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Analytical Methods

Targeted and untargeted analysis of triazole fungicides and their metabolites in fruits and vegetables by UHPLC-orbitrap-MS^2

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ABSTRACT

Two extraction methods based on solid liquid extraction and Quick, Easy, Cheap, Effective, Rugged and Safe procedure were developed for the determination of 21 triazole compounds and 5 metabolites, including triazole derivative metabolites as 1,2,4-triazole and 1,2,4-triazol 1-yl-acetic, in courgette, orange, grape and strawberry. The analysis was performed in 10.5 min, using ultra-high performance liquid chromatography coupled to Q-Orbitrap mass analyser. The proposed method was validated according to SANTE 12682/2019. Limits of quantification were $\leq$10 μg kg^{-1} for all the compounds, except for 1,2,4-triazol, 1,2,4-triazol 1-yl-acetic, difenoconazole-alcohol and prothioconazole that were 50 μg kg^{-1}. Finally, the method was successfully applied to the analysis of 30 samples. More than 30% of these samples contained residues of triazole compounds. The fungicide most frequently found was myclobutanil.

Furthermore, a suspect screening analysis was carried out to search pesticides present in the samples, detecting some of them at concentrations higher than Maximum Residue Limits.

1. Introduction

For many decades, fungicides are predominantly being used to control fungal-caused plant diseases that threaten human health and crop production (Ribas et al., 2016). Triazole compounds have been widely used since the 1980s to prevent and control fungal diseases of many crops (fruits, vegetables, nuts, grain, seed, etc.), increasing their use throughout the European market and becoming the most effective type of fungicides. The importance of the use of triazole compounds is due to their exceptional antifungal activity, relatively low resistance risk (Li et al., 2017) and their long-term stability in soil and water (Ribas et al., 2016). Their action mode is based on the inhibition of the fungal ergosterol biosynthetic pathway and inhibition of steroid demethylation (Liu et al., 2014).

Within the triazole family, the principal compounds are difenoconazole, fenbuconazole, tebuconazole, cyproconazole, myclobutanil, penconazole, propiconazole, tetraconazole, triadimenol, prothioconazole, triticonazole, bromuconazole, epoxiconazole, fluquinconazole, flutriafol, ipconazole, metconazole, paclobutrazol, flusilazole, bitertanol and triadimefon. All these compounds contain the 1,2,4-triazole moiety and are metabolized to four main common metabolites, known as triazole derivative metabolites (TDMs): 1,2,4-triazole, 1,2,4-triazol 1-yl-acetic (triazole acetic acid), triazole alanine and triazole lactic acid (Li et al., 2012; Ströher-Kolberg et al., 2016). Around 3–44% of the triazole family might transform into 1,2,4-triazole, which is one of the main metabolites. TDMs also come from other sources, such as industrial manufacturing of pharmaceuticals or nitrification inhibitors (Blondel et al., 2018). Additionally, some fungicides belonging to the triazole family have specific degradation routes. For instance, prothioconazole-desthio, difenoconazole-alcohol (CGA-205375) and tebuconazole-*tert*-butylhydroxy are the principal breakdown products of prothioconazole (Liu et al., 2017), difenoconazole and tebuconazole (University of Hertfordshire, 2021), respectively. Triadimefon may be enzymatically transformed in plants, soil and fungi by the reduction of a carbonyl group to its corresponding alcohol, triadimenol. Triadimenol, which is separately registered as a systemic fungicide, has higher fungicidal activity than triadimefon (Liang et al., 2013).

Triazole compounds are included in the list of active substances of the Commission Regulation (EU) 2017/269 (European Commission, 2017), except propiconazole, epoxiconazole, flusilazole, bitertanol,

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triadimenol and triadimefon, which were withdrawn of approval (Brancato et al., 2018). Nevertheless, triazole compounds can reach plant tissues leaving residues that might be detected in fruits and vegetables (Bordagaray et al., 2011). In order to protect consumer's health, European Union (EU) has published regulations setting the Maximum Residue Limits (MRLs) for a wide range of pesticides in different fruits and vegetables (EFSA, 2021). The established MRLs for triazole fungicides in courgettes ranged from 0.01 to 0.60 mg kg^{-1}, in oranges 0.01–9 mg kg^{-1}, in grapes 0.01–5 mg kg^{-1} and in strawberries from 0.02 to 15 mg kg^{-1}. However, the MRLs for triazole compounds do not include any of the metabolites, except the case of the prothioconazole-desthio, which is the metabolite of prothioconazole (EFSA, 2021).

Due to the complexity involved in the simultaneous analysis of triazole compounds and metabolites, some authors have developed two different analytical methods, one for the determination of the parent compounds and another for the metabolites (Blondel et al., 2018). Thus, for parent triazole compounds, pesticide multiresidue methods based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure (Liu et al., 2011; Lin et al., 2017) followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have been extensively applied, being the triple quadrupole (QqQ) the analyzer most widely used, achieving recoveries between 88 and 119%, relative standard deviation (RSD) $\leq$ 20% and limits of quantification (LOQs) lower than 10 μg kg^{-1} for most of the cases (Bordin et al., 2016; Ribeiro et al., 2018).

Furthermore, difenoconazole has been studied using gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) with QqQ in watermelon samples. QuEChERS was also used as extraction method, and cleaned up by dispersive solid-phase extraction (d-SPE) with octadecylsilane sorbent was applied. Recoveries were found between 72 and 99%, with RSD values were $<$ 20% and LOQ was 10 μg kg^{-1} (Kang et al., 2017).

Regarding TDMs, as they are high polar compounds, the QuPPe (Quick Polar Pesticides) method, with methanol as solvent, can be used for their analysis by LC-MS/MS, using Q-Trap (Quadrupole-Ion trap) as mass analyzer. In this context, 1,2,4-triazol and 1,2,4-triazol 1-yl-acetic were studied in cucumber, orange, grape and rice, finding recoveries between 74 (1,2,4-triazol 1-yl-acetic in rice) and 128% (1,2,4-triazol in orange), and RSD values ranged from 2 (1,2,4-triazol 1-yl-acetic in cucumber) to 25% (1,2,4-triazol in cucumber). The LOQs were 10 μg kg^{-1} for both compounds, except at 200 μg kg^{-1}, which was established for 1,2,4-triazol, and at 20 μg kg^{-1} for 1,2,4-triazol in rice samples (Anastassiades et al., 2017). For the determination of these compounds (1,2,4-triazol and 1,2,4-triazol 1-yl-acetic), another study was performed in orange, milk, rice and courgette samples. LC-MS/MS was used, selecting Q-Trap as analyzer. Recoveries for both metabolites were overall satisfying between 86 and 95%, and RSDs were ranged between 5 and 17% (Ströher-Kolberg et al., 2016). Despite the wide extensive use of QqQ in triazole compounds, this analyzer has some limitations, due to their few confirmation ions and possible chromatographic interferences. The mass analyzer Q-Trap has been used in the determination of prothioconazole and its metabolite prothioconazole-desthio in cucumber and pear samples. The LOQs were estimated in 0.01–0.02 μg kg^{-1} for enantiomers of prothioconazole and 0.0025–0.0075 μg kg^{-1}, for prothioconazole-desthio (Zhang et al., 2017). In this sense, high resolution mass spectrometry (HRMS) analyzers are a suitable alternative to improve the identification capability because they are able to perform accurate mass measurements and confirm requirements by the study of fragments. This kind of analyzers offers the possibility of retrospective analysis, allowing for both targeted and non-targeted approaches. Time-of-flight (TOF), hybrid quadrupole TOF (QTOF), and (Q)-Orbitrap are HRMS instruments mainly used for pesticide residue analysis in food and water matrices (López-Ruiz et al., 2019a). However, higher resolution (up to 100,000 FWHM) can be achieved using Orbitrap technology, due to it allows the detection and identification of a wide range of analytes at low levels of concentration in targeted and non-targeted analysis (López-Ruiz et al., 2016).

The aim of the present study was the development of a new, suitable and efficient analytical multiresidue method for the simultaneous determination of 26 triazole compounds, including metabolites, in fruits and vegetables (courgette, orange, grape and strawberry samples). The combination of UHPLC-Q-Orbitrap-MS^2 with an extraction procedure based on SLE or QuEChERS approaches, allows the determination of 21 parent pesticides and 5 metabolites. Additionally, due to the potential of the analytical system, a suspect screening was performed for a reliable identification of pesticides in different samples. To our knowledge, there are not any publication describing the simultaneous analysis of a high number of triazole fungicides, especially 26 (parent compounds and metabolites) in different plant matrices.

2. Materials and methods

2.1. Equipment, material and reagents

Difenoconazole (CAS registry No. 119446–68-3), fenbuconazole (CAS registry No. 114369–43-6), tebuconazole (CAS registry No. 107534–96-3), cyproconazole (CAS registry No. 94361–06-5), myclobutanil (CAS registry No. 88671–89-0), penconazole (CAS registry No. 66246–88-6), propiconazole (CAS registry No. 60207–90-1), tetraconazole (CAS registry No. 112281–77-3), triadimenol (CAS registry No. 55219–65-3) and 1,2,4-triazole (CAS registry No. 288–88-0) were acquired from Sigma Aldrich (St. Louis, MO, USA). All of them have $\geq$ 98% of purity. Prothioconazole (CAS registry No. 178928–70-6), triticonazole (CAS registry No. 131983–72-7), bromuconazole (CAS registry No. 116255–48-2), epoxiconazole (CAS registry No. 133855–98-8), fluquinconazole (CAS registry No. 136426–54-5), flutriafol (CAS registry No. 76674–21-0), ipconazole (CAS registry No. 125225–28-7), metconazole (CAS registry No. 125116–23-6), paclobutrazol (CAS registry No. 76738–62-0), flusilazole (CAS registry No. 85509–19-9), bitertanol (CAS registry No. 55179–31-2), triadimefon (CAS registry No. 43121–43-3), prothioconazole-desthio (CAS registry No. 120983–64-4), difenoconazole-alcohol (CAS registry No. 117018–19-6) and tebuconazole-*tert*-butylhydroxy (CAS registry No. 212267–64-6) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Purity of the compounds was $\geq$ 92%. The compound triazole 1,2,4-triazol 1-yl-acetic was supplied from Supelco (Buchs, Switzerland) (CAS registry No. 28711–29-7) and its purity was $\geq$98%. Individual stock standard solutions (1000 mg L^{-1}) were prepared by dissolving 10 mg of the pure compound in methanol (10 mL). Intermediate solutions (10 mg L^{-1} and 1 mg L^{-1}) were prepared with methanol and finally they were stored at ≤ -21 °C.

Methanol and acetonitrile, both LC-MS grade (99.9% of purity), were acquired from Honeywell Riedel-de-Haën (Seelze, Germany). Formic acid (>98% of purity) was obtained from PanReac AppliChem (Barcelona, Spain), ammonium formate from Fluka (Steinheim, Germany), acetic acid from Merck® (Germany), ammonium acetate from Sigma-Aldrich and water, LC-MS grade, was purchased from J.T. Baker (Deventer, The Netherlands). Magnesium sulfate (purity: 96%) and sodium chloride (purity $\geq$ 99.5%) were acquired from PanReac AppliChem, and primary secondary amine (PSA) was purchased from Scharlab (Barcelona, Spain).

A mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive) from Thermo-Fisher (Waltham, MA, USA) were employed for the accurate mass calibration of the Q-Orbitrap analyser.

2.2. UHPLC-Q-Orbitrap-MS^2 analysis

For chromatographic analysis, Thermo Fisher Scientific Vanquish Flex Quaternary LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) was used. The chromatographic system is coupled to a hybrid mass spectrometer Q-Exactive Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen,

Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive mode. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N_2, 95%), 35 (adimensional); auxiliary gas (N_2, 95%), 10 (adimensional); S-lens RF level, 50 (adimensional); heater temperature, 305 °C; and capillary temperature, 300 °C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 70,000 Full Width at Half Maximum (FWHM); AGC target = 1e6, (2) data dependent mass spectrometry fragmentation (dd-MS/MS), ESI+ (HCD on, collision energy = 30 eV), mass resolving power = 35,000 FWHM; AGC target = 1e5. The mass range in the full scan experiments was set to *m/z* 50–750. For the chromatographic separation, the column Hypersil GOLD™ aQ (100 mm × 2.1 mm × 1.9 μm particle size) was used. Nevertheless, different stationary phases were evaluated: Acclaim™ Trinity Q1 (100 mm × 2.1 mm × 3 μm particle size), Acclaim™ Trinity *P*1 (100 mm × 2.1 mm × 3 μm particle size), Hypercarb™ (100 mm × 2.1 mm × 5 μm particle size), all of them supplied by Thermo Fisher Scientific. The flow rate was set at 0.3 mL min^{-1}. The mobile phase consisted of eluent A, which was a water solution containing 4 mM ammonium formate, 0.1% formic acid and eluent B, acetonitrile. The step gradient was as follows: 0–2 min 95% A; from 2 to 7 min, it was decreasing to 5% A and then the composition was kept constant for 2 min. Finally, it returned to the initial conditions in 0.5 min and remained constant for 1 min. The total running time was 10.5 min. The column temperature was set at 30 °C and the injection volume at 10 μL.

The results were acquired using the external calibration mode and they were processed using Xcalibur™ version 4.3.73, with Quan Browser and Qual Browser (Thermo Fisher Scientific, Les Ulis, France). TraceFinder 4.0 (Thermo Fisher Scientific) was employed for suspect screening.

2.3. Sample extraction

Two extraction methods were performed for triazole compounds. The first one, which was based on QuEChERS approach (Lehotay et al., 2010), was applied to extract all analytes from orange, grape and strawberry samples, except for 1,2,4-triazol, 1,2,4-triazol 1-yl-acetic, difenoconazole-alcohol and prothioconazole. The second method based on SLE was employed for the extraction of these last 4 compounds (in orange, grape and strawberry samples) and for all compounds from courgette. An additional dispersive solid-phase extraction (d-SPE) was applied for cleaning orange extracts. Even so, all samples were crushed, as required by the current regulation (European Commission, 2019), homogenised and stored in the freezer at −21 °C for further analysis.

The first method (QuEChERS) was carried out as follows: 10 g of each type of sample and 10 mL of acetonitrile were added into a 50 mL-centrifuge tube (in the case of orange samples, 10 mL of water was likewise added before acetonitrile). Subsequently, a mixture of 4 g of anhydrous $MgSO_4$ and 1 g of NaCl was included. The tubes were shaken for 1 min and centrifuged 10 min at 3700 rpm (3061 *g*). After centrifugation, 1 mL of the supernatant was collected and injected into the UHPLC-Q-Orbitrap-MS^2.

The second method (SLE) consisted of adding 10 g of each type of sample and 10 mL of acetonitrile into a 50 mL-centrifuge tube (as the previous method, in the case of orange samples, 10 mL of water were added before acetonitrile). The tubes were shaken for 1 min and centrifuged 10 min at 3700 rpm (3061 *g*). Finally, 1 mL of the supernatant was collected and injected into the UHPLC-Q-Orbitrap-MS^2. After centrifugation, a clean-up step (d-SPE) was only applied on orange samples, adding 40 mg of PSA into a 15 mL centrifuge tube with 1.5 mL of the extract, due to this matrix is dirtier than the other matrices and their extracts must be cleaned.

2.4. Method validation

The proposed method was validated according to SANTE guidelines (European Commission, 2019). In this context, the following parameters were determined: linearity, matrix effect, LOQ, recovery and precision (intra- and inter-day values), expressed as RSD.

The matrix effect was carried out by analysing calibration curves at different fungicide concentrations in solvent (acetonitrile) and in blank matrix extract. The concentrations were 5, 25, 50, 75, 100 and 250 μg L^{-1}. The slopes were compared to assess the matrix effect and calculated using equation 1:

$$\text{Matrix effect } (\%) = \left(\frac{\text{Slope in matrix}}{\text{Slope in disolvent}} - 1\right)\cdot 100 \quad (1)$$

Consequently, matrix effect can be considered negligible if the result is equal to or lower than ± 20%, whereas values lower than −20% indicate significant matrix suppression and strong matrix enhancement can be present if values are higher than 20%.

Linearity was evaluated by least-squares regression of peak area versus concentration of the calibration standards. The linearity of the calibration curves was evaluated by determination coefficients (R^2), which must be ≥ 0.99. Moreover, it was checked that the deviation of calibration points was ≤ 20%.

The LOQ was established as the lowest spike level tested by injection of 5 replicates that provided acceptable recoveries (70–120%) and precision (RSD ≤ 20%) values. Recovery studies were performed by spiking blank samples (n = 5) at two concentrations, 10 and 100 μg kg^{-1} for courgette. For the rest of matrices, 5 μg kg^{-1} were used instead of 10 μg kg^{-1} (except for 1,2,4-triazol, 1,2,4-triazol 1-yl-acetic, difenoconazole-alcohol and prothioconazole, which were spiked at 50 μg kg^{-1}). In compliance with the SANTE guidelines (European Commission, 2019), recovery can be accepted if the range is established between 70 and 120%. Intra-day (repeatability) and inter-day (reproducibility) values for precision were studied at the same two concentrations for each analyte and were expressed as RSD (European Commission, 2019). Intra-day precision values were obtained from the analysis of 5 spiked samples on the same day. Inter-day precision values were obtained from the analysis of 5 spiked samples over 4 successive days.

For the detection and identification of triazole fungicides, a precursor ion (with a mass error lower than 5 ppm) and one fragment, at least (with a mass error lower than 10 ppm) were selected (Gómez-Pérez et al., 2014; López-Ruiz et al., 2016).

Chromatograms of blank samples for each matrix (courgette, orange, grape and strawberry) are shown in Fig. S-1 (Supplementary Material).

2.5. Sample collection

Courgette (6), orange (6), grape (6) and strawberry (12) samples were collected from greenhouses located in Almería (Spain). They were analysed using the procedure described in Section 2.3 and targeted and non-targeted analysed were carried out.

3. Results and discussion

3.1. Optimization of UHPLC-MS/MS

The first stage of the development of the analytical method consisted of the spectrometric characterization of the triazole fungicides. For that purpose, an intermediate solution of 100 μg L^{-1} of each compound was injected into the UHPLC-Q-Orbitrap-MS^2 system, using positive ionization mode (ESI^+). Full-scan mass spectra were acquired to obtain the precursor ion ($[M+H]^+$) for each triazole compound, according to exact mass and molecular formula. It is considered that the mass error had to be lower than 5 ppm (Table 1). Consequently, MS^2 spectra (at collision

Table 1
UHPLC-Q-ORBITRAP-MS^2 parameters used for the selected compounds.

Compound	RT[a] (min)	Precursor ion		Fragments		
		Theoretical exact mass (*m/z*)	Mass error (ppm)	Theoretical exact mass (*m/z*)	Molecular formula	Mass error (ppm)
1,2,4-triazol	0.98	70.03997	4.42	–	–	–
1,2,4-triazol 1-yl-acetic	1.03	128.04545	0.31	70.03997	$C_2H_4N_3$	9.70
Flutriafol	4.64	302.10994	0.75	70.03997	$C_2H_4N_3$	9.65
				123.02407	C_7H_4FO	2.60
Tebuconazole- *tert*-butylhydroxy	4.73	324.14733	0.39	70.03997	$C_2H_4N_3$	9.36
				125.01525	C_7H_6Cl	2.28
Paclobutrazol	4.80	294.13676	0.45	70.03997	$C_2H_4N_3$	9.65
				125.01525	C_7H_6Cl	3.64
Triadimenol	4.81	296.11603	0.81	70.03997	$C_2H_4N_3$	9.85
Cyproconazole	4.87	292.12111	0.11	70.03997	$C_2H_4N_3$	9.51
				125.01525	C_7H_6Cl	2.60
Triadimefon	4.87	294.10038	0.60	70.03997	$C_2H_4N_3$	9.65
				125.01525	C_7H_6Cl	3.64
Tetraconazole	4.87	372.02880	0.49	70.03997	$C_2H_4N_3$	9.63
				158.97628	$C_7H_5Cl_2$	1.74
Triticonazole	4.88	318.13676	0.51	70.03997	$C_2H_4N_3$	9.67
				121.04480	C_8H_6F	2.84
Myclobutanil	4.89	289.12145	0.13	70.03997	$C_2H_4N_3$	9.08
				125.01525	C_7H_6Cl	2.68
				151.03090	C_9H_8Cl	1.16
Epoxiconazole	4.91	330.08039	0.44	70.03997	$C_2H_4N_3$	9.22
				121.0448	C_8H_6F	2.93
Fluquinconazole	4.92	376.01627	1.94	306.9836	$C_{14}H_6Cl_2FN_2O$	0.32
				349.0054	$C_{15}H_8Cl_2FN_4O$	1.14
Difenoconazole-alcohol	4.93	350.04575	−0.02	70.03997	$C_2H_4N_3$	9.70
				125.01525	C_7H_6Cl	2.47
Prothioconazole	4.95	344.03856	2.15	125.01525	C_7H_6Cl	1.11
				326.02800	$C_{14}H_{14}N_3Cl_2S$	−0.36
Fenbuconazole	4.95	337.12145	−0.32	70.03997	$C_2H_4N_3$	9.08
				158.97628	$C_7H_5Cl_2$	−0.25
Flusilazole	4.96	316.10761	0.86	165.06968	$C_8H_8N_3F$	3.41
				187.05603	$C_9H_9N_3Si$	9.61
Prothioconazole-desthio	4.97	312.06649	0.01	70.03997	$C_2H_4N_3$	9.36
				125.01525	C_7H_6Cl	2.68
Bitertanol	4.98	338.18630	1.33	70.03997	$C_2H_4N_3$	9.42
				251.14313	$C_{18}H_{19}0$	3.17
Tebuconazole	5.00	308.15241	0.38	70.03997	$C_2H_4N_3$	9.7
				125.01525	C_7H_6Cl	1.99
				151.03090	C_9H_8Cl	1.12
Bromuconazole	5.08	375.96135	−0.70	70.03997	$C_2H_4N_3$	9.65
				158.97628	$C_7H_5Cl_2$	1.74
Metconazole	5.08	320.15241	0.63	70.03997	$C_2H_4N_3$	9.22
				125.01525	C_7H_6Cl	2.68
Propiconazole	5.13	342.07705	−0.01	158.97628	$C_7H_5Cl_2$	1.68
				204.98176	$C_8H_7O_2Cl_2$	−0.49
Penconazole	5.14	284.07157	0.63	70.03997	$C_2H_4N_3$	9.60
				158.97628	$C_7H_5Cl_2$	0.12
Ipconazole	5.17	334.16806	0.43	70.03997	$C_2H_4N_3$	9.23
				125.01525	C_7H_6Cl	2.44
Difenoconazole	5.19	406.07197	−0.25	141.01017	C_7H_6OCl	1.35
				251.00250	$C_{13}H_9Cl_2O$	1.40
				337.03930	$C_{17}H_{15}Cl_2O_3$	0.45

[a] Abbreviation: RT: Retention time.

energy of 30 eV) were studied with the aim of selecting the fragments (at least two) for each fungicide. The fragments were chosen in accordance with the most abundant ion, the retention time, which has to be equal to the corresponding of the precursor ion, and the mass error. It is considered that the mass error had to be lower than 10 ppm. Table 1 shows a summary of the MS parameters obtained for the targeted compounds, where it can be observed that at least two fragments were monitored for each triazole compound, except for 1,2,4-triazol, due to its low molecular weight.

A similar fragmentation pathway was achieved for these compounds because they belong to the same triazole family. There are common fragments as *m/z* 70.03997, *m/z* 125.01525 or *m/z* 158.97628. The fragment *m/z* 70.03997 corresponds to the protonated structure of 1,2,4-triazol. The fragment *m/z* 125.01525 (C_7H_6Cl) corresponds to a benzene composed of a chlorine and methyl groups, and the fragment *m/z* 158.97628 ($C_7H_5Cl_2$) to chlorines and a benzene with a chain carbon.

In the second stage, the chromatographic conditions were tested by studying different mobile phases, stationary phases and elution gradients, with the aim of reducing analysis time and obtaining the best peak shapes. The conditions of the mobile phase and several types of columns were evaluated. Firstly, the procedure described by Ströher-Kolberg et al. (Ströher-Kolberg et al., 2016) for the determination of 1,2,4-triazol has been evaluated, using methanol (1% acetic acid) and water (1% acetic acid + 5% methanol) as mobile phase, and a Hypercarb™ column as stationary phase. However, some parent compounds as difenoconazole, fenbuconazole, prothioconazole, tebuconazole, epoxiconazole and metconazole were not detected. Therefore, a multimode column that is suitable for very polar and non-polar compounds, Acclaim™ Trinity Q1, was evaluated. Due to this column is not compatible with alcohols, and the pH range of the mobile phase has to be between 2.5 and 7.5, with an optimum pH = 5 (ThermoScientific, 2014), acetonitrile, as organic

phase and an aqueous phase, consisted of water 5 mM ammonium formate at different level of pH (at 5, 6 and 7), were evaluated. It was observed better peak shapes at pH = 5. Thus, it will be used for further experiments, although, a poor retention of 1,2,4-triazole was observed, eluting at 1 min.

Similar conditions were checked with an Acclaim™ Trinity *P*1 column, due to the previously used was not appropriate for solvents with alcohol groups and the triazole fungicides were prepared in methanol. It was observed that the peak of 1,2,4-triazole still eluted at 1 min. Methanol was also tested as organic mobile phase, but peak shapes were better with acetonitrile. Subsequently a test in matrix (orange) was evaluated. After some analyses, a reduction of separation efficiency was observed due to this column was affected by the characteristics of the matrix studied. Despite the efforts made to find a column that provided a suitable separation for both parent and metabolite triazole compounds, for simplicity, a conventional C18 column (Hypersil gold™ aQ) was tested, using water containing 4 mM ammonium formate and 0.1% formic acid, methanol as mobile phase. Finally, it provided satisfactory results for the analysis, finding all compounds with good peak shapes, similar of the optimum conditions of the Acclaim TM Trinity P1 column.

Then, the elution gradient profile was optimized to improve the retention time of the targeted compounds. The gradient studied was one developed previously by the research group with a total time running of 22 min (López-Ruiz et al., 2019b). The mobile phase used was described previously (water containing 4 mM ammonium formate and 0.1% formic acid, as eluent A, and methanol as eluent B). Due to the long analysis time, another gradient was evaluated. Subsequently, with the aim of reducing the time of the transition of organic phase and the last stage of equilibration, a second gradient was evaluated (the total run time to determine the target analytes was 10.5 min). Due to this gradient provided suitable peak shapes for all analytes and good retention times between 1 and 5.5 min, it was selected. This analysis time was lower than the provided by other authors (Ströher-Kolberg et al., 2016), which achieved a total running time 19 min for 4 TDMs. The extracted ion chromatograms of the triazole fungicides are shown in Fig. 1, where it can be observed that bromuconazole has two peaks because each one corresponds to an enantiomer.

3.2. Optimization of extraction procedure

With the aim of developing effective extraction procedures for parent and metabolite triazole fungicides, several procedures as SLE or QuEChERS (Lehotay et al., 2010), with some modifications, were evaluated.

Due to the characteristics of orange, initially this matrix was used for the optimization of extraction procedure. It should be highlighted that the samples were fortified and quantified using calibration curves in black extract of each matrix.

Firstly, orange was studied and the SLE method proposed by Ströher-Kolberg et al. (Ströher-Kolberg et al., 2016) was tested, using methanol containing 1% formic acid as extraction solvent. However, some of the triazole compounds were not extracted (prothioconazole, bitertanol and difenoconazole-alcohol) and low recoveries, from 8 (difenoconazole) to 55% (1,2,4-triazol and 1,2,4-triazol 1-yl-acetic), were obtained for some compounds at 500 μg kg^{-1}, as it can be observed in Table S-1. Secondly, a clean-up step based on d-SPE with PSA, as sorbent, was included. In this context, recoveries were found between 22 (difenoconazole) and 62% (myclobutanil). Even though good signal to noise ratios (S/N) were obtained for all analytes, the peak shapes were not completely suitable.

Thirdly, non-buffered QuEChERS approach was evaluated, showing

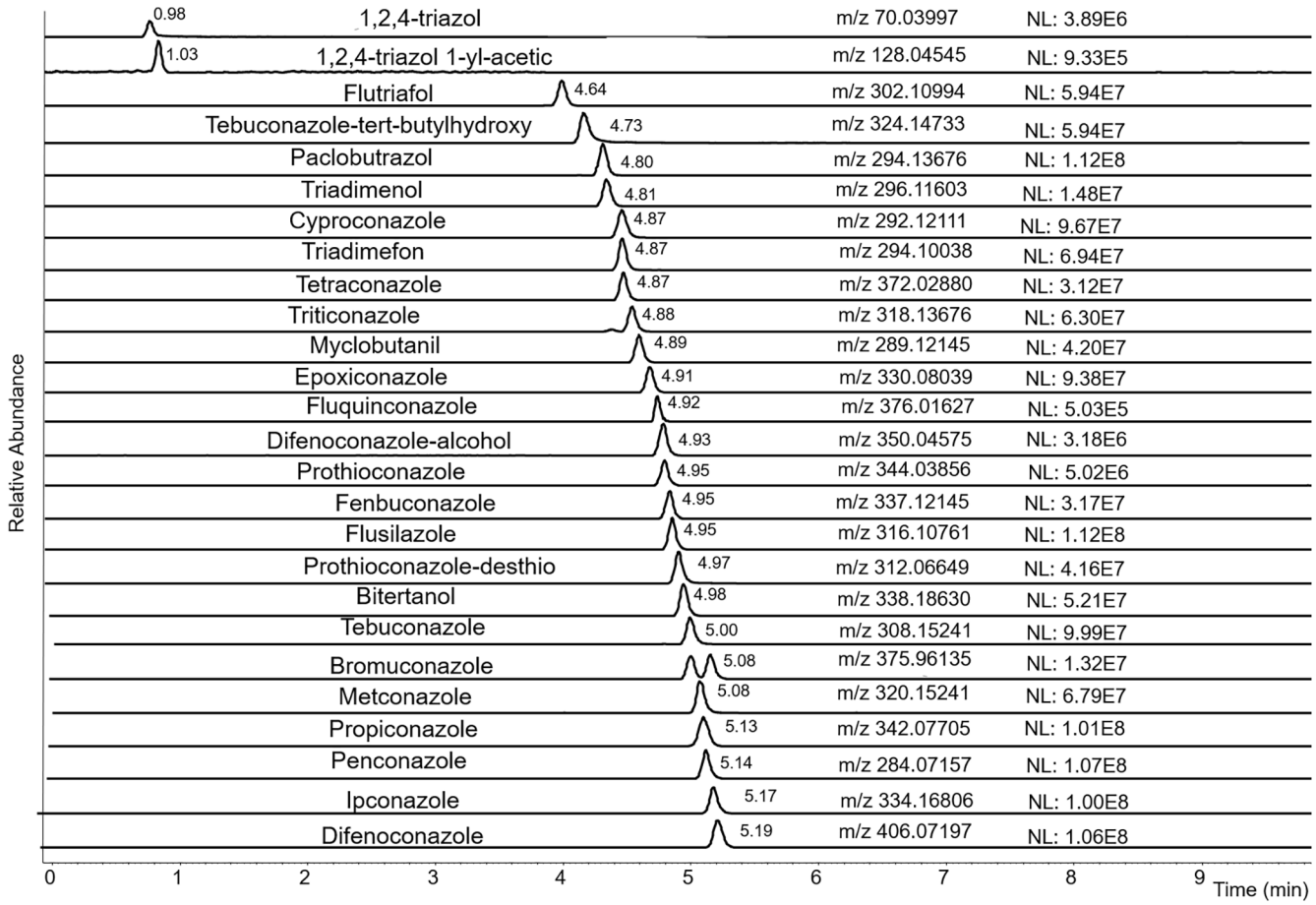

Fig. 1. Chromatograms of parent and metabolite triazole fungicides (100 μg L^{-1} in methanol).

satisfactory recoveries, with mean values ranging from 94 (difenoconazole) to 125% (fluquinconazole) at 500 μg kg^{-1}. Prothioconazole was not extracted, and poor recoveries were obtained for difenoconazole-alcohol, 1,2,4-triazol and 1,2,4-triazol 1-yl-acetic (54, 21% and 3%, respectively), as it can be observed in Table S-1. This fact shows the difficulties of the development of a unique multiresidue method for the determination of parent and metabolite triazole compounds. Thus, non-buffered QuEChERS was selected for the majority of compounds and two new methods were evaluated to improve the extraction of 1,2,4-triazol, 1,2,4-triazol acetic acid, difenoconazole-alcohol and prothioconazole: SLE with acetonitrile and SLE with acetonitrile containing 1% (v/v) formic acid previous a hydration step with 10 mL of water in both cases at 500 μg kg^{-1}. In the SLE procedure, using acetonitrile as extractant solvent, it was observed better recoveries (1,2,4-triazol and 1,2,4-triazol 1-yl-acetic, 80% and 130%, respectively) in comparison with SLE using acetonitrile containing 1% (v/v) formic acid (1,2,4-triazol and 1,2,4-triazol 1-yl-acetic, 104% and >150% respectively) as it can be observed in Table S-1. Therefore, SLE, using acetonitrile as extractant solvent, was selected for further experiments.

Finally, a clean-up step with PSA, was included to SLE in orange matrix, finding better recoveries in comparison with SLE without clean-up step, because some compounds were not recovered. For that, the clean-up step with PSA was selected as extraction procedure of 1,2,4-triazol, 1,2,4-triazol 1-yl-acetic, difenoconazole-alcohol and prothioconazole, achieving suitable results of recovery. Table S-1 shows the recoveries values.

Once the extraction procedure was optimized in orange samples, both methods (non-buffered QuEChERS approach and SLE) were evaluated in courgette, grape and strawberry matrices. In courgette samples, when non-buffered QuEChERS was used, recoveries were ranged between 25 (1,2,4-triazol) and >150% (1,2,4-triazol 1-yl-acetic). Therefore, for simplicity, SLE was evaluated and satisfactory recovery values were obtained, between 70 (paclobutrazol) and 116% (1,2,4-triazol 1-yl-acetic). Consequently, this method was selected for courgette. For grape and strawberry, SLE was employed for 1,2,4-triazol, 1,2,4-triazol acetic acid, difenoconazole-alcohol and prothioconazole as in orange samples and non-buffered QuEChERS for the rest of analytes.

Despite these exceptions, the proposed extraction procedures allow the reliable extraction of the studied analytes.

Fig. S-2 to S-5 show the extracted ion chromatograms (XICs) of fortified samples of each matrix (courgette, orange, grape and strawberry).

3.3. Method validation

The optimized method was validated in each matrix for the targeted compounds using the current SANTE Guideline (European Commission, 2019), showing the results in Tables S-2 to S-5.

The evaluation of linearity was carried out through determination coefficients (R^2) and the values were always higher than 0.98 (Tables S-2 to S-5) for all target analytes, except for 1,2,4-triazol in orange. Nevertheless, it was checked that deviation of back-calculated concentration was always equal to or lower than 20%. The working range was from 5 μg kg^{-1} (except for 1,2,4-triazol, 1,2,4-triazol 1-yl-acetic, difenoconazole-alcohol and prothioconazole, 50 μg kg^{-1}) and 250 μg kg^{-1} for triazole fungicides from orange, grape and strawberry samples. In the case of courgette, the working range was from 10 to 250 μg kg^{-1}.

For the triazole compounds, matrix effect was not detected in the 70% of the compounds in courgette, grape and strawberry. In the 20% of the cases, it was observed matrix suppression (negative), whereas strong matrix enhancement, in the 10% of matrix-analyte combinations. It should be considered that these values were similar in the three matrices (Tables S-2, S-4 and S-5). However, significant matrix effect was noted in orange for all compounds (Table S-3), specifically matrix suppression, except for bromuconazole, flutriafol, ipconazole, metconazole, penconazole and propiconazole, which did not present matrix effect.

The LOQ was established at 10 μg kg^{-1} for all triazole compounds in courgette, and at 5 μg kg^{-1} in the other matrices (orange, grape and strawberry) for the target compounds, except for 1,2,4-triazol, 1,2,4-triazol 1-yl-acetic, difenoconazole-alcohol and prothioconazole, which was set at 50 μg kg^{-1}. Additionally, it should be highlighted that LOQs are equal to or lower than the MRLs set by the EU for these matrices.

Tables S-2 to S-5 show the results obtained from the inter and intra-day recovery study. Good recoveries were obtained for the four matrices evaluated at two concentration levels, for each triazole compound. The average recoveries were ranged between 70% and 120%, as it is observed in Tables S-2 to S-5. In general, most of the analysed compounds showed satisfactory recoveries, with mean values ranging from 70 to 120%, except for prothioconazole and fluquinconazole that were not recovered at 10 μg kg^{-1} in courgette and orange samples. In these cases, the LOQ was 100 μg kg^{-1} for both matrices.

Likewise, the remarkably good precision results achieved, in terms of intra- and inter-day precision and expressed as RSD (%), are shown in Tables from S-2 to S-5. Intra- and inter- day precision values were ranged between 1 (ipconazole in orange) and 20% (1,2,4-triazol 1-yl-acetic in grape), and between 3 (paclobutrazol in strawberry) and 20% (bitertanol in courgette), respectively. As it can be observed, the RSD values were found less than or equal to 20% for the two concentrations assessed, indicating the reliability of the method.

3.4. Analysis of samples

The analytical method was applied to the determination of triazole fungicides and their metabolites in 30 samples: 6 courgettes (CRG-1 to CRG-6), 6 oranges (ORG-1 to ORG-6), 6 grapes (GRP-1 to GRP-6) and 12 strawberries (STB-1 to STB-12). To ensure the reliability of the results, an internal quality control was used. This quality control consisted of a blank sample to check the absence of interferences, a matrix calibration curve from 5 to 250 μg L^{-1} to evaluate the sensitivity and perform the quantification of samples, and fortified samples at 100 μg kg^{-1} to determine the efficiency of the extraction process. In Fig. S-1 to S-5 it can be observed the XICs of blank and fortified samples (at 100 μg kg^{-1}) in the four studied matrices.

Flutriafol, tebuconazole-*tert*-butylhydroxy, paclobutrazol, triadimenol, tetraconazole, myclobutanil, epoxiconazole, prothioconazole, flusilazole, propiconazole, penconazole and difenoconazole were detected in 10 out of the 30 analyzed samples at concentrations above the LOQ (see Table 2). In courgette samples, triazole fungicides were not detected. For orange samples, only epoxiconazole was detected in the two samples (ORG-1 and ORG-2) at 0.009 and 0.01 mg kg^{-1}, which were below the MRL (0.05 mg kg^{-1}). In grape samples (GRP-2 and GRP-3), myclobutanil was found at 0.01 and 0.05 mg kg^{-1} and prothioconazole (GRP-3) at 0.08 mg kg^{-1}, overcoming the MRLs set by EU 0.01 mg kg^{-1} for both compounds. Finally, in strawberry, flutriafol (STB-3), tebuconazole-*tert*-butylhydroxy (STB-5), myclobutanil (STB-1, STB-2 and STB-5), penconazole (STB-4) and difenoconazole (STB-1) were detected at concentration ranging from 0.005 (tebuconazole-*tert*-butylhydroxy) to 0.14 mg kg^{-1} (myclobutanil). The compounds with concentration values higher than the MRLs in strawberry were myclobutanil (0.14 mg kg^{-1}), penconazole (0.06 mg kg^{-1}) and flutriafol (0.08 mg kg^{-1}), whose MRLs were 0.05 mg kg^{-1}. Table 2 shows the results. The high percentage of residues about MRLs can be explained because samples were collected before they were ready for distribution.

Comparing all matrices, the highest concentration was detected in strawberry (STB-1), where myclobutanil was found at 0.14 mg kg^{-1}. In addition to this compound, difenoconazole (0.05 mg kg^{-1}), tetraconazole and tebuconazole-*tert*-butylhydroxy were detected in STB-1, showing the obtained chromatograms in Fig. 2. These last two compounds were detected but not quantified.

Some positive samples present in STB-1 (myclobutanil and difenoconazole) and GRP-2 (myclobutanil) are shown in the XICs of Fig. S-4 and S-5, respectively.

Regarding the triazole compounds, which have been detected at

Table 2
Concentration (mg kg^{-1}) of triazole compounds detected in different samples.[a]

Compound	Analyzed sample									
	ORG-1	ORG-2	GRP-1	GRP-2	GRP-3	STB-1	STB-2	STB-3	STB-4	STB-5
Flutriafol	–	–	–	–	–	–	<LOQ	0.08	–	–
Tebuconazole-*tert*-butylhydroxy	<LOQ	–	–	–	–	<LOQ	–	–	–	0.005
Paclobutrazol	–	–	–	–	<LOQ	–	–	–	–	–
Triadimenol	–	–	–	–	–	–	–	<LOQ	<LOQ	–
Tetraconazole	–	–	–	–	–	–	–	–	–	–
Myclobutanil	–	–	–	0.05	0.01	0.14	0.01	–	–	0.01
Epoxiconazole	0.009	0.01	–	–	–	–	–	–	–	–
Prothioconazole	–	–	–	–	0.08	–	–	–	–	–
Flusilazole	<LOQ	<LOQ	–	–	–	–	<LOQ	–	–	–
Propiconazole	–	–	–	–	–	–	–	–	<LOQ	–
Penconazole	–	–	<LOQ	–	–	–	–	–	0.06	–
Difenoconazole	–	–	–	–	–	0.04	–	–	–	–

[a]Abbreviations: ORG: Orange; GRP: Grape; STB: Strawberry; <LOQ: Compound detected below LOQ but not quantified; –: Compound not detected.

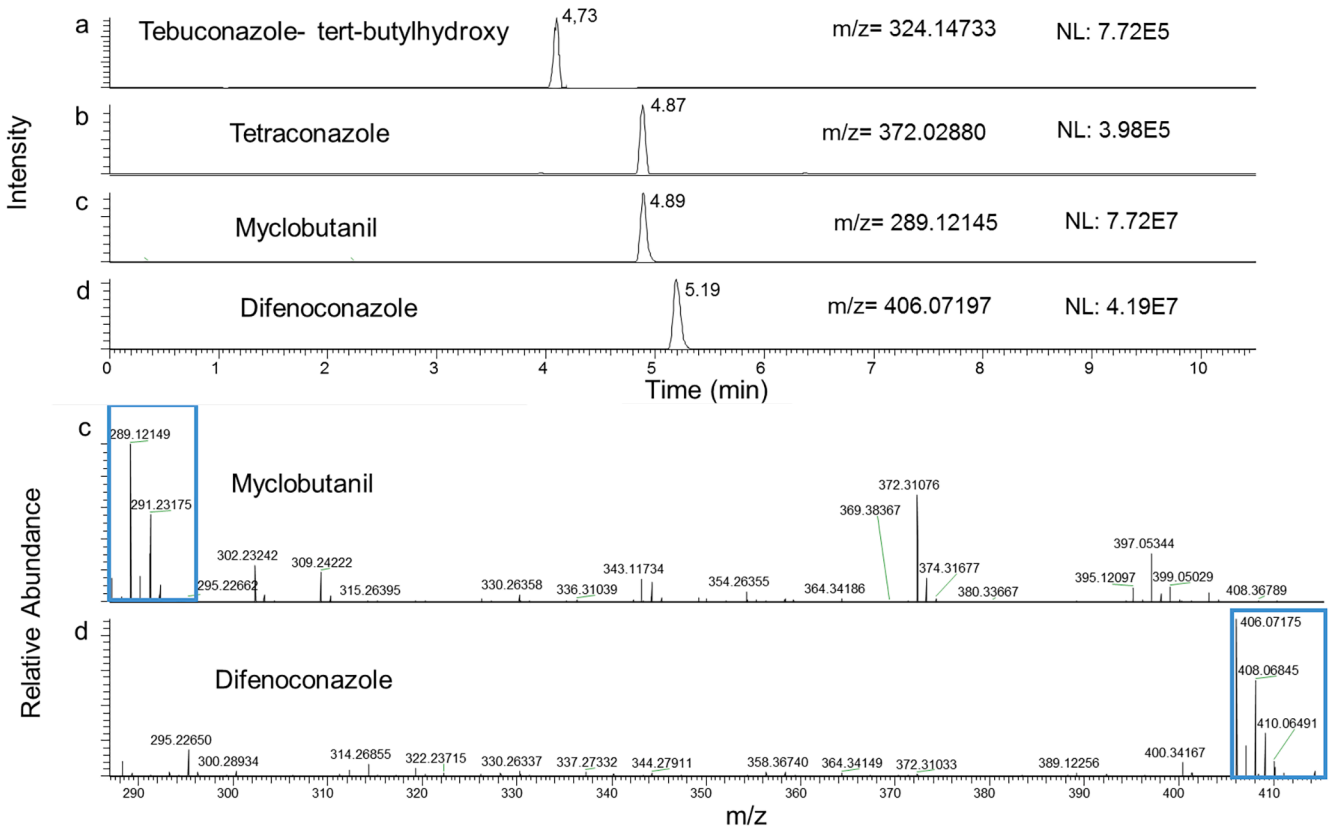

Fig. 2. Chromatograms and experimental spectra of one of the positive samples (STB-1) for: (a) tebuconazole *tert*-butylhydroxy, (b) tetraconazole, (c) myclobutanil at 0.14 mg kg^{-1} and (d) difenoconazole at 0.05 mg kg^{-1}.

concentration lower than LOQ in the analyzed samples but they could be not quantified, the precursor ion was detected.

In comparison with other studies where triazole fungicides have been analyzed, tebuconazole was found in two strawberry samples at 0.066 mg kg^{-1} and 0.21 mg kg^{-1}, overcoming the MRL of 0.02 mg kg^{-1} (European Food Safety Authority, 2021). The same fungicide was detected in one sample of grape below the MRL (Liu et al., 2011). Moreover, in another investigation 1,2,4-triazol was detected in root vegetables and citrus fruit, 63% and 28% of the samples, respectively. 1,2,4-triazol 1-yl-acetic was most found in citrus fruit (16% of the samples). In both cases, the values met the corresponding MRLs (Ströher-Kolberg et al., 2016).

3.5. Suspect analysis

In addition to the targeted analysis, a suspect screening analysis was carried out in order to discover pesticides not included in the former study. This successful implementation of non-targeted screening method to search possible pesticides has been applied in 30 samples, detecting pesticide residues in 9 out of 30 samples (1 orange, 3 grape and 5 strawberry samples).

Firstly, a suspect analysis using a homemade database, containing about 2000 pesticides was performed, according to the following criteria: exact mass (with a mass error lower than 5 ppm) and at least two fragment ions, (with a mass error lower than 10 ppm). The results of the 9 samples, where other pesticides were detected, are shown in Table 3.

Table 3
Pesticides detected in samples by suspect screening[a].

Analysed sample	Pesticide	Concentration (mg kg^{-1})	MRL (mg kg^{-1})
ORG-1	Imidacloprid	0.06	1
	Pyraclostrobin	0.48	2
	Thiamethoxam	0.03	0.15
	Clothianidin	0.04	0.06
GRP-1	Fluopyram*	0.54	0.5
	Imidacloprid	0.02	1
	Spirotetramat	0.01	1
GRP-2	Fludioxonil	0.16	10
	Cyprodinil*	0.29	0.02
	Fenhexamid*	0.04	0.01
GRP-3	Boscalid	1.1	2
	Spirotetramat	0.02	1
STB-1	Fluopyram*	0.14	0.1
	Trifloxystrobin	0.04	0.05
	Spirotetramat	0.04	0.1
STB-2	Fluopyram	0.06	0.1
	Flupyradifurone	0.02	0.05
	Trifloxystrobin	0.03	0.05
	Spirotetramat*	0.25	0.1
STB-3	Fludioxonil*	0.21	0.05
	Fluopyram	0.01	0.1
	Fosetyl-Al	0.61	500
STB-4	Fluopyram	0.05	0.1
	Flupyradifurone*	0.13	0.05
	Trifloxystrobin	0.02	0.05
STB-5	Metalaxyl*	0.08	0.05
	Formetanate*	0.26	0.05
	Indoxacarb*	0.1	0.05
	Bupirimate	0.03	0.05

[a]Abbreviations: ORG: Orange; GRP: Grape; STB: Strawberry; MRL: Maximum Residue Limit established by the European Union. *: Compound detected at concentrations higher than MRL.

Secondly, because of analytical standard of each detected pesticide was available, calibration curves (ranging from 1 to 1000 μg L^{-1}) in black extract of each matrix were prepared and injected, with the aim of confirming and quantifying the compounds tentative identified.

Additionally, positive samples were spiked with the detected compounds at 10 μg kg^{-1} to check the recovery, when the extraction method was applied. Suitable recoveries were achieved between 70 and 120%. Thirdly, the identified pesticides were quantified, finding up to 4 different pesticides in each one of the following samples: ORG-1, STB-2 and STB-5. In the case of ORG-1, imidacloprid, pyraclostrobin, thiamethoxam and clothianidin were detected. STB-2 contained fluopyram, flupyradifurone, trifloxystrobin and spirotetramat, and in STB-5, metalaxyl, formetanate, indoxacarb and bupirimate were identified. As it can be observed in Table 3, the highest concentration was detected at 1.1 mg kg^{-1} in GRP-3 for boscalid, but below the MRL (2 mg kg^{-1}) established by EU.

Finally, the identified pesticides were compared with the corresponding MRLs, observing some pesticide residues exceeding the corresponded MRLs, as it happens with cyprodinil and fenhexamid, which were detected in grape, especially in GRP-2. In addition, fluopyram (STB-1), spirotetramat (STB-2), fludioxonil (STB-3), flupyradifurone (STB-4), metalaxyl (STB-5), formetanate (STB-5) and indoxacarb (STB-5) were detected overcoming the MRLs in different strawberry samples (Table 3).

4. Conclusions

An analytical multiresidue method was developed and fully validated for the simultaneous determination of triazole fungicides and metabolites in fruits and vegetables, as courgette, orange, grape and strawberry. The developed method offers for the first time the potential of the determination of a high number of compounds (26) belong to triazole family, combining selectivity, high resolution capacity and fast analysis time (only 10.5 min) of UHPLC-Q-Orbitrap-MS^2 with the advantages of simple, rapid and reliable extraction procedures. The two extraction procedures were based on SLE or QuEChERS approach, depending on the matrix tested.

Validation criteria (linearity, matrix effect, recoveries, precision and LOQ) were performed in compliance with the SANTE 2019 guideline, ensuring the suitability of the method. Subsequently, 30 samples were analyzed and several fungicides were detected, obtaining that myclobutanil achieved the higher concentration (0.15 mg kg^{-1}) in a strawberry sample. In the tested samples, 11 parent compounds and one metabolite were detected.

Finally, a suspect screening analysis was carried out to search possible pesticides present in the samples, and some of them were identified, confirmed and quantified at concentrations higher than MRLs.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130860.

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