Determination of ETU in Tomatoes and Tomato Products by HPLC-PDA. Evaluation of Cleanup Procedures

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An HPLC-PDA method for the determination of ethylenethiourea (ETU), the main degradation product of the organic fungicides ethylene bis(dithiocarbamate)s (EBDCs), in tomatoes and tomato products is reported. Solid-matrix liquid−liquid (l−l) partitioning and separatory funnel l−l partitioning for the cleanup were examined. The effect of salt addition, pH, and phase ratio on analyte recovery at the cleanup step was studied. It was found that solid-matrix l−l partitioning afforded higher precision and more selective separation of the analyte. According to the method proposed, the samples were extracted with methanol/water (3:1, v/v) and cleaned up on an Extrelut 20 column. ETU was eluted with dichloromethane and separated on a reversed phase HPLC column. For tomato products with °Brix > 20 further purification through silica cartridge was adopted. The method was validated over the following ranges of concentrations: 0.01−0.5 mg/kg for tomatoes, 0.01−0.1 mg/kg for tomato juice, and 0.05−0.25 mg/kg for tomato paste. The accuracy (recoveries > 70%) and the precision obtained (%RSD < 10%) were satisfactory.

Keywords: *Pesticide residues; ethylenethiourea; tomato products; HPLC-PDA; solid-matrix l−l partitioning*

INTRODUCTION

Ethylenethiourea (ETU), the main degradation product of the organic fungicides ethylene bis(dithiocarbamate)s (EBDCs), has been proved to have thyreotoxic, teratogenic, and carcinogenic effects on test animals (*1*). Supervised trials studying the dissipation of EBDCs on various crops showed that no accumulation of ETU occurred due to its own further degradation. However, processing of raw agricultural products containing the parent compounds and especially cooking can give rise to increased levels of the toxic metabolite (*2*−*11*).

EBDCs are widely used to protect tomato crops against diseases and especially blights. At the same time, the tomato produce is subjected to thermal treatments such as evaporation, blanching, sterilizing, and canning in order to be consumed as juice, paste, ketchup, or canned products. In addition, home cooking of tomatoes is a common practice. Therefore, it is necessary to monitor tomatoes and tomato products for ETU residues. A simple and rapid method for the determination of ETU is of primary importance for the effective monitoring of residue levels in tomato products as well as for studying its formation during processing.

Gas chromatographic (GC) methods requiring derivatization of polar ETU tend to be superseded by high-performance liquid chromatographic (HPLC) methods. Erratic recoveries for many substrates (*12*, *13*) and conversion rates of EBDCs to ETU as high as 10% at the derivatization step (*6*) have been reported. Since the review of the methods published up to 1990 (*14*) few methods applying GC techniques have been issued. Meiring and Jong (*15*) proposed a single-step extractive derivatization GC-MS method for the analysis of water samples, whereas Dubey et al. (*16*) used GC-ECD/NPD parallel detection for the determination of ETU in various food commodities. Recently, there is a clear trend toward the application of the HPLC technique coupled with UV (*17*−*21*), MS (*22*, *23*), or electrochemical detection (*24*−*26*). Alternative techniques have been used such as the flow injection spectrofluorometric detection (*27*) or kinetic method (*28*) based on the catalytic or inhibitory properties of ETU to certain reactions. These, however, suffer from low selectivity.

HPLC methods for tomatoes or tomato products as substrates using electrochemical detection have been reported (*25*, *26*, *29*). The major merit of these detectors is increased specificity, but to date they are not widely used for routine analyses mostly because their manipulation encounters difficulties. Conversely, the HPLC-UV technique lacks selectivity. This drawback is offset by the introduction of the photodiode array (PDA) detector, which is able to check the purity of a chromatographic peak and confirm its identity by means of the UV spectra acquired during peak elution. These features increase the reliability of the analytical results.

The selectivity of the detection system applied as well as the nature of the substrate determines the cleanup procedure that should be adopted. The isolation of ETU from other polar coextractives is the critical step of the method. Cleanup based on liquid−liquid (l−l) partitioning in dichloromethane (*20*, *30*) or alternatively solid-matrix l−l partition on Extrelut columns (*12*, *18*, *19*, *25*, *26*, *31*) have been the prevalent approaches in the literature. Automation of the cleanup step via the column switching technique has been employed to a

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lesser extent, for only water (*17*) or beer samples (*32*). The majority of the methods propose a second cleanup step by solid-phase extraction (SPE) (*19*, *25*) or through an alumina column (*29*, *30*). Multiple cleanup steps, apart from being laborious and time-consuming, can result in analyte loss or the introduction of interferences due to solvent or glassware impurities. It is important that the treatment of the sample is limited to a minimum depending on the matrix.

The purpose of the present work was to develop and validate a simplified HPLC-PDA method for the determination of ETU in tomato products. In this framework, evaluation of the alternative approaches, l−l partitioning and solid matrix l−l partitioning (Extrelut columns), was conducted, and the necessity for further purification of the sample was investigated. Initially, the cleanup parameters were optimized to achieve both maximum recoveries and positive confirmation of the analyte identity through the UV spectra.

MATERIALS AND METHODS

(A) Materials. The methanol was of HPLC grade, and the water was obtained by a Nanopure UV purification device (Barnstead) supplied by demineralized water. Dichloromethane (CH_2Cl_2) was of pesticide grade (pestiscan). ETU purum quality was obtained from Fluka. Potassium fluoride (KF), ammonium chloride (NH_4Cl), sodium hydroxide (NaOH), and dipotassium hydrogen phosphate (K_2HPO_4) were of analytical grade. Anhydrous sodium sulfate (Na_2SO_4) was of pesticide free grade (pestanal) from Riedel de Häen. Alumina activity grade I was used.

Filters with a pore size of 0.45 μm and a GHP (polypropylene) membrane were from Gelman Sciences. Glass microfiber filters were from Whatman (No. 1822 70).

Extrelut columns were obtained from Merck (part 11738). Silica SPE columns, 500 mg (ISOLUTE IST, part 500-0050-B*) and strong anion exchange SAX columns, 500 mg (ISOLUTE IST, part 460-0050-C) were used.

Organically grown tomatoes and tomato products were purchased from the local market.

(B) Cleanup Parameters. The effect of three different cleanup parameters, pH, salt addition, and solvent volume, on ETU recovery from aqueous solutions was studied. Twenty milliliters of pure water (pH 6−7) containing 0.5 mg/L ETU was subjected to l−l partitioning using a separatory funnel or Extrelut column. The pH values studied were 4−5 adjusted by the addition of NH_4Cl and 7−9 by the addition of 2% NaOH. For the separatory funnel, at pH 4−5 two different volumes of dichloromethane were tested: 2 × 100 and 2 × 140 mL. Subsequently, the required salt amount was estimated by adding to the solution KF at 25, 50, and 75% w/v while the pH was held at 4−5 and the dichloromethane used was 2 × 100 mL. Solid-matrix l−l partitioning (Extrelut column) was conducted at acidic pH with KF addition at 50% w/v or without salt at basic pH. The volume of the dichloromethane used was accordingly 100 or 200 mL. The organic phase obtained after the cleanup was evaporated to dryness, and the residue was transferred quantitatively with water to a 5 mL volumetric flask prior to the HPLC-PDA analysis.

(C) Sample Analysis. *(1) Sample Preparation.* Raw tomatoes (usually 500 g) were ground in a commercial blender. Tomato juice and paste did not require preparation prior to the analysis.

(2) Extraction. A 20 g sample portion (or 5 g for samples with a soluble solids content >20 °Brix) was transferred to a 500 mL Waring blender, 160 mL of methanol/water (3:1, v/v) was added, and the mixture was homogenized for 2 min. The homogenate was filtered through a glassfiber filter in a Büchner funnel. The blender cup was rinsed with an additional portion of 40 mL of the extraction solvent. The filtrate was transferred to a flask and concentrated to 20 mL on a rotary evaporator.

(3) Cleanup. (a) Tomatoes and Tomato Products with <20 °Brix. (i) Separatory Funnel l−l Partition. After pH adjustment to 4−5 using NH_4Cl and addition of 50% w/v KF, the concentrate was tranferred to a 250 mL separatory funnel and extracted with two equal portions (2 × 100 mL) of dichloromethane. The organic phases were combined and dried through 20 g of Na_2SO_4.

(ii) Solid-Matrix l−l Partition (Extrelut Column). The cleanup conditions described in two published methods were examined. (a) In the first case (*26*), the pH of the concentrate was adjusted to 4−5 with NH_4Cl and KF was added at 50% w/v. It was then loaded to the Extrelut column and allowed to stand 10−20 min for equilibration; ETU was eluted with 100 mL of dichloromethane. (b) In the second case (*31*), the concentrate was adjusted to pH 7−9 with 2% NaOH, applied to the column, and, after equilibration, was eluted with 200 mL of dichloromethane.

The dichloromethane phase obtained by the above procedures was evaporated to dryness. The residue was transferred quantitatively with water to a 5 mL volumetric flask.

(b) Tomato Products with >20 °Brix. For those products necessitating more rigorous cleanup, the following procedures were tested.

(i) Extrelut/Alumina Combination Column. The concentrated sample extract after pH adjustment to 7−9 with 2% NaOH was transferred to a combination Extrelut/alumina column. The packing of the column consisted of a bottom layer of 5 g of alumina, in addition to the standard amount of the diatomaceous earth material that the ready-to-use product contained. Elution was conducted with 300 mL of 4% methanol in dichloromethane by gravity flow. The eluant was evaporated to dryness and the residue transferred quantitatively with water to a 5 mL volumetric flask.

Alternatively, the concentrated sample extract was cleaned up on an Extrelut column and was then further purified either by silica or by anion exchange column. The eluate of the Extrelut column obtained according to the previously described cleanup method (b) was evaporated to dryness and the analysis proceeded as follows:

(ii) Anion Exchange SAX Column. The residue was reconstituted with 3 mL of 0.05 M K_2HPO_4 buffer (pH 9). It was then applied to the SAX cartridge and allowed to elute under gravity. The column was previously solvated with 6 mL of methanol and equilibrated with 6 mL of buffer. The sample passing through the column was collected in a 5 mL volumetric flask. A second volume of 2 mL of buffer was also added and collected to the same flask.

(iii) Silica Column. The residue was dissolved in 5 mL of dichloromethane and was loaded to the silica column preconditioned with 10 mL of dichloromethane. ETU was eluted with 5 mL of 2% methanol in dichloromethane by gravity flow. The eluant was evaporated to dryness and the residue transferred quantitatively with water to a 5 mL volumetric flask.

(4) HPLC Analysis. The final aqueous solution was filtered through 0.45 μm pore size membrane filters. Twenty microliters was injected to the HPLC system. The HPLC apparatus consisted of a model 600E pump, a model 996 PDA detector, and a model 717plus autosampler (Waters). Data were processed using Millenium software (version 2.10). A Nucleosil 100 C18, 240 mm × 4.6 mm, column was used (Phase Separations Chromatography). The mobile phase was water/methanol (95:5, v/v) at a flow rate of 1 mL/min (isocratic), and the eluate was monitored at 240 nm. The mobile phase was degassed with helium constantly.

(D) Statistical Analysis. Statistical analysis was performed using Excell version 7.0 software.

RESULTS AND DISCUSSION

(A) Evaluation of the Cleanup Procedure. *(1) Effect of Cleanup Parameters on ETU Recovery from Aqueous Solutions.* The aim of this stage was to define the optimum conditions, in terms of ETU recovery, for the cleanup step based on l−l partitioning from the

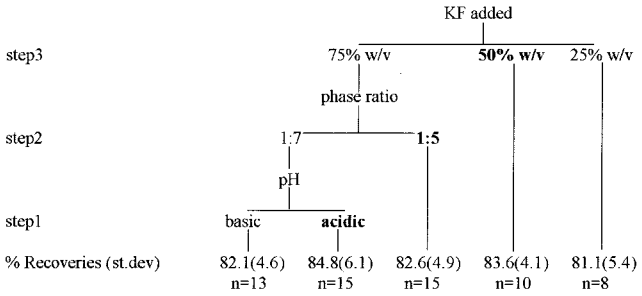

Figure 1. l−l partitioning optimization scheme and recoveries obtained. Samples: 20 mL of 0.5 mg/kg ETU aqueous standard solution. In bold characters are shown the conditions adopted in each step.

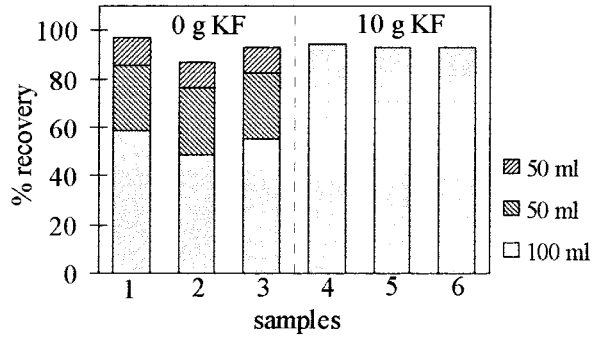

Figure 2. Effect of salt (KF) addition on the required elution volume in solid-matrix l−l partitioning. Total recoveries are given as the sum of the recoveries obtained from the first (100 mL), second (50 mL), and third (50 mL) fractions of dichloromethane. Samples: 20 mL of 0.5 mg/kg ETU aqueous standard solutions.

concentrated aqueous sample extract to dichloromethane in the presence or not of solid support. Ankumah et al. (*33*), using [^{14}C]ETU, found that ETU partition coefficients to dichloromethane from pure water and tomato sauce extracts do not differ and concluded that no association products between the analyte and food coextractives are formed. Therefore, the preliminary recovery experiments were carried out using ETU solutions in pure water at a concentration level of 0.5 mg/L.

(a) l−l Partitioning. The effect of the three parameters, namely, pH, phase ratio, and salt addition, on ETU recovery values was studied by a step-by-step optimizing scheme as indicated in Figure 1.

(i) pH. ETU is known to be stable over the pH range 5−9 (*34*). ETU is a weakly basic compound (pK_a value of 2.70; *35*). At the same time, due to ketol−thiol tautomerism, it exhibits acidic properties at pH values >10 (*15*). Therefore, the pH of the ETU solutions was adjusted to slightly acidic values of 4−5 with the aid of NH_4Cl or to slightly basic values of 7−9 with 2% NaOH. The amount of the salt (KF) added was held at 75% w/v, and the phase ratio was also held constant at 1:7. The influence of the pH on ETU recovery in the range studied was found to be insignificant ($p < 0.05$) as can been seen from Figure 1. For the ensuing steps NH_4Cl adjustment was adopted by taking into consideration that the acidic pH is closest to the pH (4.0−4.7) of the tomato matrix for which the method would be applied.

(ii) Phase Ratio. To study the effect of this parameter, the pH was adjusted to 4−5 and salt addition remained constant at 75% w/v while phase ratios of 1:5 and 1:7 were applied. The recoveries obtained presented in Figure 1 were not significantly different ($p < 0.05$). On the basis of this finding and to avoid excessive solvent consumption, the phase ratio was set subsequently at 1:5.

(iii) Salt. ETU is a highly polar compound. Due to its low partition coefficient in dichloromethane a salting-out reagent such as KF is indispensable for its quantitative removal from the aqueous phase. To assess the effect of salt, 25, 50, or 75% w/v of KF was added to the ETU solutions. The pH was adjusted with NH_4Cl, and the phase ratio was held at 1:5. As no statistical differences ($p < 0.05$) were observed from the 3-fold increase in the amount of salt added (Figure 1) and with the view to enhance the ruggedness of the method, the

Table 1. ETU Recoveries (Percent) from Fortified Tomato Samples[a] Subjected to Funnel[b] or Solid-Matrix (Extrelut)[c] l−l Partitioning (Values Are Means of Duplicate Analyses)

fortification level (mg/kg)	day	% ETU recovery	
		funnel	Extrelut
0.5	1	82.33	82.74
	2	81.35	90.63
	3	79.83	83.17
	4	71.58	83.84
	5	83.30	84.39
0.1	6	86.93	78.93
	7	82.17	73.69
	8	77.78	84.57
	9	84.20	90.44
	10	75.88	86.13
0.05	11	84.39	79.39

df = 10

$t_{0.95}$ obs = 1.17 < $t_{0.95}$ crit two-tail = 2.23

[a] Cleanup conditions: pH adjusted to 4−5, 50% w/v of KF added. [b] Solvent volume: 2 × 100 mL. [c] Eluant volume: 100 mL.

Table 2. Precision Statistics for Funnel and Column (Extrelut) l−l Partitioning Established by Replicate Analysis of ETU-Fortified Tomato Samples[a]

fortification level (mg/kg)		funnel	Extrelut
0.5	n	5	5
	s_r^2	0.0012[a]	0.00013[b]
	s_r	0.035	0.011
	%RSD$_r$	7.0	2.3
0.1	n	5	4
	s_r^2	0.000047[c]	0.000011[c]
	s_r	0.0069	0.0034
	%RSD$_r$	6.9	3.4

[a] Different superscript letters indicate significant differences ($p < 0.05$).

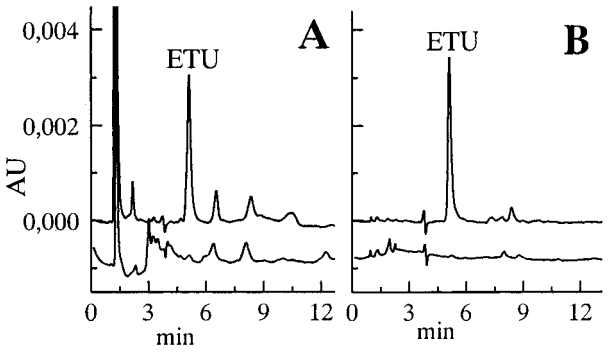

Figure 3. HPLC-PDA chromatograms (λ = 240 nm) of controls and tomato samples spiked with 0.1 mg/kg ETU and treated (A) by funnel l−l partitioning or (B) by solid-matrix l−l partitioning.

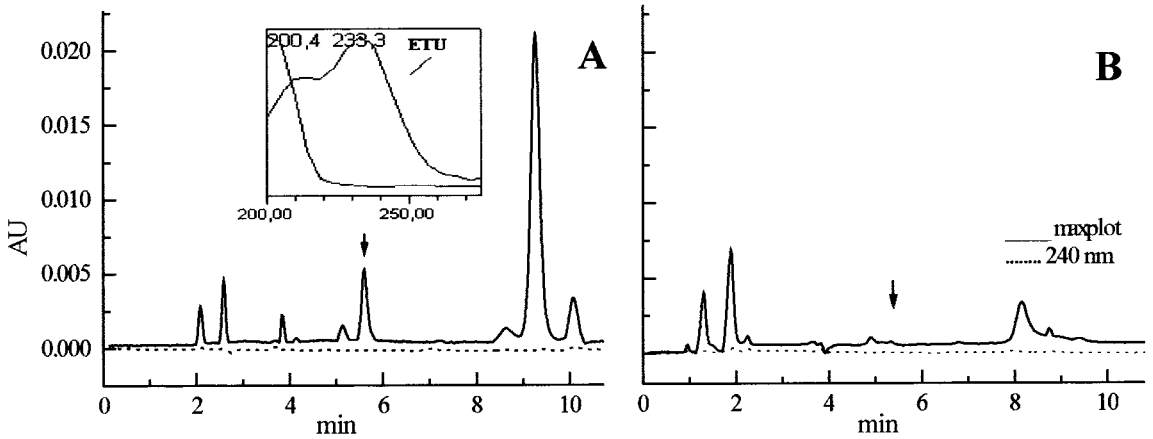

Figure 4. HPLC-PDA chromatograms of blank tomato samples subjected to Extrelut column cleanup. Conditions: (A) pH acidic, salt 50% w/v, elution with 100 mL of dichloromethane; (B) pH basic, no salt added, elution with 200 mL of dichloromethane. The arrow in (A) marks an unknown matrix interferent, and the inset box shows a comparison of this peak PDA spectrum (λ_{max} = 200.4 nm) and the spectrum of an ETU standard (λ_{max} = 233.3 nm).

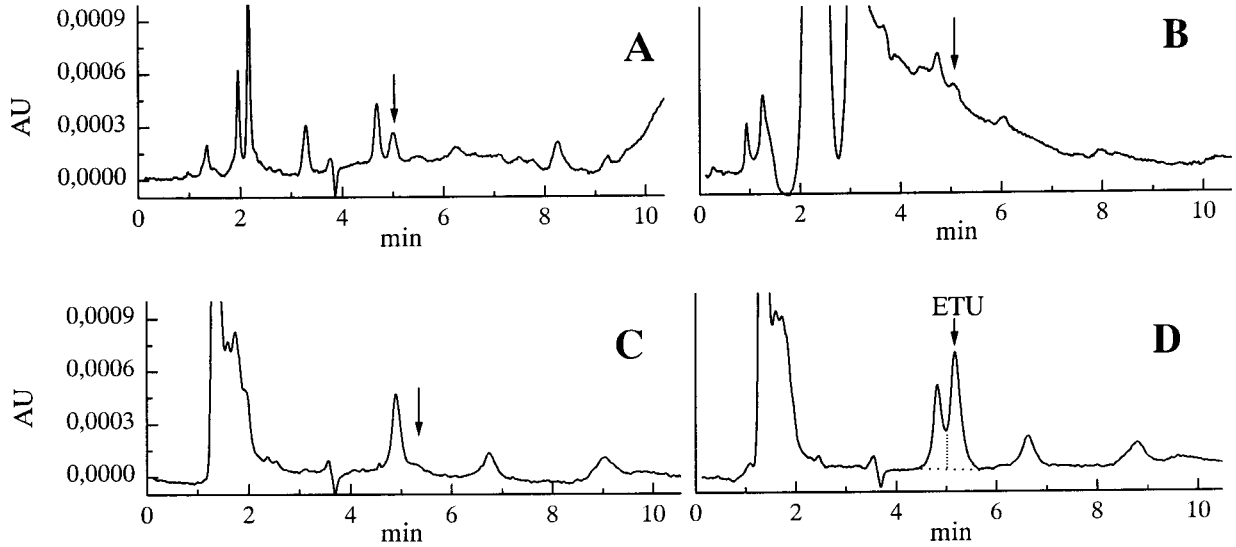

Figure 5. HPLC-PDA chromatograms of blank tomato paste subjected to (A) a combined Extrelut/alumina column cleanup, (B) Extrelut and SAX anion exchange column cleanup, or (C) Extrelut and silica SPE column cleanup and (D) of a tomato paste spiked with 0.05 mg/kg ETU (LOQ) and treated as (C).

Table 3. Repeatability (Expressed as %RSD$_r$) of the Chromatographic System Established by Replicate Injections of Standard ETU Solutions

	% RSD$_r$ (n = 5)						
	0.005 mg/kg	0.01 mg/kg	0.05 mg/kg	0.10 mg/kg	0.50 mg/kg	1.00 mg/kg	5.00 mg/kg
retention time	1.66	0.23	0.16	0.10	0.18	0.14	0.05
peak area	29.63	7.96	5.83	5.28	0.73	0.37	0.50
peak height	16.11	8.30	2.51	2.05	0.22	0.13	0.66

amount of the salt was fixed at the medium level tested, which was 50% w/v.

(b) Solid-Matrix l−l Partitioning. To evaluate the suitable conditions for effective ETU recovery, the two published methods described under Materials and Methods (section C.3.a.ii) applying Extrelut column cleanup were used as starting points. The two different cleanup procedures were applied to 20 mL aqueous solutions containing 0.5 mg/L ETU. The recoveries obtained were 93.6 ± 4.6% (n = 7) at pH values of 4−5 in the presence of KF and 90.2 ± 4.1% (n = 5) at pH values of 7−9 without salt. These recovery values do not statistically differ ($p < 0.05$).

The recovery yields of ETU for each of three consecutive dichloromethane elution fractions, consisting of 100, 50, and 50 mL, respectively, were also determined for either procedure (Figure 2). It is observed that there is an interaction effect between the salinity of the aqueous phase and the volume of the organic eluant; the salt added decreased the volume of the dichloromethane needed for the elution of ETU. At the same time, whereas 200 mL of the eluant removed ETU from the Extrelut column in the absence of salt, an equal volume (2 × 100 mL) is needed for a quantitative separatory funnel partitioning in the presence of salt. Thus, the solid support, which increases the interface of the binary solvent system, enhances the efficiency of the partitioning, rendering the addition of salt optional.

Because salt addition decreases solvent consumption and the elution time accordingly, the conditions selected for the subsequent comparison study were as follows:

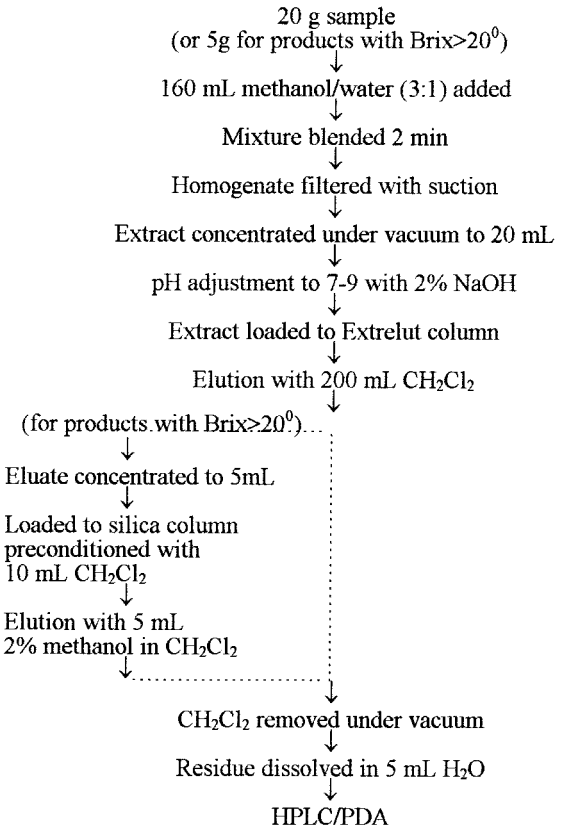

Figure 6. Validated method procedure

pH adjustment to 4−5 with NH_4Cl, addition of 50% w/v KF, and elution with 100 mL of dichloromethane.

(2) Comparison of l−l versus Solid-Matrix l−l Partitioning for ETU Recovery from Tomato Products. To compare the performance of l−l versus solid-matrix l−l partitioning, the selected values of the cleanup parameters pH, salt, and solvent volume, for which quantitative ETU recovery from aqueous solutions was obtained, were applied to the treatment of tomato extracts.

Tomato samples spiked at three different levels (0.5, 0.1, and 0.05 mg/kg) were subjected to the two cleanup techniques side by side before analysis by liquid chromatography. Statistical comparison of ETU recoveries was made by paired *t* test to offset the variation introduced by different samples and days of analysis. The percent recoveries obtained were equivalent ($p < 0.05$) and ranged from 73.7 to 90.6% for the column procedure and from 71.6 to 86.9% for the separatory funnel procedure (Table 1).

The method repeatability afforded by each technique was established by the duplicate analysis of the samples for 4 or 5 consecutive days and was found to depend, for both cases, on the concentration level (Table 2). The repeatability variance of the solid-matrix l−l partitioning was significantly lower than that of the conventional separatory funnel partitioning at the 0.5 mg/kg level. This finding probably reflects the fact that emulsion formation can occur during the separatory funnel partitioning, resulting in lower extraction efficiencies. However, $\%RSD_r$ values corresponding to both techniques are <7%.

Comparison of the chromatograms of tomato extracts purified by the different techniques revealed that the Extrelut column exhibited higher selectivity (Figure 3). As to the practicality of each cleanup procedure, it should be emphasized that the time required for l−l partitioning in comparison with solid-matrix l−l partitioning is shorter but that the latter allows simultaneous handling of several samples.

Table 4. Accuracy and Precision Statistics of the Method Determined for Tomato Samples as Substrate ($n = 6$)[a]

	fortification level			
	0.5 mg/kg	0.1 mg/kg	0.05 mg/kg	0.01 mg/kg
% recovery	79.2	74.2	79.3	86.7
s_r	0.016[a]	0.0028[b]	0.0026[b]	0.00055[c]
$\%RSD_r$	3.9	4.0	6.9	6.7
r ($2.8s_r$)	0.046	0.008	0.0074	0.0015
s_R	0.034[a]	0.0036[b]	0.0033[b]	0.00065[c]
$\%RSD_R$	7.9	5.1	8.8	7.9
R ($2.8s_R$)	0.095	0.010	0.0094	0.0018

[a] Different superscript letters indicate significant differences between the corresponding variances ($p < 0.05$).

On the basis of these results solid-matrix l−l partitioning was chosen for the purification of tomato samples and tomato products.

(3) Selectivity Optimization. The aim of this stage was to optimize the method for the analysis of tomato and tomato products in terms of specificity with the view to achieve not only reliable quantitation but also positive confirmation of the analyte identity by means of PDA detection.

(a) Tomatoes. The absorbance maximum of ETU is at 233.3 nm in 5% aqueous methanol solution. Monitoring at a slightly higher wavelength of 240 nm provided clearer chromatograms and blank samples showed no interferent peaks near the elution time of ETU. Nevertheless, when PDA detection was applied as a confirmatory tool, the analysis of various tomato samples revealed that a coeluting peak absorbing at a wavelength <210 nm distorts the analyte spectrum, making its unambiguous identification at low concentration levels problematic. Changing the chromatographic conditions (mobile phase, flow rate) did not result in better separation. Because it is not considered enough if the characteristic peak maximum is discerned in a spectrum inspected but a better than 10% match between the unknown peak spectra and the standard spectra is desirable, the efficiency of the two cleanup methods a and b described under Materials and Methods (section C.3.a.ii) was examined in the tomato extract purification.

For this purpose, blank tomato sample extracts and fortified ones at 0.5 mg/kg were subjected to Extrelut column cleanup according to both protocols. It was found that whereas percent recoveries were not significantly different (79.1 ± 3.7 and 78.4 ± 5.3, respectively, $n = 7$), selectivity was dramatically enhanced when conditions b were adopted (Figure 4). Königer (*31*), analyzing wine samples, had also reported that KF and NH_4Cl addition before an Extrelut cleanup step resulted in insufficient purification when UV detection was applied. In this study, under slightly basic conditions and no salt addition, positive identification of the analyte down to 0.01 mg/kg was possible. The lower concentration level is limited by the instrument noise and not the matrix interference. No further cleanup was necessary for samples (tomatoes and tomato juices) with <20 °Brix.

(b) Tomato Products. When complex sample matrices are treated such as sauces and tomato pastes between 20 and 30 °Brix, adjustment of the cleanup conditions does not suffice. The interferences can be compounds either present in dilution in the raw product or formed during processing. Substantial increase in the concentration of free amino acids due to denaturation and partial hydrolysis of proteins or formation of 5-hydroxymethylfurfural during thermal treatment has been

Table 5. Accuracy and Precision Statistics Determined for Tomato Juice and Tomato Paste as Substrate ($n = 3$)[a]

	tomato juice (5 °Brix)		tomato paste (30 °Brix)		
	spike level = 0.1 mg/kg	spike level = 0.01 mg/kg	spike level = 0.25 mg/kg	spike level = 0.1 mg/kg	spike level = 0.05 mg/kg
% recovery	87.6	80.0	80.5	78.5	88.5
s_r	0.0047[a]	0.0007[b]	0.014[a]	0.0081[a]	0.0039[a]
%RSD$_r$	5.4	10.1	6.7	10.3	8.6
r (2.8s_r)	0.013	0.0019	0.039	0.023	0.011

[a] Different superscript letters indicate significant differences between the corresponding variances ($p < 0.05$).

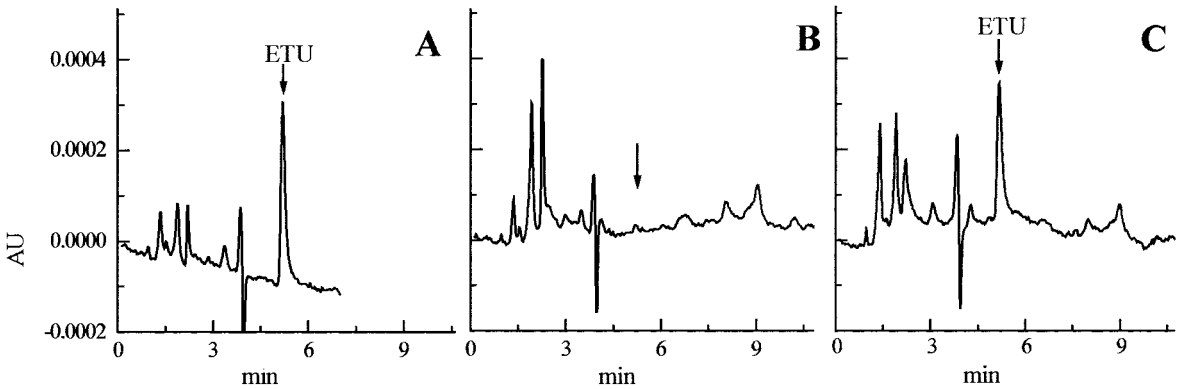

Figure 7. HPLC-PDA chromatograms of (A) ETU standard 0.04 mg/kg, (B) control tomatoes, and (C) control tomatoes spiked with 0.01 mg/kg ETU (LOQ), equivalent to 77% recovery.

reported. Therefore, a further cleanup step is needed to improve the sensitivity of the method.

In addition to the Extrelut partitioning, the use of an SPE (C18) cartridge for the further cleanup of tomato products determined with coulometric detection has been reported (*25*). This approach was not considered to be applicable for the removal of interferences eluting close to the compound of interest in the reversed phase HPLC system. On the contrary, sample pretreatment based on a different separation principle might be more appropriate. Krause (*29*) combined an alumina layer to the diatomaceous earth (Gas Chroms) solid support of a partitioning column for the pretreatment of canned tomatoes among various other products, and silica gel cleanup has been applied, too, in methods dealing with beer samples (*32*) or biological samples (*36*).

In this study, an Extrelut/alumina combination column was tested. Sample extracts were also subjected to the Extrelut column and were subsequently further purified either by an anion exchange column or by a silica column (Figure 5).

Alumina had no effect on the chromatographic profile of the sample (percent recoveries obtained were 72.9 ± 2.5, $n = 2$). The strong anion exchange column removes acidic and slightly acidic compounds. ETU was found to pass unretained through this column even when the pH was adjusted close to 9 (ETU recovered at this step = 96.2 ± 2.6%, $n = 3$). The sample extract was purer, but the coeluting interferents, although reduced, persisted. Eventually, it was found that on the silica SPE column when the strength of the eluant was adjusted for the elution of ETU (2% methanol in dichloromethane), interferent compounds were still absorbed. Thus, the silica cartridge removed the highly polar components that the reversed phase C18 HPLC column does not resolve from ETU. As a result, this step was adopted for the subsequent validation of the method for the relevant matrix.

(B) Validation of the Method. The final method proposed according to which solid-matrix l−l partitioning is adopted in conjunction with a silica column cleanup for more complex matrices is presented in Figure 6.

(1) System Suitability Check. The capacity coefficient for the column used was $k = 1.5$ and the peak asymmetry factor $A_f = 1.1$ ($A_f = b_{0.1}/a_{0.1}$). Linearity was checked in the range of 0.005−1 mg/kg. The calibration graph consisted of six levels, and each one was injected five times. The equation of the regression line was $y = 160564x - 988$, with a coefficient of correlation $r^2 = 0.999$. The percent response factor of each level versus the mean response was between 92 and 108%. The instrumental detection limit (S/N = 3:1) was found to be 0.004 mg/kg, whereas the quantitation limit (S/N = 10:1) was found to be 0.015 mg/kg. The analytical system repeatability was established by replicate injections of standards at seven concentration levels. The %RSD$_r$ values of retention time, peak area, and peak height are presented in Table 3. At the level of 0.005 mg/kg, which is very close to the detection limit, the variability, as expected, is very high. For the other concentration levels, though, the peak area and peak height %RSD$_r$ were below 10% and the retention time %RSD$_r$ below 0.2%, which are considered to be satisfactory.

(2) Accuracy and Precision. The accuracy and precision of the method were established by spiking tomatoes and tomato products at several concentration levels. Tomatoes spiked at four different levels ranging from 0.01 to 0.5 mg/kg were analyzed in triplicate on different days (Table 4). Tomato juice and tomato paste spiked at 0.01−0.1 mg/kg and at 0.05−0.25 mg/kg, respectively, were analyzed in triplicate within a day (Table 5).

Recoveries were calculated by comparison of the sample peak area and the area of the fortification solution directly diluted to the relevant volume. The concentration of the spiked samples was determined with the help of the calibration graph, and from these data the repeatability and intralaboratory reproducibility (different days) were calculated.

The mean recoveries of ETU from tomatoes, tomato juice, and tomato paste ranged between 74.2 and 86.7%, between 80.0 and 87.6%, and between 78.5 and 88.5%, respectively. Because recoveries were independent of the concentration level, there is no evidence of constant bias but rather of a proportional bias error corresponding to the partition efficiency. On the contrary, for all matrices the repeatability variance was found to be statistically different between concentration levels that differ by a factor of >5. However, for each concentration level, the variance between days did not differ significantly from the variance within days, showing that the method is under statistical control as can be seen from Table 4. In addition, the %RSD values of all levels were <10%, well below the corresponding values from the Horwitz equation (*37*).

(3) Detection and Quantitation Limits. The method limit of detection (LOD) was calculated from the standard deviation of the results of fortified samples at the lowest concentration level by multiplying with the value of Student's $t_{0.99,n-1}$. The calculated LODs were verified by the analysis of controls and control samples fortified at the appropriate level for each matrix and the observation of detectable peaks in the chromatograms at 3 times the noise level. The LODs of ETU for tomatoes, tomato juice, and tomato paste were found to be 0.002, 0.005, and 0.02 mg/kg, respectively.

The lowest concentration of the analyte in the sample that can be determined with acceptable precision and accuracy can be considered to be the limit of quantitation (LOQ) of the method. From the validation data presented in Tables 4 and 5 it can be observed that the LOQ for tomatoes and tomato juice is 0.01 mg/kg and for the tomato paste it is 0.05 mg/kg. The mean recovery values in these levels ranged between 80 and 88.5%, and the precision of the method expressed as relative standard deviation RSD_r ranged between 6.7 and 10.1. These values are acceptable according to U.S. EPA provisions (*38*). Representative chromatograms obtained from tomato and tomato paste samples fortified at the LOQ level are presented in Figures 7 and 5D, respectively. Consistent analyte identification, resulting in a better than 10% match between the UV spectra of the samples and the spectrum of a user-generated library, was achieved when sample concentrations were above the LOQs of the respective matrices.

(C) Conclusions. In the present study an HPLC-PDA method for tomatoes and tomato products was developed and validated. The focus was placed on the optimization of the cleanup step. It was demonstrated that the Extrelut column cleanup compared favorably to funnel l−l partitioning in terms of precision and selectivity. It was found that the reported addition of salt (KF) and the acidification of the aqueous extract prior to the Extrelut column cleanup were not compatible with the PDA confirmation of the analyte identity. Adequate conditions were as follows: pH adjustment of the sample extract to 7−9 without salt addition and elution through Extrelut column with 200 mL of dichloromethane. PDA purity and identity check confirmed that further purification of samples with a °Brix content <20° is not required, being able to minimize sample pretreatment and analysis time accordingly. For matrices exceeding the above-mentioned °Brix content, a further cleanup through a silica SPE cartridge was found to be efficient for the removal of interferences. The performance characteristics of the method proposed were satisfactory in terms of precision, accuracy, and detectability, and consequently the method is suitable for routine ETU monitoring in tomatoes and tomato products.

ABBREVIATIONS USED

ETU, ethylenethiourea; EBDCs, ethylene bis(dithiocarbamate)s; PDA, photodiode array (detector); l−l partitioning, liquid−liquid partitioning; RSD_r, repeatability relative standard deviation; RSD_R, reproducibility relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation.

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