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Ultrasonic or accelerated solvent extraction followed by U-HPLC-high mass accuracy MS for screening of pharmaceuticals and fungicides in soil and plant samples

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ABSTRACT

Different veterinary pharmaceuticals are used in agricultural livestock becoming a source of environment contamination. Furthermore, no regulation exists for the concentration limits of pharmaceuticals in soil or water. Monitoring programs for environment contamination with pharmaceuticals are needed, requiring new sensitive and selective screening methods. The present study focuses on developing a method for the simultaneous scanning of forty-two compounds (pharmaceuticals, azole biocides and fungicides) in soil and plant material samples. For extraction purposes the use of ultrasonic assisted and accelerated solvent extraction (ASE) were compared. The extract was purified and concentrated by applying a solid phase extraction step followed by ultra-high-performance-chromatographic separation and accurate-mass spectrometric detection using Exacte Orbitrap technology (FWHM 50,000). The effects of the different extraction solvents and conditions on the extraction efficiency were tested. Although both extraction approaches are applicable the optimal extraction efficiency was obtained by applying accelerated solvent extraction using solvent mixtures containing acetone for soil and methanol for plant samples. An ASE process has been validated for the determination of selected pharmaceuticals and fungicides in soil and in plant material. The recoveries from soil samples were >70% for more than 68% of the compounds. The levels of detection were $\leq$10 $\mu g\,kg^{-1}$ for 93% of the compounds tested. The recoveries from plant material were >70% for 64% of the compounds tested. The levels of detection were $\leq$10 $\mu g\,kg^{-1}$ for 66% of the compounds. The developed method was used to screen soil and plant material collected throughout the Netherlands and oxytetracycline residues were detected.

1. Introduction

Different classes of pharmaceutical compounds are used in agricultural livestock. More than 70% are antibiotic agents [1]. Different amounts of administered pharmaceuticals are excreted as the parent compound and/or as metabolites. The animal excrements are either directly released into the environment by grazing animals or indirectly by spreading animal manure as fertilizer onto agricultural soils. Manure application has been recognized as a source of veterinary drug contamination of soils and water [2].

Within Europe the requirement for an environmental safety part for the registration of pharmaceuticals was first described in Directives 93/39-40/EC [3], followed by EMEA Guidance: EMEA/CVMP/055/96 [4], Directive 2001/82/EC [5] as amended by Directive 2004/28/EC [6]. In accordance with those directives, a Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products, EMEA/CVMP/ERA/418282/2005 [7] was published in June 2008. For registration of a veterinary drug, additional environmental risk assessment has to be added, which take into account specific information concerning the degree of environmental exposure, the direct toxicity to aquatic and terrestrial fauna and flora, antibiotic resistance development and biodegradation of the active substance. This guideline proposes exposure models to estimate the predicted environmental concentration (PEC) of veterinary pharmaceuticals in soil and water, and propose the value of 100 $\mu g\,kg^{-1}$ in soil as a trigger value for second phase of the risk assessment. However no regulation exists for the concentration limits of pharmaceuticals in soil [8,9].

Soil contamination can lead to resistance of bacteria and fungi to qualitative and quantitative effects on resident microbial soil populations [10,11], disturbances in soil functioning [12], or to plants growth inhibition [13].

Antifungals of a class similar to those used in clinical practice are also widely used in agriculture. Multidrug resistance and

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cross-resistance to triazoles is frequently observed [14]. High concentrations of azole pharmaceuticals in sewage sludge suggest their strong tendency to be absorbed onto and persist in solids (the 1/2 life time of azoles is approx. more than one year) [15]. An accurate evaluation of the effect of those compounds on the environment by risk assessment procedures is more difficult, since these compounds are used as antifungal drugs, biocides, preserving agents and agricultural fungicides.

Eco-toxicity data for pharmaceuticals have been proven to be in the range of measured environmental concentrations [16]. In order to establish post-marketing control mechanisms, and risk management, monitoring programs for environment contamination including pharmaceuticals and fungicides are needed. Therefore, there is a need for sensitive and selective analytical methods applicable to test for a broad range of compounds.

Several chemical methods have been described for the analysis of pharmaceutical compounds in environmental matrix like soil [17]: high performance liquid chromatography, gas chromatography or capillary electrophoresis. Among the various methods liquid chromatography mass spectrometry (LC/MS) or tandem mass spectrometry MS/MS is the method of choice for estimating the low concentration of antibiotics in water and soil [18]. A new approach for multi-compound analysis is the use of the high resolution and reliable mass accuracy of Orbitrap MS systems [19].

The extraction methods mainly used are Soxhlet extraction [20], microwave assisted [21], ultrasonic assisted [17] or accelerated solvent extraction (ASE) [22] followed by an extract clean up procedure. In [23] an ASE method for seventeen pharmaceuticals in soil is compared with an ultrasonic extraction. The analysis is by LC–MS/MS.

The present study focuses on developing a method for the simultaneous determination of forty-two compounds, including pharmaceuticals, antifungals drugs, biocides and fungicides, in soil and plant material samples, comparing ultrasonic assisted and accelerating solvent extraction (ASE), followed by SPE in both cases. Different extraction condition and different solvents were tested. The analyses were performed in one single full scan MS method, using U-HPLC-Exactive Orbitrap MS at 50,000 (FWHM – full width at half maximum) resolution. The optimized method was validated and applied to a set of soil and plant samples. The advantages of full scan MS screening methods are illustrated.

2. Experimental

2.1. Chemicals, reagents and solutions

A mixture of different compounds belonging to different drugs classes: benzimidazole, tranquilizers, macrolides, sulfonamides, quinolones, penicilines, tetracyclines, non-steroidal anti-inflammatory drugs (NSAIDs), antiepileptic, lipid regulator, azole antifungals, polyene antifungals, mitotic inhibitor; azole biocides and fungicides, was chosen to develop the methods. For the compounds selected see Table 1.

All standards used were purchased from Sigma–Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) with the exception of: natamycin, which was supplied by Fluka (Buchs, Switzerland); carazolol, bromuconazole and clofibric acid, by Dr. Ehrenstorfer (Augsburg, Germany) and itraconazole, terconazole, ketoconazole and fluconazole, by EDQM (Strasbourg, France).

Methanol, acetonitrile, acetone and dimethyl sulfoxide (LC–MS grade), were supplied by Biosolve B.V. (Valkenswaard, The Netherlands). Milli-Q water (ultrapure) was obtained from a Milli-Q Gradient A10 water purification system. Acetic acid (100%), formic acid (98–100%), citric acid, and EDTA (>99%) were provided by VWR International B.V. (Roden, The Netherlands). Natrium hydroxide (Merck B.V., Schiphol-Rijk, The Netherlands), ammonia 32% (Prolab Scientific, Ontario, Canada), Ammonium formate (97%) (Sigma–Aldrich Chemie B.V., Zwijndrecht, The Netherlands), were used. Solid phase extraction (SPE) Strata-X cartridges (200 mg/6 mL) used for solid phase extraction were purchased from Phenomenex (Utrecht, The Netherlands).

2.1.1. Stock solutions

Stock solutions of 1 mg mL^{-1} were prepared by accurately weight an adequate amount of standard and dissolve in an appropriate solvent (acetonitrile for difenoconazole, terconazole and prothioconazole, acetone for penconazole and flucilazole, dimethyl sulfoxide for itraconazole, and methanol for the rest of compounds). The stock solution was stored at −30 °C. The individual stock solutions were used to prepare working mix solutions of 10, 5, 0.8 μg mL^{-1}, by diluting the appropriate volume of the stocks in methanol, in order to obtain the desired concentrations for spiking over range of 10–50 μg kg^{-1}. The correspondent working mix solutions for spiking were stored at −30 °C, under a period of 4 months.

2.2. Sample collection

Samples were selected in order to provide real environmental matrix for method development and validation. They include samples of different soils (sandy and clay) of upper 20 cm layer, crop and grass, from areas with important agricultural activities in The Netherlands. The sampling was carried out in May 2011. From each sampling point, of 1 m^2, two sub-samples were taken. The soil samples were dried in a Memmert Oven (provided by Depex, Houten, The Netherlands) at 40 °C, for 6 h, passed through a Ø 2 mm sieve and then, the sub-samples of each sampling point were homogenized. The sample was stored at 4 °C in the refrigerator until extraction. The maximum storing time was 30 days. The fresh plant material was stored at −30 °C. After defreezing the material was chopped by using a blender. The homogenized subsamples were stored in plastic containers (50 mL) at −30 °C until extraction (within 15 days).

2.3. Methods

2.3.1. Accelerated solvent extraction (ASE) procedure

ASE was performed on an ASE 200 system equipped with a solvent selector (Dionex, ASE 350, USA). Approximately 5 g of dried soil sample or 3 g of chopped plant material were weighed into 33 mL extraction cells (lined with glass microfiber filters from Whatman, Maidstone, UK) and mixed with 5 g Diatomaceous earth to increase the contact-surface between soil particles and extraction solvent and prevent clogging of the extraction cell. The optimized operating conditions were: extraction temperature 50 °C; extraction pressure 1500 psi; two cycles of 5 min each, static extraction; 50% flush volume; and a 60-s purge with nitrogen. Extraction solvent used was acetone/citric acid 0.2 M (50:50) (pH adjustment at 4.5 with sodium hydroxide) for soil sample and methanol/citric acid 0.2 M (50:50) (pH adjustment at 4.5 with sodium hydroxide) for plant material sample.

After the extraction, 100 μL Na_2EDTA 1 M was added to each sample extract and Milli-Q water was added until a final volume of 50 mL volume. The sample was centrifuged for 15 min at 2800 × *g* (Falcom 6/300 MSE Refrigerated Centrifuge, London, UK). An aliquot of the final extract, corresponding with 1 g of sample (10 mL soil extract and 16.6 mL plant material extract) was taken and diluted with Milli-Q water to a final concentration of 10% organic solvent (50 mL for soil and 85 mL for plant samples).

Table 1
Exact masses used to identify for target compounds and the retention time.

Compound	Family	Molecular formula	Rt (min)	$[M+H]^+$	$[M-H]^-$
Flubendazole	Benzimidazoles	$C_{16}H_{12}FN_3O_3$	7.1	314.09355	312.07899
Erythromycin	Macrolides	$C_{37}H_{67}NO_{13}$	6.4	734.46852	732.45396
Erythromycin (–H_2O)	Macrolides	$C_{37}H_{65}NO_{12}$	7.04	716.45795	714.44340
Dicloxacillin	Penicillins	$C_{19}H_{17}Cl_2N_3O_5S$	6.92	470.03385	468.01935
Ciprofloxacin	Quinolones	$C_{17}H_{18}FN_3O_3$	3.99	332.14047	330.12597
Sulfamethoxazole	Sulfonamides	$C_{10}H_{11}N_3O_3S$	4.12	254.05936	252.04486
Oxytetracycline	Tetracyclines	$C_{22}H_{24}N_2O_9$	4.2	461.15543	459.14093
Carazolol	Tranquilizers	$C_{18}H_{22}N_2O_2$	4.8	299.17538	297.16088
Diclofenac	NSAIDs	$C_{14}H_{11}Cl_2NO_2$	8.6	296.02394	294.00943
Meclofenamic acid	NSAIDs	$C_{14}H_{11}Cl_2NO_2$	9.27	296.02394	294.00943
Carabamazepine	Antipileptics	$C_{15}H_{12}N_2O$	6.43	237.10221	235.08771
Clofibric acid	Lipid regulators	$C_{10}H_{11}ClO_3$	7.14	215.04692	213.03242
Natamycin	Polyene antifungal	$C_{33}H_{47}NO_{13}$	6.85	666.31200	664.29750
Enilconazole	Imidazoles antifungal	$C_{14}H_{14}Cl_2N_2O$	6.98	297.05557	295.04107
Ketoconazole	Imidazoles antifungal	$C_{26}H_{28}Cl_2N_4O_4$	7.37	531.15600	529.14150
Fluconazole	Triazoles antifungal	$C_{13}H_{12}F_2N_6O$	4.57	307.11300	305.09680
Clotrimazole	Imidazoles antifungal	$C_{22}H_{17}ClN_2$	8.53	345.11528	343.10077
Miconazole	Imidazoles antifungal	$C_{18}H_{14}Cl_4N_2O$	9.39	414.99327	412.97877
Itraconazole	Triazoles antifungal	$C_{35}H_{38}Cl_2N_8O_4$	9.96	705.24656	703.23205
Griseofulvin	Mitotic inhibitor	$C_{17}H_{17}ClO_6$	6.81	353.07860	351.06410
Voriconazole	Triazoles antifungal	$C_{16}H_{14}F_3N_5O$	7.09	350.12230	348.10779
Thiabendazole	Benzimidazole fungicides	$C_{10}H_7N_3S$	4.81	202.04332	200.02882
Difenoconazole	Conazole fungicides	$C_{19}H_{17}Cl_2N_3O_3$	6.85	406.07200	404.05740
Hexaconazole	Conazole fungicides	$C_{14}H_{17}Cl_2N_3O$	9.03	314.08212	312.06762
Penconazole	Conazole fungicides	$C_{13}H_{15}Cl_2N_3$	8.8	284.07155	282.05705
Propiconazole	Conazole biocides	$C_{15}H_{17}Cl_2N_3O_2$	8.97	342.07703	340.06253
Paclobutrazol	Conazole fungicides	$C_{15}H_{20}ClN_3O$	7.91	294.13674	292.12224
Prochloraz	Conazole fungicides	$C_{15}H_{16}Cl_3N_3O_2$	9.12	376.03806	374.02356
Tebuconazole	Conazole fungicides	$C_{16}H_{22}ClN_3O$	8.85	308.15239	306.13789
Bromuconazole	Conazole fungicides	$C_{13}H_{12}BrCl_2N_3O$	8.17	375.96133	373.94683
Cyproconazole	Conazole fungicides	$C_{15}H_{18}ClN_3O$	8.01	292.12109	290.10659
Epoxiconazole	Triazole fungicides	$C_{17}H_{13}ClFN_3O$	8.44	330.08037	328.06587
Fenbuconazole	Conazole fungicides	$C_{19}H_{17}ClN_4$	8.46	337.12143	335.10692
Fluquinconazole	Conazole fungicides	$C_{16}H_8Cl_2FN_5O$	8.25	376.01624	374.00174
Flusilazole	Conazole fungicides	$C_{16}H_{15}F_2N_3Si$	8.56	316.10758	314.09308
Flutriafol	Triazole fungicides	$C_{16}H_{13}F_2N_3O$	6.81	302.10992	300.09542
Metconazole	Conazole fungicides	$C_{17}H_{22}ClN_3O$	9.1	320.15239	318.13789
Prothioconazole	Conazole fungicides	$C_{14}H_{15}Cl_2N_3OS$	8.99	344.03850	342.02400
Terconazole	Conazole biocides	$C_{26}H_{31}Cl_2N5O_3$	6.85	532.18770	350.17310
Myclobutanil	Triazole Antifungal	$C_{15}H_{17}ClN_4$	8	289.12143	287.10692
Triticonazole	Conazole fungicides	$C_{17}H_{20}ClN_3O$	8.36	318.13674	316.12224
Carbendazim	Benzimidazole fungicides	$C_9H_9N_3O_2$	4.39	192.07673	190.06222
Metalaxyl	Phenylamide fungicides	$C_{15}H_{21}NO_4$	7.07	280.15431	278.13981

2.3.2. Ultrasonic assisted extraction procedures

5 g of soil samples or 3 g of plant material sample were weighed into a 50 mL centrifuge tubes. 100 μL Na_2EDTA 1 M was added and the sample was homogenized by shaking. The analytes were extracted with 20 mL extraction solvent by placing the tube in an ultrasonic bath (Branson Ultrasonic, Danbury, USA) for 1 h, and finally shaking for another 1 h (Heidolph Instruments GmbH, Schwabach, Germany). Extraction solvent used was methanol/citric acid 0.2 M (50:50) (pH adjustment at 4.5 with sodium hydroxide). The samples were then centrifuged 15 min, at 2800 × *g*; the supernatant transferred to a 50 mL flask. Extraction procedure was repeated once, with the same solvent volume. The extracts were combined, homogenized, and Milli-Q water was added to 40 mL volume. An aliquot of extract volume, corresponding with 1 g of sample (8 mL soil extract and 13.3 mL plant material extract) was taken and diluted with water to a final concentration of 10% organic solvent (40 mL and 70 mL respectively).

2.3.3. SPE procedure

An SPE procedure was applied in order to clean and concentrate the extract, using Strata X, 200 mg/6 mL, reverse phase, SPE cartridge. The cartridge was previously preconditioned with 6 mL methanol followed by 6 mL water. Before loading the cartridge, the sample pH was adjusted to 3 with acetic acid. After sample application the cartridge was rinsed with 6 mL water, followed by 6 mL methanol/water 30% (v) and vacuum dried for 1 min. The analytes were eluted with 6 mL methanol. The eluate was concentrated by evaporation under flow of high purity nitrogen, in a water bath at 42 °C (Turbo Vap LV Evaporator, Zymark, USA) and dissolved in 25 μL methanol and 225 μL Milli-Q water.

2.3.4. Instrumentation

One LC-full scan MS configuration was used: Exactive High Performance Benchtop LC–MS Mass Spectrometer powered by Orbitrap Technology from Thermo Fisher Scientific (Breda, The Netherlands) coupled to an Ultra High Pressure Liquid Chromatography (U-HPLC) chromatograph (Accela, Thermo Fisher Scientific, Breda, The Netherlands) system. The resolution was set at 50,000 FWHM. Full scan acquisition of *m*/*z* 100–1000; scan rate used was 2 scans per second; HESI (Heated Electrospray) ion source was operated in positive and negative mode.

For separation an ultra-performance Acquity U-HPLC Column C18 (100 mm × 2.1 mm, 1.8 μm) (Waters, Etten-Leur, The Netherlands) was used. A flow rate of 0.4 mL min^{-1} was set for separation of the selected compounds. The mobile phase consisted of: eluent A, 100% water containing 2 mM ammonium formate and 160 μL formic acid; eluent B, 100% methanol containing 2 mM ammonium formate and 160 μL formic acid (pH 3.5). The column temperature was set at 40 °C. The step gradient was follow: 0–1 min 100% A; 1–2.5 min linear increase to 40% B; 2.5–10 linear increased

Table 2
HESI source parameters.

HESI source parameters	ESI (+)	ESI (−)
Spray voltage	2.8 kV	2.8 kV
Capillary temperatures	250 °C	250 °C
Capillary voltage	47.5 V	−67.5 V
Tube lens voltage	95 V	−185 V
Skimmer voltage	14 V	−46 V
Heater temperature	300 °C	300 °C

to 100% B and hold 3 min; 13–13.2 decreasing to 0% B; 13.2–15 min 100% A.

The ion-source parameters were optimized at the values presented in Table 2. The automatic gain control was set at one million and the injection time was set to 50 ms. Internal calibration was performed by multi point calibration using background ions.

Detection was based on calculated exact mass and on retention time of target compounds, presented in Table 1. Data were evaluated by the Quan Browser Xcalibur 0606 (Thermo Fisher) and Thermo ToxID (Thermo Fisher).

2.3.5. Method parameters

In order to compare the two extraction method the following parameters were considered: the absolute recovery, level of detection, selectivity and mass error (Δppm) of the measurements for the selected analytes.

The relative extraction recovery was calculated by Eq. (1):

$$\text{Recovery}\,(\%) = \frac{As}{A_{\text{ats}}}100 \quad (1)$$

where *As* is the signal (area) corresponding with spiked before sample treatment at 50 μg kg^{-1} and A_{ats} is the signal (area) corresponding of spiked after treatment sample at the same level.

The level of detection was evaluated by analyzing sample spiked at 10 and 50 μg kg^{-1} with appropriate amounts of mix-standard solutions.

Selectivity of the detection methods is strongly dependent on the chosen mass extraction window. In that case, for the results processing, a 10 ppm mass tolerance was set, leading high selectivity [14].

2.4. Validation

There are no official limits for the veterinary pharmaceutical compounds in soil or plant material. The level of interest, based on literature [7] was considered 100 μg kg^{-1}. A 50 μg kg^{-1} value was considered the screening target concentration (1/2 of level of interest).

Twenty real samples, ten different soil samples and ten plant material samples (grass and corn crop) were collected from agricultural areas within the Netherlands. Blank sample and spiked sample at 50 μg kg^{-1} level were analyzed on different days, covering all operation condition.

The validation procedures was based on the approach of Guideline of Screening Methods of Residues of Veterinary Medicines, for Community Reference Laboratories Residues 20/1/2010 [24]. This guideline document supplements Commission Decision 2002/657/EC [25] regarding the validation of screening methods, on measures to monitor certain substances and residues thereof in live animals and animal products, provided by Council Directive 96/23/EC [26]. Considering the level of measured concentration and the compounds of interest in this analysis, this guideline was found appropriate for validation the screening method for pharmaceuticals and fungicides in environmental matrix. The analyses results were evaluated in order to establish the detection capability (CCβ).

2.5. LC–MS/MS confirmation

The sample extracts suspected for containing residues of pharmaceuticals or fungicides by the screening method were (re)analyzed by LC–MS/MS for the confirmation of the proposed identity. Triple quadrupole based precursor scans were performed on a Micromass Quattro Ultima MS/MS (Waters, Milford, MA, USA) equipped with an electrospray interface, coupled to an LC-20AD (Shimadzu, USA). A Symmetry Column C18 (150 mm × 3 mm, 5 μm) (Waters, Etten-Leur, The Netherlands) was used for separation. Flow rate: 0.4 mL min^{-1}. The step gradient (eluent A, 100% water containing 1 mM ammonium acetate, pH 2.6; eluent B, 10/90% 10 mM ammonium acetate in ACN) was as follows: 0–1 min 100% A; 1–10 min linear increase to 50% B; 10–14 min linear increasing to 100% B, and hold for 4 min; 18–19 min decrease to 0% B; column temperature: 40 °C.

The following MS/MS instrument specific settings were applied: capillary voltage: 2.5 kV; cone voltage: 30 V; source temperature: 120 °C; desolvation temperature: 300 °C; cone gas flow: 207 L h^{-1}; desolvation gas flow: 615 L h^{-1}; LM1 and HM1 resolution: 15; ion energy (1): 1.0 V; entrance: 5.0 V; exit: −27 V; LM2 and HM2 resolution: 15; ion energy (2): 1.0 V; multiplier: 750 V; collision energy 28 eV.

For the final confirmation of the identity the criteria described in EU 2002/657/EC were applied, including the detection of two fragment ions with the appropriate ion-ratio.

3. Results and discussion

3.1. Strategy and optimization

The recent proposed methods for pharmaceuticals compound [17,18,27] extraction from environmental sample are accelerating solvent extraction (ASE), and ultrasonic assisted extraction. In this study ASE and ultrasonic assisted extraction were compared and different parameters affecting the extraction efficiency were investigated. Blank soils and plant material samples were spiked at 10 μg kg^{-1} and 50 μg kg^{-1} level, with appropriate amounts of mix-standard solutions, extracted and analyzed. Blank samples were extracted for each sample set.

3.1.1. Optimization the ultrasonic assisted extraction

Several previous studies [22] indicated that polar organic solvents: e.g. acetonitrile (ACN), acetone, methanol (MeOH) in water mixtures manifested the superior capability to extract pharmaceuticals from sewage sludge and soil. According with these, the mentioned solvents were tested in combination with 1% formic acid in water (50:50). Extraction was performed during 30 min with 15 mL of solvent, followed by shaking for 30 min. The procedure and repeated once. The extract was evaporated at 40 °C under nitrogen steam to the complete evaporation of organic phase. Oxytetracycline, dicloxacilline, erythromycin, ciprofloxacin, ketoconazole, natamycin, prothioconazole and itraconazole cannot be detected in any sample. Recoveries for the rest of the compounds ranged from 12% to 50%, better result were obtained by the use of ACN/H_2O for sandy soil samples and MeOH/H_2O for clay soil samples. The differences of recoveries between different soil samples were also observed and significant!

Tetracyclines form strong complexes with di- and trivalent cations in the clay mineral inter-layers or to hydroxyl-groups at the surface of the soil particles. Na_2EDTA, citric acid and oxalic acid are commonly used chelators for multivalent cations [28,29]. As a starting point, 100 μL of Na_2EDTA 0.5 M was added to the samples. Furthermore, not the total organic extract was evaporated, but an aliquot, corresponding with 1 g sample, was diluted to a final 10%

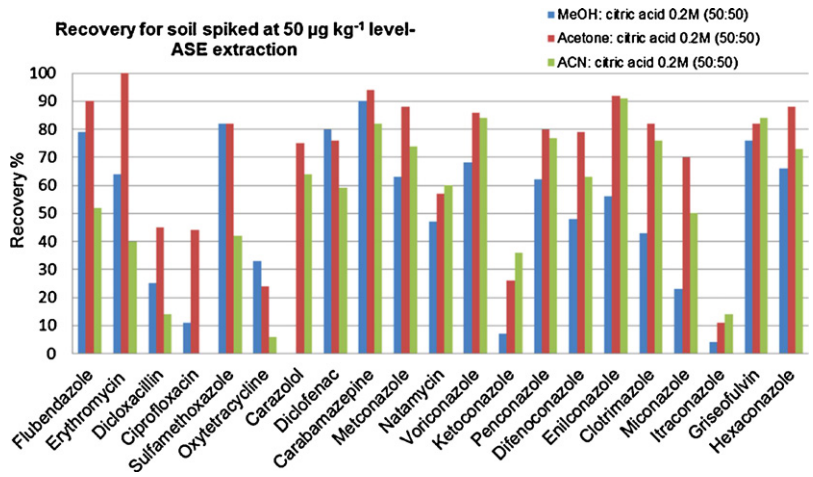

Fig. 1. Recovery twenty-one of forty-two analyzed compound in case of different organic solvents (ACN, acetone, MeOH) mixtures with citric acid 0.2 M, in soil sample, by ASE.

organic phase concentration with water. The unrecovered compounds: oxytetracycline, dicloxacilline, erythromycin now were detected, but showed very low recoveries (<10%). Also significant differences were observed between samples: in case of the clay soil higher recovery was obtain for methanol mixture as the extraction solvent (recovery ranged from 40% to 50% for 73% of compounds) and in case of the sandy soil the higher recovery was obtained for acetonitrile mixture (recovery ranged from 40% to 50% for 60% of compounds).

To improve recovery for oxytetracycline and dicloxacilline, the formic acid was replaced with citric acid 0.2 M and the concentration of Na_2EDTA solution added to the sample was increased to 1 M. Different proportions of organic solvent: ACN/citric acid 0.2 M 70:30 and 50:50 were tested, without major differences for the compounds.

Finally, ACN, acetone and MeOH in 50:50 proportions with citric acid 0.2 M were tested. 100 µL Na_2EDTA 1 M was added to every sample. Extraction time was increased to 60 min with 20 mL, followed by shaking for 60 min. After centrifugation and separation the supernatant extract the procedure was repeated. For oxytetracycline, clotrimazole and miconazole recoveries increased in case of using acetone, but for the rest of the compound better result were obtained using methanol. Recoveries ranged from 10% to 50% for all the compounds. 69% of compounds were detected at 10 $\mu g\,kg^{-1}$ level and 16% at 50 $\mu g\,kg^{-1}$ level. Ciprofloxacin, natamycin, prothioconazole and itraconazole cannot be detected in any case.

This last experiment was applied on plant material samples. Methanol seems to be the best option in that case too. The recovery was low, under 40% for all the components. 26% of compounds were detected at 10 $\mu g\,kg^{-1}$ level, and 50% of compounds at

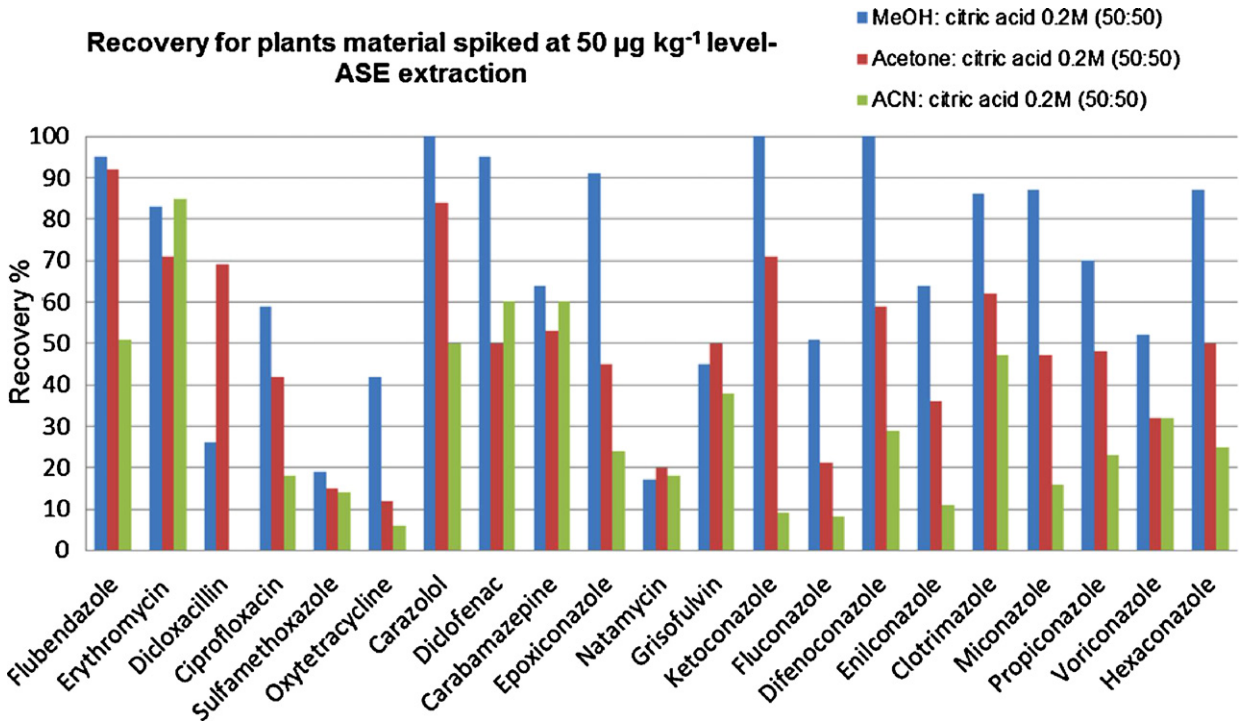

Fig. 2. Recovery for some of the analyzed compound in case of different organic solvents (ACN, acetone, MeOH) mixtures with citric acid 0.2 M, in plant material sample, by ASE.

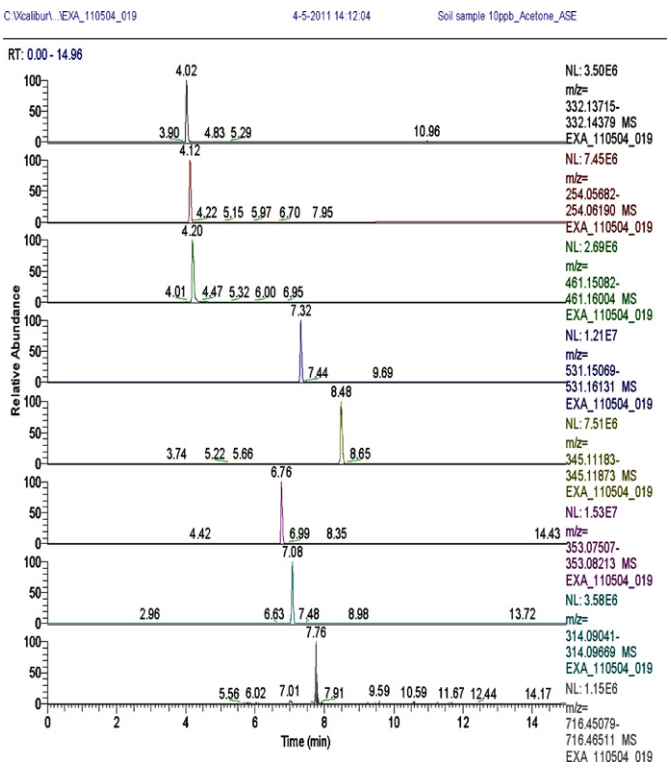

Fig. 3. The U-HPLC-Oritrap-MS chromatograms of soil sample extracted with acetone/citric acid 0.2 M (50:50) mixture, containing from the top to the bottom: ciprofloxacine, sulfamethoxazole, oxytetracycline, ketoconazole, clotrimazole, griseofulvin, erythromycin and voriconazole, at 10 μg kg^{-1} level. The ions chromatograms were extracted from TIC using a 10 ppm mass window; positive ESI ionization, resolving power 50,000.

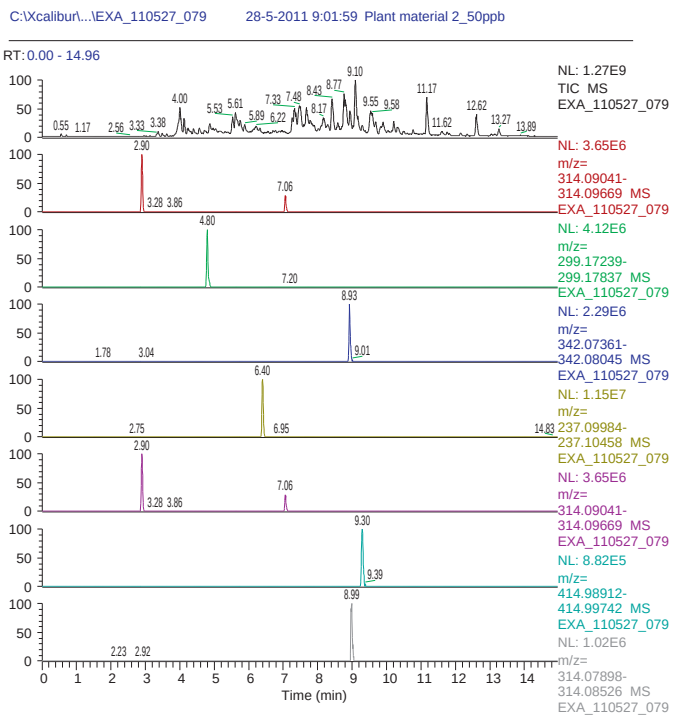

Fig. 4. The U-HPLC-Orbitrap-MS chromatograms of soil sample extracted with methanol/citric acid 0.2 M (50:50) mixture, containing from the top to the bottom: TIC, flubendazole, carazolol, propiconazole, carabamazepine, miconazole and hexaconazole, at 50 μg kg^{-1} level. The ions chromatograms were extracted from TIC using a 10 ppm mass window; positive ESI ionization, resolving power 50,000.

50 μg kg^{-1} level. Ciprofloxacin, diclofenac, fenbuconazole, bromuconazole, natamycin, prothioconazole, itraconazole ketoconazole, griseofulvin, fluqioconazole, erythromycin and dicloxacillin cannot be detected in any case.

3.1.2. Optimization the ASE extraction

According to the literature regarding ASE extractions [22,30], for method development the first solvents to be tested are one acidic, one neutral and one basic solvent. For that reason MeOH/citric acid 0.1 M (50:50) (pH adjustment at 4.5 with sodium hydroxide); ACN/H_2O (70:30); and MeOH/NH_3 0.1 M (50:50) were tested. The ASE conditions were set to three cycles of 8 min for each extraction, at 1500 psi and the temperature of 70 °C with a flush volume of 60%. The extract was evaporated at 40 °C under nitrogen steam, to the complete evaporation of organic phase. Turbid extracts were obtained, welcoming difficulties in further SPE clean-up. Oxytetracycline, dicloxacilline, erythromycin, ciprofloxacin, and fluquiconazole could not be detected in any sample. The highest recoveries were obtained in case of MeOH/0.1 M citric acid. Matrix compounds were also extracted, generating high matrix interferences during MS analysis. Conclusions of this first experiment were: to centrifuge the sample after extraction, to use a lower temperature and shorter extraction time and to use a chelate agent for improving the detection of oxytetracycline.

Considering the pK_a values of the compound, which are ranging between 3 and 7, the extraction solvent pH around 4.5 was selected because most of these compounds were expected uncharged at this value, and this increase the chance that these compounds will be extracted by the moderately polar solvents used *viz.*: methanol, acetonitrile or acetone.

Addition of Na_2EDTA in the extraction solvent was not possible because the buffer solution EDTA-citric acid, precipitated within few hours, on occasions causing blockage in the tubes and valves of the ASE system [30]. Citric acid buffer 0.2 M as complexation agent was used. The ASE extraction conditions were set to two cycles of 5 min each, pressure at 1500 psi and the temperature at 50 °C, with a flush volume of 60%. 100 μL of 1 M Na_2EDTA were added after extraction in every sample, homogenized and the extract was centrifuged. An aliquot part of extract volume, corresponding to 1 g sample, was diluted to 10% organic phase by the addition of Milli-Q water, quitting the evaporation of organic phase of the extract, in order to avoid the loss of analytes. Different proportions of organic solvent were tested (ACN/citric acid 0.2 M, 50:50 and 70:30). All the analytes were detected in the samples. For oxytetracycline the best recovery (still lower than 10%) was obtained in case of 50:50 proportion. Ketoconazole, clotrimazole, and natamycin had also low recovery around 20%. For the other compound the differences was not significant, recovery ranging between 50% and 80%. Next, three organic solvents in combination with 0.2 M citric acid/ACN, acetone, MeOH (50:50) were tested on soil and fresh grass sample. The conditions of the extraction were similar: two cycles of 5 min each extraction, at 1500 psi, 50 °C, flush volume 50%. In both matrix, soil and grass, the best results were obtained using methanol and acetone mixture (Figs. 1 and 2).

The recovery of analytes for soil matrix in the acetone mixture was between 70% and 100% for 68% of compound, between 40% and 70% for 21% of compounds and <40% for 11% of compounds. Low recovery was obtain for oxytetracycline: 33% in methanol, 24% in acetone and 6% in acetonitrile; itraconazole 4% in methanol, 11% in acetone and 14% in acetonitrile, ketoconazole: 7% in methanol, 26% in acetone and 36% in acetonitrile, and prothioconazole was detected only in acetone mixture with a recovery of 18%. All the compounds were detected: 93% of them were detected at 10 μg kg^{-1} level, and 7% at 50 μg kg^{-1} level.

Table 3
The result of validation study: CCβ, maximum recovery, average and standard deviation of mass error (Δppm) in soil and plant material sample.

Compound	Soil samples				Plant material samples			
	CCβ ($\mu g\,kg^{-1}$)	Max recovery (%)	Average (Δppm)	St dev (Δppm)	CCβ ($\mu g\,kg^{-1}$)	Max recover (%)	Average (Δppm)	St dev (Δppm)
Flubendazole	50	70	0.70	0.48	50	86	0.62	0.53
Erythromycin	50	90	3.01	3.51	50	85	0.81	033
Dicloxacillin	50	45	1.35	0.74	50	60	1.92	1.56
Ciprofloxacin	50	44	0.64	0.49	50	59	1.13	0.64
Sulfamethoxazole	50	82	1.28	0.40	50	60	1.01	1.06
Oxytetracycline	50	34	0.67	0.64	50	53	0.43	0.58
Carazolol	50	75	1.68	0.39	50	73	0.46	0.48
Diclofenac	50	76	0.94	0.72	50	95	1.26	0.97
Meclofenamic acid	50	99	1.10	0.90	50	95	1.02	1.10
Carabamazepine	50	94	1.28	0.61	50	64	0.92	0.51
Clofibric acid	50	100	1.11	0.70	50	42	0.97	0.90
Natamycin	50	57	1.52	0.72	50	52	2.03	1.45
Enilconazole	50	92	1.77	0.45	50	86	0.63	0.70
Ketoconazole	50	26	1.52	0.70	50	59	1.67	2.16
Fluconazole	50	35	0.96	0.44	50	65	0.64	0.66
Clotrimazole	50	71	1.03	0.67	50	86	1.89	1.41
Miconazole	50	70	1.84	0.44	50	87	0.85	0.59
Itraconazole	50	11	1.08	0.94	–	–	–	–
Griseofulvin	50	82	1.43	0.50	50	84	0.32	0.39
Voriconazole	50	81	1.23	0.50	50	86	0.71	0.37
Thiabendazole	50	76	1.62	0.33	50	74	0.67	0.33
Difenoconazole	50	79	2.22	0.55	50	100	0.91	0.51
Hexaconazole	50	88	1.82	0.61	50	87	1.01	0.58
Penconazole	50	80	2.13	0.53	50	93	1.12	0.92
Propiconazole	50	88	2.23	0.56	50	70	1.15	1.02
Paclobutrazol	50	69	0.81	0.63	50	80	1.12	0.62
Prochloraz	50	86	1.65	0.62	50	93	1.07	0.49
Tebuconazole	50	87	1.89	0.54	50	78	0.82	0.47
Bromuconazole	50	81	1.82	0.77	50	42	1.69	1.03
Cyproconazole	50	62	1.78	0.85	50	71	1.00	0.36
Epoxiconazole	50	82	1.92	0.45	50	91	0.69	0.82
Fenbuconazole	50	78	1.13	0.52	50	92	1.42	0.66
Fluquinconazole	50	74	0.60	0.35	–	–	–	–
Flusilazole	50	85	1.78	0.49	50	108	0.75	0.74
Flutriafol	50	82	1.04	0.46	50	65	0.51	0.27
Metconazole	50	88	1.16	0.73	50	94	0.55	0.81
Prothioconazole	50	18	1.64	0.64	–	–	–	–
Terconazole	50	51	0.98	0.59	50	84	0.71	0.87
Myclobutanil	50	67	1.24	0.58	50	47	0.32	0.28
Triticonazole	50	87	2.33	0.54	50	85	0.74	0.49
Carbendazim	50	50	1.88	0.32	50	78	0.54	0.33
Metalaxyl	50	87	1.12	0.40	50	70	1.02	0.39

Also a cleaner extract was observed in case of acetone and methanol, and as a consequence less matrix interference in the MS detection. The mass-spectrum of oxytetracycline for those three conditions showed a better S/N ratio and low mass deviation. Furthermore, the signal intensity was higher in case of acetone for 88% of compounds.

For grass sample, methanol was to be the best choice. The recoveries of analytes in the methanolic extracts were between 70% and 100% for 64% of compound, between 40% and 70% for 28% of compounds. Oxytetracyclines recovery in methanol was at an acceptable value of 53%. 66% of compound were detected at 10 $\mu g\,kg^{-1}$ for, and 28% of compounds at 50 $\mu g\,kg^{-1}$ level. Itraconazole, fluquiconazole and prothioconazole were not detected.

Among the extraction solvents tested, it appeared that higher extraction efficiencies were achieved for the mixtures containing acetone/citric acid 0.2 M (50:50) in case of soil sample and methanol/citric acid 0.2 M (50:50) in case of grass sample.

3.2. Comparison of ultrasonic and pressurized liquid extraction

Both extraction methodologies were compared, regarding absolute recovery, level of detection, and selectivity, when blank samples were spiked at 10 and 50 $\mu g\,kg^{-1}$ and the optimized procedures were carried out. ASE presents higher values of the recovery than ultrasonic extraction as well. Also, the ASE procedure allows detection of all compound of interest, most of them at 10 $\mu g\,kg^{-1}$ level.

Furthermore, ultrasonic extraction is more tedious than the ASE procedure whereas the extraction itself by ASE is faster and fully automated, offering better and repeatable results, so ASE was selected as extraction technique for the screening of pharmaceutical and fungicides in soils and plant material.

3.3. SPE cleanup procedure

Solid phase extraction is traditionally used as a clean-up technique for extracts of environmental samples, in order to reduce matrix interferences by adsorbing anionic humic particles from the soil extracts. The optimization of SPE method is described in a method development study [31], performed at RIKILT Institute, Wageningen, The Netherlands, for the same group of compounds, on surface and groundwater matrix.

3.4. Chromatographic separation

Superior chromatographic resolution as provided by U-HPLC is a very important condition for screening analyses decreasing the number of co-eluting peaks and therefore reducing matrix

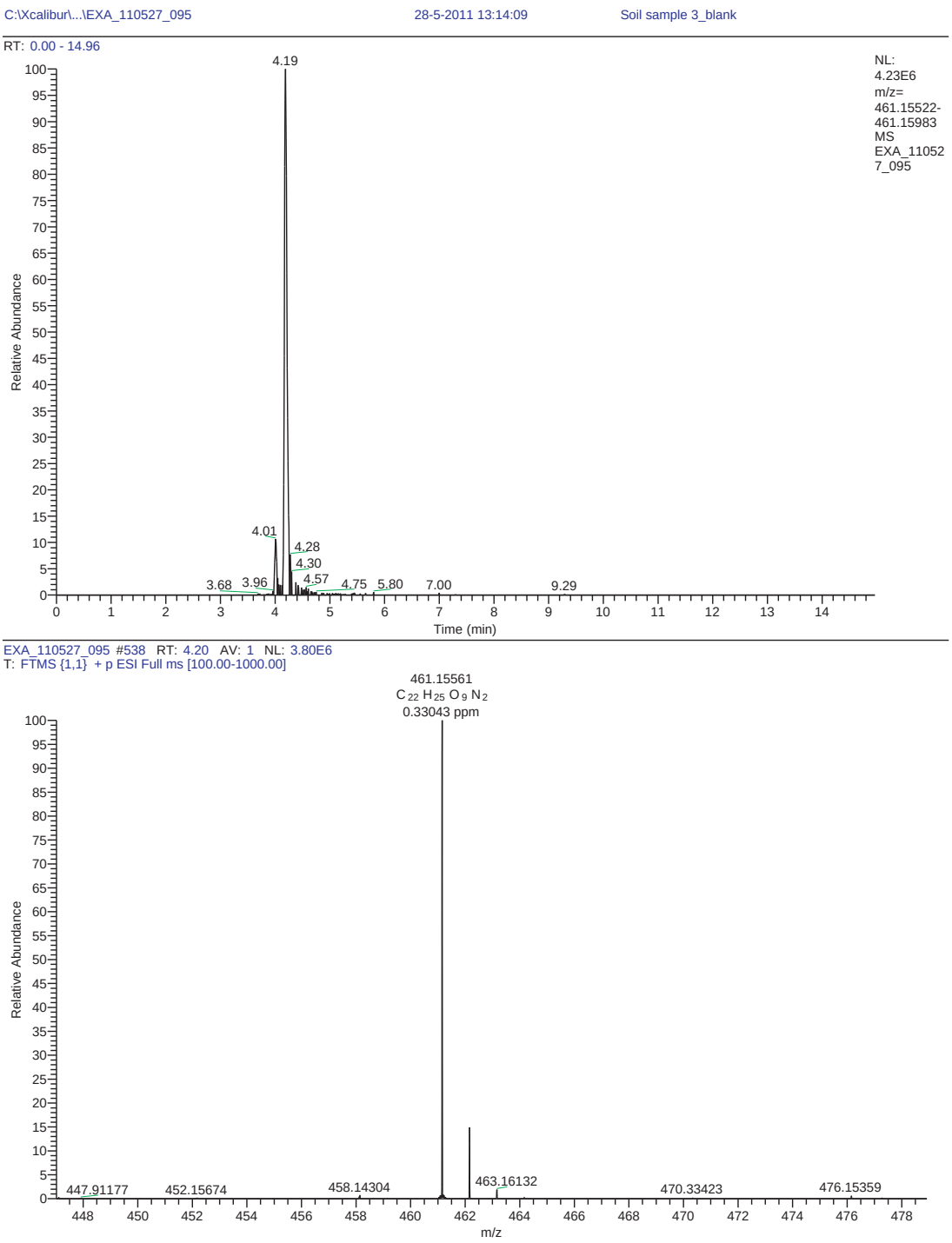

Fig. 5. The U-HPLC-Orbitrap-MS chromatogram and oxytetracycline spectra of suspect soil sample. The oxytetracycline ion was extracted from TIC using a 10 ppm mass window; positive ESI ionization, resolving power 50,000. $[M+H]^+ = 461.15543$; RT = 4.19; mass error Δppm = 0.33.

interference. Formic acid and ammonium formate, although having excellent ion pairing, solvating characteristics and being highly volatile, were used in this method development, in concentration of 160 $\mu L\,L^{-1}$ and 2 mM respectively, in both eluents (methanol and water).

3.5. MS analyses

New tendency in mass MS analysis, especially with regard to environmental monitoring programs, is post target and untargeted analysis, with offer the possibility of retrospective analysis of full-scan data, enabling laboratories to search for "unknown" contaminants after data recording. The ability of Exactive Orbitrap MS to provide full scan spectra is appropriate for post target screening, where all components eluting from chromatographic column are measured by MS and extracted afterwards from the total ion current, based on its exact mass. These characteristics allow increasing the number of detected compounds, theoretically to unlimited, without losses in sensitivity.

A common issue encountered in practice of multi-compound analysis from complex matrix, is the co-elution of the analytes with compound from matrix or even with each other. The

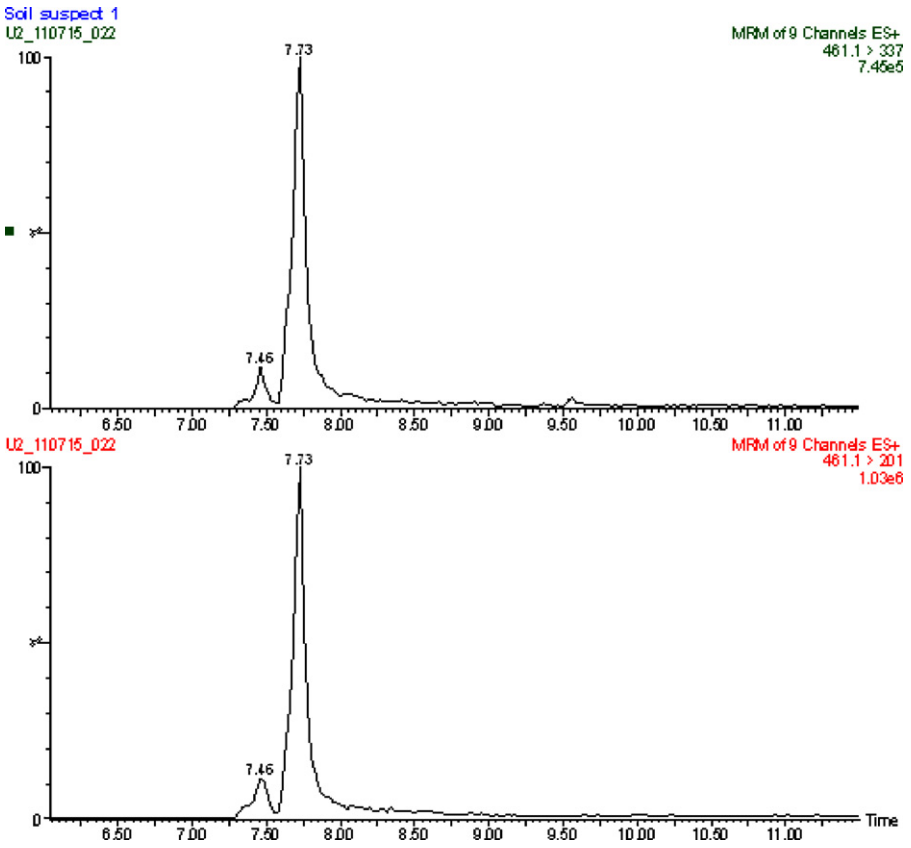

Fig. 6. LC–MS–MS chromatograms of the suspect soil sample. Track 1: m/z 461→201; RT = 7.73, peak area = 104717.06; track 2: m/z 461→337, RT = 7.73, peak area = 82964.38. For LC–MS–MS conditions, see Section 2.

differences between the exact masses of overlapping compounds, which become "visible" by applying a very narrow extraction mass window, the relative abundance and the width of the individual mass profiles, which is determined by the resolving power, are leading in the correct assignment of masses. Ultra high resolution of Exactive Orbitrap MS allows a significant sensitivity and selectivity improvement, comparable with MS triple–quadrupole [32].

In this experiment the ions were extracted by permitting a mass window of 10 ppm, and a time window of 10 s, leading in all peaks detection, without falls positives or negative results, even at low concentration of analytes. Mass accuracy was fully satisfactory, as shown in Table 3.

The MS was tuned in both ESI (+) and ESI (−) ionization mode. The positive ionization was preferred for all analytes with the exception of clofibric acid, dicloxacillin, prothioconazole, diclofenac and meclofenamic acid. The extracted ion chromatograms for some analyzed compound at 10 $\mu g\,kg^{-1}$ in soil and at 50 $\mu g\,kg^{-1}$ plant material samples are presented in Figs. 3 and 4.

4. Validation and sample analysis

The concentration of 100 $\mu g\,kg^{-1}$ (the trigger value for phase two of the risk assessment in EU regulation [7]), was set as a concentration to be relevant for the environment the screening target concentration was established at 50 $\mu g\,kg^{-1}$(1/2 of the interest value). Twenty samples of grass, crop and soil were spiked with the compounds of interest at the level of 50 $\mu g\,kg^{-1}$. These samples were analyzed by using the optimized ASE procedure for both matrices. After analyses the total ion chromatograms were checked for containing all relevant ions (see Table 1). When ≤1 sample is negative for a specific compound than the β-error at 50 $\mu g\,kg^{-1}$ is ≤5% [25]. This criteria of the β-error is set by the EU for the validation of screening methods for pharmaceutical compounds in products of animal origin [24,25].

For soil sample the evaluation of the result proves that the detection capability CCβ (concentration at with β error 5%) is lower than 50 $\mu g\,kg^{-1}$ for all analyzed compounds. For plant material sample the evaluation of the result proves that the detection capability CCβ is lower than 50 $\mu g\,kg^{-1}$ for all analyzed compounds, with the exception of itraconazole, fluquiconazole and prothioconazole. The results of validation study, including maximum recovery, CCβ, average and standard deviation of mass error (Δppm), are presented in Table 3. Validation was based on real sample which may be contaminated. Not unexpectedly, because of the intensive usage in veterinary medicine, one soil sample was founded contaminated with oxytetracycline. The soil contamination seems to be related to agricultural manuring practice (in The Netherlands manure application is allowed from March from September). The contaminated area was a field planted with corn from Harskamp and sampling was carry out in May. This finding is supported by other studies, where this substance was measured in soil one year after application [33], which could be explained by persistence and highly sorption characteristics of this compound.

For confirmatory analyses the extract of the suspect sample was reinjected in a triple quadrupole MS/MS system (Micromass Quattro Ultima), coupled with an HPLC system. Data acquisition was made in multiple reactions monitoring mode. The transitions of two product ions were monitored: m/z 461→201, and m/z 461→337. The relative intensity of ions for the sample (80%) was within ±20% of value obtained for standards (82%), complies the requirements of Directive 2000/656/EC [26]. The level of contamination was higher than 50 $\mu g\,kg^{-1}$. Figs. 5 and 6 present the U-HPLC-Orbitrap-MS and LC–MS–MS chromatogram of oxytetracycline in suspect soil sample.

The method presented in this study, which include an ASE and a SPE step of sample preparation, followed by U-HPLC high mass accuracy MS procedure is efficient for detection of selected compounds. Very high quality of data provided by the Exactive Orbitrap MS demonstrated the potential application for multi-residue analyses. The method was already applied to some real-world sample material. Oxytetracycline was detected in one soil sample, indicates that farming activities probably affect the contamination of the area by pharmaceutical compounds.

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