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Determination of ethylenethiourea in food commodities by a two-step derivatization method and gas chromatography with electron-capture and nitrogen–phosphorus detection

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Abstract

Ethylenethiourea (ETU) is a decomposition product from ethylene-bis-dithiocarbamates (EBDCs), the most widely used class of fungicides in the world. ETU has been classified as a possible human carcinogen. The maximum permitted residue level (MRL) in the European Union was set at 0.05 ppm. Gas chromatographic determination of ETU can be achieved only after derivatization. ETU is extracted from food samples and cleaned up by a combination of two-step derivatization and liquid–liquid partitioning. In the first step, ETU is derivatized with benzyl chloride to form S-benzyl ETU, which is then trifluoroacetylated to form the final product, which is amenable to GC. The determination is carried out with capillary gas chromatography using electron-capture (ECD) and nitrogen–phosphorus detection (NPD) as selective detection methods in parallel. The responses of ECD and NPD were found to be of the same order of magnitude. Therefore, the parallel response was found to be a useful criterion for peak identification down to the limit of detection. Reproducibility of the two-step derivatization of ETU to form trifluoroacetylated S-benzyl ETU was found to be satisfactory. The recoveries from apple, pear, tomato and a common baby food, at various concentration levels, were found to be between 82–92%, with a limit of detection of less than 1 ppb. Commercial samples, submitted for routine monitoring of dithiocarbamates (DTC) were also monitored in our laboratory for the presence of ETU. Four of the twenty samples found positive for DTC were also found to be contaminated with ETU in the range of 0.01 to 0.37 ppm. Three of these food samples were found to contain ETU residues above the MRL of 0.05, while those food samples containing DTC residues between 0.2 and 0.8 ppm were all below the MRL of DTC. No relation exists between the DTC residues concentration and the level of ETU. The screening data were further confirmed by electron impact mass spectrometry in selected ion monitoring mode. Chromatograms of ETU residue analyses are presented to demonstrate the extremely sensitive detection method with real food samples.

Keywords: Fruits; Vegetables; Food analysis; Pesticides; Ethylenethiourea

1. Introduction

Ethylenethiourea (ETU) is a decomposition product from ethylene-bis-dithiocarbamates (EBDCs), the most widely used class of fungicides in the world. ETU is stable in water and is readily absorbed and metabolized by plants [1,2]. The stability of ETU in crops and plants has been extensively studied and most of them showed that ETU is not very stable when exposed to food matrices [3]. ETU was, however, classified by the International Agency for Research on Cancer (IARC) as possibly being carcinogenic to humans [4]. A monitoring study of 5888 food items conducted by the US Environmental Protection Agency (EPA) showed that in 18% of the samples, residues of ETU, and in 19%, residues of

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EBDCs were detected [5]. Thus, there is an obvious need for the control of the levels of ETU residues on agricultural crops and food products of which the maximum residue level (MRL) was set as low as 0.05 ppm in the European Union [6]. Therefore, sensitive and reliable methods are required for the analysis of these residues in food commodities.

Up to now, a number of analytical procedures have been reported for the determination of ETU, including various liquid chromatographic (HPLC) and gas chromatographic (GC) methods involving complicated clean-up of extracts and chemical derivatization for GC determination [7–13]. The latter is the major technique used to achieve sensitive and selective detection [13–16]. The method first proposed by Newsome [7] was based on a good analytical principle. ETU was reacted with benzyl chloride to form the S-benzylated hydrochloride, which is soluble in water. This permitted the preliminary elimination of many non-polar interfering substances by liquid–liquid extraction with an organic solvent. After alkalization, the free base was quantitatively extracted with an organic solvent and N-trifluoroacetylated with trifluoroacetic anhydride (TFA). Sensitive detection of ETU derivative was possible with electron-capture detection (ECD), down to the concentration level of 0.01 ppm. Confirmation was performed by GC–MS. Nitz et al. [3] observed great variations in the recoveries. A modification of the extraction procedure proposed by Krause [8] enhanced the ETU recovery considerably.

In this paper, we report on the determination of ETU in agricultural crops and food products, using the Newsome technique [7] for derivatization together with an improved extraction procedure. The derivatized sample extracts were analyzed on capillary GC, splitting the eluate to two parallel detection systems of different selectivity, namely ECD and nitrogen–phosphorus detection (NPD). The response ratios of both the detectors for the selective detection of the derivatized ETU improves the certainty of its identification so that further analysis for the confirmation of the compound on MS is often not necessary. The applicability of this procedure, illustrated by a few examples, was successfully applied to fresh fruits and vegetables collected for routine monitoring from the Berlin market.

2. Experimental

2.1. Preparation of the standards

ETU (99.9%), purchased from Dr. Ehrenstorfer (Augsburg, Germany) was recrystallized from 95% ethanol to give a white crystalline product. The ETU was dissolved in water prior to being added to plant matrix. The S-benzyl ETU was prepared by refluxing ETU with benzyl chloride in 95% ethanol for 30 min. The solvent was removed on a rotary evaporator and the residues were dissolved in water. After extraction with dichloromethane, the aqueous phase was made alkaline with 1 *M* KOH and the free base was extracted with dichloromethane. The material was crystallised twice with warm dichloromethane.

A standard solution of trifluoroacetylated S-benzyl ETU was prepared by dissolving S-benzyl ETU in 10% (v/v) trifluoroacetic anhydride in toluene and diluting with toluene to the desired concentration of 1% TFA. Standard solutions were prepared immediately before analysis.

2.2. GC–ECD/NPD analysis

All GC measurements with parallel ECD and NPD were performed with an HP-5890A gas chromatograph from Hewlett-Packard (Waldbronn, Germany), equipped with an autosampler (HP-7673A); fitted with a 50 m×0.32 mm fused-silica capillary column (HP-5) with a film thickness of 0.17 μm. The carrier gas was helium (99.999% purity).

Temperature program: 100°C for 1 min, 30°C/min to 150°C, hold for 2 min; 3°C/min to 205°C, 10°C/min to 260°C and final time 20 min. Injection technique: Hot splitless at 210°C. A 1-μl volume was injected with the split closed for 0.9 min.

The outlet of the column was connected simultaneously to the two detectors, ECD and NPD, by means of a "Y-press-fit" glass connector (quick-seal splitter from Chrompack, Middelburg, Netherlands) and deactivated fused-silica capillary tubing to each detector. The GC system is linked to a personal computer running under MS DOS, via an intelligent two-channel interface (Nelson Analytical, Cupertino, CA, USA) for chromatographic data processing

using software. The carbon disulphide evolved from DTCs was estimated according to the method of Keppel [17] by heating the food matrices with hot acid.

2.3. GC–MS

All GC–MS measurements were performed with a Hewlett-Packard HP-5970 MS Detector combined with a HP-5890A gas chromatograph from Hewlett-Packard (Waldbronn, Germany) and equipped with a HP-7673 autosampler; fitted with a 25 m×0.2 mm fused-silica capillary column (HP-5) with a film thickness of 0.33 μm. The carrier gas was helium (99.999% purity). The oven temperature program was the same as that described for GC–ECD/NPD analysis in Section 2.2. Injection technique: Hot splitless at 210°C. A 2-μl volume was injected with the split closed for 0.9 min. MS measurements were performed with electron impact (EI) at 70 eV in full scan mode over the mass range of m/z 50 to 550 at 0.86 scans/s. The screening data were also confirmed in the selected ion monitoring (SIM) mode, recording the ions at m/z 288, 255 and 219, respectively.

2.4. Analytical procedure

Samples of fruits and vegetables were prepared according to the extraction method proposed by Krause [8]. Food samples (5 g) were extracted with 50 ml of methanol, 20 ml of water, 1 g of sodium acetate and 3 g of Celite 545. The mixture was blended for 2 min at high speed in a beaker, filtered through a büchner funnel into a 250-ml round-bottomed flask containing anti-bumping granules. Benzyl chloride (100 μl) was added and the contents were refluxed for 30 min. Methanol was removed with a rotary evaporator (waterbath at 47–50°C) after the addition of 0.75–1.0 ml of 6 *M* HCl. The samples were then transferred to a separation funnel with 20 ml of water and extracted twice with 10 ml of dichloromethane, which were discarded. After the addition of 1 *M* KOH (9.0 ml) to the aqueous phase and a further 10 ml of dichloromethane, the separating funnel was shaken immediately for extraction. The dichloromethane extract was dried by passage through sodium sulphate and the solvent was removed under vacuum by centrifugation. A solution containing 10% trifluoroacetic anhydride in toluene (0.5 ml) was added to the dry residue and the sample was allowed to react at room temperature for 15 min. The solvent was evaporated to dryness under a stream of nitrogen and dissolved in 1 ml of toluene for GC analysis.

3. Results and discussion

The GC analysis of ETU after derivatization for parallel ECD/NPD detection was found to be satisfactory. Trifluoroacetylated S-benzyl ETU was detected with ECD and NPD, exhibiting good sensitivity in both detectors. Fig. 1 shows the two parallel chromatograms resulting from the injection of 1 μl of a derivatized standard solution, corresponding to 50 pg of ETU. With a detector split ratio of about 1:1, a peak representing 25 pg of ETU demonstrates the good sensitivity of the method. The small difference in the retention times of chromatograms reflects the difference in the lengths of the capillaries between the analytical column and each of the two detectors and remains constant throughout the analysis. Note the good peak shape and signal-to-noise ratio at the trace concentration level employed. In the preliminary recovery experiments carried out with 5 ml of distilled water instead of food sample and spiked at 0.05 ppm ETU, the excellent sensitivity of the method was confirmed. The same procedure proved to be satisfactory in the recovery experiments with spiked fruits and vegetables.

In Fig. 2, two chromatograms obtained in parallel with ECD and NPD of an apple extract fortified with ETU at a concentration level of 0.05 ppm prior to blending are shown. Although matrix peaks are found in the chromatograms close to that of ETU, the target compound is well separated and can be identified by its corresponding signal and response ratio on both of the detectors. The recovery of ETU with the apple extract experiment was found to be 89%, with a standard deviation of 4.4%. Note that this concentration level is that of the MRL in the European Union [6].

Recovery experiments were carried out with vari-

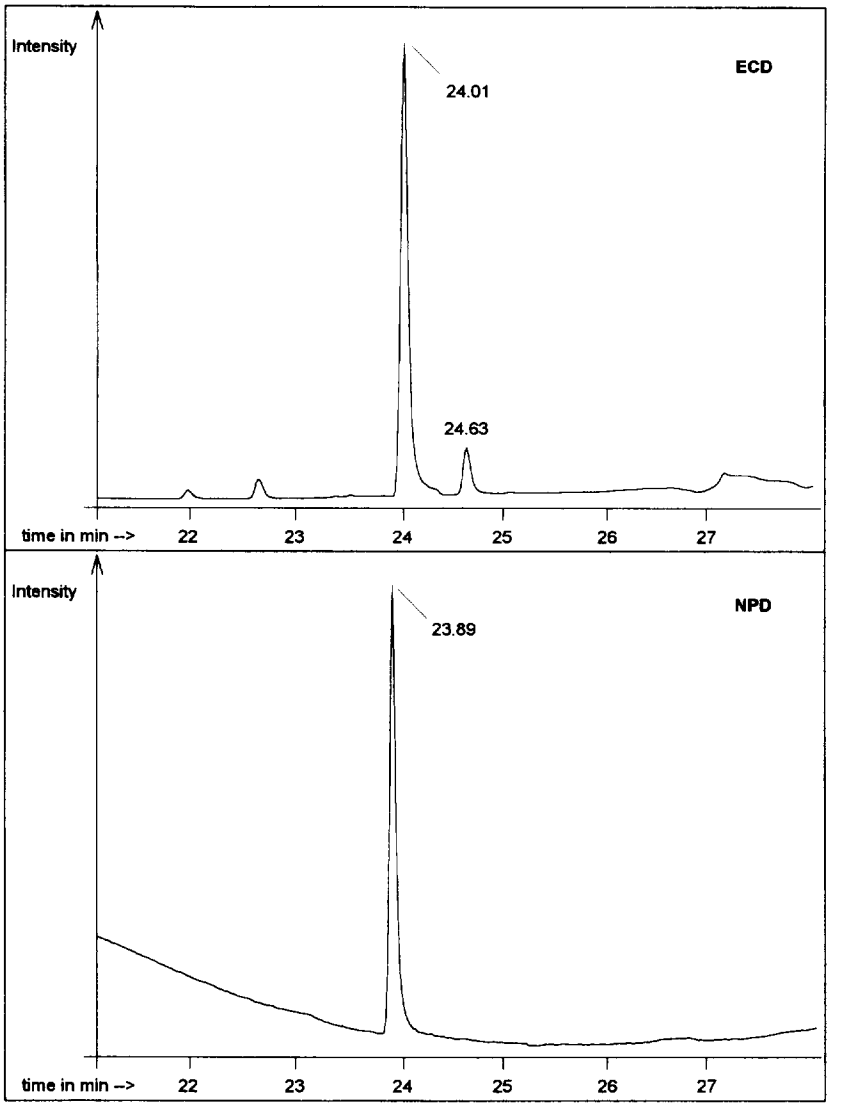

Fig. 1. Parallel chromatograms obtained with GC–ECD/NPD of a derivatized ETU standard solution (50 pg injected).

ous plant matrices at three concentration levels; 0.01, 0.05 and 0.1 ppm, respectively. The recoveries at the fortification level of 0.05 ppm were between 82–92%, with a mean of 87% and a mean standard deviation of 3.3%. The results are compiled in Table 1. As already mentioned, the parallel analysis with ECD and NPD was not only found to contribute considerably to the identification of ETU but also to the reliability of the quantitative determination. The response of both detectors varied in the course of the investigation, with the ECD being the more sensitive and stable detector. The response of NPD with a newly coated pearl was found to be almost half of the ECD response with our instrument running under conditions for routine monitoring of pesticide analysis. Therefore, the response must be calculated daily

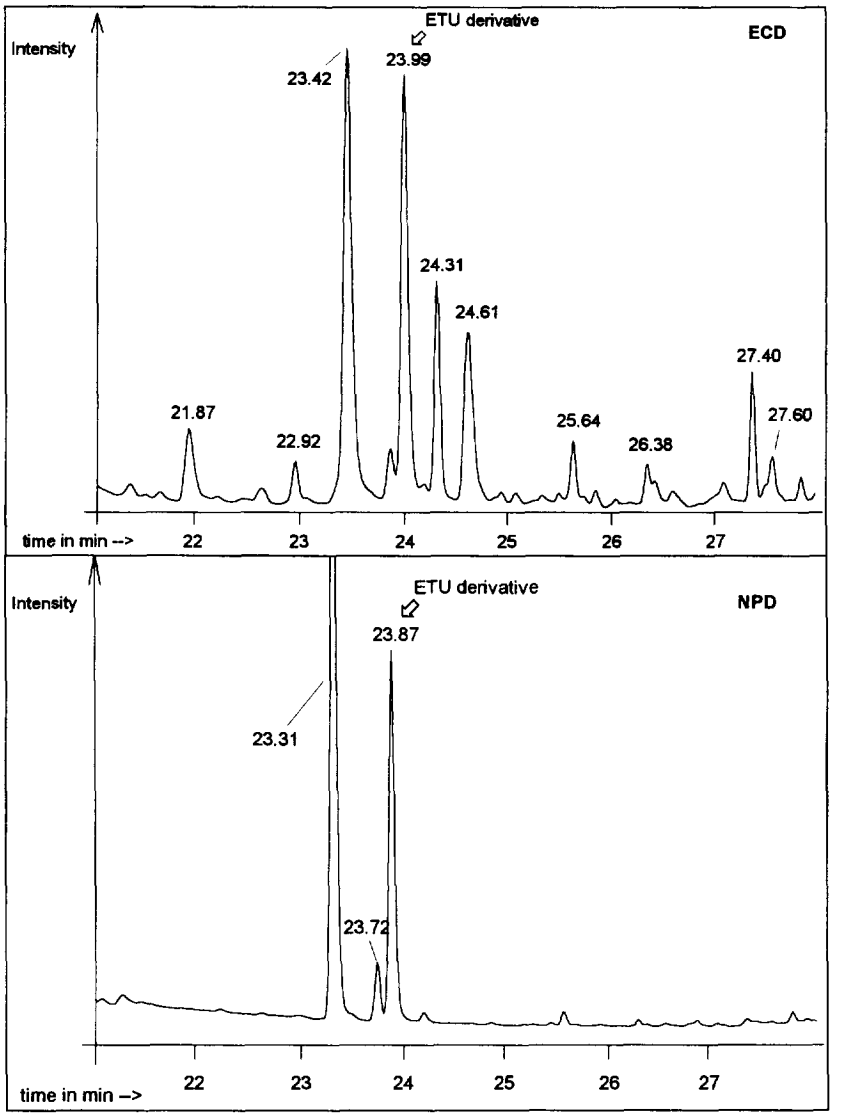

Fig. 2. Parallel chromatograms obtained with GC–ECD/NPD of an extract from an apple sample fortified with ETU at a concentration level of 0.05 ppm (50 μg/kg).

Table 1
Per cent recovery of ETU from different food commodities

Crop	ETU added (ng/g)	Mean±SD (%) (n=3)
Apple	50	89±4.4
Tomato	50	85±2.8
Pear	50	92±4.5
Milupa (baby food)	50	82±1.6

Samples were fortified just before blending.

from the calibration analysis with standards. On this basis, the response can be used as an easy check of peak purity and to improve the reliability of the quantitative data. The limit of detection (LOD) for the determination of ETU with parallel analysis with ECD and NPD (with chromatographic peaks exhibiting a signal-to-noise ratio better than three) was

calculated to be below 10 pg injected, which is equivalent to less than 1 ppb in the sample.

Gas chromatographic analysis with MS was used to check the correctness of the qualitative and quantitative results. At the trace concentrations analysed in the recovery experiments, the mass spectrometer had to be operated in the SIM mode. Ions selected for the determination of the ETU derivative were m/z 288, 255 and 219, respectively. The intensity ratio of m/z 255 to 288 was found to be 6.35 and that of m/z 219 to 288 to be 0.76. A positive confirmation of ETU was accepted when the ion intensity ratios were measured within a margin of ±20% of these values. The LOD for the determination of ETU with MS (with chromatographic peaks exhibiting a signal-to-noise ratio better than three in each of the three ion traces) was calculated to be 100 pg injected, which is equivalent to 10 ppb with the extract volume of 1 ml. In order to confirm ETU at the LOD of the ECD/NPD method, the extract volume must be reduced to 100 μl, which is easily carried out with a stream of nitrogen.

The effect of storage time and conditions on the stability of derivatized ETU in the final extract ready for injection was examined by using aldrin as the internal standard in a pear fruit extract. Storage of the final solution in a refrigerator overnight at 4°C resulted in an average degradation of more than 50% at the 0.05 ppm fortification level. Therefore, the derivatized ETU in the final extract must be determined without any delay.

The food commodities that were monitored most for residues of dithiocarbamates (DTCs) in our laboratory were apple, pear, tomato, salad, lamb's lettuce, green bean, grapes, kohlrabi and chicory, representing a cross-section of the fruits and vegetables on sale in the German market during autumn and winter. The fruit and vegetable samples that tested positive for DTCs, as determined with the CS_2 method [17], were also analysed for ETU. Four of the twenty samples monitored, found positive for DTC, were also contaminated with ETU in the range from 0.01 to 0.37 ppm. Three of these food samples were found to contain ETU residues above the MRL of 0.05 ppm. In the ETU-containing food samples, residues of DTCs were found to be between 0.2 and 0.8 ppm, all below the MRL of DTCs on CS_2 basis (the MRLs for DTCs in the European Union were set to 2 ppm for pears and 5 ppm for all types of lettuce). No relation was found between the residue concentrations of DTCs and that of ETU, as presented in Table 2. The sample with the lowest ETU residue concentration was of a pear containing 0.01 ppm ETU, which was already found to be below the MRL. In the same sample, the DTC residue concentration was 0.8 ppm, which, in contrast, was the highest DTC residue level of the four ETU-containing samples. The chromatograms of ETU residue analysis of this sample are presented in Fig. 3 in order to demonstrate the extremely sensitive detection method with a real fruit sample.

Although the data gathered are few, it seems to be meaningful to conclude that there is no relation between the residue concentration of DTCs and ETU due to variation in the degree of degradation with different food matrices and contact time. Therefore, ETU residue analysis should at least be carried out with all food samples that indicate the application of DTCs, because of non-acceptable risks to customers. The goal of the research was to develop a reliable method for the analysis of ETU residues in fruits and vegetables, with the necessary sensitivity and reproducibility. The proposed method worked completely satisfactorily in our hands for the determination of ETU in routine monitoring of samples contaminated with DTCs. Using the two selective detectors in parallel, the reliability of the identification of ETU in food matrices has proven to be very high. All positive findings in the screening analysis in the course of this study, however, have been confirmed by GC–MS in SIM mode. The response ratios of both the detectors for the selective detection of the derivatized ETU improves the reliability of its identification to a degree that further analysis for the confirmation of the compound by GC–MS is often not necessary. Therefore, we see no need in routine

Table 2
Monitoring of market samples for DTCs and ETU residues (in ppm)

Sample	Crop	DTCs[a]	ETU
361	Pear	0.80	0.010
370	Pear	0.25	0.205
382	Pear	0.20	0.047
385	Lambs lettuce	0.65	0.367

[a]Determined as CS_2.

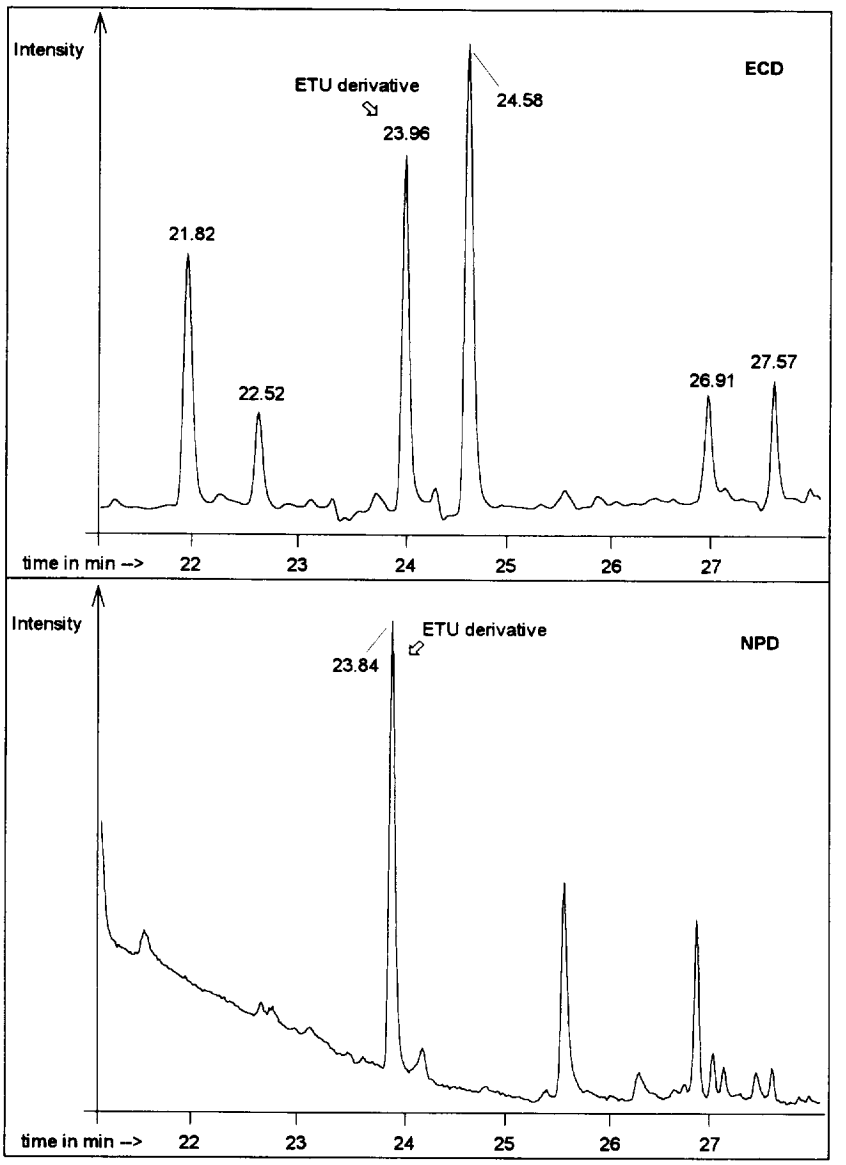

Fig. 3. Parallel chromatograms obtained with GC–ECD/NPD of an extract from a commercial pear sample (No. 361). 0.01 ppm of ETU were found in this sample.

analysis for MS confirmation of each single result obtained by GC–ECD/NPD.

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