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Determination of Glyphosate Residue in Genetically Modified Soybean by Protein Precipitator Clean-up and HPLC with OPA Post-column Derivatization

Abstract: The method of detecting glyphosate in genetically modified (GM) soybean through High Performance Liquid Chromatography (HPLC) with post-column derivatization was established and chromatography conditions were optimized in this paper. Three precipitators, such as acetonitrile, hydrochloric acid and a combination of potassium ferrocyanide and zinc acetate, were used respectively to remove protein from soybean slurry. Results show that the purifying effect of hydrochloric acid was better than others. Then the precipitated soybean solution sample after centrifugation was defatted with methylene chloride, and the supernatant was analyzed by HPLC with post-column derivatization. Sample recovery rates are between 80% and 93%. The relative standard deviations (R.S.D.) of the glyphosate content measurements are in the range 2.87-4.98%, and the detection limit is 2.5 μg/mL. So this method has high sensitivity and accuracy, and it could be applied in detecting glyphosate residue in other protein grains.

Keywords: genetically modified soybean, clean-up, glyphosate, HPLC.

1 Introduction

In developed countries, the great economic benefit had been taken with the planting of GM crops while the GM soybean was the most important one of glyphosate-resistant crops. Glyphosate, called N-(methyl phosphonic) glycine, was regarded as a kind of low toxicity, high-efficiency, broad-spectrum herbicide introduced by the Monsanto Company in the United States. It was reported that glyphosate could be transferred and gathered in fishes, amphibious animals, causing vertebrates morphological changes of organs [1,2]. Research of WHO showed that keeping in touch with glyphosate for a long time could induce various symptoms, such as the decrease of mice fetal number

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and sperm, the increasing rate of preterm birth and the promoting of abortion [3]. And Thongprakaisang et al found in 2013 that the presence of even very low concentration of glyphosate (10^{-12}-10^{-6} mol/L) could facilitate the growth of cancer cells [4]. In recent years, the residue of glyphosate and its metabolite aminomethyl phosphoric acid (AMPA) had been detected in GM soybean [5], where the content of glyphosate residue was 3.908 mg/kg and AMPA was 3.364 mg/kg. The limited content of glyphosate in soybean prescribed by US, European Union and Japan was 20 mg/kg [6]. As the largest GM soybean importer, China's soybean imports reached 63.38 million tons in 2013. More than 90% of soybean oil and blend oil containing soybean ingredient were made from GM soybean [7]. In China, with the increasing consumption and import of GM soybean, edible risk brought by the glyphosate residue in soybean was gradually enhanced. Therefore, surveillance of the glyphosate residue in GM soybean should be strengthened. Beyond that, establishing a high-efficiency and sensitive method to determinate glyphosate was an extremely urgent problem.

Hardly was glyphosate dissolved in common organic solvents and its solubility was only 1.2% in water at room temperature. Glyphosate, having chemical and physical properties of strong polarity, absence of chromophore and fluorophore, was difficult to volatilize [8]. In addition, the high content of oil and protein in soybean would interfere with UV absorption of glyphosate. Therefore, it was a challenge to detect the glyphosate accurately. At present, several methods of detecting the residual glyphosate in drinkable water, fruits and vegetables have been described. For example, glyphosate of Oolong tea, extracted through ultrasonic and solid phase extraction (SPE) technique, was detected by HPLC-MS after derivatization [9], but the equipment was expensive, and that is why its popularity was relatively low. Glyphosate residue of apple, which was extracted with deionized water and purified by ion exchange columns, was detected by GC-NPD after derivatization [10], but steps were tedious and the cost was higher. Those methods above suffered from complicated and tedious sample preparation. Glyphosate content of poisoned blood was observed by UV spectrophotometry [11]. However, the UV spectrophotometry method has poor reproducibility, more disturbed factors and low accuracy.

To obtain a suitable detection method for detecting glyphosate residue in GM soybean rich in protein and fat, HPLC method was developed in this paper. Methods of HPLC with pre-column derivatization, UV detection and post-column derivatization fluorescence detection were investigated respectively, expecting to establish an accurate and reliable method fot he detection of glyphosate residue in GM soybean.

2 Experimental

2.1 Reagents

Glyphosate standard substance was purchased from Dr. Ehrenstorfer GmbH (Germany). Transgenic soybean inputted from Columbia was provided by Nantong Laibao Oil Plant. Lithium Eluant (Cat.NO:1700-1125), O-Phthalic Aldehyde (OPA) (Cat. NO:OD104), OPA diluent (Cat.NO:O120), and THIOFLUOR™ (Cat.NO:3700-2000) were obtained from Pickering Laboratories Company (California, United States). Methanol, methylene dichloride, and acetonitrile were chromatographic grade reagents from Honeywell B&J (Morris County, New Jersey, United States). Hydrochloric acid, ferrocyanide and zinc acetate were domestic analytical reagents from Xilong Chemical Co., Ltd. (Guangdong, China). Other reagents were analytical pure. Deionized water obtained from a MilliQ water purification system (Millipore Ltd., Massachusetts, United States) was used for solution preparation.

2.2 Instrumentation

An Agilent LC-1100 HPLC (Agilent Technologies Inc., California, United States) with an online degasser, a quaternary pump, fluorescence detection (FLD) system, an auto sampler, and post-column derivatization system (Pickering Laboratories Company, California, United States).

2.3 Sample Preparation

The powdered GM soybean (5 g) was weighed thrice as three samples, placed in centrifuge tube with 30 mL deionized water and dealt with ultrasound for 30 min. Then three cleaning agents, such as 5 mL acetonitrile [12], 5 mL potassium ferrocyanide (106 g/L), and the combination of 5 mL zinc acetate (220 g/L) and 200 µL hydrochloric acid, were added into the sample solution respectively [13]. After the sample solution was diluted to 50 mL with deionized water and processed by ultrasound for 10 min, the supernatant was gotten through centrifugation (10,000 rpm) for 5 min at 0°C. Next, 10 mL supernatant was mixed with 15 mL methylene dichloride [13], vortex-mixed for 10 min and centrifuged (10,000 rpm) for 5 min at 0°C. The upper centrifugal liquid was placed to a new container and the described step was repeated twice. Finally, the purified extract liquid for HPLC determination, was prepared.

2.4 Detection of Glyphosate Content by HPLC with Pre-column Derivatization

The sample was tested using the method of nitrosation in this paper. The purified extract liquid from Step 2.3 (5 mL) was added with 2 mL sulfuric acid (100 g/L) and 0.5 mL potassium bromide (2.0 mol/L), shaken up and added with 0.5 mL sodium nitrite (50 g/L), followed by resting place for 30 min. The volume was adjusted to 100 mL with distilled water, and the sample was analyzed through HPLC after being filtrated through 0.45 μm membrane.

The analysis column was ZORBAX Eclipse XDB-C_{18} (150 mm×4.6 mm, 5 μm, Agilent Technologies Inc., California, United States). Methanol (A) and water (B) (v (methanol): v (water) = 2:98, 0.1% phosphoric acid was contained in water phase) was used as the mobile phase at the constant flow rate of 0.8 mL/min. The UV detective wavelength was 240 nm. And the column temperature was maintained at 25°C.

2.5 Detection of Glyphosate Content by HPLC with Post-column Derivatization

The sample determination was performed using an HPLC-FLD system. OPA solution used for post-column derivatization was prepared before use as follows. OPA (O-Phthalaldehyde) (63 mg) was dissolved with 2 mL methanol followed by addition of 0.42 g THIOFLUOR™. The liquid volume was adjusted to 200 mL with OPA diluent and filtrated through 0.45 μm membrane.

The analysis column was Thermo LITHIUM AMINO ACID ANALYSIS chromatography column (100 mm×4.0 mm, 5 μm, Picking Laboratories United States) and the column temperature was maintained at 40°C. Lithium Eluant was used as the mobile phase at flow rate of 0.3 mL/min. The FLD detector with wavelengths was set at 355 nm (Ex) and 465 nm (Em) and the reaction tank temperature was maintained at 44°C.

2.6 Optimization of Chromatographic Conditions and Validation of the Method

Good purification and derivatization methods were chosen in order to detect and analyze glyphosate. And the chromatographic conditions and method effectiveness were observed.

1. **Optimization of chromatographic conditions:** The glyphosate standard liquid (400 μg/mL) chromatography spectra taken respectively by the different elution flow rate of 0.2 mL/min [14] and 0.3 mL/min [9], and the different wavelength of 355 nm (Ex) [15] and 330 nm (Ex) [16] were compared to choose the suitable flow rate and detective wavelength.
2. **Validation of the method:** Four batches of spiked samples were analyzed to evaluate the performance of the method. The smashed soybean (5 g) was weighed

accurately and placed into 50 mL polypropylene centrifuge tube. The spiked levels were 5 μg/mL, 10 μg/mL, 15 μg/mL, and 20 μg/mL (n=4). The method was valid when the R.S.D was less than 5%.

3 Results and Discussion

3.1 The Purification Method

Samples disposed with different precipitators were detected by HPLC with post-column derivatization at the flow rate 0.3 mL/min and the wavelength 355 (Em) and 465 (Ex) (Fig. 1-Fig. 3). Results showed that the purifying effect of hydrochloric acid was the best because the retention time of glyphosate standard (400 μg/mL) was 3.8 min and the impurity peaks between 3.5 min and 5.0 min were relatively weak, the better was acetonitrile precipitation. Considered hydrochloric acid was the cheapest among three precipitators, this reagent was chosen to purify soybean slurry, mainly for removing protein from the sample. Because some interference could be still existed in the disposed sample (Fig. 3), dichloromethane was adopted to purify the sample further. A large amount of lipid was removed by dichloromethane and the interference to instrument was avoided, though the HPLC spectrum was not changed notably (Fig. 4). This step greatly reduced the risk of damaging the equipment.

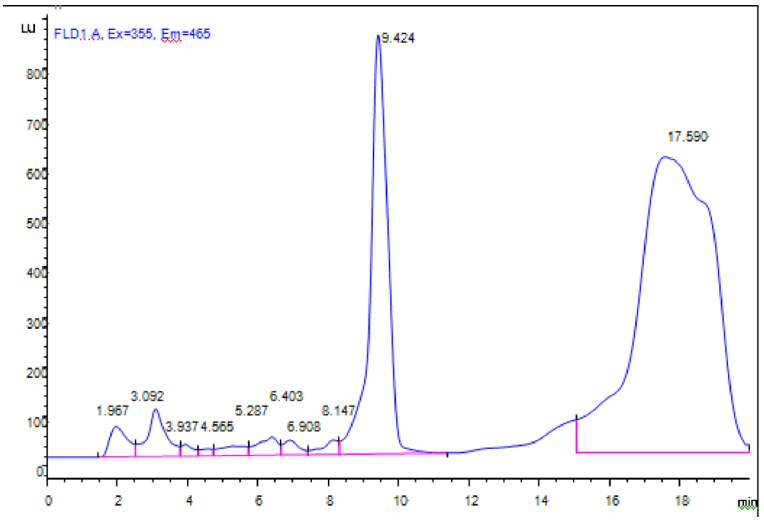

Figure 1: Chromatogram obtained from sample with acetonitrile as protein precipitator.

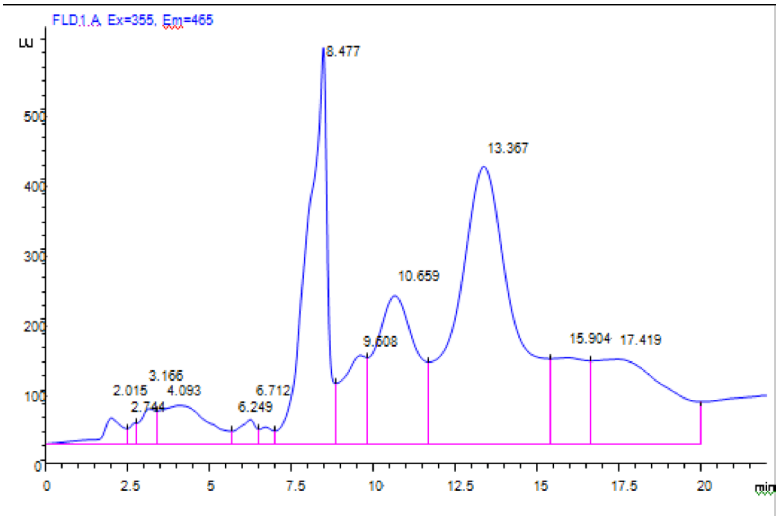

Figure 2: Chromatogram obtained from sample with ferrocyanide and zinc acetate as protein precipitator

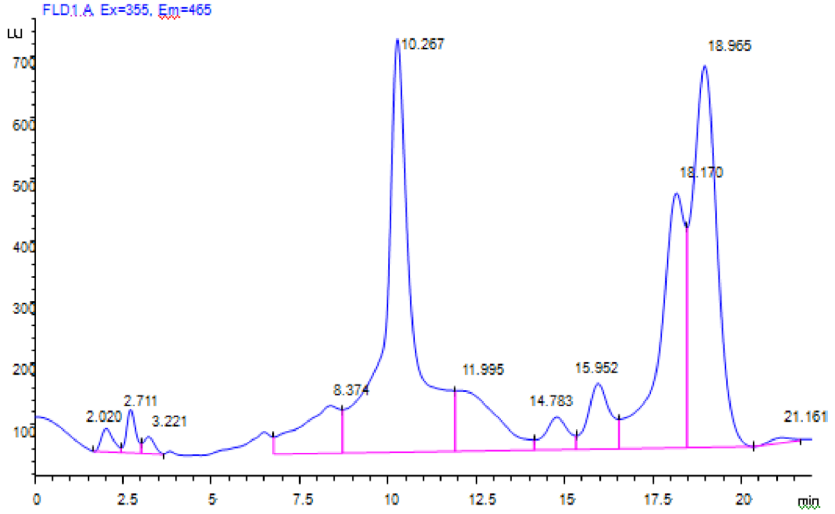

Figure 3: Chromatogram obtained from sample with hydrochloric acid as protein precipitator.

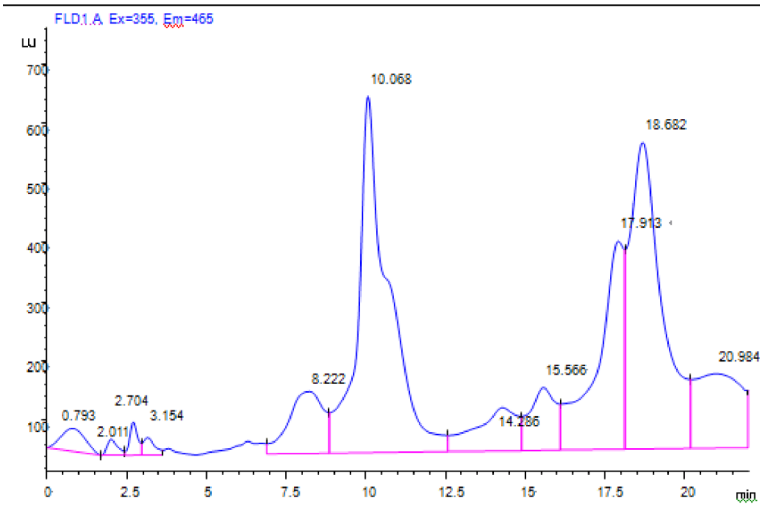

Figure 4: Chromatogram obtained from sample purified by dichloroethane with hydrochloric acid as protein precipitator.

3.2 The Derivatization Method

The spectra of glyphosate standard (400 μg/mL) by HPLC with pre-column derivatization and by HPLC with post-column OPA derivatization were illustrated in Fig. 5 and Fig. 6. The HPLC with pre-column derivatization method has been applied in determinating biogenic amines in germinated and fermented brown rice [17], and glyphosate in water and plant material [18]. Characteristic peak of glyphosate that appeared at 5.5 min was weak (Fig. 5). An interference peak at 4.8 min which was possibly the product from nitrosation derivatization had almost the same response value as glyphosate and must be a great influence on detecting glyphosate. Meanwhile, it showed that the impurity peak at 2.1 min in the spectrum from HPLC with post-column OPA derivatization (Fig. 6) was weak so its effect on the strong characteristic peak of glyphosate at 3.8 min could be neglected. Therefore, the latter method was more suitable to detect glyphosate abstracted from GM soybean than the former. It was reported that N-methylcarbamate pesticides in vegetable and water samples [19], and organothiophosphorus pesticides in water could be determined by HPLC with post-column chemiluminescence detection [20].

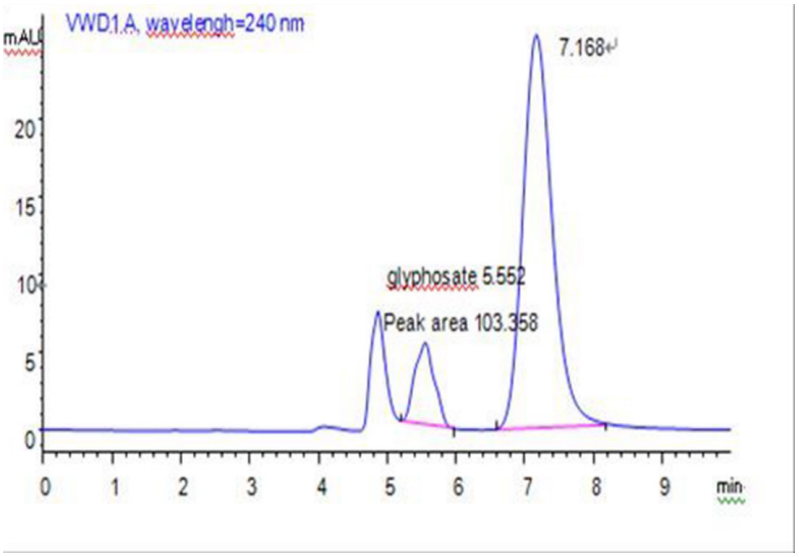

Figure 5: The chromatogram of glyphosate by HPLC with pre-column derivatization.

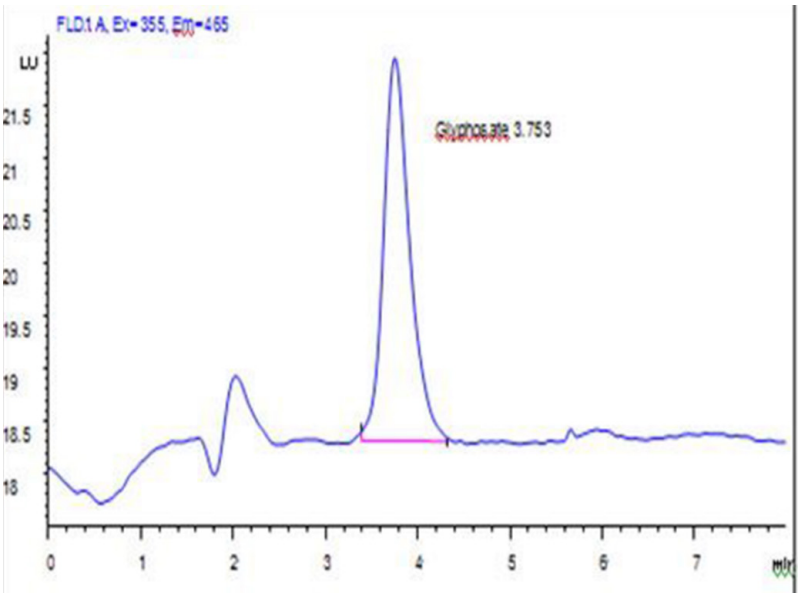

Figure 6: The chromatogram of glyphosate standard by HPLC with OPA post-column derivatization.

3.3 Optimization of HPLC post-column OPA Derivatization Chromatographic

1. **The flow rate:** The glyphosate standard was detected at the flow rate of 0.2 mL/min and 0.3 mL/min respectively (Fig. 7 and Fig. 8). The retention time of glyphosate peak was 5.45 min and peak shape was wide, the impurity peaks appeared before glyphosate had serious trailing phenomenon at the flow rate of 0.2 mL/min (Fig. 7). In contrast, the peak shape of glyphosate at the flow rate of 0.3 mL/min was sharp and kept a certain distance with impurity peaks, which reduced the interference (Fig. 8). So the flow rate 0.3 mL/min was chosen.

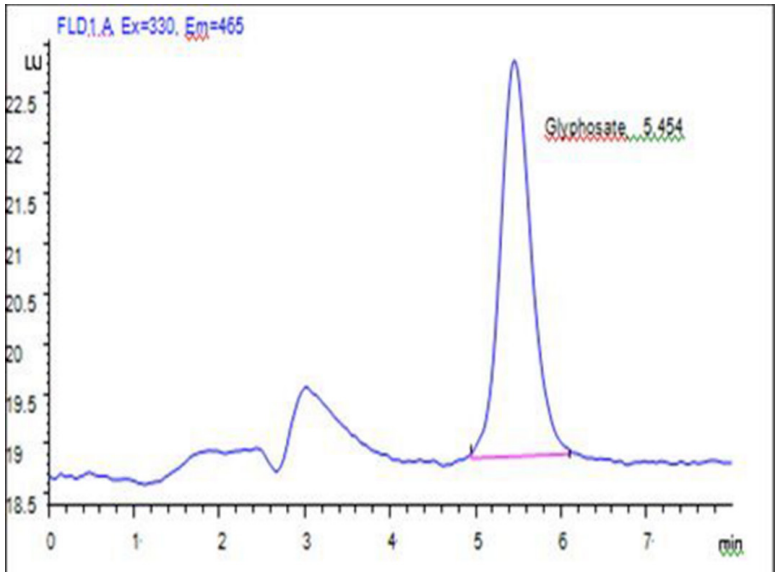

Figure 7: The chromatogram of glyphosate standard at the flow rate of 0.2 mL/min (the glyphosate concentration of 400 μg/mL)

2. **The wavelength:** The glyphosate standard (400 μg/mL) was detected at the wavelength of 330 nm (Ex) and 355 nm (Ex) respectively, which's peak area was 71.9 mAU*s and 86.7 mAU*s (Fig. 8 and Fig. 6). The response value of glyphosate at 355 nm (Ex) was higher, so the 355 nm (Ex) wavelength was chosen.

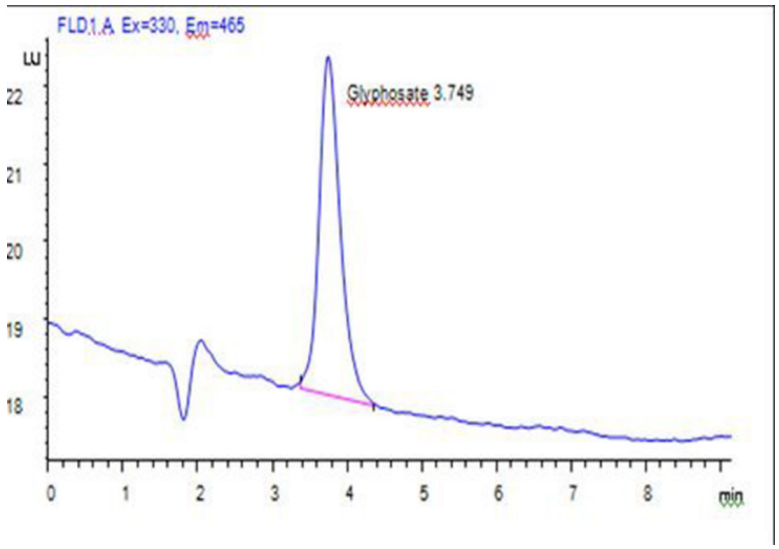

Figure 8: The chromatogram of glyphosate standard by HPLC with OPA post-column derivatization.

3.4 Detection Limit

A series of glyphosate standard samples were analyzed according to the optimized condition of HPLC. The regression statistics were calculated from the calibration curve constructed. The equation of linear regression was y=0.181x-0.440 and showed satisfactory linearity with the correlation coefficient (r2=0.9999) greater than 0.999. There was a good linear relationship in the range of 0.5-4.0 μg/mL.

The sensitivity of the method was expressed as limit of detection (LOD). The LOD was 2.5 μg/mL, calculated as three times the average baseline noise (S/N=3).

3.5 Recovery and Precision

The recovery and precision of the method were evaluated with adopting optimized purification condition and listed in Table 1. Sample recovery rates were 80.7-92.8%, R.S.D. of the glyphosate content measurement were 2.87-4.98%, which was less than 5%. So this method was high repeatability.

Overall, although the HPLC method with pre-column derivatization has been successfully applied for detecting glyphosate residue in chestnut which was obtained by extracting with water and purifying with SPE [21], the application in determining glyphosate residue in soybean was not as easy as expected.

Table 1: Recovery rates and precisions of the method

Sample No.	Matrix value (μg/mL)	Spiked concentration (μg/mL)	Recovery rate (%)	Relative standard Deviations (%)
1	n.a	5	80.7	4.98
2	n.a	10	82.3	4.48
3	n.a	15	85.9	3.49
4	n.a	20	92.8	2.87

Results showed that the glyphosate peak from pre-column derivatization was disturbed by impurity, while the glyphosate peak from post-column derivatization showed a good shape and was hardly interfered by impurity. Up to now, there have been several methods used in detecting residual glyphosate in food. HPLC-MS method was applied for detecting glyphosate residue in rice, maize and soybean samples which was ultrasonic extracted and purified with SPE [22]. Soybean sample for HPLC-MS analysis was prepared after methanol deproteinization and methylene chloride decontamination [23]. The glyphosate in fruits and vegetables was detected by mixed-mode hydrophilic interaction/weak anion-exchange liquid chromatography couples with electrospray tandem mass spectrometry [24]. Compared with above mentioned methods, the method of HPLC with post-column OPA derivatization could directly detect glyphosate residue in samples, low cost, high efficiency.

3.6 Sensitivity and High Popularity.

In this experiment, there was no residual glyphosate detected in Columbia soybean sample by HPLC with post-column derivatization under optimized conditions. This might be a reason that the glyphosate residue was less in the soybean sample used in the experiment and lower than LOD of equipment. So this batch of transgenic soybean inputted from Columbia was safe in its glyphosate content. Mo et al tested residual glyphosate in sugarcane sample extracted with acetonitrile by UPLC-MS [25]. The result suggested that residual glyphosate of samples was not being detected. Li et al. used hydrochloric acid and methylene chloride to deal with protein in soybean samples, and analyzed it by GC-MS after purifying by anion-exchange column [14]. The result was that glyphosate residue of non-GM soybean was not detected and glyphosate residue content in GM soybean was 0.08 mg/kg.

4 Conclusions

In this paper, the method of detecting glyphosate in soybean was developed. Three different precipitators, acetonitrile, potassium ferrocyanide, and the combination of zinc acetate and hydrochloric acid, were compared to choose a suitable purifying soybean slurry reagent to remove protein. Among them, hydrochloric acid had the best purification efficiency. The HPLC with OPA post-column derivatization method had more advantage than HPLC with pre-column derivatization method. The optimized conditions were the flow rate 0.3 mL/min and the wavelength 355 nm (Ex) and 465 nm (Em). This method was developed and validated for detecting glyphosate residue in soybean. In the concentration range of 0.5-4.0 μg/mL, the correlation coefficient (r^2) of glyphosate standard curve was 0.9999, the sample recovery rates were 80.7-92.8%, R.S.D. of the glyphosate content measurements were 2.87-4.98%, and the detection limit was 2.5 μg/mL. This method, as an attractive alternative to the traditional or expensive methods, allowed a rapid and direct determination of glyphosate residue in GM soybean and is expected to be applied in other high protein grains.

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Conflict of Interest: This article does not contain any studies with animal or human subject.

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