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# Determination of glyphosate residues in Hungarian water samples by immunoassay

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) for the detection of glyphosate was investigated for assay performance characteristics and was applied for determination of glyphosate contamination levels in selected surface and ground water resources in Hungary in 2010 and 2011. Advantages of the method include its simplicity (no laborious extraction) and specificity (cross-reactivity is below 0.1% for related compounds, e.g. aminomethyl-phosphonic acid, glufosinate). On the basis of our experiments, the practical limit of detection (LOD) ranged between 0.05 and 0.12 ng/ml. The standard curve was of sigmoid (logistic) characteristics, and it co-occurred with curves obtained for spiked surface water samples. Matrix effects were observed in tap water, possibly due to chlorination and/or heavy metal ions, e.g. copper and zinc. The method was applied for the analysis of 42 surface and ground water samples collected from Békés county in Hungary at 14 sampling sites in 2010 and 18 surface water samples collected from the Danube River and Lake Velencei in Hungary at 12 sampling sites in 2011. Exceedingly high glyphosate levels (nearly 1 ng/ml) were measured in 5 samples, and significant concentrations were determined in 16 cases (0.54–0.76 ng/ml) in 2010, while practically no contamination was found in 2011. The great contrast between the two sampling regimes is explained by differing agricultural locations, natural precipitation and, to a greater extent, catchment area characteristics, resulting in varying leaching or run-off of glyphosate to surface waters.



## 1. Introduction

Glyphosate (N-(phosphonomethyl)glycine or 2-[(hydroxy-oxido-phosphoryl)methylamino]acetic acid) is presently the largest selling agrochemical in the world [1,2] and its market continues to grow in line with the increase in the cultivation of glyphosate-tolerant (GT) transgenic crops [3]. It is a broad spectrum pre-emergent (and when used with GT crops, also post-emergent) herbicide dedicated to vegetation control of perennial and annual plants, broad-leaf weeds, grasses, grains, orchards and forestry, as well as aquatic weed control [4]. Although GT crop varieties are currently not cultivated in Hungary, glyphosate is commonly used. According to the annual pesticide business report of the Hungarian Central Agricultural Office, over 780 t of glyphosate was sold in Hungary in 2010, the second largest sales volume of an active ingredient after sulfur [5]. As seen with other pesticides, glyphosate, due to its high water solubility, may contaminate surface and ground waters [6], which represent 24–27% and 2% of the drinking water supplies in Hungary, obtained *via* bank filtration and shallow wells, respectively. Although glyphosate presents lower acute toxicity than other herbicides, it has been evidenced to cause toxicity and genotoxicity in aquatic organisms and amphibians [7], teratogenicity in amphibians and birds [8], and has been shown to induce endocrine disrupting effects as well [9], the latter effect being highly synergized by polyethyloxylated tallowamine (POEA), a commonly used formulating agent in glyphosate-based herbicide preparations. As an immediate consequence of the above toxicological and ecotoxicological concerns, and as these substances have proven to be persistent under typical application conditions [10], glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) are required to be regularly monitored in surface and ground waters.

The combined maximum residue level (MRL) for glyphosate and its relevant metabolites, including AMPA, in drinking water is 0.1 ng/ml in the European Union [11] and therefore, in Hungary as well. In surface and ground water, however, there are no MRLs defined according to the Water Framework Directive and its daughter directive regarding ground water protection, as they are not currently listed among the priority substances for which environmental quality standards have been set. However, both glyphosate and AMPA are subject to review for possible classification as priority substances [12–14]. The mobility of glyphosate and AMPA in soil has been debated. As glyphosate strongly adsorbs both to the organic matter and the mineral fractions (clay and/or amorphous iron and aluminum oxides) of the soil matrix, it has traditionally been considered relatively immobile in soil [4,15–19]. Yet, both compounds may move from soil to surface and

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ground waters by leaching and run-off processes [20,21] along with the prevailing water flux and may pose contamination hazards to receiving water bodies. In addition, both molecules can be transported as aggregates to soil particles, mimicking the mobility of inorganic phosphates.

Numerous analytical procedures have been published in the literature for the detection of the highly polar and amphoteric molecule of glyphosate [22], including gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), often coupled with mass spectrometry (MS). Extraction or preconcentration of this active ingredient from water samples represents a crucial point in the analytical scheme. The majority of these methods are laborious due to the high number of steps employed and derivatization required prior to GC [23] or HPLC measurements [24,25]. The most recent LC tandem MS methods using electrospray ionization (LC–ESI-MS/MS) [26] easily meet the MRL by the EU for given pesticide residues in drinking water (0.1 ng/ml), but the instrumentation demands of these methods are substantial. As a consequence of the time consuming and elaborate analytical procedures, their sample throughput is limited. In contrast, enzyme-linked immunosorbent assays (ELISAs) allow selective and sensitive determination of glyphosate without sample preparation.

In the early 1990s, it was widely considered that no effective antibodies could be produced against glyphosate and similar zwitterionic compounds due to their low immunogenicity [27]. Difficulties in immunization were overcome within a decade and sensitive ELISAs were developed [28–31]. On the basis of the immunoassay principle, sensors using glyphosate-sensitive antibodies [32] or molecularly imprinted polymers (MIPs) [33] were also developed. ELISA offers advantages over wet chemistry methods as it enables prompt environmental surveys in a cost-effective manner. Limits of detection (LODs) achieved by ELISAs are similar to or better than those obtained by GC–MS methods, although higher than those typically obtained by LC/MS/MS.

Both indirect [28] and direct [30] ELISA methods have been developed for measuring glyphosate. The direct method (based on immobilized antibodies), which includes a derivatization step with acetic anhydride, results in a substantially lower LOD (0.6 ng/ml and below) than the indirect ELISA without derivatization, and moreover has been found to be highly specific to glyphosate (without cross-reaction with its major metabolite, AMPA) as well. Even lower LOD values (0.1 ng/ml) have been reported by Lee et al. [29] in a so-called linker-assisted enzyme-linked immunosorbent assay (l'ELISA) method using derivatization with succinic anhydride. Reliability of the ELISA results was evaluated against LC–MS or LC–MS/MS [34,35] and LC–ESI-MS/MS [26] after derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) and extraction on solid phase extraction cartridges. Strong correlation ($R^2 = 0.88$) was established, although immunoassay overestimated glyphosate concentration in some cases and detected a trace level in a sample deemed uncontaminated by LC–MS/MS [26]. In contrast, due to the limits of the ELISA methods for the detection of AMPA, which under certain circumstances may be present in the absence of its parent pesticide (e.g., high use of glyphosate and vulnerable hydrogeological settings) [36], simultaneous quantification of AMPA by means of traditional instrumental analytical methods should be applied along with determination of glyphosate by ELISA [35]. The aim of the present study was to evaluate the currently available Abraxis ELISA kit for the detection of glyphosate, to explore factors affecting its analytical performance, and to test the influence of certain sample preparation steps employed prior to instrumental measurement. On the basis of performance characteristics of the ELISA method, its utility was assessed as a sensitive screening method in environmental surveys in Hungary, and is proposed as a useful tool to rapidly survey the status of aquatic environments with respect to the presence of glyphosate and also as an early warning method under different environmental conditions.

## 2. Materials and methods

### 2.1. Reagents

The glyphosate analytical standard used was Pestanal grade, from Riedel-de Haën (Seelze, Germany). Amberlite IR 120 strongly acidic cation exchange resin was purchased from Fluka (Buchs, Switzerland). All other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), unless otherwise stated.

### 2.2. ELISA

For immunoanalytical detection of glyphosate, the commercially available ELISA method (PN 500086) by Abraxis LLC (Warminster, PA, USA) was used [37]. Measurements were carried out in 96-well microtiter plates according to manufacturer's instructions. Derivatized samples or analytical standards were added to the microtiter wells along with an antibody specific for glyphosate, and the reaction mixture was incubated at room temperature. An enzyme conjugate of glyphosate was then added. After washing the microtiter plate three times with buffer, a colorimetric substrate and chromophore solution was added to each well, and a color signal (blue color) was developed until an optical density (OD) of 1.0 had been reached at 620 nm. Color development was then stopped by the addition of 0.5 M sulfuric acid, and the color signal was recorded at 450 nm. As the glyphosate conjugate was in competition with the unlabeled glyphosate content of the samples for the antibody sites, the color developed followed a sigmoid pattern, decreasing with increasing glyphosate concentrations in the samples.

### 2.3. Calibration, limits of detection and matrix effects

Calibration curves were established with standard solutions provided by Abraxis at five concentration levels between 0.075 and 4.0 ng/ml (0, 0.075, 0.2, 0.5, 1.0, 4.0 ng/ml), two replicates each. An analytical quality control solution (0.75 ng/ml) was also used. LODs, defined as glyphosate concentration causing 10% decrease in the optical assay signal, i.e. 90% $B/B_0$ (where $B/B_0$ is the signal obtained with the given sample divided by the maximum signal obtained with a sample containing no glyphosate), were determined in all experiments. For investigation of matrix effects, a stock solution of glyphosate (1.0 mg/ml) was prepared in MilliQ water. This solution was diluted to 0.1 μg/ml (spike solution). Solutions containing glyphosate at final concentrations (typically 0.075, 0.2, 0.5, 1.0, 4.0 ng/ml) were made by addition of appropriate amounts of spike solution to different water matrices.

### 2.4. Influence of sample preparation

The influence of different sample preparation steps proposed by Küsters et al. for drinking water [25] has also been investigated. Briefly, for the cleanup of spiked water samples, the cation exchange resin Amberlite IR 120 was converted to sodium form. This, and all subsequent column regeneration steps after each sample, was carried out with a 4 M sodium chloride solution. Then, 100 ml of each sample was passed through the cation exchange column, followed by washing with 30 ml of deionized water. All eluates were collected in round bottom flasks and then evaporated to dryness. The residues obtained were dissolved in 5 ml of deionized water. After each sample preparation step, concentrations of glyphosate were determined by ELISA, with concentrations obtained corrected according to the volume change.

### 2.5. Sample collection

Municipal water at the laboratory site (II. District, Budapest, Hungary) was used as tap water. Field samples were collected in 2.5 l

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amber glass bottles previously washed with aqueous hydrochloric acid (pH 2) and repeatedly rinsed with deionized water. During sampling, the bottles were rinsed twice with the water sampled, then filled and tightly capped. Samples were stored at 4 °C in the dark. In the scope of a national environmental survey, 42 water samples (6 surface water and 36 ground water samples) were obtained on September 7–8, 2010, from 14 sampling sites in Békés county, Hungary. In addition, 18 surface water samples were collected on October 1, 2011, from 11 sampling sites along the Danube River and one site at Lake Velencei, Hungary. The sampling sites are depicted on Fig. 1.

## 3. Results and discussion

### 3.1. Assay performance

The Abraxis glyphosate ELISA kit applies the principle of the competitive immunoassay, with prior sample derivatization by acetic anhydride [38]. A unique feature of the ELISA is that two key steps of the protocol are carried out simultaneously: the derivatized analyte is preincubated with glyphosate-specific antibodies, and the latter are bound to IgG-specific antibodies immobilized on the solid surface of the microwells of the ELISA plate. Therefore, the homogeneous phase process of the glyphosate-IgG reaction and the heterogeneous phase reaction of the binding of the IgG on the microwells proceed in parallel in the same incubation step. The proportion of glyphosate-specific antibodies that remain free on the solid surface are then brought into reaction with glyphosate conjugated to a reporter enzyme, the activity of which is then measured by its colorimetric substrate. As a result, the competitive ELISA provides a sigmoid (logistic) standard curve downward with increasing glyphosate concentration (Fig. 2). Typical analytical parameters of the immunoassay carried out in buffer were analyte concentration resulting in 50% inhibition of the assay signal ($IC_{50}$) at $0.66 \pm 0.16$ ng/ml, slope of the standard curve at the $IC_{50}$ at $1.52 \pm 0.76$ ng/ml, and LOD at 0.05 ng/ml. This LOD value is the 90% $B/B_0$, commonly used to indicate sensitivity, which is the estimated minimum detectable concentration based on 90% binding (10% inhibition) in the assay. The concentration of the first calibration standard was 0.075 ng/ml. Although levels between 0.05 ng/ml and 0.075 ng/ml are within the detectable range of the assay, as with any analytical technique (ELISA, GC, etc.), there must be valid calibration points on either side of a sample value to be considered a legally defensible, valid sample result. As the results for these samples were all below the first standard (0.075 ng/ml), Table 1 lists the results for the unspiked samples (with no glyphosate detected) as <0.075 ng/ml, rather than giving a (less exact) value below the calibration range of the assay.

The ELISA is highly specific for glyphosate: cross-reactivities of related compounds, including main metabolites AMPA and glycine; glufosinate, an herbicide active ingredient of related chemical structure; and glyphosine, a withdrawn fungicide active ingredient of related chemical structure, were all below 0.1% as calculated at both the LOD and at the $IC_{50}$ of each compound.

### 3.2. The effect of preincubation

Preincubation of the sample with the specific antibody is a key element in the achievable analytical sensitivity of the immunoassay. Longer preincubation of the antibodies with the free analyte (sample) allows antibody binding to approach equilibrium, and should therefore favorably affect assay sensitivity. This immunoassay was originally developed with the intention of being a commercially available

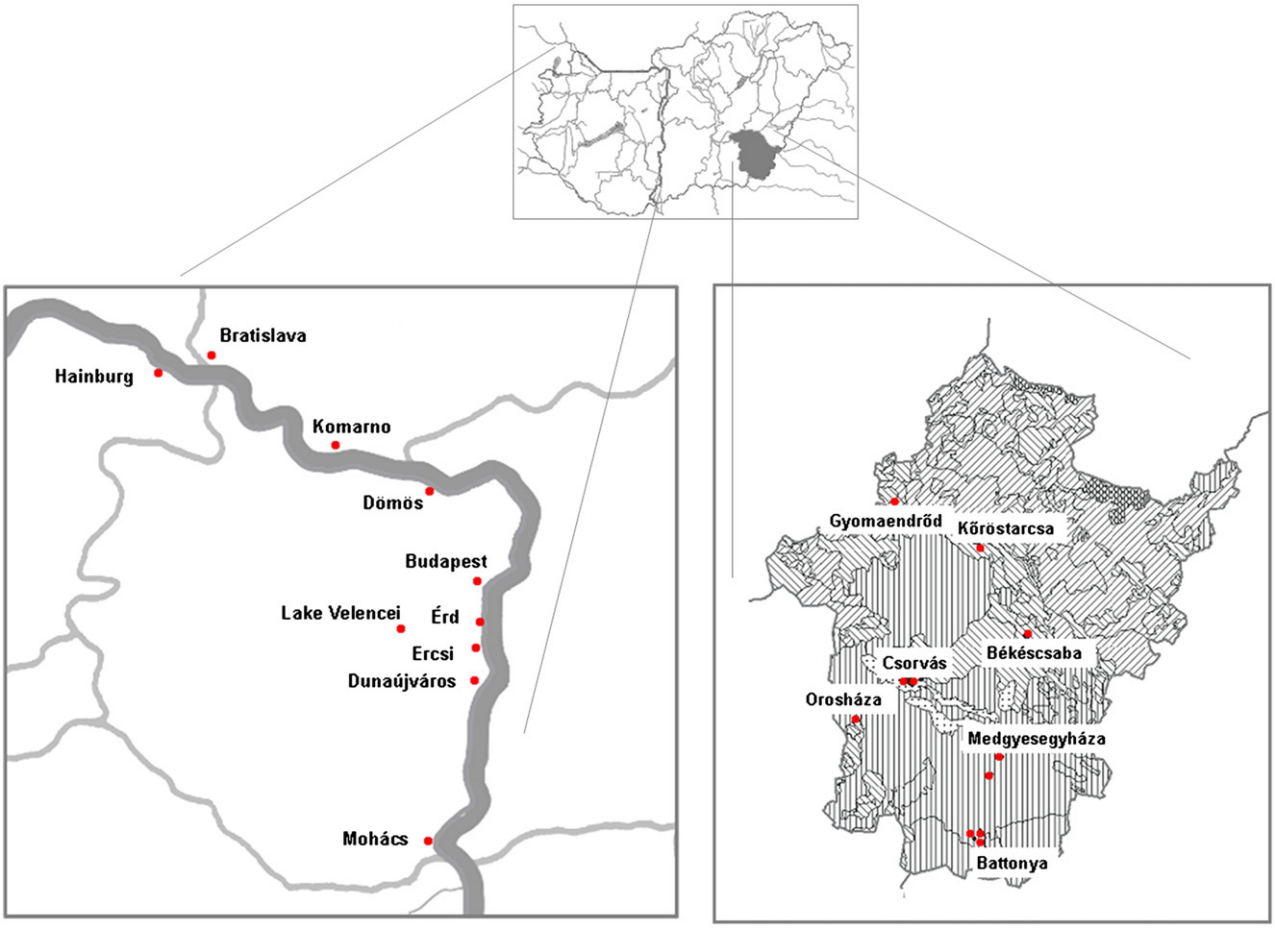


**Fig. 1.** Sampling sites in Hungary along the Danube and in Békés county.

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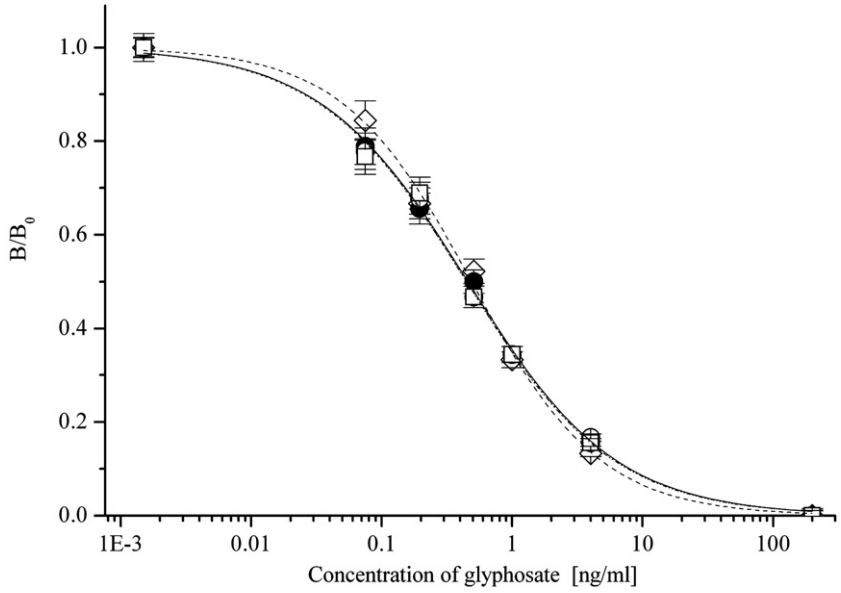


**Fig. 2.** Standard calibration curve in the glyphosate-specific competitive ELISA, and the effect of preincubation for 15 minutes (◇, dashed line), 30 minutes (●, straight line), 45 minutes (○, dotted line) and 60 minutes (□, dashed and dotted line).

product, therefore, preincubations in excess of 45 minutes were not considered desirable, and the commercial ELISA kit was developed and commercialized with 30 minute preincubation applied. Results from the present study carried out to determine whether increasing the preincubation, up to 60 minutes, would provide an increase in sensitivity which would justify the additional analysis time.

Thus, the effect of preincubation time with glyphosate at various concentrations (between 0.075 and 4.0 ng/ml) was tested. Experiments were carried out using four preincubation durations (ranging from 15 to 60 minutes) and are depicted in Fig. 2. As seen on the resulting sigmoid curves, the duration of preincubation resulted in a moderate improvement in the reproducibility of the analytical standard curves, as those obtained with 30–60 minute preincubation were practically identical to each other. In consequence, the very slight improvement noted with the 45 minute preincubation, as opposed to the 30 minute preincubation, was not considered significant enough to justify the additional analysis time. Other assay parameters did not show dependence on the preincubation time of 15–60 minutes, i.e. the $IC_{50}$ values were found to be 0.59, 0.46, 0.45 and 0.45 ng/ml at preincubation times of 15, 30, 45 and 60 minutes, respectively. Corresponding LOD values, calculated at 90% of the upper plateau of the sigmoid curve, were 0.069, 0.032, 0.025 and 0.022 ng/ml, respectively. The 30 minute preincubation resulted in a significant improvement in $IC_{50}$ and LOD when compared to the 15 minute pre-incubation.

**Table 1**
Detected glyphosate concentrations and corresponding recoveries in unspiked and spiked water samples as a measure of matrix effects on ELISA performance.

| Sample Type | Glyphosate concentration detected [ng/ml] | | Spike recovery [%] |
|---|---|---|---|
| | Unspiked | Spiked with glyphosate at 0.5 ng/ml | |
| Water matrix effect | | | |
| Deionized water | <0.075 | 0.579 | 115.8 |
| Tap water | <0.075 | 0.141 | 28.2 |
| Surface water | <0.075 | 0.636 | 127.2 |
| Tap water treated with ascorbic acid (0.125 mg/ml) | <0.075 | 0.501 | 100.2 |
| Tap water treated with sodium nitrite (0.005 mg/ml) | <0.075 | 0.383 | 76.6 |
| *Solvent effect by methanol* | | | |
| Methanol content [%] | | | |
| 0 | <0.075 | 0.620 | 124.0 |
| 20 | <0.075 | 0.656 | 131.2 |
| 40 | <0.075 | 0.550 | 110.0 |
| 60 | <0.075 | 0.446 | 89.2 |
| 80 | <0.075 | 0.581 | 116.2 |
| 100 | <0.075 | 0.450 | 90.0 |

### 3.3. Solvent effect

As methanol is often used as an extractant for soil sample preparation, its possible effect on the ELISA performance was studied. Earlier work reported methanol tolerance in the assay buffer up to 10% [30]. In the present study, solutions containing various concentrations of methanol in deionized water were analyzed unspiked and spiked with 0.5 ng/ml of glyphosate, and achievable recoveries were recorded. Methanol (applied at 0%, 20%, 40%, 60%, 80% and 100%) did not produce false positive results up to 100% in the ELISA (Table 1). This is due to the beneficial buffering effect of the assay medium allowing the use of sample solutions even in pure methanol, as the final solvent content is diluted to 20% in this case. Spiked samples with methanol concentrations up to 100% showed spike recoveries of 89.2–131.2%, with overestimation (recoveries above 100%) at low methanol content.

### 3.4. Matrix effects

As calibration curves following sigmoid (logistic) characteristics were obtained in buffer, various aqueous matrices were tested for possible matrix effects by components. According to previous results, comparison of a direct ELISA and an HPLC method for glyphosate determination in three water matrices resulted in no significant differences [30]. In our hands, indeed, no matrix effects were seen for surface water, but considerable matrix effects were observed for spiked tap water, with a curve shift towards higher concentrations (Fig. 3). This matrix effect, however, was eliminated if tap water was processed by distillation or ion exchange, changing all cations to sodium ions, and then spiked. This indicated that the component (s) causing the matrix effect in tap water can be removed. Moreover, as the standard curve in distilled water runs closely to those in ion exchanged water and assay buffer, the assay is insensitive to ionic strengths between 0 (distilled water) and 0.41 M (phosphate buffered

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saline), due to the buffering effect seen for high organic solvent (methanol) tolerance as well. In contrast, applying ion exchange after spiking could not fully eliminate the matrix effect and resulted in a standard curve with an approximately 30% lower slope than that of the standard curve in assay buffer. After evaporation of water to dryness and solution of residue the curve obtained was practically the same as for tap water itself indicating that the component possibly causing the matrix effect is non-volatile, e.g. partly non-volatile disinfection by-products of chlorination (see below).

The shift observed could be explained either by complex formation between glyphosate and multivalent cations or by oxidation of glyphosate by the active chlorine content of drinking water. On the basis of data published by the Waterworks of Budapest Corp., the concentration of free active chlorine in the tap water in the region was 0.34 ng/ml. To evaluate the possible effects of chlorine applied in water treatment, various water samples (deionized water, tap water, and surface water) were analyzed unspiked and spiked with 0.5 ng/ml of glyphosate, and achievable recoveries were recorded (Table 1). No false positives were detected in any of the water samples. Good spike recoveries were seen in deionized water and surface water samples. The spike recovery for tap water showed a biased low recovery (28.2%), due to matrix interference, possibly from chlorine. To test possible involvement of chlorine used for tap water purification, unspiked and spiked tap water samples were then treated with either ascorbic acid or with sodium nitrite (commonly used dechlorinating agents) at final concentrations of 0.125 and 0.005 mg/ml, respectively. The treated water samples were vortexed thoroughly, and were then derivatized and analyzed by the ELISA protocol. As seen from the resultant data (Table 1), treatment with ascorbic acid prior to analysis neutralized the matrix interferences from chlorinated tap water samples, allowing accurate analyte recovery. To exclude possible matrix interferences by ascorbic acid, unspiked samples were treated along with the glyphosate-spiked tap waters. These unspiked ascorbic acid treated tap water samples did not show any recovery (Table 1). Treatment with sodium nitrite also resulted in the elimination of the matrix interference and improved recovery, although to a lesser extent.

Another possible source of matrix interferences is represented by heavy metals and other chelate forming metal ions, even though the presence of calcium, magnesium and sodium at 10 mg/ml and copper at 5 mg/ml had no effect on the glyphosate immunoassay [15]. Hydrochloric acid above 0.25 M caused some interference, indicating that samples preserved with acid should be neutralized prior to evaluation using the ELISA. To further differentiate between chemical and mineral composition of the water samples used, characteristics of tap water, ion exchanged tap water, and surface water are summarized in Table 2. There is only a slight difference in the composition of surface and tap water; concentrations of copper and zinc were higher in the tap waters than in the surface waters examined. Since spiked surface water samples did not show any matrix effects, the interference observed in tap water may arise from complex formation by glyphosate with the copper or zinc content of tap water. Regarding formation constants, copper complexes are more stable than those of zinc. In our samples, concentrations of copper were 52.6 ng/ml for tap water and 12.8 ng/ml for surface water, and this 4-fold higher amount of copper is a likely explanation for elevated complex formation, possibly causing the matrix interference observed. Organic matter content in water must also be taken into account, as it may act as a limiting factor of complex formation. In principle, ascorbic acid, used as a dechlorinating agent to eliminate matrix effects by chlorine (see above), could interact with the copper content in tap water (e.g. reducing Cu (II) to Cu(I), or forming chelates with Cu(II)) as established in quantitative antioxidant capacity assays [39]. Biochemically important amino acids (alanine, glycine, aspartic acid, asparagine, glutamic acid, glutamine, phenylalanine, and histidine), however, inhibit this catalytic autoxidation of ascorbic acid due to the high conditional stability constant of their Cu-complexes. Being a phosphonate derivative of glycine, glyphosate also shows higher affinity to Cu(II) ions [40] than ascorbic acid. Therefore, an eliminatory effect of ascorbic acid on matrix effects caused by Cu(II) is not expected.

In order to eliminate complex formation and subsequent interferences, cation exchange clean-up is advised, based on the results of a Round Robin Study organized by Monsanto Europe [cited by Ref. [40]]. Another approach [41] proposed sample acidification to pH 1 and immediate neutralization. A detailed speciation study performed by Freuze et al. suggested that the complexes formed between glyphosate and cations do not dissociate during the derivatization reaction with FMOC-Cl, so only the free form is derivatized. According to equilibrium calculations [40], 1 ng/ml of glyphosate in the presence of an equivalent

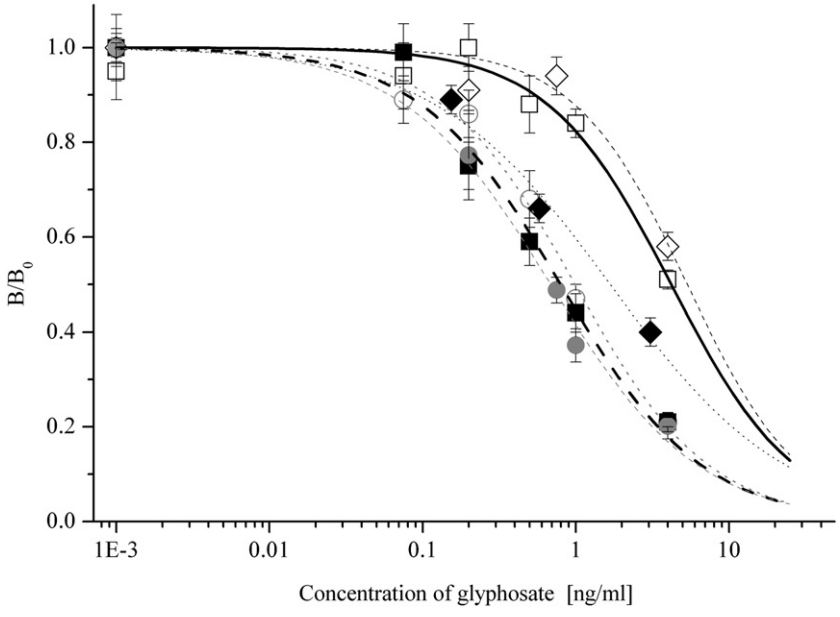


**Fig. 3.** Matrix effects in the glyphosate-specific competitive ELISA indicated by standard calibration curves obtained in assay matrix (○, gray dotted line), distilled water (●, gray dashed line), tap water (□, thick solid line), tap water treated by cation exchange and spiked (■, thick dashed line), tap water spiked and treated by cation exchange (◆, dotted line) and tap water spiked, treated by cation exchange, evaporated and resolved in distilled water (◇, dashed line).

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**Table 2**
Compositional characteristics of the water types studied.[a]

| | pH | $Ca^{2+}$ | $K^+$ | $Mg^{2+}$ | $Na^+$ | $HCO_3^-$ | $Cl^-$ | $SO_4^{2-}$ | Ba | Co | Cr | Cu | Fe | Mn | Mo | Ni | P | Pb | Sr | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/l | | | | | | | μg/l | | | | | | | | | | | |
| Tap water | 7.44 | 56.6 | 2.84 | 13.8 | 12.7 | 200 | 12.0 | 37.2 | 45.6 | <LOD | 0.5 | 52.6 | 6.4 | 14.1 | 2.0 | 1.2 | 32.3 | 4.1 | 242 | 16.5 |
| Tap water treated by ion exchange | 7.75 | 0.62 | 0.0 | 0.17 | 145 | 160 | 114 | 28.2 | 16.7 | 0.4 | 0.7 | 8.8 | <LOD | 4.8 | 1.4 | <LOD | 80.1 | <LOD | 2.7 | <LOD |
| Surface water (BA2G/FV) | 7.22 | 133 | 24.2 | 39.2 | 81.3 | 601 | 143 | 61.2 | 106 | 0.3 | 2.4 | 12.8 | 10.5 | 15.3 | 0.8 | 2.6 | 1842 | <LOD | 651 | 7.4 |
| LOD | | | | | | | | | 1.0 | 0.2 | 0.5 | 2.0 | 0.2 | 1.0 | 0.2 | 1.0 | 3.0 | 2.0 | 0.1 | 2.0 |

[a] Characteristic anion and cation concentrations were detected by titration or inductively coupled plasma atomic emission spectroscopy according to the corresponding national standard procedures (pH [55], cations [56], anions, $SO_4^{2-}$ [57], $CO_3^{2-}$, $HCO_3^-$ [58], $Cl^-$ [59]). Carbonate anions ($CO_3^{2-}$) were not detected in any of the three types of water samples. Microelement concentrations were determined by atomic absorption spectroscopy. Concentrations of Al, As, Cd, Hg and Se were below the corresponding LODs (6.0, 2.0, 0.1, 0.6, 2.0 μg/l, respectively) in all water samples.

molar concentration of free copper (0.4 ng/ml $Cu^{2+}$) will produce as much as 90% in the complex form. The extent of complexation may be significantly reduced in waters with high organic matter content, resulting in only partial complexation of interfering cations in the organic matter, but such a reduction is not expected in drinking water with low organic matter content. Matrix effects caused by calcium ions have been observed in the literature, particularly for high calcium concentration paired with low organic contents in tap waters [40]. In this study, chemical analysis indicated over twice as high $Ca^{2+}$ ion concentration in surface water than in tap water (Table 2), yet matrix effects were not seen. Therefore, $Ca^{2+}$ ions were excluded as causative agents of matrix effects.

The possible role of copper in matrix interferences raises a methodological concern as well. As copper ions are known to be bound with glyphosate through the three donor groups (amine, carboxylate, phosphonate), acylation of the amino group in the derivatization step in sample preparation may consistently be inferred. If the entire glyphosate content of the sample does not reach full derivatization, as only the free (non-complexed) form is derivatized with acetic anhydride prior to immunoassay, the apparent concentration of glyphosate detected in the ELISA may, under some circumstances, appear lower than in reality.

The attempted elimination of matrix effects in tap water by ion exchange decreased copper concentration 6-fold to 8.8 ng/ml, indicating incomplete removal of cations. Cation removal depends essentially on the rate at which the samples percolate through the column. Moreover, copper may have remained in the water phase in the form of the complex with glyphosate, if insufficient time has been allowed for the decomplexation equilibrium.

### 3.5. Analysis in field samples

Sampling of surface and ground water was carried out in Hungary in two campaigns in 2010 and 2011 (Fig. 1). In the scope of a national monitoring program, 42 surface and ground water samples were collected in 2010, on the basis of targeted sampling, from agricultural fields and on industrial sites. Among agricultural areas, three types of land usage have been involved: arable lands under intensive cultivation, arable lands under organic farming, and a pasture. The study area in the case of contamination of agricultural origin covered four settlements in Békés county (Kőröstarcsa, Medgyesegyháza, Csorvás, and Battonya). Both intensive and organic parcels were chosen in all four settlements (4 organic and 4 intensive), and the pasture was designated in Csorvás. Contamination of industrial origin was examined in three settlements in Békés county (Orosháza, Gyomaendrőd, and Békéscsaba) at five sites (Orosháza-Linamar, Orosháza-Közútkezelő, Orosháza-Üveggyár, Gyomaendrőd-Nagylapos, and Békéscsaba-Szennyvíztelep). The subsequent 2011 sampling regime focused on the Danube River and its catchment area. Altogether, 17 surface water samples were collected from the Danube River in the Middle and Lower Danube region from the Austrian–Slovakian border to the Hungarian–Croatian border, and one standing water sample from Lake Velencei.

Glyphosate content was determined in all surface and ground waters collected using the Abraxis ELISA method. The practical LOD was found to be 0.12 ng/ml as calculated from the value and standard deviation of the upper plateau of the sigmoid standard curve (as opposed to the 0.05 ng/ml LOD value determined from the 90% $B/B_0$ value and the concentration of the lowest analytical standard). A stunning difference between the results of the two sampling regimes in 2010 and 2011 was that while all samples collected in the first year contained detectable levels of glyphosate, only a slight proportion of the samples obtained in the second year had detectable glyphosate concentrations (Table 3). In 2010, exceedingly high glyphosate levels (nearly 1 ng/ml) were measured in 5 samples, significant (0.54–0.76 ng/ml) and lower (0.12–0.46 ng/ml) concentrations were determined in 16 and 21 cases, respectively. Thus, the severely or significantly contaminated samples represent half of the surface water samples obtained in the given sampling regime. In contrast, in 2011 glyphosate concentrations detected in the Danube River samples remained, in the vast majority, below the LOD of the assay (0.05 ppb) specified by the manufacturer on the basis of 90% $B/B_0$. Only the sample from Lake Velencei showed a concentration higher than the LOD (0.064 ng/ml), while two other samples from the Danube River (Dömös, Kopaszi gát) were near the LOD (0.043 and 0.035 ng/ml, respectively) (Table 3).

There are at least two characteristic differences between the two sampling regimes in 2010 and 2011: sampling location and meteorological characteristics prior to and during sampling. Both of these factors may affect detectable contamination levels. Békés county is a strongly agricultural region of Hungary, where both intensive agriculture (relying heavily on pesticide use) and ecological agriculture (prohibiting synthetic pesticides) are present. Coupe et al. [19] estimate that approximately 1% of the amount of glyphosate applied emerges as contamination in surface waters, while results of a 6-year survey in the United States [36] reported substantially higher contamination levels and frequencies in surface water than in ground water: glyphosate was detected in 9.7% and 5.8% of all surface and ground water samples analyzed, and maximum concentrations detected were 9.6 and 0.67 ng/ml, respectively. (It has to be mentioned, that AMPA was detected significantly more frequently than glyphosate, 51.5% and 32.3% in surface and ground water samples, respectively, while corresponding contamination levels for AMPA were slightly below those for glyphosate.) In contrast, findings in the 2010 campaign of the present survey did not indicate a statistically significant difference in detected glyphosate concentrations in surface and ground water: detected glyphosate concentrations in surface water were $0.422 \pm 0.271$ ng/ml (with average concentrations in individual samples ranging between 0.12 and 0.68 ng/ml), while corresponding concentrations in ground water were found to be $0.537 \pm 0.224$ ng/ml (0.5–0.98 ng/ml). Although more pronounced agricultural pesticide use is expected in Békés county than at other locations along the Danube River, considerable maize cultivation is being carried out in small plots both in Austria and in Slovakia north of the Danube. Nonetheless, in addition to agricultural usage, glyphosate was also detected in other studies in urban drainage systems and wastewater with significant contribution resulting from its urban use [21,42]. Therefore, it was a rather surprising finding that

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**Table 3**
Detected glyphosate concentrations in surface and ground water samples collected in Hungary in 2010 and 2011.

| Sample code | Type of water | Sampling site | Glyphosate concentration detected (ng/ml) |
|---|---|---|---|
| *2010 sampling regime* | | | |
| BA1F/FV | Surface water | Battonya | 0.12 ± 0.085 |
| BA1G/FV | Surface water | Battonya | 0.17 ± 0.013 |
| BA2F/FV | Surface water | Battonya | 0.27 ± 0.131 |
| BA2G/FV | Surface water | Battonya | 0.68 ± 0.090 |
| BA3F/FV | Surface water | Battonya | 0.66 ± 0.150 |
| BA3G/FV | Surface water | Battonya | 0.63 ± 0.070 |
| CSF1/TV | Ground water | Csorvás | 0.65 ± 0.130 |
| CSF2/TV | Ground water | Csorvás | 0.82 ± 0.040 |
| CS1F/TV | Ground water | Csorvás | 0.68 ± 0.120 |
| KT2F/TV | Ground water | Kőröstarcsa | 0.76 ± 0.040 |
| KT2G/TV | Ground water | Kőröstarcsa | 0.41 ± 0.040 |
| MH2F/TV | Ground water | Medgyesegyháza | 0.75 ± 0.080 |
| BSZ1A/TV | Ground water | Békéscsaba | 0.93 ± 0.080 |
| BSZ1B/TV | Ground water | Békéscsaba | 0.60 ± 0.050 |
| BSZ1C/TV | Ground water | Békéscsaba | 0.44 ± 0.001 |
| BSZ1D/TV | Ground water | Békéscsaba | 0.46 ± 0.107 |
| BSZ1E/TV | Ground water | Békéscsaba | 0.66 ± 0.100 |
| BSZ1F/TV | Ground water | Békéscsaba | 0.42 ± 0.009 |
| GYN1A/TV | Ground water | Gyomaendrőd | 0.33 ± 0.066 |
| GYN1B/TV | Ground water | Gyomaendrőd | 0.43 ± 0.069 |
| GYN1C/TV | Ground water | Gyomaendrőd | 0.98 ± 0.003 |
| GYN1D/TV | Ground water | Gyomaendrőd | 0.56 ± 0.260 |
| GYN1E/TV | Ground water | Gyomaendrőd | 0.33 ± 0.055 |
| GYN1F/TV | Ground water | Gyomaendrőd | 0.35 ± 0.012 |
| GYN1G/TV | Ground water | Gyomaendrőd | 0.63 ± 0.040 |
| GYN1H/TV | Ground water | Gyomaendrőd | 0.59 ± 0.040 |
| GYN1I/TV | Ground water | Gyomaendrőd | 0.25 ± 0.009 |
| GYN1J/TV | Ground water | Gyomaendrőd | 0.59 ± 0.110 |
| GYN1K/TV | Ground water | Gyomaendrőd | 0.87 ± 0.080 |
| OÜ1A/TV | Ground water | Orosháza | 0.39 ± 0.063 |
| OK1A/TV | Ground water | Orosháza | 0.39 ± 0.07 |
| OK1D/TV | Ground water | Orosháza | 0.38 ± 0.011 |
| OK1E/TV | Ground water | Orosháza | 0.33 ± 0.186 |
| OK1F/TV | Ground water | Orosháza | 0.35 ± 0.122 |
| OK1H/TV | Ground water | Orosháza | 0.31 ± 0.031 |
| OK1G/TV | Ground water | Orosháza | 0.66 ± 0.040 |
| OK1I/TV | Ground water | Orosháza | 0.96 ± 0.100 |
| OK1B/TV/c | Ground water | Orosháza | 0.58 ± 0.060 |
| OK1C/TV/b | Ground water | Orosháza | 0.30 ± 0.019 |
| OK1K/TV | Ground water | Orosháza | 0.33 ± 0.009 |
| OK1L/TV | Ground water | Orosháza | 0.33 ± 0.046 |
| OK1M/TV | Ground water | Orosháza | 0.54 ± 0.003 |
| *2011 sampling regime* | | | |
| DH1/FV | Surface water | Hainburg | <LOD[a] |
| DB1/FV | Surface water | Bratislava | <LOD |
| DKM1/FV | Surface water | Komarno-bridge | <LOD |
| DKM2/FV | Surface water | Komarno-bridge | <LOD |
| DDO1/FV | Surface water | Dömös-riverbank | <LOD |
| DDO2/FV | Surface water | Dömös-riverbank | 0.043 ± 0.009[b] |
| DL1/FV | Surface water | Luppa-island | <LOD |
| DL2/FV | Surface water | Luppa-island | <LOD |
| DDU1/FV | Surface water | Dunaújváros | <LOD |
| DKP1/FV | Surface water | Kopaszi gát-dam | <LOD |
| DKP2/FV | Surface water | Kopaszi gát-dam | 0.035 ± 0.017 |
| DED1/FV | Surface water | Érd | <LOD |
| DED2/FV | Surface water | Érd | <LOD |
| DER1/FV | Surface water | Ercsi | <LOD |
| DER2/FV | Surface water | Ercsi | <LOD |
| DDU2/FV | Surface water | Tököl-backwater | <LOD |
| DM1/FV | Surface water | Mohács | <LOD |
| V1/FV | Surface water | Lake Velencei | 0.064 ± 0.021 |

[a] LOD is estimated to be 0.05 ng/ml (on the basis of 90% $B/B_0$) and 0.12 ng/ml (on the basis of the value and standard deviation of the upper plateau of the sigmoid standard curve).
[b] Detected values are near the LOD (on the basis of 90% $B/B_0$).

samples from the Danube were not found to contain any significant amounts of glyphosate.

The sharp contrast between the contamination rates found in the two campaigns is likely largely due to regional differences (different catchment areas and agricultural circumstances), and partly by meteorological differences between the two years (a major difference in natural precipitation). The 2010 samples were collected in early autumn after a rainy summer. Officially reported precipitation data indicate a heavy rain event (18 and 28 mm at related locations) prior to and slight precipitation (4 and 1 mm) on the days of sampling (Sep 7–8, 2010) in the sampling region. This precipitation facilitates run-off and leaching of applied glyphosate into surface waters. This is in accordance with the results of Hanke [21] and Kjaer [43] on the occurrence of glyphosate in surface water showing strong correlation with rain events. As described [21], fast run-off from hard surfaces resulted in sharp increases in glyphosate concentrations (4.15 ng/ml) after the rain events. Such run-off of glyphosate generally exceeded those of other commonly used herbicides such as atrazine or mecoprop. A reported heavy storm event induced rapid leaching of glyphosate at concentrations reaching 2.1 ng/ml [43]. Average concentration in the drainage water during the leaching period (ca 5 months after the pesticide application) was 0.54 ng/ml. The amount leached is low compared to the applied doses. Moreover, elevated Fe and Al content in the upper soil layer may have provided sufficient sorption capacity to prevent leaching of glyphosate. In our survey in 2011, in contrast to 2010, due to the drought period and the lack of rain events prior to sampling, glyphosate applied in September most likely remained bound to soil particles and was not leached from the fields by the date of sampling (Oct 1, 2011).

These findings are in agreement with glyphosate contamination reported in environmental water contamination studies. In the United States, surface water contamination has been reported due to run-off from agricultural areas [44,45] or pesticide drift [46,47]. Glyphosate has been listed among pesticides of potential concern in surface water contamination in the Mediterranean region of Europe in the mid-nineties [48], and glyphosate and AMPA were found as contaminants in two small tributaries of the Ruhr River in North-Rhine-Westphalia, Germany at concentrations as high as 0.59 ng/ml [49]. A monitoring study in Norway [50] found frequent occurrence of glyphosate and AMPA in surface water (54% of 540 surface water samples in 1995–1999). Monitoring in Catalonia, Spain between 2007 and 2010 [36] reported a 41% contamination rate in the ground water samples analyzed. Similar finding were reported in the United States in 2002 [51] and 2006 [42], as well as in Canada in 2004–2005 (21% of 502 samples contained glyphosate or AMPA at maximum concentrations of 41 and 30 ng/ml, respectively) [23]. In France, glyphosate and AMPA were detected in 2007 and 2008 due to urban run-off [52]. Villeneuve et al. [53] found glyphosate to be one of the three herbicides most often detected in freshwater ecosystems worldwide and in French streams. Byer et al. [26] analyzed over 700 samples using the Abraxis ELISA test kit. Concentrations exceeded the method LOD (at that time) of 0.1 ng/ml in 33% of the samples collected in 2007, with a maximum concentration of 12 ng/ml. Glyphosate occurrence showed a bimodal temporal distribution with peak concentrations in late spring/early summer and fall. In another study, glyphosate was determined in surface water, soil, and sediment samples from a transgenic soybean cultivation area in Argentina [54]. As for water samples other than surface water, similar results were obtained in tile-drainage water samples from a loamy site in a Danish field experiment [43].

## 4. Conclusions

In conclusion, ELISA is a suitable and convenient method for glyphosate detection and has been successfully applied to surface and ground water samples. Although the lack of cross-reactivity with AMPA and the cost may hinder its widespread application, ELISA is still more cost-effective for routine analysis, especially in monitoring programs, as compared with traditional wet chemistry methods, if all sample preparation/measurement steps and the instrumental demand are all considered. In order to obtain more accurate results and eliminate matrix effects, characteristics of the water sample to be analyzed must be taken into account. As matrix effects

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were not experienced at all with surface water, the ELISA method appears to be readily applicable to surface water samples. Significant matrix effects were, however, experienced with tap water, indicating that the chlorine content of drinking water and/or the presence of multivalent cations may cause a considerable bias resulting in lower glyphosate content measured. Such effect was not eliminated by evaporation and subsequent resolution in water, yet was successfully eliminated by reducing agents such as ascorbic acid.

The level of glyphosate pollution in surface water detected in environmental studies may vary tremendously among locations and years of sampling, as glyphosate is strongly influenced by precipitation. Rain events result in the leaching of glyphosate from soil, due to its high water solubility. In this way, glyphosate may contaminate surface water and locations distant from its application site. This effect was seen in the current study. In spite of the fact that cultivation of GT crops is prohibited in Hungary, glyphosate was found at significant concentrations in surface water and ground water samples after a rainy period in 2010. In contrast, samples from a different catchment area, the Danube River, after a dry period in 2011 were found not to be contaminated by this target analyte.

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