Study Title

Evaluation of the mutagenic potential of GLYPHOSATE TECHNICAL by micronucleus assay in mice

Guideline Reference

OECD (Organisation for Economic Co-operation and Development). 1997. Guidelines for Testing of Chemicals. Mammalian Erythrocyte Micronucleus Test. N° 474.

Study director

[redacted]

Final Report

29/Sep/2008

Performing laboratory

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Sponsor

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Study #

3996.402.395.07

Study compliance statement

The study described in this final report was performed under my supervision according to the study plan and procedures described in the Guideline Nº 474 of the Organisation for Economic Co-operation and Development (OECD, 1997) and following the Principles of Good Laboratory Practice (GLP) as established by the OECD – Organisation for Economic Co-operation and Development (revised 1997, ENV/MC/CHEM (98) 17) and INMETRO – Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (NIT-DICLA-035-INMETRO-Dec/2007-Rev 00).

This report represents an accurate and true recording of the results obtained.

Study plan, original raw data, copy of the final report and observations referent to this study are archived at BIOAGRI Laboratórios Ltda.

[redacted]

Study Director
Phone: [redacted]

29 / Sep / 2023
dd mmm yyyy

Final report approval

The study described in this report was performed according to the Principles of Good Laboratory Practice (GLP) as established by the OECD – Organisation for Economic Co-operation and Development (revised 1997, ENV/MC/CHEM(98)17) and INMETRO – Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (NIT-DICLA-035-INMETRO-Dec/2007-Rev.00).

The documents and records of this study will be archived at BIOAGRI for a period of 10 years.

[redacted]

Test Facility Manager
Phone: [redacted]

29/Apr/2008
dd mmm yyyy

Study code: 3996.402.395.07
Study title: Evaluation of the mutagenic potential of GLYPHOSATE TECHNICAL by micronucleus assay in mice

Quality Assurance Unit Statement

This study has been audited, and the resulting final report was subsequently reviewed by the Quality Assurance Unit – BIOAGRI. The dates and phases of the audits are given below.

Audit		***Information date***	
Date	Phase	Study director	Test facility manager
13/Sep/2007	Study plan	13/Sep/2007	13/Sep/2007
25/Aug/2008	Draft report	25/Aug/2008	26/Aug/2008
29/Sep/2008	Report Final	29/Sep/2008	29/Sep/2008

The most recent process audit prior to the completion of the laboratory phase of this class of study was performed on May 14th, 2008. This audit has been record in the internal QAU document identified as RAS 041/08.

The results and observations presented in this final report are an accurate representation of the raw data generated during the conduct of this study.

[redacted]

Quality Assurance Unit
Phone: [redacted]

25 / Sep / 2008
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Summary

A mouse bone marrow micronucleus assay was carried out in order to assess the mutagenic potential of the test substance GLYPHOSATE TECHNICAL. The test substance was diluted in sterile corn oil and administered intraperitoneally twice after an interval of 24 hours at the doses of 15.62, 31.25 and 62.5 mg kg^{-1} (b.w.) corresponding to cytotoxicity analysis. Negative and positive controls were administered with the same schedule of the test substance: two intraperitoneal administrations after an interval of 24 hours. Negative control group was treated with the dilution vehicle and positive control with cyclophosphamide (25 mg kg^{-1}, b.w.). After 24 hours of the second application the animals were euthanized, their femurs excised to obtain the bone marrow cells, prepared in smears and stained on slides used for observations. The results pointed out no increase in the number of micronucleus in polychromatic erythrocytes in animals treated with the test substance when compared to the negative control. As expected, a statistically significant increase in this parameter was observed in animals treated with cyclophosphamide. In the conditions of this study, the results indicated that GLYPHOSATE TECHNICAL produced no evidence of mutagenic activity in mice.

Summary (Portuguese)

O teste do micronúcleo em medula óssea de camundongos foi conduzido para avaliar o potencial mutagênico da substância teste GLYPHOSATE TECHNICAL. A substância teste foi diluída em óleo de milho estéril e administrada por via intraperitoneal em duas aplicações, num intervalo de 24 horas, nas doses 15,62, 31,25 e 62,5 mg kg^{-1} (p.c.) correspondendo a análise de citotoxicidade. Na administração dos controles positivo e negativo empregou-se o mesmo esquema da substância teste: duas aplicações por via intraperitoneal num intervalo de 24 horas. O grupo controle negativo foi tratado com o veículo de diluição da substância teste e o controle positivo com a ciclofosfamida (25 mg kg^{-1}, p.c.). Após 24 horas da segunda aplicação os animais foram sacrificados, tendo os fêmures removidos para obtenção das células da medula óssea, preparadas em esfregaços em lâminas e corados, sendo utilizadas para as observações microscópicas. Os resultados mostraram que não houve aumento no número de micronúcleos em eritrócitos policromáticos nos animais tratados com a substância teste em comparação com o controle negativo. Um aumento estatisticamente significativo nessa variável em animais tratados com a ciclofosfamida foi observado conforme esperado. Nas condições desse estudo os resultados indicaram que a substância teste GLYPHOSATE TECHNICAL não apresentou atividade mutagênica em camundongos.

1. General information

Study initiation: 13/Sep/2007
Experimental phase initiation: 19/May/2008
Experimental phase conclusion: 13/Aug/2008
Draft Report: 26/Aug/2008
Final Report: 29/Sep/2008

2. Technical staff

Study director: [redacted]
Researcher:
Laboratory assistant:

Personnel:

Chemical researcher:
Laboratory technician:
Laboratory assistant:

3. Introduction

The micronuclei test is a mammalian in vivo test applied to evaluate the mutagenic potential of chemical agents that cause chromosomal breakage resulting in micronucleus formation (Schmid, 1975), such agents are called clastogenic. The test substance is generally administered intraperitoneally in mice and the effect is detected by microscope observation of the cell smears from the bone marrow where the micronuclei of the polychromatic erythrocytes are quantified. In most instances, micronuclei are formed from chromosome fragments (lost chromatids) that were not included in the nuclei of daughter cells during the cellular division process, and they could also be generated from entire chromosomes that were not excluded in the telophase stage (Salamone & Heddle, 1983).

When the erythroblasts extrude their nuclei while developing into erythrocytes, the micronuclei remain in the cytoplasm, where they are easily identified. During the first 10 to 24 hours, the young polychromatic erythrocytes stained blue, not red (normochromatic erythrocytes). Thus, such cells are easily identified and they have a relatively short and defined lifetime. The micronuclei from polychromatic erythrocytes that are counted are those formed under the last mitosis by induction of the substance administered to the animal. The spontaneous polychromatic erythrocyte with micronuclei is very low, around 3 per thousand of cells. The test detects an increase in the frequency of micronucleated polychromatic erythrocytes in the bone marrow of treated animals compared to a negative control.

The ratio of polychromatic erythrocytes to normochromatic erythrocytes is also used for the evaluation of results and it is expected to be 1:1 in mice, therefore some variations have been detected and cited by many researchers and they are expected to be between 48.5 and 70% (Müller & Streffer, 1994). The toxicity of a substance to the bone marrow results in lower values to this ratio. Mavournin et al (1990) consider ratios lower than 5% unsuitable.

4. Definition

Micronuclei – are small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division.

5. Objective

The aim of the study is to evaluate the mutagenic potential of the test substance GLYPHOSATE TECHNICAL in mice when administered intraperitoneally. The obtained results give information about the clastogenic potential of this test substance to induce an increase in micronucleus number in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCEs) of the bone marrow in mice.

6. Material and methods

6.1 Test substance information

Test substance:	GLYPHOSATE TECHNICAL
Received on:	20/Aug/2007
BIOAGRI code:	AGR-0790/07
Common name:	glyphosate
Chemical name (IUPAC):	*N*-(phosphonomethyl)glycine [1,2]
CAS RN:	1071-83-6 [1,2]
Batch nº:	20070606
Declared concentration of a.i. (Sponsor):	min. 980.0 g kg^{-1}
Analyzed concentration of a.i. (Bioagri):	980.0 g kg^{-1}
Analysis certificate:	CA-708/08 R1
Homogeneity data:	Homogeneous sample (TH-115/08 R1)
Molecular formula (a.i.):	$C_3H_8NO_5P$ [1,2]
Molecular weight (a.i.):	169.1 [1]
Water solubility (a.i.):	10.5 g L^{-1} (pH 1.9, 20°C) [1]
Stability data (a.i.):	Glyphosate and all its salts are non-volatile, do not photochemically degrade in buffered water and are stable in air. Glyphosate is stable to hydrolysis at pH 3, 6 and 9 (5-35°C). [1]
Sponsor:	Jingma Chemicals Co., Ltd.
Supplier:	Jingma Chemicals Co., Ltd.

Reference:

[1] TOMLIN, C. D. S. 2006-2007.
[2] Information provided by the sponsor.

6.2 Reference substance information

Common name: Cyclophosphamide monohydrate, 97%
Batch number: A0164185001
Brand: Acrós
CAS number: 6055-19-2
Expiry date: 18/April/2016

6.3 Equipment

Equipment	BIOAGRI code
Analytical Balance	B#16, B#18, B#28 and B#30
Centrifuge	CE#10
Freezer	FR#17 and FR#19
Magnetic stirrer	AG#03
Micropipette	MA#127 and MA#132
Microscope	MP#12, MP#18, MP#19 and MP#20
Refrigerator	GE#17
Semi-analytical balance	B#15
Drying stove	EST#06
Termohygrometer	HT#45 and HT#48
Chromatograph (HPLC)	CL#01 and CL#38
Ultra-sound	US#01
Mechanical stirrer	AT#06

6.4 Solvents and reagents

Substance	Batch	Expiry date
Water	LFQ1 045/08	12/Aug/2008
	LFQ1 046/08	18/Aug/2008
Sterilized corn oil	0108	24/May/2008
	03 2902	05/Jul/2008
Phosphate buffer solution	01/Apr/2008	12/Jul/2008
	04/Jul/2008	04/Oct/2008
Wright solutions	28/Mar/2008	28/Jul/2008
	06/Jun/2008	06/Oct/2008
Giemsa solutions	28/Mar/2008	28/Mar/2011
Alcohol 70%	18/Apr/2008	18/Jul/2008
	12/Jun/2008	12/Sep/2008
Fetal calf serum	003/08	30/Jan/2010
	006/08	29/Apr/2010
Salt solution sterilized 0.9%	16/Jul/2008	16/Oct/2008
Acetonitrile – 99.9% - Tedia	710033R	Oct/2012
Methanol – 100% - J. T. Baker	B08E54	Feb/2015
KH_2PO_4 - Vetec	044830	Aug/2008
Phosphoric acid – 99.7% - Quimex	30391	20/Dec/2008
Gliphosate standard solution	395-135A	Feb/2011

6.5 Treatment solutions

The test substance in the definitive test was dissolved at 3 concentrations: 1.04(2A), 2.08(3A) and 4.16(4A) mg mL^{-1}, in sterile vehicle (corn oil) in a final volume of 15 mL for each dose. The cyclophosphamide, used as the positive control (5A) was dissolved in sterile physiological solution and, was administered twice at a concentration of 1.66 mg mL^{-1} corresponding to 25 mg kg^{-1} of body weight.

6.6 Test system

Swiss albino male and female, healthy young adult mice (Mus musculus), aged 7-12 weeks and approximate weight 30 g, acquired from selected animal husbandry unit (Anilab Animais de Laboratório Criação e Comércio Ltda.), were used after inspection and acclimation (SOP-M 0007-Rev 11 and SOP-M 0700 Rev 04) to the laboratory conditions for 5 days. Animals from both sexes, 5 males and 5 females, were used for each treatment. Animal's selection was made randomly and formed a group in each treatment keeping the mean weight within ±20% calculated separately for males and females.

6.7 Test system selection justification

Mouse is an universally used model for evaluation of the mutagenic potential of various classes of chemicals and for which there is a large historical database. It is the recommended species by various regulatory agencies and has demonstrated sensitivity to detect agents that cause structure or numerical chromosome aberrations, according to the OECD 474 (1997).

6.7.1 Housing

The animals were maintained in groups of 5 per sex and per polypropylene cage closed with a metallic grid and suspended. The cages were padded out with sterile sawdust (30 minutes at 121 °C). Housing hygienic conditions and sanitation cares were in accordance with the patterns advised by SOP-M 0007 - Rev 11. Each cage was properly identified with the study number, sample code, sex and dose that was administered.

6.7.2 Environmental conditions

The animals were maintained in a room with controlled and monitored temperature (20 to 24 °C), humidity (50-70%), light cycles (12 hours day^{-1}) and room ventilation (10 to 15 air changes $hour^{-1}$), using specific equipment. Daily data of temperature and humidity were printed at least once a day.

6.7.3 Feeding

The animals were provided with a balanced diet and water *ad libitum*; pellets of commercial food (Commercial name: Purina Labina, Brand: Agribrands Purina do Brasil Ltda.) were used as diet. The results obtained for chemical composition, microbiological and mycotoxins analysis were within the expected range for feeding. The available water meets the standard quality for human consumption.

6.7.4 Animal welfare compliance statement

This study complied with the applicable rules for animal welfare and human use and care of laboratory animals (Brazilian Federal Law, Nº 6.638 of May 8th, 1979). Wherever procedures were used to avoid or minimize discomfort, distress and pain to animals.

6.8 Experimental procedure

6.8.1 Preliminary test

A preliminary test was conducted with the doses of 62.5, 125, 250, 500 and 1000 mg kg^{-1} of body weight in six animals per dose (03 males and 03 females). The test substance was administered by two intraperitoneal injections over a 24 hours interval. The preliminary test evaluated the cytotoxicity of test substance in surviving animals by counting 200 polychromatic erythrocytes/normochromatic erythrocytes.

6.8.2 Test substance, positive control, negative control and used doses

The doses for the definitive test were: 15.62(2A), 31.25(3A) and 62.5(4A) mg kg^{-1}, in final volume of 15 mL per each dose. The cyclophosphamide, used as the positive control (5A) in sterile physiological solution, was administered twice at a concentration of 1.66 mg mL^{-1} corresponding to 25 mg kg^{-1} of body weight. In the negative control (1A), the sterile vehicle diluent only was administered at the same quantity (volume) as used for each one of the tested doses. Table 01 shows animal group and treatments.

Table 01. Animals groups and treatments

Groups	Animals		Treatments	Sampling time (h) after second injection
	Males	Females		
1A	5	5	Negative control (dilution vehicle)	24
2A	5	5	Test substance (lower dose)	24
3A	5	5	Test substance (middle dose)	24
4A	5	5	Test substance (higher dose)	24
5A	5	5	Positive control (cyclophosphamide)	24

6.8.3 Experimental description

This test was conducted in compliance with BIOAGRI Laboratórios' Standard Operating Procedure SOP-M 0069 – Rev 10 (Micronucleus test – 402).

- Exposure route: the test substance was administered by intraperitoneal injections.

- Justification for the exposure route: The intraperitoneal injections are more used, because they maximize absorption and are better indicated for the test.

The test was carried out with ten animals (five males and five females) per tested dose. The animals were treated twice with intraperitoneal injections over a 24 hours interval, with volumes defined in accordance with the body weight of each animal. They were euthanized 24 hours after the second test substance application. The same procedures were used for negative and positive controls SOP-M 0069 – Rev 10.

6.8.4 Bone marrow preparation

The following protocol is an adaptation of the procedure described by SCHMID (1975). Mice were sacrificed using a CO_2 euthanasia chamber, at 24 hours after the second injection. Immediately both femurs were totally excised and cleaned outside; the distal epiphyseal portion and the proximal end of the femur were removed with a scissors until the marrow was visible. The bone marrow cells were successively flushed out with fetal calf serum (Cultilab) administered with a sterile syringe and needle. The flushed bone marrow was centrifuged for 5 minutes, at 1000 rpm. The bone marrow cells were resuspended in fetal calf serum and smeared on microscope slides (2 slides for each animal) that were air-dried. After drying, the slides were fixed in 70% alcohol for 10 minutes, and stained with Wright's concentrated solution (3 minutes); then they were placed in a Wright's phosphate buffer solution (pH 6.0, 1:1) and rinsed. After that, they were immersed for 10 minutes in Giemsa's buffer – deionized water solution. The stained slides were washed in stream water, dried and assembled in Permount (SOP-M 0069 – Rev 10).

6.8.5 Microscopic analysis

The slides were coded and observed with a 1000X magnification objective in an Olympus microscope. Codification was done in such a manner that technicians were unable to know the corresponding treatment to the slide. For each animal, 2,000 polychromatic erythrocytes (PCEs) and present micronuclei (MN) were counted. At the same time, normochromatic erythrocytes (NCEs) and MN were examined and recorded. The ratios of PCEs/NCEs were determined when the first 2,000 PCEs had been computed.

6.8.6 Statistical analysis

Differences in the incidence per animal of MNPCEs and MNNCEs per 2,000 cells and the relation PCEs/NCEs were compared using the U Mann-Whitney´test and the K´test for independent variable in accordance with Kruskal & Wallis Test cited by Conover (1980). The samples were compared to the negative control. The results were evaluated with two levels of statistical significance ($p \leq 0.05$ and $p \leq 0.01$).

For determining a positive result with a test substance, the following criteria were met: a dose-related increase in the number of micronucleated polychromatic erythrocytes statistically significant ($p \leq 0.05$) when compared to the negative control; a reproducible and statistically significant positive response for at least one of the test points.

6.8.7 Concentration verification

After the end of the test, the remaining samples of the stock solutions used in the definitive test, corresponding to doses of group 2A, 3A, 4A and 5A, were taken to determine the test substance concentration. The samples were kept in a refrigerator (approximately 5°C) until the analysis being performed. The test substance concentration in the test and positive control solutions were determined by analyzing the solution for the active ingredient with a validated analytical method following the VM-040/08 and SOP-M 0456 – Rev. 02. The analyses were carried out using a high performance liquid chromatograph (HPLC) with an ultra violet (UV) absorption detector, under the following conditions:

Test substance

Chromatograph:	HP 1050 (CL#01) POP-E 0018 Rev.04
Detector:	UV
Wavelength:	195 nm
Column:	Sax (250 mm x 4.6-mm x 5 µm)
Injected volume:	20 µL
Mobile phase:	H_2O/Met/KH_2PO_4 (960 mL + 40 mL + 0.8435 g) pH 2.0 phosphoric acid
Flow of mobile phase:	1.5 mL minute^{-1}

Cyclophosphamide (positive control)

Chromatograph:	Finnigan (CL#38) POP-E 0352 Rev.01
Detector:	UV
Wavelength:	194 nm
Column:	C18 (250 mm x 4.6-mm x 5 µm)
Injected volume:	10 µL
Mobile phase:	acetonitrile/water (40:60)
Flow of mobile phase:	1.0 mL minute^{-1}

The analyses were performed in the Physical Chemistry Laboratory within the Chemistry Division of Bioagri Laboratórios Ltda., SP.

6.8.7.1 Calculations

The analyzed concentration of the test substance in the test solution was calculated using the following equation:

$C_{ts} = (C_f \times F \times 100/C_{ca})$

Where:

C_{ts} = analyzed concentration of the test substance (mg mL^{-1})

C_f = concentration of the a.i. (test substance) determined by HPLC/UV (ng µL^{-1})

F = dilution factor (for some concentrations of the a.i. it was necessary to dilute the test solutions prior to analysis by HPLC/UV, so that the concentration following dilution was within the range over which the system response varied linearly with the a.i. concentration - Table 3)

C_{ca} = concentration of the a.i. in the test substance (%), indicated on the Analysis Certificate

The deviation of the analyzed test substance concentration from the nominal concentration was calculated using the following equation:

$Dev = (C_{ts}-C_{nom}) \times 100/C_{nom}$

Where:

Dev = deviation of the analyzed concentration from the nominal concentration (%).

C_{nom} = nominal concentration of the test substance (mg mL^{-1})

6.8.8 Positive and negative control historical data

Table 2. Positive and negative control historical data (mean number of micronucleus in polychromatic erythrocytes (MNPCE) and relation of PCEs/NCEs).

Evaluated statistical parameters (Mean values)	Males				Females			
	Positive Control		Negative Control		Positive Control		Negative Control	
	PCEs/NCEs (1)	MNPCEs (2)	PCEs/NCEs (1)	MNPCEs (2)	PCE/NCEs (1)	MNPCEs (2)	PCEs/NCEs (1)	MNPCEs (2)
Mean	1.31	9.30	1.43	0.87	1.35	9.20	1.54	0.76
Average Amplitude	0.69	-	0.72	-	0.65	-	0.68	-

Positive control: Cyclophosphamide – Batch number: A0164185001.
PCE: Polychromatic erythrocyte, NCE: Normochromatic erythrocyte, MNPCE: micronucleated polychromatic erythrocyte
(1): Mean obtained of 5 animals. Data from January to December of 2007.
***Note:** These data are referred to historical data of the laboratory, but change could occur in accordance to literature data.
The spontaneous polychromatic erythrocyte with micronuclei rate is very low, around 3 per thousand of cells, and ratio PCEs/NCEs is expected to be 1:1 in mice. Therefore, some variations have been detected and cited by many researchers and they are expected to be between 48.5 a 70% (Müller & Streffer, 1994).

7. Results

7.1 Analytical results

The results of nominal and analyzed concentrations of GLYPHOSATE TECHNICAL and the deviations (%) are shown in Table 3. The deviations were within the 20% of the maximum tolerated limit.

An example of test substance analyzed concentration calculation is shown in Appendix 01.

Table 3. Test substance and positive control (cyclophosphamide) nominal and analyzed concentrations and their respective deviation, dilution factor and chromatogram results.

Identification sample	Nominal concentration (mg mL^{-1})	Concentration of a.i. found in the chromatogram (ng μL^{-1})	Dilution factor	Analysed concentration* (mg mL^{-1})	Deviations** (%)
TS	1.04	2032.370	2	0.96	7.69
TS	2.08	1880.016	1	2.07	0.48
TS	4.16	1952.673	2	3.98	4.33
PC	1.66	56.764	30	1.76	6.02

TS: test substance; PC: positive control, cyclophosphamide.
* Analyzed concentration was determined according to the equation (1) described in page 15 and exemplified in page 26.
** Dev% = $(C_{ts} - C_{nom}) \times 100 / C_{nom}$

7.2 Preliminary test

The doses chosen to the preliminary test were: 62.5, 125, 250, 500 and 1000 mg kg^{-1}. The obtained results of mortality in the preliminary test are shown in Table 4.

Table 4. Mortality of the animals in the preliminary test.

Dose (mg kg^{-1} of body weight)	Sex	Mortality/ Nº of animals
62.5	Male	0/3
	Female	0/3
125	Male	0/3
	Female	0/3
250	Male	0/3
	Female	0/3
500	Male	1/3
	Female	2/3
1000	Male	3/3
	Female	3/3

7.2.1 Cytotoxicity evaluation of the preliminary test

The results of cytotoxicity obtained in the preliminary test are shown in Table 5.

Table 5. Cytotoxicity results in the preliminary test (mean values obtained of three animals)

Dose (mg kg^{-1})	Sex	PCEs	NCEs	PCEs/NCEs
62.5	Male	228.6	127.6	1.7911
	Female	231.0	159.0	1.4528
125	Male	241.0	196.6	1.2254
	Female	248.0	149.0	1.6644
Negative control	Male	213.3	125.6	1.6976
	Female	274.3	155.0	1.7698
250	Male	217.0	257.6	0.8423
	Female	256.0	199.0	1.2864
500	Male	239.6	220.0	1.0890
	Female	*	*	*
Negative control	Male	229.3	174.3	1.3155
	Female	225.0	213.6	1.0533

PCE: Polychromatic erythrocyte; NCE: Normochromatic erythrocyte; * = died

The dose of 125 mg kg^{-1} was chosen to the definitive test because cytotoxicity was not observed when compared to the negative control (ratio of polychromatic erythrocytes to normochromatics lower than 20%).

7.3 Definitive test

The data were analyzed all together and also by sex (Tables 6, 7 and 8). The statistical analysis of the results pointed out that the test substance did not induce an increase in micronuclei number in polychromatic erythrocytes of the bone marrow when compared to the negative control, at any evaluated concentrations. No adverse effect was observed in the ratio of polychromatic erythrocytes to normochromatics in animals treated with the test substance GLYPHOSATE TECHNICAL, at any evaluated concentrations. A significant statistical increase of micronucleated cells in polychromatic and normochromatic erythrocytes was observed in animals treated with cyclophosphamide, as expected.

Table 6. Individual data obtained in the definitive test.

Obs	Sex	Dose	Weight (g)	Average of weight (g) and Standard Deviation(%)[1]	MNPCE	MNNCE	PCE	NCE	PCE/NCE
1	Male Group	Negative control	30	31.0 SD: 3.95	0	0	2025	1172	1.72782
2			31		0	0	2031	1109	1.83183
3			30		0	0	2080	1207	1.72328
4			31		0	0	2015	1065	1.89202
5			33		0	0	2046	1179	1.73537
6		15.62 mg kg^{-1}	30	29.2 SD: 7.42	0	0	2017	1115	1.80897
7			31		0	0	2040	1159	1.76014
8			26		0	0	2081	1069	1.94668
9			31		0	0	2170	1314	1.65145
10			28		0	0	2041	1219	1.67432
11		31.25 mg kg^{-1}	32	29.6 SD: 6.14	0	0	2062	1226	1.68189
12			28		0	0	2079	1197	1.73684
13			28		0	0	2024	1132	1.78799
14			29		1	0	2073	1202	1.72463
15			31		0	0	2031	1137	1.78628
16		62.5 mg kg^{-1}	28	29.4 SD: 7.83	0	0	2055	1232	1.66802
17			27		0	0	2038	1170	1.74188
18			30		0	0	2065	1273	1.62215
19			29		3	0	2040	1141	1.78791
20			33		0	0	2089	1205	1.73361
21		Positive control	32	30.4 SD: 5.50	22	0	2107	1441	1.46218
22			30		30	2	2100	1390	1.51079
23			28		26	0	2125	1588	1.33816
24			30		8	0	2038	1159	1.75841
25			32		29	0	2043	1221	1.67322
26	Female Group	Negative control	26	26.6 SD: 9.44	0	0	2049	1164	1.76031
27			26		0	0	2033	1154	1.76170
28			31		0	0	2032	1147	1.77158
29			25		0	0	2040	1152	1.77083
30			25		0	0	2070	1153	1.79532
31		15.62 mg kg^{-1}	24	26.8 SD: 7.18	0	0	2070	1222	1.69394
32			26		0	0	2045	1092	1.87271
33			27		0	0	2045	1083	1.88827
34			29		0	0	2036	1064	1.91353
35			28		0	0	2036	1283	1.58691
36		31.25 mg kg^{-1}	31	28.0 SD: 7.99	0	0	2056	1277	1.61002
37			28		0	0	2076	1168	1.77740
38			25		0	0	2065	1132	1.82420
39			27		0	1	2063	1095	1.88402
40			29		0	0	2089	1224	1.70670
41		62.5 mg kg^{-1}	26	27.4 SD: 6.11	0	0	2065	1149	1.79721
42			26		0	0	2007	1098	1.82787
43			27		0	0	2067	1205	1.71535
44			30		0	0	2030	1135	1.78855
45			28		0	0	2025	1122	1.80481
46		Positive control	27	27.0 SD: 5.86	10	0	2036	1124	1.81139
47			29		11	0	2023	1077	1.87837
48			26		7	0	2053	1204	1.70515
49			28		26	1	2060	1238	1.66397
50			25		7	0	2090	1320	1.58333

PCE: Polychromatic erythrocyte; NCE: Normochromatic erythrocyte. MNPCE: micronucleated polychromatic erythrocyte; MNNCE: micronucleated normochromatic erythrocyte. (1) Relative standard deviation = SD x 100 / average (considering 30 g ± 20%).

Table 7. Statistical analysis of the obtained results after administration of the test substance and positive and negative controls on micronucleus number of polychromatic erythrocytes (MNPCEs) and on the ratio of polychromatic (PCEs) to normochromatic erythrocytes (NCEs) from bone marrow cells in mice (mean values obtained from 5 animals of each sex/dose).

Treatments	Sex	MNPCEs/ 2,000 cells	PCEs	NCEs	PCEs/NCEs
Negative control	M	0.0	2039.4	1146.4	1.78197
TS (15.62)	M	0.0	2069.8	1175.2	1.76831
TS (31.25)	M	0.2	2053.8	1178.8	1.74353
TS (62.5)	M	0.6	2057.4	1204.2	1.71071
Positive control	M	23.0**	2082.6	1359.8	1.54855
Negative control	F	0.0	2044.8	1154.0	1.77195
TS (15.62)	F	0.0	2046.4	1148.8	1.79107
TS (31.25)	F	0.0	2069.8	1179.2	1.76047
TS (62.5)	F	0.0	2038.8	1141.8	1.78676
Positive control	F	12.2**	2052.4	1192.6	1.72844

* $p \leq 0.05$ and ** $p \leq 0.01$: difference statistically significant from negative control (dilution vehicle), by Mann-Whitney (Kruskal & Wallis Test).
Key: M-males, F-females, TS = test substance (mg kg^{-1} of body weight); positive control = cyclophosphamide at 25mg kg^{-1} (b.w.).

Table 8. Statistical analysis of the obtained results after administration of the test substance and positive and negative controls on micronucleus number of polychromatic erythrocytes (MNPCEs) and on the ratio of polychromatic (PCEs) to normochromatic erythrocytes (NCEs) from bone marrow cells in mice (mean values obtained from 10, males and females/dose).

Treatments	MNPCEs/ 2,000 cells	PCEs	NCEs	PCEs/NCEs
Negative control	0.0	2042.1	1150.2	1.77696
TS (15.62)	0.0	2058.1	1162.0	1.77969
TS (31.25)	0.1	2061.8	1179.0	1.75200
TS (62.5)	0.3	2048.1	1173.0	1.74874
Positive control	17.6**	2067.5	1276.2	1.63850

* $p \leq 0.05$ and ** $p \leq 0.01$: difference statistically significant from negative control (dilution vehicle), by Mann-Whitney (Kruskal & Wallis Test).
Key: TS= test substance (mg kg^{-1} of body weight); positive control = cyclophosphamide at 25 mg kg^{-1} (b.w.).

8. Conclusion

According to the obtained results and with the test conditions, the test substance GLYPHOSATE TECHNICAL did not induce positive effects dose related or isolated group ($p \leq 0.05$). Therefore, it could be concluded that the test substance did not show a mutagenic potential activity in mice.

9. References

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Annexes

Annex 01 – Analysis certificate

BIOAGRI LABORATÓRIOS

Analysis Certificate
CA – 708/08 R1

Company:	Jigma Chemicals Co., Ltda
Address:	No. 50 BaaTa Road Longyou, Zhejiang, China
Sample information:	
Commercial Name:	**GLYPHOSATE TECHNICAL**
Common Name:	glyphosate
Chemical Name (IUPAC):	N-(phosphonomethyl)glycine
Declared concentration:	min. 980.0 g/kg
Batch:	20070606
Bioagri code:	AGR 0790/07
Analysis Information:	
Test start:	29/May/2008
Test end:	29/May2008
Analysis certificate conclusion:	02/Jul/2008
Methodology used:	SOP M 0118 – Rev.: 06 – 26/Jul/2007
Equipment used:	Liquid Chromatograph (HPLC) HP 1100 (CL#26)

RESULTS OF THE ANALYSIS

Concentration of a.i. glyphosate: 980.0 g kg^{-1}

Observations: The results mentioned above are only referred to the submitted sample.
The documents and records of this certificate will be archived at BIOAGRI for a period of 10 years.

[redacted]

Responsible Analyst

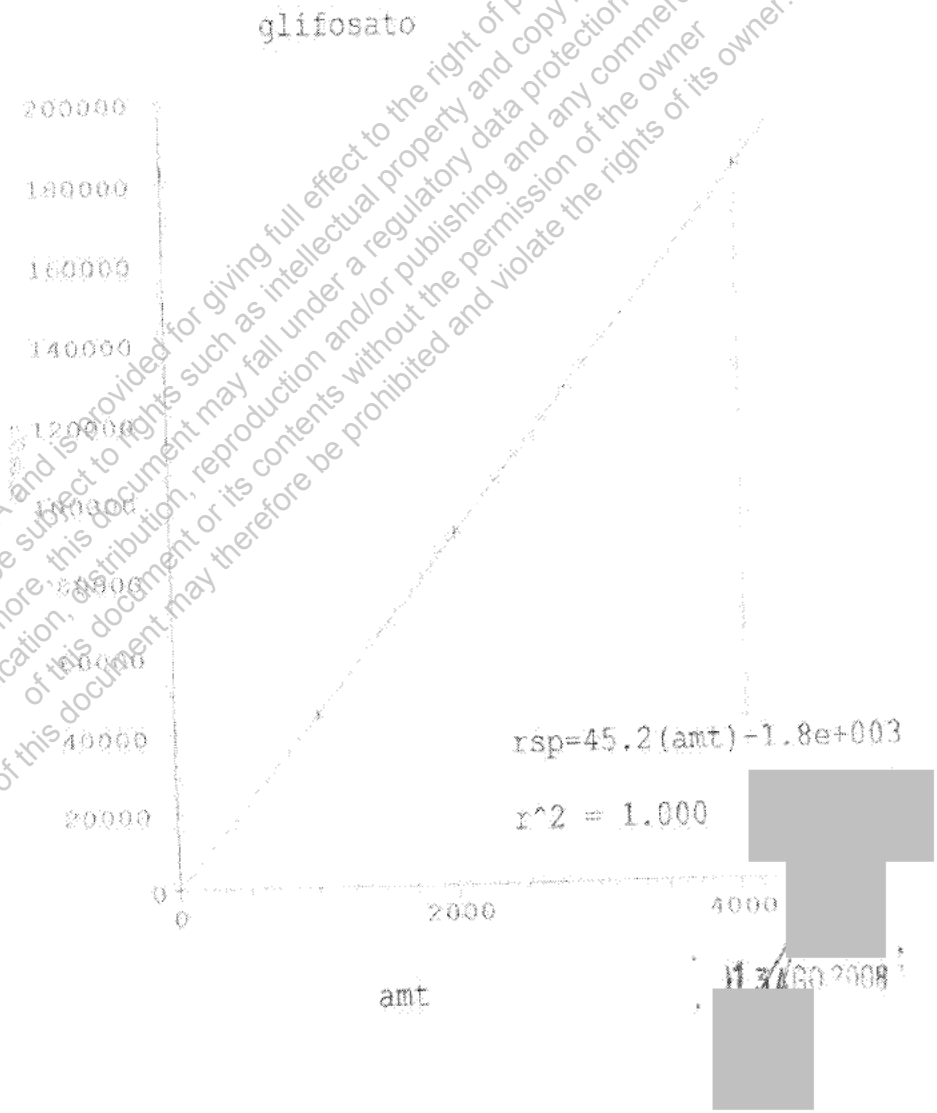
[redacted]

Responsible Technician

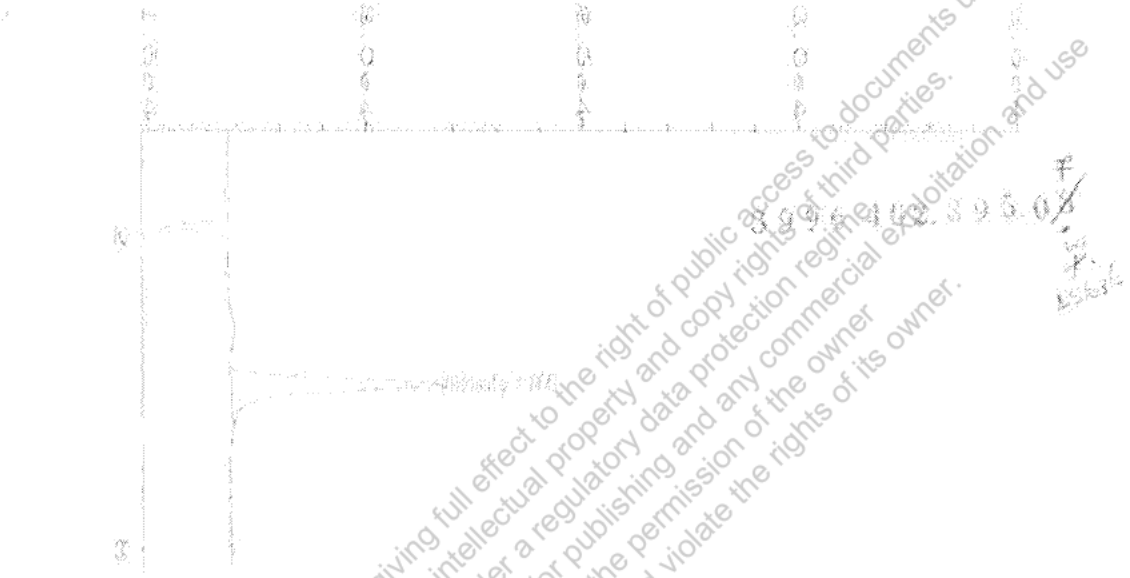
This Analysis Certificate CA-708/08 R1 cancels and substitutes the previously issued one CA-708/08.

Annex 02 - HPLC/UV chromatograms obtained from test solutions analysis

a) Linearity;



b) Standard;



External Standard Report

Data File Name : C:\HPCHEM\2\DATA\PUREZA\12088093.D
Operator : [redacted]
Instrument : HPLC 1050
Sample Name : Pad
Run Time Bar Code:
Acquired on : 13 Aug 08 07:03 AM
Report Created on: 13 Aug 08 10:49 AM
Last Recalib on : 13 Aug 08 10:37 AM
Multiplier : 1

Page Number : 1
Vial Number : 28
Injection Number : 2
Sequence Line : 5
Instrument Method: GLIFOSAT.MTH
Analysis Method : GLIFOSAT.MTH
Sample Amount : 0
ISTD Amount :

Sig.2 in C:\HPCHEM\2\DATA\PUREZA\12088093.D

Ret Time	Area	Type	Width	Ref#	ng/ul	Name
4.866	85460	BBA	0.262	1	1952.673	glifosato

[redacted]

13/08/2008

[redacted]

c) Test substance solution (4.16 mg mL^{-1}) results.

External Standard Report

```
Data File Name   : C:\HPCHEM\2\DATA\PUREZA\12088026.D
Operator         : [redacted]                       Page Number       : 1
Instrument       : HPLC 1050                        Vial Number       : 7
Sample Name      : pad07                            Injection Number  : 2
Run Time Bar Code:                                  Sequence Line     : 1
Acquired on      : 12 Aug 08  08:25 PM              Instrument Method : GLIFOSAT.MTH
Report Created on: 13 Aug 08  10:38 AM              Analysis Method   : GLIFOSAT.MTH
Last Recalib on  : 13 Aug 08  10:37 AM              Sample Amount     : 0
Multiplier       : 1                                ISTD Amount       :

Sig. 2 in C:\HPCHEM\2\DATA\PUREZA\12088026.D
Ret Time    Area      Type Width Ref#  ng/ul      Name
|-------|-----------|----|-----|-----|---------|------------------------------|
  4.848      182486 BBA  0.253  1    4077.249 glifosato
```

[redacted]

Appendix

Appendix 01 - Example of test substance's analyzed concentration calculation

$C_{ts} = C_f \times F \times 100/C_{ca}$
Cf = 1952.673 ng µL^{-1} or 1952,673 mg mL^{-1}
F = 2
Cca = 980.0 g kg^{-1} or 98%

C_{ts}= 1952,673 x 2 x 100/98
C_{ts}= 3.98 mg mL^{-1}

Where:
Cts = analyzed concentration of the test substance (mg mL^{-1})
Cf = concentration of the a.i. (test substance) determined by HPLC/UV (ng µL^{-1})
F = dilution factor (for some concentrations of the a.i. it was necessary to dilute the test solutions prior to analysis by HPLC/UV, so that the concentration following dilution was within the range over which the system response varied linearly with the a.i. concentration - Table 3)
Cca = concentration of the a.i. in the test substance (%), indicated on the Analysis Certificate

The deviation of the analyzed test substance concentration from the nominal concentration was calculated using the following equation:

$Dev = (C_{ts}-C_{nom}) \times 100/C_{nom}$
Dev = (3.98-4.16) x 100/4.16
Dev = 4.33%

Where:

Dev = deviation of the analyzed concentration from the nominal concentration (%)
C_{nom} = nominal concentration of the test substance (mg mL^{-1})

Appendix 02 - Endorsement of Compliance with the OECD Principles of Good Laboratory Practice

[illegible]

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on [illegible]

Bioagri Laboratórios Ltda.
Rodovia Rio Claro/Piracicaba Km 24
Piracicaba - SP - Brazil

It is hereby confirmed that the above-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Physical-chemical testing; Mutagenicity studies; Environmental toxicity studies; Studies on behaviour in water and soil; Analytical chemistry testing.

Den Haag, 19 December 2006

[redacted]

Manager GLP Compliance Monitoring Program

[illegible] Product Safety Authority (VWA)
[illegible] 2, 2595 AL Den Haag
Postbus 19506, 2500 CM Den Haag
The Netherlands

Appendix 03 - INMETRO GLP Accreditation

Federal Republic of Brazil
Ministry of Development, Industry and Foreign Trade
National Institute of Metrology, Standardization and Industrial Quality - INMETRO

(General Coordination for Accreditation)

Accreditation Certificate

Accreditation n° CLA 0002

Initial Accreditation: April 25th, 2000

BIOAGRI LABORATÓRIOS LTDA
RODOVIA SP, 127 - km 24 - GUAMIUM
PIRACICABA - SP

The General Coordination for Accreditation of Inmetro – CGCRE/INMETRO – grants accreditation to the above-mentioned Laboratory, according to the requirements establishED in NIT-DICLA-035 (General Requeriments for Laboratories, according to the Principles of Good Laboratory Practice - GLP). This accreditation contitutes the formal expression of recognition of the laboratory's competence to carry out studies as described in the scope of Accreditation.

Issue Date: March 25th, 2008

General Coordinator for Accreditation

Valid To: April 25th, 2010