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Residues of glyphosate and aminomethyl phosphonic (ampa) in genetically modified soybean[1]

Resíduos de glyphosate e aminometilfosfônico (ampa) em soja geneticamente modificada

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Abstract - Successive and arbitrary application of glyphosate may cause cases of intoxication in humans by indirect ingestion, since transgenic soybeans present levels above the permitted for this herbicide. The objective of this study was to evaluate the glyphosate and aminomethyl phosphonic acid (AMPA) levels in genetically modified soybeans tolerant to glyphosate. The chosen experimental design was composed by random blocks of eight treatments and four replications. Treatments consisted in glyphosate, applied only once and singly in doses of 720 and 960 g a.i. ha^{-1}, glyphosate at 720 and 960 g a.i. ha^{-1} in mixtures, respectively, with com chlorimuron-ethil at 10 g a.i. ha^{-1}, sequential applications of glyphosate in doses of 720/720; 960/720; 960/720/720 g a.i. ha^{-1} and a control group weeded throughout the entire cycle. The samples were analyzed by high efficiency liquid chromatography (HELC). Evaluated characteristics were: residue levels (mg kg^{-1}) of glyphosate and its metabolite AMPA. The highest glyphosate residue level found in the soybeans was 0.92 mg kg^{-1}, less than the highest permitted, which is 10.00 mg kg^{-1}. Residues of AMPA were also found, with the highest level being 1.53 mg kg^{-1}, indicating glyphosate metabolization.
Keywords: herbicide; transgenic; chromatography

Resumo - Aplicações indiscriminadas e sucessivas podem ocasionar casos de intoxicação nos seres humanos por ingestão indireta de glyphosate devido ao fato dos grãos de soja transgênica apresentarem níveis de resíduos desse herbicida acima do permitido. Objetivou-se com este trabalho avaliar os níveis de resíduos de glyphosate e ácido aminometilfosfônico (AMPA) nos grãos de soja geneticamente modificada tolerante ao glyphosate. O delineamento experimental utilizado foi em blocos casualizados com 8 tratamentos e 4 repetições. Os tratamentos foram constituídos por glyphosate, aplicados uma única vez e isoladamente nas dosagens de 720 e 960 g.i.a ha^{-1}, glyphosate a 720 e 960 g.i.a ha^{-1} em mistura, respectivamente, com chlorimuron-ethil a 10 g.i.a ha^{-1}, aplicações sequênciais de glyphosate nas dosagens de 720/720; 960/720; 960/720/720 g.i.a ha^{-1} e testemunha capinada durante todo o ciclo da cultura. As amostras foram analisadas por cromatografia líquida de alta eficiência (CLAE). As características avaliadas foram os níveis de resíduos (mg kg^{-1}) de glyphosate e do seu metabólito AMPA. O nível máximo de resíduo de glyphosate encontrado nos grãos de soja foi de 0,92 mg kg^{-1}, estando abaixo do permitido que é de 10,00 mg kg^{-1}. Também foram encontrados resíduos de AMPA, máximo de 1,53 mg kg^{-1}, indicando que houve metabolização do glyphosate.
Palavras-chaves: herbicida; transgênico; cromatografia

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Introduction

With the release of the commercial planting of genetically modified soybeans in Brazil, glyphosate became the main herbicide used in the control of weeds in agricultural production system. Indiscriminate and successive applications may cause intoxication cases in humans by indirect intake of glyphosate, due to the fact that transgenic soybeans contain residues of this herbicide above permitted by the national sanitary vigilance Agency (Anvisa, 2003).

Glyphosate, N-(phosphonomethyl) glycine, is a non-selective systemic herbicide of low acute toxicity. Its oral Ld50 varies between 1950 to 5000 mg kg^{-1} of body mass in mice, rats, and goats. It is registered in Brazil since the end of 70`s, and is used to control weeds in various environments, cultures and non-agricultural uses like roadsides in highways and railways, control of vegetation under transmission lines etc. (OMS, 2008).

Glyphosate-based herbicides have been used in world agriculture for over 30 years and in the brazilian for over 25 years, where it began to be used in 1978 and produced industrially in 1984. Glyphosate acts as inhibitor of the enzyme 5-enolpiruvil-shikimato-3-phosphate synthetase (EPSPs), which synthesizes aromatic amino acids (tryptophan, phenylalanine and tyrosine), and is metabolized into aminomethyl phosphonic acid (AMPA) by plants and microorganisms (Reddy et al., 2004).

Studies on the analysis of glyphoste and AMPA levels in genetically modified soybeans are scarce. In this way, the objective of this stufy is to assess the levels of glyphosate and aminomethyl phosphonic acid (AMPA) residues in genetically modified soybeans tolerant to glyphosate.

Material and Methods

The experiment was installed in the experimental area, municipality of Engenheiro Coelho (SP), in the agricultural year of 2006/07. Climate in the region is classified as Cwa (Köppen), characterized by dry winter. The genetically modified soybean cultivar used was the BRS-Valiosa RR. Sowing was carried out in December 28th 2006, at 14 to 18 plants density/linear meter and 0.5 m spacing between lines.

The experimental design was randomized in blocks with 8 treatments and 4 replications. The experimental plots were set up by 6 rows of 5.0 m long with 4 repetitions. The four centerlines with 4.0 m long were considered as the usable area, disregarding 0.5 m at each end of the parcel (borders). The treatments were constituted by glyphosate, commercial product Roundup Ready® containing 480 g AI L^{-1} of equivalent acid, applied once and separately in the dosages of 720 and 960 g a.i. ha^{-1}, 720 and 960 g of glyphosate a.i. ha^{-1} in combination with ethyl chlorimuron-the 10 g a.i. ha^{-1}, following applications of glyphosate in the dosages of 720 and 720; 720 and 960; 960, 720 and 720 g a.i. ha^{-1} and control group weeded throughout the cycle of culture. The unique and isolated applications of glyphosate were made 15 days after emergence (DAE) of soybean culture in the phenological stage V2 (three knots) when the soy plants were with a pair of unifoliate leaves and two pairs of trifoliate leaves with the edges of the leaves fully deployed (not touching anymore). The following glyphosate applications were performed at intervals of 15 days, i.e. at 15 and 30 (DAE) with soybeans in phenological stages V2 (three knots) and V5 (five knots), respectively, and at 15, 30 and 45 (DAE) with soy in phenological stages V2 (three knots), V5 (five knots) and V8 (eight knots), respectively, according to Fehr et al. (1971) classification. The applications were made within glyphosate`s waiting period, which is 56 days before the soybeans harvest. Herbicides were applied always in the morning and without wind to avoid the drift, with the aid of a constant pressure knapsack sprayer (CO2), adjusted for volume of 300 L ha^{-1} and bar with flat fan nozzles (110-SF-05).

The experimental plots were set up by 6 rows of 5.0 m long with 4 repetitions. The four centerlines with 4.0 m were considered as the usable area, disregarding 0.5 m at each end of the parcel (borders).

Soy grain samples were collected in the two central lines within the area of each parcel (+ 2 kg) and sent to the laboratory for analysis of residues of glyphosate and AMPA. In the lab, 25 g aliquots of each sample of soybeans that have previously been homogenized were taken, crushed and kept in freezer ± 18°C until the time of analysis.

In oder to extract glyphosate and AMPA, Chloroform and a solution of 0.1 N HCl were used. These extracts were applied on columns containing resin Chelex-Fe (III). These columns were washed with 0.1 N of HCl solution. Then, the glyphosate and AMPA were eluted with 6 N aliquots of HCl. Concentrated HCl was added to the eluate, the extract was homogenaized and applied in an ion exchange column AG1-X 8. In the column, 6 N of HCl were added, and the extract which was concentrated collected under vacuum until dryness. The residue was resuspended in 5 mL of mobile phase and filtered (0.45 μm membrane).

Glyphosate and AMPA were separated by ion-exchange chromatography and quantified by fluorescence after reaction with o-phthaldialdehyde (OPA) and mercaptoethanol (MERC). The glyphosate was oxidized to glycine with calcium hypochlorite prior to the reaction with OPA-MERC. Fluorescent derivatives formed were detected in a fluorimeter with excitation wavelength of 330 nm and emission of 465 nm.

For chromatographic analysis a system of high performance liquid chromatography (HPLC) was used, containing furnaces to chromatographic columns and post-column reaction and fluorescence detector. Column: Aminex A-9 300 x 150 x 4.6 mm d.i. and 4.6 mm d.i., Bio Rad Laboratories at 50^{o}C, temperature of reaction with oxidizing spiral to 38° C, mobile phase: an aqueous solution of potassium dihydrogen phosphate (KH2PO4) 0.005 mol L^{-1}, 4% methanol, pH, with a flow rate of 1.9 0.6 mL min^{-1}, flow of oxidizing solution Ca (ClO) 2 of 0.1 mL min^{-1}, OPA solution flow of 0.6 mL min^{-1} and injected volume 20 μL. The retention time of the glyphosate was around 17 minutes and AMPA around 35 minutes. Excitation wavelength of 350 nm and emission wavelength of 440 nm.

After collecting and tabulating data, these were submitted to analysis of variance using the ASSISTAT program, with the averages compared by Tukey test at 5% probability level.

Results and Discussion

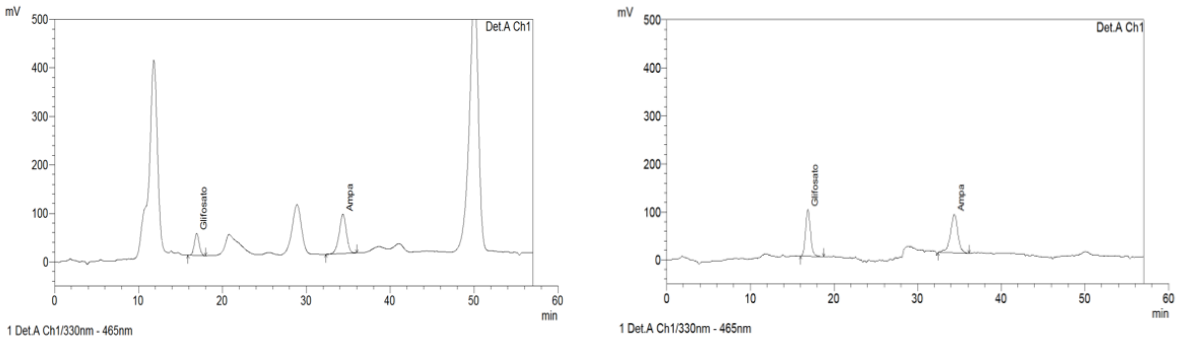
Recoveries for glyphosate and AMPA (Table 1), answered the method`s criteria of acceptability of 70 to 120%, according to Inmetro's guidelines (2003). The chromatograms obtained from soybean dry extract fortified with 0.10 mg kg^{-1} of glyphosate and 0.20 mg kg^{-1} of AMPA, are shown in Figure 1 (A and B), respectively, and a chromatographic peak was clearly distinguished for both.

Table 1. Glyphosate and AMPA recovery in transgenic soybean matrix.

Fortification (mg kg^{-1})		Average recovery*(%)	
Glyphosate	AMPA	Glyphosate	AMPA
0.1	0.2	81.8 $\pm$ 3.0	87.5$\pm$ 4.9
10	10	89.7$\pm$ 3.3	88.0$\pm$ 4.9

* Average of four determinations

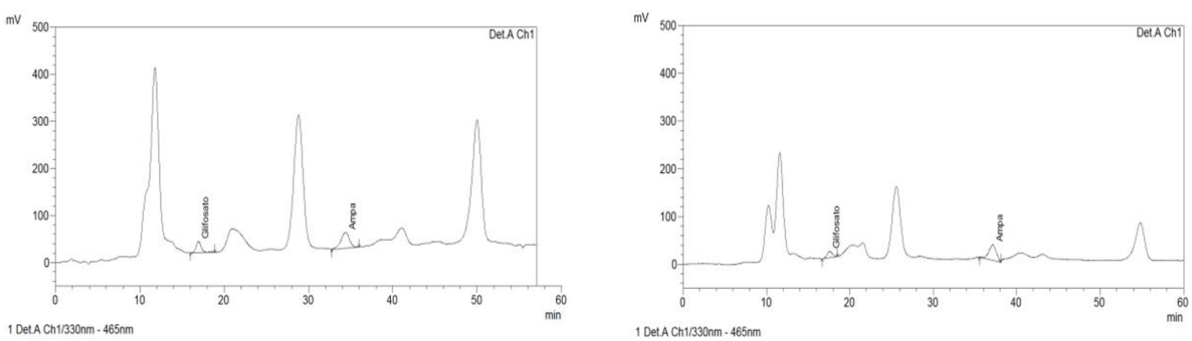
The chromatograms with analytical data of transgenic soybeans samples subject to unique and isolated applications of glyphosate (720 and 960 g a.i. ha^{-1}), glyphosate mixture in tank with chlorimuron-ethyl (720 + 10 and + 10 960 g a.i. ha^{-1}), following applications of glyphosate and control group (720/720 and 960/720 g a.i. ha^{-1}) and (960/720/720 and 0 g a.i. ha^{-1}) are inserted in Figures 2 (A and B), 3 (A and B), 4 (A and B) and 5 (A and B), respectively.



A B

Figure 1. Chromatograms obtained from dry soybean extract fortified with glyphosate and AMPA acid. (A) Fortification 0.10 mg kg^{-1} of glyphosate and 0.20 mg kg^{-1} of AMPA. (B) Strengthening 10.00 mg kg^{-1} of glyphosate and AMPA.

The residue levels observed were between 0.14 to 0.92 mg kg^{-1} (Table 2), close to the values reported by Arregui (2003) that examined glyphosate residue in soybeans from 1997 to 1999, in the region of Argentina, and found quantities of glyphosate between 0.10 to 1.80 mg kg^{-1}. The presence of a metabolite (substance produced by metabolism) generated by the transformation of glyphosate in AMPA (aminomethyl phosphonic) was also detected, indicating that glyphosate was methabolized. Reddy et al. (2004) also observed that the formation of the AMPA can derive in damage in the cultivation of genetically modified glyphosate-tolerant soybean, since its metabolite also features phytotoxic character.



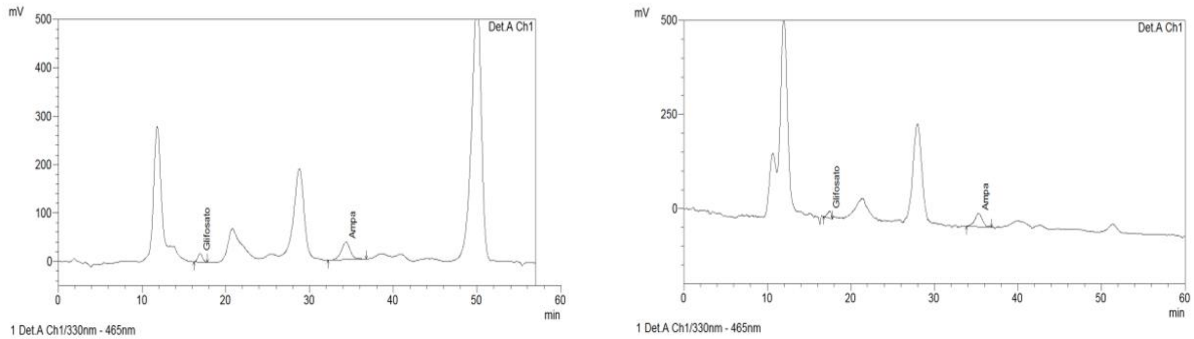
A B

Figure 2. Chromatograms of samples from soybeans subjected to unique and isolated applications of glyphosate in the dosages of 720 (A) and 960 g a.i. ha^{-1} (B).

In this study, the amount of AMPA was greater than glyphosate. A possible explanation for this is that the biodegradation of glyphosate is faster. According to Araújo et al. (2003) and Gimsing et al. (2004), who studied the biodegradation of these molecules in the soil, glyphosate`s half-life is shorter than that of the AMPA, which may explain the higher levels of AMPA and lower of glyphosate. Higher concentrations of glyphosate and its metabolite, aminomethyl phosphonic acid (AMPA), has been found in new foliage, Amarante Jr et al. (2002).

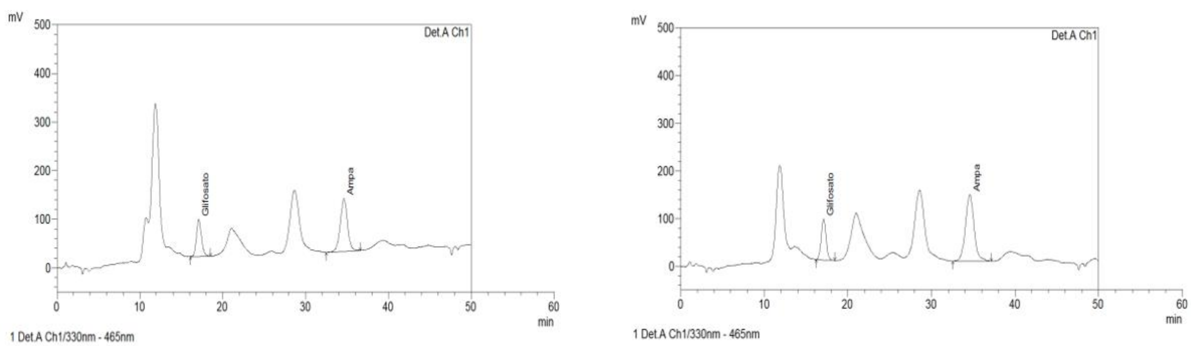
Treatments with unique and isolated applications of glyphosate and in a tank containing chlorimuron-ethyl, residue levels were below the limit of quantification (LOQ) for both glyphosate and AMPA. However the treatments in that soybean was subjected to sequential applications of glyphosate, i.e.

situations in which soybeans received 1440 g a.i. ha^{-1} (720/720), 1680 g a.i. ha^{-1} (960/720) and 2400 g a.i. ha^{-1} (960/720/720), the levels of glyphosate and AMPA residues were above the LOQ. The higher the amount of active ingredient applied the major was the residue levels found in grains. The treatment T7 (2400 g a.i. ha^{-1}) showed increased amounts of glyphosate and AMPA, values of 0.92 and 1.53 mg kg^{-1}, respectively, differing significantly from other sequential treatments T5 (1440 g a.i. ha^{-1}) and T6 (1680 g a.i. ha^{-1}) which showed levels of residues of glyphosate and 0.14 0.14 mg kg^{-1} and AMPA 0.25 and 0.29 mg kg^{-1}, respectively.



A B

Figure 3. Chromatograms of samples from soybeans submitted the application of glyphosate in mixture in tank with chlorimuron-ethyl in dosages of 720 + 10 (A) and 960 + 10 g a.i. ha^{-1} (B).



A B

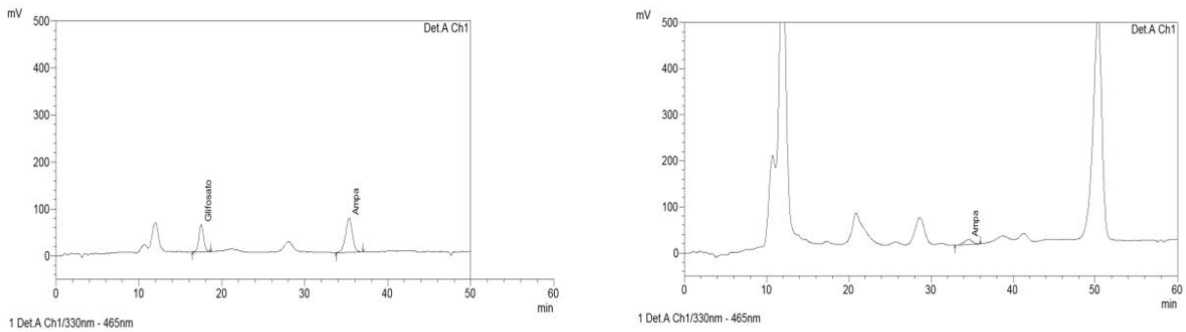
Figure 4. Chromatograms of soybean samples undergoing following application of glyphosate in the dosages of 720/720 (A) and 960/720 g a.i. ha^{-1} (B).

The unanimous conclusion of the main regulatory agencies of various countries and specialists is that the commercial use of glyphosate does not represent risk to the environment or to humans and animals when used in accordance with the usage record and the recommendations contained in the package insert and on the product label (Agriculture Canada, 1991; US EPA, 1993; Who 1994; FAO/WHO 2004, Giesy et al., 2000; Grossbard and Atkinson, 1985; Franz et al., 1997; Malik et al., 1989).

Bohm et al. (2008) assessing glyphosate residues in grains, also found the presence of glyphosate molecule and in proportion to the dosage applied. The treatment in which an application of glyphosate (960 g a.i. ha$_{-1}$) was performed, at 28 days after planting (DAP), the concentration was 19.00 mg kg^{-1}, while in that with two applications (960 g a.i. ha^{-1}) at 28 and 56 (DAP), the concentration increased to 36.00

mg.kg^{-1}. These results are concerning, since these residue levels found exceeded the maximum limit established by Anvisa (2003) and Agrofit (2007).

The same authors, while monitoring the concentrations of AMPA, noticed that the behavior was similar, i.e. in soybeans treated with glyphosate (960 g a.i. ha^{-1}) at 28 days after planting (DAP) had a residue of 9.00 mg kg^{-1} and in plots treated twice, (960 g a.i. ha^{-1}) at 28 and 56 (DAP), they detected 12.00 mg kg^{-1}.



A **B**

Figure 5. Chromatograms of samples of soybeans (diluted extract 1:10) and submitted to sequential application of glyphosate at the dosage of 960/720/720 g a.i. ha^{-1} (A) and control group (B).

Table 2. Glyphosate and AMPA residues in transgenic soybean.

Treatments (g i.a. ha^{-1})		Glyphosate mg kg^{-1}	AMPA mg kg^{-1}
Glyphosate (720)	15	< LOQ	< LOQ
Glyphosate (960)	15	< LOQ	< LOQ
Glyphosate + clorimuron ethil (720 + 10)	15	< LOQ	< LOQ
Glyphosate + clorimuron ethil (960 + 10)	15	< LOQ	< LOQ
Glyphosate + glyphosate (720 / 720)	15/30	0.14 ^{b}	0.25 ^{b}
Glyphosate/ glyphosate (960 / 720)	15/30	0.14 ^{b}	0.29 ^{b}
Glyphosate / glyphosate / glyphosate (960 / 720 / 720)	15/30/45	0.92 ^{a}	1.53 ^{a}
Control group	---	ND	< LOQ

ND = non detectable; LOQ = limit of qualification (glyphosate = 0.10 mg kg^{-1}) and (AMPA = 0.20 mg kg^{-1}). In columns, data averages with the same letters did not differ statistically by Tukey test ($p<0.05$).

Duke et al. (2003) have detected lower glyphosate residues in grains (3.08 mg kg^{-1}), but those of AMPA were elevated (25.00 mg kg^{-1}) in genetically modified soybeans treated with the herbicide Roundup Ready® at the dosage of 1260 g a.i. ha^{-1}, applied 8 weeks after planting (56 days). According to Duke et al. (2003), the causes of lower levels of glyphosate residue in these grains, although, were not demonstrated, but it is known that molecule metabolization in plants is dependent on, in addition to the genotype, soil and climate conditions during cultivation, these conditions may be the cause of the differences

Conclusions

Residues of glyphosate were found in soybean grains in levels between 0.14 and 0.92 mg kg^{-1}, but these values are within the limit allowed by Anvisa (2003) which is 10 mg kg^{-1}. Residues of AMPA Were also found, meaning that there was metabolizing of glyphosate in levels between 0.25 and 1.53 mg kg^{-1}.

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