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Accurate Determination of Pesticide Residues in Milk by Sonication-QuEChERS Extraction and LC-LTQ/Orbitrap Mass Spectrometry

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Abstract: A modified, quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction procedure combined with sonication and Ultra-High Performance Liquid Chromatography–Orbitrap-Mass Spectrometry (UHPLC–Orbitrap-MS) was developed as a sensitive and reliable methodology for the determination of multiclass pesticides in full-fat milk. Different amounts of EMR-lipid sorbent were assayed for the cleanup step in order to achieve both acceptably high recoveries and low co-extractives in the final extracts. Accurate mass measurements of the analyte's pseudo-molecular ions and tandem MS fragmentation were used to quantify and identify the target pesticides. Analytical performance characteristics of the method, such as linearity, recovery, precision, the limit of detection (LOD) and quantification (LOQ), matrix effects (ME), and expanded uncertainty, have been determined for method validation fulfilling all criteria for its use as a validated routine method. The method was successfully applied to real samples (by local farms and commercial), revealing the presence of carbendazim in one milk sample at a concentration level below the maximum residue limits.

Keywords: pesticides; milk; QuEChERS; EMR-lipid; Liquid Chromatography–LTQ Orbitrap Mass Spectrometry; uncertainty

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1. Introduction

Milk is considered an essential source of nutrients, especially for infants, children, and the elderly, and is consumed as both raw and dairy products. According to the latest report of the Food and Agriculture Organization of the United Nations, milk production worldwide in 2021 reached about 928 million tons, exhibiting an increase of 1.5% compared to 2020 [1]. Milk global production percentage of different milk-producing animals follows the trend of cow milk at 81%, buffalo at 15%, goat at 2%, sheep at 1%, and camel at 0.5% [2]. Pesticides are widely applied in agriculture; thus, milk-producing animals may accumulate pesticide residues from contaminated feed, including grass and corn silage or from other sources such as top-layer soil, water, and air [3]. These accumulated pesticide residues can be transferred to the final product of milk [4,5] and consequently to human beings.

The European Union (EU) has established maximum residue limits (MRLs) of pesticides permitted in products of plant origin or animals that are consumed by humans or animals in order to minimize the risks associated with the consumption of food containing pesticide residues [6]. Several previous studies have reported that pesticide residues were found in milk products [7–11]. Therefore, it is important to develop fast, sensitive, and selective analytical methods that enable the determination and quantification of multiresidues of multiclass pesticides in milk in a single analytical process.

There are various components in milk, including fat and proteins, which can interfere with or prevent proper analysis of pesticides [12]. For this reason, in recent years, many analytical methodologies have been developed for determining pesticides in milk. In

milk sample preparation, the most commonly used methods for the analysis of pesticide residues in milk were based on liquid–liquid extraction (LLE) [13,14], however requiring a high amount of solvent; therefore, they were replaced by other approaches, such as solid-phase extraction (SPE) [15,16], solid-phase microextraction (SPME) [17–19], dispersive solid-phase extraction (DSPE) [20], dispersive liquid–liquid microextraction (DLLME) [21], Matrix Solid-Phase Dispersion (MSPD) extraction [22].

These methods can be complicated in terms of time and labor consumption, low recovery of some analytes, reproducibility, and method optimization [23–25], therefore, in recent years, the so-called quick, easy, cheap, effective, rugged, and safe (QuEChERS) method was applied in pesticide residue analysis of milk [26,27]. Various modified QuEChERS procedures have been reported in the literature for the analysis of pesticide residues in milk by using acetonitrile or ethyl acetate as extraction solvents [27,28] and various sorbents, or a combination of them (e.g., PSA, GCB, C18, florisil), in the clean-up approach [26,29–33]. Due to the high concentration of proteins and fat in milk, processes should be capable of decreasing their content or even entirely removing them from the final extract. Recently, a new generation of sample preparation sorbents has been developed, the Enhanced Matrix Removal-Lipid (EMR-Lipid) sorbent that is used as a dispersive SPE (dSPE) agent and promises highly selective lipid removal without analyte retention. EMR-lipid has been initially applied for the determination of pesticides in fatty vegetable matrices [34]. Thereafter, it has been evaluated for the determination of multiple target analytes from different categories to other fatty matrices, such as salmon, kale, pork, avocado [35], bovine tissues (kidney, liver, and muscle) [36], and milk [37].

Furthermore, sonication is a treatment utilized to decrease the size of milk fat globules (MFG) [38] and improve rennet gelation properties [39]. The lipids in milk exist in the form of MFG. When ultrasound travels through a liquid medium, it generates numerous microbubbles by "acoustic cavitation". The violent collapse of these microbubbles induces localized strong shear forces and turbulence, which cause disruption of MFG. Various studies on the use of ultrasonic processing of cow's milk have been published [39–42]. Complete purification of the analytes in a single extraction step may induce difficulties, especially for very complex matrices and multiclass pesticide residues. Thus, the combination of treatment techniques may be crucial to purify the analytes. Some studies have reported the combination of QuEChERS extraction with ultrasonic-assisted extraction to analyze pesticides and pharmaceutical drugs in complex food sample matrices [43,44].

Thus far, final extracts of milk were mostly analyzed using gas chromatography (GC) and liquid chromatography (LC) systems coupled to mass spectrometry, such as ion trap GC (LC) –MS (IT) or triple quadrupole GC(LC)-MS/MS (QqQ) [26,30,45–48]. During the last years, high-resolution (HR) mass spectrometer instruments providing accurate high-resolution mass data received increasing importance for pesticide residue and pesticide metabolite analysis in food matrices [49]. Hybrid HR mass spectrometers such as quadrupole-time of flight (Q-TOF) and linear ion-trap or quadrupole Orbitrap (LIT-Orbitrap or Q-Orbitrap) offers the advantages of both accurate mass measurements and MS/MS confirmation. In particular, hybrid LC-Orbitrap MS instruments allow for the confirmation and quantification of an unlimited number of analytes in a single injection, even in complex matrices, where matrix components of similar mass and polarity may be co-eluted. In addition, the combination of high mass accuracy and high resolution limits the risk of false identifications and, along with the uncompromised sensitivity, scan speed and high-quality MS/MS fragmentation—allows diverse applications in target and non-target analysis [50]. Based on the forenamed advantages of QuEChERS extraction and hybrid HR-Orbitrap mass spectrometry, their combination can provide simple, fast, reliable, and cost-effective analytical alternatives for the simultaneous multiresidue detection of pesticides in milk or other food commodities. Finally, some of the method validation parameters have been reported in previous studies on milk residue analysis with LC-MS or GC-MS, but barely the overall uncertainty of the analytical result, i.e., the range within

which a reported or experimental result can be expected to lie with a certain degree of confidence [51].

Following the considerations above, the target of this study was to develop a sensitive and fast analytical method to determine multiclass pesticides in milk using a modified QuEChERS procedure ("AOAC 2007.01") combined with sonication and liquid chromatography coupled to hybrid linear ion trap-Orbitrap MS system. Different amounts of Enhanced Matrix Removal-Lipid (EMR-Lipid) sorbent were assayed as dispersive SPE (dSPE) agents for the determination of pesticides. The analytical method has been fully validated, and expanded uncertainty was also determined. Eventually, twenty milk samples commercially available in Greek markets and local farms were analyzed with the optimized method.

2. Materials and Methods

2.1. Chemicals and Reagents and Samples

High purity (>98%) analytical standards were purchased from Sigma-Aldrich (Steinheim, Germany) and LGC Standards, formerly Dr. Ehrenstorfer (Augsburg, Germany). Individual stock solutions of each compound were prepared in methanol and kept in amber glass vials in the dark (−20 °C). Solvents, methanol, acetonitrile and water (LC–MS grade) were purchased from Fisher Scientific (Leicestershire, UK). Formic acid and acetic acid (purity, 98–100%) were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure deionized water was produced in the lab by the Milli-Q water purification system (Millipore, Temecula, CA, USA).

The salts/sorbents used in QuEChERS were anhydrous magnesium sulfate ($MgSO_4$), purchased from Merck (Darmstadt, Germany), sodium acetate (NaOAc) and sodium chloride (NaCl) purchased from Riedel-de Haën (Hannover, Germany), Enhanced Matrix Removal-Lipid (EMR-Lipid) sorbent (containing 1 g), purchased from Agilent Technologies (Waldbronn, Germany). Syringe filters (polytetrafluoroethylene, 0.22 μm) were purchased from Millipore (Cork, Ireland), while propylene centrifuge tubes of 50 mL and 15 mL were also used.

All samples analyzed were produced in Greece. Full-fat (3.5%) milk was used for the optimization and validation of the analytical method. Cow and goat milk samples (20 samples in total, all full-fat) that were further analyzed were purchased from various local supermarkets, markets and local farms in Ioannina, Epirus region, northwest Greece. The samples, once transferred to the laboratory for analysis, were stored at 4 °C in amber glass bottles until analysis.

2.2. QuEChERS Extraction Procedure

The "AOAC 2007.01" QuEChERS method with some modifications was selected for the determination of pesticide residues in milk. Five grams of the milk sample were weighed into a 50 mL polypropylene centrifuge tube (Tube 1), 10 mL acetonitrile + 1% acetic acid was added, and the tubes were immediately shaken for 1 min. Then, 10 mL of Milli-Q water was added into a centrifuge tube (Tube 1) and immediately shaken for 1 min. The mixture was placed in a sonication bath (100 W, 37 kHz, Elmasonic P, Singen, Germany) for 20 min. Afterward, extract salts were added, shaken for 1 min, and centrifuged for 10 min at 4000 rpm. Meanwhile, the dSPE tube (Tube 2) containing 0.5 or 1 g of EMR-lipid sorbent was conditioned with 2.5 mL or 5 mL of Milli-Q water, respectively, by shaking for 30 s. An amount of 5 mL of the organic layer (Tube 1) was added to the prepared EMR-Lipid dSPE tube (Tube 2) and vortexed for one minute, followed by centrifugation at 4000 rpm for 15 min. All the organic and liquid phase was transferred to an EMR-Lipid Polish tube (Tube 3) (containing 1.6 g $MgSO_4$ and 0.4 g NaCl) and vigorously shaken for 1 min. Finally, it was centrifuged again at 4000 rpm for 20 min. 1 mL of the supernatant was transferred to a glass testing tube, evaporated to dryness under a gentle stream of nitrogen at 40 °C and then reconstituted into 1 mL of water: methanol, 90:10 (v/v) + 0.1% formic

acid. The sample was filtered through syringe membrane filters (polytetrafluoroethylene, 0.22 μm) prior to the injection into the UHPLC-LTQ/Orbitrap MS system.

2.3. UHPLC-Orbitrap MS Analysis

An UHPLC–hybrid LTQ Orbitrap Accela LC system (Thermo Fisher Scientific, Bremen, Germany) was used for standard solution and sample analysis. The UHPLC system was equipped with an Accela quaternary gradient UHPLC pump (model 1.05.0900) and an Accela AS autosampler (model 2.1.1). A reversed-phase Fortis C18 (Fortis Technologies, Neston, UK) analytical column (50 × 2.1 mm, 1.7 μm) was used for target analytes separation. The column oven temperature was maintained at 35 °C during analysis. Solvent A (water + 0.1% formic acid) and solvent B (MeOH + 0.1% formic acid) were chosen as the mobile phase elution system. The elution gradient started with 95% solvent A, remained at 95% for 1 min, changed to 30% in 1 min, then decreased to 0% after 3 min, where it stayed for 2 min, and finally returned to the initial conditions. The total run time was 10 min. The injection volume was 5 μL, and the flow rate was 250 μL/min.

The LC system was coupled to a hybrid LTQ Orbitrap XL Fourier transform mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an Ion Max electrospray ionization probe. A full scan was performed in positive (PI) ionization mode with a mass range of 120–1000 Da and a mass resolving power of 60,000 FWHM. Furthermore, the identification and quantification of target compounds were carried out using extracted ion chromatograms. Additionally, the data-dependent acquisition (full MS/dd-MS^2) mode was applied based on Collision-Induced Dissocation (CID) to obtain the unique fragmentation pattern of each analyte. For that purpose, ions were isolated in the LTQ ion trap and were fragmented at 35% normalized collision energy (NCE) with a resolution set at 15,000 FWHM. The mass tolerance window was set to 5 ppm (Table 1). The main parameters of the mass spectrometer were a spray voltage of 4 kV, auxiliary gas flow rate of 10 arbitrary units (au), sheath gas flow rate of 35 au, tube lens of 90 V, and capillary temperature of 320 °C. Moreover, the automatic gain control (AGC) was set at 4×10^5 ions. Data processing was performed using the Thermo Xcalibur 2.1 software (Thermo Electron, San Jose, CA, USA).

Table 1. UHPLC–LTQ Orbitrap mass spectrometry analysis data. Target pesticides, use, retention times, and parameters for full MS/dd-MS^2 analysis.

Pesticides	Use *	t_R (min)	Pseudo-Molecular Ion $[M+H]^+$	Theoretical Mass (m/z)	Experimental Mass (m/z)	Mass Accuracy (ppm)	Ring Double Bond Equivalent (RDBE)	Fragment Ion 35% NCE
Acetamiprid	In	3.44	$C_{10}H_{13}ClN_4$	230.7450	230.7430	−0.90	6.5	126.0102/187.0972/196.0627
Azinphos-methyl	In	4.28	$C_{10}H_{14}N_3O_3PS_2$	318.0130	318.0135	1.429	6.5	261.1.306
Azoxystrobin	Fu	4.26	$C_{22}H_{19}N_3O_5$	404.1240	404.1245	0.997	15.5	372.0974
Benalaxyl	Fu	5.17	$C_{20}H_{25}NO_3$	326.1751	326.1751	0.092	9.5	148.1119/208.1331
Boscalid	Fu	4.42	$C_{18}H_{14}Cl_2N_2O$	343.0399	343.0401	0.452	12.5	307.0626/139.9898
Bupirimate	Fu	4.57	$C_{13}H_{26}N_4O_3S$	317.1642	317.644	0.669	3.5	166.0973/237.2070
Carbaryl	In	3.90	$C_{12}H_{12}NO_2$	202.0862	202.0861	−0.273	7.5	147.0647
Carbendazim	Fu	3.07	$C_9H_{11}N_3O_2$	192.0767	192.0768	0.244	6.5	160.0505/132.0556
Cymoxanil	Fu	3.49	$C_7H_{12}N_4O_3$	199.0826	199.0827	0.669	4.5	128.0462
Cyprodinil	Fu	4.88	$C_{14}H_{17}N_3$	226.1339	226.1342	1.441	8.5	210.1024/185.1074/144.0806
Dichlorvos	In	3.83	$C_4H_9Cl_2O_4P$	220.9532	220.9533	0.556	0.5	144.9813/127.0151/109.0045
Dimethoate	In	3.41	$C_5H_{14}NO_3PS_2$	230.0069	230.0067	−0.859	0.5	170.9697
Fenthion sulfoxide	In	3.86	$C_{10}H_{17}O_4PS_2$	295.0222	295.0223	0.294	3.5	264.0033/201.0400/279.9983
Imidacloprid	In	3.33	$C_9H_{12}ClN_5O_2$	256.0595	256.0596	0.083	6.5	175.0976/209.0587
Iprovalicarb	Fu	4.64	$C_{18}H_{30}N_2O_3$	321.2172	321.2174	0.407	5.5	119.0851
Metalaxyl	Fu	4.15	$C_{15}H_{29}NO_4$	280.1543	280.1544	0.233	5.5	248.1281/220.1332
Myclobutanil	Fu	4.58	$C_{15}H_{19}ClN_4$	289.1215	289.1217	0.862	8.5	220.0882/125.0148
Tebuconazole	Fu	5.08	$C_{16}H_{24}ClN_3O$	308.1524	308.1523	−0.378	6.5	151.0306/290.1402
Thiacloprid	In	3.52	$C_{10}H_{11}ClN_4S$	253.0309	253.0309	−0.084	7.5	126.0102
Thiamethoxam	In	3.20	$C_8H_{12}ClN_5O_3S$	292.0266	292.0265	−0.220	5.5	180.9578/139.0325

* In—insecticide, Fu—fungicide.

2.4. Method Validation

The optimized method was validated according to Documents No. SANTE/11813/2017 and No. SANTE/12682/2019 [51,52]. The following parameters were determined sensitivity/linearity, recovery (as a measure of trueness), precision (repeatability and within laboratory reproducibility), the limit of detection (LOD), limit of quantification (LOQ) matrix effect (ME), and measurement uncertainty (MU).

Linearity was investigated over the 1–250 μg kg^{-1} range, evaluated by weighted least squares regression and expressed as a determination coefficient (r^2). Blank samples of organic milk were spiked with a pesticide mixture at fortification levels of 10, 25, 100, and 200 μg kg^{-1} to assess recovery. Six replicates were prepared for each spike level (n = 6), and recoveries were estimated by comparing the concentrations obtained after the extraction with the initial spiking level in all cases. Precision was expressed as repeatability %RSDr (intra-day) and within-laboratory reproducibility $\%RSD_{WR}$ (inter-day) of the method. Mean recoveries should be within the range of 70 to 120%, with an associated RSD $\leq$ 20%, for all analytes, according to the SANTE guidance documents. In extraordinary circumstances, mean recovery rates outside the range of 70 to 120% can be accepted if the RSD value is equal to or below 20%, but the mean recovery must not lie below 30% or above 140%. The limits of quantification (LOQs) and limits of detection (LODs) were calculated on the basis of a signal-to-noise ratio $\geq$10 and $\geq$3, respectively and the corresponding analyte concentration. The LOQ values should be $\leq$ MRLs in order to be in accordance with the SANTE/12682/2019 guideline. Matrix effects (ME) for target analytes were estimated by comparing a calibration curve prepared in milk extracts and a calibration curve prepared in a solvent at the same concentration range, according to the following equation:

$$\%ME = \frac{\text{slope of calibration curve in matrix}}{\text{slope of calibration curve in solvent}} - 1 \times 100 \quad (1)$$

Values of the matrix effect lying between 0 and 20% are considered as low, between 20 and 50% as medium and $\geq$50% as strong [50].

The expanded MU was evaluated based on six replicate analyses on different days, at levels 10, 25, and 100 μg kg^{-1}, in the present study. A default expanded MU of 50% should not be exceeded (corresponding to a 95% confidence level and a coverage factor of 2).

Finally, the Horwitz ratio (HorRat) was also determined as a simple performance parameter of the measurement precision. When the ratio equals 1, there is an exact correspondence; when the ratio is <1, the precision is better than expected and poorer if it is greater than one [53].

3. Results and Discussion

3.1. Optimization of QuEChERS Extraction

Acetonitrile was chosen as the extraction solvent being the most widely used organic solvent in the QuEChERS methods. Furthermore, acetonitrile offers the possibility to extract pesticides of different polarities. The addition of 1% acetic acid in acetonitrile as well as the addition of sodium acetate, promotes the salting-out effect and buffers the extract. The use of sodium acetate is helping the extraction of low, pH-sensitive pesticides or those that present stability problems [50,54].

The optimization of the cleanup approach of the proposed acetate method was then performed. The sorbent evaluated was EMR-lipid. The following approach concerning the extracts cleanup step were assayed: (a) 1 g EMR-lipid sorbent and (b) 0.5 g EMR-lipid sorbent. Recoveries obtained after the above combinations are presented in Figure 1. Both (a) and (b) cleanup approaches showed similar recoveries, as illustrated in Figure 1. The same number of pesticides have recovery values lower than 60% (2 pesticides; benalaxyl and myclobutanil), between 60–70% (two pesticides; iprovalicarb and tebuconazole) and between 70–120% (16 pesticides); in both cases, regardless of the amount of EMR-Lipid sorbent used in this study (Figure 1). The two investigated clean-ups revealed minor differences concerning recoveries of the same pesticide, as shown in Figure 1. Moreover,

the results indicated that in both cases (a) and (b), the co-extracted matrix components of milk can be efficiently removed and the effectiveness of cleanup was also confirmed by the absence of interfering peaks on the chromatogram. Taking these results into account and for cost reasons, cleanup step (b) with 0.5 g EMR-Lipid was chosen. The results revealed satisfactory recoveries and clean chromatograms. As a consequence, milk samples were prepared according to the previously described and optimized QuEChERS procedure.

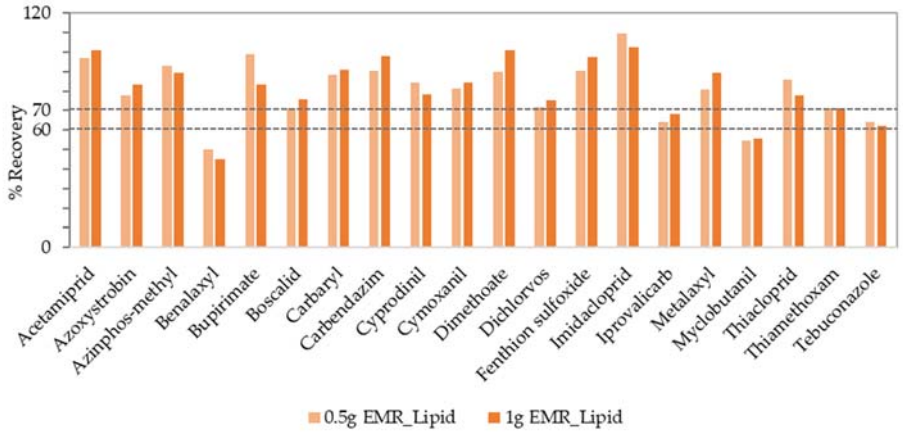

Figure 1. Recoveries (%) obtained for different amounts of sorbent used in cleanup step (0.5 g and 1 g EMR-lipid) at 20 μg kg^{-1} spiking concentration level in milk.

3.2. Validation of the Proposed Method

Validation parameters for milk are presented in Table 2. Matrix matched-standard calibration curves prepared in 90:10 (v/v) water: MeOH + 0.1% formic acid were used for the quantification of target analytes. Blank milk samples were treated for the construction of matrix matched-calibration curves. The final extracts were spiked with the selected compounds in the appropriate concentrations. Organic milk samples were used in order to secure that no target analytes existed. Extracted Ion Chromatogram of target compounds at a concentration of 500 μg kg^{-1} and the relevant one of the blank milk samples are shown in Figure 2. The method linearity proved to be excellent in the tested concentration ranges, with correlation coefficient values of $\geq$0.9918, in all cases, obtained from a calibration curve constructed following least-squares linear regression analysis.

The method's trueness and precision were determined via recovery studies with spiked samples at four concentration levels (10, 25, 100, and 200 μg kg^{-1}) analyzed six times on the same day and on six consecutive days, also (Table 2). The recoveries at the 10, 25, 100, and 200 μg kg^{-1} levels for most of the compounds were within the range of 70–120% with associated RSDs of <20%. More specific recoveries ranged between 79.5% (boscalid) and 119.5% (tebuconazole), with the exception of two investigated compounds (myclobutanil and iprovalicarb) at low concentration levels, with relative recoveries <70%, but with RSD < 11.7%. Relative standard deviation (RSD) values never exceeded 11.7% in all cases. Intra-day precision in milk ranged from 1.0% for tebuconazole to 11.7% for tebuconazole, and inter-day precision in milk ranged from 0.6% for dichlorvos to 11.7% for myclobutanil.

Method LOD and LOQ values were determined as described in Section 2.4. The LODs and the LOQs of the method are also presented in Table 2. LODs ranged between 0.2 μg kg^{-1} for metalaxyl and 8.1 μg/kg^{-1} for thiamethoxam, while LOQs ranged between 0.61 μg kg^{-1} for metalaxyl and 24.8 μg kg^{-1} for thiamethoxam.

Table 2. Method validation parameters: linearity (1–250 μg kg^{-1} range), limits of detection (LODs), limits of quantification (LOQs), mean relative recoveries (%), and relative standard deviations (RSDs) (%) of four different spiked levels (n = 6) studied for intra and inter-day, as well as maximum residue levels (MRLs), for pesticide residues in milk.

Pesticides	Linearity (r^2)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	MRL (μg kg^{-1})	Relative Recoveries and RSDs (n = 6)													
					Intra-Day								Inter-Day					
					10 μg kg^{-1}		25 μg kg^{-1}		100 μg kg^{-1}		200 μg kg^{-1}		10 μg kg^{-1}		25 μg kg^{-1}		100 μg kg^{-1}		200 μg kg^{-1}	
					Rec%	RSD_r%	Rec%	RSD_r%	Rec%	RSD_r%	Rec%	RSD_r%	Rec%	$RSDw_R$%	Rec%	$RSDw_R$%	Rec%	$RSDw_R$%	Rec%	$RSDw_R$%
Acetamiprid	0.9950	5.8	17.8	50	-	-	80.2	7.8	111.6	6.1	116.3	6.3	-	-	93.4	7.7	105	6.2	119.2	3.6
Azoxystrobin	0.9918	1.56	4.7	5	79.6	6.8	80.4	9.6	81	6.1	82.0	3.6	83.9	1.4	86.7	2.8	87.4	3.0	90.3	4.7
Azinphos-meth	0.9948	2.1	6.4	10	79.6	2.8	80.4	2.5	89	5.7	91.2	5.6	84.1	2.6	86.3	1.1	87.0	7.0	95.3	6.8
Benalaxyl	0.9936	4.69	14.2	50	-	-	60.0	6.4	99	4.1	113.8	10.3	-	-	60.9	8.8	110.0	4.6	115.1	7.4
Bupirimate	0.9940	1.05	3.2	50	84.9	7.7	85.0	8.7	102.5	4.8	103	11.6	90.2	6.2	90.5	6.8	98.3	7.5	102.6	10.4
Boscalid	0.9959	3.3	10	20	79.5	2.3	80.3	6.4	105.5	6.4	112.3	9.4	84.1	2.6	87.1	3.5	100.0	9.1	107.4	5.6
Carbaryl	0.9942	2.29	6.9	50	84.3	8.0	85.0	6.2	98.6	6.8	100.3	7.1	88.8	5.5	89.0	6.1	101.3	6.4	102.0	4.1
Carbendazim	0.9933	6.8	20.5	50	-	-	79.9	2.8	103.5	6.0	109.8	9.9	-	-	86.5	7.1	92.5	6.4	103.2	6.9
Cyprodinil	0.9922	2.79	8.46	20	81.7	9.3	84.2	6.7	85.6	6.9	95.5	11.5	82.4	1.1	86.0	1.7	86.9	2.1	91.6	3.6
Cymoxanil	0.9968	2.3	7.0	10	86.1	8.6	86.0	5.5	95.7	7.1	96	3.9	85.0	3.8	86.6	3.2	91.4	4.8	92.3	2.5
Dimethoate	0.9958	3.3	9.8	10	80.4	6.2	80.5	8.5	97.0	7.6	98.1	10.2	85.9	5.9	88.6	5.4	100.4	4.4	101.0	2.0
Dichlorvos	0.9954	1.4	4.3	10	81.6	2.4	82.5	5.8	94.85	6.0	99.2	7.6	85	0.6	86.7	3.1	87.4	6.2	100.8	7.0
Fenthion sulfoxide	0.9975	3.3	8.7	10	81.2	3.1	81.7	2.2	95.5	10.5	107.8	10.6	86.1	7.5	86.6	1.8	93.2	6.8	119.2	6.1
Imidacloprid	0.9935	5.3	16.1	100	81.8	3.6	100.0	5.5	100.2	5.2	102.5	6.6	87.4	4.9	93.8	5.1	94.0	7.5	96.7	4.6
Iprovalicarb	0.9950	1.3	4.0	10	60	4.4	62.2	7.0	87.1	5.9	88.4	2.4	66.1	8.94	71.1	2.8	83.2	4.2	88.9	6.7
Metalaxyl	0.9961	0.2	0.61	10	84.5	9.8	84.9	4.9	102.0	6.0	103.0	5.1	87.4	5.7	87.7	5.1	95.4	8.7	98.5	5.6
Myclobutanil	0.9941	3.3	10.0	10	50.0	7.2	60.9	11.3	74.10	7.5	112.0	7.2	50.0	7.04	59.4	8.4	80.5	5.0	107.1	11.7
Thiacloprid	0.9935	5.4	16.3	50	-	-	82.6	4.1	105.1	8.2	107.3	2.7	-	-	90.1	5.8	100.6	5.2	110.2	5.4
Thiamethoxam	0.9964	8.1	24.8	50	-	-	80.2	2.9	97.02	9.8	98.0	5.5	-	-	85.5	6.9	90.2	7.9	95.6	5.6
Tebuconazole	0.9963	4.0	12.1	20	-	-	80.2	1.0	98.0	11.7	119.5	5.2	-	-	86.0	1.7	100.5	6.4	110.5	8.0

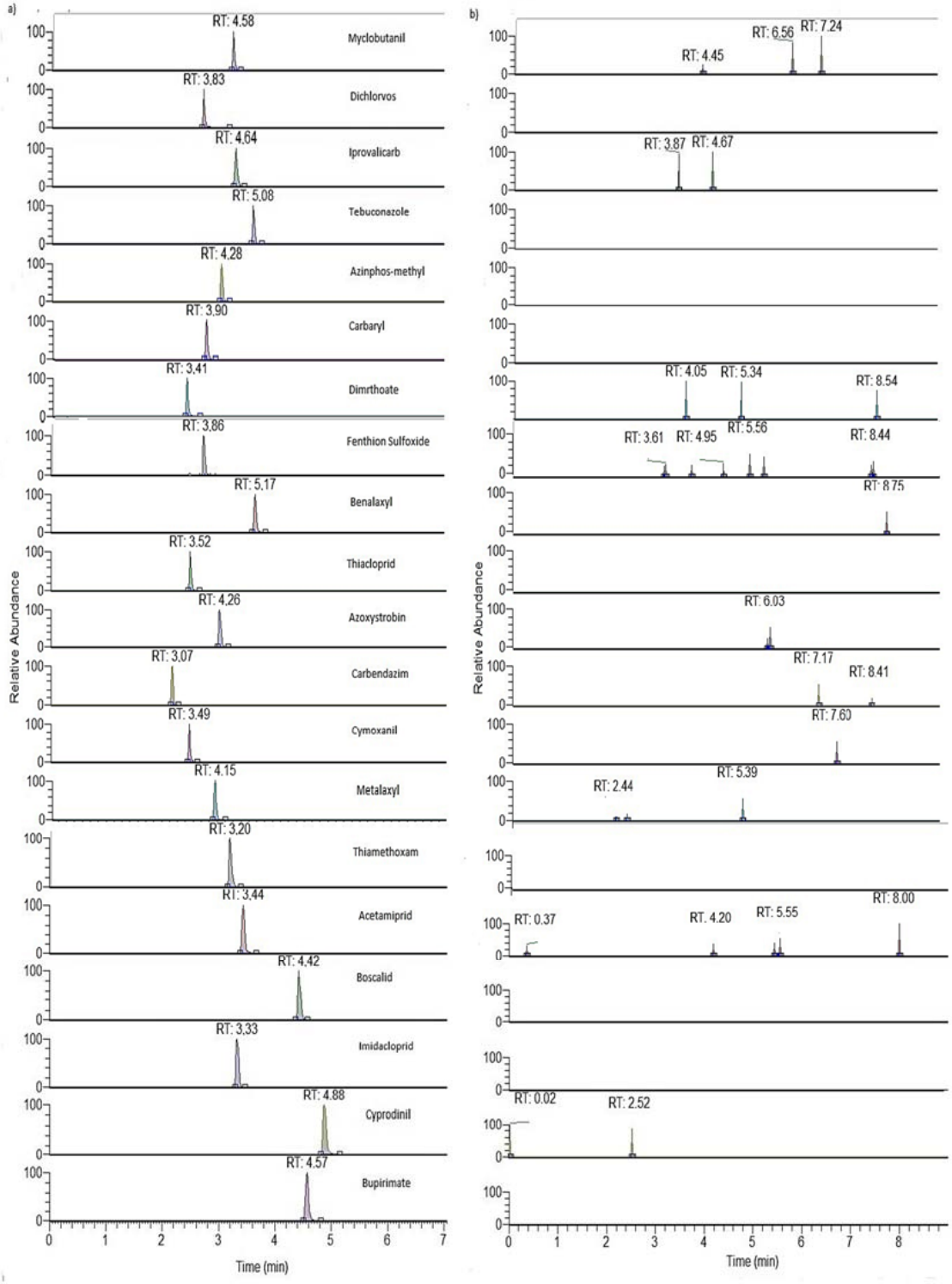

Figure 2. Extracted ion chromatogram (XIC) obtained for target compounds at (**a**) a concentration level of 500 μg kg^{-1} and (**b**) a blank sample of milk.

The evaluation of matrix effects, determined as signal suppression or enhancement, is shown in Figure 3. Potential interferences on analyte ionization may arise by the matrix components, which can be eluted at the same retention time as the target compounds.

In milk, approximately 30% of the investigated compounds revealed negative matrix effects, demonstrating that matrix components suppress their instrumental signals. Seven pesticides presented a low matrix effect (between −20% and 17.4%), and the rest

of the compounds presented a medium matrix effect (12 compounds, from −46.6% to 45.8%). A strong matrix effect was found for carbendazim with a matrix effect value of −55%. Strong suppression has also been reported for carbendazim (−55%) in previous studies [55,56]. To conclude, the use of matrix-matched calibration curves allows the problems of quantification accuracy to be overwhelmed in a single step.

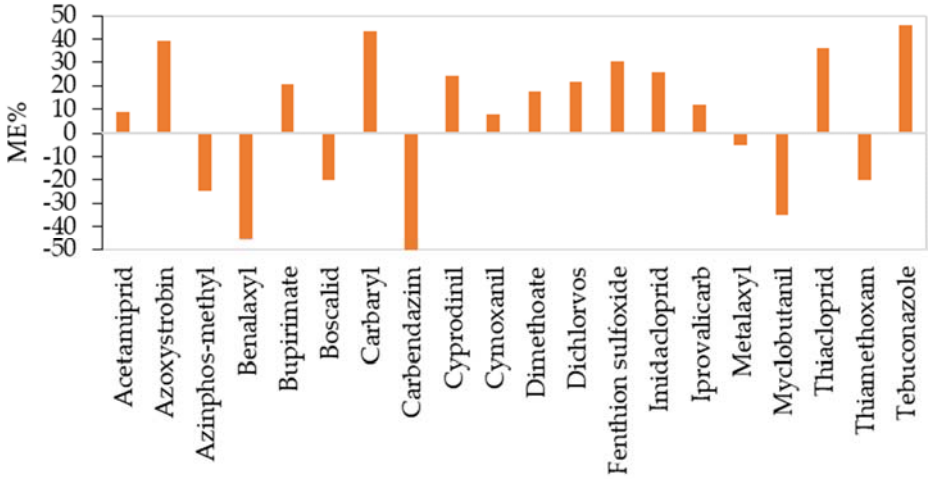

Figure 3. Matrix effects (%ME) calculated from the slopes of the solvent and matrix-matched calibration curves in milk.

The main parameters in previously published methods concerning QuEChERS extraction in combination with LC-MS techniques for the evaluation of pesticide residues, the presence in milk could be compared with the proposed methodology conditions in the present study. Table 3 presents modifications of the QuEChERS procedure, but regarding the clean-up step and LC-MS techniques, only the present study uses EMR-lipid sorbents for the clean-up procedure and high-resolution orbitrap mass spectrometry analysis. Similar recoveries (between 70 and 120%) were found in most of the studies. In terms of the LODs and LOQs, higher values were determined by Wu et al. [33], while lower values were found by Zheng et al. [27] and Jadhav et al. [57]. The linearity for the studied compounds was found to be $\geq$0.99, with the exception of some pesticides such as spinosad, temephos, and piperonyl butoxide, while linearity was found to be $\geq$0.98 in Zheng et al. [27]. Concerning the ME, the values (%) in Rejczak and Tuzimski [58] ranged between −19% and 68%, while the ME values (%) in Zheng et al. [27] were in the range of −40% to 2%.

Table 3. Main characteristics of recent studies regarding QuEChERS extraction and LC-MS techniques for the analysis of pesticides in milk.

Milk	Compounds	Amount	QuEChERS		LC-MS Technique	Recovery (%)	LODs	LOQs	Linearity	Reference
			Extraction	Clean-Up						
milk	20 pesticides	5 g	6 g $MgSO_4$/1.7 g NaOAC	EMR-lipid	UHPLC-LTQ/Orbitrap MS	79.5–119.5%	0.2–8.1 μg kg^{-1}	0.61–24.8 μg kg^{-1}	0.9918	This study
yogurt/milk	8 herbicides	5 mL	NaCl	PSA	HPLC–DAD	78.9–99.9%,	0.15–0.35 ng mL^{-1}	0.51–1.16 ng mL^{-1}	0.9999	[59]
milk	7 carbamates	10 g	5 g NaCl	SPE Bond Elut amino propyl (NH2) cartridges	UPLC-MS/MS	85.4–110.9%	0.010–0.068 μg kg^{-1}	0.033–0.23 μg kg^{-1}	0.990	[9]
milk	30 pesticides	20 mL	2 g NaCl/8 g $MgSO_4$	PSA / Z-Sep/Z-Sep Plus	HPLC–DAD	35–131%		5–50 ng mL^{-1}	0.9994	[58]
milk	Organophosphorus-Organochlorine pesticides	1 g	100 mg NaCl/400 mg $MgSO_4$	$MgSO_4$/PSA/ C18/ CarbonX Plus	LC-ESI/MS/MS	74.0–137		0.20–2.2 mg kg^{-1}	0.990	[31]
milk/dairy products	167 pesticides	15 mL	6 g $MgSO_4$/1.5 g NaCl	$MgSO_4$/PSA/C18	LC-MS/MS	80.4–117.3%	0.3–3.9 μg kg^{-1}	1.1–13.1 μg kg^{-1}	0.990	[29]
eggs/ milk	pesticides/Spinosad, temephos and piperonyl butoxide	2 mL	4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate tribasic dihydrate and 0.5 g sodium citrate dibasic sesquihydrate	C18/$MgSO_4$	LC–MS/MS	71–105%	0.1–1.6 μg kg^{-1}	0.3–4.4 μg kg^{-1}	0.980	[27]
milk	sulfoxaflor	2 g	1 g NaCl/2 g $MgSO_4$	C18/PSA/GC /$MgSO_4$	UPLC-MS/MS	86.6–91.7%	1.8 μg kg^{-1}	5 μg kg^{-1}	0.999	[60]
bovine milk	238 pesticides	5 g	2 $MgSO_4$/ 0.5 g NaCl	C18	UFLC-MS/MS	70–120%		0.1–10 ng g^{-1}	0.990	[57]
milk	195 pesticides	2 g	4 g $MgSO_4$, 1 g NaCl, 0.5 g disodium citrate, and 1 g trisodium citrate	$MgSO_4$/C18	LC-Q-TOF/MS	70–120%		0.1–50 μg kg^{-1}	0.99	[33]
bovine milk	209 Veterinary Drugs, Mycotoxins, and Pesticides	5 g	NaCl	C18	UHPLC-MS-MS	51.20–129.76%		0.05–5 μg kg^{-1}	0.99	[61]

The expanded MU was calculated for each pesticide as twice the value of the uncertainty (k = 2, confidence level 95%) and the resulting values are summarized in Table 4 and depicted in Figure 4. The MU values ranged from 23.71% (bupirimate) to 50.0% (myclobutanil) at a concentration of 10 μg kg^{-1} and from 16.64% (imidacloprid) to 48.07% (benalaxyl) at a concentration of 25 μg kg^{-1}, being in accordance with the requirement (50%) of the EU guidance documents SANTE/11813/2017 and SANTE/12682/2019 [51,52]. At a concentration of 100 μg kg^{-1}, the expanded uncertainties ranged between 9.67% (dimethoate) and 37.20% (myclobutanil). The MU% values calculated by Danezis [62] for 28 xenobiotics (polar and hydrophilic compounds) ranged from 3.5% to 39% for milk, whereas MU% values calculated by Golge [29] for 167 pesticides in milk revealed values lower than 50%, with some compounds presented with expanded measurement uncertainties up to 40%, ranged from 7.8% (phosphamidon) to 46.4% (diafenthiuron) in milk.

Table 4. Measurement uncertainty (MU %) for milk in concentrations 10, 25, and 100 μg kg^{-1} (k = 2, confidence level 95%) as well as HorRat values in 10 and 25 μg kg^{-1}.

Pesticide	Concentration Levels (μg kg^{-1}) in Milk				
	10 μg kg^{-1}		25 μg kg^{-1}		100 μg kg^{-1}
	MU (%)	HorRat	MU (%)	HorRat	MU (%)
Acetamiprid	_	_	21.38	0.52	16.85
Azinphos-methyl	32.00	0.14	27.47	0.07	30.70
Azoxystrobin	32.00	0.08	27.24	0.18	26.02
Benalaxyl	_	_	48.07	0.40	22.39
Boscalid	32.00	0.14	26.77	0.21	19.79
Bupirimate	23.71	0.35	24.03	0.44	16.63
Carbaryl	24.90	0.41	25.66	0.39	14.19
Carbendazim	_	_	26.18	0.34	20.43
Cymoxanil	30.96	0.20	27.59	0.19	20.09
Cyprodinil	32.00	0.03	27.73	0.10	26.08
Dichlorvos	30.00	0.03	27.34	0.19	28.46
Dimethoate	30.93	0.31	25.50	0.34	9.67
Fenthion sulfoxide	32.00	0.40	26.93	0.11	19.94
Imidacloprid	27.30	0.27	16.64	0.34	20.36
Iprovalicarb	47.57	0.14	46.28	0.23	34.80
Metalaxyl	28.01	0.31	27.00	0.32	21.03
Myclobutanil	50.00	0.33	47.40	0.44	37.21
Tebuconazole	_	_	27.86	0.09	14.03
Thiacloprid	_	_	23.36	0.37	11.30
Thiamethoxam	_	_	26.51	0.37	25.98

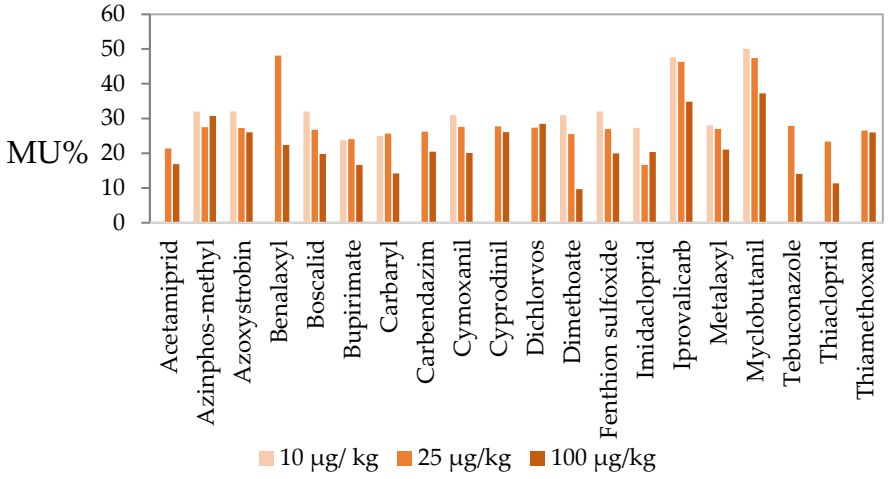

Figure 4. Measurement uncertainty (MU%) for milk in concentrations 10, 25, and 100 μg kg^{-1}.

Calculation using the Horwitz equation in 10 μg kg^{-1} fortification level revealed an acceptable $PRSD_R$ = 32% and an acceptable $PRSD_R$ = 27.88% in fortification level 25 μg kg^{-1}. Table 4 shows the HorRat value, which was calculated for each one of the pesticides. The HorRat ratio value for 10 μg kg^{-1} fortification level was <1 in all cases and varied between 0.03 (cyprodinil and dichlorvos) and 0.41 (carbaryl). Consequently, the method's precision is better than the maximum allowed.

3.3. Application to Real Milk Samples

Milk samples obtained from the Greek markets and local farms were analyzed using the optimized method. In 20 samples in the total analyzed, 13 samples of goat's milk (give samples of milk from local markets and eight from local farms) and seven samples of cow's milk (five samples of milk from local markets and two from local farms) were included.

The results revealed the occurrence of one fungicide in one milk sample provided by a local farmer (goat's milk). More specifically, carbendazim was detected only in one sample in concentration below the quantification limit and far below the MRLs set by the EU for milk. Full scan and MS/MS data for both the standard solution at concentration level of 50 μg kg^{-1} and the real milk sample where carbendazim was detected are shown in Figure 5. Carbendazim has been used as a fungicide in many crops, but nowadays has been abolished as a fungicide and approved as an existing active substance for use in certain biocidal products of product type 7 (film preservatives) and product type 10 (masonry preservatives) [63]. Nevertheless, residues of the pesticide carbendazim have been detected in the milk sample. It is commonly known that carbendazim is a main metabolite of thiophanate-methyl [64,65], a fungicide of the benzimidazole family, which is extensively applied pre- and post-harvest to control fungal pathogens in many crops [66]. The occurrence of carbendazim in milk s may be due to the utilization of thiophanate methyl in animal feed and may lead to the presence of carbendazim in edible tissue or milk and consequently into the human body through the food chain.

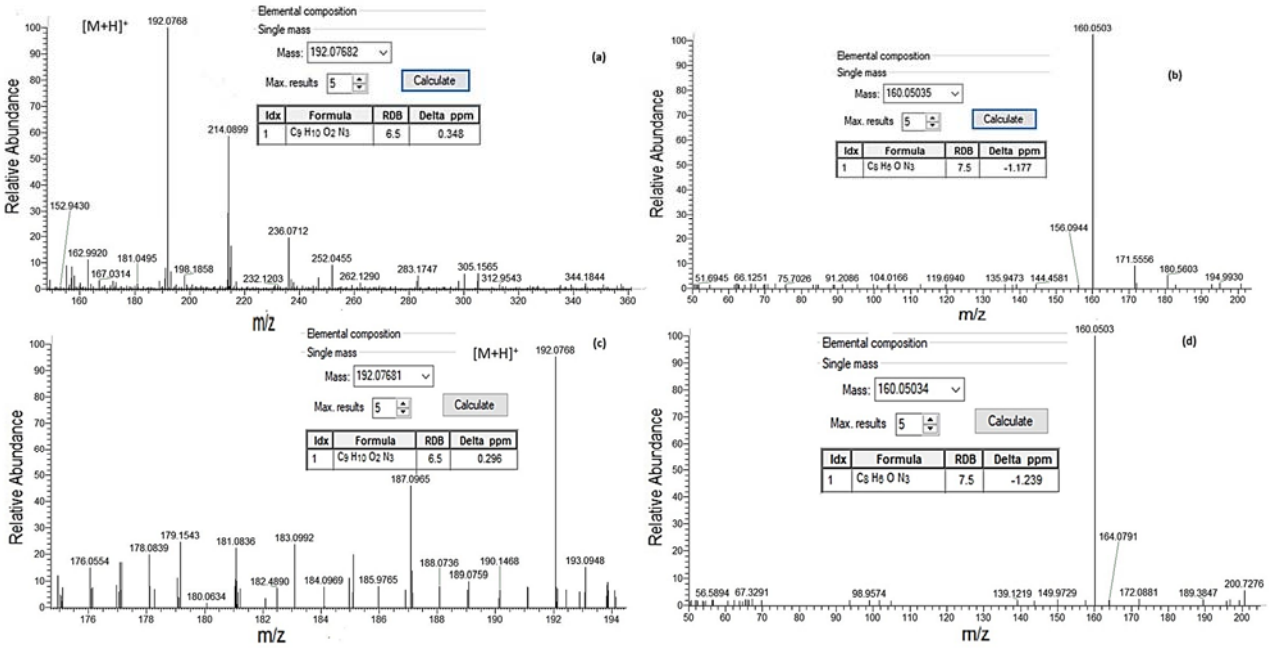

Figure 5. (**a**) Full scan accurate mass parent ion spectrum and (**b**) MS/MS data obtained for carbendazim in standard solution at a concentration of 50 μg kg^{-1}. (**c**) Full scan mass parent ion spectrum and (**d**) MS/MS data obtained for carbendazim in a milk sample from a local farm.

4. Conclusions

A methodology combining the QuEChERS extraction method, sonication, and the UHPLC Orbitrap MS/MS technique has been successfully established for the determination of

pesticides in milk samples. The acidified acetate method was chosen, and the optimization of the cleanup step with a new generation of sample purification sorbents (EMR-Lipid) used as a dispersive SPE (dSPE) agent was investigated and validated. The optimized modified QuEChERS is a dynamic, simple, and fast procedure with few analytical steps, minimizing errors. The use of accurate mass screening of UHPLC Orbitrap MS with ESI in positive mode, provided satisfactory selectivity and high sensitivity. The method was validated in terms of linearity, precision, trueness, LOD and LOQ, and the values achieved were highly satisfactory. The method's expanded uncertainty was acceptable according to the SANTE guidelines, while the HorRat values showed that the precision was better than permitted. Furthermore, validation data, including expanded uncertainty plus the HorRat values, were reported, providing the necessary data to demonstrate the efficiency of the method for milk analysis. The developed method was successfully applied to analyze real milk samples, revealing the presence of one pesticide in milk. To conclude, the modified method is recommended as an effective approach for monitoring a variety of pesticide residues in milk.

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