Research Article

Multiscreening LC-MS/MS Designed for Ten Pesticide and Six Antimicrobial Residues in Eggs

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A multiscreening method was experimentally validated for pesticide and antimicrobial residues in spiked eggs at levels of 5, 50, and 500 $\mu\text{g L}^{-1}$. The residue extraction was performed with acetonitrile and 1% formic acid. The extracted material was stored at -70°C until detection and quantitation within 24-hour period by LC-MS/MS. Pesticide and antimicrobial residue recovery performed adequate standard deviation and the results were from 94.10% (ampra) to 9.20% (carbaryl) for pesticides and from 95.70% (enrofloxacin) to 46.20% (sulfathiazole) for antimicrobial. The method was highly accurate, considering the linearity for all pesticide and antimicrobial residues ($r^2 > 0.99$), with quantitation limits ranging from 0.78 to 6.25 $\mu\text{g L}^{-1}$ for pesticides and 0.78 to 3.13 $\mu\text{g L}^{-1}$ for antibiotics. A highly correlation coefficient ($r > 0.99$) was found, except for carbaryl. Although time extraction and carbaryl recovery remain to be improved, the method has shown a rapid pesticide and antimicrobial residue detection.

1. Introduction

Although pesticides have been the main global tool for weed, pest, and disease in agricultural control, detection methods of pesticide residues in animal products remain antiquated compared to agricultural and environmental methodologies [1]. In recent years, Brazilian agriculture has experienced exponential growth, as well as the country becoming the world's top consumer of pesticides [2, 3].

Eggs have been considered an inexpensive and nutritive food source with worldwide production of 68,262 thousand tons in 2013 [4], with antimicrobial residues in food security having been uncertain in developing countries, but detection methodology established and routinely used [5–8].

Whereas European Union has listed veterinary drug presence in two alert notifications (trimethoprim, sulfonamide, enrofloxacin, and toltrazuril) between January, 2013, and

September, 2016, in eggs and egg products, no pesticide notification has been made during this period [9]. Moreover, no specific regulation has been globally provided for egg and egg products, although maximum levels of pesticide and antibiotic residues in food were established worldwide [10, 11].

Since Brazil has been also listed as the seventh world biggest egg producer [12], a rapid, simple, and reliable multiresidue detection would provide current sanitary status, food control, and prevention. Accordingly, the aim of the present study was to validate a multiscreening method based on LC-MS/MS for ten pesticide and six antimicrobial residues in egg contents.

2. Material and Methods

2.1. Egg Samples. Egg samples were provided by two high-production egg farms certified by the Brazilian Inspection

Service in Sao Paulo State, Brazil: Farm A with both conventional and free-range systems and Farm B with conventional system. There were 10 eggs from each system, totaling 30 eggs. These samples were stored at under frost conditions (-20°C) in a Falcon tube until analysis.

2.2. Equipment and Residues. Analyses were carried out by a LC-MS/MS system, composed by commercially available High Performance Liquid Chromatograph (Proeminence UFLC, Shimadzu Co., Sao Paulo, Brazil) coupled to a mass spectrometer (3200QTRAP, Applied Biosystems, Sao Paulo, Brazil). The triple quadrupole analysis mode, which is Q3 only defined to detect one especial mass and known fragment from the intact molecule of identified mass filtered by Q1, was selected and used [13, 14].

Pesticide residues of aminomethylphosphonic acid (ampa), epoxiconazole, fipronil, metolachlor, malathion, flutriafol, pyraclostrobin, carbendazim, glyphosate, and carbaryl, complying with the maximum residue limits (MRL) established by *Codex Alimentarius* for soy and corn grains [15], were selected and tested. These residues represented the most common pesticides applied in corn and soy crops, the worldwide main ingredients for laying hen feeding.

Antimicrobials residues of enrofloxacin, sulfamethazine, sulfadimethoxine, sulfadiazine, chloramphenicol, and sulfathiazole, complying with the maximum residue limits (MRL) established for eggs [15], were selected and tested. In addition, comparisons were made with a previous study of drug use in poultry in Paraná State [18] and worldwide [19, 20].

2.3. Validation of Liquid Chromatography Analysis and Mass Spectrometry. To validate the methodology, several experimental studies for analytical curve linearity, limit of detection and quantitation, precision (repeatability, and intermediate precision), and accuracy [21, 22] were considered.

To optimize mass spectrometry conditions, direct injections to the equipment were performed with standard analytical solution of 1 mg L^{-1} from each compound, individually. The analytical standards for tests were commercially acquired with 98% purity or higher (Sigma-Aldrich, Sao Paulo, Brazil). From infusions, ESI (electrospray ionization) was picked as the ionization source, which produces analyte ions on liquid phase prior to entering mass spectrometer. Negative ionization mode was utilized to glyphosate and ampa. For other compounds, positive ionization was done.

Chromatographic conditions used for compound quantifications on negative ionization mode were a chromatographic column (Gemini 5 micrometers C18 110 Å 150 mm $\times$ 4.6 mm) with phase A (PA) = 5 mM of ammonium acetate in water and phase B (PB) = 5 mM of ammonium acetate in methanol on mobile phase, 0–4 min = 10% PB and 90% PA; 4–6 min = 95% PB and 5% PA; 6–8 min = 95% PB and 5% PA; 8–10 min = 10% PB and 90% PA with 12 min stop on mobile phase gradient and on mobile phase flow rate 0.500 mL min^{-1} .

Chromatographic conditions used for compound quantifications on positive ionization mode were a chromatographic column (Synergi 2.5 micrometers Hydro-RP 110 Å 50

$\times$ 4.60 mm) with phase A (PA) = 0.1% formic acid in water and phase B (PB) = 0.1% formic acid in methanol on mobile phase, 0–0.5 min = 10% PB and 90% PA; 0.5–4.5 min = 95% PB and 5% FA; 4.5–7.5 min = 95% PB and 5% PA; 7.5–9.5 min = 10% PB and 90% PA with 11 min stop on mobile phase gradient and on mobile phase flow rate 0.400 mL min^{-1} .

Monitoring more than one ion was necessary to avoid erroneous identifications of the composing quantification. LC-MS/MS use enabled control of molecular ion and ionic transition from substance fragmentation, ensuring sensitivity and specificity for an accurate measurement of weak traces of analytes, sometimes present in highly complex matrices [13, 14].

2.4. Analytical Curve. The analytical curve is the quantification tool most commonly used. It consists in determining response of any particular instrument to various concentrations of a tested substance. Commonly chromatographic analytical techniques use a first-order linear association between detector response (y) and concentration (x) of the analyte [23, 24]. This mathematical relation must also have a high correlation coefficient ($r > 0.9999$) and may be represented by the linear regression equation described below [25]:

$$y = ax + b, \quad (1)$$

where b is the intersection of the calibration curve and a is the slope.

2.5. Residue Extraction. The congealed egg content samples were defrosted into a refrigerator for 24 hours. Then, 2.50 g of homogenate albumen and yolk were weighted and homogenized into a Falcon tube with 5 mL of ultrapure water by Turrax. After, 15 mL of acetonitrile containing 1% formic acid was aggregated, generating a 20-fold dilution of the sample. Diluted sample was maintained under continuous and intense stirring for one hour. The tube was centrifuged (10 min/2000 rcf) (Rotanta 460R), and 0.50 mL of the supernatant was sifted through $0.45\text{ }\mu\text{M}$ Millex HV filters (Millipore), with a 13 mm durapore membrane, and packaged in a 9 mm amber vial (Flow Supply), for subsequent quantification by LC-MS/MS (Mol et al. 2008 adapted).

For checking the recovery, a sample enrichment was performed by a sample solution containing a mixture of the analytical standards representing itself contents of 5, 50, and $500\text{ }\mu\text{g L}^{-1}$ from each compound. Four replications were performed. The average recovery and standard deviation were calculated by matching enriched samples versus calibration matrix standards. Recovery outcome reflected on the recuperation of extraction process. Results were calculated by comparing response obtained from each component with the matching matrix pattern.

3. Results and Discussion

3.1. Validation of Liquid Chromatography Analysis and Mass Spectrometry. The molecular mass and fragments produced from each pesticide molecule, respectively, were ampa 109.99 (62.90, 78.90, and 80.80), epoxiconazole 330.15 (121.00, 101.10, and 75.10) fipronil 436.91 (368.00, 251.10, and 263.10),

metolachlor 284.21 (252.30, 176.20, and 91.10), malathion 331.01 (99.00, 127.10, and 125.00), flutriafol 302.10 (70.00, 123.20, 95.00), pyraclostrobin 388.13 (163.20, 194.30, and 104.20), carbendazim 192.25 (160.20, 132.30, and 105.10), glyphosate 167.92 (63.10, 78.80, and 150.20), and carbaryl 202.15 (145.10, 127.10, and 117.20), and, for each antimicrobials, respectively, were enrofloxacin 360.23 (342.30, 245.30, and 203.20), sulfamethazine 279.01 (108.20, 92.20, and 156.30), sulfadimethoxine 311.01 (155.90, 108.20, and 64.90), sulfadiazine 250.87 (156.20, 64.90, and 108.20), chloramphenicol 322.84 (275.00, 165.10, and 83.20), and sulfathiazole 256.04 (92.10, 156.10, 65.20).

The detection and separation of the substances were performed in two intervals, a negative mode (12 minutes) and a positive mode (11 minutes). The retention time for each compound in the chromatographic column was 3.65 min for ampa, 6.65 min for epoxiconazole, 6.53 min for fipronil, 6.76 min for metolachlor, 6.46 min for malathion, 6.04 min for flutriafol, 6.86 min for pyraclostrobin, 3.98 min for carbendazim, 4.02 min for glyphosate, 5.95 min for carbaryl, 4.20 min for enrofloxacin, 4.50 min for sulfamethazine, 5.24 min for sulfadimethoxine, 3.97 min for sulfadiazine, 5.35 min for chloramphenicol, and 4.03 min for sulfathiazole. There is an example of analytical standard chromatogram ($6.25 \mu\text{g L}^{-1}$) of sulfadimethoxine under conditions described (Figure 1).

3.2. Analytical Curve. The analytical curves were constructed for compounds resulting in r^2 and linear interval, respectively: ampa (0.98 and $1.56\text{--}200 \mu\text{L}^{-1}$); epoxiconazole (0.99 and $0.78\text{--}200 \mu\text{L}^{-1}$); fipronil (0.99 and $0.78\text{--}100$); metolachlor (0.99 and $0.78\text{--}100$); malathion (0.99 and $0.78\text{--}200$); flutriafol (0.99 and $0.78\text{--}200$); pyraclostrobin (0.99 and $0.78\text{--}100$); carbendazim (0.98 and $0.78\text{--}100$); glyphosate (0.99 and $0.78\text{--}200$); carbaryl (0.98 and $0.78\text{--}100$); enrofloxacin (0.98 and $1.56\text{--}200$); sulfamethazine (0.99 and $0.78\text{--}200$); sulfadimethoxine (0.98 and $0.78\text{--}200$); sulfadiazine (0.99 and $0.78\text{--}100$); chloramphenicol (0.99 and $3.13\text{--}200$); and sulfathiazole (0.99 and $1.56\text{--}100$). The linear model used in the equations model showed to be very suitable [21, 22, 25].

3.3. Residue Extraction. The average concentration recovery for studied pesticides was 5.62 to 99.40% and for antimicrobial 63.80 to 100%, except for sulfathiazole, with 49.00% (Table 1). Since minimum limit of quantification (MLQ) is of 1.56 of sulfathiazole, it is possible to qualify the residue recovery by MRL ($10 \mu\text{g L}^{-1}$) hypothetical extrapolation accepted by the Ministry of Agriculture (Brasil, 2013). So, although the lower range of recovery, there is linearity on results.

Minimum limits of quantification (MLQ) were 0.78 to $6.25 \mu\text{g L}^{-1}$ for pesticides and 0.78 to $3.13 \mu\text{g L}^{-1}$ for antimicrobials. This complies the needs for residue screening, considering MRL established by national and international legislation [10, 15], except for chloramphenicol, whose MRL is $0.30 \mu\text{g L}^{-1}$ [10], and its MLQ was $3.13 \mu\text{g L}^{-1}$.

For pesticides, average recovery was more variable, although linearity has also been observed in all the compound recuperation. Average recovery of ampa, epoxiconazole, fipronil, and metolachlor was considered excellent (56.17

FIGURE 1: Chromatograms of sulfadimethoxine and fragments generated at concentration of $6.25 \mu\text{g L}^{-1}$, Botucatu/SP, 2013.

to 94.08%). Analyzed substances in the medium recovery range from 24.17 to 48.05% were considered of moderate efficiency (malathion, flutriafol, pyraclostrobin, and cabendazim). Low average recovery was observed for glyphosate and carbaryl (19.23 and 9.20%, resp.). None of these compounds has MRL established for egg, only for corn and soybeans [15].

On considering the minimum quantitation limits, high correlation coefficient ($r > 0.99$), and the large number of evaluated compounds, this residue extraction method through acetonitrile, with added 1% formic acid, was considered efficient for quantifying all compounds assessed in this study, except for carbaryl. In this case, an alternative extraction method should be performed. In summary, even touch time extraction and carbaryl recovery persist to be enhanced, and this multiscreening method for egg residues has shown a rapid pesticide and antimicrobial residue detection.

Additional Points

Practical Applications. We described the validation of a LC-MS/MS method to simultaneous determination of ten pesticides and six antimicrobial. The methodology may be applied in laboratory routine.

TABLE 1: Evaluated compounds and results to validate multiresidue method of pesticides and antimicrobials.

Injection concentration	MRL ^a [10]	MRL ^b [15]	MQL ^c ($\mu\text{g L}^{-1}$)	R/CcA ^d (%)	R/CpA ^e (%)	RA ^f ($\mu\text{g L}^{-1}$) $\pm$ SD	CI ^g	Lower and upper limit ($\mu\text{g L}^{-1}$)
Pesticides								
Ampa								
5 $\mu\text{g L}^{-1}$	—	—	—	99.40	—	4.97 $\pm$ 0.09	0.09	4.88–5.06
50 $\mu\text{g L}^{-1}$	—	—	6.25	87.40	94.08	43.70 $\pm$ 4.09	4.00	39.70–47.70
100 $\mu\text{g L}^{-1}$	—	—	—	95.43	—	95.43 $\pm$ 4.41	4.32	91.12–99.75
Epoxiconazole								
5 $\mu\text{g L}^{-1}$	—	—	—	91.00	—	4.55 $\pm$ 0.57	0.56	3.99–5.10
50 $\mu\text{g L}^{-1}$	—	—	0.78	64.86	77.58	32.43 $\pm$ 2.03	1.99	30.44–34.42
100 $\mu\text{g L}^{-1}$	—	—	—	76.87	—	76.87 $\pm$ 20.36	19.95	56.92–96.81
Fipronil								
5 $\mu\text{g L}^{-1}$	—	0.01–0.02 mg kg ⁻¹	—	69.00	—	3.45 $\pm$ 0.05	0.05	3.41–3.50
50 $\mu\text{g L}^{-1}$	—	—	1.56	55.60	60.00	27.80 $\pm$ 5.87	5.75	22.05–33.55
100 $\mu\text{g L}^{-1}$	—	—	—	55.40	—	55.40 $\pm$ 10.43	10.22	45.18–65.62
Metolachlor								
5 $\mu\text{g L}^{-1}$	—	—	—	69.20	—	3.46 $\pm$ 0.60	0.59	2.87–4.05
50 $\mu\text{g L}^{-1}$	—	—	0.78	55.20	56.17	27.60 $\pm$ 6.99	6.85	20.75–34.45
100 $\mu\text{g L}^{-1}$	—	—	—	44.10	—	44.10 $\pm$ 6.79	6.65	37.45–50.75
Malathion								
5 $\mu\text{g L}^{-1}$	—	0.05 mg kg ⁻¹	—	64.8	—	3.24 $\pm$ 1.09	1.07	2.17–4.31
50 $\mu\text{g L}^{-1}$	—	—	0.78	43.26	48.05	21.63 $\pm$ 4.88	4.78	16.85–26.42
100 $\mu\text{g L}^{-1}$	—	—	—	36.10	—	36.10 $\pm$ 7.20	7.05	29.05–43.15
Flutriafol								
5 $\mu\text{g L}^{-1}$	—	—	—	38.00	—	1.90 $\pm$ 0.78	0.76	1.14–2.66
50 $\mu\text{g L}^{-1}$	—	—	0.78	22.94	32.25	11.47 $\pm$ 3.14	3.08	8.39–14.55
100 $\mu\text{g L}^{-1}$	—	—	—	35.80	—	35.80 $\pm$ 10.34	10.13	25.67–45.93
Pyraclostrobin								
5 $\mu\text{g L}^{-1}$	—	0.02–0.05 mg kg ⁻¹	—	13.60	—	0.68 $\pm$ 1.09	1.07	0.00–1.75
50 $\mu\text{g L}^{-1}$	—	—	0.78	36.74	25.43	18.37 $\pm$ 5.47	5.36	13.01–23.72
100 $\mu\text{g L}^{-1}$	—	—	—	25.95	—	29.25 $\pm$ 4.26	4.18	21.77–30.13

TABLE 1: Continued.

Injection concentration	MRL ^a [10]	MRL ^b [15]	MQL ^c ($\mu\text{g L}^{-1}$)	R/CcA ^d (%)	R/CpA ^e (%)	RA ^f ($\mu\text{g L}^{-1}$) $\pm$ SD	CI ^g	Lower and upper limit ($\mu\text{g L}^{-1}$)
Carbendazim								
5 $\mu\text{g L}^{-1}$	—	0.05–0.10 mg kg ⁻¹	0.78	22.40		1.12 $\pm$ 0.05	0.05	1.07–1.17
50 $\mu\text{g L}^{-1}$				25.20	24.17	12.60 $\pm$ 0.27	0.26	12.34–12.86
100 $\mu\text{g L}^{-1}$				24.90		24.70 $\pm$ 0.75	0.74	24.16–25.34
Glyphosate								
5 $\mu\text{g L}^{-1}$				17.60		0.88 $\pm$ 0.27	0.27	0.61–1.45
50 $\mu\text{g L}^{-1}$	—	0.05–150.00 mg kg ⁻¹	0.78	20.08	19.23	10.04 $\pm$ 2.80	2.74	7.29–12.78
100 $\mu\text{g L}^{-1}$				20.00		20.00 $\pm$ 3.54	3.47	16.53–23.47
Carbaryl								
5 $\mu\text{g L}^{-1}$				10.00		0.50 $\pm$ 0.68	0.66	0.00–1.16
50 $\mu\text{g L}^{-1}$	—	15.00–250.00 mg kg ⁻¹	0.78	5.62	9.24	2.82 $\pm$ 1.15	1.13	1.70–3.95
100 $\mu\text{g L}^{-1}$				12.10		12.10 $\pm$ 4.60	4.51	7.59–16.61
Antimicrobials								
Enrofloxacin								
5 $\mu\text{g L}^{-1}$				100.00		5.00 $\pm$ 0.57	0.56	4.44–5.56
50 $\mu\text{g L}^{-1}$	10.00 $\mu\text{g L}^{-1}$	Not recommended for use in laying hens [16].	1.56	94.00	95.70	47.80 $\pm$ 8.37	8.21	39.59–56.01
100 $\mu\text{g L}^{-1}$				93.10		93.10 $\pm$ 13.52	13.24	82.86–109.34
Sulfamethazine								
5 $\mu\text{g L}^{-1}$				95.00		4.75 $\pm$ 0.86	0.84	3.92–5.59
50 $\mu\text{g L}^{-1}$	10.00 $\mu\text{g L}^{-1}$	Not recommended for use in laying hens [17].	0.78	92.20	92.50	46.10 $\pm$ 3.59	3.51	42.59–49.61
100 $\mu\text{g L}^{-1}$				90.30		90.30 $\pm$ 2.14	2.09	88.21–92.39
Injection concentration	MRL ^a [10]	MRL ^b [17]	MQL ^c ($\mu\text{g L}^{-1}$)	R/CcA ^d (%)	R/CpA ^e (%)	RA ^f ($\mu\text{g L}^{-1}$) $\pm$ SD	CI ^g	Lower and upper limit ($\mu\text{g L}^{-1}$)
Antimicrobials								
Sulfadimethoxine								
5 $\mu\text{g L}^{-1}$				79.80		3.99 $\pm$ 0.15	0.15	3.84–4.14
50 $\mu\text{g L}^{-1}$	10.00 $\mu\text{g L}^{-1}$	—	1.56	83.40	79.73	41.70 $\pm$ 8.25	8.09	33.61–49.79
100 $\mu\text{g L}^{-1}$				75.98		75.98 $\pm$ 5.55	5.44	70.54–81.41
Sulfadiazine								
5 $\mu\text{g L}^{-1}$				66.76		3.38 $\pm$ 1.63	1.60	1.78–5.06
50 $\mu\text{g L}^{-1}$	10.00 $\mu\text{g L}^{-1}$	—	1.56	69.94	72.15	34.96 $\pm$ 4.15	4.07	30.90–47.70
100 $\mu\text{g L}^{-1}$				79.75		79.75 $\pm$ 10.46	10.25	69.50–90.00
Chloramphenicol								
5 $\mu\text{g L}^{-1}$				71.74		3.59 $\pm$ 2.01	1.95	1.07–4.97
50 $\mu\text{g L}^{-1}$	0.30 $\mu\text{g L}^{-1}$	There is no safe level of residues or its metabolites in food.	3.13	64.93	66.82	32.47 $\pm$ 2.31	2.27	30.20–34.73
100 $\mu\text{g L}^{-1}$				63.80		63.80 $\pm$ 6.28	6.15	57.65–69.95
Sulfathiazole								
5 $\mu\text{g L}^{-1}$				41.80		2.09 $\pm$ 0.53	0.52	1.57–2.61
50 $\mu\text{g L}^{-1}$	10.00 $\mu\text{g L}^{-1}$	—	1.56	49.00	46.24	24.33 $\pm$ 5.07	4.97	19.36–29.31
100 $\mu\text{g L}^{-1}$				47.93		47.93 $\pm$ 11.87	11.63	36.30–59.57

^{a,b}MRL: maximum residue limits; ^cMQL: maximum quantification limits; ^dR/CcA: recovery/concentration averaged; ^eR/CpA: recovery/compound average; ^fRA: recovery average; ^gCI: confidence interval.

Disclosure

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Competing Interests

The authors declare that they have no competing interests.

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