield 1994).

ETU is a common impurity of EBDC formulations (Bontoyan and Looker 1973; Farrington and Hopkins 1979) and concentrations have been shown to increase with storage (Farrington and Hopkins 1979). Data on the accumulation of ETU in living plants resulting from degradation or metabolism of EBDCs (reviewed by Lentza-Rizos 1990) is rather contradictory, but is consistent with rapid formation of ETU followed, in some cases, by rapid further degradation. However, cooking and other processing of plant material contaminated with EBDCs has been shown to increase levels of ETU (Newsome and Laver 1973; Watts et al. 1974; Marshall 1977), presumably by promoting its formation while deactivating the plant enzymes or microbial action responsible for further degradation. ETU and i-PTU are thus of greatest significance in processed foods.

In Europe, ETU and i-PTU are not themselves regulated by the same system of maximum residue levels (MRLs) as the pesticides. However, infant foods are an important class of processed food and specific rules on the presence of pesticides residues in processed cereal-based foods and baby foods are set out in Commission Directive 99/39/EC of 6 May 1999 which states that such foods shall not contain residues of individual pesticides

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at levels exceeding 0.01 mg/kg. We have therefore adopted 0.01 mg/kg as the target reporting limit for the alkylenethioureas.

Many papers have described methods for the determination of ETU and the earlier literature has been reviewed by Bottomley et al. (1985) and Lentza-Rizos (1990). However, other workers have often commented on the unsatisfactory performance of many of the reported methods (Nitz et al. 1982; Krause 1989a; Ahmad et al. 1995). Extraction from plant material has most frequently been accomplished with MeOH, EtOH or MeOH-water, although $CHCl_3$–EtOH has also been employed (Bottomley et al. 1985; Lentza-Rizos 1990). The AOAC Official Method (AOAC 1995) involves extraction by blending sample and diatomaceous earth with MeOH-water after the addition of NaCl. Subsequent steps in this method are clean-up with further diatomaceous earth and alumina. Improved recovery from diatomaceous earth has been achieved by pH adjustment (Krause 1989a; Bolzoni et al. 1993) and salting-out with KF combined with pH buffering (Nitz et al. 1982; Maruyama 1994; Ahmad et al. 1995). Oxidation of ETU during extraction has been noted to be a cause of poor recovery and either sodium ascorbate (Otto et al. 1977) or cysteine hydrochloride (Sack 1995) have been employed as antioxidants during extraction. Preliminary investigation also suggested that Na_2SO_4 was effective (Sack 1995). Impurities in DCM used in the method have also been shown to cause losses of ETU (Sack et al. 1993).

ETU and i-PTU are of high polarity and low volatility. Although GC of underivatized ETU is possible, most workers attempting GC have found that reliable determination is possible only after derivatization, S-butylation having been widely employed, as, for example, in the AOAC method (AOAC 1995). The use of derivatization, however, requires additional preparation steps which increase the time taken for analysis, and may also increase the possibility of errors and of low recoveries (Bolzoni et al. 1993). HPLC has the advantage that derivatization may be unnecessary. However, ETU has only a weak chromophore, with an absorption maximum at about 230 nm. Several workers have reported methods based on UV detection (Lehotay et al. 1992; Ahmad et al. 1995; Kontou et al. 2001; Garcinuno et al. 2004) but detection limits have usually been greater than 0.01 mg/kg. Electrochemical detection has also been employed and affords good sensitivity (Krause 1989a; Bolzoni et al. 1993; Matuyama et al. 1994) but these methods still involve tedious clean-up steps.

A few reports have appeared describing the use of LC-MS. Following early use of particle-beam (Doerge and Miles 1991), and thermospray in our laboratory (unpublished) and elsewhere (Kurttio et al. 1992), atmospheric pressure ionization techniques have recently been explored. Electrospray (ES) has been applied to the determination of ETU in urine (Sottani et al. 2003) and ES has been compared with atmospheric pressure chemical ionisation (APCI) for determination of dithiocarbamates and their metabolites in a variety of fruits and seeds (Blasco et al. 2004). The latter paper also compares matrix solid-phase dispersion and solid-phase-extraction methods, but recovery was not evaluated at levels below 0.25 mg/kg which corresponded to the LOQ of the method with the single quadrupole mass spectrometer employed.

We describe here a modification of a simple and rapid extraction procedure that has been used in our laboratory for some years, in which ETU and PTU are extracted directly into DCM and, after solvent exchange, determined by APCI-LC-MS with a double-focusing mass spectrometer at a resolution of 5000, with the use of $^2H_4$-ETU and *n*-PTU as internal standards.

Materials and methods

Chemicals

Dichloromethane (DCM), methanol, and water of HPLC grade, ammonium acetate and ascorbic acid of analytical grade, and anhydrous sodium carbonate and anhydrous sodium sulphate of laboratory reagent grade were from Fisher Scientific (Loughborough, UK). Thiourea (A.C.S. reagent grade) and *n*-PTU were obtained from Aldrich (Poole, UK). ETU (99.5%) and i-PTU (97%) for use as standards were purchased from QM_x Laboratories (Thaxted, UK). $^2H_4$-ETU (minimum isotopic enrichment 98 atom%) for use as internal standard was from CDN Isotopes (Quebec, Canada). Cyclohexylamine and cyclooctylamine were obtained from Aldrich (Poole, UK) and used as a solution in MeOH of approximately 0.05 mg/ml).

Materials

For use in method development and validation studies and to serve as blanks, foods (labelled as of organic origin when available) were purchased from retail outlets and subsequently shown by the method described herein not to contain detectable residues of ETU or i-PTU, nor to give rise to interferences with the measurement of the internal standards.

Preparation of standard solutions

Stock solutions of ETU, $^2H_4$-ETU, i-PTU and *n*-PTU of 1 mg ml^{-1} were prepared in water and were kept for up to six months at 4°C. Stability over this period was demonstrated by comparing the responses of stored standards with freshly-prepared solutions. Working standard solution containing both ETU and i-PTU, and internal standard solution containing both $^2H_4$-ETU and *n*-PTU, all at 1 μg ml^{-1}, were prepared by dilution in water. Matrix-matched calibration solutions were freshly prepared for each experiment using extracts of blank samples, similar to those to be analysed, which had previously been shown not to contain detectable residues of ETU or i-PTU. Aliquots of 5–50 μl of working standard solution and 20 μl of internal standard solution were diluted to 1 ml with blank extract.

Sample preparation

Infant foods and yoghurt were removed from the packaging in which they were sold and mixed well. Pizza and potato products were homogenized with a food processor. Prepared samples were stored at -18 ± 2°C until required for analysis.

Extraction

Without complete thawing, 10 ± 0.1 g of the test material was placed in a 250 ml borosilicate-glass screw-capped bottle together with 1 ml of 15 mg ml^{-1} aqueous thiourea solution and 10 ml of 0.05 g ml^{-1} aqueous ascorbic acid solution. For the determination of recovery, spiking by addition of appropriate volumes of working standard solution was performed at this stage. A 0.2 ml aliquot of internal standard solution was added to all extractions, except the blank to be used for preparation of calibration solutions.

100 ml of DCM was added and the mixture blended using an Ultra-Turrax T-25 homogenizer at 8000 rpm while 20 g anhydrous sodium carbonate and 50 g anhydrous sodium sulfate were added, and then for a further 30 s at 20 500 rpm. The extract was filtered through a cotton wool plug in a large glass funnel. An aliquot of 50 ml of filtrate was transferred to a round-bottom flask and 0.5 ml of water added before evaporating on a rotary evaporator at 300 mbar and 35°C to a final volume of approximately 0.5 ml. Water (2.5 ml) was added to the flask and the extract transferred to a glass tube. The flask was rinsed with a further 2 ml of water which was also added to give an extract volume of approximately 5 ml.

To remove co-extracted fats, a 2 ml aliquot of the extract was transferred to a centrifuge tube together with 0.5 ml isooctane, and vortex mixed for approximately 2 s. The mixture was centrifuged at 3000 rpm for 5 min and then all of the upper, organic layer was removed with a Pasteur pipette and discarded.

The extract was passed through a nylon syringe filter (0.45 μm pore size, 13 mm diameter) prior to the HPLC-MS determination.

LC-MS analysis

Analysis was performed using a P200 binary HPLC pump, AS300 autosampler and MAT 95T reverse-geometry double-focusing instrument (Thermoquest, Hemel Hempstead, UK) fitted with a Finnigan API II heated capillary (0.4 mm bore) atmospheric pressure ionisation interface. A Genesis AQ HPLC column of 150×4.6 mm (Jones Chromatography, Hengoed, UK) was used. Isocratic conditions were employed with a mobile phase consisting of 90% water and 10% MeOH supplied at 1 ml min^{-1}. The injection volume was 20 μl and samples were injected at 8 min intervals.

For tuning and to provide a lock mass during data acquisition a solution containing cyclohexylamine and cyclooctylamine (0.05 mg ml^{-1} in MeOH) was introduced at 5 μl min^{-1} from a Harvard 22 syringe pump (Harvard Apparatus, USA) through a Jour PEEK mixing tee (Capital HPLC, Broxburn, UK) installed into the tubing between the column and mass spectrometer. The resolution of the instrument was set to 5000 (10% valley definition) and the APCI and source parameters adjusted for maximum intensity of the protonated cyclohexylamine peak (*m*/*z* 100.1126). Typical optimized APCI voltage settings were: heated capillary 6 V; tube lens 50 V; skimmer 1V, and octapole lens −7 V. Mass calibration of the electric sector was carried out using the $[M+H]^+$ ions of the two amines (*m*/*z* 100.1126 and 128.1439).

Compound specific conditions were set following optimization using $[M+H]^+$ of *n*-PTU, introduced as a solution at a flow rate of 10 μl min^{-1} into solvent flow from the HPLC pump via the mixing tee. Following this initial optimization the following settings were used: APCI corona 5 μA; vaporiser temperature 300°C; heated capillary temperature 200°C; sheath gas pressure 50 psi; auxiliary gas rotameter setting 30 (arbitrary units). The electron multiplier was operated at voltages between 1.5 and 2.0 kV.

Initial recording of complete mass spectra was by magnetic scanning from *m*/*z* 50 to 800 at 1 s/decade. For trace analysis, ETU and PTU were

detected by selected ion monitoring (SIM) of $[M+H]^+$ ions at *m/z* 103.0330 (ETU), 107.0577 ($^2H_4$-ETU) and 117.0486 (PTU) with *m/z* 100.1126 and 128.1439 defined as lock and calibration mass, respectively. The SIM cycle time was 0.5 s.

Results and discussion

The positive ion APCI mass spectra of ETU, i-PTU and *n*-PTU obtained under the conditions described contained, essentially, only $[M+H]^+$ (*m/z* 103 for ETU and 117 for PTU). In contrast electrospray gave both $[M+H]^+$ and $[M+Na]^+$, consistent with the findings of Blasco et al. (2004), and with considerably less sensitivity than APCI under the conditions employed. The selected column and conditions gave good chromatographic peak shapes and retention times of approximately 2.6, 3.1, and 4 min for ETU, *n*-PTU and i-PTU respectively, corresponding to capacity factors (k') of 0.49, 0.78 and 1.3, and excellent signal-to-noise performance was obtained at 0.01 mg kg^{-1} with the mass spectrometer resolution (10% valley definition) adjusted to 5000 (see Figure 1). At the mass spectrometer resolution used the method gave excellent selectivity such that no extraneous peaks were visible in chromatograms for any of the commodities tested.

The response curve of the instrument was shown to be linear over the range 0.005–0.05 mg kg^{-1} (Figure 2) with r^2 values > 0.99 when internally standardized. Absolute responses with this instrument showed some drift with time, necessitating the use of internal standards for accurate results. Repeated injections of aqueous ETU at a concentration equivalent to 0.01 mg kg^{-1} gave an RSD of 12% for the measured peak area while the RSD of the area ratio was improved to 6%. In accord with EU guidelines for pesticide residue monitoring (Hill 1997), matrix-matched calibration solutions were used. Although the isotope dilution method used for ETU should compensate for any matrix effects, i-PTU was quantified with the use of its

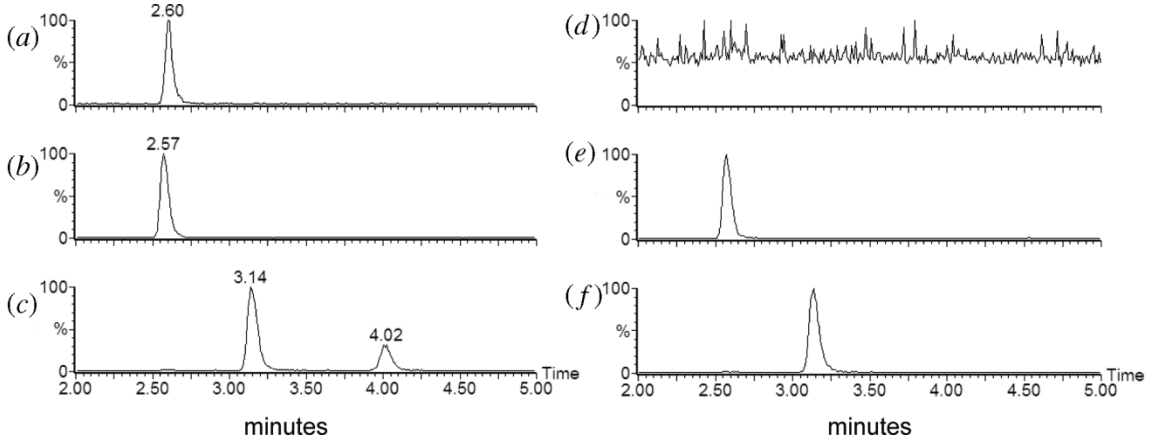

Figure 1. Selected ion monitoring chromatograms for ETU, $^2H_4$-ETU and i- and n-PTU in a–c an extract of fruit-based infant food spiked with 0.01 mg kg^{-1} ETU and i-PTU before extraction and d–f from the unspiked blank. Chromatograms a and d are *m/z* 103 (ETU), b and e are *m/z* 107 ($^2H_4$-ETU), and d and f are *m/z* 117 (PTU).

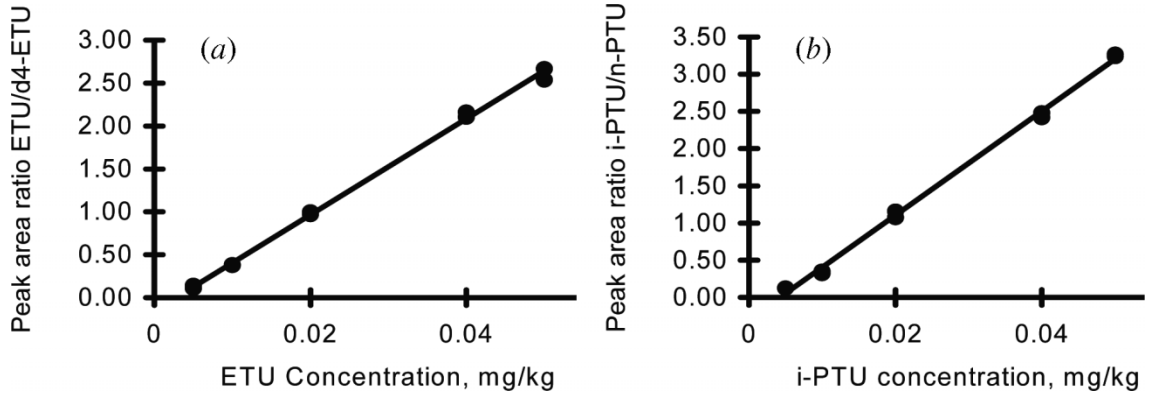

Figure 2. Internally standardized response curves for (a) ETU and (b) i-PTU. The pairs of points at each concentration were separated by 10 injections.

Table I. Recovery (level found as percentage of level spiked) and relative standard deviation for determination of ETU and i-PTU in various foods. ETU internally standardised with $^2H_4$-ETU and i-PTU internally standardized with *n*-PTU.

				ETU		i-PTU	
Commodity	Type of precision[a]	*n*	Level, mg/kg	mean	RSD, %	mean	RSD, %
Fruit-based infant food	r	7	0.01	94	5.43	101	5.16
Cereal-based infant food	r	7	0.01	96	7.10	91	12.3
Cereal-based infant food	R	5	0.01	92	7.07	94	6.64
Meat-based infant food	r	7	0.01	90	3.81	93	4.66
Meat-based infant food	R	12	0.01	94	3.18	101	11.9
Pizza	r	7	0.02	99	4.00	108	5.46
Pizza	R	4	0.1	94	9.31	95	9.51
Potato chips	r	7	0.01	101	3.90	99	13.1
Potato tinned	r	7	0.01	95	3.60	93	3.80
Yoghurt	R	4	0.05	94	9.24	99	5.42

[a]r: repeatability conditions–replicates analysed in a single batch. R: internal reproducibility conditions–from single recovery determinations in multiple batches analysed on different occasions.

isomer, *n*-PTU, as internal standard and as these compounds are chromatographically resolved the internal standard cannot correct for matrix effects, should they occur.

Recoveries from infant foods and some other commodities calculated by the internal standard method are shown in Table I, and demonstrate good method accuracy at $0.01\,mg\,kg^{-1}$, giving mean recoveries of 95% for ETU and 97% for i-PTU with acceptable RSDs. Internal standardization with addition of standards before extraction, as incorporated in this method, corrects for analyte losses but the uncorrected analyte yield remains of importance as it effects the limit of detection. As noted above, some cyclic drift in instrument sensitivity necessitated internal standardization, and internal standardization also makes it possible to allow some variation in the final extract volume so that a method designed for optimal use of an internal standard cannot simply be recalculated by the external standard method. However, in this case final volumes were sufficiently consistent to allow yield of analytes and internal standards to be estimated with reasonable confidence. Yields throughout, at $0.01\,mg\,kg^{-1}$, were > 80%.

A number of publications have reported erratic recoveries of ETU. Using MeOH extraction in the presence of Na acetate, Krause (1989b) obtained satisfactory recovery from lettuce, beans, potatoes and mushrooms, but not from celery. The use of DCM in the clean-up has been found to produce erratic recoveries (0–106%) from vacuum rotary evaporation of the solvent, recoveries being dependent on the bottle of solvent used, not on supplier or lot (Sack et al. 1993). Losses of ETU were prevented by passing the solvent through a column containing sodium sulfate, sodium carbonate and alumina. Sack (1995) subsequently showed that ETU is prone to oxidative degradation during extraction and proposed the use of cysteine hydrochloride as a protectant. In tests with 53 food items the lowest recovery when cysteine hydrochloride was used was 68% while in its absence some recoveries were as low as 20%.

Other antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and vitamins A or C were not as effective. However, it was found that sodium sulfate may be even more effective. In our procedure anhydrous sodium sulfate is used in the extraction together with additions of both ascorbic acid and excess thiourea and this combination clearly provides effective protection from oxidation and other losses.

The described method is convenient and quick, and provides excellent performance making it very suitable for fast turnaround food monitoring. The validated quantitation limit of $0.01\,mg\,kg^{-1}$ is far lower than that of previously reported MS-based methods. In our experience low resolution LC-MS cannot always provide sufficient selectivity at the measurement levels required. LC-MS/MS with a triple quadrupole instrument provides an obvious alternative, and such instruments are more widely available than high resolution mass spectrometers. We have found that multiple reaction monitoring of the transitions $m/z\,103 \rightarrow 44$ and $117 \rightarrow 58$ for ETU and PTU respectively, also provides suitable performance.

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