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RESEARCH ARTICLE

Development and Validation of an UPLC-MS/MS Method for Quantification of Glyphosate in Urine

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SUMMARY

For the last four decades, the herbicide glyphosate has been the most widely used herbicide worldwide, including in Turkey, with the assumption that it has insignificant effects on the human and environmental health. However, particularly for the last decade, global concerns have escalated about the potential direct and indirect health risks it may pose to humans and ecosystems due to its high-volume use. Due to these increasing health concerns, especially cancer, the development of methods to detect traces of this herbicide in biological materials is of great importance for the protection of public health. To assess glyphosate exposure levels, it is crucial to have a selective, sensitive, and precise analytical procedure that can determine low glyphosate concentrations, especially in complex biological matrices. In the presented study, we developed and validated an Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) procedure for the accurate and sensitive determination of glyphosate levels in urine. It is based on the "dilute and shoot" technique with no derivatization procedure and is quantitatively determined by UPLC-MS/MS. The advantages of this methodology are simplicity, minimal analyte loss, and high sample yield. The calibration curve for glyphosate was linear in the concentration range of 0.1–50 ng/mL. The limit of quantification (LOQ) for glyphosate was 0.1 ng/mL. This validated method can be applied very quickly and easily in the analysis of spot urine samples collected from healthy people.

Key Words: *Glyphosate, herbicide, urine, UPLC-MS/MS*

İdrarda Glifosatın Kantitatif Analizi için bir UPLC-MS/MS Yönteminin Geliştirilmesi ve Doğrulanması

ÖZ

Son 40 yıldır, Glifosat, çevre ve insan sağlığı üzerinde ihmal edilebilir etkileri olduğu varsayımıyla Türkiye de dahil olmak üzere tüm dünyada en yaygın kullanılan herbisittir. Ancak, özellikle son on yılda, glifosatın yüksek miktarlarda kullanımı nedeniyle insan sağlığı ve ekosistemler üzerindeki potansiyel doğrudan ve dolaylı etkileri konusunda küresel olarak endişeler artmıştır. Başta kanser olmak üzere, artmış sağlık endişeleri nedeniyle insan biyolojik örneklerinde bu herbisitin kalıntılarını tespit etmeye yönelik yöntemlerin geliştirilmesi halk sağlığının korunması açısından büyük önem taşımaktadır. Glifosat maruziyet düzeylerini değerlendirmek için, özellikle karmaşık örnek matrislerinde düşük glifosat konsantrasyonlarını tespit edebilen seçici, hassas ve doğru bir analitik yönteme sahip olmak önemlidir. Sunulan çalışmada, idrardaki glifosat seviyelerinin doğru ve hassas bir şekilde belirlenmesi için UPLC-MS/MS yöntemi geliştirilmiş ve valide edilmiştir. Hiçbir türevlendirme prosedürü olmayan ve ultra performanslı sıvı kromatografisi-tandem kütle spektrometrisi ile kantitatif olarak belirlenen, "seyrelt ve enjekte et" tekniğine dayanmaktadır. Bu metodolojinin avantajları basitlik, minimum analit kaybı ve yüksek numune verimidir. Glifosata ait kalibrasyon eğrisi 0.1- 50 ng/mL konsantrasyon aralığında doğrusaldı. Glifosat için tayin limiti (LOQ) 0,1 ng/mL dir. Valide edilmiş/doğrulanmış bu yöntem, sağlıklı kişilerden toplanan spot idrar örneklerinin analizinde çok hızlı ve kolay bir şekilde uygulanabilme özelliğindedir.

Anahtar Kelimeler: *: Glifosat, Herbisit, İdrar, UPLC-MS/MS.*

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INTRODUCTION

The ever-increasing human population and decreasing agricultural lands require the development of new strategies for effective crop management. At the forefront of these strategies are herbicide applications, which aim to eliminate small plants and weeds that cause serious losses in agriculture. Glyphosate (N-(phosphonomethyl) glycine) ($C_3H_8NO_5P$) is the active ingredient developed by Monsanto under the name Roundup in the 1970s. It quickly became one of the most widely used herbicides in many communities and regions of the World. It is the most popular organophosphate herbicide and has become indispensable in the agricultural sector in more than 140 countries and regions due to its non-selectiveness, high efficiency, and compatibility with genetically modified agricultural products (Jiang et al., 2025). Today, it is an herbicide with widespread post-emergence application in Türkiye to control grass and broadleaf weed species both in agricultural and non-agricultural areas such as gardens, parks, etc.

Humans are exposed to this herbicide through several routes, including oral, dermal, and inhalation, as well as through the food chain, soil, air, water, and surrounding fauna and flora. For example, dust ingestion is a significant exposure route, especially in indoor environments near agricultural regions (Galli et al., 2024; Ben Khadda et al., 2025).

There is a great debate both in the scientific community and among various health authorities regarding the toxic effects of exposure to glyphosate in humans. Studies conducted in recent years in particular, have reported that glyphosate exposure may cause atherosclerosis, neurological effects, and alterations in the intestinal microbiome. Furthermore, links have also been found for increased risks of allergic respiratory symptoms and follicular lymphoma. Thus, both in the scientific community and in the general population, glyphosate is suspected to act as a mutagenic, carcinogenic, and a neurotoxic substance (Van Bruggen et al., 2018). The International Agency for Research on Cancer (IARC) (IARC, 2016) has classified glyphosate as "probably carcinogenic to humans" in Group 2A. However, the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) have classified glyphosate as "non-carcinogenic to humans" (ECHA, 2017; EFSA, 2015), and the U.S. Environmental Protection Agency (EPA) has classified it as Category IV, meaning practically non-toxic and non-irritating (EPA, 2016). In 2017, the European Commission (EC) permitted the use of glyphosate for another five years. The EC's decision on the continued use of glyphosate was suspended in 2022. Later, in November 2023, the Commission approved the use of glyphosate in herbicide applications until 2033 (EC, 2023). However, member states are allowed to implement different rules at the national level. While no EU country has currently banned glyphosate entirely, some, such as Austria, France, the Netherlands, Belgium, Luxembourg, and Germany, have introduced partial bans that prohibit its use in certain regions. In addition, the risk of glyphosate causing soil and water pollution increases, especially if applied incorrectly. Therefore, exposure to glyphosate residue levels through environmental routes such as drinking water, surface water, and groundwater can pose a significant threat to human health and ecosystems. For example, information is increasing on the negative effects of glyphosate on marine organisms in the aquatic ecosystem, including fish and mollusks (Parlapiano et al., 2021; Ames, Miragem, Cordeiro, Cerezer & Loro, 2022).

The accumulating evidence of the adverse effects of glyphosate on both ecosystem integrity and human health highlights that the use of this herbicide is becoming a serious public health crisis not only locally but also globally. Recent scientific findings, in particular, suggest that strict restrictions on the use of this chemical and, ultimately, its ban are critical steps

that must be taken without delay. For these reasons, there is a need for sensitive, rapid, and reliable analysis methods that can facilitate the biomonitoring of human exposure to glyphosate and assess the health risks that may develop due to exposure. In the presented study, an improved UPLC-MS/MS analysis procedure with the specified features has been developed for this need.

MATERIALS AND METHODS

Chemicals

Glyphosate and 1,2-$^{13}C_2$ ^{15}N-Glyphosate were obtained from HPC Standards GmbH. Methanol, acetonitrile, formic acid, and ultrapure water were obtained from Sigma Aldrich. All solvents used in this study were HPLC grade. The representative chemical structures of the target compounds are presented in Figure 1.

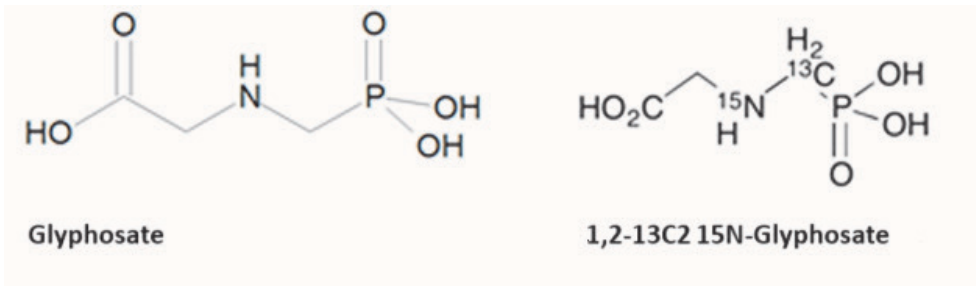

Figure 1. The representative chemical structures of glyphosate and 1,2-$^{13}C_2$ ^{15}N-Glyphosate

Instrumentation

Identification and quantification of the target analytes were carried out using a liquid chromatography triple quadrupole/trap mass spectrometer, QTRAP 5500 (Applied Biosystem, SCIEX, Singapore).

Sample preparation

1,2-$^{13}C_2$ ^{15}N-Glyphosate (≥ 95%) was used as an internal standard (IS) for glyphosate determination and was added to the samples at the beginning of the analysis. The urine samples (900 µL) were mixed with water containing 5 µg/mL 1,2-$^{13}C_2$ ^{15}N-Glyphosate (100 µl) as an IS before vortex mixing. After that, the sample was diluted 1:10 with water. It was transferred to a polypropylene vial, and a 10 µL aliquot was used for the UPLC-MS/MS application.

Analytical conditions

The liquid chromatography operating conditions established for this study were as follows: A Torus DEA (Waters Corporation, Milford, MA, USA; 2.1 mm x 100 mm; 1.7 µm) column was preferred to separate the analytes from matrix components while the column oven was held at 40 °C. The mobile phase A consisted of water with 1.2% formic acid. The mobile phase B consisted of acetonitrile with 0.6% formic acid. The analysis for glyphosate was performed using the following gradient (%B) program at a flow rate of 0.5 mL/min: 0 min, 90%; 0.5 min, 90%; 1.5 min, 20%; 4.5 min, 10%; 17.5 min, 10%; and 17.6 min, 90%. The re-equilibration of the column to the initial conditions lasted 6 min. The total run time was 24 min.

The mass spectrometry conditions were as follows: A Mass spectrometer coupled with electrospray ionization (ESI) interfaces was used in a negative ion mode. Nitrogen (N_2) was used as a nebulizer gas (55 psi). The source temperature and ion spray voltage were fixed at 550 °C and 4000 V, respectively. Optimization results for each analyte in multiple reaction monitoring scan mode are presented in Table 1. Glyphosate and 1,2-$^{13}C_2$ ^{15}N-Glyphosate were assayed by quantifying the multiple reaction monitoring transition of the [M + H]- ion of glyphosate at m/z 167.9→63.0 and 1,2-$^{13}C_2$ ^{15}N-Glyphosate at m/z 170.9→63.0 (Table 1).

Table 1. *Optimal mass spectrometry parameters provided for the Glyphosate and 1,2-$^{13}C_2$ ^{15}N-Glyphosate*

Compounds	Precursor ion (m/z)	Product ion (m/z)	Declustering potential [V]	Entrance potential [V]	Collision energy [V]	Cell exit potential [V]	Dwell time [ms]
Glyphosate Quantifier Ion	167.9	63.0	85	10	-32.0	-9.0	500
Glyphosate Qualifier Ion	167.9	150.0	85	10	-14.0	-11.0	500
1,2-$^{13}C_2$ ^{15}N-Glyphosate	170.9	63.0	85	10	-32.0	-9.0	500

RESULTS

Method validation

The method was validated according to the US FDA guidelines for bioanalytical methods (FDA, 2018). Due to the lack of a matrix, the method validation was carried out with the help of synthetic urine. The synthetic urine used in our study was prepared following the guidelines of the Centers for Disease Control and Prevention (CDC). One liter of synthetic urine was prepared by adding 24.5 g urea, 8.5 g sodium chloride, 3.8 g potassium chloride, 1.4 g creatinine, 1.03 g citric acid, 1.18 g potassium phosphate, 0.64 g sodium hydroxide, 0.47 g sodium bicarbonate, 0.34 g ascorbic acid, and 0.28 mL sulfuric acid in deionized water (NCEH, 2010).

Standard solutions, calibration standards, and quality control samples

The standard stock solution of glyphosate was prepared in water (1 mg/mL). The working solutions of glyphosate were prepared by proper dilution of these stock solutions in synthetic urine with 0.1 % formic acid, which included 2 ng/mL 1,2-$^{13}C_2$ ^{15}N-Glyphosate (1 mL). The calibration standards, quality control (QC) samples (at low, medium, and high concentrations), samples at the lower limit of quantification (LOQ), and limit of detection (LOD), were prepared by spiking the synthetic urine with a known quantity of compounds. The set of concentrations of the analytes in the quality control samples and of calibration curve are shown in Table 2.

A calibration curve was prepared for glyphosate to enable quantitative analyses. For this purpose, glyphosate standards were added to synthetic urine samples corresponding to concentrations of 0.1, 0.5, 1, 5, 10, 25, and 50 ng/mL. These concentrations were injected into the LC-MS/MS device three times to achieve the best possible values. Concentration-dependent responses were calculated from glyphosate standards prepared in synthetic urine and measured with a 7-point calibration curve having a linear range of 0.1–50 ng/mL. The regression equation for glyphosate was obtained as y = 0.23774x- 0.01121 (R^2 = 0.9934).

Table 2. *Selected concentrations of analytes in quality control samples and calibration curve ranges (ng/mL).*

	Calibration range	Lower limit of quantification (LOQ)	Limit of detection (LOD)	Low-quality control (QC1)	Medium-quality control (QC2)	High-quality control (QC3)
Glyphosate	0.1–50	0.1	0.03	1	10	50

For intraday and interday precision, QC samples and LOQ were prepared by spiking the synthetic urine with a known quantity of analyte standards. Each sample was analyzed ten times to determine intraday precision. Each sample was analyzed once on five separate days within 2 months for interday precision.

The precision and accuracy of the LOQ and QC samples obtained in this method development study are presented in Table 3. The intraday accuracy ranged from 98.60 to 100.81%, whereas the interday accuracy ranged from 96.67 to 100.38%. The precision was expressed as a CV% that varied depending on the con-

centration. It was determined that intraday precision varied between 1.06% and 2.49%, while interday precision varied between 2.01% and 3.58%.

Glyphosate chromatograms shown for blank samples (synthetic urine), synthetic urine spiked with the LOQ level (0.1 ng/mL), and synthetic urine spiked with 1 ng/mL are shown in Figure 2 and Figure 3. The internal standard, 1,2-$^{13}C_2$ ^{15}N-Glyphosate (2 ng/mL) (m/z 170.9→63), was used in the analysis.

Table 3. *Intraday and interday accuracy and precision were obtained for the analytes in synthetic urine.*

	Lower limit of quantification (LOQ)	Low-quality control (QC1)	Medium-quality control (QC2)	High-quality control (QC3)
Intraday accuracy [%]				
Glyphosate	98.60	100.33	100.37	100.81
Intraday precision [%]				
Glyphosate	2.35	2.49	1.06	1.67
Interday accuracy [%]				
Glyphosate	96.67	100.14	100.07	100.38
Interday precision [%]				
Glyphosate	3.58	2.85	2.01	2.02

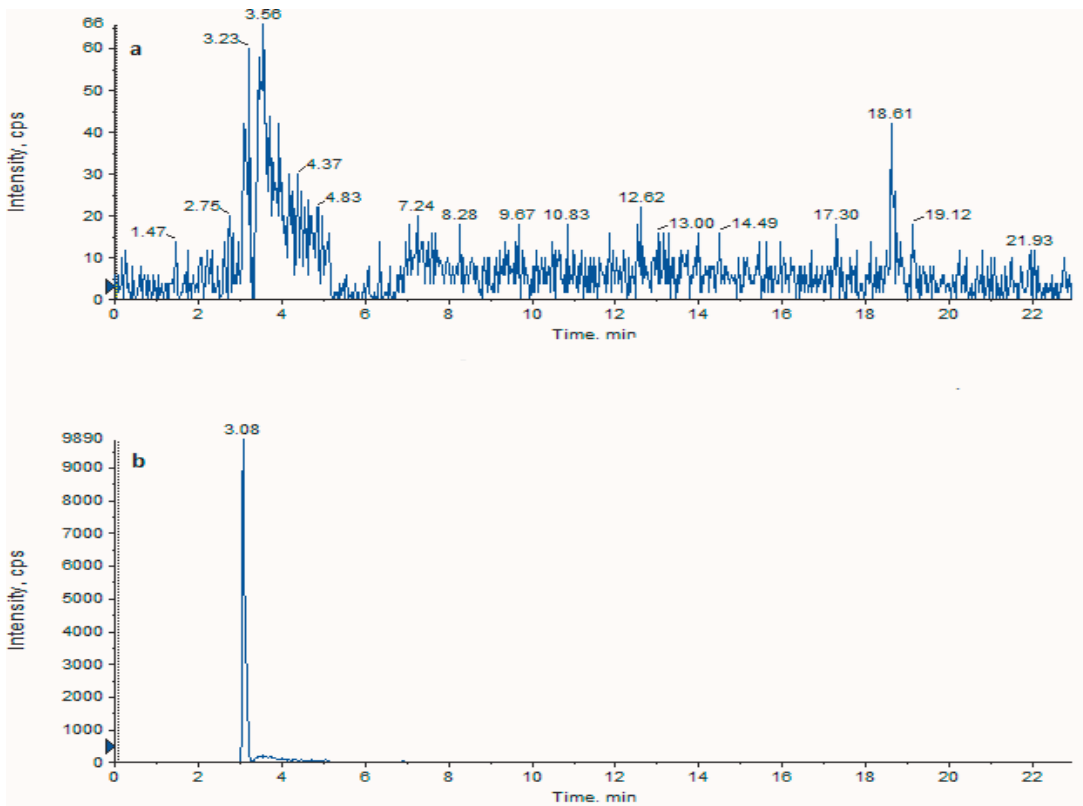

Figure 2. Chromatograms of the ion transitions: **(a)** glyphosate blank (synthetic urine) monitored at m/z 167.9→63, **(b)** 1,2-13C2 15N-Glyphosate (IS) (2 ng/mL) monitored at m/z 170.9→63.

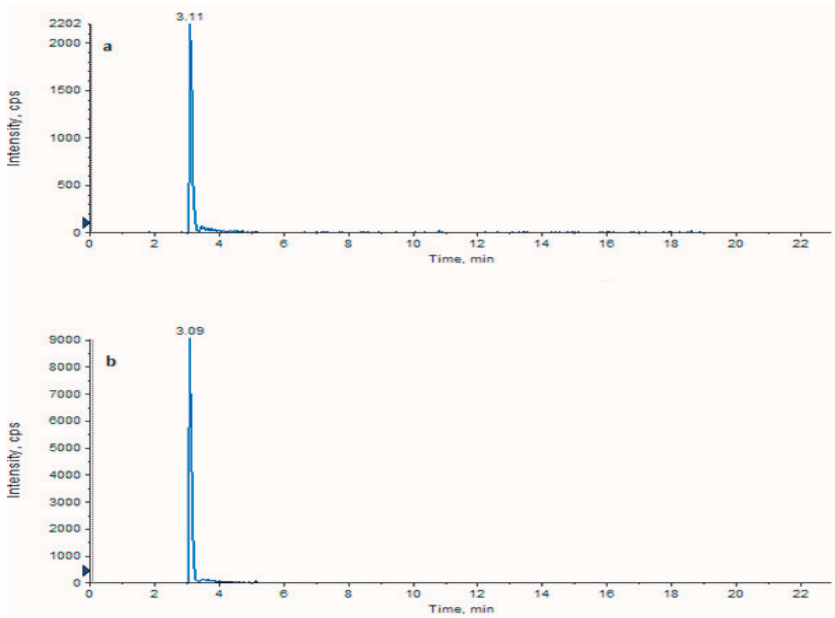

Figure 3. Chromatograms of the ion transitions: **(a)** for 1ng/ml glyphosate monitored at m/z 167.9→63, **(b)** 1,2-13C2 15N-Glyphosate (I.S) (2 ng/mL) monitored at m/z 170.9→63.

DISCUSSION

Pesticides are chemicals developed to control or eliminate pests primarily in agricultural areas and are the most important chemicals used to increase the yield of agricultural products. Herbicides are a group of pesticides used most widely and in large volumes in all societies. Due to this widespread use, unwanted/unexpected risks may occur to humans and the environment due to exposure from application areas or residues in the product obtained. Glyphosate, which is used as an herbicide in many countries, is currently the highest volume herbicide used globally in both agricultural applications and non-agricultural areas to control invasive weeds.

Concerns about various health risks caused by glyphosate are increasingly disturbing societies. Therefore, in this presented study, a fast UPLC-MS/MS analytical procedure was developed and characterized for the quick separation and quantification of glyphosate in urine for routine applications for assessment of exposure levels. Quantification of xenobiotics such as pesticides themselves or their metabolites in biological samples such as urine or blood (i.e., biomonitoring) allows for the assessment of exposure levels to these chemicals and the health risks that may arise at these levels (Barr, 2008).

Glyphosate does not undergo extensive biotransformation in the human body and, the parent compound can be detected in urine. Glyphosate is one of the polar pesticides. The high water solubility of glyphosate and its low affinity for organic matter (Log Kow < 3.4) (Acquavella et al., 2004) make urine the most widely used biological material for monitoring human exposure to glyphosate. In order to determine human exposure to this herbicide, glyphosate and its major degradation product, aminomethylphosphonic acid (AMPA), can also be detected in other biological materials, such as blood/serum, breast milk, and meconium. AMPA has a level of toxicity comparable to glyphosate and is therefore considered to be of similar toxicological importance (EFSA, 2022). Glyphosate is excreted in both urine and feces as an unchanged compound in greater amounts than its parent metabolite AMPA (Leblanc, Breton, Léveillé, Tessier, & Pelletier, 2024; Peillex & Pelletier, 2020). The number of studies providing quantitative data on the AMPA metabolite is quite scarce.

Regardless of the analytical strategy followed, the most critical step is method validation. Validation is the entirety of the processes performed to demonstrate that the success of a device, method, or system complies with the specified conditions. It is a test and measurement process performed according to a set of variables to determine the performance of a method. Many decisions in various fields are made based on the results of the measurements made. To make the right decision, it is desired that the analytical measurement result is accurate and reliable (repeatable) (FDA, 2018). In this step, a wide range of experiments should be performed to obtain appropriate selectivity, sensitivity, accuracy, recovery, and precision. In our study, we tried to contribute to the most reliable results that can be obtained in human biomonitoring studies of glyphosate by performing these steps for method validation.

Different methods are used for extraction in glyphosate determination studies in urine. For example, while some studies performed liquid-liquid extraction for pretreatment, many studies preferred solid phase extraction (SPE) in method development. In addition to the methods that use a derivatization process, some studies perform the analysis with dilution methods. There are also studies utilizing immunoassay methodology, which is considered a less reliable method for measuring glyphosate levels due to lower sensitivity and higher false positive rates, and the LOD in these studies ranged from 0.9 to 7.5 ng/mL (Acquavella et al., 2004; Curwin et al., 2007).

Other studies have performed glyphosate analysis in urine with low LOD values (0.15-2.0 ng/ml) using the more sensitive GC-MS or HPLC/MS techniques. The analytical methods that have been developed for the detection of glyphosate were reviewed and discussed by Wei et al. (2024). Among these different methods, MS analysis quickly provides accurate results. Being selective, sensitive, and accurate at even trace amounts of glyphosate concentrations and requiring no derivatization in the method, LC-MS/MS is the primary used LC technique for specifying glyphosate and for glyphosate-related evaluation of biological samples. On the other hand, electrospray ionization (ESI), one of the ionization modes used to determine the fragmentation patterns of ions and among the modern analysis techniques used today, is one of the most sensitive techniques for mass spectrometry detection and one of the most successful interfaces used in LC-MS configurations. Configuration of LC-MS with ESI represents fragmentation data for structural verification and provides an effective method for the analysis of complex systems (Kumar, Dinesh, & Rini, 2016).

In the present study, the sample preparation process was realized by dilution. The "dilute and shoot" method is based on simple sample dilution instead of extraction, and the biological sample is diluted with an IS-containing solution before being injected into the Torus DEA column. It is suitable for liquid samples containing low amounts of protein, such as urine and saliva. The dilution method provides superiority over the methods reported above because it is easy, faster, and more cost-effective to perform analyses.

The LOQ value in this work was calculated as 0.1 ng/mL for glyphosate. The values obtained with the methods we developed in this study are more sensitive than the results of many other studies reported previously. For example, in two studies where the dilution method was used and analysis was performed with LC-MS/MS (Jaikwang et al., 2020; Trasande et al., 2020), similar to this study, the LOQ values were 0.33 ng/mL and 5 ng/mL. On the other hand, in three separate studies that attempted to determine the amount of glyphosate in urine, the LOQ level of 0.1 ng/ml that we obtained in our study was reached. In these studies, unlike the present study, solid-phase extraction and liquid-liquid extraction techniques were used in sample preparation processes. However, in the study, we used the dilution method, which is much simpler, less time-consuming, and more cost-effective for the same LOQ value. On the other hand, the LOQ values of five studies in the literature were lower than in the present study (Fagan, Bohlen, Patton, & Klein,

2020; Nova, Calheiros, & Silva, 2020; Soukup et al., 2020), but the recovery and precision data of these methods were not specified. All of the LOQ, recovery, and reproducibility data obtained in the method used in the study reveal that it was more sensitive and reliable when compared with studies using urine as biological material.

CONCLUSIONS

The findings obtained from this study show that the presence of glyphosate at very low concentrations in urine samples can be detected with the help of the dilution method we used and the UPLC-ESI MS/MS method. In this method developed for glyphosate analysis in urine, sensitivity, linearity, and, recovery were evaluated and all of these parameters meet the criteria in the literature. We believe that by using the method we developed, determination of glyphosate exposure in urine will be easier, cost less, and save time. It is considered that this method we developed will make significant contributions by facilitating glyphosate biomonitoring studies.

AUTHOR CONTRIBUTION RATE STATEMENT

Concept and Design (İÇ), Data Collection (GKM), Analysis (GKM), Interpretation (GKM, İÇ), Writing (İÇ).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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