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Method Article

Determination of biomarkers of exposure to boscalid, captan, folpel, mancozeb and tebuconazole in urine and hair samples

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A B S T R A C T

In order to develop a tiered approach to identify relevant biomarkers and matrices for assessing pesticide exposure in residents living close to vineyards, five priority pesticides (boscalid, captan, folpel, mancozeb and tebuconazole) and their metabolites were analyzed in urine and hair samples from the biobank of a French national prevalence study conducted between 2014 and 2016. To do this, several analytical methods based on gas chromatography coupled with tandem mass spectrometry (GC/MS/MS) were developed by relying on the expertise of the laboratory and the scientific literature, in particular on a paper describing the use of gas chromatography-mass spectrometry for the determination in human urine samples of ethylene thiourea (ETU), a metabolite of mancozeb, after a supported liquid extraction followed by a derivatization step [1]. The main adaptations carried out as part of this study concerned:

- the determination of ethylene urea (EU), another metabolite of mancozeb, at the same time as ETU in urine samples
- the determination of all substances of interest including boscalid, EU and ETU, folpel and one of its metabolite (phthalimide), tebuconazole and one of its metabolite (hydroxytebuconazole), and tetrahydrophthalimide (metabolite of captan) in organic hair extracts by GC/MS/MS after a derivatization step

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Resource availability;	N.A.

Method details

Reagents and chemicals

Acetone and dichloromethane (DCM) (PESTIPUR - For pesticide analysis) were purchased from CARLO ERBA Reagents S.A.S (Val-de-Reuil, France). Acetonitrile and methanol (MeOH) absolute (ULC/MS - CC/SFC) was purchased from Biosolve Chimie SARL (Dieuze, France). Acetic acid (For LC-MS) was purchased from CARLO ERBA Reagents S.A.S (Val-de-Reuil, France). Ammonium hydroxide (28–30% solution in water) was purchased from Acros Organics (NJ, USA). Ammonium chloride (For analysis) was purchased from Merck KGaA (Darmstadt, Germany). Nitric acid (67–69% - For trace metal analysis) was purchased from VWR International (Radnor, PA, USA). Potassium fluoride (≥99%), anhydrous sodium acetate (For molecular biology, ≥99%), N-methyl-N-(trimethylsilyl)trifluoroacetamide) (MSTFA) for GC derivatization (≥98.5%), β-glucuronidase from limpets (Patella vulgata) and sulfatase from Helix pomatia were purchased from Sigma Aldrich (Saint Louis, MO, USA). Strata-X cartridges (6 mL, 200 mg) were purchased from Phenomenex France (Le Pecq, France). Chem Elut cartridges (3 mL, unbuffered) were purchased from Agilent Technologies (Folsom, CA, USA).

Certified standards of boscalid, ethylene thiourea (ETU), ethylene urea (EU), folpel, phthalic acid, phthalimide and tebuconazole were purchased from LGC Labor GmbH (Augsburg, Germany). Certified standard of hydroxytebuconazole (TEB-OH) was purchased from Sigma Aldrich (Saint Louis, MO, USA). Certified standard of tetrahydrophthalimide (THPI) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Standards of phthalic acid D_4 and THPI D_6 were purchased from Chiron AS (Trondheim, Norway). Standards of boscalid D_4 and phthalimide D_4 were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Standard of TEB-OH D_6 was purchased from Alsachim (Illkirch, France). Standard of ETU D_4 was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Standard of EU D_4 was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Standard of folpel D_4 was purchased from LGC Labor GmbH (Augsburg, Germany). The purity of native standards was above 98% and that of labeled standards above 95%. Individual standard stock solutions (1 g/L) were prepared in acetone or MeOH by accurately weighing 10 mg (± 0.1 mg) of standards using a Sartorius Cubis MSE 225P semi-micro balance (Sartorius AG, Göttingen, Germany) into 10-mL volumetric flasks, and stored at −18 °C. Acetone solution (100 mg/L) of tebuconazole D_6 was purchased from LGC Labor GmbH (Augsburg, Germany). Intermediate and spiking solutions were prepared in acetone by appropriate dilution of individual standard stock solutions and commercial solutions.

Pooled urine and hair samples for quality controls were obtained by donation. Surine™ Negative Urine Control was purchased from Sigma Aldrich (Saint Louis, MO, USA).

Determination of ETU and EU in urine samples

Preparation of urine samples

After adding 10 µL of nitric acid to 2 mL of urine in a 10-mL tube, the sample was agitated and transferred in a 8-mL tube containing 70 mg of ammonium chloride and 1 g of potassium fluoride that were used to adjust pH and ion strength, respectively. The tube was then vortexed before adding the labeled ISTDs.

Extraction of ETU and EU from urine samples

Supported liquid extractions (SLE) were performed using a Supelco Visiprep DL (disposable liner) vacuum manifold and a GAST vacuum pump. The pre-treated urine sample (2 mL), previously vortexed to ensure the dissolution of salts, was loaded on a Chem Elut cartridge (3 mL). After sample percolation (5 to 10 min), analytes were then extracted by passing through the column 10 × 2 mL of DCM. Organic extracts collected in 20-mL gauged glass tubes were then concentrated to 0.5 mL at 30 °C under a nitrogen stream using a N-EVAP 111 Organomation Nitrogen Evaporator and adjusted to 500 μL of DCM prior to be transferred into 2 mL amber glass vials. After adding 40 µL of the derivatization agent (MSTFA), concentrated extracts were incubated at 40 °C overnight (at least 16 h) prior to analysis by GC/MS/MS.

GC/MS/MS analysis

Analyses were performed using a 7890A GC system coupled to a 7000C GC/MS Triple Quad (Agilent Technologies, Santa Clara, California, United States) operated in electron impact ionization (EI) mode (70 eV). The GC system was equipped with a Gerstel MPS (MultiPurpose Sampler) robotic autosampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) and a multimode inlet (MMI) fitted with a single taper ultra-inert glass liner with glass wool. Calibration solutions and SLE extracts were injected (2 µL) in the splitless mode (purge flow to split vent: 60 mL/min at 1.8 min) with the following injector temperature program: 37 °C (hold 0.1 min), ramp at 500 °C/min to 325 °C (hold 10 min). Helium was used as the column carrier gas at a constant flow rate of 1.0 mL/min. Chromatographic separation was performed on a Rtx®-PCB capillary column (30 m length × 0.25 mm I.D., 0.25 µm film thickness) supplied by Restek Corporation (Bellefonte, Pennsylvania, United States) with the following oven temperature program: 37 °C (hold 2 min), first ramp at 37 °C/min to 250 °C (hold 0 min), second ramp at 15 °C/min to 325 °C (hold 1 min to reach an analysis time of 13.8 min). The MSD transfer line, ion source and quadrupole temperatures were fixed at 325, 280 and 180 °C, respectively. The mass spectrometer (triple quadrupole) was operated in multiple reaction monitoring (MRM) mode. The two most intense and specific MRM transitions of each compound (quantifier and qualifier transitions) were monitored for identification, confirmation and quantification. They were selected using the pesticides and environmental pollutants MRM database provided by Agilent Technologies for the compounds present in the database or following the usual procedure for others. Analytical characteristics of measured compounds are reported in Table 1. MassHunter software (10.0) was used for instrument control, data acquisition and quantification.

Quality assurance and quality control (QA/QC)

The limit of detection (LOD) was defined as the lowest concentration of a substance that can be distinguished from the absence of that substance. LODs were estimated from the replicate analysis of a blank sample. The limit of quantification (LOQ) was defined as the lowest concentration of a substance for which the relative standard deviation (RSD) of the raw signal ($n = 5$) was lower than or

Table 1
Analytical characteristics of measured compounds.

	Compound[a]	CAS number	Chemical Family	ISTD	Time segment	t_R (min)	Quantifier MRM transition Precursor > Product (CE (V))	Qualifier MRM transition Precursor > Product (CE (V))	LOD urine[b] (µg/L)	LOQ urine[b] (µg/L)	LOD hair[c] (ng/g)	LOQ hair[c] (ng/g)
Target compounds												
1	EU	120–93–4	Carbamates	EU D_4	1	6.5	214.8 > 133.0 (15)	214.8 > 147.0 (15)	0.2	0.5	40	80
2	THPI	85–40–5	Phthalimides	THPI D_6	2	7.4	222.8 > 192.0 (10)	222.8 > 207.0 (5)	1.3	2.5	20	40
3	ETU	96–45–7	Carbamates	ETU D_4	2	7.6	230.7 > 159.7 (5)	245.7 > 231.1 (10)	0.4	1.0	20	40
4	Phthalimide	85–41–6	Phthalimides	Phtalimide D_4	2	7.6	203.8 > 102.0 (35)	203.8 > 130.0 (25)	0.5	1.0	10	20
5	Phthalic acid	88–99–3	Phthalimides	Phthalic acid D_4	3	8.1	294.9 > 147.1 (5)	294.9 > 73.0 (40)	2.5	5.0	–	–
6	Folpel	133–07–3	Phthalimides	Folpel D_4	4	9.8	259.7 > 130.0 (30)	259.7 > 102.0 (30)	–	–	40	80
7	Tebuconazole	107534–96–3	Triazoles	Tebuconazole D_6	5	11.1	249.7 > 125.0 (25)	249.7 > 153.0 (10)	1.3	2.5	10	20
8	TEB-OH	212267–64–6	Triazoles	TEB-OH D_6	5	11.8	187.9 > 157.1 (5)	187.9 > 98.1 (5)	1.3	2.5	20	40
9	Boscalid	188425–85–6	Carboxamides	Boscalid D_4	5	13.2	341.6 > 203.9 (10)	166.7 > 140.1 (15)	1.3	2.5	40	80
Labeled ISTDs												
a	EU D_4	n/a	Carbamates		1	6.5	218.6 > 134.0 (10)	–				
b	THPI D_6	203578–24–9	Phthalimides		2	7.4	228.8 > 214.1 (10)	–				
c	ETU D_4	352431–28–8	Carbamates		2	7.6	234.8 > 163.1 (35)	–				
d	Phthalimide D_4	60161–31–1	Phthalimides		2	7.6	133.9 > 106.0 (15)	–				
e	Phthalic acid D_4	87976–26–9	Phthalimides		3	8.1	224.5 > 222.1 (10)	–				
f	Folpel D_4	1327204–12–5	Phthalimides		4	9.8	265.7 > 134.1 (20)	–				
g	Tebuconazole D_6	n/a	Triazoles		5	11.0	254.6 > 156.0 (10)	–				
h	TEB-OH D_6	n/a	Triazoles		5	11.8	193.8 > 160.0 (10)	–				
i	Boscalid D_4	n/a	Carboxamides		5	13.2	170.8 > 142.1 (30)	–				

[a] Compounds listed in order of retention times.
[b] For a 2-mL sample of urine.
[c] For a 25-mg sample of hair.

equal to 20%, the signal-to-noise ratio (S/N) was greater than or equal to 10, and the raw signal was greater than or equal to 5 times the signal of the blank sample.

Several labeled substances were selected to best cover the physical and chemical properties of the targeted analytes. ETU D_4 and EU D_4 were added prior to the extraction step and used as internal standards (ISTDs). All compounds were quantified using the appropriate ISTD (Table 1) to compensate for the variability associated with the analytical procedure, from calibration curves generated for each compound by analyzing at least four different calibration samples. A quadratic fit (origin ignored, no weighting) was used to compensate for the nonlinearity of the instrument response over a wide working range.

Each batch included: i) up to 32 urine samples (2 mL), ii) one procedural calibration blank sample and six procedural calibration samples prepared from Surine™ and analyzed as regular samples to assess whether contamination may have occurred during analysis and to generate quadratic calibration curves intended for quantification, respectively, and iii) at least three matrix procedural QC samples (2-mL pooled urine sample non-spiked and spiked at the LOQ level and at an intermediate level) analyzed as regular samples to check for method accuracy.

Targeted substances were identified by comparing retention times and MRM transition ratios (±30%) between calibration and urine samples. The data validation protocol included several conditions: i) the determination coefficient of the calibration curve had to be greater than 0.99, ii) the concentration of a substance measured in the procedural calibration samples had to be within ±50% of its theoretical concentration value at the LOD and LOQ levels and ±25% at all other levels, iii) the response of a substance (ISTD response ratio) in the procedural blank samples had to be lower than 50% of that in the procedural calibration sample at the LOQ level, iv) the concentration of a substance measured in the matrix procedural QC samples prepared from pooled urine samples had to be within ±50% of its theoretical concentration value at the LOQ level and ±30% at the intermediate level (results are reported in Table 2), and v) the concentration of a substance measured in the urine samples had to be within the method working range without exceeding 110% of the concentration of the most concentrated calibration samples. If all these conditions were not met, results were not validated and samples were reanalyzed if possible.

Determination of the other targeted substances in urine samples

Preparation of urine sample (enzymatic hydrolysis)

After adding 2 mL of a 0.2 M sodium acetate buffer at pH 4.8 to 2 mL of urine, the labeled internal standards (ISTDs) were added and the pH of the urine sample adjusted to pH 5 with the commercial solution of ammonium hydroxide or a solution of nitric acid diluted to one tenth. Then, 200 µL of a solution of β-glucuronidase from limpets (Patella vulgata) (25 g/L/25 units/µL) and sulfatase from Helix pomatia (2.5 g/L/0.025 units/µL) prepared in a 0.2 M sodium acetate buffer at pH 4.8 were added and the sample was subsequently incubated and agitated for 2 h at 50 °C. After return to room temperature, 16 mL of ultra-pure water and 40 µL of nitric acid were added to reach a final volume of approximately 20 mL at pH 2.

Extraction of the target substances from urine samples

Solid phase extractions (SPE) were performed using a Gilson GX-274 ASPEC automatic extraction system (Gilson, Middleton, WI, USA). The Strata-X cartridge was conditioned with successively 10 mL of DCM, 10 mL of MeOH mixture and 10 mL of ultra-pure water. Then, the pre-treated urine sample (20 mL) was loaded. The cartridge was washed with 10 mL of acidified ultra-pure water (pH 2) and subsequently completely dried for 15 min. Analytes were eluted with 4 × 2.5 mL of a 90% DCM / 10% MeOH mixture. Organic extracts were then evaporated to dryness at 30 °C under a nitrogen stream using a N-EVAP 111 Organomation Nitrogen Evaporator and reconstituted in 500 μL of DCM prior to be transferred into 2 mL amber glass vials and stored at −18 °C. 40 µL of the derivatization agent (MSTFA) were added prior to analysis by GC/MS/MS.

Table 2
Determination of ETU and EU in urine samples: interday method accuracy and precision assessed by analyzing several matrix procedural QC samples at different concentration levels (8 batches).

Compound	Range (µg/L)	R^2	Matrix procedural QC sample[a] LOQ level				Matrix procedural QC sample[a] Intermediate level			
			N	Theoretical concentration (µg/L)	Measured concentration (µg/L)	Accuracy (%) (precision (%))	N	Theoretical concentration (µg/L)	Measured concentration (µg/L)	Accuracy (%) (precision (%))
ETU	0.4 - 20	> 0.9999	8	1	1.13	113 (11)	8	10	10.6	106 (11)
EU	0.2 - 10	> 0.9997	8	0.5	0.46	92 (19)	8	5	4.98	100 (20)

[a] 2-mL pooled urine sample spiked at the LOQ level and at an intermediate level.

GC/MS/MS analysis

Analyses were performed using a 7890A GC system coupled to a 7000C GC/MS Triple Quad (Agilent Technologies, Santa Clara, California, United States) operated in electron impact ionization (EI) mode (70 eV). The GC system was equipped with a Gerstel MPS (MultiPurpose Sampler) robotic autosampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) and a multimode inlet (MMI) fitted with a single taper ultra-inert glass liner with glass wool. Calibration solutions and SPE extracts were injected (2 µL) in the splitless mode (purge flow to split vent: 60 mL/min at 1.8 min) with the following injector temperature program: 37 °C (hold 0.1 min), ramp at 500 °C/min to 325 °C (hold 10 min). Helium was used as the column carrier gas at a constant flow rate of 1.0 mL/min. Chromatographic separation was performed on a Rtx®-PCB capillary column (30 m length × 0.25 mm I.D., 0.25 µm film thickness) supplied by Restek Corporation (Bellefonte, Pennsylvania, United States) with the following oven temperature program: 37 °C (hold 2 min), first ramp at 37 °C/min to 250 °C (hold 0 min), second ramp at 15 °C/min to 330 °C (hold 3 min to reach an analysis time of 16.1 min). The MSD transfer line, ion source and quadrupole temperatures were fixed at 325, 280 and 180 °C, respectively. The mass spectrometer (triple quadrupole) was operated in multiple reaction monitoring (MRM) mode. The two most intense and specific MRM transitions of each compound (quantifier and qualifier transitions) were monitored for identification, confirmation and quantification. They were selected using the pesticides and environmental pollutants MRM database provided by Agilent Technologies for the compounds present in the database or following the usual procedure for others. Analytical characteristics of measured compounds are reported in Table 1. MassHunter software (10.0) was used for instrument control, data acquisition and quantification.

Quality assurance and quality control (QA/QC)

The limit of detection (LOD) was defined as the lowest concentration of a substance that can be distinguished from the absence of that substance. LODs were estimated from the replicate analysis of a blank sample. The limit of quantification (LOQ) was defined as the lowest concentration of a substance for which the relative standard deviation (RSD) of the raw signal ($n = 5$) was lower than or equal to 20%, the signal-to-noise ratio (S/N) was greater than or equal to 10, and the raw signal was greater than or equal to 5 times the signal of the blank sample.

Several labeled substances were selected to best cover the physical and chemical properties of the targeted analytes. Boscalid D_4, phthalic acid D_4, phthalimide D_4, TEB-OH D_6, tebuconazole D_6 and THPI D_6 were added prior to the extraction step and used as internal standards (ISTDs). All compounds were quantified using the appropriate ISTD (Table 1) to compensate for the variability associated with the analytical procedure, from calibration curves generated for each compound by analyzing at least four different calibration samples. A quadratic fit (origin ignored, no weighting) was used to compensate for the nonlinearity of the instrument response over a wide working range.

Each batch included: i) up to 18 urine samples (2 mL), ii) one procedural calibration blank sample and seven procedural calibration samples prepared from Surine™ and analyzed as regular samples to assess whether contamination may have occurred during analysis and to generate quadratic calibration curves intended for quantification, respectively, and iii) two matrix procedural QC samples (2-mL pooled urine sample non-spiked and spiked at an intermediate level) analyzed as regular samples to check for method accuracy.

Targeted substances were identified by comparing retention times and MRM transition ratios (±30%) between calibration and urine samples. The data validation protocol included several conditions: i) the determination coefficient of the calibration curve had to be greater than 0.99, ii) the concentration of a substance measured in the procedural calibration samples had to be within ±50% of its theoretical concentration value at the LOD and LOQ levels and ±25% at all other levels, iii) the response of a substance (ISTD response ratio) in the procedural blank samples had to be lower than 50% of that in the procedural calibration sample at the LOQ level, iv) the concentration of a substance measured in the matrix procedural QC samples prepared from pooled urine samples had to be within ±30% of its theoretical concentration value (results are reported in Table 3), and v) the concentration of a substance measured in the urine samples had to be within the method working

Table 3
Determination of the targeted substances other than ETU and EU in urine samples: interday method accuracy and precision assessed by analyzing several matrix procedural QC samples at an intermediate concentration level (7 batches).

Compound	Range (µg/L)	R^2	Matrix procedural QC sample[a]			
			N	Theoretical concentration (µg/L)	Measured concentration (µg/L)	Accuracy (%) (precision (%))
Boscalid	1.25 - 125	> 0.9997	6	25	28.0	112 (33)
Phthalic acid	2.5 - 250	> 0.9999	6	50	53.7	107 (4)
Phthalimide	0.5 - 50	> 0.9999	6	10	10.3	103 (11)
TEB-OH	1.25 - 125	> 0.9998	6	25	26.0	104 (13)
Tebuconazole	1.25 - 125	> 0.9999	6	25	19.3	77 (18)
THPI	1.25 - 125	> 0.9998	6	25	26.4	106 (21)

[a] 2-mL pooled urine sample spiked at an intermediate level.

range without exceeding 110% of the concentration of the most concentrated calibration samples. If all these conditions were not met, results were not validated and samples were reanalyzed if possible.

Determination of the targeted substances in hair samples

Preparation of hair sample (pulverization)

Approximately 20 to 100 mg of hair sample were accurately weighed (± 0.1 mg) on a piece of paper and transferred to a 5-mL stainless steel grinding jar with one 10 mm ball per jar. Grinding was performed in the mixer mill MM400 (Retsch GmbH, Haan, Germany) for 10 min at 25 Hz. The hair powder, after recovery on a piece of paper, was transferred into an amber glass vial that was then sealed and stored at −18 °C until analysis.

Extraction of the targeted substances from hair samples

Solvent extractions were performed using a mixer mill MM400 (Retsch GmbH, Haan, Germany). 25 mg of hair powder, the labeled internal standards (ISTDs), three 3 mm stainless steel micro balls previously rinsed with dichloromethane and 400 µL of acetonitrile were successively added in a 1.5-mL Eppendorf tube then placed on the mixer mill in a 24-position rack. Agitation/grinding was performed for 1 min at 25 Hz. The organic extract was then centrifuged at 3500 rpm for 20 min at 20 °C using a micro centrifuge accuSpin™ Micro 17R. The supernatant was transferred to a second Eppendorf tube. The procedure was repeated twice from the addition of acetonitrile and the three supernatants were combined in the second Eppendorf tube before a final centrifugation (3500 rpm for 20 min at 20 °C). The final supernatant was transferred to a third Eppendorf tube and stored at −18 °C prior to the evaporation step. Acetonitrile extracts were then evaporated to dryness at 30 °C under a nitrogen stream using a N-EVAP 111 Organomation Nitrogen Evaporator and reconstituted in 500 μL of DCM prior to be transferred into 2-mL amber glass vials. After adding 40 µL of the derivatization agent (MSTFA), concentrated extracts were incubated at 40 °C overnight (at least 16 h) prior to analysis by GC/MS/MS.

GC/MS/MS analysis

Analyses were performed using a 7890A GC system coupled to a 7000C GC/MS Triple Quad (Agilent Technologies, Santa Clara, California, United States) operated in electron impact ionization (EI) mode (70 eV). The GC system was equipped with a Gerstel MPS (MultiPurpose Sampler) robotic autosampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) and a multimode inlet (MMI) fitted with a single taper ultra-inert glass liner with glass wool. Calibration solutions and organic extracts were injected (5 µL) in the solvent vent mode (vent flow: 100 mL/min; vent pressure: 0 psi until 0.1 min; purge flow to split vent: 60 mL/min at 1.5 min) with the following injector temperature program: 37 °C (hold 0.1 min), ramp at 500 °C/min to 325 °C (hold 10 min). Helium was used

Table 4
Determination of the targeted substances in hair samples: interday method accuracy and precision assessed by analyzing several matrix procedural QC samples at different concentration levels (5 batches).

Compound	Range (ng/g)	R^2	Matrix procedural QC sample[a] LOQ level				Matrix procedural QC sample[a] Intermediate level				Matrix procedural QC sample[a] High level			
			N	Theoretical concentration (ng/g)	Measured concentration (ng/g)	Accuracy (%) (precision (%))	N	Theoretical concentration (ng/g)	Measured concentration (ng/g)	Accuracy (%) (precision (%))	N	Theoretical concentration (ng/g)	Measured concentration (ng/g)	Accuracy (%) (precision (%))
Boscalid	40 - 1600	> 0.9995	5	80	74.4	93 (14)	5	400	375	94 (5)	5	1600	1439	90 (4)
ETU	20 - 800	> 0.9998	5	40	45.5	114 (15)	5	200	246	123 (3)	5	800	1145	143 (20)
EU	40 - 1600	> 0.9998	5	80	91.9	115 (5)	5	400	411	103 (2)	5	1600	1695	106 (5)
Folpel	40 - 1600	> 0.9998	4	80	114	143 (4)	4	400	600	150 (15)	–	1600	–	–
Phthalimide	10 - 800	> 0.9998	5	40	44.2	111 (6)	5	200	199	100 (7)	5	800	756	95 (6)
TEB-OH	20 - 800	> 0.9996	5	40	38.5	96 (17)	5	200	202	101 (6)	5	800	820	102 (8)
Tebuconazole	10 - 400	> 0.9998	5	20	21.1	105 (10)	5	100	110	110 (6)	5	400	436	109 (5)
THPI	20 - 800	> 0.9997	5	40	41.5	104 (10)	5	200	210	105 (7)	5	800	796	99 (4)

[a] 25-mg real hair sample spiked at the LOQ level, at an intermediate level and at a high level.

as the column carrier gas at a constant flow rate of 1.0 mL/min. Chromatographic separation was performed on a Rtx®-PCB capillary column (30 m length × 0.25 mm I.D., 0.25 µm film thickness) supplied by Restek Corporation (Bellefonte, Pennsylvania, United States) with the following oven temperature program: 37 °C (hold 2 min), first ramp at 37 °C/min to 250 °C (hold 0 min), second ramp at 15 °C/min to 330 °C (hold 3 min to reach an analysis time of 16.1 min). The MSD transfer line, ion source and quadrupole temperatures were fixed at 325, 280 and 180 °C, respectively. The mass spectrometer (triple quadrupole) was operated in multiple reaction monitoring (MRM) mode. The two most intense and specific MRM transitions of each compound (quantifier and qualifier transitions) were monitored for identification, confirmation and quantification. They were selected using the pesticides and environmental pollutants MRM database provided by Agilent Technologies for the compounds present in the database or following the usual procedure for others. Analytical characteristics of measured compounds are reported in Table 1. MassHunter software (10.0) was used for instrument control, data acquisition and quantification.

Quality assurance and quality control (QA/QC)

The limit of detection (LOD) was defined as the lowest concentration of a substance that can be distinguished from the absence of that substance. LODs were estimated from the replicate analysis of a blank sample. The limit of quantification (LOQ) was defined as the lowest concentration of a substance for which the relative standard deviation (RSD) of the raw signal ($n = 5$) was lower than or equal to 20%, the signal-to-noise ratio (S/N) was greater than or equal to 10, and the raw signal was greater than or equal to 5 times the signal of the blank sample.

Several labeled substances were selected to best cover the physical and chemical properties of the targeted analytes. Boscalid D_4, ETU D_4, EU D_4, folpel D_4, phthalimide D_4, TEB-OH D_6, tebuconazole D_6 and THPI D_6 were added prior to the extraction step and used as internal standards (ISTDs). All compounds were quantified using the appropriate ISTD (Table 1) to compensate for the variability associated with the analytical procedure, from calibration curves generated for each compound by analyzing at least four different calibration samples. A quadratic fit (origin ignored, no weighting) was used to compensate for the nonlinearity of the instrument response over a wide working range.

Each batch included: i) up to 15 hair samples (25 mg), ii) one procedural calibration blank sample and six procedural calibration samples analyzed as regular samples to assess whether contamination may have occurred during analysis and to generate quadratic calibration curves intended for quantification, respectively, and iii) four matrix procedural QC samples (25-mg real hair sample non-spiked and spiked at the LOQ level, at an intermediate level and at a high level) analyzed as regular samples to check for method accuracy.

Targeted substances were identified by comparing retention times and MRM transition ratios (±30%) between calibration and hair samples. The data validation protocol included several conditions: i) the determination coefficient of the calibration curve had to be greater than 0.99, ii) the concentration of a substance measured in the procedural calibration samples had to be within ±50% of its theoretical concentration value at the LOD and LOQ levels and ±25% at all other levels, iii) the response of a substance (ISTD response ratio) in the procedural blank samples had to be lower than 50% of that in the procedural calibration sample at the LOQ level, iv) the concentration of a substance measured in the matrix procedural QC samples prepared from real hair samples had to be within ±30% of its theoretical concentration value (results are reported in Table 4), and v) the concentration of a substance measured in the hair samples had to be within the method working range without exceeding 110% of the concentration of the most concentrated calibration samples. If all these conditions were not met, results were not validated and samples were reanalyzed if possible.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Reference

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