Review

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Glyphosate as Contaminant in Cereals: Chromatographic Methods for Its Determination. Discussion about Occurrence, Accumulation, Fate, Degradation, and Regulatory Status

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Abstract: The European Union's recent decision to renew the authorization for the use of glyphosate until 15 December 2033 stimulates the scientific discussion all around the world regarding its toxicity or otherwise for humans. Glyphosate is a chemical used by millions of tons in the last 50 years worldwide to drying weeds in cultivated fields, greenhouses, roadsides. Concern has been raised in many quarters about the possible presence in the food chain and the consequent adverse effects on health: both aspects that argue in favor of toxicity and those that instead may indicate limited toxicity of glyphosate are here discussed. The great debate that has been generated requires further investigations and field measurements to understand its fate once dispersed in the environment and its concentration in the food chain. Hence the need for validated analytical methods that are available to analysts in the field. In the present review, methods for the analytical determination of glyphosate and its main metabolite, AMPA, are discussed, with a specific focus on chromatographic techniques applied to cereal products. The experimental procedures are explained in any detail, including cleanup, derivatization, and instrumental conditions to give the laboratories involved enough information to proceed with the implementation of this line of analysis. The prevalent chromatographic methods used are LC-MS/MS, GC-MS/SIM, and GC-MS/MS but sufficient indications are also given to those laboratories that wish to use the simpler HPLC-FLD, HPLC-UV, GC-NPD or GC-FPD techniques for screening purposes. Concentrations from literature measured in wheat, corn, barley, rye, oats, soybean, and cereal-based foods are reported together with the regulatory status in various parts of the world and the accumulation mechanism. As for the accumulation in cereals, available data show that glyphosate tends to accumulate more in wholemeal flours than in refined ones, that its concentration in the product strictly depends on the treatment period (the closer it is to the time of harvesting, the higher the concentration) and that in cold climates the herbicide tends to persist in the soil for a long time.

Keywords: glyphosate; analytical methods; sample preparation; liquid chromatography; gas chromatography; mass spectrometry; cereals; occurrence; accumulation mechanism; regulatory status

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1. Introduction

Glyphosate, IUPAC name N-(phosphonomethyl)glycine also called 2-(phosphonomethylamino)acetic acid (Figure 1), is the most used herbicide in the world. It was introduced on the market in 1974 under the trade name of Roundup® for weed control in agriculture or for eliminating roadside weeds as well as in orchards, forests, parks, squares, railways. In traditional agriculture, glyphosate was used only in the pre-emergence phase but after the introduction of genetically modified organisms (GMOs) resistant to Roundup®, it also began to be used in post-emergence phase. In 1996, genetically modified soybean, corn, and cotton plants, called

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'Roundup® Ready' plants, were introduced onto the market. Since then, the use of genetically modified organisms and the use of glyphosate have grown to unprecedented levels: about 660 million kg of global glyphosate use in 2011 and 826 million in 2014 are reported [1–3].

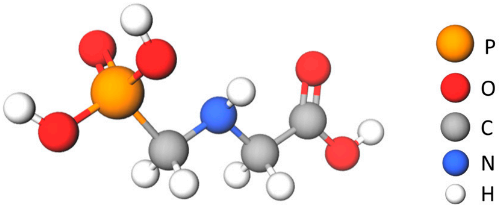

Figure 1. Glyphosate.

However, the improper use of this herbicide in the pre-harvest phase gave rise to a heated debate. This controversial application is carried out directly on the crop a short time before harvesting for drying purposes, to optimize yield [3,4]. This happens especially in climates that are not fully suitable for some cultivations. In the US, in Canada and elsewhere there is a practice of using glyphosate to desiccate crops by spraying the maturing plants, in order to speed up and make the 'maturation' of the crop more uniform, thereby facilitating harvest [5–12]. This may add to the residue levels of glyphosate, as shown in field pea, barley and flax seed. Particularly if the plant is still growing, translocation of the herbicide within the plant may result in its accumulation in the seed, both for GM and unmodified soy [13]. Even Mediterranean countries imports significant quantities of wheat from those countries that use glyphosate in the pre-harvest phase [14]. Plants translocate this systemic herbicide to their roots, shoots, seeds, and fruits where it causes the accumulation of shikimic acid and hinders the enzymatic conversion of shikimic acid to anthranilic acid by inhibiting the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase [15–17]. Crops treated with glyphosate slowly die over a period of days or weeks, and because the chemical is transported throughout the plant, no part survives [18]. Because plants absorb glyphosate, it cannot be completely removed by washing or peeling produce or by milling, baking, or brewing grains [1] although in this way its content can be somewhat reduced [14]. In the environment, the free pesticide degrades rapidly, but when it comes into contact with the soil it adsorbs to soil particles and degrades very slowly: sometimes it remains undegraded and inactive in the soil for years [18,19] dissociating only into its main degradation product [20], namely aminomethylphosphonic acid (AMPA) which has comparable toxicity to glyphosate and which must always be analytically determined together with the latter.

In 2017 the IARC, the International Agency for Research on Cancer, classified this pesticide in Group 2A 'probably carcinogenic to humans' [21]. On the contrary, the European Commission in 2016 established that 'on the basis of the information currently available, no hazard classification for carcinogenicity is justified for glyphosate' [22] and therefore in December 2017 it renewed the use permit for five years [22,23]. In 2022 the decision about the use of glyphosate was postponed and in November 2023 the Commission renewed the use permit until 2033 [24] also based on an opinion delivered by the European Food Safety Authority (EFSA) [25]. Some studies declare toxicity of glyphosate. New research indicate that glyphosate causes leukemia in early life of rats administered with the herbicide via drinking water, at doses currently considered safe by regulatory agencies [26]. The same considered-safe doses showed endocrine toxicity in rats [27], later confirmed in a human population of mothers and newborns exposed to glyphosate during pregnancy [28]. A very recent review of Lacroix and Kurrasch is less conclusive. They observe that co-formulants in Glyphosate preparations can greatly amplify toxicity, indeed, the co-formulants themselves may be more toxic than Glyphosate itself [29]. Therefore, the question of toxicity remains open.

In this context, the availability of reliable analytical methods is crucial. The highly topical debate on an herbicide declared in the past to be of little danger currently requires more careful evaluations and a greater number of analytical measurements to understand its fate once used in the field and how much of it passes into the various final products intended for consumption, with the consequent degree of exposure by consumers. Due to some molecular characteristics (absence of UV absorbance, low volatility, high hydrophilicity) [1,30–33] the quantitative determination of glyphosate both in crops, in soils and in waters is challenging. Chromatography is the most successful and the most used technique, which, very frequently, exploits derivatization (mandatory in gas chromatography). When Liquid Chromatography with derivatization is used the prevalent method is with FMOC-Cl (9-fluorenylmethyl chloroformate). FMOC-Cl reacts with glyphosate and AMPA to give the corresponding derivatives [33–42]. The determination of glyphosate by liquid chromatography is also possible without derivatization: in such a case Ion Exchange Liquid Chromatography is used. As regards gas chromatography (GC) a largely used derivatization is that using perfluoroalcohols plus trifluoroacetic anhydride [43–52]. Perfluoroalcohols used are trifluoroethanol (TFE) or heptafluorobutanol (HFB). In addition, another GC derivation method exists, which is used to a lesser extent. In the present review all methods for determining glyphosate and AMPA in cereals via liquid and gas chromatography will be discussed in detail.

2. Glyphosate Degradation Pathways

In general, glyphosate degradation proceeds by either of two pathways [53–56] as shown in Figure 2. They are also called ‘AMPA pathway’ and ‘sarcosine pathway’.

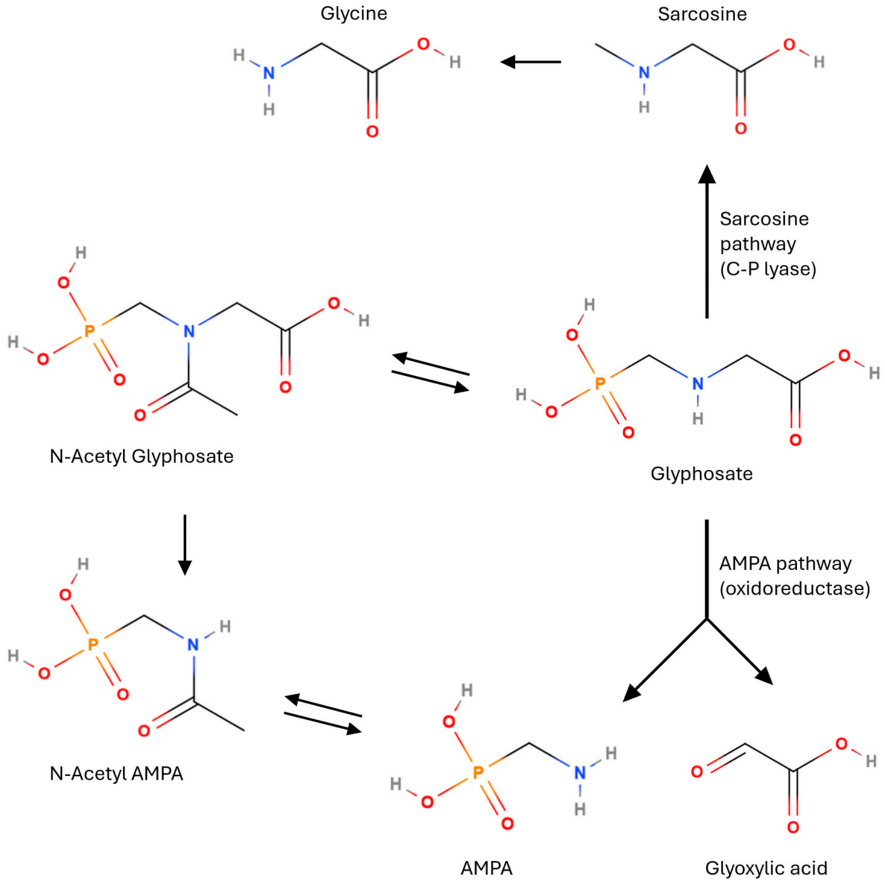

Figure 2. The two main degradation pathways of glyphosate.

Glyphosate is either transformed into AMPA and glyoxylic acid by oxidoreductase or into sarcosine by C-P lyase [57]. Sarcosine is then converted to glycine by sarcosine oxidase [55], while glyoxylic acid in turn is converted to glycine and carbon dioxide by the glyoxylic acid cycle [53,55]. AMPA is presumed to be converted to methylamine [54]. Among the degradation products of glyphosate, AMPA is the only persistent compound. From the analytical point of view, it is of primary importance to always monitor AMPA together with glyphosate since AMPA is the main metabolite of glyphosate [58,59]: from some research it appears to have equal or greater toxicity than glyphosate itself [59,60]. Degradation of AMPA is generally slower than glyphosate possibly because AMPA may adsorb onto soil particles more strongly than glyphosate and/or because it may be less likely to permeate the cell walls or membranes of soil microorganisms [55]. In Figure 2 they are shown N-Acetyl derivatives. N-acetyl glyphosate is a metabolite formed after glyphosate application to glyphosate tolerant GM crops [55].

3. Occurrence Data, Accumulation, Fate

Table 1 shows an overview of Glyphosate and AMPA levels measured in cereals.

Table 1. Glyphosate and AMPA concentrations measured in cereals and related foods.

Cereals and cereal-based foods	Glyphosate concentration (mg kg^{-1})	AMPA concentration (mg kg^{-1})	Reference
Barley	< 0.45	n.a. [1]	[61]
Oats	< 0.08	n.a. [1]	[61]
Rye	< 0.04	n.a. [1]	[61]
Durum wheat	0.421 (max.)	0.0247 (max.)	[62]
Wheat	< 0.13	n.a. [1]	[61]
Wheat	6.1 – 11.1	n.a. [1]	[4]
Wheat bran	< 0.7	n.a. [1]	[61]
Wheat flour	0.02	n.a. [1]	[61]
Bread	0.0458 (max.)	traces	[62]
Breakfast cereals	0.291 (max.)	0.01 (max.)	[62]
Flour and baking mixtures	0.133 (max.)	traces	[62]
GM soybean	0.4 – 8.8	0.7 – 10	[13]
GM corn	0.15	0.49	[63]
Wheat	0.373	0.034	[5]
Barley	2.15	0.041	[5]
Whole grain	0.0257	n.a. [1]	[64]
White bread	0.0149	n.a. [1]	[64]
Soy-based infant formulas	0.03 – 1.08	0.02 – 0.17	[65]
GM soybean	0.1 – 1.8	0.9 (max.)	[66]
Corn flour	0.0052 – 0.3 [2]		[67]
Breakfast cereals	0.006 – 0.034	n.a. [1]	[68]
Wheat flour	< 0.03	n.a. [1]	[69]
Wheat bran	1.62 (max.)	n.a. [1]	[70]

[1] not available. [2] sum of glyphosate and AMPA.

3.1. Glyphosate Concentration in Straight Grade Flour vs. Concentration in Wholemeal Flour

It was observed that glyphosate is more present in bran and less present in white flour. Granby et al. have sampled grains from Danish mills and major producers in 1998-2001 and carried out the analysis of glyphosate residues. The average results for wheat showed that the glyphosate content in bran was concentrated compared with the grain while the content in flour was somewhat lower than in grain [61]. It may be reasonable to further consider that the application of glyphosate is generally sprayed directly onto the crops and that the extractable glyphosate is then directly correlated to the presence of the outer bran. Reasonably, the differences of glyphosate in whole wheat where the bran remains and the refined flours where the bran is removed may explain the differences observed in extractable glyphosate in commercially available food samples [71]. Tittlemier et al. demonstrated that 50% of the total glyphosate mass resides in the outer 17% of the kernels and that 81% of the total glyphosate mass in wheat was associated with the bran, shorts, and feeds milling fractions. They argue that glyphosate concentrations in bread made from straight grade flour will be approximately 4× lower than that made from whole grain flour [72]. The results of a further one-year study showed that glyphosate residues were more concentrated on top of grain/seed layers, rather than inside, and that the highest concentrations of glyphosate residues were found in bran [5]. Other studies confirm such trend [4,64,73,74] with Ashley-Martin et al. who observed a dose-response relationship between consumption of whole grain bread and higher urinary glyphosate concentrations [75]. Obviously crops that were grown without the use of glyphosate do not show any trace of residue in any part of the grain, not even in the bran [5,13]: this last statement is important for the recognized beneficial health effects related to the consumption of whole grains [76–80].

3.2. Glyphosate Accumulation in Crops

The period of crop treatment with the herbicide is critical for the concentration of glyphosate in the final harvested product.

Gélinas et al. applied Roundup® before harvest to some wheat varieties in the amount of 0.82 kg ha^{-1}, as recommended on the label, and observed a glyphosate residue of 11.1 mg kg^{-1} in one wheat variety and 6.1 mg kg^{-1} in another variety. This high residue content was attributed to the date of treatment, very close to the harvest time [4]. Bøhn et al. reported that GM soybean treated with Roundup® during the growing season accumulated glyphosate and AMPA at concentrations of 0.4-8.8 mg kg^{-1} and 0.7-10 mg kg^{-1}. In contrast, conventional and organic soybean crops did not contain glyphosate and AMPA [13].

Therefore, they confirmed the hypothesis that GM-soybeans may contain high residue levels of glyphosate and AMPA due to repeated spraying of the plants with glyphosate-based herbicides throughout the production season (Figure 3).

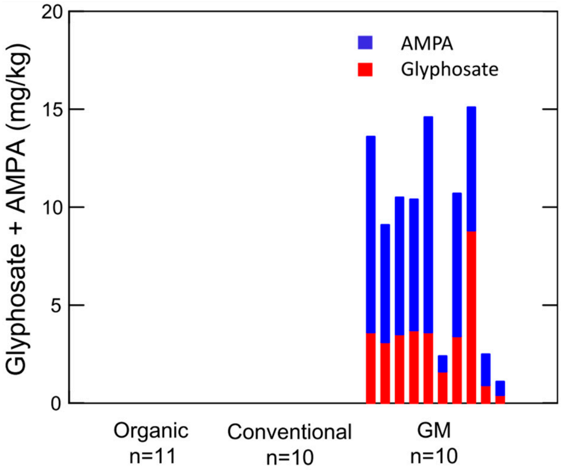

Figure 3. Residues of glyphosate and AMPA in individual soybean samples (n = 31). Excerpted from the work of Bøhn et al. 2014 [13].

Kadžienė et al. applied a study protocol to test for glyphosate accumulation in cereals following the pre-emergence treatment (1 week after sowing) and the pre-harvest treatment (14-10 days before harvest). From their study, repeated for two seasons, it was found that the pre-emergency application has no impact on the final contamination of cereals (no residues). Instead, pre-harvest application resulted in maximum concentrations of 2.15 mg kg^{-1} for glyphosate and 0.04 mg kg^{-1} for AMPA [5].

3.3. Fate and Degradation of Glyphosate

Studies carried out in crops, and in products derived from them, indicates that washing wheat grains is able to reduce the concentration of glyphosate. Also grain processing can reduce the concentration of the herbicide [71] as well as decortication, i.e. the removal of the external layers of the grains, even if glyphosate cannot be eliminated completely as it is absorbed by the entire plant. It was demonstrated that the preparation of dried pasta from semolina and flour milling fractions reduced concentrations by a factor of 1.8 for glyphosate [73]. In the same research, concentrations in cooked pasta decreased significantly with cooking time: after 15 min of cooking, approximately 73% of the total glyphosate mass had transferred from pasta to cooking water [73]. Malting of barley results in gross loss of residues: glyphosate residue levels in beer were found about 4% of original levels in barley [74].

Conflicting results are available on glyphosate degradation resulting from dough fermentation during the breadmaking process [4,81]. As regards storage it was found that when glyphosate was applied at pre-harvest period at the rate indicated by the label its level in grain remains steady during a 2-year storage time [5].

Given the hydrophilicity of glyphosate, water bodies adjacent to a treated field are frequently polluted by glyphosate and AMPA [82–89] with associated possible human health diseases [90]. Dissipation rates of the herbicide in the field are influenced by soil properties, application methods and environmental conditions. Greater persistence has been observed in colder climates [91]: in northern climates with seasonally frozen soils, field studies have shown clear persistence of glyphosate through the winter. After applications in June and July at two Finnish sites, approximately 10-20% of the applied Glyphosate was detected in June of the following year, demonstrating that the time for dissipation of 90% of glyphosate (DT 90) was about 11 months. In warmer climates, glyphosate did not persist beyond the growing season, even after 15 consecutive annual applications [92].

4. Regulatory Status

The toxicity of glyphosate for human health is still under study and this can be deduced from Table 2. In the case of wheat, for example, FAO/WHO and EPA set the MRL for glyphosate at 30 mg kg^{-1}, while European Union and Health Canada has set 10 and 5 mg kg^{-1}, respectively. Considering the controversy on glyphosate maximum residue level in foodstuff [1] there is a possibility that the current MRLs could be modified in the coming years: future research will have to take into consideration even very low levels of intake and follow chronic exposures, to ascertain the possible negative effect on health of this herbicide.

Table 2. Maximum Residue Levels (MRLs) for glyphosate in cereals as established by international organizations (mg kg^{-1}). Values updated as of December 2023.

Cereals and related crops	European Union [93,94]	FAO / WHO Codex [94,95]	U.S. EPA [1] [96]	Health Canada [97]
Barley	20	30	30	10
Buckwheat	0.1	30	30	
Maize/corn grains	1	5	5	3
Millet	0.1	30	30	
Oats	20	30	30	15
Rice	0.1		0.1	
Rye	10	30	30	
Sorghum	20	30	30	
Soya beans	20	20	20	20
Wheat	10	30	30	5

[1] MRLs are referred to as ‘Tolerances’ in U.S.

From the analytical point of view mass spectrometry as a chromatographic detector is the solution of choice for research studies on glyphosate given its high sensitivity and specificity. Other detectors more affordable than mass spectrometry in terms of costs, maintenance, and learning, also exist. We are talking about the much simpler and historically widely used HPLC-Fluorimetry (HPLC-FLD) [98–101] or HPLC-UV [39,42,87] which have less sensitivity and specificity than mass spectrometry but would still be suitable for checking the compliance of many of the cereal products listed in Table 2. The same apply to gas chromatography with nitrogen phosphorus detection (NPD) or with flame photometric detection (FPD) which are more affordable than gas chromatography – mass spectrometry but with less sensitivity and specificity [51,102].

5. Chemical Analytical Methods

5.1. Liquid Chromatography

The analysis of Glyphosate and AMPA in water has been performed for a long time by liquid chromatography with FMOC derivatization, subsequently also codified by the ISO 16308:2014 standard [103,104]. The ISO 16308:2014 standard involves the use of tandem mass spectrometry as detector, but the method was originally based on HPLC-Fluorimetry. By switching to mass spectrometry as a detector, better sensitivity was obtained. The method uses the derivatization of both molecules with 9-fluorenylmethyl chloroformate (FMOC-Cl) as shown in Figure 4.

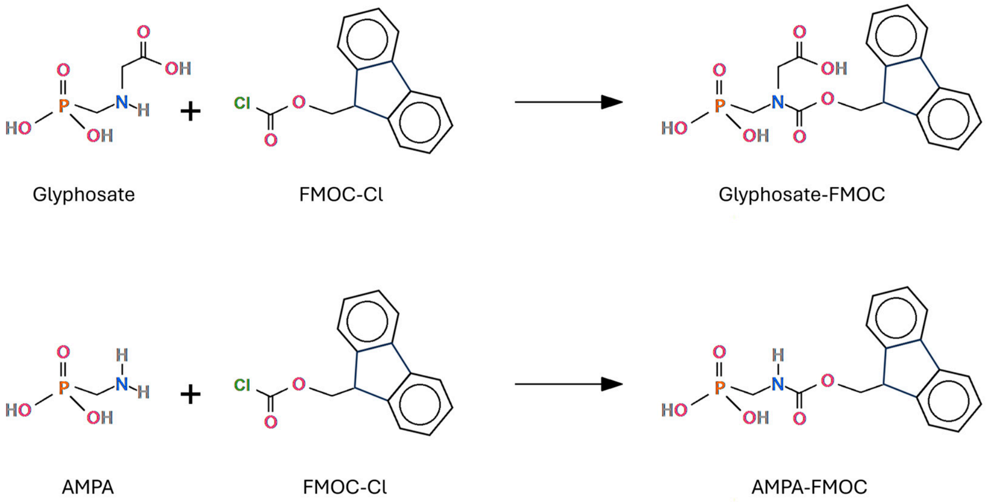

Figure 4. FMOC derivatization of glyphosate and AMPA for determinations carried out by liquid chromatography.

This is needed due to the high polarity of the compounds as such, which would make analysis in reversed-phase liquid chromatography difficult. From the analysis of water, the liquid chromatography method via FMOC Derivatization was quickly extended to the analysis of foods. In a 2015 interlaboratory ring test for the analysis of glyphosate in wheat flour, all the laboratories that used liquid chromatography with derivatization exploited FMOC derivatization and almost all used tandem mass spectrometry (LC-MS/MS) as the detection technique [1]. It must be said that out of 12 laboratories, 5 did not use derivatization: this is possible if ion-exchange liquid chromatography is used. The performance of the laboratories that participated in the study indicates that LC/MS/MS has a sensitivity of 0.03-0.05 mg per kg of product. Some recent works claim even better performance [35,62].

5.1.1. FMOC Derivatization

Pre-column FMOC-Cl derivatization has been proven to be simple and successful. The complete reaction of glyphosate with FMOC-Cl guarantees stability and successful chromatographic separation on LC columns [33]. Glyphosate-based compounds are commonly determined using the reversed-phase technique. The derivatization takes place at alkaline pH: generally, pH is adjusted to 9 by adding borate buffer and by adding EDTA. With LC-MS/MS the parent ions to be monitored are 390 m/z for glyphosate-FMOC and 332 m/z for AMPA-FMOC [33,34]. Daughter ions are frequently 150 m/z for glyphosate-FMOC and 110 m/z for AMPA-FMOC, so the transitions of interest are generally 390 → 150 m/z and 332 → 110 m/z [34,38] but also 168 → 63 m/z and 110 → 63 m/z in those cases where derivatization is not used [62]. Detection can be carried out in positive electrospray mode [36] or in negative electrospray ionization mode [33,34,38,62]. Eluting solvents are often ammonium acetate in LC−MS grade water, and acetonitrile (ACN). Martin et al. evaluated the influence of borate addition to the derivative step and the response of the various borate buffer concentrations (w/v). They found that the reaction did not occur in the control, for which no borate buffer was added, while when borate buffer was added to the reaction medium, the peak intensities of the derivative products were greater: a significantly higher response was obtained with 5% borate addition, compared to the control, than the other variables [33]. Reactivity of glyphosate's amino group was enhanced by increasing the buffer concentration, which improved the derivatization reagent's solubility.

Before carrying out the derivatization step, the extraction of glyphosate and AMPA from the sample must be performed. In the next section, an overview of sample preparation will be given with some practical examples.

5.1.2. Sample Preparation Used with FMOC Derivatization

A good practical example of analysis of glyphosate in cereals via liquid chromatography is the work of 2021 by Cruz and Murray from U.S. National Institute of Standards and Technology (NIST) who analyzed oat products (oatmeal, oat-based cereals, oat flour, both conventional and organic). They used a Solid Phase Extraction (SPE) step. To the ground cereal sample (1 g) the internal standard aqueous solution was added (isotopically labeled glyphosate and AMPA) then an aliquot of a 50/50 mix of acidified H_2O (0.1% formic acid) and MeOH was added, and a centrifugation step followed. After centrifuging, the sample was transferred into a polypropylene centrifuge tube containing the borate buffer reagent. After homogenization, the FMOC reagent was added, and the sample was left in the dark during the derivatization reaction. Then the SPE step was carried out by adjusting pH, centrifugating, loading the supernatant onto the SPE cartridge and eluting with a $MeOH/NH_4OH$ solution mix in a 2 mL glass vials. The eluate was evaporated, and H2O was added to bring a final volume of 500 μL. Extracts were filtered and stored at 6 °C until LC–MS/MS analysis. The separation was carried out on a C18 column, injection volume was 20 μL and the monitored transitions for quantification were 390 → 168 m/z for glyphosate-FMOC and 332 → 110 m/z for AMPA-FMOC [105].

Similar preparation was used for soy protein isolate [36], cereal flour samples by means of HPLC-FLD [98], tea samples [34], and beebread samples [38].

From the examination of the works cited it can be deduced that the use of a cleanup step via SPE is almost indispensable for the determination of glyphosate and AMPA in food matrices such as cereals, which present a certain complexity (while it is not necessary for sample preparation of drinking water or groundwater, for example, which are quite clean themselves).

5.1.3. Direct vs. Indirect Determination

As regards FMOC derivatization it must be noted that originally the determination of glyphosate and AMPA via liquid chromatography took place mainly with fluorimetry and that in the absence of derivatization the two molecules had practically no absorption (analysis not possible). A precolumn derivatization step with FMOC-Cl yielded highly fluorescent derivatives of the analytes [106]. Subsequently, even with the use of mass spectrometry, the standard ISO 16308:2014 has foreseen FMOC derivatization with the use of triple quadrupole analyzer together with a C18 column [104]. Derivatization (indirect determination) is intended to improve at least one of the principal analytical parameters, namely, detection sensitivity or separation selectivity, by modifying the hydrophobic/hydrophilic character of the target molecule [107]. Glyphosate and AMPA are very small and polar compounds that lack chromophores or other heteroatoms that can facilitate their sensitive detection. In addition, the amphoteric nature of these agents makes their concentration and purification by normal phase or RP SPE very difficult [108].

Nevertheless, several works analyzing glyphosate via LC-MS/MS do not make use of derivatization. These are methods using the direct determination, i.e. without derivatization, which can work properly if the Ion Chromatography technique (with an ion-exchange analytical column) is used [109–112]. Direct determination is sometimes also exploited for the analysis of cereal samples [61]. Obviously, from what has been said, determinations of glyphosate and AMPA with C18 columns work properly in indirect mode only.

5.1.4. Glyphosate and Glufosinate

It is possible to observe that many of the research papers aimed at analytically determine glyphosate and AMPA include the compound glufosinate (2-amino-4-[hydroxy(methyl)phosphoryl]butanoic acid) in the determination. This is due to the similarity in the chemical structure of glyphosate and glufosinate but there are no other links between them, although both can be used as herbicides. Glufosinate is a natural compound [113] that was first isolated from the bacteria *Streptomyces viridichromogenes* or *Streptomyces hygroscopicus* [114] while glyphosate was first discovered as a synthetic compound.

5.2. Gas Chromatography

Apart from liquid chromatography, the determination of glyphosate and AMPA can also be carried out with other instrumental techniques [103,115–117], among which gas chromatography stands out, with derivatization as a mandatory preliminary step. Initially, various types of detectors were used, such as FPD, NPD, and ECD [118–120]. Subsequently, the use of mass spectrometry (GC-MS) became increasingly established, even if the simpler NPD and FPD detectors are still currently exploited in some cases [51,102].

5.2.1. Sample Preparation and Perfluoroalcohol/TFAA Derivatization

From the first works to the present day, the largely prevalent derivatization for determining glyphosate in gas chromatography is that using perfluoroalcohols and perfluorinated anhydrides, with trifluoroacetic anhydride (TFAA) used almost universally. In this way all phosphonic and carboxylic acid groups are esterified, and all amino groups are acylated as shown in Figures 5 and 6.

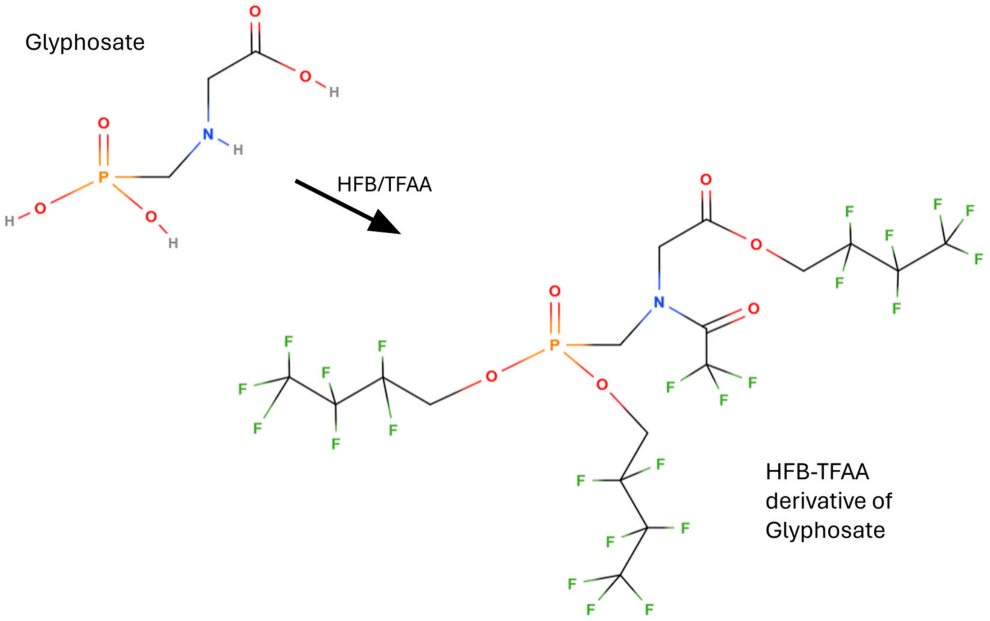

Figure 5. Derivatization of glyphosate with 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB) and trifluoroacetic anhydride (TFAA) for determinations carried out by gas chromatography.

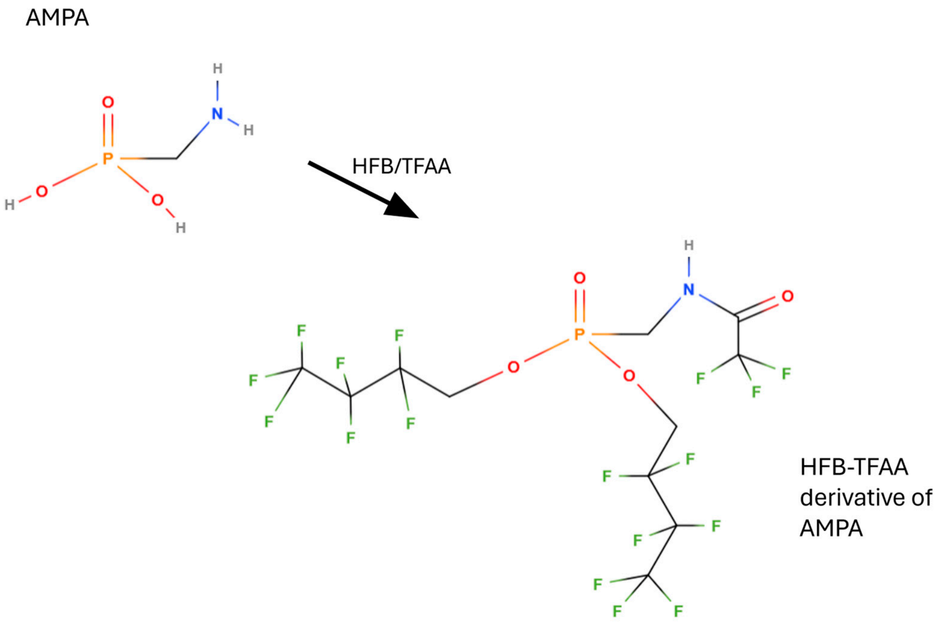

Figure 6. Derivatization of AMPA with 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB) and trifluoroacetic anhydride (TFAA) for determinations carried out by gas chromatography.

Such derivatization was first applied by Deyrup et al. in 1985 [118]. This is also the procedure codified in the AOAC Official method 2000.05 ‘Determination of glyphosate and AMPA in crops, gas chromatography with mass-selective detection’ [121–123]. A variant of the derivatization in Figures 5 and 6 is the use of 2,2,2-trifluoroethanol (TFE) instead of HFB [46,48–51]. The crops tested in the interlaboratory study supporting acceptance of the AOAC method were field corn grain, soya forage, and walnut nutmeat, but it is applicable to a wide variety of crops and processed commodities: over 100 matrices were successfully analyzed with virtually no method modifications required.

Sample preparation involves steps of extraction, purification, and derivatization.

In summary extraction of grains requires homogenization with water, centrifugation, addition of dichloromethane, a second centrifugation, addition of an acid modifier and a third centrifugation. The supernatant is so sent for a cation exchange cleanup.

The cleanup is achieved with a cationic exchange SPE column on which the supernatant from the previous step is loaded. Then elution with a specific mobile phase is carried out.

Derivatization is carried out with HFB and TFAA kept at low temperature until added to the eluate for the reaction, which takes place at 85-90°C. After the addition of a citral reagent the solution is ready for the gas chromatographic analysis.

5.2.2. Instrumental

The apparatus to be used is a gas chromatograph equipped with a capillary column 0.25 mm ID × 30 m, 0.50 μm film thickness of cross-linked 95% methyl - 5% phenyl silicone phase. Helium is used as a carrier gas at a flow rate of about 30 cm/s at 180°C, equal to about 40–50 kPa (6–7 psi) at column head. Suggested column oven temperature program is the following: initial temperature 90°C, hold for 1.5 min, increase to 300°C at 30°C/min (20°C/min if limited by instrument capabilities), and hold at 300°C for 4 min. An alternative program for increased resolution is the following: initial temperature 60°C, hold for 1.5 min, increase to 120°C at 10°C/min, hold at 120°C for 1.0 min, increase to 300°C at 30°C/min, and hold at 300°C for 4 min. Injection port temperature 200°C, injection volume: 2–5 mL in splitless mode.

The AOAC method requires that mass spectrometry is used as a detection technique by means of a quadrupole instrument, capable of providing electron impact mass spectra over an amu range up to m/z 650 operated in selected-ion monitoring (SIM) low-resolution mode. Method qualification data were obtained exclusively with quadrupole instrumentation only, but some participant laboratories in the development of the AOAC method proposed the use of the ion trap as a viable alternative after that some minor method modifications were applied. These included elimination of the citral reagent in the final ethyl acetate extract, the use of a programmed variable temperature GC inlet, and the use of methylene chloride as a keeper during evaporation of the derivatization reagents [122]. MS/MS determinations are also possible. Royer et al. reported a successful ion-trap tandem MS application of the HFB/TFAA derivatization procedure to the analysis of glyphosate and AMPA in water, black currents, and hazelnuts [47].

The major ion fragments that can be used for the determination of the glyphosate and AMPA derivatives via GC-MS, SIM mode, are indicated in the AOAC method. They are 611.5, 584, and 460 m/z for glyphosate and 502, 446, and 372 m/z for AMPA. Although 611.5 and 446 m/z provide the greatest response for the glyphosate and AMPA derivatives, respectively, the alternative ions can be used for confirmatory analyses. The alternative ions may also be helpful for eliminating/reducing problematic interferences. The ion 611.5 m/z for the glyphosate derivative can be explainable as follows. The molecular weight of the glyphosate derivative in Figure 5 is 811 amu. The need to monitor the ion 611.5 m/z after ionization results from the loss of an ion of about 199 m/z seen that 811 – 611.5 = 199.5 m/z. The fragment being losing is the group 2,2,3,3,4,4,4-heptafluoro-1-buthoxy-, as shown in Figure 7, which is indeed a fragment of about 199 m/z. Such fragmentation mechanism is the same when 2,2,2-trifluoroethanol (TFE) id used in place of HFB but in such a case there is the loss of a 2,2,2-trifluoroethoxy- group (99 m/z). Therefore, with TFE, one of the main ions to be monitored for the glyphosate derivative (of 511 amu) is 412 m/z.

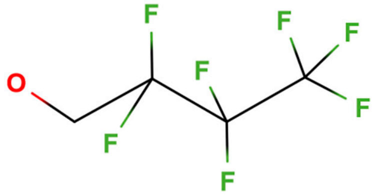

Figure 7. Fragment 2,2,3,3,4,4,4-heptafluoro-1-buthoxy- deriving from the EI ionization of the glyphosate derivative in Figure 5.

Other ions of interest are those deriving from the loss of a CF_3 group (69 m/z) which, for the HFB derivative of AMPA (571 amu), results in the ion fragment 502 m/z, while, for the TFE derivative of AMPA (371 amu), yields a 302 m/z ion fragment [124]. Other relevant ions in SIM mode or transitions that are exploited in tandem mass spectrometry are available from literature [43–52].

5.2.3. Alkylsilyl Derivatization

The alkylsilyl derivatization is a little used procedure: it is mainly exploited in the gas chromatographic analysis of glyphosate and AMPA in biological fluids, such as serum and urine [125–128]. Furthermore, in some cases the sensitivity is lower with respect the derivatization with perfluoroalcohols and perfluorinated anhydrides. However, alkylsilyl derivatization is reported here for the sake of completeness. By far the most used reagent is N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) as shown in Figure 8. Such reagent is capable of replacing three hydrogens of the glyphosate and AMPA molecules with three tBDMS groups.

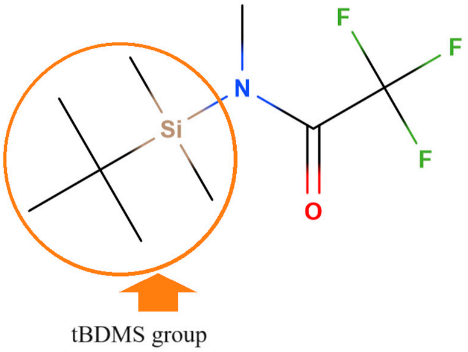

Figure 8. The derivatizing agent MTBSTFA. The tBDMS group is indicated in the circle; tBDMS stands for tert-butyldimethylsilyl-.

The chemical reaction yields a glyphosate derivative of 511 amu and an AMPA derivative of 453 amu. As reported by Tsunoda [129] when MTBSTFA is used the ions of interest to be monitored in GC-MS analysis are MW-15 and MW-57, therefore the ions to be monitored are 496 and 454 m/z for glyphosate, while for AMPA they are 438 and 396 m/z [126,127,129].

Other proposed alkylsilyl derivatizations involve the use of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [130,131] or N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) [132] which replace the hydrogens of glyphosate and AMPA with a trimethylsilyl group (TMS group).

6. Conclusions

An overview of current research on glyphosate is provided.

Studies that support toxicity and those that do not are reported, including the fact that some works attributes much of glyphosate's toxicity to the secondary ingredients present in commercial preparations (co-formulants).

It was demonstrated that most of the total glyphosate mass resides in outer kernel layers with higher concentrations in bread from whole grain flour. Given the claimed health benefits of wholemeal flours such finding could be of primary importance.

The controversial use of glyphosate in pre-harvest phase (and its consequences) is reviewed as well as to what extent post-harvest treatments (storage, processing) may or may not reduce the herbicide content in the final product.

The degradation pathways of the herbicide are exposed, and it is highlighted in which cases the molecules N-Acetyl-glyphosate and N-Acetyl-AMPA are formed.

It is explained the difference between glyphosate and glufosinate and why in research works they are often mentioned together.

Relevant analytic information is reported in the present paper with a deeply overview of the methods available. It is exposed when and why to apply an SPE cleanup step during the sample purification. The use of derivatization is discussed, and it is clarified in which cases, and for what reasons, it could be not carried out for liquid chromatography and what are, instead, the advantages of its use. The sample preparation and the instrumental conditions are treated in any detail.

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