2,4-D (020)

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EXPLANATION

Herbicide 2,4-D, 2,4-dichlorophenoxyacetic acid, is currently registered in a variety of salt, amine, and ester formulations for control of broadleaf weeds on a variety of food and feed uses. As a synthetic auxin herbicide, 2,4-D causes disruption of plant hormone responses. It is currently registered in many countries.

It was first evaluated by JMPR in 1970. It was subsequently evaluated in 1986, 1987, 1996, 1997, 1998 and 2001. The 1998 JMPR evaluated it under the Periodic Reevaluation Programme. Its specification was established by the Joint FAO/WHO Meeting on Specifications in 1994. The present ADI is 0–0.01 mg/kg bw for sum of 2,4-D and its salts and esters expressed as 2,4-D and ARfD is unnecessary. The present residue definition established by the 1998 JMPR is 2,4-D for enforcement of MRLs and for dietary intake estimation for plant and animal commodities. The residue is not fat-soluble.

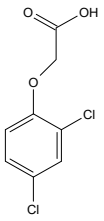
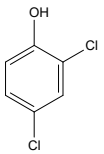
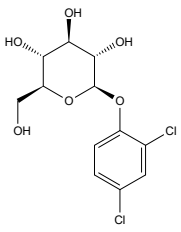
2,4-D was scheduled for JMPR evaluation in the year 2017 for consideration of residues arising from use on a GM cotton crop. There are a number of Codex MRLs but no MRL has been set for cotton seed.

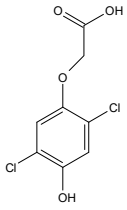
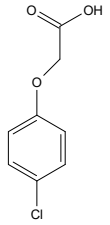
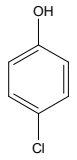
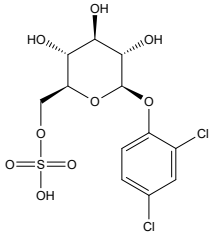
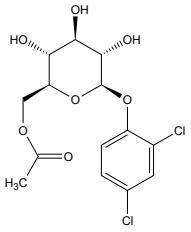
METABOLISM AND ENVIRONMENTAL FATE

JMPR has previously considered a number of plant and animal metabolism studies, studies on rotational crops and environmental fate. The current Meeting received information on a GM cotton, in which expression of the aryloxyalkonoate dioxygenase-12 (AAD-12) protein confers tolerance to 2,4-D and associated increased metabolism of 2,4-D (hereafter referred to as AAD-12 cotton).

Following table described the structure, chemical name and the common or code name of each of residue components identified in the study.

Table 1 Identification of metabolites from 2,4-D metabolism study on AAD-12 cotton

Common name/code no.	Chemical name	Major or minor	Chemical structure
2,4-D	2,4-dichlorophenoxyacetic acid	Major	
2,4-DCP	2,4-dichlorophenol	Major	
2,4-DCP glucose conjugate	2,4-dichlorophenyl beta-D-glucopyranoside	Major	

Common name/code no.	Chemical name	Major or minor	Chemical structure
4-OH-2,5-D	(2,5-dichloro-4-hydroxyphenoxy) acetic acid	Minor	
4-CPAA	4-chlorophenoxyacetic acid	Minor	
4-CP	4-chlorophenol	Minor	
2,4-DCP-glucose-sulfate conjugate	2,4-dichlorophenyl beta-D-glucopyranoside-sulfate	Minor	
2,4-DCP-glucose-acetate conjugate	2,4-dichlorophenyl beta-D-glucopyranoside-acetate	Minor	

AAD-12 Cotton (Rotondaro, S.L. et al, 2015; Study No. 140024)

Cotton plants have been genetically modified to express the aryloxyalkanoate dioxygenase (AAD-12) protein. The AAD-12 protein provides rapid metabolic detoxification mediated by an α-ketoglutarate-dependent dioxygenase enzyme and thereby provides protection of the plant to certain phenoxy auxins, such as 2,4-D. The enzyme facilitates degradation of 2,4-D to the corresponding inactive phenol (2,4-DCP).

[^{14}C-]-2,4-D choline (2,4-dichlorophenoxy acetic acid choline salt labelled at phenyl ring) was applied to a plot of AAD-12 cotton at the maximum seasonal rate in the USA of 3.3 kg ai/ha (three applications each 1.1 kg ai/ha at pre-emergence, BBCH 61, and BBCH 65 with an interval of 12 days). The 2,4-D choline was formulated as a soluble concentrate (GF-2654). The cotton was grown outdoors to maturity. Plot maintenance simulated typical cultural practices, with the exception that the harvest occurred when the lower bolls were mature, whereas commercial production is when all bolls are mature to obtain maximum yield.

Mature seeds and associated trash (e.g., leaves and open bolls) samples were collected 56 days (DAT) after the third and final application (138 days after the pre-emergent application).

Aliquots (10 × approximately 0.05 g) of the milled treated samples were analyzed by oxidative combustion to determine the radioactive residues in the samples.

A portion of these tissues was sequentially extracted beginning with hexane to remove oils (seeds only), a neutral methanol/water, then a basic methanol, a base reflux, and an acid reflux (seeds only). Extracts analyzed by liquid sintilation counter and HPLC after clean-up with an SPE cartridge. LC-MS or LC-MS/MS was used for identification/confirmation. The remaining post-extracted solids were subjected to determination of unextracted residue such as pectin, lignin, acid-detergent fiber, and cellulose.

TRR levels in the treated samples, expressed as mg/kg of 2,4-D acid equivalents are shown below.

Table 2 Total radioactive residues (TRRs) in cotton seed and trash

Plant part	Application			DAT (days)	TRR (mg eq/kg)
	No.	Method	Timing		
Treated Seed	3	Foliar	Preemergence, BBCH 61 and BBCH 65	56	1.18
Treated Trash	3	Foliar	Preemergence, BBCH 61 and BBCH 65	56	39. 8

Seeds and trash contained 1.18 mg/kg and 39.8 mg/kg 2,4-D acid equivalents (mg eq/kg), which, due to the shortened DAT, would be near maximum predicted residue levels.

The distribution of the residues in each seed and trash sample among the fractions generated following extraction, expressed both as a percentage of the total sample residue and as mg/kg of 2,4-D acid equivalents, is shown below.

Table 3 Distribution of the parent and the metabolites in AAD-12 cotton matrices after applications of [^{14}C-]-2,4-D choline

	Seed		**Trash**	
	%TRR	mg eq /kg	%TRR	mg eq /kg
TRR	**100.0**	**1.181**	**100.0**	**39.78**
Hexane extract	3.6	0.042	—[a]	—
MeOH:H_2O 90:10 extract	21.3	0.251	74.7	29.73
MeOH:1M NaOH 90:10 extract	7.5	0.088	8.2	3.24
2 N NaOH extract (1 replicate)	31.8	0.375	11.9	4.75
2 N HCl extract (1 replicate)	2.9	0.034	--	--
Total Extracted[b]	**67.0**	**0.791**	**94.8**	**37.72**
Total Unextracted[c]	**31.2**	**0.368**	**3.4**	**1.36**
Pectin	0.6	0.007	2.2	0.87
Lignin	15.1	0.178	0.5	0.19
ADF	0.9	0.011	0.3	0.14
Cellulose	4.7	0.056	0.1	0.039
Accountability[d]	**98.2**	**1.159**	**98.3**	**39.08**

[a] — = Not Applicable

[b] Includes exhaustive extractions of hexane (seed only), methanol/water (90/10), methanol/1 N NaOH (90/10), 2 N NaOH, and 2 N HCl (seed only)

[c] Residues remaining after exhaustive extractions

[d] Accountability = (Total extracted + Total unextractab le)/(TRRs from combustion analysis) × 100

The pellet remaining after the neutral extraction was next extracted with methanol/1 N NaOH (90/10, v/v). Approximately 8% of the seed and trash TRR was further extracted using this procedure.

An aliquot of each basic methanol extract was prepared and analyzed by HPLC. Seed and trash SPE recoveries were good (90–100%); however, only 75–90% of the applied radioactivity was recovered in SPE elution EL1. For the seed, only EL1 was concentrated and analyzed by HPLC. For the trash, all three phases (load/wash, EL1, and EL2) were concentrated and analyzed by HPLC. Concentration recoveries were good for the load/wash and EL1, but lower for EL2. The total amount analyzed by HPLC in all three phases is reported below. Overall, less than 2% of the TRR was unaccounted due to extract preparation procedural recoveries; 5.8% out of 7.5% of the seed extracted residues and 7.9% out of 8.2% of the trash extracted residues were analyzed.

The pellet from replicate A remaining after the methanolic base extraction was next extracted with 2 N NaOH. An additional 32% of the seed and 12% of the trash TRR was removed using this procedure.

An aliquot of each sodium hydroxide extract was prepared and analyzed by HPLC. Seed and trash SPE recoveries were good (90–100%); however, approximately 25% of the radioactivity was recovered in the load/wash, 60–65% was recovered in SPE elution EL1, and 6–11% was recovered in EL2 (methanol/1 M NaOH [90/10, v/v] for trash and methanol/2 M NaOH [50/50, v/v] followed by methanol/10 M NaOH [90/10, v/v] for seed). All three of the trash SPE phases were concentrated with good recovery, and analyzed by HPLC. The trash load/wash (3% of the TRR) contained only non-retained polar material (not shown), whereas EL1 and EL2 (< 1% of the TRR, not shown) contained a range of non-resolved compounds eluting from 2–22 minutes; 2,4-D and 2,4-DCP were observed each at < 1.5% of the TRR. The total of these three trash SPE phases is provided below.

Concentration of the seed SPE phases proved to be more difficult. The seed load/wash was concentrated and a precipitate formed, which was partially dissolved in methanol and partially in hydrochloric acid. Ultimately, approximately 5% of the seed TRR in the load/wash was analyzed by HPLC (of the 8.1% originally in the load/wash), and the majority of the radioactivity eluted near the solvent front. Seed EL1, containing approximately 20% of the TRR, was initially adjusted to pH 7–8 then concentrated using a Turbovap evaporator (40°C) with approximately 80% recovery; therefore, approximately 4% of the TRR was not recovered and not analyzed by HPLC. Another aliquot of the seed EL1 was concentrated on a rotary evaporator, collecting the condensate (data reported below). In this case, the concentration recovery was >85%; however, nearly 15% (3% of the TRR) was volatile material that was collected in the condensate. The HPLC profile of the seed EL1 SPE phase concentrated by rotovap was similar to concentration by Turbovap. In addition, the pH of the condenser sample was adjusted to >9, then reconcentrated with almost 70% recovery, analyzed by HPLC, and shown to be primarily 2,4-DCP. Seed EL2 contained approximately 3% of the TRR. A precipitate was formed upon concentration of the seed EL2. HPLC analysis of the supernatant, which contained 1% of the TRR, showed entirely non-retained polar component(s).

The amount of unextracted radioactivity was approximately 30% of the seed TRR and less than 5% of the trash TRR. Total accountability after all the extraction and hydrolysis procedures was approximately 98%.

The residues remaining unextracted were further evaluated, and the results are also shown above. In seed, the majority of the unextracted residue was associated with the lignin (approximately 15% of the TRR), plus lesser amounts of cellulose. In trash, pectin contained 2% of the TRR, and other bound fractions each accounted for less than 1% of the TRR.

Residues in both seed and trash methanol/water extract were multicomponent, with the major residues identified as parent 2,4-D and conjugates of 2,4-DCP. There was very little polar residue. Additional low level metabolites, <5% of the TRR, were also observed. Upon acid hydrolysis, many of the low-level peaks (labeled as conjugates) were converted to primarily 2,4-DCP. Levels of 2,4-D did not increase after hydrolysis, indicating that the conjugates were not of 2,4-D. Levels of 4-CP did increase slightly; however, they remained below 1% of the TRR (seed) or 3% of the TRR (trash). Minor non-polar residues were also released upon hydrolysis. In the trash, a component was detected at 27 minutes, but this was shown to consist of multiple low-level components when the gradient was extended. Alternatively, the non-polar product was not reproducible. In either case, the non-polar product is not relevant to the residue profile.

Table 4 Characterization of methanol/water extract of AAD-12 Cotton seed and trash

	Seed MeOH/water average		Seed hydrolyzed MeOH/water replicate A EL1		Trash MeOH/water average		Trash hydrolyzed MeOH/water replicate A EL1	
	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg
TRR	100%	1.181	100%	1.181	100%	39.778	100%	39.778
% Extracted	21.3%	0.251	19.7%	0.232	74.7%	29.731	76.4%	30.395
% Analyzed by HPLC	21.0%	0.248	18.0%	0.213	74.7%	29.731	70.6%	28.068
Polar (multi-component)	0.1%	0.001			0.5%	0.215		
Unknown conjugate	0.6%	0.008			0.5%	0.203		
Unknown conjugate	0.7%	0.008			3.0%	1.186		
Sulfate glucose conjugate of 2,4-DCP	4.7%*	0.055*			*	*		
4-OH-2,5-D	2.0%*	0.024*	*	*	2.8%*	1.094*	*	*
DCP glucose conjugate	2.2%*	0.026*	0.2%*	0.003*	**22.6%***	**8.995***	*	*
Unknown conjugate	0.3%	0.004			0.6%	0.235		
Unknown conjugate	1.0%	0.012			0.3%	0.135		
Unknown conjugate	1.8%	0.022			4.1%	1.623		
4-CPAA	*	*	*	*	3.3%*	1.320*	0.8%*	0.309*
2,6-D	0.5%	0.005	0.8%	0.009	1.1%	0.427		
4-CP	< 0.1%*	0.001*	0.3%*	0.004*	0.3%	0.113	2.8%*	1.128*
DCP acetyl glucose conjugate	0.1%*	0.002*	*	*	*	*	*	*
Unknown 1	0.2%	0.002	1.8%	0.021			0.7%	0.264
2,4-D	4.8%*	0.057*	4.1%*	0.048*	**31.0%***	**12.326***	**24.6%***	**9.796***
2,4-DCP	0.2%	0.003	8.3%*	0.099*	1.7%	0.672	**33.4%***	**13.282***
Unknown 2			1.1%	0.013				
Unknown 3			1.1%	0.013				
Unknown 4					0.7%	0.295	6.8%‡	2.723‡
DCA							0.5%	0.205
Conjugated DCP or 4-CP (sum)	**13.5%**	**0.159**	0.2%	0.003	**33.9%**	**13.471**	ND	ND

* Identified by LC-MS.

‡ Multiple component or non-reproducible.

As shown below, residues in the methanolic base extract of both seed and trash were multicomponent, with the major residues tentatively identified as parent 2,4-D and glucose conjugates of 2,4-DCP, as was observed in the neutral organic extracts. There was very little polar residue, even though the load fraction was entirely non-retained polar material (<1% of the TRR, not shown). Upon acid hydrolysis of the SPE methanol/water elution (EL1) (only one replicate was hydrolyzed for trash), many of the low-level peaks were converted to primarily 2,4-DCP, although levels remained less than 3% of the TRR. Levels of 2,4-D did not increase significantly, indicating that the conjugates were likely not of 2,4-D. Levels of 4-CP did increase slightly; however, they remained well below 1% of the TRR. Minor non-polar residues were also released upon hydrolysis.

Table 5 Characterization of methanol/NaOH extract of AAD-12 Cotton seed and trash

	Seed MeOH/NaOH average		Seed hydrolyzed MeOH/NaOH average EL1		Trash MeOH/NaOH average		Trash hydrolyzed MeOH/NaOH replicate A EL1	
	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg
TRR	100%	1.181	100%	1.181	100%	39.778	100%	39.778
% Extracted	7.5%	0.088	7.5%	0.088	8.2%	3.242	8.1%	3.230

	Seed MeOH/NaOH average		Seed hydrolyzed MeOH/NaOH average EL1		Trash MeOH/NaOH average		Trash hydrolyzed MeOH/NaOH replicate A EL1	
	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg
% Analysed by HPLC	5.8%	0.068	5.3%	0.062	7.9%	3.129	6.1%	2.434
Polar (multi-component)	0.4%	0.004			0.4%	0.174		
Unknown conjugate	0.2%	0.002			0.1%	0.037	< 0.1%	0.019
Unknown conjugate	0.3%	0.003			0.1%	0.052		
Sulfate glucose conjugate of 2,4-DCP	0.6%	0.007			< 0.1%	0.019		
4-OH-2,5-D	0.9%	0.010			0.2%	0.077	< 0.1%	0.012
DCP glucose conjugate	2.0%	0.023	< 0.1%	< 0.001	2.3%	0.919	< 0.1%	0.011
Unknown conjugate	0.4%	0.004	< 0.1%	< 0.001	0.1%	0.025	< 0.1%	0.026
Unknown conjugate	0.1%	0.001			< 0.1%	0.019	< 0.1%	0.022
Unknown conjugate	0.1%	0.001			0.2%	0.060		
4-CPAA	< 0.1%	0.001	< 0.1%	< 0.001	0.4%	0.164	0.2%	0.070
2,6-D	< 0.1%	0.001	0.2%	0.002			< 0.1%	0.024
4-CP	< 0.1%	< 0.001	< 0.1%	0.001	< 0.1%	0.013	0.3%	0.105
DCP acetyl glucose conjugate	< 0.1%	0.001	< 0.1%	< 0.001	0.1%	0.040		
Unknown 1			0.8%	0.009	< 0.1%	0.014		
2,4-D	< 0.1%	0.001	0.2%	0.002	2.5%	0.988	2.5%	0.995
2,4-DCP			2.7%	0.032	< 0.1%	0.020	2.5%	0.975
Unknown 2			0.3%	0.004				
Unknown 3			0.3%	0.003				
Unknown 4					0.2%	0.098	0.1%	0.057
DCA								
Conjugated DCP or 4-CP (sum)	4.5%	0.054	0.1%	0.002	3.1%	1.238	0.2%	0.091

The total amount of the 2 N NaOH extract analyzed by HPLC (all three SPE phases) is reported below. Overall, less than 2% of the TRR was unaccounted due to extract preparation procedural recoveries from the trash and approximately 9% from the seed. Extensive efforts were made to recover all of the radioactivity in the seed 2 N NaOH extract. As shown below, residues in both seed and trash 2 N NaOH extract are multicomponent, with the major residues characterized as parent 2,4-D and glucose conjugates of 2,4-DCP, as was observed in the neutral and basic organic extracts, plus polar radioactivity.

The SPE methanol/water elution (EL1) was acid hydrolyzed. For trash, the procedural recoveries were nearly 100%. For the seed, several attempts resulted in procedural recoveries of nearly 80%; however, without centrifugation to remove the precipitate that formed, no signal was observed in the RAM trace. With centrifugation, despite significant effort to recovery the radioactivity that precipitated, ultimately less than 4% of the TRR could be analyzed by HPLC. Apparently, hydrolysis products were strong associated with the precipitate or otherwise insoluble in solvent. After hydrolysis, the low levels of seed that were analyzed contained only 2,4-D; 2,4-DCP; and 2,6-D; each at <2% of the TRR. The trash analyses showed a similar profile to the sample before hydrolysis: a large “hump” of non-resolved radioactivity eluted from 2–22 minutes.

Table 6 Characterization of 2N NaOH extract of AAD-12 Cotton seed and trash

	Seed 2N NaOH replicate A		Seed hydrolyzed 2N NaOH replicate A EL1		Trash 2N NaOHL replicate A		Trash hydrolyzed 2N NaOH replicate A EL1[a]	
	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg
TRR	100%	1.181	100%	1.181	100%	39.778	100%	39.778
% Extracted	31.8%	0.375	31.8%	0.375	11.9%	4.748	11.9%	4.748
% Analyzed by HPLC	22.6%	0.267	3.8%	0.045	10.2%	4.042	7.7%	3.079
Polar (multi-component)	9.3%	0.110	31.8%	0.375	3.8%	1.503	1.2%	0.464
Unknown conjugate			3.8%	0.045			0.2%	0.078
Unknown conjugate							0.3%	0.105
Sulfate glucose conjugate of 2,4-DCP					0.2%	0.068		

	Seed 2N NaOH replicate A		Seed hydrolyzed 2N NaOH replicate A EL1		Trash 2N NaOHL replicate A		Trash hydrolyzed 2N NaOH replicate A EL1[a]	
	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg	% TRR	mg eq /kg
4-OH-2,5-D	0.4%	0.004					0.3%	0.130
DCP glucose conjugate	0.7%	0.009			0.3%	0.126	0.4%	0.144
Unknown conjugate	2.4%	0.028			0.2%	0.088		
Unknown conjugate	0.3%	0.004			0.1%	0.051	0.2%	0.098
Unknown conjugate	0.3%	0.003			0.2%	0.066	0.2%	0.081
4-CPAA					< 0.1%	0.004	0.1%	0.048
2,6-D	0.7%	0.008	0.6%	0.007	0.4%	0.145	0.3%	0.129
4-CP	0.2%	0.002			0.3%	0.110	0.2%	0.060
DCP acetyl glucose conjugate	0.3%	0.004			< 0.1%	0.035	0.4%	0.144
Unknown 1	0.2%	0.002	1.5%	0.017	0.2%	0.070	0.2%	0.093
2,4-D	0.2%	0.002			1.2%	0.473	1.4%	0.556
2,4-DCP	2.9%	0.035	0.6%	0.007	0.8%	0.325	0.7%	0.291
Unknown 2								
Unknown 3								
Unknown 4					0.1%	0.057		
Conjugated DCP or 4-CP (sum)	4.4%	0.052	ND	ND	1.1%	0.444	2.0%	0.779

[a] There was a non-resolved “hump” of radioactivity eluting from 2.83–21.67 min.

For the trash, >90% of the TRR was extracted and analyzed by HPLC. The major residues in trash were 2,4-D and conjugated 2,4-DCP, both at approximately 35% of the TRR, as shown below. Acid hydrolysis was effective at releasing the 2,4-DCP from the conjugates, which were known or suspected glucose conjugates.

For the seed, nearly 70% of the TRR was extracted and approximately 50% was analyzed by HPLC. The major residue in seed was conjugated 2,4-DCP at approximately 22% of the TRR. Acid hydrolysis was effective at releasing the 2,4-DCP from the conjugates, which were known or suspected glucose conjugates.

Table 7 Total characterized from extracts of AAD-12 Cotton Seed and Trash

	Seed MeOH/water Total		Seed Hydrolyzed Total		Trash MeOH/water Total		Trash Hydrolyzed Total	
	% TRR	mg eq/kg	% TRR	mg eq/kg	% TRR	mg eq/kg	% TRR	mg eq/kg
TRR	100%	1.181	100%	1.181	100%	39.778	100%	39.778
% Extracted	67.0%	0.791	58.9%	0.696	94.8%	37.721	96.5%	38.373
% Analyzed by HPLC	49.4%	0.583	27.1%	0.320	92.8%	36.903	84.4%	33.581
Polar (multi-component)	9.8%	0.116			4.8%	1.892	1.2%	0.464
Unknown conjugate	0.8%	0.010			0.6%	0.241	0.2%	0.097
Unknown conjugate	0.9%	0.011			3.1%	1.239	0.3%	0.105
Sulfate glucose conjugate of 2,4-DCP	5.3%	0.062			0.2%	0.088		
4-OH-2,5-D	3.2%	0.038			2.9%	1.171	0.4%	0.142
DCP glucose conjugate	4.9%	0.058	0.3%	0.003	**25.2%**	**10.040**	0.4%	0.155
Unknown conjugate	3.1%	0.036	< 0.1%	< 0.001	0.9%	0.348	< 0.1%	0.026
Unknown conjugate	1.5%	0.017			0.5%	0.205	0.3%	0.120
Unknown conjugate	2.2%	0.026			4.4%	1.750	0.2%	0.081
4-CPAA	< 0.1%	< 0.001	0.6%	< 0.001	3.7%	1.488	1.1%	0.427
2,6-D	1.2%	0.014	1.5%	0.018	1.4%	0.572	0.4%	0.153
4-CP	0.3%	0.004	0.4%	0.005	0.6%	0.237	3.3%	1.294
DCP acetyl glucose conjugate	0.5%	0.006	< 0.1%	0.001	0.2%	0.074	0.4%	0.144
Unknown 1			4.0%	0.048	0.2%	0.084	0.9%	0.357
2,4-D	5.1%	0.060	4.3%	0.050	**34.7%**	**13.788**	**28.5%**	**11.347**
2,4-DCP	3.2%	0.037	**11.7%**	**0.138**	2.6%	1.017	**36.6%**	**14.548**
Unknown 2			1.5%	0.017				
Unknown 3			1.4%	0.017				

	Seed MeOH/water Total		Seed Hydrolyzed Total		Trash MeOH/water Total		Trash Hydrolyzed Total	
	% TRR	mg eq/kg	% TRR	mg eq/kg	% TRR	mg eq/kg	% TRR	mg eq/kg
Unknown 4					1.1%	0.450	7.0%‡	2.780
DCA							0.5%	0.205
Conjugated DCP or 4-CP (sum)	**22.5%**	**0.265**	0.4%	0.005	**38.1%**	**15.153**	2.2%	0.870

‡ Multiple components or not reproducible.

The analytical method uses a similar extraction method of methanol/1.0 N NaOH (90/10, v/v), demonstrating effectiveness toward extracting as well as stability of 2,4-D and 2,4-DCP using this solvent. Radiovalidation with this extraction solvent has shown it to be effective for conventional crops, AAD-1 corn and AAD-12 soy. Furthermore, the analytical method includes a similar acid hydrolysis as was used in this study: 1.7 N HCl at 90°C for 1 hour.

Several components in both the neutral organic extracts and the hydrolyzed extracts have been identified. The specificity of mass spectrometry (HPLC retention time and known molecular mass when compared to a reference standard) allowed for identification of several low level components, including some which were not observed by the radioactive HPLC detection methods used for quantitation purposes.

In both the seed and trash, the mono-glucose (major metabolite), glucose-acetyl, and glucose-sulfate conjugates of 2,4-DCP were confirmed by mass spectrometry. Additional minor metabolites that were confirmed by mass spectrometry included 4-OH-2,5-D (chlorine migration and hydroxylation), 4-CPAA (loss of a chlorine), and 4-CP (loss of acetic acid to phenol from 4-CPAA). Additional conjugates were suspected due to decreased levels after acid hydrolysis, e.g., the peaks eluting at 16, 17, and 17.7 minutes. The majority of these unknown conjugates are of 2,4-DCP, as shown by the significant increase of 2,4-DCP after hydrolysis, but slight increases of 4-CP may indicate 4-CP conjugates were also present at low levels in the original extracts.

The low levels of free DCP compared to conjugated forms indicated that conjugation was rapid and the preferential route of metabolism. Radioactivity was further incorporated into natural plant constituents demonstrating extensive metabolism of the applied 2,4-D. In neither nor AAD-12 soybeans was hydroxylation and subsequent chlorine migration observed; however, the overall metabolism is consistent with the metabolic pathway of 2,4-D in conventional (non-genetically modified) plants.

Metabolic pathway

The primary route of metabolism proceeds through loss of the acetic acid side chain to form the phenol. The phenol is then rapidly conjugated with glucose, which is subsequently further conjugated. Metabolism proceeds through natural incorporation of the radiolabeled carbon into natural plant constituents, such as lignin and cellulose. The minor metabolism pathways include de-chlorination and chlorine migration with hydroxylation, to form 4-CPAA and 4-OH-2,5-D, respectively. Additional minor degradation of 2,4-DCP and 4-CPAA may then occur to form 4-CP by de-chlorination.

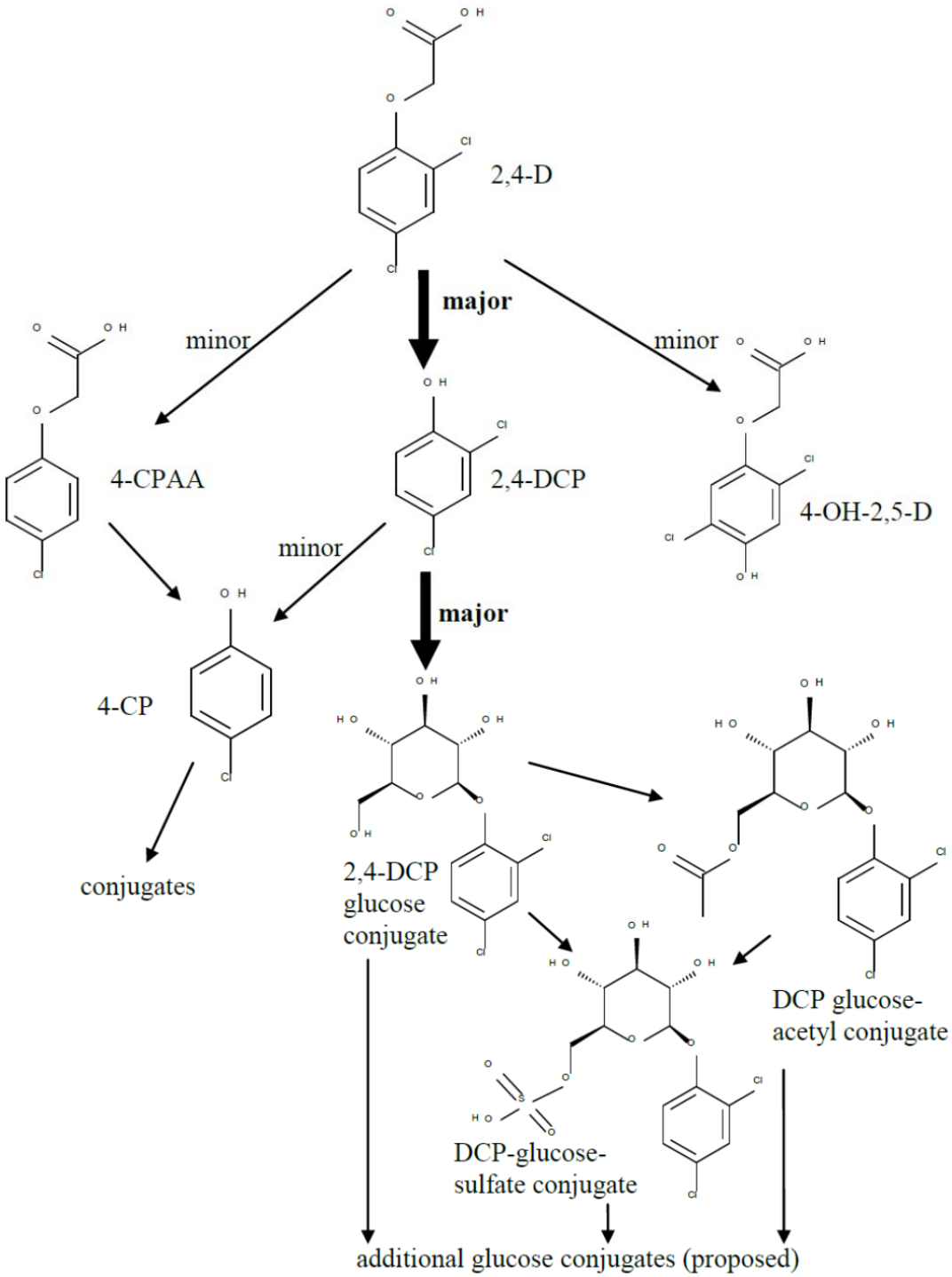


Figure 1 Proposed metabolic pathway of 2,4-D in AAD-12 cotton

RESIDUE ANALYSIS

Analytical methods

Analytical method for 2,4-D and its metabolite (2,4-DCP) in undelinted seed and gin by-products of AAD-12 cotton (Vespestad, D. 2014a, study No. 120430; Vespestad, D. 2014b, study No. 120431)

The analytical method "Procedure for the Analysis of 2,4-D and 2,4-DCP in Cotton Matrices by LC-MS/MS" was concurrently validated within the scope of the above two trial studies for residues of 2,4-D and its metabolite 2,4-DCP. The validated LOQ of the method was 0.01 mg/kg for 2,4-D and 2,4-DCP in undelinted cotton seed and cotton gin by-products.

Procedure: Residues of 2,4-D and 2,4-DCP were extracted from the sample matrices by shaking with a methanol:1.0M NaOH (90:10, v/v) solution. For Study No. 120430, an aliquot of each sample was taken and mixed with 5 mL of hexane. For both studies, after centrifuging, an aliquot was taken from the aqueous layer of the mixture, and a solution containing 0.1 mg/L of the mixed stable-isotope internal standards was added. The samples were mixed and concentrated to near dryness. Next, 500 µL of 2M HCl was added, and the acidified samples were incubated at 90 ± 5°C for a minimum of 60 minutes. After cooling, 500 µL of methanol was added to the hydrolyzed samples; samples were mixed by vortex and rolled, then filtered into vials. The samples were analyzed using an online reverse-phase polymeric solid-phase extraction (SPE) cartridge with liquid chromatography with negative-ion electrospray tandem mass spectrometry (LC-MS/MS). Tables 8 through to Table 11 summarize the recovery data from these method validation studies.

Table 8 Summary of 2,4-D Recovery Data for Validation of Method (Study No. 120430)

	Recovery Results				
Fortification (µg/g)	n	Range (%)	Mean (%)	Standard Deviation (%)	% RSD[a]
Undelinted Cotton Seed					
0.01	10	88–103	95	4.4	4.6
0.1	8	89–99	92	3.5	3.8
0.5	2	94	NA[b]	NA	NA
Overall	**20**	**88–103**			
Gin By-products					
0.01	8	87–108	94	6.1	6.5
0.5	4	88–94	91	2.7	3.0
0.7	3	94–100	97	3.0	3.1
Overall	**15**	**87–108**			

[a] RSD = Relative standard deviation

[b] NA = Not applicable

Table 9 Summary of 2,4-DCP Recovery Data for Validation of Method (Study No. 120430)

	Recovery Results				
Fortification (µg/g)	n	Range (%)	Mean (%)	Standard Deviation (%)	% RSD[a]
Undelinted Cotton Seed					
0.01	10	92–114	103	7.1	6.9
0.1	8	92–103	98	3.4	3.5
0.5	2	98–103	NA[b]	NA	NA
Overall	**20**	**92–114**			
Gin By-products					
0.01	8	78–106	92	8.8	9.6
0.5	4	92–97	94	1.9	2.1
0.7	3	88–105	95	8.9	9.4
Overall	**15**	**78–106**			

[a] RSD = Relative standard deviation

[b] NA = Not applicable

Table 10 Summary of 2,4-D Recovery Data for Validation of Method (Study No. 120431)

Fortification (µg/g)	Recovery Results n	Range (%)	Mean (%)	Standard Deviation (%)	% RSD[a]
Undelinted Cotton Seed					
0.01	4	81–96	90	6.6	7.3
0.5	4	84–97	90	5.1	5.6
Overall	**8**	**81–97**			
Hulls					
0.01	4	86–100	92	6.2	6.7
0.5	4	88–91	90	1.2	1.4
Overall	**8**	**86–100**			
Untoasted Meal					
0.01	4	82–100	92	8	8.6
0.5	4	82–91	87	4	4.6
Overall	**8**	**82–100**			
Toasted Meal					
0.01	4	84–97	91	6.7	7.3
0.5	4	75–94	84	10.4	12.4
Overall	**8**	**75–97**			
Crude Oil					
0.01	4	80–91	83	5.2	6.3
0.5	4	82–86	84	1.9	2.3
Overall	**8**	**80–91**			
Refined Oil					
0.01	4	79–95	87	7.5	8.6
0.5	4	89–100	95	5.3	5.6
Overall	**8**	**79–100**			

[a] RSD = Relative standard deviation

Table 11 Summary of 2,4-DCP Recovery Data for Validation of Method (Study No. 120431)

Fortification (µg/g)	Recovery Results n	Range (%)	Mean (%)	Standard Deviation (%)	% RSD[a]
Undelinted Cotton Seed					
0.01	4	76–107	95	14	14.7
0.5	4	90–98	96	3.5	3.7
Overall	**8**	**76–107**			
Hulls					
0.01	4	94–123	107	13.4	12.5
0.5	4	92–101	96	4.2	4.4
Overall	**8**	**92–123**			
Untoasted Meal					
0.01	4	103–112	108	4.4	4.1
0.5	4	92–99	95	3.1	3.2
Overall	**8**	**92–112**			
Toasted Meal					
0.01	4	101–116	107	6.7	6.2
0.5	4	84–105	93	10.1	10.8
Overall	**8**	**84–116**			
Crude Oil					
0.01	4	105–110	108	2.2	2.1
0.5	4	95–106	100	4.8	4.8
Overall	**8**	**95–110**			
Refined Oil					
0.01	4	98–112	107	6.2	5.9
0.5	4	94–96	95	0.7	0.8
Overall	**8**	**94–112**			

[a] RSD = Relative standard deviation

Stability of Pesticide Residues in Stored Analytical Samples

Freezer storage stability of 2,4-D and 2,4-DCP in samples of transgenic cotton and its processed fractions were studied at the fortitification level of 0.10 mg/kg at -20 °C (nominal) for a duration of 3 to 8 months. The residue levels of transgenic cotton and its processed fractions were analyzed after 1 month-, 2 month and 3 month storage. At additional interval of 6 months and 8 month for the undelinted seed and 6 months for gin by products storage stability was determined (Gesell, J.T., Smith, K. A. 2014; Report No. 130518). Samples were analysed using the same method as that employed in the two residue trial studies (Study No. 120430 and 120431, "Procedure for the Analysis of 2,4-D and 2,4-DCP in Cotton Matrices by LC-MS/MS").

The results of the study indicate that residues of 2,4-D and 2,4-DCP are stable in cotton gin by-products for at least 6 months and stable in cotton hulls, untoasted meal, toasted meal, crude oil, and refined oil for at least 3 months when stored at -20 °C. Additionally, the results of the study indicate that residues of 2,4-D and 2,4-DCP experienced some observable degradation in cotton undelinted seed during storage. (Tables 12 and 13).

Table 12 Summary Results of Frozen Storage Stability Samples for 2,4-D

Length of Frozen Storage (Months)	Average Uncorrected 2,4-D (mg/kg)	Percent Remaining	Procedural Recovery (%)
Seed			
0	0.092	100	92
1	0.064	70	98
2	0.061	66	95
3	0.065	71	94
6	0.062	67	89
8	0.063	68	93
Gin by-products			
0	0.090	100	88
1	0.090	100	96
2	0.091	101	92
3	0.086	96	89
6	0.091	101	89
Hulls			
0	0.092	100	96
1	0.079	86	96
2	0.083	90	96
3	0.087	94	100
Untoasted meal			
0	0.097	100	93
1	0.091	94	90
2	0.101	104	101
3	0.104	107	100
Toasted meal			
0	0.099	100	100
1	0.098	99	95
2	0.102	103	100
3	0.105	106	100
Crude oil			
0	0.093	100	92
1	0.094	101	88
2	0.102	110	100
3	0.105	113	104
Refined oil			
0	0.102	100	98
1	0.106	104	97
2	0.103	101	100
3	0.103	101	101

Table 13 Summary Results of Frozen Storage Stability Samples for 2,4-DCP

Length of Frozen Storage (Months)	Average Uncorrected 2,4-DCP (μg/g)	Average Uncorrected Percent Remaining	Procedural recovery (%)
Seed			
0	0.099	100	96
1	0.075	76	95
2	0.074	75	94
3	0.072	73	94
6	0.062	63	88
8	0.056	57	96
Gin by-products			
0	0.090	100	92
1	0.096	107	98
2	0.093	103	94
3	0.096	107	90
6	0.096	107	98
Hulls			
0	0.098	100	100
1	0.097	99	104
2	0.090	92	102
3	0.090	92	106
Untoasted meal			
0	0.104	100	102
1	0.104	100	95
2	0.103	99	101
3	0.103	99	97
Toasted meal			
0	0.102	100	100
1	0.112	110	104
2	0.102	100	100
3	0.105	103	102
Crude oil			
0	0.106	100	107
1	0.114	108	104
2	0.103	97	101
3	0.104	98	96
Refined oil			
0	0.103	100	102
1	0.105	102	107
2	0.102	99	100
3	0.105	102	102

USE PATTERN

2,4-D is an herbicide in the phenoxyacetic acid family that mimics the naturally occurring plant auxins and overloads the plant's auxin balance affecting vital processes such as cell division and elongation, resulting in abnormal growth and control of susceptible plants. It is used postemergence for selective control of broadleaf weeds in a number of crops and also in other sites including pasture and rangeland. 2,4-D is currently registered for use on a number of crops in many countries.

Many of the 2,4-D formulations in use contain the amine salts of 2,4-D, which are more water soluble than the acid, or the ester derivatives, which are readily dissolved in an organic solvent. There are two formulations containing 2,4-D in the USA related to the use on AAD-12 cotton. The two formulations contain 2,4-D in the form of 2,4-D choline, which is a quaternary ammonium salt.

Table 14 presents a summary of the GAP for use of 2,4-D choline salt in the in the USA in AAD-12 cotton. The application rate for 2,4-D in the GAP table as well as in the summary of supervised residue trials is presented based on 2,4-D acid (acid equivalent or a.e. basis). All the uses are ground broadcast spray in the field.

Table 14 Registered uses of 2,4-D for cotton

Crop	Country	Conc., g ai/L, Form	Application						PHI days
			Timing	g ae/hL	L/ha	g ae/ha	No.	Interval day	
AAD-12 Cotton	USA	455 SL	Preplant/preemergence + Postemergence (from crop emergence to full flowering; BBCH 65)	570-1130	94-140	800-1060	1 + 2	12 (between the 2nd and 3rd)	≥30
Cotton	USA	192 SL	Preplant/preemergence + Postemergence (from crop emergence to full flowering; BBCH 65)	570-1130	94-140	780-1060	1 + 2	12 (between the 2nd and 3rd)	≥30

RESULTS OF SUPERVISED RESIDUE TRIALS ON CROPS

AAD-12 Cotton

The critical GAP for residue trials with 2,4-D conducted in AAD-12 cotton in the USA is summarized in Table 15. As indicated in this summary table, the critical GAP is based on a total of three applications of 2,4-D, each at a rate of 1060 g eq/ha with one application made preemergence and two applications postemergence with the first postemergence application targeted at 12 days prior to the time when the crop would reach BBCH 65 (mid-bloom) when the final/second postemergence application would occur.

Table 15 Critical GAP for 2,4-D in USA Cotton Expressing AAD-12

Type of Application	Max. no. of Applications	Max. Rate (g ae/ha)	Min. Interval between Applications	Maximum Growth Stage at Final Application
Preemergence + Postemergence (Ground broadcast spray)	1 Preemergence + 2 Postemergence applications (Total of 3 applications for the season)	1060 (seasonal maximum of 3180)	12 days between postemergence applications	BBCH 65 (mid-bloom growth stage)

A total of 16 trials were carried out in the USA, where cotton is grown commercially, with 2,4-D on AAD-12 cotton during 2012 (Study ID 120430, Vespestad, 2014a). AAD-12 cotton contained the inserted Aryloxyalkanoate Dioxygenase-12 (*aad-12)* gene, which expresses the AAD-12 protein and is a genetic modification added to increase tolerance of cotton to the herbicide 2,4-D. The crop was grown according to typical commercial production practices at the trial sites. The application / use pattern of 2,4-D in these trials was in compliance with the critical GAP listed in Table 15.

Each trial included two plots: an untreated control plot and a treated plot that received three applications of an SL formulation of 2,4-D choline salt (formulation number GF-2654), at the target application rate of 1120 g acid equivalent (ae) per ha for each application, which is within the range of ± 25% of the critical GAP application rate of 1060 g ae/ha. the SL formulation was applied at pre-emergence, post-emergence at approximately 12 days before application 3, and post-emergence at the BBCH 65 growth stage (mid-bloom) at all site locations. Applications were performed with pressurized ground broadcast spray equipment, using a tractor-mounted boom or a backpack sprayer with a hand-held boom.

Seed cotton was collected from all plots at earliest crop maturity suitable for commercial harvest and was ginned to separate undelinted seed and cotton gin by-products from the lint within approximately 24 hours of harvest. Two independently collected replicate samples of seed cotton were obtained from the treated plot at each sampling interval, and one sample was obtained from

control plots, with each sample being obtained from at least 12 separate areas of the plot. Raw Agricultural Commodity (RAC) samples of undelinted cottonseed were collected from all trials and cotton gin by-products were collected from five selected trials in Oklahoma and Texas (120430 OK-2, TX-2, TX-3, TX-4, and TX-5). All cotton gin by-product samples were collected using cotton stripper harvest (USA EPA guidelines require data for cotton gin by-products on only stripper cotton from three field trials; but data from picker cotton are not required). At decline trials, undelinted cottonseed and cotton gin by-product samples were collected at five timing intervals; at 7 days prior to when cotton was expected to first reach maturity suitable for commercial harvest, at earliest crop maturity suitable for commercial harvest, and approximately 7 days, 14 days, and 21 days following earliest crop maturity sampling. Only undelinted cottonseed was collected from decline trial 120430 AR-1.

The samples of undelinted cottonseed and cotton gin by-products were placed into frozen storage generally within 4 hours after collection. Samples were stored frozen at field sites, and then shipped frozen to the analytical laboratory, where they were homogenized and held in frozen storage (maximum storage interval of 145 days for undelinted cottonseed and 161 days for cotton gin by-products) until analyzed for 2,4-D and 2,4-DCP. Results from a frozen storage stability study with 2,4-D and 2,4-DCP indicated at least 92%, if corrected for concurrent recovery) of both compounds remained in cotton gin byproducts for a period of 6 months/184 days (Gesell, 2014), which fully covers the storage period in the magnitude of residue study.

The LC-MS/MS method used for analysis of 2,4-D and 2,4-DCP was validated concurrently within the scope of the residue study. The analytical method had a limit of quantification (LOQ) of 0.01 µg/g, and a limit of detection (LOD) of 0.003 µg/g, for both 2,4-D and 2,4-DCP for the matrix types analyzed. Individual concurrent recoveries for undelinted cottonseed and cotton gin by-products ranged from 87 to 108% for 2,4-D and 78 to 114% for 2,4-DCP over all levels.

A summary of the 2,4-D and 2,4-DCP residue data for undelinted cottonseed and for cotton gin by-products for each of the sixteen trials conducted in the USA is presented in Table 16 and Table 18, respectively. Residue values from both replicate samples collected from each treated plot are presented, as well as an average of the two replicate values. Residue concentrations in study samples are reported as the uncorrected concentration. Analyte residues were detected in both treated sample matrices but were generally highest in cotton gin by-products. Decline data indicated that residues of 2,4-DCP in undelinted cottonseed and gin by-products generally decreased at longer days after the last application. In the decline trials, the samples collected at earliest crop maturity suitable for commercial harvest (second sampling interval) were selected as the samples used to represent the GAP residue values for that trial since this sample collection interval aligns with the interval targeted in other trials (i.e. at earliest crop maturity suitable for commercial harvest) and generally provided the highest level of residues.

In addition to results from the 16 critical GAP-compliant trials conducted in the USA, there are results from five trials conducted in Brazil during 2014-2015 that are also included in the residue trial summary presented in Table 16 (Study ID numbers 141060/141060.01). Registration of 2,4-D on GM cotton in Brazil is not anticipated until 2018, these trials were included in this document.

The targeted use pattern for the five trials conducted in Brazil (Study ID numbers 141060/141060.01) was as follows: a total of four applications of 2,4-D with each at a nominal rate of 1368 g ae/ha; one application was preplant (approximately 15 days before planting), one application preemergence, and two applications postemergence. Therefore, the total seasonal application rate for the Brazil trails targeted 5472 g ae/ha (4×1368 g ae/ha) with 2,736 g ae/ha (2×1368 g ae/ha) of the total seasonal rate applied after crop emergence (postemergence). By comparision, the critical GAP for the U.S. is for a total seasonal application rate of 3,180 g ae/ha (3×1060 g ae/ha) with 2,052 g ae/ha (2×1060 g ae/ha) applied after crop emergence.

The formulation applied in the Brazil trials was 455 SL. Applications were performed with pressurized ground broadcast spray equipment, using a backpack sprayer with a hand-held boom. There was a single untreated and treated plot at each trial site.

A single sample of seed cotton was collected from each the untreated and treated plot at sampling and the seed was mechanically separated, providing at least 1 kg of seed per sample. Samples of cotton gin by-products were not collected from these trials. Decline samples were collected in three of the five trials conducted in Brazil. Samples were analysed within 84 to 118 days of sample collection using the same LC-MS/MS analytical method used for the USA trials.

The sums of residues of 2,4-D and 2,4-DCP in undelinted cottonseed and cotton gin by products from the field residue study are presented in Table 17 and

Table 19, respectively.

In Table 16 and 18, 2,4-DCP residues are expressed (uncorrected) as 2,4-DCP.

Table 16 Summary of 2,4-D Residue Trial Data in Undelinted Cotton Seed from Cotton Expressing AAD-12

Trial ID / Country / Year	Form-ulation	No. of Apps.	Rate (g ae/ha)	DALA (days)	2,4-D (mg/kg)		2,4-DCP (mg/kg)*		Reference (Study ID No.)
					Rep. Samples	Avg. Residue [a]	Rep. Samples	Avg. Residue [b]	
120430 AR-1 / USA / 2012	GF-2654	3	1122 + 1121 + 1124	51	< 0.01	< 0.01	0.093	0.092	120430
					< 0.01		0.090		
				58 [c]	< 0.01	**ND**	0.061	*0.074*	
					ND		0.086		
				65	0.012	< 0.01	0.043	0.043	
					ND		0.043		
				72	ND	ND	0.041	0.044	
					ND		0.046		
				79	ND	ND	0.042	0.034	
					ND		0.025		
120430 CA-1 / USA / 2012	GF-2654	3	1147 + 1128 + 1143	81	< 0.01	**ND**	0.076	0.079	120430
					ND		0.082		
120430 CA-2 / USA / 2012	GF-2654	3	1113 + 1140 + 1096	113	ND	**ND**	0.049	0.041	120430
					ND		0.033		
120430 CA-3 / USA / 2012	GF-2654	3	1129 + 1115 + 1146	79	0.011	**0.016**	0.075	0.070	120430
					0.020		0.065		
120430 CA-4 / USA / 2012	GF-2654	3	1126 + 1127 + 1122	57	0.055	**0.070**	0.093	0.099	120430
					0.084		0.105		
120430 GA-1 / USA / 2012	GF-2654	3	1150 + 1133 + 1126	74	ND	**ND**	0.050	0.048	120430
					ND		0.046		
120430 MO-1 / USA / 2012	GF-2654	3	1122 + 1128 + 1125	79	ND	**ND**	0.012	0.012	120430
					ND		0.012		
120430 MS-1 / USA / 2012	GF-2654	3	1137 + 1149 + 1113	77	ND	**ND**	0.024	0.024	120430
					ND		0.023		
120430 MS-2 / USA / 2012	GF-2654	3	1079 + 1124 + 1121	84	ND	**ND**	0.032	0.032	120430
					ND		0.031		
120430 NC-1 / USA / 2012	GF-2654	3	1117 + 1120 + 1139	84	ND	**ND**	0.076	0.082	120430
					ND		0.087		
120430 OK-1 / USA / 2012	GF-2654	3	1131 + 1096 + 1098	87	ND	**ND**	0.039	0.035	120430
					ND		0.030		
120430 OK-2 / USA / 2012	GF-2654	3	1139 + 1101 + 1106	70	ND	**ND**	0.116	0.152	120430
					ND		0.188		
120430 TX-	GF-	3	1124 +	81	< 0.01	**< 0.01**	0.158	0.140	120430

Trial ID / Country / Year	Form-ulation	No. of Apps.	Rate (g ae/ha)	DALA (days)	2,4-D (mg/kg)		2,4-DCP (mg/kg)*		Reference (Study ID No.)
					Rep. Samples	Avg. Residue [a]	Rep. Samples	Avg. Residue [b]	
2/ USA / 2012	2654		1132 + 1128		< 0.01		0.122		
120430 TX-3 / USA / 2012	GF-2654	3	1127 + 1126 + 1116	61	< 0.01	< 0.01	0.069	0.064	120430
					< 0.01		0.058		
				69 [c]	< 0.01	**< 0.01**	0.033	0.034	
					< 0.01		0.034		
				76	ND	ND	0.026	0.029	
					ND		0.031		
				83	< 0.01	< 0.01	0.046	0.040	
					< 0.01		0.034		
				90	< 0.01	< 0.01	0.041	0.040	
					< 0.01		0.039		
120430 TX-4 / USA / 2012	GF-2654	3	1119 + 1111 + 1112	79	< 0.01	ND	0.140	0.124	120430
					ND		0.108		
				86 [c]	< 0.01	**0.014**	0.163	*0.148*	
					0.022		0.132		
				93	< 0.01	< 0.01	0.134	0.148	
					< 0.01		0.162		
				100	< 0.01	< 0.01	0.196	0.172	
					< 0.01		0.148		
				107	< 0.01	< 0.01	0.228	0.211	
					< 0.01		0.194		
120430 TX-5 / USA / 2012	GF-2654	3	1112 + 1130 + 1125	82	ND	ND	0.080	0.074	120430
					ND		0.068		
				89 [c]	ND	**ND**	0.091	*0.081*	
					ND		0.071		
				96	ND	ND	0.052	0.051	
					ND		0.050		
				103	ND	ND	0.041	0.041	
					ND		0.041		
				111	ND	ND	0.052	0.058	
					ND		0.064		
GO1 / Brazil / 2014	GF-3073	4	1382 + 1315 + 1363 + 1356	125	ND	—	ND	—	141060/ 141060.01
MG1 / Brazil / 2014	GF-3073	4	1361 + 1334 + 1505 + 1368	125	ND	—	ND	—	141060/ 141060.01
SP1 / Brazil / 2014	GF-3073	4	1402 + 1327 + 1343 + 1382	111	ND	—	0.024	—	141060/ 141060.01
				117	ND	—	0.026	—	
				125	ND	—	0.028	—	
				132	ND	—	0.035	—	
				139	ND	—	0.031	—	
SP2 / Brazil / 2014	GF-3073	4	1334 + 1348 + 1382 + 1368	111	ND	—	ND	—	141060/ 141060.01
				117	ND	—	ND	—	
				125	ND	—	ND	—	
				132	ND	—	ND	—	
				139	ND	—	0.013	—	
SP3 / Brazil / 2014	GF-3073	4	1368 + 1348 + 1320 + 1402	119	ND	—	0.016	—	141060/ 141060.01
				125	ND	—	ND	—	
				131	ND	—	ND	—	
				139	ND	—	ND	—	

* Including conjugates.

[a] 2,4-D values underlined are considered compliant with the critical GAP and are selected for use in MRL calculation

[b] 2,4-DCP values shown in italics in decline trials are those for the sampling that is the earliest mature harvest

[c] Sampling interval for crop maturity at normal commercial harvest in decline trials

Table 17 Sum of 2,4-D and 2,4-DCP Residues in Undelinted Cotton Seed, Expressed as 2,4-D Equivalents—Results from USA Trials

Field Trial ID[a]	Rep	Residues (mg/kg)				
		2,4-D	2,4-DCP	2,4-DCP in 2,4-D Equivalents[b]	Sum of 2,4-D and 2,4-DCP[c]	Trial Site Average[d]
120430 CA-1	1	< 0.01	0.076	0.103	0.113	0.117
	2	ND	0.082	0.111	0.121	
120430 CA-2	1	ND	0.049	0.066	0.076	0.066
	2	ND	0.033	0.045	0.055	
120430 CA-3	1	0.011	0.075	0.102	0.113	0.110
	2	0.02	0.065	0.088	0.108	
120430 CA-4	1	0.055	0.093	0.126	0.181	0.204
	2	0.084	0.105	0.142	0.226	
120430 GA-1	1	ND	0.050	0.068	0.078	0.075
	2	ND	0.046	0.062	0.072	
120430 NC-1	1	ND	0.076	0.103	0.113	0.121
	2	ND	0.087	0.118	0.128	
120430 MO-1	1	ND	0.012	0.016	0.026	0.026
	2	ND	0.012	0.016	0.026	
120430 MS-1	1	ND	0.024	0.033	0.043	0.042
	2	ND	0.023	0.031	0.041	
120430 MS-2	1	ND	0.032	0.043	0.053	0.053
	2	ND	0.031	0.042	0.052	
120430 OK-1	1	ND	0.039	0.053	0.063	0.057
	2	ND	0.030	0.041	0.051	
120430 OK-2	1	ND	0.116	0.157	0.167	0.216
	2	ND	0.188	0.255	0.265	
120430 AR-1	1	< 0.01	0.061	0.083	0.093	0.110
	2	ND	0.086	0.117	0.127	
120430 TX-2	1	< 0.01	0.158	0.214	0.224	0.200
	2	< 0.01	0.122	0.165	0.175	
120430 TX-3	1	< 0.01	0.033	0.045	0.055	0.055
	2	< 0.01	0.034	0.046	0.056	
120430 TX-4	1	< 0.01	0.163	0.221	0.231	0.216
	2	0.022	0.132	0.179	0.201	
120430 TX-5	1	ND	0.091	0.123	0.133	0.120
	2	ND	0.071	0.096	0.106	

[a] Field Trial ID and residue data for 2,4-D and 2,4-DCP in undelinted cottonseed taken from Study No. 120430.

[b] 2,4-DCP in 2,4-D Equivalents was calculated by multiplying the 2,4-DCP residue (including conjugates) value by the ratio of the molecular weights of 2,4-D and 2,4-DCP, which is 221 / 163 or 1.3558.

[c] Sum of 2,4-D and 2,4-DCP expressed in 2,4-D equivalents. When the 2,4-D residue was ND (not detected, < limit of detection of 0.003 mg/kg) or < 0.01 mg/kg, a value of 0.01 mg/kg (equal to the LOQ) was assigned as a conservative approach for calculation of the sum of 2,4-D and 2,4-DCP, expressed in 2,4-D equivalents.

Cotton gin by-products

Table 18 Summary of 2,4-D Residue Trial Data in Cotton Gin By-Products from Cotton Expressing AAD-12

Trial ID / Country / Year	Form-ulation	No. of Apps.	Rate (g ae/ha)	DALA (days)	2,4-D (mg/kg)		2,4-DCP (mg/kg)*		Reference (Study ID No.)
					Rep. Samples	Avg. Residue [a]	Rep. Samples	Avg. Residue [b]	
120430 OK-2 / USA / 2012	GF-2654	3	1139 + 1101 + 1106	70	ND	**ND**	2.94	2.99	120430
					ND		3.04		
120430 TX-2 / USA / 2012	GF-2654	3	1124 + 1132 +	81	0.574	**0.557**	14.6	14.7	120430
					0.539		14.7		

Trial ID / Country / Year	Form-ulation	No. of Apps.	Rate (g ae/ha) 1128	DALA (days)	2,4-D (mg/kg)		2,4-DCP (mg/kg)*		Reference (Study ID No.)
					Rep. Samples	Avg. Residue [a]	Rep. Samples	Avg. Residue [b]	
120430 TX-3 / USA / 2012	GF-2654	3	1127 + 1126 + 1116	61	0.018	0.022	0.526	0.440	120430
					0.025		0.353		
				69[c]	0.040	**0.039**	0.374	*0.307*	
					0.038		0.239		
				76	0.040	0.044	0.215	0.217	
					0.047		0.219		
				83	0.040	0.034	0.179	0.174	
					0.027		0.169		
				90	0.034	0.034	0.162	0.161	
					0.033		0.159		
120430 TX-4 / USA / 2012	GF-2654	3	1119 + 1111 + 1112	79	0.182	0.180	5.56	5.63	120430
					0.177		5.70		
				86[c]	0.334	**0.282**	8.80	*7.77*	
					0.230		6.74		
				93	0.161	0.256	6.08	8.89	
					0.350		11.7		
				100	0.266	0.282	8.36	7.78	
					0.298		7.20		
				107	0.270	0.263	5.92	6.69	
					0.256		7.46		
120430 TX-5 / USA / 2012	GF-2654	3	1112 + 1130 + 1125	82	0.224	0.121	2.40	2.57	120430
					0.018		2.74		
				89[c]	0.113	**0.108**	2.24	*2.45*	
					0.103		2.66		
				96	0.252	0.166	1.55	1.45	
					0.079		1.34		
				103	0.119	0.093	1.09	1.09	
					0.067		1.08		
				111	0.087	0.136	1.97	1.75	
					0.184		1.52		

* Including conjugates.

[a] 2,4-D values underlined are considered compliant with the critical GAP and are selected for use in MRL calculation

[b] 2,4-DCP values shown in italics in decline trials are those for the sampling that is the earliest mature harvest

[c] Sampling interval for crop maturity at normal commercial harvest in decline trials

[d] The Trial Site Average is the average of the two independently collected replicate samples from the treated plot at each trial site. The residue values are based on those for the sum of 2,4-D and 2,4-DCP, expressed in 2,4-D equivalents. The maximum value across these trial site averages is considered as the HAFT for the sum of 2,4-D and 2,4-DCP, expressed in 2,4-D equivalents.

Table 19 Sum of 2,4-D and 2,4-DCP Residues in Cotton Gin By-products, Expressed as 2,4-D Equivalents—Results from USA Trials

Field Trial ID[a]	Rep	Residues (µg/g)				
		2,4-D	2,4-DCP	2,4-DCP in 2,4-D Equivalents[b]	Sum of 2,4-D and 2,4-DCP[c]	Trial Site Average[d]
120430 OK-2	1	ND	2.94	3.986	3.996	4.064
	2	ND	3.04	4.122	4.132	
120430 TX-2	1	0.574	14.6	19.795	20.369	20.419
	2	0.539	14.7	19.931	20.470	
120430 TX-3	1	0.040	0.374	0.507	0.547	0.455
	2	0.038	0.239	0.324	0.362	
120430 TX-4	1	0.334	8.80	11.931	12.265	10.817
	2	0.230	6.74	9.138	9.368	
120430 TX-5	1	0.113	2.24	3.037	3.150	3.430

	2	0.103	2.66	3.607	3.710	

[a] Field Trial ID and residue data for 2,4-D and 2,4-DCP in cotton gin by-products taken from Study No. 120430.

[b] 2,4-DCP in 2,4-D Equivalents was calculated by multiplying the 2,4-DCP residue value by the ratio of the molecular weights of 2,4-D and 2,4-DCP, which is 221 / 163 or 1.3558.

[c] Sum of 2,4-D and 2,4-DCP (including conjugates) expressed in 2,4-D equivalents. When the 2,4-D residue was ND (not detected, < limit of detection of 0.003 mg/kg) or < 0.01 mg/kg, a value of 0.01 mg/kg (equal to the LOQ) was assigned as a conservative approach for calculation of the sum of 2,4-D and 2,4-DCP, expressed in 2,4-D equivalents.

[d] The Trial Site Average is the average of the two independently collected replicate samples from the treated plot at each trial site. The residue values are based on those for the sum of 2,4-D and 2,4-DCP, expressed in 2,4-D equivalents. The maximum value across these trial site averages is considered as the HAFT for the sum of 2,4-D and 2,4-DCP, expressed in 2,4-D equivalents.

FATE OF RESIDUES IN STORAGE AND IN PROCESSING

Cotton

The potential for concentration of 2,4-D and its metabolite 2,4-DCP in processed commodities produced from undelinted cottonseed was evaluated in a study conducted in the USA using two field trial sites (Study ID no. 120431, Vespestad, 2014b). In this study, the two field trial sites were located in the states of Georgia (GA) and Texas (TX) and the trials were carried out during the 2012 growing season. The field trials were used to to produce the undelinted cottonseed Raw Agricultural Commodity (RAC) samples used for generating the processed commodities.

One untreated control plot and two treated plots were established at both trial site locations using AAD-12 cotton. The treated plots received three exaggerated rate applications each of a SL formulation containing 2,4-D choline salt (formulation number GF-2654). The target rate for each application of the first treated plot was 2240 g ae/ha (2×, Plot T2), and 4480 g ae/ha (4×, Plot T3) in each of three applications was the target rate for the second treated plot. An application rate higher than the 4× rate was not used due to the concern over the potential for crop injury. The timing/crop growth stage for the three applications in the two treated plots at both trial site locations were scheduled as follows: (1) pre-emergence, (2) post-emergence at approximately 12 days before Application 3, and (3) Application 3 targeted at the BBCH 65 (mid-bloom) growth stage. Applications were performed with pressurized ground boom broadcast spray equipment, using a tractor-mounted boom.

At each site, one bulk undelinted cottonseed sample was collected from each of the untreated and treated plots at both test sites for generating processed commodities. The bulk samples of undelinted seed were shipped frozen from the field trial facilities to a facility for processing.

The bulk undelinted cottonseed samples were separately processed using simulated industrial processing procedures to produce commercially representative samples of the processed commodities hulls, meal (toasted meal as well as untoasted presscake meal from cold press extraction), crude oil, and refined oil. Prior to generating the processed commodities, two replicate samples of undelinted seed were collected from each bulk sample of seed for residue analysis since the residue concentration in these samples were to be used for comparison with residue concentration in the processed commodities. The samples of undelinted seed as well as samples of processed commodities collected for residue analysis were stored frozen at the processing facility and then were shipped frozen for analysis.

The undelinted cottonseed and processed commodity samples were analyzed for residues of 2,4-D and 2,4-DCP using the same LC-MS/MS method used for analysis of field residue trials in study no. 120430.

The undelinted cottonseed samples collected at the processing facility just prior to beginning processing and processed commodity samples were stored frozen, from collection to extraction for analysis, for a maximum of 91 days. Although the total storage period for the undelinted seed from the time of harvest until extraction for analysis was 234 days (7.8 months), the storage period from collection of the undelinted seed at the processing facility until extraction for analysis is considered as

the more relevant storage period since residue values in processed fractions are compared to residues in the undelinted RAC seed samples collected at the processing facility. Results from a frozen storage stability study with 2,4-D and 2,4-DCP indicated an average of 69% (corrected) 2,4-D and 76% (corrected) 2,4-DCP remained in undelinted seed after frozen storage for 3 months/90 days (Gesell, 2014). In the processed fractions (hulls, meal, and oil), the average corrected amount of 2,4-D and 2,4-DCP remaining was at least 88% and 84%, respectively. Therefore, for purposes of determining level of transfer or concentration of residues from the undelinted cottonseed to process fractions, it is considered that there was sufficient storage stability for both 2,4-D and 2,4-DCP.

A summary of the residue results for seed and processed commodities produced from the seed from both the 2× and 4× rate treatments for seed from both trial sites is presented in Table 20. A summary of mean residue values by trial and treatment for the undelinted cottonseed samples collected at the processor and the processed commodities, and associated Processing Factors (PFs) is presented in Table 21.

This study provides evidence indicating that significant concentration of 2,4-D residues in processed products produced from cottonseed is not expected. Although 2,4-D was not detected in most of the samples of undelinted seed in this study, it was detected at < 0.01 mg/kg in seed from a 4X rate trial conducted in Texas. Since no residue of 2,4-D was detected in processed products (hulls, meal or oil) produced from this seed, the study is considered to provide evidence of no significant concentration of 2,4-D in the processed products hulls, meal or oil. With regard to 2,4-DCP, although residues did not concentrate in oil and residues were slightly higher than seeds in hulls (overall average Processing Factor of 1.16 in hulls, typical of any chemical used on cotton). There was concentration of residues in meal with an overall average Processing Factor across both treatments and both trial sites for untoasted meal (meal from cold press extraction) and toasted meal of 2.10 and 1.86, respectively. These processing factors could be used in estimation of livestock dietary burden if 2,4-DCP is included in the livestock diet for purposes of estimating human dietary exposure and risk

Table 20 Summary of Processing Study Residue Data for 2,4-D in/on Cotton Expressing AAD-12 (Study No. 120431)

Field Trial ID (City, State)	Appl. Method	No	Spray Vol L/ha	Appl. Rate[a,b] g ae/ha	Spray Int. (days)	DALA (day)	Cotton Commodity / Matrix	2,4-D mg/kg[c]	2,4-D Site Mean, mg/kg[c,d]	2,4-D mg/kg[c]	2,4-DCP Site mg/kg[c,d]
GA-1	Preemergence	3	230	2263	-	107	Undelinted seed	ND	ND	0.0113	(0.0094)
(Sycamore, GA)	Foliar		228	2243	54	107	Undelinted seed	ND		(0.0075)	
2× rate treatment	Foliar		231	2246	11		Hulls	ND	ND	0.0155	0.0153
		Seasonal Total =		6752			Hulls	ND		0.0150	
							Untoasted meal	ND	ND	0.0204	0.0240
							Untoasted meal	ND		0.0276	
							Toasted meal	ND	ND	0.0250	0.0262
							Toasted meal	ND		0.0274	
							Crude oil	ND	ND	(0.0049)	(0.0051)
							Crude oil	ND		(0.0053)	
							Refined oil	ND	ND	ND	ND
							Refined oil	ND		ND	
GA-1	Preemergence	3	229	4507	-	107	Undelinted seed	ND	ND	0.0121	0.0133
(Sycamore, GA)	Foliar		227	4483	54	107	Undelinted seed	ND		0.0145	
4× rate treatment	Foliar		231	4485	11		Hulls	ND	ND	0.0267	0.0227
		Seasonal Total =		13,475			Hulls	ND		0.0187	
							Untoasted meal	ND	ND	0.0346	0.0376
							Untoasted meal	ND		0.0405	
							Toasted meal	ND	ND	0.0367	0.0368
							Toasted meal	ND		0.0368	
							Crude oil	ND	ND	(0.0066)	(0.0074)
							Crude oil	ND		(0.0082)	
							Refined oil	ND	ND	ND	ND
							Refined oil	ND		ND	

Field Trial ID (City, State)	Appl. Method	No	Spray Vol L/ha	Appl. Rate[a,b] g ae/ha	Spray Int. (days)	DALA (day)	Cotton Commodity / Matrix	2,4-D mg/kg[c]	2,4-D Site Mean, mg/kg[c,d]	2,4- mg/kg[c]	2,4-DCP Site mg/kg[c,d]
TX-1	Preemergence	3	248	2259	-	112	Undelinted seed	ND	ND	0.0393	0.0485
(Groom, TX)	Foliar		249	2275	44	112	Undelinted seed	ND		0.0576	
2× rate treatment	Foliar		256	2336	12		Hulls	ND	ND	0.0284	0.0303
		Seasonal Total =		6870			Hulls	ND		0.0321	
							Untoasted meal	ND	ND	0.0565	0.0615
							Untoasted meal	ND		0.0664	
							Toasted meal	ND	ND	0.0359	0.0368
							Toasted meal	ND		0.0376	
							Crude oil	ND	ND	ND	0.0155
							Crude oil	ND		0.0309	
							Refined oil	ND	ND	ND	ND
							Refined oil	ND		ND	
TX-1	Preemergence	3	248	4514	-	112	Undelinted seed	ND	(0.0042)	0.0455	0.0685
(Groom, TX)	Foliar		245	4492	44	112	Undelinted seed	(0.0083)		0.0915	
4× rate treatment	Foliar		246	4480	12		Hulls	ND	ND	0.0461	0.0475
		Seasonal Total =		13,486			Hulls	ND		0.0488	
							Untoasted meal	ND	ND	0.107	0.121
							Untoasted meal	ND		0.135	
							Toasted meal	ND	ND	0.0791	0.0785
							Toasted meal	ND		0.0778	
							Crude oil	ND	ND	0.0726	0.0734
							Crude oil	ND		0.0741	
							Refined oil	ND	ND	ND	ND
							Refined oil	ND		ND	

Residues of 2,4-DCP include conjugates.

[a] GF-2654, a SL formulation containing 2,4-D, choline salt, was applied in each of three applications at a target rate of 2242 g ae/ha (2×rate) or 4483 g ae/ha (4× rate) with the first application made preemergence and two foliar applications in approximately a 12-day interval with the final application at mid-bloom (BBCH 65).

[b] Each application included a non-ionic surfactant (NIS) in the spray mixture at approximately 0.25% (v/v)

[c] ND = Not detected; less than the LOD (< 0.003 mg/kg); Values displayed in parentheses indicate a concentration < LOQ (0.01 mg/kg) and ≥ LOD (0.003 mg/kg)

[d] To facilitate calculation of average values, residues that were "ND" (<LOD of 0.003 mg/kg) were assigned a value of zero and residues that were detected (i.e. ≥ LOD of 0.003 mg/kg), but were < LOQ of 0.01 mg/kg were assigned the reported numerical residue value (i.e. values displayed within parentheses)

Table 21 Summary of Processing of Cotton Seed (2,4-D and 2,4-DCP)

Commodity	Plot T2 (2× Application Rate) Mean Residues (mg/kg)[a]		Processing Factor[b]		Plot T3 (4× Application Rate) Mean Residues (mg/kg)[a]		Processing Factor[b]	
	2,4-D	2,4-DCP	2,4-D	2,4-DCP	2,4-D	2,4-DCP	2,4-D	2,4-DCPP
Cotton Trial 120431 GA-1								
Undelinted Cotton Seed RAC[c]	ND	<LOQ (0.0094)	-	-	ND	0.0133	-	-
Hulls	ND	0.0153	NC	1.63	ND	0.0227	NC	1.71
Untoasted Meal	ND	0.0240	NC	2.55	ND	0.0376	NC	2.83
Toasted Meal	ND	0.0262	NC	2.79	ND	0.0368	NC	2.77
Crude Oil	ND	<LOQ (0.0051)	NC	0.54	ND	<LOQ (0.0074)	NC	0.56
Refined Oil[d]	ND (0.0015)	ND (0.0015)	NC	0.16	ND (0.0015)	ND (0.0015)	NC	0.11
Cotton Trial 120431 TX-1								
Undelinted Cotton Seed	ND	0.0485	-	-	<LOQ (0.0042)	0.0685	-	-

Commodity	Plot T2 (2× Application Rate) Mean Residues (mg/kg)[a]		Processing Factor[b]		Plot T3 (4× Application Rate) Mean Residues (mg/kg)[a]		Processing Factor[b]	
	2,4-D	2,4-DCP	2,4-D	2,4-DCP	2,4-D	2,4-DCP	2,4-D	2,4-DCPP
RAC[c]								
Hulls	ND	0.0303	NC	0.62	ND	0.0475	NC	0.69
Untoasted Meal	ND	0.0615	NC	1.26	ND	0.1210	NC	1.77
Toasted Meal	ND	0.0368	NC	0.76	ND	0.0785	NC	1.15
Crude Oil	ND	0.0155	NC	0.32	ND	0.0734	NC	1.07
Refined Oil[d]	ND (0.0015)	ND (0.0015)	NC	0.03	ND (0.0015)	ND (0.0015)	0.36	0.02

Residues of 2,4-DCP include conjugates.

[a] "ND" indicates residues below the analytical method LOD of 0.003 mg/kg. "<LOQ" indicates residues between the LOD and LOQ (0.01 mg/kg). For calculation of Processing factors (PF), "ND" values in individual samples were assumed to equal zero, and values <LOQ were assumed to equal the estimated value reported.

[b] PFs were not calculable where the concentration in the RAC is below the LOD (indicated as ND), and in the table described as "NC".

[c] Mean undelinted cottonseed values are the average of processor collected RAC samples

[d] These values have been adjusted using value a of ½ LOD (0.0015 μg/g) for ND values.

Table 22 Summary of Processing Factors of Cotton Seed Processing (2,4-D and 2,4-DCP)(Based on Table 21)

	2,4-D		2,4-DCP	
	Precessing factors	Best estimate	Precessing factors	Best estimate
RAC: Undelinted Cotton Seed				
Hulls	NC, NC, NC, NC	NC	0.62, 0.69, 1.63, 1.71	1.16
Untoasted Meal	NC, NC, NC, NC	NC	1.26, 1.77, 2.55, 2.83	2.16
Toasted Meal	NC, NC, NC, NC	NC	0.76, 1.15, 2.77, 2.79	1.96
Crude Oil	NC, NC, NC, NC	NC	0.32, 0.54, 0.56, 1.07	0.55
Refined Oil[d]	NC, NC, NC, 0.36	NC	0.02, 0.03, 0.11, 0.16	0.07

RESIDUES IN ANIMAL COMMODITIES

Livestock Feeding Studies

Relevant studies were previously reviewed by JMPR.

As presented in the 1998 JMPR Report, groups of three cows were dosed at four dose levels equal to 1446, 2890, 5779, and 8585 ppm 2,4-D ae in the diet on a dry weight basis for 28 to 30 consecutive days. Two further groups were treated at the high dose level for 28 days and slaughtered 3 or 7 days after the last dose. The highest residues were in the kidneys, followed in decreasing order by liver, fat, muscle and milk. This relationship was generally consistent in all four dose groups. The residue levels were generally dose-dependent, except in fat where the mean residue in the high dose group was slightly lower than that in the medium-high group, indicating that a plateau level had been reached in fat.

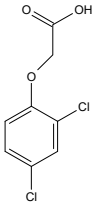
As presented in the 1998 JMPR Evaluation, residues in the tissues, eggs, and excreta were measured in laying hens (three groups of five, each bird weighting 1.5 kg) dosed orally for 7 days with radiolabelled capsules of 2,4-D approximately equivalent to 18 ppm in the feed intake (112–119 g/bird/day). The eggs and excreta were collected throughout the 7 days, and the birds were killed 22–24 hours after the final dose. In hens dosed orally with [^{14}C] 2,4-D, about 90% of the dose was recovered from the excreta. The edible tissues and eggs each contained < 0.1% of the total dose.

APPRAISAL

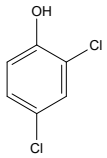
The herbicide 2,4-D, 2,4-dichlorophenoxyacetic acid, is registered in a variety of salt, amine, and ester formulations for control of broadleaf weeds in a variety of food and feed uses. As a synthetic auxin herbicide, 2,4-D causes disruption of plant hormone responses. It is currently registered in many countries.

It was first evaluated by JMPR in 1970. It was subsequently evaluated in 1986, 1987, 1996, 1997, 1998 and 2001. The 1998 JMPR evaluated it under the Periodic Reevaluation Programme. Its specification was established by the Joint FAO/WHO Meeting on Specifications in 1994. The present ADI is 0–0.01 mg/kg bw for sum of 2,4-D and its salts and esters expressed as 2,4-D, and ARfD is unnecessary. The present residue definition established by the 1998 JMPR is 2,4-D for enforcement of MRLs and for dietary intake estimation for plant and animal commodities. The residue is not fat-soluble.

2,4-D was scheduled for JMPR evaluation in the year 2017 for consideration of residues arising from a GM cotton crop. There are a number of Codex MRLs but no MRL has been set for cotton seed.



2,4-D



2,4-DCP

Plant metabolism

The 1998 JMPR evaluated plant metabolism studies on apple, lemon, potato and wheat. The predominant residue was 2,4-D in these plants, except in the apple study in which radioactivity was too low to identify.

The current Meeting received information on GM cotton, in which expression of the α-ketoglutarate-dependent aryloxyalkonoate dioxygenase-12 (AAD-12) protein confers tolerance to 2,4-D and associated increased metabolism of 2,4-D (hereafter referred to as AAD-12 cotton).

A plot of AAD-12 cotton was treated with [^{14}C-]-2,4-D choline (2,4-dichlorophenoxy acetic acid choline salt labelled at phenyl ring) at the maximum seasonal rate in the USA of 3.3 kg ai/ha (three applications each at 1.1 kg ai/ha at pre-emergence, BBCH 61, and BBCH 65 with an interval of 12 days). The 2,4-D choline was formulated as a soluble concentrate. The cotton was grown outdoors to maturity. Cotton bolls were harvested when the lower bolls were mature, unlike in commercial production.

Total radioactive residue (TRR) 56 days after the last application was 1.2 mg eq/kg in cotton seed and 40 mg eq/kg in cotton trash.

A series of extractions with hexane, methanol/water (9:1) and methanol/2M NaOH extracted 32% TRR in seed and 83% TRR in cotton trash (no hexane extraction). Refluxing in 2M NaOH further extracted 32% TRR in seed and 12% TRR in trash; and refluxing in 2M HCl extracted 3% TRR from seed. After these treatments, the unextracted radioactivity was 31% TRR in the seed and 3.4% TRR in trash. The unextracted radioactivity was attributed to pectin, lignin, acid-detergent fiber and cellulose. Overall, 98% of the applied radioactivity was recovered from the seed and trash.

2,4-D was metabolized into numerous components and a number of radioacitive components were identified. Major residues identified were parent 2,4-D and conjugates of 2,4-DCP. Other metabolites were also identified but at lower rates of <5% TRR.

Cotton seed: In the methanol/water extract, 2,4-D accounted for 4.8% TRR (0.057 mg eq/kg) and free and conjugated 2,4-DCP accounted for 8.3% TRR (0.099 mg eq/kg), mostly in the form of sulphate-glucose conjugate (4.7% TRR). In the methanol/NaOH extract, 2,4-D accounted for less than 0.1% TRR (0.001 mg eq/kg) and free and conjugated 2,4-DCP accounted for around 2.7% TRR (0.032 mg eq/kg). In these extracts, hydrolysis released free 2,4-DCP effectively and hydrolysed 2,4-D to a certain extent to produce 2,4-DCP. 2,4-D and free and conjugated 2,4-DCP were below 2% TRR in the 2M NaOH extract.

Cotton trash: In the methanol/water extract, 2,4-D accounted for 31% TRR (12 mg eq/kg) and free and conjugated 2,4-DCP accounted for 33% TRR (13 mg eq/kg), mostly in the form of sulphate-glucose conjugate (23% TRR). In the methanol/NaOH extract, 2,4-D accounted for 2.5% TRR (0.99 mg eq/kg) and free and conjugated 2,4-DCP accounted for around 2.5% TRR (0.98 mg eq/kg). In these extracts, hydrolysis released free 2,4-DCP effectively and hydrolysed 2,4-D to a certain extent to produce 2,4-DCP. 2,4-D and free and conjugated 2,4-DCP were below 2% TRR in the 2M NaOH extract.

The metabolism of 2,4-D in conventional crops (lemon, potato and wheat) evaluated by the 1998 JMPR and that in 2,4-D tolerant transgenic cotton (AAD-12 cotton) were qualitatively similar. Quantitatively, while in conventional crops 2,4-D was the predominant residue with 2,4-DCP as one of the minor components, in AAD-12 cotton 2,4-DCP and its conjugates were detected at similar or even at higher ratios of the TRR. However, 2,4-DCP may occur in or on the plants not only from the use of 2,4-D, but also from its presence in water and the environment and, therefore, it was not considered appropriate to include this compound in the residue definition.

The main route of metabolism of 2,4-D in AAD-12 cotton is the rapid loss of the acetic acid side chain to produce 2,4-DCP. 2,4-DCP is also rapidly conjugated with glucose, which is subsequently further conjugated. Metabolism proceeds through natural incorporation of the radiolabelLed carbon into natural plant constituents, such as lignin and cellulose.

Due to its chemical structure, 2,4-D is not expected to concentrate in cotton seed oil, crude or refined. It was not possible to calculate processing factors as 2,4-D was <LOQ in cotton seed and not detected in crude or refined oil.

Methods of analysis

Analytical methods for 2,4-D and 2,4-DCP in undelinted seed and gin by-products of AAD-12 cotton was submitted to the current Meeting. It was concurrently validated within the supervised residue trial studies.

The analytical procedure involves extraction of 2,4-D and 2,4-DCP with methanol/1M NaOH (90:10, v/v). After addition of stable-isotope internal standards, and concentration to near dryness, the sample solution was incubated at 90 ± 5 °C for a minimum of 60 minutes in the presence of 2M HCl. The samples were analysed using an online reverse-phase polymeric solid-phase extraction (SPE) cartridge with liquid chromatography with negative-ion electrospray tandem mass spectrometry (LC-MS/MS).

The validated LOQ of the method was 0.01 mg/kg for 2,4-D and 2,4-DCP in undelinted cotton seed and cotton gin by-products.

Stability of residues in stored analytical samples

Freezer storage stability of 2,4-D and 2,4-DCP in samples of AAD-12 cotton and its processed fractions were studied at the fortification level of 0.10 mg/kg at -20 °C for a duration of 3 to 8 months.

The results of the study indicate that residues of 2,4-D and 2,4-DCP are stable in cotton gin by-products for at least 6 months and stable in cotton hulls, untoasted meal, toasted meal, crude oil,