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CROP PROTECTION

Residues of glyphosate and aminomethylphosphonic acid (AMPA) in genetically modified glyphosate tolerant soybean, corn and cotton crops

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ABSTRACT: *Glyphosate is a broad-spectrum herbicide capable of controlling a wide range of weed. It uses could cause the occurrence of residues in conventional or genetically modified crops. For this purpose, grains (soybean and corn) and cotton seeds were sampled from commercial areas in 2012/2013 to 2017/2018 seasons in different Brazilian agricultural regions to monitor glyphosate residues levels and its metabolite (aminomethylphosphonic acid - AMPA) after different managements. The glyphosate residues levels in genetically modified corn (GM) ranged from no detected (ND) to 0.15 mg kg^{-1}, in GM soybean values ranged from ND to 2.81 mg kg^{-1} and in GM cotton ranged from ND to 1.78 mg kg^{-1}. AMPA residues levels indicated a correlation with the glyphosate residues. Glyphosate residues levels in soybean and corn grains and cotton seeds were within the Maximum Residue Limits (MRLs) established by ANVISA and Codex Alimentarius.*
Key words: *monitoring, soybean, corn, cotton, commercial areas.*

Resíduos de glifosato e ácido aminometilfosfônico (AMPA) em culturas de soja, milho e algodão geneticamente modificados tolerantes ao glifosato

RESUMO: *O glifosato é um herbicida de amplo espectro capaz de controlar uma grande diversidade de ervas daninhas e seu uso pode acarretar na ocorrência de resíduos, seja em culturas convencionais ou geneticamente modificadas. Sendo assim, amostragens de grãos de soja e milho e de sementes de algodão foram realizadas em áreas comerciais nas safras de 2012/2013 a 2017/2018 em diferentes regiões agrícolas brasileiras com o objetivo de monitorar os níveis de resíduos de glifosato e seu metabólito (ácido aminometilfosfônico - AMPA) após diferentes manejos. Os níveis de resíduos de glifosato em milho geneticamente modificados (GM) tolerante ao glifosato variaram desde não detectados (ND) a até 0,15 mg kg^{-1}, em soja GM tolerante ao glifosato os valores variaram de ND a 2,81 mg kg^{-1} e em algodão GM tolerante ao glifosato os resultados se estabeleceram entre ND a 1,78 mg kg^{-1}. Os valores de resíduos de AMPA indicaram correlação com os resíduos de glifosato. Os níveis de resíduos de glifosato em grãos de soja e milho e sementes de algodão ficaram dentro dos Limites Máximos de Resíduos (LMRs) preconizados pela ANVISA e Codex Alimentarius.*
Palavras-chave: *monitoramento, soja, milho, algodão, áreas comerciais.*

INTRODUCTION

The commercialization of agricultural products derived from biotechnology, or Genetically Modified (GM) crops, started in 1996, and in 2017 the cumulative area planted in the World was of approximately 2.3 billion hectares, including the main agricultural crops (soybean, corn, cotton and canola). In the period between 1996 and 2016, GM crops were adopted by more than 18 million farmers, 90% of them small farmers in emerging countries. In 2017, 24 countries planted GM crops, of which 19 are emerging countries. In the same year, the area cultivated with GM crops in the World grew 3% and totaled approximately 190 million hectares, and the main countries have adopted this technology are the United States, Brazil and Argentina, which had more than 75% of their agriculture area planted with GM crops (ISAAA, 2017).

In Brazil, the total of 50.2 million hectares planted with GM crops in 2017, approximately 34 million hectares were GM soybean, being 40% of them were for herbicide tolerant cultivars (HT) and 60% to insect resistant and herbicide tolerant cultivars (IR/HT). GM corn was cultivated at approximately 15.6

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million hectares with most of the area (75%) being cultivated with IR/HT hybrids. GM cotton;conversely, was cultivated in 0.94 million hectares distributed among cultivars IR, HT and IR/HT, with 11%, 30% and 59% of area, respectively. Based on these data, Brazil has cultivated approximately 400 million hectares of GM crops cumulatively since the approval of the first GM crop in the country (ISAAA, 2017).

Glyphosate is a broad-spectrum herbicide capable of controlling a great diversity of weed, including dicotyledons, monocotyledons and perennials. Its lack of residual activity, low cost and safe environmental and toxicological profile (EPA, 1993; EPA, 2013b; EPA, 2017; EFSA, 2017; PMRA, 2017) make this herbicide the most used in the world (DUKE & POWLES, 2008; JAN et al. 2009; DILL et al. 2010; POLLAK, 2011; SZEḰÁCS & DARVAS, 2012; BENBROOK, 2016).

Glyphosate could leave residues after its use for weed control, whether in conventional crops or GM crops, and for this reason, international and national regulatory agencies have registered agrochemicals products and its use to ensure their safety to environment and human and animal health. The CODEX ALIMENTARIUS (2018), for example, established MRLs (Maximum Residue Limits) for soybean at 20.0 mg kg^{-1}, corn at 5.0 mg kg^{-1} and cotton at 40.0 mg kg^{-1}. In Brazil, the National Agency of Sanitary Surveillance – ANVISA established the following MRLs: 10 mg kg^{-1} in soybean grains; 1.0 mg kg^{-1} in corn grains; and 3.0 mg kg^{-1} in cotton seeds (ANVISA MRLs pattern were used as the main threshold in this study). The quantification of agrochemicals residues levels is relevant in GM soybean, corn and cotton crops mainly to economic importance that these biotechnology products have in Brazil. Together with glyphosate residues data assessment, residues evaluations of its metabolite AMPA (aminomethylphosphonic acid) are commonly performed (EPA, 2013b), being these levels assessment equally important.

The aim of this study was to measure the glyphosate and AMPA metabolite residues in GM glyphosate tolerant soybean and corn grains and cotton seeds sampled in commercial areas in Brazil. The monitoring of GM glyphosate tolerant cotton, corn and soybean were conducted between 2012/2013 and 2017/2018 seasons.

MATERIALS AND METHODS

Experimental design

The samples (grains and seeds) were collected in commercial producing areas of soybean, corn and cotton located in different Brazilian agricultural regions. The experimental design is described in figure 1 and table 1.

Two approaches were considered: the first to monitor events expressing Bt proteins (MON 531, MON 15985, MON 89034, MON 88017 and MON 87701); and the second to monitor glyphosate tolerant events (MON 1445, MON 88913, MON 89788, MON 88017). But, the term “control treatment” does not imply the non-adoption of the herbicide glyphosate to weed control. In general, it means having a treatment without insect resistance (IR) technology and; therefore, controls 1, 2, 3, 4 and 5 are here designated as non-Bt cultivars or hybrids.

The studies with GM soybean and corn were composed of two treatments distributed in a randomized complete block design (CBD) with three replicates, evaluated during four to five consecutive seasons. The management for weed control in MON 87701 × MON 89788 soybean used from a single application of the herbicide in the pre-planting (PP) phase up to four applications of this herbicide, including PP and post-emergency (PE) phase and with great variability of applied doses (Table 2).

In the corn study, treatments MON 89034 × NK603, control 1, MON 89034 × 88017 and control 2, the glyphosate application was carried out in PP and PE of the crop (Tables 3 and 4).

The GM cotton technologies studies were conducted in single block trials per treatment. Sampling was done in two areas for MON 15985 × MON 88913 (Table 5) and three areas separately for MON 531 × MON 1445 in the five monitored seasons (Table 6). Glyphosate application was carried out in PP and PE of cotton crop (Tables 5 and 6).

The studies were conducted in areas with more than 12 hectares, with individual plots of 2.0 to 7.0 hectares, depending on the study site and the crop. Both the production area of the farm and the experimental area of the study received the same agricultural practices and inputs. In each plot, the monitored technologies and control treatments, three collection points were defined. From each point, a radius of 20.0 meters was established for random plant sampling to be performed. In this way, matured soybean plants and corn ears were collected to compose a sample of 2.0 kg of grains/plot. For cotton crop, seeds (fiber + seed) were collected to compose the same sample of 2.0 kg of seeds/plot. The three subsamples collected for each treatment were homogenized, allowing the subsequent withdrawal of two composite aliquots of 1.0 kg for cotton seeds and 0.5 kg for corn and soybean grains. Each sample received in the

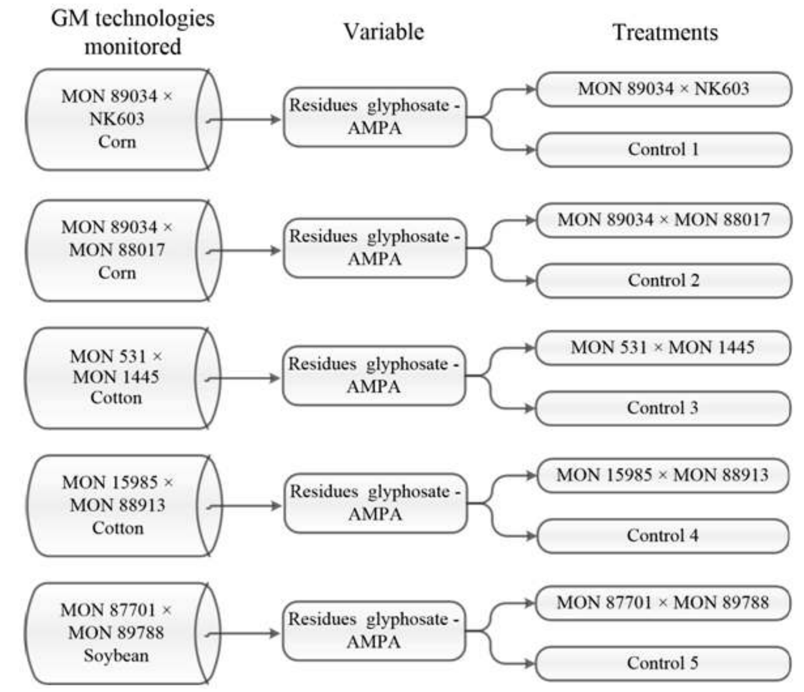

Figure 1 - Schematic presentation of data sets of the following GM products: MON 89034 × NK603 corn (VT PRO2®), MON 89034 × MON 88017 corn (VT PRO3®), MON 531 × MON 1445 cotton (Bollgard RR®), MON 15985 × MON 88913 cotton (Bollgard II RR FLEX®) and MON 87701 × MON 89788 soybean (Intacta RR2 Pro®).

laboratory was individually packaged in sealed plastic bags and identified with coded labels. Subsequently, the samples were stored and conditioned at - 20 °C ± 5 °C until the laboratory analysis.

Glyphosate and AMPA residue analysis

Glyphosate and AMPA reference standards were purchased from Sigma-Aldrich (USA), with purity 99.8 and 98.7%, respectively. Individual stock

Table 1 - Information about the treatments, crops and study sites.

----------Treatments----------		Crop	Season	Study sites[a]
GM Technology	Control			
MON 89034 × NK603	Conventional Hybrids/glyphosate tolerant (Control 1)[b]	Corn	2012/2013 to 2016/2017	ROL and MVD
MON 89034 × MON 88017	Hybrids glyphosate tolerant (Control 2)	Corn	2014/2015 to 2017/2018	ROL and MCT
MON 531 × MON 1445	Conventional Cultivars/glyphosate tolerant (Control 3)[b]	Cotton	2012/2013 to 2016/2017	LEM, CAV and PVL
MON 15985 × MON 88913	Cultivars glyphosate tolerant (Control 4)	Cotton	2013/2014 to 2016/2017	LEM, CAV and PVL
MON 87701 × MON 89788	Cultivars glyphosate tolerant (Control 5)	Soybean	2013/2014 to 2017/2018	MCT and PVL

[a] MCT: Mato Castelhano, RS; SLG: São Luiz Gonzaga, RS; ROL: Rolândia, PR; PGR: Ponta Grossa, PR; LON: Londrina, PR; MVD: Montividiu, GO; SHG: Santa Helena de Goiás, GO; LEM: Luís Eduardo Magalhães, BA; BAR: Barreiras, BA; RON: Rondonópolis, MT; SOR: Sorriso, MT; PVL: Primavera do Leste, MT; CAV: Campo Verde, MT; SGO: São Gabriel do Oeste, MS.
[b] control 1: conventional hybrids were used in the 2012/2013 and 2013/2014 seasons, RR hybrids were used as control 1 in the other seasons; control 3: conventional cultivar was used in the 2012/2013 season, RR cultivars were used as control 3 in the other seasons.

Table 2 - MON 87701 × MON 89788 soybean: study sites, seasons, treatments, application number, time of application and doses.

Study sites/Seasons[a]	Treatments	Applications number[b]	Times[c]	Doses (g a.e ha^{-1})[d]
MCT 2013/2014	MON 87701 × MON 89788 soybean	3	PP; PE	1016; 1080 e 360
	Control 5	3	PP; PE	1016; 1080 e 360
PVL 2013/2014	MON 87701 × MON 89788 soybean	3	PP; PE	1650; 1364 e 909
	Control 5	3	PP; PE	1650; 1364 e 909
MCT 2014/2015	MON 87701 × MON 89788 soybean	1	PE	1189
	Control 5	1	PE	1189
PVL 2014/2015	MON 87701 × MON 89788 soybean	2	PP; PE	975; 911
	Control 5	2	PP; PE	975; 911
MCT 2015/2016	MON 87701 × MON 89788 soybean	2	PP; PE	654; 654
	Control 5	2	PP; PE	654; 654
PVL 2015/2016	MON 87701 × MON 89788 soybean	4	PP; PE	1060; 1300, 845 e 845
	Control 5	4	PP; PE	1060; 1300, 845 e 845
MCT 2016/2017	MON 87701 × MON 89788 soybean	2	PP; PE	1000; 1080
	Control 5	2	PP; PE	1000; 1080
PVL 2016/2017	MON 87701 × MON 89788 soybean	3	PP; PE	1170; 650 e 650
	Control 5	3	PP; PE	1170; 650 e 650
MCT 2017/2018	MON 87701 × MON 89788 soybean	2	PP; PE	576; 1030
	Control 5	2	PP; PE	576; 1030

[a] MCT: Mato Castelhano, RS; PVL: Primavera do Leste, MT.
[b,c,d] PP: pre-planting; PE: post-emergence; the management used with glyphosate was decided by each farmer responsible for the area in which MON 87701 × MON 89788 soybean study was installed. (g a.e ha^{-1}): *grams* of *equivalent acid* per hectare.

Table 3 - MON 89034 × NK603 corn: study sites, seasons, treatments, application number, time of application and doses.

Study sites/Seasons[a]	Treatments	Applications number[b]	Times[c]	Doses (g a.e ha^{-1})[d]
ROL 2012/2013	MON 89034 × NK603 corn	2	PP; PE	722; 981
	Control 1*	1	PP	722
MVD 2012/2013	MON 89034 × NK603 corn	2	PP; PE	711; 872
	Control 1	1	PP	711
ROL 2013/2014	MON 89034 × NK603 corn	2	PP; PE	1081; 893
	Control 1	1	PP	1081
MVD 2013/2014	MON 89034 × NK603 corn	1	PE	667
	Control 1	-	-	-
ROL 2014/2015	MON 89034 × NK603 corn	2	PP; PE	1440; 1440
	Control 1	1	PP	1440
MVD 2014/2015	MON 89034 × NK603 corn	1	PE	1636
	Control 1	1	PE	1636
ROL 2015/2016	MON 89034 × NK603 corn	2	PP; PE	785; 1011
	Control 1	1	PP	785
MVD 2015/2016	MON 89034 × NK603 corn	2	PP; PE	1200; 1470
	Control 1	1	PP	1200
ROL 2016/2017	MON 89034 × NK603 corn	1	PE	2774
	Control 1	1	PE	2774
MVD 2016/2017	MON 89034 × NK603 corn	1	PE	960
	Control 1	1	PE	960

[a] ROL: Rolândia, PR; MVD: Montividiu, GO.
[b,c,d] PP: pre-planting; PE: post-emergence; the management used with glyphosate was decided by each farmer responsible for the area in which MON 89034 × NK603 corn study was installed. (g a.e ha^{-1}): grams of equivalent acid per hectare.
[*]control 1: conventional hybrids were used in the 2012/2013 and 2013/2014 seasons, RR hybrids were used as control 1 in the other seasons.

Table 4 - MON 89034 × MON 88017 corn: study sites, seasons, treatments, application number, time of application and doses.

Study sites/Seasons[a]	Treatments	Applications number[b]	Times[c]	Doses(g a.e ha^{-1})[d]
MCT 2014/2015	MON 89034 × MON 88017 corn	2	PP; PE	1025; 793
	Control 2	2	PP; PE	1025; 793
ROL 2014/2015	MON 89034 × MON 88017 corn	2	PP; PE	1440; 1440
	Control 2	1	PP	1440
MCT 2015/2016	MON 89034 × MON 88017 corn	2	PP; PE	720; 654
	Control 2	2	PP; PE	720; 654
ROL 2015/2016	MON 89034 × MON 88017 corn	2	PP; PE	785; 654
	Control 2	2	PP; PE	785; 654
ROL 2016/2017	MON 89034 × MON 88017 corn	1	PE	2774
	Control 2	1	PE	2774
MCT 2017/2018	MON 89034 × MON 88017 corn	2	PP; PE	781; 936
	Control 2	2	PP; PE	781; 936
ROL 2017/2018	MON 89034 × MON 88017 corn	2	PP; PE	518; 2160
	Control 2	2	PP; PE	518; 2160

[a] MCT: Mato Castelhano, RS; ROL: Rolândia, PR.
[b,c,d] PP: PP: pre-planting; PE: post-emergence; the management used with glyphosate was decided by each farmer responsible for the area in which MON 89034 × MON 88017 corn study was installed. (g a.e ha^{-1}): grams of equivalent acid per hectare.

solutions were prepared by weighing 20 mg of each standard in 20 mL volumetric flasks. The volume flask was complete with water and the solutions were stored at -20 ± 5 °C. Standard working solutions were prepared by diluting mobile stock solutions in the concentration range of 0.05 to 75 µg mL^{-1}.

Methanol and HPLC grade chloroform and hexane grade PA were purchased from JT Baker

Table 5 - MON 531 × MON 1445 cotton: study sites, seasons, treatments, application number, time of application and doses.

Study sites/Seasons[a]	Treatments	Applicatios number[b]	Times[c]	Doses (g a.e ha^{-1})[d]
LEM 2012/2013	MON 531 × MON 1445 cotton	1[**]	PE	1817
	Control 3[*]	-	-	-
PVL 2013/2014	MON 531 × MON 1445 cotton	3	PP; PE	1440; 1111 e 1111
	Control 3	3	PP; PE	1440; 1111 e 1111
PVL 2014/2015	MON 531 × MON 1445 cotton	3	PP; PE	1430; 975 e 1441
	Control 3	2	PP; PE	1430; 975
LEM 2015/2016	MON 531 × MON 1445 cotton	2	PP; PE	1500; 1250
	Control 3	2	PP; PE	1500; 1250
CAV 2015/2016	MON 531 × MON 1445 cotton	2	PP; PE	900; 900
	Control 3	2	PP; PE	900; 900
LEM 2016/2017	MON 531 × MON 1445 cotton	3	PP; PE	1500; 1000 e 1000
	Control 3	3	PP; PE	1500; 1000 e 1000
CAV 2016/2017	MON 531 × MON 1445 cotton	2	PP; PE	1296; 1440
	Control 3	2	PP; PE	1296; 1440

[a] LEM: Luís Eduardo Magalhães, BA; PVL: Primavera do Leste, MT; CAV: Campo Verde, MT.
[b,c,d] PP: pre-planting; PE: post-emergence; the management used with glyphosate was decided by each farmer responsible for the area in which MON 531 × MON 1445 cotton study was installed. (g a.e ha^{-1}): *grams* of *equivalent acid* per hectare.
[*]control 3: conventional cultivar was used in the 2012/2013 season; RR cultivars were used as control 3 in the other seasons. [**]Partial information obtained from the farmer.

Table 6 - MON 15985 × MON 88913 cotton: study sites, seasons, treatments, application number, time of application and doses.

Study sites/Seasons[a]	Treatments	Applications number[b]	Times[c]	Doses (g a.e ha^{-1})[d]
LEM 2013/2014	MON 15985 × MON 88913 cotton	1	PP	797
	Control 4	2	PP; PE	797; 960
LEM 2015/2016	MON 15985 × MON 88913 cotton	2	PP; PE	1500; 1250
	Control 4	2	PP; PE	1500; 1250
CAV 2015/2016	MON 15985 × MON 88913 cotton	2	PP; PE	900; 900
	Control 4	2	PP; PE	900; 900
CAV 2016/2017	MON 15985 × MON 88913 cotton	2	PP; PE	1296; 1440
	Control 4	2	PP; PE	1296; 1440

[a] LEM: Luís Eduardo Magalhães, BA; PVL: Primavera do Leste, MT; CAV: Campo Verde, MT.
[b,c,d] PP: pre-planting; PE: post-emergence; the management used with glyphosate was decided by each farmer responsible for the area in which MON 15985 × MON 88913 cotton study was installed. (g a.e ha^{-1}): grams of equivalent acid per hectare.

(USA). Water was obtained from a Milli-Q water system (Millipore, Bradford, MA, USA) with a resistivity of 18.2 MΩ cm-1. O-Phthaldialdehyde (OPA) was purchased from Sigma (USA); monopotassium phosphate, calcium hypochlorite, 2-mercaptoethanol, sodium chloride, sodium hydroxide and hydrochloric acid were purchased from Merck (Germany). Chloride resins AG1 X8 mesh 200-400 and in the sodium form Chelex-100 100-200 mesh were purchased from Bio-Rad (USA).

Samples were processed, homogenized and conditioned in double plastic bags, and then kept in freezer -20 ± 5 °C until the moment of the analysis. Sample extraction was based on COWELL et al. (1986). Aliquots of 25.0 g of soybean and corn grains and cotton seeds were transferred to 250 mL centrifuge tubes. The soybean grains were prepared with 50 mL of hexane for 30 seconds and centrifuged for 5 min at 16000 g (18 to 23 °C). This step was carried out to remove the oils and fat in soybean grains. Then, aliquots of 25.0 g of soybean grains, cotton seeds and corn were extracted with 150 mL of 0.1 mol L^{-1} HCl and 50 mL of chloroform for 90 seconds and centrifuged for 20 min at 16000 g in refrigerated centrifuge (18 to 23 °C). The supernatant was transferred to another 250 mL centrifuge tube, the pH adjusted between pH 3 and 4 and then centrifuged again under the same conditions.

Twenty grams of Chelex-100 resin impregnated with Fe^{3+} solution (COWELL et al. 1986) were placed in the glass column. The collected extracts containing glyphosate and AMPA (120 mL for corn and 80 mL for cotton and soybean) were percolated through the column and washed with 100 mL of 0.1 mol L^{-1} HCl solution. Then, glyphosate and AMPA were eluted with 5 aliquots of 5 mL of 6 mol L^{-1} HCl. At this point, 10 mL of concentrated HCl was added to the eluate and the extract was homogenized and applied to another glass column with 15 grams of AG1-X8 resin. After eluting the cleaned extract free of impurities or interferences, it was vacuum dried and re suspended in a suitable volume of mobile phase, filtered through a Millex HV filter (0.45 µm, Millipore) and analyzed by HPLC-FD.

Analyzes were performed on a Shimadzu LC 20A HPLC system with RF10 AxL fluorescence detector (Tokyo, Japan). The chromatographic separation was performed using two ion exchange columns in the potassium form A-9 (300 x 4.6 mm, and 150 x 4.6 mm, 5µ) obtained from Bio Rad (USA). The temperature of the column oven was maintained at 50 °C. An isocratic flow of 0.6 mL min^{-1} was employed with the composition of the mobile phase in 0.005 mol L^{-1} of monopotassium phosphate (KH_2PO_4) with 4% in methanol and pH adjusted to 1.9. The injection volume was 50 µL. The post-column reaction was performed using a solution of OPA with 2-mercaptoethanol (MERC) and calcium hypochlorite, prepared according COWELL et al. (1986). Fluorescent derivatives were formed at 38 °C in post-column oven and detected at excitation wavelength of 330 nm and emission of 465 nm.

For the soybean and cotton analyzes the Shimadzu FCV-20AH2 valve (Tokyo, Japan) was also used in conjunction with an LC 20 AD pump to perform the cleaning of the analytical column.

The previously described method was validated for corn, soybean and cotton crops (SANTE, 2018). During the analysis, several internal quality criteria were applied, such as: the use of a control sample (without herbicide application) in each batch of samples analyzed; fortified samples.

The linearity was determined from calibration standard solutions obtained from triplicate analysis of glyphosate and AMPA diluted mobile HPLC phase. Results were analyzed by the least squares method and linearity was expressed by the coefficient of determination (R^2). The outliers were evaluated by the Hubber test and the homoskedasticity by the Cochran test. The analysis of the residues with normal distribution of the points of the analytical curve was performed obeying the criteria of deviation of the calculated concentration back from the actual concentration (± 20%) according the SANTE / 11813/2017 guide (SANTE, 2018). The recovery data were calculated by comparing the amount added and the concentration obtained after the analysis. At the end, the average of each treatment was calculated for glyphosate residue, requiring the transformation of categorical variables into numerical ones. Therefore, the results <LOQ (Limit of Quantification) and ND (Not Detected) were transformed into values equal to 0.05 and 0 (WHO, 2002), respectively. Correlations (r) between glyphosate residues, number of applications and period between the last application of the herbicide and the harvest were calculated to explain possible variations in the residual values. All the figures were generated by TIBCO Spotfire® and Microsoft Visio® Softwares.

RESULTS

Analyzing the different experimental sites, with different numbers of applications and doses, most of the samples showed no herbicide residues. Results regarding corn grains showed residual concentrations of glyphosate ranging from ND up to 0.15 mg kg^{-1} (Table 7). The average levels of glyphosate residues were 0.05 mg kg^{-1}, 0.02 mg kg^{-1}, 0.07 mg kg^{-1} and 0.07 mg kg^{-1}, corresponding to treatments MON 89034 × NK603, control 1, MON 89034 × 88017 and control 2, respectively (Figure 2). The AMPA metabolite levels varied between ND at 0.49 mg kg^{-1} (Table 7).

The results of glyphosate residues in cotton seeds for treatments MON 531 × MON 1445, control 3, MON 15983 × MON 88913 and control 4 ranged from ND to 1.78 mg kg^{-1} (Table 8). The averages were 0.13 mg kg^{-1} for the treatment MON 531 × MON 1445, 0.15 mg kg^{-1} for control 3, 0.08 mg kg^{-1} for MON 15983 × MON 88913 and 0.06 mg kg^{-1} for control 4 (Figure 2). AMPA metabolite values varied between ND at 0.06 mg kg^{-1} (Table 8).

The results of glyphosate residues in soybean treatments (MON 87701 × MON 89788 and control 5) showed a variation from ND to 2.81 mg kg^{-1} (Table 9). The averages of residues in soybean in treatments MON 87701 × MON 89788 and control 5 were 0.87 mg kg^{-1} and 0.79 mg kg^{-1}, respectively (Figure 2). AMPA metabolite values varied between ND at 3.38 mg kg^{-1} (Table 9).

The averages of glyphosate residues, in all the crops studied, were clustered according the number of application and by the period between the last application and harvest, and they evidenced that residues can vary depends on crop and also due to the variables previous cited. The averages estimated were between 0.04 and 0.59 mg kg^{-1}, when analyzed the number of applications (Figure 3a), and 0.05 and 2.07 mg kg^{-1} for the period between last application and harvest (Figure 3b).

In general, considering the averages of all treatments for glyphosate residues and its metabolite AMPA, higher values were evidenced for MON 87701 × MON 89788 and control 5 (soybean crop), but lower than the MLR standardized for this crop (10 mg kg^{-1}). In the remaining treatments averages lower than 0.15 mg kg^{-1} for Glyphosate and 0.06 mg kg^{-1} for AMPA (Figure 2) were observed.

DISCUSSION

The MRL established by ANVISA in Brazil to glyphosate in corn is 1 mg kg^{-1} (ANVISA, 2015). Considering the results presented for this crop, glyphosate levels residues in the MON 89034 × NK603 treatment were 20 times below the MRL, as were control 1 (50 times), MON 89034 × 88017 (14 times) and control 2 (14 times). For cotton, which the glyphosate MRL established by ANVISA is 3 mg kg^{-1}, the treatments MON 531 × MON 1445, control 3, MON 15983 × MON 88913 and control 4 present results were 23, 20, 38 and 50 times below the MRL, respectively. For soybeans, ANVISA has established the MRL of 10 mg kg^{-1}, then the averages were 11.5 times below the MRL for MON 87701 × MON 89788 and 13 times below of this value for control 5. In a study conducted by ABAKERLI et. al (2014), eight areas from commercial soybean areas with SCV/HCV + SRR/HCV (Roundup Ready and conventional soybean with application of conventional herbicides) treatment and SRR/HRR (Roundup Ready soybean with application of Roundup Ready herbicide) treatment were monitored during five seasons. The

Table 7 - Glyphosate and AMPA residues levels in corn grains from treatments MON 89034 × NK603, control 1, MON 89034 × MON 88017 and control 2.

Treatments	Study sites/Seasons[a]	----------------------Residues (mg kg^{-1})------------------	
		Glyphosate	AMPA
MON 89034 × NK603	ROL 2012/2013	ND	ND
Control 1*		ND	0.16
MON 89034 × NK603	MVD 2012/2013	ND	ND
Control 1		ND	<LOQ
MON 89034 × NK603	ROL 2013/2014	0.11	0.49
Control 1		ND	0.05
MON 89034 × NK603	MVD 2013/2014	0.11	ND
Control 1		ND	ND
MON 89034 × NK603	ROL 2014/2015	<LOQ	<LOQ
Control 1		<LOQ	<LOQ
MON 89034 × NK603	MVD 2014/2015	ND	ND
Control 1		ND	ND
MON 89034 × NK603	ROL 2015/2016	<LOQ	ND
Control 1		<LOQ	ND
MON 89034 × NK603	MVD 2015/2016	0.06	<LOQ
Control 1		ND	ND
MON 89034 × NK603	ROL 2016/2017	ND	ND
Control 1		<LOQ	0.05
MON 89034 × NK603	MVD 2016/2017	0.07	<LOQ
Control 1		ND	ND
MON 89034 × MON 88017	MCT 2014/2015	0.09	<LOQ
Control 2		0.11	<LOQ
MON 89034 × MON 88017	ROL 2014/2015	<LOQ	ND
Control 2		<LOQ	ND
MON 89034 × MON 88017	MCT 2015/2016	ND	ND
Control 2		ND	ND
MON 89034 × MON 88017	ROL 2015/2016	<LOQ	<LOQ
Control		0.05	<LOQ
MON 89034 × MON 88017	ROL 2016/2017	<LOQ	ND
Control 2		<LOQ	ND
MON 89034 × MON 88017	MCT 2017/2018	0.06	0.01
Control 2		0.09	0.02
MON 89034 × MON 88017	ROL 2017/2018	0.15	<LOQ
Control 2		0.14	<LOQ

[a] MCT: Mato Castelhano, RS; ROL: Rolândia, PR; MVD: Montividiu, GO.
LOQ: Limit of Quantification (0.05 mg kg^{-1}); ND: Not Detected.
*control 1: conventional hybrids were used in the 2012/2013 and 2013/2014 seasons; RR hybrids were used as control 1 in the other seasons.

results were below the ANVISA´s soybean MRL in 200 and 33 times to SCV/HCV + SRR/HCV and SRR/HRR treatment, respectively.

DUKE et al. (2003) also showed residues below the MRL in soybeans, within the range of 0.08 to 3.08 mg kg^{-1}. Similarly, BØHN et al. (2014) reported glyphosate residues levels between 0.4 and 8.8 mg kg^{-1} in GM soybean plants, considering in this case the use of soybean for silage (SEFER et al. 2004; GOBETTI et al. 2011; RIGUEIRA et al. 2015). The studies conducted by DUKE et al. (2003) and BØHN et al. (2014) followed the MRL established by the Codex Alimentarius (20 mg kg^{-1}). Considering the MRLs recommended by the Codex Alimentarius for soybean, corn and cotton crops, the glyphosate residues levels detected in the present study are at

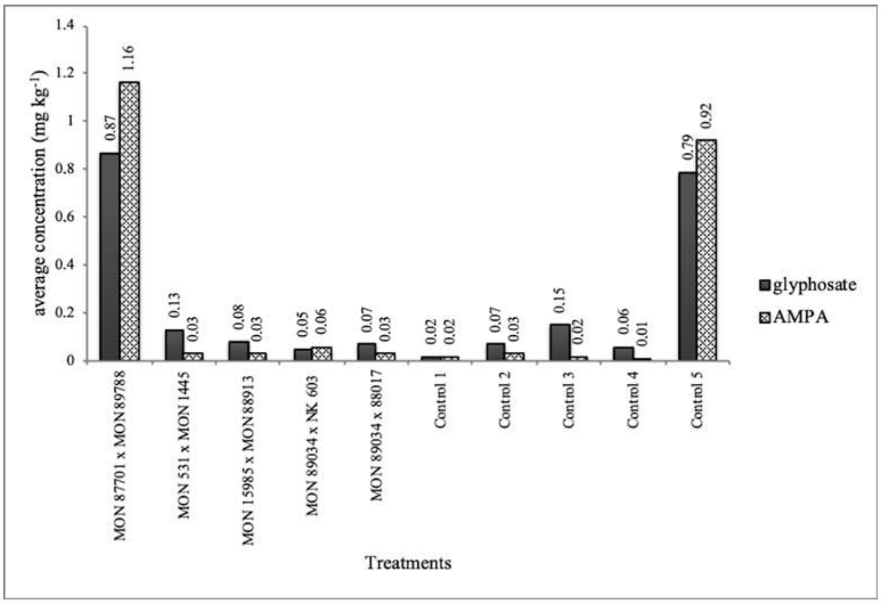

Figure 2 - Averages of glyphosate and AMPA (mg kg $^{-1}$) residues in treatments MON 89034 × NK603, MON 89034 × MON 88017, MON 531 × MON 1445, MON 15985 × MON 88913, MON 87701 × MON 89788, control 1, control 2, control 3, control 4 and control 5.

Table 8 - Glyphosate and AMPA residues in cotton seeds from treatments MON 531 × MON 1445, control 3, MON 15985 × MON 88913 and control 4.

Treatments	Study sites/Seasons[a]	---------------------Residues (mg kg^{-1})-------------------	
		Glyphosate	AMPA
MON 531 × MON 1445	LEM 2012/2013	1.78	0.06
Control 3[*]		<LOQ	ND
MON 531 × MON 1445	PVL 2013/2014	0.08	ND
Control 3		ND	ND
MON 531 × MON 1445	PVL 2014/2015	<LOQ	<LOQ
Control 3		<LOQ	<LOQ
MON 531 × MON 1445	LEM 2015/2016	<LOQ	<LOQ
Control 3		0.1	ND
MON 531 × MON 1445	CAV 2015/2016	0.46	<LOQ
Control 3		0.05	<LOQ
MON 531 × MON 1445	LEM 2016/2017	<LOQ	ND
Control 3		0.62	ND
MON 531 × MON 1445	CAV 2016/2017	0.11	ND
Control 3		0.07	ND
MON 15985 × MON 88913	LEM 2013/2014	ND	ND
Control 4		ND	ND
MON 15985 × MON 88913	LEM 2015/2016	0.07	<LOQ
Control 4		0.1	ND
MON 15985 × MON 88913	CAV 2015/2016	0.13	<LOQ
Control 4		0.05	<LOQ
MON 15985 × MON 88913	CAV 2016/2017	<LOQ	ND
Control 4		0.07	ND

[a]LEM: Luís Eduardo Magalhães, BA; CAV: Campo Verde, MT; PVL: Primavera do Leste, MT.
LOQ: Limit of Quantification (0,05 mg kg^{-1}); ND: Not Detected.
[*]control 3: conventional cultivar was used in the 2012/2013 season; RR cultivars were used as control 3 in the other seasons.

Table 9 - Glyphosate and AMPA residues in soybean grains from treatments MON 87701 × MON 89788 and control 5.

Treatments	Study sites/Seasons[a]	---------------------Residues (mg kg^{-1})------------------	
		Glyphosate	AMPA
MON 87701 × MON 89788	MCT 2013/2014	0.25	0.5
Control 5		0.23	0.53
MON 87701 × MON 89788	PVL 2013/2014	0.72	1.02
Control 5		0.36	0.61
MON 87701 × MON 89788	MCT 2014/2015	0.33	0.45
Control 5		0.35	0.5
MON 87701 × MON 89788	PVL 2014/2015	0.75	0.76
Control 5		1.02	0.59
MON 87701 × MON 89788	MCT 2015/2016	0.17	0.59
Control 5		0.20	0.44
MON 87701 × MON 89788	PVL 2015/2016	2.81	3.38
Control 5		1.42	2.21
MON 87701 × MON 89788	MCT 2016/2017	0.42	0.29
Control 5		0.46	0.55
MON 87701 × MON 89788	PVL 2016/2017	1.88	2.69
Control 5		2.26	1.69
MON 87701 × MON 89788	MCT 2017/2018	0.50	0.80
Control 5		0.82	1.12

a MCT: Mato Castelhano, RS; PVL: Primavera do Leste, MT.

least 23 times below the recommended MRL for soybean (20 mg kg^{-1}), 71 times below the MRL for corn (5 mg kg^{-1}) and 267 times below the established MRL for cotton (40 mg kg^{-1}).

Currently, in glyphosate tolerant GM crops, weed control with this herbicide can also be performed after the emergence of the main crop, varying the number of applications from one to three times (BEENBROK, 2016). In addition, in soybean it is considered that sequential application of glyphosate (two applications) should be used in the PE of RR (Roundup Ready) soybean in areas with high infestation and/or uneven germination of weed. Thus, it was verified that in a single site (PVL - soybean 2015/2016) this criterion was not followed, and three PE applications were performed in treatments MON 87701 × MON 89788 and control 5 by decision of the producer due to particularities of the area, techniques and high density of weed. Despite all the management carried out for weed control, the variation in the number of herbicide applications during the development of the soybean crop had a low correlation (r=0.39) with the herbicide residues in grains. The same results were observed for corn (r=0.28) and cotton (r=0.18). Because it is a positive correlation, the increase in applications number should result in an increase in the residue variable of glyphosate; however, this increase was very low or almost null, even decreasing in some cases (Figure 3a).

Considering the relationship between AMPA and glyphosate average concentrations, three analysis can be exploited: *i*) the correlation between the averages was high (r=0.94) (Figure 2), it means and reinforce that AMPA came from glyphosate metabolization; *ii*) the ratio were 1.35 in soybean, 0.90 in maize and 0.11 in cotton. The ratio greater than 1 for soybean can be explained due to the higher average of AMPA instead of glyphosate (Figure 2), which in turn is related with slow metabolization of AMPA, compared with glyphosate metabolization (ABAKERLI et. al., 2014). *iii*) an additional result inferred by this study goes through the correlations between the glyphosate residues and interval of application until the harvest. Analyzing the results for soybean (r = -0.48), maize (r = -0.25) and cotton (r = -0.21), they showed that the greater amount of interval between applications and harvests, the values of soybean and corn glyphosate residues and cotton seeds are lower (Figure 3b), which indicated the existence of an inversely proportional relation, validating the metabolism of both substances over time.

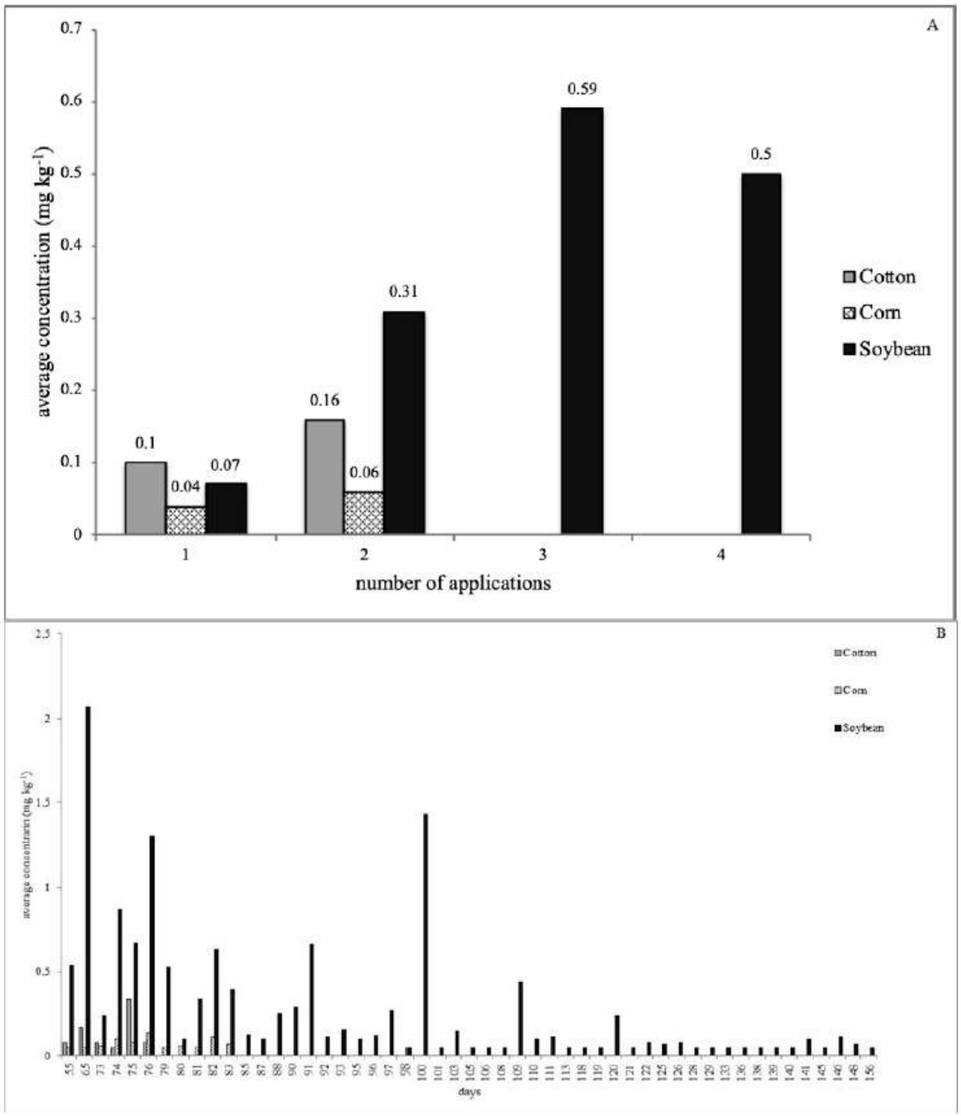

Figure 3 - Average of glyphosate residues (mg kg^{-1}) in soybean and corn grains and cotton seeds. (A) By the number of applications of the herbicide in pre-planting (PP) and post-emergence (PE) of the crop and (B) by the period between last application and harvest (days).

CONCLUSION

Considering the variation of factors such as the different experimental sites, number of applications and the interval between the last application of the herbicide and the harvest studied in corn, cotton and soybeans crops, all results were below the Maximum Residue Limits (MRLs) established by ANVISA for glyphosate to each one of these crops in Brazil. In this study the observation of AMPA residue values indicated a correlation with glyphosate residues.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

GUB, LRGF and ACC conceived and designed experiments. GBU, LRGF, DPVB, ACC and PPPM supervised the experiments, NRR and APFS carried out the lab analyses. PPPM performed statistical analyses of experimental data. PPPM, DPVB, NRR and APFS prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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