DETERMINATION OF ALIPHATIC ORGANO-PHOSPHORUS COMPOUNDS IN ENVIRONMENTAL SAMPLES BY INTEGRATED PULSED AMPEROMETRIC DETECTION-ION CHROMATOGRAPHY

by

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Dedication

This thesis is dedicated to the author's parents, Alan and Jaime Rayford. Thank you for the support during this critical juncture of their sons' lives. This thesis was also completed with the help and support of James Rayford.

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ABSTRACT

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Robert Rayford
University of Houston-Clear Lake, 2021

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Glyphosate (*N*-(phosphonomethyl) glycine) is globally the most commonly used herbicidal active ingredient. Public concerns have elicited environmental agencies to monitor the persistence and occurrence of glyphosate. Recent environmental assessments quantify its annual mean concentration well within the parts per trillion range for US natural water sources. The environmental metabolites of glyphosate, aminomethyl phosphonic acid (AMPA), and the herbicidal active ingredient glufosinate are also frequently analyzed alongside glyphosate due to their similar chemical structures. However, their determination is labor intensive and not amenable to typical analytical methods due to the polyprotic and photo-inactive properties of these compounds. These compounds are typically analyzed by derivatization-based single residue methods

(SRMs), where a few analytes undergo quantitative analysis by Liquid Chromatography-Tandem Mass Spectrometry method only (LC-MS/MS). The United States Geological Survey (USGS) has developed a method for glyphosate determination using online SPE-LC-MS/MS. Yet, studies utilizing similar LC-MS/MS methods have suggested that derivatizing steps are susceptible to salt-associated matrix effects. Ion Chromatography-Integrated Amperometric Detection (IC-IPAD) offers simple, direct analysis of aliphatic organo-phosphorus compound without derivatization steps. In this study, an offline SPE-IC-IPAD method was developed and validated for the determination of glyphosate and other aliphatic organophosphorus compounds in environmental water samples. The linear range was found to be 3-750 µg L^{-1} (R^2 = 0.9973, 0.9998, 0.9983) and the limits of detection (LOD) were found to be 0.950, 0.402, and 0.252 µg L^{-1} for glyphosate, glufosinate, and AMPA in reagent water, respectively. The offline solid phase extraction (SPE) method provided excellent recovery values (104-131%) by standard addition of spiked glyphosate in Horsepen Bayou water. Although our SPE-IC-PAD method was not able to detect glyphosate, glufosinate, and AMPA in several surface water samples collected within the Houston area, a satisfactory recovery value of 96.8% was achieved for certified reference material containing glyphosate. Further, our study on optimal sample storge conditions suggested that glyphosate is stable in acidified surface water for 36 days. Our study recommends acidified or refrigerated conditions following sample collection intended for glyphosate determination. Optimal method development parameters and development strategies were also discussed with the intention to further lower LOD values and improve precision in natural water matrices.

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1. Introduction

Background information concerning the scientific and regulatory interest toward glyphosate occurrence will be reviewed in this section. Chemistry and physical properties of AMPA, glufosinate, and glyphosate will be provided. The metabolic pathway concerning glyphosate will be reviewed. The working methods developed by the United States Geological Survey (USGS) and U.S. Environmental Protection Agency will also be reviewed as a means of comparison to our offline Solid Phase Extraction-Ion Chromatography – Integrated Pulsed Amperometric Detection (SPE-IC-IPAD) method. Finally, the amperometric detector and electrochemical process behind how this method detects glyphosate will also be reviewed.

1.1 Background

Glyphosate has been regarded as one of the most frequently used herbicidal active ingredients currently on the market. Glyphosate is a systemic and post-emergence herbicidal active ingredient used primary as weed management for its non-selective action (Benbrook, 2016). Its market release in 1974 was promptly increased with the development of glyphosate resistant crops, accelerating both the market value of the active ingredient and its application rates.

Environmental concerns over the widespread use of glyphosate were raised when toxicological assessments provided insights into its environmental and human health impact. Glyphosate has exhibited overland runoff and spray draft which leads to environmental exposure of non-target organisms (Giesy et al., 2000). Non-target plants occupying crucial ecological niches are susceptible to its mode of action, inhibiting 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in the shikimic acid pathway essential for aromatic amino acid synthesis (Annett et al., 2014). While this pathway is largely absent in animal cells, glyphosate-based herbicides are suspected to induce

toxicity toward aquatic life through the polyethoxylated tallow amine (POEA), a surfactant commonly included in their herbicidal formulation (Dill et al, 2010). Figure 1 provides a list of aliphatic organophosphorus compounds and their metabolites which are of interest to environmental analyses due to their implication in non-point source pollution.

Concerns were further elevated when the International Agency on Cancer Research (IARC), the authority of cancer research under the World Health Organization (WHO), categorized glyphosate as a probable carcinogen (Group 2A) based on reports provided from occupational exposure and animal experimental studies (Benbrook, 2019; IARC, 2017). Following this evaluation, the U.S. Environmental Protection Agency (EPA) re-evaluated its classification of glyphosate as a carcinogen, reaffirming its prior classification as a non-carcinogen when following safety guidelines (EPA). The discrepancy in categorizing whether glyphosate acts as a carcinogen has prompted both a scientific and regulatory re-assessment of the safety regarding glyphosate and other organophosphorus compounds as a herbicidal active ingredient.

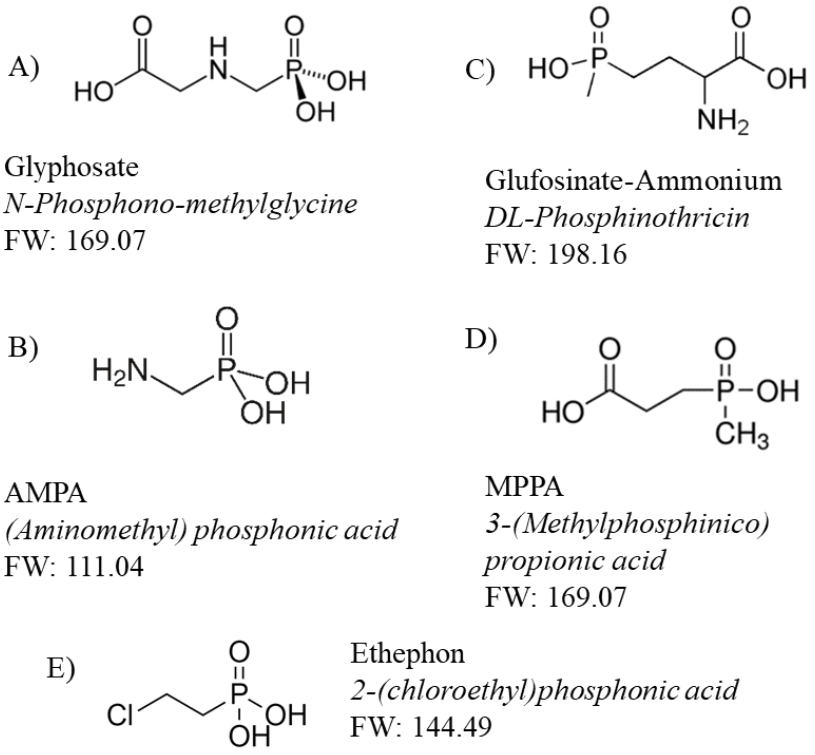

Figure 1. A selected list of aliphatic organophosphorus compounds frequently studied in environmental analysis. The compounds presented are as follows: A. Glyphosate, B. Amino-methyl phosphonic acid (AMPA), C. Glufosinate (Phosphinothricin) depicted in its acid form, D. 3-(Methylphosphinico)propionic acid (MPPA), and E. Ethephon.

Furthermore, while glyphosate's role as a global leading active ingredient can be accurately assessed through market figures and application data, records on its occurrence and persistence in the environment and the effects of low dose chronic exposure on the general population through dietary uptake are less certain (Benbrook, 2016). Environmental assessments of glyphosate and its major metabolite, amino-methyl phosphonic acid (AMPA), have been primarily conducted by the United States Geological Survey (USGS) as part of its efforts to evaluate non-point source pollution of US natural water sources (Lee et al., 2001). Whereas, the U.S. Food and Drug Administration (FDA) has only recently included glyphosate and glufosinate, a structurally similar herbicidal active ingredient, in its pesticide residue monitoring for foods prepared for consumption as of 2017 (U.S. FDA, 2017). While these environmental

and health assessments provide exhaustive scientific reports on glyphosate and AMPA occurrence, the chemical and physical properties of glyphosate and other organophosphorus compounds restrict their monitoring to specially-equipped laboratories such as LC-MS/MS. Calls for more glyphosate monitoring regarding environmental, eco-toxicological, and food analysis have been published (EFSA, 2015; Huhn, 2018). More affordable analytical methods capable of providing robust and accurate assessment of glyphosate and other organophosphorus compounds in natural sources will be needed to meet these challenges.

1.2 Chemical and Physical Properties

Glyphosate (*N*-(phosphonomethyl)glycine) is an organophosphorus compound which shares moieties with glycine. Glufosinate (phosphinothricin) and its metabolite, 3-(methylphosphinico)propionic acid (MPPA), are also characterized by their similar chemical structures to glyphosate, and glufosinate is routinely included in glyphosate determination due to shared physical properties and use. The polyprotic acidic and amphiprotic nature of glyphosate is attributed to its phosphonate, carboxylate, and amine moieties. Chemical and physical properties are listed in Table 1. While the protonation sequence of these moieties is somewhat in dispute, the widely accepted dissociation constants provide insight into its behavior under aqueous conditions. The dissociation constants are as follows; pK_{a1} 0.78 (first phosphonic acid), pK_{a2} 2.29 (carboxylate), pK_{a3} 5.96 (second phosphonic acid), and pK_{a4} 10.98 (amine) (Chamberlain, 1996). Consequently, glyphosate exhibits zwitterionic in a wide range of pH values. In environmentally relevant pH values between 5-9, the zwitterion of glyphosate obtains net negative charges of -1 at pH values approximately between 5-6 and -2 at pH values approximately between 6-9. Under alkaline conditions, glyphosate behaves as a trivalent anion, providing a chemical property to be exploited by anion exchange-based methods

(Mallat and Barceló, 1998; Borggard et al., 2008; EFSA, 2015; Läubli et al., 2016; Rigobello-Masini, 2019).

Table 1. Physical and chemical properties of aliphatic organophosphorus compounds

Chemical Name	Glyphosate	AMPA	Glufosinate
Chemical Abstracts Service (CAS) No.	1071-83-6	1066-51-9	77182-82-2
Molecular Wt.	169.1 g mol^{-1}	111.04 g mol^{-1}	198.2 g mol^{-1}
Melting point	189 °C	120 °C	215°C
Decomposition Temperature	200-230 °C	200-230 °C	245-305 °C
Relative Density	1.74 g cm^{-3}	-	1.32 g cm^{-3}
Vapor Pressure	1.31×10^{-5} Pa	-	3.1×10^{-2} Pa
Henry's law constant	2.1×10^{-7} Pa m^3 mol^{-1}	-	4.48×10^{-9} Pa m^3 mol^{-1}
Water Solubility	10.5-15.7 g L^{-1}	5.8-10.5 g L^{-1}	1370 g L^{-1}
Surface tension	72.2 mN m^{-1}	-	72.2 mN m^{-1}
Partition co-efficient: Log K_{OW}	-3.2	-2.36	-4.81
UV-Vis absorption (λ_{max})	-	-	193 nm
Sorption partition coefficient: (K_d)	3-1,188	-	-
Sorption partition coefficient: (K_{OC})	9-60,000	9,749[b]	9.6-1,229
Dissociation Constants (pK_{a1}, pK_{a2}, pK_{a3}, pK_{a4})	0.78, 2.26, 5.96, 10.98	0.9, 5.6, 10.2	<2, 2.9, 9.8
Half-life ($DT_{50, water}$)	7–142 days 2-91 days	-	12-70 days
Half-life ($DT_{50, soil}$)	2 – 215 days	76 -240 days[c]	-

a. Properties related to glyphosate, glufosinate, and AMPA were taken directly from Giesy et al. (2000), MacLachlan (2013), and Traas and Smit (2003), respectively.
b. EFSA (2015)
c. Annett et al. (2014)

1.3 AMPA Metabolic Pathway in the Environment

The environmental degradation of a contaminant is considered the transformation of the parent compound to the metabolite by abiotic and biotic processes. The degradation has been well-established through a microbe-mediated pathway in soil as its primary degradation process. The C-N bond cleavage of glyphosate produces its most widely occurring metabolite, amino-methyl phosphonic acid (AMPA), in the

environment. AMPA was detected as the primary metabolite by 13.3 to 50.1% applied radioactivity in soil degradation studies (EFSA, 2015). AMPA occurs as the primary metabolite under both aerobic and anaerobic conditions. Abiotic processes such as photolysis are typically regarded as minor compared to the microbial processes (Mercurio et al., 2014; EFSA, 2015). Comprehensive hydrological assessments by the USGS determined that AMPA persisted in greater concentrations than its parent, glyphosate, depending on the soil conditions that control the degradation processes (Battaglin et al., 2014) However, it is well understood that AMPA occurrence also results from non-point source pollution of other phosphonates such as EDTMP (ethylenediamine tetra (methylene-phosphonic acid)) and ATMP (amino tri-methylene-phosphonic acid), shown in Figure 2. Phosphonates have a wide range of applications including industrial detergents, anti-scaling agents in waste water treatment plants (WWTP), anti-corrosives, fire retardants in manufacturing, and dispersants in ceramics and the cement industry (Studnik et al., 2015; Grandcoin et al., 2017). Phosphonates are thought to undergo metal oxide-catalyzed photo-degradative processes in soil as their primary degradation pathway, as these compounds possess poor biodegradability due to the strength of its C-P bond (Grandcoin et al., 2017). While tracing AMPA occurrence through each respective parent species has yet to be shown as feasible, the same hydrological assessment by the USGS detected AMPA 17.9% of the time without glyphosate as compared to when glyphosate was detected 2.3% of the time without AMPA (Grandcoin et al., 2017; Battaglin et al., 2014). This USGS assessment was conducted from 2001-2010 with 3,732 samples across 38 U.S. hydrological sites.

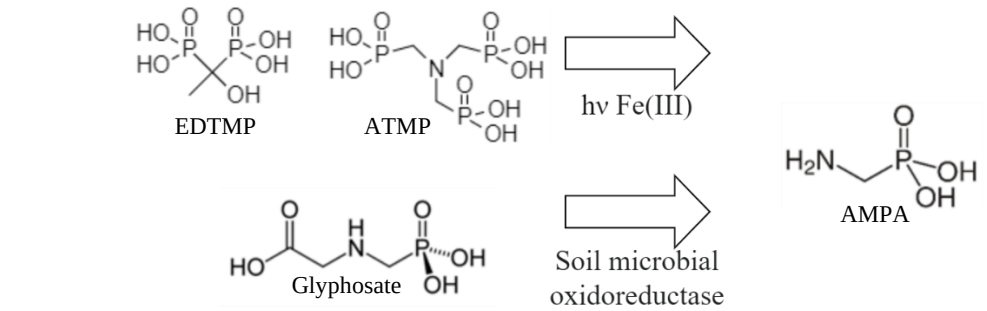

Figure 2. The primary degradation pathways for industrial detergents and glyphosate contributing to AMPA occurrence in the environment. Industrial detergents undergo metal-catalyzed photodegradation as their main pathway, whereas glyphosate undergoes soil biodegradation through microbial oxidoreductase (Annett et al., 2014; Grandcoin et al., 2017).

Glyphosate can also undergo a C-P lyase pathway which is regarded as far more chemically benign due to the formation of non-toxic metabolites, sarcosine and phosphate. A limited number of toxicological assessments have concluded that AMPA possesses no greater concern than glyphosate (Battaglin et al., 2014). Thus, AMPA is routinely included in environmental analyses of glyphosate to monitor the environmental occurrence and persistence of glyphosate.

1.4 Glyphosate Determination by the United States Geological Survey

US EPA and USGS have established regulatory methods for glyphosate determination in drinking water and typical natural water sources, respectively. US EPA Method 547 targeted glyphosate in drinking water, establishing the maximum concentration limit (MCL) in natural water sources at 700 μg L^{-1}. As with many amino acid determinations, this HPLC-FLD method utilized a post-column derivatization step with *O*-phthalaldephyde (OPA) to oxidize glyphosate for fluorescence quantitation. The method detection limit (MDL) for Method 547 was dependent on the sample matrix, where reagent water, ground water, and dechlorinated tap water provided method detection limit (MDL) values of 6.00, 8.99, and 5.99 μg/L, respectively (Winfield et al., 1990). The USGS Organic Geochemical Research Laboratory (OGRL) has established

two LC-MS/MS methods in its efforts to monitor non-point source pollution of hydrological sites throughout the US (Lee et al., 2001). The first OGRL developed method, known as method O-2136-01, utilized an automated online SPE LC-MS method for quantification of glyphosate, AMPA, and glufosinate in ground and surface water, where each analyte received a method reporting level (MRL) of 0.1 µg L^{-1}. Method development for these natural water sources necessitated the implementation of a clean-up, pre-concentration, and derivatization step filiated with 9-fluroenylmethylchloroformate (FMOC), a derivatization agent commonly utilized in amino acid determination. Quantification was accomplished by standard-addition with isotopically labeled (2-^{13}C, ^{15}N) glyphosate, as isotopically labelled internal standard (ILIS) spiking compensated for variability introduced by matrix effects and MS fragmentation (Lee et al., 2001). This variability was observed after the derivatization and MS fragmentation sequence. A collaborative analysis utilizing LC-MS/MS modified O-213-01 to provide a lower MRL value of 0.02 µg L^{-1}, where quantification used isotope dilution analysis for glyphosate and AMPA and pseudo-isotope dilution for glufosinate. This method was later validated and published as USGS method O-2141-09.

Method O-2141-09 provided considerably lower detection limit to glyphosate monitoring, as a 30% greater detection frequency in stream samples was determined when compared to method O-213-01 (Meyer et al., 2009). This method was also utilized to re-evaluate glyphosate concentrations in column water and bed-sediment core samples collected from an Oregon reservoir when a Monsanto developed HPLC-FLD method provide reporting values as high as 130 mg kg^{-1}. Upon re-evaluation, all samples were found to be well below the reporting level of 0.02 µg L^{-1} (Fosness et al., 2013). Regional distribution of glyphosate and AMPA in stream water samples across the greater US was

accomplished by this method, reporting detection frequencies above 74% for 70 US streams (Medalie et al., 2019).

Non-point source pollution of glyphosate and AMPA in runoff is typically regarded as limited due to their high affinity toward soil represented as their high adsorption coefficients, K_d and K_{oc} (Sprankle et al., 1975; Giesy et al., 2000). Glyphosate is considered persistent in the environment due to its soil-bound half-life which varies between days to months compared to its half-life in natural water sources of 7-91 or 2-142 days (EFSA, 2015). However, environmental assessments have frequently detected these aliphatic organophosphorus compounds in natural water sources, providing annual mean concentrations ranging from 0.02 to 0.16 μg L^{-1} by recent USGS assessments for glyphosate across US regions (Medalie et al., 2020).

1.5 Challenges in Method Development

Environmental analysis is contingent on developing a working method which meets a series of criteria set by regulatory and scientific communities. A full assessment of the physical and chemical properties of glyphosate can provide insight into the challenges surrounding method development for environmental analysis of aliphatic organophosphorus compounds (i.e., Table 1). Namely, these compounds are incompatible with typical chromatographic methods such as Reverse Phase-Liquid Chromatography (RP-LC) and Gas Chromatography (GC) methods due to their insolubility in organic solvents, low volatility, and thermal liability (Skeff et al., 2016). Their compatibility with typical spectroscopic methods is also limited due to their absence of chromophores and fluorophores. Both challenges are typically handled by derivatization procedures, as these derivatization steps are considered necessary for GC and largely unavoidable for LC analyses (Arkan and Molnár-Perl, 2015). Pre-column derivatization by 9-fluorenylmethyl chloroformate (FMOC-Cl) for fluorometric, UV, MS, and MS/MS makes up

approximately two-thirds of published methods for glyphosate by certain estimates (Koskinen et al., 2015). While derivatization procedures are routine for many established methods, these procedures have their drawbacks. These methods are typically regarded as time-consuming and require intensive offline bench top procedures. Briefly, natural water samples are typically spiked with 5% borate buffer and adjusted to pH 9 before spiking in concentrated FMOC-Cl reagent (Ibáñez et al., 2006). Reaction time for this derivatization have ranged from 2 hours to overnight under 30°C.

1.5.1 Challenges in Method Development: Salt-associated Matrix Effects

Variations in instrument response across multiple injections can be attributed to instrument drift and matrix effects experienced throughout the duration of analysis. Thus, method development should account for matrix effects on derivatization procedures of the sample matrices. In the case of aliphatic organophosphorus compounds, these matrix effects have been reported to affect the derivatization steps for glyphosate by HPLC-FLD, LC-MS, and LC-MS/MS methods (Ibáñez et al., 2006; Skeff et al., 2016; Gros et al., 2019). An interlaboratory study (ILS) commissioned by Monsanto Europe provided poor recovery values for ground water samples as low as 15% for glyphosate (Ibáñez et al., 2004). Upon re-evaluating of natural water sources with a modified LC-MS/MS method amended with an acidification to a final concentration of 120 mM HCl for an approximate pH value of 1, the mean recovery values for the analysis of groundwater and surface water samples improved to 100% and 91%, respectively (Ibáñez et al., 2006). A total of 13 glyphosate-positive samples retained from the 30-lab ILS analysis were also re-analyzed with the modified LC-MS/MS method. The acidification step provided these samples with increased response factor of 1 to 14 in detected concentrations suggesting in-field acidification would prevent salt-associated matrix effects which accrue during storage (Ibáñez et al., 2006). Ibáñez et al. attributed this increase in response factor to

coordinated metal-glyphosate complexes which release glyphosate upon acidification. Ibáñez and coworkers further postulated that certified QC samples of aliphatic organophosphorus compounds in natural water sources would attenuate the difference in instrument response between the spiked standards and stored real samples, as the spiked standards do not undergo comparable storage times experienced by real samples necessary to replicate the known matrix effects.

Skeff et al. (2016) assessed the salt-associated matrix effects on the detection of aliphatic organophosphorus compounds with Heated Electrospray Ionization (HESI)-LC-MS/MS, documented these matrix effects in the form of complex associated retention time shifts and quantitative changes in peak area ratios between analytes and ILISs when constituting these compounds in salt water of various concentrations. Matrix effects on HESI-MS/MS response for 5 µg L^{-1} glyphosate in artificial salt water at concentrations between 2 to 10 g L^{-1} were quantified by a 181% increase in glyphosate signal compared to glyphosate constituted in LC-MS grade water. This was attributed to the differential solvent compositions surrounding the ionization of glyphosate and glyphosate-metal complexes by the HESI. AMPA, glufosinate, and 2-AEP demonstrated low to moderate signal suppression across the artificial seawater concentrations. Skeff et al. also investigated the use of surrogate isotopically labeled internal standards of glyphosate and AMPA for the other aliphatic organophosphorus compounds, and their results revealed that these surrogates are only suitable under salt concentrations in the range of low g L^{-1}. AMPA-IS was recommended as a surrogate over glyphosate-IS due to its signal variation at greater salt concentrations (Skeff et al., 2016).

1.5.2 Challenges in Method Development: Solid Phase Extraction

Solid Phase Extraction (SPE) remains necessary if not unavoidable for LC method development in regards to glyphosate determination in most natural water sources and

agricultural products (Rigobello-Mashini et al., 2019). Method development may require SPE steps for matrix clean-up and pre-concentration purposes. As with the LC analytic column phases, the efficiency of SPE columns will be predicated on the compatibility of a particular SPE phase toward the aliphatic organophosphorus compounds intended for analysis. Thus, in the case of glyphosate, SPE and LC phases often share physical characteristics to target the physical and chemical properties of this analyte or its derivatives (Rigobello-Mashini et al., 2019; Koskinen et al., 2015). For example, the USGS method O-2141-09 utilizes Oasis HLB mode online-SPE cartridges following FMOC derivatization for simple extraction from the sample matrix. This online-SPE mode was also utilized by Ibáñez and coworkers to elucidate the matrix interferences responsible for unsatisfactory results of ground water analysis (2006). Hydrophobic (C18), Hydrophilic Lipophilic Balance (HLB), and other phases compatible with RP-LC methods follow FMOC-derivatization, and these phases are typically utilized for both enrichment and cleanup. Whereas, SAX SPE methods were effective for pre-concentration of glyphosate before derivatization (Mallat and Barceló, 1998; Jiang et al., 2007; Corbera et al., 2015; Rigobello-Mashini et al., 2019). SCX clean-up was found to be necessary for AGG determination by HPLC-FLD, as AGG became undetectable at concentrations below 100 ng L^{-1} in drinking water samples. Küsters and Gerhartz (2010) attributed this loss of AGG signal to the highest concentrated divalent cation, Ca^{2+}, determined to be present at 20.9-80.6 mg L^{-1} by atomic absorption spectroscopy (AAS). The presence of Cl^-, SO_4^{2-}, and HCO_3^- were also attributed to this matrix effect (Küsters and Gerhartz, 2010).

These SPE steps can be labor-intensive due to the need for precise pH adjustments (Jiang et al., 2007). For example, the authors found difficulties in performing pH adjustments necessary for FMOC derivatization after concentrations greater than 10 mM

HCl were used to elute glyphosate from SAX preconcentration columns (Jiang et al., 2007). Thus, analytical methods which are less pH sensitive throughout each step of preparation could facilitate quick and reliable monitoring programs. Moreover, workload for nation-wide studies conducted by government agency such as the USGS could benefit from reduced sample preparation by way of direct glyphosate determination rather than the determination of its derivatized species (Meyer et al., 2009).

1.6 Voltammetric Detection Methods

As mentioned earlier, LC-MS and LC-MS/MS have reported matrix effects in the analysis of glyphosate and other aliphatic organophosphorus compounds (Skeff,et al, 2016; Lee et al.; 2001). While these LC-MS and LC-MS/MS methods provided reliable and accurate detection of glyphosate, electrochemical analysis provides an opportunity to develop a highly selective analytic method for these compounds which does not depend on labor intensive derivatizing steps and ILISs susceptible to matrix effects.

A three-electrode electrolytic cell is a routinely used electrolytic cell set-up for voltammetric systems to measure the current generated by electrochemical processes occurring at the electrode surface. These electrochemical processes in an electrolytical cell only occur when a potential necessary for their forward reaction is applied (LaCourse, 1997). The electrolytic cell for a voltammetric detector is composed of a working, reference, and auxiliary electrode. The working electrode is responsible for measuring the electrochemical processes occurring at its surface and amplifying the resulting signal. A reference electrode provides a constant potential, facilitating an applied potential across the working electrode due to the potential difference between the working and reference electrodes. An auxiliary electrode completes the circuit, and a current is established between working and auxiliary electrodes (LaCourse, 1997).

Current, nA, is typically measured with respect to time at the necessary redox potential, mV.

Linear Sweep Voltammetry (LSV) scans a range of potentials in an ascending sequence of values, measuring the current response provided by the electrochemical processes on the electrode surface. Cyclic Voltammetry (CV) scans the potential range across an ascending sequence of potential values (i.e., forward scan) followed by a descending sequence (i.e., reverse scan). The reverse scan facilitates reductive electrochemical processes in electrolytical cells. The peak current (i_p), proportional to the concentration, can be described by the Randles-Sevcik equation (LaCourse, 1997):

$$i_p = \left(2.69 \times 10^5\right) n^{3/2} A\, C\, D^{1/2} v^{1/2} \quad (1)$$

where n is the number of electrons transferred in the redox event, A is the electrode area (cm^2), C is the concentration in the bulk solution (mol cm^3), D is the diffusion coefficient ($cm^2\ s^{-1}$), and v is the potential scan rate ($V\ s^{-1}$). These techniques are used to determine the potential value, E°, of electroactive species which is necessary for other electrochemical detection techniques e.g., amperometry.

Voltammetric methods are highly sensitive due to the unique voltammetric response measured for each electrochemical process present at the working electrode surface (LaCourse, 1997). However, these methods have poor resolution when applied to complex mixtures beyond dilute salt solutions. Thus, the excellent resolving power of LC methods can be coupled to voltammetric detectors for analysis of electroactive compounds (LaCourse, 1997).

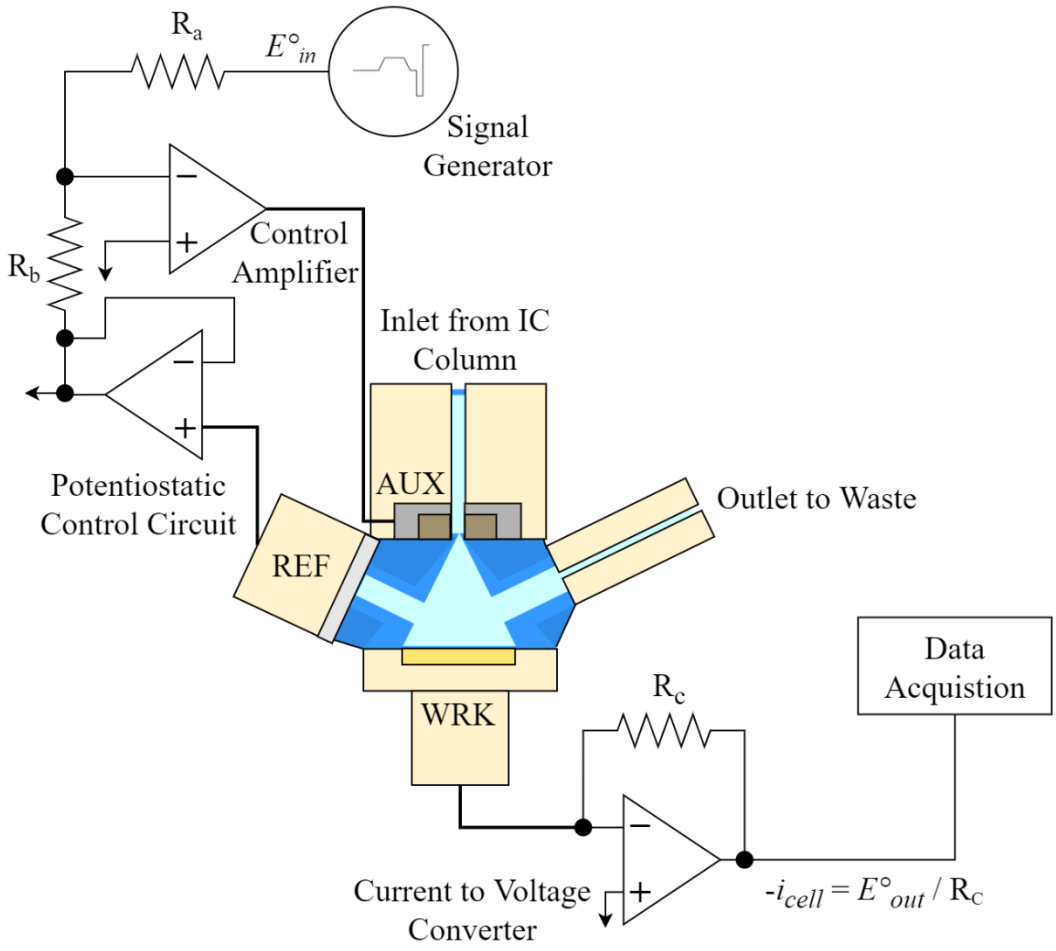

Figure 3. The wall jet configuration utilized by the Metrohm amperometric detection. This cell design provides the greatest signal-to-noise ratio when compared to other cell configurations. The notation of each electrode was as follows; WRK for the gold working electrode, REF for the palladium reference electrode, and AUX for the stainless-steel electrode. Eluent flows from the inlet line, establishing a jet stream when emitted from the auxiliary electrode surface. The jet stream then bombards the working electrode surface before making contact with the downstream reference electrode. The eluate proceeds to the outlet after sustaining contact with the reference electrode surface. Output signal for the electrochemical cell is then measured as current (nA) in the flexIPAD mode. Resistance components are listed as Ra, Rb, and Rc. A schematic illustrating the potentiostat design supporting the electrochemical cell was also provided (Adapted from LaCourse, 1997; Kelly, R.S.).

1.7 Amperometric Detector

Amperometry is a voltammetric technique where the electrode surface catalyzed electrochemical processes behind detection occur at only ≥5% conversion efficiencies

(LaCourse, 1997). Thus, amperometry can be regarded as a non-destructive analysis, as only a maximum of 5% of the analyte would be consumed by the analytical method, allowing for theoretical recovery of the analyte after analysis. The electrolytic cell can be manufactured in a few configurations (LaCourse, 1997). A notable design is the Wall-Jet Cell which has a peak current, often called the limiting current in this case, described by the following equation:

$$i_p = 0.898\ n\ FC\ D^{\frac{2}{3}}\ \nu^{-\frac{5}{12}}\ a^{-\frac{1}{2}}\ A^{\frac{3}{8}}\ V^{\frac{3}{4}} \quad (2)$$

where n is the number of electrons transferred in the redox event, A is the electrode area (cm^2), a is the diameter of the jet stream (cm), C is the concentration of the electroactive analyte in the bulk solution (mol cm^3), D is the diffusion coefficient ($cm^2\ s^{-1}$), F is the Faraday Constant (96485 C mol^{-1}), V is the volumetric flow rate ($cm^3\ s^{-1}$), and ν is the kinematic viscosity ($cm\ s^{-1}$). The advantage of this cell design includes improved rates of mass transfer, smaller effective dead volume, and greatest signal-to-noise ratio associated with its electrode surface area when compared to other cell designs (LaCourse, 1997).

Integrated Pulsed Amperometric Detection (IPAD) applies a cycle of multi-step potentials in series over 800 to 1000 ms to the working electrode known as the waveform, facilitating surface catalyzed anodic detection of electroactive species. Pulsed Amperometric Detection (PAD), the precursor amperometric detection method, simply utilizes a single detection potential compared to the trapezoidal waveform highlighted in Figure 4. This study will utilize the IPAD detection method. Major features of the waveform include a zero potential, a detection potential, and a recursive negative potential, and positive potential (Figure 4). Each potential value corresponds to an essential electrochemical process necessary for detection. The zero potential is necessary

for non-spontaneous adsorption of aliphatic species. The detection potential, which is typically determined by CV to be the optimal anodic potential for the oxidation of an electroactive species, provides the input signal to elicit the detector response. The negative potential typically restores the activity of the electrode surface, while the positive potential cleans organic buildup responsible for electrode fouling, a byproduct of oxidation.

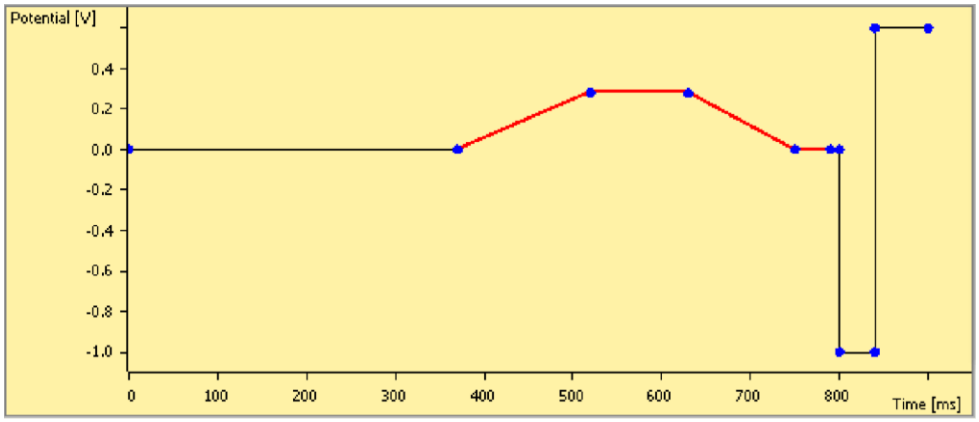

Figure 4. The potential waveform pre-set in the MagIC Net software controlling the IC.

A detail scheme showing the detection of glyphosate in IPAD is provided in Figure 5. Briefly, glyphosate and other nitrogen containing aliphatic organophosphorus compounds provide an anodic signal for detection by the following series of electrochemical processes. Firstly, the nitrogen containing moiety must undergo a spontaneous adsorption onto the electrode surface, where its nonbonded electron pair occupies the d-orbitals of the gold surface (Fedorowski and LaCourse, 2014). Following this adsorption process, the potential-dependent formation of gold oxide (typically 280-300 mV) facilitates the activation and transfer of oxygen species onto the adsorbed aliphatic compound. The oxidized product then desorbs from the electrode surface, providing an anodic detection signal (LaCourse et al, 1997).

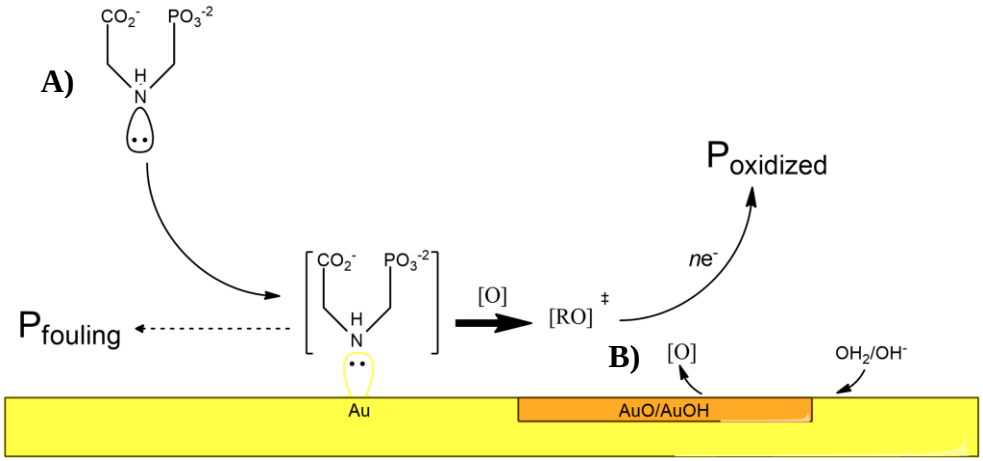

Figure 5. The oxidation of glyphosate at the gold electrode surface generating amperometric signal. A) The spontaneous absorption and the surface-stabilized oxidation of glyphosate by oxygen transfer on the gold surface. B) The oxygen transfer facilitated by gold oxide formation and anodic discharge of H_2O. (Adapted from LaCourse., 1997).

1.8 Use of Ion Chromatography for the Analysis of Glyphosate, Glufosinate, and AMPA

Ion chromatographic techniques have been coupled with PAD and IPAD methods to provide the fundamental instrumental parameters necessary for the quantitative analysis presented in this work (Sato et al., 2001; Sánchez-Bayo et al., 2010; Sukzuki et al., 2019). Each of these studies utilized a quaternary ammonium SAX analytical column for separation. As of our knowledge, Sato et al. (2001) were the first to apply and develop an IC-PAD method to glyphosate, glufosinate, and AMPA, noting these nitrogen containing compounds as ideal for the gold surface catalyzed detection of PAD. Cyclic voltammetry was also performed to provide its gold oxide-dependent oxidation potential at 200 mV and confirm the detection process. Peak area-electrode potential curves were also obtained to determine the potential value necessary for optimal detector response of these analytes which was found to be 230 mV. The LOD and linear range for this method are listed in Table 2. Recovery values of glyphosate spiked in human urine and serum at

8.5 mg/L and 8.5 µg/L were also found to be between 98-101%, illustrating the feasibility of method development with complex matrices. A LC-PAD method was also developed to evaluate the extraction of glyphosate, AMPA, and Amitrole from passive samplers intended for environmental monitoring (Sánchez-Bayo et al., 2010). Sánchez-Bayo et al. optimized their IC-PAD method along with similar instrumental parameters to Sato et al. (i.e. CV scans, detection-signal-electrode potential curve, mobile phase composition, etc.), and its LOD and linear range values are provided in Table 2. Only AMPA and Amitrole provided good recovery values when extracted from the reverse phase-based passive samplers. A more recent method provided a detailed analysis with an Metrohm Vario IC system equipped with the Metrohm Amperometric Detector for the quantification of glyphosate, glufosinate, and AMPA in environmental samples (Suzuki et al., 2019). Sukzuki et al. demonstrated that the IPAD mode provided better detection for these compounds compared to PAD (Suzuki et al., 2019). SPE resins were also evaluated with this method for environmental analysis of reservoir water; however, AMPA and glufosinate experienced poor recovery for the H^+ and Na^+ SCX SPE resins. Thus, IC-IPAD methods intended for glyphosate determination need further development through their sensitivity, detection limit, and extraction step.

Table 2. Ion Chromatography – Pulsed Amperometric Detection methods compared to US regulatory methods for Glyphosate, Glufosinate, and AMPA Quantification

Compound	Method	Column (Length/I.D., mm)	Vol. Injection (µL)	Detection	MDL (µg/L)	Linearity (µg/L)	r^2	Mean RSD (%)
Glyphosate	Sato et al.	IonPac AS 15 (250.0/4.0)	25	IPAD	**51**	100-50,700	0.996	1.7
	Sanchez-Bayo et al.	IonPac AS 15 (250.0/4.0)	25	PAD	**320**	1,000-180,000	0.996	-
	Sukzuki et al.	Carb 2 (250.0/4.0)	250	IPAD	**1.45**	-	-	3.2
	EPA 547	Aminex A-9 (250.0/4.0)	200	FLD	**6.00**	-	-	-
	O-2136-01	Phenomenex Prodigy C-18 (250.0/3.0)	10,000	MS	**0.084**	0.1-2.0	>0.999	14
	O-2141-09	Luna C-18 (150/3.0)	-	MS/MS	**0.024**	0.02-5.0	>0.999	17.2
AMPA	Sanchez-Bayo et al.	IonPac AS 15 (250.0/4.0)	25	PAD	**50**	100-50,000	0.99	-
	Sukzuki et al.	Carb 2 (250.0/4.0)	250	IPAD	**0.18**	-	-	1.16
	O-2136-01	Phenomenex Prodigy C-18 (250.0/3.0)	10,000	MS	**0.078**	0.1-2.0	>0.999	12
	O-2141-09	Luna C-18 (150/3.0)	-	MS/MS	**0.022**	0.02-5.0	>0.999	14.9
Glufosinate	Sato et al.	IonPac AS 15 (250.0/4.0)	25	IPAD	**18.0**	100-45,300	0.9965	3
	Sukzuki et al.	Carb 2 (250.0/4.0)	250	IPAD	**0.43**	-	-	1.03
	O-2136-01	Phenomenex Prodigy C-18 (250.0/3.0)	10,000	MS	**0.057**	0.1-2.0	>0.999	9
	O-2141-09	Luna C-18 (150/3.0)	-	MS/MS	**0.017**	0.02-5.0	>0.999	11.2

MDLs for O-2141-09 were determined from samples (n = 36) with surface, ground, and reagent water matrices.
MDLs reported in literature were determined from a signal-to-noise ratio greater than 3 (i.e., $S/N \geq 3$).
The correlation coefficient, r^2, for USGS methods must be >0.999 to be accepted.

2. Objectives

The overall objective of this work was to develop a working IC-IPAD method for the direct analysis of glyphosate, glufosinate, and AMPA in water within acceptable analytical accuracy, precision, and limit of detection. Specific objectives were (a) to optimize instrument and operational parameters in achieving the method detection limit of aliphatic organophosphorus compounds which are competitive to existing mass spectrometry-based instrument methods but with significantly reduced cost using IC-IPAD; (b) to achieve desired high level of recovery of these compounds in the natural water matrices as well as the precision evaluated by standard deviation measured from replicate measurements; (c) to develop a simple SPE method as a necessary component of sample preparation without derivatization for subsequent direct injection of natural water samples with the improved detection limit, and essential accuracy and precision; (d) to examine several sample storage methods essential for the determination of Maximum Holding Time (MHT) of these three pesticides.

3. Materials and Methods

The method and materials will be provided for the method development and environmental analysis performed by the offline SPE-IC-IPAD method for glyphosate determination. Calibration curves necessary for method development were injected in triplicate. The instrumental parameters typical to method development and environmental analysis are provided below. Procedures for the offline SPE method can also be found below. Environmental sampling, stability study procedure, and data analysis will also be provided.

3.1 Materials

The analytical standards of glyphosate PESTANAL® (≥98.0%), (Aminomethyl) phosphonic acid, PESTANAL® (≥98.0%), and glufosinate-ammonium PESTANAL® (≥98.0%) were purchased from Sigma Aldrich (St. Louis, MO). ACS sodium acetate anhydrous and citric acid monohydrate anhydrous ACS (99%) were purchased from VWR International (Solon, OH). Fifty mL polypropylene tubes were purchased from VWR International (Radnor, PA). Sodium hydroxide (reagent ACS, pellets ± 97%) was purchased from VWR through its manufacturer, Acros Organics (Geel, Belgium). Falcon^{TM} 15 mL conical centrifuge tubes were purchased from Fisher Scientific (Waltham, MA). SiliaPrepX polymeric SPE cartridges (SPE-P0005-06C; SPE-P0010-06C) and the polymer-based SiliaPrepX SPE Cartridges Development Kit (SPE-K0050-03BB) used in this work were purchased from Silicycle Inc. (Quebec, Canada). Ultrapure water typically measuring at 18.2 ΩM cm^{-1} was received from the Milli-Q Quantum system on the University of Houston-Clear Lake campus for blank sets and the preparation of standard solutions. Certified reference material (product number: QC1435-2ML; Lot number: LRAD0897) was purchased from Sigma Aldrich as a 2 mL ampule of

diquat (20.9 ± 0.6 µg/L), paraquat (20.6 ± 0.6 µg/L), and glyphosate (495 ± 15 µg/L) constituted in drinking water.

3.2 Instrumental Method

The instrument for this study was 940 Professional IC Vario IC system equipped with an Metrosep Carb 2 – 100.0 /4.0 column (100mm length, 4.0mm ID) and a Metrohm amperometry detector (Pasadena, TX). This electrochemical detector (2.850.9110) was designed with a Wall-Jet electrolytical cell. The typical IC instrumental parameters were provided below in Table 3.

Table 3. Instrumental parameters and eluent conditions for analysis

Ion Chromatograph	940 Professional IC Vario
Column	Metrosep Carb 2 – 100/4.0
Detector	IC amperometric detector
Mobile Phase	300 mM CH_3COONa 15 mM NaOH 1 mM citric acid monohydrate
Flow Rate	0.5 mL min^{-1}
Water Quality	18.2 MΩ cm^{-1}

3.3 Preparation of Standard Solutions

Glyphosate, AMPA, and glufosinate-ammonium were each individually weighted out at approximately 100 mg. Masses were recorded and subsequently dissolved in 50 mL centrifugal tubes containing ultrapure water to obtain the stock solution with pre-determined concentrations. Stock solutions were then stored in 4°C under typical refrigerated conditions. Aliquots of these stock solutions were then diluted according to the desired concentrations necessary for calibration point preparation. Standard solutions were dissolved to the necessary concentrations for calibration curve preparation.

3.4 Surface Water Sample Collection

Environmental samples were collected locally from Houston area surface water sources with a Wildco stainless steel Kemmerer sampler (Figure 6). The sampler was washed after each sampling event. The sampler was typically rinsed with 500 mL of ultrapure water before sampling which would be analyzed as a blank to monitor quality control. Environmental samples were typically collected over bridges with the exception of the Brazos River sampling events. Samples collected from the Brazos River were collected from near riverbank by hand. Samples were transported to the laboratory within the same day as collection and stored under typical refrigerated conditions at 4°C. Samples were only considered fit for analysis within the one to two weeks of their collection. Samples kept beyond their second week of analysis were acidified to pH 2-3 with a final concentration of 12 mM HNO_3 if necessary.

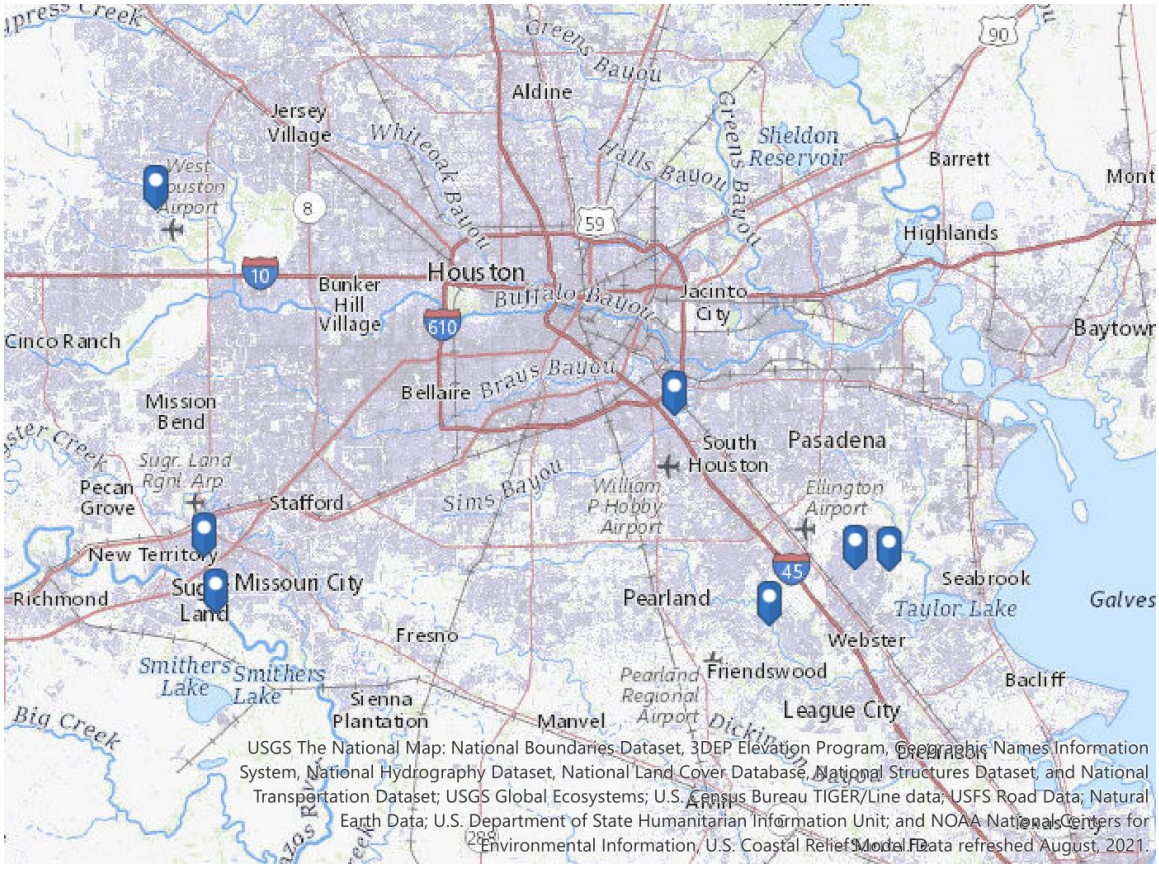

Figure 6. A USGS map depicting the sampling events across the Houston area. Sampling events are indicted by the markers corresponding to their geographic coordinates (i.e., Table 4). The map was provided by Texas Commission on Environmental Quality (TCEQ et al., 2021).

Samples were collected from the following locations: Bear Creek (n = 2, 250 mL), Clear Creek (n = 1, 250 mL), Sims Bayou (n = 2, 250 mL), and Telfair Lake in the Sugarland area (n = 1, 500 mL). Horsepen Bayou was sampled more frequently than the other sampling sites, as this site served as a basis for initial SPE method development (i.e. the development outlined for Table 10). However, nearly all sampling dates crucial to the data presented in this work are provided in Table 4.

Table 4. Water quality of collected natural water samples

	Date Sampled	Location	pH	Conductivity µS/cm
Bear Creek	9-08-21	-95.6866, 29.8310	7.66	215
Clear Creek	9-08-21	-95.1965, 29.5422	7.56	619
Sims Bayou	9-08-21	-95.2723, 29.6882	7.27	1805
	9-21-21		7.13	332
Telfair Lake	9-21-21	-95.6482, 29.5898	7.24	234
Horsepen Bayou	9-24-21	-95.1012, 29.5800	7.77-8.04	549
Horsepen Bayou	9-24-21	-95.1280, 29.5810	7.89-8.31	587
Brazos River	9-01-21	-95.6387, 29.5509	7.37-8.17	736
	9-21-21		6.94	978

3.5 Solid Phase Extraction (SPE)

A SPE preparation kit was purchased to assess the appropriate SPE cartridge stationary phase. The SCX and SAX cartridges were selected as the SPE stationary phases for assessment due to literature supporting their treatment of natural water samples in the detection of glyphosate (Mallat and Barceló, 1998; Jiang and Lucy, 2006; Küsters and Gerhartz, 2010). Early SPE method development was accomplished on the SPE columns with the sample volume of 3 mL and a 60 mg bed size (i.e., SPE treatments detailed by Table 10). Once the SCX and SAX cartridge phases were selected, typical method development was accomplished on SiliaPrepX polymeric SPE cartridges. Both the Tosic Acid-based SCX cartridges and TMA (Tetra-methyl ammonium) Chloride-based SAX cartridges had a particle size of 85 µm and pore size of 60 Å (Silicycle Inc., 2016). These SPE cartridges had a sample volume of 6 mL with 100 mg bed size.

Environmental water samples were filtered through 0.45 µm syringe filters in large volumes before SPE treatment during method development. Vacuum SPE extractions were performed in a fume hood under the vacuum function of the fume hood or the vacuum provided by a benchtop metered motor pump. SPE cartridges were conditioned with 4-6 mL methanol followed by 6 mL ultrapure water. The procedure for loading and extraction was dependent on the phase of SPE. Acidified samples (12-20 mM HNO_3 or pH 2-3) were percolated through SCX columns for SPE SCX treatment. SCX treatment was performed on all environmental samples, whereas SAX treatment was only evaluated by the SAX treatment assessment (i.e., Figure 13). Samples were loaded under neutral conditions before a 4-6 mL methanol wash followed by a 6 mL ultrapure water wash. Extraction from SAX cartridges was then performed with 30 mM HNO_3. Samples were then neutralized with 40-50 µL 6 M NaOH before injection to IC.

3.6 Stability Study

Two sample storage methods were conducted; each under a typical storage condition of environmental samples prior to analysis. The first method was conducted under dark, refrigerated (4°C) conditions, and the other was conducted under sunlight, ambient (25 °C) temperature conditions. For the method under refrigerated conditions, 2 sample sets were prepared at 250 µg L^{-1} AGG in 15 mL conical centrifugal tubes in Brazos River water, where one set was stored in 4°C under acidified conditions by way of 20 mM HNO_3 (pH 2-3) and the other set under neutral pH values. The neutral sample set was acidified by 50 µL 6 M HNO_3 (final pH 2.21) before the subsequent SPE clean-up step. These sample sets were made in triplicate and intended for single injection to avoid IC contamination. All refrigerated samples were stored in an empty 30 mL syringe cardboard box as a container to minimize light exposure. For the samples stored under ambient conditions, two sample sets were prepared under the Sims Bayou and Horsepen

Bayou matrices. The Sims Bayou water was collected from a connected stream surrounding the Houston Botanic Garden. 50 mL samples were prepared at 250 µg L^{-1} AGG in 50 mL centrifugal tubes, and 10 mL was collected from each sample periodically to determine AGG recovery within each sample. Samples were stored on a window seal in a temperature controlled chemical storage room. All samples were processed by the following sequence: SPE treatment, neutralization with 40-50 µL 6 M NaOH depending on sample volume, and filtration through 0.45 µm syringe filter before IC injection. All samples under ambient temperature were acidified to pH 2-3 before storage to prevent bacteria growth, as the caustic-acetate eluent of the instrument is easily susceptible to bacterial contamination by non-sterilized injections. Kill standards were prepared using $HgCl_2$ at 500 µg/L.

3.7 Method Validation

The contents of 2 mL of the certified reference material sample were decanted into an empty 50 mL centrifugal tube and spiked with 167 µL 6M HNO_3 to achieve a final concentration of 20 mM HNO_3. The centrifuge tube was then filled to the 50 mL graduated line with ultrapure water, and this sample was treated as a stock solution. This stock solution was then stored under refrigerated conditions at 4°C. One mL of diluted reference material was then added to a separate 50 mL centrifuge tube and spiked with 167 µL 6 M HNO_3 before the centrifuge tube was filled to 40 mL graduated line. An aliquot of 10 mL was then collected from each 40 mL sample and underwent SCX treatment before neutralization with 45 µL 6 M NaOH. This was accomplished in triplicate. Each sample of 10 mL eluate was then filtered through a 0.45 µm syringe filter before IC injection. IC conditions were the instrument method outlined above. Each sample was injected in triplicate, resulting in a total of nine injections.

3.8 Reporting Values

Typical chemical analysis requires the precision and accuracy of a method be reported in terms of relative standard deviation (RSD) and percent recovery, respectively (Christian et al., 2013). Recovery is the numerical agreement between a value obtained by an analytical method and the true value of the measurement.

$$\% \text{ Recovery} = \frac{\text{Analytical Value}}{\text{True Value}} (100) \tag{3}$$

Acceptable measurements are typically within the 80-120% range. Precision is the mutual agreement among replicate injections or samples. Standard deviation is provided below in the following equation

$$s = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n - 1}} \tag{4}$$

where n is the number of replicates, x_i is the analytical value of a replicate and $\bar{x}$ is the mean value. The RSD value can be obtained by

$$\text{RSD} = \frac{s}{\bar{x}}(100) \tag{5}$$

where s represents the standard deviation value respective to the set of replicates and $\bar{x}$ represents the mean value. The limit of detection (LOD) or method detection limit (MDL) is the minimum detectable concentration by the instrumental method and is typically 3 times the value of the signal-to-noise ratio (S/N) for the respective instrument. These LOD values will be used to assess optimal instrumental conditions and compare the detection limit between interlaboratory methods. LOD values presented in this work were calculated from the following equation:

$$\text{LOD} = s \times t_{(n-1,1-\alpha=0.99)} \quad (6)$$

where *s* represents the standard deviation, *t* represents the Student's t value at n–1 degrees of freedom. Student's t-value were obtained from the *t*-distribution at the appropriate 1–α (99 %) confidence level (EPA, 2016). All calculations were performed using MS Excel spreadsheets.

4. Results

The offline SPE-IC-IPAD method development for glyphosate determination was optimized by its column length, selected eluent modifying agent, and injection volume. Four injection volumes (i.e., 20, 31, 303, and 1001 µL) were assessed during method development. SPE SCX method development was also provided below, as this column phase was determined to provide matrix cleanup for natural water sources. SAX was assessed, as well. The MHT value and optimal storage conditions from the stability study of glyphosate stored in natural water sources can also be obtained below.

4.1 Method Development

4.1.1 Effects of Column Length on Retention Times

The Vario IC system was fitted with a pre-installed sample injection loop of 20 µL and the working column was Metrosep Carb 2.0 150.0/4.0. Initial eluent conditions were outlined in the Metrohm white paper and are as follows: 300 mM sodium acetate and 10 mM sodium hydroxide (Läubli et al., 2016). These parameters underwent adjustments throughout the course of method development for the IC instrument.

The effect of column length on method performance was investigated between the Carb 2.0 100.0/4.0 (length, mm/ ID, mm) and the Carb 2.0 150/4.0 columns. Both columns were packed with a quaternary ammonium phase explicitly for its well-established selectivity toward glyphosate (Läubli et al., 2016; Suksuki et al., 2019). Table 5 features the capacity and selectivity factors for each analyte when eluding from each respective column. The capacity factor or retention factor is regarded as the ratio of resident times of an analyte distributed between the two phases responsible for its separation (Christian et al., 2013). The capacity factor (k) can be described by the following equation:

$$k = \frac{T_R - T_m}{T_m} \quad (7)$$

where T_R represents the retention time and T_m is the dead time for the respective column. The dead time were 1.2 and 1.6 mins for the 100 mm and 150 mm Carb 2.0 columns (Figure 7), respectively. The selectivity factor (α) is the measure of relative retention of analyte onto column (Christian et al., 2013).

$$\alpha = \frac{k_2}{k_1} = \frac{T_{R,1} - T_m}{T_{R,2} - T_m} \quad (8)$$

where $T_{R,1}$ and $T_{R,2}$ denote the retention times for sequentially eluded compounds and T_m is the dead time for the respective column.

Table 5. Method selectivity as a function of column length.

Carb 2.0 150/4.0	Retention (min)	Capacity factor, k	Selectivity factor, α
AMPA	3.69	1.31	1
Glufosinate	4.48	1.8	1.38
Glyphosate	12.46	6.79	3.77
Carb 2.0 100/4.0	Retention (min)	Capacity factor, k	Selectivity factor, α
AMPA	4.27	2.56	1
Glufosinate	4.77	2.98	1.16
Glyphosate	9.88	7.23	2.43

Injection volume for the Carb 2.0 150/4.0 and Carb 2.0 100/4.0 was 31 µL and 1.00 mL, respectively.

These values were only provided to support the sufficient resolution provided by the 100 mm column observed in Figure 7. The comparison between these selectivity factors illustrates the reduced resolving power by the 100.0/4.0 column, suggesting that the 150 mm column would be the better choice for resolution. However, in conjunction with the observable baseline separation in Figure 7, the 100.0 mm column demonstrated

sufficient resolution of AMPA and glufosinate necessary for further analysis. The Carb 2.0 100.0/4.0 column was selected as the analytical column for the following analysis.

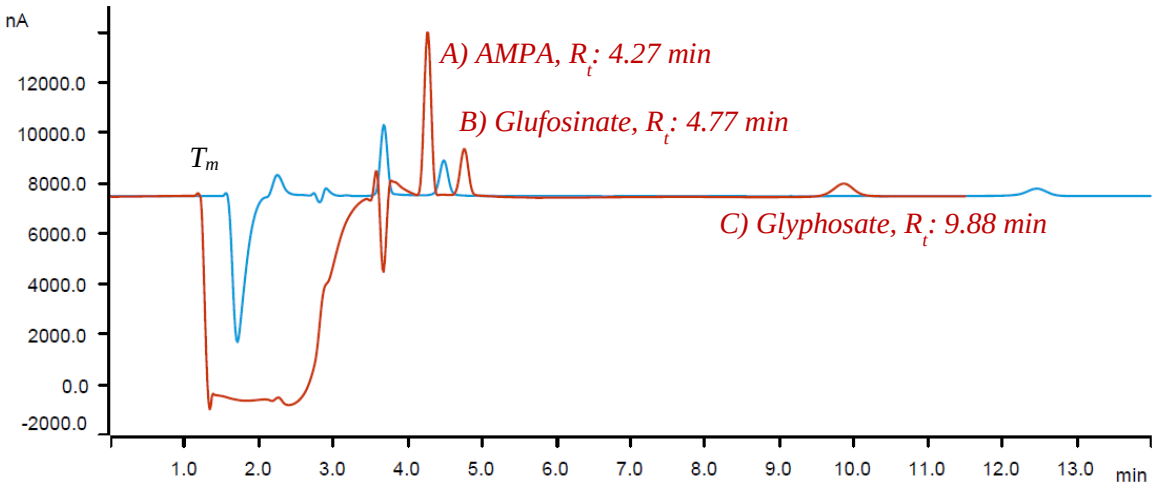

Figure 7. The retention time shifts demonstrated between 1) initial injection of 1 mg L^{-1} AGG unto the Carb 2.0 150.0/4.0 column (blue chromatogram) and 2) an injection of 0.3 mg L^{-1} AGG unto the Carb 2.0 100.0/4.0 column (red chromatogram). Injection sample loop volumes were 20 µL and 300 µL, respectively. The peaks identified were as follows: A) AMPA, B) glufosinate, and C) glyphosate. Flow rate was 0.6 mL min^{-1}. The eluent was prepared with 300 mM sodium acetate, 15 mM sodium hydroxide, and 1 mM citric acid.

4.1.2 Effects of Modifying Agents (Citric Acid) in Eluents

Citric acid-monohydrate was investigated as an eluent additive to improve peak shape (Forsman et al., 1986). However, retention time shifts were observed because of its addition. Figure 8 illustrates the observed retention time shifts: 4.2 to 3.69 min, 5.22 to 4.5 min, and 21.42 to 12.44 for AMPA, glufosinate, and glyphosate, respectively. Citric acid did not provide improved method performance in the form of sharper peaks. However, eluent prepared with this modifier significantly reduced the retention time for glyphosate, as the trivalent citrate ion had a greater affinity toward the SAX analytical column than the analytes in alkaline conditions, resulting in shorter retention times. Thus, eluent was prepared with 1 mM citric acid for all subsequent analyses.

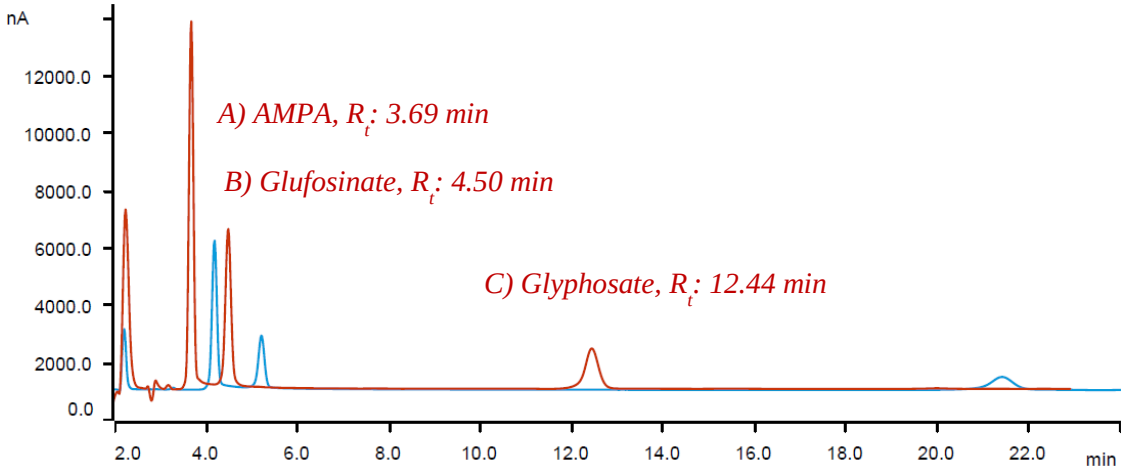

Figure 8. The retention time shifts demonstrated between 1) initial injection without 1 mM citric acid modifier in eluent (blue chromatogram) and 2) an injection after the addition of 1 mM acid modifier to the eluent (red chromatogram). The peaks identified were as follows: A) The system peak, B) AMPA, C) glufosinate, and D) glyphosate. Flow rate was 0.6 mL min^{-1}. Injection sample loop volume was 20 µL. The eluent was prepared with 300 mM sodium acetate, and 15 mM sodium hydroxide.

4.1.3 Test of Linear Range

Early calibration curves were typically prepared between concentrations of 5-50 mg L^{-1} for AGG, as detailed in Figure 9. Correlation coefficients (R^2) were all ≥ 0.99 and considered acceptable linearity at the injection value. Furthermore, the lower detectable concentrations of these instrumental parameters were not competitive when compared to the USGS and other working methods analyzing environmental relevant concentrations of glyphosate. For comparison, the LOD value for glyphosate obtained by the USGS method O-2141-09 was 0.024 µg/L.

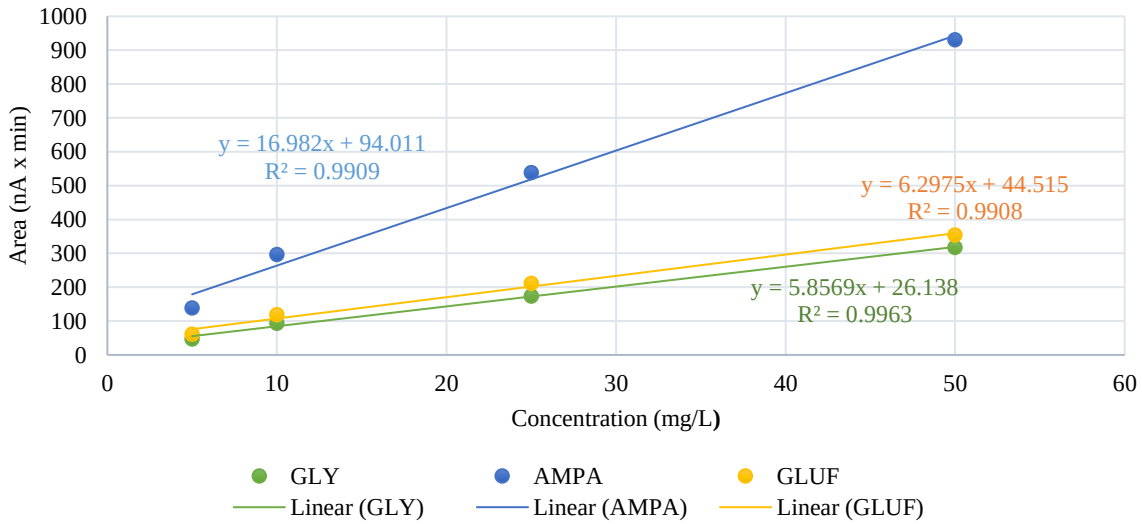

Figure 9. An initial calibration curve generated from glyphosate, AMPA, and glufosinate standard dissolved in ultrapure water. Sample loop was 25 µL at the time of injection. The dilution series necessary to generate this calibration curve was 5, 10, 25, and 50 mg L^{-1}. This calibration curve was taken before any instrumental and method adjustments were made. The analytical column was the Carb 2.0 150.0/4.0. Flow rate was 0.6 mL min^{-1}. Injection sample loop volume was 20µL. The eluent was prepared with 300 mM sodium acetate, 15 sodium hydroxide, and 1 mM citric acid.

A 31 µL injection volume was installed to determine the effect of injection volume on LOD values and detector response, as Suksuki and coworkers (2019) attributed low LOD values obtained for glyphosate, glufosinate, and AMPA by IC-IPAD to their 250 µL injection volume. To assess the concentration ranges which provide the best linearity for a 31 µL injection volume, standard concentrations were prepared under the following values: 0.25, 0.5, 0.75, 1, 5, 10, 20, 25, 50, and 75 mg L^{-1}. Peak distortions began to appear as high as 50 mg L^{-1}, indicating column overload at these concentrations. Thus, 50 mg L^{-1} and 75 mg L^{-1} AGG concentrations were discarded as calibration points for this behavior. The correlation coefficients for each curve can be seen in Figure 10 and Table 6 where 0.25 mg L^{-1} to 5 mg L^{-1} provided the best linear response across a concentration range.

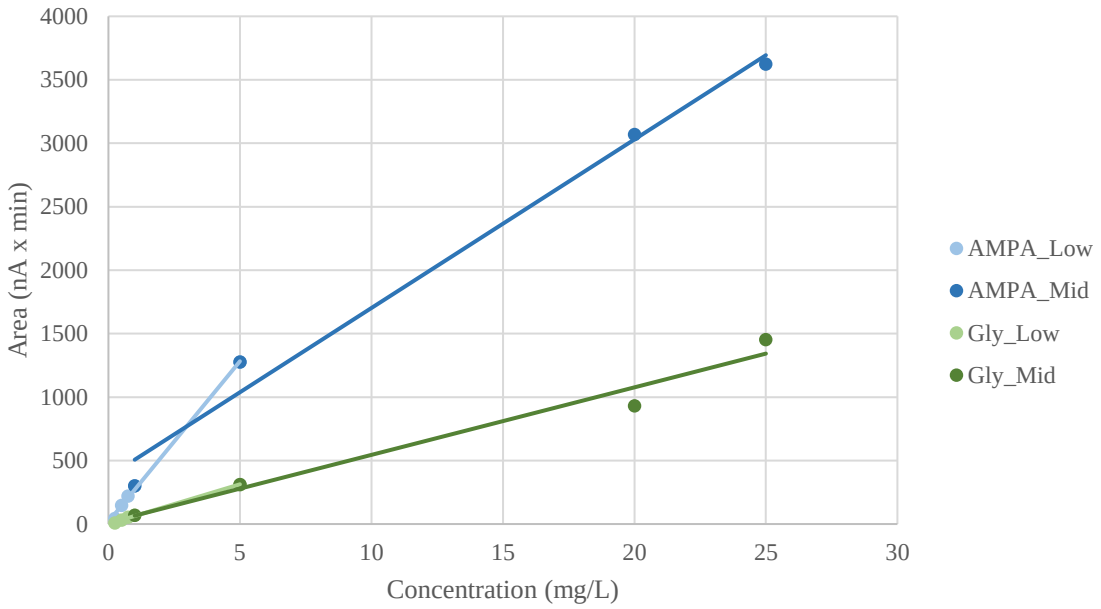

Figure 10. Calibration curve for AMPA (blue) and glyphosate (green) between 0.25 to 25 mg L^{-1}. The calibration curve for glufosinate was not included for clarity. The curve was divided into 2 different concentration ranges to determine the better linear range. These ranges were 0.25-5 mg L^{-1} and 1-25 mg L^{-1}. The flow rate was 0.6 mL min^{-1}. Injection sample loop volume was 31µL. The eluent was prepared with 300 mM sodium acetate, 15mM sodium hydroxide, and 1 mM citric acid.

Table 6. Best fit equations for calibration curve of a 31 µL sample loop made in the range of 0.25 – 75 mg L^{-1}.

Compound	Range (mg L^{-1})	Best fit Eq	R^2
AMPA	0.25 - 5.0	y = 252.89x + 17.848	0.9975
	1.0 - 25.0	y = 132.79x + 373.91	0.9852
Glufosinate	0.25 – 5.0	y = 75.672x + 31.358	0.9584
	1.0 – 25.0	y = 48.692x + 129.75	0.9962
Glyphosate	0.25 - 5.0	y = 62.391x + 0.9742	0.9980
	1.0 - 25.0	y = 53.198x + 12.729	0.9707

A follow-up calibration curve was prepared at 0.003, 0.01, 0.05, 0.1, 0.3, and 0.5 mg L^{-1} to extrapolate the lowest detectable concentration provided the sample injection loop of 31 µL. At the sample loop volume of 31 µL, 0.05 mg L^{-1} was the lowest

concentration which returned signal from standard glyphosate. The calibration curves of this concentration series can be viewed in Figure 11 and Table 7.

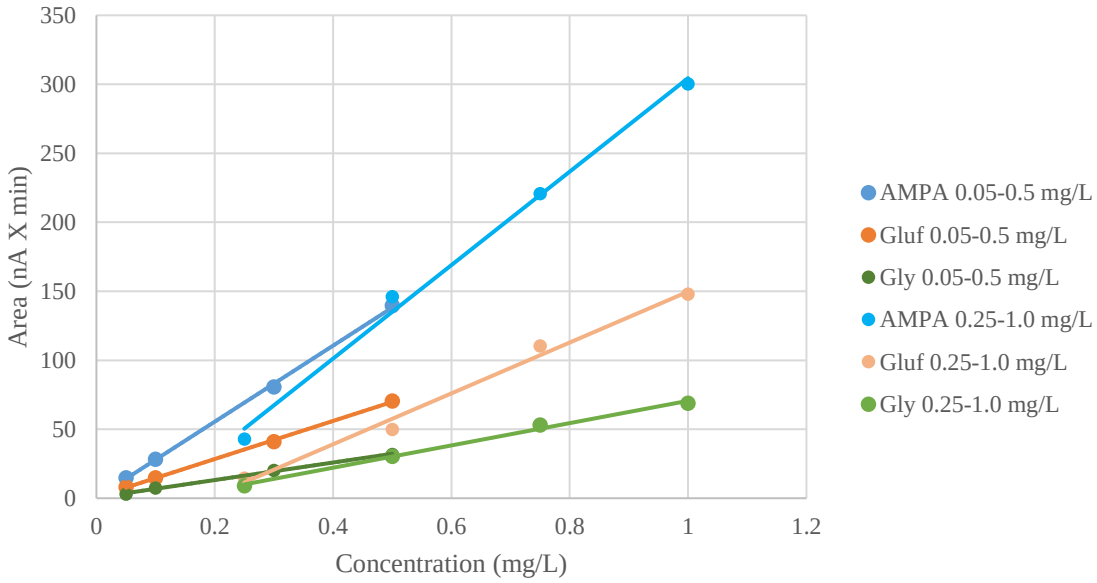

Figure 11. Assessment of best fit curves across the lower concentration range detectable for a 31 µL sample loop.

Table 7. Best fit equations for calibration curve of a 31 µL sample loop made from lower concentrations (i.e. 0.25 -1 mg L^{-1})

Compound	Range (mg L^{-1})	Eq of best fit	R^2
AMPA	0.05 - 0.5	y = 275.52x + 0.3671	0.9991
Glufosinate	0.05 - 0.5	y = 138.01x + 0.8108	0.9992
Glyphosate	0.05 - 0.5	y = 63.448x + 0.4928	0.9971
AMPA	0.25 - 1.0	y = 338.81x - 34.322	0.9946
Glufosinate	0.25 - 1.0	y = 184.35x - 34.626	0.9890
Glyphosate	0.25 - 1.0	y = 80.823x - 10.217	0.9946

4.1.4 Effects of Sample Injection Volumes (303 µL vs. 1.00 mL)

Method sensitivity was improved compared to MDL values when greater injection loops were installed. Initially, an injection volume of 303 µL (ID 0.75 mm and length 68.58 cm) was installed to provide lower MDL values. Seven replicate injections of 50

µg L^{-1} AGG and 8 injections of 10 µg L^{-1} AGG were made to obtain MDL values for comparison with the previous MDL values obtained from the 31 µL injection volume (Table 9). Due to the high RSD values seen for glyphosate (32.7%), 8 more injections were made the following day to see if the high RSD would be reproduced. The RSD from the second series of injections was 20.7%. A final adjustment of the sample injection volume of 1000.7 µL (i.e.,1 mL) was reached through the installment of a capillary tube of ID 0.75 mm and length 226.5 cm. Figure 12 and Table 8 illustrates the change in detector response along the respective calibration curves for the sample loop volumes. After the 1 mL sample loop was installed, best fit equations for calibration curves were calculated with (0,0) intercept moving forward to avoid negative concentrations upon converting area response. Replicate injections (n = 7-9) were made to obtain MDL values from these calibration curves.

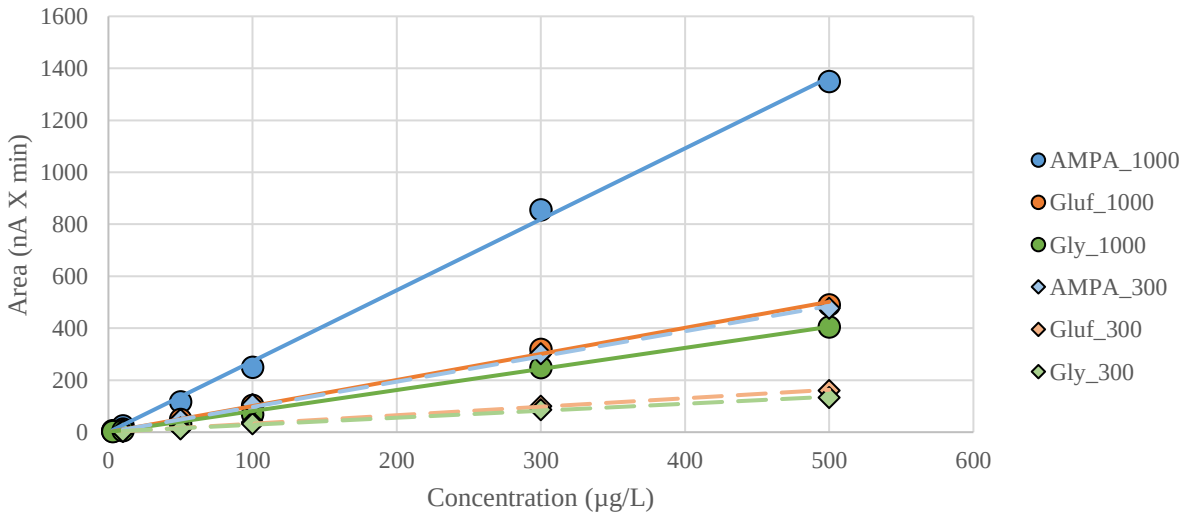

Figure 12. Calibration curves between 303 and 1000 µL sample loops.

Table 8. Best fit equations for calibration curves of the 300 and 1000 µL sample loops made from 10 -500 µg L^{-1}.

Compound	Injection Loop (µL)	Eq of best fit	R^2
AMPA	300	y = 0.971x	0.9992
Gluf	300	y = 0.326x	0.9989
Gly	300	y = 0.274x	0.9979
AMPA	1000	y = 2.73x	0.9991
Gluf	1000	y = 1.00x	0.9986
Gly	1000	y = 0.811x	0.9989

The MDL values for AMPA, glufosinate, and glyphosate injected with a 303 µL sample loop ranged from 0.654-5.68, 1.59-9.25, and 7.80-14.0 µg L^{-1} depending on spiked concentration and number of injections, respectively. These values were improved to 0.252-0.722, 0.366-1.88, and 0.950-1.44 µg L^{-1} for AMPA, glufosinate, and glyphosate with a 1.00 mL sample loop, respectively. MDL values were found to be significantly reduced when comparing 303 µL and 1.00 mL sample loop volumes.

Table 9. MDLs (µg L^{-1}) for glyphosate, glufosinate, and APMA between 300 and 1000 µL injection volume

Injection Vol.	*n*	Spiked Concentration (µg L^{-1})	AMPA	Glufosinate	Glyphosate
31 µL	8	50	12.3	44.2	37.1
303 µL	7	50	5.68	9.25	14.0
	8	10	1.51	2.25	5.69
	8	10	0.654	1.59	7.80
1.00 mL	8	10	0.722	1.88	1.44
	8	5	0.409	0.366	1.03
	9	3	0.252	0.402	0.950

The slope of the best fit curves obtained from the glyphosate calibration curves were 0.274 and 0.811 for the 303 µL and 1.00 mL, respectively. Greater slope values were also observed for AMPA and glufosinate. The slope values of each injection volume can be considered the measure of the sensitivity of respective method. Thus, the 2.97-fold

increase in injection volume from 303 to 1000 µL provided an increase in response factor of 3. The ratios of slope values between the 303 µL and 1.00 mL were 2.81, 3.07, 2.96 for AMPA, glufosinate, and glyphosate, illustrating the increase in sensitivity of method upon the installation of the greater injection volume. While both the sensitivity and MDL values of the method were improved by the 1.00 mL injection volume, the increase in injection volume was proportional to the increase in response factor. Also, the LOD value reported by this IC-IPAD at 0.950 µg L^{-1} for glyphosate is comparable to Suksuki et al. (2019) at 1.45 µg L^{-1}, suggesting that there may be an attenuative effect of injection volume on the sensitivity of the method. Thus, 1.00 mL was selected as the injection volume for this IC-IPAD method.

4.1.5 Effects of SPE (SCX) Cleanup Treatment on Recovery for Glyphosate, Glufosinate, and AMPA

Selectivity of the SPE column phase toward the intended set of analytes or interferents is crucial to providing effective SPE extraction. To this end, the SPE SCX column phase provided by Silicycle Inc., was assessed for its effectiveness as a cleanup step for an offline SPE method. SPE SCX was suspected to be a candidate for sample cleanup due its capacity to remove cationic interferences such as naturally occurring divalent metal ions. SPE SCX demonstrated good recovery and precision by the mean values of 3 separate SPE treatment runs. SPE SCX was assessed in triplicate with 500 µg L^{-1} AGG prepared in bayou water acidified to a final concentration of 12 mM HNO_3 (pH 2-3). The recoveries for all 3 SPE SCX elution were between 81.6% and 115.8% and can be viewed in Table 10. However, the run labelled SCXL_SAXL provided a mean glyphosate recovery value of 130.9% and the runs labelled SCXL and SCXL_2 provided mean glufosinate recovery values of 73.9% and 72.9%, respectively. While RSD values indicate good reproducibility, these recovery values are typically considered not as

acceptable as those found within the 80-120% recovery acceptability window. The mean values were regarded as acceptable, despite these recovery values of these runs. SCXL_SAXL was conducted differently from the following 2 runs, as this run was initially intended to assess the effect of both SCX and SAX on AGG in acidified bayou water. The following SAX column did not seem to affect the AGG concentration as the sample was acidic throughout SCX and SAX elution, leading to minimal AGG retention on the SAX column. SAX column phases were intended to retain AGG, but were found to provide no retentive power under acidic conditions. The concentrations obtained from this run were considered acceptable to include with the SCXL and SCXL_2 runs. Thus, concentrations obtained under the labelled SCXL_SAXL were considered only to be treated by SCX SPE due to the minimal SAX retention. Concentrations and recoveries for the SPE SCX method are listed below in Table 10. This illustrates the potential of SCX as the SPE method for removing cationic interferences. A slight interference can occur between the HNO_3 associated peak and the AMPA associated peak. The IC flow rate was adjusted to 0.5 mL/min to provide better resolution between AMPA and this interferent. The flow rate for the IC was maintained at a 0.5 mL/min for all subsequent IC injections. Taken altogether, SCX SPE is recommended for sample clean-up of surface water samples.

Table 10. Concentrations and recoveries from 500 µg L^{-1} AGG in acidified bayou water eluded through SPE SCX.

Sample ID	Analyte	Concentration	RSD (%)	Recovery (%)
SCXL_SAXL	AMPA	586.7 ± 10.9	1.86	117.3
	Glufosinate	490.8 ± 17.8	3.62	98.2
	Glyphosate	654.7 ± 42.2	6.44	130.9
SCXL	AMPA	585.2 ± 9.9	1.70	117.0
	Glufosinate	369.4 ± 7.6	2.06	73.9
	Glyphosate	528.5 ± 11.5	2.18	105.7
SCXL_2	AMPA	564.8 ± 9.8	1.74	113.0
	Glufosinate	363.1 ± 2.5	0.678	72.6
	Glyphosate	535.3 ± 19.9	3.72	107.1
Mean Values	AMPA	578.9 ± 13.8	2.39	115.8
	Glufosinate	407.8 ± 63.1	15.5	81.6
	Glyphosate	572.8 ± 66.0	11.5	114.6

4.1.6 Effect of Preconcentration (SAX)

Strong Cation Exchange (SCX) showed sample clean-up capacity in Table 10 by providing both the sufficient recoveries between 81.6 to 115.8%. Theoretically, SCX can be followed with Strong Anion Exchange (SAX) to provide sample enrichment, which SCX does not have the capacity to do in the cases of glyphosate, AMPA, and glufosinate under acidic conditions. None of these analytes develop a net positive charge across the pH scale, making them unlikely to be selectively associated with the stationary phase of SPE SCX for enrichment purposes. Methods for glyphosate determination have been developed which provide SAX enrichment following SCX cleanup (Mallat and Barceló, 1998; Jiang and Lucy, 2006; Küsters and Gerhartz, 2010). However, these methods were not applied to IC-IPAD for analysis of glyphosate, glufosinate, and AMPA without derivatizing agent. Thus, the data below attempts to implement SAX as an enrichment step following SCX clean-up (Figure 13). SAX was not found to retain AMPA or

glufosinate effectively under these basic conditions. These compounds provided poor recovery values compared to glyphosate.

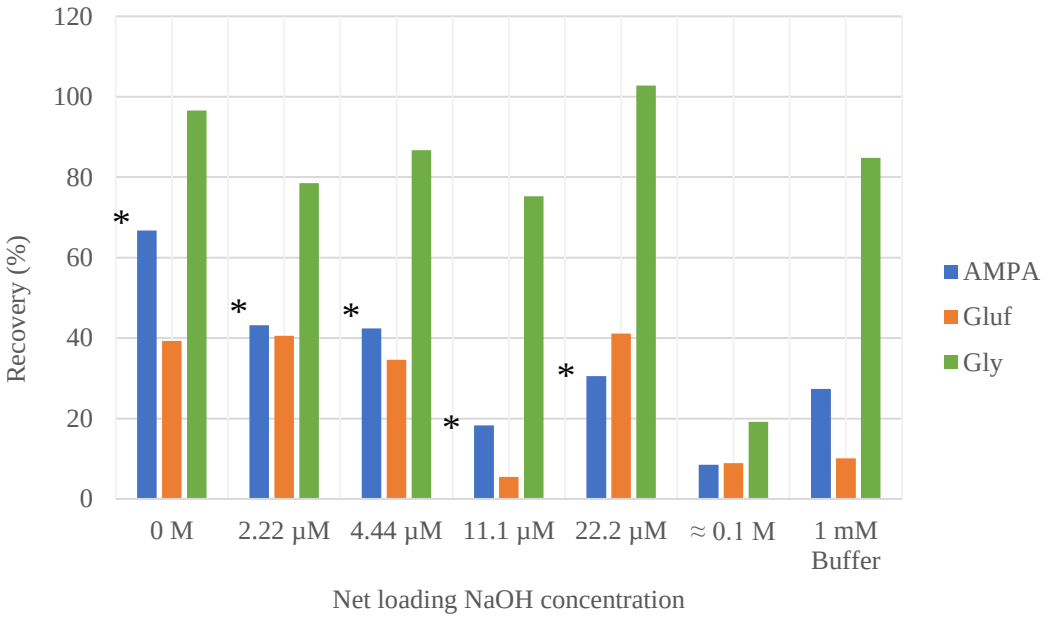

Figure 13. Neutral to basic loading conditions for SAX retention (n = 1) by glyphosate, AMPA, and glufosinate. The asterisks denote unresolved AMPA elution which affected its determination.

Silicylce Inc. has recommended that SAX columns be loaded under neutral to basic conditions at pH 7-8 (Silicycle, Inc., 2021). Thus, the sensitivity of SAX retention to the loading NaOH concentration was evaluated. AGG samples were prepared in bayou water at a concentration of 250 µg L^{-1} before the acidification to a final concentration of 12 mM HNO_3 (pH 2-3) and SCX clean-up treatment. Samples were then neutralized with various volumes (0.54 mL, 0.5401 mL, 0.5402 mL, 0.5405 mL, and 0.541 mL) of 200 mM NaOH before SAX loading. A final sample was prepared by adding 0.1 mL 6 M NaOH to a 500 µg L^{-1} AGG bayou sample treated with SCX. A buffered solution was also spiked into a neutralized 500 µg L^{-1} AGG bayou sample to better maintain the neutral pH value and assess its effect on SAX retention. K_2HPO_4 - citric acid was chosen

as the buffer system due to its buffer range within pH 3-8 (Dawson, 1960). The final buffered sample concentration was 1 mM K_2HPO_4 and 0.5 mM citric acid (pH 7.26) to attenuate its effect on LC behavior. Figure 13 shows the retention of each analyte compared to the net loading NaOH concentrations for the particular sample. Only glyphosate demonstrated moderate to excellent recovery values under most loading conditions (i.e.,75.2 to 103%), whereas AMPA and glufosinate recoveries were 8.48 to 66.8% and 5.44 to 41.1%, respectively. The poor recovery values for AMPA and glufosinate could be explained by their proton equilibria under near neutral conditions. AMPA does not possess a carboxylate moiety, and glufosinate possesses the monoprotic phosphinic moiety. Thus, both AMPA and glufosinate only develop a net negative charge of -1, whereas glyphosate can develop a net divalent negative charge under these pH values. The dianion state of glyphosate in near neutral conditions may explain its excellent retention on the SAX columns compared to AMPA and glufosinate. Regardless, the SAX enrichment step was not deemed suitable for further analysis under these conditions.

AMPA could not be resolved from an unidentified peak under most of these loading conditions. As such, AMPA concentrations were labelled with asterisks, as these concentrations may have been affected by the unresolved interference. All 5 samples exhibiting this interference were performed on the same SCX column, as each SCX column can provide clean-up treatment to a large volume of sample. It is uncertain if the interference originated from a matrix component or column contaminant. The origin of this interference could not be determined. The 0.1 M NaOH loaded sample or buffered sample did not experience this interference. Earlier SPE chromatograms did not exhibit this behavior, as well. Also, the appearance of a negative peak was observed for the buffered solution when injected into the IC. Controls were collected from the SCX

treatment which demonstrated good recoveries for glyphosate, AMPA, and glufosinate. When taken together, SPE enrichment following SCX clean-up will need further assessment to be properly amended to this method.

4.1.7 Standard Addition Study in Sample Matrix

Quantitation methods have been employed to analyzes AGG in milk, urine, Baltic seawater, and estuarine water, respectively (Jensen et al., 2015; Wirth et al, 2021). Wirth et al. used Mandel's test of linearity to assess the fitness of their matrix-constituted calibration curves within seawater and estuarine water (2021). Thus, the accuracy, precision, and linearity were assessed for standard addition curves in the Horsepen Bayou water source. A similar approach to the one used by Wirth and coworkers was adopted to see if the signal response and precent recovery values were sufficient for further AGG analysis. Correlation coefficients for AGG in bayou water treated with SPE SCX were 0.9985, and 0.9848, and 0.9817 which appear sufficient for further analysis, but several recovery values for AGG across the standard addition curve were not within the 80-120% range as shown in Tables 11 and 12. Figure 14 compares the calibration curves between AGG in ultrapure water (5-500 µg L^{-1}) and the standard addition curve of AGG in Horsepen bayou (5-500 µg L^{-1}) samples treated with SPE SCX.

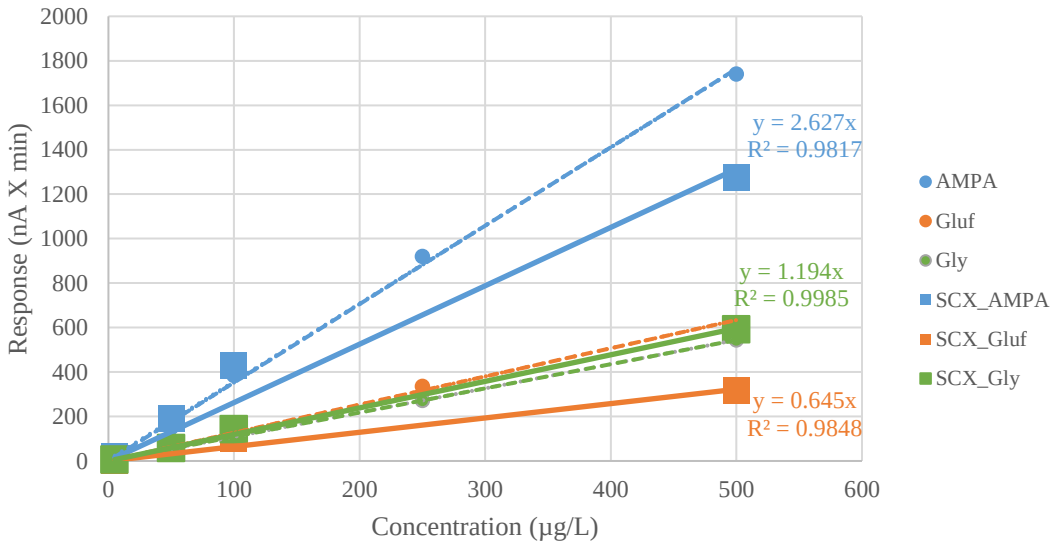

Figure 14. Effect of SCX treatment on standard addition concentrations. The calibration curves prepared by standards without SCX treatment are represented by dashed lines. The calibration curves prepared in bayou water and treated with SCX are represented by solid lines.

The analysis for AGG in SCX treated bayou samples are listed in Tables 10, 11, and 12, and the recovery values for each concentration of AGG are visualized in these tables. Acceptable recoveries were 105.4% and 99.1% for 5 µg L^{-1} and 50 µg L^{-1} AMPA; 85.4% for 50 µg L^{-1} glufosinate; and 111.4%, 106.6%, and 108.9% for 5, 50, and 500 µg L^{-1} glyphosate. 10 of the 36 injections of AMPA were affected by an unresolved unidentified peak which did not interfere at the greater concentration of 500 µg L^{-1} AGG. Also, an injection of 50 µg L^{-1} AGG demonstrated an unstable baseline resembling that of an acidified sample. The experiment was reproduced over greater concentrations of AGG to assess if linearity and recovery would hold, as seen in Table 12. Thus, matrix constituted calibration curves for glyphosate can provide good linearity and excellent recovery values for further environmental analysis. Despite these results, subsequent calibration curves were prepared in ultrapure water to continue analysis of AMPA and glufosinate.

4.2 Analysis of AGG in Surface Water Samples

4.2.1 SPE Pre-treatment of Environmental Samples

The Brazos River was targeted as a natural water source for this analysis due to the recent analysis conducted by the USGS (Medalie et al., 2020). Thus, the accuracy and precision were assessed for the effect of SPE SCX treatment provides to the Brazos River matrix. Three separate Brazos River water samples were collected and filtered with 0.45 µm syringe filters. Five hundred µg L^{-1} AGG was spiked into 3 separate aliquots of 15 mL and acidified to a final concentration of 12 mM HNO_3 (pH 2-3) before SPE treatment. Three aliquots of Brazos River samples were acidified to 12 mM HNO_3 (pH 2-3) without receiving a 500-µg L^{-1} spike to see if AGG is present in sample matrix. These non-spiked sample would serve to assess if AGG pollution was present in the Brazos River samples. A sample volume of 9-10 mL from each of the 6 samples underwent SPE treatment and then neutralized with 35 µL 6 M NaOH before injection. The remaining 5-6 mL from AGG spiked samples were combined and neutralized with less than 35 µL of 6 M NaOH, and these 5-6 mL functioned as an untreated sample to compare to the SPE treated samples.

Table 11. Recovery and concentrations for SCX treated Horsepen Bayou samples spiked with AGG

Compound	Spiked Concentration ($\mu g\ L^{-1}$)	Measured Concentration ($\mu g\ L^{-1}$)	RSD (%)	Recovery (%)
AMPA	5	5.27 ± 2.23	42.2	105.4
	50	49.6 ± 11.3	22.8	99.1
	100	122.3 ± 69.7	57.0	122.3
	500	361.0 ± 16.7	4.6	72.2
Glufosinate	5	2.72 ± 0.8	27.9	54.3
	50	42.7 ± 14.9	34.8	85.4
	100	77.3 ± 36.5	47.3	77.3
	500	247.4 ± 24.4	9.8	49.5
Glyphosate	5	5.57 ± 1.04	18.7	111.4
	50	53.3 ± 18.2	34.2	106.6
	100	131.1 ± 15.3	11.7	131.1
	500	544.3 ± 22.1	4.1	108.9

Table 13 illustrates the accuracy and precision of the IC method on the Brazos River matrix with and without SPE SCX treatment. Glyphosate and AMPA had excellent recovery values (115-96.4%) in the Brazos River water under both SPE treated and untreated samples. Glufosinate saw an improved recovery in Brazos River water which was not treated with SPE. The RSD values fell for SPE treated Brazos River water compared to water not treated with SPE. While the samples without AGG spiking provided no convincing evidence (i.e., peaks) of non-point source pollution of AGG, these results illustrated that glyphosate can be detected with sufficient accuracy and precision in Brazos River water without SPE SCX treatment when the present concentration is within detectable limits. However, due to the variability in matrix throughout samples collected at various times, SPE treatment is still recommended.

Table 12. Recovery for SCX treated Horsepen Bayou samples spiked with AGG at 250 to 1000 µg L^{-1}.

Compound	Spiked Concentration (µg L^{-1})	Measured Concentration (µg L^{-1})	RSD (%)	Recovery (%)
AMPA	250	244.9 ± 59.8	24.4	98.0
	400	382.5 ± 91.8	24.0	95.6
	500	377.9 ± 123.7	32.7	75.6
	750	458.9 ± 84.6	18.4	61.2
	1000	571.9 ± 37.0	6.46	57.2
Glufosinate	250	151.8 ± 15.3	10.0	60.7
	400	424.9 ± 66.1	15.6	106
	500	267.7 ± 107.9	40.3	53.5
	750	339.4 ± 98.8	29.1	45.2
	1000	433.8 ± 44.0	10.1	43.8
Glyphosate	250	269.4 ± 17.3	6.41	108
	400	417.1 ± 136.2	32.6	104
	500	628.7 ± 132.4	21.0	126
	750	801.1 ± 176.8	22.1	107
	1000	1121 ± 103.3	9.22	112

Natural water sources across the Greater Houston area were sampled and evaluated for non-point source pollution. AGG was not detected in samples collected from Bear Creek (n = 2, 250 mL), Clear Creek (n = 1, 250 mL), Sims Bayou (n = 2, 250 mL), Horsepen Bayou (n = 2, 250 mL), and Telfair Lake in the Sugarland area (n = 1, 500 mL). Note that these were often one-time collection sites, and further sampling at other times and locations may provide more accurate assessment once further method development provides lower LOD values.

Table 13. Percent recovery and RSD values of 500 µg L^{-1} AGG spiked in Brazos River Water

No. of Injections	SPE treatment	Compound	Mean Concentration (µg L^{-1})	RSD (%)	Recovery (%)
9	SCX	AMPA	482.9 ± 42.7	8.85	96.6
		Gluf	257.2 ± 81.4	31.7	51.4
		Gly	575.3 ± 50.0	8.70	115
3	None	AMPA	482.2 ± 75.4	15.6	96.4
		Gluf	383.5 ± 125.1	32.6	76.7
		Gly	536.2 ± 89.8	16.8	107

4.2.2 Stability Study (MHT)

The stability of environmental samples intended for analysis has been a concern for analytes suspected to undergo degradative processes while in storage. Stability studies have been performed to determine the half-life values for glyphosate under typical storage conditions (Mallat and Barceló, 1998; Kylin, 2013). However, few assessments provide the maximum holding time (MHT) for environmental samples suspected of glyphosate concentrations. To this end, a stability study was developed to provide MHT values necessary for adequate storage of glyphosate.

Storage samples were constituted by spiking 250 µg L^{-1} AGG into the following storage conditions; 1) Brazos River water stored under refrigerated conditions (4 °C), 2) Brazos River water acidified to approximately pH 2 and stored under refrigerated conditions (4 °C), 3) Sims Bayou water acidified to approximately pH 2 by a final concentration of 20 mM HNO_3 and stored under ambient conditions in direct sunlight (25 °C), and 4) Horespen Bayou water acidified to approximately pH 2 (20 mM HNO_3) and stored under ambient conditions in direct sunlight (25 °C). Temperature was not measured by the window seal during the time of storage. Refrigerated samples were prepared in 15 mL conical centrifuge tubes, and 10 mL were typically collected and

treated with the SPE procedure previously outlined in Section 3.6. Samples stored in ambient conditions were prepared in 50 mL centrifuge tubes and 10 mL was collected from each sample at the time of analysis. These samples also underwent the SPE procedure outlined in Section 3.6. Typical kill standards are constituted in 500 µg L^{-1} $HgCl_2$ which serves as a biocide against potential microbial growth during storage. Kill controls were intended for this study, however, the kill standards constituted for analysis were found to be incompatible with the SPE method, as SPE treated solution of the kill control produced chromatograms with poor baseline stability.

Figure 15 illustrates the temporal concentrations of glyphosate during the initial 36 days of storage of a stability study. AMPA and glufosinate exhibited interferences which resulted in recovery values as low as 44.6% for AMPA and 32.6% for glufosinate. Thus, this method did not provide adequate evaluation for the stability of AMPA and glufosinate. Results shown in Figure 15 suggests that SPE was still considered necessary for all matrices despite lower conductivity values measured for the Sims and Horsepen Bayou matrices compared to their prior sampling events. These values for Sims and Horsepen bayou measured at 332 µS/cm and 574 µS/cm. Regardless, concentrations of glyphosate demonstrated variability through the storage analysis. The greatest standard deviation values were observed at 242±99 µg L^{-1} with a RSD value of 26.6 % and 243±59 µg L^{-1} with a RSD value of 40.9% for the second and seventh day of storage for glyphosate stored at 4°C in non-acidified Brazos River water matrix, respectively. This standard deviation resulted in an inability to model its stability through a linear or exponential fit. All coefficient values were well below 0.90, illustrating no trend in degradation. Thus, this method provided a maximum holding time of 36 days under both acidified and refrigerated conditions for surface water. Longer MHTs are likely; however, the experiment was ceased.

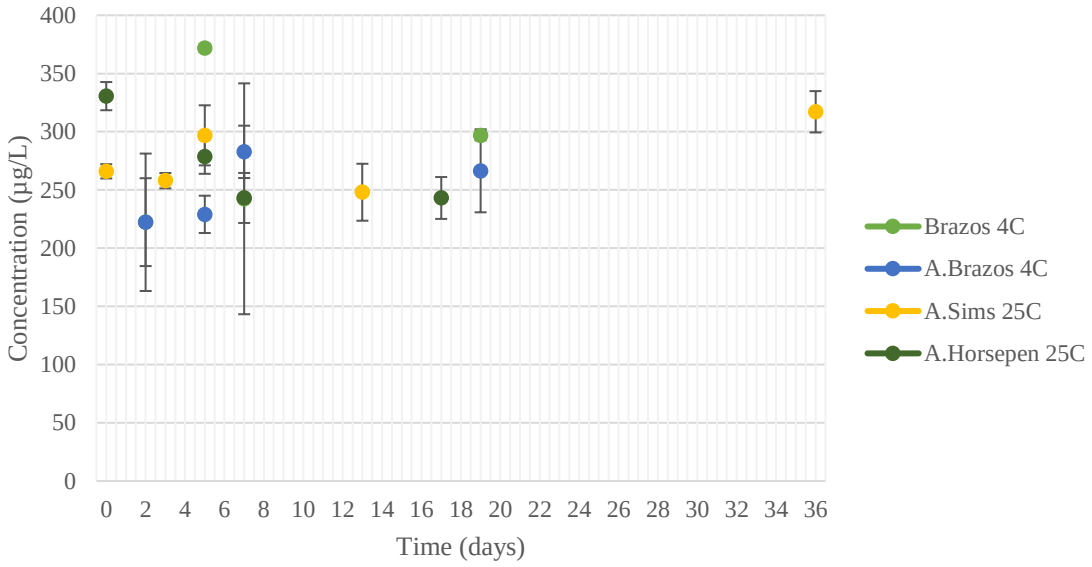

Figure 15. Stability study over the initial 36 days of storage for glyphosate. Each sample was stored in triplicate (n =3) and underwent single IC injection. A represents acidification by 20 mM HNO_3.

5. Discussion

The IPAD-IC method development and detector sensitivity will be discussed below. The SPE method development will also be discussed below. The method was found to provide excellent accuracy and precision when validated by a certified reference material. Lastly, the IPAD-IC method will be compared to existing methods as a means of providing further method evaluation and development strategies.

5.1 IPAD-IC Method Development

Method parameters were investigated through much of this work, including column selection, modifier agent in the mobile phase, sample loop size, sample preparation using SPE, matrix effects, for analytical precision, accuracy, detection limit and linear calibration range. Carb 2.0 series has been reported as a suitable analytical column for glyphosate determination, and this study showed that the length of 100 mm column has sufficient resolution power for glyphosate, glufosinate, and AMPA due to the high selectivity of the solid phase for glyphosate compared to AMPA. While greater column length would improve selectivity toward glufosinate, the lack of co-eluting interferents suggests that the selectivity toward glyphosate and glufosinate may already be sufficient. AMPA was found to be the least selective toward this phase, experiencing co-elution with interferents during SPE method development, possibly due to the presence of inorganic anions such as SO_4^{2-}, Cl^-, and F^- in the environmental samples. However, for these inorganic anions to interfere, they must also be electroactive to voltammetric methods, as these co-eluting interferents were detected by the amperometric detector throughout this work (Figure 13). Sukzuki et al. (2019) also attributed unidentified peaks observed under experimental instrumental parameters to unidentified, common inorganic anions. Küsters and Gerhartz (2010) provided

concentrations of common ions in drinking water samples through IC, AAS, and ICP-MS, highlighting dissolved ions mostly likely to interfere with glyphosate, glufosinate, and AMPA determination. More multi-instrumental analysis may be needed to sufficiently address the interferents responsible for the poor recovery of AMPA, glufosinate, and glyphosate in natural water samples.

5.2 Method Validation

Whether a developed method meets the required analytical performance criteria can be validated through inter-laboratory analysis using a third-party-prepared certified samples of similar matrix with known concentrations or blind samples without given concentration. To this end, the concentration of the certified reference material was not known at the time of analysis, as the certified reference material was treated as an unknown sample suspected of glyphosate pollution. The sample preparation outlined by Sigma Aldrich suggested diluting the 2 mL certified material into 1L of reagent water. This sample preparation approach risked dilution below our LOD value of glyphosate. Thus, the dilution series outlined in section 3.7 was adopted to ensure the diluted certified reference would be within a concentration range which could be determined by our IC method.

A total of nine IC injections were made for method validation with the certification material provided by Sigma Aldrich. This method provided a mean concentration of 478.82 ± 18.67 µg L^{-1} and a RSD value at 3.90. The certified values provided by Sigma Aldrich were obtained after analysis at 495 ± 15 well within the acceptable window. The resulting recovery value was found to be 96.7%. Thus, while drinking water was not thoroughly investigated in this work, this method has been deemed suitable for analysis of water sources by this validation process. Reconstituting certification material in natural

water sources would provide further elucidation of matrix effects on certificated samples, as postulated by Ibáñez et al. (2007).

5.3 Detector Sensitivity

A lower LOD value was initially thought to be achievable by loading greater volumes of sample onto the detector, as classically IC possesses a greater loading capability than RP-LC (Kromidas, 2016). However, while LOD values for 1.00 mL were determined to be 6.50-fold lower than the previous injection volume at 303 µL for glyphosate determination, Figure 12 illustrates the improved sensitivity of the method with this approximately three-fold injection volume increase. The lowest LOD value obtained through the method presented in this work was 0.950 µg L^{-1} for glyphosate. These LOD values were calculated from replicate injections at concentrations near the lower limit of the calibration curve, whereas an alternative method of calculating LOD values utilizes the signal to noise ratios (S/N) as a method development parameter. These values would have provided further validation to the lower detection limit of this method. Regardless, the loading capacity of IC-IPAD was determined to be a limited approach to improving LOD values, but rather provided greater sensitivity toward glyphosate, glufosinate, and AMPA.

Amperometric detection of glyphosate and other aliphatic organophosphorus compounds is not as thoroughly studied as LC-MS/MS detection for their determination (Koskinen, 2019; Valle. et al., 2019). A handful of studies illustrate the signal response of amperometric detectors coupled to LC, substantiating the 280-300 mV detection potential window for aliphatic organophosphorus compounds (Sato et al, 2001; Sanchez-Bayo et al., 2010; Suksuki et al., 2019). For amperometric detectors to compete with existing MS/MS methods in terms of glyphosate determination, electrochemical parameters such as the electrode surface area (see Eq. 1 and Eq. 2) and chemical modification of the

working electrode surface may need to be evaluated (Wang, 2006). Furthermore, glyphosate determination by IPAD-IC methods may also see improved LOD values when utilizing tandem electrochemical detectors which was demonstrated for amino-acid determination (Welch et al., 1990).

5.4 SPE Method Development

From the results provided above, SPE SCX provided reliable sample cleanup for glyphosate determination. However, Tables 11, 12, and 13 demonstrated that glufosinate and AMPA retained to the SCX column phase, receiving moderate to poor recovery values outside of the initial SPE assessment (Table 10). Suksuki and coworkers (2019) also reported a similar behavior where moderate or poor recovery was observed for AMPA and glufosinate when evaluating Na^+ and H^+ form SPE SCX cartridges. The facilitated matrix cleanup through the coupling of SAX enrichment after SCX has been reported (Jiang and Lucy, 2007; Mallat and Barceló, 1998); however, SAX demonstrated poor recovery values for AMPA and glufosinate over a NaOH concentration gradient (Figure 13). Anion exchange phases were found to be less retentive toward AMPA compared to glyphosate in the case of recovery values (Corbera et al., 2015). Improved AMPA and glufosinate recovery values for SPE clean-up and enrichment will be necessary to compete with the current USGS method.

5.5 Comparison to Existing Methods

IC-IPAD methods have been developed for glyphosate determination due to its unique challenge related to its physical and chemical properties. However, more method development work is warranted to further reduce the LOD values competitive with existing methods required for low concentration environmental samples. Table 2 illustrates the LOD values for methods utilized by USGS and other environmental laboratories. USGS method O-2141-09 reported a LOD value lower than Sukzuki et al.

by a factor of 60, providing a MRL value of 0.02 µg L^{-1} compared to the lowest MDL value reported for an IC-IPAD method at 1.45 µg L^{-1} (Sukzuki et al, 2019). This method utilized an MS/MS detection compared to the detection provided by the Metrohm amperometric detector utilized by Suzuki et al. The USGS recently determined the median and time-weighted annual mean concentrations for glyphosate to be 1.39 µg L^{-1} (n = 70) and 0.05 µg L^{-1} (n = 3,204) in hydrological sites throughout US watersheds. The maximum detected concentration of glyphosate for this nationwide survey was 8.1 µg L^{-1}, whereas prior nationwide analyses provided maximum glyphosate concentrations at 427 µg L^{-1} for ditches and drains in Iowa, Idaho, Indiana, Kansas, Mississippi, Washington, and Wisconsin (Battaglin et al., 2014). While USGS frequently detected glyphosate concentrations in concentrations near or below the LOD values found in this IC-IPAD method, these maximum concentrations provide the motive for exposure assessments or environmental analysis of in-land natural water previously reported as non-point source pollution by concentrations greater than the annual mean values.

Earlier IC-PAD and IC-IPAD methods reported greater linear ranges than both reported USGS methods. These linear ranges were 100-50,700 µg L^{-1} and 1,000-180,00 µg L^{-1} for the IC-IPAD and IC-PAD methods, respectively (Sato et al., 2001; Sánchez-Bayo et al., 2010). These early methods demonstrate narrow linear ranges have not been shown to be characteristic of this technique compared to other electrochemical detection methods such as electron capture (EC). The narrow linear range demonstrated by the IC-IPAD method presented here was found to be 3-750 µg L^{-1}. Peak fronting was considered an artifact of column overload at 2 mg L^{-1}, and the next lowest concentration was considered the higher limit of detection.

6. Summary and Conclusion

The offline SPE-IC-IPAD method presented in this work has been developed and validated for environmental analysis of glyphosate in surface water. Column length of 100 mm was found to be sufficient for analysis with the Carb 2.0 100.0/4.0 column. The injection volume was optimized to 1.00 mL with a 6.50-fold improved detection limit and a 2.97-fold improved sensitivity for glyphosate compared to the 303 µL injection. In reagent water, the IC-IPAD method provided suitable analysis for glyphosate, glufosinate, and AMPA in the form of LOD values of 0.950, 0.402, and 0.252 µg L^{-1}, respectively. These values are competitive with the previously reported IC-IPAD method (Suksuki et al., 2019). The linear range in reagent water for this method was 3-750 µg L^{-1} for glyphosate, glufosinate, and AMPA, providing excellent linearity with correlation coefficients of 0.9973, 0.9998, and 0.9983, respectively. One mM citric acid was also determined to be a suitable eluent modifying agent due to the retention shifts of AMPA, glufosinate, and glyphosate to shorter retention times. An offline SCX SPE method was developed to cleanup cationic interferents which provided recovery values for glyphosate between 104-131% along two standard addition curves constituted in surface water. The offline SPE-IC-IPAD achieved good linearity in the concentration range of 5-500 µg L^{-1} with a correlation coefficient of 0.9985. The validation of this method was conducted with a certified standard of glyphosate constituted in drinking water. A mean value of 479 ± 19 µg L^{-1} was obtained from 9 injections and the certified value provided by the manufacturer was 495 ± 15, resulting in a recovery value of 96.7% and a RSD value of 3.97%. Our method found that glyphosate can be stored with a MHT value of 36 days in acidified to a final concentration of 20 mM HNO_3 or refrigerated conditions at 4°C.

A major drawback inherent to the electrochemical method developed in this study was the lack of required detection limit comparable to environmentally relevant concentrations of these three test compounds. As a result, this method did not detect the presence of glyphosate, glufosinate, or AMPA in grab surface water samples collected at the time and space of our sample collection. It is likely that these samples did not represent the time and areas with high concentration of glyphosate such as the case of first flush after rain or point of pesticide use. The method did not approach the annual mean values for glyphosate provided by the USGS or the LOD values reported for the USGS working methods. Perhaps with more samples to be collected at other seasons or locations, certain detection frequency will likely be obtained. Thus, our method currently will be limited to applications where point source pollution and potential exposure is suspected to be within the 3-750 µg L^{-1} range to provide the most accurate and reliable analysis. More work is warranted to further improve the detection limit of this promising technique using IC-IPAD.

7. References

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GLOSSARY

AGG	AMPA, Glufosinate, Glyphosate
AMPA	Amino methyl-phosphonic acid
CV	Cyclic Voltammetry
ESI	Electrospray Ionization
FMOC-Cl	9-Fluorenylmethyl chloroformate
HESI	Heated Electrospray Ionization
IC	Ion Chromatography
ILIS	Isotopically Labelled Internal Standard
ILS	Interlaboratory Study
IPAD	Integrated Pulsed Amperometric Detection
LC	Liquid Chromatography
LOD	Limit of Detection
LSV	Linear Sweep Voltammetry
MHT	Maximum Holding Time
MDL	Method Detection Limit
MS	Mass Spectrometry
PAD	Pulsed Amperometric Detection
RP	Reverse Phase
SAX	Strong Anion Exchange
SCX	Strong Cation Exchange
TMA	Tetra-methyl ammonium Chloride
UV	Ultraviolet
WWTP	Waste Water Treatment Plant