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Application of a quick, easy, cheap, effective, rugged and safe-based method for the simultaneous extraction of chlorophenols, alkylphenols, nitrophenols and cresols in agricultural soils, analyzed by using gas chromatography–triple quadrupole-mass spectrometry/mass spectrometry

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ABSTRACT

Due to the different physico-chemical properties of phenols, the development of a methodology for the simultaneous extraction and determination of phenolic compounds belonging to several families, such as chlorophenols (CPs), alkylphenols (APs), nitrophenols (NTPs) and cresols is difficult. This study shows the development and validation of a method for the analysis of 13 phenolic compounds (including CPs, APs, NTPs and cresols) in agricultural soils. For this purpose, a quick, easy, cheap, effective, rugged and safe (QuEChERS)-based procedure was developed, validated and applied to the analysis of real samples. A derivatization step prior to the final determination by gas chromatography (GC) coupled to a triple quadrupole analyzer operating in tandem mass spectrometry (QqQ-MS/MS) was performed by using acetic acid anhydride (AAA) and pyridine (Py). The optimized procedure was validated, obtaining average extraction recoveries in the range 69–103% (10 $\mu g\,kg^{-1}$), 65–98% (50 $\mu g\,kg^{-1}$), 76–112% (100 $\mu g\,kg^{-1}$) and 76–112% (300 $\mu g\,kg^{-1}$), with precision values (expressed as relative standard deviation, RSD) $\leq$ 22% (except for 4-chlorophenol) involving intra-day and inter-day studies. Furthermore, 15 real soil samples were analyzed by the proposed method in order to assess its applicability. Some phenolic compounds (e.g. 2,4,6-trichlorophenol or 4-tert-octylphenol) were found in the samples at trace levels (<10 $\mu g\,kg^{-1}$).

1. Introduction

The presence of phenolic compounds in agricultural soils is due to different sources. They can be found in formulations of phytosanitary products [1] or biocides [2], they can appear as transformation products of some pesticides and herbicides [3], by their formation in the atmosphere [4], by anthropogenic emissions [5] or by the use of wastewater effluents treated with aerobic or anaerobic microorganisms [6]. Some phenols show high toxicity (e.g. chlorophenols, CPs) [2] and estrogenic, anti-androgenic and vasodilatory activity (e.g. nitrophenols, NTPs) [4]. Furthermore, they can mimic natural hormones by interaction with the estrogenic receptors acting as endocrine disrupters (e.g. alkylphenols, APs) [7].

Nowadays, the intensive agriculture industry is taking great efforts to reduce the use of phytosanitary products and the contamination generated by their residues by the application of integrated pest management programs (IPMs), which significantly reduce the amount of applied pesticides [8]. However, the relatively high stability of some of these compounds enables their persistence in soil as residues [9] due to their application during decades. The monitoring of the levels of phenolic compounds in soils is therefore of high interest.

Phenolic compounds can be classified into a variety of families showing very different physico-chemical properties. The most studied families are CPs, NTPs, cresols (phenols with methyl groups ortho, meta or para-substituted) and APs [10]. The United States Environmental Protection Agency (US EPA) classifies CPs, NTPs and APs as priority pollutants [11]. Moreover, the Spanish legislation has established maximum residue levels (MRLs) for 2,4,6-trichlorophenol (2,4,6-triCP, 0.9 $mg\,kg^{-1}$), 2,4,5-trichlorophenol (2,4,5-triCP, 10 $mg\,kg^{-1}$), 2,4-dichlorophenol (2,4-diCP, 0.1 $mg\,kg^{-1}$), 2-chlorophenol (2-CP, 1 $mg\,kg^{-1}$) and pentachlorophenol (PCP, 0.01 $mg\,kg^{-1}$) in different types of soils [12]. Therefore, the development of analytical methodologies for

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the simultaneous determination of phenols belonging to different groups (namely, CPs, NTPs, cresols and APs) is needed in order to provide a complete overview of the contamination of agricultural soils by these types of compounds (specially those soils working under IPMs) in accordance with current legislation.

A variety of extraction techniques have been reported in the literature for the analysis of phenols in soils, although most of them have only been used for the simultaneous analysis of one or few phenols belonging to the same family. For instance, ultrasonic assisted extraction (USE) has been applied for the analysis of 4-tert-octylphenol (4-tertOP) [13], stir bar sorptive extraction (SBSE) for CPs and APs [13], Soxhlet extraction for nonylphenol (NP) [14], CPs and APs [15], and pressurized liquid extraction (PLE) for CPs, NTPs, cresols [16] or APs [17]. In the last few years, a new procedure so-called QuEChERS method (acronymic name from quick, easy, cheap, effective, rugged and safe) [18] has been applied for the simultaneous extraction of pesticides with a wide polarity range (polar and non-polar pesticides) from fruits and vegetables. This method consists in an extraction with acidified acetonitrile followed by an induced liquid–liquid partition after the addition of salts. The QuEChERS method has a low cost per sample and the possibility of increasing the sample throughput as well as other advantages defined by its name. Although this procedure has been used for the extraction of other compounds (e.g. pesticides or mycotoxins) from several types of matrices, such as milk [19] and soil [20], up to our knowledge, this method has not been applied for the extraction of different families of phenols from soils.

In relation to the determination techniques, different approaches have been reported for the analysis of phenols, although gas chromatography (GC) [21–23] and liquid chromatography (LC) [17,24,25] have been mainly used. Currently, the use of GC and LC coupled to mass spectrometry detectors (MS) [26,27] is widespread in environmental analysis because they provide high selectivity and sensitivity [28], especially when operating in the selected-reaction monitoring (SRM) mode [29].

Phenol analysis by GC coupled to MS working in tandem mass spectrometry (MS/MS) is difficult due to the polarity of some of these compounds (e.g. 2-CP, $\log K_{OW} = 2.17$ or 2-nitrophenol (2-NTP) $\log K_{OW} = 1.77$), resulting in poor chromatographic peaks [13]. Therefore, a derivatization step is mandatory to improve the chromatographic performance and sensitivity, offering better results than LC–MS/MS, which normally does not include a derivatization step [25].

The most used derivatization reagents are bis(trimethylsilyl) trifluoroacetamide (BSTFA) [30], pentafluorobenzyl chloride (PFBCl) [31], pentafluorobenzyl bromide (PFBBr) [32] and acetic acid anhydride (AAA) [33]. Nevertheless, certain derivatizing agents, such as BSTFA, are prone to degradation in aqueous medium, they are expensive (e.g. PFBBr, PFBCl) or they require long derivatization times (e.g. BSTFA). In this sense, the use of AAA in basic conditions (e.g. K_2CO_3, NaOH, pyridine, (Py)) offers several advantages since the derivatization reaction can take place at room temperature in a few minutes (even in water) [22], and it is cheaper than the other derivatizing reagents.

In this study, a new method has been developed for the simultaneous extraction and determination of different phenolic families in agricultural soils, namely, CPs, NTPs, cresols and APs. A QuEChERS-based procedure was applied for the extraction stage using AAA with Py as derivatization reagents. The final determination was carried out by GC–MS/MS. Finally, the developed method was fully validated and applied for the analysis of real soil samples from intensive agricultural areas working under IPM systems in Southern Spain.

2. Experimental

2.1. Chemicals and materials

2-NTP, 4-nitrophenol (4-NTP), 2,4-dimethylphenol (2,4-DMP), 2-CP, 4-chlorophenol (4-CP), 2,4-diCP, 2,4,5-triCP, 2,4,6-triCP and 4-n-nonylphenol (4-NP) were obtained from Fluka (Buchs, Switzerland). 3-Nitrophenol (3-NTP), 4-chloro-3-methylphenol (4-C-3-MP), 4-tertOP and PCP were supplied by Supelco (Bellefonte, PA, USA). Purities were always >97%. A standard solution (100 mg L^{-1}) of isotopically labeled pentachlorophenol, [$^{13}C_6$]-PCP, was used as internal standard (IS) and it was obtained from Dr. Erhenstofer (Augsburg, Germany). Stock standard solutions of the individual compounds (with concentrations ranging from 200 to 450 mg L^{-1}) were prepared by exact weighing of the powder or liquid and dissolved in 50 mL of acetone. These solutions were then stored under refrigeration ($T < 5\,^{\circ}C$). A working standard solution of the 13 phenolic compounds (2 mg L^{-1} of each compound) was prepared by appropriate dilution of the stock solutions with acetone, and it was stored under refrigeration ($T < 5\,^{\circ}C$). A working standard solution of [$^{13}C_6$]-PCP (22 mg L^{-1}) was prepared by appropriate dilution of the stock solution with acetone and stored under the aforementioned conditions. AAA (99.9%) and Py (99.8%) were purchased from Sigma–Aldrich (Madrid, Spain). Triethylamine (>99%) and glacial acetic acid (99.7%) were obtained from Riedel de Haen (Seelze-Hannover, Germany) and Panreac (Barcelona, Spain), respectively. Acetone, *n*-hexane, ethyl acetate (EtOAc), methanol (MeOH), sodium acetate (NaOAc) and sodium chloride (NaCl) were obtained from J.T. Baker (Deventer, Netherlands), cyclohexane from Fluka, acetonitrile (AcN) from Merck (Darmstadt, Germany), and isopropanol and anhydrous magnesium sulphate ($MgSO_4$) from Panreac. Hydromatrix was supplied by Varian (Palo Alto, CA, USA). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). 50-mL polypropylene tubes and 2-mL microtubes were available for extraction purposes. 30-mm cellulose filters were obtained from Whatman (Maidstone, England).

2.2. Apparatus

A GC system Varian 3800 (Varian Instruments, Sunnyvale, CA, USA) equipped with electronic flow control was interfaced to a 1200 L triple quadrupole (QqQ) mass spectrometer. Samples were injected into an SPI/1079 split/splitless programmed-temperature injector using a Combi Pal (CTC Analytics AG, Zwingen, Switzerland) with a 100-μL syringe. A fused-silica untreated capillary column (2 m × 0.25 mm i.d.) from Supelco was used as a pre-column connected to a VF-5 ms Factor Four capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) purchased from Varian. Helium was used as carrier gas (99.9999%) at a constant flow rate of 1 mL min^{-1}, and argon (99.999%) was used as collision gas. The mass spectrometer was operated in electron ionization (EI) at 70 eV. The mass spectrometer was calibrated every four days with perfluorotributylamine. Varian Workstation software was used for instrument control and data analysis.

A Reax-2 rotary agitator from Heidolph (Schwabach, Germany) was used for extraction in the optimized method. Centrifugations were performed in a high-volume centrifuge from Centronic (Barcelona, Spain). Other apparatus also used during the optimization of the extraction process are described. PLE was performed using an ASE 100 accelerated solvent extraction system (Dionex, Sunnyvale, CA, USA) equipped with 34-mL stainless steel extraction cells. Extractions using a high-speed homogenizer were performed using a Polytron (mod. PT2100, Kinematica A.G., Littan/Luzern, Switzerland). Ultrasonic extractions were carried out in a J.P. Selecta sonication bath (Selecta, Barcelona, Spain).

Table 1
GC–QqQ-MS/MS conditions for the derivatized phenols.

Compound	Family	M.W.[a] (amu)	M.W. of derivatized compound (amu)	RTW[b] (min)	Segment	SIM ion (*m/z*)[c]	Precursor ion (*m/z*)	Product ions, *m/z* (collision energy, eV)[d]
2-CP	CP	128.5	170.5	7.60–7.88	1	170	128	92 (10), 100 (5)
4-CP	CP	128.5	181.0	7.86–8.15	1	170	128	65 (15), 100 (5)
2,4-DMP	Cresol	122.0	164.0	7.88–8.15	1	164	122	77 (20), 107 (5)
4-C-3-MP	Cresol	142.5	184.5	8.60–8.84	2	184	142	77 (10), 79 (5)
2,4-DiCP	CP	163.0	205.0	8.68–8.92	2	205	162	98 (15), 126 (10)
2-NTP	NTP	139.0	181.0	8.97–9.21	2	181	139	81 (10), 109 (10)
2,4,6-TriCP	CP	197.5	239.5	9.32–9.55	2	239	196	132 (15), 160 (10)
3-NTP	NTP	139.0	181.0	9.38–9.62	2	181	139	81 (5), 93 (5), 111 (10)
4-NTP	NTP	139.0	181.0	9.54–9.77	2	181	139	93 (15), 109 (5)
2,4,5-TriCP	CP	197.5	239.5	9.71–9.94	2	239	196	97 (25), 132 (15), 160 (5)
4-TertOP	AP	206.0	248.0	10.82–11.10	3	248	135	77 (20), 95 (10), 107 (5)
PCP	CP	266.5	308.5	11.54–11.78	3	308	266	167 (20), 202 (10), 230 (10)
[$^{13}C_6$]-PCP	CP (IS)[e]	272.2	314.2	11.60–11.72	3	314	272	172 (25)
4-NP	AP	220.0	262.0	12.28–12.52	3	262	107	77 (30), 81 (15), 95 (10)

[a] Molecular weight.
[b] Retention time window.
[c] Ions used for identification/confirmation of derivatized compounds.
[d] Ions used for quantification of phenolic compounds.
[e] IS: Internal standard.

An analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) and a rotary evaporator R-114 (Büchi, Flawil, Switzerland) were used during extraction and standard preparation.

2.3. Sampling

Soil samples were collected from agricultural areas working under IPM practices in Almeria province (Southern Spain). They were dried at room temperature during two days and sieved, obtaining particle sizes <2 mm. Finally, the soil was stored at 4 °C. Phenol free samples obtained from an organic farm were checked by our laboratory and they were used for the optimization and validation procedure.

2.4. GC–QqQ-MS/MS

Aliquots of 10 μL were injected into the GC system operating at a syringe injection flow rate of 10 μL s^{-1}. The injector temperature program was as follows: 70 °C (hold for 0.5 min) → 310 °C (100 °C min^{-1}, hold for 10 min). The injector split ratio was initially set at 10:1. Splitless mode was switched on at 0.5 min until 3.5 min. At 3.5 min, the split ratio was 100:1 and at 10 min, 20:1. The column oven program was as follows: 70 °C (hold for 3.5 min) → 300 °C (20 °C min^{-1}) → 300 °C (hold 4 min). Cryogenic cooling with CO_2 was applied when the injector temperature was 170 °C in order to reach the initial injector temperature as fast as possible before continuing with the next injection. The total running time was 19 min.

The QqQ mass spectrometer was mainly operated in the SRM mode, although some SIM reactions were also monitored for confirmation purposes. The electron multiplier was set +200 V above the optimal value indicated by the software instrument. The temperatures of the transfer line, manifold, and ionization source were set at 300, 40, and 265 °C, respectively. The optimal values for the scan time were found to be 0.144 s (segment 1), 0.240 s (segment 2) and 0.132 s (segment 3). Peak widths of *m/z* 2.0 and 1.5 were set in the first (Q1) and third quadrupole (Q3), respectively. The optimized MS/MS parameters are indicated in Table 1.

2.5. QuEChERS extraction and derivatization procedure

10 g of sample were weighed into a 50-mL polypropylene tube. Then, 10 mL of AcN (acetic acid 1%, *v/v*) and 5 mL of Milli-Q water were added, and the mixture was shaken end-over-end for 1 h in a rotary shaker. Afterwards, 1.7 g of NaOAc, 6 g of $MgSO_4$ and 4 g of NaCl were added and the tubes were shaken immediately for 1 min. After centrifugation at 5000 rpm (4136 × *g*) for 5 min, 1.5 mL of the AcN layer were transferred into a 2-mL polypropylene microtube containing 0.75 g of $MgSO_4$ in order to remove residual water. 860 μL of extract was put into a 1-mL vial; then, 20 μL of [$^{13}C_6$]-PCP (IS), 20 μL of Py and 100 μL of AAA were added. The vial was vigorously shaken in a vortex for 1 min to carry out the derivatization reaction, which was performed at room temperature in a few seconds. 10-μL aliquots were then injected in the GC–QqQ–MS/MS system.

3. Results and discussion

3.1. GC–QqQ-MS/MS optimization and derivatization procedure

For the optimization of the chromatographic analysis and MS characterization, standard solutions of each phenol in acetone were used. It is well-known that underivatized phenols (except some APs) show poor peak shapes and sensitivity because of the hydroxyl polar group (Fig. 1). For this reason, their determination by GC–MS/MS requires a derivatization stage in order to minimize their polar character. Among the different derivatization reagents commented in previous sections, AAA in basic conditions was selected due to its advantages, such as simplicity or low-cost, but also because it can be carried out at room temperature in short times, which facilitate its application in routine analysis. During the derivatization reaction, the hydrogen atom from the –OH moiety is substituted by an acetyl group. Fig. 1 shows the substantial differences in peak shape and sensitivity observed for 4-NP (1a) and 2,4,6-triCP (1b) when the derivatization step is performed. Generally, the derivatized compounds present peaks with higher intensity and S/N ratios, which improve the sensitivity of the method. It is important to notice that the spectra of the derivatized and non-derivatized phenols are practically equal, and the ion corresponding to the derivatized phenol shows an extremely low relative abundance (Fig. 1). Identification of each compound was carried out by analysis of all derivatized compounds in the full scan mode. For extraction experiences and analysis of real samples, the molecular ions of the derivatized compounds (represented as *m/z* [M+42]) were used for the identification of each derivatized compound when sample and reference spectra were compared, and

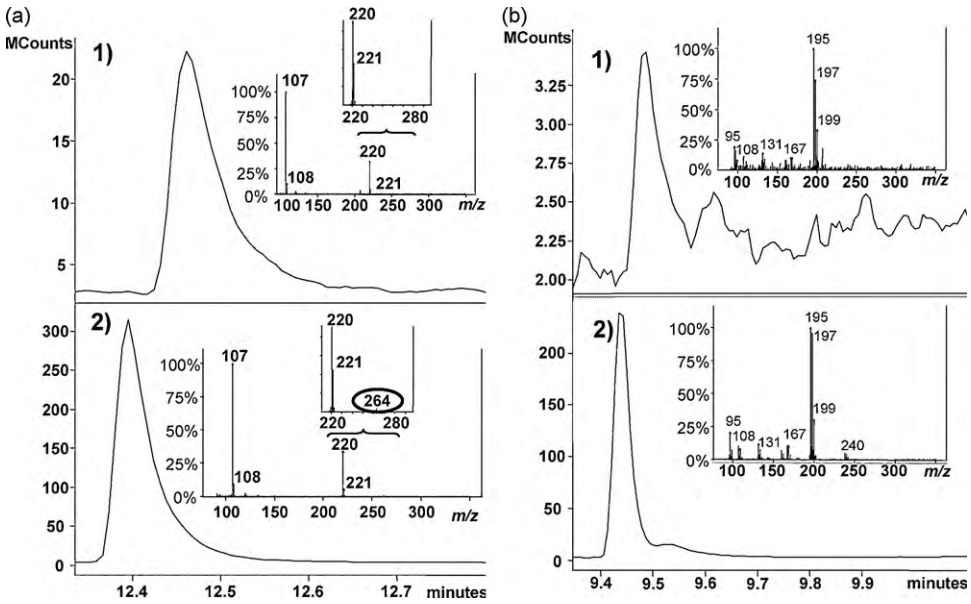

Fig. 1. Full scan chromatogram and the corresponding spectrum of: (a.1) 4-NP underivatized and (a.2) 4-N P derivatized, and (b.1) 2,4,6-triCP underivatized and (b.2) 2,4,6-triCP derivatized.

SRM ions were used for quantification (Table 1). As it can be seen in Fig. 1 a2 and b2, the spectra plots and retention times (differences lower than 0.1 min) are very similar for derivatized and underivatized compounds, which can make difficult to distinguish between both types of compounds. However, the fragments corresponding to *m/z* 264 and *m/z* 240 confirmed the presence of the derivatized 4-NP and 2,4,6-triCP, respectively. It must be noticed that 4-tertOP and PCP did not show the identification ion, *m/z* [M+42], which means that these compounds were not derivatized. Despite this, sensitivity was not affected, obtaining adequate signals and peak shapes.

For the derivatization step, the conditions described by Regueiro et al. [21] and Pérez-Pavón et al. [33] were considered as the basis for the following studies. For the optimization of the derivatization, different volumes of AAA (100, 200 and 300 μL) and Py (20, 40 and 60 μL) were tested, always keeping a constant ratio of AAA/Py (5:1, *v/v*). Py was selected to provide basic conditions instead of NaOH or K_2CO_3 because these salts are prone to form precipitates in organic solvents, which could increase the need for instrument maintenance or deterioration of certain chromatographic parts. The best results were obtained when 100 μL of AAA and 20 μL of Py (data not shown) were used. Besides, a study of the stability of the derivatized compounds was performed by injecting matrix-matched standards of the derivatized phenols at 200 μg L^{-1}, which were submitted to two different storage conditions: room temperature and −20 °C. In general, derivatized phenolic compounds are stable up to 4 days after storage at room temperature and at −20 °C (Fig. 2); as a consequence, samples were always injected within the following 3 days after the extraction.

Finally, a total ion chromatogram (TIC) of the derivatized phenolic compounds is shown in Fig. 3, observing that the use of MS/MS improves the sensitivity and selectivity of the method.

3.2. Extraction method

As it has been commented, sample preparation is critical during the development of multi-class methods of phenols due to their different structure and polarity. With this goal, several extraction methods were evaluated in order to develop simultaneous extraction conditions for CPs, NTPs, APs and cresols. Three solid–liquid extraction techniques (SLE) were evaluated with fortified samples at 25 μg kg^{-1}: PLE, USE and SLE using high-speed homogenizers (Polytron). APs, 2,4,5-triCP and 2,4,6-triCP were recovered with optimum values when PLE was used, whereas only APs were recovered satisfactorily with USE, and APs and 4-CP showed adequate results when SLE using Polytron was applied. Eventually, despite that Soxhlet extraction was initially discarded because of its high solvent-consumption and analysis time, it was also tested, obtaining acceptable results for APs, PCP, 2-CP, 4-CP, 2,4,5-triCP and 2,4-diCP. Different solvents showing a wide range of polarity, such as acetone, *n*-hexane, acetone/*n*-hexane (1:1, *v/v*), cyclohexane, isopropanol (triethylamine 5%, *v/v*), AcOEt, MeOH, AcOEt/MeOH (3:1, *v/v*) and AcN were tested in the aforementioned extraction techniques. In spite of that these solvents and techniques have been previously applied in phenol analysis or in wide-scope pesticide analysis, satisfactory results were not achieved for all the families under study, with recoveries around 40–50%, except for APs, whose recoveries were in the range of 70–120% in all cases. Table 2 shows a summary of the efficiencies of the extraction methods tested for the selected compounds. It can be observed that PLE, USE, SLE using Polytron and Soxhlet are only valid for the extraction of APs and

Table 2
Recovery values (%) obtained for the different SLE procedures tested on the extraction of phenols from soils (spiked samples at 25 μg kg^{-1}).

Compound	PLE[a]	Soxhlet	USE[b]	Polytron	QuEChERS[c]
2-CP	50	69	54	32	90
4-CP	64	83	36	62	102
2,4-DMP	34	39	22	N.E.[d]	101
4-C-3-MP	77	67	45	13	100
2,4-DiCP	69	71	25	44	103
2-NTP	15	N.E.[d]	N.E.[d]	24	96
2,4,6-TriCP	80	55	38	35	107
3-NTP	36	N.E.[d]	28	17	84
4-NTP	22	N.E.[d]	27	7	116
2,4,5-TriCP	69	86	47	17	108
4-TertOP	73	69	71	43	97
PCP	N.E.[d]	71	344	8	102
4-NP	69	76	82	48	99

[a] PLE: pressurized-liquid extraction.
[b] USE: ultrasonic assisted extraction.
[c] QuEChERS: acronymic name from quick, easy, cheap, effective, rugged and safe.
[d] N.E.: not extracted.

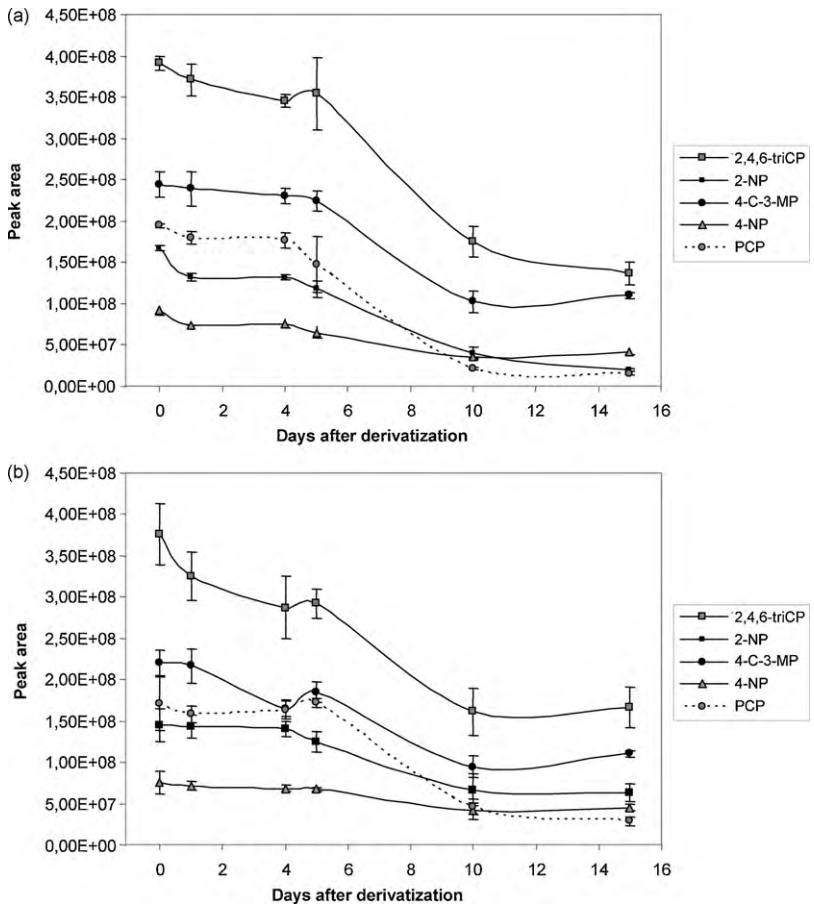

Fig. 2. Study of the stability of the representative derivatized phenols in two storage conditions: (a) room temperature and (b) −20 °C.

some CPs, but they are not able to achieve the simultaneous extraction of APs, CPs, NTPs and cresols, which is the main aim of this study.

In order to improve the results obtained with the previous techniques, the QuEChERS method was then evaluated. This procedure has been widely used for the simultaneous extraction of compounds with a wide polarity range, such as pesticides. This characteristic let us to evaluate its possible application to multifamily analysis of phenols in soil. Two different experimental conditions were checked, based on the official methods developed in Europe (citrate-based method) [34] and the US (acetate based-method) [35]. The citrate-based method was performed as follows: 10 g of soil spiked with 20 $\mu g\,kg^{-1}$ of phenolic compounds, were extracted with 10 mL of AcN (acetic acid 1%, *v/v*) and 5 mL of Milli-Q water, and shaken end-over-end for 1 h. Then, 0.5 g of disodium citrate, 1 g of trisodium citrate, 4 g of $MgSO_4$ and 4 g of NaCl was added. The mixture was centrifuged at 5000 rpm (4136 × *g*) during 5 min. Finally, a 1.5-mL aliquot was transferred to a 2-mL microtube containing 0.75 g of $MgSO_4$ to ensure the complete removal of water. On the other hand, the acetate-based method was applied as described for the citrate method but adding a different mix of salts: 1.7 g of NaOAc, 4 g of $MgSO_4$ and 4 g of NaCl. The best results were obtained with the acetate-based method (Fig. 4), but when these conditions were evaluated at different days, high recoveries (>120%) were found for most of the analytes (data not shown). A possible explanation of the high recovery values observed in the acetate-based method is due to an insufficient amount of $MgSO_4$ used in the partition step, which may cause an incomplete removal of water. Since an additional amount of $MgSO_4$ was added in the microtube, the remaining water could be then absorbed by the salt, reducing the aliquot volume and increasing the concentration of the phenolic compounds and their recovery values. Consequently, the acetate-based method was modified by adding more amount of $MgSO_4$ (from 4 to 6 g total) to ensure the complete removal of water (Fig. 4).

Bearing in mind that the optimized QuEChERS method does not require an evaporation step, contrary to the extraction techniques mentioned above (PLE, Soxhlet, etc.), the performance of concen-

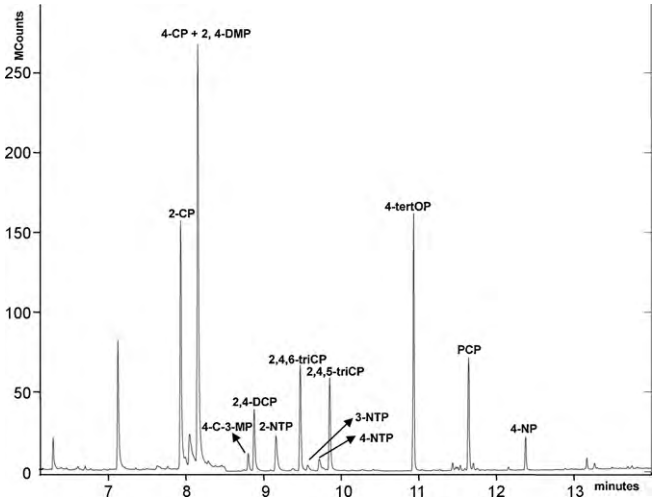

Fig. 3. Total ion chromatogram (TIC) of a soil sample spiked with the target compounds (200 $\mu g\,kg^{-1}$).

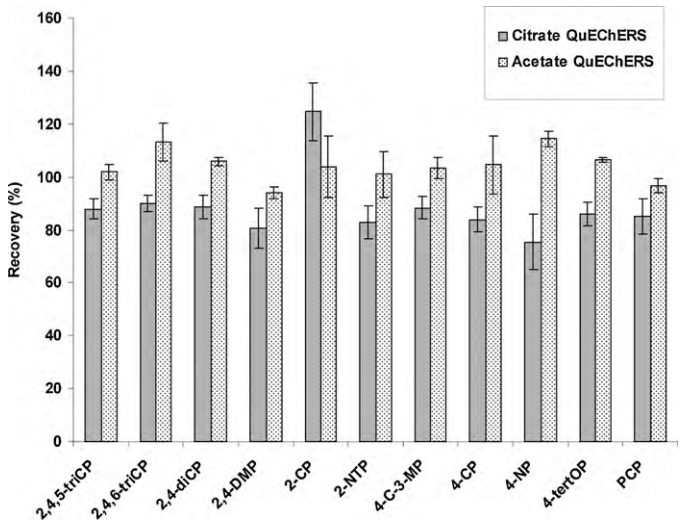

Fig. 4. Recovery results obtained after the application of two different QuEChERS-based procedures for the extraction of phenols from soils (spiked samples at 25 μg kg^{-1}).

tration and/or evaporation stages could explain the low recoveries obtained with those techniques for many phenols. Besides, this new approach demonstrates the suitability of the QuEChERS method for the simultaneous extraction of different phenol families.

3.3. Method validation

A validation protocol of the optimized procedure was carried out in order to establish the performance characteristics of the method, ensuring the adequate identification, confirmation and quantification of the target compounds. Several parameters such as linearity, trueness (expressed as recovery), intra-day precision, inter-day precision, limits of detection (LODs) and quantification (LOQs) were studied.

Identification and confirmation of the target compounds in GC–QqQ-MS/MS was performed by the use of retention time windows (RTWs), which were defined as the retention time (RT) average plus or minus 3 times the standard deviation (SD) of the RT (RT ± 3 SD) when five spiked samples at 50 μg kg^{-1} were injected. Confirmation in GC–QqQ-MS/MS was carried out by comparing the sample spectrum with a reference spectrum obtained with a spiked blank soil sample (mid-level calibration standard). Then, a forward search compared both spectra, obtaining a value named FIT ranging from 1 to 1000 (arbitrary units, a.u.). FIT ≥ 700 (a.u.) was used to confirm the identity of the compound.

For the quantification of the target compounds, it must be taken into account that soil is a complex matrix that has a large amount of compounds that can interfere in the analyte signal, providing matrix effect. Consequently, this effect was studied to ensure bias-free analytical results, analyzing standard solutions of the phenolic compounds in solvent (from 1 to 300 μg L^{-1}) and matrix-matched standards at the same concentrations. The calibration curves obtained using matrix-matched standards were significantly different from that obtained by the use of standard solutions in solvent observing a matrix effect for all compounds (data not shown). Therefore, in order to compensate this effect, matrix-matched standard calibration was used for quantification purposes.

Linearity was evaluated in the range 10–300 μg L^{-1} except for 2-NTP and 4-C-3-MP (range 50–300 μg L^{-1}), and 3-NTP and 4-NTP (range 100–300 μg L^{-1}). Linear calibration graphs were plotted by least-squares regression of relative peak area (analyte/IS) versus concentration of the calibration standards. Determination coefficient (R^2) ranged from 0.9841 to 0.9966. (Table 3). Bearing in mind the limits established for some phenolic compounds, if a sample presents a concentration higher than the upper limit, it must be diluted in order to work within the linear range of the proposed method and increase the reliability of the quantification process.

Trueness was estimated in terms of recovery by evaluating four different concentration levels in a wide range (10, 50, 100 and 300 μg kg^{-1}), due to the variety of MRLs established by legislation [12], except for 2-NTP and 4-C-3-MP (50, 100 and 300 μg kg^{-1}) and 3-NTP and 4-NTP (100 and 300 μg kg^{-1}). Five blank soil samples were processed at each fortification level. Recoveries (Table 3) were in the range 69% (2,4-DMP)-103% (2-CP) at 10 μg kg^{-1}, 65% (4-CP)-98% (2,4,5-triCP) at 50 μg kg^{-1}, 91% (2-NTP)-113% (2,4,6-triCP) at 100 μg kg^{-1}, and 76% (4-CP)-102% (4-tertOP) at 300 μg kg^{-1}. At the lower concentration level (10 μg kg^{-1}), some phenols did not show adequate recoveries values (e.g. NTPs and 4-C-3-MP). Nevertheless, considering the maximum permitted concentrations defined by the current legislation, the method was still fitted to purpose. In the

Table 3
Validation parameters of the optimized method.

Compounds	Linearity range (μg kg^{-1})	Linearity (R^2)	Recovery (RSD intra-day, %)[a]				RSD inter-day (%)[b]		LOD (μg kg^{-1})	LOQ[c] (μg kg^{-1})
			10 μg kg^{-1}	50 μg kg^{-1}	100 μg kg^{-1}	300 μg kg^{-1}	10 μg kg^{-1}	50 μg kg^{-1}		
2-CP	10–300	0.9948	103 (16)	86 (8)	102 (5)	95 (11)	11	11	3	5
4-CP	10–300	0.9939	71 (**29**)[d]	65 (7)	96 (11)	76 (11)	16	12	3	5
2,4-DMP	10–300	0.9965	69 (15)	89 (10)	100 (9)	80 (12)	13	14	3	10
4-C-3-MP	50–300	0.9841	N.Q.[e]	82 (3)	101 (4)	102 (3)	N.Q.	7	10	50
2,4-DiCP	10–300	0.9966	81 (13)	77 (10)	104 (5)	94 (3)	11	7	5	10
2-NTP	50–300	0.9927	N.D.[f]	80 (8)	91 (12)	87 (8)	N.D.	15	20	50
2,4,6-TriCP	10–300	0.9953	102 (9)	95 (7)	113 (2)	94 (2)	7	13	3	10
3-NTP	100–300	0.9845	N.D.	N.Q.	78 (22)	99 (3)	N.D.	24[g]	50	100
4-NTP	100–300	0.9915	N.D.	N.Q.	110 (20)	87 (15)	N.D.	21[g]	20	100
2,4,5-TriCP	10–300	0.9934	90 (14)	98 (3)	108 (4)	98 (4)	8	17	3	10
4-TertOP	10–300	0.9917	98 (4)	97 (3)	94 (2)	102 (4)	14	10	0.1	5
PCP	10–300	0.9864	102 (6)	96 (2)	94 (5)	99 (2)	18	9	0.5	1
4-NP	10–300	0.9956	89 (12)	90 (1)	98 (10)	99 (9)	16	10	1	5

[a] $n = 5$; RSD: relative standard deviation.
[b] $n = 5$.
[c] In order to simplify the routine quality controls, the LOQ was established at 10 μg kg^{-1} for all compounds, except for 4-C-3-MP (50 μg kg^{-1}), 2-NTP (50 μg kg^{-1}), 3-NTP (100 μg kg^{-1}) and 4-NTP (100 μg kg^{-1}).
[d] Figures in bold indicate that the values are outside the limits (recovery and precision) established in the validation requirements.
[e] NQ: Not quantified.
[f] ND: Not detected.
[g] Estimated at 100 μg kg^{-1}.

case of PCP, whose MRL set by legislation is 10 μg kg^{-1}, adequate performance values were obtained (Table 3).

Precision (expressed as relative standard deviation, RSD) was evaluated by performing intra-day and inter-day precision studies. Intra-day precision was studied analyzing 5 spiked soil samples, which were extracted during the same day, at the same concentration levels evaluated in the recovery studies for each compound. In this case, RSD values ranged from 1% (4-NP) to 29% (4-CP) for the selected compounds. Inter-day precision was studied by analyzing 5 spiked samples at the lowest concentrations (10 and 50 μg kg^{-1}) for all compounds, except for 2-NTP and 4-C-3-MP (50 μg kg^{-1}) and 3-NTP and 4-NTP (100 μg kg^{-1}), and extracted in different days. The range of RSD obtained was 7% (2,4,6-triCP)-18% (PCP) at 10 μg kg^{-1} and 7% (4-C-3-MP)-17% (2,4,5-triCP) at 50 μg kg^{-1}.

LODs and LOQs were estimated analyzing blank spiked samples at decreasing concentrations. Both were calculated as the concentrations for which S/N ratios were 3 and 10, respectively. The ranges of LOD and LOQ in soils for phenols were from 1 to 100 μg kg^{-1}. However, bearing in mind that certain phenolic compounds did not show adequate performance characteristics at low concentrations, and in order to simplify the subsequent routine quality controls, the LOQ was established at 10 μg kg^{-1} for all compounds (except for 4-C-3-MP, 2-NTP, 3-NTP and 4-NTP, whose LOQ was set at 50, 50, 100 and 100 μg kg^{-1}, respectively), assuring that recoveries ranged from 70% to 110% and precision was lower than 20% [36]. It is important to notice that the established limits were referred to concentrations in soil prior to the extraction procedure, and consequently, they could be described as method detection and method quantification limits (MDL, MQL). It must be emphasized that the established LOQs allow the determination of the target phenols below the maximum permitted concentrations in the current legislation.

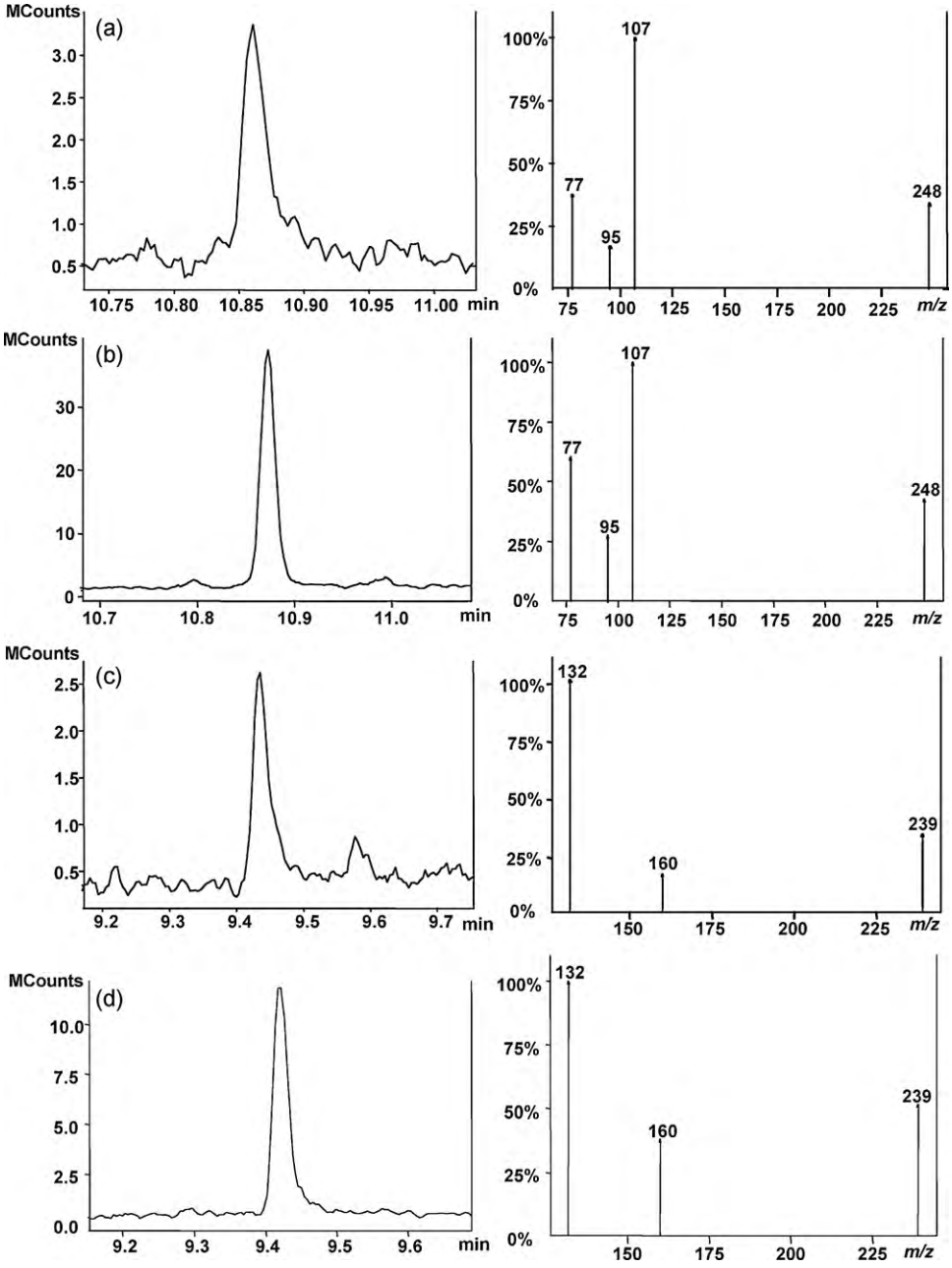

Fig. 5. SRM chromatogram and the corresponding spectrum of: (a) 4-tert OP in a real soil sample; (b) 4-tertOP in a 20 μg L^{-1} matrix-matched standard; (c) 2,4,6-triCP in a real soil sample and (d) 2,4,6-triCP in a 20 μg L^{-1} matrix-matched standard.

4. Application to real samples

The developed method was applied for the analysis of 15 real different agricultural soil samples. In order to avoid errors and assure the quality of the results, an internal quality control (IQC) was carried out. This IQC was based on the use of a blank extract, that eliminated false positives caused by a contamination in the extraction procedure or by the presence of a interference; a reagent blank (obtained by performing the whole procedure without sample), which removed any possibility of false positive due to contamination in the instruments or reagents employed; a spiked blank sample at 50 $\mu g\,kg^{-1}$ except for 3-NTP and 4-NTP (100 $\mu g\,kg^{-1}$), to assess the extraction efficiency; and a matrix-matched standard calibration curve to check linearity and sensitivity.

Traces of phenols were detected in five of the analyzed samples. Less than 3 phenols were detected simultaneously in the same soil sample except in one sample, which showed traces of 2,4,6-triCP, 2-CP and 4-tertOP. 2,4,5-triCP, 2,4,6-triCP and 4-tertOP were detected in two samples, whereas 2-CP was only detected in one soil. Fig. 5 shows two phenols (2,4,6-triCP and 4-tertOP) detected in two real samples at concentrations below the corresponding LOQ.

5. Conclusions

A new method has been developed for the simultaneous extraction of 13 phenolic compounds, including CPs, NPs, APs and cresols, in a single extraction. Typical SLE techniques are not adequate for the simultaneous extraction of a wide range of phenols. A QuEChERS-based procedure was applied for the extraction without any evaporation step and a simple and fast derivatization stage using AAA and Py was performed before the chromatographic determination of these compounds by GC–QqQ-MS/MS. The use of a simultaneous extraction step for the different families allows the reduction of sample-handling and sample pre-treatment, increasing sample throughput, because a large number of samples can be extracted simultaneously. The proposed method has been validated allowing a reliable determination of the selected phenols with recoveries in the range 65–113%. Inter-day precision, expressed as RSD were in the range 7–24%. The MDLs and MQLs achieved allow the analysis of the selected phenols with adequate results at concentrations lower that the maximum levels set by the current legislation. Phenolic compounds such as 4-NP, 4-tertOP and PCP were validated with LOQs < 10 $\mu g\,kg^{-1}$, which is the lowest MRL established [12]. This method has been validated for agricultural soils and was also applied to 15 real samples. Phenolic compounds were not found with concentration over the MQL. Some compounds, such as 2,4,6-triCP and 4-tertOP, were found at levels under the LOQ established in the method.

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