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Optimization of a matrix solid-phase dispersion method with subsequent clean-up for the determination of ethylene bisdithiocarbamate residues in almond samples

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Abstract

A matrix solid-phase dispersion (MSPD) method with subsequent clean-up has been developed to isolate the ethylene bisdithiocarbamate (EBDC) main metabolites (ethylenethiourea, ETU, and ethylenebis [isothiocyanate] sulphide, EBIS) in almond samples. The optimized experimental set-up configuration involved 0.2 g of almond sample, washed sand as MSPD support and NaOH as defatting agent. A subsequent purification step on alumina using acetonitrile as extraction solvent was enough to remove all interferent matrix components, including the fatty material, and provide clean extracts. Quantitative analysis was performed by reversed phase liquid chromatography (RPLC) with diode-array ultraviolet absorbance (DAD UV) detector. Analytes recoveries were between 76 and 85% with relative standard deviations ranging from 3 to 12%. The low limits of quantification of 0.05 and 0.07 mg kg^{-1} achieved for ETU and EBIS, respectively, make the method useful for the determination of EBDC residues on almond samples.

Keywords: Food analysis; Almonds; Matrix solid-phase dispersion; Ethylene bisthiocarbamate

1. Introduction

Ethylene bisdithiocarbamates (EBDCs) are among the most widely used fungicides to control a broad spectrum of fungi and their associated plant diseases. Maneb belongs to this group of fungicides used for the treatment of almonds, stone fruits and a large range of crops [1]. Due to its low acute toxicity, combined with strong activate, low cost production and short environment persistence, the amount of maneb and other EBDCs used is increasing world-wide.

The EBDCs are noted for their instability in the environment [2]. They are generally unstable in the presence of moisture, oxygen and in biological systems [3]. Its main products for hydrolysis and photolysis are ethylenethiourea (ETU) and ethylenebis(isothiocyanate) sulphide (EBIS) and other minority degradation products such as ethyleneurea (EU), which is further degraded to CO_2 under aerobic conditions [4,5] and glycine. ETU and EBIS are considered to be the main responsible for the toxic effects of this pesticide group. ETU has been proved to have teratogenic carcinogenic, immunotoxic and mutagenic effects [6] and EBIS is perinatally toxic and causes peripheral paralysis and thyroid dysfunction [7]. Therefore, the development of simple and sensitive analytical methods for fast monitoring of EBDC residues in fruits and vegetables is of relevant interest.

The maximum residue limits (MRLs) established by The Codex Alimentarius for EBDCs and their metabolites expressed as carbon disulphide (CS_2) is 0.1 mg kg^{-1} [8] on almonds. Up to now, most of the methods for determining EBDCs are based on the quantification of CS_2 after acidic digestion of the sample. Carbon disulphide can be analyzed either by gas chromatography [9–12] or by absorption spectrophotometry [13,14]. Although this approach is used by authorities in Europe and USA to determine the presence of EBDCs and their metabolites in crops, they are insuffi-

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ciently specific to distinguish between residues of individual species.

The determination of ETU using gas chromatographic (GC) methods require derivatization of the analyte [15] and suffer from erratic recoveries and time consuming [16]. As a consequence, there is a clear tendency toward the application of liquid chromatography coupled with UV (LC–UV) [17–20], mass spectrometry (LC–MS) [21,22] or electrochemical detectors [23,24]. Previous experiments have demonstrated the suitability of coupling LC–UV for the simultaneous determination of ETU and EBIS in tomatoes. However, isolation of these analytes from almonds is a complicated and laborious task because of the fatty nature of the matrix. To the best of our knowledge, no reliable methods to determine individual EBDCs residues in almonds have been reported. Classical methods for the extraction of herbicide residues from almond samples are based on Soxhlet extraction and steam distillation, are usually time consuming (more than 12 h), involve relatively large amounts of solvents and often several subsequent treatments, like centrifugation, filtration and evaporation [25]. These highly manipulative methodologies have often lead to low recovery of the target compounds or poor reproducibility.

Matrix solid-phase dispersion (MSPD) combines both sample homogenization and a possible preliminary clean-up of the compounds of interest. The method involves the dispersion of the sample over a solid support, followed by a preliminary purification and the subsequent elution of the analytes with a relatively small volume of solvent. The obtained extracts are generally ready for analysis, although if necessary, they can easily been subjected to direct extra purification [26–28]. That is, MSPD efficiently contributes to simplify the extraction and clean-up steps, reduces sample manipulation and so speed up the total analytical process.

MSPD has been applied to the analysis of several residues of pesticides in fruit and vegetables [29–31]. However up to date, MSPD has not been used for the analysis of EBDC residues in almonds. This paper reports a new MSPD procedure for sequential selective extraction and sorption clean-up of ETU and EBIS from almond samples, followed by separation and quantification by LC–DAD UV detection. The different experimental parameters affecting the analytical process were optimized.

2. Materials and methods

2.1. Chemicals and materials

Solid stock standard of ETU was obtained from Sigma–Aldrich (Steinheim, Germany). Maneb was obtained from Riedel-de-Häen (Seelze, Germany). EBIS solution (no commercial standards of EBIS are available) was obtained from the degradation of maneb in a 1:1 deionized water–acetonitrile solution using an UV lamp (CN-6T Vilber Lourmat, France) at a wavelength of 312 nm. Sodium hydroxide (NaOH) was obtained from Merck (Darmstadt, Germany) and sodium sulphate anhydrous (Na_2SO_4) from Panreac (Barcelona, Spain). Different sorbents were tested for MSPD and clean-up of the extracts including alumina (Merck), Florisil (60–100 mesh), AnalytiCals Carlo Erba (Milan, Italy), silica gel (0.063–0.200 mm, 70–230 mesh), Fluka–Chemika (Milan, Italy), which were previously activated at 130 °C for 15–18 h, octadecyl-funcionalized silica gel (C-18), Aldrich (Madrid, Spain) and washed sea sand (0.25–0.30 mm), Panreac. Glass wool was purchased from Panreac. Acetonitrile and methanol supergradient LC grade were also from Sigma–Aldrich. All reagents used were of analytical grade or better. Deionized water was obtained using a Milli-Q water system (Millipore Ibérica, Madrid, Spain).

The glass solid-phase extraction (SPE) cartridges (100 mm × 9 mm i.d.) used for packing the dispersed sample and the sorbents used for further clean-up were from J.T. Baker (Deventer, The Netherlands).

Almonds were purchased from a local commercial food market in Madrid (Spain). After removing the hulls, a representative amount of the whole fruit plus peel (200 g), were homogenized using a food processor and mixed thoroughly.

2.2. Analytical procedures

2.2.1. Optimization of the clean-up step

Some preliminary experiments were carried out to optimize the clean-up step. In this part of the study different polar and non-polar bounded solid-phases such as florisil, silica gel, alumina and C-18 were tested. Acetonitrile, methanol and 1:1 (v/v) acetonitrile/methanol were assayed as eluents.

The selected sorbent, 1 g, was packed in the SPE cartridge plugged with a silanized glass wool. In all cases, the SPE column was washed with 4 ml of the solvent tested, which was drained until the meniscus reached the top of the sorbent. A volume of 500 μL of analytes aqueous solution was then applied to the conditioned column and eluted with a prefixed volume of the selected solvent. Several collected fractions of 4 mL were subsequently evaporated to dryness under a gentle stream of argon at room temperature. Finally, each separate residue was redissolved in 500 μl of 1:1 water/acetonitrile and analyzed by LC–DAD UV to construct the corresponding elution profile curves of the analytes from each sorbent–solvent combination.

2.2.2. Extraction procedure A

A portion of 0.4 g of the homogenized sample was placed into a glass mortar and gently blended with 0.4 g of C-18 and 1.2 g of sodium sulphate for 5 min using a pestle to obtain a homogeneous mixture. C-18 was used without any previous treatment.

The homogenized mixture was packed into an SPE column plugged with silanized glass wool and containing 1 g of either C-18 or alumina at its bottom part. Acetonitrile was used as extraction solvent in these experiments, in which,

after the whole column was wet by the solvent, the flow through the column was stopped for a 5 min static extraction. After this static extraction step, the organic solvent (4 mL in total) was allowed to elute from the SPE column dropwise by gravity. Two additional 5 min static extraction steps using 2 mL of solvent on each extraction were carried out. A teflon valve was used to obtain a constant flow rate of approximately 1 mL min^{-1}. The eluate was collected as three separate 2-mL fractions which were collected in graduated conical tubes (10 mL). A volume of 500 μL of methanol was then added to the eluents to act as a keeper and the mixture evaporated to dryness under a gentle stream of argon at room temperature. Finally, the residues were redissolved in 250 μL of 1:1 (v/v) water/acetonitrile and 20 μL were injected into the LC–DAD UV chromatographic system.

2.2.3. Extraction procedure B

After several experiments, in procedure B, a homogeneous mixture containing 0.2 g of sample and 0.55 g of sand was prepared into a glass mortar by gently blended for 5 min using a pestle. The sand was used without any previous treatment.

The homogenized mixture was packed into an SPE column plugged with silanized glass wool in its bottom part. The sample was firstly defatted with NaOH 0.02 M. This step was carried out by three consecutive 2 min static extractions with 1 mL of NaOH 0.02 M. Afterwards, additional 1 mL of NaOH (equivalent to the dead volume of the column) was added to the MSPD column before starting the extraction step. The hydrolyzed fat was allowed to elute dropwise by gravity flow. The teflon valve was used to obtain a constant flow of approximately 1 mL min^{-1}. The fatty extracts were discarded and this SPE column directly coupled to another one containing 1 g of alumina, plugged with a silanized glass–wool and previously conditioned with 4 mL of acetonitrile. A volume of 6 mL of acetonitrile was enough for complete sample preparation.

Further treatment and analysis of the eluted extract was identical to that of extract from procedure A. Otherwise specified, experiments were carried out in duplicate.

2.3. Preparations of standards and spiked samples/columns

Standard stock solutions of maneb (250 mg L^{-1}) were prepared by dissolving 12.5 mg of the fungicide in 50 mL of acetonitrile. Standard stock solutions of ETU (1000 mg L^{-1}) were prepared by dissolving 10 mg of each analyte in 10 mL of acetonitrile. The EBIS solution concentration (approximately 7.4 mg L^{-1}) was calculated as the difference between the original total amount of maneb minus the amounts calculated for the rest of the degradation compounds formed (ETU and EU) after UV degradation of a 25 mg L^{-1} maneb solution in 1:1 (v/v) water/acetonitrile for 36 h. All solutions were stored in amber glass bottles at −20 °C in the dark until use. Working solutions were prepared daily in ultrapure Milli-Q water by appropriate dilution. For recovery determinations, experiments were performed by spiking on the sample column head 100 μL of the corresponding diluted solutions containing ETU and EBIS at a concentration level of 0.20 and 0.25 mg kg^{-1}, respectively. The spiked columns were maintained at room temperature for 15 min before extraction to allow the solution to penetrate the homogenized test sample.

2.4. Instrumentation

Analyses were performed using an HP 1050 Series liquid chromatographic system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a quaternary pump, a column compartment, a vacuum degasser and a diode-array detector. The instrument control and data processing utilities included Hewlett-Packard CHEMSTATION software (Hewlett-Packard). The stainless analytical column used was packed with LiChrosorb RP-18 5 μm (25 cm × 9.6 mm i.d.) from Supelco (Bellefonte, PA, USA). Samples were manually injected through a sample injection valve (Rheodine Inc., model 7725; Hewlett-Packard) in which a 20 μL loop was mounted.

2.5. LC–DAD UV analysis

Analyte separation was performed by gradient elution at a flow rate of 1 mL min^{-1}. The initial conditions were 95% water solution plus 5% acetonitrile followed by a linear gradient to 30% aqueous solution, 70% ACN within 5 min, and a postrun time of 7 min. Quantitative measurements of the peak areas by LC–DAD UV were carried out at 232 nm.

3. Results and discussion

3.1. Optimization of the clean-up procedure

Experiments were carried out in order to optimize the clean-up step. The feasibility of different sorbents, i.e. Florisil, alumina, silica gel and C-18, for selective retention of the coextracted material was investigated. In all cases, acetonitrile, methanol and 1:1 (v/v) acetonitrile/methanol ranging from 4 to 6 mL were tested for quantitative extraction of the pesticide residues from the SPE cartridge.

The best recoveries of ETU and EBIS were obtained within the range of 95–105% using C-18 or alumina as sorbents and 4 mL of acetonitrile. Consequently, alumina and C-18 were selected for further validation of the method with the almond sample.

3.2. Optimization of the extraction procedure

3.2.1. Extraction procedure A

An analytical method for the determination of pesticide residues in a complex matrix, such as almonds, requires the

extraction of the residues from the matrix and the clean-up of the extracts before chromatographic determination. The goal of this part of the work was to disperse the sample over a large surface area sorbent to achieve a preliminary fat removal by retention on the solid-phase material. Theoretically, an appropriate solvent or solvent mixture should allow the elution of analyte free of matrix components [32].

Because of the fatty nature of almonds, C-18 was chosen as apolar sorbent to disperse the sample and for preliminary fat removal. As quoted above, alumina and C-18 were checked as clean-up sorbent. Acetonitrile, methanol and a 1:1 (v/v) acetonitrile/methanol mixture were tested for extraction of the MSPD column, which was prepared as described in Section 2.2. A total solvent volume of 8 mL was used in all experiments and the eluate from the columns was collected as three separated fractions of 2 mL. Rather similar results were obtained with the two clean-up sorbents tested, alumina and C-18. Among solvents tested, acetonitrile provided the cleanest extracts. However, none of the results were fully satisfactory. In all cases, the first two fractions collected contained large amount of fat as evidenced by the colour of the residue obtained after solvent evaporation. This fact prevented from LC analysis of the extracts, even after an additional clean-up step using C-18 or alumina, depending on the experiment. The analytes recoveries in the third fraction were between 25–33 and 5–12% for ETU and EBIS, respectively.

3.2.2. Extraction procedure B

The main analytical difficulty during almond samples pretreatment is fat removal (fat content 55–70%). In order to solve this problem, due to the limited capacity of fat retention of alumina and C-18, the sample was firstly deffated by alkaline hydrolysis with NaOH. The different experimental parameters affecting the efficiency of this step were optimized.

Untreated sand, an inert material, was chosen as solid support to disperse the sample in the MSPD column in this part of the study. Firstly, experiments were carried out using 0.4 g of almond and 1.1 g of sand as commented in Section 2.2. According to previous results, two subsequent clean-up approaches were tried, adsorption chromatography on either alumina or C-18. The target compounds were eluted from the upper SPE column with 6 mL of acetonitrile, which were dropped on top of the selected clean-up SPE column. This configuration allowed to minimize the total solvent consumption as the solvent extracting the pesticide residues from the upper SPE cartridge was also used for the elution of the analytes through the clean-up column. The consequent reduction of the time required for sample preparation (e.g. by eliminating the concentration step of the fatty extracts previous to its application to the clean-up column) and the minimization of the risk of contamination by reducing the sample manipulation were considered additional benefits of this configuration. Fig. 1B and C shows the DAD UV chromatogram of the extracts obtained from non-spiked almond samples after being cleaned up on alumina and C-18, respectively. The results showed that alumina was more efficient in removing interferences from almond sample than C-18. Some interference peaks were also found in the chromatograms at ETU retention time which could obscure the determination and quantification of this analyte when C-18 was used as clean-up sorbent. Consequently, a column containing alumina was preferred and used throughout method validation for clean-up, because it gave the best recovery averages of the compounds, the best repetitively and the cleanest extract.

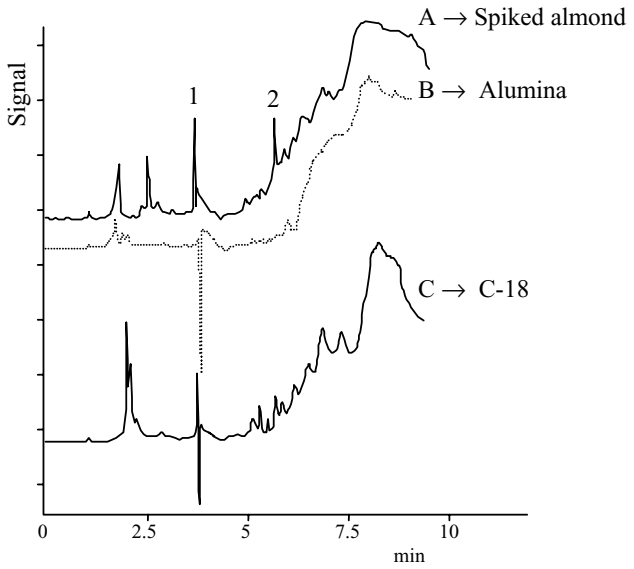


Fig. 1. Typical comparison of LC–DAD UV chromatograms obtained for a spiked (A); a non-spiked almond sample after extraction of the analytes plus clean-up using alumine (B); and C-18 (C). Peak identification: ETU (1); EBIS (2). Experiments were carried out using 0.2 g of almond sample and spiking at concentrations close to the LOQ.

The parameters affecting the MSPD process were then optimized. After several experiments, a sample/sand ratio of 1:3 (w/w) was concluded to provide the best flow solvent control with the proposed arrangement and, consequently, selected for further method validation. Under these experimental conditions, sample amount up to 0.4 g could be reproducibly packed and eluted. However, a sample amount of 0.2 g was found to provide clear analyte signals at a spiking level of 0.20 and 0.25 mg kg^{-1} of ETU and EBIS, respectively, while allowing a significant reduction of the volumes of solvents required for fat hydrolysis and quantitative extraction and clean-up of the target compounds. Thereby, a 0.2 g sample was selected to proceed with method development.

The efficiency of a dynamic alkaline hydrolysis for fat removal compared to that of a static hydrolysis, i.e. the hydrolysis mode, was evaluated. In this part of the study, the static time and the number of extraction cycles, as well as the concentration and volume of NaOH were optimized. All the extractions were performed at room temperature. Firstly,

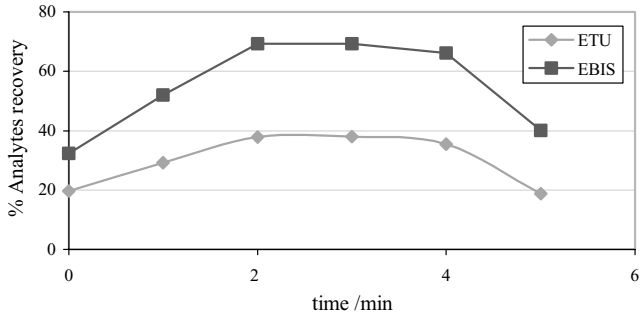


Fig. 2. Influence of the extraction time of the cycle in the analytes recovery using 6 mL of NaOH 0.01 M in five extraction cycles.

the influence of the static extraction time on the fat removal was investigated using 6 mL of NaOH 0.01 M through five extraction cycles (1 mL per cycle). Extraction times from 0 to 5 min (time 0, corresponding to the dynamic mode), were investigated. As can be seen in Fig. 2, the analytes recovery increased with the static extraction time up to 2 min which provided the highest recoveries. Static time longer than 2 min did not really improve the yield which decreased when times longer than 4 min were selected. Under these conditions a five cycles extraction provided a complete removal of fat. However, the saponification reaction could have broken down the analytes and extraction cycles of two minutes were run in further experiments. The feasibility of reducing the concentration of NaOH and the number of extraction cycles to prevent analytes degradation was then investigated. The influence of the NaOH concentration within the range 0.005–0.1 M in the extraction efficiency was also studied. The recovery of the analytes increased with the NaOH concentration up to 0.02 M and remained almost constant for higher concentrations for ETU but not for EBIS for which the recovery decreased from 80 to 50%. A concentration of 0.02 M was chosen as optimum and used in further experiments.

To determine the effect of the number of static extraction cycles, analytes were extracted varying this parameter from two to nine. The influence of the number of repeated extraction cycles on the recoveries is shown in Fig. 3. Running only two extraction cycles, analytes were extracted along the MSPD column with an important amount of fatty material that prevented from further LC–DAD UV analysis of the extracts. Increasing the number of static extraction cycles from more than three did not improve the extraction efficiency. Consequently, three 2-min extraction cycles corresponding to a total volume of 4 mL of NaOH 0.02 M was chosen as optimum.

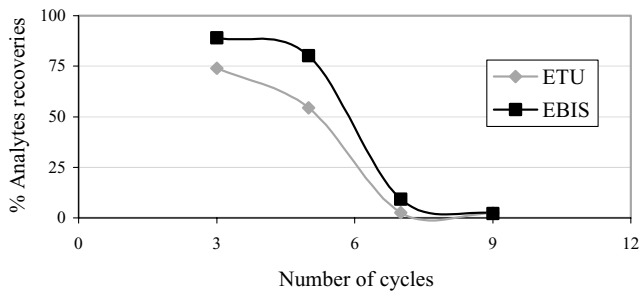


Fig. 3. Effect of the number of 2 min extraction cycles on the extraction yields of the analytes.

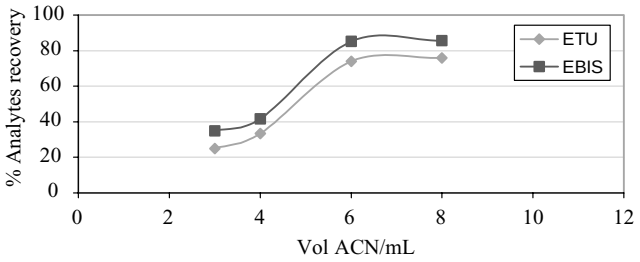


Fig. 4. Effect on the analyte recoveries of the extraction volume of acetonitrile used.

The analytes extraction from the sample and along the coupled SPE clean-up columns was optimized varying the volume of acetonitrile added to the column. Fig. 4 shows the extraction recoveries obtained using volumes of acetonitrile in the range 3–8 mL. As expected, recoveries tended to increase with the volume of solvent used. An acetonitrile volume of 6 mL was found to provide quantitative recoveries of both analytes, 76% for ETU and 85% for EBIS and considered as the optimum value.

3.3. Analytical performance and application

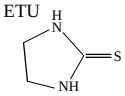
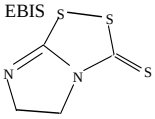
The *linearity* of the proposed method (sample preparation plus instrumental analysis) for ETU and EBIS was studied. Calibration curves were prepared by spiking the analytes on the head column sample in triplicate with increasing concentrations of the target compounds (which is in some cases the less favourable experiment [33]). This study was performed using concentration ranges of 0.012–0.300 mg kg^{-1} for ETU and 0.025–0.350 mg kg^{-1} for EBIS. The peak area values were plotted against concentrations. Linear regression analysis showed a good linear relationship for the analytes ($R^2 > 0.999$). Table 1 shows the analytical characteristics for both contaminants.

Recovery experiments were carried out at three spiked levels, between 0.16 and 0.36 mg kg^{-1} and reliable data were obtained in all instances (Table 1).

Repeatability was evaluated by spiking three samples at concentrations of 0.16 and 0.18 mg kg^{-1} of ETU and EBIS, respectively. The results expressed as relative standard deviation (R.S.D.) were between 3 and 10%. Reproducibility between days ($n = 3$) was also evaluated at the same concentration as for the repeatability analysis. Results were between 5 and 11%.

The quantification limits of the method (LOQs) were calculated as the lowest concentration where the R.S.D. is less than 5%. Taking into account the preparation of samples, LOQs were set at 0.05 and 0.07 mg kg^{-1} for ETU and EBIS,

Table 1
Analytical characteristics obtained by the proposed MSPD method for ETU and EBIS

Analyte	Concentration ($mg\,kg^{-1}$)	Percent recovery	R.S.D.	Concentration range ($mg\,kg^{-1}$)	R^2	Regression equation	LOQ ($mg\,kg^{-1}$)
ETU 	0.16	74.0	10.3	0.012–0.300	0.9997	$y = 0.1872x + 1.1825$	0.05
	0.20	76.9	4.7				
	0.32	75.9	2.7				
EBIS 	0.18	77.9	8.9	0.025–0.350	0.9992	$y = 0.1365x - 0.0129$	0.07
	0.25	86.7	4.6				
	0.36	89.5	3.6				

respectively, in almonds. These LOQ values are far below the (calculated) equivalent MRLs for ETU and EBIS, 0.27 and $0.15\,mg\,kg^{-1}$, respectively, corresponding to the legislated MRLs for total EBDCs residues in almonds as mg CS_2 kg^{-1}. The LOQ values obtained are comparable to those published for ETU in various plant tissues [20,34], making the method suitable for routine analysis. From a critical point of view, the proposed method represents a valuable alternative to more laborious methods based on MSPD or SPE, which involves larger amounts of sample and organic solvents and reports lower recoveries for the test compounds [18,22]. As an illustration, Fig. 1 shows a typical chromatogram of almond sample spiked at a concentration level close to the LOQs.

4. Conclusions

A novel method for the determination of ethylene bisdithiocarbamate residues, ETU and EBIS, in almond samples has been developed. The combination of the deffating of the almonds dispersed on sand followed by the clean-up of the extracts on alumina allowed a successful extraction and purification of ETU and EBIS to yield extracts ready for HPLC–DAD UV determination and quantification. Reliable recovery data (mean values 76 and 85% for ETU and EBIS, respectively, with R.S.D. better than 10%) were found at various concentrations after spiking samples and appropriate LOQs were attained. The columns arrangement proposed allowed to minimize the total solvent consumption as the solvent extracting the analytes from the MSPD column was also used for the elution of the analytes through the clean-up column. Complete sample preparation was achieved using only 4 mL of NaOH 0.02 M and 6 mL of acetonitrile. Additional advantages of the configuration proposed were the reduction of the total time required for sample preparation (e.g. by eliminating the concentration step of the extracted analytes after purification) and the minimization of the contamination risk by reducing the sample manipulation. The presented MSPD-based method provides a rapid alternative to conventional methods (total sample preparation can be done in about 20 min and up to six samples can be simultaneously processed), which makes it to be considered a promising approach for the determination of ETU and EBIS in almonds at ppb level.

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