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DECAGLIP - Colorimetric Detection of Glyphosate Herbicide

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Abstract: The project addresses the issue of the verification of the presence of pesticide N-(phosphonomethyl) glycine, commonly known as glyphosate, in small amounts through an innovative method of colorimetric detection. The purpose of this project is to find a facilitator of glyphosate residues' identification in apple samples, which may be used in the inspection of the same. The importance of the research is precisely to detect glyphosate in human consumption, since this pesticide is considered probable carcinogen in humans by IARC (*International Agency for Research on Cancer*), and its use is growing in an exceptional way in the whole world. The current methods for detection of glyphosate are based in High Performance Liquid Chromatography (HPLC), which is more expensive and complex than the colorimetric analysis. The proposed method involves the formation of glyphosate complexes with copper (II) ions in aqueous media; followed by the colorimetric detection which is based on the enzymatic activity of copper (II) in peroxidase reactions. Copper catalyzes the peroxidase reaction that occurs between the chromogen 3,3,5,5-tetramethylbenzidine (TMB) and H_2O_2, producing a blue color change in the solution. When copper is complexed with glyphosate, it occurs the inhibition of catalysis and hence lesser intensity of the color produced. Thus, the concentration of glyphosate in solution can be determined by the formation of copper-glyphosate complexes, which prevent the color development. According to the results obtained so far, including the successfully glyphosate extraction from apple samples using the QuEChERS extraction method, it is possible to observe color changes in the solutions distinguished by naked eye and absorbance changes at very low concentrations, up to 1.185 ppm of glyphosate, by the method of UV–visible Molecular Absorption Spectroscopy using 655 nm of wavelength. Therefore, the proposed method shows high sensitivity, besides its simplicity of procedure and immediate response.

Keywords: Pesticides, Glyphosate, Residue analysis, Colorimetric detection method.

I. INTRODUCTION

In the last ten years, the growth rate of the world pesticides market was about 93%, while in the brazilian pesticides market the growth reached almost 190% [1]. Glyphosate, besides being one of the most used pesticides in Brazilian agriculture, has the highest production volume between all herbicides. It was classified as a carcinogenic for human beings by the world's biggest institution for research on cancer: *IARC*, agency member of the World Health Organization (WHO) [2]. The consumption of this herbicide, although its toxicity is not classified at the most dangerous categories, it is restricted by law and its acceptable daily ingestion is extremely low: 0.042 mg/kg/day in Brazil [3].

This project focus on creating a new method to facilitate the identification of glyphosate in small quantities, presenting a high importance to the industrial, social and alimentary fields, once that this herbicide use have

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grown exponentially in rural cultures [4]. Health agencies, responsible for the fiscalization of pesticides in food, could use this method as an alternative to control the constantly growing glyphosate rate. Thereby, the benefit expands also to consumers in general, due to the possible wide access of controlling the herbicide presence on their own nourishments through intensify of fiscalization.

In this research, it was chosen primary to work with apple samples for the extraction method due to its satisfactory pigmentation and wide spread of glyphosate on its crops.

Given above, a new and simple alternative to detect the herbicide can be developed, aiming to reduce the complexity of glyphosate identification, seeking a way to turn this process cheaper, more accessible and proposing an improvement to the current public health conditions.

II. GOALS

Develop an identification method of small quantities of glyphosate residues in water and food samples, focusing in apples, through an innovative colorimetric detection method which involves copper-glyphosate complexes. Also verify its sensibility and efficiency of detection in lower concentrations.

III. MATERIALS AND METHODS

The project has the character of experimental research, it has started in February of 2016 and is still in progress. The research methodology was performed at the dependencies of Fundação Liberato Salzano Vieira da Cunha (FETLSVC).

Commercial Glyphosate Herbicide/ Glyphosate Dilutions

The commercial glyphosate herbicide used as standard pursue two main components: glyphosate and AMPA (aminomethylphosphonic acid), its metabolite. It was chosen to use this specific herbicide in the research due to its commercial use at large scales in rural settings around the world. The concentration is 44.5% w/v glyphosate diammonium salt and 37% w/v the acid equivalent. The Brazilian legislation establishes that active ingredient in commercial pesticides must follow the maximum variation of 5%, considering the value declared in registration process [5]. Thus, the concentration of glyphosate in the commercial herbicide was considered 37 (±1.85) % w/v.

The concentrations used were diluted from the acid equivalent, which has 3700 (±185) ppm. The concentrations obtained were: 1850 (±92.5) ppm; 185 (±9.25) ppm; 92.5 (±4.63) ppm; 74 (±3.7) ppm; 37 (±1.85) ppm; 18.5 (±0.93) ppm; 9.25 (±0.46) ppm; 3.7 (±0.19) ppm; 0.925 (±0.046) ppm; 0.370 (±0.0185) ppm and 0.185 (±0.009) ppm. These concentrations were compared to the values of millimolar concentrations of glyphosate. In the course of this research, it was chosen to maintain the concentrations in parts per million (ppm) as a reference, due to the fact that this unit of concentration is currently used as a standard in Brazilian legislation and by Brazilian surveillance agencies.

Chromogens

The chromogens were obtained through donations from institutions and laboratories that apply the ELISA method in their tests. These reagents are generally used for the detection of diseases such as HIV, toxoplasmosis, etc. The chromogenic substrates generate soluble products with certain specific coloration, almost always blue or yellow. According to Bender [6], the peroxidase reaction occurs between hydrogen peroxide and a chromogen, being one of the most currently used the tetramethylbenzidine (TMB).

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Colorimetric Detection

The colorimetric reaction in which this project was based on occurs due to the peroxidase reaction of chromogen 3,3,5,5-tetramethylbenzidine (TMB) with copper (II) ions, which have a peroxidase-like activity and catalyze the oxidation of TMB, producing a blue coloration. However, when glyphosate is available in the solution, the formation of copper-glyphosate complexes occurs. Glyphosate has a strong propensity to form complexes with metals in aqueous solution [7]. The formation of these complexes leads to the inhibition of the enzymatic activity of copper (II) in the peroxidase reaction, once copper will be in complexed form. Thus, glyphosate can be detected by the color variation color of the solution: the higher the glyphosate concentration, the greater the number of copper-glyphosate complexes formed and, consequently, the lower the blue color intensity.

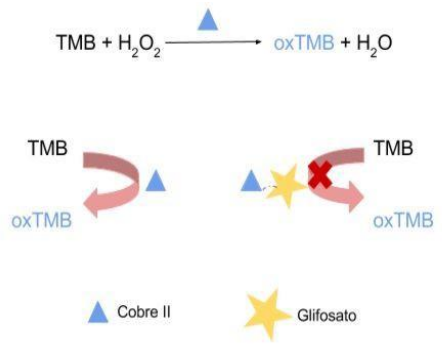

Fig. 1. Scheme of colorimetric reaction.

The basic principle of the colorimetric detection is demonstrated in Fig. 1. In the absence of glyphosate, copper imitates an enzymatic activity and is able to catalyze the reaction, forming a strong blue coloration. But when glyphosate is added, copper and glyphosate react generating complexes. These, by their turn, inhibit the enzymatic activity performed by copper, producing a correspondingly weaker color in the solution to the increase of the glyphosate concentration. The increases of glyphosate concentration decreases the color intensity.
The reactional characteristics and amount of reagents were used according to Chang [8]. Initially, 440 µL of acetic acid and sodium acetate buffer (pH 4.0) were volumetrically added to the mini test tubes and 20 µL of glyphosate of 3.7 ppm concentration and 20 µL of 2.5 mM $CuSO_4$ were added in this solution. After 5 minutes at room temperature, 10 µL of TMB chromogen and 10 µL of 1M H_2O_2 were added, followed by heating at 40°C for 20 minutes. As a validation control of the presented method, a test was carried out to verify the occurrence of the peroxidase reaction and color formation considering the present variables.

Table 1. Test to verify the occurrence of the reaction by varying the reagents.

A	TMB + H_2O_2
B	TMB + H_2O_2 + Cu_2+
C	TMB + H_2O_2 + Cu_2+ + Glyp
D	TMB + H_2O^2 + Glyp

Thus, different mechanism conditions were tested counting on the absence or presence of each reagent, as shown in the Table 1.

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UV–Visible Molecular Absorption Spectroscopy

The coloration produced by the peroxidase-like reaction was detected through molecular absorption spectroscopy, using a BEL Photonics Spectrophotometer SP1102. This analytical method allows the measurement of the trials' absorbances right after the peroxidase reaction, identifying then the darker and lighter colorations, which correspond to the glyphosate concentration on the respective sample [8]. The wavelength used on the spectrophotometer was 655 nm and the measurements were conducted at room temperature. Glyphosate concentration on the trials varied from 0,185 ppm to 1850 ppm.

Extraction of Glyphosate Residues

The extraction method applied to the extraction of glyphosate residues on apple samples was the QuEChERS method: a new and recent method introduced to the analytical chemistry in order to enable the extraction of pesticides from a wide range of polarities through the use of acetonitrile as a solvent [9]. QuEChERS is currently used as a simple extraction method of pesticides which will be posteriorly analyzed by HPLC (High Performance Liquid Chromatography) [10].

Firstly, the apple was crushed until an extract consisting, then heated until achieving a temperature between 70°C and 90°C with the purpose of eliminate the apple's active enzymes. Then, the apple extract was separated into four trials, of 1.0 gm each and all of them were fortified with known concentrations of glyphosate.

In each trial, it was added 4 mL of acetonitrile - whose function is to extract the glyphosate from the samples - and anhydrous salts - whose function is to withhold the water and therefore ameliorate the extraction of glyphosate. The quantity of salts used were 0.8 gm of $MgSO_4$ and 0.2 gm of NaCl, followed by agitation and centrifugation on a centrifuge FANEM Baby I Centrifuge Mod 206 for 5 minutes in 2000 rpm.

The next step is called low temperature partitioning: the centrifuged supernatant is freezed for 48 hours allowing a liquid-liquid partition of water and solvent, once the water melting point is 0°C and the acetonitrile one is -20°C [11]. Then, the remained water in the solution freezes, creating a heterogeneous solution that makes possible the extraction of glyphosate along with the acetonitrile surnageant.

After the glyphosate extraction from the apple samples, the proposed colorimetric method is applied on the same quantities and conditions as described in 3.3. The whole process was repeated thrice. The difference, thus, is that now the colorimetric method is assigned also to the apple samples besides water ones.

IV. RESULTS AND DISCUSSIONS

Glyphosate Detection

It was possible to determine the best conditions for the colorimetric reaction by testing reagents quantities and it was found that the best ratio is 60 µL of glyphosate, copper and TMB solution to 40 µL of hydrogen peroxide solution.

In this condition, it was possible to differentiate, by naked eye, the coloration of the control solution (right), without addition of glyphosate, and the solution with a concentration of 37 ppm of glyphosate (middle). In addition, it was observed that no color formation occurred in the 3700 ppm glyphosate solution (left) (Fig. 2).

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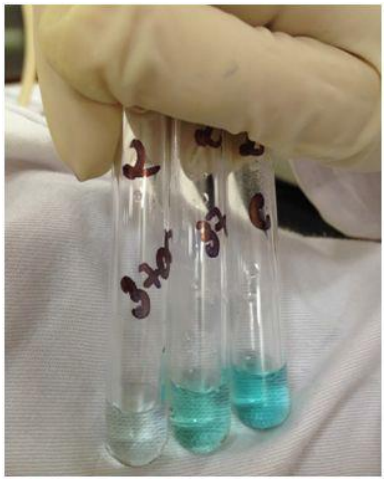

Fig. 2. Colors obtained by concentrations of 3700 ppm, 37 ppm and control.

Among the other variables tested, interferences from exposure to light, time and temperature were found out. The reaction occurs preferably out of direct contact with light, and this happens because the chromogen is sensitive to it. The ideal heating time in the water bath varies between 10 and 20 minutes, since after this time, the chromogen starts to degrade. Finally, the optimum temperature in the tests was 37 (±2)°C. There is no reaction occurring at room temperature and above 50°C the formed color weakens and disappears.

The test developed for verification of the reaction mechanism presented results that confirm the confiability of the method (Fig. 3).

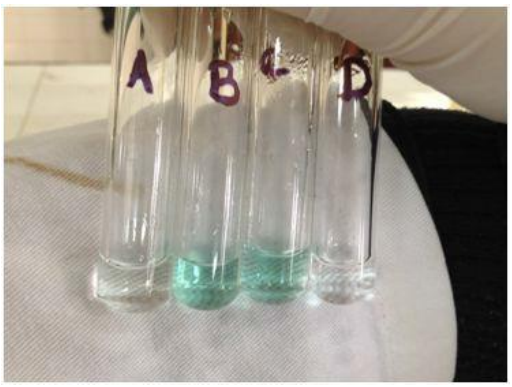

Fig. 3. Colorations formed by the test by varying the reagents.

In tubes A and D, which do not have copper (II) ions in the solution, the formation of blue color did not occurred. Among the tubes with the formation of blue staining, it is possible to notice a higher color intensity in tube B, due to the presence of only copper ions and the absence of glyphosate. Tube C shows a weaker coloration, due to the low concentration of glyphosate added to the solution. Tube B is equivalent to the trials control and tube C to the common trials to detect glyphosate.

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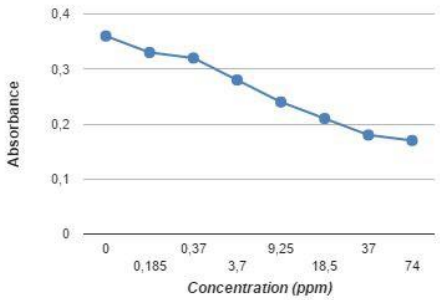
Confirmation through Molecular Absorption Spectrophotometry

The next step to the colorimetric detection method consisted on verification using UV-visible Molecular Absorption Spectrophotometry. In this step, it was measured through the equipment the absorbances relative to the color generated in the peroxidase reaction at each sample of prepared glyphosate concentrations. Using the same conditions as the previous tests, a pretest was performed to analyze the absorbance variation (Table 2).

Table 2. Pretest concentrations and respective absorbances.

Trial/Glyp Concentration	Absorbance
Trial Control (0 ppm)	0.59
Trial 1 (37ppm)	0.37
Trial 2 (185ppm)	0.27

The pretest presented an increasing and possibly proportional change in the absorbances. Posteriorly, a larger scale of concentrations were applied into the method, following the same procedural conditions, and the obtained absorbances were inserted into a graph. This procedure was repeated twice.



Graph 1: Absorbance × Glyp concentration in water samples.

The graph indicates that there is an absorbance decrease as concentration increase in the trials. It was observed that the values obtained by Graph 1 show a huge absorbance difference up to the concentration of 37 ppm, and above this, they started to present more constant values. In other words, the method does not differentiate the coloration produced. According to Chang [8], the chromogens have efficiency at low concentrations, with a limit of 200 µM. The calculations performed indicate that the concentration of 200 µM is equivalent to 33.82 ppm. Thus, the linearity can be up to concentrations of approximately 30 ppm and does not invalidate the colorimetric method, since it continues to identify colorings that can be differentiated from control.

Extraction in Apple Samples

The efficiency of QuEChERS extraction method was also confirmed by spectrophotometry, using the same spectrometer and conditions as mentioned above. The differences in coloration were also visible by naked-eye, when comparing the control trial - without any addition of glyphosate - and the ones with increasing concentrations of glyphosate. The Table 3 shows the concentrations of glyphosate on the trials and their respectives absorbances (an arithmetic mean of the values obtained on each repetition).

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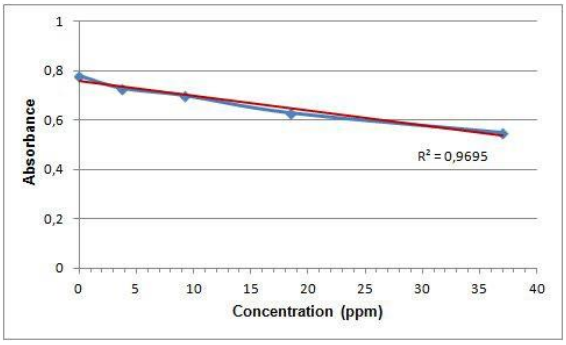
Table 3. QuEChERS extraction trials' absorbance.

Trial/Glyp Concentration	Absorbance
Trial Control (0 ppm)	0.78
Trial 1 (3.7 ppm)	0.73
Trial 2 (9.25 ppm)	0.7
Trial 3 (18.5 ppm)	0.63
Trial 4 (37 ppm)	0.55
Trial 5 (74 ppm)	0.54

As it can be seen from the table, there is a decrease in the absorbance according to the increase in the concentration, proving the efficiency of the proposed method. Graph 2 compares glyphosate concentrations and absorbances.



Graph 2. Absorbance × Glyp concentration in apple samples.

The graph linearity, indicated by the correlation R^2=0.9656, means that the method presents a good functionality in detecting the approximated glyphosate concentration in apples samples, through the colorimetric detection followed by QuEChERS extraction.

Considerations and Perspectives

After the obtained results, some considerations can be made. When it comes to the colorimetric method, its high sensibility was confirmed once it shows good results on low concentrations. Moreover, the method cost of application is much lower than the current methods applied on industry, acquiring then an economical and procedimental viability to be implemented in large scale.

V. CONCLUSION

Considering the studies and experiments developed in this project, it was possible to verify that low concentrations of glyphosate can be detected through the proposed method, forming soluble copper-glyphosate complexes which were later identified by the inhibition of the peroxidase reaction of TMB. Thereby, the use of this colorimetric method was

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able to detect the presence of glyphosate residues in water and apple samples. It can be stated that, after some method aprimoration and new confirmatory tests, it will be possible to detect glyphosate in other fruits besides apples.

By the end of this step, it is then verified that the initial goals were successfully achieved. Overall, the project conception presented an alternative to the detection of glyphosate residues, being this of high sensibility, immediate response and efficiency of procedure.

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