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Analytical method development for polar pesticides using liquid chromatography

Master's Thesis
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Abstract

Pesticides are well-known and widely spread substances that have been heavily used for many decades, especially in the agricultural sector. Throughout these decades, the alarming ecological and toxicological effects of the pesticides were also noticed, often forcing a strong response, such as international ban of a specific compound and financing of numerous ecological investigations. However, complexity of organic chemicals and equipment limitations may often provide a challenge for reliable qualitative and quantitative analysis. Thus, it may often be crucial to look into different analytical methods and their development, in order to add up to variability, that could help further ecotoxicological investigations.

The aim of this master's thesis was to develop analysis methods for three commonly used and known pesticides, namely glyphosate, glufosinate and aminomethylphosphonic acid (AMPA). Two chromatography techniques were mainly used for these analysis procedures - hydrophilic interaction liquid chromatography with ultra-high performance liquid chromatography and tandem mass spectrometry (HILIC-UHPLC-MS/MS) and high-performance liquid with ultraviolet detection (HPLC-UV). The resulting methods were also compared with each other in order to evaluate their pros and cons.

During our experiments we managed to successfully develop methods for qualitative and quantitative analysis of glyphosate, AMPA, and glufosinate using HILIC-UHPLC-MS/MS and HILIC-HPLC-UV in the range of 1-50 µg/ml. Our methods demonstrated suitable analyte separation and produced well defined peaks for both derivatized and non-derivatized pesticides. Additionally, although HILIC-HPLC-UV method showed to be especially easy to operate and has been relatively consistent throughout the tests, non-derivatized pesticide analysis with HILIC-UHPLC-MS/MS seemed to provide more reliable results. Taking this into account, there is still room for improvement, which could be done in future experiments.

Tiivistelmä

Torjunta-aineet ovat hyvin tunnettuja ja laajalti levinneitä aineita, joita on käytetty runsaasti useiden vuosikymmenten ajan, erityisesti maatalousalalla. Näiden vuosikymmenten aikana on myös havaittu torjunta-aineiden hälyttäviä ekologisia ja toksikologisia vaikutuksia, mikä on usein johtanut esim. tietyn yhdisteen kansainväliseen kieltoon ja lukuisten ekologisten tutkimusten rahoittamiseen. Orgaanisten kemikaalien monimutkaisuus ja laitteiden rajoitukset voivat kuitenkin usein aiheuttaa haasteita luotettavalle laadulliselle ja määrälliselle analyysille. Siksi on usein tärkeää tarkastella erilaisia analyyttisiä menetelmiä ja niiden kehitystä, jotta voidaan lisätä vaihtelua, mikä voisi edelleen auttaa ekotoksikologisia tutkimuksia.

Tämän pro gradu -tutkielman tavoitteena oli kehittää analyysimenetelmiä kolmelle yleisesti käytetylle ja tunnetuille torjunta-aineelle - glyfosaatille, glufosinaatille ja aminometyylifosfonihapolle (AMPA). Näissä analyysimenetelmissä käytettiin pääasiassa kahta kromatografiatekniikkaa - hydrofiilistä vuorovaikutusnestekromatografiaa, ultrakorkean suorityskyvynnestekromatografia-tandemmassaspektrometrialla (HILIC-UHPLC-MS/MS) sekä korkean suorituskyvyn nestekromatografiaa UV-spektroskopialla (HPLC-UV). Menetelmiä myös vertailtiin keskenään niiden etujen ja ongelmien arvioimiseksi.

Kokeilujemme aikana onnistuimme kehittämään menetelmiä glyfosaatin, AMPA:n ja glufosinaatin kvalitatiiviseen ja kvantitatiiviseen analysointiin käyttämällä HILIC-UHPLC-MS/MS- ja HILIC-HPLC-UV 1–50 µg/ml alueella.

Menetelmät mahdollistivat analyyttien erottamisen ja havaitsemisen sekä derivatisoiduille että ei-derivatisoiduille torjunta-aineille. Lisäksi vaikka HILIC-HPLC-UV-menetelmä osoitti olevan erityisen helppokäyttöinen, oli suhteellisen johdonmukainen toimivuudessaan sekä tuloksissa koko testien aikana, HILIC-UHPLC-MS/MS:llä tehty johdannainen torjunta-aineanalyysi näytti tuottavan luotettavampia tuloksia. Tämä huomioon ottaen, parantamisen varaa on edelleen, mikä voitaisiin tehdä tulevissa kokeissa.

Foreword

This master's thesis was done as a part of the Master's degree program in the University of Jyväskylä at The Department of Chemistry. Experimental work was conducted in mass spectrometry laboratory YO309, between 17.11.2023-17.05.2024. The thesis was written until the end of May 2024. Thesis literature was searched via Web of Science, Reaxys and Google Scholar, and the literature review was limited to the topics which were relevant to the experimental project.

The work was instructed by Dr. Elina Kalenius to whom I owe my greatest thanks. In addition, I'd like thank Ph.D. student Janne Frimodig, who provided the glyphosate samples for the tests and Dr. Anniina Kiesilä, for helping with the measurements and providing me with advice on a weekly basis during the whole project. Finaly, I would also like to thank my friends and family for the moral support.

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Yury Devitte

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Abbreviations and terms

Abbreviations	Meaning
AMPA	Aminomethylphosphonic acid
Gly	Glyphosate
Glu	Glufosinate
SFC	Supercritical fluid chromatography
HILIC	Hydrophilic interaction liquid chromatography
UHPLC	Ultra-high performance liquid chromatography
MS/MS	Tandem mass spectrometry
HRMS	High-resolution mass spectrometry
BBB	Blood–brain barrier
BPA	Bisphenol-A
GBH	Glyphosate-based herbicides
LC-MS	Liquid chromatography mass spectrometry
HPLC	High-performance liquid chromatography
GC	Gas chromatography
FID	Flame ionization detector
ECD	Electron capturing detectors
MSD	Mass selective detectors
FPD	Flame photometric detectors
NPD	Nitrogen phosphorus detectors
ELISA	Enzyme-linked immunosorbent assay
FL	Fluorescence
SERS	Surface-enhanced Raman scattering
LOD	Limit of detection
LOQ	Limit of quantitation
NP-LC	Normal phase liquid chromatography
RP-LC	Reversed phase liquid chromatography
CID	Collision-induced dissociation
MRM	Multiple reaction monitoring
SPME	Solid phase microextraction
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
MSPD	Matrix solid-phase dispersion

1 Introduction

The modern agricultural prosperity and development can hardly be looked at without the mention of agrochemicals, such as fertilizers or pesticides.[1] Pesticides, comprising of a wide variety of different complex compounds, have been found to be useful in many applications of pest control, not only on commercial agricultural level, but also in private gardens and other public areas, and are one the biggest groups of manufactured chemicals.[2] In order to meet the global food demand, they are especially used in protecting food plants, and can be divided in four main categories, depending on their main target – herbicides (weeds), fungicides (fungi), rodenticides (rodents) and insecticides (insects).[1] Pest control itself may be applied as elimination of a specific pest, or its eviction from a protected region.[3]

Although the use of pesticides is often seen as inevitable, in order to sustain high food production, they are also known to cause a lot of environmental stress.[4] Due to their overwhelming global usage, pesticides often act as an omnipresent environmental pollutant, that impacts biodiversity, water and soil,[5] watershed's, as well as human health.[1] It is estimated that around 64% of global agricultural land (24.5 million km^2) is facing a pesticide contamination risk by multiple active compounds, and in 31% of agricultural land this risk is considered to be high.[1] At the same time, around 61.7% (2.3 million km^2) of this high-risk region is consisting of European agricultural land and 34% is located in high-biodiversity area, where the risk is especially concerning in the watersheds regions of South Africa, India, Argentina and China. Additionally, pesticide-related studies are often exclusive to a specific site, and thus their pollution effects on more global scale remains mostly unknown.[1] Nevertheless, minimizing pesticide pollution is crucial for the biodiversity, which has a direct impact for example on soil health and function, which again is contributing to food production.[6] The usage of pesticides, however, will most likely only increase in the future, due to the population growth, which may drive questions regarding pesticides impact on a global scale even further.[7]

Newly emerging global problem with pesticide pollution, is the occurrence of pesticide mixtures, where combination of different pesticides might synergize their toxicity towards non-target organisms, leading to chronic and acute toxicity.[8] World Health Organization estimates that around 1 000 000 people are annually under acute poisoning due to the contact with pesticides, and at the same time, repeated exposure to lower levels of pesticides is associated

with numerous mediums and long term syndromes, that include different types of nervous system disorder and tumors.[2]

Considering the environmental and toxicological problems that revolve around pests and pesticides, finding a way to increase pesticides safety and selectivity became an important task, especially with pesticides that cover a broad spectrum of different insect and herbal pests, and decrease the quality and quantity of food production.[2] However, it is also important to be able to qualitatively and quantitatively analyze pesticides from different samples, in order to better understand their effect in distinct environments and in specific concentration. This is crucial not only for environmental protection, but also for pesticide compliance regulations and food safety.

Pesticide analysis usually involves their formulation, physical-chemical properties, as well as residues.[9] Through formulation, pesticides qualitative and quantitative analysis reveals the active ingredient, as well as possible impurities, thus showing the overall composition and quality. Analysis of the pesticide's residue happens through quantitative and qualitative analysis of biological and agricultural samples, like food and soil. At the same time, analyzed compounds might be for example pesticides parent chemicals, or be a result of toxic metabolites, degradation, or chemical reaction, since pesticides tend to remain in the environment for a long period of time.[9,10] Often it is needed to monitor both pesticides and their metabolites, for risk assessment. Nowadays, one of the most popular types of multi-componential and non-targeted analysis methods are high-resolution mass spectrometry (HRMS) and liquid chromatography coupled with mass spectrometry (LC-MS). Still, new analysis methods are being constantly developed, with the intension of more rapid and economical detection, higher sensitivity and on-site testing.[9]

From the new analytical method, two seemed to be especially on the rise. Supercritical fluid chromatography (SFC) is known for its impressive performance and robustness, while at the same time maintaining good separation and sensitivity.[11] More selective, hydrophilic interaction liquid chromatography with ultra-high performance liquid chromatography (HILIC-UHPLC) method, on the other hand, has been found to be especially useful when analyzing multiple pesticides, with the quantitation limits in the low microgram per kilogram.[12]

Through this master's thesis, we will first take a look at analyzed pesticides, their properties and characteristics, as well as investigate different methods these pesticides have been analyzed with. We will also develop a procedure for sample treatment and method validation. In the

experimental part, using gained knowledge, we will comprise different methods for glyphosate, glufosinate and aminomethylphosphonic acid (AMPA) analysis. First method will consist of hydrophilic interaction liquid chromatography with ultra-high performance liquid chromatography and tandem mass spectrometry (HILIC-UHPLC-MS/MS) and the second one of high-performance liquid chromatography with UV-spectroscopic detection (HPLC-UV). This will help us better understand which analytical method is better to be used in a specific situation, and what are the possible problems that may occur along the way.

2 Pesticides

2.1 Global use of pesticides

In 2021 around 4.2 million metric tons of pesticides were used worldwide as a cheap and effective way to ensure growing food yield and its quality. At the same time, this usage is expected to grow up to 4.4 tones by the end of 2026.[13] From the Figure 1 we can see that till 2020, one of the important contributors to pesticide usage were USA, China and Brazil.[14]

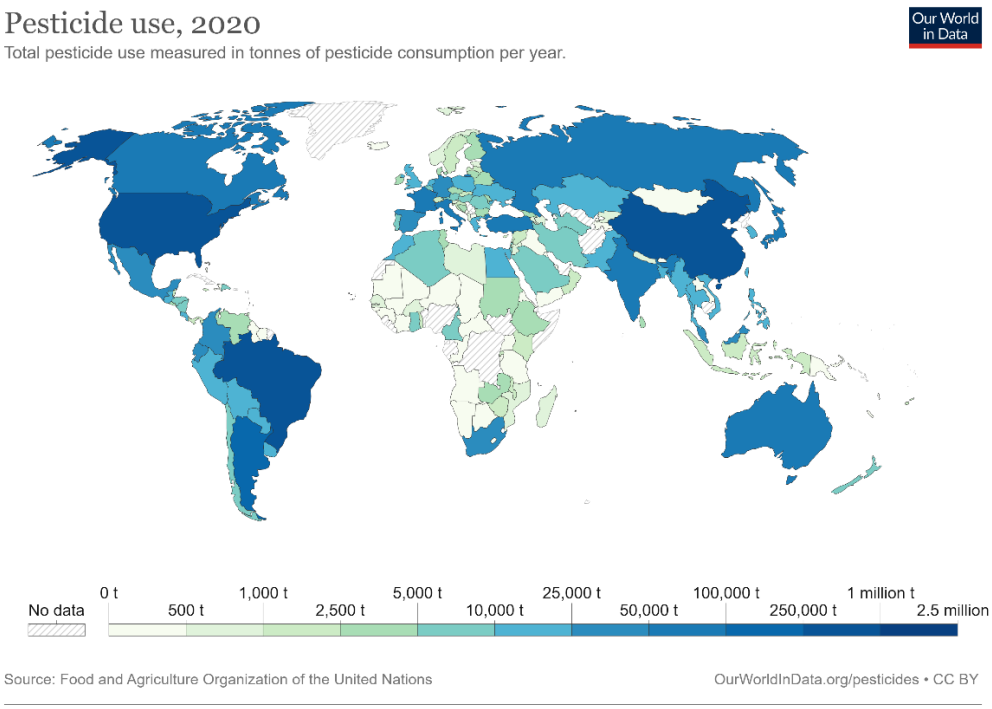


Figure 1. Pesticide usage around the world in 2020.[14]

For now, it is unlikely that the usage of pesticides will slow down, especially when considering that about 45% of agricultural yield is annually lost to pests.[15] Alternative food-protective

methods have been under development for some time, like application of pest-resistant crop or biopesticides, however, chemical pesticides are still more commonly used.[16] One of the most frequently used types of pesticides are herbicides (around 47.5%), insecticides (29.5%) and fungicides (17.5%).[17] Additionally, pesticides are useful not only for food and commercial product protection, but also in public health programs, were they can impact disease vectors, such as fleas and mosquitoes, which can often be crucial in more vulnerable regions.[3] This again may make it more difficult to consider other method of pest control, which may take more resources and time.

Although pesticide usage and manufacturing are usually regulated by the agencies and government, overall risk assessments and monitoring of environmental impact are limited by the pesticide's active ingredients and additives of its formulations.[18] This is especially worrying, considering that each year around 200,000 people worldwide die and another 3 million are poisoned due to the pesticide exposure, with alarming 95% of the cases coming from developing countries.[19]

2.1.1 Pesticide Types

Pesticides can be separated into two categories – natural and synthetical and are usually named depending on the type of targeted organism and functional group in the active ingredient (e.g., triazine herbicides, organophosphate insecticides).[20] Most of the synthetic pesticides are made of organic chemicals, which can be separated into pyrethroids, organochlorines, organophosphates and carbamates groups. At the same time, natural pesticides (biopesticides) are a product of living organisms, like fungus and plants.[21] Additionally, pesticides can also be classified depending on their methods of entry. For example, systemic pesticides can be absorbed through tissue or skin and transferred further into organism via vascular system. On the other hand, non-systemic pesticides, also known as contact pesticides, are able to produce damage to the targeted organism simply through surface contact. Stomach toxicants need to be digested and absorbed from the inside of target organism body, and fumigants are used in the volatile, gaseous form, which is then absorbed through respiratory system. Lastly, repellents are usually used simply to keep pests away from the targeted area.[22]

It is, however, important to remember that commercial pesticides usually consist of different mixtures of active and other compounds.[20] The major role for these compounds may be for

example efficiency increase, so that the product is more cost-effective, and details about them are rarely publicly available, due to product confidentiality.[23] This may sometimes make it difficult to estimate commercial pesticides effects as a pollutant, since active compound and the other compounds can have their own effect separately and combined. Thus, although both in-vitro and in-vivo toxicological tests have helped to recognize potentially dangerous pesticides for commercial practices, post-market epidemiological analysis of pesticide is still needed, to ensure environmental wellbeing.[23] For example, through post-market monitoring, it is estimated that around 25 million workers in agricultural sector are unintentionally poisoned by the pesticides every year worldwide[24] and at the same time many commercially available pesticides may lead to cancer in humans.[20]

2.1.2 Environmental Impact and Fate of Pesticides

After pesticides are applied to the area, due to the different microbial and physicochemical conditions, they can migrate even further into the environmental ecosystem, where active and other ingredients can show different effects on non-targeted organisms. Physicochemical processes might involve for example temperature, oxygen and moisture, which degrade pesticides into their metabolites.[21] Depending on the chemical mixture, these metabolites can be non-toxic or quite hazardous, which again forces deep epidemiological analysis, in order to ensure pesticides safety.[25] At the same time, using adsorption, volatilization, or leaching and surface runoff, both pesticides and comprising metabolites can be carried off from targeted region to another. This is further escalated by attraction force between soil particles and pesticides, due to the soil texture and its organic matter, which keeps pesticides in soil for longer period of time, causing damaging effects to surrounding ecosystem.[26]

Pesticides, however, are not the only chemicals that act as manmade pollutants, which may provide a challenge when distinguishing specifically pesticides related risk on human health and the environment. Industrial complexes often intentionally or accidentally provide large amount chemical waste into the environment.[2] Still, it is estimated that agricultural pesticide usage does have a significant impact on water quality and other environmental factors.[27] For example in US, 10.4% of public and 4.2% of rural wells have concerning amounts of one or more pesticides.[28] The contamination of area by pesticide might occur via leaching into groundwater, absorption by plants, as well as volatilization. At the same time, many non-

targeted organisms can experience short- and long-term toxicological effects directly from the pesticide's application onto targeted area.[3]

Although pesticides accumulate in air, soil and biota[16], especially concerning seems to be pesticides aquatic pollution, since they tend to accumulate in aquatic organisms and sediments, which more easily provides a risk to the human. Aquatic pollution usually occurs through industrial wastewater and agricultural fields runoffs, where soluble pesticides are taken by the water current and then seep further into the soil layers, sometimes reaching surface waters and groundwater, especially during the rain. Not only does this decrease the quality of water, but it also reduces drinkable water supply, since even low concentrations of pesticides, under long-time exposure might escalate into non-carcinogenic health risks.[29] Additionally, ingestion of pesticide infected water may disrupt hormone equilibrium, lower immunity, cause reproductive issues and even cause problems in children's instinctual development.[19]

Soil pollution by pesticides usually happens directly from agricultural applications or through unintentional contamination of air. It becomes a good storage for pesticides, due to previously mentioned soils organic chemicals and its high affinity[16], increasing the chance of toxicological exposure to soils organisms, which can again accumulate in the food chain.[30] At the same time, pesticides activity in a soil depends greatly on its chemical and physical properties, like polarizability, molecular weight, volatility and ionizability, which further impacts abiotic and biological transformation. For example, pesticides adsorption is often based on van der Waals forces or electronic interactions.[21]

2.2 Pesticide Influencing Factors, Mechanisms, and Health Risks

As mentioned in the previous chapter, many factors can have an impact on a toxicity level in the individual, such as pesticides nature, concentration in the environment, duration of exposure as well as pH, temperature, humidity and so on. For example, the majority of used pesticides act as nerve toxins. In the case of acetylcholinesterase inhibitors, their inhibition of acetylcholinesterase in neuromuscular junctions and brain cholinergic synapses leads to acetylcholine neurotransmitter build up, and thus ongoing acetylcholine receptors stimulation, which can cause constant muscle contraction and other harmful effects.[31] Some pesticides, like organophosphates, can also affect acetylcholine itself, by phosphorylating it, thus preventing acetylcholine enzyme ability to break it and leading to acetylcholine build up in central and

peripheral nervous systems. Since acetylcholine works as major neurotransmitter in brain and muscles, this can cause acute cholinergic syndrome, which include insomnia, drowsiness, headache and giddiness.[32]

In humans this may lead to chronic, subacute or even acute toxicity, with possible neurological follow-up, which is especially worrying, since these neurological pesticides can be inhaled or even absorbed via skin and mucous membrane, after arial spray. And although toxicological effects can be reversible over time, after isolation from exposure, they can still inflict permanent damage.[31] Some pesticides can also change hormones levels in different organisms by affecting hormones discard, production, operation, transportation, metabolism and release.[33] Other pesticides can for example affect reactive oxygen species levels in the organism because of their autoxidation through molecular oxygen, which is linked to cancer, as wells as cardiovascular, inflammation and neurodegenerative diseases.[34]

Many herbicides, which are usually applied against weeds, shrubs, trees and even aquatic plants, tend to work also through other mechanisms. This can be growth regulation, pigment and lipid biosynthesis inhibition, photosynthesis, and amino acid synthesis inhibition, as well as seeding growth inhibition and cell membrane disruption. Thus, in a targeted organism, herbicides usually tend to focus on plant's specific metabolic pathway, which in ideal conditions is exclusive to the pest in a chosen area and does not affect other non-target organisms.[35] Still, herbicides popularity, which only increased in the following years[36], has shown that its improper usage can still pose danger to the human health and the environment. Especially since for many herbicides the toxicity mechanism itself in non-targeted organisms is largely unknown.[35] It is thus important to further investigate toxicological mechanisms in non- and target organisms, in order to have a better understanding of pesticides' functioning.

2.2.1 Mechanisms of Pesticide Toxicity

Previously mentioned growth regulators (e.g., 2,4-dichlorophenoxiacetic acid, glyphosate in small quantities),[37] which are often used for example on broadleaf weeds, affect plants growth by changing its hormone levels, since they act as a natural hormone. For example, 2,4-dichlorophenoxiacetic acid acts like auxin – a plants growth hormone and binds to its receptor. Since auxin modulates gene expression, which leads to cell differentiation, division and elongation, this can lead to prolong effects in plants, like uncontrolled cell division, root growth

inhibition and plant death.[38] On the other hand, seedling growth inhibitors (e.g., molinate, metachlor) block the development of stem and roots, thus negatively affecting plat growth. Cell division inhibitors are also in this category and often work by preventing mitosis in roots and stems.[35]

Photosynthetic inhibitors (e.g., simazine, copper-containing pesticides) tend to damage cell membranes with highly active molecules, which then weakens plants photosynthesis and may even lead to plants death, after active molecule build up. Inhibition can also happen through plants photosystem II protein complex, which is an enzymatic multi-subunit complex, located in thylakoid membranes and is essential for the oxygenic photosynthesis reactions in plants. Many photosynthetic inhibitors tend to compete with native plastoquinone molecules, which have a crucial role in the electron transport chain, by binding to the photosystems II D1 protein´s specific QB site.[39] This blocks electrons transportation, which stops carbohydrate synthesis and also leads to carbon dioxide buildup in plant cell, thus bringing nutrient imbalance, reduced plant growth makes it more vulnerability to stress.[35]

Inhibitors of lipid biosynthesis (e.g., sethoxydim, fluazifop) degrade plants' ability to form biological membranes, by inhibiting its lipid biosynthesis, and are used mostly as grass suppression post-treatment. Inhibition usually happens through acetyl-CoA carboxylase enzyme, which plays a vital role in fatty acid biosynthesis and its suppression provides a broad range of negative effects on plant cells, like disruption of membrane integrity and growth impairment.[35]

Cell membrane disrupters (e.g., diquat, paraquat) on the other hand are used more as post-emergence herbicides and are applied when undesirable pests are already actively growing, since this pesticide activates better on an actively growing tissue. Activation itself happens through light exposure, which helps oxygen compound formations, like hydrogen peroxide. These compounds can then rupture plants tissue and membrane, resulting in tissue necrosis and cell leakage, as well as plant death.[35]

Inhibitors of pigment biosynthesis (e.g., clomazone, tranexamic acid), which are also used as post-emergence herbicides, suppress photosynthetic pigments, such as carotenoids, biosynthesis. This may happen for example through pesticides inhibition of phytoene desaturase enzyme, which plays an important role in carotene and carotenoid pigment production. Normally, carotenoids tend to protect chlorophylls destruction by light, and since

chlorophylls are often essential in plants photosynthesis, their destruction leads to leaf bleaching, as well as plant starvation.[40]

Finally, amino acid biosynthesis inhibitors (e.g., glyphosate, aminomethylphosphonic acid) function by stopping the biosynthesis of a specific amino acid. Because of this, in theory they usually tend to have low toxicity towards mammals, since they tend to lack similar biosynthesis mechanisms.[41] In the case of glyphosate, plants biosynthesis of aromatic amino acids like phenylalanine and tyrosine is interrupted.[35] This happens through glyphosate inhibition of 5-enolpyruvylshikimate-3-phosphate synthase enzyme, which can be seen in a Figure 2 below.

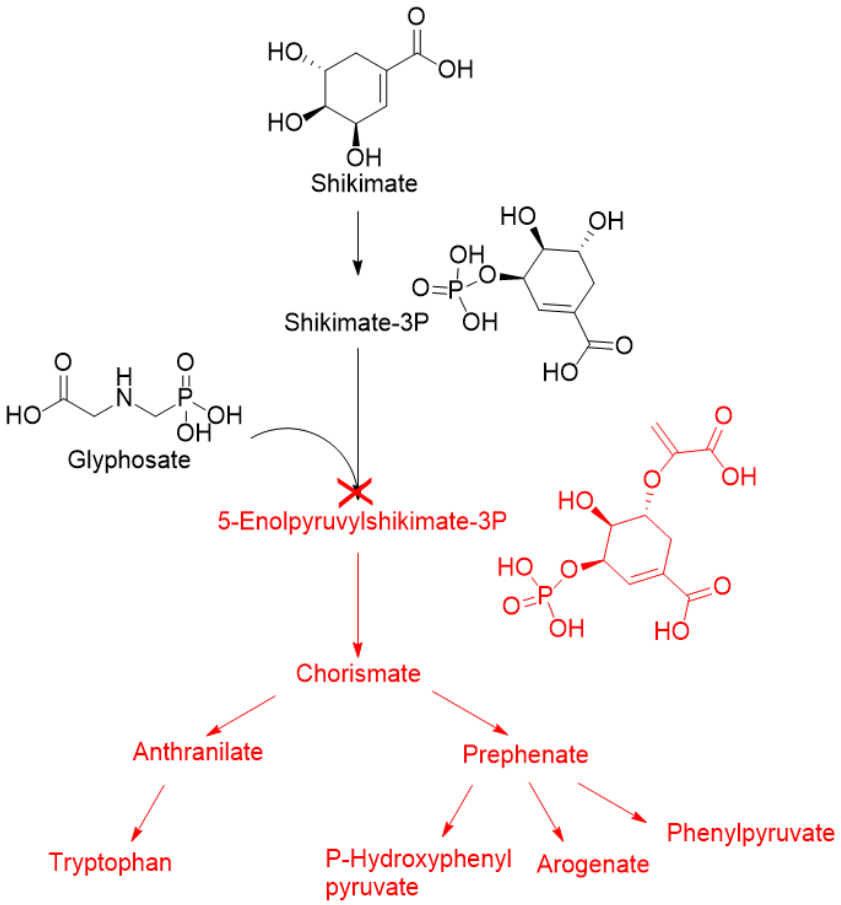


Figure 2. Glyphosate inhibition of 5-enolpyruvylshikimate-3-phosphate synthase.[41]

This enzyme is heavily included in synthesis of these aromatic amino acids. The consequences of glyphosate interruption are thus seen in damaged carbon metabolism, induced fermentation in roots, which causes carbohydrate accumulation in leaves, as well as overall plants growth arrest and slow death. However, it is not yet fully understood what exactly causes plant failure after synthase inhibition.[41] At the same time, other compounds, such as glufosinate, are glutamine synthetase and glutamate decarboxylase inhibitors, which are crucial enzymes that

catalyzes biosynthesis of ammonia into glutamate and glutamate into gamma-aminobutyric acid respectively, that may further lead to a fatal multiorgan failure.[42]

2.2.2 Toxicological Effects and Risks

In the case of glyphosate and its other commercial formulations, its toxicological effects are seen in many non-targeted organisms, including invertebrates, fish and humans. Exposure to glyphosate seems to result in numerous neurotoxic effects, as well as disrupt specific signaling pathways and thus negatively affect normal cell development. At the same time, glyphosate is able to force oxidative stress and mitochondrial malfunction, which can often result in necrosis, neuronal death and even behavioral disorders.[43] Glyphosate, together with its metabolite - aminomethylphosphonic acid (AMPA), showed to have a negative effect on human cells integrity and cause damaging effect in central nervous system, including cells apoptosis and necrosis. For example, they tend to increase blood–brain barrier (BBB) permeability by affecting proteins, that moderate hermetic junctions in BBB endothelial cells. This can further lead to brains increased glucose uptake, changing neurons metabolic activity and affecting central nervous system neuronal development in humans.[44]

Development of neuronal cytoskeleton and axonal growth cones was also found to be affected, since glyphosate and AMPA can negatively impact synthesis of its proteins by inhibiting expression of TUBB3 and CAP43 genes.[45] Additionally, glyphosate itself tends to increase methodological defects in neurons by increasing levels of Wnt messenger RNA, like mRNA-Wnt3a. Taking all this into account, glyphosate can cause different neurocognitive developmental disorders, like autism, even in children whose mothers were in contact with glyphosate during pregnancy.[43]

Similar to glyphosate and AMPA, glufosinate was also found to be neurotoxic, although specific details about its mechanism are not known and, because of glufosinate structural similarity with glyphosate, it thought to cause likewise mitochondrial degradation. Consumption of glufosinate containing solution, even at concentrations of 0.6-0.8 ml/kg, causes acute poisoning which can last for several days and negatively affects central nervous system and heart rhythm.[42]

Direct effects from pesticides in non-target organisms are seen especially well in aquatic organisms, such as fishes. Fishes are often important indicator of aquatic ecosystem quality, since they are relatively low in the aquatic food chain, and tend to easily accumulate heavy metals and pesticides, for example by consuming high amounts of pesticide-polluted aquatic plants, and algae. Pesticide effects are usually seen in higher mortality rates after acute poisoning and damaging changes in lower doses. These changes could be for example hematological, affecting red and white blood cells, as well as serum and plasma levels, which caused histological irregularities and further damages to kidneys, liver, brain, muscles and guts.[21] Some pesticides also had genotoxic effects on fishes, affected their behavior (e.g., increased non-responsiveness, hyperexcitability), or even body color and growth rate.[42] Pyrethroids have been found especially harmful to fishes reproductive capabilities[42], and herbicides are often seen to cause developmental abnormalities.[46]

For humans, exposure to pesticides can occur for example through the agricultural usage of herbicides, where contact with skin, mouth eyes and respiratory track can often happen, especially if safety guidelines are not followed correctly. This, depending on the pesticides, can cause different reactions, such as rashes on the skin, headache, vomiting and sneezing. Exposure can also happen via vegetable and fruit consumption, which are contaminated by pesticides due to soil and water pollution. As a result, chronic diseases can emerge, such as cancer, asthma, diabetes, reproductive disorder and so on.[47]

2.3 Glyphosate

N-(Phosphonomethyl)glycine, also known as glyphosate, also seen in a Figure 3 bellow, is one of the most known pesticides, discovered back in 1970 by John Franz, and is still most commonly used in today's agricultural sector, as an organophosphorus, non-selective systemic herbicide with a broad-spectrum activity.[48]

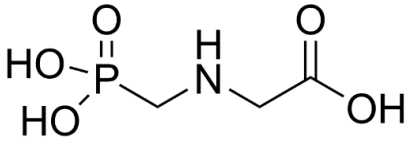


Figure 3. Chemical structure of glyphosate.

It has also been commercially divorced into hundreds different glyphosate-based herbicides (GBH) products, spreading in more than 100 countries around the world and accounting for

approximately 60% of all non-selective herbicides on the market.[49] Commercial position of GBH is also thought to only grow in the future, especially after development and spread of glyphosate resistant, genetically modified crops, which provide more economic benefits to the agricultural sector.[50] For example, each year approximately 600.000 – 700.000 tons of glyphosate are used, which is thought to only grow up to 920.000 tons by the end of 2025.[43]

Glyphosate is quite common in agricultural, industrial and forestry sectors, where it is usually intensively applied, for example onto plant leaves to control broadleaf weeds and grasses growth. The most noticeable use of glyphosate is seen in agricultural growing of field corn, soybeans and hay, however, in smaller quantities it sometimes used as plant growth regulator.[48] After applying herbicide to plant it usually undergoes growth and tissue degradation, leaf green color bleaching, deformation and shrinking, as well as death, which can occur after 4-20 days.[50]

The trace of glyphosate has since been especially noticeable in water, soil and plants, with the ability to persist in the environment for many days and even months. This again raises concern about environmental health, especially after its was found to be also in food products and human urine.[49] Usually, the degradation of glyphosate happens through microorganisms, after which its concentration in the soil tends to be low, however, its biodegradation can still vary drastically, depending on a soil's bio- and physico-chemical properties.[43]

Additionally, glyphosate can stay biologically inactive by strongly binding to its constituent particles, and thus its high usage has shown to noticeably decrease biodegradation rates, increasing its groundwaters pollution chances, because of its high water solubility.[51] Similarly to soil, glyphosate half-life in water may also vary from a few days to a couple of months, although in marine water it can remain for as long as 315 days. Glyphosate also tends to stay in vegetation up to several days and for as long as a year in different foods and crops, which again raises concern about its accumulating and possible risks.[43]

There are different methods for synthesis of glyphosate on industrial level, that vary in starting materials and their application, however, most of these methods use precipitation of product from water or aqueous alcohol. For example, one of the most common methods uses iminodiacetic acid as a base for glyphosate synthesis, with relatively simple and efficient transformations. In Figure 4 below we can see an example of iminodiacetic acid, with a monochloroacetic acid as a starting material, as well as iminodiacetic acid phosphonomethylation and oxidization to glyphosate.[52]

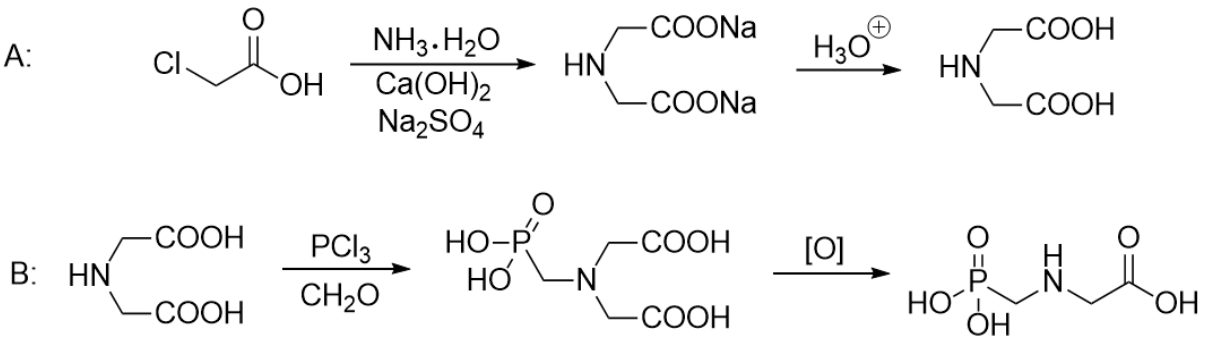


Figure 4. Iminodiacetic acid synthesis for glyphosate (A) and Phosphonomethylation and oxidation of iminodiacetic acid (B).[52]

Glyphosate, being glycine derivative and having amine, carboxylate and phosphonate functions, is a zwitterion. Depending on the surrounding conditions, it can possess positive or negative charges, and react as either acid or base and because of its structure it can chelate with quadrivalent and trivalent atoms. Chemical and physical characteristics of glyphosate are presented in Table 1 bellow.

Table 1. Glyphosate chemical and physical characteristics.[53]

Characteristic	Value
IUPAC name	N-(phosphomethyl) glycine
Molecular formula	$C_3H_8NO_5P$
Molecular weight	169.07 g/mol
Exact mass	169.01400935 Da
Solubility (water)	10.5 g/l at 20 °C and pH 1.9
Melting point	189 °C
Dissociation constant	pKa_1 = 2.0; pKa_2 =2.6; pKa_3 = 5.6; pKa_4 =10.6
Formal charge	0
Topological Polar Surface Area	107.0 $Å^2$

Here we can see that glyphosate, taking to account its hydrophilicity, is relatively well soluble in water and has high adsorption, as well as compatibility with other compounds. However, because of its high polarity, it is not well soluble in organic solvents, like acetone and ethanol. At room temperature glyphosate is an odorless white powder, that also lacks UV absorption capability and tends to have low ionization and volatility.[49]

2.3 Aminomethylphosphonic acid

Aminomethylphosphonic acid, also called AMPA, is mostly known as a degradation product of amino-polyphosphonate compounds and glyphosate. Thus, AMPA usually tends to stay in the same environment, and at the same time is also often detected together with its parent chemical.[54] Chemical structure of AMPA can be seen in Figure 6 below.

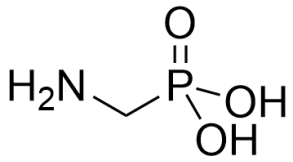


Figure 6. Chemical structure of aminomethylphosphonic acid.

Over the years AMPA was found to be on the rise in the environment, especially in water and agricultural areas, after usage of its parent chemicals became more common. Additionally, AMPA can be found in the air near agricultural areas, and although its concertation might be insignificant, it can still come down during heavy rainfall. AMPA also tend to accumulate in sediments and occur in groundwater. Overall AMPA sources can vary greatly from urban to agricultural and industrial, which can provide a challenge when trying to investigate its pollution origins.[54]

An especially common source of AMPA is glyphosate, which has a wide application, mentioned in the previous chapter. However, phosponates are also very commonly used, for example in industrial and domestic sectors, especially in textile industry as complexing agents and in water treatment as membrane anti-fouling agents.[55] Data from 1998 and 1999 shows that worldwide around 56.000 tons of phosphonates were used, with 16.000 tons coming from Europe.[54] However, these numbers most likely only grew throughout the years, especially because of newly emerging phosphonate product and their applications.[56]

Similar to glyphosate, AMPA is also well absorbed by soil particles, and degrades relatively slowly in soil, usually concentrating in the upper layers. Thus, it is rarely able to reach deep groundwater, although contamination of shallow groundwater can occur, especially during rainfall runoff when AMPA, being attached to the particles, is moving towards the stream. In more urban areas it usually occurs from wastewater, due to glyphosate and phosphonate usage, and although wastewater treatment plants tend to remove considerable amounts of AMPA from

water, there is only limited amount of epidemiological data considering its exposure on water and toxicological effects.[54]

The occurrence of AMPA can happen for example in soil and sediments, due to microbial activity with glyphosate, or from photo- or biodegradation or amino-polyphosphonate compounds in water.[54] Phosphonates are often resistant to degradation due to their C-P bond, even though they can be used by microbes, such as *Bacillus megaterium* and *Pseudomonas stutzeri,* as a source of phosphorus. Thus, they tend to accumulate for longer time in the environment.[57] More often occurs phosphonates photodegradation, which is metal-catalyzed, and in case of most commercially popular phosphonates products like nitrilotris-methylenephosphonic acid, diethylenetriaminepenta-methylenephosphonic acid and hexaethylenediamine-tetramethylenephosphonic acid produced AMPA as a main degradation product.[58]

Glyphosate, on the other hand, mainly degrades through two different pathways. The first pathway, which tends to occur first is glyphosate C-P bond lyase. It is linked to microbial activity and leads to the formation of sarcosine and glycine. The second pathway is glyphosate oxidoreductase, seen better in Figure 6 (A), which is thought to happen after absence of other nutrients for microbes, and results in formation of AMPA and glyoxylate.[54] Additionally, AMPA can be formed through glyphosate degradation directly in the corps and weed, as well as transferred into soil from plant residues.[59] It tends to appear more frequently in soil with better oxygenic conditions, since glyphosate is degraded more easily there. Same can also be said for aquatic environments with rich metallic ions concentrations, like Cu^{2+}, that tend to boost degradation of glyphosate.[54]

Degradation of AMPA itself, also seen in Figure 7 (B) can happen in the range from 23 to 958 days and is often thought to be slower than its parent chemical, like glyphosate.[43] It is mainly biodegraded through microbial activity with C-P lyase, which result in the formation of phosphate and methylamine and is noticeably affected by soil moisture and temperature, with higher heat and moisture correlating causing faster degradation.[54]

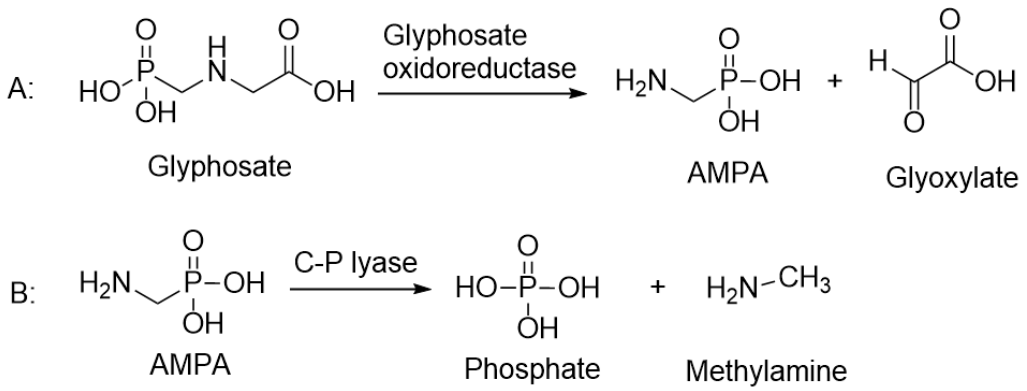


Figure 7. Decomposition of glyphosate (A) and aminomethylphosphonic acid (B).[54]

AMPAs chemical and physical characteristics, which can also be found in Table 2 below, are quite similar to glyphosate.

Table 2. AMPAs chemical and physical characteristics.[60,61]

Characteristic	Value
IUPAC name	(Aminomethyl)phosphonic acid
Molecular formula	CH_6NO_3P
Molecular weight	111.04 g/mol
Exact mass	111.00853005 Da
Solubility (water)	56.0 g/l at 20 °C [62]
Melting point	277 - 281 °C
Dissociation constant	pKa_1 = 0.9; pKa_2 =5.6; pKa_3 = 10.2
Formal charge	0
Topological Polar Surface Area	83.6 $Å^2$

It is noticeably more water soluble than glyphosate, however, it is also not well dissolved in common organic solvents. Additionally, it also appears as white crystals or powder and being zwitterion, tends to have high polarity.[62]

2.4 Glufosinate

Phosphinothricin, also known as glufosinate, is a popular and commonly used non-selective herbicide with fast acting properties, that target glutamine synthetase. In 2014 alone, around 12 million hectares were treated with glufosinate worldwide.[63] Commercially it is usually sold as an ammonium salt, with areas of application ranging from agriculture sector to domestic gardens, and concentration of glufosinate itself varying from 14 to 30%.[42] Glufosinate, which

can be seen with its ammonium salt counterpart in a Figure 8 below, is quite similar to glyphosate, with its structure and organophosphorus nature, which is why it has found to be especially useful against glyphosate-resistant weeds.[64]

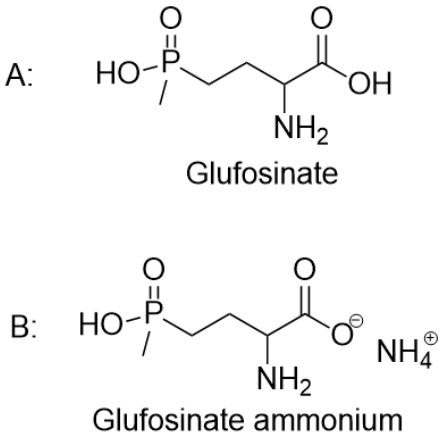


Figure 8. Chemical structure of glufosinate (A) and glufosinate ammonium (B).

Glufosinate is especially often applied in North and South America, onto glyphosate-resistant soybean and cotton plantations respectively. It is also widely used across the globe on rice, vineyards orchards and non-agricultural areas, although its appearance in Europe since 2018 was decreasing, due to European Commission toxicological concerns. Still, worldwide glufosinate usage will only continue to increase, due to the spread of glyphosate-resistant weed, that will continue to evolve, in order to resist other herbicides as well. At the same time, genetically modified herbicide-resistant crops are being more available, while over non-selective herbicides with better efficiency are yet to be found.[63]

Compared to glyphosate, glufosinate is often used on far smaller areas, due to its function inconsistency. Glufosinate, for example, can be greatly affected by its application methods, weed species and surrounding conditions. Because of its high hydrophilicity, glufosinate also does not transfer well in plants, causing difficulties in its control with non-targeted species.[63] As a result of its long and popular usage, glufosinate has been occasionally found in water, soil, vegetables, as well as food and even in humans. This exposure can happen for example through tea, since its cultivation is often heavily controlled by herbicides like glufosinate.[64]

Just like with any other pesticides, glufosinate activity in soil for the most part depends on soil's properties, and as soils clay and organic content increases, glufosinate leaching decreases. For example, it is possible for glufosinate to contact shallow groundwaters in sandy soils, unlike in silty loam soils.[65] However, pollution risk itself is still thought to be minimal.[63] At the same time, glufosinate can move to surface water through eroding soil adsorption or runoff water,

especially after rain. This can be seen even outside of the target area, since glufosinate is able to move quite far via drift during its application.[65]

After glufosinate is applied in the field, there is usually enough time for dispersing of its residues. Even when considering plant leaves, that can possess some glyphosate residue, which also help its absorption in the soil after leaves fall, final concentration of glufosinate in the crops can still be less than 0.3% of the total applied amount.[66] Unlike many other herbicides, glufosinate has a relatively low half-life, between 1 and 7 days, and thus rarely accumulates in the soil, or even in the food chain. Especially since neither glufosinate nor its metabolites assemble in fatty tissue of living organisms. Degradation itself happens mainly in soil through the rapid aerobic activity of microorganisms, for example via acetylation, oxidation or transamination, and resulting glufosinate remains usually do not cause residual activity or limit crop rotation.[63] Still, glufosinate can be noticeably mobile outside applied area and cause acute and chronic toxicity to both targeted and non-targeted terrestrial, as well as aquatic organisms.[65,67]

Commercial production of glufosinate typically results in a racemic mixture of D,L-phosphinothricin. Although several methods exist for the enantioselective synthesis of L-phosphinothricin, which is believed to be the main active compound, the cost of large-scale production remains significantly higher than that of producing the racemic mixture.[63] One example of D,L-phosphinothricin synthesis, which can be seen in a Figure 9 below, uses 2-oxophosphinothricin and ruthenium-carbon composite with ammonia in methanol and hydrogen.[68]

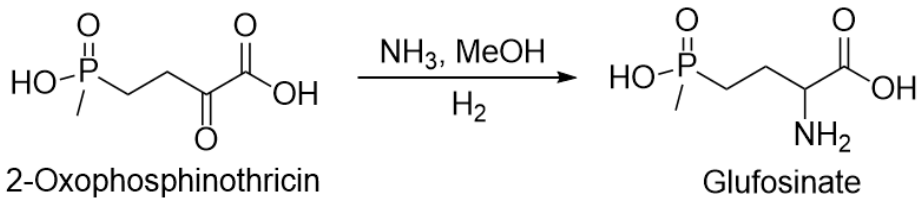


Figure 9. Glufosinate synthesis using 2-oxophosphinothricin.[68]

Similar to glyphosate, glufosinate, being phosphinic acid and having alpha amino acid, also possesses acid-base properties, and can occur both as cation or anion.[64] Chemical and physical characteristic of glufosinate can be seen in a Table 3 below.

Table 3. Chemical and physical characteristics of glufosinate.[63,69]

Characteristic	Value
IUPAC name	(2RS)-2-amino-4-[hydroxy(methyl)phosphinoyl]butanoic acid
Molecular formula	$C_5H_{12}NO_4P$
Molecular weight	181.13 g/mol
Exact mass	181.05039486 Da
Solubility (water)	1370.0 g/l
Melting point	229 – 231 °C
Dissociation constant	$pKa_1 = 2.0$; $pKa_2 = 2.8$; $pKa_3 = 9.8$
Formal charge	0
Topological Polar Surface Area	101 $Å^2$

Glufosinate and its metabolites are usually stable under light and do not evaporate easily. Additionally, glufosinate is highly soluble in water, especially in the form of ammonium salt, but not well in organic solvents. It is easily hydrolyzed in water with the pH of 5-9 due to the amine and hydroxyl groups.[63,69]

3 Analytical methods for pesticides

Since pesticides often tend to bioaccumulate in the environment, polluting its soil, water and air, as well as different living organisms, causing various toxicological effects, it is important to be able to analyze them quantitatively and qualitatively. This monitoring helps to evaluate contamination levels and act accordingly, at the same time providing more information about the substance action and effects in different environments, which further improves its management.

The presence of pesticides in food and vegetables has been especially under precise analysis over the last years, due to rising pesticides usage in agriculture. The analytical methods need to be accurate and robust, as well as sensitive, in order to ensure that pesticides levels are below the allowed limits. There are several different methods that have proved to be useful with their own pros and cons, however, it is still necessary to investigate new analytical techniques that could help pesticide monitoring.[10]

One of the most traditional analytical methods, that detect pesticides in food, are based on liquid or gas chromatography combined with various detection methods. These methods usually consist of physical separation, where compounds are distributed by stationary and mobile phases, which is moving in a specific direction.[70] Some examples of such techniques are liquid chromatography with mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC) as well as ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), or gas chromatography coupled with mass spectrometry (GC-MS).[10]

GC and LC methods became especially popular due to the commonly appearing complexity of analyzed sample matrix, which makes it difficult to directly measure pesticide concentrations. And even though there are somewhat utilized methods of direct pesticides detection from samples, such as enzyme-linked immunosorbent assay (ELISA) and capillary electrophoresis (CE), chromatography methods are still one the most used, since they provide good and reliable separation, identification, as well as sensitivity.[10]

Gas chromatography is thought to be especially reliable when working with easily vaporized and volatile pesticides. For example, it can be used with flame ionization detector (FID), when analyzing organophosphorus pesticides even in complex samples like apple juice or onion, as well as pyrethroids in vegetable oils. The flexibility of GC also allows to use different detectors, like electron capturing detectors (ECD), mass selective detectors (MSD), flame photometric detectors (FPD) or nitrogen phosphorus detectors (NPD), in order to maximize the sensitivity.[10]

Recently, especially on the rise is the application of mass spectrometer detectors, which are as popular alternative for pesticide analysis as previously mentioned detectors. Using gas chromatography tandem mass spectrometry (GC/MS-MS) it is possible to monitor up to 381 different pesticides in grapes.[71] At the same time, it is often possible to use the same GC-MS modifications, with around 95% of separations happening in a fused silica column with a nitrogen or helium as a carrier gas. Still, the application of GC techniques can become more difficult in the future, due to the rising popularity of more polar pesticides, which are extremely volatile and have low thermal stability. This is because reduced thermal stability can cause a compound to degrade or break down in a high temperature of GC, thus possibly leading to incomplete and inaccurate results.[10] Although, analyte derivatization can often, insure successful analysis by improving analyte response and chromatographic separation.[72] High polarity on the other hand may cause poor separation in a column since polar compounds tend to react more strongly for example with a stationary phase.

Despite their wide and popular usage, chromatographic and mass spectrometry methods are often costly and require highly professional team, as well as a lot of time. Thus, cheaper and faster on-site analysis methods are often in high demand. One of these methods is enzyme-linked immunosorbent assay (ELISA). It is a biochemical test, that is usually done on a microplate, and which uses enzymes or antibodies for compound identification. ELISA has proven to be especially useful when detecting diverging groups of pesticides, like organophosphorus pesticides, fungicides or neonicotinoids in different food matrices.[73]

Another relatively easy to use on-site detection methods are often linked to colometry, like fluorescence (FL) and surface-enhanced Raman scattering (SERS).[10] Through fluorescence spectroscopy, it is possible to analyze molecules by its electromagnetic radiation, which can be especially useful when examining modern food components, as well as its additives and contaminants.[74] SERS on the other hand enhances and examines scattered light, that is produced after photons interaction with compounds molecular vibrations, which results in highly selective and sensitive analysis.[75] Overall, these methods have been especially on the rise since the development of new nanomaterials, that can for example enhance fluorescence detection, as well as provide more sensitivity and selectivity. Additionally, this kind of optical detection does not require sample preparation and can even be linked to smartphones, which can often help with data analysis.[10]

Some electrochemical methods were also found to be useful in detecting pesticides, like paraquat. These kinds of methods usually utilize electrical current monitoring, for example near electrodes during electrochemical reactions, and tend to be quite versatile. Although conventional unmodified electrodes are not usually reliable, it can still be portable, and its sensitivity and selectivity improved.[76] Examples of different previously mentioned analytical methods for pesticides in foods, with their limits of detection (LODs) and quantitation (LOQs) can be seen in Table 4 below.

Table 4. Detection methods for pesticide analysis in fruits and vegetables.[77]

Detection method	Pesticide class	LODs (mg/kg)	LOQs (mg/kg)
GC-MS	Multiclass pesticides (80)	0.0025-0.02	0.01-0.1
GC-MS/MS	Multiclass pesticides (140)	0.006-0.008	0.01
LC-MS/MS	Multiclass pesticides (128)	0.00012-0.0021	0.0004-0.0071
HPLC-UV	Trichlorfon and monocrotophos	1.2-4.2	-
UHPLC-MS/MS	Pyrazole	<0.0001	<0.006
UHPLC-MS(TOF)	Multiclass pesticides (60)	0.3-3.7	0.8-11.8
ELISA	Chlorpyrifos and fenthion	0.0002 and 0.0005	-
Electrochemical biosensor	Carbamate	0.0004 mol/l	-

Overall, although there are different analytical methods for pesticide detection, with their own advantages, we will take especially close look at the hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-UHPLC-MS/MS), as well as supercritical fluid chromatography with ultraviolet detection (SFC-UV).

3.1 Liquid Chromatography

The main concept of chromatography lies in the separation of mixture into individual components, in order to better understand the mixture and compounds that it contains. The compounds in the investigated mixture are usually distributed differently between two phases, that are moving relative to each other - one of the phases stays in place (stationary phase), while the other moves in a certain direction (mobile phase). This allows compounds to be separated from each other, when sample is carried through the stationary phase, i.e. elution.[78]

One of the most frequently used chromatography techniques, that separates compounds into individual parts, is liquid chromatography (LC). It is found to be especially useful, for example in separation, identification and quantitation of medication, amino acids, environmental toxins and carbohydrates.[79] In liquid chromatography mobile phase consists of a liquid, while stationary phase is either a solid or a liquid immobilized on its surface. LC stationary phase typically contains small (approx. 5 or less μm in diameter) spherical particles, which are highly

porous. This creates a large surface to which an 18 carbon atoms long alkyl chain can also be bonded.[80]

During analysis, the sample is dissolved in a mobile phase and eluted through stationary phase. Chromatographic separation itself mainly occurs due to the different levels of affinity between compounds and mobile, as well as stationary phases. This difference can originate from physicochemical properties, such as polarity and adsorption, which tend to vary between different compounds and both phases. Stronger interaction results in longer retention, while weaker interaction allows compounds to move faster. Since analytes are often being retained mainly by the surface of the stationary phase pores, stationary phase material plays a crucial role in compound separation. Combined differences in specific compounds interaction with the stationary and mobile phases results in different detection time for each analyte, which can be used for further investigation.[80]

The measured time that analyte spends in column (retention time) can for example be used in compound identification, by comparing analytes retention time to the known standards and databases. Additionally, intensity of the signal can be used for analyte quantitation, for example by comparing the area of the peak with the calibration standards. Chromatographic analysis results are thus usually presented in the form of chromatogram, where X-axis is the acquisition time and Y-axis is the strength of a signal.[70] An example of LC chromatogram can be seen in a Figure 10 below.

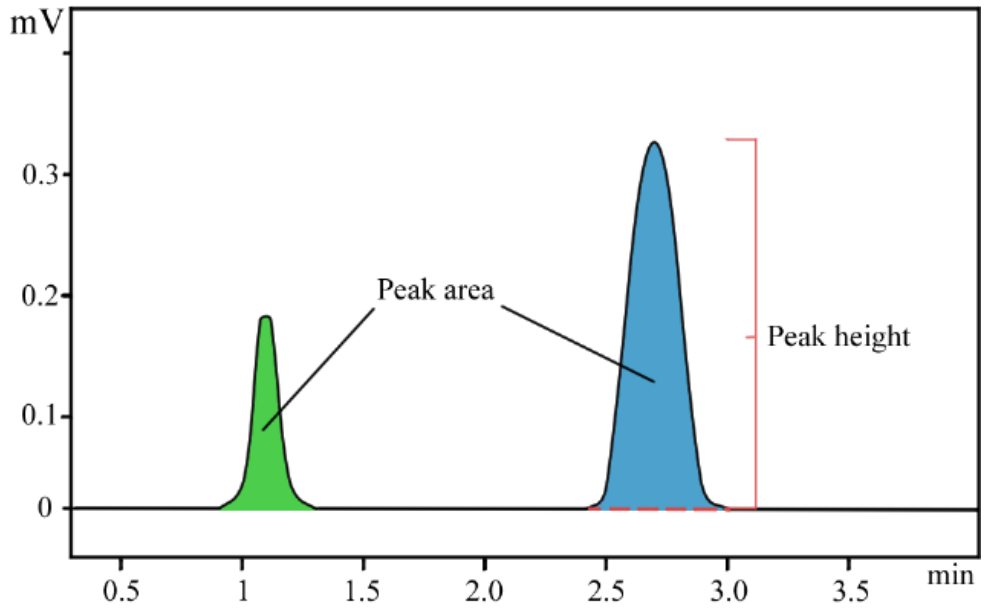


Figure 10. Example of a chromatogram from LC analysis. Here, two analytes produced separate peaks, from which blue one seems to give stronger signal, based on its peak height and area.

Although liquid chromatography can often be divided into High Performance Liquid Chromatography (HPLC), Ultra-High Performance Liquid Chromatography (UHPLC) as well as liquid chromatography coupled with mass spectrometry (LC-MS) and other mods, they all usually consist of the same structure. This structure mainly contains injector, mobile and stationary phases, pump, column, detector, as well as data system.[70]

First, sample, usually in the form of liquid containing analyzed mixture, is accurately and precisely introduced into mobile phase via injection. Mobile phase, which contains a solvent or a mixture of solvents, then carries out the sample through the system, including stationary phase. Pump plays a crucial role in the movement of mobile phase through LC-system, which is done at a controlled flow rate. The stationary phase itself is packed into a column, and after chromatographic separation, components are eluted from the column at a different time. This is then observed by a UV-Vis spectrophotometer, mass spectrometer or other detector, depending on the analysis requirements. Finally, the data system processes collected signals from the detector and generates a chromatogram, which is essential for qualitative and quantitative analysis of the sample.[70] An example containing HPLC-system can be seen in the Figure 11 bellow.

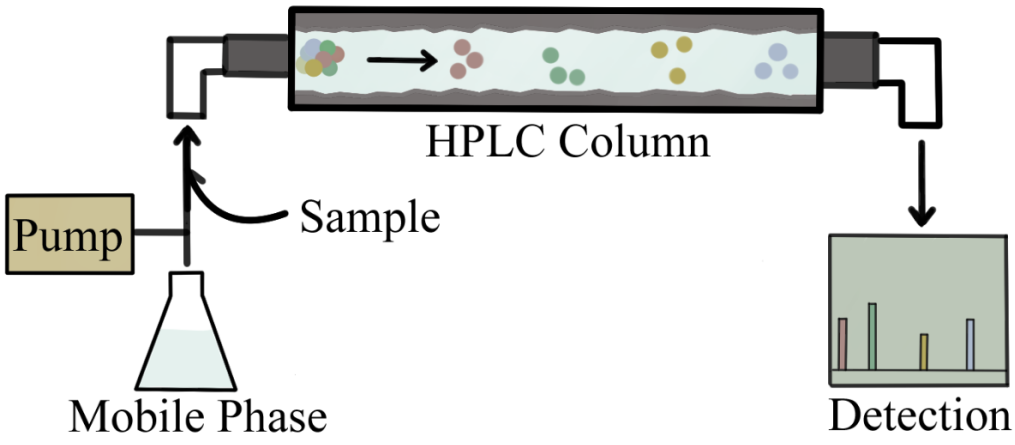


Figure 11. HPLC column compound separation and detection.[81]

Here in Figure 11 example, we can see that blue components have stronger affinity in a mobile phase, and are thus detected first, while red ones are more fixed to the stationary phase. In liquid chromatography correct combination of both mobile and stationary phases is important for efficient separation of samples compounds.[82]

When choosing mobile phase, it is crucial to consider its polarity and how it is relative to the stationary phase and the sample. For instance, mobile phase can be adsorbed to the surface of stationary phase, if both are strongly polar, which can reduce the separation, since sample is

eluted too quickly.[80,79] Additionally, elution itself can be isocratic, or gradient elution. During isocratic elution, the composition of the mobile phase mixture is consistent throughout the analysis. On the other hand, during gradient elution, composition of the mobile phase changes during measurements, which affect compounds separation. Gradient elution technique is frequently done for example in HPLC or UHPLC.[82,80]

An example of quite often used stationary phase adsorbent materials is silica. The silica stationary phase usually consists of bare silica, silica that is modified with different polar functional groups or polymer-based stationary phase. Silica particles are usually incompressible, rigid, and contain acidic silanol groups on their surface, which can cause strong retention with analytes carrying amino groups, as well as other polar groups (OH, SH, CO, etc.).

Bare silica gel can be divided into two main types - fine pore silica gel (type A) and large pore silica gel (type B), from which type B silica usually provides better separation. This is because type A silica is often acidic and causes asymmetric peaks, as well as strong retention, due to the contamination by specific metals which can form complexes with chelating solutes by activating surface silanol groups. This can be especially crucial for basic compounds that are positively charged, since silanol groups tend to ionize under high pH values, after which retention is strongly affected by cationic exchange.[83]

Type B silica on the other hand is less acidic, containing only a small amount of metals in it, and at the same time is highly purified, with better stability in medium and high pH values. Thus, samples applied to Type B silica are usually well separated. There is also a type C silica gel, that can be utilized in HILIC mode for base or acid separation, usually with buffer mobile phase that contains around 50-70% organic solvent. It is also less polar normal silica gel, since its surface is hydrosilated with nonpolar silicon hydride groups.[83,84]

Organic solvents, such as hexane and acetonitrile are often used as a mobile phase, when utilizing silica based columns. Water, acetonitrile and alcohols are usually considered to be strong solvents, due to the binding to silanol groups, which deactivates silica and minimizes chromatogram peaks “tailing”. Alkanes on the other hand are usually viewed as a weak solvent. When utilizing silica stationary phase, mobile phase solvents are also often changed to elute sample components in different polarities and thus enhance the separation.[80]

3.1.1 High- and Ultra High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a wildly used form of liquid chromatography, due to the enchantments in sensitivity, analysis time, resolution, and versatility, that it presents. Compared to LC system, HPLC utilizes pump with high pressure (up to 400 bar), which allows it to use smaller particles in the chromatography column. These particles are usually porous throughout, and their pore size can range from 6-30 nm, depending on the size of the analyzed molecule. This makes separation faster and more efficient, because of the increased surface area, as well as reduced diffusion of compounds. Furthermore, another column can be added, to increase efficiency.[80]

Ultra high-performance liquid chromatography (UHPLC) can be viewed as an improvement to HPLC, with even faster separation and higher resolution. Compared to the HPLC system, UHPLC can operate in up to 1200 bars, and requires a special column, which contains even smaller, totally porous particles - less than 2 μm in size, which further improves separation and reduces analysis time. High sensitivity makes it especially useful for analysis of complex mixtures, for example in environmental monitoring and pharmaceutical research.[80]

3.1.2 Normal and Reverse Phase Liquid chromatography

Normal and reversed phases chromatography are some of the most popular mods that can be used during LC. As mentioned in the previous chapter, compound separation and adsorption depend on the two main intermolecular interactions: attraction between solute and the stationary phase surface, as well as attraction between solute and the mobile phase. In normal phase liquid chromatography (NP-LC), which can be seen in Figure 12, mobile phase is less polar than stationary phase. For example, NP-LC mobile phase often consists of hexane with some polar solvents, like acetone, while stationary phase surface commonly consists of polar silica or alumina.

Because of the polarity of stationary phase and less polar nature of mobile phase, polar compounds tend to have stronger adsorption, compared to nonpolar, which remain in the mobile phase and thus tend to have faster retention time. Additionally, since retention is heavily dependent on compounds interaction with stationary phase surface, geometry of these interactions also plays a crucial role, and NP-LC can be quite sensitive to the relative position of molecules functional groups.[79]

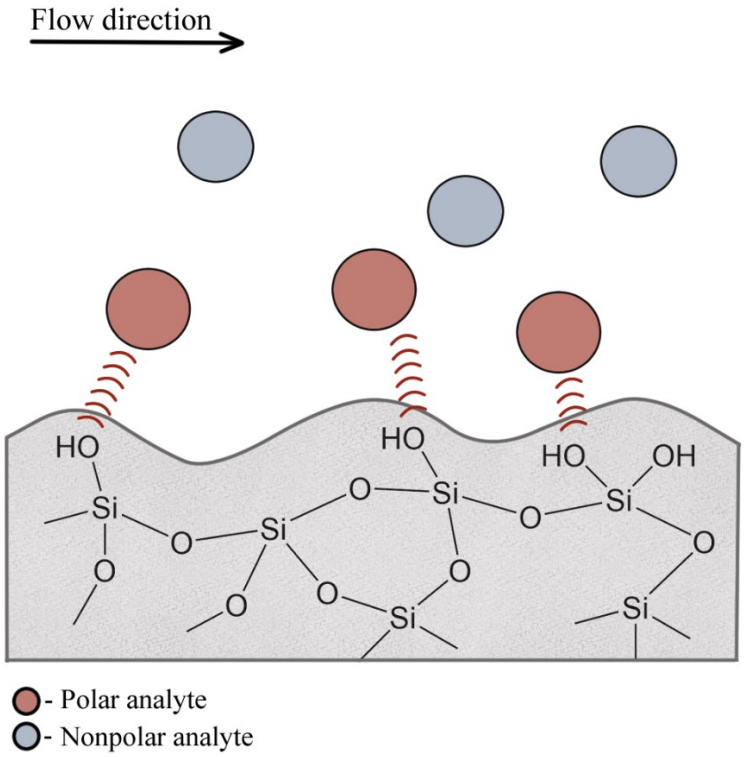


Figure 12. Normal phase liquid chromatography. In this example, silica based stationary phase is used to which polar analytes are adsorbed, while less polar remains in the mobile phase. This leads to less polar analytes faster elution, and polar analytes longer retention time.[79]

Contrary to the NP-LC, where retention is mainly caused by compounds adsorption onto surface of the stationary phase, in reversed-phase liquid chromatography (RP-LC) retention is caused by compound absorption into a liquid-like stationary phase, which is coated on the surface of support particles. During RP-LC, mobile phase is more polar, compared to stationary phase, which can be weakly polar or even nonpolar. Because of this, analytes with stronger polarity are eluted first and less polar compounds tend to have higher retention time.[79]

Reversed-phase liquid chromatography is especially often used in HPLC.[83] During RP-LC gradient elution, water and organic phase (e.g. methanol or acetonitrile) are usually used. Here, organic solvent elutes analytes more rapidly through the column, and is thus considered the stronger mobile phase. When utilizing polarity gradient for solvent, it is proceeded from the most to the least polar solvent and often used mixtures for polar solvents include for example acetonitrile, water, or methanol.[85]

RP-LC stationary phase is relatively nonpolar and organic in nature, and its silica surface can for example consist of 18-carbon-long hydrocarbons (C18). Because of these C18 chains, stationary phase can be liquid-like, and have solute diffuse in it, which furthermore leads to

solutes partitioning between stationary and mobile phases. When analytes are divided between stationary and mobile phases, the hydrophobic ones are retained to the stationary phase for a longer time, compared to less hydrophobic.

On the other hand, compounds with stronger polarity will move more rapidly with the mobile phase. Thus, in the case of C18 column, if faster retention of polar analytes is needed, more polar mobile phase can be used. Vice versa, if analyte is less polar, mobile phase can contain higher amounts of organic modifier.[86] An example of reversed-phase liquid chromatography utilizing C18 column can be seen in Figure 12 below.

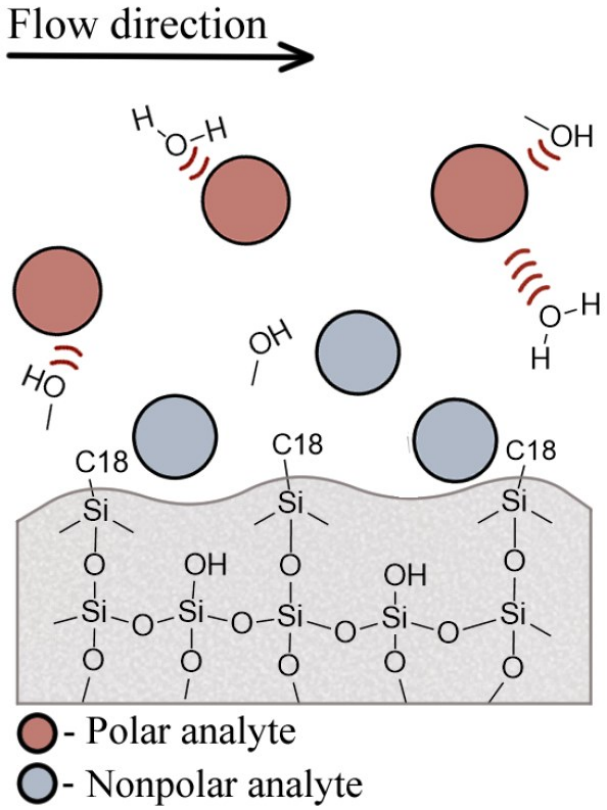


Figure 13. Reversed phase liquid chromatography. Stationary phase consists of silica, covered with C18 chains. Mobile phase consists of methanol and water. Polar analyte, due to its polarity, is strongly attracted to polar mobile phase. Contrary, nonpolar analyte can interact with mobile phase only through weak dipole-dipole interaction and is better adsorbed to non-polar stationary phase. Thus, polar analyte will be eluted first.[79]

RP-LC is quite versatile analysis method for various applications, such as environmental monitoring, pharmaceutical and forensic analysis, as well as food testing. With the help of MS

detection technique, RP-LC method has also become more convenient and rapid, including a wide spectrum of possible extraction formats.[87]

3.1.3 Hydrophilic interaction liquid chromatography

Hydrophilic compounds are well soluble in water and tend to dissolve in it and stick to its surface, which is also true for polar organic substances, since they have hydrophilic regions. Because of this phenomenon, with the help of hydrophilic interaction chromatography (HILIC) it is possible to separate extremely polar compounds, like peptides and pharmaceuticals. Thus, it is often viewed as a useful variation of reversed-phase liquid chromatography, especially when dealing with strongly polar compounds that are highly water soluble.[79]

In HILIC, more hydrophilic analytes tend to be retained for a longer period of time, while at the same time separation between compounds can be based on their polarity and degree of solvation. Solvation with stationary phase surface can for example result in different solvent concentrations near stationary and mobile phase. Overall, retention of the sample depends on different intermolecular interactions between analyte, stationary- and mobile phases, as well as interactions within stationary and mobile phases themselves. For example, weak electrostatic interactions can occur, as well as hydrogen donating.[83]

HILIC stationary phases are usually strongly polar, containing for example silica or amino groups. Some HILIC column types also consist of zwitterionic materials (ZIC-HILIC), that have an equal amount of negatively and positively charged functional groups. Even though it is possible to separate small polar molecules with great efficiency using polar stationary phases, the properties of this stationary phase often greatly impact the choice of mobile phase, as well as buffer pH and ion strength. Additionally, stationary phases in HILIC are always coated with thin water layer and polar analyte partition in it, sometimes even directly interacting with polar stationary phase.[83]

HILIC mobile phases on the other hand are more similar to those in RP-LC and contain around 60-97% aprotic organic solvent (acetonitrile, methanol, ethanol etc.), which is always mixed with aqueous buffer.[79] During HILIC separation, mobile phase composition can be isocratic, utilizing high concentrations of organic solvent. Another HILIC separation method can also utilize gradients, with gradient elution going from weak to strong mobile phase - starting from solvents with high organic concentrations and ending with highly aqueous ones.[84] It is

important to remember that water is one of the strongest eluents in HILIC, and thus large volumes of aquatic sample solutions can result in broad and unreliable peaks. Additionally, higher concentration of organic solvent usually weakens the polar analyte solubility in the mobile phase.[70]

The separation mechanism of HILIC can occur through several different pathways, such as previously mentioned partitioning of sample between mobile and stationary phase, sample adsorption onto adsorbent surface, mobile phase modifier selective adsorption onto the surface of adsorbent, and ion exchange.[83] Depending on the characteristics of solute, stationary phase structure and mobile phase content, each of these separation mechanism have their own relative contribution to the analytes retention time.[79]

Partitioning is thought to be especially important in HILIC retention phenomenon, since separation often occur due to differential dispersion of analyzed molecules between organic mobile solvent and hydrophilic stationary phase water layer.[83] For example, when using bare silica, water from the mobile phase is attracted and held by Si-O-H groups via hydrogen bonding. This liquid layer, formed on top of the stationary phase, may hold more water than mobile phase, and thus attract and retain even more polar analytes. Additionally, the effect of this liquid layer if further increased, the more hydrophilic analyte is, resulting in stronger partitioning and retention. It is still also possible for specific analytes to interact directly with silanol groups via hydrogen bonding.[80] An example of such HILIC elution can be seen in Figure 14 below.

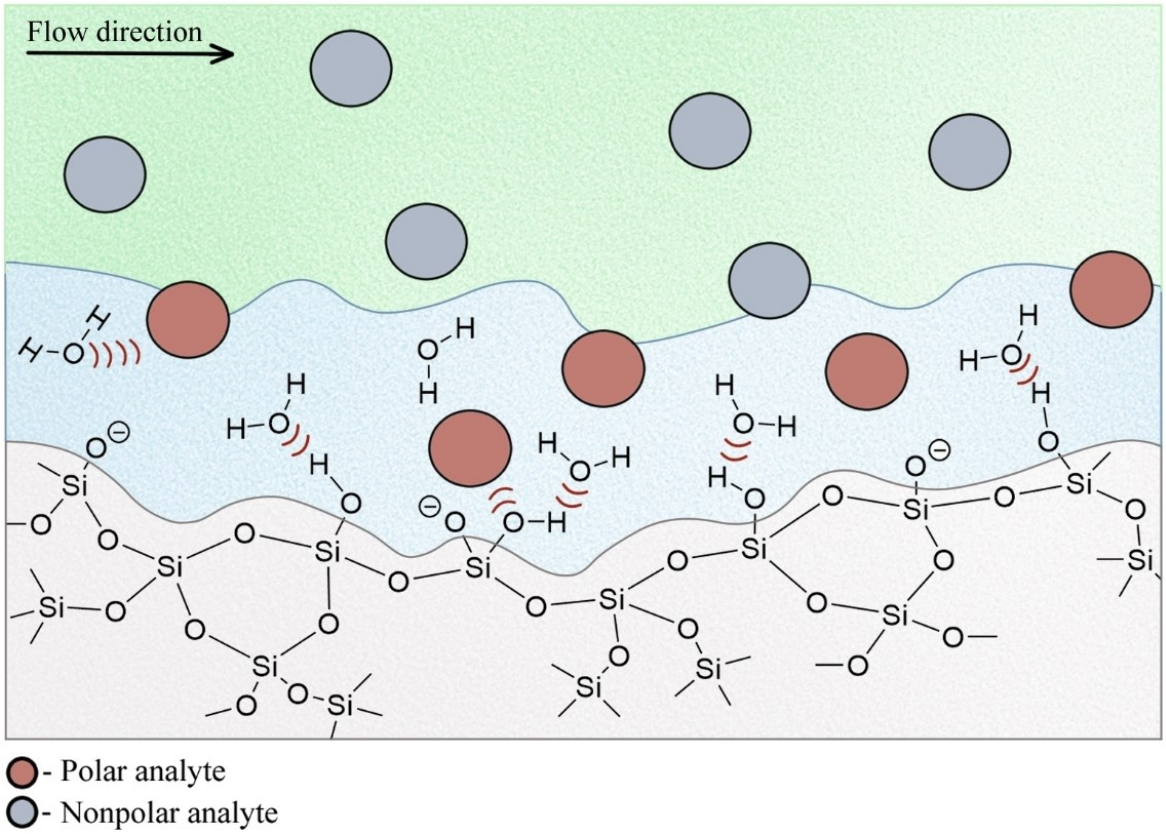


Figure 14. HILIC liquid chromatography. Grey area represents silica stationary phase, blue area – water rich layer, green area – organic-rich layer.[79]

In this example, polar and nonpolar samples are added to column with a mobile phase consisting of 10% water and 90% acetonitrile. Stationary phases consist of bare silica, on top of which a water-rich layer is formed, due to water and Si-O-H group hydrogen bonding. Since polar compounds tend to be more water soluble, polar analyte partitions into this water-rich layer, from organic-rich acetonitrile layer. Additionally, polar analyte may be adsorbed directly to stationary phase, again through hydrogen bonding. On the other hand, nonpolar analyte is better soluble in organic-rich layer, and thus is eluted faster.[79]

An ion-exchange mechanism often relies on the acidic nature of bare silica, with its pK_a being around 4.5, and the following deprotonation of Si-O-H groups, when pH values are near and above this value. Resulting negatively charged surface can act as a cation exchange site, attracting and retaining positively charged compounds from the mobile phase. In case of amino-groups used in stationary phase, its surface can become positively charged at pH values below their pK_a, and thus act as anion-exchange site. Additionally, HILIC columns with zwitterionic materials are able to utilize both phases, which can provide multiple retention mechanisms.[79]

Ions, such as ammonium formate and ammonium acetate can be added to control the pH and ion strength of the mobile phase, as well as analyte polarity, which can noticeably affect its

retention. For example, when dealing with ionizable analytes, it is important to control the pH and ensure that the analyte is kept at a single ionic form. Additionally, when ion exchange affects the retention, raising buffer concentration may reduce it. However, when ion exchange is not utilized in HILIC, the opposite occurs, and if buffer concentration and pH are not adjusted, asymmetric peak shapes and poor recovery from stationary phase may occur.[83]
When analyzing neutral polar compounds, like carbohydrates, buffer might not be needed. In order to achieve elution, salts, like sodium perchlorate, that can be diluted into organic mixture, can be utilized to increase mobile phase polarity. Nevertheless, when using MS detector, this can provide difficulties, since used salts might not be volatile.[83]

3.2 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) can often be viewed as an alternative to high-performance liquid chromatography, combining the advantages of both HPLC and GC. It is a form of normal phase chromatography, with supercritical fluid as a mobile phase. Supercritical fluid consists of a compressed gas, surrounded by critical temperature and pressure, with characteristics between gas and liquid. It is usually denser than gas, but has higher diffusivity and lower viscosity, compared to liquid, which enhances the solubilization and movement of solutes through the column.[70] Additionally, supercritical fluid mobile phase tends to cause less column backpressure, compared to a liquid.[80]

The instrument setup used in SFC, also seen in Figure 18 below, is quite similar to the HPLC, with alike stationary phases and column types. It consists mainly of an injector, pump, column, compartment that heats the column, restrictor that maintains pressure, and detector. SFC columns also use coating materials similar to HPLC columns, with open-tubular columns being usually preferred one and resembling HPLC silica-fused columns. However, there are some additions to SFC, like heat and pressure control mechanisms since it is crucial to keep supercritical fluids in the right condition.[80,88]

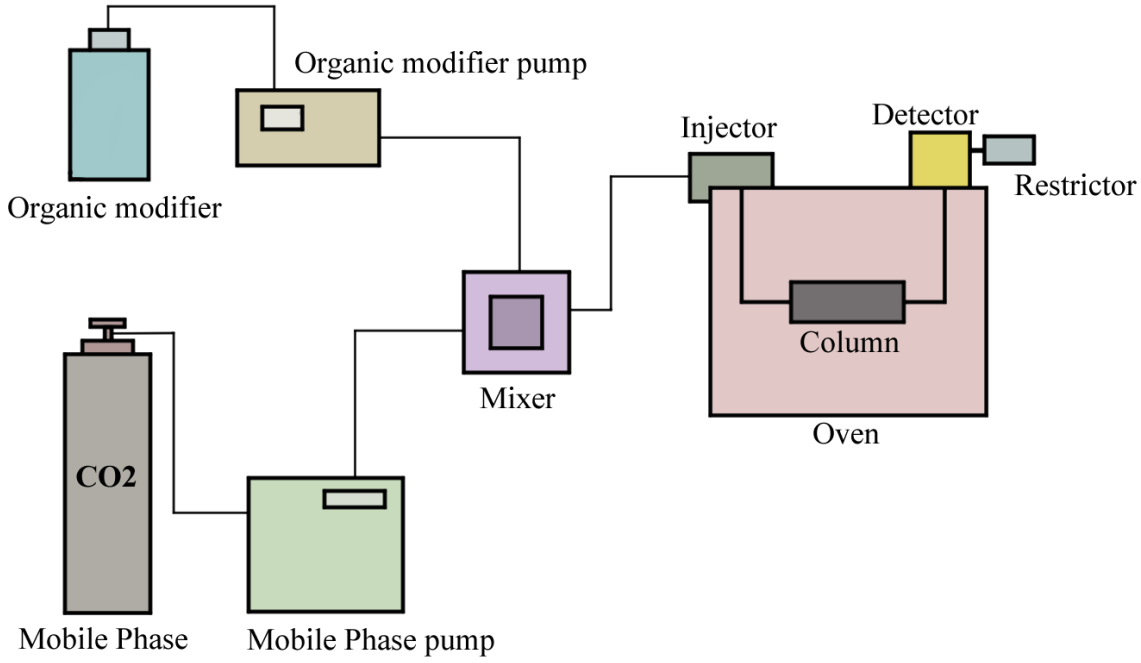


Figure 18. The instrumentation of supercritical fluid chromatography. In this example, restrictor is placed after the detector and packed column is used.[80]

The mobile phase, consisting of e.g. CO_2, is transferred from a pressurized tank by a dip tube. On the way towards the injector, the mobile phase is warmed up, and organic modifier, such as methanol can be added by mixing both streams. After sample injection, mobile phase is pushed through the column, where analytes are separated and further detected.[80]

Although many different GC detectors can be used with SFC, often they provide lower sensitivity, compared to GC, due to CO_2 high background noise. However, when utilizing flame ionization detector (FID), CO_2 does not provide a response, which is why it is often used with SFC. Additionally, others quite often used detectors for SFC include ultraviolet detectors (UV) and mass spectrometer. Previously mentioned restrictor that helps to maintain pressure at the set values, depending on the detector can be placed either in front (FID, MS) or after (UV) the detector.[80]

SFC is more often utilized as a normal phase technique, where polar-polar interaction between polar stationary phase and solutes takes place, with mobile phase advancing from low to high polarity. The mobile phase of SFC usually consists of organic solvent and CO_2 mixture, since its supercritical point is relatively easy to achieve. CO_2 has a critical temperature of 31 °C and critical pressure of 73 bars, with its density being around 0.47g/ml at critical point. However, a combination of CO_2 and polar modifier usually has higher critical temperature and pressure.[80]

One of the most common modifiers for CO_2 mobile phase is methanol, which increases polar compound solubility. Other alcohols, such as isopropanol and ethanol can also be used. Thus, although CO_2 is a weak solvent on its own, when mixed by different organic solvents it can dissolve a wide variety of compounds.[70] It is important to note however, that a mobile phase of CO_2 and methanol cannot be used, when utilizing FID.[80]

During chromatography, steric hindrance has a significant impact on retention time and for instance molecules with unhindered polar functionality will have a longer retention, compared to the molecules were nonpolar moieties surround polar functional group. Good analytes for SFC separation are thus usually well soluble in methanol or other less polar solvents. On the other hand, less suitable analytes tend to require more aqueous buffered solutions or comprise of large biomolecules.[89]

Temperature and the density of the fluid also plays a crucial role for the analyte solubility in a supercritical fluid, as well as both analyte and fluid polarities. For example, as density or temperature of the fluid increases, so does the analyte solubility. It is important to note that increasing fluid temperature reduces its density, and thus both of these effects can work in the opposite way. Additionally, changes in density have more effect closer to the critical point, and the changes in temperature have a stronger impact on solubility at higher pressures. Taking this into account, gradient elution in SFC can be performed by increasing pressure, temperature or the concentration of polar modifiers, with pressure increase being a more commonly used technique.[80]

SFC columns usually consist of either open or packed capillary columns. Open capillary columns are especially often applied with neat CO_2 as well as flame ionization detector (FID) or MS. They are usually 50-100 mm in inner diameter, 10m long and are well suited for analytes with low polarity and high molecular mass. SFC packed columns on the other hand can be either packed capillaries columns, or conventional HPLC columns. They usually have higher loadability compared to open columns, with usual length being between 15 and 25 cm. It is more suitable for natural product components, chiral compounds as well as small industrial polymers. In this case, often used mobile phase is the mix of CO_2 and methanol, with the use of gradient elution, and UV or MS detectors.[80]

Highly pure and porous bare silica is often used in SFC as polar stationary phase, as well as porous silica with bonded phases. SFC columns are usually not expose to ultrahigh pressures, and thus particle strength is not a crucial factor, with the most common particle being spherical

and having diameter around 1.7 and 5 µm. For polar solutes, it is better to use fully porous particles, with pore size around 60 to 120 Ångstroms and surface area > 350 m^2/g.[89]

In comparison to HPLC, SFC not only provides more speed and safety, but also higher flexibility and resolution, due to the higher coefficients for diffusion of analytes in supercritical fluids. Supercritical fluids can also dissolve nonvolatile solutes, which is why SFC is especially used for separation of nonvolatile and thermally labile substances, that cannot be analyzed with LC or GC. Additionally, SFC can be utilized with other techniques, like nuclear magnetic resonance (SFC-NMR), as well as mass spectrometry (SFC-MS), which further enchases the analyte determination capabilities.[70,11] The comparison between SFC, HPLC and HILIC preferred analytes is shown in Figure 19 bellow.

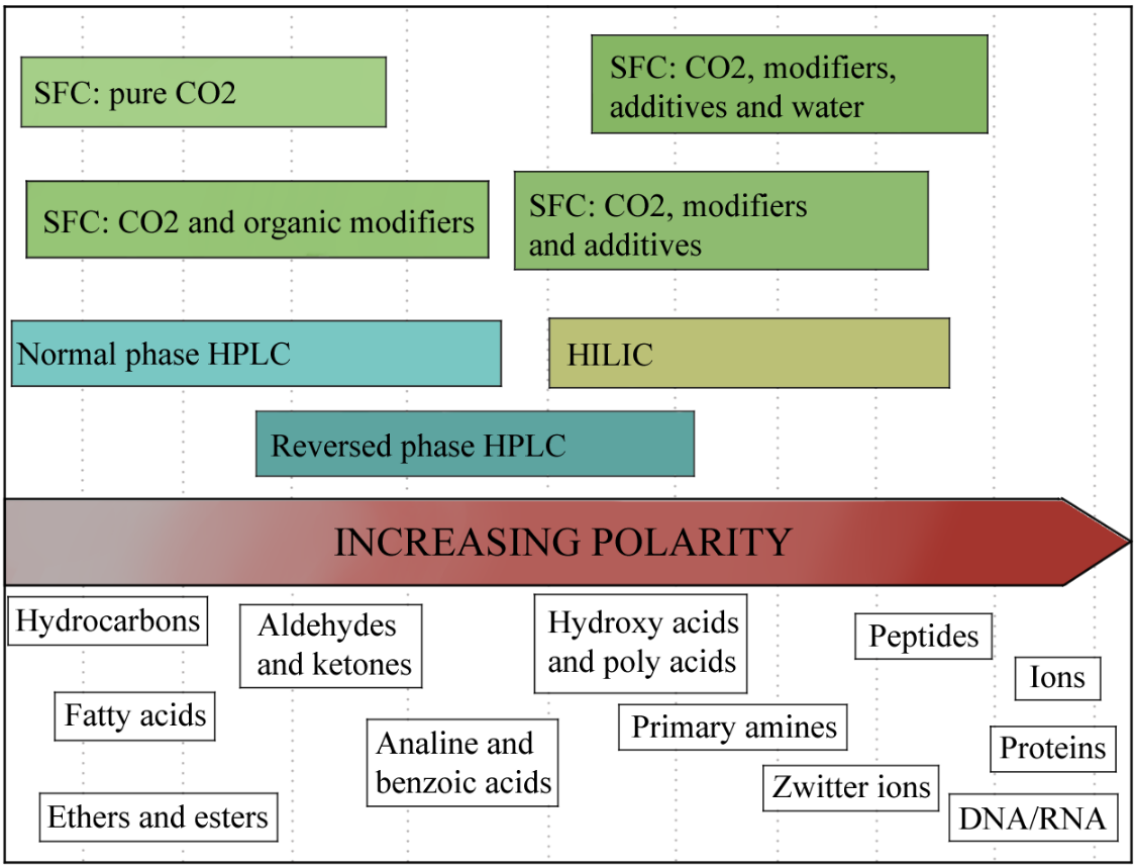


Figure 19. Preferred analytes for SFC, HPLC and HILIC.[89]

SFC is often used in pharmacy since it can separate many different pharmaceutical solutes and is helpful in preparative chromatography of chiral compounds. However, because of its novelty and the need for special equipment, its application in clinical laboratories is often limited. [88] SFC is also often utilized in food and environmental industries, for example in the analysis of pesticides, polymers or explosives.[88,11]

3.3 Mass spectrometry

One of the most important analytical tools for chromatography, that provides qualitative and quantitative information with high sensitivity, is mass spectrometry (MS). It is used to analyze the mass of atoms, molecules, or fragments of molecules, and consists mainly of ion source, mass analyser and detector.

During MS analysis, sample is first ionized with the help of an ion source. It is important to ionize the sample, in order to achieve its manipulation by external electric and magnetic fields and depending on the compound characteristics as well as experimental requirements, either positively or negatively charged ions can be made. Formed ions are then accelerated further through an electric field and are separated based on their mass-to-charge ratio (*m/z*), which is followed by a detection. The typical MS spectrum thus shows the amount of ions being detected at different mass-to-charge ratios. Additionally, mass of the ion with the charge of ±1 is equal to ions m/z value, while in other cases, *m/z* value will only be a fraction its mass - for example, an ion with a charge of ±2 will have an *m/z* value that is half of its mass.[70]

3.3.1 Ionization

Although there are several different ionization methods, with their own unique versatility and applications, in this chapter we will focus on the more significant for the pesticide analysis, which are electron ionization (EI) and electrospray ionization (ESI).

During EI, high energy electrons, that are emitted from a heated filament, interact with sample molecules or atoms, which can result in a loss of an electron and formation of positively charged ions. These ions are then pushed further by repeller plate with similar potential. Additionally, after interacting with electrons, ions often have enough internal energy to further break down into fragments. Because of this, it is considered to be “hard ionization”, and can be used only with volatile and thermally stable compounds. The resulting smaller fragments can also be used for structural determination of a compound.[70]

Another quite popular ionization method is electrospray ionization (ESI), which can be seen in Figure 15. Contrary to the EI, electrospray ionization is a “soft” ionization method, that usually does not produce fragments, and can be done under atmospheric pressure. During ESI, high voltage is used to produce ions directly from the solution. It is quite versatile method, suitable for stable and thermally labile compounds, and is often used for compounds with polar groups.

Because of this, the ESI method is especially often used when MS is coupled with LC. Under suitable conditions, neutral polar compounds can donate or accept protons, resulting in positive or negative ions already in the mobile phase. For example, ionization of acids and bases can occur by adjusting the pH of the mobile phase.[80]

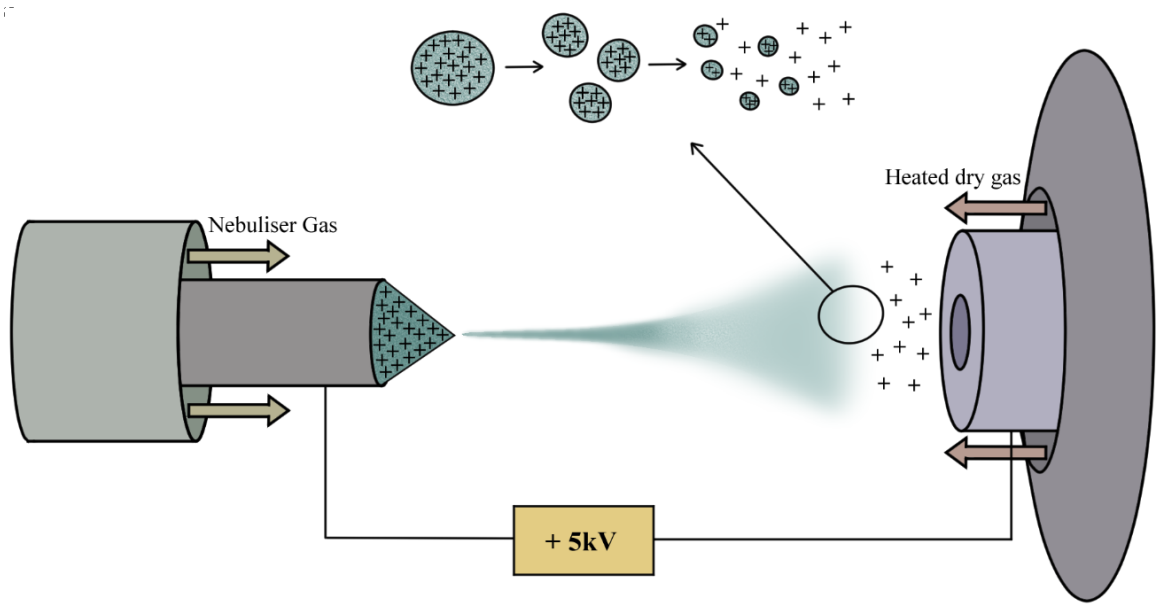


Figure 15. The structure of electrospray ionization method. On the left, the mobile phase containing sample is ejected from thin capillary under a high voltage. After ions in gas phase are formed, they move further towards mass analyzer.[80]

At the start of the ESI process, the mobile phase containing sample is pushed through a thin capillary with a high voltage (e.g. 5kV), which promotes the formation of sample ions. At the end of the capillary, nebulizing gas, usually consisting of N_2, is applied in order to assist the formation of droplets. Additionally, heated dry gas can be applied in the opposite direction to the ion flow, to evaporate solvent from droplets. Because of the accumulated ions, these droplets are highly charged, and decrease is size as they move towards mass analyzer.

This repetitive partitioning is mostly due to the repulsive forces of ions exceeding the surface tension the of drop, resulting in the formation of smaller and smaller drops, until ions in the gas phase are formed. Ions themselves can then be analyzed and detected in either positive or negative mode and can consist of intact analyte ions, protonated/deprotonated ions, as well as adduct ions.[80]

3.3.2 Mass analysis

After ionization of sample compounds, formed ions move further through skimmers and lenses, that focus this ion beam and divide mass spectrometer into different parts. Throughout these parts, vacuum pressure levels are lowered, the closer they are to the mass analyzer, creating a gradually increasing vacuum system. Vacuum conditions are necessary for the MS analysis, since air molecules might prevent ions from reaching the analyzer.[80]

The performance of mass analyzer itself can be viewed based on its mass resolution, mass accuracy, scan speed and sensitivity. Mass resolution (R) shows the ability of mass analyzer to separate different m/z values from each other, with higher R resulting in better separation for closely related m/z values. Mass accuracy (E) on the other hand shows the difference between theoretical and measured m/z value, with smaller E indicating more precise measured results. Scan speed describes the time needed to scan ions during mass analysis and sensitivity shows the signal-to-noise ratio.[80]

One example of mass analyzer is quadrupole mass analyzer, which can be seen in Figure 16. It consists of four identical rods that are placed parallel to each other, with opposite rods pairs being connected electrically. This enables the creation of an oscillating electric field, when specific direct current (DC) and radio frequency (RF) are applied to one pair, and the opposite DC and RF to the other pair. After ions enter this oscillating electric field, they will oscillate in the x-and y- axis. Because of this, ions with unstable trajectory will collide with the quadrupole, while only the stable ones will reach the detector. Taking this into account, when DC and RF are adjusted to a specific value, only ions with certain m/z will be able to pass the quadrupole.[80]

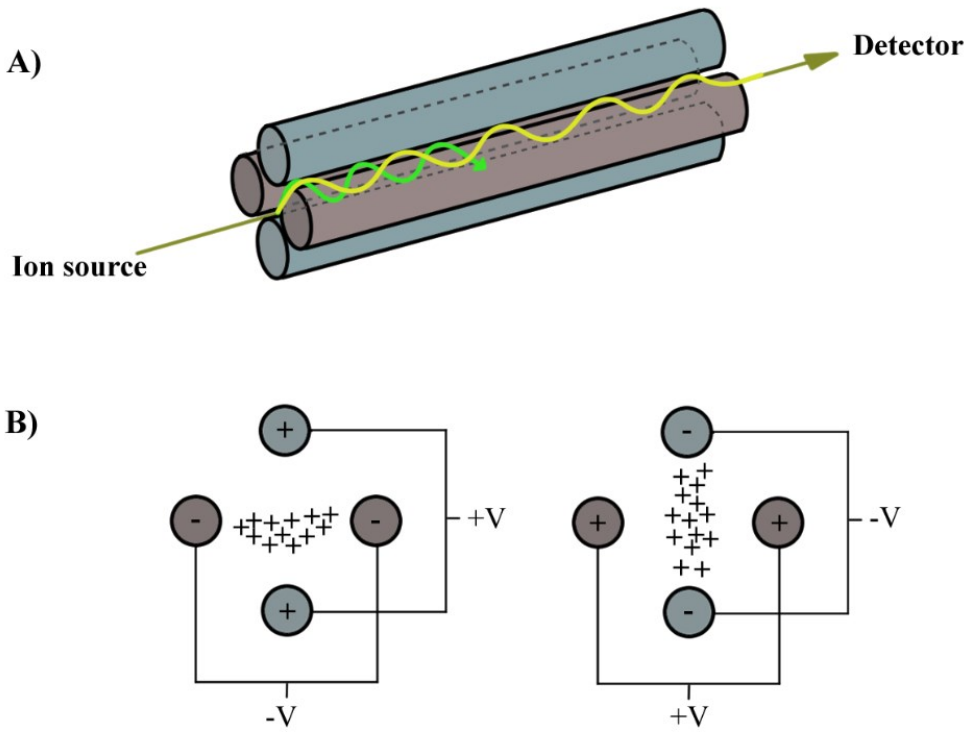


Figure 16. Quadrupole mass analyzer. A) Ions travel from the source towards detector. Only stably oscillating ions (yellow line) will manage to pass through quadrupole and reach the detector. Unstable ions (green line) will collide with the quadrupole rods. B) Different current and radio frequency are applied to rod pairs, resulting in oscillating electric field.[80]

An important mass analysis technique in MS is tandem mass spectrometry (MS/MS). During MS/MS, specific ions - precursor ions, are selected and isolated based on their m/z values, after which fragmentation takes place, for example through collision-induced dissociation (CID). During CID, ions interact with collision gas, impact of which cases the fragmentation. Generated fragments - product ions, that are detected, or used in further fragmentation. These fragments can then be used to achieve detailed information about the structure and composition of a compound.[90,91]

This tandem MS technique is often used for example in triple-quadrupole mass spectrometer (QqQ). In QqQ the first quadrupole (Q1) acts as a mass filter, allowing only ions with a specific mass-to-charge ratio (m/z) to pass further. Second quadrupole (Q2), also called collision cell, generates more analyte fragmentation, for example through collision-induced dissociation (CID). Third (Q3) quadrupole again acts as mass filter and allows only specific ions generated from Q2 to pass through, enabling selective measurements of a product ions. Taking this into account, QqQ can be extremely useful for the analysis of compound structure, or quantitation.[92]

During structural QqQ mass analysis several scan modes can be utilized, such as product ion scan, precursor ion scan, neutral loss scan, as well as multiple reaction monitoring (MRM). Product ion scan is often used to obtain compounds structural information, as well as quantitative target analysis. During product ion scan, Q1 is set to allow compound with only certain m/z value, after which this precursor ion is fragmented in Q2, and its product ions are analyzed through Q3. Resulting mass spectrum thus shows all product ion signals that are formed from the chosen precursor ion.[70, 91]

When applying precursor ions scan, Q3 is set to pass product ion with a specific mass, and Q1 is then scanning "upwards" from that mass. This time only those compounds that provide certain product ion are detected, which can be useful when multiple compounds produce the same fragment. Precursor ions scan spectrum thus contains all precursor ions which could fragment into selected product ions. On the other hand, in neutral loss scan, Q1 and Q3 scans are applied at a specific difference in m/z ratio. This can be used to track the loss of a neutral fragment during CID, and only those compounds that produce fragments with a specific loss are being detected. Neutral loss scan can be used for example when multiple compounds produce the same loss.[74]

Multiple reaction monitoring (MRM), also known as selective reaction monitoring (SRM), requires a selecting of precursors ions, as well as their products. During MRM, both mass analyzers are set to a specific mass, and several different transitions of precursor to product ion are monitored. First, Q1 filters the chosen precursor ions to the Q2, where they are fragmented, and product ions are further transmitted to the Q3, where they are analyzed. Thus, the resulting signal is the reaction monitoring for several selected compounds and their fragments.[93] MRM technique is especially sensitive, highly specific and allows for quantitative analysis of compounds. It is especially often used when analyzing protein, peptides, as well as drugs and their metabolites. However, in order for this to work, analyte needs to have specific precursor ion and specific product ions, which can be linked to it.[94]

Compared to some other techniques, like time-of-flight mass spectrometry (TOF-MS), QqQ does not have as wide mass range and high resolution, however, it still noticeably increases selectivity and accuracy, as well as lowers the limits of analyte quantitation, while being low-cost option and relatively easy to operate. Because of these characteristics, it is quite popular method that is especially used in pharmaceutical, food and environmental sectors.[92]

The previously mentioned time-of-flight method for mass spectrometry (TOF-MS), is also a frequently used method e.g., in pharmaceutical sector, but it utilizes slightly different analysis mechanics. Since ions with different mass-charge ratio (m/z) and the same kinetic energy have separate velocities in a constant electric field, their detection can help determine the composition and structure of a compound. An example of time-of-flight mass spectrometer analysis can be seen in Figure 17 below.

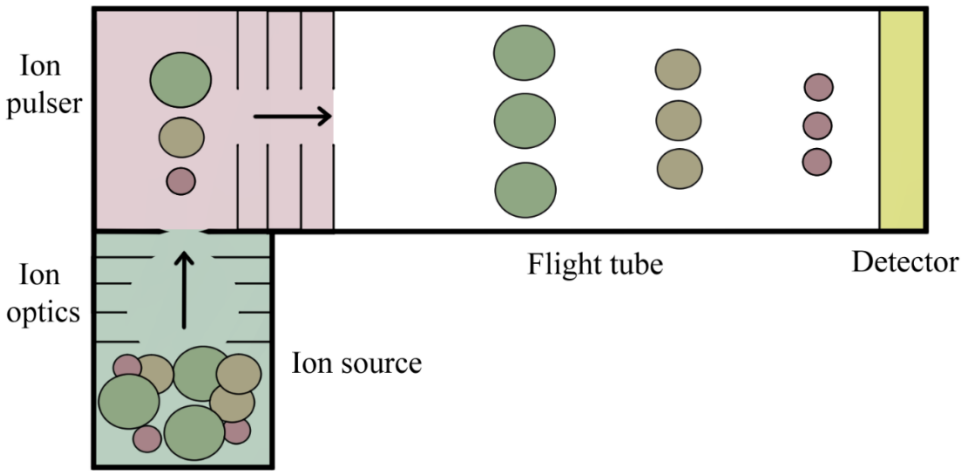


Figure 17. Linear time-of-flight mass spectrometer analysis.[95]

Detection usually occurs after accelerated and positively charged ions travel in a flight tube and hit the detector. Depending on their m/z ratio, they will reach detector at different times, from low m/z to high. TOF works in a theoretically unlimited mass range as a non-scanning technique, and thus full mass spectrum can be obtained in one shot, with insignificant distortion even if composition changes with time during sample stream in MS. Additional positive attributes of TOF include rapid acquisition time, high resolution, sensitivity and accuracy. Modern TOF-MS methods has been especially useful in confirming compounds molecular formula, as well as unknown compound identification.[96]

3.3.3 Detectors

There are many different types of detectors for mass spectrometers, such as electron multiplier, photon multiplier, and microchannel plates (MCP). Each of detectors is better for a specific mass analyzer and can provide different levels of sensitivity as well as response time. For example, microchannel plates detector (MCP) is used with TOF analyzer, while electron multiplier and photon multiplier detectors are used with quadrupole mass analyzer.[74]

The electron multiplier is one of the most commonly used detectors, especially when detection of both negative and positive ions is needed on the same instrument. It operates mainly based on dynodes and secondary emission. Dynode, being an electrode in a vacuum, emits electrons (secondary emission), after ion with enough kinetic energy interacts with it. Additionally, since the electron multiplier itself consists of multiple series of dynodes, these secondary emissions occur repeatedly and are amplified exponentially. Resulting accumulated charge can be measured as a voltage pulse.[97]

Photon multiplier works in a similar way as electron multiplier, with ions also striking dynode, and the following electron emission. However, these electrons then interact with phosphorous screen, resulting in the release of photons. Photons are then exponentially amplified, similarly to the electron multiplier, and are converted to an electrical signal. The microchannel plates detector is also slightly similar to electron multiplier, however, instead of having single channel of amplification, MCP consists of several parallel channels, which are positioned across a plane. Thus, it can also provide spatial information, that can be crucial for example for time-of-flight mass spectrometry.[97]

Overall, mass spectrometry is quite often coupled with liquid chromatography (LC-MS, HPLC-MS etc.), which greatly enhances qualitative and quantitative analytical capabilities. After LC separation, grouped sample components are introduced into MS, whereby providing samples spectral information, MS helps in each compound identification, while being sensitive and selective. MS can also cover a wide range of analytes, which is why it is often used, for example, in food processing, environmental monitoring, metabolomics and with other complex compounds in a biological matrix.[98]

Samples well suitable for LC-MS are usually thermally unstable and non-volatile, as well as large, polar or ionic. These can be for example peptides, alcohols, hormones or fatty acids. However, as with MS, LC-MS utilization also requires precise sample preparation, depending on its chemical and physical characteristics.[98]

3.4 Sample treatment

There are several different methods for the treatment of samples, such as pesticides, in order to achieve their determination from various different matrixes, like food, soils and water. Even though there are no globally accepted standard procedures, pesticide analysis usually follows

two main steps – analyte extraction from the matrix (fruits, vegetables, etc.) with cleaning, and then determination by preferred analysis technique.[10]

Initial material is often compromised to slightly smaller sub-samples, that are cleaned up and homogenized in a mixer, which can enhance the separation. Various solvents can be used for analyte separation from the rest of the matrix with high efficiency, depending on the analyzed compound and the matrix. For example, solvents, such as methanol, acetonitrile and toluene are often used to extract pesticides from vegetables and fruits. It is important for pesticides to be well soluble in the solvent, while not chemically reacting with it, and co-extractives to have low solubility in the solvent. Additionally, different solvent mixes can be used, in order to enhance the procedure.[10]

These extractions also can be done through several different standardized techniques, which include cleaning processes. However, it is important to consider different chemical changes that might occur to the pesticide during extraction and reduce them to a minimum. For the most part, co-extracts are removed through chemical and physical application, while moisture can be disposed during sample analysis. One of the most used methods for analyte extraction from the sample matrix are liquid-liquid extraction (LLE), solid-phase extraction (SPE), rapid, easy, cheap, effective, robust and safe extraction (QuEChERS), matrix solid-phase dispersion (MSPD) and solid-phase microextraction (SPME).[10]

Partitioning, also known as liquid-liquid extraction (LLE), is based on analyzed compound distribution between two immiscible solutions phases, like aqueous phase and organic phase, which depends on analyte equilibrium distribution coefficient and solubility in each phase. Additionally, extraction effectiveness can be assumed based on donor and acceptor phases partition coefficient, and for example medium-polarity solvents (e.g ethyl acetate, aminopurine) can increase nonpolar solvents polarity, and at the same time reduce polar solvent polarity.[99] Example of liquid-liquid extraction technique can be seen in a Figure 20 below.

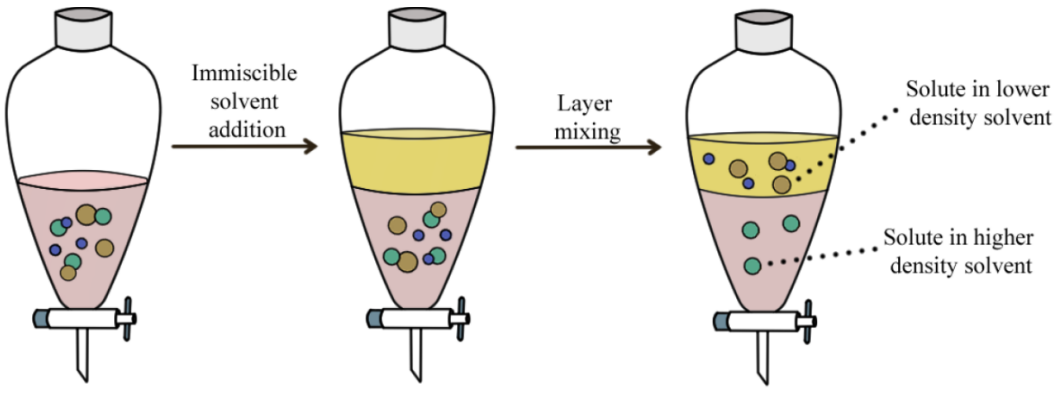


Figure 20. Liquid-liquid extraction technique.[10]

In LLE compounds can be first transferred to one solvent, after which second, immiscible solvent is added. Pesticide tends to dissolve well in an immiscible solvent, which is why several organic solvents, such as hexane, acetonitrile and chloroform can be used, when analyzing them from environmental and food samples. It is also possible to separate several different pesticides, like cyhalothrin, deltamethrin and chlorpyrifos from the same sample, for example using the mix of acetonitrile and ethyl acetate.[99]

Overall, LLE is a well-known, highly efficient and selective method, well suitable for quantitative measurements, and can be utilized for many different analysis techniques, like HPLC and LC-MS/MS. However, utilization organic solvents can often cause difficulties in process automatization, due to hazardous leftovers as well as emulsion, that are hard to break up, making LLE quite costly and time-consuming procedure.[10,99]

Another widely used extraction procedure, solid-phase extraction (SPE), is also known for its ability to separate various samples from complex matrixes, with great speed and recovery, while being relatively simple to use. It usually exploits packing columns or cartridges, where solid-phase material is chosen based on its adsorption selectivity with target analyte. It can consist for example of bonded silica, C18 columns or florisil columns. Before SPE extraction, the column is washed with specific solvent (e.g., water, organic solvent), which cleans the sorbent and enhances the separation.[100] Example of solid-phase extraction technique can be seen in a Figure 21 below.

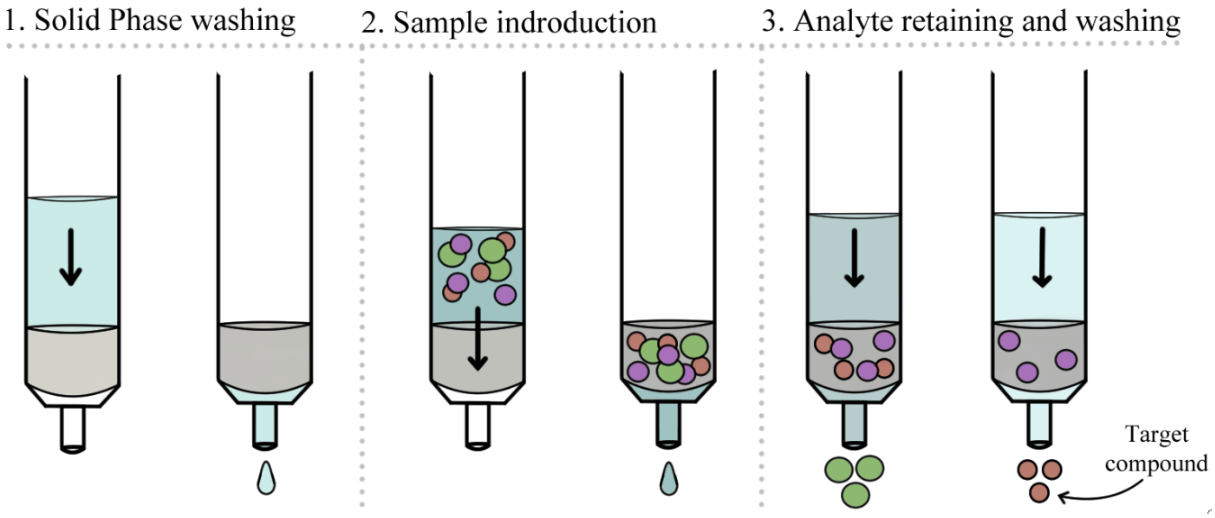


Figure 21. Solid-phase extraction technique.[10]

As sample passes through column, target compounds interact with solid-phase and are retained based on their affinity, which can be controlled with solvent and pH. Thus, it is also crucial to maintain the stability of pesticide. Unwanted compounds move through the column more

quickly and can also be washed away with suitable solvent (usually organic), which further purifies the target compound. Finally, elution with a solvent (e.g., methanol. acetone, hexane), which can be chosen based on the properties of pesticides, is used to collect the compound from solid phase. SPE can also be paired up with magnetic nanoparticles (MNPs), especially when dealing with complex biological matrices, which further boosts selectivity, speed and recovery or the SPE technique. Additionally, when dealing with highly polar pesticides, multiwalled carbon nanotubes (MWCNTs) can be used as a sorbent for SPE.[10,100]

Generally, SPE uses relatively small amounts of solvent, does not generate emulsions, achieves analyte concentration faster and is easy to automate. Its dispersive solid-phase extraction (dSPE) variation, where sorbent is mixed into the sample extract, is relatively easier to use and compared to LLE, while also being more cost-effective. Because of this, it has become especially useful in pesticide analysis, for example from food samples. However, when analyzing multiple pesticides with varying chemical and physical properties, it can be difficult to find suitable adsorbents, as well as elution solvents. Additionally, most commercially available SPE cartridges are not reusable, which can affect the experimentation cost.[10]

Contrarily to the solid-phase extraction, solid-phase microextraction (SPME), which can be seen in Figure 22, does not utilize solvents in sample preparation. Additionally, extraction phase volume is much smaller, compared to the total sample volume. Extraction itself happens due to analyte adsorption, for example from a liquid sample to a fiber, with resulting equilibrium between analyte in the liquid and in extraction phase. Many different stationary phases can be used in fiber, however, is usually consists of fused silica and can be coated with polymers. It can be, for example, located inside the tip of a hallow needle, with the ability to move in and out using the plunger.[80]

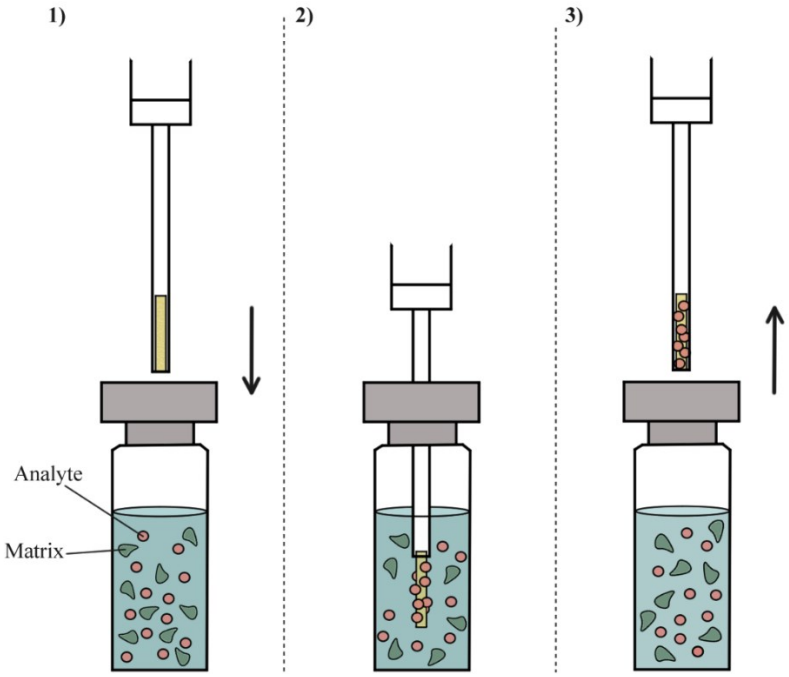


Figure 22. Solid-phase microextraction. Fiber is pushed into the sample solution (1), where analyte adsorption occurs (2), after which needle is pulled out from the solution.[80]

Upon fiber introduction into the sample solution, adsorption equilibrium can take from 2 to 30 minutes, after which fiber is withdrawn back into the needle. The needle is then removed from the sample vessel and the analyte is usually directly injected into an analytical system, such as HPLC or GC. Overall, SPME is quite a useful technique due to its easy automation, reusable fibers, as well as solvent-free way of operating.[80]

Quick, easy, cheap, effective, rugged, and safe method (QuEChERS) is a sample treatment procedure that has also been used for pesticide analysis from different food matrices. It is known for its simplicity, low cost and efficiency. Additionally, it uses only a minimal amount of organic solvents, while providing consistent quantitative results and is relatively low time-consuming, since its mostly based on liquid-liquid extraction, followed up by solid-phase extraction. Example of QuEChERS extraction technique can be seen in Figure 23 below.

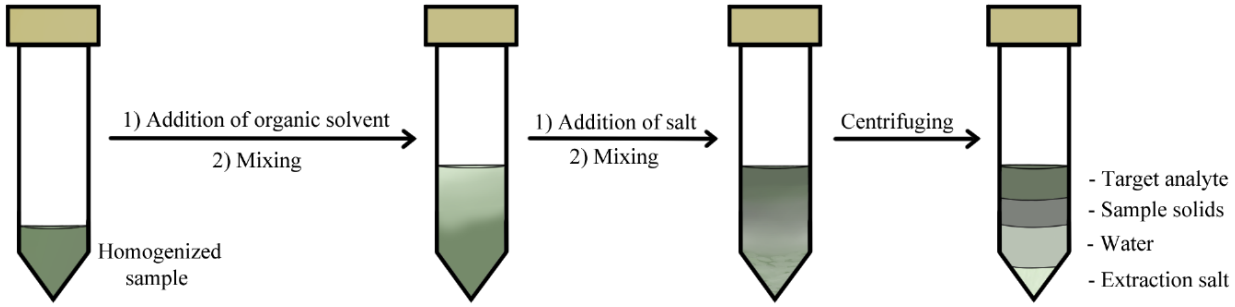


Figure 23. QuEChERS extraction technique.[101]

A homogenized sample is first mixed together with water-miscible organic solvent (e.g., acetonitrile), in order to dissolve the target analytes. Inorganic salts (e.g., sodium chloride, magnesium sulfate) are then added to enhance the separation and partitioning of analytes into organic phase. Salts also stabilize the organic phase and aid in water removal.[102]After mixing sample solution, for example by tube shaking, analytes are further partitioned into organic phase and the tube can be centrifuged, to facilitate the phase separation.

Analytes are usually contained in the upper organic phase, which can be easily transferred for further analysis. In case there are multiple different phase layers, solution cleaning can also be done with the previously mentioned dSPE, after which analysis can be done, for example via GC and LCMS. One of the recent uses of QuEChERS with UHPLC-MS/MS allowed for the analysis of 310 pesticide residues from different food samples, like brown rice, spinach and oranges.[10,102]

Compared to previously mentioned sample treatment techniques, Matrix Solid-Phase Dispersion (MSPD) has a single phase, which includes extraction and cleaning, making MSPD simple and less time consuming. It also produces less waste, does not use as much solvent, and was found to be especially useful in analysis of pesticides from different food samples (e.g., fish, fruit, eggs).[10]

A sample, usually consisting of homogenous powder is placed in a column filled with solid-phase adsorbent (e.g., silica, alumina), which is chosen based on its affinity with target compound and will serve as dispersion medium. Sample and sorbent can also be mixed together, after which they are packed within a column in a single layer, to insure efficient extraction. After elution solvent (e.g., methanol, acetonitrile) is added to the column, it passes through the sample-sorbent layer and selectively dissolves target analyte, carrying it out of the matrix.[103]

It is crucial to utilize suitable solvent composition, in order to achieve pesticide desorption from the adsorbent. For example, the combination of ethyl acetate and alumina columns was effective for extraction of organochlorine and pyrethroid insecticides, unlike hexane and alumina columns. Eluent, containing extracted target compound, can then be collected and further concentrated through evaporation and filtration, or analyzed. Overall MSPD is a rapid and low-cost technique, that can be selective, flexible and particularly useful in treatment of solid, semi-sold and viscous organic samples.[10,103]

3.5 Method validation

In order to ensure the acceptability and reliability of analytical procedures, it is crucial to conduct a method validation process. The validation process might include several tests for analytical methods for various attributes, which provide useful information that can be used further. It is important to conduct the validation in normal test conditions, and the results will be specific to the procedures and parameters such as sample treatment, analyte concentration range and analyte matrix. The validation test itself can include studies on method accuracy, specificity, linearity, range, precision, accuracy, robustness, as well as limit of detection (LOD) and limit of quantification (LOQ).[70,104]

Specificity and selectivity, describes the ability to distinguish specific analyte from other compounds that might be in the sample. The more selective a method is, the more accurately it can measure an analyte in a mix with other compounds. Usually, baseline separation is required, where in the spectrum, after analyte detection peak, detector signal returns to baseline before the next compound is detected. Additionally, selectivity can be examined through chromatographic blanks, in the time windows for the expected analyte peak. Another criterion can also be, for example, that possible impurities do not affect the analyte assay by more than 0.5%.[70,104]

The range of analytical methods describes the interval at which precision, linearity and accuracy are acceptable. It often consists of a concentration range which involves a linearity test. Before analyzing the target compound from a sample, a calibration curve is usually made. The linearity of a calibration curve thus shows how well it will be proportional to the quantity of analyte. Calibration curve is usually made of five standard solutions with the range of 0.5 to 1.5 times the expected analyte concentration, with each standard being prepared and analyzed three separate times, resulting in 15 standard solutions and three blanks. Suitable linearity for calibration curve usually requires square of the correlation coefficient (R^2) to be at least 0.999 or more.[70]

Accuracy of the method shows the relation between methods test results and the true value. It can be determined by analyzing a certified reference material in a similar matrix, using the same precision. It is also possible to utilize different analytical methods, and then compare the results. Another common method of accuracy validation is sample spiking. It involves the addition of a known amount of specific compound or analyte to a sample, after which it is again analyzed, and the results are compared with expected or known concentration.[70,104]

Precision describes the similarities between replicated measurements, and can be expressed as standard uncertainty, standard deviation or confidence interval. When replicating individual sample measurements with the same procedure in the same environment and using the same instruments, ideally results should be quite similar. Precision itself can also be divided into repeatability and reproducibility. Reproducibility shows the spread in the results when method is being proceed using same procedure but at different time, by different people in different labs with different instruments. Repeatability on the other hand shows the dispersion of the results when the method is proceeded in the same environments, by the same people and equipment.[70] Precision as relative standard deviation (%rsd) can be calculated from measured values, by utilizing standard deviation (SD) and mean values, as seen in the following equation 1:[104]

$$\%\text{rsd} = \frac{SD}{Mean\ values} \times 100 \quad (1)$$

Additionally, reproducibility precision can be calculated using Horwitz equation, which shows the standard deviation reproducibility ($\%RSD_R$) between collaborative measurements, using concentration of analyte as dimensionless mass fraction:[104]

$$\%\text{RSDR} = 2^{(1-0.5logC)} \quad (2)$$

Limits of detection (LOD) and quantitation (LOQ) describes the smallest concentration of analyte that can be reliably distinguished from the background or noise, and the lowest concentration of analyte that can be reliably quantified with acceptable accuracy and precision. LOD and LOQ can be calculated for instrument sensitivity using following equations 3 and 4:[104]

$$LOD = 3 \times \frac{Noise}{Signal} \times lowest\ concentration\ of\ the\ linearity\ samples \quad (3)$$

$$LOQ = 10 \times \frac{Noise}{Signal} \times lowest\ concentration\ of\ the\ linearity\ samples \quad (4)$$

LOD and LOQ can also be calculated for a specific method using following equations 5 and 6:

$$LOD = 3{,}3 \times \frac{\sigma}{s} \quad (5)$$

$$LOQ = 10 \times \frac{\sigma}{s} \quad (6)$$

Where σ is the is error of the y-axis intersection of the calibration curve and s is a slope of the calibration curve. Usually, when a signal is 3 times greater than the background or noise, it is detectable, but not well suited for accurate measurements. However, if a signal is 10 times

greater, compared to the noise, it can be defined as the lowest limit of quantitation. Additionally, instrument detection limit can be achieved by replicating seven or more measurements of one sample aliquots, as well as methods detection limit by analyzing seven or more individual samples.[70,104]

Finally, robustness describes analytical methods' ability to remain unchanged, even after small changes to methods procedure. These factors could be for example, slight variations in mobile phase content, temperature, detection wavelength or eluent pH.[70] The study of robustness thus includes purposeful changes in method parameters within a known range, and determination of the following results. Theoretical modeling software can be utilized in robustness prediction, which can then be verified experimentally. Additionally, different statistical designs can be used in method variables control, like Fractional Factorial and Plackett-Burman design.[105]

When analyzing with chromatographic methods, it is also often useful to calculate the retention factor and separation factor of the analytes, in order to better understand methods characteristics. The Retention Factor (k), seen in equation 7 below, describes the retention of an analyte in the column relative to the non-retained compound, such as mobile phase.[106]

$$k = \frac{(t_r - t_m)}{t_m} \tag{7}$$

Here t_r is the retention time of the analyte, and t_m is the void time - the time required to elute non-retained solutes. Although the void time t_m can be determined from the chromatogram, it can also be calculated using the following equation 8:[106]

$$t_m = \frac{\left(\frac{\left(\pi \times \left(\frac{D}{2}\right)^2 \times L \times Pv\right)}{1000}\right)}{FR} \tag{8}$$

Were D and L being the diameter and length of the column in mm respectively, Pv is the pore volume of the stationary phase, and FR is the flow rate in ml/min. Usually, suitable retention factor k ranges from 1 to 10, with lower values (< 1) indicating weak retention and fast elution, while higher values (>20) indicate much stronger retention, which could lead to broader peaks and longer analysis times.[106]

The separation factor, also known as selectivity r (a), describes how well chromatographic systems distinguish between different analytes. This is usually calculated by comparing the retention factors of two peaks, as seen in an equation 9 below:[106]

$$a = \frac{k_B}{k_a} \quad (9)$$

Where k_B is retention factor of solute with longer retention time. Thus, for example selectivity a value of 1 indicates the compounds are not separable and are co-elution, while high a value indicates efficient separation between analytes.[106]

3.6 Previous pesticides analysis

By examining previous methods for pesticides analysis, especially regarding glyphosate (GLY), glufosinate (GLU) and aminomethylphosphonic acid (AMPA), we can better understand conditions, required for reliable quantitative and qualitative analysis with HILIC-UHPLC-MS/MS, as well as SFC-UV. Although in general different methods were utilized with different sample matrixes, used dissimilar equipment and provided varying validation values, they still have a lot of important factors in common.

For instance, when utilizing HILIC LC-MS/MS method, ion-pair reagents are required, which is more time consuming and deteriorates robustness. This is because polar and amphoteric pesticide result in weak peak shape and poor separation, when analyzed without derivatization or ion-pair.[107] Thus, when analyzing glyphosate, glufosinate and AMPA, HILIC column is quite useful, since it does not require derivatization. Several columns can be used, however, highly polar mixed-mode Obelisc N and Anionic Polar Pesticides columns provided especially good results.[107,108] For HILIC mobile phase, 0.1% formic acid in acetonitrile was proven to be especially effective, together with isocratic elution.[107] However, the combination of 99:1 (v/v) water with 0.1% formic acid, together with acetonitrile can also work, providing suitable retention. Retention time can also be increased by increasing acetonitrile concentration.[109] Additionally, all three pesticides can be analyzed with MS in negative ion mode, even though glufosinate in positive-ion mode is more sensitive.[107,109] Examples of different LC analysis methods can be seen in a Table 5 below.

Table 5. Different liquid chromatography analysis methods and their parameters.

Method	Pesticides	LOD	LOQ	Separation conditions
HILIC (LC–MS/MS)[107]	GLY GLU AMPA	0.03 µg/ml 0.03 µg/ml 0.02 µg/ml	-	Column: Obelisc N Elution: Isocratic MP: 0.1% formic acid in acetonitrile (80:20, v/v) Column temperature: 40 ˚C
HILIC (LC–MS/MS)[108]	GLY GLU AMPA	-	0.125 µg/L 0.125 µg/L 0.125 µg/L	Column: Anionic Polar Pesticides Elution: Gradient MP (A): 0.1% formic acid in ultrapure water MP (B): 0.1% formic acid in acetonitrile Column temperature: 50 ˚C
HILIC (LC- MS/MS)[109]	GLY GLU	0.20 ng 0.16 ng	0.02 mg/kg 0.05 mg/kg	Column: Venusil XBP-C18 Elution: Isocratic MP: 0.1% formic acid in water and acetonitrile (99:1) Column temperature: 25°C
UHPLC-MS/MS[110]	GLY AMPA	0.01 mg/kg 0.01 mg/kg	0.02 mg/kg 0.02-0.03 mg/kg	FMOC derivation Column: Acquity UPLC BEH C18 1.7 µm-columns Elution: Gradient MP (A): 5 mM ammonium acetate with water and 2.5% methanol MP (B): 5 mM ammonium acetate solution in methanol Column temperature: 40 ˚C

One of the analyses of glyphosate, glufosinate and AMPA utilized HILIC (LC–MS/MS). The sample was taken in form of a human serum, which was filtrated through ultrafiltration membrane, and washed with chloroform. Analysis resulted in successful determination, with no derivatization or solid phase extraction clean-up, RSDs – 5.9% and LOD – 0.02 µg/ml (AMPA), 0.03 µg/ml (GLY, GLU). Obelisc N column (150 mm × 2.1 mm I.D., 5 m) under 40 ˚C was used for separation, with mobile phase consisting of 0.1% formic acid in acetonitrile (80:20, v/v) and injection of 0.2 ml/min for 2 l. Mass spectrometry utilized multiple reaction monitoring (MRM) with negative electrospray ionization and the desolvation and source temperatures were kept at 350 ˚C and 125 ˚C respectively. Extraction, capillary and multiplier voltages were set at 3.0 kV, 3.0 V, and 650 V, respectively. [107] Additional information regarding MS/MS can be seen in a Table 6 below.

Table 6. MS/MS analysis of glyphosate, glufosinate and AMPA.[107]

Compound	MW	Cone voltage V	Quantitation	
			Collision energy (eV)	MRM transition
Glyphosate	169	20	10	168 > 150
Glufosinate	181	25	20	180 > 85
AMPA	111	30	10	110 > 81

Another analysis of glyphosate, glufosinate and AMPA in household dust utilized Anionic Polar Pesticides (APP) column (130 Å, 5 µm, 100 mm × 2.1 mm) together with a guard column (130 Å, 5 µm, 5 mm × 2.1 mm). This time gradient elution was utilized, with a mobile phase A containing ultrapure water with 0.9% formic acid, and mobile phase B acetonitrile with 0.9% formic acid. Flow rate was kept at 0.5 ml/min and the temperature at 50 ˚C. ESI was used for MS, as well as negative mode.[108] The parameters for this ESI-MS/MS analysis can be seen in Table 7 below.

Table 7. ESI-MS/MS analysis parameters for glyphosate, glufosinate and AMPA.[108]

Compound	Time segment (min)	ESI mode	Precursor ion (m/z)	Quantifying/qualitative ions (m/z)	Cone voltage (V)	Collision energy (eV)	Dwell time (s)
Glyphosate	5.0-8.5	(-)	167.8	62.9	15	16	0.2
				80.8	15	14	0.2
				150.0	15	10	0.2
Glufosinate	4.0-5	(-)	179.9	84.9	20	18	0.06
				94.9	20	14	0.06
				118.9	20	16	0.06
AMPA	2.5-5	(-)	109.7	62.9	26	18	0.06
				79.0	26	15	0.06
				80.8	26	12	0.06

Although there are not a lot of SFC analysis specifically for glyphosate, glufosinate and AMPA, there are many studies simultaneously investigating a group of pesticides with similar polarities and chemicals characteristics. Different pesticides analysis with SFC can be seen in Table 8 below.

Table 8. Analysis of pesticides with SFC and its characteristics.

Method	Pesticides	LOD	LOQ	Separation conditions
SFC/MS[11]	17 different pesticides	0.1 – 3.7 ppb	0.3 – 12.5 ppb	Columns: Agilent ZORBAX Rx-SIL Agilent ZORBAX SB-CN Agilent ZORBAX NH2 Modifiers: Methanol, ethanol SFC flow: 3 ml/min Column temperature: 40 ˚C
SFC-UV [111]	7 Pyrethroids	0.31 - 0.62ppm	-	Columns: Hypersil APS Modifiers: Methanol SFC flow: 1-3 ml/min Column temperature: 60°C
SFC-UV [111]	10 Pesticides	-	-	Columns: Torus 2-PIC Modifiers: Ethanol SFC flow: 1.5 ml/min Column temperature: 40°C
SFC-DAD[111]	6 Herbicides	4.3-8.6 µg/kg	-	Columns: HSS C18 SB Modifiers: Methanol SFC flow: 1.5 mL/min Column temperature: 60°C

SFC analysis is applied with CO_2 as a mobile phase, often coupled with MS, and usually utilizes silica-based columns that are highly porous and pure. However, amino- and cyano- columns can also be used. Methanol, acetonitrile and ethanol are often applied as modifiers to increase polarity.[11] Additionally, QuEChERS sample preparation technique seems to be especially well compatible for SFC.[111] Additionally, even though SFC gradient separation with CO_2 and organic solvent modifier can work well with many compounds, highly polar analytes can exhibit strong retention which further results in poor separation.[112]

The analysis of highly polar pesticides can thus be optimized for example by adding water into the gradient system, which can enhance peak shape and analyte chromatographic behavior.[111] Water as a modifier can be mixed for example with methanol in 1-7% range, which has been found to be especially useful in ultra-high performance supercritical fluid chromatography (UHPSFC). Addition of water usually helps to increase mobile phase elution strength, however, other additives, such as ammonia, ammonium acetate and trifluoroacetic acid can be useful.[112]

Overall SFC seems to be rapid and reliable even when monitoring a high number of pesticides with different polarities, especially when coupled with MS and tandem MS.[111]

4. Experimental part

During the experimental part, our main task was to develop a method for quantitative and qualitative analysis of glyphosate, AMPA and glufosinate, using HILIC-UHPLC-MS/MS and HPLC-UV. This was achieved by testing different columns, mobile phases, gradients, injection volumes, flow rates and analyte concentrations. By comparing the quality of analyte response, we then managed to select settings optimized for our analytical purposes suited for our analytical purposes.

4.1 Materials

For the analysis of glyphosate, AMPA, and glufosinate with HILIC-UHPLC-MS/MS and HPLC-UV following reagents were used, seen in Table 9 below. All reagents were HPLC grade.

Table 9. Reagents used in HILIC-UHPLC-MS/MS and HPLC-UV analysis.

Reagent	Molecular formula	Manufacturer	Purity
Glyphosate	$C_3H_8NO_5P$	Supelco	Analytical standard
AMPA	CH_6NO_3P	Supelco	Analytical standard
Glufosinate-Ammonium	$C_5H_{12}NO_4P$	SIGMA-ALDRICH	Analytical standard
Acetonitrile	C_2H_3N	Honeywell	99.9%
Ammonium bicarbonate	NH_4HCO_3	ACROS ORGANICS	99%
Formic acid	CH_2O_2	SIGMA-ALDRICH	>98%
Trifluoroacetic acid	CF_3CO_2H	SIGMA-ALDRICH	>99%
9-Fluorenylmethyl chloroformate	$C_{15}H_{11}ClO_2$	SIGMA-ALDRICH	>99%
Sodium borate	$Na_2[H_{20}B_4O_{17}] \cdot 8H_2O$	SIGMA-ALDRICH	>99%
Water	H_2O	SIGMA-ALDRICH	HPLC grade

4.2 Methods

Throughout the UHPLC-MS/MS analysis, Agilent Technologies 1290 Infinity was used for UHPLC and Agilent Technologies 6460 Triple Quadrupole for MS/MS. It has a *m/z* range of 5-3000, with a resolution of 0.5 Da (FWHM) and mass accuracy of 0.1 Da (m/z 5-1000). Additionally, Agilent Jet Steam ESI (AJS) was used as an ion source. While utilizing MS, several scan modes were available, such as scan, product ion and MRMs. The minimum MRM dwell time is 1 ms, with 450 MRM transitions per time segment. It is important to note that during some first measurements, UHPLC had a leak in one of its pumps, possibly influencing certain results.

During measurements, negative polarization and MRM were mostly used, with total ion chromatogram (TIC) chromatogram type. The monitored transitions of the compounds were mainly the following: glyphosate m/z 168 ->63, AMPA m/z 110 -> 63, glufosinate 180 -> 63. The mass spectrometry parameters used during experiments can be seen in Table 10 below.

Table 10. Technical parameter of mass spectrometry.

Parameter	Value
Ion Source Type	ESI
Fragmentor	135 V
Collision energy	20 V
Cell accelerator voltage	7 V
Polarity	Negative
Capillary	3000 V
Nozzle Voltage	500 V
Seath Gas temperature	300 °C
Seath Gas temperature flow	11 l/min
Nebulizer	30 psi
Gas Temperature	300 °C
Gas Flow	11 l/min
Drying Gas	N_2

For HPLC-UV analysis, Agilent 1260 SFC-HPLC was used. It utilizes a DAD detector with a wavelengths analysis between 190 and 950 nm. During sample analysis, signal wavelength was kept mainly at 264 nm, and a reference wavelength at 360 nm. The HPLC system has a maximum operating pressure of 600 bar, and flow range from 0.001 to 5.0 ml/min.

Columns used for analyte separation consisted of Waters XBridge Premier BEH Amide 2.5 µm VanGuard FIT 2.1x50 mm Column (HILIC-Amide) and Agilent InfinityLab Poroshell 120 HILIC-Z 2.1x100mm 2.7 Micron Column (HILIC-Z). Agilent Technologies Inc. MassHunter software, such as Workstation Data Acquisition (version B.07.00), Quantitative Analysis (B.06.00) and Qualitative Analysis (B.07.00) were used for data processing.

4.3 Sample preparation

Analytes were weighed using Mettler Toledo analytical balance scale and diluted with water. The structure of analyte, as well as their molecular weight can be seen in Figure 24 below.

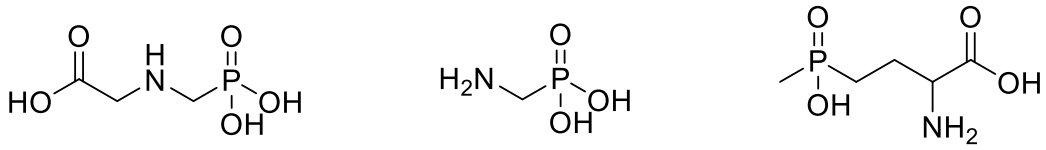


Figure 24. Chemical structure of Glyphosate, AMPA and Glufosinate and their molar masses.

When handling pesticides, only plastic containers were used. For the analyte measurement as well as calibrations, 1.0 mg/ml stock solution of each analyte (Gly, AMPA, Glu) were made, using measurement flask and water as a solvent. Additionally, 0.33 mg/ml, 0.25 mg/ml as well as 1.0 mg/ml stock solutions containing all three analytes were made. For UHPLC measurements, 250-400 µl plastic vials were used.

After method development, its final version was tested on glyphosate filtered solutions, as well as diluted, commercially available herbicide - Roundup Bio, in order to quantitatively analyze the amount of glyphosate in it and compare the results between different methods. Filtration itself was conducted through different materials, such as polyamide-12 (PA12), polystyrene (PS), thermoplastic polyurethane (TPU) as well as activated polyamide-12 carbon (ACPA12). It is assumed that the filtrated solutions, which at the time of measurement were more than a year old, contained around 1.45 ppm of glyphosate, and the diluted Roundup Bio solution around 5.76 ppm. Taking this into account, by comparing our measurement results we can roughly estimate finalized methods precision and reliability.

4.3.1 Sample derivatization

Analyte derivatization prior to the HPLC analysis can often noticeably enchase its response and chromatographic separation, by making analyte more sensitive to detection, as well as chemically stable.[80] Because of this, it was decided to conduct experiments with both derivatized and non-derivatized analytes, and at the same time compare the results. In order to achieve derivatization of glyphosate, glufosinate and AMPA, 9-fluorenylmethyl chloroformate (FMOC) was used. The example of analyte derivatization reaction with FMOC can be seen in Figure 25 below.

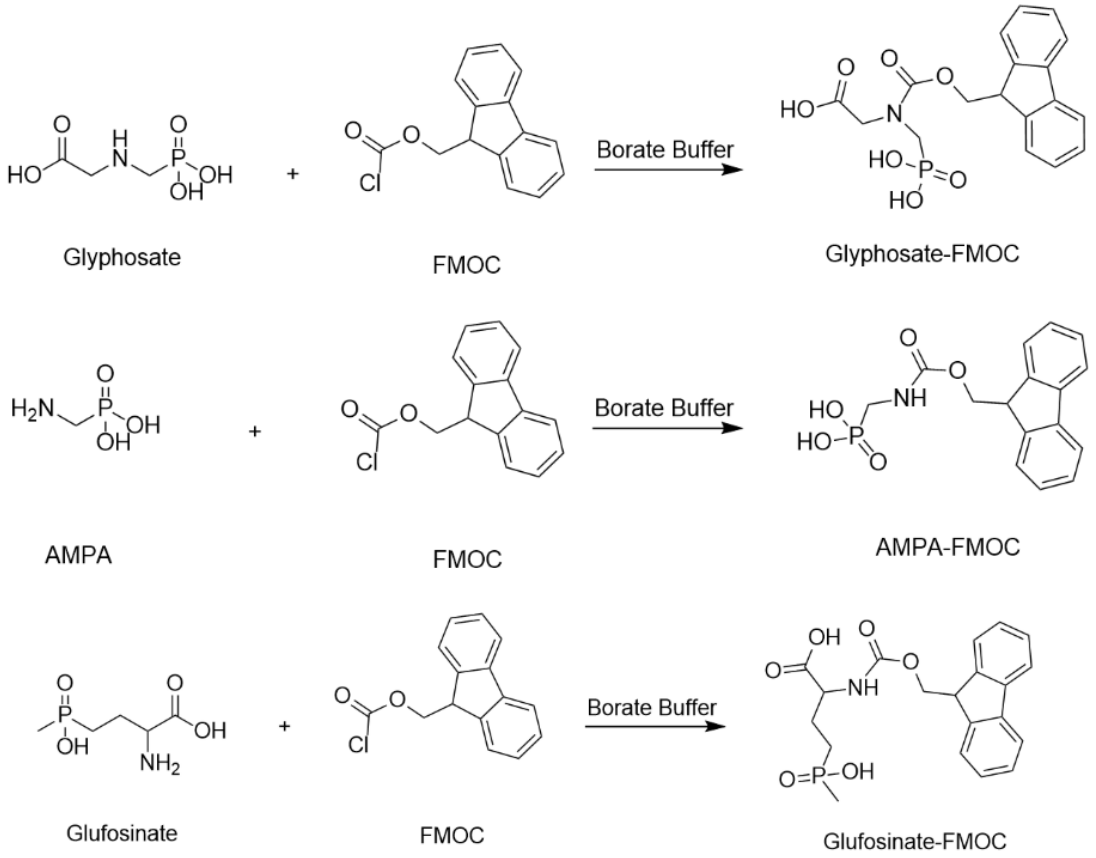


Figure 25. Derivatization of Glyphosate, AMPA and Glufosinate with FMOC.[113]

First, 20 mM FMOC solution was made in MeCN and H_2O (50:50). The 50mM Sodium borate buffer was then made in water. FMOC, borate buffer and 1 mg/ml sample solution were all mixed in 1:2:1 ratio respectively, and vortexed for 1 hour, resulting in 0.250 mg/ml sample solution. Diethyl ether was used to wash away unreacted FMOC-Cl, after which water layer was collected, filtered through syringe with filter, and further diluted for the UHPLC-MS/MS and HPLC-UV analysis.

5 Results and discussion

5.1 HILIC-UHPLC-MS/MS analysis

During glyphosate (Gly), AMPA and glufosinate (Glu) analysis using HILIC-UHPLC-MS/MS, different columns, mobile phases, mobile phase gradients, collision energy (CE) values, flow rates, injection volumes and analyte concentrations were tested, to achieve more defined and clearly separated peaks, suitable for the calibration curve and further quantitative and qualitative determination of pesticides.

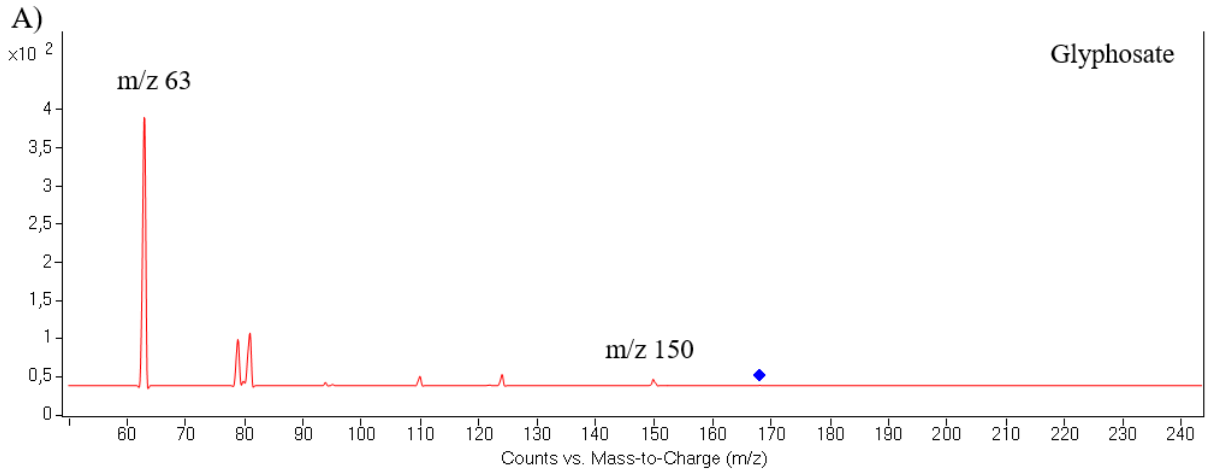
In order to insure that analyte degradation over time does not have a noticeable effect on measured results, the same sample containing glyphosate was tested with the same method after a few weeks. As we can see in Table 11 below, after a 22 day period, glyphosate sample analysis did not reveal significant differences in analytes concentration.

Table 11. Measured differences between 22 days old glyphosate sample.

	Peak area	Percentage difference
First glyphosate measurement	140 073	0.97%
Glyphosate measurement after 22 days	138 714	

Thus, it is relatively safe to say that during our experiments, resulted values were not greatly affected by the degradation of analyte in solution.

In order to correctly identify and quantify our target molecules, as well as ensure selective monitoring with MRM, the fragment ions for analytes were chosen based on negative product ion analysis, which can be seen in Figure 26 below.



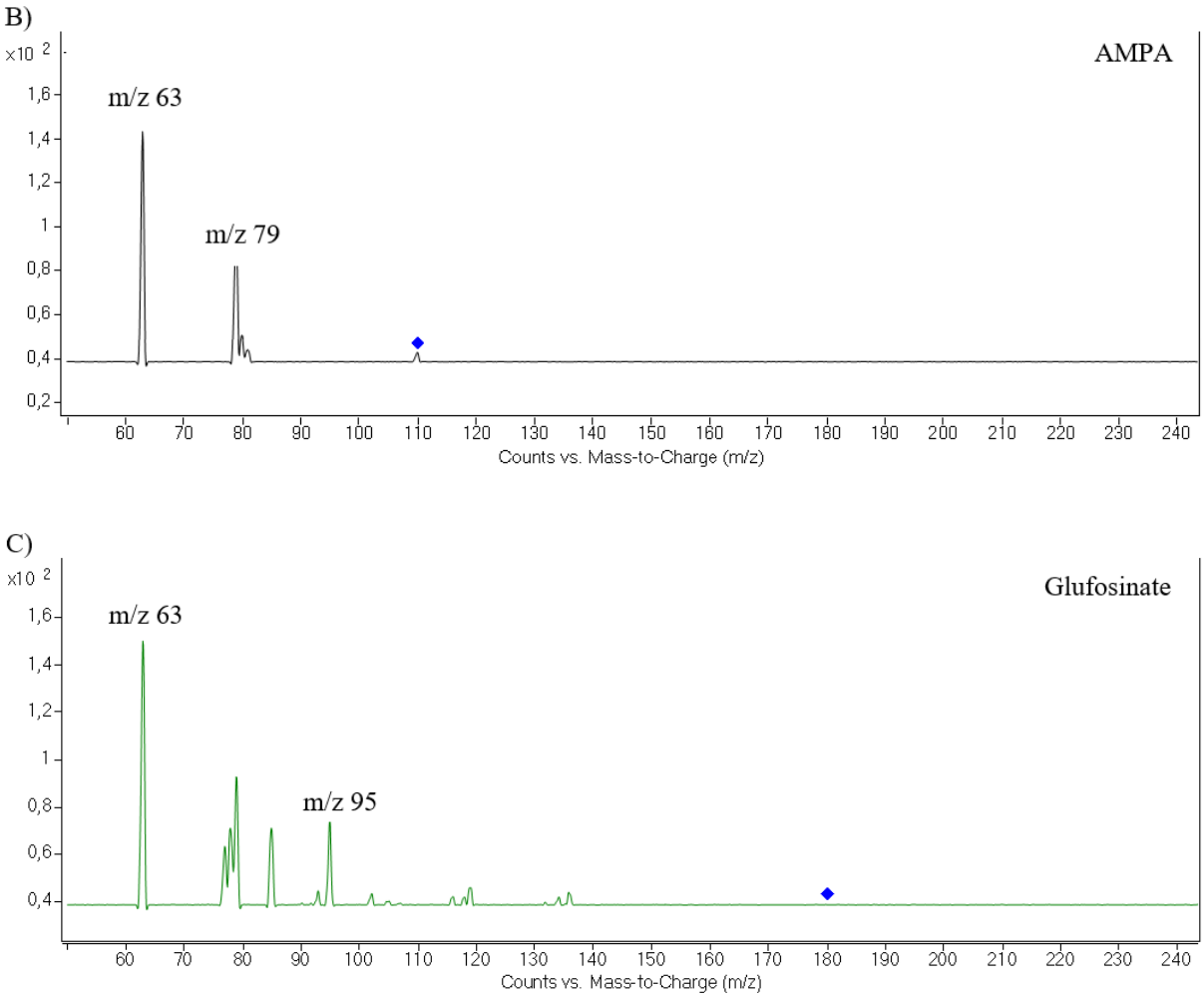


Figure 26. MS/MS measurements for Glyphosate (A), AMPA (B), and Glufosinate (C). The Collision Energy values for all samples is 20 V.

Although there are several different fragments, only two were further chosen for the MRM analysis of each analyte. Namely *m/z* 63 and *m/z* 150 for Glyphosate, *m/z* 63 and *m/z* 79 for AMPA as well as *m/z* 63 and *m/z* 95 for Glufosinate. These fragments and their structure can also be seen in Figure 27 below.

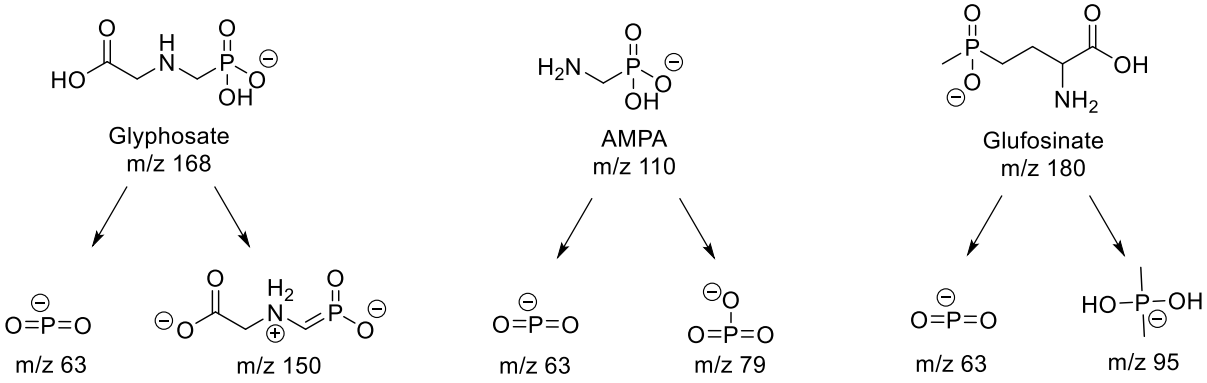


Figure 27. Glyphosate, AMPA and glufosinate fragments chosen for the MRM analysis.

However, for simplicity reasons, further illustrations will mostly consist of a single 63 *m/z* fragment for each pesticide, since it is common among all of them and results in intense peaks.

5.1.1 HILIC Amide and HILIC-Z columns

Previously mentioned Waters XBridge Premier BEH Amide 2.5 µm VanGuard FIT 2.1 x 50 mm Column (HILIC-Amide) and Agilent InfinityLab Poroshell 120 HILIC-Z 2.1 x 100mm 2.7 Micron Column (HILIC-Z), were both tested with MRM method, under similar conditions, such as mobile phase, mobile phase gradients and flow rate, with the same samples, in order to find more suitable column for further method development.

Both HILIC-Amide and HILIC-Z columns were designed for the retention and separation of small, highly polar compounds. Because of these characteristics, HILIC was overall chosen as a chromatographic method for the analysis of our pesticides. The stationary phase of HILIC-Amide is comprised of BEH (ethylene bridged hybrid) particles, to which the amide group is attached. Its column is slightly shorter than HILIC-Z, which could provide faster analysis times. Additionally, the HILIC-Amide column works withing a pH range of 2-11 and has a pore diameter of 130 Å.[114]

Compared to HILIC-Amide, HILIC-Z column, due to its length and thus increased separation path, could provide a better resolution when analyzing complex mixtures. It utilizes zwitterionic stationary phase, that has both negatively and positively charged functional groups. HILIC-Z column also has a slightly smaller pore diameter – 100 Å and operates within a similar pH range of 2-12.[115]

After several tests it was noticed that HILIC-Amide column indeed provided faster retention time, compared to HILIC-Z column, which on the other hand provided slightly better peak separation. This can be seen for example in Figure 28 below, where 0.9% formic acid (FA) was used in both A and B mobile phase components - A: H_2O/0.9%FA, B: ACN/0.9%FA.

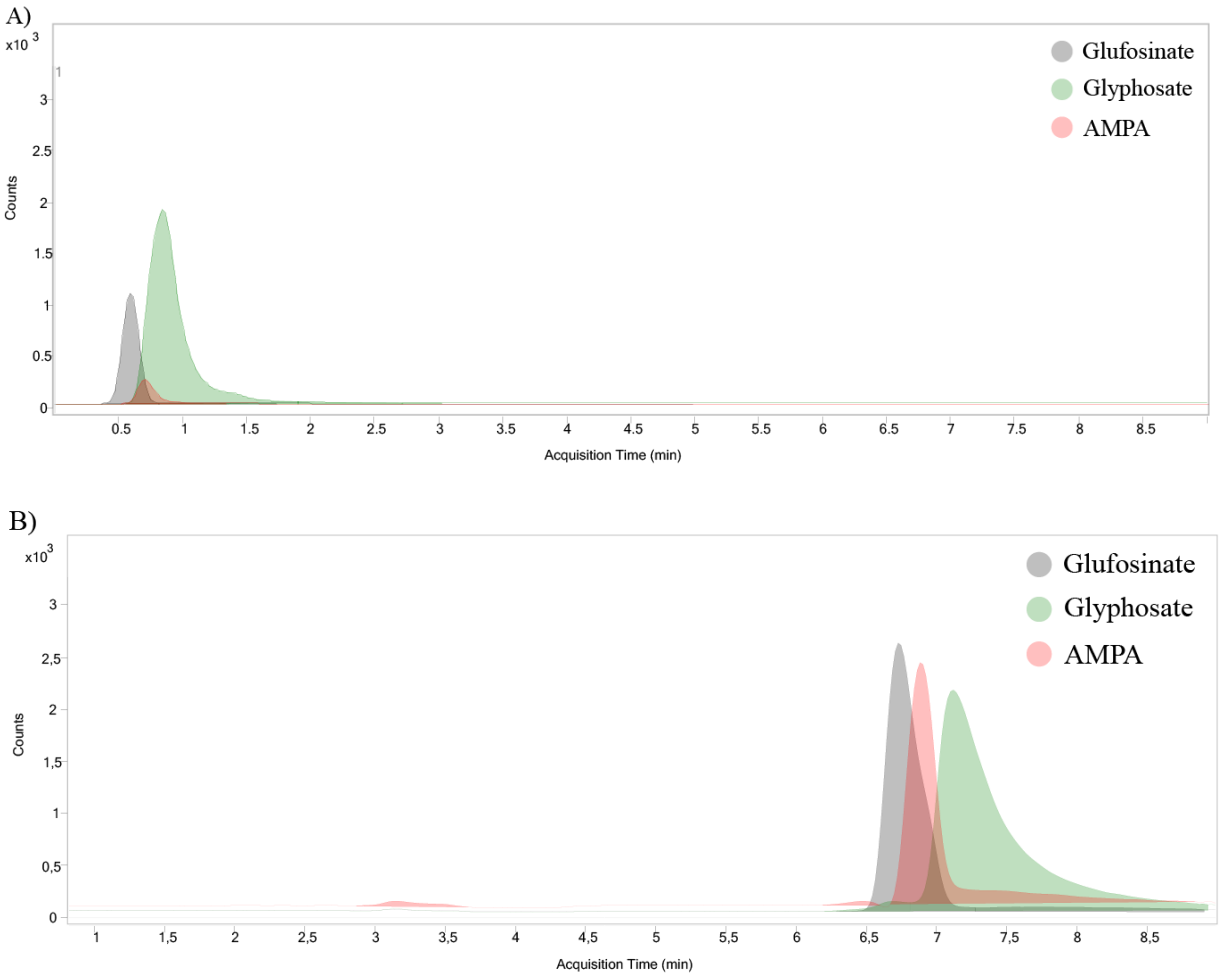


Figure 28. MRM, HILIC-Amide (A) and HILIC-Z (B) column comparison, utilizing H_2O/0.9% FA, and ACN/0.9% FA mobile phase with 0.1 mg/ml pesticide mix.

Here we can see that when utilizing HILIC-Amide column, analytes are detected around 0.8 min, while in HILIC-Z their retention time is increased to around 7 min. Additionally, HILIC-Z column seems to cause higher intensity for each analyte peak, as well as longer peak tailing, compared to HILIC-Amide column. Longer retention of analytes in HILIC-Z column could be for example due to their stronger adsorption to the stationary phase, where ion exchange could play an important role.[79]

On the other hand, peak tailing usually occurs due to continuing interactions between analyte and stationary phase, because of the multiple mechanisms of analyte retention present. More precisive reasons can consist of uncoated sites of the stationary phase, unsuitable mobile phase pH level or column overload with analyte.[80]

Similar results can also be seen when mobile phase was changed to 0.9% trifluoroacetic acid (TFA) – A: H_2O/0.9%TFA, B: ACN/0.9%TFA was used in mobile phase, which can be seen in Figure 29 below.

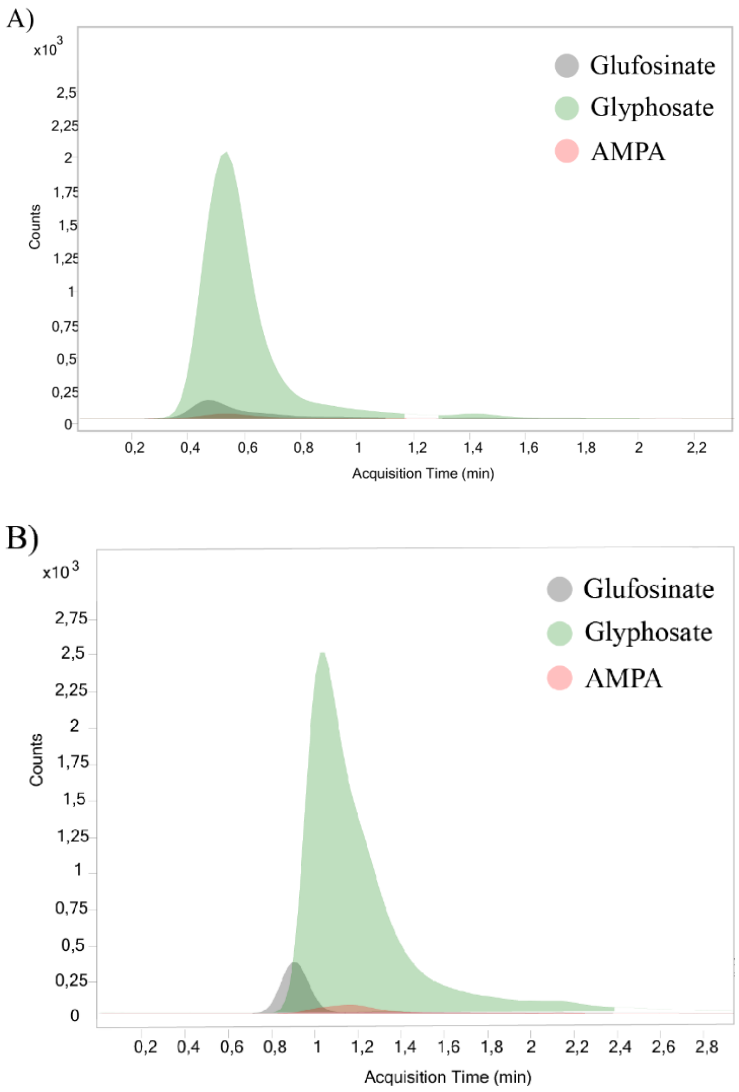


Figure 29. MRM, HILIC-Amide (A) and HILIC-Z (B) column comparison, utilizing A: H_2O/0.9% TFA, B: ACN/0.9% TFA mobile phase with 0.1 mg/ml pesticide mix.

Although this time both columns resulted in relatively fast retention time for analytes, HILIC-Z column (B) had slightly longer retention time, and the analytes were slightly more separated, with higher intensities, compared to HILIC-Amide column (A). Taking all this into account, HILIC-Z column was chosen for the further analysis tests, since it provided better peak separation and intensities.

5.1.2 Mobile phases and gradients

While utilizing HILIC-Z column, four different mobile phases were tested. It was important to investigate several mobile phases, since their differences, such as pH and the proportions between organic and aquatic phases could play a crucial role in analyte retention and peak shape. All of the tested mobile phases contained water and acetonitrile, with a specific combination of different additives, such as ammonium bicarbonate, 0.5 and 0.9% formic acid (FA) and 0.9% trifluoroacetic acid (TFA). Each mobile phase was also tested at different gradient levels, in order to achieve better peak separation, when analyzing a sample containing all three pesticides. At the same time, other values such as flow rate, injection volume and analyte concentration were kept the same between different mobile phases, for more precise comparison.

MRM measurements with ammonium bicarbonate (pH 9) – A: 20mM NH_4HCO_3 in water, B: ACN/H_2O (95/5-%), example of which is seen in a Figure 30 below, showed that although it produced clear peaks with only small tailing from AMPA, all these peaks are closely stacked together, complicating the distinction between analytes.

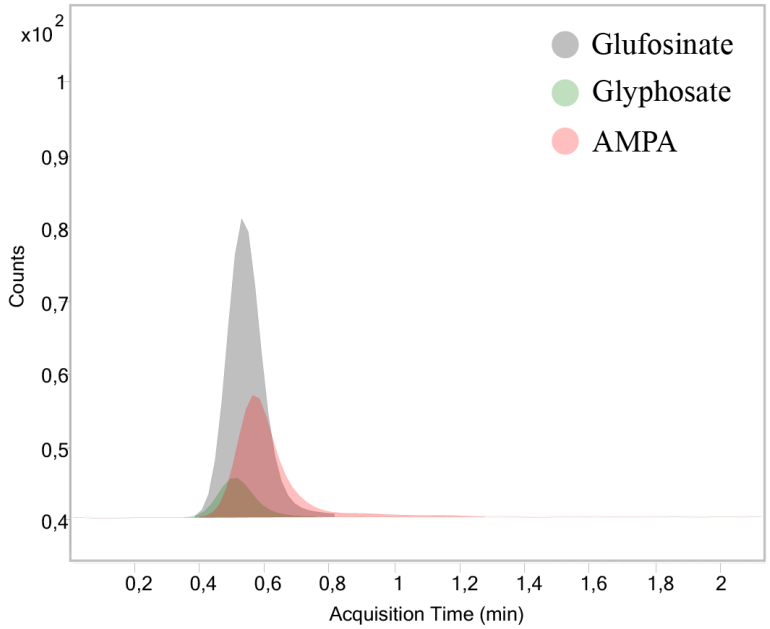


Figure 30. MRM, HILIC-Z analysis utilizing A: H_2O/NH_4HCO_3 B: ACN/H_2O mobile phase with 0.1 mg/ml pesticide mix.

This stacking could be explained by fast retention time of each analyte – around 0.5 min, which could not provide enough noticeable difference for individual analyte separation. Analytes themselves also seem to provide proportionally different peak intensities. Ammonium bicarbonate is a highly polar compound, and thus it could result in analyte faster retention when

present in a mobile phase. It is also important to note that all three pesticides are highly similar in polarity and size, and thus often end up having similar retention times.

After changing mobile phase to 0.5% formic acid – A: H_2O/0.5%FA, B: ACN/0.5%FA, example of which can be seen in Figure 31 below, it resulted in peaks with much higher intensity, but also made them much broader. Considering noticeably lower pKa of formic acid - 3.75, compared to previous ammonium bicarbonate solution - 9.25, higher intensity could occur due to enhances deprotonation of the analyte. At the same time, broader peaks could indicate unstable interaction between analyte and stationary phase.

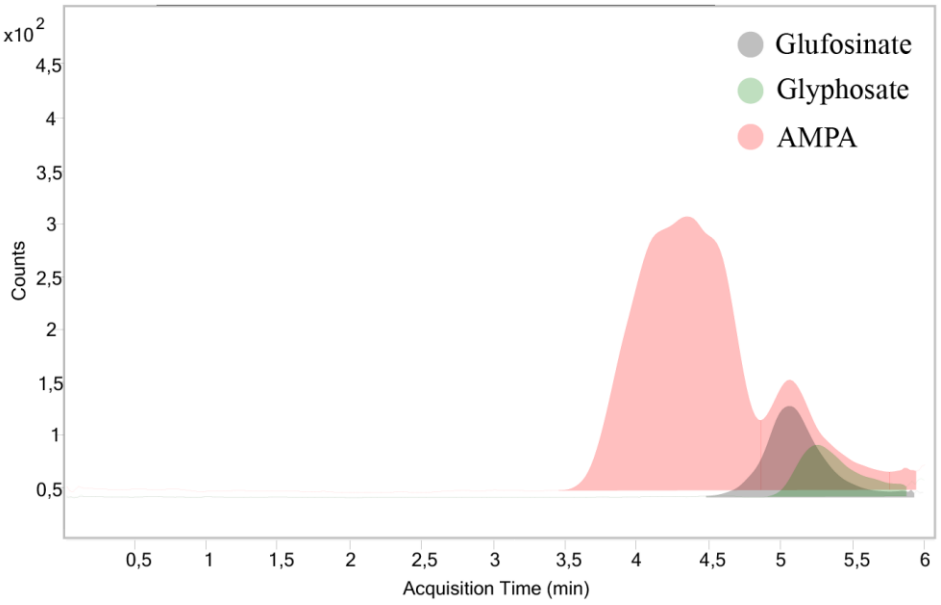


Figure 31. MRM, HILIC-Z analysis utilizing A: H_2O/0.5%FA, B: ACN/0.5%FA mobile phase with 0.1 mg/ml pesticide mix.

Although peaks had slightly different retention times between them, and overall retention time was longer, compared to ammonium bicarbonate mobile phase, the presence of AMPA double peaks and Gly, as well as Glu tailing, made results less reliable. This time, analytes were retained in column for longer period, but the retention itself seems to be inconsistent. Resulting AMPA double peak could occur due to unsuitable pH level of the mobile phase, as well as high values of injection volume.

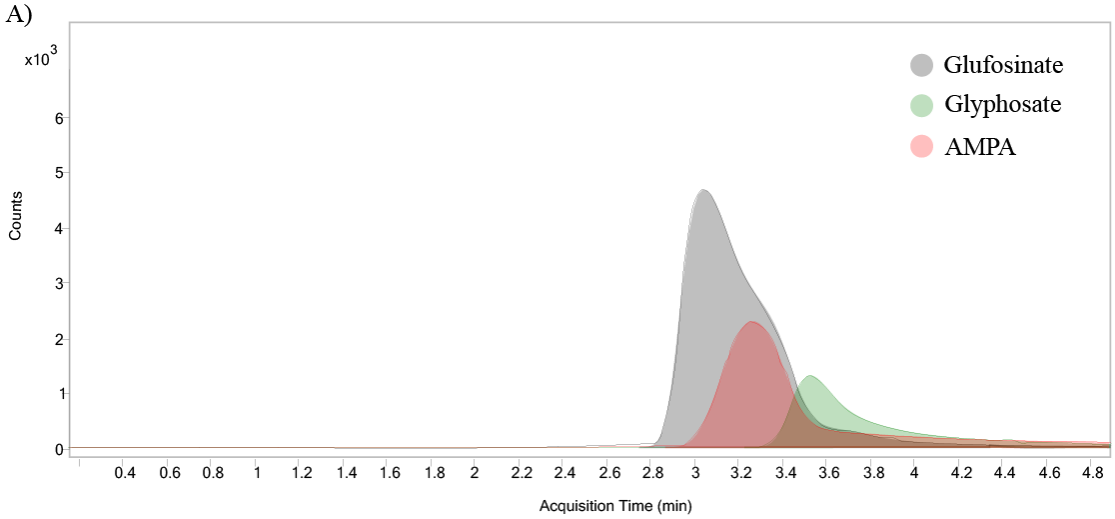
When increasing the concentration of formic acid from 0.5% to 0.9% - H_2O/0.9%FA, B: ACN/0.9%FA, as seen previously in Figure 25 B), the individual compound separation was slightly more distinguishable from each other, with similar peak intensities. Although, peak tailing was again quite noticeable, mostly due to previously mentioned mobile phase pH level or column overload with analyte.

Changing mobile phase to trifluoroacetic acid - A: H_2O/0.9% TFA, B: ACN/0.9% TFA, as seen in previously shown Figure 26 B), resulted in much faster retention time and poor peak separation, compared to formic acid mobile phases. This is probably due to TFA being stronger acid, resulting in polar analyte higher occurrence in the mobile phase. Glyphosate intensity also seems to be much higher compared to other analytes, with AMPA intensity being extremely low.

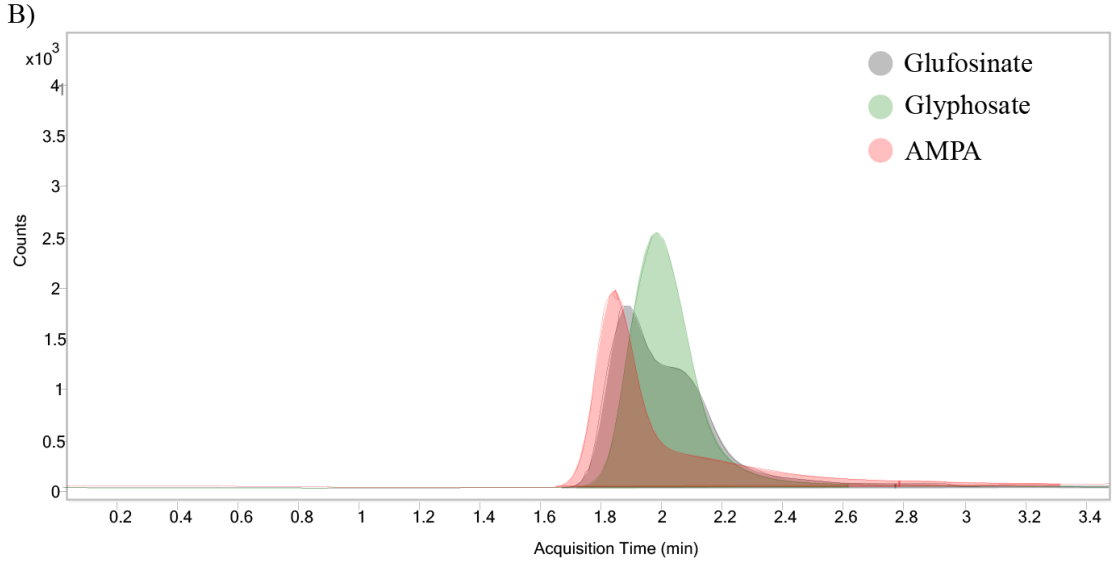
It is important to note that several different gradient tests were also done with four previously mentioned mobile phases, however, overall differences between them remained the same. Taking all this into account, it was decided to continue to work with 0.9% FA mobile phase and further test the effect of different gradients.

Different settings of 0.9% formic acid mobile phase seemed to have a noticeable impact on each analyte separation and the intensity of its peak, examples of which can be seen in Figure 32 below.

A)



B)



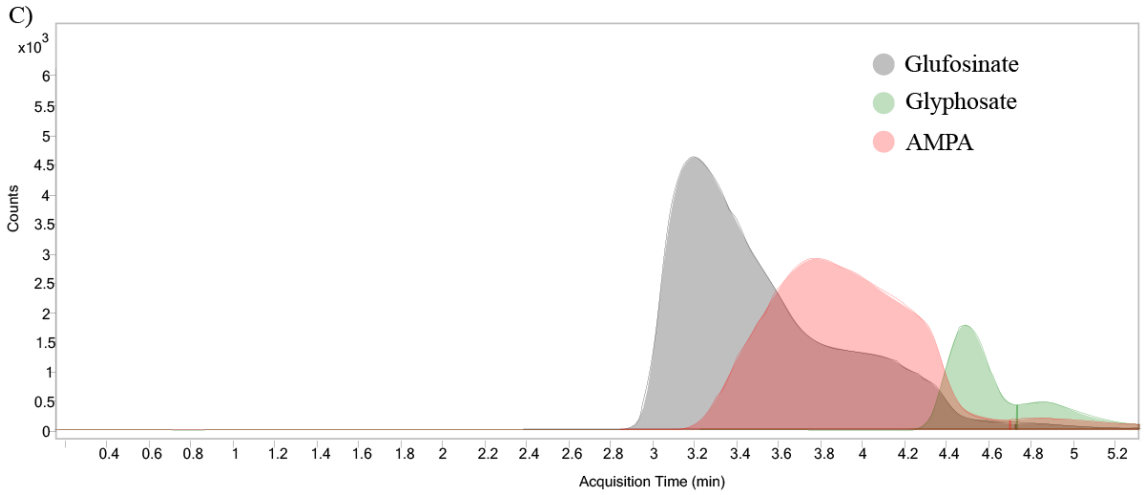


Figure 32. MRM, HILIC-Z analysis using different gradient settings for mobile phase A: H_2O/0.9%FA, B: ACN/0.9%FA with 0.3 mg/ml pesticide mix.

Setting used for each A), B) and C) tests can also be seen in Table 12 below. As mentioned previously in HILIC chapter 3.1.3, HILIC gradient elution usually goes from weak to strong mobile phase, in other words from highly organic mobile phase to highly aquatic one. Although this can be partly seen in test A) and C) other gradients were also tested. For example, in test B) elution started with strong mobile phase and ended with weak one, resulting relatively faster retention time, compared to other gradient setting, and more grouped peaks.

Table 12. Mobile Phase gradient setting for Figure 30 tests A, B and C.

Time (min)	The percentage of ACN/0.9% Formic Acid in the mobile phase		
	A	B	C
0	65%	20%	75%
2	15%	20%	20%
4	10%	50%	75%
6	60%	70%	75%
8	65%	80%	75%

Even though both A) and C) tests gradient settings were quite similar, it seems that the presence of higher percentage of aqueous phase in A) test again resulted in peaks faster retention and stacking. Overall, one of the most successful peak separations was achieved with test C), which first utilized elution with weak mobile phase, followed by a stronger one, and then again highly organic. Thus, although resulting peaks where broad, with noticeable tailing, it was decided to utilize this gradient in further measurements.

5.1.3 Collision Energy

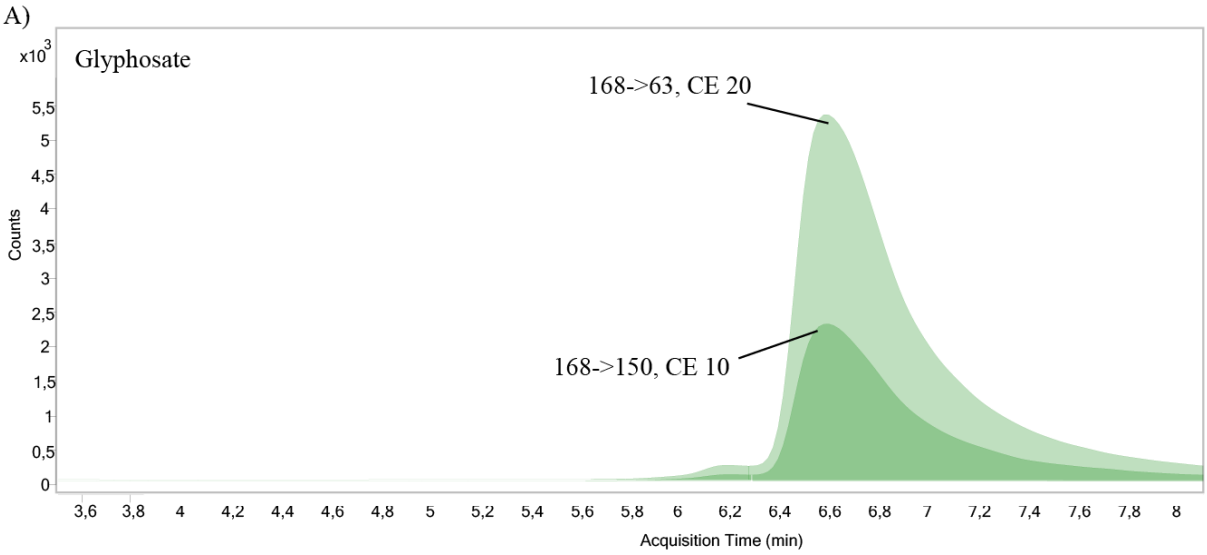
Since our method utilized tandem mass spectrometry, where analyte detection relies heavily on collision-induced fragmentation, it was important to properly adjust the collision energy (CE) settings for each analyte, in order to ensure high response values. Thus, several different CE values were tested for each analyte, ranging from 10 to 60 V.

Resulting peaks with the highest intensities can be seen in Figure 33 below. Additionally, collision energy setting for each glyphosate, AMPA and glufosinate fragments can be seen in Table 13 below.

Table 13. Optimal collision energies Gly, AMPA and Glu fragments, negative ionization.

Analyte	Fragment	Collision energy (V)
Glyphosate	168->63	20
	168->150	10
AMPA	110->63	20
	110->79	30
Glufosinate	180->63	40
	180->95	20

These values seem to be quite similar to those found in other studies[116], although in this case, for example CE values of glufosinate 180->63 fragmentation seem to be higher.



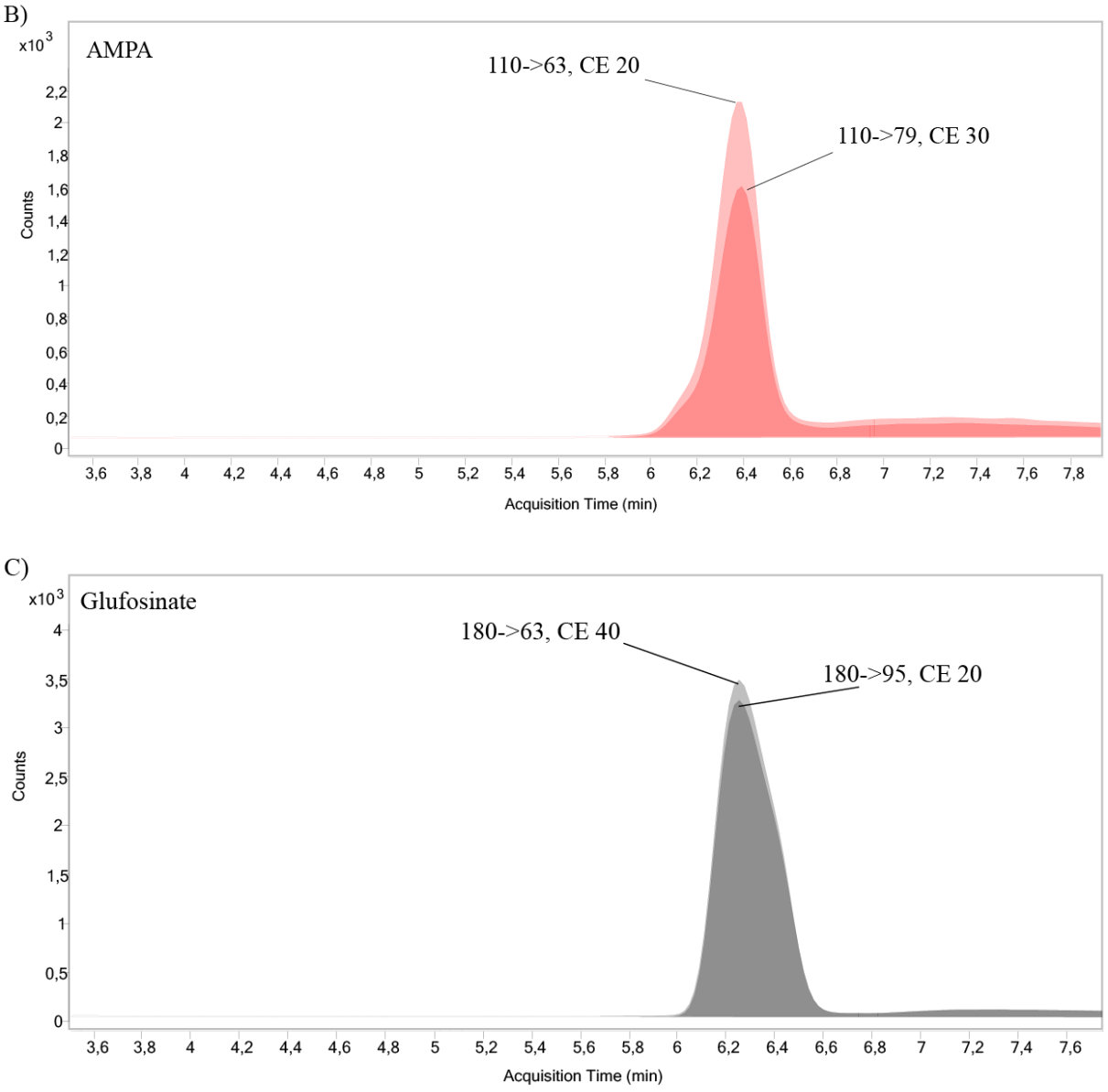


Figure 33. MRM, HILI-Z analysis using different CE value settings. Mobile phase A: H_2O/0.9%FA, B: ACN/0.9%FA with 0.1 mg/ml pesticide mix.

Here we can see that the fragment at *m/z* 63 for each analyte produces peak with higher intensities, compared to other fragments, which is especially noticeable with glyphosate A). From the individual peaks, it is also seen that glyphosate produces the most noticeable tailing, compared to glufosinate and AMPA.

5.1.4 Injection volume and Flow Rate

Both injection volume and flow rate values are often modified, in order to ensure peak separation and achieve better peak shapes. Thus, several different MRM analysis tests were done utilizing HILIC-Z column and Mobile Phase A: H_2O/0.9%FA, B: ACN/0.9%FA, with Table 13 gradient settings C. An example of flow rate test can be seen in Figure 34 below.

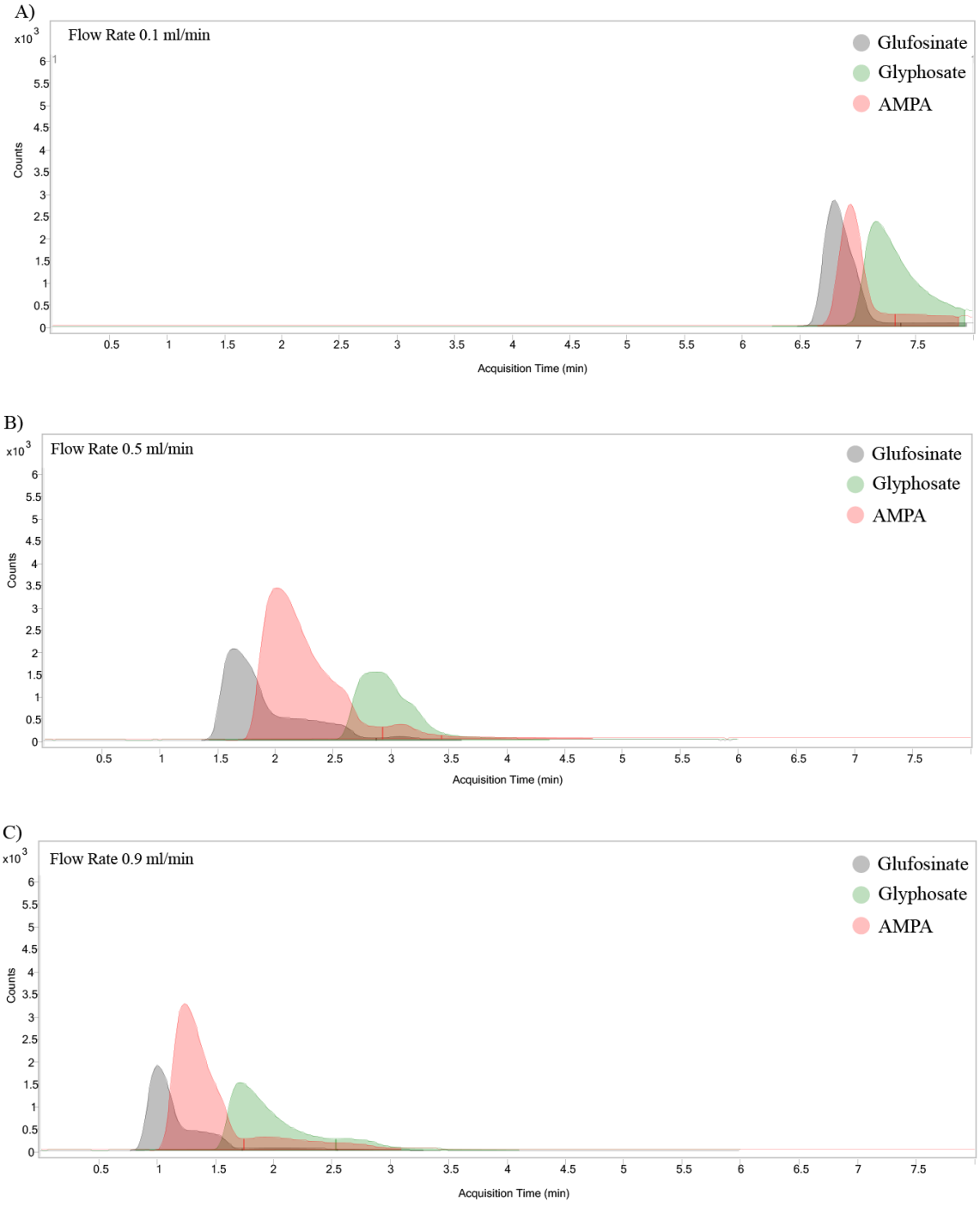
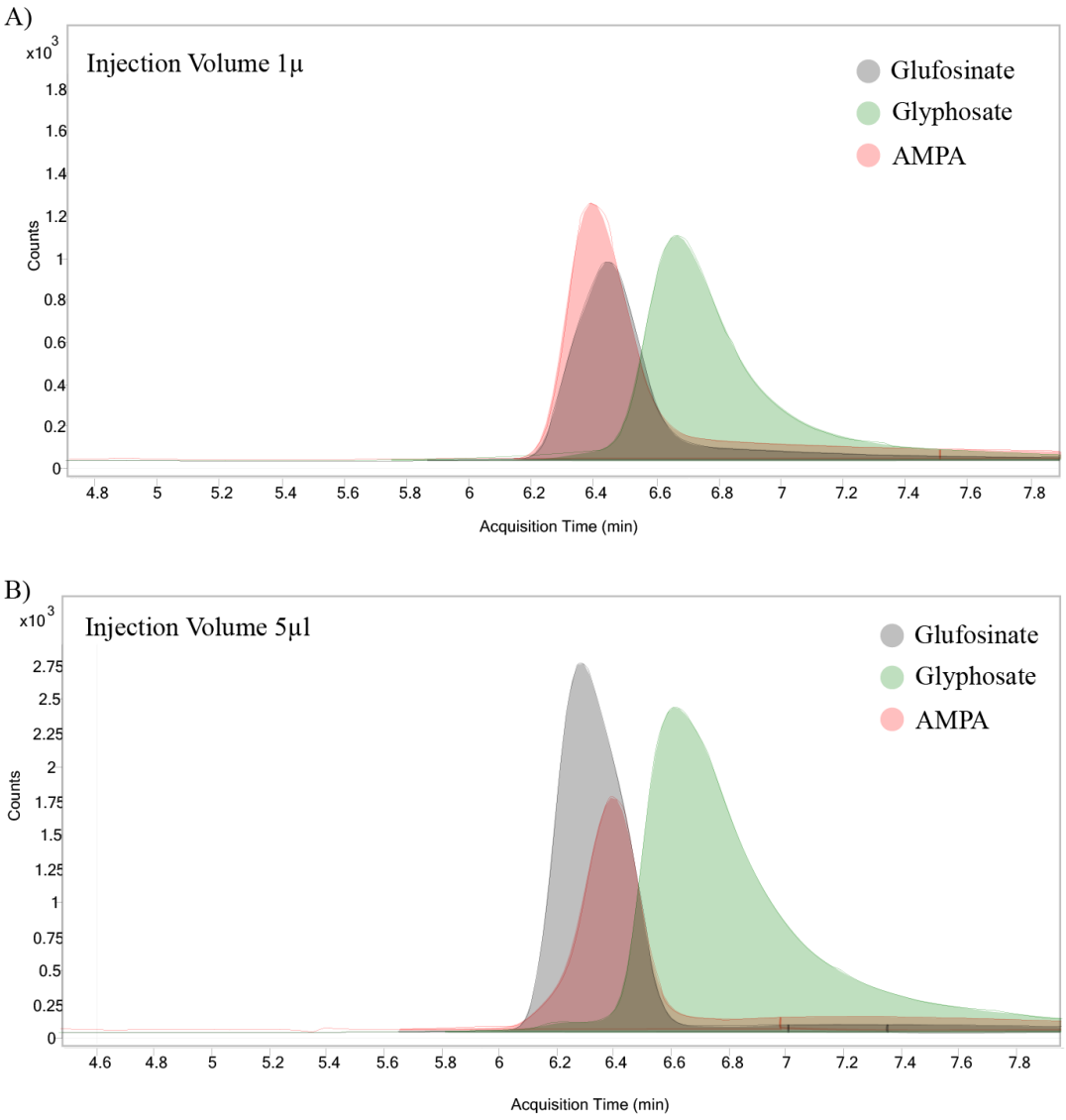


Figure 34. MRM, HILIC-Z 0.1 mg/ml pesticide mix analysis using different flow rate settings.

Based on the results, we can see that slower flow rate, such as 0.1 ml/min (A) provides slightly better peak shapes and less tailing, compared to 0.5 (B) and 0.9 ml/min (C) flow rates. Although, at the same time, it also seems to somewhat reduce peak separation. Still, we decided to continue with a slower flow rate 0.1 ml/min, which provided better peaks overall. This could occur because lower flow rate usually decreases column outlet retention factor, which results in narrower peaks.[117]

Based on injection volume tests (see Figure 35), it seems that lower injection volume values, such as 1 µl (A) and 5 µl (B) produced better peaks, compared to 9 µl (C), which appears to cause negative effect on peak shapes and resulted in double peak from AMPA.



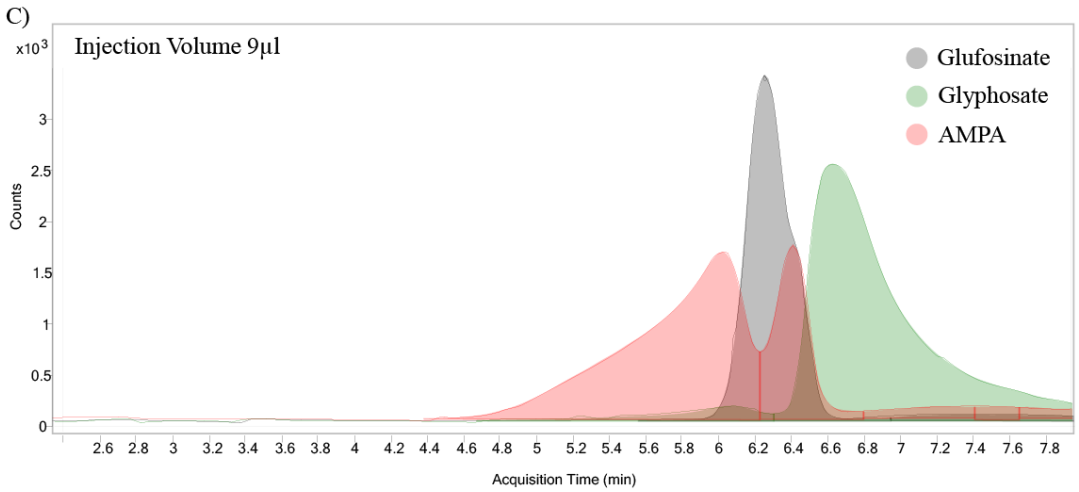


Figure 35. MRM, HILIC-Z 0.1 mg/ml pesticide mix analysis using different injection volume settings.

This could be explained by column overloading with sample, which often decreases retention time and causes harmful impact on column efficiency.[117] Taking this into account, it was decided to continue with 5 µl injection volume, since it performed well in both peak shape and intensity, compared to other injection volume values.

5.1.5 Concentration and calibration

As mentioned previously, peak shape could be heavily affected by the concentration of analyte in the solution due to the possibility of column overload, which negatively affects its performance. Because of this, different pesticide mix concentrations were tested, in order to achieve more reliable peaks and construct a calibration curve within that concentration range.

Overall, several tests were conducted, ranging from 1.0 µg/ml to 330 µg/ml. Despite some inconsistencies during measurements, it seems that lower concentrations of pesticides, in the range of 1 to 50 µg/ml seemed to result in slightly better peaks, compared to stronger solutions as seen from as seen in Figure 36 bellow.

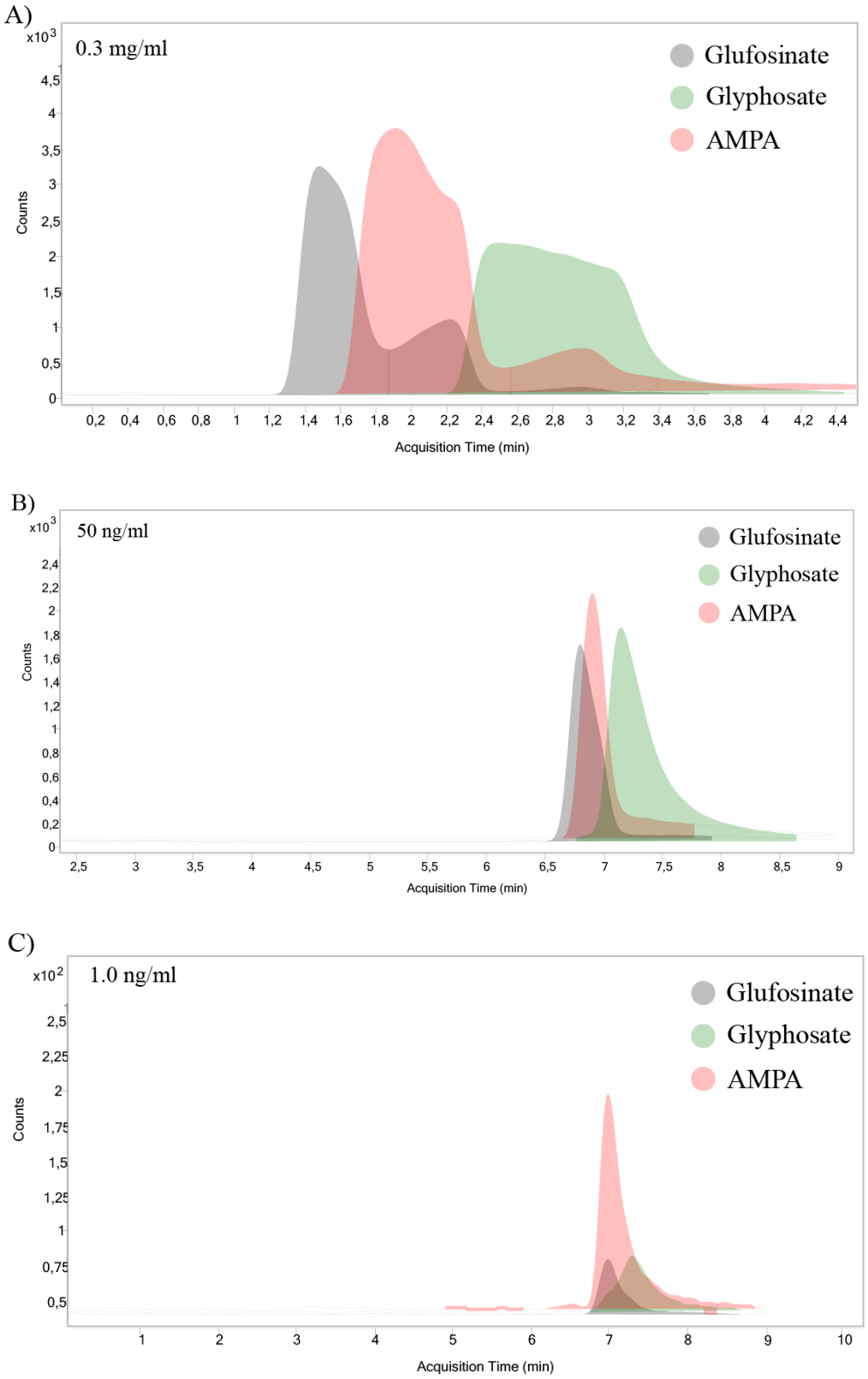


Figure 36. MRM, HILIC-Z 0.3 mg/ml (A), 50 µg/ml (B) and 1 µg/ml (C) pesticide mix analysis. Mobile Phase A: H_2O/0.9%FA, B: ACN/0.9%FA.

From test (A) with 0.3 mg/ml pesticide mix, we can see that resulting peaks are broad, with noticeable tailing and double peaks from Glufosinate and AMPA. This kind of peak

deformation most likely occurred due to the column overloading, since more diluted samples – 50 µg/ml (B) and 1.0 µg/ml (C) did not produce such noticeable tailing or double peaks. From 1.0 µg/ml test (C) we can also see that AMPAs response seems to grow in smaller concentrations. This could be explained by column overall sensitivity towards AMPA, which is especially noticeable in lower concentrations.

It is important to note that these measurements were conducted in a single day, and some of the resulting values differ from previous analysis of similar analytes, as seen from 0.3 mg/ml pesticide mix measurement in Figure 29 test (C) and 0.3 mg/ml pesticide mix measurement in Figure 33 test (A). However, it was still decided to continue calibration tests within 1-50 µg/ml concentration range, since it produced more stable and reliable peaks, and the calibration line itself seems to go down towards stronger concentrations. The relative standard deviation (%RSD) for 10 µg/ml glyphosate can be seen in Table 14 below.

Table 14. Relative standard deviation for 10 µg/ml glyphosate area after five measurements.

Measurement	Peak Area 168->63 m/z
1	3068
2	3170
3	3019
4	3107
5	2990
Relative standard deviation %	2.32 %

Formed calibration curve, which can be seen in Figure 37, consisted of 6 calibration points from 1 to 50 µg/ml, with an R^2 value of 0.9982.

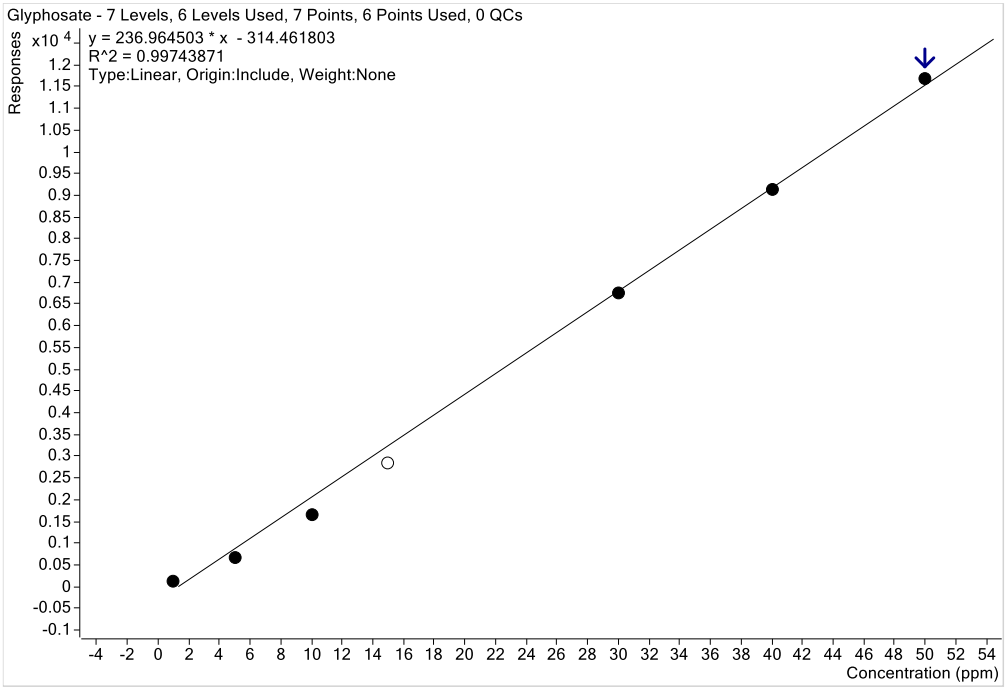


Figure 37. MRM, HILIC-Z, Glyphosate 1-50 µg/ml calibration curve. The curve equation is y = 236.96503*x-314.461803.

Based on the equations (5) and (6) its LOD and LOQ was calculated to be 2.1 µg/ml and 6.3 µg/ml respectively. Further experiments with filtrated glyphosate samples and commercially available herbicide using this calibration curve can be seen in chapter 5.3.

5.1.6 Derivatization

One of the methods that usually improves chromatographic performance of analytes is derivatization, which is why FMOC derivatization was utilized in glyphosate, AMPA and glufosinate analysis. By modifying chemical structure of the analyte and increasing its hydrophobic nature, it is often possible for example to enchase separation and detectability.[118] Based on the previous tests, we decided to continue to work with HILIC-Z column and the mobile phase containing 0.9% formic acid. However, due to the analyte structural changes, it was also necessary to conduct new test regarding target compounds product ions, collision energy values, as well as UHPLC method flow rate, injection volume and mobile phase gradients. The results containing negative product ion tests can be seen in Figure 38 below.

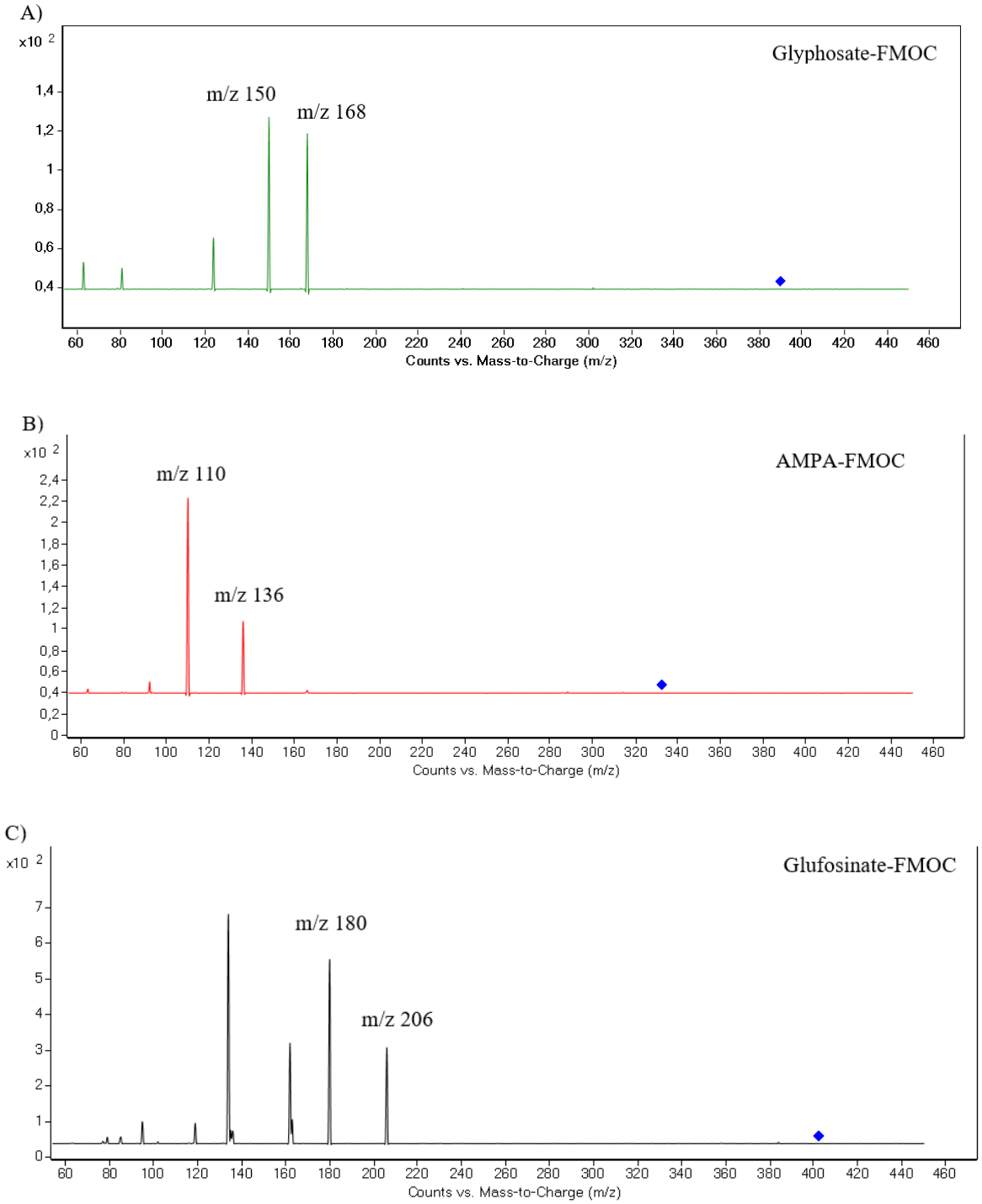


Figure 38. MS/MS measurements for Glyphosate-FMOC (A), AMPA-FMOC (B), and Glufosinate-FMOC (C).

These values turned out to be similar compared to those in previous studies[119,120] and the precursor ion chosen for Glyphosate (A), AMPA (B) and Glufosinate (C) can also be seen in the Table 15 below, together with optimized Collision Energy values. It seems that all analyte produced peaks with higher intensities, compared to non-derivatized counterparts, and at the same time required less collision energy.

Table 15. Collision energies for Gly-FMOC, AMPA-FMOC and Glu-FMOC fragments, negative ionization.

Analyte	Fragment	Collision energy (V)
Glyphosate-FMOC	390->150	10
	390->168	20
AMPA-FMOC	332->136	10
	332->110	10
Glufosinate-FMOC	402->206	10
	402->180	10

Following several tests regarding mobile phase gradients, as well as flow rate and injection, showed that derivatized analytes peaks separated well during – 0.2 ml/min flow rate. Smaller injection volume – 1 µl also seemed to result in better shape peaks. Optimized peak separation test can be seen in Figure 39 below. Additionally, the gradient setting for this test can be seen in Table 16.

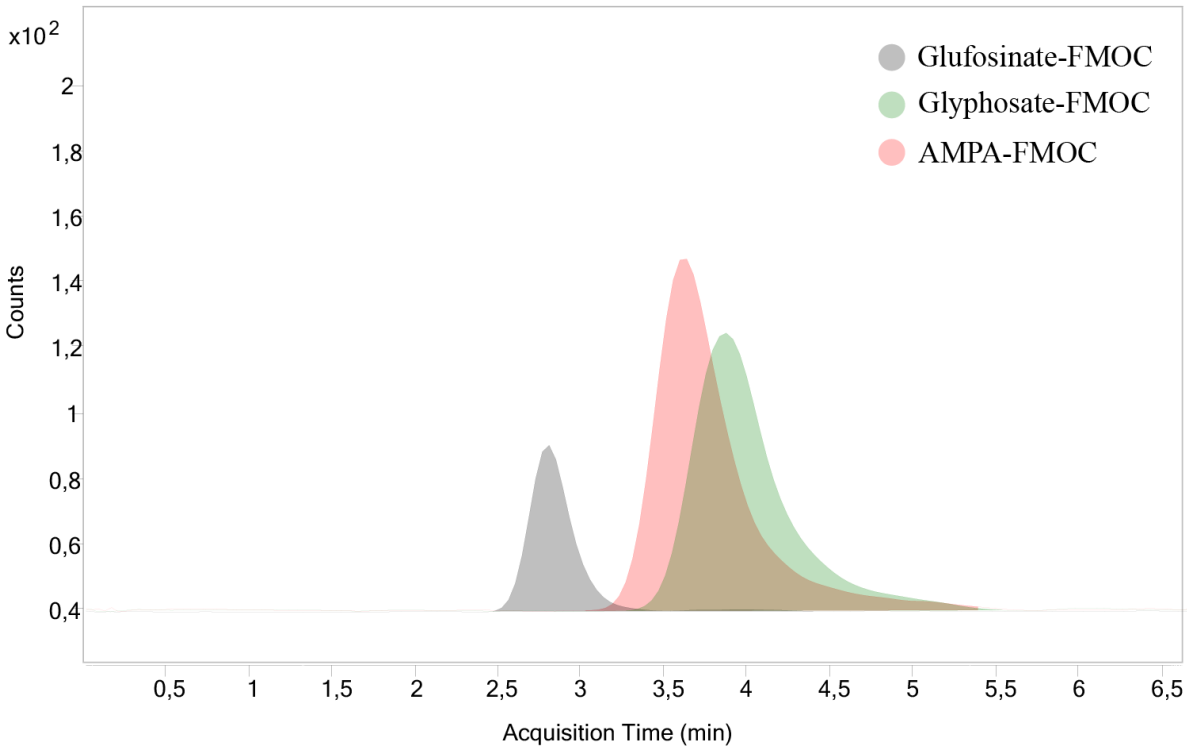


Figure 39. MRM, HILIC-Z 50 µg/ml derivatized pesticide mix analysis. Mobile Phase A: H_2O/0.9%FA, B: ACN/0.9%FA.

Derivatization with FMOC seemed to enhance the peak shape, reducing tailing and improving intensity. Additionally, analyte peak separation was also slightly upgraded. This could be

occurring due to derivatized analytes having better stability and stronger adsorption towards stationary phase, resulting in higher sensitivity.

Table 16. Mobile phase gradient settings for 50 µg/ml derivatized pesticide mix.

Time (min)	The percentage of ACN/0.9% Formic Acid in the mobile phase
0	95
2	70
4	30
6	30
8	80
14	80

During UHPLC-MS/MS analysis with derivatized analytes, solution was also tested for non-derivatized analytes, results of which can be seen in Figure 40.

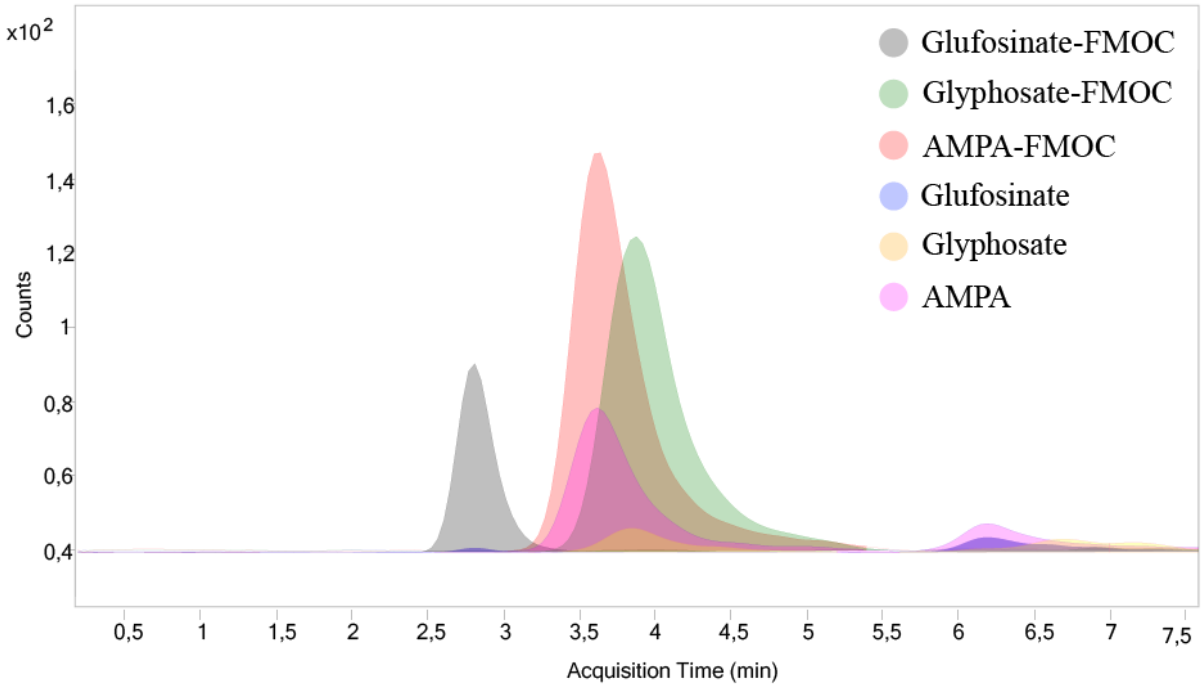


Figure 40. MRM, HILIC-Z 50 µg/ml derivatized pesticide mix analysis. Comparison between derivatized and non-derivatized analytes. Mobile Phase A: H_2O/0.9%FA, B: ACN/0.9%FA.

Here we can see that there are some unreacted analytes, especially AMPA, however, overall derivatization went well. Since the method was meant to be applied for glyphosate analysis, several repeatability tests were done for glyphosate-FMOC and its non-derivatized counterpart, results of which can be seen in Table 17 below.

Table 17. Relative standard deviation for 10 µg/ml Glyphosate and Glyphosate-FMOC.

Measurements	Glyphosate-FMOC Peak Area 390 -> 168 m/z	Glyphosate Peak Area 168 -> 63 m/z
1	17261	1109
2	14000	932
3	13617	828
4	14385	877
5	15433	1117
%RSD	10,96 %	13,71 %

Based on these results, we can also determine that the average Glyphosate-FMOC solution contains on average around 6% of its non-derivatized counterpart. Glufosinate-FMOC had a slightly lower value than its non-derivatized counterpart - around 2%. AMPA-FMOC on the other hand seemed to have the most non-derivatized counterpart – around 39%.

Utilizing previously mentioned settings, it was decided to conduct a calibration curve in the same concentration range as with non-derivatized analytes – from 1 to 50 µg/ml. Resulting calibration curve can be seen in Figure 41 below.

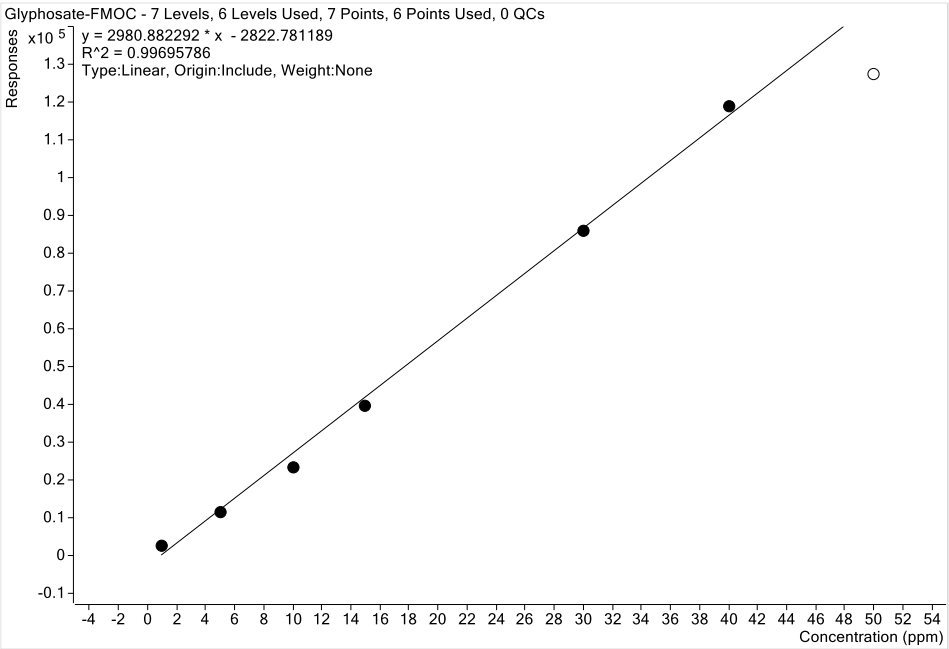


Figure 41. MRM, HILIC-Z, Glyphosate 1-50 ppm calibration curve. The curves equation is y = 2980.882292*x-2822.781189.

It consists of 6 calibration points from 1 to 50 µg/ml, with an R^2 value of 0.9970. Based on the equations (5) and (6) its LOQ and LOD were calculated to be 1.8 µg/ml and 5.4 µg/ml

respectively. Further analysis of filtrated glyphosate samples and commercially available herbicide using this calibration line can be seen in chapter 5.3.

5.2 HILIC-HPLC-UV analysis

Both non-derivatized and derivatized analytes were tested during HILIC-HPLC-UV analysis, utilizing the same mobile phase and gradient elution settings mentioned in the previous chapter (Table 16). However, early tests with different UV wavelengths values showed that non-derivatized analytes did not provide a reasonable response, and their results were close to blank. Because of this, it was decided to continue only with derivatized analytes, where FMOC noticeably enhanced UV detection.

It is important to note that the overall chromatographic settings, such as gradient elution (see Table 16) and flow rate were kept the same as in previous derivatization chapter. As for detection, glyphosate-, AMPA- and glufosinate-FMOC all showed to have the most noticeable UV response in a similar area of 254-264 nm, as seen in Figure 42 below, which is similar to the values mentioned in the previous studies.[121,122]

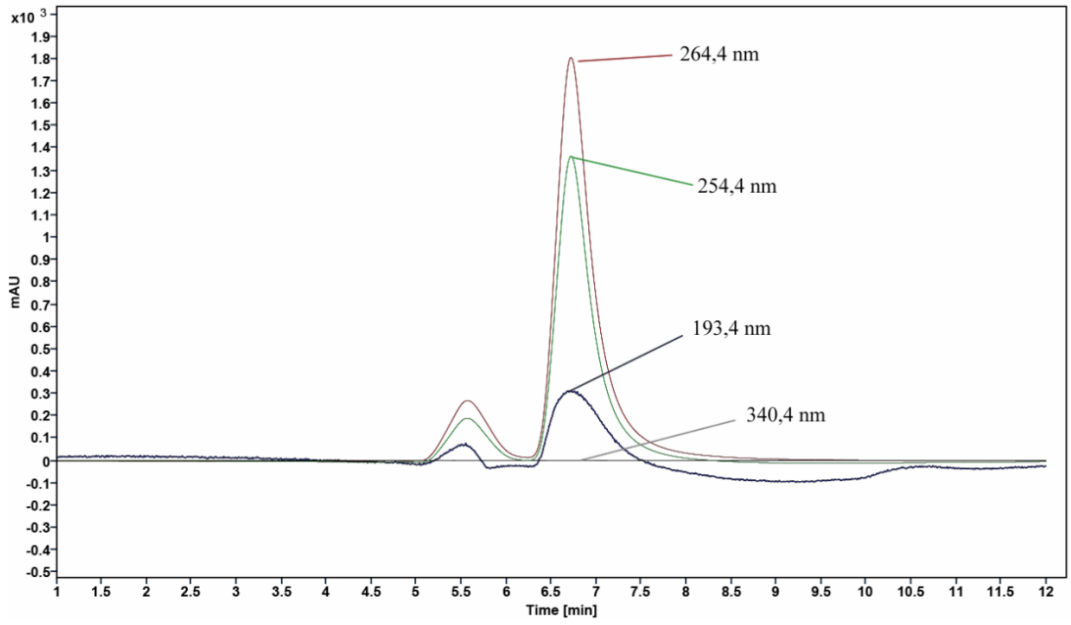


Figure 42. HPLC-UV, HILIC, 50 µg/ml derivatized pesticide mix analysis using different wavelength values.

Because of these similarities, the pesticides in the mix could only be distinguished from each other by the retention time. As seen in Figure 43 below, the retention time for Glufosinate

showed out to be around 5.7 min, while for both AMPA and Glyphosate turned out to be quite similar – 6.8 and 6.7 min respectively, making their separation challenging.

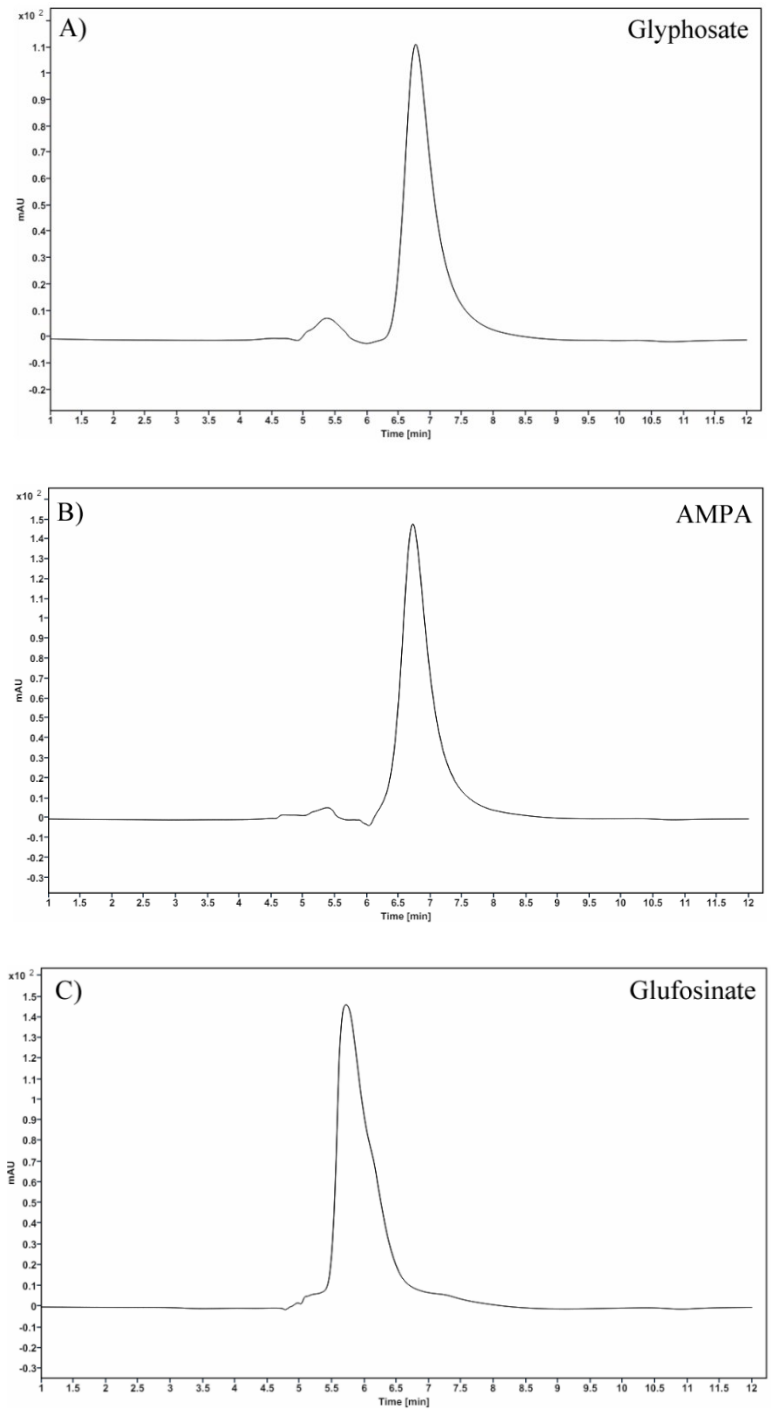


Figure 43. HPLC-UV, HILIC, 15 µg/ml derivatized Glyphosate (A), AMPA (B) and Glufosinate (C) analysis at 264 nm.

Thus, it was decided to continue mainly with glyphosate and glufosinate, since their retention time makes it possible to distinguish one from another in the mix. In order to enhance glyphosate peak shape, especially at lower concentrations, several tests were performed, were

sample solution was modified with formic acid in the range of 0.5 to 2.5%. The results of this can be seen in Figure 44 below.

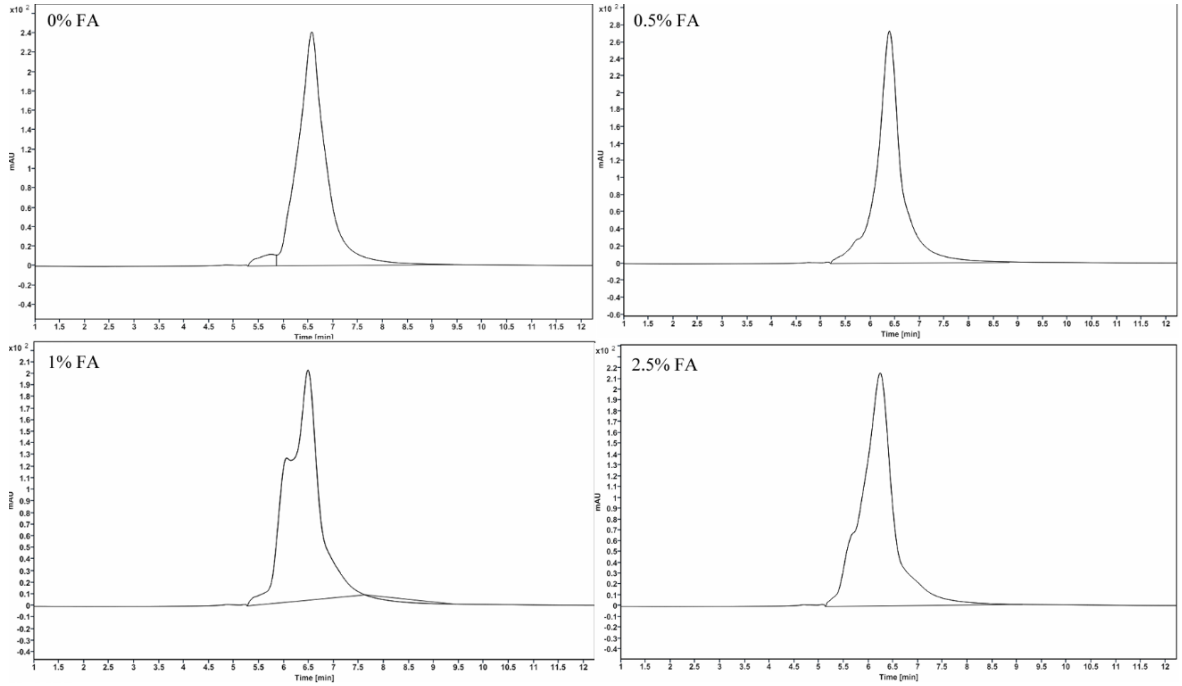


Figure 44. HPLC-UV, HILIC, 25 µg/ml derivatized Glyphosate analysis in 264 nm using 0.5-2.5% formic acid solution.

Here we can see the addition of 0.5% formic acid produced a slightly better peak shape, compared to 1 and 2.5% formic acid which can be especially helpful during peak integration. Because of this, the following glyphosate solutions were done with addition of 0.5% formic acid. Both glyphosate-FMOC and glufosinate-FMOC analytes also showed relatively good repeatability, as seen in Table 18 bellow.

Table 18. Relative standard deviation for 25 µg/ml Glyphosate- and Glufosinate-FMOC.

Measurements	Glyphosate Peak Area	Glufosinate Peak Area
1	9487.2	6850.5
2	9442.6	6737.2
3	9353.9	6907.6
4	9510.8	6696.0
5	9470.9	6737.0
Relative Standard Deviation	0.64 %	1.31%

Similar to the UHPLC-MS/MS, the calibration range for HPLC-UV analysis was also chosen to be between 1 and 50 µg/ml, as seen in a Figure 45 below. It consists of 7 calibration points from 1 to 50 µg/ml, with an R^2 value of 0.99821.

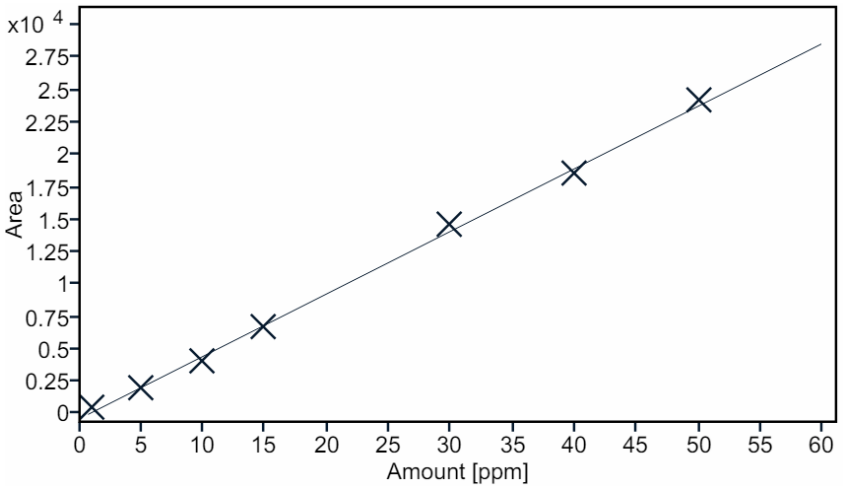


Figure 45. HILIC HPLC-UV Glyphosate-FMOC 1-50 µg/ml calibration curve. The curves equation is y= 483.71117*x-342.51668.

Based on the equations (5) and (6) its LOQ and LOD were calculated to be 1.5 ppm and 6.2 ppm respectively. Further analysis of the filtrated glyphosate samples and commercially available herbicide using this calibration line can be seen in the next chapter.

5.3 Measured results

After comprising a calibration curve for the HILIC-UHPLC-MS/MS method with non-derivatized glyphosate (see Figure 37), relatively low LOD and LOQ values of 2.1 µg/ml and 6.3 µg/ml respectively showed that further measurements of filtrated samples provided unreliable results, since most of the solutions seemed to have around 1.8-8.3 µg/ml of glyphosate, as seen in a Figure 46 below

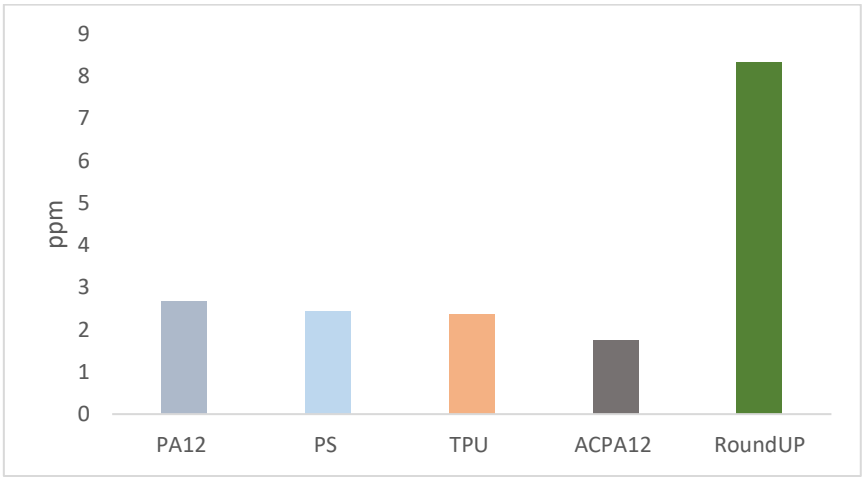


Figure 46. HILIC-UHPLC-MS/MS, the analysis of Glyphosate from different filtrated solutions as well as diluted RoundUp Bio herbicide.

Compared to assumed glyphosate concentration – around 1.45 ppm in filtrated solutions and 5.76 in diluted Roundup Bio solution, it seems that our measurements provided noticeably higher values, with around 2.2 ppm of glyphosate in filtrated solutions and 8.3 in diluted Roundup Bio solution. Still, these values are in a reasonable range from each other, and the difference could be explained by peculiarities of our analysis method, as well as the fact that analytes were stored for a relatively extended period of time, possible affecting their concentration. Additionally, based on these results, there are no noticeable differences between filter materials, although ACPA12 provided slightly lower concentrations of glyphosate.

Experiments using HILIC-UHPLC-MS/MS method with FMOC-glyphosate calibration curve (see Figure 41), revealed that calculated LOD and LOQ - 1.8 µg/ml and 5.4 µg/ml respectively, is also relatively low. Similar to non-derivatized samples analysis, these LOD and LOQ values again showed that filtrated samples provided unreliable results, since most of the solutions seemed to have around 0.96 µg/ml of glyphosate in filtrated, and 1.01 µg/ml in diluted RoundUP solution, as seen in Figure 47 below. It is important to note that before measurements, herbicide and its filtered solutions were also derivatized, with addition of 0.5% formic acid.

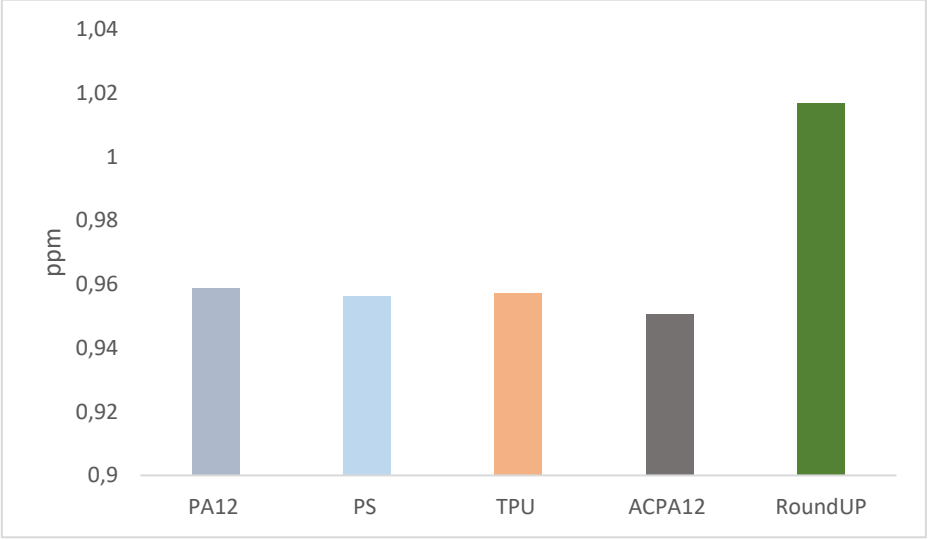


Figure 47. HILIC-UHPLC-MS/MS, the analysis of Glyphosate-FMOC from different filtrated solutions as well as diluted RoundUp Bio herbicide.

Compared to assumed glyphosate concentration – around 1.45 ppm in filtrated solutions and 5.76 in diluted Roundup Bio solution, it seems that this time our measurements provided noticeably lower values, with around 0.96 ppm of glyphosate in filtrated solutions and 1.0 in diluted Roundup Bio solution. These results greatly differ from the previous measurements with non-derivatized samples, which could be explained by possible errors during sample derivatization, where some of the Glyphosate-FMOC could be for example removed during

washing process. Overall, based on these measurements results, utilized filter materials did not have noticeable differences between themselves in glyphosate removal.

Experiments using HILIC-HPLC-UV method with FMOC-glyphosate calibration curve (see Figure 45), also revealed that calculated LOD and LOQ - 1.5 µg/ml and 6.2 µg/ml respectively, is also relatively low, similarly to the previous MS/MS tests. This noticeably reduced the reliability of the measured values, seen in Figure 48 below, since analyzed solutions reveled 1.45 µg/ml of glyphosate in filtrated solutions and 5.76 in µg/ml Roundup Bio solution. Additionally, before measurements, herbicide and its filtered solutions were derivatized, with addition of 0.5% formic acid.

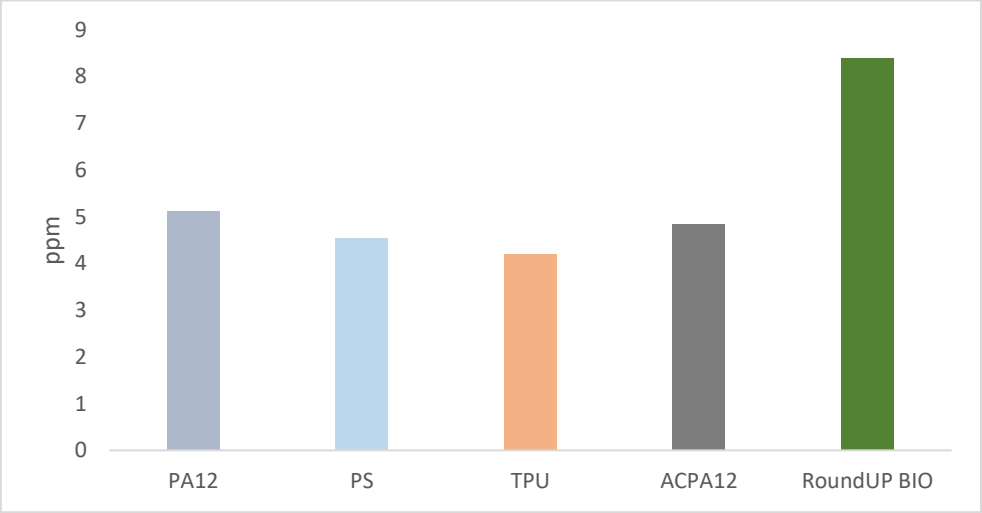


Figure 48. HILIC-HPLC-UV, the analysis of Glyphosate-FMOC from different filtrated solutions as well as diluted RoundUp Bio herbicide.

Compared to the assumed glyphosate concentration – around 1.45 ppm in filtrated solutions and 5.76 in diluted Roundup Bio solution, it seems that this time measurements provided slightly higher values, with around 4.7 ppm of glyphosate in filtrated solutions and 8.4 in diluted Roundup Bio solution. Although it might be difficult to estimate the reasons for such differences, these values are in a reasonable range from each other, and although there are no noticeable differences between filter materials, TPU for example provided slightly lower concentrations of glyphosate, compared to the PA12.

6 Conclusions

During our experiments, we managed to achieve a suitable method for glyphosate, AMPA and glufosinate qualitative and quantitative analysis using HILIC-UHPLC-MS/MS as well as HILIC-HPLC-UV. Both methods seemed to work well with InfinityLab Poroshell 120 HILIC-Z 2.1x100mm 2.7 Micron Column (HILIC-Z), most likely due to more stable interactions between analytes and the stationary phase. For the mobile phase, water and acetonitrile with 0.9% formic acid (A: H_2O/0.9%FA, B: ACN/0.9%FA) produced suitable peaks in both methods, with noticeable separation among all three analytes. Overall, it seems that 0.9 % formic acid creates a suitable pH environment, which improves analytes solubility and retention.

Unfortunately, HILIC-HPLC-UV tests could only be performed with FMOC-derivatized samples, due to the lack of reliable response with non-treatable analyte, and the glyphosate and AMPA could not be separated from each over, due to similar retention time. Overall, derivatization with FMOC seemed to noticeably improve peak shapes for analytes and intensity, for both methods, most likely by enhancing the interaction with the stationary phase, increasing analytes hydrophobicity and improving its detection sensitivity. At the same time, derivatization itself seemed to be quite successful, with around 94% of glyphosate being derivatized. The gradient elution also seemed to work well for both non-derivatized and derivatized analytes, with only a small difference between them, as seen in Table 19 bellow:

Table 19. Mobile phase gradients for non-derivatized and derivatized analytes.

Time (min)	Mobile Phase B% (Non-derivatized)	Mobile Phase B% (Derivatized)
0	75	95
2	20	70
4	75	30
6	75	30
8	75	80
14	-	80

Here we can see that elution starts with a weak mobile phase, followed by a more aquatic one, and then again highly organic. It seems that derivatized samples utilize slightly longer analysis method overall, even though the analytes themselves are eluted faster, compared to their non-derivatized counterparts. This could be explained by the slightly longer stabilization of the HILIC column when utilizing derivatized analytes. Additionally, the addition of 0.5% formic acid to the derivatized samples slightly enhanced analytes peak shape, and thus the following

peak integration. This pH adjustment most likely enhanced peak shape for example by suppression analyte ionization, improving their solubility as well as retention consistency.

Relatively slow flow rate of 0.1 ml/min and small injection volume of 1-5 µl also seemed to work well for HILIC-UHPLC-MS/MS as well as HILIC-HPLC-UV methods, which could be explained by column overloading, when moving towards higher values. This is also seen when testing different analytes concentration, with 1-50 µg/ml being and optimal range for qualitative and quantitative analysis of glyphosate, AMPA and glufosinate. Taking this into account, calibration tests were also done in the 1-50 µg/ml range. Additionally, before testing main samples, 4-5 quality standards were measured and their relative standard deviation (%RSD) calculated, in order to evaluate measurements accuracy and repeatability.

Main samples themselves consisted of glyphosate solutions, which were filtrated through different filters (PA12, TPU, PS, ACPA12) as well as diluted and commercially available herbicide - Roundup Bio. Filtrated solutions, as well as diluted Roundup Bio solution were assumed to contain around 1.4 and 5.7 ppm of glyphosate respectively. However, as we can see from Figure 49 below, our methods showed noticeably varying values for glyphosate concentration.

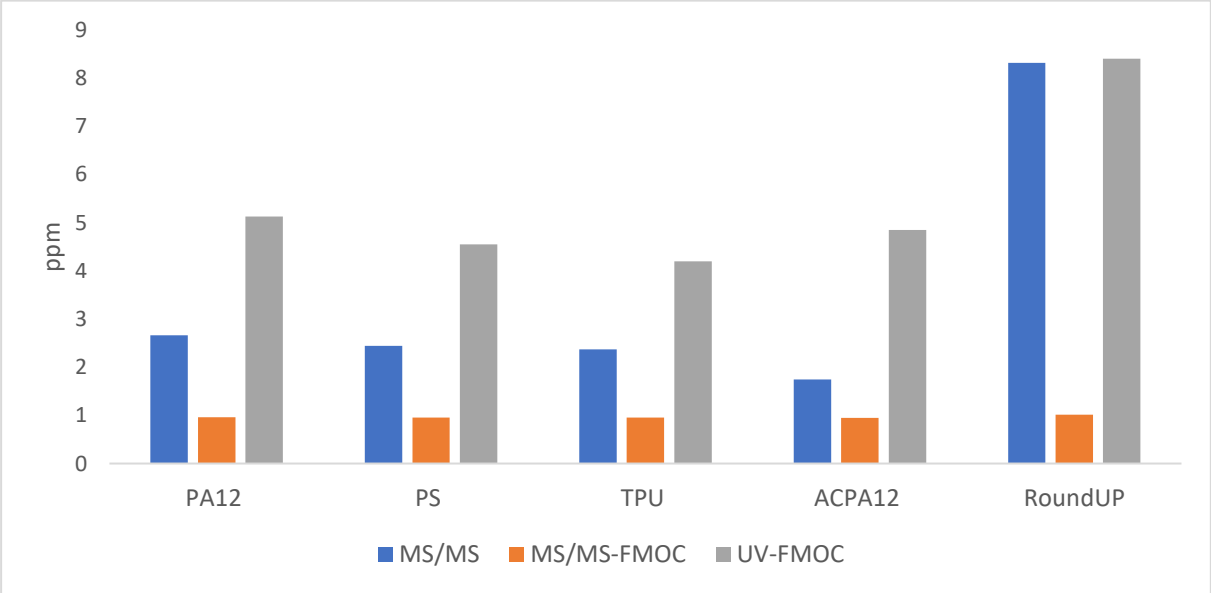


Figure 49. The analysis of Glyphosate and Glyphosate-FMOC from filtrated and RoundUp Bio solutions, utilizing HILIC-UHPLC-MS/MS as well as HILIC-HPLC-UV methods.

Here we can see that our first method, consisting of glyphosate HILIC-UHPLC-MS/MS analysis (MS/MS), compared to assumed glyphosate concentration provided noticeably higher values, with around 2.2 ppm of glyphosate in filtrated solutions and 8.3 in diluted Roundup Bio solution. Nevertheless, these values are in a reasonable range from each other, and the

difference could be explained by the relatively low accuracy of our method, as well as overall differences in analytical technique and method calibration. Additionally, although the %RSD of five quality standards for this measurement was acceptable (2.32 %), the LOD and LOQ were calculated to be 2.1 µg/ml and 6.3 µg/ml respectively, which makes our measured results unreliable. It is important to note there are different ways to calculate LOD and LOQ, and if, for example, equations (3) and (4) were utilized, LOD and LOQ for this measurement would be 1.3 and 4.4 µg/ml respectively. Still, we decided to use equations which utilize calibration curves slope, as well as error of its y-axis intersection (equations 5 and 6), which is usually more reliable and precise.

Our second method, consisting of glyphosate-FMOC, HILIC-UHPLC-MS/MS analysis (MS/MS-FMOC), showed noticeably lower concentration of analyte, compared to other measurements - around 0.96 µg/ml of glyphosate in filtrated solution, and 1.01 µg/ml in diluted RoundUP solution. It is challenging to estimate the reason for such noticeable change in concentration, especially when considering that the same samples were used in glyphosate-FMOC, HILIC-HPLC-UV analysis (UV-FMOC), were the concentration of analyte is much higher - around 4.7 µg/ml of glyphosate in filtrated solution, and 8.4 µg/ml in diluted RoundUP solution.

This could be occurring for example due to the errors in sample derivatization process, where perhaps some of the glyphosate-FMOC was removed during diethyl ether washing, lowering the overall concentration. Both MS/MS-FMOC and UV-FMOC measurements also experienced the same low-reliability problem as MS/MS, since their LOD and LOQ are noticeably higher than the acquired measurement values. Taking this into account, the sample preparation procedure could be extremely important when analyzing derivatized samples. One possible improvement to this method could be the addition of internal standard, in order to correct possible loss of analyte, for example during derivatization process.

To better understand the chromatographic parameters of our method, Retention Factor (k) and Selectivity (a) were calculated using equations (7), (8) and (9) for both HILIC-UHPLC-MS/MS (see Table 20) and HILIC-HPLC-UV (see Table 21).

Table 20. Retention factor and selectivity for analytes during HILIC-UHPLC-MS/MS

Sample	Retention Factor (k)	Selectivity (α)
Glyphosate	2.84	1.05^1
Glyphosate-FMOC	1.24	1.13^1
AMPA	2.70	1.02^2
AMPA-FMOC	1.09	1.76^2
Glufosinate	2.64	1.02^2
Glufosinate-FMOC	0.62	1.76^2

Where a^1 is selectivity between Glyphosate and AMPA, and α^2 between AMPA and Glufosinate.

As we can see, most derivatized and non-derivatized analytes have a suitable retention factor values between 1.09 and 2.84, meaning that the analyte experiences enough retention. It seems that derivatized analytes have an overall lower retention factor value, especially Glufosinate-FMOC with k value of 0.62, which could indicate a weak retention. From analytes selectivity factor we can also see that all analytes are not very well separated from each other, with α values being close to 1.0.

This kind of trend with separation and selectivity factors is also seen in HILIC-HPLC-UV method, as seen in Table 21 below

Table 21. Retention factor and selectivity for analytes during HILIC-HPLC-UV

Sample	Retention Factor (k)	Selectivity (α)
Glyphosate-FMOC	3.15	1.13^1
AMPA-FMOC	2.79	1.20^2
Glufosinate-FMOC	2.32	1.20^2

Where α^1 is selectivity between Glyphosate and AMPA, and α^2 between AMPA and Glufosinate.

Compared to the previous HILIC-UHPLC-MS/MS method, we can see that the retention factor is slightly higher, which could be explained by lower flow rate, resulting in longer retention times. Selectivity on the other hand is again close to 1.0 with all analytes, thus showing low separation between them.

Overall, although derivatization noticeably helps with analytes sensitivity, improves its peak shape and retention, it seems that in our case the most reliably analysis method consisted of non-derivatized glyphosate analysis with HILIC-UHPLC-MS/MS, since its measured values were closest to the assumed ones, and the samples themselves have not been modified, minimizing possible errors. Additionally, although there are slight differences in filters during

a specific method analysis, when compared to the results from other methods, there are no clear distinction between them. This could be occurring due to the overall differences between analytical techniques, instrument calibration, sensitivity and condition, as well as sample preparation, making overall repeatability quite low.

Throughout the experiments we have faced several challenges regarding methods repeatability as well as their reliability. Still, both HILIC-UHPLC-MS/MS and HILIC-HPLC-UV methods showed to be able to detect and quantify pesticide in a relatively small concentration range of 1-50 µg/ml. Although glyphosate analysis with HILIC-HPLC-UV method showed to be especially easy to operate and has been relatively consistent throughout the tests, glyphosate analysis with HILIC-UHPLC-MS/MS seemed to provide more reliable results. There is also room for improvement for both methods, which could noticeably reduce possible errors and enhance the reliability of the measured values. For example, internal standards could be used in order to improve accuracy and precision of quantification. The pesticide derivatization method could also be further tested and modified, ensuring its repeatability.

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