Article

Multiresidue Determination and Uncertainty Analysis of Pesticides in Soil by Ultrafast Liquid Chromatography Coupled to Mass Spectrometry

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Este trabalho apresenta os resultados da validação de um método simples, rápido e de baixo custo de preparação de amostras para a determinação de resíduos de pesticidas em solo por cromatografia líquida ultrarrápida acoplada a espectrometria de massas com ionização por *electrospray*. O método se baseia na extração com acetonitrila assistida por ultrassom, centrifugação e evaporação, sem etapa de purificação adicional. As recuperações foram avaliadas a partir de dois tipos de solo, apresentando resultados na faixa de 70-110%, com desvio padrão relativo menor que 20%. Os coeficientes de correlação para as curvas analíticas de calibração foram maiores do que 0,99. Os resíduos de pesticidas foram detectados em solos no intervalo de 1-62 ng g^{-1}, dependendo do composto. A incerteza do método foi estimada a partir de diferentes experimentos de adição e recuperação e materiais certificados. O processo de extração foi identificado como a principal fonte de incerteza e as incertezas expandidas para cada pesticida, utilizando-se um fator de cobertura de 2, situaram-se entre 8,4 e 33%. O método proposto foi aplicado para a determinação de pesticidas em amostras reais.

This work presents the validation results of a simple, fast and low-cost sample preparation method for the determination of pesticide residues in soil by ultrafast liquid chromatography coupled to electrospray ionization mass spectrometry. The method uses acetonitrile ultrasound-assisted extraction, centrifugation and evaporation step, without further cleanup. Recovery studies were performed for two soil blanks, presenting results in the range of 70-110% with relative standard deviation lower than 20%. The correlation coefficients for analytical calibration curves were higher than 0.99. Pesticide residues were detected in soils in the range of 1-62 ng g^{-1}, depending on the compound. The uncertainty of the method was estimated from different addition-recovery experiments and calculations from certificates. The extraction process was identified as the main source of uncertainty, and the expanded uncertainties for each pesticide, using a coverage factor of two, were between 8.4 and 33%. The proposed method was applied to the determination of pesticides in real samples.

Keywords: pesticide, mass spectrometry, liquid chromatography, soil

Introduction

Soil is a valuable natural resource that needs to be preserved and, if possible, its quality and productive capacity improved. The agricultural soils of several regions in the world are known to store a wide range of contaminants, especially pesticides and heavy metals.[1,2] Once a pesticide is released into the soil matrix, its effects will depend on both its characteristics and those of the soil.[3,4] The organic matter is the key soil component in retaining pesticides. The binding of pesticides to the organic matter decreases its potential for downward movement through the soil.[5]

The type of physicochemical interactions with the mineral and organic components of the soil, and other variables such as the mobility, stability and physicochemical properties of the pesticides determine its persistence and pollution risk. When the pesticides are used repeatedly in each crop season, the chemicals can bind and accumulate for longer periods of time causing environmental pollution.[6] Thus, the monitoring of the levels of pesticides in soils is of high interest.

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The need to determine as many compounds (e.g., pesticides) as possible in an environmental sample has fostered the development of multiresidue analysis protocols, in which attention must be devoted to the sample preparation and chromatographic determination steps.[7] Current methods for determining pesticides in soil often require continuous[8] and discontinuous liquid solid extraction,[9] supercritical fluid extraction,[10] solid phase microextraction,[11,12] solid-phase extraction,[13,14] pressurized liquid extraction,[15,16] ultrasonic-assisted extraction and other procedures.[17,18] The main problem with these techniques is the low selectivity towards the analytes. During the extraction step, many interfering components are co-extracted from soil samples together with target analytes, requiring a cleanup to remove those that could affect the accuracy and precision of the method.[7] However, due to recent technological developments, such as quads, ion traps and other mass filters, mass spectrometry has acquired a high specificity and selectivity in a wide mass range. This trend has led to an almost complete elimination of interferences at the target signal entry while keeping a suitable sensitivity. Such advances have turned out into the development of analytical techniques requiring the least possible number of steps in sample processing and a minimal chromatographic retention, such as the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method.[19]

In this work, the full laboratory validation of a multiresidue analysis method based on ultrasound-assisted extraction followed by an evaporation step is reported. A simultaneous determination of 30 currently used pesticides in soils by ultrafast liquid chromatography coupled to mass spectrometry (UFLC-MS) was carried out with excellent selectivity, high sensitivity and a wide application scope. Finally, to allow adequate interpretation of the analytical results obtained with the developed method, an innovative uncertainty estimation method[20] was applied for the first time to the analysis of contaminants in soils.

Experimental

Chemicals and reagents

Pesticide reference standards (all > 95% purity) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and Chemservice (West Chester, PA, USA). The pesticides used in this study were chosen for their different physicochemical properties, some have been reported as soil pollutants in the literature[21] and are widely used in Colombia, especially in potato and tomato crops, which are staples in the country.[22] Triphenylphosphate and tributylphosphate were used as surrogate and internal standard (I.S.), respectively. All solvents used were of HPLC grade JT Baker (Phillipsburg, NJ, USA). Sodium acetate and anhydrous sodium sulfate (dried at 650 ℃ for 4 h) were both of analytical grade. Distilled water was provided by a Milli-Q water purification system from Millipore (Bedford, MA, USA). Formic acid (99%) was purchased from Carlo Erba (Milan, Italy) and ammonium acetate was obtained from JT Baker.

Standard solutions

Standard stock solutions were prepared in a concentration around 1000 µg mL^{-1}, using either acetonitrile or methanol as solvent, and were stored in amber glassware under appropriate conditions such as –20 °C, exclusion of moisture and light. Mixture standard stock solutions were prepared by dilution of the corresponding stock solution with methanol according to the UFLC-MS response. The obtained solutions were stored in a refrigerator at −20 ℃.

Soil samples

Two soils were used for this study and their physicochemical characterization was performed at the Soils Laboratory at Jorge Tadeo Lozano University (Colombia). Soil A (Boyaca, Colombia) had a clay content of 400 g kg^{-1}, an organic matter content of 40.3 g kg^{-1} and a cation exchange capacity of 21.2 cmol kg^{-1}. A second soil B (Cundinamarca, Colombia) had a clay content of 190 g kg^{-1}, an organic matter content of 194 g kg^{-1} and a cation exchange capacity of 51.8 cmol kg^{-1}. The soil samples were dried at room temperature for 2 days and sieved, obtaining particle sizes lower than 2 mm. Blank matrices obtained (soils A and B) were checked to be free of the target pesticides before spiking.

Sample preparation

A 5 g sample was weighed in a 50 mL polypropylene centrifuge tube with screw caps. Recovery studies for validation were carried out by adding appropriate volumes of the working standard solution to blank samples. Then, 10 mL of acetone were added, and the samples were homogenized by mixing and shaking for 30 min on a horizontal shaker. After the bulk of the solvent evaporated at room temperature, the soil samples were stored at 4 ℃ in a centrifuge tube for 3 days to allow the interaction between the compounds and the soils, according to Garcia *et al.*[23]

Sodium acetate (2 g) and 5 mL of water were added to the tubes containing the spiked samples. Afterwards,

the samples were vigorously shaken for 15 min at 25 °C in a horizontal shaker. Then, 20 mL of acetonitrile were added, and the sample was vigorously shaken by hand for 2 min after screwing the tube caps. Next, the sample was sonicated for 20 min in an ultrasonic bath working at 50/60 Hz and 100 W from Branson. After this, 4 g of anhydrous magnesium sulfate ($MgSO_4$) were added. The tubes were capped and immediately shaken by hand for 2 min and centrifuged for 5 min at 4500 rpm.

On the other hand, aiming to improve the sensitivity of the method, a concentration step upper layer (acetonitrile) was needed. So, 15 mL of this extract were left in a 50 mL polypropylene centrifuge tube and, placed into a vacuum speed concentrator, in which the volume was reduced to *ca.* 500 µL. After, this extract was quantitatively transferred into a 1 mL volumetric flask, 20 µL of I.S. were added, and acetonitrile was employed to complete to volume. Finally, the supernatant was filtered through a 0.22 µm PTFE filter. At the initial step, triphenylphosphate (TPP) was spiked to reach a 0.3 µg mL^{-1} concentration in the UFLC-MS extract. The internal standard was used at a concentration of 0.5 µg mL^{-1}.

Instrumentation

The chromatographic analyses were performed on an ultra-high speed liquid chromatograph Shimadzu Prominence™ coupled to an LCMS-2020 mass selective detector (Maryland, CA, USA). The chromatograph was equipped with an automatic sampler, a binary high pressure pump, an online degasification system and an oven to control the column temperature. An ABN2ZE Peak Scientific (Billerica, MA, USA) nitrogen generator was employed. The data acquisition, control and processing were performed using the Lab Solutions software version 3.5. The instrumental conditions were previously optimized.[23]

A Shim Pack C_{18} column (75 mm × 2 mm i.d., 2.1 µm particle size and stationary phase C_{18}), a linear gradient mode consisting of 0.1% m v^{-1} formic acid and 5 mmol L^{-1} ammonium acetate in Milli-Q grade water (A) and acetonitrile (B) were used to perform the separation. The program used expressed as B percentage was: first, 0% at 0.2 min, afterwards, a rising to 40% at 1 min, and then, again to 100% in the next 7 min, and finally, a hold time of 1 min. Thereafter, the initial condition was restored in 2 min, and held for 5 min to reach the equilibrium. The flow rate of the mobile phase was 0.3 mL min^{-1} at a column temperature of 40 ºC. An aliquot of 5 µL of the extracts was injected into UFLC system.

A built-in DUIS (ESI, APCI) interface operating in ESI (electrospray ionization) mode, a drying gas flow rate of 15 L min^{-1} and a nebulizer gas flow rate of 1.5 L min^{-1} were used. The temperatures of the heating block and of the desolvation line were 200 and 250 °C, respectively. The analysis was carried out in both positive and negative modes; the applied voltages at the capillary were 4500 and −4500 V, respectively. Selected ion monitoring (SIM) of the most abundant ions of each compound was used. However, when a compound gave matrix interferences, another product ion was selected.

Validation and uncertainty

The validation procedure was performed following SANCO/825/2010.[24] Linear dynamic range, precision, recovery, lower limits, selectivity, ruggedness and uncertainty were evaluated for the analytical methodology developed. For linear dynamic range, the calibration samples were prepared by appropriate dilution of the stock solution in blank matrix extract in order to avoid matrix effects. Calibration solutions, at concentrations ranging between 2 and 1640 ng mL^{-1}, were used.

Intra-assay precision and recovery were assessed using spiked blank samples at two concentration levels, at twice the first level of the calibration curve and at 80% the highest level. On a set of replicated (n = 6) samples, the relative standard deviation (RSD) and recovery values were calculated. The limits of detection (LOD) and quantification (LOQ) were calculated according to IUPAC recommendation.[25]

The presence of potential interferences in the chromatograms from the analyzed samples was monitored by running control blank samples on each calibration. The absence of any chromatographic components at the same retention times as target pesticide suggested that no chemical interferences occurred.

Uncertainty on pesticide measurements was evaluated based on a hybrid bottom up/top down approach, assuming that they comprise the total analytical procedure.[20] Six replicates of soil were fortified at 50 ng g^{-1} at different stages of the analytical procedure: extraction, evaporation and UFLC analysis. The uncertainties of the gravimetric and volumetric measurements, as well as the standard purity, were estimated and integrated in the calculation of the total combined uncertainty. Then, the most of the contributions were obtained from the statistical analysis of repeated measurements to estimate the combined uncertainty and some sources were obtained from calibration certificates. Uncertainty was further divided into the following sources: (*i*) calibration solutions, (*ii*) extraction, (*iii*) evaporation, (*iv*) UFLC calibration and (*v*) instrumental analysis. After the estimation of all sources of uncertainty, they

were combined according to the law of propagation of uncertainties, obtaining the combined standard uncertainty. The expanded uncertainty (U) is obtained by multiplying the relative uncertainty by a coverage factor k, assuming a normal distribution of the measurand.

Results and Discussion

Method optimization

Based on our previous work and other publications, the key factors that affect the extraction efficiency in ultrasound-assisted extraction methods were investigated here in detail.[26] Firstly, ultrasound-assisted extraction of 5 g of soil was initially carried out for 15 min with 10 mL of four organic solvents with different polarity, methanol, acetonitrile, ethyl acetate and their mixture, used as extractants to investigate their impact on recoveries. High recoveries (68.7-115.2%) were obtained when acetonitrile was used as extractant. It is attributed to the wide range of physicochemical properties of the analytes (polarity, solubility, volatility, etc.), and in addition, the relatively low polarity of ethyl acetate does not allow adequate recovery of some compounds, such as methamidophos, acephate, thiocyclam, carbendazim, dimethoate, etc. Furthermore, the use of methanol in the extraction step does not help in the physical separation of the phases because it is very miscible with the water, being discarded in the extraction. Secondly, the effect of the addition of water (i.e., 2, 3, 4 and 5 mL) was assayed. A volume of 5 mL of water was chosen as the optimum volume because the final extract provided the best recoveries and lowest noise in the chromatograms. This noise reduction provides a higher signal-to-noise ratio, being attributed to the addition of water, which functions as liquid-liquid partition cleanup, removing the water-soluble interference. The acetonitrile volume (10-30 mL) effect on the recoveries was also tested. The results showed that recoveries reached their maximum when the extraction was performed with 20 mL. To obtain the optimal ultrasound time, the sample was sonicated for 0, 10, 20 and 30 min. The results showed that the extraction efficiency increased at first by increasing the ultrasound time up to 20 min, and then did not change with further increase of time. Thus it was concluded that these conditions exhibited excellent extraction capabilities; therefore, no further optimization would be needed. All experiments were performed in quintuplicate.

Validation: specificity

To investigate specificity, 10 different soil samples were extracted and analyzed. All samples had low intensity peaks in the region of interest (± 0.5% of the expected retention time of the pesticides). Although there was an apparent presence of famoxadone and chlorfenapyr, their presences were discarded because the relative intensities of fragment ions did not correspond to reference values. Given the efficient separation obtained from the ultrafast liquid chromatography and the high selectivity of mass spectrometric analysis, the absence of significant interferences was not surprising.

Validation: matrix effects and linearity

The matrix effect was studied by comparison of the slopes of the analytical calibration curves in solvent and matrix. The matrix/solvent slope ratio for each compound was obtained (Table 1) considering a signal enhancement or suppression effect as acceptable if the slope ratio ranged from 0.8 to 1.2. A value < 1 indicates signal suppression due to the matrix, while values > 1 involve enhancing effect of the matrix on the analyte signal. Slope ratios higher than 1.2 or lower than 0.8 indicate a strong matrix effect. It can be observed that a significant matrix effect was noticed for chlorfenapyr, famoxadone, hexaconazole, imazalil, methomyl, methoxyfenozide, oxamyl and spinosad, whereas tolerable matrix effect was observed for the rest of the compounds. As illustrated in Table 1, most compounds showed signal suppression in the matrices investigated; only spinosad had increase or signal enhancement.

Calibration curves were obtained from matrix-matching calibration solutions. The lowest concentration level in the calibration curve was established according to signal-noise ratio (S/N > 10). Linearity was evaluated by the calculation of a five-point linear plot with three replicates, based on the linear regression and squared correlation coefficient (r^2) which should be > 0.9900. The model was validated using a regression analysis of variance (ANOVA). Table 1 shows the linear range for each pesticide and r^2 values above or equal to 0.9903.

Validation: LOD and LOQ

Limits of detection and quantitation were calculated as three and ten times the standard deviation of five blank soil samples, respectively. Limit of detection and quantification were calculated according to the IUPAC recommendation.[25] Table 1 shows LODs obtained by SIM mode detection; these limits were verified by determining the signal-to-noise ratio and in all cases, it was greater than 3. Table 1 shows that the limits of detection of the method varied from 1 to 65 ng g^{-1} for the pesticides. The highest values (62 and 47 ng g^{-1}) were for famoxadone and chlorfenapyr due to

Table 1. Linear range, slope ratios between matrix-matched and solvent calibration, determination coefficient (r^2), limit of detection (LOD) and limit of quantitation (LOQ)

Compound	Linear range / (ng mL^{-1})	Matrix/Solvent slope ratios	r^2	LOD / (ng g^{-1})	LOQ / (ng g^{-1})
Acephate	7.3-88	0.98	0.9962	3.0	11.0
Azoxystrobin	3.3-40	0.83	0.9944	2.0	5.0
Benalaxyl	2.0-24	0.82	0.9913	1.0	3.0
Carbendazim	2.0-24	0.93	0.9909	1.0	3.0
Carbofuran	3.3-40	0.82	0.9928	2.0	5.0
Chlorfenapyr	105-1264	0.75	0.9949	47.0	158.0
Cymoxanil	101-1216	0.99	0.9980	46.0	152.0
Difenocolazole	3.3-40	0.94	0.9903	2.0	5.0
Dimethoate	3.3-40	0.82	0.9975	2.0	5.0
Tiabendazole	2.0-24	0.89	0.9987	1.0	3.0
Dimetomorph	2.0-24	0.86	0.9985	1.0	3.0
Famoxadone	137-1640	0.74	0.9932	62.0	205.0
Hexaconazole	18-216	0.76	0.9903	8.0	27.0
Imazalil	7.3-88	0.80	0.9949	3.0	11.0
Imidacloprid	48-576	0.98	0.9927	22.0	72.0
Indoxacarb	4.7-56	0.98	0.9924	2.0	7.0
Metalaxyl	2.0-24	0.88	0.9926	1.0	3.0
Methomyl	3.3-40	0.75	0.9986	2.0	5.0
Methoxyfenozide	6.7-80	0.76	0.9908	3.0	10.0
Monocrotophos	6.7-80	0.82	0.9942	3.0	10.0
Pyrimethanil	2.0-24	0.94	0.9930	1.0	3.0
Tebuconazole	15.3-184	0.90	0.9965	7.0	23.0
Thiocyclam	28-336	0.81	0.9913	12.0	42.0
Propanil	9.2-109	0.79	0.9922	4.1	13.7
Oxamyl	8.8-106	0.77	0.9930	4.0	13.3
Thiodicarb	9.6-115	0.96	0.9925	4.0	14.3
Atrazine	9.0-108	0.87	0.9970	4.1	13.5
Pirimicarb	9.1-109	0.97	0.9917	4.1	13.6
Spinosad	22.1-265	1.25	0.9942	9.9	33.2
Diazinon	9.5-115	0.97	0.9971	4.3	14.3

problems related to low chromatographic response of these pesticides and the presence of interferences with retention time near these compounds.

The limits of quantitation were determined as the lowest concentration of pesticide that gives a response that could be quantified with acceptable precision and accuracy under the stated operational conditions of the method. Table 2 shows the average recoveries obtained from six spiked samples at LOQ. The recovery rates were adequate, ranging from 70 to 120% with an RSD below 15% (Table 2).

Validation: trueness and precision

Trueness of the analytical procedure was determined by evaluating the recovery of spiked analyte from two soils. Recoveries and relative standard deviations obtained for six replicate samples of soils A and B, spiked at two different concentration levels, are reported in Table 2. Recoveries were in the range of 68.7-115.2%, which is a satisfactory performance. Trueness of the method was proven using a significance test (Student's *t*-test) with 5% statistical significance level. According to the current European Union guidelines on pesticide residue analytical methods, the recoveries obtained during the validation of the method should be within 70-120%.[24,27] However, in certain justified cases, typically with multiresidue methods, recoveries outside this range may be accepted, especially if they are consistent. All pesticides showed particularly good recoveries in the studied soils, most of them ranging from 100 to 120%. The lowest recoveries

Table 2. Recovery values (R) and relative standard deviations (RSDs) of the target compounds in two soils

| Compound | Soil A | | Soil B | | | |
| | R[a] / % (RSD[b]$_r$ / %) | | R / % (RSD$_r$ / %) | | RSD$_R$[c] / % | |
	LOQ[d]	5 × LOQ[d]	LOQ	5 × LOQ	LOQ	5 × LOQ
Acephate	101.2(7.1)	95.8(6.9)	117.1(16.6)	116.2(12.2)	20.8	12.9
Azoxystrobin	93.4(4.2)	93.9(2.5)	75.7(7.0)	102(9.4)	8.5	10.5
Benalaxyl	106.3(5.1)	101.3(3.7)	75.2(6.2)	81.5(9.4)	7.2	11.1
Carbendazim	81.3(2.1)	99.4(4.6)	102.6(5.2)	112.3(10.0)	7.1	11.1
Carbofuran	105.1(5.5)	95.1(4.5)	83.7(6.4)	97.9(7.3)	8.5	8.7
Chlorfenapyr	88.5(9.9)	95.1(5.5)	91.5(9.2)	82.5(9.5)	11.4	10.2
Cymoxanil	96.5(3.6)	101.1(4.2)	78.7(6.4)	91.7(5.6)	7.7	6.1
Difenocolazole	100(4.2)	103.4(3.4)	81.1(8.4)	71.9(12.3)	10.9	13.8
Dimethoate	101.5(4.9)	100.1(3.2)	76.9(4.1)	97.4(7.7)	6.1	8.9
Tiabendazole	71.9(3.6)	70.5(5.6)	68.5(6.9)	95.1(8.3)	5.0	8.9
Dimetomorph	96.5(4.9)	100(3.6)	109.7(6.8)	102.9(11.8)	8.2	12.8
Famoxadone	83.8(4.0)	88.9(7.0)	61.2(14.8)	90.3(12.0)	17.9	13.9
Hexaconazole	106.4(3.8)	104.6(3.9)	74.7(4.8)	85.5(10.2)	6.2	11.1
Imazalil	97.3(4.2)	102.6(4.4)	65.7(9.4)	75(11.8)	12.2	14.1
Imidacloprid	91.6(4.6)	94.6(5.2)	77.1(5.1)	87.4(6.5)	6.8	7.6
Indoxacarb	88.5(10.2)	96.9(9.5)	101.8(13.5)	86.9(10.9)	16.7	12.8
Metalaxyl	100.8(3.8)	107(4.1)	107.6(11.6)	114(7.2)	15.7	8.3
Methomyl	93.8(5.8)	88.2(3.1)	85.1(7.9)	102.7(6.1)	9.9	6.5
Methoxyfenozide	106.4(5.0)	104.8(4.0)	70.7(6.0)	89.3(9.9)	8.3	11.7
Monocrotophos	92.3(5.0)	101.4(4.6)	85.6(5.1)	95.5(6.3)	5.9	7.3
Pyrimethanil	88.9(5.2)	95.3(5.3)	108.8(5.3)	95.6(11.3)	6.8	12.5
Tebuconazole	92.9(10.0)	100.9(4.0)	72.3(3.4)	80.1(10.2)	11.2	11.1
Thiocyclam	85.2(6.9)	92.4(7.0)	101.7(9.0)	110.7(7.8)	10.6	8.4
Propanil	93.4(3.6)	101.4(4.4)	92.6(5.2)	99.9(6.3)	6.8	7.1
Oxamyl	100.5(7.6)	78.6(9.9)	86.5(12.2)	101.3(9.9)	15.7	10.9
Thiodicarb	84.9(5.4)	79.5(4.4)	81.3(7.1)	74.6(5.9)	8.2	6.4
Atrazine	98.2(5.5)	91.5(5.4)	95.2(6.9)	90.6(5.9)	9.0	6.9
Pirimicarb	99.5(5.1)	105.6(4.9)	90.6(6.1)	100.3(5.8)	7.6	6.7
Spinosad	76.6(9.0)	96.2(6.8)	17.7(31.4)	20.9(36.8)	40.4	40.5
Diazinon	100.8(7.4)	103.7(2.0)	92.4(7.1)	111.3(7.0)	10.0	7.5

[a]R: Recovery; [b]RSDr: repeatability; [c]RSD$_R$: intermediate precision; [d]fortification level.

were those achieved for spinosad in soil B, being consistent with that reported in literature in which the physicochemical properties of soil affect the extraction of some pesticides.[28]

The results obtained from the trueness experiments were used to estimate the precision of the method. The precision of the method is evaluated by its repeatability (variation of the results obtained in a same series), expressed as relative standard deviation or RSDr, and its intermediate precision (variation of the results obtained in different series) is also expressed as a relative standard deviation, often called RSD$_R$. The RSD$_r$ values, presented in Table 2, were less than 15% for all concentration levels tested, except for the lowest one, in which the RSD$_r$ value was 36.8%. For

intermediate precision, an increase in the values of RSD$_R$ was found, but any pesticide exceeded the 20%, except acephate and spinosad in soil B. The behavior of spinosad may be attributed to an irreversible adsorption in the soil, being consistent with the high content of organic matter that this soil presented.

Validation: ruggedness

The ruggedness was investigated using a Youden experimental design, introducing minor reasonable variations by the laboratory and by observing their consequences. Such a design allows the study of a maximum of seven factors (variables) with eight

experiments. It was established that the water volume, temperature, concentration and the pH of the mobile phase had the greatest effects on the robustness of the method. The factors over which it was not necessary to exercise strict control were the oven temperature, sodium acetate quantity, ultrasound time and $MgSO_4$ quantity.

Uncertainty

Demonstrating the reliability of the analytical results is essential in order to interpret them correctly, and method validation is only the first step to achieve this. Indeed, it is not enough if one aims at interpreting and comparing results correctly. For this reason, the global uncertainty was determined for all pesticides at the level of 50 ng g^{-1}, except imidacloprid, cymoxanil, chlorfenapyr and famoxadone which were evaluated at LOQ. Five different sources of uncertainty were taken into account, namely, the calibration graph (u_1), the preparation of the calibration standard solutions (u_2), the extraction procedure (u_3), the evaporation process (u_4) and the instrumental analysis (u_5). The overall uncertainty (u_s) was given by equation 1.

$$u_s = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2} \tag{1}$$

Finally, this value was reported as expanded uncertainty, which is twice the value of the overall uncertainty. The principle and calculations in the present study are similar to the calculations made by Štěpán *et al.*[20]

u_1 and u_2: calibration graph and solution preparation

The uncertainty u_1 was given by equation 2:

$$u_1 = \frac{s}{b_1} \times \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(C_0 - ç)^2}{S_{XX}}} \tag{2}$$

where s is the standard deviation of the residuals of the calibration curve, b_1 is the slope of the calibration curve, p is the number of measurements of the unknown, n is the number of points used to establish the calibration curve, C_0 is the calculated concentration of the analyte from the calibration curve, ç is the average of all standards used to obtain the analytical calibration curve, and S_{XX} is calculated as follows: $S_{XX} = \Sigma(c_j - ç)^2$, where j = 1, 2, ..., n. c_j is the concentration of each calibration standard used to build up the analytical calibration curve.

The u_2, associated with the preparation of the calibration standard solutions, is a combination of the uncertainty of the pesticide concentration (u_{pest}) given in the certificate of purity by the manufacturer at 95% confidence level (k = 2), the uncertainty from weighing given in the calibration

certificate of the analytical balance (u_{weight}), and the uncertainty coming from the volumetric flasks and pipettes used for dilution (u_v). Finally, u_2 was given by equation 3.

$$u_2 = \sqrt{u_{pest}^2 + u_{weight}^2 + u_v^2} \tag{3}$$

u_3, u_4 and u_5: extraction, evaporation and UFLC analysis

In the present study, uncertainty of each analytical step consists of random and systematic error components; thus, each component was quantified and incorporated into the combined standard uncertainty. For this purpose, the random errors of extraction, evaporation and UFLC analysis steps were approximated by the relative standard deviations which were calculated from repeated determinations of analytes (n = 6) at different stages of the analytical process. To determine the random components of uncertainty which can be separately assigned to extraction ($u(r)_{Extr}$), evaporation ($u(r)_{Evap}$) and UFLC ($u(r)_{UFLC}$) steps, the following equations were used:

$$u(r)_{UFLC} = \sqrt{RSD_3^2} \tag{4}$$

$$u(r)_{Evap} = \sqrt{RSD_2^2 - u(r)_{UFLC}^2} \tag{5}$$

$$u(r)_{Extr} = \sqrt{RSD_1^2 - u(r)_{Evap}^2 - u(r)_{UFLC}^2} \tag{6}$$

Systematic components of uncertainty ($u(s)_n$) were estimated on the basis of the recovery for each stage of the method (extraction, evaporation and UFLC analysis). It is calculated by first estimating the uncertainty associated with the recovery from each fortification step, and then, using equations similar to equation 4-6. Uncertainties of these apparent recoveries were derived from a rectangular distribution using equation 7:

$$u(s)_n = \frac{0.5\,(100 - R_n)}{\sqrt{3}} \tag{7}$$

where n is the stage (extraction, evaporation or UFLC analysis) and R (%) is the recovery at the respective step. Then, random and systematic components of uncertainty of each analytical step were used for the calculation of the combined uncertainty associated with the analytical procedure (extraction, evaporation, UFLC, equations 8-10)

$$u_3 = \sqrt{u(s)_{Extr}^2 + u(r)_{Extr}^2} \tag{8}$$

$$u_4 = \sqrt{u(s)_{Evap}^2 + u(r)_{Evap}^2} \tag{9}$$

$$u_5 = \sqrt{u(s)_{UFLC}^2 + u(r)_{UFLC}^2} \tag{10}$$

The calculated expanded standard uncertainties (u) for this method are given in Figure 1. This figure shows that the global uncertainty of the test pesticides varied up to 10%, with the exception of some compounds such as thiocyclam, oxamyl and methoxyfenozide. These results indicated that the method performance could be considered satisfactory for the whole range of the tested pesticides.
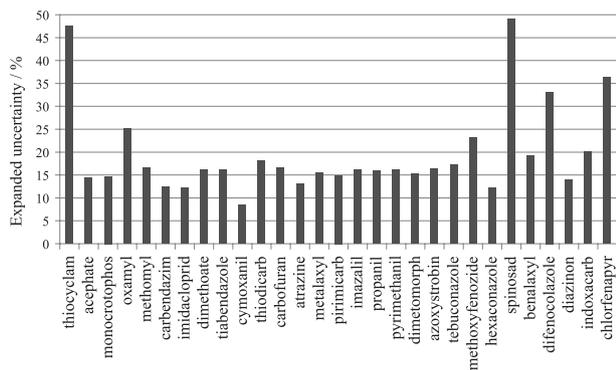
Figure 1. Expanded uncertainty (%) for each pesticide (coverage factor, k = 2).

Using this method, the major contributor to the total combined uncertainty was the extraction step, with contributions ranging between 22 and 93%. Figure 2 shows the individual contributions of each step in the total uncertainty. The uncertainty related to calibration graph and
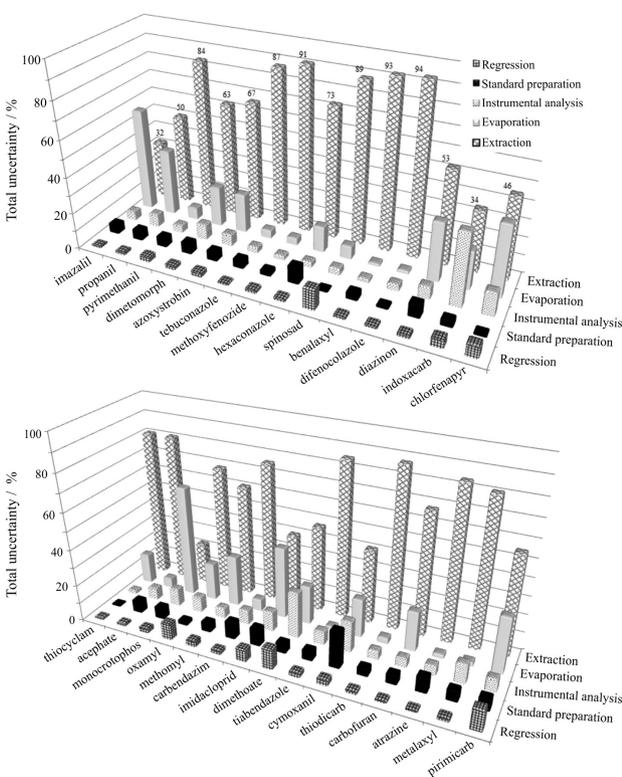
Figure 2. Contribution of each source of uncertainty to the total uncertainty.

solution preparation did not contribute significantly to the total uncertainty estimate. The curve calibration contributed with less than 12% for the global uncertainty. The influence of standard preparation and instrumental analysis were quite similar between each other for most of the pesticides. Some analytes such as monocrotophos, imidacloprid, imazalil, propanil and chlorfenapyr had a high variance during the evaporation step. This behavior suggests that these compounds may have decomposition or volatilization losses.

Application to real samples

Twenty representative soil samples were collected from a farm located in Boyacá Department, Colombia (N 5°61'970''; O 73°59'302''). It is a farm dedicated to the production of greenhouse tomato, soils are clayey, median compaction, pH 5.5 and electrical conductivity of 0.685 S cm^{-1}. The farm has a conventional agricultural management that includes pesticide application for managing different pests and diseases among which stand out the whitefly (*Trialeurodes vaporariorum*), gray mold (*Botrytis cinerea*) and blight (*Phytophthora infestans*). The samples were collected during ten weeks at each harvest date and were stored in plastic (high density polyethylene) containers (ca. 1 kg). The samples were stored in the dark at −18 °C until required for residue analysis. All samples were analyzed in duplicate.

Those samples were determined by the method established above and concentrations of the main pesticide residues are shown in Table 3. In this study, none pesticide residue was detected for the remaining weeks (2, 3, 5, 6, 7, 9 and 10). The results indicated that the presence of pesticide residues in soils of this crop is random, which may be due to many factors, such as environmental, crop management, stability of the pesticides, among other. All compounds detected were identified by comparing the retention times and product ion masses in both samples and matrix matched standards. For pyrimethanil, it was found that the S/N ratio in all cases was greater than three.

Table 3. Mean values (ng g^{-1}) of pesticide concentrations determined in soil samples

Week	Pesticide	Concentration and U^a / (ng g^{-1})	LOD / (ng g^{-1})	LOQ / (ng g^{-1})
1	pyrimethanil	< LOQ	1.0	3.0
4	carbendazim	40.0 ± 5.0	1.0	3.0
8	pyrimethanil	< LOQ	1.0	3.0

^{a}Expanded uncertainty (coverage factor, k = 2).

Comparison with previous works

Numerous methods for determining pesticide residues have previously been described in matrices, such as fruits, vegetables, cereals, water and others, but, until some years ago, in case of soils, many current pesticides are not included in a single method.[29] On the other hand, most reported methods have a restricted range of applicability because they have been developed mainly for the analysis of just organochlorine pesticides,[30,31] herbicides,[28] or carbamates[32] and there are few reports of simultaneously determining multiresidues in soils.[33] For example, a recent study proposed headspace single drop micro-extraction for extraction of only five organophosphorus pesticides in soil.[34] Although, it is noteworthy that the ultrasound use improved and accelerated the extraction of the analytes from the sample. On the other hand, a similar method has been reported for the analysis of cyantraniliprole and its metabolites by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS), however this study is limited to only four compounds.[26] Thus, the proposed procedure allows for screening of a much broader range of currently used pesticides (carbamates, organophosphorus, azoles and others) in just one analytical process. In addition, this method has great advantages in comparison with others, such as using a minimal amount of solvent without using any complex instrumentation, applicability to a broad range of pesticides and the method used for uncertainty estimation allows identifying the real weaknesses of the method compared to other approaches.

Conclusions

Using UFLC coupled to mass spectrometry, a method of analysis was developed for multiresidue determination of pesticides in soil. For a quantitative evaluation, matrix effects were evaluated by comparing the slopes of the matrix matched and solvent-based calibration curves. The matrix effects displayed by most pesticides and the matrix matched calibration were used to minimize any matrix effects. The sensitivity of the UFLC-MS method is sufficient to be applied to the determination of pesticide residues in soils. The LOD values obtained were lower than 5 ng g^{-1} for 22 pesticides, lower than 50 ng g^{-1} for 6 pesticides, and higher than 50 ng g^{-1} for only one compound. The low limits of detection, reliable linearity, precision and accuracy of the developed method exceed the requirements for assessing adherence to limits established by the current European regulations in pesticide contamination of soil. Finally, the uncertainty of the method was estimated from different experiments on spiked samples and calculations from certificates. The extraction process was identified as the main source of uncertainty and the expanded uncertainties for each pesticide, using a coverage factor of two, were between 8.4 and 33%. Finally, this method was successfully applied to the analysis of pesticides in soils from a farm dedicated to the production of tomato.

Supplementary Information

Supplementary data (Tables and Figures) are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgements

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Supplementary Information

Multiresidue Determination and Uncertainty Analysis of Pesticides in Soil by Ultrafast Liquid Chromatography Coupled to Mass Spectrometry

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Table S1. Identification parameters of targeted pesticides obtained by UFLC/ESI-MS. Retention time (t_R), target ion (TI) and qualifiers (Q1 and Q2)

Compound	t_R / min	TI, *m/z*	Q1, *m/z*	Q2, *m/z*
Positive ionization mode				
Acephate	2.625	184	202	389
Azoxystrobin	6.531	404	405	372
Benalaxyl	7.655	326	327	
Carbendazim	3.257	192	234	
Carbofuran	4.886	222	280	
Difenocolazole	7.756	408	406	
Dimethoate	3.419	230	232	171
Tiabendazole	3.479	202	203	
Dimetomorph	6.093	390	388	
Famoxadone	6.861	369	313	
Imazalil	5.395	297	299	
Indoxacarb	8.459	528	550	
Metalaxyl	5.329	280	281	
Methomyl	2.995	163	180	
Methoxyfenozide	6.956	369	313	
Monocrotophos	2.864	241	224	242
Pyrimethanil	6.153	200	201	
Tebuconazole	6.914	308	310	
Thiocyclam	2.540	182	183	
Propanil	4.210	218	220	
Oxamyl	2.889	237	238	
Thiodicarb	4.697	355	357	
Atrazine	5.249	216	218	
Pirimicarb	5.064	239	240	
Spinosad	6.712	747	748	733
Diazinon	8.095	305	306	
TPP	7.879	344	327	
Negative ionization mode				
Imidacloprid	3.405	256	297	254
Hexaconazole	7.100	358	360	
Cymoxanil	3.848	197	233	
Chlorfenapyr	7.813	349	347	

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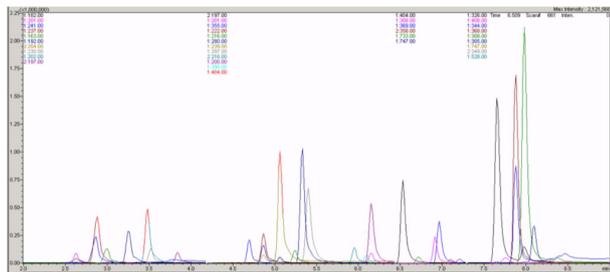
Figure S1. Chromatograms of target ions of pesticides analyzed by UFLC-MS at the concentration corresponding to LOQ.

Figure S3. Chromatograms of target ions in real sample (week 4). Signal in ca. 3.25 min is carbendazim.

Figure S2. Chromatograms of target ions in blank samples (with addition of TPP).
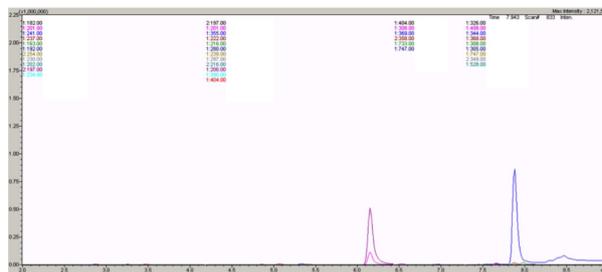
Figure S4. Chromatograms of target ions in real sample (week 1). Signal in ca. 6.15 min is pyrimethanil.