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The impact of altered herbicide residues in transgenic herbicide-resistant crops on standard setting for herbicide residues

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Abstract

The global area covered with transgenic (genetically modified) crops has rapidly increased since their introduction in the mid-1990s. Most of these crops have been rendered herbicide resistant, for which it can be envisaged that the modification has an impact on the profile and level of herbicide residues within these crops. In this article, the four main categories of herbicide resistance, including resistance to acetolactate-synthase inhibitors, bromoxynil, glufosinate and glyphosate, are reviewed. The topics considered are the molecular mechanism underlying the herbicide resistance, the nature and levels of the residues formed and their impact on the residue definition and maximum residue limits (MRLs) defined by the Codex Alimentarius Commission and national authorities. No general conclusions can be drawn concerning the nature and level of residues, which has to be done on a case-by-case basis. International residue definitions and MRLs are still lacking for some herbicide–crop combinations, and harmonisation is therefore recommended.

Keywords: herbicides; transgenic crops; genetic modification; herbicide resistance; pesticide residues; maximum residue limits; regulatory affairs; international harmonisation

1 INTRODUCTION

Since genetically modified (GM) crops were introduced commercially on a large scale in the mid-1990s, the area covered with these crops worldwide, as well as the number of countries where these crops are grown, has steadily increased. In 2010, this area reached 148 million ha, divided over 29 countries, predominantly the United States (45%), Brazil (17%), Argentina (15%), India (6%), Canada (6%), China (2%), Paraguay (2%), Pakistan (2%) and South Africa (1%).[1] The most important GM crops are soybean, cotton, maize and canola, while other GM crops, such as alfalfa, papaya, squash, sugar beet, sweet pepper and tomato, are grown to a more limited extent at present.

The main traits with which the major GM crops have been modified are herbicide resistance and insect resistance. Herbicide resistance allows for 'over-the-top' (direct to the growing crop) sprays of herbicides that could otherwise be toxic to the plant. The resistance traits introduced into GM herbicide-resistant crops can facilitate weed management in various ways,[2] for example by:

- obviating the need for weed tillage or directed spraying on weeds between crop plants;
- allowing for the use of non-residual herbicides that enable certain crop rotations that would otherwise be impossible owing to the phytotoxicity of residues of herbicides remaining in the soil;
- replacing combinations of conventional post-emergence herbicides targeting grass and broadleaf weeds with a single broad-spectrum herbicide;
- avoiding crop injury that may be caused by conventional post-emergence herbicides;
- allowing for more flexible timing of applications with the target herbicide.

In sum, herbicide resistance provides more flexibility in weed management.

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Abbreviations used: *ALS, acetolactate synthase; AMPA, aminomethylphosphonic acid; CAC, Codex Alimentarius Commission; CCPR, Codex Committee on Pesticide Residues; DBHA, 3,5-dibromo-4-hydroxybenzoic acid; DNA, deoxyribonucleic acid; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; EU, European Union; FAO, Food and Agriculture Organisation; FDA, Food and Drug Administration; FSANZ, Food Standards Australia New Zealand; GAT, glyphosate N-acetyltransferase; GM, genetically modified; GOX, glyphosate oxidoreductase; HRAC, Herbicide Resistance Action Committee; IUPAC, International Union for Pure and Applied Chemistry; JMPR, Joint FAO/WHO Meetings on Pesticide Residues; MHB, 4-methyl-phosphinico-2-hydroxy-butanoic acid; MPA, methyl-phosphinico-acetic acid; MPB, 4-methylphosphinico-butanoic acid; MPP, 3-methyl-phosphinico-propanoic acid; MRL, maximum residue limit; NAG, N-acetyl-L-glufosinate; NAFTA, North American Free Trade Agreement; OECD, Organisation for Economic Cooperation and Development; PAT, L-phosphinothricin N-acetyltransferase; PPO, 4-methyl-phosphinico-2-oxo-butanoic acid; PRAPeR, Pesticide Risk Assessment Peer Review; STMR, supervised trial median residue; WHO, World Health Organisation.*

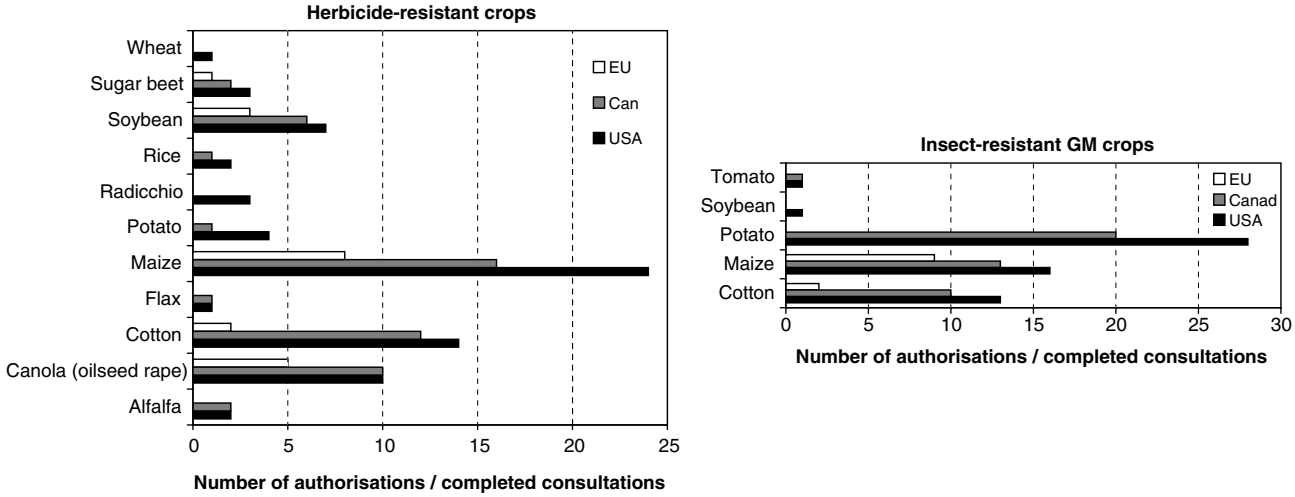


Figure 1. Summary of authorisations (Canada, EU) and completed consultations (United States) for the use of GM herbicide-resistant and insect-resistant crops as food. Information sources: European Commission,[6] FDA[7] and Health Canada;[8] status of authorisations and completed consultations as of 1 January 2011. 'Stacked' versions of GM crops in which multiple traits from single-trait GM crops are combined have not been included as they are commonly not authorised separately in the United States and Canada.

The other main trait is insect resistance, which has been achieved mainly through the introduction, via genetic modification, of insecticidal proteins that naturally occur in crystalline, parasporal inclusions of the soil bacterium *Bacillus thuringiensis*. The proteins, designated 'Cry proteins' or '*Bt* proteins', are toxic towards specific insect species but not to humans or domestic animals. The resistance trait of insect-resistant GM crops may therefore replace the applications of insecticides against the pest insects that are sensitive towards the action of the particular Cry protein that has been introduced into the crop *via* genetic modification.

Given that the herbicide and insect resistance traits aim at facilitating the management of crop pests and weeds, thereby replacing or supplementing other conventional management practices, they are likely to impact on pesticide use on these crops. Previous research by the present authors' team, which was carried out within the framework of a project of the International Union for Pure and Applied Chemistry (IUPAC), explored the data that are available on the types and quantities of pesticide that are used on GM crops as compared with their conventional counterparts. Because each pesticide may have its own specific effects, dose–effect relationships and environmental behaviour, the observed changes in quantities of pesticide used on these crops were also interpreted in terms of the overall predicted environmental impact associated with these changes. The outcomes of this research showed a general decrease both in the quantities of pesticides used on GM crops compared with non-GM crops and in their predicted environmental impact.[3,4] Another prospective study by the present authors' team focused on the potential impacts on pesticide usage and environmental impact were glyphosate-resistant crops to be introduced in Europe. It was concluded that, under certain circumstances, favourable environmental impacts would be feasible.[5] The present article will further focus on GM herbicide-resistant crops, as explained below.

Further questions that may be posed with regard to the impact of the altered pesticide usage on GM crops is whether this may bring changes to crop residue levels, and what these changes might be. Several factors must be taken into account here:

1. GM herbicide-resistant crops can change the way that herbicides can be used on these crops, for example:
   (a) post-emergent over-the-top applications (i.e. on the crop itself) instead of directed sprays, avoiding herbicide contact with the crop; or
   (b) pre-emergent and preharvest applications made to the conventional crop and not, or in different quantities, to the GM crop.
2. The residue profile of the applied pesticide may have been altered on the basis of the nature of the modification.
3. The overall pattern of pesticides applied to the particular crop may have been altered, leading to different exposure to pesticide residues overall.

In the present article, the background of the herbicide resistance traits will be highlighted first, including the traits in GM crops that have already received authorisation for commercial use. The molecular mechanisms by which resistance has been achieved and the impacts that the modification may have on the metabolism and profile of crop residues will be indicated. The issue of residue definition and appropriate residues levels in GM and non-GM crops will be dealt with.

2 AUTHORISATIONS OF GM CROPS

To provide an impression of the range of crops into which herbicide and insect resistance traits have been introduced and that have been authorised around the world, Fig. 1 summarises the GM herbicide- and insect-resistant crops that have been authorised for commercial use as food and feed by regulatory authorities in Canada and the European Union (EU), and for which the consultation has been completed in the United States. There is no formal authorisation of a GM food per se in the United States. The Food and Drug Administration (FDA) urges developers to consult the administration before commercialisation of GM foods to ensure that all regulatory matters are resolved. In the following, the use of the terms 'authorisation' and 'authorised' also implicitly refers to these completed consultations with the FDA, if applicable. As can be seen in the graphs, GM varieties of commodity crops such as soybean, maize, canola and cotton have authorised uses

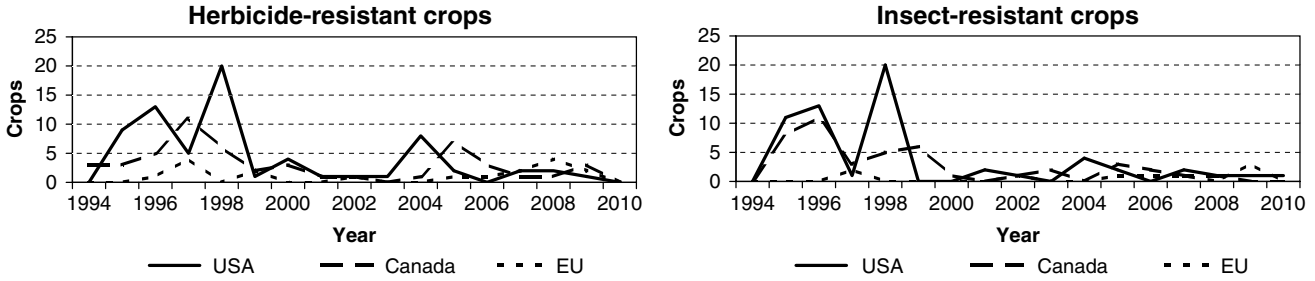


Figure 2. Chronology of the authorisations/completed consultations for use of GM herbicide-resistant and insect-resistant crops as food. Same references as for Fig. 1.

in all of the three sets of legislation, while a number of other GM crops, such as potato, have only been authorised so far in the United States and Canada. Although the number of authorisations appears to be higher in the United States and Canada as compared with the EU, the timeline of authorisations (Fig. 2) shows that the main part of the authorisations in the United States and Canada took place in the 1990s, while the annual numbers of authorisations in the last 10 years appear to converge for all three legislations.

These authorisations of GM crops do not include the authorisation of the target herbicide to be applied to the GM herbicide-resistant crops; this usually falls under parallel legislation that, in some situations, is handled by a separate agency. Also, for the intrinsic 'pesticide' introduced via genetic modification, additional assessments may be needed, which may be linked with the assessment of the GM crop, such as by the US Environmental Protection Agency (EPA) and the Australian Pesticides and Veterinary Medicines Authority.

3 HERBICIDE-RESISTANT CROPS

With regard to the herbicide-resistant crops that have been authorised for food use, the herbicides to which these crops have been rendered resistant fall into four different categories, namely the acetolactate synthase (ALS) inhibitors, bromoxynil, glufosinate and glyphosate.

3.1 Overview of herbicide resistance mechanisms

There are basically two mechanisms through which resistance to herbicides has been achieved in GM crops. Firstly, for herbicides that target an enzyme in plant cells, an insensitive variant of this enzyme can be introduced by genetic modification, allowing the plant cells to function in the presence of otherwise inhibitory levels of herbicide. This insensitive variant of the enzyme can either be a mutant of the native enzyme, which carries a mutation in one or more amino acid residues that are involved with the inhibitory action of the herbicide, or an analogous enzyme from a different organism, such as herbicide-resistant microorganisms isolated from soil or other environments that contain environmental levels of the pertinent herbicide.

Another means of conferring herbicide resistance is by introducing an enzyme that detoxifies the herbicide by metabolising it to a less phytotoxic or non-phytotoxic compound. Usually, such enzymes have been discovered in microorganisms, and the genes encoding these enzymes have subsequently been elucidated and cloned into GM plants in order to render them resistant against the herbicide.

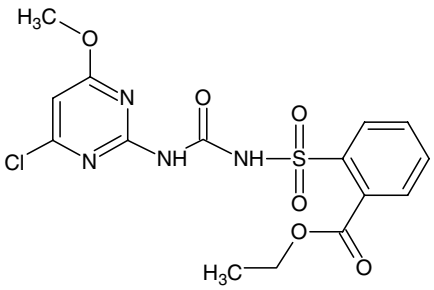
3.1.1 ALS inhibitor resistance

ALS inhibitors fall into category B of the Herbicide Resistance Action Committee's (HRAC) classification, which includes herbicides of the imidazolinone, sulfonylurea, triazolopyrimidine, pyrimidinyl(thio)benzoate and sulfonylaminocarbonyltriazolinone chemical families.[9] What these herbicides have in common is their inhibition of plant ALS enzymes, which convert pyruvate to 2-acetolactate, or 2-ketobutyrate to 2-aceto-2-hydroxbutyrate. This constitutes an important step in the biosynthesis of several branched amino acids, i.e. valine and isoleucine, as well as leucine. While different groups of herbicides, such as imidazolinones and sulfonylureas, act on ALS, this does not necessarily mean that resistance to one chemical family confers cross-resistance to another family (e.g. p. 203 of Duke[10]).

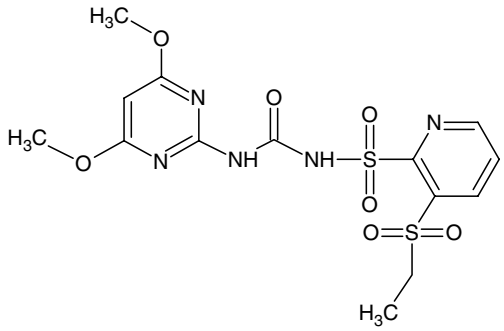
Resistance to ALS inhibitors in crops has been achieved through mutation of ALS enzymes in one or more of their amino acid residues, yielding enzymes that are still active but insensitive towards the inhibiting action of the herbicides. Both non-GM and GM crops with mutated ALS have been created, the first through somaclonal variation or chemical mutagenesis, for example, and the latter through introduction of new, mutated-ALS-encoding genes via recombinant DNA techniques. Under Canadian regulations on 'novel foods', for example, which include foods derived from 'plants with novel traits', non-GM imidazoline-resistant canola, lentil, maize, rice, sunflower and wheat have received favourable decisions for marketing as foods. ALS-inhibitor-resistant GM crops that have received favourable decisions include flax, maize and soybean in the United States and Canada, as well as resistant cotton in the United States.[7,8]

The documentation provided by companies that have developed these GM crops that are resistant towards ALS-inhibiting herbicides describe the usage of herbicides containing specific ALS inhibitors, of which most are sulfonylureas. For GM cotton, post-emergence applications of the sulfonylurea thifensulfuron methyl (synonymous to M6316) and another ALS inhibitor, pyrithiobac, are described.[11] Interestingly, with regard to pyrithiobac, various publications in the scientific literature mention that pyrithiobac is generally considered safe for post-emergence use 'over the top' in non-GM cotton, and also describe the resistance of non-GM cotton towards the phytotoxic action of pyrithiobac observed in experiments (e.g. Harrison *et al.*[12]). The post-emergence application of pyrithiobac to cotton is also described on the herbicide label.[13] It therefore appears that the ALS inhibitor resistance of GM cotton allows for the additional inclusion of thifensulfuron methyl as active ingredient in post-emergence herbicide sprays.

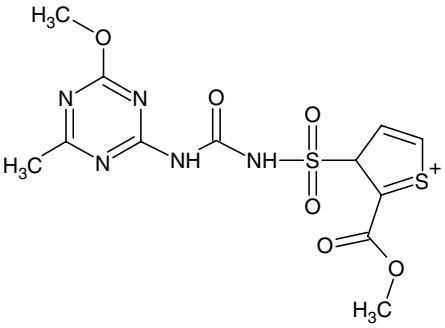
For one of the two GM soybean lines, it is mentioned that the ALS resistance trait has only been used as a selection marker during the creation of the GM crop, while not being



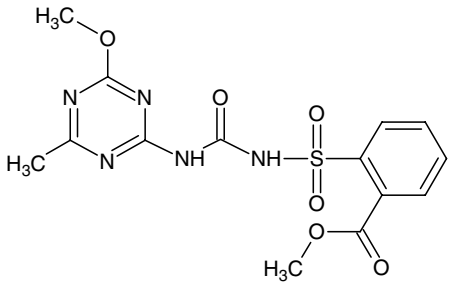
Chlorimuron ethyl



Rimsulfuron



Thifensulfuron methyl



Tribenuron methyl

Figure 3. Structures of ALS-inhibiting herbicide active ingredients applied to GM ALS-inhibitor-resistant maize and soy.

intended as a herbicide resistance trait for field use.[14] With regard to the other soybean line, it is resistant to both ALS inhibitors and glyphosate. According to the company that has developed this line, various sulfonylurea-containing herbicides can be applied either pre-emergence or post-emergence to this soybean line. The sulfonylurea active ingredients of these herbicides include chlorimuron ethyl, rimsulfuron, thifensulfuron methyl and tribenuron methyl (Fig. 3).[15] Similar to this soybean line, a GM maize line has been developed by the same company, featuring both the ALS inhibitor and glyphosate resistance traits, which can be combined with herbicides containing the same sulfonylureas as used on soybean.[16]

While the ALS inhibitor resistance in GM cotton, maize and soybean enables the application of ALS-inhibiting herbicides to the pertinent crop, the resistance in GM flax enables it to be grown in the presence of the otherwise toxic soil residues of ALS inhibitors resulting from application of herbicides to the crop grown previously in the rotation. More specifically, the developer of this GM flax mentions resistance against residues of the sulfonylureas triasulfuron and metsulfuron methyl.[17]

Besides GM crops modified with ALS inhibitor resistance trait, a range of conventional crops with this trait also exist. For example, sulfonylurea-resistant soybeans have been obtained through exposure of soybean seeds to a mutagenic compound and subsequent selection of seeds and subsequent generations of plants for resistance towards sulfonylureas.[18] These sulfonylurea-resistant soybeans were subsequently brought to the market. In addition, a range of imidazolinone-resistant crops, including brassica (canola), maize, rice, sunflower and wheat, have been obtained through breeding of lines containing either mutagenesis-derived mutations or naturally occurring mutations selected in wild relatives, and have subsequently been commercialised.[19] Mutations of various specific amino acid residues that are located around the active site within the ALS enzyme contribute to resistance towards ALS inhibitors in these conventionally bred crops.[19]

3.1.2 *Bromoxynil resistance*

Bromoxynil (3,5-bromo-4-hydroxybenzonitrile) (Fig. 4) and derived esters are cyano (nitrile)-group-containing active substances of herbicides that belong to the HRAC group C3 of compounds inhibiting plant photosynthesis at photosystem II,[9] thereby causing the formation of reactive oxygen species. A bromoxynil-degrading enzyme, i.e. bromoxynil nitrilase, was identified in bacterial isolates of *Klebsiella pneumonia* var. *ozaenae* from bromoxynil in soil from previous use. This enzyme converts the cyano (CN) group of bromoxynil into a carboxyl (COOH) group, thereby rendering the herbicide inactive. The putative natural function of this enzyme is its contribution to the degradation of aldoxime compounds secreted by plants into soils (reviewed by Kleter *et al.*[20] and by Duke[10]).

Genes coding for the bromoxynil nitrilase enzyme have been introduced into canola and cotton, which have been authorised for food use in Canada, the United States, Australia and New Zealand, among others.[7,8,21,22] In addition, bromoxynil-resistant canola has been given authorisation for cultivation in Canada, while bromoxynil-resistant cotton can be grown in the United States.[23,24] As explained in Section 3.3.2, the marketing of seeds of these crops in the United States and Canada has been discontinued.

3.1.3 *Glufosinate resistance*

Glufosinate [DL-homoalanin-4-yl (methyl)phosphonic acid] (Fig. 5), the ammonium salt of which is usually included in herbicide

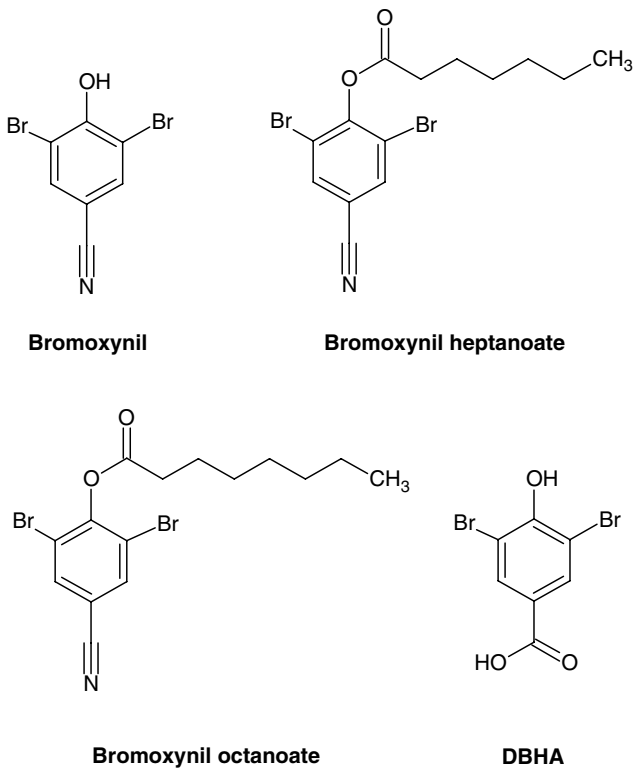


Figure 4. Structure of bromoxynil, its heptanoate and octanoate esters and its metabolite DBHA.

formulations, is a synthetic analogue of the L-phosphinothricin molecule. L-Phosphinothricin is a component of natural antibiotics such as bialaphos and phosalacin produced by streptomycetes, which are soil microorganisms. Glufosinate ammonium is an ingredient of broad-spectrum herbicides, while it is also used as preharvest desiccant for a number of crops.

Phosphinic acids such as glufosinate and bialaphos fall into HRAC group H of glutamine-synthase-inhibiting herbicides. Glutamine synthase is an enzyme with an important role in the metabolism of nitrogen in prokaryotes and eukaryotes. Glutamine synthase catalyses the condensation of ammonium ions and glutamic acid into glutamine. Inhibition of glutamine synthase by L-phosphinothricin or L-glufosinate leads to accumulation of ammonium ions and depletion of glutamine and other amino acids in the plant cell, which ultimately leads to a decrease in photosynthetic activity and other phytotoxic effects.[25] The D-enantiomer (i.e. D-glufosinate) is also part of technical glufosinate-containing formulations containing the racemic mixture with both the D- and L-enantiomers, but is not herbicidally active.

Resistance to L-glufosinate and L-phosphinothricin has been achieved in GM crops through the introduction of genes encoding for L-phosphinothricin *N*-acetyltransferase (PAT) enzymes. Two PAT enzymes have been introduced into GM crops, which occur naturally in *Streptomyces hygroscopicus* (encoded by the *bar* gene) and in *Streptomyces viridochromogenes* (encoded by the *pat* gene). These enzymes share a high degree (85%) of identical residues when both their amino acid sequences are aligned using computer algorithms. They catalyse the acetylation of the L-phosphinothricin/glufosinate molecule, with a relatively high affinity compared with other acetyltransferases, using acetyl coenzyme A as a cosubstrate, thereby inactivating the herbicidal activity of L-phosphinothricin/glufosinate.[26]

A number of glufosinate-resistant GM crops that have been modified with the PAT enzyme encoded by either the *pat* gene or the *bar* gene have been authorised in the United States, Canada and the EU. These crops include canola, cotton, maize and soybean, while also rice and sugar beet have been authorised in the United States and Canada.[6–8] Also, for GM glufosinate-resistant radicchio,

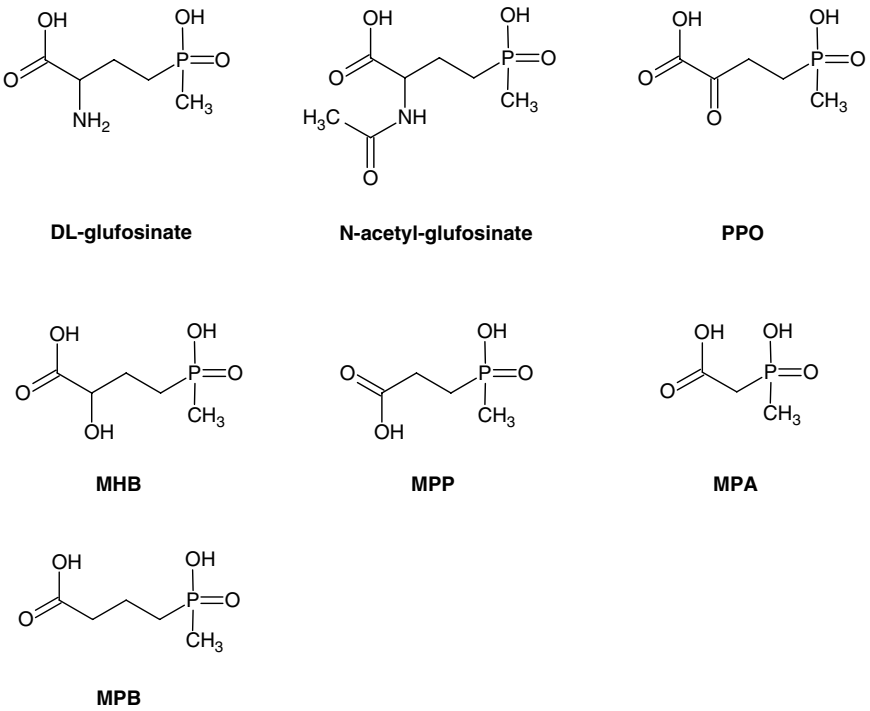


Figure 5. Structure of glufosinate and its metabolites NAG, PPO, MHB, MPP, MPA and MPB.

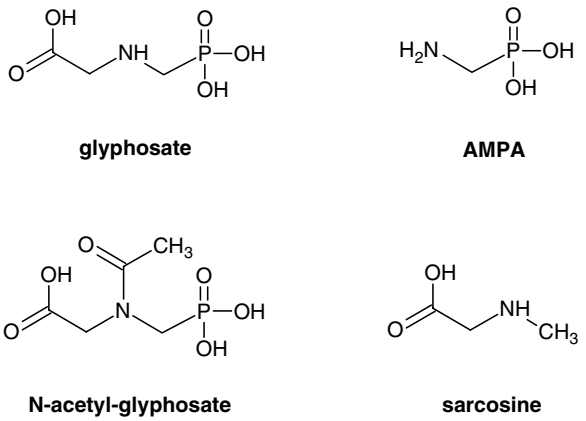


Figure 6. Structure of glyphosate and its metabolites AMPA, *N*-acetyl-glyphosate and sarcosine.

a consultation has been concluded in the United States,[6] although the company developing this GM crop has indicated that it will not commercialise these GM varieties.[27]

3.1.4 Glyphosate resistance

The herbicide active ingredient glyphosate [*N*-(phosphonomethyl)-glycine] (Fig. 6) is a glycine derivative that falls into HRAC group G of compounds that inhibit 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS occurs in a wide range of organisms, including microorganisms (bacteria, fungi) and plants. It is an enzyme that catalyses the formation of 5-enolpyruvylshikimate-3-phosphate from shikimate-3-phosphate and phosphoenolpyruvate. This is one of the steps in the 'shikimate pathway', which is responsible for the biosynthesis of aromatic precursors for compounds such as aromatic amino acids (e.g. tyrosine, phenylalanine) and lignin.[10]

Two strategies have been followed by developers of GM crops to confer glyphosate resistance to these crops. The first strategy involves the introduction, through genetic modification, of EPSPS enzymes that are insensitive towards inhibition by glyphosate. For this purpose, genes encoding the CP4 EPSPS enzymes from a glyphosate-degrading soil bacterium, *Agrobacterium* strain CP4, have been introduced into a range GM crops, including alfalfa, canola, cotton, maize, potato, soybean, sugar beet and wheat, and, for lawns and golf courses, creeping bentgrass (reviewed by Duke[28] and by Kleter *et al*.[20]). Also, a mutated EPSPS from maize has been used for genetic modification of cotton and maize.

The second strategy involves the enzymatic inactivation of glyphosate. For example, GM canola that has been modified with CP4 EPSPS also contains glyphosate oxidoreductase (GOX), which was identified in the glyphosate-degrading bacterium *Achromobacter* LBAA isolated from sludge. The latter enzyme catalyses breakage of the carbon–nitrogen (C–N) bond in glyphosate, yielding amino-methylphosphonic acid (AMPA) (Fig. 6) and glyoxylic acid as products.[10,20] A relatively novel example of this strategy is the use of the enzyme glyphosate *N*-acetyltransferase (GAT), which catalyses the acetylation of glyphosate into *N*-acetyl-glyphosate (Fig. 6), which is non-herbicidal. The gene sequences coding for GAT enzymes isolated from *Bacillus licheniformis* have been used for 'DNA shuffling' in order to create novel sequences with optimal catalytic activity towards glyphosate.[29] GAT-expressing crops that have been authorised for food use include maize and soybean.[7,8]

3.2 The profile of herbicide residues and their metabolites in herbicide-resistant crops

It can be envisaged that herbicide resistance, which allows crops to sustain exposure to foliar or residual applications with the target herbicides, will affect not only the levels but also the nature of the residues of the herbicide active ingredient in the crop's tissues. Besides various research studies published in the scientific literature, additional residue data from field trials and processed products of herbicide-resistant and non-resistant crops have been considered by organisations such as the Organisation for Economic Cooperation and Development (OECD) and the Joint Food and Agriculture Organisation (FAO)/World Health Organisation (WHO) Meetings on Pesticide Residues (JMPR). The JMPR advise the Codex Alimentarius Commission (CAC) and in particular the Codex Committee on Pesticide Residues (CCPR) dealing with pesticide residue issues, including the establishment of CAC maximum residue limits (MRLs). This section summarises the data available on the levels and metabolites of residues of the herbicides to which commercialised GM crops have been rendered resistant, i.e. imidazolinones, sulfonylureas, bromoxynil, glufosinate and glyphosate.

3.2.1 ALS inhibitors

The group of ALS-inhibiting active ingredients of herbicides comprises a broad range of substances, as mentioned in Section 3.1.1 above. The main two largest categories of these ALS inhibitors are the imidazolinone and sulfonylurea types of substance. The ability of conventional crops rapidly to metabolise these substances often relates to the selectivity of herbicides being phytotoxic to particular weeds but non-phytotoxic to the crop. Whereas the nature and importance of a given metabolic reaction with an imidazolinone or sulfonylurea in plants depends on both the specific active ingredient and the plant species to which it is applied, it is still possible to infer generalisations.

For imidazolinones, for example, the most important reactions in plants in general include: the hydroxylation of the alkyl substituent of the aromatic ring (e.g. phenyl ring) attached to the imidazolinone molecule; the subsequent conjugation of this hydroxyl group with a glycosyl group; the hydrolysis of the imidazolinone group itself; and the cyclisation following the condensation of the carboxylate group (attached to the aromatic ring) with a nitrogen in the imidazolinone ring.[30]

Sulfonylureas comprise a broad range of substances, most of which contain an aromatic pyrimidinyl or triazinyl group, i.e. an aromatic six-atom ring including two or three nitrogen atoms, respectively, linked to the urea side of the sulfonylurea linkage. Metabolic reactions that are observed with sulfonylureas in plants include: hydroxylation of aliphatic and aromatic carbon atoms followed by conjugation with glycosides; dealkylation of alkoxy groups to form hydroxyl groups; de-esterification of ester groups; demethylation of nitrogen (N) atoms; conjugation with glutathione; hydrolysis of the sulfonylurea linkage; and contraction/rearrangement of the sulfonylurea group.[31]

3.2.2 Bromoxynil

A limited number of scientific reports have appeared on the metabolism and residue formation of bromoxynil in crops. Given that bromoxynil is used in many cereals, which are resistant towards its toxicity, many of these investigations have focused on the residues formed in these crops, in particular wheat.

Bromoxynil within currently marketed herbicide formulations is either present as such, which is also referred to as the 'phenol'

form, or as an ester of the phenolic group with heptanoic or octanoic acid. Regulatory authorities have evaluated these active ingredients within the framework of a single evaluation, which is based on the rapid hydrolysis of the esters to the 'phenol' form of bromoxynil and the organic acids. In wheat seedlings, for example, 0.4% of radiolabelled bromoxynil octanoate applied to these seedlings remained unhydrolysed within 7 days.[32]. Cummins *et al*.[33] isolated an esterase enzyme with a molecular mass of 45 kDa and an isoelectric point of pI 4.6 from the shoots of wheat seedlings. While this esterase was capable of hydrolysing bromoxynil octanoate, the authors estimated that this enzyme could only account for 0.4% of the total activity hydrolysing this substrate in wheat extracts. In a follow-up study, esterase activity towards bromoxynil octanoate as a substrate was observed in the extracts of various crop species, in particular maize and also alfalfa, flax, rice, sorghum and soybean, besides a number of weeds. It also showed that the repertoire of esterase enzymes differed among plant species, based on the activity of separated enzymes and their sensitivity towards inhibition.[34]

The metabolism of bromoxynil was found to be more extensive in non-GM resistant plant species, such as barley, than in sensitive species, such as tomato.[35] Among the metabolites found in barley seedlings were the parent compound, differently substituted hydroquinones and glycosides of both the parent and the hydroquinone. The presence of hydroquinones indicated that the nitrile (cyano) group of bromoxynil must have been replaced by a hydroxyl group.[36] In wheat seedling leaves, a wide range of metabolites was observed besides the parent compounds bromoxynil octanoate and bromoxynil.[32] The authors concluded that the nature of the major metabolites was indicative of various reactions, including the substitution of the nitrile group with a carboxyl or amide group, the substitution of bromide with a hydroxyl group or a hydrogen atom and decarboxylation of the metabolites containing a carboxyl group replacing the nitrile group.[32]

In line with the observation by Schaller *et al*.,[36] who observed that bromoxynil and its metabolites are glycosylated *in planta*, are the outcomes of a study on a glycosyltransferase enzyme from strawberry. A recombinant form of this enzyme was found to be able to glycosylate 3,5-dichloro-4-hydroxybenzoic acid, which is a chlorine analogue of 3,5-dibromo-4-hydroxybenzoic acid (DBHA) (Fig. 4), one of the main metabolites of bromoxynil. Glycosylation was observed to take place both at the carboxyl group, i.e. ester formation, and at the phenol group, i.e. *O*-glucoside formation.[37]

With regard to the formation of residues in the crop parts used as food, various experiments indicate that little or no residues are present in the crops that have been studied. No or very little radioactivity was present, for example, in grains that had been harvested from field-grown wheat plants 4 months after application of radiolabelled bromoxynil octanoate at 3–4 times the recommended application rate.[38] Also, other studies specifically analysing the content of bromoxynil in seeds of cereals failed to show its presence, such as in triticale and wheat following early post-emergence applications with bromoxynil esters, which were made 2.5 months before harvest in wheat.[39–41]

Whereas JMPR has not evaluated data on residues of bromoxynil, the authorities of two nations where GM bromoxynil-resistant crops are allowed onto the market, the United States and Canada, have done this at a national level. The US EPA, for example, has registered the use of bromoxynil and its heptanoate and octanoate esters on GM bromoxynil-resistant cotton, besides a range of other food and feed crops, such as alfalfa, flax, garlic, grass, onion, peppermint, spearmint and a number of cereals, including barley, maize, oat, rye, sorghum and wheat. When defining the residue, the EPA makes a distinction between these other crops and bromoxynil-resistant cotton.[42] The latter is designated only as cotton in the Code of Federal Regulations, while this obviously applies to bromoxynil-resistant cotton. For crops other than cotton, MRLs ('tolerances') have been established for the residue of bromoxynil, while for cotton seeds, gin byproducts and hulls, as well as for a number of animal-derived products, MRLs have been established for the sum of the residues of bromoxynil and DBHA.[43] Besides bromoxynil, DBHA has also been included in the residue definition for animal-derived products because it may occur in feed commodities from which it can be transferred to animal products after intake of these commodities by the animals (p. 37 of EPA[44]).

Similarly to the situation in the United States, bromoxynil has also been registered in Canada as the active ingredient of herbicides that can be applied to a range of crops, such as alfalfa, flax, garlic, grass grown for seed and a number of cereals, including barley, maize, millet, oats, rye, sorghum and wheat. In addition, it has been registered for use on GM bromoxynil-resistant canola.[45] With regard to the definition of the residues of bromoxynil in crops, this is defined by Health Canada as bromoxynil plus DBHA, except for dry bulb onions, millet and sorghum, for which the definition only includes bromoxynil.[46] For bromoxynil in onion, residue field trial data indicated that the residues of bromoxynil were below the limit of quantitation (0.02 ppm).[47]

Food Standards Australia New Zealand (FSANZ) has also evaluated the safety of imported food products, including oil and linters derived from GM bromoxynil-resistant cotton and oil from GM bromoxynil-resistant brassica (canola).[21,22] In its assessment of the GM cotton products, FSANZ also mentions that significant amounts of DBHA can be present in bromoxynil-resistant cotton, whereas it was not clear to what extent residues could also occur in linters and oil derived from this cotton. In the assessment of GM canola, it notes that residue field trials showed that the seed did not contain quantifiable levels of residues of bromoxynil and DBHA, which were just above the limit of detection, and that none was detectable in processed canola fractions (oil and meal). Both assessments by FSANZ of cotton and canola also note that no MRL has been set for bromoxynil in these commodities, and that therefore residues in these products should be below detectable levels (pp. 9–10 of FSANZ;[21] p. 8 of FSANZ[22]).

3.2.3 *Glufosinate*

In laboratory-grown experimental plants, including tobacco, carrots and alfalfa that had been genetically modified with a PAT enzyme, Dröge-Laser *et al*.[48] studied the metabolism of [^{14}C]-radiolabelled glufosinate applied to plant leaves.[48] These authors observed that two parallel routes exist for metabolism of glufosinate in plants.

One of these two routes is specifically linked with the modification of GM crops with a PAT enzyme, which causes the appearance of the metabolite *N*-acetyl-L-glufosinate (NAG) (Fig. 5), besides the parent compound glufosinate. This formation of the *N*-acetylated metabolite corresponds to the enzymatic activity of the PAT enzyme.

The second route, which is linked to glufosinate metabolism in non-modified plants, involves the initial deamination of glufosinate to 4-methyl-phosphinico-2-oxo-butanoic acid (PPO) (Fig. 5), which was found to be prone to further metabolism. For example, PPO was found to be further decarboxylated to

3-methyl-phosphinico-propanoic acid (MPP) (Fig. 5). PPO was also found to re-form glufosinate through amination by plant metabolism. An additional metabolite consisting of the hydroxyl form of glufosinate, 4-methyl-phosphinico-2-hydroxy-butanoic acid (MHB) (Fig. 5), was also found to be present in non-modified plants.

The authors further concluded that both routes compete with each other for the metabolism of glufosinate. This could, for example, account for the observation that metabolites of the route linked with intrinsic plant metabolism, such as PPO, MHB and MPP, are still observed in GM plants with comparatively low PAT activity.[48]

In line with these findings are other reports in the scientific literature that describe the metabolism of DL-glufosinate and/or L-glufosinate in experimentally grown crop plantlets, including glufosinate-resistant brassica (canola), maize, cotton and soybean expressing PAT.[49–53] These studies show that, in the treated leaves and other parts of glufosinate-resistant plants, L-glufosinate is metabolised to a significant extent. NAG has been demonstrated to be the main metabolite of the parent compound.[49,52,53] In glufosinate-resistant maize and brassica (canola), Ruhland *et al*.[53] also reported the additional presence of MPP and MHB, as well as 2-methyl-phosphinico-acetic acid (MPA) (Fig. 5) and 4-methylphosphinico-butanoic acid (MPB). For MPA and MPB, the authors concluded that they could not exclude the possibility that these metabolites are possibly derived from the metabolism of L-glufosinate by microorganisms under the experimental outdoor conditions. D-Glufosinate applied to maize was only found to be absorbed to a limited extent, after which it was partially metabolised to both NAG and methylphosphinico-substituted organic acids.[53] In an experiment in which maize and brassica (canola) plants had been harvested 4 months after application of DL- and L-glufosinate to leaves of either crop (and D-glufosinate to maize), comparatively minor levels of residues, including the parent compound, NAG and methylphosphinico-substituted organic acids, occurred in the grains of these crops.[53]

The JMPR's evaluation of glufosinate that was published in 1998 includes a summary of studies on the metabolism of glufosinate in glufosinate-resistant plants, including brassica (canola), maize, soybean, sugar beet and tomato.[54] The outcomes of these studies are consistent with those reported in the scientific literature discussed above, as they show that L-glufosinate is rapidly metabolised by the plants to NAG, while D-glufosinate is relatively stable. In many cases, the outcomes also show that, besides the parent compound and NAG, additional metabolites of glufosinate are present within plant tissues, including MHB and methylphosphinico-substituted organic acids, in particular MPP and sometimes also MPA and MPB. MPP is the most predominant residue in grain of GM glufosinate-resistant maize, and also a relatively important metabolite of glufosinate in beans of GM glufosinate-resistant soybean. NAG is an important residue in the seed, grains or fruits from glufosinate-resistant brassica (canola), maize, soybean, sugar beet and tomato.[55]

A report that had previously been published by JMPR in 1991 concluded that, in conventional non-glufosinate-resistant crops, MPP is the main metabolite of the parent compound.[56] By that time, JMPR defined the residue used for the setting of the MRL as the sum of the residues of glufosinate-ammonium and MPP expressed as free glufosinate.[56]

In the preamble to its report of 1998, JMPR noted that the different agricultural practice and residue patterns in glufosinate-resistant crops that had been newly developed by that time required new MRLs. The JMPR then proposed to redefine the residue as the sum of glufosinate-ammonium, MPP and NAG expressed as free glufosinate. The extension of the residue definition with NAG was based on two considerations: (1) NAG is a major metabolite formed from glufosinate in glufosinate-resistant crops; (2) NAG and glufosinate give rise to the same chemical derivative that is prepared for the analysis of glufosinate residues. This newly proposed definition could not yet be adopted then, pending the completion of the evaluation of the toxicity of NAG.[54]

After the completion of the toxicological evaluation in 1999, the JMPR adopted the newly proposed residue definition, which included glufosinate-ammonium, NAG and MPP being expressed as free glufosinate. Moreover, JMPR considered this definition as being suitable for MRL setting and for estimating the dietary intake of glufosinate and its metabolites.[57]

3.2.4 *Glyphosate*

Until the 1990s, a generally held belief was that glyphosate is not metabolised by crops to which it is phytotoxic, whereas the metabolism of glyphosate to AMPA by soil microorganisms was well described by that time. Several scientific reports in the early 1990s showed that AMPA was formed from glyphosate as its main metabolite in sterilised cultures of crop cells or tissues, ruling out a possible contribution of microbiological activity.[58,59] Moreover, a group of Canadian authors showed that AMPA occurred in a range of experimentally field-grown conventional, non-GM crops to which glyphosate had been applied as a preharvest application, including barley, brassica (canola), crested wheatgrass, field pea and wheat. AMPA levels in the seeds, straw and/or foliage of these crops generally was a fraction of the levels of glyphosate, i.e. up to approximately 7% in seed or straw of barley. The levels of AMPA by and large followed the same pattern as those of glyphosate with regard to their dependency on application rates, stage of application and other environmental factors.[60–63] These authors also mentioned that, in several different crops investigated in other studies, such as strawberry, no formation of AMPA had been observed. Obviously, the formation of AMPA from glyphosate through decarboxylation is caused by an intrinsic GOX-like activity in crop plants, the exact source and nature of which have not yet been elucidated.

Sarcosine (*N*-methylglycine) (Fig. 6) is another metabolite that is formed from glyphosate by various environmental microorganisms isolated from soil and water. It can involve the cleavage of the C–P bond in glyphosate.[64] There are several accounts of the presence of sarcosine in glyphosate-treated plants, including weeds[65,66] and trees,[67] although it appears to be absent from crop plants, i.e. glyphosate-treated, GM glyphosate-resistant canola.[68] However, these results should be interpreted with care, as the sarcosine measured in plants may have been derived either from contaminations of the applied glyphosate preparation[66] or from microbiological C–P lyase activity within plants.[69]

With regard to metabolism of glyphosate in GM glyphosate-resistant crops, AMPA was detected in seeds harvested from field-grown GM glyphosate-resistant soybean carrying a gene coding for the glyphosate-insensitive CP4 EPSPS enzyme, which had received post-emergence applications of glyphosate.[70,71] Residues of both compounds were also detected in stems and leaves of the soybean plants.[71] The levels of AMPA and glyphosate were found to be variable, depending on the timing and/or number of applications, with later applications generally giving rise to higher levels of AMPA and glyphosate.[70,71] Experiments with greenhouse-grown

young plantlets of conventional non-resistant and GM glyphosate-resistant soybean and brassica (canola) also showed the presence of AMPA besides glyphosate in these plantlets after glyphosate application.[72–74] AMPA was not detected in maize plantlets, which had received relatively low application rates of glyphosate.[74] The GM glyphosate-resistant brassica (canola) plantlets studied by Nandula *et al*.[72] also contained GOX, the enzyme producing AMPA from glyphosate, besides CP4 EPSPS. The AMPA levels found in these plantlets were not particularly high compared with those in glyphosate-resistant soybean without GOX. This led the authors to conclude that, in canola, AMPA must be further metabolised.[72]

The JMPR evaluated data on the metabolism of radiolabelled glyphosate in a number of non-GM, non-glyphosate-resistant crops in 2005, following a previous evaluation in 1997.[75,76] These crops included coffee, cotton, maize, soybean and wheat, as well as mixtures of grass and legumes (i.e. alfalfa and clover), which extended upon the wide range of crops exposed to glyphosate in soil that had already been considered in 1986. In general, the data evaluated by the JMPR show that glyphosate is the main compound present in non-GM crops to which it has been applied at subherbicidal doses, while its metabolite AMPA is present to a limited extent. An exception was noted for maize forage, in which a relatively high level of AMPA had been observed, at a level comparable with that of glyphosate. Furthermore, the uptake of glyphosate from soil, which, for example, is possible after pre-emergence applications, had only been observed to occur to a very limited extent. Further metabolism of glyphosate and AMPA includes the conjugation of AMPA with natural compounds, such as organic acids, as well as degradation to one-carbon elements that are incorporated into intrinsic crop compounds.[75,76]

The evaluations that JMPR carried out in 1997 and 2005 also considered data on glyphosate-resistant crops, including cotton, maize, soybean and sugar beet.[75,76] Contrary to the way that glyphosate is applied in-crop to conventional, non-resistant plants, it can be applied to resistant GM crops at higher dosage and directly to the crop plants. After pre-emergence application of glyphosate, residue levels in GM crop tissues are low, similar to the levels observed in glyphosate-sensitive non-GM crops. Following post-emergence application of glyphosate, the glyphosate-resistant GM crops contain glyphosate and AMPA as the main residues. In the various tissues of resistant cotton, soybean and sugar beet, the level of AMPA is relatively minor compared with that of glyphosate, except for the seed of soybean, in which AMPA exceeds glyphosate levels.[76] AMPA reaches substantial levels in glyphosate-resistant maize that has been genetically modified with both a glyphosate-insensitive EPSPS and the GOX enzyme, the latter converting glyphosate to AMPA.[75] Also, trace levels of conjugates of glyphosate and AMPA have been detected in these crops.[75,76]

With regard to the residue definition of glyphosate, the JMPR's report that was published in 2005 concluded that this definition would continue to include the parent compound glyphosate for compliance with MRLs.[77] The JMPR also noted that this was in line with the definition that is generally used in national systems. In addition, it was concluded that both glyphosate and AMPA are the residues of toxicological concern, based on the observation that AMPA is the main metabolite of glyphosate in plants. For the estimation of the supervised trial median residue and the estimated dietary intake, the JMPR defined the residue as the sum of the levels of glyphosate and AMPA. For this purpose, the level of AMPA has to be converted to 'glyphosate equivalents' by multiplication by a factor of 1.5, and subsequently has to be added to the level of glyphosate, i.e. glyphosate + 1.5 × AMPA.[77]

As mentioned in Section 3.1.4, recently approved lines of GM maize and soybean express the GAT enzyme, which converts glyphosate to *N*-acetyl-glyphosate. In order to take account of the potential presence of *N*-acetyl-glyphosate residues in GAT-expressing maize and soybean that are grown in the United States, the US EPA has included this metabolite of glyphosate into its residue definition for glyphosate in maize and soybean, as well as meat and milk.[78,79] The US definition thus includes both glyphosate and *N*-acetyl-glyphosate in these agricultural products. This is because the US EPA assumes that *N*-acetyl-glyphosate is equally toxic as glyphosate, while noting that this is a conservative assumption because *N*-acetyl-glyphosate is probably less toxic than glyphosate. AMPA is not included in the US residue definition because the US EPA has previously concluded that AMPA is of no toxicological concern. Moreover, the US EPA considers the toxicity of *N*-acetyl-AMPA, another metabolite formed in GAT-expressing crops, to be low and of limited concern.[79]

The presence of *N*-acetyl-glyphosate and *N*-acetyl-AMPA in GAT-expressing maize and soybean has also been considered by FSANZ in the safety assessment of these GM crops, import commodities of which were notified for marketing approval in Australia and New Zealand (pp. 86–105 of FSANZ[80]).[81] Based on weight of evidence comprising toxicological data provided for *N*-acetyl-glyphosate and *N*-acetyl-AMPA, as well as the outcomes of residue trials with the GAT-expressing crops, the assessments conclude that these two metabolites of glyphosate are not toxicologically significant and therefore do not have to be included in the residue definition. The Australian residue definition does not include AMPA either, because glyphosate is considered to be the only toxicologically significant compound among the four substances (*N*-acetyl-glyphosate, glyphosate, AMPA, *N*-acetyl-AMPA) that occur as residues in GAT-expressing crops.[80,81]

In the EU, the issue of the occurrence of *N*-acetyl-glyphosate and other metabolites in GM maize and soybean expressing GAT has been considered by the European Food Safety Authority's (EFSA) Pesticide Risk Assessment Peer Review (PRAPeR).[82] A distinction was made between the residue definitions used for risk assessment and for enforcement of pesticide residue MRLs. For risk assessment, it was considered necessary to cover, besides glyphosate, the metabolites formed from glyphosate, including *N*-acetyl-glyphosate, AMPA and *N*-acetyl-AMPA, in all plant and animal food commodities. For enforcement, three options for changes to the residue definition of glyphosate residues in maize and soybean were offered to risk managers. The first option was not to change the current definition comprising only glyphosate, while the second option was to include *N*-acetyl-glyphosate in the definition of glyphosate, i.e. the sum of glyphosate and *N*-acetyl-glyphosate. The third option was to establish a separate MRL for *N*-acetyl-glyphosate, with the definition covering *N*-acetyl-glyphosate only. EFSA noted that glyphosate residues only would not be a good marker for monitoring compliance because it had been observed that *N*-acetyl-glyphosate was the major residue in maize grain and soybean seeds of the GAT-expressing GM crops. On the other hand, it noted that creating separate residue definitions for maize and soybean could create confusion in enforcement laboratories. Similarly to EPA, EFSA concluded that the metabolites AMPA, *N*-acetyl-glyphosate and *N*-acetyl-AMPA are of no higher toxicological concern than glyphosate itself, and therefore, for risk assessment purposes, the ADI of glyphosate can be used.[82]

3.3 Impact of GM herbicide-resistant crops on MRL setting

In 2001, the CCPR considered the possibility of establishing separate MRLs for pesticide residues in GM crops.[83] In particular, herbicide-resistant crops were considered. The experience of three members, namely the United States, Canada and Mexico, were reviewed. This showed that these three nations approached the herbicides applied to GM herbicide-resistant crops in the same way as they approached the herbicides applied to conventional crops. For example, an in-depth study on the metabolism of the herbicide in the resistant crop will be required if this is expected to be different from the metabolism in conventional crops, in addition to data from supervised field trials on the levels of residues of the herbicide and its metabolites. Also, the residue definition would have to be broadened in order to incorporate any specific metabolites of concern that are formed in the resistant crop but not in the conventional one. Because the commodities from the resistant crop may be mingled with those from the conventional counterpart in practice, a single residue definition and MRL for a given herbicide will be applied to both types of crop, i.e. no distinction will be made between herbicide-resistant and herbicide-susceptible crops. In a more general sense, the CCPR concluded that there was general agreement that separate MRLs should not be elaborated for GM and conventional crops.[83]

As explained in Section 3.2, the altered metabolism of herbicide residues in various GM herbicide-resistant crops has led national governments and/or CAC to revise, in a number of cases, the definition of the residue so as to accommodate novel metabolites of interest that are specifically formed from the herbicides applied to these crops. Besides the definition of the residue, there is a theoretical possibility that also the level of residues of the herbicide and its metabolites may have increased so that the maximally tolerable levels of residues present within agricultural commodities, i.e. MRLs, need to be adjusted, taking into account the safety of these residues. In this section, the impact that the introduction of GM herbicide-resistant crops has had on the setting of MRLs by CAC is explored in more detail. In the absence of CAC MRLs for a given herbicide, the national MRLs set by the US EPA have been reviewed.

3.3.1 ALS inhibitors

As mentioned in Section 3.1.1, the company that has developed GM maize and soybean that are resistant towards herbicide active ingredients that inhibit the ALS enzyme mentions the possibility of combining these crops with the use of various sulfonylurea-containing herbicides. The active ingredients of these herbicides include chlorimuron ethyl, rimsulfuron, thifensulfuron methyl and tribenuron methyl.[15,16] Currently, neither a review by JMPR nor a CAC MRL is available for these ALS inhibitors. The US EPA has assessed whether new or amended MRLs should be adopted to cover the use of three of these sulfonylureas on GM ALS-inhibitor-resistant maize and soybean. The US EPA's assessment followed a petition by the company for amendment of pesticide MRLs for the pertinent use of the ALS inhibitors on GM maize and soybean.[84–86] For the fourth sulfonylurea, i.e. thifensulfuron methyl, no such petition was apparently made, while MRLs for thifensulfuron methyl in maize and soybean had already been established by that time.

The residue definition of chlorimuron ethyl, rimsulfuron and tribenuron methyl includes the parent compounds only. The new data on field trials with these three sulfonylureas applied to GM ALS-inhibitor-resistant maize and soybean showed that, according to the proposed usage of these sulfonylureas, their residues were below the limit of quantitation (<0.01 mg kg^{-1}) in maize grain and soybean seeds. Residues were detectable in plant parts used for animal feeding, such as maize and soybean forage, maize stover and soybean hay. Moreover, processing studies showed that these sulfonylureas were concentrated in the aspirated grain fractions of both crops. Rimsulfuron and tribenuron methyl were also found to concentrate in soybean hulls.[84–86]

MRLs that corresponded to the limit of quantification (0.01 mg kg^{-1}) were thus recommended by the US EPA for these sulfonylureas present in maize grains and soybean seed. Because the existing higher MRL for chlorimuron ethyl in soybean seed aligned with the corresponding MRL in Canada (0.05 mg kg^{-1}), and that for rimsulfuron in field maize grain with the corresponding MRL in Mexico (0.1 mg kg^{-1}), these MRLs were sustained. The latter is relevant within the framework of the North American Free Trade Agreement (NAFTA) among the United States, Canada and Mexico, which also strive towards harmonising MRLs. Specific MRLs were recommended for each of the three sulfonylureas in maize forage, maize stover, soybean hay, soybean forage, soybean seed and aspirated grain fractions, as well as for rimsulfuron and tribenuron methyl in soybean hulls. Based on studies on animals, no additional MRLs were considered necessary for edible animal products including meat, milk and eggs.[84–89] A comparison of national MRLs for these three sulfonylureas in maize and soybean shows that the MRL for rimsulfuron in maize grain (0.1 mg kg^{-1}) exceeds the EU threshold of 0.05 mg kg^{-1}, which is not a specific MRL but the lower limit of analytical determination used specifically to ensure that all sulfonylureas are regulated to a common standard.[90]

In Section 3.1.1 it is noted that assessment reports on GM ALS-inhibitor-resistant crops also mention the suggested use of thifensulfuron methyl on resistant cotton, and that of triasulfuron and metsulfuron methyl in resistant flax. Whereas an MRL has been established by EPA for the usage of thifensulfuron methyl in cotton, the proposal for this MRL only mentions the preplant use of this compound, which may be possible in both non-GM sensitive and GM ALS-inhibitor-resistant cotton, rather than the post-emergence 'over-the-top' use in resistant cotton alone.[91,92] Whereas triasulfuron and metsulfuron methyl were envisaged as soil residues in fields where GM ALS-inhibitor-resistant flax was to be grown subsequently in the rotation, no specific MRLs in flax have been set for these sulfonylureas by Canada and the United States. Instead, both nations have established MRLs for thifensulfuron methyl and tribenuron methyl in flax (linseed grown for fibre). Tribenuron methyl acts as a precursor of metsulfuron methyl, to which it is initially metabolised by plants that have absorbed it. The supporting data for the petition that was filed by the herbicide-producing company for the setting of MRLs by EPA describe post-emergence broadcast applications of both thifensulfuron methyl and tribenuron methyl to GM ALS-inhibitor-resistant flax at an early stage of crop development. No residues were found in seed (linseed) above the limit of quantification, i.e. 0.02 mg kg^{-1} in both cases, which was then used as the MRL for thifensulfuron methyl and tribenuron methyl in linseed.[91–94] The same MRLs have been established in Canada, while the EU has set no specific MRLs but has taken the lower limit of analytical determination for thifensulfuron methyl (0.05 mg kg^{-1}) and tribenuron methyl (0.01 mg kg^{-1}) in linseed, the latter being lower than the MRL in Canada and the United States.[90] The product label of the herbicide formulation containing both sulfonylureas does not, however, mention the use of this herbicide on flax but on cereals

that are naturally resistant. Flax is mentioned as one of the crops that can be planted after the herbicide has been applied, with a minimum crop rotation interval of 2 months.[95]

3.3.2 Bromoxynil

As explained in Section 3.2.2, no residue definition and MRL have been defined by CAC for bromoxynil, while definitions and limits for its residues on bromoxynil-resistant cotton and brassica (canola) have been put in place by the US and Canadian authorities respectively. The US EPA adopted an interim decision for a temporary MRL of 0.04 ppm for bromoxynil in seeds of bromoxynil-resistant cotton, valid until 1998.[96] This coincided with the commercial introduction of bromoxynil-resistant cotton in US cotton-growing areas in 1995, being the first GM cotton to be commercialised there. The further chronology of the regulation of MRLs of bromoxynil in cottonseed highlights the criticality of the estimation of cancer risk derived from intake of bromoxynil residues in food and drinking water.

At that time, an additional study on the carcinogenicity of bromoxynil tested in mice still had to be reviewed.[97] Such a study had previously been requested by EPA independently from the petition for the MRL in cotton, extending the dose range of bromoxynil that had been used in a study in mice in which tumour formation had been identified in livers of male animals. Bromoxynil had also been tested positive in several but not all *in vitro* mutagenicity/genotoxicity studies, while not being carcinogenic in rats *in vivo*. Bromoxynil had been classified as a group C carcinogen, which is EPA's category for carcinogens for which there is limited evidence of carcinogenicity based on animal data in the absence of human data. EPA also mentioned, in its proposed rule, that residues of bromoxynil at the proposed MRL levels in cottonseed would contribute insignificantly, i.e. 1/1000th, to the estimated overall cancer risk and long-term toxicity of bromoxynil caused by aggregate exposure. The risks were estimated on the basis of a worst-case scenario in which consumers consumed crop foods containing residues of bromoxynil at its maximally permissible levels (i.e. MRLs) in 100% of all crops for which it had been registered.

Following this interim decision, EPA published a rule on bromoxynil in 1997, in which a number of changes were made compared with the previous decision as a result of the usage of bromoxynil in cotton. New MRLs were set for the residues of bromoxynil in cottonseed, while the range of commodities with MRLs was also enlarged to include two cottonseed byproducts and additional animal products (EPA, 1997b). Moreover, the residue definition for bromoxynil in cotton and animal products was extended with DBHA (see Section 3.2.2). The applicant had also indicated that it wanted to expand the acreage for which the usage of bromoxynil was registered from 200 000 acres (80 937 ha) to 400 000 acres (161 875 ha). The proposed MRL for bromoxynil plus DBHA in undelinted cottonseed was higher in 1997, namely 7 ppm, than previously set for bromoxynil in cottonseed in 1995. MRLs were also proposed for cotton gin byproducts at 50 ppm and for hulls at 21 ppm.[42] The question as to whether the MRLs for animal products had to be changed was considered, given the inclusion of cotton gin byproducts in animal feed. In addition, the cancer risk assessment also considered the findings from the second carcinogenicity study in mice, which showed the formation of carcinomas in livers in both male and female mice (pp. 14–15 of EPA[44]).[98]

The new risk assessments for the various kinds of potential toxicity in 1997 were based on a different scenario than the worst-case scenario used in 1995. In the scenario that was used in 1997, anticipated residues were determined on the assumption that 3% of the crop was treated at an application rate (1.5 lbs AI acre^{-1}, or 1.7 kg ha^{-1}) that was lower than the field-tested application rate (4.5 lbs AI acre^{-1}, or 5.0 kg ha^{-1}) but that corresponded to the rate for which the applicant had filed its petition for registration. Also, the contribution by residues in drinking water to consumers' exposure was taken into account, as well as the safety for infants and children.[98] No risks of non-carcinogenic toxicity in the short and the long term were identified. The upperbound cancer risk from food intake was estimated to be 1.5×10^{-6}, and that from drinking water intake 6.3×10^{-7} at most. Based on these values, which were close to the EPA's common reference of negligible risk of 1×10^{-6}, the EPA considered the cancer risks to be negligible, also taking into account the uncertainties inherent in the extrapolation of data and the safety factors used, as well as the overestimations of the residue levels in food and drinking water.[98]

A new risk assessment was also performed by the EPA in 1998, based on new residue data that had been provided by the applicant after its petition, and also because of the applicant's proposal to expand the acreage of cotton where bromoxynil could be applied from 400 000 acres (161 875 ha) to 1 300 000 acres (526 093 ha). The latter area corresponded to 10% of the US cotton area in 1998, which was also considered in the new scenario used for the risk assessments.[99] At that time, in 1998, the adoption of bromoxynil-resistant cotton was indeed on the rise, reaching its peak in 1999 when 7.8% of the total US cotton area was planted with this crop.[100] After that, its share declined, before eventually being pulled from the market in 2005.[100,101] The MRLs thus established in 1998 differed from those proposed in 1997. The new MRL in delinted cottonseed was set at 1.5 ppm, while the MRLs for gin byproducts and hulls were set at 7.0 and 5.0 ppm respectively.[99] Outside the United States, Mexico has also set an MRL of 1.5 ppm for bromoxynil in cottonseed, while the EU, Norway and Israel have defined an MRL of 0.1 ppm, which corresponds to the lower limit of analytical determination.[90]

In Canada, the use of bromoxynil is permitted on GM bromoxynil-resistant brassica (canola) (see Section 3.1.2). The Canadian authorities have defined an MRL of 0.1 ppm for bromoxynil in canola, as well as in a number of animal products (eggs, meat and milk). In addition, a general MRL of 0.1 ppm is valid for products for which no specific MRL has been specified.[45] Outside Canada, no specific MRLs have been established for bromoxynil in brassica (canola) in the United States, while the EU has set the threshold at the lower limit of analytical determination, i.e. at 0.1 ppm, which aligns with the Canadian MRL.[43,102] Bromoxynil-resistant canola was on the Canadian market until 2001, after which it was discontinued for economic reasons.[103]

3.3.3 Glufosinate

As explained above in Section 3.2.3, the JMPR reconsidered the residues of glufosinate ammonium present in crops on the basis of new data from GM glufosinate-resistant crops in 1998. One of the changes that JMPR considered necessary was to broaden the residue definition, which at that time included the parent glufosinate and MPP expressed as free glufosinate, with NAG. It also considered it necessary to change a number of MRLs so as to cover the altered levels of residues in GM glufosinate-resistant crops. Because the review of toxicity data on NAG could not be completed at that time, no formal recommendations could be made for these changes by JMPR to the CAC.[54] In 1999, JMPR was

able to complete its assessment of the toxicity of NAG, which is the product that is formed from glufosinate by the PAT enzyme introduced in glufosinate-resistant crops. The JMPR thus noted that, among others, the lowest level of NAG that showed no toxicity when administered to animals in animal trials was higher than that previously observed for glufosinate. Additional data on the toxicity of MPP had been provided as well.[104] These data led the JMPR to conclude that the toxicity of NAG and MPP was comparable with or less than that of glufosinate, and that the acceptable daily intake (ADI) for glufosinate could be sustained.[57]

In 1999, the MRL that JMPR recommended for glufosinate in seeds of glufosinate-resistant brassica (canola) was based on data from supervised field trials in Canada. Its value of 0.3 mg kg^{-1} fell below the MRL of 5 mg kg^{-1} that had previously been recommended, in 1994, on the basis of data from Canadian and European field trials with non-resistant brassica (canola). The higher MRL of 5 mg kg^{-1} was therefore maintained.[57,105]

Data on residues of glufosinate in glufosinate-resistant maize from supervised field trials in North America and Europe were considered by the JMPR in 1998, following its previous assessment of data on glufosinate in non-resistant maize in 1994. Most residues in grain of glufosinate-resistant maize were below the limit of determination, with a few values reaching above this limit but still below the MRL previously established for non-resistant maize, namely 0.1 mg kg^{-1}. The MRL estimated by JMPR for glufosinate-resistant maize was equivalent to that for non-resistant maize. Conversely, for maize forage, the previously established MRL of 0.02 mg kg^{-1} had to be raised to an estimated value of 5 mg kg^{-1} because of the fact that glufosinate residues were detectable in forage of glufosinate-resistant maize, whereas it had been below the limit of detection in forage from non-resistant maize. In 1998, an additional MRL was estimated for maize fodder at 10 mg kg^{-1}.[55,105]

With regard to soybean, an MRL for glufosinate used as desiccant in soybeans was set at 0.5 mg kg^{-1} in 1991.[56] In 1994, following abandonment of the use of glufosinate as a desiccant for soybean, this MRL was lowered to 0.1 mg kg^{-1} on the basis of field trial data in which glufosinate had been used as herbicide. In 1998, the JMPR considered new data on residues of glufosinate used as herbicide in GM glufosinate-resistant soybean in supervised trials, which also had shorter preharvest intervals than glufosinate used in conventional non-resistant soybean. The outcomes, showing maximum and median residue levels that exceeded the existing MRL, led the JMPR to raise the MRL for glufosinate to 2 mg kg^{-1} in 1999.[54]

In a processing study, sugar from roots of glufosinate-resistant sugar beets had been shown to contain no detectable residues of glufosinate, while the residues appeared to be concentrated in the molasses. Oils from brassica (canola), maize and soybean did not contain detectable levels of glufosinate, whereas some byproducts, such as the meal of these crops, were found to contain residues.[54]

MRLs were also established for animal products (e.g. meat, milk and eggs) at the respective lower limits of analytical determination in 1999.[57]

Besides brassica (canola), maize, soybean and sugar beet considered by JMPR for residue chemistry in GM glufosinate-resistant varieties, other crops have also been rendered glufosinate-resistant through genetic modification. GM glufosinate-resistant crops that have not been reviewed by JMPR for residue chemistry and that have been authorised for food use include cotton and rice in the United States and Canada, as well as radicchio in the United States.[7,8] No MRLs have been established by CAC for residues in these three crops,[106] while EPA has established MRLs for cottonseed (4.0 mg kg^{-1}) and rice grain (1.0 mg kg^{-1}). Until 2007, these two MRLs were part of a list of MRLs in the US Code of Federal Regulations that had specifically been assigned to transgenic crops, including brassica (canola), cotton, maize, rice, soybean and sugar beet. A separate list contained MRLs for crops not being designated as transgenic. The residue definition was the same for the crops in both lists, i.e. glufosinate, NAG and MPP. Cottonseed and cotton gin byproducts were included in the lists of both non-transgenic and transgenic crops with the same MRLs.[107] The company that produced both the herbicide and the transgenic crops filed a petition to bring these lists together. One of the reasons for this was that the company wanted to extend the registration of glufosinate to preplant burndown applications in both transgenic and non-transgenic varieties of three crops that had previously been listed as transgenic, namely brassica (canola), maize and soybean. In EPA's final rule on this petition, it was concluded that the MRLs did not have to be raised because of these preplant applications, given that the total amount to be applied during a season was retained and because post-emergence foliar applications would cause higher residue levels than the preplant ones. In these cases, it was therefore the transgenic varieties that determined the MRLs. Both lists were thus merged and appeared in the next edition of the Code of Federal Regulations.[108]

3.3.4 *Glyphosate*

In 1997, the JMPR evaluated data from supervised field trials that had been carried out in the United States on glyphosate residues in both conventional cotton and GM glyphosate-resistant cotton. The Good Agricultural Practice (GAP) in both non-GM and GM cotton allowed for the pre-emergence, directed post-emergence and preharvest applications of glyphosate to cotton. In GM cotton, post-emergence applications, i.e. over-the-top applications, were also allowed. The JMPR noted that the preharvest applications were considered not to contribute to residues in the cottonseed because of lack of translocation of the absorbed glyphosate to the seed in senescent cotton plants. The JMPR considered that the residue data from both types of cotton showed similar distributions and therefore combined these data in order to derive the supervised trial median residue (STMR; 2.0 mg kg^{-1}) and maximum residue (6.0 mg kg^{-1}) for glyphosate in cotton. An MRL of 10 mg kg^{-1} was thus established.[109]

Subsequently, in 2005, the JMPR reconsidered the residues of glyphosate in cotton. Residue data had been received on both susceptible and GM resistant cotton, while the data on susceptible cotton were found not to comply with GAP and therefore only the data on resistant cotton were considered.[109] The data on glyphosate-resistant cotton not only included the data that had already been considered in 1997 but also the outcomes of supervised field trials with the 'second generation' of glyphosate-resistant cotton plants, to which glyphosate can still be applied over the top at a late stage of crop development without causing damage to the cotton bolls. The tables with data on residues in these second-generation plants show that the level of residues is higher than that in first-generation plants.[76] Considering the combined data on glyphosate-resistant cotton, including a higher STMR (5.2 mg kg^{-1}) and maximum residue (28 mg kg^{-1}) than in 1997, it was recommended that the MRL be raised from 10 to 40 mg kg^{-1} for total residues in cottonseed.[77]

Data on residues in glyphosate-resistant maize had been provided to the JMPR in 2005 but were not used for establishing an STMR, maximum residue or MRL because the field trials were found

not to comply with the GAPs for these crops.[76] The supervised field trials that had been carried out with conventional maize, i.e. non-GM susceptible maize, were found to comply with GAP and were therefore used for further evaluation by the JMPR. While the GAP for conventional maize included pre-emergence, spot and preharvest treatments, it was concluded by the JMPR that the preharvest and spot treatments would not substantially contribute to the residue in the crop. The STMR (0.12 mg kg^{-1}) and maximum residue (3 mg kg^{-1}) observed in conventional maize grain obtained from these field trials led the JMPR to propose an MRL of 5 mg kg^{-1} for total residues.[77] The levels of residues that the JMPR had previously considered for conventional and glyphosate-resistant maize in 1997 would also fall below this MRL.[109]

With regard to soybean, the JMPR considered data on residues from supervised field trials with both non-GM susceptible and GM glyphosate-resistant soybean. While the GAP for both types of soybean included pre-emergence and preharvest applications, the GAP for glyphosate-resistant soybean also allowed for post-emergence directed sprays. Preharvest sprays were not supposed to contribute to residue formation owing to limited translocation of absorbed residues to seed during senescence. The JMPR therefore considered those field trials that included pre-emergence and in-crop sprays to comply with GAP. Because the residue populations of both types of soybean were similar, these populations were combined for the determination of the STMR (5.0 mg kg^{-1}) and the maximum residue (20 mg kg^{-1}) measured in the field trials.[76] The JMPR thus concluded that these data confirmed the previously established MRL of 20 mg kg^{-1}.[77]

In 2005, the JMPR also summarised the data from processing studies on commodities containing glyphosate, which showed, for example, that glyphosate and AMPA were not detectable in oil derived from various crops, such as cottonseed, maize, rapeseed and soybean. It was found to be concentrated in several other products such as bran of cereals and hulls of soybean.[77]

Whereas data on glyphosate-resistant sugar beet had been presented to the JMPR in 2005, these data were not considered to represent GAP owing to deviant preharvest applications.[77]

Studies with food-producing animals showed that glyphosate was detectable in the kidneys and livers of cattle, pigs and poultry, while it was not detectable in meat, milk or eggs, at feeding levels of glyphosate corresponding to the estimated dietary burden. The JMPR therefore recommended MRLs for meat, milk and eggs that corresponded to the minimum threshold for analytical determination, and specific MRLs for edible offal from cattle, pigs, other mammalians and poultry.[77]

Besides the crops for which CAC has considered the residues in GM glyphosate-resistant varieties, i.e. cotton, maize and soybean, various other GM glyphosate-resistant crops have been authorised for use as food as well, including brassica (canola) and sugar beet in the United States, Canada and the EU, alfalfa and potato in the United States and Canada and wheat in the United States.[6–8] CAC has established MRLs for glyphosate in alfalfa (fodder; 500 mg kg^{-1}), brassica (rapeseed; 20 mg kg^{-1}) and wheat bran and straw (20 and 300 mg kg^{-1} respectively).[106] No CAC MRLs have been established for alfalfa seed (United States, 0.5 mg kg^{-1}), sugar beet (United States and Canada, 10 mg kg^{-1} in roots) and potato (United States, vegetable, root and tuber, group 1, 0.2 mg kg^{-1}).

As described in Section 3.2.4, EFSA's PRAPeR considered three options for the residue definition of glyphosate, following the assessment of data on GM glyphosate-resistant crops containing the GAT enzyme, in which the novel metabolite *N*-acetyl-glyphosate is formed. One of these options was to establish a separate MRL for *N*-acetyl-glyphosate. PRAPeR also recommended MRL values for *N*-acetyl-glyphosate under this option, including one for maize grain (0.3 mg kg^{-1}) and for soybean seed (7 or 10 mg kg^{-1}).[82]

4 CONCLUSIONS

In the previous sections, the herbicide resistance traits that are carried by GM herbicide-resistant crops that have been approved for food use in the United States, Canada and/or the EU have been reviewed with regard to their potential impact on the nature and level of residues present within food crops. One of the rationales behind this project was to explore the possibility that asynchronous authorisations of GM crops and differences in national legislations on herbicide residues could lead to potential barriers to trade in crop commodities.

Four categories of herbicide resistance traits that have been introduced into GM crops have been reviewed, including resistance to ALS inhibitors, bromoxynil, glufosinate and glyphosate. Two different molecular mechanisms underlie herbicide resistance, namely the introduction in the GM crop of (1) a mutated version of the enzyme that is targeted and inhibited by the herbicide or (2) an enzyme that converts the herbicide active ingredient to less toxic or non-toxic forms. As has been observed for GM crops containing enzymes of the latter group, which convert the herbicide active ingredient, the formation of new metabolites has led JMPR, which reports to CAC, or national authorities to redefine the residue within crops. This has been observed for bromoxynil-resistant crops, which contain DBHA formed from bromoxynil by bromoxynil nitrilase, for glufosinate-resistant and some glyphosate-resistant crops, in which *N*-acetylase enzymes deactivate the herbicide active ingredient by forming the *N*-acetylated derivatives NAG and *N*-acetyl-glyphosate, and for glyphosate-resistant crops expressing glyphosate oxidoreductase, which converts glyphosate to AMPA.

In line with the approach followed by CAC, national authorities usually do not specify specific MRLs for GM herbicide-resistant crops, but for the particular crop in general. The residue definition of glufosinate has been amended by JMPR with the addition of NAG, while other authorities have amended the definitions of bromoxynil and glyphosate on the basis of the metabolism of herbicides in GM crops. For example, the US residue definition for bromoxynil in cotton includes DBHA, besides the parent compound, while that for glyphosate in maize and soybean includes *N*-acetyl-glyphosate, besides glyphosate, based on the metabolism of these active ingredients of herbicides applied to GM herbicide-resistant crops. For these cases involving herbicide-metabolising enzymes, it can be concluded that the genetic modification has had an impact on the definition of residues. Conversely, the introduction of the GOX enzyme into a range of crops has not changed the way that the glyphosate residue is defined because AMPA is also a major metabolite of glyphosate in non-GM crops.

With regard to herbicide-insensitive mutants of enzymes inhibited by the herbicide active ingredient, ALS-inhibitor-resistant crops expressing mutant ALS enzymes and glyphosate-resistant crops expressing insensitive forms of EPSPS, either a mutant or a bacterial analogue, have been authorised for food use. The chemistry of the residues formed within these crops has not led to a change in the residue definitions. In addition, for ALS-inhibitor-resistant crops, the distinction between GM and non-GM crops may not be justified, as conventionally bred crops can also carry ALS mutants that confer resistance to ALS inhibitors, such

as for a range of commercialised imidazolinone-resistant crops and sulfonylurea-tolerant soybean. Interestingly, herbicides of the latter category are applied at low application rates, giving rise to residues of the parent compound and its metabolites below the limit of quantification, and are generally of low toxicological concern. Authorities therefore tend to limit the residue definition for these herbicides to the parent compound, notwithstanding the fact that the metabolites may also be toxic (pp. 22–23 of OECD[110]).

No general trend of an increase or decrease in the levels of herbicide residues in GM herbicide-resistant crops has been observed in supervised field trials performed within the framework of herbicide registration. It is therefore not possible to infer generalisations on the impact of these GM crops on the MRLs that are to be set for the residues of the herbicide active ingredients, but this has to be considered on a case-by-case basis. This is also reflected in the changes in MRLs following the registration of herbicides for use on GM herbicide-resistant crops. Increases in the MRLs were recommended by JMPR in a few cases, for example for glufosinate in soybean and for glyphosate in cottonseed, while in many other cases this was not recommended.

While residue data from experimental studies have been used to establish the residue tolerances for the herbicide–crop combinations described above, it would be interesting to compare these tolerances with what is actually measured in the field, i.e. in commercially produced foods. No measurement of the herbicides of interest in the particular crop foods in question is apparently carried out by the centralised or federal pesticide residue monitoring programmes of the EU, the United States and Canada. The ALS inhibitors (chlorimuron ethyl, rimsulfuron, thifensulfuron methyl and tribenuron methyl), bromoxynil, glufosinate and glyphosate, for example, do not feature on the lists of the most recent monitoring data of the US FDA's Pesticide Program,[111] the Canadian Food Inspection Agency's National Chemical Residue Monitoring Program[112] and the EU Coordinated Programme.[113] While bromoxynil and chlorimuron ethyl are measured under the US Department of Agriculture's Pesticide Data Program, this is done in water only.[114] Interestingly, various EU member states' national programmes, which operate in parallel to the EU coordinated programme, measure glyphosate residues. In particular, the UK Pesticide Residues Committee and the Norwegian National Food Authority (Mattilsynet) monitor glyphosate in cereals and infant foods. The UK Pesticide Residue Committee also measured glyphosate in foods derived from two crops of interest, namely soybean and maize, in the years 2002 and 2006. The limit of reporting for glyphosate residues in these studies was 0.1 mg kg^{-1}. In tofu, a protein-rich soybean product, 11 out of 60 samples were positive for glyphosate, with residue levels ranging between 0.1 and 1.1 mg kg^{-1}.[115] Polenta, a food product derived from maize, was found to contain glyphosate in six out of 23 samples, all at a level of 0.1 mg kg^{-1}.[116] In soy milk and popcorn samples, no glyphosate residues at or above the reporting limit were found.[116,117] For glyphosate in the maize and soybean products containing residues, no MRLs had been established in the United Kingdom at that time (since then, EU-wide MRLs for pesticide residues in food, including those of glyphosate, have become established under EU regulations). The intake of glyphosate resulting from consumption of the products containing residues was estimated by the committee to be below the acute reference dose of glyphosate and therefore did not raise safety concerns. In addition, Harris and Gaston[118] report that the residues in maize grain reported in dossier applications and measured during monitoring in the United Kingdom were similar (i.e. median value 0.1 mg kg^{-1}) (Tables 5 and 6 of Harris and Gaston[118]).

It has also been observed that no CAC MRLs exist for a number of herbicide–crop combinations that are applicable to a number of GM herbicide-resistant crops and corresponding herbicides that have been authorised by national authorities. Not only does this pertain to ALS inhibitors and bromoxynil, for which CAC has no MRLs at all, but also to certain crops that can be combined with applications of glufosinate or glyphosate, such as glufosinate-resistant rice and glyphosate-resistant brassica (canola). In a number of cases, national authorities, such as the US EPA, have considered whether the residue definition or MRL should be changed to cover the usage on the GM crop.

For a number of residues, including those of bromoxynil and ALS inhibitors, it has been observed in the present review that North American countries strive towards harmonisation under NAFTA. In addition, it has also been noted that national authorities take into account other nation's evaluations of herbicides being applied to GM crops, such as for residues of GAT-expressing crops producing *N*-acetyl-glyphosate from glyphosate, for which both FSANZ and EFSA referred to the US EPA when assessing the residue and toxicity data. It can be envisaged, however, that, without international harmonisation under CAC, different definitions and thresholds may still be employed by food control inspections, which, in turn, may lead to impediments in trade. It is therefore recommended that the international harmonisation of residue definitions and thresholds be continued to extend the range of herbicide–crop combinations so as to cover the use of herbicides on GM herbicide-resistant crops.

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