

Glyphosate attachment on aminoactivated carriers for sample stabilization and concentration

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Abstract. Glyphosate (N-phosphonomethylglycine) is the most widely used non-selective postemergence herbicide for weed and vegetation control. The need for monitoring glyphosate levels in environmental samples and agricultural products proceeds from its extensive use due to the unregulated application and contradictory information about its toxicity on living organisms. In order to achieve high sensitivity and reliability of glyphosate assessment, stabilization and preconcentration steps are generally required for its determination in different samples. The aim of the present study was to develop and optimize possibilities for effective glyphosate stabilization and concentration using aminoactivated nano- and microparticles of different materials. The results show that the usage of aminoactivated iron (II,III) oxide magnetic nanoparticles for the attachment and concentration of glyphosate is a prospective option to be integrated with *in situ* analytical technologies (e.g. biosensors), as the whole processes of glyphosate attachment was efficiently and reproductively carried out within 20 minutes.

Key words: glyphosate, stabilization, concentration, analysis, amino-activated, magnetic iron oxide nanoparticles, silica.

INTRODUCTION

Glyphosate (N-phosphonomethylglycine; trade name Roundup) is the most widely used non-selective postemergence herbicide for weed and vegetation control. Glyphosate is commonly regarded to be safe to the environment, but several studies point out its potential toxicity on living organisms (Marc et al., 2004; Relyea, 2005a; Relyea, 2005b; Benachour & Séralini, 2008; Paganelli et al., 2010; Jones et al., 2011). Due to the unregulated application, the occurrence of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) in soil, ground and surface water has raised the need of continuous monitoring of glyphosate levels both in environment and agricultural products (Torstensson et al., 2005; Kolpin et al., 2006; Mörtl et al., 2013).

Limits have been set for glyphosate levels in drinking water and food. The maximum concentration of glyphosate in drinking water in the European Union should not exceed 0.1 µg l⁻¹ (Council Directive 98/83/EC); in US, the tolerable level is much higher, being 700 µg l⁻¹ (EPA, 2014). The maximum residue level of glyphosate in foods and feeds of plant and animal origin is currently set between 0.05–50 mg kg⁻¹, depending on the particular object (MRL Database, 2015).

The quantitative analyses of glyphosate and AMPA are commonly carried out using high performance liquid chromatography (HPLC). Although the sensitivity of HPLC is very high and the detection limits of glyphosate and AMPA are as low as $0.1 \mu\text{g l}^{-1}$ (Lee et al., 2002), there is an urgent need for the development of analytical methods for continuous monitoring of these compounds in real-time. A promising option for rapid analyses is the application of biosensors. In order to achieve high sensitivity and reliability of detection, stabilization and preconcentration of glyphosate samples are generally required as glyphosate is highly soluble in water and is readily carried into surface water, streams and lakes (Council Directive 91/414/EC; Sanchís et al., 2012; Ding & Yang, 2013).

At present, only a limited number of glyphosate biosensors have been proposed. Sandwich-type immunosensors for glyphosate based on Au or CoB magnetic nanoparticles coupled with a fluorescence marker have been developed by Lee et al (2010; 2013). These systems are quite complex with limits of glyphosate detection (LOD) 0.01 mg l^{-1} and $0.046 \mu\text{g l}^{-1}$, respectively.

Glyphosate is readily degraded by microorganisms and its half-life depends on environmental conditions. The median half-life of glyphosate in water varies from a few days to 91 days (NPIC). To enhance the reliability of its quantitative analyses, preliminary glyphosate stabilization has been additionally used. Glyphosate has been covalently immobilized on amino-activated solid carriers through (N-[3-dimethylaminopropyl]-N'-ethylcarbodiimide hydrochloride) / (N-hydroxysuccinimide) or EDC/NHS chemistry. Ding & Yang (2013) used this method to immobilize glyphosate on amino-activated glass beads and developed a surface plasmon resonance biosensor with a glyphosate detection limit $98 \mu\text{g/l}$. In this biosensor, silica beads served as a solid support to prevent the loss of glyphosate during screening procedures with oligopeptides used for glyphosate biorecognition (Ding & Yang, 2013).

In addition, glyphosate molecule acts as an anion within relevant pH ranges in water and it can be bound onto solid surfaces by sorption (Borggaard & Gimsing, 2008). Due to the high specificity of alumina towards phosphate groups, alumina-coated iron oxide nanoparticles as the affinity adsorbent for glyphosate and AMPA in aquatic solutions have been reported. This micro-scale solid phase extraction has been used in combination with electrochemical detection of glyphosate and AMPA with LODs 0.3 and $30 \mu\text{g l}^{-1}$ respectively (Hsu & Whang, 2009). The presence of negatively charged groups of glyphosate has been also used in glyphosate analyses with the positively charged amino groups of cysteine-modified gold nanoparticles (Cs-AuNPs), where glyphosate stimulates the aggregation of Cs-AuNPs, detectable photometrically with LOD $9.9 \mu\text{g l}^{-1}$ (Zheng et al., 2013). For a more selective adsorption of soluble glyphosate, molecularly imprinted microspheres (MIMs) have been synthesized. However, the application of MIMs in integration with the measurement of chemiluminescence resulted in higher LOD values for glyphosate: $46 \mu\text{g l}^{-1}$ (Zhao et al., 2011). As it can be seen, the detection limits of glyphosate with biosensors are quite close to the glyphosate levels set by EU in drinking water. However, there is still a need for more sensitive analyses, which can be achieved by sample stabilization and preconcentration.

The aim of the present study was to develop rapid and reliable methods for glyphosate attachment on aminoactivated nano- and microparticles, which enable to improve the sensitivity and reliability of glyphosate analyses and can be used in further quantitative detection with biosensors or other systems (Fig. 1). Glyphosate was

immobilized onto two different types of aminoactivated solid beads – silica microparticles and magnetic iron oxide nanoparticles, which can be easily extracted after immobilization from mixtures using bead injection or magnetic separation techniques, respectively.

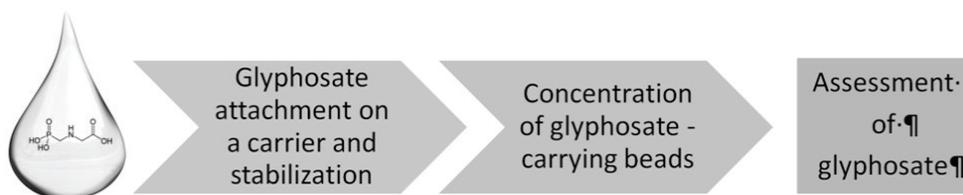


Figure 1. Steps of glyphosate analyses.

MATERIALS AND METHODS

Aminosilasorb (aminoactivated silica beads or ASB, \varnothing 30 μm) was purchased from Chemapol (Brno, Czech Republic) and aminopropyl silane coated magnetic iron oxide nanoparticles (AFeNP, \varnothing 14 nm) from GreenBead OÜ (Tartu, Estonia). Both materials were fabricated with the help of 3-aminopropyltriethoxysilane and had similar chemically active groups on surface (Fig. 2).

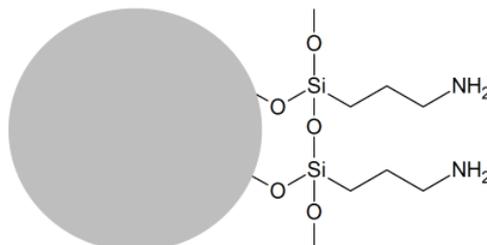


Figure 2. The surface of aminoactivated beads.

The analytical standard of glyphosate (N-phosphonomethylglycine) (99.7%) was purchased from Sigma. We also used the commercial preparation of glyphosate (36% solution in water) (Monsanto Europa S.A.). EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (\geq 98%) and NHS (N-Hydroxysuccinimide) (98%) were purchased from Sigma; all other reagents used were of analytical grade from different producers. For preparation of all solutions the deionized water was used.

Glyphosate immobilization on the aminoactivated beads was carried out using EDC/NHS chemistry according to the reaction scheme shown on Fig. 3.

The activation of carboxyl groups of glyphosate was carried out at room temperature in 10 mM HEPES buffer (pH 7.0) at EDC and NHS concentrations 0.2 M and 0.05 M, respectively. EDC reacts with a carboxylic-acid group of glyphosate forming an amine-reactive O-acyl isourea intermediate. The addition of NHS convert it to an aminereactive NHS-ester, thus increasing the efficiency of EDC-mediated coupling reaction (Bart et al., 2009). After 10 min of incubation under constant stirring, 10–40 μl

of the solution, containing activated glyphosate was mixed with aminoactivated carrier beads. The immobilization time at room temperature (10 mM HEPES buffer, pH = 7.0) was 1–120 minutes. After immobilization, the beads were washed with 0.1 wt % SDS solution and DI water to remove unbound glyphosate. The concentration of bound glyphosate was determined photometrically measuring the light absorption of phosphorus-complexes at $\lambda = 880$ nm with spectrometer UV-1800 (Shimadzu) after treatment of glyphosate-bead complexes according to ascorbic acid method (Greenberg et al., 1985).

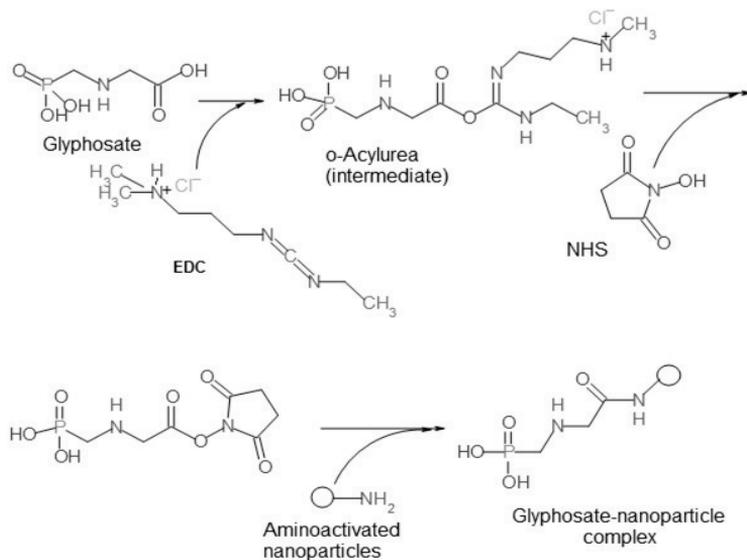


Figure 3. Immobilization of glyphosate onto aminoactivated beads.

RESULTS AND DISCUSSION

Glyphosate was immobilized on the aminoactivated silica beads and magnetic iron oxide nanoparticles using EDC/NHS chemistry. The optimization of immobilization process was focused on the generation of reproducible amounts of bound glyphosate within minimal time. For these studies, the concentrations of ASB and AFeNP were 200 and 20 mg l⁻¹, respectively.

As the first step, we determined the minimal number of washing procedures to remove all unbound glyphosate. Aspiration was used for ASB and magnetic extraction for AFeNPs to separate the insoluble particles with attached glyphosate from mixtures. It was found that in case of ASB, the minimal number of washing procedures to remove all the unbound glyphosate was 10 and in the case of AFeNPs 1 (data not shown). This difference is the result of effective separation of AFeNPs with magnets, placed outside the reaction tube; at the same time, the separation of ASBs from solution was carried out with the help of vacuum filtering. The abovementioned numbers of washing procedures were applied in further studies of optimizing the immobilization time and building the calibration curves for the bound glyphosate.

The main factor of decreasing the time of the overall stabilization and concentration procedure of glyphosate is the time required for its immobilization on a carrier. The

recovery rates of glyphosate bound to ASB or AFeNP after 1–120 minutes of immobilization is shown on Fig. 4.

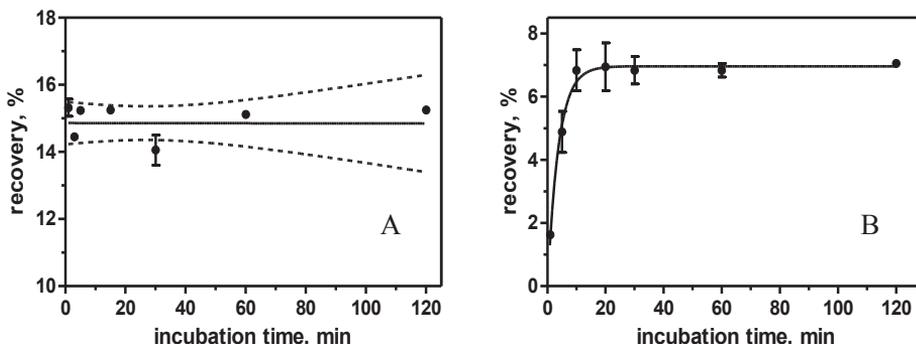


Figure 4. Glyphosate recovery at different immobilization times. The immobilization was carried out at room temperature in 10 mM HEPES buffer at pH 7.0. **A:** onto aminoactivated silica beads, [glyphosate] = 8 mg l⁻¹; [ASB] = 200 mg l⁻¹; **B:** onto AFeNP, [glyphosate] = 4 mg l⁻¹; [AFeNP] = 20 mg l⁻¹.

The measured maximal recovery of glyphosate was twice bigger with ASB than AFeNP, (14.9 ± 0.3) % and (7.0 ± 0.2) % on the initial amount of glyphosate in solution, respectively (Fig.4). Based on the obtained data, we calculated the maximum specific load of immobilized glyphosate per 1 mg carrier (ML). The value of ML was (6.0 ± 0.1) µg glyphosate/mg ASB and (14.0 ± 0.4) µg glyphosate/mg AFeNP. These ML values were also used to estimate the concentration of active amino groups on the surface of the used particles. These calculated concentrations were 0.036 µM mg⁻¹ for ASB and 0.083 µM mg⁻¹ for AFeNP. Taking into consideration the estimated number of particles per mg and the Avogadro number, we found that both carriers used had over 10³ amino groups per particle available for glyphosate immobilization.

The maximum immobilization yield was achieved already within 1 minute incubation with ASB and there was no change in recovery during the next 119 minutes. So, using ASB, the incubation time for immobilization can be as short as 1 minute. Shorter incubation times were not studied in the present work due to technical reasons, but if ASB will be used in automated analysis system, even shorter immobilization times can be considered. For AFeNPs, the shortest immobilization time yielding maximum attachment (Fig. 4B) was 10 minutes (it was also similar for other glyphosate concentrations, data not shown).

Comparing the immobilization of glyphosate on ASBs and AFeNPs, the total time required as a sum of immobilization and washing procedures is considerably lower for AFeNPs due to the very effective separation of unbound glyphosate. For further decrease of the immobilization time, higher temperatures can be suggested.

The concentration of ASBs used for immobilization was taken as proposed by Ding & Yang (2013). As there was no data available for AFeNPs, the influence of the concentration of AFeNPs with the aim of improving the sensitivity of the system was studied. As it can be seen on Fig. 5, the detected recovery of glyphosate increases in a linear mode as a function of the concentration of AFeNPs (shown with 95% confidence band).

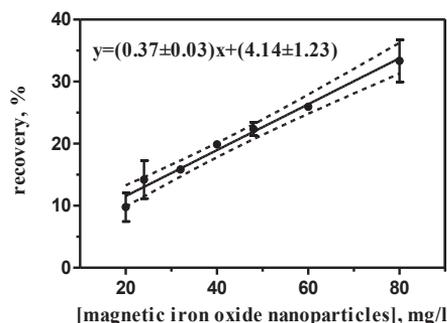


Figure 5. The recovery of glyphosate after immobilization as a function of the concentration of magnetic iron oxide nanoparticles. The initial concentration of glyphosate was 4 mg l⁻¹. The immobilization was carried out at room temperature in 10 mM HEPES buffer at pH 7.0 for 10 min.

Increasing the concentration of AFeNPs to 80 mg l⁻¹ raised the immobilization yield to over 30% of the theoretical. Higher recoveries lead to lower glyphosate detection limits, which can be further improved by combining the glyphosate attachment with more sensitive detection methods.

The glyphosate calibration curves with 95% confidence bands, using ASB or AFeNP at concentrations 200 and 20 mg l⁻¹ respectively, are shown on Fig. 6.

The calibration plots were linear in the studied concentration ranges. The line functions for ASB and AFeNP systems were following:

$$y = (0.022 \pm 0.001)x + (0.069 \pm 0.006) \quad (\text{ASB})$$

$$y = (0.023 \pm 0.002)x + (0.085 \pm 0.005) \quad (\text{AFeNP})$$

The high detection limits were resulting from the detection limit of phosphorus by ascorbic acid method, being 0.1–1 mg l⁻¹ (Greenberg et al., 1985). The slope of calibration curve, defining the sensitivity of detection, was similar for ASB and AFeNP systems, although the concentration of aminoactivated silica beads was 10 times higher. As shown on Fig. 5, the slope for AFeNP system can be increased easily by increasing the concentration of AFeNPs. The y-intercepts of these calibration plots indicate the background signal of the used photometric detection.

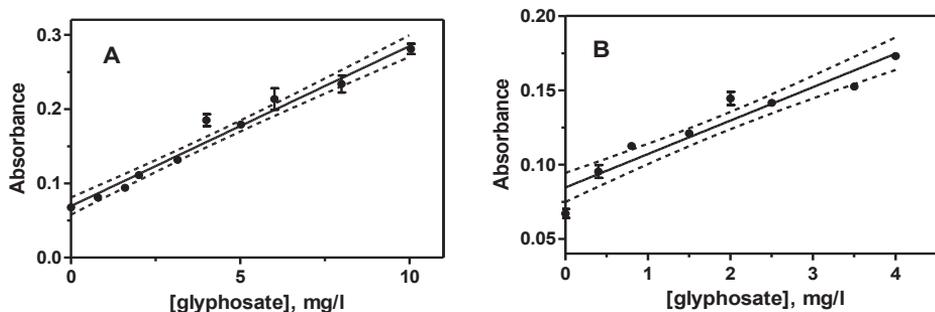


Figure 6. Glyphosate calibration curves for ASB and AFeNP systems. The immobilization of glyphosate was carried out at room temperature in 10 mM HEPES buffer at pH 7.0. **A:** with aminoactivated silica beads; [ASB] = 200 mg l⁻¹; **B:** with AFeNP; [AFeNP] = 20 mg l⁻¹.

CONCLUSIONS

The study indicates that the application of AFeNPs for the stabilization and concentration of glyphosate-containing probes has a high potential to be integrated with *in situ* analyses for continuous monitoring of glyphosate, as all necessary procedures (incl. glyphosate activation, immobilization and concentration) can be carried out with high efficiency within 20 minutes. For the development of methods for practical analyses, the detection of the bound on AFeNPs glyphosate will be further carried out with immunobiosensing system, enabling to achieve high selectivity and low detection limits, which are in line with the established glyphosate concentrations in drinking water and food.

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