UNIVERSITY OF LATVIA

FACULTY OF CHEMISTRY

DEVELOPMENT AND APPLICATION OF MASS SPECTROMETRIC METHODS FOR THE DETERMINATION OF GLYPHOSATE AND ITS METABOLITES IN PLANT AND ANIMAL ORIGIN PRODUCTS AND ENVIRONMENTAL OBJECTS

DOCTORAL THESIS

MASSPEKTROMETRISKO METOŽU IZSTRĀDE UN PIELIETOŠANA GLIFOSĀTA UN TĀ METABOLĪTU NOTEIKŠANAI AUGU UN DZĪVNIEKU IZCELSMES PRODUKTOS UN APKĀRTĒJĀS VIDES OBJEKTOS

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RĪGA

2021

Promocijas darbs izstrādāts Pārtikas drošības, dzīvnieku veselības un vides zinātniskā institūtā "BIOR" un Latvijas Universitātē laika posmā no 2017. līdz 2020. gadam.

LATVIJAS UNIVERSITATE
ANNO 1919

Darbs sastāv no ievada, 3 nodaļām, secinājumiem, literatūras saraksta, 5 pielikumiem.

Darba forma: disertācija ķīmijas nozarē, analītikās ķīmijas apakšnozarē

Darba zinātniskie vadītāji: Prof., *Dr. chem.* Vadims Bartkevičs
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ABBREVIATIONS

24-h EC_{50}	half-maximal effective concentration after 24-hour exposure
ADI	acceptable daily intake
AMPA	aminomethylphosphonic acid
ARfD	acute reference dose
AS	activated sludge
CFU	colony-forming units
CI	confidence interval
d	dilution factor
DAD	diode array detector
dSPE	dispersive solid phase extraction
DT_{50}	degradation time half-life
EDTA	ethylenediaminetetraacetic acid
EEF	extraction efficiency
EFSA	The European Food Safety Authority
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)
ESI	electrospray ionisation
FMOC-Cl	fluorenylmethoxycarbonyl chloride
GBH	glyphosate-based herbicide
GCB	graphitized carbon black
GHS	Globally Harmonised System (for Classification and Labelling of Chemicals)
GLP	Good Laboratory Practice
GM	genetically modified
GMHT	genetically modified herbicide-tolerant
GMO	genetically modified organism
GNPLs	graphene nanoplatelets
HILIC	hydrophilic interaction chromatography
IARC	International Agency for Research on Cancer
ILIS	isotopically labelled internal standards
k	retention factor
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LOD	limit of detection

LOQ	limit of quantification
m/z	mass-to-charge ratio
ME	matrix effect
MOE	margin of exposure
Nano-LC-MS	nanoflow liquid chromatography coupled to mass spectrometry
NOAEL	no observed adverse effect level
NOEC	no observed effect concentrations
PPP	plant protection product
PPT	protein precipitation
PVDF	polyvinylidene fluoride
QC	quality control
QuPPe	Quick Polar Pesticides method
RMS_{bias}	root-mean square bias standard deviation
RP	reversed phase
RSD	relative standard deviation
RSD_{WR}	within-laboratory reproducibility relative standard deviation
S/N	signal-to-noise ratio
SAX	silica-based anion exchange sorbent
SPE	solid phase extraction
s_{pooled}	pooled standard deviation
SRM	selected reaction monitoring
TEAB	triethylammonium bicarbonate
U	expanded uncertainty
u	standard uncertainty
UHPLC	ultra-high performance liquid chromatography
WW	wastewater
WWTP	wastewater treatment plant
XA	Strata-X polymeric anion exchange sorbent
XC	Strata-X polymeric cation exchange sorbent

ABSTRACT

Development and application of mass spectrometric methods for the determination of glyphosate and its metabolites in plant and animal origin products and environmental objects. Jansons M., scientific supervisors *Dr. chem.*, Prof. Bartkevičs V. and *Dr. Chem.*, Pugajeva I. Doctoral thesis in analytical chemistry, 101 pages, 19 figures, 7 tables, 105 literature references, 5 annexes. In English.

The aim of the doctoral thesis is to optimise and develop sensitive procedures for mass spectrometric determination of glyphosate and its metabolites in different plant and animal origin products, as well in biological and environmental objects. Glyphosate and its degradation products are compounds with polar moieties, determination of which is challenging, therefore effective sample preparation and analysis procedures are needed in order to reduce matrix effects and improve the detection of these compounds.

In this study, the possibility to improve detection capability and quantification of glyphosate and aminomethylphosphonic acid has been investigated in three main directions – unsuppressed ion chromatography coupled to mass spectrometry, multi-stage sample preparation based on solid phase extraction sorbents, and derivatization and analysis of samples using tandem mass spectrometry or high resolution Orbitrap mass spectrometry. Applicability of ion chromatography has been evaluated on different electrospray ionisation sources, as well as the changes in sensitivity and matrix effects depending on the composition of eluent have been investigated. A sensitive method for determination of glyphosate and aminomethylphosphonic acid using solid phase extraction has been developed. A novel and reliable method for sample preparation by derivatization with dansyl chloride and determination of derivatized glyphosate, aminomethylphosphonic acid and glufosinate has been developed and validated. The dansyl derivatives were characterised for the first time using liquid chromatography and electrospray ionisation tandem mass spectrometry and high resolution Orbitrap mass spectrometry.

The optimised and developed methods were applied for analysis of glyphosate and aminomethylphosphonic acid in various food and environmental samples.

GLYPHOSATE, AMINOMETHYLPHOSPHONIC ACID, ION CHROMATOGRAPHY, SOLID PHASE EXTRACTION, DERIVATISATION, TANDEM MASS SPECTROMETRY, HIGH RESOLUTION MASS SPECTROMETRY, ORBITRAP

ANOTĀCIJA

Masspektrometrisko metožu izstrāde un pielietošana glifosāta un tā metabolītu noteikšanai augu un dzīvnieku izcelsmes produktos un apkārtējās vides objektos. Jansons M., zinātniskie vadītāji *Dr. chem.,* Prof. Bartkevičs V. un *Dr. chem.* Pugajeva I. Promocijas darbs, 101 lappuse, 19 attēli, 7 tabulas, 105 literatūras avoti, 5 pielikumi. Angļu valodā.

Promocijas darbā optimizētas un izstrādātas, kā arī pielietotas metodes glifosāta un tā sabrukšanas produktu noteikšanai dažādos augu un dzīvnieku izcelsmes produktos, kā arī bioloģiskajos un vides objektos, izmantojot šķidruma hromatogrāfiju apvienojumā ar masas spektrometriju. Glifosāts un tā sabrukšanas produkti ir mazmolekulāri savienojumi ar polārām funkcionālajām grupām, tāpēc to noteikšana ir problemātiska, un noteikšanai ir nepieciešamas efektīvas paraugu sagatavošanas un analīzes metodes, ar mazinātiem matricas efektiem un uzlabotu noteikšanas spēju.

Promocijas darbā trijos galvenajos virzienos izpētītas iespējas uzlabot glifosāta un aminometilfosfonskābes detektēšanu un kvantificēšanu – jonu hromatogrāfijā un masas spektrometrijā bez supresora, vairāku stadiju paraugu sagatavošanā ar cietfāžu ekstrakcijas sorbentiem, kā arī paraugu sagatavošanā ar derivatizāciju un paraugu analīzē ar tandēma masspektrometru vai augstas izšķirtspējas Orbitrap masspektrometru. Tika novērtēta jonu hromatogrāfijas pieletojamība uz dažādiem elektroizsmidzināšanas jonizācijas avotiem, izmaiņas jutībā un matricas efektos atkarībā no izmantotā eluenta. Tika izstrādāta jutīga metode glifosāta un aminometilfosfonskābes noteikšanai, izmantojot cietfāžu ekstrakciju. Tika izstrādāta un validēta inovatīva un uzticama paraugu sagatavošanas un analīzes metode glifosāta, aminometilfosfonskābes un glifosināta noteikšanai pēc derivatizācijas ar dansilhlorīdu. Pirmo reizi raksturoti ar dansilhlorīdu derivatizētie nosakāmie savienojumi, izmantojot šķidrumu hromatogrāfiju un elektroizsmidzināšans jonizācijas tandēma masspektrometriju.

Izstrādātās un optimizētās metodes tika pielietotas glifosāta un aminometilfosfonskābes noteikšanai sarežģītos pārikas un vides paraugos.

GLIFOSĀTS, AMINOMETILFOSFONSKĀBE, JONU HROMATOGRĀFIJA, CIETFĀŽU EKSTRAKCIJA, DERIVATIZĀCIJA, TANDĒMA MASSPEKTROMETRIJA, AUGSTAS IZŠĶIRTSPĒJAS MASSPEKTROMETRIJA, ORBITRAP

INTRODUCTION

Glyphosate (*N*-(phosphonomethyl)glycine) is a systemic non-selective herbicide – sufficiently soluble in water, resulting in uptake and circulation inside plant tissue, and affecting any plants that may absorb it. It is the active ingredient in glyphosate-containing herbicide formulations widely used in agriculture, gardening, and industrial sites for weed control after emergence. In the past decade, glyphosate, its degradation product aminomethylphosphonic acid (AMPA) and other polar and acidic pesticides, including glufosinate, have been particularly studied due to concerns over their globally wide and intensive use.

The lack of selectivity towards weeds was the main reason for its applications being very limited since discovery of the herbicidal activity of glyphosate in 1970, until genetically modified herbicide-tolerant (GMHT) crops were introduced in 1996 – crops designed to be resistant towards specific herbicides and to be grown with the application of the associated herbicides in order to eliminate growth of weeds. Due to the rapid adoption of GMHT crops by farmers, the estimated global consumption of glyphosate experienced rapid growth from approximately 67 thousand tonnes in 1995 to 826 thousand tonnes in 2014 (including non-agricultural uses). Glyphosate has also pushed out of use some other herbicides in the USA [1], [2]. Taking into account the rate of application (0.15–0.2 tonnes km^{-2} in 2014), it would have been sufficient for treating 20–30% of the cropland cultivated globally, which makes it the most widely and intensively used pesticide in history [1].

Glyphosate is the most widely and intensively applied pesticide globally [1], [2] and its safety for human health and environmental biomes has recently come under scrutiny, in part due to the reapproval process in the European Union for the active substance glyphosate. Due to ionic chemical structure, non-volatility, absence of chromophores, and strong metal complexing properties, determination of it presents significant analytical challenges. As a result, studying glyphosate has become a growing trend in scientific research, especially with focus on development and application of sensitive and reliable laboratory testing methods for determination of glyphosate, its degradation products and other related pesticide residues.

The practical relevance of the problem

Currently, monitoring of pesticide residues is regarded as a research priority to enforce safer use of pesticides and to improve the knowledge of pesticide properties, occurrence, and effects on public health and biota. Different analytical methods may be used to achieve this, but due to the low concentration of analytes and complex samples, mass spectrometry and liquid chromatography are of the most important residue analysis techniques. However,

chromatography and mass spectrometry-based procedures require elaborated sample preparation protocols and rigorous validation for reliable quantitative results.

Innovative approaches to determination of analytes should be regarded as an important area of scientific efforts due to the challenges presented by the wide range of chemical properties of pesticides. Taking into account the chemical properties of polar and acidic pesticides such as glyphosate and its main degradation product – aminomethylphosphonic acid (AMPA), special methods, like derivatization or some form of ion exchange chromatography, are needed for analysis in plant and animal origin products. These approaches, however, present a problem of compatibility with ESI-MS due to the presence of salts and other compounds with low volatility. Also, these special methods, which usually are not part of a wide scope multi-residue method, result in higher costs of laboratory testing. Improvements in sample preparation, sensitivity and reliability of the analytical procedure are therefore especially useful to advance efforts at studying the occurrence and properties of these pesticides.

The aim of the work

The following aims were proposed during this thesis:

i. Investigation of ecotoxicity, biodegradability and soil mobility of glyphosate from glyphosate containing formulations and its degradation products.

ii. Investigation of the applicability of different separation science techniques and development of the novel and sensitive mass spectrometric methods for determination of glyphosate in different plant, animal and biological origin products, and environmental objects.

iii. Estimation of the occurrence of glyphosate in products being at risk for contamination on the Latvian market, as well as environmental objects being at risk for contamination.

The approach used

The following objectives have been set in order to fulfil the aims of the thesis:

i. To optimise a LC-MS/MS instrumental method for the determination of glyphosate and aminomethylphosphonic acid in environmental objects with low measurement uncertainty at levels appropriate for laboratory model experiments.

ii. To review the literature and perform laboratory model experiments on ecotoxicity, biodegradability and soil mobility of glyphosate and glyphosate containing formulation and its degradation products.

iii. To assess experimentally solid phase extraction procedures in order to achieve the highest sensitivity for determination of glyphosate at low $\mu g\ kg^{-1}$ levels in food and environmental samples.

iv. To review the literature and assess experimentally different sample preparation and extraction procedures and different stationary phases for liquid chromatography in order to achieve the highest sensitivity for reliable LC-MS/MS determination of glyphosate at low μg kg^{-1} levels in food.

v. To assess experimentally different mobile phases for unsuppressed ion chromatography coupled to LC-MS/MS for determination of glyphosate at low μg kg^{-1} levels in food with low matrix effect, as well as to assess experimentally electrospray ionisation sources with respect to compatibility with unsuppressed ion chromatography.

vi. To develop a novel and reliable derivatisation-based LC-MS/MS analytical method for the analysis of glyphosate and aminomethylphosphonic acid in complex food matrices.

vii. To characterise the derivatised analytes using LC-MS/MS.

viii. To compare the performance of the novel derivatisation-based procedure with the performance of the QuPPe procedure in combination with the recently invented diethylamino stationary phase for anionic pesticide analysis from Waters™.

ix. To apply the developed and optimised analytical methods for the analysis of glyphosate and its degradation products to objects of plant, animal or environmental origin at risk for contamination.

Scientific novelty

i. Knowledge on the applicability of unsuppressed ion chromatography coupled to tandem mass spectrometry for simultaneous analysis of ionic and acidic pesticides and the comparative assessment of different mobile phases and electrospray ionisation sources.

ii. Development and application of a sensitive multi-step solid phase extraction-based LC-MS/MS method for determination of glyphosate and aminomethylphosphonic acid in food of plant and animal origin, and the comparative assessment of different combinations of solid phase extraction sorbents and preconcentration factors, and the comparative assessment of different analytical columns.

iii. Development of a novel and reliable dansyl chloride derivatisation-based LC-MS/MS method for determination of glyphosate, aminomethylphosphonic acid and glufosinate in foods of plant and animal origin.

iv. Characterisation of dansyl chloride derivatives of glyphosate, aminomethylphosphonic acid and glufosinate has been reported for the first time using electrospray ionisation LC-MS/MS in combination with high resolution Orbitrap mass spectrometry.

v. Detection of dansyl chloride derivatized glyphosate, aminomethylphosphonic acid and glufosinate using nanoflow liquid chromatography coupled to mass spectrometry, which could be applied for analysis of small samples, has been reported for the first time.

vi. Knowledge on the origin and extent of matrix effects in the novel and reliable dansyl chloride derivatisation-based LC-MS/MS method for determination of glyphosate, aminomethylphosphonic acid and glufosinate, and the QuPPe procedure in combination with the recently invented diethylamino-based stationary phase from Waters™.

vii. Knowledge on the method performance of the novel and reliable dansyl chloride derivatisation-based LC-MS/MS method for determination of glyphosate and aminomethylphosphonic acid in foods of plant and animal origin in comparison with the QuPPe procedure in combination with the recently invented diethylamino-based stationary phase from Waters™.

viii. Knowledge on the occurrence of glyphosate and aminomethylphosphonic acid in products being at risk for contamination on the Latvian market, as well as environmental objects being at risk for contamination, obtained by application of the sensitive multi-step solid phase extraction-based LC-MS/MS method.

Practical application of the work

The optimised and developed analytical methods and the comparative assessments provide a range of well characterised, practical and reliable options for performing measurement of glyphosate, aminomethylphosphonic acid and selected other pesticides depending on the matrix in question and sample size. The methods can be applied for extended monitoring of the occurrence or scientific studies on these chemicals.

Scientific publications

1. **Jansons, M.**; Pugajeva, I.; Bartkevics, V; Karkee, H. B. LC-MS/MS characterization and determination of dansyl chloride derivatized glyphosate, aminomethylphosphonic acid (AMPA) and glufosinate in foods of plant and animal origin. **Under review at Journal of Chromatography B**[1], **2020**.
2. **Jansons, M.**; Pugajeva, I.; Bartkevics, V. Evaluation of selected buffers for simultaneous determination of ionic and acidic pesticides including glyphosate using anion exchange chromatography with mass spectrometric detection. *Journal of Separation Science*[2], **2019**, *42*(*19*), 3077-3085.
3. Bērziņš, A.; **Jansons, M.**; Kalneniece, K.; Shvirksts, K.; Afanasjeva, K.; Kasparinskis, R.; Grube, M.; Bartkevičs, V.; Muter, O. Modeling the mobility of glyphosate from two contrasting agricultural soils in laboratory column experiments. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*[3], **2019**, *54*(*7*), 539-548.
4. Reinholds, I.; **Jansons, M.**; Pugajeva, I.; Bartkevičs, V. Recent Applications of Carbonaceous Nanosorbents in Solid Phase Extraction for the Determination of Pesticides in Food Samples. *Critical Reviews in Analytical Chemistry*[4], **2019**, *49*(*5*), 439-458.
5. **Jansons, M.**; Pugajeva, I.; Bartkevičs, V. Occurrence of glyphosate in beer from the Latvian market. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*[5], **2018**, *35*(*9*), 1767-1775.
6. Birsa, T.; Kalneniece, K.; **Jansons, M.**; Kalniņš, M.; Ozolina, I.; Bērziņš, A.; Švinka, V.; Švinka, R.; Bartkevičs, V.; Muter, O. Evaluation of glyphosate ecotoxicity and biodegradability in the municipal wastewaters. *Key Engineering Materials*[6], **2018**, *762*, 75-80.

[1] *Peer reviewed journal, imprint of Elsevier (IF=3.004 (2019)), ISSN:1570-0232*
[2] *Peer reviewed journal, imprint of John Wiley & Sons (IF=2.878 (2019)), ISSN:1615-9314*
[3] *Peer reviewed journal, imprint of Taylor & Francis (IF=1.697 (2019)), ISSN:1532-4109*
[4] *Peer reviewed journal, imprint of Taylor & Francis (IF=4.568 (2019)), ISSN:1547-6510*
[5] *Peer reviewed journal, imprint of Taylor & Francis (IF=2.340 (2019)), ISSN:1944-0057*
[6] *Peer reviewed journal, imprint of Trans Tech Publications (IF=0.350 (2020)), ISSN:1662-9795*

Conferences

1. International scientific symposium "Science to strengthen sustainable and safe food systems", Riga, Latvia, Chemistry Section, Riga, Latvia, 30-31 January 2020. **Jansons, M.**; Pugajeva, I.; Bartkevičs, V. Reliable LC-MS/MS determination of dansyl chloride derivatized glyphosate, AMPA and glufosinate (in book of abstracts and poster presentation);
2. The 78th International Scientific Conference of the University of Latvia, Chemistry Section, Riga, Latvia, 2020. **Jansons, M.**; Pugajeva, I.; Bartkevičs, V. Reliable LC-MS/MS determination of dansyl chloride derivatized glyphosate, AMPA and glufosinate (in book of abstracts and oral presentation).
3. The 77th International Scientific Conference of the University of Latvia, Chemistry Section, Riga, Latvia, 2019. **Jansons, M.**; Pugajeva, I.; Bartkevičs, V. Evaluation of select buffers for simultaneous determination of ionic and acidic pesticides by ion chromatography and mass spectrometry (in book of abstracts and oral presentation);
4. European Union Laboratories for Residues of Pesticides – Single Residue Methods Training Contribution Session, Chemisches und Veterinäruntersuchungsamt, Stuttgart, Germany, 15-17 October 2018. **Jansons, M.** Determination of glyphosate in eggs (oral presentation);
5. The 76th International Scientific Conference of the University of Latvia, Chemistry Section, Riga, Latvia, 2018. **Jansons, M.**; Pugajeva, I.; Bartkevičs, V. Occurrence of glyphosate in beer from the Latvian market (in book of abstracts and oral presentation).

1. LITERATURE REVIEW

1.1. Glyphosate properties and applications

Glyphosate (*N*-(phosphonomethyl)glycine), a phosphonomethyl derivative of glycine amino acid, is a systemic, i.e., absorbed by the internal tissues of the plant, and a non-selective herbicide, i.e., that affects all plants, and it is the active ingredient in some herbicide formulations since its invention in 1970.

Pure glyphosate appears as colourless crystalline solid. The melting point is 190 °C, decomposition occurs at 230 °C. The dissociation constants are – pK_{a1} = 2.0 (phosphate), pK_{a2} = 2.6 (carboxylic acid), pK_{a3} = 5.6 (secondary amine), pK_{a4} = 10 (phosphate), as a result it exists only in ionic forms in dilute aqueous solution, and logP = -3.4 The solubility of pure glyphosate in water is only 10.5 g L^{-1}, however, solubility of its triethylammonium salt is 1050 g L^{-1} at 20 °C. The solubilities of ammonium and alkali metal glyphosate salts are > 19% mass concentration of an aqueous solution at 20 °C [3]. The pH of a 1% glyphosate solution in water is 2.5 [4].

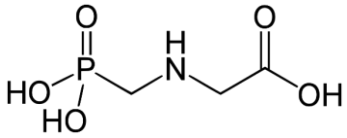

Figure 1.1. **The chemical structure of glyphosate.**

Because of the solubility properties, glyphosate is produced and applied in various salt forms. The identity of the counter ion or adjuvant is an important part of the formulation of glyphosate-based plant protection products (PPPs). PPPs containing glyphosate are widely used in agriculture, gardening and industrial sites for weed control post-emergence, as well as for applications before crop planting. Compared to contact herbicides, glyphosate may be applied merely on a small part of the foliage of the weed in order to be effective, as the herbicide is translocated to underground parts and distant foliage [5].

Glyphosate can compete for binding with the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (EPSPS) ubiquitously found in plants, bacteria and fungi, but not animals [6]. The enzyme is involved in a metabolic pathway (shikimate pathway) for synthesis of aromatic amino acids. Therefore, glyphosate causes impaired protein biosynthesis, which then results in the EPSPS-dependent organism dying [7].

Glyphosate was synthesized for the first time in 1950 by Dr. Henri Martin, a Swiss chemist who worked for a small pharmaceutical company. At the time, glyphosate was not reported in the literature and no applications were found for the newly synthesized chemical

compound. A decade later, the pharmaceutical company was acquired, and the samples of synthesized glyphosate were sold out as fine chemicals, however, no reports on the biological activity were made. In 1970 glyphosate was synthesized in the agricultural division of the Monsanto company (USA) after many efforts at synthesis and testing of structurally similar potential aminomethylphosphonic acid-based herbicides. Glyphosate was found to have the strongest herbicidal effect [3].

However, the high level of phytotoxicity of glyphosate made its agricultural applications difficult, as only very small amount of unintended application could result in significant damage to the crops being protected against weeds [5]. This was the main reason for glyphosate applications being very limited since discovery of the herbicidal activity of glyphosate in 1970, only until genetically modified herbicide tolerant (GMHT) crops were introduced in 1996. GMHT crops are engineered resistant towards specific herbicides and intended to be grown with the application of the associated herbicides in order to eliminate growth of weeds. Due to the rapid adoption of GMHT crops by farmers, the estimated global consumption of glyphosate experienced rapid growth from approximately 67 thousand tonnes in 1995 to 826 thousand tonnes in 2014 (including non-agricultural uses). Glyphosate has also pushed out of use some other herbicides in the USA [1], [2]. Taking into account the rate of application (0.15–0.2 tonnes km^{-2} in 2014), it would have been sufficient for treating 20–30% of the cropland cultivated globally, which makes it the most widely and intensively used pesticide in history [1]. The maximum application rate of glyphosate in any 12-month period across representative uses equivalent to the sum of pre-plant, pre-harvest and post-harvest applications assuming the worst-case scenarios has been reported to be 0.432 tonnes km^{-2}. This application rate has been taken into account in the latest risk assessments by the European Food Safety Authority (EFSA), which have been carried out as part of the approval renewal process for the active substance glyphosate in the European Union. The global average application rate of glyphosate is below the maximum application rate [8].

1.2. Glyphosate applications in Latvia

The cereal cropland in Latvia consisted of 64% wheat in 2017, the remainder consisted of oat, barley, rye and other cereals [9]. According to a survey on the use of pesticides in 2017 [10], the pesticides used for treating the cereal crops were plant growth regulators (44.8%), herbicides (30.2%), fungicides (24.4%), and insecticides (0.5%). Glyphosate accounted for 12.1% of the total weight of the active substances used on the crops. Winter barley were treated with pesticides at 0.053 tonnes km^{-2} and winter wheat were treated with 0.071 tonnes km^{-2}. Taking into account the share of glyphosate among other pesticides, applications of glyphosate

in Latvia are at least an order of magnitude below the global average. However, none of the agricultural crops sown in Latvia are glyphosate tolerant.

The main genetically modified (GM) corps are maize, soybean, oilseed rape and cotton. Although, many other crop plants have been modified for novel traits, such as resistance to glyphosate and glufosinate herbicides. Only Spain and Portugal have so far grown significant amounts of GM crops, particularly the MON 810 maize, as GM plants can be authorized for cultivation in accordance to the directive 2001/18/EC, however, the EU has made it mandatory to label GMO products [11].

Therefore, the main purposes for applications of glyphosate in Latvia exclude taking advantage of glyphosate resistance of GMHT crops, as there are currently no GMHT crop seeds commercially available for wheat, barley, rye, and oats, which are the main cereal crops in Latvia. However, to the best of our knowledge, farmers may sometimes use glyphosate for pre-harvest treatment of cereals. Pre-harvest applications in certain cases may be carried out in order to prevent the growth of weeds and is considered to be in line with good agricultural practices. However, the pre-harvest application of glyphosate must not be carried out for the desiccation of the crops with the sole intention to control the time of harvest and to optimise threshing, which may be considered not in line with good agricultural practices [12].

Taking into account the most likely environmental fate of glyphosate, pre-harvest applications could be the main source of detectable glyphosate residues in barley, wheat and rye products in Latvia due to the unavailability of GMHT versions of these crops and the restrictions that apply to GMHT in the EU in general.

1.3. Glyphosate and glyphosate-based herbicide toxicity and carcinogenicity

Toxicity of glyphosate has already been extensively studied and reviewed taking into account numerous studies [6], [13]. A summary of toxicity of glyphosate is given in Table 1.1.

Table 1.1.

Summary of chronic toxicity of glyphosate to various taxa and species of animals [6]**.**

Taxa / species	Route	Evaluation	Value	Unit
Birds	Diet	NOAEL	93	mg kg^{-1} d^{-1}
Rabbits	Oral	NOAEL	175	mg kg^{-1} d^{-1}
Mice	Diet	NOAEL	507 – 1890	mg kg^{-1} d^{-1}
Fish	Environment	NOEC	26 – 52	mg L^{-1}
Daphnia magna	Environment	NOEC	50	mg L^{-1}
Aquatic microorganisms	Environment	NOEC	0.28 – 33.6	mg L^{-1}
Soil microorganisms	Environment	NOEC	5.0	mg kg^{-1}

The no observed adverse effect levels (NOAEL) and no observed effect concentrations (NOEC) are in the mg kg^{-1} range for pure glyphosate. Under the assumption that these values have been determined under conditions representing chronic exposure, margin of exposure approach (MOE) can be applied – the toxicity exposure ratio (TER), which can be estimated by taking into account a realistic estimate of exposure, can be considered acceptable if equal to or greater than 100. The TER values have been shown to greatly exceed 100 for glyphosate, taking into account the highest potential exposure for human adults and children [13].

According to the conclusions drawn in the peer review of the pesticide risk assessment of the active substance glyphosate conducted by EFSA, which is based on expert reviews on mandatory regulatory Good Laboratory Practice (GLP) studies, other relevant studies and peer-reviewed scientific studies, glyphosate is not classified as toxic according to the Regulation (EC) 1272/2008 for classification, labelling and packaging of chemicals, also known as the Globally Harmonised System of Classification and Labelling of Chemicals (GHS), as the acute toxicity of glyphosate is low when administered orally, dermally or by inhalation. The toxicological evaluation adopted an approach whereby the conclusions are drawn from a large number of valid studies instead of relying on a single key study. An overall long term NOAEL of 100 mg kg^{-1} d^{-1} was obtained from long term studies in rats [8].

The peer review of the pesticide risk assessment of glyphosate conducted by EFSA also concluded that glyphosate does not present genotoxic potential and no evidence of carcinogenicity was observed in rats or mice. However, during the peer review process the International Agency for Research on Cancer (IARC) published an article classifying glyphosate as a probable carcinogenic to humans. Differences were pointed out in the approaches to evaluation adopted by EFSA and IARC, particularly that the IARC reported on a large number of studies with negative results for glyphosate but positive results for glyphosate-based PPPs. Surfactants frequently used in glyphosate-based PPPs, such as polyethoxylated tallow amines are orders of magnitude more cytotoxic than glyphosate due to their cell membrane damaging effects [14]. The EFSA peer review was focused on the active ingredient and one representative formulation containing isopropyl ammonium glyphosate. The IARC conclusions were evaluated by EFSA, and it was concluded that due to lack of consistency in animal studies and lack of statistical significance in pair-wise comparison tests and slightly increased incidences only at very high doses that are not suitable for mutagenicity studies, no carcinogenic classification is justified [8]. However, it should be pointed out that the goal of the evaluations performed by IARC has a different scope, which are not directly connected to risk management decisions. The IARC classifications are aimed to provide the

first step to alerting on the carcinogenicity potential of a broad range of agents [14]. Numerous authors have suggested that the recent discussions over carcinogenicity of glyphosate have been controversial [5], [15], [16], [17], [18]. Nevertheless, the metabolism of glyphosate has recently been investigated at high doses (200 mg kg^{-1} d^{-1}) in mice, where it was shown that, contrary to previous research claiming that glyphosate is not metabolized in vivo, it was shown to metabolize to glyoxylate, which reacted with many protein targets, particularly cysteine-reactive protein targets, and led to elevated levels of fat and cholesteryl esters in the liver, a major lipid dysregulation [16].

1.4. Environmental fate of glyphosate

Glyphosate has low mobility in soil, low uptake by plants through roots and low toxicity in general [6], however recent studies have raised some concerns regarding some possible emerging environmental effects. Concerns have been raised that as a result of the selection pressure caused by use of glyphosate with GMHT crops the steady rise in prevalence of glyphosate resistant weeds in different locations observed between 1998 and 2014 may further drive up the consumption of glyphosate due to diminishing efficiency [2].

Although glyphosate strongly adsorbs to soil and is degraded predominantly by bacteria, concerns have been raised that unfavourable weather conditions, such as frequent dry and cold, may bring about accumulation of glyphosate in soil [19], which in turn may bring unwanted changes in soil chemistry [20]. It can take anywhere from 8 to 280 days depending on soil and weather conditions for 90 % of the initial amount of glyphosate to degrade [19]. Soil retention capacity depends on soil mineral content, pH and phosphate content [21]. The transfer of glyphosate to freshwater and groundwater is largely controlled by its sorption on soils and sediments [22], [23]. Amorphous oxides, e.g., aluminium, iron, and the crystal lattice edge sites of clay minerals have been suggested as the dominant sites for glyphosate adsorption [24], [25].

It has been estimated that the glyphosate-derived phosphorus, that is being added to the environment as a result of glyphosate applications, has recently reached an arguably significant > 1% of fertilizer-derived phosphorus. This may have implications for phosphate losses to freshwater, as the competition for sorption sites in soil between glyphosate and phosphate could potentially cause greater phosphate losses from soils, as glyphosate is a stronger anion [21].

Most known bacteria have been found to break down glyphosate by oxidoreductases to aminomethylphosphonic acid (AMPA) and glyoxylate. AMPA can be excreted to the environment or consumed as a source of phosphorus catalysed by C-P lyase, producing methylamine as by-product. Glyoxylate can further be degraded to carbon dioxide, and methylamine can further be degraded to ammonia and carbon dioxide. Alternatively, in the

presence of glyphosate-specific C-P lyases, the primary degradation product is sarcosine, which can further be degraded to formaldehyde and glycine by sarcosine oxidases. The sarcosine pathway rarely occurs in natural environments unless there is a condition of phosphorus deficiency. Most glyphosate degrading bacterial strains excrete AMPA to the environment, however, a number of strains can use AMPA as source of phosphorus [6], [26].

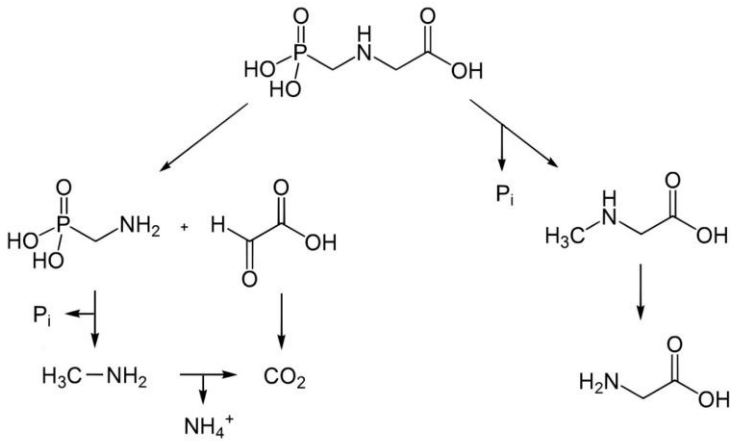

Figure 1.2. **The main degradation pathways of glyphosate in the environment** [13]**.**

The largest survey of glyphosate's environmental occurrence up to date was conducted in the United States from 2001 to 2010 and established that glyphosate occurs widely in the environment. Samples were collected from diverse hydrologic settings and a wide range of geographic locations. Glyphosate was detected in 34 % of surface and groundwater samples, 70.9 % of ditch and drain water samples at 0.20 μg L^{-1} median and 60 % of soil and sediment samples at 9.6 μg kg^{-1} median [27].

1.5. Glyphosate and AMPA residues in soil and leaching from soil

The cation exchange capacity and clay content have been shown to influence sorption of glyphosate across soils and sediments [22]. The content of soil organic matter has been shown to positively correlate with sorption of glyphosate, depending on the polarity, electron density at the binding sites of the organic matter molecules relevant for the interaction with glyphosate [28]. Surface area of mineral phase has been suggested as more important for adsorption of glyphosate, compared to the amount of organic carbon [29]. The sorption of glyphosate, mineralization and persistence in conventional tillage and non-tillage soil systems have been shown to be similar [30]. In turn, other authors indicated that the risk of leaching of aged glyphosate and AMPA residues from soil is greater in fertilized soil. It was demonstrated in a leaching study using phosphate solution as an extraction agent [31].

Along with sorption processes, different mechanisms of glyphosate transport can take place in soil and water, which can vary depending on biota conditions. Soil type has been shown

to affect glyphosate leaching to a larger extent than the experimental treatments [32]. Particle facilitated transport of glyphosate may also occur. In an 8-month field study, the particle facilitated transport (particles >0.24 μm) accounted only for a 13–16% of the observed glyphosate [33]. AMPA persisted longer in soil than glyphosate [19]. The degradation half-life DT_{50} values of 9 days for glyphosate and 32 days for AMPA have been reported [31]. Long persistence of glyphosate has been shown in boreal soils. In particular, 19% of glyphosate and 48% of AMPA relative to the applied glyphosate amount was detected in the topsoil after 20 months [34]. Seasonal changes in the hydraulic regime in summer during the vegetation period and in winter, when the soil freezes, affect pesticide losses through surface runoff [35]. The highest glyphosate concentrations in the surface runoff were detected during the periods of snow melting and soil thawing in the first winter following an autumn application [34]. The non-extractable residues later become available for biodegradation and leaching [36], [37], [38].

Apart from the soil characteristics and changes in hydraulic regime, the application rate of glyphosate is one of the most important risk factors for leaching. Also, the cultivation of GMHT crops has led to increased application of glyphosate-based herbicides, which in turn has contributed to widespread growth of glyphosate resistant weeds. Thus, specific combinations of geographical, geological, meteorological and agronomical factors can increase the risks of glyphosate occurrence in streams, groundwater and drinking water.

In this study we have compared the behaviour of glyphosate in the agriculturally relevant sandy and loamy sand soils after spiking with concentrations of glyphosate from GBH and bioaugmentation, followed by weathering for 40 days and a consistent three-stage leaching in a laboratory column experiment. This represents an alternative approach to those described by other authors. Addition of the microbial consortium with a high degradation potential towards the glyphosate-based herbicide contaminated soil is expected to reveal the role of microbial activity in the mobility of glyphosate and AMPA, as well as the overall ecotoxicity of the GBH in these agricultural soils after 40 days since the herbicide application.

1.6. Glyphosate and AMPA residues in wastewater

The main source of pesticides in the environment is the surface runoff from agricultural areas [39], however, the urban contributions have also been appreciated, originating from non-agricultural uses of pesticides, such as grass management, vegetation control, forestry and horticulture [40]. With increasing population and industrial activities, and therefore freshwater resources becoming ever more precious, the wastewater (WW) treatment efficiency with

respect to various classes of contaminants, such as pharmaceuticals, pesticides, persistent organic compounds, plastic residues and metals, has become an important area of research [41].

Glyphosate or AMPA has been detected in 67 % of the wastewater treatment plant (WWTP) effluents (median concentration of glyphosate was 0.1 μg kg^{-1} and concentration of AMPA was 0.7 μg kg^{-1}) sampled in year 2002 from 10 locations across the USA representing a variety of climatic conditions, population densities and treatment practices. A part of the AMPA detections, however, may have originated from phosphonate detergent uses.

In this study we have evaluated the removal efficiency and ecotoxicity of a GBH added to the municipal raw wastewater (WW) in a laboratory model column experiment. The effect of oxide ceramics, as well as activated sludge and nutrients has been compared. The ecotoxicity was performed as evaluated as whole effluent toxicity towards *Daphnia magna*. Whole effluent toxicity towards test organisms, such as phytoplankton, zooplankton, fish, *Daphnia magna* and algae is considered one of the main criteria for evaluating the efficiency of wastewater treatment plants [41].

1.7. Glyphosate residues in food

In 2015 EFSA had applied an acute reference dose (ARfD) value of 0.5 mg kg^{-1} of body weight for glyphosate, which is an estimate of the amount of a chemical substance that can be ingested over a short timeframe, without facing a health risk. Also, an acceptable daily intake (ADI) value of 0.5 mg kg^{-1} d^{-1} has been set. Further safety assessments for evaluation of the relevance of glyphosate residues in food and setting of maximum residue values in food are based on these estimated values.

In GMHT crops tolerant to glyphosate, additional metabolites such as *N*-acetyl-glyphosate and *N*-acetyl-AMPA could be formed, which have recently been introduced into the glyphosate residue definition. For risk assessment, a general residue definition covering both conventional and genetically modified crops has been proposed as the sum of glyphosate, AMPA, *N*-acetyl-glyphosate and *N*-acetyl-AMPA, expressed as glyphosate [42], [43]. No toxicological data has been provided for evaluation to EFSA on *N*-acetyl-glyphosate and *N*-acetyl-AMPA. This has been recognized as a data gap [8].

The number of glyphosate quantifications in the EU in 2016 was below median among the quantified pesticides in organic food, yet 50% of quantifications exceeded the maximum residue limit (MRL) and glyphosate was among the top 10 pesticides most frequently exceeding MRL [54].

1.7.1 Glyphosate resides in raw agricultural commodities and beer

Considering the comprehensive risk assessment covering all uses and residues in imported foods planned to be carried out by the EU [14], in this study the focus is placed on a data gap on occurrence of glyphosate in cereal derived alcoholic beverages [17], [44]. Beer is an important cereal derived alcoholic beverage, therefore, occurrence data for glyphosate in beer produced in Latvia would provide a useful insight. According to an industry report, the consumption of beer in Latvia was estimated at 78.2 L of beer per capita in 2014, approximately a third of the top beer consuming country, therefore beer is an important commodity in Latvia [45].

According to the current EU Regulation (EC) No 396/2005, the maximum residue limits (MRL) set for glyphosate in raw agricultural commodities are 20 mg kg^{-1} in barley, 10 mg kg^{-1} in wheat and rye, and 0.1 mg kg^{-1} in hops, which are the commodities used in preparation of beer. No MRL is set for glyphosate in beer. When there are no relevant authorisations or import tolerances agreed at the EU level, the default MRL of 0.01 mg kg^{-1} may be considered.

Glyphosate as an ionic water-soluble compound is expected to be significantly carried over from cereals to beer during brewing. The carry-over of glyphosate has been estimated at >90% of spiked amount during each stage of brewing [46]. A broad range of other hydrophilic pesticides having log P<0 showed significant carry-over to beer ranging from 20 to 80% [47].

To the best of our knowledge, only one method has been published for the determination of glyphosate in beer with a LOQ of 10 μg kg^{-1} [44]. Considering the insufficient availability of occurrence data, and the lack of a sufficiently sensitive method, a superior analytical procedure for the determination of glyphosate in beer has been developed and the occurrence data has been provided by analysing 100 samples of beer within this study.

1.7.2 Determination of glyphosate in raw agricultural commodities and beer

The determination of glyphosate at low levels in other matrices such as cereals, maize, and soybeans has been reported. Glyphosate has been quantified in cereals at 30 μg kg^{-1} using a combination of reversed phase chromatography and suppressed conductivity ion chromatography with mass spectrometric detection [48]. Detection of glyphosate at 2 μg kg^{-1} level in soybeans has been demonstrated using a novel fluorescent labelling reagent [49]. Direct determination in soybeans and corn using mixed mode analytical column with minimal solid phase extraction (SPE) clean-up provided a method quantification limit of 42 μg kg^{-1} [50]. In another study a LOQ of 30 μg kg^{-1} in rice, 20 μg kg^{-1} in maize, and 400 μg kg^{-1} in soybeans was reported using a similar procedure [51]. A method employing two-step SPE extraction achieved 20 μg kg^{-1} LOQ in soybeans, corn, carrots, apples, and cabbage [52]. Although these results are

not directly comparable to the less concentrated beer matrix, the procedures used to achieve these results clearly illustrate that the determination of glyphosate at low levels is challenging and requires special approaches such as sophisticated columns and complicated sample extraction.

1.7.3 Determination of glyphosate in matrices of animal origin and honey

Due to the cost of laboratory testing, a gap in knowledge on the occurrence in the food chain of polar pesticides and their degradation products, including glyphosate and aminomethylphosphonic acid, formed in the past decade, particularly in products of animal origin, where determination thereof in such matrices presents an analytical challenge [53]. Recently, however, with the re-evaluation of glyphosate for approval in the EU and the increase in monitoring efforts of glyphosate and AMPA, mainly enabled by the recent invention of stationary phases for chromatography, such as porous graphitic carbon and polar bonded phases, the number of analysed samples had increased from 5329 to 9573, during 3 years since 2015 in the EU, while the detection of glyphosate above the limit of quantification ranged from 3.1% to 1.9% [42], [54]–[56]. Due to high rates of occurrence and 3.2% of samples exceeding the maximum residue limit (MRL), it has been recommended in the EFSA report to include glyphosate in monitoring programmes analysing honey, which is a new analytical challenge to be solved [56]. Honeybees are known to prefer drinking from agricultural and urban runoff, therefore pesticides such as glyphosate may occur in honey [57], thus analysis of honey samples can also provide additional information on occurrence of various agricultural residues from the environment if multi-residue analysis or non-targeted analysis methods are employed.

1.8. Sample preparation and instrumental analysis methods for determination of glyphosate and aminomethylphosphonic acid

Due to ionic chemical structure, non-volatility, absence of chromophores, and strong metal complexing properties of glyphosate, determination of it presents significant analytical challenges. As a result, studying glyphosate has become a growing trend in scientific research, especially with focus on monitoring programmes and development of sensitive and reliable laboratory testing methods for determination of glyphosate, its degradation products and other related pesticide residues.

1.8.1. Carbonaceous sorbent-based sample preparation for analysis of glyphosate

A study on the application of carbonaceous sorbent for extract clean-up prior to LC-MS/MS analysis of glyphosate, AMPA, *N*-acetyl AMPA, glufosinate, *N*-acetyl glufosinate, chlormequat, diquat, trimethylsulfonium, maleic hydrazine, mepiquat and paraquat has been reported for several food matrices (onion, wheat, potato and pea). The dSPE method was tested using graphene nanoplatelets (GNPLs), and compared to dSPE clean-up with graphitized carbon black (GCB), C_{18}, Florisil® and Chitosan® [58]. During the clean-up step, 5 mL of the supernatant in the acidic methanol-water solution (1:1, v/v) were transferred to a centrifuge tube containing oxidized GNPLs or the conventional clean-up sorbents (C_{18}, GCB, Florisil®, Chitosan®). We have summarized the average matrix effect across all analytes and matrices in Figure 1.3.

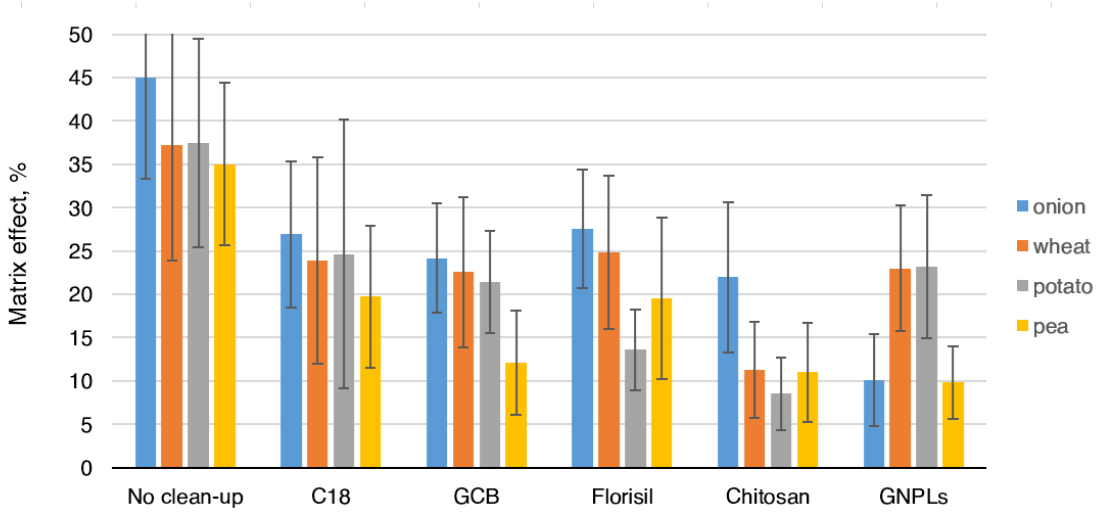

Figure 1.3. **Average matrix effects and the respective 68% confidence intervals calculated from reported validation data across the polar pesticides analysed by Kaczyński [58] for onion, wheat, potato and pea matrices during LC-MS/MS measurement after application of different clean-up sorbents.**

Recoveries of the analytes were in the 64–97% range, indicating that no significant sorption of the analyte occurred from the acidic extracts to the sorbents. The average matrix effect was reduced depending on the sorbent and matrix combination, however, only for some matrix and sorbent combinations the reduction in average matrix effect is significant. Since there is a need to desorb the analytes from complex matrices with strong buffers, acids or bases, application of carbonaceous sorbents for solid phase extraction of polar pesticides would probably be difficult.

1.8.2 Ion chromatography-based analysis of glyphosate and simultaneous analysis of acidic pesticides

Ion chromatography is a relevant approach to the separation of underivatised ionic and acidic pesticides and would offer an alternative chromatographic selectivity to that of reversed phase columns. Determination of ionic pesticides such as glyphosate and its metabolites with non-ion chromatography methods requires derivatisation [59] or the use of sophisticated columns for successful separation [60]. Numerous methods using liquid chromatography or gas chromatography for the determination of either derivatised or non-derivatised glyphosate and its metabolites have been published, mostly improving on the detection capability and resolving matrix effects by modified sample preparation for specific matrices or the use of sophisticated analytical columns or extraction techniques such as online solid phase extraction and liquid-liquid microextraction [61]. Acidic pesticides are also considered a specific case, because those are usually extracted under special conditions using the acidified Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction approach where acetonitrile with 1% formic acid is used to suppress the ionisation of acidic pesticides and to facilitate their transfer to the organic solvent. Low mobile phase pH during chromatographic separation must be used for acidic pesticides to ensure retention on ordinary C_{18} stationary phases [62].

Ion chromatography-based methods have been published where complex buffer suppression technology was used before the introduction of eluate into the ion source of the mass spectrometer [63]–[65]. Dilution of the eluate with organic solvent after suppression increased detection sensitivity by a factor of 3 [63]. The lowest validated limit of quantification was 10 $\mu g\ kg^{-1}$ in food matrices [64] and 5 $\mu g\ L^{-1}$ in water [65]. Analyte recoveries were found to be affected by solvent acidification during the analyte extraction procedure. When methanol acidified with 1% of formic acid was used as per the Quick Polar Pesticides (QuPPe) extraction method [66], the median recovery across analytes was about 64%. The recoveries improved to the median value of 96% if methanol was not acidified [63].

Analyte loss during electrolytic suppression has been observed and compared between commercially available and novel electrolytic suppressors [67]. Analyte loss was attributed to hydrophobic adsorption, precipitation and permeation through the suppressor membranes. With some hydrophobic acids, such as ibuprofen, significant loss and peak broadening were observed during the electrolytic suppression. High levels of organic solvent (60-80 %) were required in the eluent to effectively eliminate the losses, however, long-term operation under such conditions led to the deterioration of membranes [67]. While consistent losses could be taken into account by appropriate calibration or internal standards, it is the matrix from real samples that would inflict major deterioration of suppressor membranes during long-term operation.

A simplified technique for simultaneous determination of ionic pesticides including glyphosate and acidic pesticides not requiring additional equipment other than an ion exchange column and a standard liquid chromatography – mass spectrometry (HPLC-MS/MS) setup would be an alternative approach.

Typical ion exchange eluents are considered incompatible with the electrospray technique, however, carbonate, sulphate, oxalate, and citrate eluents have been successfully applied in ion chromatography with mass spectrometric detection (IC-MS) using the HP 59987A electrospray interface (Hewlett-Packard, USA) and the results have been compared to suppressed IC-MS. Citrate eluent in non-suppressed IC was found to be most suitable because of its high elution strength. The signals were found to be the highest if the concentration of citrate was <0.5 mM. Minor contamination of the electrospray interface was observed. Suppressed IC-MS provided limits of detection that were better by about an order of magnitude [68].

Volatile eluents are preferred for ion chromatography, however, even with a polymeric weak ion exchange stationary phase, the required eluent concentration can be considered excessive for mass spectrometry. A method for direct determination of glyphosate and aminomethylphosphonic acid (AMPA) using the Shodex Asahipak NH2P-50 4E column achieved an LOQ of 5 μg kg^{-1} for glyphosate and 50 μg kg^{-1} for AMPA in fruit and vegetable matrices. The analytes were effectively eluted using a 70 mM ammonia solution in 30% aqueous acetonitrile within 50 min. However, significant signal suppression by 38% for glyphosate and 44% for AMPA was reported, which could be compensated for by prolonged gradients or use of isotope-labelled internal standards [69].

Volatile eluents have been applied in chromatography using porous graphitic carbon (PGC), which has been shown to retain ionic analytes [70], [71]. Also, PGC columns have been coated permanently with ion-pairing reagents to improve retention of anions [72]. Ion-pairing chromatography methods using ordinary C_{18} stationary phases with volatile ion-pairing reagents have been reported for separation of amino acids and other zwitterionic species using perfluorinated carboxylic acids [73]–[75] and for separation of oligonucleotides with anionic character using aliphatic amines [76]. Heptafluorobutyric acid provided significant increases in retention factors over trichloroacetic acid [75]. Whether such ion-pairing reagents can be applied in mass spectrometric determination of ionic and acidic pesticides with low matrix effect needs to be studied further.

Considering the literature, determination of acidic and ionic pesticides with low matrix effects is difficult. Also, to the best of our knowledge, the use of non-volatile buffers in mass spectrometry is not well described in the literature. In this study one of the aims is to evaluate

selected buffers for simultaneous mass spectrometric trace determination of 22 ionic and acidic pesticides without using sophisticated buffer removal equipment. The practical aspects of buffer application such as compatibility with ionisation source, spectral and chromatographic interferences, analyte sensitivity, selectivity, and recovery from spiked matrices are characterised.

1.8.3. Derivatization-based analysis of glyphosate

Glyphosate is often derivatised to reduce its polarity and increase retention on reversed phase sorbents, enabling better separation from matrix components and improving ionisation in the electrospray ionisation source [77]. Glyphosate derivatised with fluorenylmethoxycarbonyl chloride (FMOC-Cl) can also be detected with a fluorescence detector, for example, a method has been reported employing FMOC-Cl derivatisation, reversed phase separation and fluorescence detection, providing 10 $\mu g\ kg^{-1}$ LOQ in processed foods including green tea. However, SPE clean-up was needed for the analysis of sauce and red wine matrices [78]. A method using derivatisation with FMOC-Cl and preconcentration with SPE achieved 0.2 $\mu g\ kg^{-1}$ LOD for the determination of glyphosate in surface waters with high content of organic matter [77]. A recently reported novel method employing *in situ* derivatisation, dispersive liquid-liquid microextraction, and mass spectrometric detection demonstrated 1 $\mu g\ kg^{-1}$ LOQ in irrigation water [79]. The advantage of reversed phase compatible methods based on derivatisation compared to other approaches such as separating underivatised glyphosate on appropriate stationary phases is not obvious. Even with derivatisation there may still be a need for SPE to achieve low LOQ in complex matrices.

Due to the lack of retention with reversed phase chromatography, analysis of underivatized glyphosate, AMPA and glufosinate at the lower $\mu g\ kg^{-1}$ level requires use of stationary phases of alternative selectivity. Analysis of underivatized glyphosate using flow injection mass spectrometry may deliver only low sensitivity in the $mg\ kg^{-1}$ range and low selectivity due to the matrix effects [80], [81]. Although, for achieving high selectivity and sensitivity, alternative, but less common, methods may be used, such as gas chromatography coupled to pulsed flame photometric detector [82] or ion chromatography mass spectrometry – a rather complex setup due to the need for an eluent suppressor device [83], more readily available options mostly include derivatization [59] or some form of ion exchange chromatography [69], [84], all of which present a problem of compatibility with ESI-MS detection, limitations with respect to multi-analyte capability [81], [85] or incompatibility with the chromatographic separation itself [81], as extraction procedures may also require use of additives to the solvent to limit complexing with metal ions and improve recovery [18].

Analysis of underivatized analytes using porous graphitic carbon, although sensitive and selective, may not deliver fully reproducible results due to drifting of retention time and the need for frequent conditioning of the stationary phase to maintain peak shape and retention time [86], [87]. Procedures described in the scientific literature for determination of glyphosate, AMPA and glufosinate by derivatization mostly deal with analysis in water [59], [88]. It is expected that regardless of derivatization agent used, there may be significant problems with derivatization-based approaches that need to be addressed in case of more complex food matrices, such as the possibility of contamination of the analytical columns and mass spectrometers by derivatization agent by-products present in great excess [89] and interferences from matrix in the case of foods of plant and animal origin [90]. Many FMOC-Cl derivatization-based analytical procedures have been validated using online solid phase extraction (online-SPE) systems to perform preconcentration or avoid contamination from derivatization reagent by-products [90]–[92]. Significantly interfered chromatograms were observed for corn matrix after FMOC-Cl derivatization and analysis using online-SPE-LC-MS/MS at the 10 $\mu g\ kg^{-1}$ level [90], and contamination of analytical columns and mass spectrometers has been reported during analysis with ordinary LC-MS/MS, although an LOQ of 120 $\mu g\ kg^{-1}$ was achieved for yam vegetable matrix [93].

Some of these issues may be resolved by performing derivatization with a different reagent, such as dansyl chloride (5-(dimethylamino)naphthalene-1-sulfonyl chloride), and an appropriate choice of solution for extraction and derivatization. Dansyl chloride derivatization of glyphosate and AMPA has been demonstrated before [94], however, the derivatization products have not been characterized with ESI-MS or targeted with LC-MS/MS analysis. It is expected that a procedure for derivatization of amino acids using dansyl chloride demonstrated before [95] should be appropriate for the analysis of trace level glyphosate, AMPA and glufosinate as well.

Therefore, in this study a simple procedure for derivatization of glyphosate, AMPA and glufosinate has been developed and validated for selected matrices, as well as the separation of analytes from the derivatization agent by-products has been demonstrated. Analytical performance of the proposed procedure has been compared to that of the QuPPe procedure [96] using the Waters™ diethylamino-based stationary phase for the determination of anionic pesticides [97], which has been invented recently and is expected to be widely used by analytical laboratories in the future due to the improved chromatographic selectivity to anionic pesticides.

2. EXPERIMENTAL PART

2.1. Chemicals and materials

The solvents used in the different experiments were ultra-pure water prepared by Milli-Q system (MilliporeSigma, MA, USA), HPLC grade methanol and acetonitrile (Merck, Germany) and pesticide grade acetone (J.T. Baker, USA).

The following chemicals were used for adjustments of pH – formic acid (98%), acetic acid (98%), 36% hydrochloric acid, aqueous 25% ammonia solution, sodium carbonate (99.9%) and sodium hydrogen carbonate (99.9%) obtained from Merck (Germany).

The following chemicals were used as part of mobile phases – formic acid (98%), acetic acid (98%), ammonium hydrogen carbonate (99% assay) obtained from Sigma-Aldrich Chemie (Germany), ammonium carbonate (99% assay), triethylammonium bicarbonate (TEAB) buffer solution (1.0 mol L^{-1}), edetic acid (99.4% assay), oxalic acid obtained from Merck (Germany), anhydrous citric acid (99.5% assay) obtained from Penta (Czech Republic) and salicylic acid (99% assay) obtained from Dr. Ehrenstorfer (Germany),

The following chemicals were used as part of solutions for extraction – ethylenediaminetetraacetic acid disodium salt dihydrate with a purity of 99% obtained from Sigma-Aldrich (Germany) and potassium hydroxide (90%) obtained from AG Chemi Group s.r.o. (Czechia).

Dansyl chloride reagent for derivatization (5-(dimethylamino)naphthalene-1-sulfonyl chloride) with a purity of 99% was obtained from Merck (Germany).

The following standards used throughout the study, with purities ranging from 91% to 98% and uncertainties of assay ranging from 2.0 to 3.9%, were – aminomethyl phosphonic acid (AMPA), aminomethyl phosphonic acid-$^{13}C^{15}N$, glyphosate, glyphosate-$^{13}C_2{}^{15}N$ and glyphosate-$^{13}C_2{}^{15}N$ that were obtained from Dr. Ehrenstorfer (Germany), as well as glufosinate and glufosinate-D_3 that were obtained from TRC (Canada). Solutions of these standards were prepared in 10% aqueous acetonitrile and stored in Nalgene™ plastic bottles at 4°C.

For the comparative assessment of different mobile phases for unsuppressed ion chromatography coupled to LC-MS/MS, the following acidic pesticides and their metabolite standards, with purities ranging from 97% to 99.9% and uncertainties of assay ranging from 0.5 to 5%, were used – flonicamid, fludioxonil, haloxyfop, fuberidazole, florasulam, mecoprop (MCPP), 2,4-D, quinmerac, bentazone, clopyralid, fluroxypyr, aminopyralid, ethephon, and glyphosate, which were obtained from Dr. Ehrenstorfer (Germany), and fluazifop-P, flonicamid

metabolite TFNG, flonicamid metabolite TFNA, chlorate, bromate, and glyphosate degradation product AMPA, which were obtained from Sigma-Aldrich (Germany), as well as thiabendazole and MCPA, which were obtained from AccuStandard (CT, USA). Solutions of these standards were prepared in 50% aqueous acetonitrile.

For the comparative assessment of solid phase extraction procedures, the SPE cartridges used were – Strata-X polymeric polar reversed phase (RP), Strata-XC polymeric strong cation exchange (XC) resin, Strata-XA polymeric strong anion exchange (XA) resin, and Strata SAX silica-based strong anion exchange (SAX) medium, obtained from Phenomenex (CA, USA), each with sorbent mass of 500 mg and cartridge volume of 6 mL.

The filters used for filtering of sample extracts and solutions of chemicals throughout the study were – centrifugal filters (0.22 μm pore size, PVDF) and Luer lock syringe filters (0.45 μm pore size, regenerated cellulose). No loss of analytes due to filtering was observed.

2.2. Microorganisms and microbiological testing

A consortium of endophytic bacteria and fungi was used to augment soil samples in laboratory model experiments. The suspension, obtained from oilseed and barley bacterial isolate in the University of Latvia, consisted of 10^8 CFU of endophytic bacteria *Brevibacillus, Enterobacter, Kytococcus, Lactococcus, Micrococcus, Pantoea, Pseudomonas, Serratia, Stenotrophomonas,* and fungi *Cutaneotrichosporon, Mucor, Wickerhamomyces* that are expected to have high degradation activity towards glyphosate. The moisture of soil was maintained at 60% of water holding capacity and the incubation was performed in triplicate for 40 days at 23°C with mixing twice a week.

Toxicity of glyphosate containing herbicides and wastewater samples in laboratory model experiments were tested on *Daphnia magna* (Daphtoxkit F MAGNA, Belgium) and expressed as percentage of immobile individuals or half-maximal effective concentration after incubation with the test portion for 24 hours (24-h EC_{50}).

The number of CFU in wastewater samples was determined by plating decimal dilutions of soil suspension on tryptone glucose yeast extract agar (Sifin, Germany). The plates were incubated at 37°C for 72 h.

2.3. Wastewater experimental treatment setup

Wastewater (WW) samples were collected in May 2016 from the WW influent basin of central WWTP *Daugavgriva* in Riga, Latvia. All samples were collected in clean 1 L amber glass bottles and kept at 4 °C during transportation and then at -20 °C until further usage for experiments. Activated sludge (AS) was sampled from the aeration tank of the same WWTP.

Oxide ceramics were manufactured as reported by Muter et al. [98]. Prior to the experiments the carriers were rinsed with deionised water and autoclaved at atmospheric pressure for 15 min.

The laboratory-scale column model system for WW treatment consisted of 7 columns (340 mm × 75 mm). An Resun® AC-1500 air pump (Shenzhen, People's Republic of China) provided continuous aeration with a mean air flow of 0.80-0.95 L min^{-1}. The columns were filled with one litre filtered WW. 500 g of oxide ceramics were added to column B, while 3 mL of nutrient composition and 700 µL concentrated AS was added to column C. Nutrient composition consisted of diluted sugar beet molasses containing 40% sucrose (final concentration 0.1%), previously autoclaved for 20 min at 1 bar, and 500 µL cabbage leaf extract, prepared according to Le et al. [99] and sterilized by filtering through hydrophilic Minisart® syringe filter (Sartorius AG, Germany). The final concentration of AS in the columns C was 500 mg L^{-1} total suspended solids. After incubating for 48 h with continuous aeration at 23 °C, 100 mg L^{-1} of glyphosate-based herbicide (GBH) Klinik® (Nufarm, Austria) was added to the columns A, B and C. After incubating for 72 h, 50 mL WW of was sampled from each column for testing, and after that 100 mg L^{-1} GBH was added again to the columns A, B and C. The column D was not given any treatment, except aeration. After 72 h incubation, 50 mL WW was sampled from each column. Samples were immediately frozen until testing. The treatments A, B and C were performed in duplicate, treatment D was performed once without replicates.

2.4. Experimental setup for modelling mobility of glyphosate from two contrasting agricultural soils

The average soil samples obtained from three parallel pots after incubation for 40 days were thoroughly mixed and filled into columns with 15 mm diameter and 650 mm height. The columns (two parallel experiments) were each filled with a 60 g soil sample with dry matter content of 90–97%. The height of the layer depended on the soil structure and reached 28–31 cm. Prior to the leaching experiment the columns were slowly pre-saturated by capillary action with a 0.01 M $CaCl_2$ solution. The columns were leached with 50 mL of 0.01 M $CaCl_2$ solution and the eluents were collected in two consistently sampled 25 mL aliquots. The leaching was performed once a day for a total of three times. The aliquots were immediately frozen and stored at-25 °C until testing. The leachates collected from soils with different treatments were labelled by specific combination of letters meaning – "S" for sandy soil; "L" for loamy sand soil; "G" for added 445 mg kg^{-1} of glyphosate from GBH – Klinik® (Nufarm,

Austria) to the soil 40 days prior to the leaching experiment; "M" for soil bioaugmented with microorganisms.

2.5. Instrumental analyses

2.5.1. Parameters of the LC-MS/MS method for laboratory model experiments

Glyphosate and AMPA in soil, soil leachates and wastewater was analyzed using an Acquity UHPLC system (Waters, MA, USA) coupled to a QTrap 5500 (AB SCIEX, MA, USA) tandem mass spectrometer equipped with Turbo V™ electrospray ionisation (ESI) source. The following conditions were used for mass spectrometric analysis: curtain gas pressure 30 psi; nebulizer gas pressure 40 psi; heater gas pressure 60 psi; electrospray voltage -4500 V; source temperature 700°C. Detection was carried out in selected reaction monitoring (SRM) mode with declustering potential of -50 V, collision exit potential of -17 V and collision energy of -20 V. The following selected reactions were monitored for glyphosate: *m*/*z* 168 → 63 (quantifier ion), *m*/*z* 168 → 150 (qualifier ion), *m*/*z* 171 → 63 ($^{13}C_2{}^{15}N$ internal standard ion). The following selected reactions were monitored for AMPA: *m*/*z* 110 → 63 (quantifier ion), *m*/*z* 110 → 79 (qualifier ion), *m*/*z* 112 → 63 ($^{13}C^{15}N$ internal standard ion).

Chromatographic separation was carried out on Hypercarb™ (Thermo Scientific, MA, USA) analytical column (100 × 2.1 mm) containing 3 μm porous graphitic carbon particles. The mobile phase was 1% aqueous acetic acid delivered isocratically at 0.3 mL/min. The injection volume was 10 μL.

2.5.2. Parameters of the LC-MS/MS method for experimental assessment of solid phase extraction and sample preparation procedures and different stationary phases

Glyphosate was analyzed using an Acquity UHPLC system (Waters, MA, USA) coupled to a QTrap 5500 (AB SCIEX, MA, USA) tandem mass spectrometer equipped with Turbo V™ electrospray ionisation source. The following conditions were used for mass spectrometric analysis: curtain gas pressure 30 psi; nebulizer gas pressure 30 psi; heater gas pressure 20 psi; electrospray voltage -4500 V; source temperature 300°C. Detection was carried out in selected reaction monitoring mode with declustering potential of -70 V, collision exit potential of -17 V. The monitored SRM transitions were: *m*/*z* 168 → 63 (quantifier ion); *m*/*z* 168 → 81 (qualifier ion); *m*/*z* 171 → 63 ($^{13}C^{15}N$ internal standard ion).

Several different analytical columns were tested with the appropriate mobile phases and gradient programmes that were expected to deliver sufficient retention and peak quality (Table 2.1).

Table 2.1.

Experimentally assessed analytical columns, mobile phases and gradient programmes.

Analytical column	Mobile phases	Gradient programme
1 – Obelisc® R (SIELC, IL, USA) 5 μm mixed phase (150 × 2.1 mm)	A – 20 mmol L^{-1} ammonium formate adjusted to pH 3, B – acetonitrile	isocratic, 80% A at 0.5 mL min^{-1}
2 – Luna® SCX (Phenomenex, USA) 5 μm cation exchange phase (50 × 4.6 mm)	A – 1% aqueous acetic acid, B – methanol	gradient, 0–5 min 10% to 90% A at 0.3 mL min^{-1}
3 – Hypercarb™ (Thermo Fischer Scientific, USA) 5 μm porous graphitic carbon (100 × 2.1 mm)	1% aqueous acetic acid phase	isocratic at 0.3 mL min^{-1}
4 – Luna® NH_2 (Phenomenex, USA) 3 μm aminopropyl phase (150 × 3 mm)	A – 10 mmol L^{-1} aqueous ammonium hydrogen carbonate adjusted to pH 10 with ammonia, B – acetonitrile	gradient, 0–8 min 20% to 50% A, 8–10.5 min 90% A, 10.5–15 min 20% A at 0.75 mL min^{-1}
5 – Luna® NH_2 (Phenomenex, USA) 3 μm aminopropyl phase (100 × 1 mm)	A – 10 mmol L^{-1} aqueous ammonium hydrogen carbonate adjusted to pH 10 with ammonia, B – acetonitrile	gradient, 0–15 min 20% to 50% A, 15–17 min 90% A, 17–25 min 20% A at 0.11 mL min^{-1}

The HPLC columns were maintained at ambient temperature (20±2 °C); autosampler temperature was set at 8 °C.

The finalized method, which was applied to experimental assessment of solid phase extraction and sample preparation procedures, and analysis of real samples, included chromatographic separation carried out on Luna® NH_2 (Phenomenex, CA, USA) analytical column (100 × 1 mm) containing 3 μm end-capped aminopropyl silica particles. Binary pump provided the gradient for separation at a flow rate of 0.110 mL min^{-1} by mixing acetonitrile with aqueous 10 mmol L^{-1} ammonium hydrogen carbonate solution adjusted to pH 10 with aqueous ammonia. The following gradient program was used (% of aqueous mobile phase given): 0 min – 20%, 15 min – 50%, 15.5 min – 90%, 16.5 min – 90%, 17 min – 20%, 25 min – 20%. The injection volume was 2 μL.

A six-point matrix-matched calibration with stable isotope labelled internal standard normalisation was used in the range from 0.2 μg kg^{-1} to 25 μg kg^{-1} with the internal standard at 25 μg kg^{-1}.

2.5.3. Parameters of the LC-MS/MS for determination of glyphosate and aminomethylphosphonic acid in unsuccessful eggs from wild birds

Glyphosate and AMPA in unsuccessful eggs from wild birds was analyzed using an Acquity UHPLC system (Waters, MA, USA) coupled to a QTrap 5500 (AB SCIEX, MA, USA) tandem mass spectrometer equipped with Turbo V™ electrospray ionisation (ESI) source. The following conditions were used for mass spectrometric analysis: curtain gas pressure 30 psi; nebulizer gas pressure 40 psi; heater gas pressure 60 psi; electrospray voltage -4500 V; source temperature 700°C. Detection was carried out in selected reaction monitoring (SRM) mode with declustering potential of -50 V, collision exit potential of -17 V and collision energy of -20 V. The following selected reactions were monitored for glyphosate: *m*/*z* 168 → 63 (quantifier ion), *m*/*z* 168 → 150 (qualifier ion), *m*/*z* 171 → 63 ($^{13}C_2{}^{15}N$ internal standard ion). The following selected reactions were monitored for AMPA: *m*/*z* 110 → 63 (quantifier ion), *m*/*z* 110 → 79 (qualifier ion), *m*/*z* 112 → 63 ($^{13}C^{15}N$ internal standard ion).

Chromatographic separation was carried out on Hypercarb™ (Thermo Scientific, MA, USA) analytical column (100 × 2.1 mm) containing 3 μm porous graphitic carbon particles. The mobile phase was 1% aqueous acetic acid delivered isocratically at 0.3 mL/min. The injection volume was 10 μL.

Under these conditions glyphosate eluted in 2.2 min and AMPA eluted in 0.75 min during a 10 min run. A one-point calibration, containing 10 μg L^{-1} of each analyte and 20 μg L^{-1} of the respective internal standards, was used for screening of the prepared samples. The ratio of response factors for the analytes and the respective internal standards was determined and taken into account together with the instrumental LOQ in order to estimate the LOQ in each individual sample.

2.5.4. Parameters of the method for experimental assessment of different mobile phases for unsuppressed ion chromatography coupled to LC-MS/MS and assessment of two different electrospray ionisation sources

Pesticides were analysed using an Acquity UHPLC system (Waters, MA, USA) coupled to a QTRAP 5500 (AB SCIEX, MA, USA) tandem mass spectrometer operated in SRM mode with a Turbo V™ ionisation source in ESI mode.

Chromatographic separation was carried out on a Metrosep® (Metrohm, Switzerland) A Supp 5 anion exchange column (150 × 4 mm) with 5 μm polyvinyl alcohol particles containing quaternary ammonium groups.

For qualitative comparison of electrospray ionisation sources with respect to compatibility with unsuppressed ion chromatography using the edetate buffer, an Ion Max API

source housing with HESI-II probe in ESI mode coupled to an Orbitrap™ mass spectrometer with UltiMate 3000 UHPLC system (Thermo Scientific, MA, USA) was also used.

The anion exchange column was maintained at ambient temperature (20±2°C); the autosampler temperature was set at 10°C. The injection volume was 10 μL.

The following ionisation parameters were applied on the Turbo V™ ionisation source: curtain gas pressure 50 psi; nebuliser gas pressure 40 psi; heater gas pressure 40 psi; source temperature 550°C; source voltage -3500 V; declustering potential -20 V. The micrometer for vertical adjustment of probe was set to 5 mm. A survey of the fragmentation of analytes and the optimisation of collision energies of selected transitions was carried out by delivering eluents through the analytical column to the mass spectrometer at the analytical conditions with a post-column infusion of the standard solutions. Two transitions with the lowest background noise were selected and optimised for each analyte. The monitored SRM transitions together with the chemical structures and properties of analytes are given in Annex 1.

The following ionisation parameters were set for the Ion Max source: sheath gas pressure 75 psi; auxiliary gas flow 20 units; vaporizer temperature 500 °C; ion transfer tube temperature 400 °C; source voltage -3500 V.

Spectral interferences from the system were studied by delivering eluents through the analytical column to the mass spectrometer at the analytical conditions and by scanning the precursor ion mass spectrum from *m/z* 50 to 300.

The appropriate concentrations of buffers and chromatographic gradient programmes were found experimentally in order to elute all analytes within 30 min and to ensure that the peaks are covered by a sufficient number of data points. Re-equilibration of initial conditions was ensured in all cases (Table 2.2). Product ions with the lowest background noise were chosen for each analyte (Annex 1).

Chromatography parameters and mobile phases are given in Table 2.2, with parameters relevant to the detection periods only, given that sufficient re-equilibration of initial conditions was ensured in all cases. Mobile phases were pH adjusted using an InoLab 730 pH meter with a SenTix 42 pH electrode (WTW, Germany). Before dilution with organic solvent, all buffers used in this study were adjusted to pH 10.0.

For the preparation of mobile phases A-F, the appropriate amount of citric or edetic acid was dissolved rapidly in a small volume of 100 mmol L^{-1} aqueous ammonium carbonate (pH 10.0). The solutions were diluted with deionised water, adjusted to pH 10.0 with ammonia and diluted with deionised water and acetonitrile to the appropriate concentrations. Citrate and edetate were an order of magnitude stronger eluents than carbonate, therefore the effect of ammonium carbonate on retention was negligible. For the preparation of mobile phases I-K,

triethylammonium bicarbonate (TEAB) buffer (1.0 mol L^{-1}) was diluted with deionised water, adjusted to pH 10.0 with triethylamine, and diluted with deionised water and acetonitrile to the appropriate concentrations. Mobile phase H was prepared by mixing equal volumes of A and I and diluting the mixture with an equal volume of mobile phase G.

Table 2.2.

The experimentally assessed mobile phases for unsuppressed ion chromatography coupled to LC-MS/MS and the respective gradient programmes.

Procedure name	Mobile phases	Parameters
0.6 mM citrate	A – 0.6 mmol L^{-1} citric acid and 1.25 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile	isocratic, 25 min at 0.9 mL min^{-1}
0.3 mM citrate	B – 0.3 mmol L^{-1} citric acid and 1.25 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile	isocratic, 25 min at 0.9 mL min^{-1}
0.25-0.75 mM citrate	C – 0.25 mmol L^{-1} citric acid and 1.25 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile D – 0.75 mmol L^{-1} citric acid and 1.25 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile	gradient, 0-15 min 100 % C to 100 % D, 15-17.5 min 100 % D at 0.5 mL min^{-1}
0.25-0.75 mM edetate	E – 0.25 mmol L^{-1} edetic acid and 1.25 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile F – 0.75 mmol L^{-1} edetic acid and 1.25 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile	gradient, 0-15 min 100 % E to 100 % F, 15-17.5 min 100 % F at 0.5 mL min^{-1}
12.5 mM TEAB + 0.15 mM citrate	G – 50 % aqueous acetonitrile H – 12.5 mmol L^{-1} triethylammonium bicarbonate (TEAB) and 0.15 mmol L^{-1} citric acid and 0.63 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile	gradient, 0-5 min 90 % G, 5-10 min 10 % to 100 % H, 10-15 min 100 %H, 15-22 min 100 % to 10 % H at 0.9 mL min^{-1}
50 mM TEAB	I – 50 mmol L^{-1} triethylammonium bicarbonate in 50 % aqueous acetonitrile	isocratic, 10 min at 0.5 mL min^{-1}
12.5-50 mM TEAB	J – 12.5 mmol L-1 triethylammonium bicarbonate in 50 % aqueous acetonitrile K – 50 mmol L-1 triethylammonium bicarbonate in 50 % aqueous acetonitrile	gradient, 0-12.5 min 100 % J, 12.5-27.5 min 0 to 100 % K, 27.5-32.5 min 100 % K at 0.5 mL min^{-1}

A five-point solvent-based calibration was used in the range from 2.5 μg kg^{-1} to 50 μg kg^{-1}, corresponding to a range from 10 μg kg^{-1} to 200 μg kg^{-1} in the spiked matrix extracts. Solvent-based calibration solution was prepared by adding 5 μL of the standard solution for the respective spiking level to 1000 μL of 50% aqueous acetonitrile. The analyte peaks were confirmed by the presence of a peak with overlapping retention time in the

chromatogram of a different product ion. Peaks with a signal-to-noise ratio (S/N) ≥10 were quantified. The evaluation of LOD was based on S/N≥3. LOQ was defined for each analyte as the lowest spiked level with recovery within the range from 80% to 120%. Quantification was performed using 1/*x* weighted linear regression model. For estimating the mean recovery and measurement uncertainty, a total of 5 procedural replicates were analysed over two different days. A 25 μg L^{-1} standard solution was analysed on both days throughout the sample sets to monitor the stability of response. The measurement uncertainty was used as the control limit.

Beer and oat flour were selected as two types of cereal-derived products that were expected to show significant matrix effects. Samples were obtained from the local markets and stored refrigerated at 5°C in darkness until the analysis. The materials were analysed in-house prior to the present study and were not found to contain any residues of the pesticides concerned in this study.

2.5.5. Parameters of a novel and reliable derivatization-based LC-MS/MS method for determination of glyphosate, aminomethylphosphonic acid and glufosinate in foods of plant and animal origin

The dansyl chloride derivatized glyphosate, AMPA and glufosinate were analyzed using an UltiMate 3000 UHPLC system (Thermo Scientific, Waltham, MA USA) coupled to Thermo Scientific Quantis™ triple quadrupole mass spectrometer and Thermo Scientific Q Exactive Orbitrap™ mass spectrometer with heated electrospray ionisation probes.

Chromatographic separation was carried out on a Luna® (Phenomenex, Torrance, CA, USA) column (150 × 2 mm) with 3 μm C_{18} bonded silica particles. The analytical column was thermostated at 40°C; the autosampler was thermostated at 8°C; injection volume was 40 μL. The mobile phases were 0.1% formic acid in ultra-pure water (A) and 0.1% formic acid in acetonitrile (B). The gradient was: 0–14 min 15% B–25% B; 14–22 min 97.5% B; 22–30 min 15% B. The flow rate was set to 0.2 mL/min during 0–18 min and 0.35 mL/min during 18–30 min.

The following parameters were used with the ionisation sources of both mass spectrometers: sheath gas 35 units; aux gas 7 units; sweep gas 0 units; probe heater temperature 275°C; ion transfer capillary temperature 300°C; spray voltage at least ±3 kV. The adjustable positions of the probes in the ionisation sources were set to medium values. A diverter valve was used with both mass spectrometers and was programmed to allow flow to the mass spectrometer only during 8 to 18 min.

With the triple quadrupole mass spectrometer, at least 2 transitions for each analyte were monitored across 3 elution windows in both polarities. The minimum dwell times ranged from 165 to 248 ms per transition.

With the Orbitrap™ mass spectrometer precursor ions were isolated by an isolation window of 0.4 m/z at the respective elution windows in the parallel reaction monitoring mode in negative polarity and fragmented at the average optimum collision energy of all identified fragments of the respective precursor ion. Fragments were detected simultaneously at resolution of 140 000. The AGC feature was not used and the ion injection time was set to 1000 ms. With both mass spectrometers at least 10 scans per peak were obtained. Detection parameters are given in Annex 3.

Table 2.3.

Mass spectrometric detection parameters for determination of dansyl chloride derivatized glyphosate, glyphosate-$^{13}C_2{}^{15}N$, AMPA, AMPA-$^{13}C^{15}N$, glufosinate and glufosinate-D_3.

Detector	Analyte	Polarity	Precursor m/z	CE (V)
Thermo Scientific Q Exactive Orbitrap™	Dansyl AMPA	Negative	343.0523	15
	DansylAMPA-$^{13}C^{15}N$	Negative	345.0527	15
	Dansyl glyphosate	Negative	401.0578	27
	Dansyl glyphosate-$^{13}C_2{}^{15}N$	Negative	404.0615	27
	Dansyl glufosinate	Negative	413.0942	20
	Dansyl glufosinate-D_3	Negative	416.1130	20

Performance of the proposed analytical procedure was compared for all matrices to analysis of underivatized analytes using a 100 × 2.1 mm, 5 μm particle column containing the diethylamino stationary phase with proprietary endcapping from Waters™, Milford, MA, USA (Waters™ anionic polar pesticide column) after extraction with the Quick Polar Pesticides (QuPPe) procedure. The mobile phases were 1.2% formic acid in ultra-pure water (A) and 0.5% formic acid in acetonitrile (B). The gradient was: 0–1.25 min 90% B; 1.25–3.75 min 90% B–20% B; 3.75–11.25 min 20% B–10% B; 11.25–45 min 10% B; 45–60 min 90% B. The flow rate was set to 0.2 mL/min during 0–45 min while the diverter valve allowed flow to the mass spectrometer and 0.5 mL/min during 45–60 min while the diverter valve diverted to waste. Detection was carried out with the triple quadrupole mass spectrometer. At least 10 scans per peak were obtained. Detection parameters are given in Annex 4.

Table 2.4.

Mass spectrometric detection parameters for determination of dansyl chloride derivatized glyphosate, glyphosate-$^{13}C_2{}^{15}N$, AMPA, AMPA-$^{13}C^{15}N$, glufosinate and glufosinate-D_3.

Detector	Analyte	Polarity	Precursor m/z	Fragment m/z	CE (V)
Thermo Scientific Quantis™	Dansyl AMPA	Negative	343.1	63	23
		Positive	345.0	252	15
	Dansyl AMPA-$^{13}C^{15}N$	Negative	345.1	63	23
		Positive	347.1	252	20
	Dansyl glyphosate	Negative	401.0	148	22
		Positive	403.1	257	15
	Dansyl glyphosate-$^{13}C_2{}^{15}N$	Negative	404.1	124	20
		Positive	406.1	260	10
	Dansyl glufosinate	Negative	413.1	134	20
		Positive	415.1	164	20
	Dansyl glufosinate-D_3	Negative	416.1	137	20
		Positive	418.1	167	20

A five-point standard addition calibration was performed in the range from 10 μg kg^{-1} to 250 μg kg^{-1} on each matrix and reagent blanks. Isotopically labelled internal standards (ILIS) were added at 50 μg kg^{-1} prior to extraction.

Honey, milk, cucumber, milk porridge baby formula, bovine liver and kidney were selected as examples of challenging matrices due to content of salts and amino acids or proteins, which may interfere with the derivatization of analytes. Samples were obtained from the local market and were stored at 5°C in darkness until the analysis.

2.6. Sample preparation procedures

2.6.1. Sample preparation for analysis of soil from laboratory model experiment

3.00 g portions of each received sample were extracted with 30 mL of 0.6 mol L^{-1} potassium hydroxide solution for 2 h, using a rotary shaker. Soil samples spiked at 100 μg kg^{-1} were analyzed for evaluation of uncertainty. The extracts were centrifuged for 1 h at 4500 rpm and diluted in two steps with deionised water in order to obtain results that fit into the calibration range from 10 to 100 μg L^{-1}. Stable isotope labelled internal standards for glyphosate and AMPA were added to the final dilution to a concentration of 10 μg L^{-1}. The moisture content was determined for 3.00 g of sample using analytical balance and oven drying at 105 °C for 24 h. The processed samples were analyzed using the LC-MS/MS method described in 2.5.1.

2.6.2. Sample preparation for analysis of wastewater from laboratory model experiments

The wastewater aliquots were centrifuged for 1 h at 4500 rpm and diluted with deionised water after addition of the stable isotope labelled internal standards for glyphosate and AMPA to a concentration of 10 μg L^{-1}. The wastewater samples were diluted in order to obtain results that fit into the calibration range from 10 to 100 μg L^{-1}. The processed samples were analyzed using the LC-MS/MS method described in 2.5.1.

2.6.3. Sample preparation for experimental assessment of different mobile phases for unsuppressed ion chromatography coupled to LC-MS/MS

Beer and oat flour were selected as two types of cereal-derived products that were expected to show significant matrix effects. Samples were obtained from the local markets and stored refrigerated at 5°C in darkness until the analysis. The materials were analysed in-house prior to the present study and were not found to contain any residues of the pesticides concerned in this study. It was found that the acidity of the extracts influenced the retention times and peak shapes if the QuPPe extracts (extracted with 1% formic acid in methanol) were analysed with <1 mmol L^{-1} mobile phases. Poor recoveries have been reported for acidified QuPPe extracts [63], but in this study only peak shape distortions and retention time shifts due to lack of buffering capacity were observed. No retention time shifts or peak shape distortions were observed with the 12.5-50 mM TEAB procedure (Table 2.2) when analysing QuPPe extracts. To avoid these problems and to prevent the need for further dilution, which would require large sample loop injections to maintain signal, extraction was carried out with a basic buffer similar in strength to the mobile phases.

The present study focuses on matrix effects originating within the ionisation source and the analytical column, therefore, spiking was performed after the extraction. A 5.00 g sample of each matrix was weighed and 15 mL of 20 mmol L^{-1} ammonium carbonate in 50 % aqueous acetonitrile was added to beer, while 20 mL of the same ammonium carbonate solution was added to oat flour. The mixtures were shaken thoroughly for 15 min and centrifuged for 30 min at 4500 rpm and 4°C. The final dilution factor for both matrices was 4. The supernatant was filtered and aliquots of filtrate were spiked with the standard solutions prepared separately for each corresponding spiking level. A 5 μL portion of the respective standard solution was added to 1000 μL of the extract.

2.6.4. Sample preparation for determination of glyphosate, aminomethylphosphonic acid and glufosinate with the novel derivatization-based LC-MS/MS method

Honey, milk, cucumber, milk porridge baby formula, bovine liver and kidney were selected as examples of challenging matrices due to content of salts and amino acids or proteins, which may interfere with the derivatization of analytes. Samples were obtained from the local market and were stored at 5°C in darkness until the analysis.

1.00 g portions of each matrix in 15 mL polypropylene tubes were spiked to 50 μg kg^{-1} with 20 μL of a solution containing all isotopically labelled internal standards (ILIS) and spiked, depending on the calibration level, with up to 50 μL of a solution containing all analytes. In addition, reagent blanks, samples containing only ILIS and samples not containing any standard additions were processed for each matrix. 2 mL of ultra-pure water and 3 mL of saturated aqueous ethylenediaminetetraacetic acid disodium salt dihydrate solution (approx. 10%) were added and the mixtures were shaken for 10 min and centrifuged at 4500 rpm. 500 μL of supernatants were filtered through centrifugal filters (0.22 μm pore size, PVDF) and 180 μL of filtrates were transferred to 2 mL screw cap glass vials. 20 μL of saturated sodium carbonate solution and 240 μL of dansyl chloride solution (1.5% mass concentration) freshly prepared in 40% acetone in acetonitrile was added to the vials. The vials were screw capped, shaken and stored at 40°C for 30 min. 1300 μL of 1.5% formic acid in ultra-pure water were added to each vial and shaken. The mixtures were filtered, transferred to vials and 40 μL were injected for analysis of derivatized analytes using Luna® C_{18} column (Phenomenex, Torrance, CA, USA) and triple quadrupole or Orbitrap™ mass spectrometer. The processed samples were analyzed using the LC-MS/MS method described in 2.5.5.

2.6.5. Sample preparation for determination of glyphosate, aminomethylphosphonic acid and glufosinate with the QuPPe (Quick Polar Pesticides) procedure for comparison of method performance

1.00 g portions of each matrix in 15 mL polypropylene tubes were spiked according to the aforementioned procedure. In addition, reagent blanks, samples containing only ILIS and samples not containing any standard additions were processed for each matrix. 4 mL of 50% methanol containing 1.5% formic acid and 100 μL of saturated aqueous ethylenediaminetetraacetic acid disodium salt dihydrate solution (approx. 10%) were added and the mixtures were shaken for 10 min and centrifuged at 4500 rpm. 500 μL of supernatants were filtered through centrifugal filters (0.22 μm pore size, PVDF) and transferred to vials, and 10 μL were injected for the analysis of underivatized analytes using the Waters™ anionic polar

pesticide column and triple quadrupole mass spectrometer. The processed samples were analyzed using the LC-MS/MS method described in 2.5.5, in combination with the Waters™ anionic pesticide column.

2.6.6. Sample preparation for analysis of beer samples

A total of 100 different bottled and canned beer samples were purchased from local supermarkets. The samples were stored refrigerated at 5 °C in darkness until analysis. The samples taken for analysis were shaken, filled into polypropylene tubes and degassed by sonication in ultrasonic bath and centrifuged for 15 min at 4500 rpm. A 10.0 g portion of each sample was weighed and 10 μL of glyphosate internal standard solution was added to a concentration of 25 μg kg^{-1}. The glyphosate standard solutions to be added to the corresponding level of calibration and quality control (QC) samples were prepared separately for each calibration level and 10 μL of the respective solution were added to the weighed sample. The Strata-X SPE cartridges were conditioned with 3 mL of methanol and 6 mL of ultra-pure water. Each sample (1 mL) followed by 4 mL of ultra-pure water was slowly passed through the cartridge at approximately 1 mL min^{-1} and the eluent was collected. The remainder of the liquid was collected from the cartridges with suction. The Strata-XA cartridges were conditioned with 3 mL of methanol and 6 mL of ultra-pure water. The previously purified extracts were quantitatively transferred to the conditioned Strata-XA cartridges and slowly passed through them. The cartridges were rinsed with 1 mL of ultra-pure water and 5 mL of methanol. Glyphosate was eluted from the Strata-XA cartridges with 5 mL of methanolic 10 mmol L^{-1} HCl solution and the eluted solutions were evaporated to dryness at 50 °C under purified air stream. The samples were reconstituted in 200 μL of ultra-pure water. The processed samples were analyzed using the finalized LC-MS/MS method described in 2.5.2.

2.6.7. Sample preparation for analysis of unsuccessful eggs from wild birds

1.00 g portion of each sample was spiked with 10 μL of the internal standard for glyphosate and AMPA to 10 μg kg^{-1} in 15 mL polypropylene tubes, and 5 mL of 11.7 mol L^{-1} methanolic hydrochloric acid were added. The mixtures were shaken for 10 min in rotating shaker and centrifuged for 10 min at 4500 rpm. The supernatant liquid was poured into a clean 15 mL tube and evaporated to dryness at 60 °C under a stream of nitrogen. The dry residues were reconstituted in 3 mL of deionised water and 50 μL of saturated sodium hydrogencarbonate. The tubes were vortexed for 15 min. The reconstituted residues were passed slowly through Strata-X SPE cartridges and collected in 15 mL tubes. The collected purified extracts were then passed slowly through Strata-XA SPE cartridges to waste. The

Strata-XA cartridges were washed with 5 mL of methanol and dried under vacuum. The Strata-XA cartridges were eluted with 3 mL of 1.2 mol L^{-1} methanolic hydrochloric acid, and the eluate was evaporated to dryness under a stream of nitrogen at 60 °C. The dry residues were reconstituted in 200 µL of deionised water. All SPE cartridges were previously conditioned by passing 3 mL of methanol and 6 mL of deionised water through the cartridges. A total of 57 unsuccessful eggs from wild birds were analyzed. The processed samples were analyzed using the LC-MS/MS method described in 2.5.3.

2.7. Calculations

Matrix effect and extraction efficiency are terms often confused with apparent recovery. The distinction should be made between apparent recovery values that show the amount of analyte recovered during analysis with respect to a calibration set (usually procedurally and matrix-wise matched), and recovery values that represent the actual extraction efficiency, i.e. extraction recovery. The apparent recovery may also show the matrix effect, if the calibration set consists of injections that contain only the analytes and no matrix. In this study, the apparent recovery will be referred to as recovery.

The matrix effects were evaluated by comparing analyte peak areas obtained during analysis under the same conditions (and at the same on-column analyte mass) of a sample extract (preferably not containing the analyte) that has been spiked post-extraction and a solvent composition containing only the analyte.

Extraction efficiency or extraction recovery was evaluated by comparing analyte peak areas obtained during analysis under the same conditions (and at the same per-sample analyte mass) of a sample extract (preferably not containing the analyte) that has been spiked post-extraction and the same sample that has been spiked pre-extraction. In such case it is assumed that matrix effects are compensated, and the ratio of peak areas refers to the extraction efficiency.

The matrix effect for each analyte was estimated according to Equation 1:

$$ME = |\, 100\,\% \cdot A_{matrix}/A_{solvent} - 100\,\% \,| \qquad (1)$$

where $A_{matrix}/A_{solvent}$ is the ratio of analyte response from spiked matrix to the analyte response from standard in solvent. A situation free of matrix effect is defined as having the *ME* value equal to 0%.

The extraction efficiency was estimated according to Equation 2:

$$EEF = 100\ \% \cdot A_{spiked\ sample} / A_{spiked\ aliquot} \qquad (2)$$

where $A_{spiked\ sample}/A_{spiked\ aliquot}$ is the ratio of analyte response from a blank matrix spiked prior to the extraction to the analyte response from a spiked extract aliquot from a sample not containing the analyte.

In the development of a novel and reliable dansyl chloride derivatization-based LC-MS/MS method for determination of glyphosate, aminomethylphosphonic acid and glufosinate in complex food matrices, the matrix effect for underivatized analytes was estimated according to Equation 3:

$$ME_{underivatized} = 100\ \% \cdot (A_{matrix} / A_{solvent} - 1) \qquad (3)$$

where $A_{matrix}/A_{solvent}$ is the ratio of analyte response from spiked blank matrix to the analyte response from standard in solvent.

The matrix effect originating in the LC-MS stage for derivatized analytes was estimated according to Equation 4:

$$ME_{derivatized} = 100\ \% \cdot \left(A_{M+(RB+A)} / A_{RB+(RB+A)} - 1\right) \qquad (4)$$

where $A_{M+(RB+A)}/A_{RB+(RB+A)}$ is the ratio of analyte response from a mixture in equal ratio of a processed blank matrix and reagent blank containing the analytes to the analyte response from a mixture of a processed reagent blank not containing the analytes and a reagent blank containing the analytes. *ME* value equal to 0% is defined as no observable matrix effect.

Pooled standard deviations were calculated according to Equation 5:

$$s_{pooled} = \sqrt{\frac{(n_1-1)s_1^2+(n_2-1)s_2^2+\cdots+(n_i-1)s_i^2}{n_1+n_2+\cdots+n_i-i}} \qquad (5)$$

where n_i is the number of measurements within the group, s_i is the standard deviation within the group to be pooled and i is the number of groups.

The measurement uncertainty at 95% CI was estimated according to Equation 6:

$$U = k \cdot \sqrt{RSD_{WR}^2 + RMS_{bias}^2 + {u_{C_{ref}}}^2 + {bias_{carry-over}}^2} \quad (6)$$

where k is the coverage factor, RSD_{WR} is the within-laboratory reproducibility and RMS_{bias} is the root-mean-squared bias, u_{Cref} is the relative uncertainty of the certified value for the analyte standard, $bias_{carry\text{-}over}$ is the absolute estimated carry-over after the acquisition of the highest calibration level divided by the nominal concentration of the level for which the uncertainty is estimated. The $bias_{carry\text{-}over}$ was estimated whenever carry-over effect could be detected.

In case of sensitivity estimates, the measurement uncertainty was estimated within two standard deviations from the mean value. In case of sums of matrix effects, the worst estimated relative uncertainty of the mean recovery was assigned.

The sensitivity of the analytical procedure towards the analytes was estimated as the S/N ratio calculated during processing of chromatograms with AB SCIEX Analyst 1.6 software. All other calculations were performed with Microsoft Excel.

Chromatograms from AB SCIEX mass spectrometers were processed using the Analyst 1.6 software (AB SCIEX, USA) and all other calculations were performed in Microsoft Excel. Chromatograms from Thermo Scientific mass spectrometers were processed using the Qual Browser Thermo Xcalibur 4.1 software (Thermo Scientific, MA, USA). Chromatograms from Thermo Scientific UV detector were processed using the Chromeleon 7.2.6 software (Thermo Scientific, MA, USA).

Mann-Whitney U tests were carried out with MYSTAT statistics software (Systat Software, USA) to evaluate the significance of the difference between the results of two groups. For statistical analysis, sample results below the LOD were assigned a value of zero, and the results above the LOD were unchanged. Regression analysis was carried out with the Minitab statistics software (Minitab, Inc., USA). The findings of $p<0.01$ were interpreted as a strong evidence against the null hypothesis.

Unpaired *t*-tests were carried out with the MYSTAT software (Systat Software, San Jose, CA, USA) to evaluate the difference between the results of two groups. The findings of $p<0.05$ were interpreted as a strong evidence against the null hypothesis.

3. RESULTS AND DISCUSSION

Experiments were performed in three main directions, in which capability of detection and quantification of glyphosate and AMPA could be potentially improved – unsuppressed ion chromatography, multi-stage solid phase extraction-based sample preparation, and derivatisation-based sample preparation.

Unsuppressed ion chromatography coupled to electrospray ionisation mass spectrometry was investigated by comparing sensitivity and matrix effects while using volatile triethylammonium bicarbonate buffer or non-volatile carboxylic acid eluents, furthermore, the applicability of an unsuppressed ion chromatography procedure was investigated with respect to compatibility with particular ionisation sources. Two differently designed ionisation sources were qualitatively compared with respect to applicability and stability of the method.

Multi-stage solid phase extraction-based sample preparations were investigated with respect to sensitivity gains compared to dilution-based sample preparation while combining different solid phase extraction sorbents – polymeric reversed phase, silica-based anion exchanger, polymeric anion exchanger, polymeric cation exchanger, as well as varying the preconcentration factor. The elaborated multi-stage SPE method was validated and applied for analysis of beer samples. Next, the extraction step was optimised by applying methanolic hydrochloric acid for analysis of egg samples. Different analytical columns were also compared at different stages of the procedure. The variability of the limit of quantification was also investigated after analysis of a large number of unsuccessful eggs from wild birds.

A novel and reliable dansyl chloride-based derivatization sample preparation method was developed and validated for several matrices – honey, milk, cucumber, porridge formula, bovine liver, bovine kidney. These matrices can be considered challenging due to their high content of salts, proteins and carbohydrates that could interfere with the analysis. The developed procedure was compared with the QuPPe procedure in combination with the recently invented diethylamino stationary phase from Waters™, in order to ascertain the extent of matrix effects and possible advantages of the novel method.

An optimised porous graphitic carbon-based LC-MS/MS method was applied for accurate determination of glyphosate and AMPA in soil and wastewater, which were in this instance considerably less challenging matrices, due to higher concentrations of the analytes present from laboratory model experiments on the soil and wastewater.

3.1. Experimental assessment of solid phase extraction and sample preparation procedures and different stationary phases

Different SPE stationary phases from Phenomenex® were tested to maximise the analytical method sensitivity for glyphosate: RP, XC, XA, SAX, and some combinations thereof, as well as protein precipitation (PPT) with 10 mM HCl in methanol (PPT) at different ratios and the QuPPe method (instead of SPE extraction the spiked 10.0 g samples were diluted with 10 mL of 1% formic acid in methanol). The comparison was based on monitoring the normalised glyphosate signal, defined here as the ratio of response factors: peak area per μg kg^{-1} in blank spiked prior to the extraction to the peak area per μg kg^{-1} in standard solution (deionised water). The concentration of solutions for elution of glyphosate retained on SPE cartridges was optimised by preparing sequential ten-fold dilutions and selecting the weakest dilution that completely eluted glyphosate. For this purpose, fractions from the SPE extraction steps were monitored for the presence of glyphosate. The results of the signal comparison for different sample preparations is shown in Figure 3.1. The aim of this comparison is to identify the sample preparations that provided the highest sensitivity. In addition to the selectivity, also the selection of SPE cartridges or solvents used for the extraction, as well as the dilution factor, here defined as the ratio of sample volume after reconstitution to the volume taken for the extraction, are important parameters to be optimised, because they strongly affect the sensitivity and matrix effects. Under the conditions of d=1, the strongest signal was obtained by the RP-XA method, therefore preconcentration was attempted to further increase the sensitivity. Excessive preconcentration (d=0.067 and d=0.033) resulted in the lowest observed signals due to signal suppression. The strongest signal was observed after purification with RP, followed by preconcentration with XA when d=0.2 (Figure 3.1). The estimated matrix effect of the final SPE procedure was -66%. The matrix effect was compensated with matrix-matched calibration and the use of a stable isotope labelled internal standard.

Five different columns and appropriate mobile phases according to Table 2.1. were tested in order to optimise the chromatographic conditions at the same mass spectrometric parameters as in the final method. All of the tested columns retained glyphosate with the respective retention factors *k*=6.5 (Column 1), 4.0 (Column 2), 0.7 (Column 3), 3.8 (Column 4), and 3.9 (Column 5). Under these conditions the peak shapes obtained from standard solutions on Obelisc® R and Luna® SCX columns were extremely broadened. Hypercarb™ and Luna® NH_2 columns produced tailing peaks. The selectivity obtained with spiked samples prepared using the final SPE procedure was tested on Hypercarb™ and Luna® NH_2 columns. The Hypercarb™ column produced distorted flat top peaks, possibly due to the insufficient

retention factor. The Luna® NH_2 (100 × 1 mm) column was chosen for the final method based on the satisfactory peak shape and retention factor, as well as the low solvent consumption.

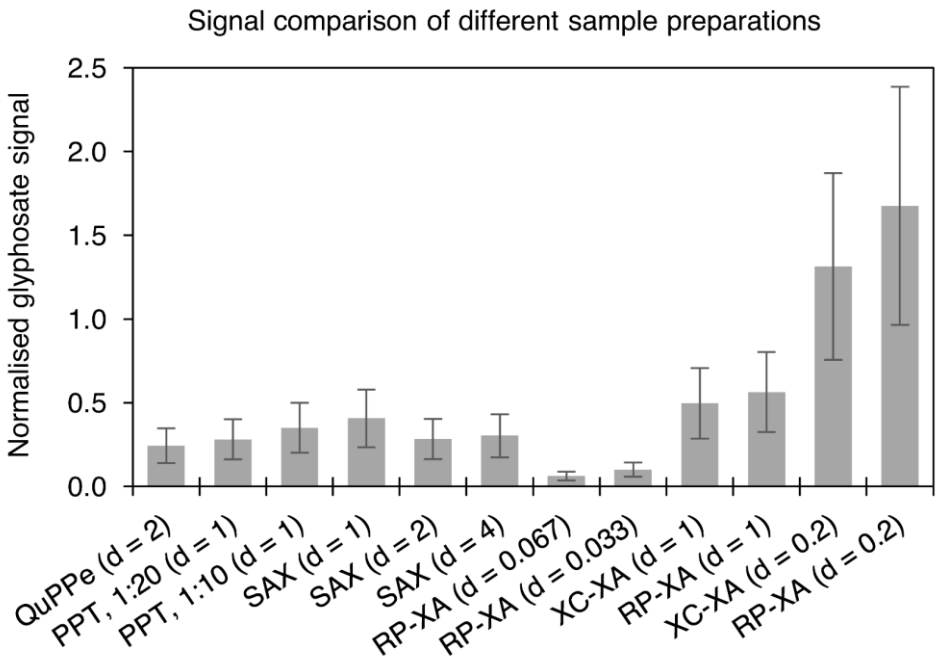

Figure 3.1. **Signal comparison of different sample preparations, where ' d ' represents the dilution factor, defined as the volume of sample after reconstitution to the volume of sample taken for extraction.**

The gradient rate is an important parameter that can affect resolution. A beer sample spiked at 10 μg kg^{-1}, prepared using the final SPE procedure, was analysed at different gradient rates: 0.1% min^{-1}, 0.5% min^{-1}, 1% min^{-1}, 2% min^{-1}, 3% min^{-1}, 3.75% min^{-1}, and 7% min^{-1}. The S/N ratio of the quantitative ion peak reached maximum and levelled out at 2% min^{-1} gradient rate. The S/N ratio of the confirmatory peak (*m/z* 168→81) reached maximum at 2% min^{-1} and decreased markedly with increasing gradient rate. The optimised gradient rate was 2% min^{-1}. In the final method, glyphosate eluted at 10.8 min (Figure 3.2).

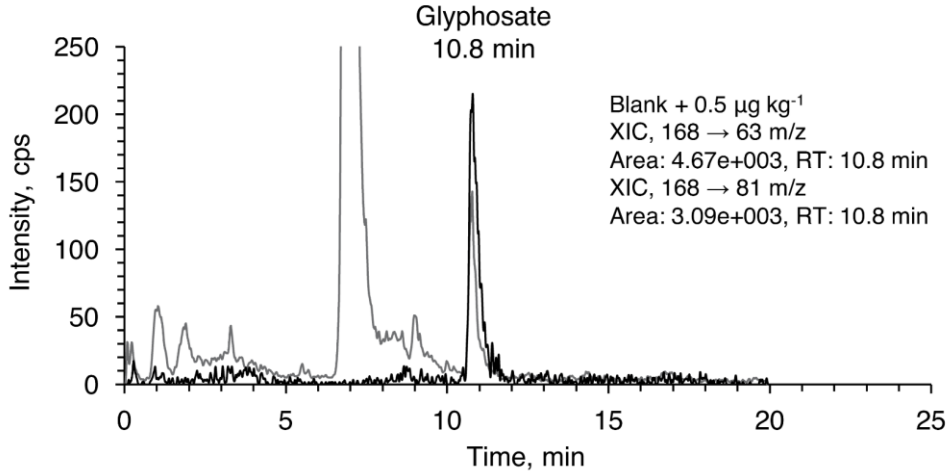

Figure 3.2. **Chromatogram of a blank beer sample spiked with glyphosate at 0.5 µg kg^{-1}.**

3.2. Experimental assessment of different mobile phases for unsuppressed ion chromatography coupled to LC-MS/MS

3.2.1. Effect of mobile phase composition on the retention times of pesticides

Among the non-volatile buffers, salicylate and oxalate were not useful below 2.5 mmol L^{-1} due to poor elution strength. Ammonium carbonate and TEAB eluted all analytes within 15 min at 40 mmol L^{-1}. Citrate and edetate buffers eluted all analytes, except ioxynil, within 15 min at 0.6 mmol L^{-1}. Therefore, the recovery of ioxynil was not studied with the citrate and edetate mobile phases. Ammonium carbonate is a widely used volatile buffer in mass spectrometry, however it may be difficult to maintain retention time stability between batches of buffer. The use of TEAB, citrate, and edetate buffers in anion exchange for determination of pesticides, to the best of our knowledge, is not well described in the scientific literature, therefore these buffers were selected for characterisation with respect to quality of quantification.

The retention factors were calculated and compared to find whether the choice of eluent influenced the selectivity. The acidic pesticides eluted with retention factors ranging from 1.9 to 4.1 with 0.3 mM citrate mobile phase under isocratic conditions, while the retention factors of the ionic pesticides AMPA, bromate, chlorate, glyphosate, ethephon, and ioxynil were 2.4, 2.7, 3.2, 6.7, 7.1, and 19.0, respectively. The retention factors decreased by about 10% if 0.6 mM citrate mobile phase was used, except for the pesticides in azolide (fludioxonil) and azanide forms (flonicamid, fuberidazole, thiabendazole), which did not respond to an increase in eluent strength, and also glyphosate and ethephon, for which the retention factor was reduced

by 30%. The same selectivity was observed with the edetate mobile phases, except for AMPA, which gave a 10% increase in retention factor. With the 12.5-50 mM TEAB procedure, the acidic pesticide retention factors ranged from 1.7 to 5.3, the retention factors of the ionic pesticides chlorate, bromate, ioxynil, ethephon, AMPA, and glyphosate were 4.0, 4.2, 10.0, 13.2, 13.4, and 14.5, respectively. Retention factors of AMPA, glyphosate, and ethephon were significantly higher with the 12.5-50 mM TEAB gradient than with 0.3 mM citrate or edetate buffer, however, the retention factor of ioxynil was significantly lower. In all cases, only flonicamid was poorly retained, with a retention factor of 1.1. TEAB and edetate eluents were selected for characterisation with respect to quality of quantification.

3.2.2. Effect of mobile phase composition on mass spectrometric signals

Triethylammonium bicarbonate (TEAB), ammonium carbonate, and eluents based on citric, edetic, salicylic, and oxalic acid were tried with the Turbo V™ ionisation source. The precursor ion mass spectrum indicated interference by extremely broad and intense bands of peaks around *m/z* 90, 150, 180, and 220 with the salicylate eluent. Suppression of signals originating from the system in the precursor ion mass spectrum was observed and suppression of AMPA signal occurred with the oxalate eluent. With citrate and edetate buffers the precursor ion mass spectrum showed minimal interference. The top 5 signals occurred at *m/z* 59, 60, 62, 76, and 89 when using the edetate eluent. The molecular ion of edetic acid at *m/z* 291 was not observed with the Turbo V™ ionisation source, however, with the Ion Max source it was the major ion in the precursor ion mass spectrum. The most significant effect of mobile phase composition on the background signal was observed in the case of clopyralid product ions. The background level in counts per second (cps) was increased from about $2 \cdot 10^2$ cps to $5 \cdot 10^3$ cps for *m/z* 190 → 146 with edetate buffer and *m/z* 192 → 148 with citrate buffer. The elevated background level appeared unstable during prolonged operation. Unstable background was also observed for glyphosate (*m/z* 168 → 63) with the citrate mobile phase.

Sensitivity comparisons were performed (Figure 3.3) for the pesticides in standard solutions analysed with the mobile phases and procedures given in Table 2.2. The sensitivity of analysis for ethephon, glyphosate, and AMPA was significantly lower (Figure 3.3, A) with eluents based on carboxylic acids in comparison to a 12.5-50 mM gradient with the TEAB eluent. The highest median sensitivity across all analytes was also achieved with the same procedure (Figure 3.3, B). The median sensitivity in the determination of acidic pesticides appeared to increase with lower buffer concentrations and decrease if gradient conditions were applied, and for ionic pesticides it was the highest in case of using 12.5 mM isocratic hold during the gradient program with TEAB buffer. A combined eluent of TEAB and citrate

significantly decreased the sensitivity to ionic pesticides (unpaired *t*-test, $p<0.05$). The sensitivities for ionic pesticides were similar with procedures using carboxylic acid eluents, however, significantly lower than with the TEAB gradient (unpaired *t*-test, $p<0.01$). The carboxylic acid in the eluent may compete with the analytes for the limited excess charge or surface area on the droplets formed during the electrospray process, thus resulting in a lower sensitivity.

3.2.3. The influence of mobile phase composition on matrix effects

The procedures using gradient with edetate buffer and gradient with TEAB were selected for comparison with respect to matrix effects, because the median sensitivity for ionic pesticides was the highest for edetate among non-volatile buffers and the highest for TEAB among volatile buffers (Figure 3.3, B).

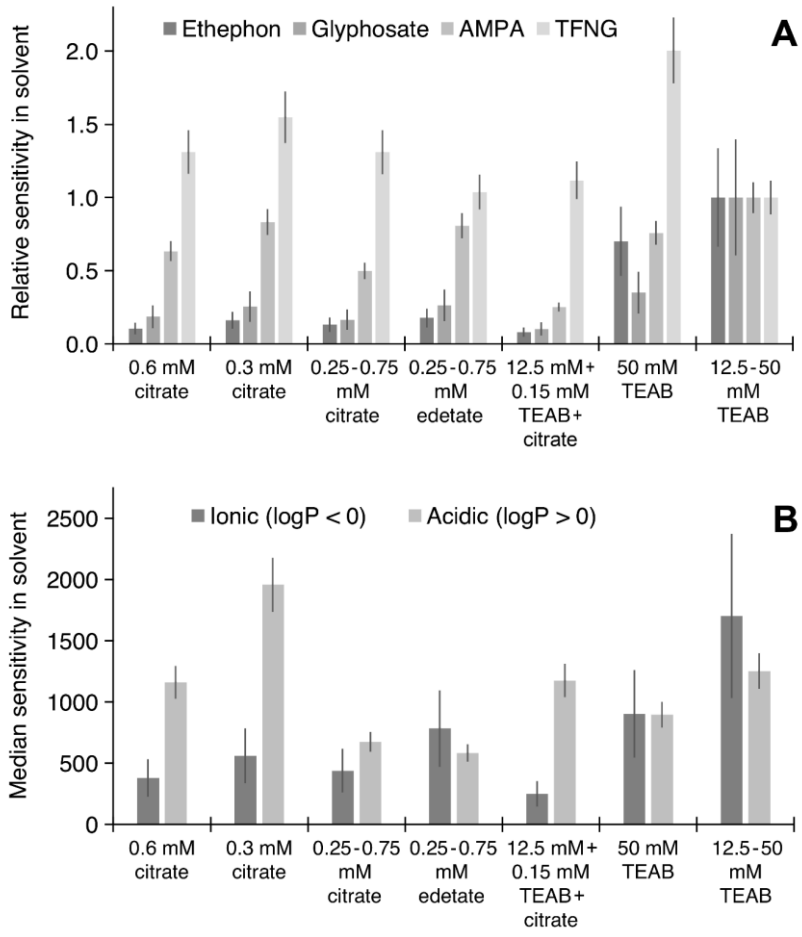

Figure 3.3. **Sensitivity comparisons based on the signal-to-noise ratios of signals from standard solutions analysed with different mobile phases and procedures (Table 2.2) – (A) relative sensitivity for AMPA, ethephon, glyphosate, and TFNG, (B) median sensitivity for ionic and acidic pesticides. AMPA, bromate, chlorate, ethephon, and glyphosate were considered to be ionic, the rest of the pesticides were considered to be acidic.**

In addition, to the best of our knowledge, the application of such mobile phases for the determination of pesticides is not well described in the scientific literature. Considering the improvement in sensitivity with TEAB discussed previously and its volatility, it was hypothesised that TEAB would provide acceptable recoveries with improved sensitivity, however, the opposite was observed. The 12.5-50 mM TEAB and 50 mM TEAB procedures resulted in poor analyte recoveries in the case of spiked extract injections, while the 0.25-0.75 mM edetate procedure provided acceptable recoveries within the 80-110 % range (Figure 3.4, A) for both matrices.

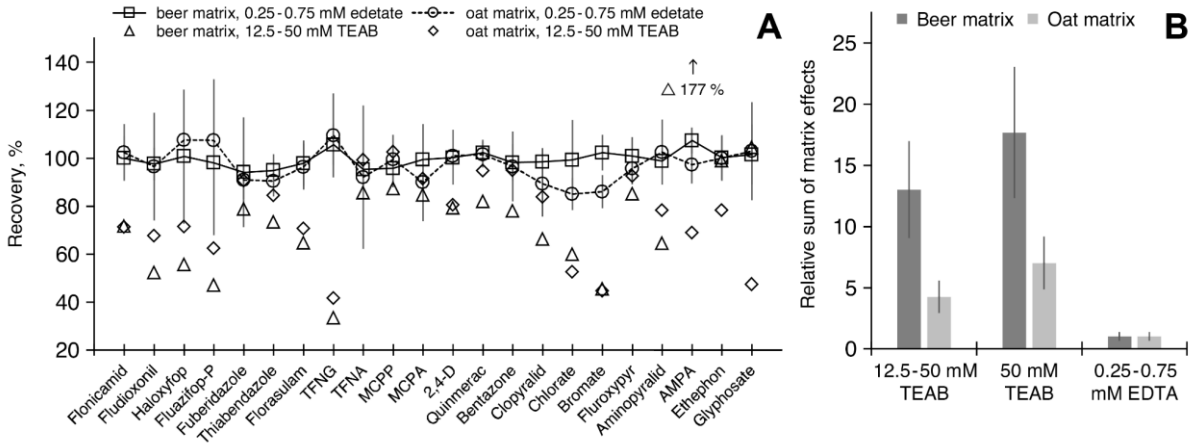

Figure 3.4. **A comparison of matrix effects in spiked oat flour and beer extracts analysed with different mobile phases and procedures (Table 2.2) – (A) analyte recoveries, with analytes shown in the order of elution according to the 0.25 0.75 mM EDTA procedure, (B) the relative sum of matrix effects. Error bars for the recoveries with TEAB omitted for clarity.**

Furthermore, most pesticides were eluted in the 12.5 mM isocratic hold part of the gradient program with TEAB, which can be considered an acceptable buffer concentration for mass spectrometry methods in general. It was observed that the use of TEAB gradient for elution, where most pesticides eluted at 12.5 mM, significantly improved the sensitivity for solvent-based standard solutions, but led to enhanced matrix effects in the cases of spiked oat and beer sample extracts. Therefore, it may be concluded that the higher concentration of TEAB in mobile phase adversely impacted the ionisation efficiency of selected analytes in the presence of matrix, which led to poor analyte recoveries. However, this may be compensated using isotope-labelled internal standards. A sum of matrix effects was calculated for the results from each procedure for spiked beer and oat matrices (Figure 3.4, B) and relatively increased matrix effects were observed with procedures using the TEAB buffer. Furthermore, the matrix effects with TEAB were about two-fold greater in the case of beer matrix. This pattern of matrix effects

was confirmed by analysing the same vial of spiked extract using different chromatographic procedures.

3.2.4. Qualitative comparison of Turbo V ™ and Ion Max ionisation sources

Two differently designed ionisation sources – Turbo V™ and Ion Max were compared qualitatively with respect to the effects from application of a non-volatile buffer. Turbo V™ and Ion Max sources are different in the angle of spray relative to the vacuum interface and delivery of heated gas. In the Ion Max source, the spray is directed at an angle to a point below the orifice, however, in Turbo V™ the spray is orthogonal to the orifice and directed towards an exhaust chimney. The ionisation sources were thoroughly cleaned before use. After prolonged operation using the 0.25-0.75 mM edetate procedure with a total of 250 mL of mobile phases C and D consumed, the ionisation source interior and the front-end components of the mass spectrometers were inspected for contamination. An insignificant amount of a transparent thin-film-like solid residue, less than typically obtained after an overnight analysis of QuPPe extracts, was observed on the Turbo V™ curtain plate. The amount of residue was slightly increased if the procedure was repeated with the source temperature lowered from 550 to 350 °C. The inner surfaces of the exhaust chimney located below the probe electrode were coated with burnt residue, similar to that obtained during normal routine operation with injections containing matrix from the samples. Citrate buffers showed similar outcomes. Variation of curtain gas pressure did not affect the analyte signals; the maximum curtain gas pressure of 50 psi was applied in order to prevent contamination of the orifice. With the Ion Max source at the maximum temperature of 500°C, severe contamination with inhomogenous crystalline and burnt residue was observed on all inner surfaces of the source housing and to a greater extent on the front-end components of the mass spectrometer. The overall amount of residue observed in the Ion Max source was greater than in the Turbo V™ source, suggesting that thermal decomposition of the buffer was more pronounced in Turbo V™ source. However, this may possibly be optimised by reducing the flow rate to the Ion Max source.

Glyphosate, being a poorly ionising analyte, was selected for verifying the stability of response. The peak areas of glyphosate from the analysis of the calibration check standard on QTRAP 5500 using the Turbo V™ source were within the control limits (Figure 3.5).

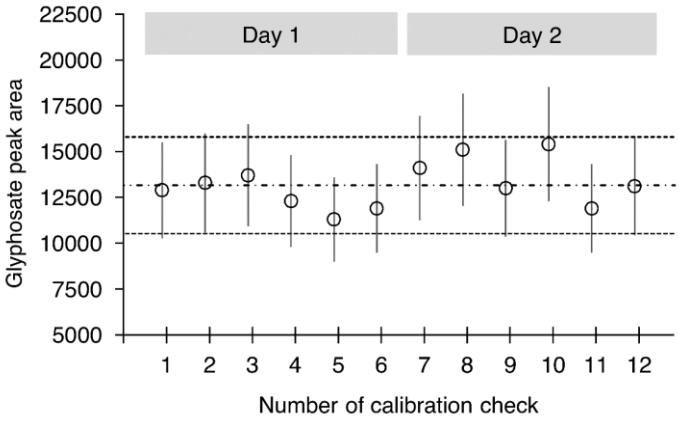

Figure 3.5. **The glyphosate peak area from the analysis of calibration check standard throughout the sample sets over two days using the 0.25-0.75 mM EDTA procedure on a QTRAP 5500 instrument with Turbo V™ source. The control limits were based on the measurement uncertainty for glyphosate.**

Due to the excessive noise in chromatograms, stability could not be verified for the Ion Max source under the described conditions. This may be due to differences in ionisation, as the molecular ion of edetic acid at *m/z* 291 was not observed with Turbo V™ source, but it was the major signal with Ion Max source, possibly concomitant with suppression of other signals.

3.2.5. Quantitative results

Acceptable quantitative results were obtained with the 0.25-0.75 mM edetate procedure. The recoveries of all analytes were in the 80-110 % range based on calibration with standards in solvent. The median recovery across analytes was 99% from beer matrix and 97% from the oat flour matrix. The measurement uncertainties of the mean recoveries ranged from 5 to 30%. The median uncertainty across analytes was 8% for beer matrix and 11% for the oat flour matrix. For the calibration model, R^2>0.99 was observed for all of the analytes. LOQ of 20 μg kg^{-1} was obtained for aminopyralid, clopyralid, fluroxypyr, and thiabendazole, while LOQ of 10 μg kg^{-1} was obtained for 2,4-D, AMPA, bentazone, bromate, chlorate, ethephon, flonicamid, florasulam, fluazifop-P, fludioxonil, fuberidazole, glyphosate, haloxyfop, MCPA, MCPP, quinmerac, TFNA, and TFNG (Annex 2). Interfering peaks in the chromatograms of spiked matrix injections were observed only for the product ions of glyphosate (*m/z* 168 → 79) and MCPP (*m/z* 213 → 141) in the oat flour matrix, nevertheless, they were resolved from the analyte peak.

We have obtained acceptable quantitative results for pesticide determination in spiked oat flour and beer by separation with 0.25-0.75 mM edetate buffer gradient and detection with

a AB SCIEX QTRAP 5500 tandem mass spectrometer with Turbo V™ ionisation source. The recoveries of analytes were in the 80-110 % range based on external calibration with standards in solvent. The LOQ ranged from 10 μg kg^{-1} to 20 μg kg^{-1}. Therefore, it may be concluded that determination with low matrix effects may be possible using mobile phases that contain <1 mmol L^{-1} of buffer. Additional validation efforts are necessary before non-volatile buffers can be used, because they may interfere with the precursor ion mass spectrum leading to the high and unstable background levels for some transitions, and non-volatile buffers may not be suitable for all electrospray ionisation sources. Considering the successful recovery of all analytes from oat flour and beer matrices, the present approach offers a useful alternative to suppressed IC-MS. Further optimisation is needed, including the selection of analytical column with the appropriate dimensions and adjusting the chromatographic conditions in order to maximise the sensitivity and minimise the delivery of non-volatile buffer to the ionisation source. The present approach, however, may only be used with ionisation sources that are designed to withstand significant amounts of non-volatile residue from the eluate spray. Complete thermal decomposition of eluate with matrix to volatile products cannot be expected in the electrospray process, therefore, the arrangement of the Turbo V™ source, where the electrospray is directed to an exhaust chimney, may be the only feasible design for successful prolonged application of non-volatile buffers.

3.3. Development of a novel and reliable dansyl chloride derivatization-based LC-MS/MS method for determination of glyphosate, aminomethylphosphonic acid and glufosinate in complex food matrices

3.3.1. ESI LC-MS and MS/MS characterization of dansyl derivatized analytes

Spiked reagent blanks were used for characterization of the dansyl derivatized analytes. The dansyl derivatized and chromatographically separated analytes were characterized in high resolution using the Orbitrap™ mass spectrometer in parallel reaction monitoring mode in both polarities. Fragments were confirmed by overlap of the extracted chromatogram peak with the precursor ion peak, and the optimum collision energy was found by re-injection from the same vial at all collision energies in steps of 5 units. The mass spectra are given in Figure 3.6. and the table of peaks together with the optimum collision energies is given in Annex 5.

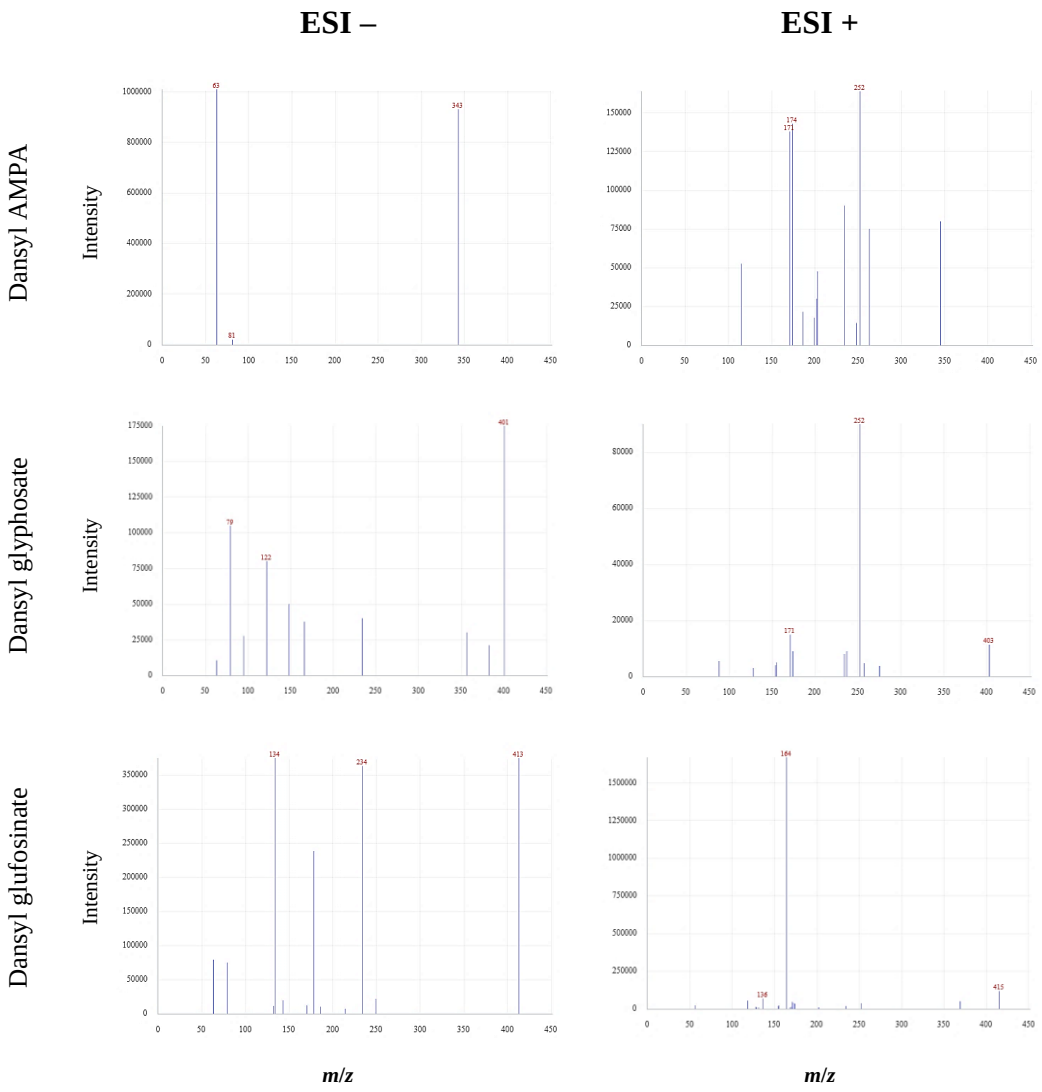

Figure 3.6. **Collision-induced dissociation (CID) mass spectra of dansyl AMPA, dansyl glyphosate and dansyl glufosinate acquired using electrospray ionisation in positive and negative mode with collision energy (CE) ramping from 10 V to 60 V.**

Retention factors under full gradient range condition were estimated (2.5–99% B at 1.93% B/min gradient rate) using nano-LC-MS with column dimensions expected to provide separation capacity equivalent to that of the column in the proposed procedure. Nano-LC-MS was used to obtain a perspective on the applicability of nano-LC-MS. The estimated retention factor *k* was 7.7, 7.8 and 8.0 for AMPA, glufosinate and glyphosate, respectively, and were determined in reagent blanks spiked with the analytes.

Additional sample preparation steps were needed in order to introduce the sample into the nano-LC-MS system. Reagent blank spiked with the analytes to 100 μg L^{-1} and prepared according to the proposed procedure was further diluted with acetonitrile to a dilution factor of 6.66 to precipitate salts. 50 μL of the supernatant was diluted to 2000 μL with ultrapure water containing 0.1% formic acid and filtered (0.22 μm pore size, PVDF).

The following conditions for nano-LC-MS detection were applied – 1 µL was injected to an EASY-Spray™ C_{18} nano-LC column (150 mm × 75 µm) containing 3 µm particles and detected with Orbitrap™ mass spectrometer in parallel reaction monitoring mode. The gradient started at 2.5% ultrapure water and reached 99% acetonitrile in 50 min at 225 nL/min (both solvents contained 0.1% formic acid).

The resulting chromatograms are given in Figure 3.7. Validation using nano-LC-MS was not pursued due to lower sensitivity than conventional LC-MS as a result of the needed additional sample preparation steps.

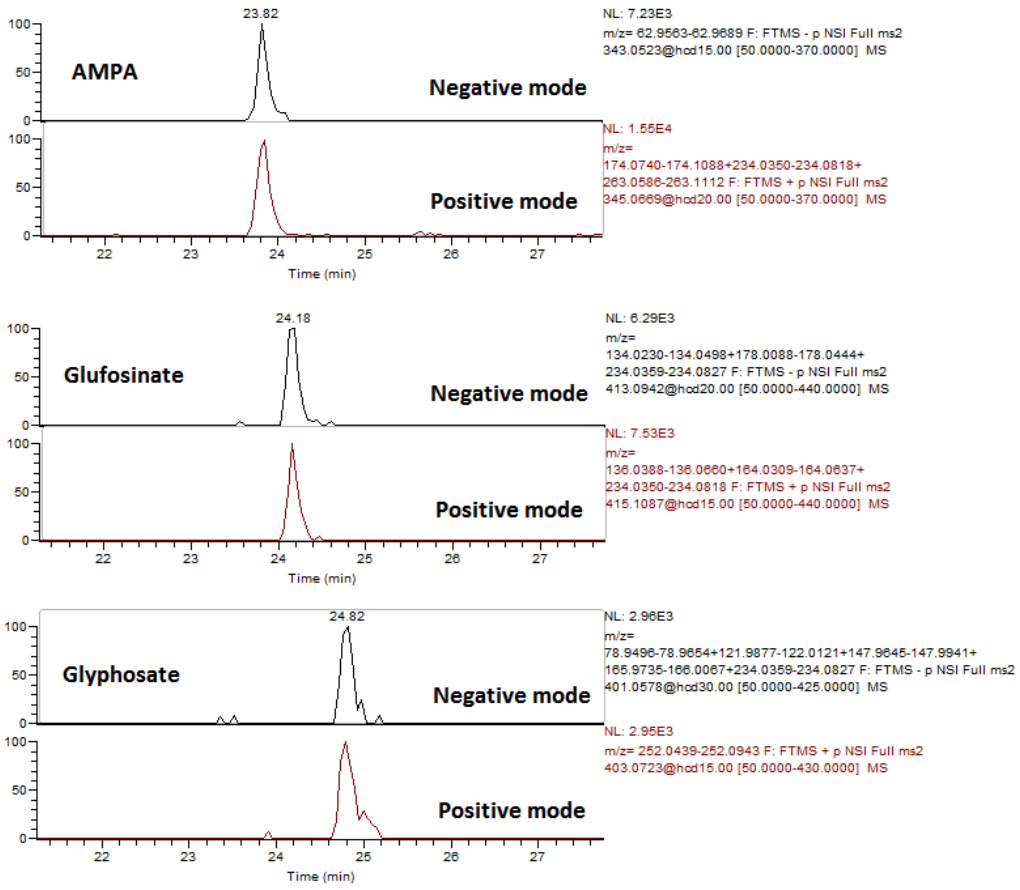

Figure 3.7. **Nano-LC-MS detection of peaks corresponding to AMPA, glyphosate and glufosinate derivatized with dansyl chloride in a spiked reagent blank. The estimated retention factor *k* was 7.7, 7.8 and 8.0 for AMPA, glufosinate and glyphosate, respectively.**

3.3.2. Choice of the reversed phase column and the mobile phase

Separation of derivatization reagent by-products from the derivatized analytes was demonstrated on standard solutions in the mg L^{-1} range by UV absorption detection using Thermo Scientific DAD-3000 diode array detector (DAD) at 254 nm wavelength. The separation was initially attempted with a Phenomenex Kinetex® (50 × 3 mm, 3 μm C_{18} bonded silica particles) column and 5 mM ammonium acetate mobile phase at pH 5.8, however, complete separation of the analytes could not be achieved at any gradient rate. The best retention and resolution of the derivatized analytes was achieved with a Phenomenex Luna® (150 × 2 mm, 3 μm C_{18} bonded silica particles) column, however, a derivatization reagent by-product present in great excess still interfered with the derivatized analytes at the baseline level. Also, the background signal intensity was not stable across chromatograms from repeated injections. The best separation of the derivatized analytes from derivatization reagent by-products and a stable low baseline height was achieved with mobile phases of 0.1% formic acid in acetonitrile and water (Figure 3.8).

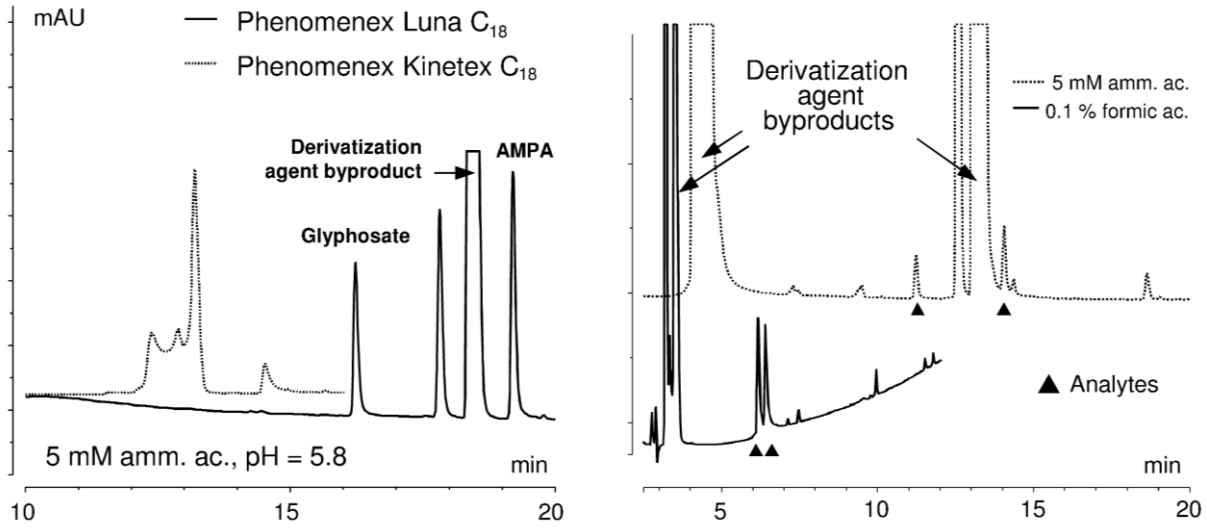

Figure 3.8. **Separation of dansyl chloride derivatized AMPA and glyphosate from dansyl chloride derivatization agent by-products on different octadecyl bonded silica phases and with different mobile phase additives. A standard solution containing 100 mg L^{-1} of analytes was derivatized and analyzed.**

3.3.3. Optimization of sample preparation with dansyl chloride derivatization

The composition of solution for extraction was optimized to achieve the highest signal in porridge formula matrix, which was rich in calcium. A two-fold increase in signal of the analytes was observed if the concentration of EDTA, which is a strong anion needed for

desorption of the analytes from matrix, was increased from 2 to 6% in the solution for extraction. Addition of 1% formic acid or 0.36% sodium carbonate to the solution for extraction resulted in a significant decrease of signals and difficult filtering of extracts, indicating that the unadjusted pH of the EDTA solution is optimal. The volume of solution for extraction was optimized to obtain a sufficiently homogenous liquid extract after centrifugation. 5 mL were found to be the smallest feasible volume.

3.3.4. Evaluation of extraction efficiency and matrix effects

The extraction efficiency was evaluated for each matrix according to Equation 2. In the case of underivatized analytes, matrix effects were evaluated according to Equation 3. In the case of derivatized analytes, matrix effects originating in the LC-MS stage were evaluated according to Equation 4. Extraction efficiencies and matrix effects originating in the LC-MS stage are given in Figure 3.9.

Evaluation of matrix effects originating in the derivatization stage was carried out by comparison of standard addition calibration curve slopes with and without use of internal standards. A ten-fold change in calibration curve slope was observed without use of internal standards for bovine kidney in comparison to reagent blank. The matrix effect in the derivatization stage ranged from -19 to -91% as reduction of calibration curve slope. The magnitude of matrix effects increased as follows: milk < cucumber < honey < porridge formula < bovine kidney < bovine liver. The matrix effects were compensated if internal standards were used. The mean relative standard deviation of calibration curve slopes across all matrices was 5% for derivatized analytes with the Luna® C_{18} column and 11% for underivatized analytes with the Waters™ column.

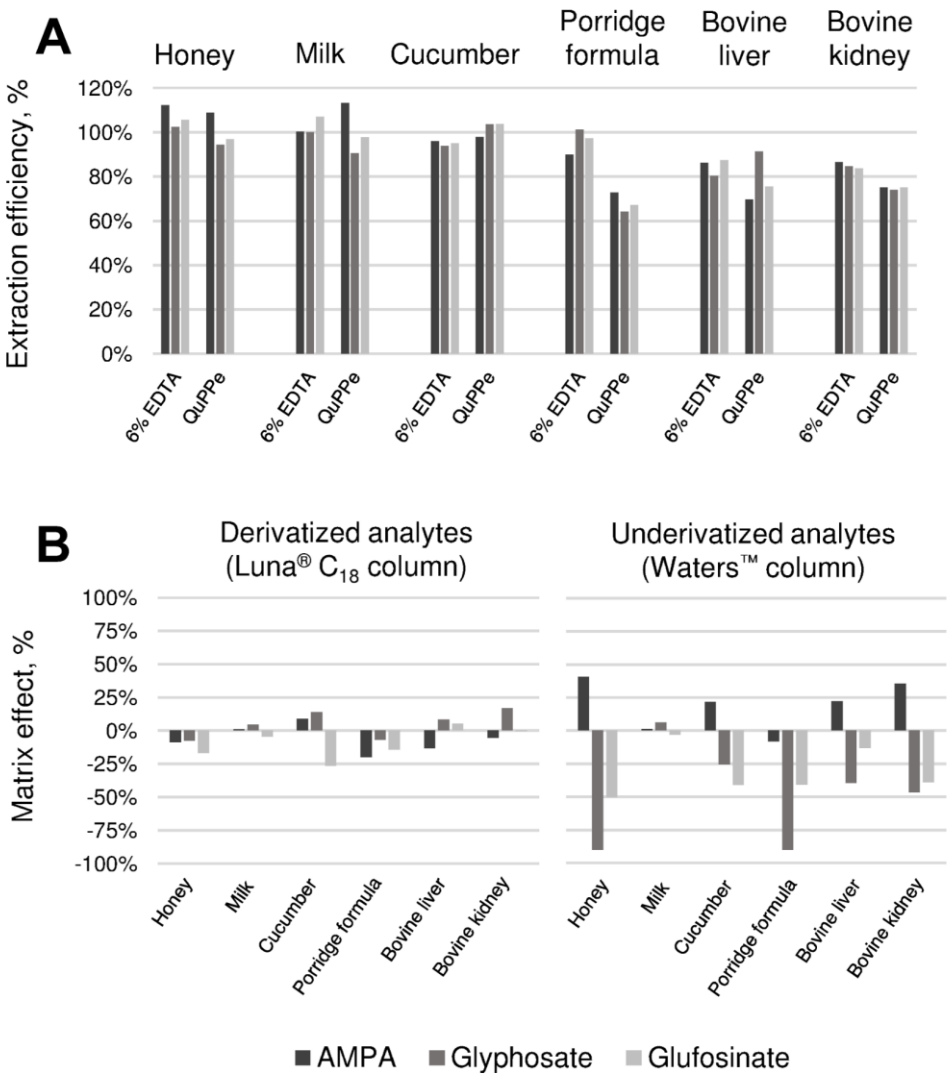

Figure 3.9. **Comparison of extraction efficiencies and matrix effects – (A) extraction efficiencies of AMPA, glyphosate and glufosinate for different matrices with aqueous 6% EDTA and with the QuPPe extraction procedure (50% methanol containing 1.5% formic acid and 0.25% EDTA), (B) matrix effects originating in the LC-MS stage in the case of dansyl chloride derivatized analytes (Luna® C_{18} column) and underivatized analytes (Waters™ anionic polar pesticide column).**

3.5.5. Quantitative results

The proposed procedure using derivatization, as well as determination of underivatized analytes using the Waters™ anionic pesticide column was validated by evaluating the mean recovery of analytes from spiked matrix, LOQ and expanded uncertainty. Validation results are given in Table 3.1.

The recoveries of all analytes were in the 80-120 % range based on calibration with isotopically labelled internal standards prepared in reagent blanks. The mean recovery across

all analytes and matrices was 104%. The measurement uncertainties ranged from 4 to 44%. For the calibration model, R^2>0.98 was observed for all analytes. LOQ of 10 µg kg^{-1} and chromatograms free of interference were observed for all underivatized analytes from all matrices with the Waters™ anionic pesticides column. For derivatized analytes the LOQ's ranged from 10 to 250 µg kg^{-1}, if detected with the triple quadrupole mass spectrometer, however, if detected with the Orbitrap™ mass spectrometer, chromatograms free of interference and LOQ's in the range from 10 to 25 µg kg^{-1} were observed. Exemplary chromatograms are given in Figure 3.10.

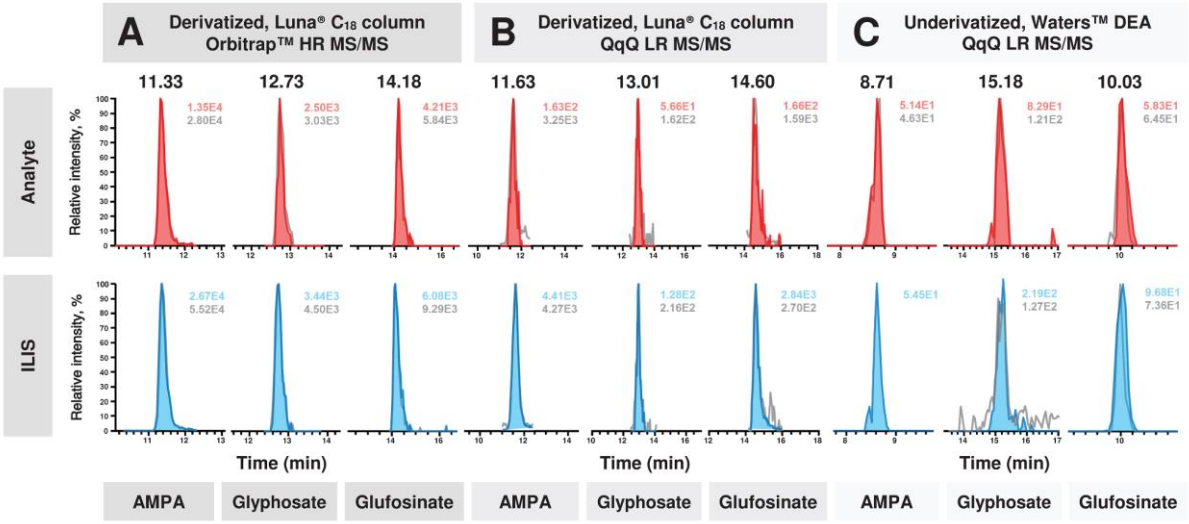

Figure 3.10. **Peaks corresponding to AMPA, glyphosate, glufosinate and the respective isotopically labelled internal standards from milk porridge baby formula spiked to 50 µg kg^{-1} – (A) dansyl chloride derivatized and detected with high resolution Orbitrap™ detection, (B) dansyl chloride derivatized analytes detected with low resolution tandem mass spectrometric detection, (C) underivatized analytes extracted using the QuPPe extraction procedure and detected with low resolution tandem mass spectrometric detection. Peak height given next to the respective peaks.**

Table 3.1.

Validation results for determination of dansyl chloride derivatized glyphosate, glyphosate-$^{13}C_2{}^{15}N$, AMPA, AMPA-$^{13}C^{15}N$, glufosinate and glufosinate-D_3 with high resolution Orbitrap™ and low resolution tandem mass spectrometric detection and for determination of the underivatized analytes using the Waters™ anionic pesticide column with low resolution mass spectrometric detection.

			Honey	Porridge formula	Bovine liver	Bovine kidney	Milk
Derivatized AMPA	HR MS/MS Luna® C_{18} column	Mean recovery, %	99	104	95	103	102
		Uncertainty, %	6	10	23	22	4
		LOQ, µg kg^{-1}	10	10	10	10	10
	LR MS/MS Luna® C_{18} column	Mean recovery, %	101	115	113	111	107
		Uncertainty, %	5	38	26	24	15
		LOQ, µg kg^{-1}	10	10	10	10	10
Underivatized AMPA	LR MS/MS Waters™ column	Mean recovery, %	104	99	98	101	110
		Uncertainty, %	17	25	28	28	17
		LOQ, µg kg^{-1}	10	10	10	10	10
Derivatized glyphosate	HR MS/MS Luna® C_{18} column	Mean recovery, %	109	103	102	104	105
		Uncertainty, %	19	19	8	17	16
		LOQ, µg kg^{-1}	10	25	25	25	10
	LR MS/MS Luna® C_{18} column	Mean recovery, %	98	104	115	109	113
		Uncertainty, %	44	41	23	29	24
		LOQ, µg kg^{-1}	25	50	250	250	10
Underivatized glyphosate	LR MS/MS Waters™ column	Mean recovery, %	111	109	111	104	97
		Uncertainty, %	15	18	20	30	25
		LOQ, µg kg^{-1}	10	10	10	10	10
Derivatized glufosinate	HR MS/MS Luna® C_{18} column	Mean recovery, %	106	103	99	101	98
		Uncertainty, %	25	19	9	12	33
		LOQ, µg kg^{-1}	10	10	10	10	25
	LR MS/MS Luna® C_{18} column	Mean recovery, %	105	105	104	103	102
		Uncertainty, %	15	25	18	23	14
		LOQ, µg kg^{-1}	10	10	10	10	10
Underivatized glufosinate	LR MS/MS Waters™ column	Mean recovery, %	98	118	97	99	103
		Uncertainty, %	19	26	29	20	19
		LOQ, µg kg^{-1}	10	10	10	10	10

3.6. Optimization and application of LC-MS/MS method for determination of glyphosate in laboratory model experiments

The optimised method described in 2.5.1. was used for analysis of samples from laboratory model experiments. The samples were analysed with prior knowledge of the approximate concentration of the analytes being in the mg kg^{-1} range, therefore, dilution of extracts was performed. Retention time drift during analysis with the Hypercarb column was observed, a known and often reported problem in polar analyte analysis with porous graphitic carbon [87]. The drifting retention time could be explained by low content of matrix in the injections. that otherwise could stabilize the retention on porous graphitic carbon. As proposed by Anastassiades et al., the Hypercarb columns need to be primed by a matrix rich injection derived from a spinach extract according to the QuPPe protocol [66]. However, to the best of our experience, this treatment of the column is only necessary when gradient elution with organic solvents is performed, as this facilitates removal of matrix compounds from the column. Therefore, the procedure was optimised under isocratic and fully aqueous conditions. As a result, the observed retention time drift did not result in loss of signal, and the matrix effects were compensated by using stable isotope labelled internal standards, resulting in good reproducibility (RSD_{WR} < 6%).

Under these conditions glyphosate eluted in the average retention time of 3.5 min and AMPA eluted in the average retention time of 0.9 min during a 10 min run. A six-point linear calibration was used for quantification in the range from 5 μg L^{-1} to 100 μg L^{-1}, with stable isotope labelled internal standards at 10 μg L^{-1}. For the calibration, an R^2>0.999 was observed. The estimated carry-over was <0.9%. The measurement uncertainty for glyphosate and AMPA, estimated from spiking experiments, was 11% (k=2, 95% confidence interval).

3.6.1. Evaluation of biodegradability and ecotoxicity of glyphosate-based herbicide in wastewater

Results from the column experiment showed a slight inhibition effect of GBH on heterotrophic microorganisms in WW in concentration range from 100 to 200 mg L^{-1}. Additional ecotoxicological testing of the GBH was performed in order to characterize this herbicide in a broader concentration range. The CFU number in WW after incubation for 7 days in the presence of 0-300 mg L^{-1} GBH varied in the range from $4.2 \cdot 10^7$ to $3.4 \cdot 10^8$ CFU mL^{-1}. No inhibition effect of GBH on heterotrophic microorganisms in WW was found under tested conditions. Effect of the GBH on *Daphnia magna* was tested. After 24-h incubation of *Daphnia magna* in standard freshwater spiked with 1 mg L^{-1}, 10 mg L^{-1}, 50 mg L^{-1} and 100 mg L^{-1} of GBH, the immobility rate was 20%, 40%, 100% and 100%, respectively, with

24-h EC_{50} = 22 mg L^{-1}. According to earlier studies of other authors, toxicity of GBHs for *Daphnia magna* can vary in a broad range. The 24-h EC_{50} was reported to be 5 mg L^{-1}, 96 mg L^{-1} and 190 mg L^{-1} of GBH [100], [101]. Obviously, differences in crustaceans' response to the GBH occur due to different physicochemical characteristics of aquatic environments. Additional testing was performed in raw WW, in order to reveal possible side effects of WW compounds on the toxicity of GBH towards *Daphnia magna*. The GBH possessed the stronger toxic effect on *Daphnia magna* when incubated in WW compared to standard conditions. In particular, an immobility rate of *Daphnia magna* at 10 mg L^{-1} of GBH was significantly higher ($p<0.05$) in WW, than in standard freshwater.

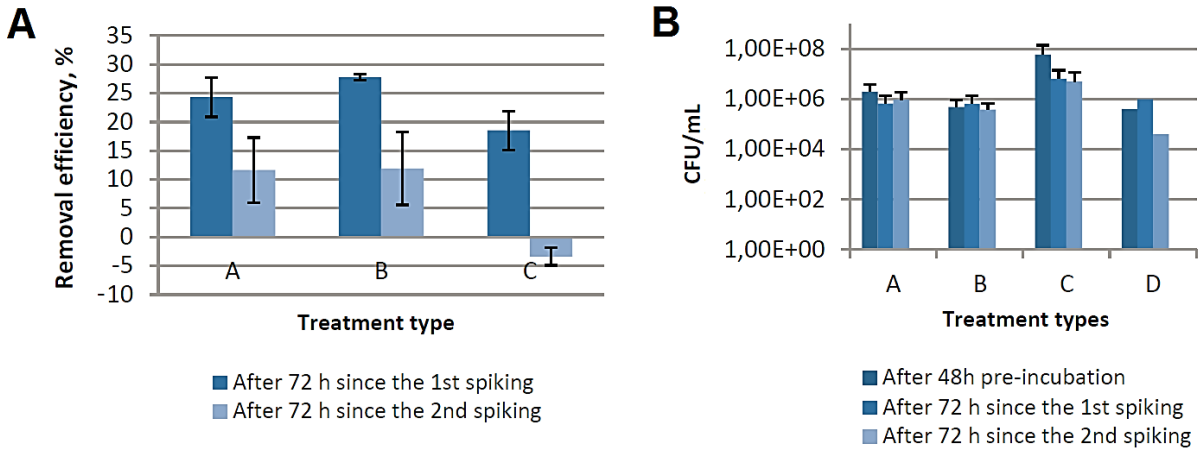

Figure 3.11. **(A) – Efficiency of glyphosate removal from WW determined using the optimized LC-MS/MS method described in 2.5.1., (B) – the number of colony-forming units (CFU) in WW. Treatment A – non-amended WW, treatment B – WW + 500 g oxide ceramics, treatment C – WW + nutrient composition and activated sludge, treatment D – only aeration (no replicates). WW were spiked with 100 mg L^{-1} of glyphosate-based herbicide on day 3 and 6.**

The treatment of raw municipal WW in the presence of oxide ceramics (Figure 3.11.A, treatment B) demonstrated the highest removal efficiency of glyphosate compared to nutrient and activated sludge amended WW (treatment C) and non-amended WW (treatmen A). For treatment C the glyphosate addition rate due to spiking exceeded the glyphosate removal rate, therefore, negative removal efficiency was observed after the 2nd spiking. The improved removal efficiency with treatment B effect could be explained by combined sorption and biodegradation processes. The 24-h EC_{50} for the GBH under standard conditions and in raw WW was calculated to be 22 mg L^{-1} and 6 mg L^{-1} of GBH, respectively. The CFU counts in WW treated in the column experiments with two spikings of GBH at 100 mg L^{-1} for 4 days did not show any considerable decrease. Similar results were shown after incubation of raw WW for 7 days in the presence of GBH in the concentration range from 10 mg L^{-1} to 300 mg L^{-1}.

These data along with the results on glyphosate removal indicate that microorganisms in WW have a comparatively high resistance to GBH. Therefore, WW microorganisms represent a source for glyphosate-based herbicide biodegradation in WW.

3.6.2. Evaluation of mobility of glyphosate from two contrasting agricultural soils

The remaining concentrations of glyphosate in soils after spiking with the GBH followed by a pot weathering experiment for 40 days, was compared for bioaugmented and non-bioaugmented soils. The LC-MS/MS analysis of the base extracted samples revealed non-significant ($p>0.05$) differences among the tested soil treatments, where the glyphosate concentration decreased during the 40-day experiment from 445 mg kg^{-1} to 241–273 mg kg^{-1}.

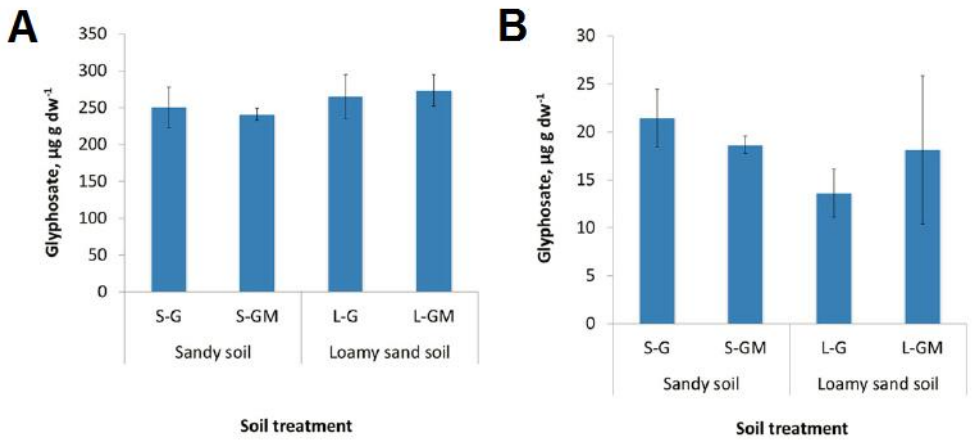

Figure 3.12. **Concentration of glyphosate in soils after the 40-day incubation determined using the optimized LC-MS/MS method described in 2.5.1. (A) – base extract, (B) – deionised water extract. Abbreviations of the soil treatments describe the conditions of soil treatment with glyphosate-based herbicide (G) and microorganisms (M). Error bars represent the 95% confidence interval (*N* = 3).**

The leaching of glyphosate from two types of soils pre-treated with GBH and microorganisms were compared according to the total amount collected during three leaching stages (Figure 3.13). The total amount of glyphosate in leachates after three stages varied in the range from 2380 to 4808 µg L^{-1} with the highest value found for "S-GM". Comparison of the total glyphosate amount in leachates between bio-augmented soils and soils without added microorganisms showed notable differences between these two soil types. Thus, in sandy soil the addition of microorganisms led to a significant ($p<0.01$) increase of glyphosate leaching, compared to the non-augmented soil (4808 and 3834 µg L^{-1}, respectively). Conversely, in the case of loamy sand soil, the bio-augmented soil was characterized by a lower amount of

glyphosate leached, as compared to the control sample (2380 and 2740 μg L^{-1}, respectively). These changes for loamy sand soil, however, were not statistically significant.

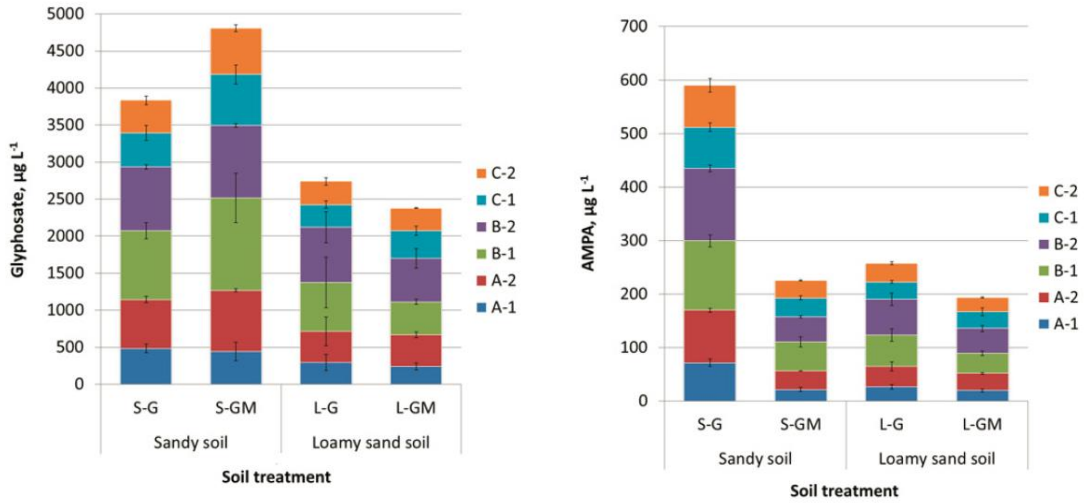

Figure 3.13. **Glyphosate and AMPA concentration in leachates determined using the optimized LC-MS/MS method described in 2.5.1. The leachates were obtained from the treated soil samples after three leaching stages performed with 50 mL of 0.01 M $CaCl_2$ solution at each stage. Abbreviations of the soil treatments describe the conditions of soil treatment with glyphosate-based herbicide (G) and microorganisms (M) for 40 days before the leaching experiments. Leachates A, B and C were obtained on day 1, 2 and 3, respectively, while 1 and 2 represent two consistently sampled 25 mL aliquots of leachates from parallel experiments.**

Additional testing of leachates was performed for the evaluation of their overall ecotoxicity, choosing the immobilization of *Daphnia magna* for testing. The presence of glyphosate-based herbicide in both soil types under tested conditions increased the toxicity of leachates from 10–13% up to 50–54%. No significant differences in the ecotoxicity of bioaugmented and non-bioaugmented soils were found.

The comparison of CFU count in soils after leaching did not reveal any significant changes associated with different treatments. A slight increase of CFU count was detected in the glyphosate treated loamy sand soil, compared to the control. In turn, a comparison of the two soil types showed that sandy soil contained higher CFU count than a loamy sand soil, i.e. $2 \cdot 10^5$ and $2 \cdot 10^4$ CFU g^{-1} on average, respectively. No significant differences in CFU counts along the leaching columns (sampling from the bottom to top of the column) were found.

Leaching of glyphosate and AMPA from sandy soil was considerably higher compared to loamy sand soil. This could be explained by macropores in sandy soil. Also, results obtained in different studies on glyphosate leaching can be explained by a specificity of the experiment setup, particularly, by soil disturbance [102]. Comparatively high mobilization

and transport of glyphosate has been detected in minimally disturbed soil [103]. Our experimental setup represents a disturbed soil because the soil that was weathered for 40 days was thoroughly mixed prior to the column filling.

The analysis of leachates in this study revealed notable differences in the concentrations of glyphosate and AMPA depending on the soil type and bioaugmentation. The present study results indicate the presence of a complex combination of environmental factors, which finally determines microbial activity towards glyphosate and AMPA. In this respect, the role of bioaugmentation in degradation and mobility of glyphosate and AMPA is not clarified and, therefore, needs to be studied in further experiments.

3.7. Validation and application of the developed methods to analysis of products at risk for contamination with glyphosate.

3.7.1. Validation and application of SPE extraction-based LC-MS/MS method for the estimation of the occurrence of glyphosate in beer from the Latvian market

A total of 100 different bottled and canned beer samples were purchased from local supermarkets. The samples were stored refrigerated at 5 °C in darkness until analysis. The selected samples represented the majority of available brands and varieties of beer sold in local supermarkets by 24 different producers and distributors from Latvia. All available information about the samples was recorded: constituents, country of production, beer type by colour, type of packaging, presence of precipitate, disclosed use of pasteurisation or filtering. There were 70 samples of light beer, 19 samples of dark beer, and 11 samples of specialty beer. A total of 71 samples were produced from barley malt only, 10 samples were produced from combined malt (wheat, rye or rice), and 9 samples also contained other ingredients such as honey, fruit juices or aromatisers.

The analysis of 100 beer samples revealed that the glyphosate content in beer ranged from below LOQ up to 150 μg kg^{-1}, with 2.9 μg kg^{-1} median value, 7.5 μg kg^{-1} average value, and the standard deviation of 16.6 μg kg^{-1}. The exponential distribution of glyphosate occurrence in beer is shown in Figure 3.14. Only in eight samples the concentration of glyphosate was below LOD. Glyphosate was detected below LOQ in nine samples. Glyphosate concentrations up to 30 μg L^{-1} have been reported previously [17]. This study shows that glyphosate content up to 150 μg kg^{-1} can occur.

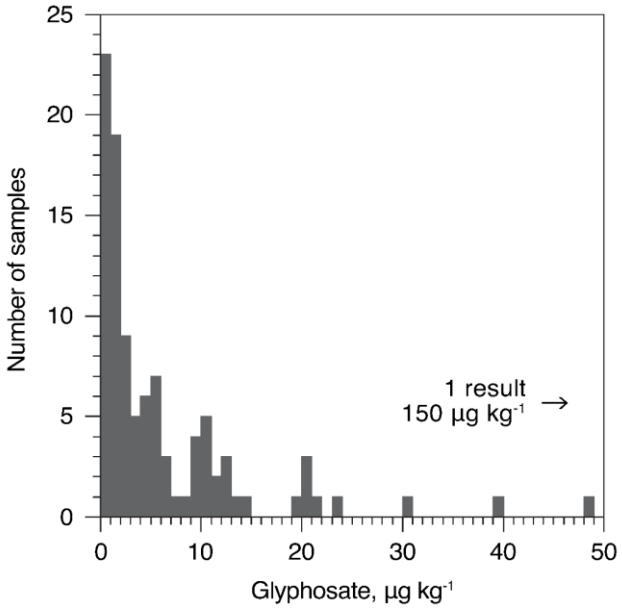

Figure 3.14. **Glyphosate content in 100 samples of beer from Latvia**

No correlation of glyphosate content with alcohol content was observed. No significant difference in glyphosate content between large and small producers was observed.

The results were categorised in following groups: by malt type (barley or combined/other), country of production (local or undisclosed origin), beer type by colour (light or dark), type of packaging (canned or bottled), presence of precipitate (precipitate or no precipitate), filtration (filtered or not filtered), pasteurisation (pasteurised or not pasteurised). The beer samples originated from a total of 24 different producers and distributors in Latvia. Of these, 25 beers originated from distributors, 3 of which were imported, and 22 of which had no country of production disclosed on the labelling. Some producers operate within consortia with several manufacturing or bottling plants across different countries and therefore choose not to disclose the country of production, which is legal.

No significant difference was observed between the groups by malt type, beer type by colour, presence of precipitate, filtration, and pasteurisation ($p>0.1$).

A significant difference in content of glyphosate was observed between locally produced and beer of undisclosed origin ($p<0.01$) with 6.7 µg kg^{-1} median value in 25 beers of undisclosed origin and 1.8 µg kg^{-1} median value in 75 locally produced beers, and between canned and bottled beer ($p<0.01$) with 6.8 µg kg^{-1} median value in 16 canned beers and 2.2 µg kg^{-1} median value in 82 beers sold in glass bottles. Among beers of undisclosed origin 52% of samples were canned and 81% of canned beer were of undisclosed origin, therefore the beers of undisclosed origin were categorised in two groups by type of packaging and the significance of difference tested. No significant difference was observed between canned (6.9 µg kg^{-1} median value) and bottled (5.6 µg kg^{-1} median value) beer among the beers of undisclosed

origin (p=0.586). This suggests that beer sold by distributors from Latvia with no country of production disclosed on the labelling may contain significantly higher content of glyphosate than the locally produced beer.

Taking into account the consumption of barley to produce a kilogram of beer (processing factor of approx. 0.09) and assuming complete carry-over of glyphosate to beer, even the maximum glyphosate residue quantified does not lead to suspicion that the current MRL [104] in barley or wheat used in the production could have been exceeded.

Validation of the SPE extraction-based LC-MS/MS method

The calibration levels are equidistant on a logarithmic scale, therefore a transformation must be applied, otherwise the highest levels would have a high degree of leverage leading to poor accuracy for the lowest levels and inflated R^2. Regression analysis using ANOVA was performed on data from each validation day consisting of 7 repetitions at each calibration level. The lack-of-fit *F*-tests suggested that a linear model did not fit the data ($p<0.01$). Plots of residuals were constructed in the case of linear and quadratic calibration model. The residual plot was satisfactory only in the case of quadratic calibration model where the residuals were distributed randomly around the horizontal axis. For the log-log linear calibration model, $R^2>0.99$ was observed. For the log-log quadratic calibration model, $R^2>0.999$ was observed. The peak area for internal standard deviated from the peak areas for calibrants by -53% to 30%, therefore the calibration using stable isotope labelled internal standard should properly compensate for the matrix effects because the deviation does not exceed one order of magnitude. The peak area deviation for the internal standard appeared random and did not reveal any relationship with the type of beer or other known parameters of beer samples. One QC sample at the 0.5 μg kg^{-1} level was analysed after every 10 samples. The estimated concentrations of blank samples were 0.15, 0.08, 0.12, 0.11, and 0.08 μg kg^{-1}, and the standard deviation of the blank concentrations was 0.03 μg kg^{-1}. The recoveries of QC samples were: 119, 119, 113, 123, 90, 91, 87, 90, 99, and 93%. The QC sample recoveries were within the estimated method uncertainty. The estimated carry-over effect was 0.21% and was taken into account in the uncertainty estimation. The estimated LOD was 0.2 μg kg^{-1} and the LOQ was 0.5 μg kg^{-1}. The repeatability RSD_R ranged from 4.1 to 1.6%, decreasing with higher analyte concentration. The RSD_{WR} was 9.5%, with little variation and no trends observed over the concentration range. The estimated uncertainty due to the bias of measurements at 0.5 μg kg^{-1} and above was 13%. The estimated measurement uncertainty at and above the LOQ of 0.5 μg kg^{-1} was 32% (k=2 at 95% CI), calculated as the average over the calibration levels. The analytical procedure developed and applied in this study has a superior sensitivity compared to

the currently known methods for the determination of glyphosate in beer, however, the sample preparation procedure is not particularly cheap or simple.

The samples where no glyphosate could be detected (below the LOD) were used as blanks for calibration and bias estimation. A maximum permissible deviation of ±30% from the average quantitation and confirmation peak area ratio over all levels of the calibration set was selected as the confirmation criterion. The evaluation of LOD was based on the signal-to-noise ratio for the analyte (S/N≥3), the evaluation of LOQ was based on S/N≥10 and repeatability RSD_R≤20%. A six-point matrix-matched calibration with stable isotope labelled internal standard normalisation was used in the range from 0.2 μg kg^{-1} to 25 μg kg^{-1} with the internal standard at 25 μg kg^{-1}. Quantification was performed using a log-log quadratic model. Samples with glyphosate concentration above the calibration range were reanalysed with calibration at the levels of 25, 75, 125, and 175 μg kg^{-1}. Due to lack of a true blank material, blank subtraction was applied uniformly across the calibration of each batch by subtracting the blank (low level sample) peak area. The 100 samples were analysed in 5 batches of 20 samples. Different blank samples were used for calibrating each batch and a blank sample different from the calibration was used to prepare the QC samples. The criterion for acceptable QC recovery was the estimated method uncertainty. Validation was carried out by analysing 5 different blank samples (B_1, B_2, B_3, B_4, and B_5) each spiked at the calibration levels of 0.2, 0.5, 1, 5, 10, and 25 μg kg^{-1}. The blank samples were light beers of different origin and malt type. Three of the blanks were of barley malt, two of the blanks were of wheat malt, and three of the blanks had a precipitate. This way, some bias due to the differences in matrix composition are included in the uncertainty estimation. On each validation day, one blank was used to prepare the calibration samples at each level. Selected other blanks spiked at each level were analysed by subjecting the samples to the whole analytical procedure. On the first validation day, B_5 was used as calibration, with three repetitions of B_5, one repetition of B_1, B_2, and B_3 analysed. On the second validation day, B_4 was used as calibration, with three repetitions of B_1, one repetition of B_2, B_3, and B_4 analysed. On the third validation day, B_2 was used as calibration, with three repetitions of B_2, one repetition of B_3, B_4, and B_5 analysed. The uncertainty was estimated according to the Nordtest guidelines [105] modified to take the carry-over effect into account (Equation 6). The carry-over effect was estimated by quantifying the glyphosate peak in solvent injection (deionised water) acquired after an injection of the 25 μg kg^{-1} calibration level. Pooled standard deviation was used to estimate the repeatability (RSD_R) and within-laboratory reproducibility (RSD_{WR}), expressed as relative standard deviation. For estimation of repeatability, *RSD* of measurements performed on the same sample on the same day were pooled with the pooled standard deviation, resulting in 9 repetitions for each calibration level.

For estimation of within-laboratory reproducibility, measurements performed on the same sample on different days were pooled, resulting in 12 repetitions for each calibration level. Bias was estimated as the root-mean-square of a total of 11 bias determinations, expressed as relative differences from 100% recovery for 5 different blank samples at each level, after excluding the samples used as calibrants on the respective days.

A sensitive LC-MS/MS method consisting of SPE extraction and detection using tandem mass spectrometry has been developed and validated to determine the content of glyphosate in 100 samples of beer sold in local supermarkets in Latvia. The analytical procedure developed and applied in this study has a superior sensitivity compared to the currently published methods for the determination of glyphosate in beer. The content of glyphosate in beer varied from below the LOD of 0.2 μg kg^{-1} up to 150 μg kg^{-1}, with a median value of 2.9 μg kg^{-1} and an average value of 7.5 μg kg^{-1}. The selected samples represented most of the beer brands and varieties sold in local supermarkets by producers and distributors from Latvia. Our results show that glyphosate content up to 150 μg kg^{-1} can occur in beer, which is higher than previously reported, however, even the maximum glyphosate residue quantified does not lead to suspicion that the current MRL [104] in barley or wheat used in the production could have been exceeded. Malt type, beer type by colour, presence of precipitate, type of packaging, use of filtration and pasteurisation in manufacturing were not found to correlate with the content of glyphosate in beer. Our results show that the glyphosate content was significantly higher ($p<0.01$) in those samples of beer that did not have the country of production disclosed on the label and were sold in local supermarkets by distributors from Latvia (1.8 μg kg^{-1} median in locally produced beer, 6.7 μg kg^{-1} median in beer of undisclosed origin).

3.7.2. Validation and application of the novel derivatization-based LC-MS/MS method to analysis of food of plant and animal origin

The proposed derivatization-based procedure was validated with acceptable quantitative results, and in the case of Orbitrap™ detection LOQ's in the range from 10 to 25 μg kg^{-1} were observed. During the method optimization and validation for honey samples, several samples were found to contain glyphosate residues below the LOQ – approx. 5.9 and 7.0 μg kg^{-1}, indicating on a good applicability of the proposed method. With low resolution tandem mass spectrometric detection, in the case of complex matrices such as liver and kidney, the LOQ's increased from 10 up to 250 μg kg^{-1}. This increase in LOQ can be explained by increase in the background signal due to low resolution mass spectrometry. Therefore, high resolution mass spectrometry can be used to resolve interferences and significantly improve the LOQ of derivatized analytes in complex food matrices.

It was concluded that high resolution mass spectrometric detection is preferable for analysis of the derivatized analytes, in order to achieve the lowest LOQ's regardless of matrix. Along with improved signal-to-noise of peak profiles, the absolute peak intensities were higher, and the median measurement uncertainty and standard deviation of calibration curve slope was lower in the case of the derivatization-based procedure, which could be explained by better stability of the electrospray process and lower ion suppression with the mobile phases containing 0.1% formic acid, instead of 0.5-1.2%, which must be used with the Waters™ anionic pesticides column. Most of matrix effect was found to originate in the derivatization step with the dansyl derivatization-based determination, while the matrix effect in the LC-MS stage was low. Matrix effects increased as follows: milk < cucumber < honey < porridge formula < bovine kidney < bovine liver. The proposed procedure constitutes a useful and reliable alternative for the determination of glyphosate, AMPA and glufosinate, particularly because the sample preparation is simple, does not require preconcentration, and the main by-products of the derivatization reagent present in large excess have been demonstrated to elute before the derivatized polar analytes and, therefore, would not contribute to an increasing baseline between repeated injections, and can be diverted away from the mass spectrometer.

3.7.3. Validation and application of the optimized LC-MS/MS method to analysis of unsuccessful eggs from wild birds

A total of 57 unsuccessful eggs from wild birds were analyzed for glyphosate and AMPA. The egg samples from different locations and few species of birds, including stork and eagle, were obtained and provided for analysis by ornithologists.

Due to foraging behaviour of birds, such as stork and eagle, and due to the widespread use of glyphosate containing herbicides near or at the natural habitats of these bird species, there is a risk for consumption of contaminated prey that could result in detectable glyphosate residues in the eggs of these bird species. A recent controlled long-term experimental study in a bird model revealed that consumption of glyphosate containing herbicide contaminated feed, at lower concentration levels than applied in older studies (200 mg kg^{-1} of glyphosate from glyphosate-based herbicide) and without use of direct injections to the eggs, resulted in detectable glyphosate residues in the eggs. Although a significant, approx. 500-fold reduction in residue concentration from feed to eggs was observed, it was found that overall embryonic development was affected, while no differences in egg quality were observed. Oxidative stress biomarkers were determined, of which higher lipid damage expression in the embryonic brain tissue for the exposed group was the only significant finding. It should be noted that these findings do not distinguish the observed effects between effects from glyphosate and the

adjuvants in glyphosate-based herbicides [15]. The toxicity exposure ratio values have been shown to be insufficiently high for some non-target terrestrial vertebrates, indicating that there is some risk for exposure to glyphosate and glyphosate-based herbicides above the recommended level.

The unsuccessful egg samples received for analysis varied in their age, state of freshness, amount and species. Therefore, it was expected that the reproducibility and limits of detection may be specific to the individual samples depending on their properties.

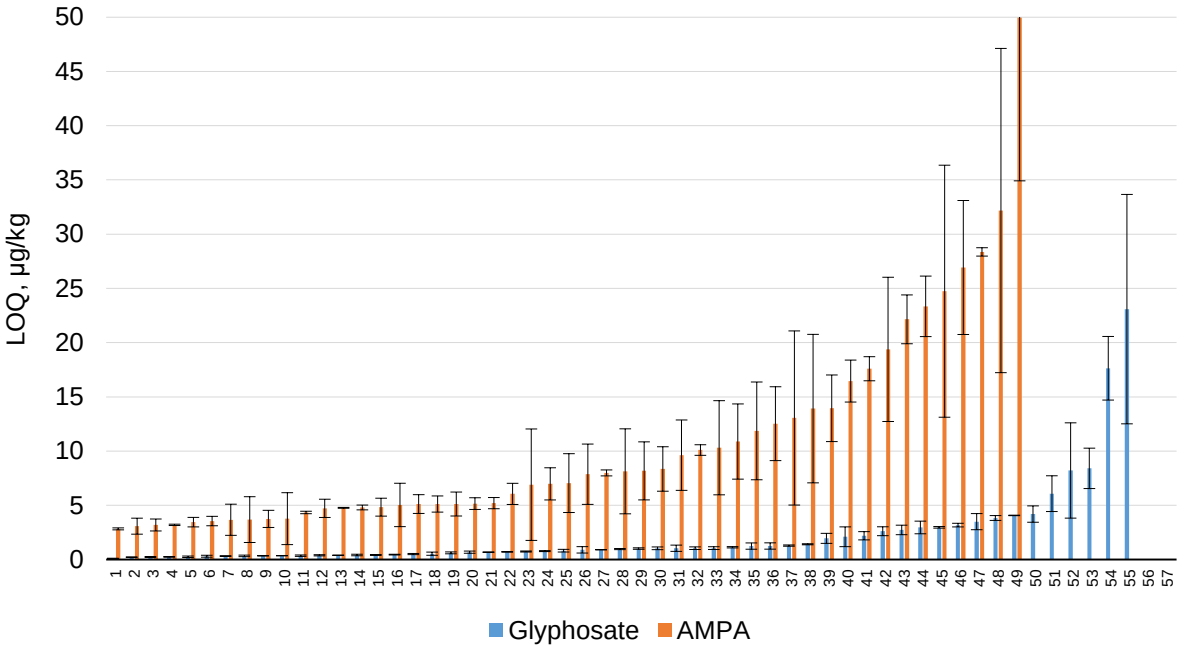

Figure 3.15. **Sample-specific estimated LOQ's for glyphosate (blue) and AMPA (red) in unsuccessful eggs from wild birds. The error bars represent the uncertainty of LOQ from triplicate measurements. For estimation of LOQ 8 samples were out of measurement range for AMPA, and 2 samples were out of measurement range for glyphosate. The analytes were not detected in any of the samples.**

Under the conditions described in 2.6.7, glyphosate eluted in 2.2 min and AMPA eluted in 0.75 min during a 10 min run. Drifting of retention time, a known and often reported problem for polar analytes analysed with porous graphitic carbon [87], was not observed, indicating that sufficient amount of matrix was present in the final extracts for sufficient coating of the active sites on the porous graphitic carbon stationary phase. A one-point calibration, containing 10 µg L^{-1} of each analyte and 20 µg L^{-1} of the respective internal standards, was used for screening of the prepared samples. Glyphosate and AMPA was not detected in any of the samples, and the chromatograms were free of interfering peaks. The ratio of response factors for the analytes to the respective internal standards was determined and taken into account together with the instrumental LOQ in order to estimate the LOQ in each individual sample.

Recovery of the analytes was validated first-hand by procedural analysis of different samples spiked to 100 μg kg^{-1} with glyphosate and AMPA, and the respective internal standards before the extraction. Four chicken eggs aged at room temperature and 5 fresh chicken eggs, as well as a random selection of 5 of the received unsuccessful eggs from wild birds were selected for validation of recovery. The average recovery was 106% for glyphosate and 118% for AMPA. The individual recoveries ranged from 101% to 112% for glyphosate and from 105% to 145% for AMPA. The values of internal standard corrected recovery were distributed homogeneously across the different samples and were not dependent on the nature of the sample, however the estimated LOQ was significantly lower in all chicken eggs. The uncertainties of LOQ were consistent with the measurement uncertainty estimated from spiking experiments. The increase in LOQ without an associated deterioration in reproducibility could be explained by extraction losses in the SPE stage and matrix effects in the LC-MS stage. Matrix effect was evaluated for a mixture of different samples by comparison of response factor in spiked blank sample and standards in solvent. The average matrix effect was –83%. The resulting decrease in signal due to extraction losses and matrix effects was compensated by quantification against the internal standards, which were added to the samples before extraction. The median LOQ was 1.0 μg kg^{-1} for glyphosate and 7.1 μg kg^{-1} for AMPA in the unsuccessful eggs from wild birds. The LOQ's for glyphosate ranged from 0.11 to 23 μg kg^{-1}, and the LOQ's for AMPA ranged from 2.8 to 50 μg kg^{-1}. The large differences in LOQ between different samples could be explained by different ionic strength of the samples loaded to the anion exchange column after the RP column clean-up.

Measurement uncertainty was estimated from the spiking experiments at 6% for glyphosate and 24% for AMPA. LOQ's for glyphosate and AMPA in unsuccessful eggs from wild birds given in Figure 3.15. The measurements for estimation of LOQ were performed in triplicate, following the full sample preparation procedure described in 2.6.7. Glyphosate and AMPA was not detected in any of the unsuccessful egg samples.

Three different sample preparation procedures were compared – dilution with 1% formic acid in methanol; methanolic hydrochloric acid extraction followed by evaporation and reconstitution; and methanolic hydrochloric acid extraction followed by solid phase clean-up and extraction. The latter procedure showed the highest signal, therefore the signal was compared with three different analytical columns and appropriate mobile phases, of which a quaternary ammonium-based ion exchange column from Metrohm showed the highest signal (Figure 3.16).

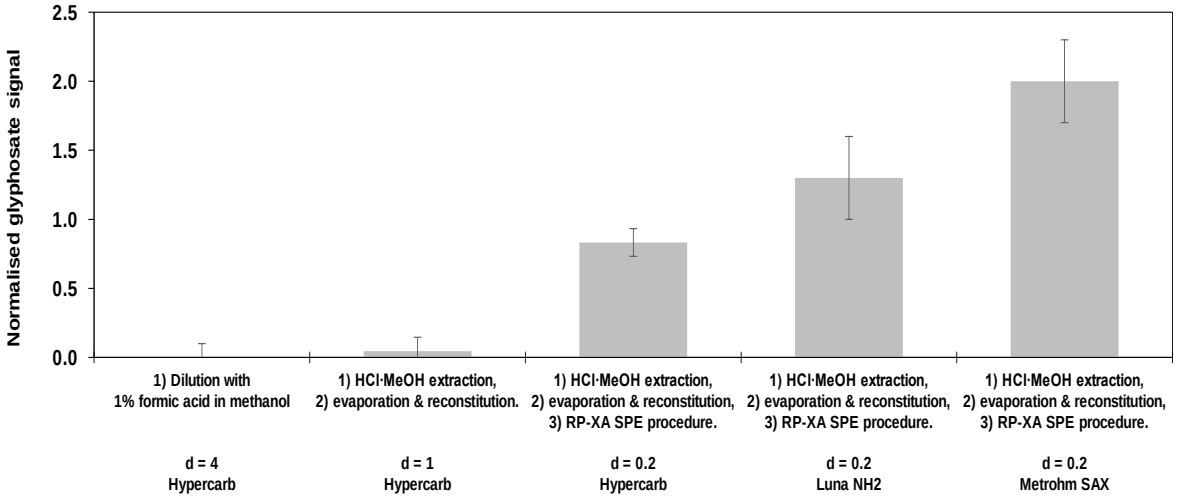

Figure 3.16. **Comparison of glyphosate response factors in egg samples with the SPE-based sample preparation given in 2.6.7, analyzed with different stationary phases and appropriate mobile phases. Results with sample preparation consisting of simple dilution and extraction without use of the SPE procedure are given for comparison. The matrix effect was -83% with the Hypercarb stationary phase and -60% with the Metrohm SAX stationary phase.**

Hypercarb was chosen for analysis due to a more LC-MS compatible mobile phase and the possibility to perform analysis in isocratic mode with reduced retention times compared to the other columns. However, the matrix effect was higher with the Hypercarb column, than with the ion exchange column. The matrix effects were compensated using internal standards. The retention factors of glyphosate increased in the following order with the tested analytical columns: Hypercarb << Luna NH_2 < Metrohm SAX. The reduction in matrix effect with the Metrohm SAX column can be explained by improved separation of the analyte from interferences, however, an influence due to the different mobile phases cannot be excluded.

The large differences in glyphosate signal between non-SPE and SPE-based sample preparation, taking into account the dilution factor, can be explained by improved clean-up in case of SPE-based procedures. The low signal in case of non-SPE procedures could be explained by interferences from the matrix co-eluting with the analyte, as the retention factor of glyphosate and AMPA is low with the Hypercarb column.

3.8. Overview of the optimised and developed methods for determination of glyphosate and AMPA.

Generally, regardless of analytical columns and the mobile phases that were tested, the LOD for glyphosate in standard solution with a typical injection volume to the analytical column coupled to a mass spectrometer was approx. 3 $\mu g\ L^{-1}$ for glyphosate and AMPA. The derivatization-based procedure, as described in 2.6.4, was found to be more sensitive in

combination with Orbitrap mass spectrometer, where the LOD was approx. 0.1 μg L^{-1} for AMPA and 1 μg L^{-1} for glyphosate and glufosinate in drinking water.

As it was shown experimentally, preconcentration of underivatized glyphosate at preconcentration factors > 5 may only be feasible for samples of very low ionic strength, due to signal suppression by pre-concentrated matrix components or the possibility that salts and matrix components present in the sample extract could act as eluent, decreasing recovery and detectable signal. This could be compensated to some extent by using ILIS. Furthermore, preconcentration of extracts from solid samples is even more difficult and an evaporation step may be necessary before SPE, as described in 2.6.7, due to the need to desorb strongly anionic glyphosate from matrix components during extraction. This desorption can be achieved by strong bases, acids or high ionic strength, e.g., as performed using potassium hydroxide in 2.6.1. or EDTA in 2.6.4 and 2.6.5. Thus with non-derivatization methods, injection of minimally diluted samples would be preferable to ensure highest signal in the mass spectrometer. If the analytical column is such, that it needs to be operated under relatively weak or dilute buffers, acids or bases, injection of such strongly basic or acidic extracts will result in altered peak shapes and retention times. A dilution or neutralization step in such case could be performed, however, at the expense of analyte signal.

The recently invented diethylamino-based stationary phase from Waters™ currently offers, according to the obtained results, the best sensitivity for glyphosate and AMPA detection in undiluted highly acidic EDTA-containing extracts from complex matrices. The only drawbacks are, arguably, the HILIC retention mechanism, which lead to peak tailing, and the mobile phases containing 0.9% formic acid, which may suppress ion signals in mass spectrometry, compared to conventional mobile phases.

The novel dansyl chloride derivatization-based method showed better overall precision and higher sensitivity in drinking water, compared to the diethylamino column. However, the highest sensitivity in complex matrices was obtained only with high resolution Orbitrap detection, indicating that the increase in LOQs with triple quadrupole mass spectrometer for complex matrices was due to resolution of peaks in mass spectra and not due to reduced derivatization reaction yield.

The limits of detection in real samples of food or environmental origin, depended on the sample preparation procedure and the nature of samples. The optimised and developed methods described in this study and the ranges of LOQs are summarised in Table 3.2. together with remarks on the limitations, advantages and important observations.

Table 3.2.

Summary on the optimised and developed methods.

Column, eluent and detector	Sample preparation	LOQ (glyphosate)	Important observations
Hypercarb (1% aqueous acetic acid) (QqQ mass spectrometer)	Extraction in aqueous potassium hydroxide and dilution *Section 2.6.1 and 2.6.2*	Soil extract, soil leachate – 0.2 mg kg^{-1} Wastewater – 1 mg L^{-1}	– Drifting of retention time (30%) during long sequences
Hypercarb (1% aqueous acetic acid) (QqQ mass spectrometer)	Extraction in methanolic hydrochloric acid and 2-step solid phase extraction and preconcentration *Section 2.6.7*	Chicken eggs – 0.11 μg kg^{-1} Unsuccessful wild bird eggs – 0.11-20 μg kg^{-1}	– Limits of quantitation depend on nature of sample
Luna NH_2 (10 mmol L^{-1} aq. amm. hydrogen carbonate at pH 10 and acetonitrile) (QqQ mass spectrometer)	2-step solid phase extraction and preconcentration *Section 2.6.6*	Beer – 0.5 μg kg^{-1}	– Column life expectancy may be reduced due to high eluent pH
Metrohm SAX (0.25-0.75 mM aq. edetate pH 10 and acetonitrile) (QqQ mass spectrometer)	Extraction in mobile phase *Section 2.6.3*	Oat flour – 10 μg kg^{-1} Beer – 10 μg kg^{-1}	– Compatibility of the non-volatile eluent with the ionisation source and particular settings of the mass spectrometer needs to be validated – Injection of acidic extracts will distort peaks and shift retention
Waters™ anionic pesticide column (0.5-1.2% formic acid in acetonitrile and water) (QqQ mass spectrometer)	QuPPe extraction protocol (1.5% formic acid in 50% methanol, + 0.25% EDTA) *Section 2.6.5*	Honey, porridge formula, bovine liver, bovine kidney, milk – 10 μg kg^{-1} Drinking water – 3 μg L^{-1}	+ Excellent selectivity towards the analytes regardless of tested matrix – Precision may be worse due to HILIC type elution, ion suppression due to eluent and grater matrix effects in the LC-MS/MS stage
Luna C_{18} column (0.1% formic acid in acetonitrile and water) (QqQ mass spectrometer)	Extraction in aqueous EDTA and derivatization with dansyl chloride *Section 2.6.4*	Milk – 10 μg kg^{-1} Honey – 25 μg kg^{-1} Porridge formula – 50 μg kg^{-1} Bovine liver, bovine kidney – 250 μg kg^{-1} Drinking water – 1 μg L^{-1}	– Limits of quantitation depend on nature of sample
Luna C_{18} column (0.1% formic acid in acetonitrile and water) (Orbitrap mass spectrometer)	Extraction in aqueous EDTA and derivatization with dansyl chloride *Section 2.6.4*	Honey, milk – 10 μg kg^{-1} Porridge formula, bovine liver, bovine kidney – 25 μg kg^{-1} Drinking water – 1 μg L^{-1}	+ Excellent selectivity towards the analytes + Improved precision due to reversed phase retention and more stable electrospray with lower ion suppression due to eluent and low matrix effects in the LC-MS/MS stage

CONCLUSIONS

1. A novel and reliable dansyl chloride derivatisation-based LC-MS/MS method for the determination of glyphosate, AMPA and glufosinate in foods of plant and animal origin has been developed and validated. The method showed good performance in combination with high resolution Orbitrap mass spectrometry, with LOQs in complex food samples ≤ 25 μg kg^{-1}, and improved precision, with expanded uncertainties $\leq 25\%$, compared to analysis with the best currently available column – the recently invented diethylamino stationary phase-based procedure. The improvement in precision could be explained by better stability of the electrospray process and lower ion suppression with the mobile phases used with reversed phase chromatography containing 0.1% formic acid, instead of 0.5-1.2%, which must be used with the Waters™ anionic pesticides column.
2. The investigation into the extent of matrix effect in the novel and reliable dansyl chloride derivatisation-based LC-MS/MS method for the determination of glyphosate, AMPA and glufosinate in foods of plant and animal origin showed that most of matrix effect originated in the derivatization step, while the matrix effect in the LC-MS stage was low. Matrix effects increased as follows: milk < cucumber < honey < porridge formula < bovine kidney < bovine liver.
3. It was concluded that high resolution mass spectrometric detection is preferable for analysis of the dansyl chloride derivatized analytes, in order to achieve the lowest LOQ's in all matrices, indicating that the increase in LOQs with triple quadrupole mass spectrometer for complex matrices was due to resolution of peaks in mass spectra and not due to reduced derivatization reaction yield.
4. The investigation into the applicability of unsuppressed ion chromatography coupled to tandem mass spectrometry showed that determination of ionic and acidic pesticides with low matrix effects may be possible using mobile phases that contain <1 mmol L^{-1} of buffer. It was concluded that additional validation efforts are necessary before non-volatile buffers can be used, because they may interfere with the precursor ion mass spectrum leading to high and unstable background levels for some transitions, and that non-volatile buffers may not be suitable for all electrospray ionisation sources.

5. Multi-stage solid phase extraction-based sample preparations were investigated with respect to sensitivity gains compared to dilution-based sample preparation while combining different solid phase extraction sorbents. The analytical procedure developed and applied had a superior sensitivity compared to the currently published methods for the determination of glyphosate in beer. The elaborated multi-stage solid phase extraction method was validated and applied for analysis of 100 beer samples from Latvian market with an LOQ of 0.5 μg kg^{-1}.
6. Study results showed glyphosate content up to 150 μg kg^{-1} in beer, higher than previously reported, however, even the maximum glyphosate residue quantified does not lead to suspicion that the current maximum residue limit in barley or wheat used in the production could have been exceeded. Malt type, beer type by colour, presence of precipitate, type of packaging, use of filtration and pasteurisation in manufacturing were not found to correlate with the content of glyphosate in beer in any way. Study results showed that the glyphosate content was significantly higher ($p<0.01$) in those samples of beer that did not have the country of production disclosed on the label and were sold in local supermarkets by distributors from Latvia (1.8 μg kg^{-1} median in locally produced beer, 6.7 μg kg^{-1} median in beer of undisclosed origin).
7. The elaborated multi-stage solid phase extraction method with optimised extraction step was applied for analysis of unsuccessful eggs from wild birds. The variability of the limit of quantification was investigated after analysis of a large number of unsuccessful eggs from wild birds. The LOQ's for glyphosate ranged from 0.11 to 23 μg kg^{-1}, and the LOQ's for AMPA ranged from 2.8 to 50 μg kg^{-1}. The large differences in LOQ between different samples could be explained by different ionic strength of the samples loaded to the anion exchange column after the reversed phase column clean-up.
8. The treatment of raw municipal wastewater in the presence of oxide ceramics demonstrated the highest removal efficiency of glyphosate, compared to other wastewater treatment methods. Results on glyphosate removal indicated that microorganisms in wastewater have a comparatively high resistance to glyphosate-based herbicide and represent a source for glyphosate-based herbicide biodegradation in wastewater.
9. In sandy soil the addition of microorganisms led to a significant ($p<0.01$) increase of glyphosate leaching, compared to the non-augmented soil. The presence of glyphosate-based herbicide in sandy soil and loamy sand soil types under tested conditions increased the toxicity of soil leachates from 10–13% up to 50–54%. No significant differences in the ecotoxicity of bioaugmented and non-bioaugmented soils were found.

ACKNOWLEDGEMENTS

I sincerely thank my scientific supervisors, associate professor, *Dr. chem.* Vadims Bartkevičs and *Dr. chem.* Iveta Pugajeva for valuable guidance, allocation of resources and infrastructure for the development of this thesis and the critical revision of scientific manuscripts produced as a result of this work. I sincerely thank the co-authors with whom the scientific publications were produced as a result of this thesis and the publications, to which this thesis provided a contribution.

A part of the research was funded by the National Research Program "Agricultural Resources for Sustainable Production of Qualitative and Healthy Foods in Latvia" (AgroBioRes) project No. 4 "Sustainable use of local agricultural resources for qualitative and healthy food product development".

A part of the research was funded by the project "Sustainable use of natural resources in the context of climate change" (No. ZD2016 AZ03).

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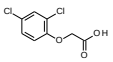
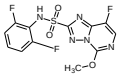
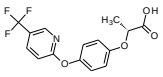
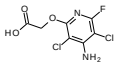
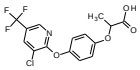
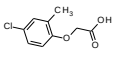
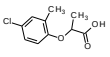
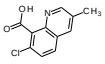
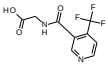
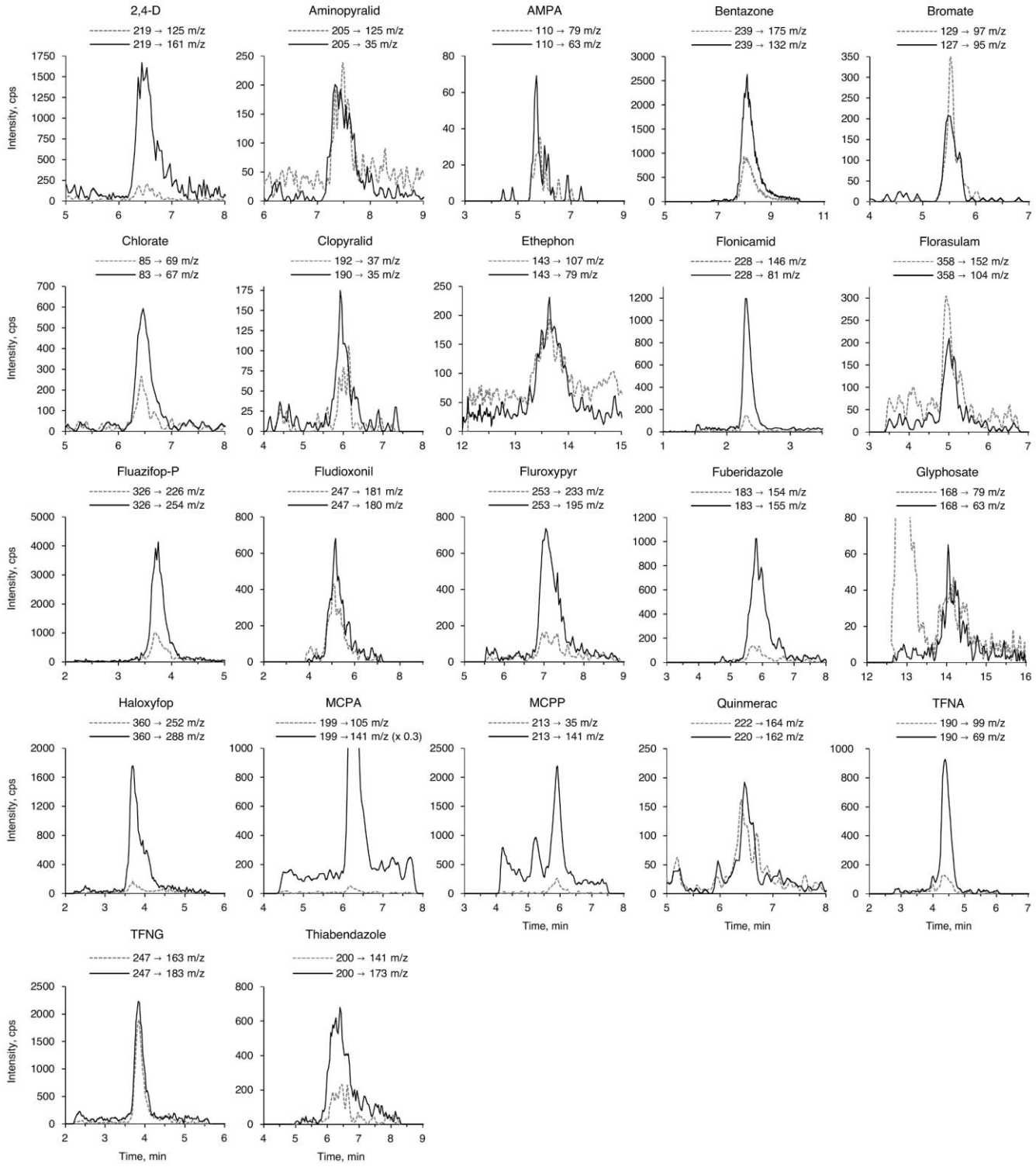
ANNEXES

Annex 1

MS/MS transitions, collision energies, chemical structures and properties of the analyzed pesticides.

Analyte	Quantification product ion (m/z)	Collision energy (V)	Confirmation product ion (m/z)	Collision energy (V)	Chemical structure of analyte	pK_a	logP
2,4-D	219 → 161	-20	219 → 125	-35		3.4	2.6
Aminopyralid	205 → 35	-50	205 → 125	-25		2.6	1.6
AMPA	110 → 63	-25	110 → 79	-40		0.9	-2.8
Bentazone	239 → 132	-35	239 → 175	-30		3.5	2.8
Bromate	127 → 95	-40	129 → 97	-40		-2	-
Chlorate	83 → 67	-30	85 → 69	-30		-1	-
Clopyralid	190 → 35	-35	192 → 37	-35		2.0	1.3
Ethephon	143 → 79	-25	143 → 107	-12		2.8	-1.4
Flonicamid	228 → 146	-30	228 → 81	-15		11.6	0.8
Florasulam	358 → 104	-55	358 → 152	-45		8.8	1.6
Fluazifop-P	326 → 254	-25	326 → 226	-35		3.1	2.9
Fludioxonil	247 → 180	-40	247 → 181	-30		14.1	2.6
Fluroxypyr	253 → 195	-20	253 → 233	-10		2.9	1.8
Fuberidazole	183 → 155	-30	183 → 154	-40		10.1	2.2
Glyphosate	168 → 63	-20	168 → 79	-60		0.8	-2.4
Haloxyfop	360 → 288	-20	360 → 252	-35		2.9	4.2
Ioxynil	370 → 127	-50	370 → 215	-45		5.6	3.6
MCPA	199 → 105	-40	199 → 141	-20		3.7	2.5
MCPP	213 → 35	-60	213 → 141	-20		3.1	2.8
Quinmerac	220 → 162	-20	222 → 164	-20		4.3	1.8
TFNA	190 → 69	-45	190 → 99	-40		2.6	1.5
TFNG	247 → 183	-20	247 → 163	-25		2.8	0.4
Thiabendazole	200 → 173	-30	200 → 141	-42		10.3	2.5

The analyte peaks from spiked oat flour extract obtained with the 0.25-0.75 mM EDTA procedure (Table 2.2). The spiked concentration was 20 µg kg^{-1} of aminopyralid, clopyralid, fluroxypyr, and thiabendazole, and 10 µg kg^{-1} of 2,4-D, AMPA, bentazone, bromate, chlorate, ethephon, flonicamid, florasulam, fluazifop-P, fludioxonil, fuberidazole, glyphosate, haloxyfop, MCPA, MCPP, quinmerac, TFNA and TFNG.



Mass spectrometric detection parameters for determination of dansyl chloride derivatized glyphosate, glyphosate-$^{13}C_2{}^{15}N$, AMPA, AMPA-$^{13}C^{15}N$, glufosinate and glufosinate-D_3.

Detector	Analyte	Polarity	Precursor m/z	CE (V)	Electrospray ionization source and detection parameters
Thermo Scientific Q Exactive Orbitrap™	Dansyl AMPA	Negative	343.0523	15	Sheath gas flow rate = 35 Auxiliary gas flow rate = 7 Sweep gas flow rate = 0 Capillary temperature = 300 °C Auxiliary gas temperature = 275 °C Spray voltage = -3.00 kV Parallel reaction monitoring mode Maximum IT = 1000 ms Precursor isolation window = 0.4 m/z Resolution = 140 000
	Dansyl AMPA-$^{13}C^{15}N$	Negative	345.0527	15	
	Dansyl glyphosate	Negative	401.0578	27	
	Dansyl glyphosate-$^{13}C_2{}^{15}N$	Negative	404.0615	27	
	Dansyl glufosinate	Negative	413.0942	20	
	Dansyl glufosinate-D_3	Negative	416.1130	20	

Detector	Analyte	Polarity	Precursor m/z	Fragment m/z	CE (V)	Electrospray ionization source and detection parameters
Thermo Scientific Quantis™	Dansyl AMPA	Negative	343.1	63	23	Sheath gas flow rate = 35 Auxiliary gas flow rate = 7 Sweep gas flow rate = 0 Capillary temp. = 300 °C Auxiliary gas temp. = 275 °C Spray voltage = 5.00 |kV| Selected reaction monitoring mode Cycle time = 2 s Resolution (Q1 & Q3) = 0.7 CID gas = 1.5 mTorr
		Positive	345.0	252	15	
	Dansyl AMPA-$^{13}C^{15}N$	Negative	345.1	63	23	
		Positive	347.1	252	20	
	Dansyl glyphosate	Negative	401.0	148	22	
		Positive	403.1	257	15	
	Dansyl glyphosate-$^{13}C_2{}^{15}N$	Negative	404.1	124	20	
		Positive	406.1	260	10	
	Dansyl glufosinate	Negative	413.1	134	20	
		Positive	415.1	164	20	
	Dansyl glufosinate-D_3	Negative	416.1	137	20	
		Positive	418.1	167	20	

Mass spectrometric detection parameters for determination of underivatized glyphosate, glyphosate-$^{13}C_2{}^{15}N$, AMPA, AMPA-$^{13}C^{15}N$, glufosinate and glufosinate-D_3.

Detector	Analyte	Polarity	Precursor m/z	Fragment m/z	CE (V)	Electrospray ionization source and detection parameters
Thermo Scientific Quantis™	AMPA	Negative	110	63	18	Sheath gas flow rate = 35 Auxiliary gas flow rate = 7 Sweep gas flow rate = 0 Capillary temp. = 300 °C Auxiliary gas temp. = 275 °C Spray voltage = 3.00 |kV| Selected reaction monitoring mode Dwell time = 90 ms Resolution (Q1 & Q3) = 1.2 CID gas = 1.5 mTorr
		Negative	110	81	12	
	AMPA-$^{13}C^{15}N$	Negative	112	63	16	
	Glyphosate	Negative	168	63	22	
		Negative	168	81	15	
	Glyphosate-$^{13}C_2{}^{15}N$	Negative	171	63	22	
		Negative	171	126	9	
	Glufosinate	Negative	180	63	35	
		Negative	180	85	18	
	Glufosinate-D_3	Negative	183	63	44	
		Negative	183	98	16	

Precursor and fragment ions of dansyl chloride derivatized glyphosate, glyphosate-$^{13}C_2{}^{15}N$, AMPA, AMPA-$^{13}C^{15}N$, glufosinate and glufosinate-D_3 in high resolution at 50 µg L^{-1}. Ions written in bold letters were used in validation of the dansyl chloride derivatization based procedure.

Analyte	Polarity	Precursor m/z (theoretical)	Precursor m/z (observed)	Δm/z (ppm)	Fragment m/z	Collision energy (V)	Ion intensity
Dansyl AMPA	Negative	**343.0523**	343.0530	+2.04	**62.9626**	-20	$1.01 \cdot 10^6$
					343.0523	-10	$9.30 \cdot 10^5$
					80.9732	-20	$2.00 \cdot 10^4$
Dansyl AMPA-$^{13}C^{15}N$	Negative	**345.0527**	345.0536	+2.61	**62.9626**	-20	$9.56 \cdot 10^5$
					345.0527	-10	$7.63 \cdot 10^5$
					80.9732	-20	$2.08 \cdot 10^4$
Dansyl AMPA	Positive	345.0669	345.0662	-2.03	252.0690	15	$1.64 \cdot 10^5$
					174.0914	15	$1.43 \cdot 10^5$
					171.1043	15	$1.38 \cdot 10^5$
					234.0584	15	$9.00 \cdot 10^4$
					345.0669	10	$8.00 \cdot 10^4$
					263.0849	15	$7.50 \cdot 10^4$
					115.0545	60	$5.25 \cdot 10^4$
					203.0942	40	$4.75 \cdot 10^4$
					202.0865	15	$3.00 \cdot 10^4$
					186.0914	15	$2.15 \cdot 10^4$
					199.1231	15	$1.78 \cdot 10^4$
					248.0616	30	$1.43 \cdot 10^4$
Dansyl AMPA-$^{13}C^{15}N$	Positive	347.0673	347.0666	-2.02	174.0916	15	$1.25 \cdot 10^5$
					252.0690	15	$1.25 \cdot 10^5$
					171.1044	15	$1.13 \cdot 10^5$
					234.0585	15	$6.88 \cdot 10^4$
					347.0673	10	$5.56 \cdot 10^4$
					265.0857	15	$5.00 \cdot 10^4$
					115.0546	60	$4.88 \cdot 10^4$
					203.0943	40	$4.38 \cdot 10^4$
					202.0864	15	$2.50 \cdot 10^4$
					186.0915	15	$2.00 \cdot 10^4$
					254.0650	15	$1.63 \cdot 10^4$
					201.1236	15	$1.25 \cdot 10^4$
					250.0620	30	$1.06 \cdot 10^4$

Analyte	Polarity	Precursor m/z (theoretical)	Precursor m/z (observed)	Δm/z (ppm)	Fragment m/z	Collision energy (V)	Ion intensity
Dansyl AMPA-$^{13}C^{15}N$	Positive	347.0673	347.0666	-2.02	236.0540	15	$8.75 \cdot 10^3$
Dansyl glyphosate	Negative	**401.0578**	401.0589	+2.74	**401.0578**	-10	$1.75 \cdot 10^5$
					78.9575	-60	$1.05 \cdot 10^5$
					121.9999	-20	$8.00 \cdot 10^4$
					147.9793	-20	$5.00 \cdot 10^4$
					234.0593	-30	$4.00 \cdot 10^4$
					165.9901	-15	$3.75 \cdot 10^4$
					357.0687	-20	$3.00 \cdot 10^4$
					94.9889	-40	$2.75 \cdot 10^4$
					383.0481	-15	$2.10 \cdot 10^4$
					62.9626	-60	$1.05 \cdot 10^4$
Dansyl glyphosate-$^{13}C_2{}^{15}N$	Negative	**404.0615**	404.0622	+1.73	**404.0615**	-10	$1.25 \cdot 10^5$
					78.9575	-60	$8.75 \cdot 10^4$
					124.0003	-20	$5.31 \cdot 10^4$
					150.9831	-20	$3.50 \cdot 10^4$
					234.0593	-30	$3.13 \cdot 10^4$
					359.0691	-20	$2.56 \cdot 10^4$
					94.9889	-40	$1.81 \cdot 10^4$
					386.0518	-15	$1.69 \cdot 10^4$
					167.9905	-20	$8.13 \cdot 10^3$
Dansyl glyphosate	Positive	403.0723	403.0712	-2.73	252.0691	15	$9.00 \cdot 10^4$
					171.1044	15	$1.48 \cdot 10^4$
					403.0723	10	$1.13 \cdot 10^4$
					237.0455	50	$9.00 \cdot 10^3$
					174.0915	30	$9.00 \cdot 10^3$
					234.0585	15	$8.00 \cdot 10^3$
					88.0399	15	$5.50 \cdot 10^3$
					155.0731	50	$5.00 \cdot 10^3$
					257.1286	15	$4.75 \cdot 10^3$
					154.0652	60	$4.00 \cdot 10^3$
					275.0850	15	$3.75 \cdot 10^3$
					128.0623	60	$3.00 \cdot 10^3$
Dansyl glyphosate-$^{13}C_2{}^{15}N$	Positive	406.0761	406.0751	-2.46	252.0691	15	$7.50 \cdot 10^4$
					171.1045	15	$1.13 \cdot 10^4$
					406.0761	10	$1.00 \cdot 10^4$
					174.0916	30	$7.50 \cdot 10^3$

Analyte	Polarity	Precursor m/z (theoretical)	Precursor m/z (observed)	Δm/z (ppm)	Fragment m/z	Collision energy (V)	Ion intensity
Dansyl glyphosate-$^{13}C_2{}^{15}N$	Positive	406.0761	406.0751	-2.46	234.0585	15	$7.50 \cdot 10^3$
					237.0447	40	$7.50 \cdot 10^3$
					91.0436	15	$4.94 \cdot 10^3$
					155.0730	60	$4.69 \cdot 10^3$
					260.1322	15	$4.25 \cdot 10^3$
					154.0652	60	$4.13 \cdot 10^3$
					128.0623	60	$3.13 \cdot 10^3$
					277.0856	15	$2.69 \cdot 10^3$
Dansyl glufosinate	Negative	**413.0942**	413.0925	-4.11	**134.0364**	30	$3.75 \cdot 10^5$
					413.0951	10	$3.75 \cdot 10^5$
					234.0593	10	$3.63 \cdot 10^5$
					178.0266	20	$2.38 \cdot 10^5$
					62.9626	60	$7.92 \cdot 10^4$
					78.9939	50	$7.50 \cdot 10^4$
					249.0706	30	$2.13 \cdot 10^4$
					143.0490	40	$1.92 \cdot 10^4$
					170.0839	50	$1.17 \cdot 10^4$
					132.0207	40	$1.13 \cdot 10^4$
					186.0916	40	$1.00 \cdot 10^4$
					214.0868	40	$7.08 \cdot 10^3$
Dansyl glufosinate-D_3	Negative	**416.1130**	416.1141	+2.64	**416.1139**	10	$2.70 \cdot 10^5$
					137.0552	30	$2.50 \cdot 10^5$
					181.0455	20	$1.60 \cdot 10^5$
					234.0594	30	$6.50 \cdot 10^4$
					62.9626	60	$5.70 \cdot 10^4$
					82.0127	60	$3.30 \cdot 10^4$
					78.9575	50	$1.80 \cdot 10^4$
					249.0704	30	$1.50 \cdot 10^4$
					143.0490	40	$1.30 \cdot 10^4$
					170.0839	50	$8.60 \cdot 10^3$
					186.0916	40	$8.60 \cdot 10^3$
					135.0395	40	$7.40 \cdot 10^3$
Dansyl glufosinate	Positive	415.1087	415.1085	-0.48	164.0473	15	$1.67 \cdot 10^6$
					415.1088	10	$1.17 \cdot 10^5$
					136.0524	15	$6.67 \cdot 10^4$
					118.0420	40	$5.42 \cdot 10^4$

Analyte	Polarity	Precursor m/z (theoretical)	Precursor m/z (observed)	Δm/z (ppm)	Fragment m/z	Collision energy (V)	Ion intensity
Dansyl glufosinate	Positive	415.1087	415.1085	-0.48	369.1034	15	$5.00 \cdot 10^4$
					171.1044	15	$4.58 \cdot 10^4$
					172.1122	15	$3.79 \cdot 10^4$
					252.0690	15	$3.67 \cdot 10^4$
					174.0915	30	$3.38 \cdot 10^4$
					56.0503	60	$2.21 \cdot 10^4$
					155.0730	60	$2.17 \cdot 10^4$
					234.0584	15	$1.83 \cdot 10^4$
					154.0652	60	$1.54 \cdot 10^4$
					128.0623	60	$1.19 \cdot 10^4$
					170.0965	50	$1.17 \cdot 10^4$
					129.0700	50	$9.58 \cdot 10^3$
					202.0865	30	$9.17 \cdot 10^3$
					131.0732	60	$8.79 \cdot 10^3$
					168.0809	60	$7.08 \cdot 10^3$
Dansyl glufosinate-D_3	Positive	418.1276	418.1268	-1.91	167.0662	15	$1.20 \cdot 10^5$
					418.1277	10	$9.20 \cdot 10^4$
					139.0712	15	$4.80 \cdot 10^4$
					372.1223	15	$3.60 \cdot 10^4$
					171.1044	15	$3.20 \cdot 10^4$
					121.0608	40	$3.80 \cdot 10^4$
					172.1122	20	$1.00 \cdot 10^4$
					252.0691	15	$2.50 \cdot 10^4$
					174.0916	30	$2.50 \cdot 10^4$
					56.05030	60	$1.68 \cdot 10^4$
					234.0583	15	$1.30 \cdot 10^4$
					155.0730	60	$1.45 \cdot 10^4$
					154.0652	60	$9.60 \cdot 10^3$
					128.0623	60	$9.30 \cdot 10^3$
					129.0701	50	$7.50 \cdot 10^3$
					170.0966	50	$8.30 \cdot 10^3$
					202.0865	30	$6.50 \cdot 10^3$
					131.0732	50	$6.70 \cdot 10^3$
					146.0966	40	$4.70 \cdot 10^3$
					168.0810	60	$5.00 \cdot 10^3$

The research for doctoral thesis **"Development and application of mass spectrometric methods for the determination of glyphosate and its metabolites in plant and animal origin products and environmental objects"** was carried out at the Institute of Food Safety, Animal Health and Environment "BIOR" and University of Latvia from 2017 to 2020.

I hereby confirm that I have written the doctoral thesis independently, that I have not used other sources or facilities that the ones mentioned and that the submitted electronic copy of the work is identical to printed version.

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September 24th, 2020

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