Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 838 (2006) 96–106
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

LC–ESI-MS/MS determination of phenylurea and triazine herbicides and their dealkylated degradation products in oysters

E. Bichon *, M. Dupuis, B. Le Bizec, F. André

LABERCA—LABoratoire d'Etude des Résidus et Contaminants dans les Aliments, Ecole Nationale Vétérinaire de Nantes, route de Gachet, BP 50707, 44307 Nantes cedex 3, France

Received 3 February 2006; accepted 14 April 2006
Available online 24 May 2006

Abstract

A method was developed for the determination of several phenylurea and triazine herbicides and their transformation products in oysters at the low μg/kg level. Pressurised liquid extraction (PLE) of lyophilisated samples had required successive SPE combined with a liquid/liquid extraction to provide relatively clean extracts for the determination in LC–MS/MS. This procedure was validated according to the 2002/657/EC analytical decision. Efficiency of the analytical method led to confirmatory CCα values ranging from 0.1 to 14 μg/kg with an R.S.D. value ranging from 14% to 66% and a recovery yield ranging from 32% to 46% for phenylureas and from 29% to 75% for triazines.

Keywords: Liquid chromatography; Electrospray ionization; Tandem mass spectrometry; Herbicides; Phenylurea; Triazine; Transformation products; Oysters; Validation

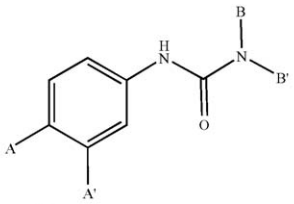
1. Introduction

Phenylurea (PU) and triazine (TZ) compounds are widely used in several European countries as selective or non-selective herbicides to control weeds on crops, railways and gardens. They can potentially contaminate all the non-target environment especially aquatic organisms, such as shellfish [1,2]. Indeed, bivalves are directly exposed and can be used as bio-indicator organisms in order to evaluate environment contamination. Such studies have been yet initiated on polycyclic aromatic hydrocarbon (PAH), polychlorobiphenyls (PCB) or organochlorated compounds (OC) [3]. The current evaluation of the existing method dedicated to phenylurea and triazine shows a lack of efficient validated method especially in edible tissues [4]. In order to assess potential presence of PU and TZ residues in oysters, it appears necessary to develop a sensitive and specific analytical method. Because parent compounds are widely metabolised, the monitoring of degradation such as dealkylated and demethoxylated is more fit for purpose to evaluate triazine and phenylurea contamination [5–8]. The molecules studied here are chlortoluron, diuron, isoproturon, linuron, 1-(3-chloro-4-methylphenyl)urea, 1-(3,4-dichlorophenyl)-3-methylurea, 1-(3,4-dichlorophenyl)-urea, 1-(4-isopropylphenyl)-3-methylurea, 1-(4-isopropylphenyl)-urea, atrazine, simazine, terbuthylazine desethylatrazine, desisopropylatrazine and desethylterbuthylazine (cf. Fig. 1).

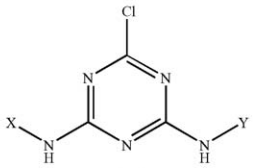
Crouch and Barker [3] have studied supercritical fluid extraction (SFE) and matrix solid-phase extraction (MSPD) preparation. The first technique is very efficient because of its selectivity, but conditions must be adapted for each analyte and therefore the technique cannot be applied to a wide range of residues. The second technique is relevant for tissue analysis, such as beef fat, catfish muscle or oysters. Matrices are blended with C18 or Florisil phases before analyte elution with an adequate solvent. The major drawback of this procedure is the manual preparation which complicates the routine application. Nowadays, new extraction techniques like microwave-assisted solvent extraction (MASE) [9,10] and pressurised liquid extraction (PLE) [12–14] are used for the contaminant residue control. Due to their thermolability, PU would be extracted at

* Corresponding author. Tel.: +33 240687880; fax: +33 240687878.
E-mail address: laberca@vet-nantes.fr (E. Bichon).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.04.019



Phenylurea



Triazine

Group	Name	A	A'	B	B'	X	Y
Phenylurea	Chlortoluron	CH_3	Cl	CH_3	CH_3		
	1-(3-chloro-4-methylphenyl)-3-methylurea	CH_3	Cl	CH_3	H		
	1-(3-chloro-4-methylphenyl)-urea	CH_3	Cl	H	H		
	Diuron	Cl	Cl	CH_3	CH_3		
	1-(3-4-dichlorophenyl)-3-methylurea	Cl	Cl	CH_3	H		
	1-(3-4-dichlorophenyl)-urea	Cl	Cl	H	H		
	Linuron	Cl	Cl	CH_3	OCH_3		
	Isoproturon	$(CH_3)_2CH$	H	CH_3	CH_3		
	1-(4-isopropylphenyl)-3-methylurea	$(CH_3)_2CH$	H	CH_3	H		
	1-(4-isopropylphenyl)-urea	$(CH_3)_2CH$	H	H	H		
Triazine	Desisopropylatrazine					CH_3CH_2	H
	Desethylatrazine					H	$(CH_3)_2CH$
	Simazine					CH_3CH_2	CH_3CH_2
	Desethylterbuthylazine					H	$(CH_3)_3C$
	Atrazine					CH_3CH_2	$(CH_3)_2CH$
	Terbuthylazine					CH_3CH_2	$(CH_3)_3C$

Fig. 1. Main molecules belonging to phenylurea and triazine groups.

relatively low temperature. Therefore, we have chosen to use PLE at relatively low temperature. Moreover, this technique uses low solvent volume during automatic and fast extraction (generally below 30 min). Therefore, the main drawback remains the wide range of co-extracted compounds, this lack of specificity leading to more purification steps. In this work, we chose to adapt this technology for our compounds of interest.

Compared to GC–MS [15,16], LC–MS/MS measurement is probably the most adapted technique to PU and TZ analysis because of their thermolability and polarity. In studies of interest, detection of PU and TZ was performed by HPLC coupled with different detectors [5,11,17–20]. The LC–MS/MS instrument was chosen for its specificity. Therefore, liquid chromatography coupled to tandem mass spectrometry, despite conventional beliefs, requires relevant sample purification for a repeatable specific measurement (for instance, to avoid signal extinction in electrospray ionisation). Berger [18] described a purification method with two successive HPLC steps with octadecyl bond silica stationary phases to purify soil extracts. The more specific purification method is probably the immunoaffinity chromatography [21,22]. Therefore, automatisation is difficult, cross contamination is sometimes observed and the cost is relatively high.

In this context, we studied various approaches regarding detection, purification and extraction. This document describes the different investigations leading to the final method as well as the determination of the method performance according to the 2002/657/EC decision (implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results) [23]. Finally, the results of an inter-comparison study between three laboratories will be presented.

2. Experimental

2.1. Reagents and chemicals

Pestipur Quality Solvents were purchased from Solvents Documentation Synthesis (SDS, Peypin, France), the other reagents were of analytical grade quality and were provided by Merck (Darmstadt, Germany). Solid-phase extraction (SPE) columns were delivered by Interchim for silica/cyanopropyl bonded silica (1:0.5, w/w; 6 mL) (Montluçon, France), by Waters for OASIS MCX (150 mg, 6 mL) (Milford, MA, USA) and by Supelco for octadecyl bonded silica (1 g, 6 mL) (St. Quentin Fallavier, France). Fluorometholone (external standard) and 4-bromoacetanilide (internal standard) were from Sigma–Aldrich (l'Isle d'Abeau Chesnes, France). Phenylurea and triazine standards such as chlortoluron, 1-(3-chloro-4-methylphenyl)urea, diuron, 1-(3,4-dichlorophenyl)-3-methylurea, 1-(3,4-dichlorophenyl)urea, isoproturon, 1-(4-isopropylphenyl)-3-methylurea, 1-(4-isopropylphenyl)urea, linuron, atrazine, desethylatrazine, desisopropylatrazine, simazine, desethylterbuthylazine and terbuthylazine were purchased from Promochem (France) and 1-(3-chloro-4methylphenyl)urea was provided by Makhteshim-Agan (Issy-les-Moulineaux, France). Standard solutions were prepared in Pestipur® grade methanol and were stored below −16 °C.

2.2. Biological samples

The oysters used as blank samples were collected far from the coast to avoid any pesticide contamination linked to an herbicide treatment. After collection, the tissues were pooled before freezing below −16 °C. These samples have been used as control

samples (blank and surrogate). For the validation, the oysters were bought on the market and came from 10 different sites to be representative of the various influences of the environment.

2.3. Material

The extraction was performed using an automatic pressure liquid extractor (ASE 300, Dionex, Sunnyvale, USA).

An alliance 2690 HPLC pump with automatic injector was used (Waters, Milford, MA, USA). A reversed-phase liquid chromatography was performed on octadecyl bonded silica Uptispher ODB stationary phase (50 mm × 2 mm, 3 μm) (Interchim, Montluçon, France) with a guard column (Uptispher ODB, 10 mm × 2 mm, 3 μm). Elution solvents were acetonitrile (A) and acetic acid in water (0.5:99.5, v/v) (B). The mobile phase composition (A:B, v/v) was 10:90 (2 min), 50:50 (8–15 min) and 100:0 at 25 min. The gradient was linear and flow rate was set at 0.2 mL/min. The injected volume was 10 μL.

The data were acquired in the positive electrospray mode using a QuattroLC® triple quadrupole analyser (Micromass, Manchester, UK). Nitrogen was used as nebulisation and desolvatation gas, at 90 and 600 L/h flow rates, respectively. The transitions were optimized in direct introduction in order to determine all parameters of capillary, cone and collision voltages. The potential applied onto the capillary was 4 kV. The cone potential was optimised for each molecule (15–40 V). In the collision cell, argon was used as collision gas at $4.5.10^{-4}$ mbar; collision energy varied from 15 to 40 V. For each molecule (M), acquired transitions included the $[M+H]^+$ as precursor ion and two fragments as product ions (Table 1).

The detection optimisation was carried out on GC–MS. It was composed of a gas chromatograph with a capillary column (OV1-Agilent) (length = 30 m, internal diameter = 0.25 mm, film thickness = 0.25 μm), a split/splitless injector set at 250 °C, a programmable temperature oven started at 70 °C (2 min) up to 300 °C (5 min) at 15 °C/min (Agilent-6890) and an automatic injector. The mass spectrometer was a quadrupole low resolution analyser (Agilent-MSD 5973) allowing electronic impact ionisation.

Table 1
List of screened analytes, internal and external standards (acquired transitions, ESI and MS/MS conditions and LC retention time)

Group	Name	Parent ion	Daughter ion	Cône (v)	Collision (v)	Retention time (min)
Phenylureas	Chlortoluron	213	46	35	20	10.35
			72	35	20	
	1-(3-Chloro-4-methylphenyl)-3-methylurea	199	142	30	20	9.92
			107	30	35	
	1-(3-Chloro-4-methylphenyl)-urea	185	141	35	15	9.32
			107	35	25	
	Diuron	233	46	35	20	10.90
			72	35	25	
	1-(3-4-Dichlorophenyl)-3-methylurea	219	162	35	20	10.41
			127	35	35	
	1-(3-4-Dichlorophenyl)-urea	205	167	35	20	9.81
			126	35	35	
	Linuron	249	181	30	20	12.48
			160	30	20	
	Isoproturon	207	46	35	25	10.74
			72	35	25	
	1-(4-Isopropylphenyl)-3-methylurea	193	151	35	15	10.24
			94	35	25	
	1-(4-Isopropylphenyl)-urea	179	137	35	15	9.76
			94	35	30	
Triazines	Desisopropylatrazine	174	131	40	20	3.69
			96	40	20	
	Desethylatrazine	188	104	40	25	6.94
			146	40	20	
	Simazine	202	124	40	20	9.16
			132	40	20	
	Desethylterbuthylazine	202	104	30	30	9.65
			146	30	15	
	Atrazine	216	96	40	25	10.52
			174	40	20	
	Terbuthylazine	230	96	40	30	12.21
			174	40	15	
External standard	Fluorometholone	377	339	15	40	10.79
			278	20	40	
Internal standard	4-Bromoacetanilide	216	174	35	15	9.59
			93	35	30	

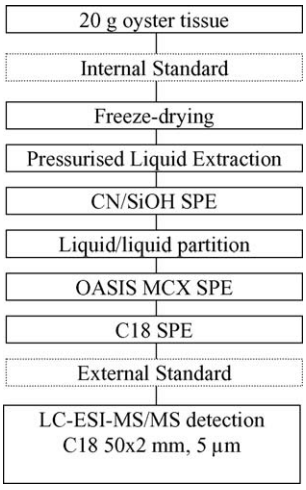


Fig. 2. General analytical procedure (SPE: solid-phase extraction).

2.4. Sample preparation (cf. Fig. 2)

The oyster sample (20 g) was freeze-dried, ground to a powder and transferred into a 34 mL ASE cell. Dispersing materials such as Fontainebleau sediment and Celite were used to fill cells. The extraction program consisted in three successive extractions with methylene chloride and acetone (50:50, v/v) (5 min of each). The temperature and the pressure were set at 60 °C and 100 bar, respectively. The extract was transferred into a flask and evaporated to dryness below 40 °C with a rotary evaporator. The dry residues were finally dissolved in 2 mL cyclohexane.

2.5. Herbicide residues clean-up (cf. Fig. 2)

The extract dissolved in cyclohexane was passed through an SPE CN/SiOH cartridge previously conditioned with 20 mL cyclohexane (until translucent phases were obtained). After elimination of interfering molecules with 6 mL cyclohexane/diethylether (80:20, v/v), the analytes were eluted with 6 mL ethyl acetate and 6 mL ethyl acetate/methanol (80:20, v/v). The solvent was evaporated to dryness under nitrogen stream below 40 °C. The extract was reconstituted in 3 mL methanol and 1 mL water. The sample was then washed twice with 2 mL hexane to discard fatty co-extracted molecules. Methanol in the aqueous phase was evaporated under nitrogen stream below 40 °C. The extract was reconstituted in 2 mL water and applied onto an OASIS MCX cartridge previously conditioned with 3 mL methanol followed by 3 mL water. The interferences were discarded with 3 mL water, 3 mL water/methanol/NH_4OH (78:20:2, v/v/v) and 3 mL sodium acetate buffer (2 M, pH 5.2). Analytes were eluted with 3 mL methanol followed by 3 mL methanol/NH_4OH (98:2, v/v). Methanol was evaporated under nitrogen stream below 40 °C and sample was reconstituted in 2 mL water. The last purification was performed on an octadecyl bonded silica SPE column to eliminate residual salts which can interfere during the further electrospray ionisation. After conditioning of the cartridge with 6 mL methanol followed by 6 mL water, the extract was applied. Cartridges were rinsed with 6 mL water and 6 mL water/methanol (80:20, v/v) before target analyte elution with 6 mL water/methanol (20:80, v/v). The extract was then evaporated (until 100 μL approximately) under nitrogen stream below 60 °C; 500 ng of external standard was then added in 150 μL of methanol/water (30:70).

2.6. Method validation

In order to control all the purification steps, an internal standard was added at the beginning of the sample preparation. The molecule chosen must have the same physicochemical properties as the analytes. An external standard was also used in each sample to control the LC–MS/MS performances.

The evaluation of the performances of the previously described analytical method was done according to the 2002/657/EC decision. This decision provides rules for the analytical methods to be used in the testing of official samples and specifies common criteria for the interpretation of analytical results of official control laboratories for such samples. In this way, a calibration curve, 10 blank samples and the same 10 spiked samples were analysed with the protocol described above. Specificity, sensitivity, accuracy (precision and trueness) and recovery were evaluated (cf. Table 2).

2.6.1. Decision limit CCα

The decision limit is the smallest result of measure that permits to decide if the analyte is present in the sample, with a risk of error equal to α ($\alpha = 1\%$). CCα is calculated with the following expression:

$$\mathrm{CC}\alpha = \frac{I_{\mathrm{CC}\alpha} - b}{a} = \frac{\mu_B - b + 2.33\sigma_B}{a}$$

Table 2
Validation procedure

Nature of samples	Number of samples	Objectives	Requirements
Different blank samples	10		False negative results avoided
Blank samples fortified at 0, 1, 3, 6 and 9 time the detection limit	1	Detection, identification	Highest specificity False positive results avoided
Blank samples fortified at the theoretical CCβ	10		Repeatability > 80 % Recovery known
Blank samples fortified at the 0;0.5; 0.75; 1; 1.25; 1.5; and 2 time the theoretical CCβ	1	Quantification	Identification > 90 % Trueness > 80% Linearity $R^2 > 0.98$

where μ_B and σ_B represent the mean-value and the standard deviation of the relative noise intensity (compared to the internal standard intensity) at the analyte retention time on the 10 batches of oysters. Values a and b represent the slope and the intercept, respectively, of the calibration curve achieved from spiked samples. These samples were prepared from a pool of the 10 selected batches of oysters in order to be representative of a maximum of interferences.

2.6.2. Detection capacity $\sigma_{CC\beta}$

Ten batches of oysters were supplied with a quantity permitting to approach the theoretical CCβ value for every analyte. This value has been predetermined on the most abundant transition in previous experiments for a signal to noise ratio included in-between 10 and 30 for each analyte. The spiking was realized in methanol before the freeze-drying step.

The detection capacity CCβ is the smallest result of measure that permits to identify and to quantify the analyte in the sample, with a risk of error equal to β ($\beta = 5\%$). CCβ is calculated with the following expression:

$$CC\beta = \frac{I_{CC\beta} - b}{a} = \frac{\mu_B - b + 2.33\sigma_B + 1.64\sigma_{CC\beta}}{a}$$

where $\sigma_{CC\beta}$ represent the mean-value and the standard deviation of the signal of the analyte in the 10 batches of oysters. Values a and b represent the slope and the intercept, respectively, of the calibration curve achieved from spiked samples.

The ruggedness of the method has been evaluated through five samples spiked between 10 and 50 $\mu g\,kg^{-1}$. Three laboratories were included in this interlaboratory study.

3. Results and discussion

3.1. Mass spectrometry measurement

GC–MS was firstly used to measure phenylurea residues, but because of their thermolability, isocyanate degradation products were observed (cf. Figs. 3 and 4). Isocyanates are specific of the aniline fragment and cannot be used to differentiate several substances with the same substituted aniline such as linuron, diuron and their demethylated and demethoxylated metabolites. Similarly, aniline isocyanates are non-specific and can be generated by other interfering compounds. Moreover, the transformation of phenylurea into isocyanate compound is incomplete and may cause a bias for quantification. For these reasons GC–MS analysis appears clearly less adapted than LC–MS analysis. A new method was also developed. Positive electrospray mode was chosen because of the efficient and sensitive ionisation of phenylureas and triazines. Elution of analytes on octadecyl bonded silica column was found optimal with an acetonitrile/water gradient (cf. Fig. 5). Methanol was compared to acetonitrile in the mobile phase but less polar triazine such as terbuthylazine and atrazine were not removed from the column, even after rinsing it with 100% methanol. Injection with acetonitrile decreases this phenomenon but precautions have to be taken in-between two injections when highly contaminated samples are analysed (cf. Fig. 5).

3.2. Sample preparation

In the documentation available, phenylurea and triazine residues are generally extracted from sediments after centrifugation with methanol [7,13]. This technique was compared with Folch, Soxhlet or PLE in terms of extraction efficiency and time consumption [24]. At first, Soxhlet was tested with two solvents: dichloromethane and a mixture of dichloromethane/acetone (50:50, v/v) and compared with methanol extraction and Folch extraction with water/methanol/chloroform (1:4:8, v/v/v). The best recoveries were obtained with soxhlet, using a dichloromethane/acetone mixture. The second step consisted in automatising this step and comparing soxhlet with PLE in the same conditions ($T°$ fixed at 60 °C to avoid phenylurea degradation). Pressure set at 100 bar allowed extract solvent to be kept liquid. The results were similar after three consecutive cycles of PLE. Pressure liquid extraction has been chosen because the extraction time was very short (30 min per sample), the recovery yield of this step was almost 100% and solvent volumes used were limited. Results are presented in Table 3. Best recoveries were obtained with three consecutive extraction cycles of 5 min.

3.3. Sample purification

The behaviour of 10 phenylurea compounds was studied on six different stationary phases qualified as “normal”: diol, acidic and basic alumina, cyanopropyl/silica double phase, silica and florisil. All profile elutions are represented in Fig. 6. The best profile was obtained with the CN/SiOH column, which

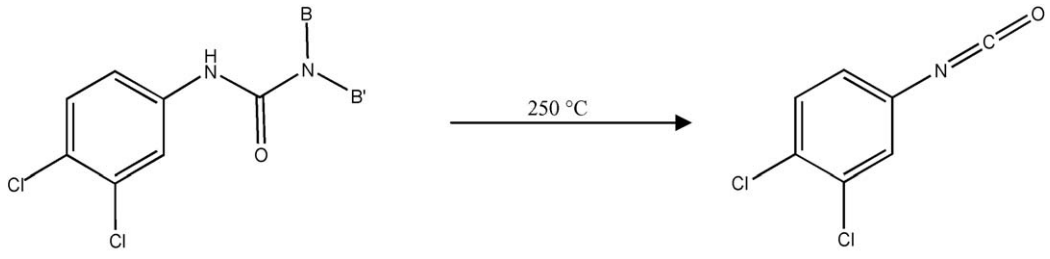


Fig. 3. Pitfalls in gas chromatograph injector: isocyanate observation for phenylurea molecules. (B and B′ correspond to different substituents presented in Fig. 1.)

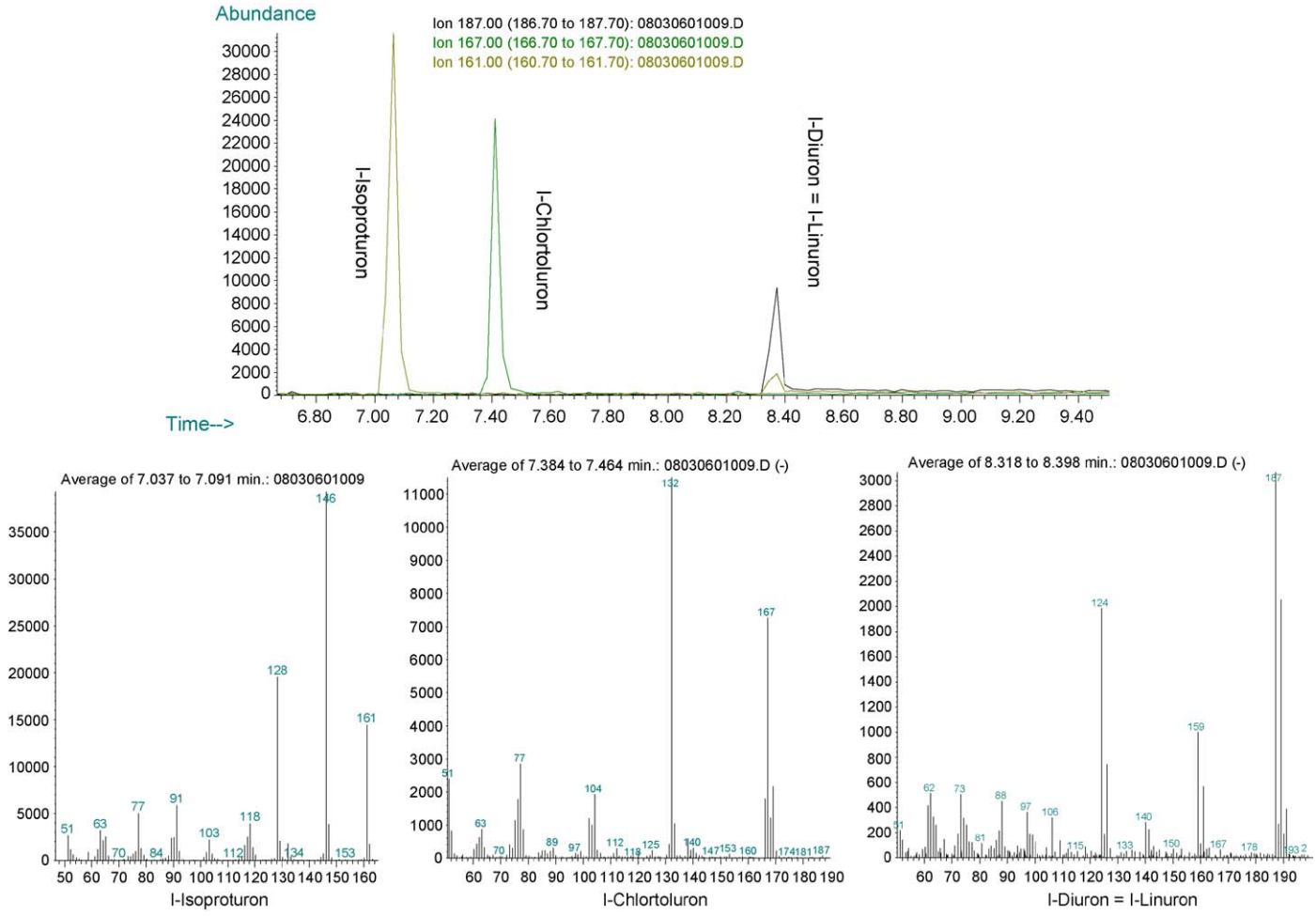


Fig. 4. Isocyanate side products of phenylurea herbicides after injection in the splitless mode at 250 °C (I-Isoproturon: 1-(4-isopropylphenyl)-isocyanate; I-Chlortoluron: 1-(3-chloro-4-methylphenyl)-isocyanate; I-Linuron = I-Diuron: 1-(3,4-dichlorophenyl)-isocyanate).

allowed for an efficient rinsing of the stationary phase, eliminating lipophilic molecules. Triazine residues, because of their higher polarity, needed a more eluotropic mobile phase (ethyl acetate/methanol; 80:20, v/v) to be eluted.

To improve the purification (elimination of polar interferences) six reversed stationary phases have been compared (cf. Fig. 7). The best profile was obtained with C18 cartridge, which permitted to eliminate most interferences with methanol/water mixture before analyte elution.

Because of their nitrogen atoms, phenylurea and triazine residues could be retained on cation exchange columns such as OASIS MCX (Waters). This specific phase was composed of an HLB (Hydrophilic Lipophilic Balance) polymer on which a controlled sulfonation was carried out in order to insert cation exchange groups. All residues of TZ and PU could be retained both by the HLB polymer and the exchange ion sites. After sample loading onto the cartridges, water was used to discard polar anionic molecules. Then, one washing step involving 3 mL $H_2O/CH_3OH/NH_4OH$ (78:20:2) permitted elimination of cationic interferences. Application of a 3 mL acetate buffer (2 M) broke ionic affinity and analytes were finally eluted with 3 mL of $H_2O/CH_3OH/NH_4OH$ (18:80:2).

When the purification step with the three successive cartridges described above (CN/SiOH, MCX and C18 consecutive cartridges) was tested, complexity of extracts led to a saturation of cation exchange cartridge. This phenomenon was the

Table 3
Comparative recovery observed with various extraction techniques

Extraction	Solvent used	Mean recovery (%)	Time of extraction
Folch	Water/methanol/chloroform, 1:4:8	4.5	2 h
Centrifugation	Methanol	8.6	2 h
Soxhlet	Dichloromethane	8.1	6 h
Soxhlet	Dichloromethane/acetone, 50:50	11.4	6 h
PLE	Dichloromethane/acetone, 50:50	35.3	30 min

Mean values are obtained on 10 different molecules. Each recovery yield includes the respective extraction and the same basic and non-optimised purification.

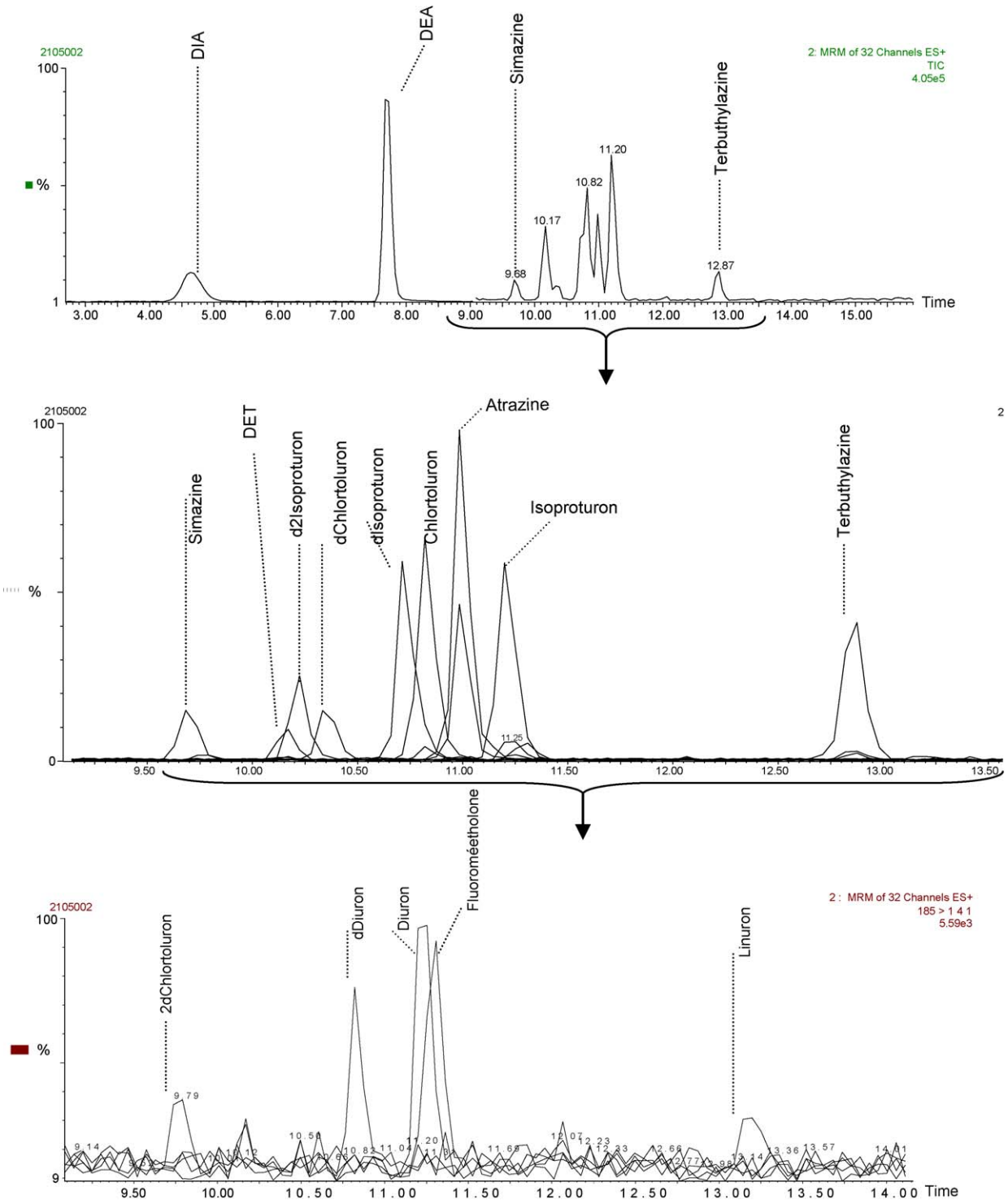


Fig. 5. Specificity and sensitivity of LC–MS/MS detection in the MRM mode (20 ng of each molecule were injected) (MRM: multiple reaction monitoring; DEA: desethylatrazine; DIA: desisopropylatrazine; DET: desethylterbuthylazine; dDiuron: 1-(3-4-dichlorophenyl)-3-methylurea; dChlortoluron: 1-(3-chloro-4-methylphenyl)-3-methylurea; 2dChlortoluron: 1-(3-chloro-4-methylphenyl)-urea; dIsoproturon: 1-(4-isopropylphenyl)-3-methylurea; d2Isoproturon: 1-(4-isopropylphenyl)-urea).

result of a bad dissolution of dry extract in water. The most lipophilic molecules must be avoided. A liquid/liquid partition with hexane had also been carried out. Therefore, to keep the analyte in aqueous phase, an addition of methanol was required.

This protocol led to a mean recovery of 30% with the worse recoveries obtained for terbutylazine and 1-(3,4-dichlorophenyl)urea. Both analytes represent respectively the most apolar and the most polar of the compounds monitored.

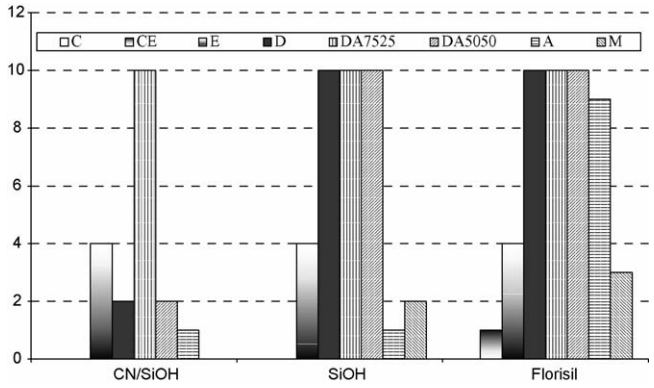


Fig. 6. Elution profile of phenylurea depending on stationary and mobile phases (C: cyclohexane; CE: cyclohexane/ether (80:20); E: ether; D: dichloromethane; DA7525: dichloromethane/ethyl acetate (75:25); DA5050: dichloromethane/ethyl acetate (50:50); A: ethyl acetate; M: methanol).

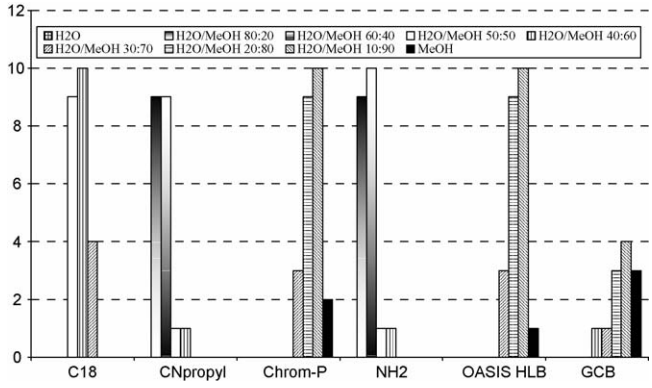


Fig. 7. Number of eluted molecules (total of 10) depending on the elution solvent.

3.4. Validation

3.4.1. Specificity

The analysis of 10 blank samples gave the guarantee of the good specificity of the method, since no interferences could be detected at the retention time of the analytes (cf. Fig. 8). However, a recurrent interference (batches 1, 4, 5, 6, 8, 9 and 10) appeared on a simazine transition (200 > 132).

3.4.2. Decision limit and detection capacity

CCα were included between 0.1 and 14.4 μg kg^{-1} (cf. Table 4 and Fig. 8). CCβ were included between 0.2 and 31.5 μg kg^{-1} (cf. Table 4 and Fig. 8) (CCβ spiked value available in Table 4). In terms of sensitivity, the identification limit of the methodology described above was fixed at around 3 μg kg^{-1} because all the CCα values were below this concentration (except for 1-(4-isopropylphenyl)-urea).

3.4.3. Accuracy

3.4.3.1. Repeatability. The repeatability of the ratio analyte/internal standard characterizes the adequacy of the internal standard with the target molecules.

The ratio repeatability was found to be 29% ($n = 10$) (Table 5). Four substances were found not to be in adequacy with the internal standard: *N*,*N*-bisdemethylchlortoluron, *N*,*N*-bisdemethyldiuron, *N*,*N*-bisdemethylisoproturon and terbutylazine. These substances correspond to the more polar phenylurea residues and the less polar triazine residue. This result permits to conclude that the internal standard (bromoacetanilide) has guaranteed the efficient control of the analytic process for the sixteen molecules, in spite of bad mimesis for four of them.

3.4.3.2. Trueness. The error of trueness was calculated on 10 different spiked samples (cf. Table 5). Average trueness error value was found to be 26%. Four substances were significantly far from this value, *N*,*N*-bisdemethylchlortoluron, *N*,*N*-bisdemethyldiuron, simazine and terbutylazine.

Table 4
Determination of the decision limit CCα and the determination capacity CCβ (ND: not determined)

Molecules	CCα (μg/kg) for the more sensitive transition	CCα (μg/kg) for the less sensitive transition	Levels of spiking	CCβ (μg/kg) for the more sensitive transition	CCβ (μg/kg) for the less sensitive transition
Chlortoluron	0.1	0.1	0.5	0.3	0.4
1-(3-Chloro-4-methylphenyl)-3-methylurea	0.6	0.4	1.0	0.9	0.7
1-(3-Chloro-4-methylphenyl)-urea	1.0	2.8	10.0	7.0	14.5
Isoproturon	0.5	0.8	1.2	0.8	1.0
1-(4-Isopropylphenyl)-3-methylurea	0.9	1.8	3.0	1.8	3.0
1-(4-Isopropylphenyl)-urea	4.8	14.4	20.0	21.6	25.9
Diuron	1.9	0.7	6.0	4.1	3.6
1-(3-4-Dichlorophenyl)-3-methylurea	ND	0.1	0.5	0.2	0.3
1-(3-4-Dichlorophenyl)-urea	0.2	0.2	0.5	0.3	0.4
Linuron	3.9	1.4	2.5	4.9	2.7
Desisopropylatrazine	0.2	0.3	1.6	0.9	1.1
Desethylatrazine	0.1	0.2	0.7	0.4	0.6
Simazine	1.3	ND	1.7	9.1	0.9
Atrazine	0.8	0.5	4.0	2.3	2.1
Desethylterbutylazine	0.1	0.3	3.0	1.5	1.6
Terbutylazine	3.9	5.5	30.0	31.5	30.4
Mean value (μg/kg)	1.3	1.8		5.5	5.6

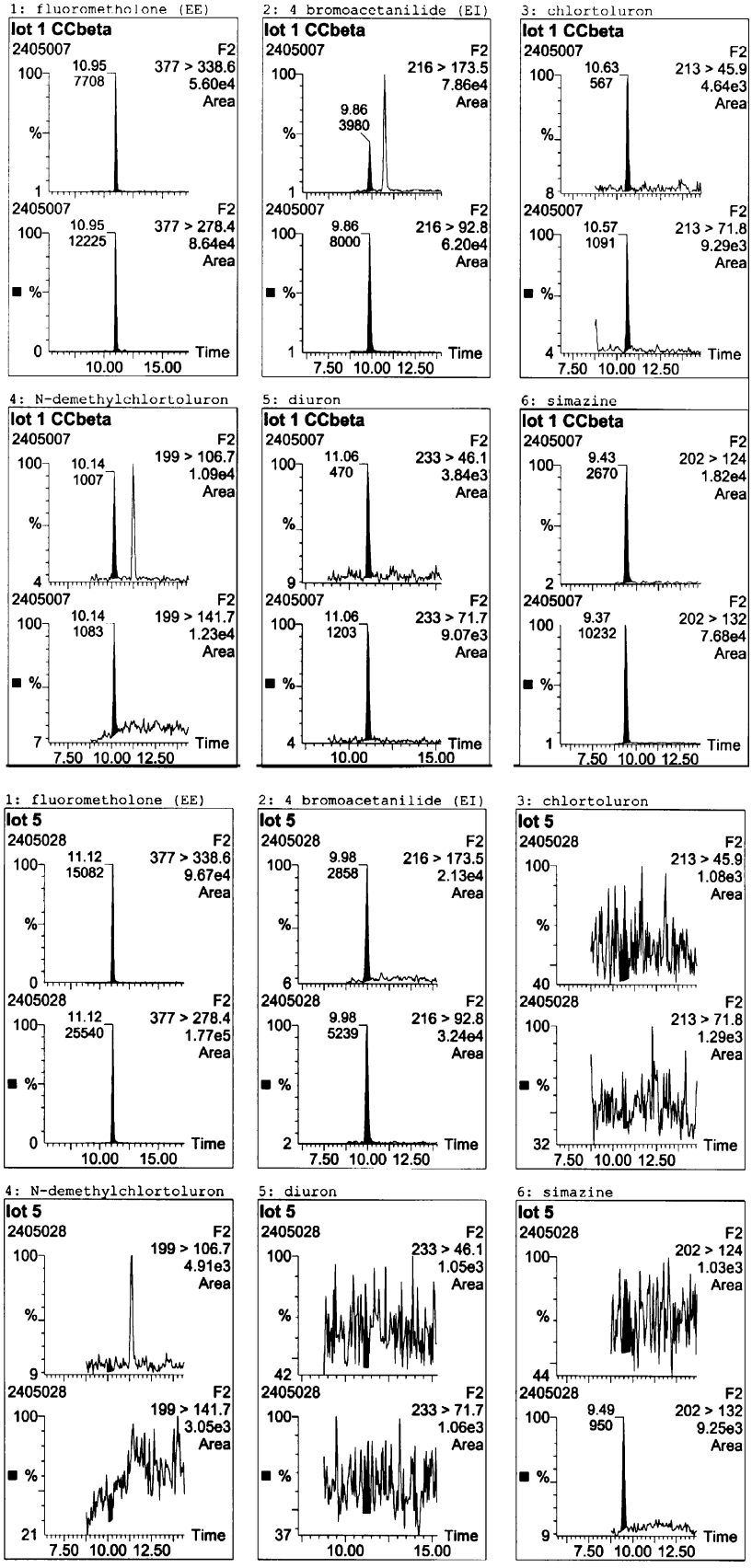


Fig. 8. LC–MS/MS profiles of the two main diagnostic transitions of four phenylurea and internal and external standards, in a blank sample (down) and in a spiked sample (up) (from the left to the right and up to down: external standard, internal standard, 0.5 μg/kg for chlortoluron, 1.0 μg/kg for *N*-demethylchlortoluron, 6.0 μg/kg for diuron and 1.7 μg/kg for simazine).

Table 5
Recapitulative information regarding quantitative performances ($n = 10$)

Molecules	Spiking level (μg/kg)	Repeatability error (%)	Trueness error (%)	Recovery (%)	R^2 (on most intense transition)
Chlortoluron	0.5	30	26	44	0.977
1-(3-Chloro-4-methylphenyl)-3-methylurea	1.0	32	22	46	0.971
1-(3-Chloro-4-methylphenyl)-urea	10.0	46	49	44	0.817
Isoproturon	1.2	14	13	37	0.961
1-(4-Isopropylphenyl)-3-methylurea	3.0	23	19	46	0.787
1-(4-Isopropylphenyl)-urea	20.0	45	37	39	0.865
Diuron	6.0	22	17	40	0.844
1-(3-4-Dichlorophenyl)-3-methylurea	0.5	20	17	46	0.996
1-(3-4-Dichlorophenyl)-urea	0.5	19	28	45	0.986
Linuron	2.5	38	24	32	0.734
Desisopropylatrazine	1.6	24	19	74	0.972
Desethylatrazine	0.7	26	19	75	0.998
Simazine	1.7	22	55	69	0.824
Atrazine	4.0	20	15	29	0.827
Desethylterbutylazine	3.0	24	21	63	0.973
Terbutylazine	30.0	66	44	29	0.844
Average value		29	26	47	0.899

3.4.4. Recovery yield

The recovery yield was calculated on 10 different spiked samples (cf. Table 5). Average recovery yield value was found to be 47%. This result was not used during quantification step because each signal of analyte was reported to the internal standard in the sample and compared to the same ratio in the spiked sample. Indeed, the recovery yield was automatically calculated in each sequence of analysis.

3.5. Intercomparison study

The 16 molecules studied were unambiguously identified in all spiked samples (between 10 and 50 μg/kg) according to the 2002/657/EC decision. The uncertainty of trueness evaluated on this intercomparison corresponds to a mean-value of 5.5% for phenylurea residues and 6.8% for triazine residues ($n = 6$). These values are very low and confirm the trueness of the analytical method between 10 and 50 $\mu g\,kg^{-1}$. Linearity of the method was concluded satisfactory ($R^2 > 0.98$) (cf. Table 6) and well adapted for the further quantification of PU and TZ in oysters. The average recoveries calculated on the set of the ten phenylureas and the six triazines in the five spiked samples are 60% and 63%, respectively.

Table 6
Interlaboratory validation: evaluation of R^2

Phenylurea residues	R^2	
	Other laboratories	LABERCA
Chlortoluron	0.973	0.999
1-(3-Chloro-4-methylphenyl)-3-methylurea	0.965	0.999
1-(3-Chloro-4-methylphenyl)-urea	0.966	0.994
Diuron	0.932	0.997
1-(3-4-Dichlorophenyl)-3-methylurea		0.945
1-(3-4-Dichlorophenyl)-urea	0.972	0.953
Linuron	0.979	0.988
Isoproturon	0.996	0.998
1-(4-Isopropylphenyl)-3-methylurea	0.952	0.997
1-(4-Isopropylphenyl)-urea	0.962	0.993
Mean value	0.966	0.986
Triazine residues		
Desethylatrazine	0.995	0.979
Desethylterbutylazine	–	0.984
Simazine	0.984	0.994
Atrazine	0.970	0.988
Desisopropylatrazine	–	0.992
Terbutylazine	0.980	0.993
Mean value	0.982	0.988

4. Conclusion

Pressurised liquid extraction combined with adjusted purification and LC–MS/MS analysis was demonstrated an efficient method for the determination of PU and TZ in molluscs tissues. Performances of the developed methodology are very relevant in terms of sensitivity, specificity, accuracy and linearity. Nevertheless, some molecules like demethylated phenylurea, simazine and terbuthylazine must be only semi-quantified because of the demonstrated weak accuracy. In terms of sensitivity, the identification limit of the methodology described above is set at around 3 $\mu g\,kg^{-1}$. This method will be used to evaluate new metabolites, which can be used as relevant bio-indicator molecules.

Acknowledgments

This work was supported by Bretagne and Pays de la Loire regions (program number 1034). Similarly, the authors would like to thank the different partners involved in the interlaboratory study. Finally, Makhteshim-Agan company and particularly Miss Jeanne Roederer are thanked for having provided chlortoluron metabolite.

References

[1] M. Garmouna, M. Blanchard, A. Chesterikoff, Water Res. 31 (1997) 1489.
[2] D. Michael Revitt, J. Bryan Ellis, N.R. Llewellyn, Urban Water 4 (2002) 13.
[3] M.D. Crouch, S.A. Barker, J. Chromatogr. A 774 (1997) 287.
[4] I. Muñoz, N. Rosés, Water Res. 34 (2000) 2846.
[5] D. Barcelo, K. Martinez, Fresenius J. Anal. Chem. 370 (2001) 940.
[6] M.A. El-Dib, H.F. Abou-Waly, Water Res. 32 (1998) 1881.
[7] I. Scheunert, S. Reuter, Environ. Pollut. 108 (2000) 61.
[8] T. Vroumsia, R. Steiman, F. Seigle-Murandi, J.L. Benoit-Guyod, A. Khadrani, Chemosphere 33 (1996) 2045.
[9] S. Jayaraman, R.J. Pruell, R. McKinney, Chemosphere 44 (2001) 181.
[10] C. Molins, E.A. Hogendoorn, E. Dijkman, H.A.G. Heusinkveld, R.A. Baumann, J. Chromatogr. A 869 (2002) 487.
[11] R. Dommarco, A. Santilio, L. Fornarelli, M. Rubbiani, J. Chromatogr. A 825 (1998) 200.
[12] E. Concha-Graña, M.I. Turnes-Carou, S. Muniategui-Lorenzo, P. López-Mahía, E. Fernández-Fernández, D. Prada-Rodríguez, J. Chromatogr. A 1047 (2004) 147.
[13] U. Dbrowski, H. Giergielewicz-Moajska, M. Biziuk, J. Gaca, J. Namienik, J. Chromatogr. A 957 (2002) 59.
[14] P. Suchan, J. Pulkrabová, J. Hajlová, V. Kocourek, Anal. Chim. Acta 520 (2004) 193.
[15] H. Berrada, G. Font, J.C. Molto, J. Chromatogr. A 890 (2000) 303.
[16] M.C. Gennaro, E. Marengo, V. Gianotti, V. Maurino, J. Chromatogr. A 910 (2001) 79.
[17] E. Baltussen, H. Snijders, H.G. Janssen, P. Sandra, C.A. Cramers, J. Chromatogr. A 802 (1998) 285.
[18] B. Berger, J. Chromatogr. A 769 (1997) 338.
[19] C. Crescenzi, A. Di Corcia, E. Guerriero, R. Samperi, Environ Sci. Technol. 31 (1997) 479.
[20] J.Y. Hu, T. Aizawa, Y. Magara, Water Res. 33 (2) (1999) 417.
[21] S. Ben Rejeb, N. Fischer Durand, A. Martel, B. Le Pouennec, J.F. Lawrence, M.C. Hennion, F. Le Goffic, Anal. Chem. Acta 376 (1998) 41.
[22] J.F. Lawrence, C. Menard, M.C. Hennion, V. Pichon, F. Le Goffic, N. Durand, J. Chromatogr. A 732 (1996) 277.
[23] J.P. Antignac, B. Le Bizec, F. Monteau, F. André, Anal. Chim. Acta 483 (2003) 325.
[24] T. Gallina Toschi, A. Bendini, A. Ricci, G. Lercker, Food Chem. 83 (2003) 551.