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Article

Simultaneous Determination of Herbicides in Rice by QuEChERS and LC-MS/MS Using Matrix-Matched Calibration

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The main objective of the present work was to validate a chromatographic method to determine herbicides commonly applied in the irrigated rice farming. For this, matrix-matched calibration was employed along with the extraction and clean-up of the samples by quick, easy, cheap, effective, rugged and safe (QuEChERS) method and determination of the analytes by high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization in the positive mode. By this method, it was possible to achieve the ionization and detection of a total of 18 herbicides, with quantification of 12 of them. The method presented adequate precision and accuracy according to the European Commission and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines for analytes in low concentrations. The limits of quantification ranged from 0.015 μg g^{-1} for oxadiazon to 0.165 μg g^{-1} for imazapyr. The method showed good linearity with $R^2 > 0.99$ and recovery values from 92 to 103%. The proposed protocol is adequate for monitoring bispyribac-sodium, cyclosulfamuron, cycloxydim, clomazone, ethoxysulfuron, fenoxaprop-*p*-ethyl, imazapic, imazapyr, imazethapyr, metsulfuron-methyl, oxadiazon and thiobencarb in rice grains in concentrations up to 109 times lower than the maximum residue limits established by the Brazilian Health Surveillance Agency (ANVISA) for these compounds in rice samples.

Keywords: liquid chromatography tandem mass spectrometry, LC-MS/MS, herbicides, multiresidue, ANVISA

Introduction

Pesticides, including herbicides, are widely used to guarantee large-scale food production and to support the demand worldwide.[1] Considering their high toxicity for humans, quality control agencies establish a maximum residue limit (MRL) for each pesticide on each food.[2] In Brazil, the Brazilian Health Surveillance Agency (ANVISA) is responsible to establish these limits.[3,4] Considering the rice production, among the current 71 pesticides regulated in Brazil, 26 are herbicides, corresponding to 35% of all. According to ANVISA, the MRL for herbicides range from 0.01 to 2.0 μg g^{-1}. Thus, the development of sensitive multiresidue analytical protocols is necessary to monitoring food products in a short time with low limits of quantification (LOQ). Although the determination of pesticide residues in rice has been traditionally based on gas chromatography with mass spectrometry (GC-MS),[5] nowadays high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) is widely employed due to the new classes of pesticides used in the production.[5-9]

Rice grain is considered a complex matrix. Thus, the extraction and clean-up steps must be carefully studied. The most frequent strategies are based on liquid-liquid; solid-phase; as well as supercritical-fluid extraction; and quick, easy, cheap, effective, rugged and safe (QuEChERS) method; with the latest being the most frequently employed.[10]

Pareja *et al.*[8] studied four different QuEChERS-based protocols in the analysis of 16 commonly applied herbicides and 26 other pesticides in polished rice by LC-MS/MS.

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The best results were found for the method that used acetic acid in the extraction with no clean-up. Recoveries ranged from 70% to 120% for most samples.[8,11] Kruve *et al.*[12] studied three different sample preparation methods for pesticide analysis in fruits and vegetables by LC-MS/MS: Luke method, QuEChERS and matrix solid-phase dispersion (MSPD). Among these, QuEChERS provided the best recoveries. However, the matrix-effect was less pronounced by using the Luke method.[12]

Even employing extraction and clean-up steps and using one of the most specific detectors coupled to liquid chromatography, such as tandem mass spectrometry (MS/MS), it is known that multiresidue analysis of pesticides in food is commonly affected by the matrix.[12,13] When caused by those co-eluting components in sample extracts that have similar ions in the MS/MS experiment, the interference can be solved by using non-interfering single reaction monitoring (SRM) transitions associated to an extensive sample clean-up provided by QuEChERS. However, matrix effects may also be caused by interactions between pesticides and co-extractives in the prepared sample that could suppress or enhance the ionization in the mass spectrometry interface, leading to errors in the measurement. Among the possibilities to minimize these matrix effects, standard addition, internal standards and/or matrix-matched calibration are commonly used to compensate these signal suppression or enhancement.[14] In fact, the letter is recommended by the European Commission Health & Consumer Protection Directorate-General, since the preparation of standard solutions in all possible matrixes is unrealistic.[13]

Based upon the aforementioned aspects, this paper describes the use of both external and matrix-matched calibration to determine a total of 12 herbicides in rice grains by employing modified QuEChERS-based methodology met to LC-MS/MS using SRM mode.

Experimental

Reagents and chemicals

All analytical standards of pesticides (2,4-dichlorophenoxyacetic acid, azimsulfuron, bentazon, bispyribac-sodium, cyclosulfamuron, cycloxydim, cyhalofop-butyl, clomazone, ethoxysulfuron, fenoxaprop-*p*-ethyl, glyphosate, imazapic, imazapyr, imazethapyr, metsulfuron-methyl, oxadiazon, oxyfluorfen, pendimethalin, penoxsulam, picloram, propanil, pyrazosulfuron-ethyl, quinclorac, thiobencarb, triclopyr and trifluralin purity > 98%), and sulfamethoxazole (purity > 98%) were purchased from Sigma Aldrich (St. Louis, USA). High-performance liquid chromatography (HPLC) grade methanol, acetonitrile and formic acid (49-51%, m/m) were obtained from Merck (Darmstadt, Germany). Anhydrous magnesium sulfate (99.8%), anhydrous sodium acetate (99%) and primary and secondary amine (PSA) 40 μm (Agilent Technologies, Santa Clara, USA) were purchased from J.T. Baker (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore, Bedford, USA).

Individual pesticide and sulfamethoxazole (used as internal standard, IS) stock solutions were prepared separately (1000 mg L^{-1}) in methanol or acetonitrile and stored at −4 °C. From the stock solutions, a mixture of all pesticides containing different concentrations based on the MRL of each one was prepared in water. Immediately after its preparation, this solution was used to prepare the working standard solutions in acetonitrile, including the analytical standards as well as the fortified solutions. The final concentration of IS was 9.5 mg mL^{-1} in all working solutions.

Samples

Rice grain samples free of pesticides were used as blank control. They were harvested, peeled and ground in particles with 0.2-1.0 mm by the Company of Agriculture Research and Rural Extension of Santa Catarina (EPAGRI), and then provided to us.

Eight commercial rice samples were analyzed in this work. All of them were purchased in local market and were provided from different suppliers. Before analysis, samples were ground using an industrial mixer, resulting in particles with size varying from 0.2 to 1.0 mm.

Liquid chromatographic separation

All analyses were performed on an Agilent HPLC series 1200 system, equipped with a quaternary pump, a membrane degasser, a thermostatted column compartment and an automatic injector (Agilent Technologies, Santa Clara, USA). The chromatographic separation was performed on a Synergi Polar-RP column (150 mm, 2.0 mm inner diameter, 4 μm particle size) and a guard column has been set between the injector and analytical column (Phenomenex, Torrance, USA). The mobile phase components were (A) acetonitrile:water (95:5, v/v) and (B) aqueous solution of formic acid 0.1% used in gradient mode according to the method developed by Rebelo *et al.*[15]

Tandem mass spectrometric detection

A triple quadrupole-linear ion trap mass spectrometer QTrap 3200 equipped with an electrospray ionization

(ESI) source (Applied Biosystems/MDS Sciex, Foster City, USA) was used coupled to the chromatographic system. All analyses were performed in positive ion mode. The capillary needle was maintained at +5500 V. For operation in the MS/MS mode, the follow parameters were set: curtain gas (N_2) 10 psi; temperature 600 °C, gas 1 (argonium) 18 psi, gas 2 off and collisionally activated dissociation (CAD) gas (nitrogen) high. The analytes were monitored and quantified using SRM.

Optimization of the MS/MS conditions, identification of the parent and product ions, as well as the selection of the cone and collision voltages, were performed with direct infusion of their individual standard solutions employing the positive ion mode. Every individual standard herbicide solution was prepared in the concentration of 1 mg mL^{-1} in water. The Analyst software version TF1.5.1 was used for the LC-MS/MS system control and data analysis. After the optimization of the collision cell energy of the triple quadrupole, two different *m/z* transitions were selected for each analyte, one for quantification (QIT) and one for confirmation (CIT).

For seven herbicides, which were validated by the external calibration developed by Rebelo *et al.*,[15] the parameters of quantification, qualification and fragmentation, were kept the same. For the other analytes these same parameters were optimized in this work.

Sample preparation

Considering the good results obtained by using the modified QuEChERS method to prepare samples of rice grains before the chromatographic separation of pesticides, this methodology was chosen for application in this work. As previously reported by our research group,[15] the following procedure was applied: 1.0 mL of a solution containing the internal standard (142 mg mL^{-1}) was added to 5 g of sample previously ground in particles of 0.2-1.0 mm. After 30 min kept interacting in a closed falcon tube and in the absence of light, 14 mL of acetonitrile with 1.0% acetic acid, 2.0 g of anhydrous magnesium sulfate and 0.5 g of sodium acetate were added in the mixture. Thereafter, it was vortex stirred for 1.0 min and then centrifuged at 4000 rpm for 1 min. For the clean-up step, 1.5 mL of the liquid phase was extracted, placed in a falcon tube, in which was added 150 mg of anhydrous magnesium sulfate and 50 mg of PSA. The tube was stirred in a vortex system for 1.0 min and centrifuged at 4000 rpm for one more minute. An aliquot of 1.0 mL was collected from the supernatant, which was placed directly into vials for automatic injection into the chromatographic system. All procedures were performed in triplicate.

Method validation by matrix-matched calibration

Analytical curves used to evaluate the performance of the method were obtained by the matrix-matched calibration. For this, 5 g of the blank control previously ground were used to prepare each concentration level. Sulfamethoxazole, acetonitrile, acetic acid and a mixture of standards were then added. After that, the QuEChERS procedure was employed as previously described.

The proposed method was evaluated in terms of linearity (slope of the external standard analytical curves and their determination coefficients, R^2); precision (instrumental, repeatability, or intra-assay, and inter-assay) obtained for the intermediate concentration of each linear range (by dilution of concentrations corresponding to the MRL values established by ANVISA); limits of detection (LOD) and quantification (LOQ), obtained from the signal to noise ratio, 3:1 and 10:1, respectively;[16] and accuracy. To evaluate the accuracy, recovery assays using grains of rice in the absence of pesticides (blank control) were used.[12,13] This procedure was performed in triplicate by addition of the internal standard (final concentration of 9.5 mg mL^{-1}) and five concentration levels of each analyte in 5 g of rice, before the addition of acetonitrile and salts used in the QuEChERS extraction. The five concentrations used in the recovery assays represented the entire linear ranges and varied from 0.004 to 0.070 mg mL^{-1} depending on each linear range.

Matrix-effect by LC-MS/MS

The matrix-effect was evaluated according to the European Commission,[11] by comparison of the response of each herbicide obtained from the standard solution with the response of the enriched sample. For this, the ratio between the slope obtained from the matrix-matched calibration curve and that obtained by the external calibration curve was calculated for all herbicides. Assays were performed in triplicate.

Results and Discussion

MS/MS optimization parameters

For the simultaneous determination of herbicides, the same ionization mode was applied. The positive mode was chosen because it is the most suitable for the majority of the analytes. The optimized MS/MS parameters for 18 of the 26 herbicides studied in this work are shown in Table 1. In the case of cyclosulfamuron, cycloxydim, clomazone, ethoxysulfuron, metsulfuron-methyl,

oxadiazon, thiobencarb and sulfamethoxazole, the same MS/MS parameters previously reported by Rebelo *et al.*[15] were applied.

From the 26 herbicides studied and employed in rice production, 18 presented mass spectra in positive ionization mode and 8 did not show mass spectra under the studied conditions. Molecules containing alkanoic acid groups, such as the 2,4-dichlorophenoxyacetic (2,4-D) acid[17] and cyhalofop-*p*-butyl, are considered proton-donor, as well as the glyphosate, which is easily transformed to aminomethylphosphonic acid.[18] Thus, they are usually analyzed in the negative ion mode. According to Demoliner *et al.*[19] in a multiresidue method developed to determine pesticides in water by LC-MS/MS, 7 of the 20 compounds analyzed were ionizable in the negative mode only (metsulfuron-methyl, quinclorac, bentazone, penoxsulam, fipronil, pyrazosulfuron-ethyl and 2,4-D).

In a method proposed for the determination of 44 pesticides in rice grains, the compounds propanil and bentazone were ionizable in the negative mode only. Azimsulfuron, bensufuron, bromacil and imazosulfuron were ionizable in both, negative and positive modes. All other analytes were analyzed in the positive ion mode only.[5,17] However, in this work the ionization of azimsulfuron was not observed in the positive mode. This phenomenon can be explained by the premature fragmentation of the molecule in the ESI source. It is already known that compounds which present the urea group can be broken in the ionization process, preventing the correct fragmentation, filtering and detection by the mass analyzers.[20] In another study aiming to determine diphenyl ether herbicides in water using LC-MS/MS, the negative ionization mode was successfully employed.[21] In the same study, oxifluorfem was also included as one of the analytes. However, its determination was not accomplished since its mass spectrum in the positive ion mode was not obtained. The same behavior was observed in our study not only for oxifluorfem, but also for etofemproxi, a diphenyl ether as well.

The compound trifluralin did not show ionization in positive mode. In fact, other authors have obtained the ionization of this analyte using ESI by protonating $[M + H]^+$. However, it presented high LOD values due the weak ionization in positive mode. The mass spectrum showed no fragmentation but the presence of the *quasi*-molecular ion with *m/z* 336.11.[22,23] The chemical structure of trifluralin presents high stability and dissociation colision-induced,

Table 1. Tandem mass spectrometry (MS/MS) parameters optimized for each herbicide

Pesticide	SRM[a] transition / (*m/z*)		DP[d] / V	CE[e] / eV		EP[f] / kV	CEP[g] / kV	CXP[h] / V		Dwell time / ms
	QIT[b]	CIT[c]		QIT[b]	CIT[c]			QIT[b]	CIT[c]	
Bentazon	241.05 > 199.10	241.05 > 107.10	51	13	35	3.0	8	6	4	10
Bispyribac-sodium	431.03 > 275.00	431.03 > 413.00	21	19	19	5.0	18	6	6	10
Cyclosulfamuron	422.03 > 260.90	422.03 > 218.00	31	21	31	5.0	18	6	4	10
Cycloxydim	326.11 > 280.10	326.11 > 180.00	31	17	25	4.0	16	6	4	10
Clomazone	241.15 > 126.00	241.15 > 125.00	36	25	25	7.0	14	4	4	10
Ethoxysulfuron	399.05 > 260.70	399.05 > 218.00	41	23	39	2.5	34	4	4	10
Fenoxaprop-*p*-ethyl	363.06 > 289.00	363.06 > 77.10	106	19	79	4.0	18	6	4	10
Imazapic	276.12 > 231.10	276.12 > 163.00	56	21	39	4.5	14	4	4	50
Imazapyr	262.15 > 217.00	262.15 > 69.10	41	21	39	6.5	14	4	4	50
Imazethapyr	290.12 > 86.10	290.15 > 245.00	46	41	21	5.0	16	4	4	10
Metsulfuron-methyl	382.10 > 167.00	382.10 > 141.00	31	19	21	5.5	28	4	4	10
Oxadiazon	346.06 > 304.00	346.06 > 184.90	31	17	37	6.5	28	6	4	10
Picloram	242.97 > 224.90	242.97 > 196.90	31	15	21	6.5	14	4	4	10
Pirazosulfamuron	415.01 > 182.00	415.01 > 83.00	31	23	71	4.5	22	4	4	10
Quinclorac	243.00 > 162.00	243.00 > 224.90	26	15	43	3.0	14	6	4	10
Pendimenthalin	282.00 > 211.90	282.00 > 91.20	26	15	31	4.0	15	4	4	10
Triclopyr	257.99 > 211.80	257.99 > 147.90	41	17	37	3.0	12	4	4	10
Thiobencarb	259.05 > 126.00	259.05 > 125.00	26	21	21	5.5	14	4	4	10
Sulfamethoxazole (IS[i])	254.02 > 156.00	254.02 > 108.10	31	19	33	5.5	14	4	4	10

[a]Single reaction monitoring; [b]quantitation ion transition; [c]confirmation ion transition; [d]de-clustering potential; [e]collision energy; [f]entrance potential; [g]collision cell entrance potential; [h]collision cell exit potential; [i]internal standard.

especially after protonation, being necessary high energy for its fragmentation. Electron ionization employed in gas chromatography is inappropriate for many compounds due the high energy applied. However, it allows the fragmentation of compounds with high stability, including trifluralin.[24]

Table 1 also shows the dwell time optimized for each analyte. Low dwell times may decrease the detectability of the analyte. On the other hand, high dwell times may interfere in other mass transitions and lead to poor resolution of chromatographic peaks. Thus, this parameter must be optimized for obtaining the lower time for adequate monitoring with no loss in resolution and also keeping the higher detectability. In this work, the dwell time of 10 ms was appropriate for most of the compounds, but 50 ms was needed for imazapir and imazapic.

Analytical method evaluation

Precision and accuracy parameters, evaluated for the proposed method, are shown in Table 2 with employing the matrix-matched calibration. The results for precision, recovery and matrix-effect are only for those compounds which presented relative standard deviation (RSD) lower than 20% and R^2 at least 0.99, indicating adequate linearity of the method in the studied concentration ranges. Linearity was obtained from the matrix-matched calibration curves prepared in triplicate.

Bentazon, triclopyr, quinclorac, pyrazosulfuron, picloram and pendimethalin did not show adequate precision and accuracy during the validation procedures. According to the literature, all of them are analyzed in the negative ionization mode.[19,25,26] Although bentazon presents an amphoteric character, its proton donation capacity is quite pronounced.[25] Bentazone is an herbicide with molar mass of 240.28 g mol^{-1}. In the method applied in this work, a positive ion products $[M + H]^+$ with *m/z* of 199.10 and 107.10 were selected for the QIT and CIT, respectively. Previous studies showed that the ionization of bentazone by protonation is less stable than its ionization in the negative mode. Using ESI in the negative mode, the ion products formed present *m/z* 197 and 175.[25,27] The same behavior is expected for triclopyr,[26] quinclorac and picloram, which present a carboxylic group in their structures. Furthermore,

Table 2. Performance parameters of the method

Pesticide	Linear range / (μg mL^{-1})	Precision (RSD)			Linearity	Recovery	
		Instrumental[a]	Repeatability[b]	Inter-assay precisions[c]		%	RSD
Bispyribac-sodium	0.0240-0.1679	19.53	8.77	10.59	y = 4.837x + 0.018 r^2 = 0.9932	101.76	1.46
Cyclosulfamuron	0.0238-0.1666	19.15	11.32	18.50	y = 1.724x – 0.005 r^2 = 0.9919	95.82	6.07
Cycloxydim	0.0208-0.1456	14.86	18.23	18.50	y = 3.057x + 0.038 r^2 = 0.9934	102.68	3.65
Clomazone	0.0040-0.0280	17.64	16.72	19.57	y = 1.305x + 0.003 r^2 = 0.9914	91.76	11.06
Ethoxysulfuron	0.0238-0.1666	13.32	14.86	15.36	y = 1.589x – 0.025 r^2 = 0.9915	99.68	8.79
Fenoxaprop-*p*-ethyl	0.0222-0.1551	12.92	20.24	20.28	y = 0.060x + 0.005 r^2 = 0.9900	101.20	9.29
Imazapic	0.0262-0.1833	17.16	20.19	21.25	y = 0.387x – 0.005 r^2 = 0.9918	98.20	5.12
Imazapyr	0.0246-0.1721	14.04	13.94	17.59	y = 0.227x – 0.002 r^2 = 0.9930	100.80	5.26
Imazethapyr	0.0202-0.1413	15.82	21.33	30.22	y = 1.963x – 0.033 r^2 = 0.9943	101.75	4.95
Metsulfuron-methyl	0.0210-0.1470	12.86	16.61	18.93	y = 6.974x – 0.077 r^2 = 0.9991	99.60	1.95
Oxadiazon	0.0296-0.2071	12.19	15.58	22.45	y = 0.713x + 0.001 r^2 = 0.9960	101.98	5.65
Thiobencarb	0.0025-0.0176	19.46	18.11	19.40	y = 0.787x + 0.001 r^2 = 0.9905	101.52	5.04

[a]Ten consecutive injections of the intermediate concentration level (prepared by dilution of concentrations corresponding to the maximum residue limit values established by ANVISA) of the calibration curves; [b]obtained by 8 preparations of the intermediate level of the calibration curves and injected in the same day; [c]obtained by 8 new preparations of the intermediate level of the calibration curves injected another day and compared with the results obtained in the repeatability assay; RSD: relative standard deviation.

the picloram polarity does not allow partition in organic solvents resulting in poor recovery.[28] On the other hand, the precision of pendimethalin was probably affected by its decomposition.[29]

As it can be seen in Table 2, instrumental precision (n = 10) was better than 20% for all analytes. Repeatability (n = 8) and inter-assay precision (n = 8) were also better than 20% for most compounds. However, imazethapyr presented inter-assay precision around 30%. This result is above the limit established by ANVISA.[30] However, according to the European Commission and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines it is acceptable due to its very low concentration as well as sample complexity.[11,16,31]

The method based on matrix-matched calibration curves presented LOD and LOQ in the range of 0.003-0.048 μg g^{-1} and 0.015-0.165 μg g^{-1}, respectively (Table 3). These results indicate that the analytes can be detected in concentrations 9-109 times lower than the MRL established by ANVISA.[3] Thus, this analytical protocol can be applied for monitoring 12 herbicides commonly used in the rice production.

Recovery and matrix-effect

Recovery assays were performed to evaluate the accuracy and matrix-effect. Five concentration levels for all analytes were studied comprising the entire analytical curves. The results are shown in Table 3 as the average obtained for all concentration levels with RSD lower than 12%. The mean recovery percentage varied from 92 to 103% for all analytes, showing higher accuracy in comparison with the results obtained by using external calibration.[15] Furthermore, 5 of the studied compounds only presented adequate recovery when matrix-matched calibration was employed, demonstrating the advantage of using this procedure when a very complex matrix, such as rice, is analyzed.

Values below 1.0 for matrix-effect indicate suppression, while values above 1.0 indicate enhancement of the signal. Thus, values close to 1.0 demonstrate a low or no interference of the matrix in the determination of the analyte.[32] According to Sampaio *et al.*,[33] results between 0.9 and 1.1 demonstrate low matrix-effect, but values higher than 1.37 and lower than 0.47 show a pronounced signal enrichment or signal suppression, respectively. In this work, both suppression and signal enhancement were observed depending on each compound. All determinations presented matrix-effect being the best results found for clomazone and ethoxysulfamuron, which presented values of 1.07 and 1.13, respectively. These results were previously expected considering the high complexity of the matrix and they demonstrate the need of matrix-matched calibration, in order to achieve reliable results.

According to Kruve *et al.*[12] and Jiménez *et al.*,[34] the matrix-effect by complex samples with low concentration of the analytes is higher and demand the use of matrix-matched calibration. The use of this procedure has been necessary to guarantee the validation of multiresidue methods, due the greater accuracy attained by this way, making some methods able to determine higher amount of analytes.[35]

Figure 1 shows the chromatograms obtained for each compound, which could be determined with adequate precision and accuracy, and also for the sulfamethoxazole.

Table 3. Matrix-effect, determination coefficients (R^2), limit of detection (LOD), limit of quantification (LOQ) and recoveries for the evaluated compounds

Pesticide	Matrix-matched calibration				External calibration	Matrix-effect[d]
	R^2	LOD[a] / (μg g^{-1})	LOQ[a] / (μg g^{-1})	Recovery[b]	Recovery[c]	
Bispyribac-sodium	0.9932	0.048	0.159	101.76	48.2	0.81
Cyclosulfamuron	0.9919	0.045	0.150	95.82	95.0[e]	1.72
Cycloxydim	0.9934	0.039	0.129	102.68	111.8[e]	1.33
Clomazone	0.9914	0.009	0.027	91.76	96.3[e]	1.07
Ethoxysulfuron	0.9915	0.048	0.159	99.68	96.3[e]	1.13
Fenoxaprop-*p*-ethyl	0.9900	0.048	0.162	101.20	46.5	0.04
Imazapic	0.9918	0.048	0.156	98.20	138.6	0.26
Imazapyr	0.9930	0.048	0.165	100.80	210.6	0.12
Imazethapyr	0.9943	0.036	0.120	101.75	250.8	0.47
Metsulfuron-methyl	0.9991	0.012	0.036	99.60	100.3[e]	1.26
Oxadiazon	0.9960	0.003	0.015	101.98	87.2[e]	0.65
Thiobencarb	0.9905	0.006	0.018	101.52	103.0[e]	1.48

[a]LOD and LOQ were determined from the signal to noise ratio, 3:1 and 10:1, respectively. To obtain the results in μg g^{-1}, the mass of sample and also the volume of solvent used in the extraction procedure were considered; [b]recovery values calculated for 5 concentration levels. Results expressed as average; [c]recovery values calculated for 4 concentration levels. Results expressed as average;[15] [d]results expressed as the ratio between the slopes obtained from the matrix-matched calibration and external calibration curves; [e]data obtained from Rebelo *et al.*[15]

Finally, the method was validated for a total of 12 herbicides, all presenting different retention time, being the last compound eluted in 12 minutes.

Application in real samples

The proposed method was applied to eight different samples of rice grains; including brown, parboiled and white rice; all prepared and analyzed in triplicate. All samples presented at least one herbicide in their compositions; however, they are all in concentrations lower than the LOQ of the method and also in concentrations below the MRL established by ANVISA. Among the herbicides detected, cycloxydim, fenoxaprop-*p*-ethyl, metsulfuron-methyl and oxadiazon have been found in all samples tested. These results indicate that the studied herbicides were properly employed in the production of the evaluated rice grains.

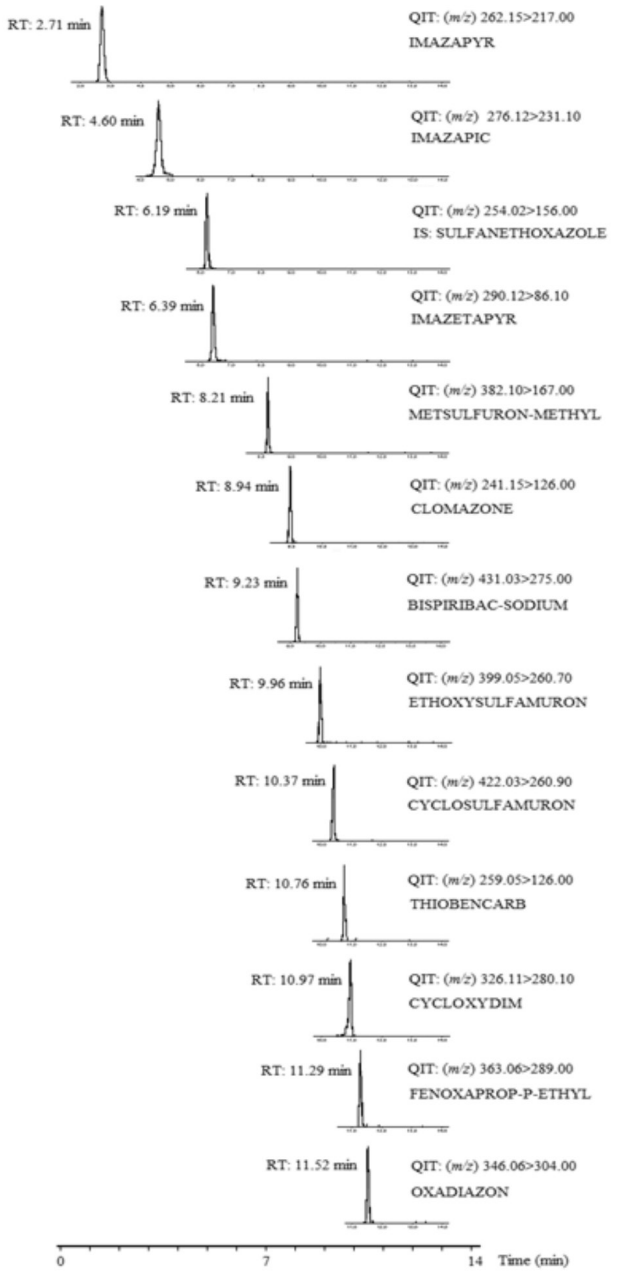

Figure 1. Chromatograms obtained by liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) in positive ionization and single reaction monitoring (SRM) mode for the herbicides, which were validated using the method by matrix-matched calibration. QIT and RT correspond to the quantification ion transition and retention time, respectively.

In 2001, after several warnings from health, agriculture and research institutes, the Program on Pesticide Residue Analysis in Food (PARA) was created in Brazil. However, only in 2008 the MRL of pesticides in rice were established by ANVISA, which allowed its inclusion in the program.[36] By disclosing the results of analyses and also due to the consumer demands, farmers and cooperatives are recently working to avoid pesticides concentrations above those MRL established by ANVISA and other international organizations.

Conclusions

The method using matrix-matched calibration proposed in this work has shown to be necessary for the determination of twelve herbicides commonly deployed in rice farming, which limits were established by the Brazilian regulatory agency, ANVISA. Without any changes in instrumentation or spectrometric conditions, the use of the matrix-matched calibration expands the applicability of a method based upon external calibration, such as the one recently publish by our research group.[15]

An important aspect is that, for the samples tested, none of the studied analytes was found in concentration higher than those established by ANVISA. However, these findings do not mean neither that rice produced in Brazil presents this profile nor that it is totally free of herbicide residues. Firstly, because an appropriate sampling is needed for mapping it in Brazil; secondly, because new and consequently not yet legislated products have been used in rice farming; and lastly because the legislation does not have limits established for degradation products for the regulated compounds.

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