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Compositional and Nutritional Safety Assessment of Stacked Trait Products and their Lower Order Combinations

Establishing the safety of food and feed produced from biotechnology-derived crop products that contain a single trait relies mainly on the comparative safety assessment process. This comparative safety assessment process focuses on the assessment of any differences between the GM crop (and/or food and feed) and its conventional counterpart; and includes detailed molecular, compositional, phenotypic, and agronomic analyses. By carefully conducting these comparative safety assessment studies, there is a high degree of confidence in determining both intended and unintended consequences that might impact the safety and nutritional quality of food and feed from a biotechnology-derived crop product. The use of comparative safety assessments has been widely adopted by the regulatory authorities globally and successfully delivered biotechnology-derived products to the market for two decades which have proven to be compositionally and nutritionally equivalent to the conventional comparator.

Based on scientific rationale, the safety of breeding stacks of biotechnology derived traits (stacked trait products) is not different from the safety of breeding stacks of conventional (non-biotechnology derived) traits and therefore safety risk assessment of these products is, in most cases, unnecessary (CLI, 2016). Where crops containing individual single events have been determined to be as safe as their conventional counterparts, it can generally be concluded, based on the knowledge and experience with conventional breeding, that the breeding stack of the single events is also as safe as the stacked product of non-biotechnology-derived traits in conventional varieties (Steiner *et al*., 2013; Pilacinski, *et al*., 2011, Kok *et al*., 2014). These conclusions are applicable to the safety assessment of breeding stacks regardless of number of events in the stacked product. Many global regulatory agencies recognize this principle and require minimal or no additional safety assessment of breeding stack products.

To date multiple stacked trait products have been approved (www.biotradestaus.com) and there are many examples of approved lower order stacks or sub-stacks that have subsequently been incorporated into larger breeding stacks (see Table 1 as an example). The multiple assessments concluding that these products are safe demonstrates that the risk assessment of the highest order stacked trait product is applicable to and covers all smaller sub-stacks of those events. For example, safety data submitted in support of the stacked trait product A x B x C x D should be sufficient to inform on the safety of all possible sub-stacks of events (e.g., A x B x C, A x C x D, A x D, etc.). This practice has been adopted by regulatory agencies in some countries that regulate stacked trait products (e.g., Argentina, Brazil, European Union (for segregating crops), Japan, Philippines) where specific safety assessments for commercial sub-stacks are not required. These countries have arrived at this position through careful consideration of scientific and historical data generated for stacked products. Given that each approved single event is thoroughly assessed and determined to be safe and that conventional breeding of biotech events has an established history of safe use (HOSU), an assessment of the highest order stacked trait product evaluates any possibility of an effect of, or interaction among, the traits and the unlikely scenario that stacking them would cause any unintended effect that would impact safety. Another factor that has led these countries to adopt this position is experience; countries such as Japan, that had previously evaluated sub-stacks and not identified any safety concerns (Table 1), have accepted the HOSU for these sub-stacks and regulations have evolved to no longer require additional assessments of stacked agronomic traits products for food and feed approvals. Also, considered in this determination was that segregating crops, such as corn, would produce sub-stacks in the F2 grain of the F1 seed used to produce the commercial crop; therefore, it becomes redundant to have additional approvals for the sub-stacks. Current scientific data continues to support this position; recently Kramer et al. (2016) reported data representing 15 years of field and laboratory data on higher order stacked trait products and their sub-stacks demonstrating that no substantial changes occurred in composition when stacking the single traits through conventional breeding into stacked trait products. (Kramer et al., 2016). Additional global examples of where higher order stacks and lower order sub-stacks have been assessed and approved are included in Appendix 1.

Table 1. Examples of multiple stacked trait products containing higher and lower order stacks that have been independently assessed and approved in Japan

Highest Order Stack Approved	Lower Order Stacks approved
DAS-01507-1 X DAS-59122-7 X MON-00810-6 X SYN-IR604-5 X MON-00603-6	DAS-01507-1 X DAS-59122-7 X MON-00810-6 X MON-00603-6
	DAS-01507-1 X MON-00810-6 X MON-00603-6
	DAS-01507-1 X SYN-IR604-5 X MON-00603-6
	DAS-01507-1 X DAS-59122-7 X MON-00603-6
	DAS-01507-1 X DAS-59122-7
	DAS-01507-1 X MON-00603-6
	DAS-59122-7 X MON-00603-6
	MON-00603-6 X MON-00810-6

As part of the assessment of single trait events, some countries may require animal feeding studies with high levels of the traited whole food in the diet. However, such *in vivo* work should only be conducted on a case-by-case basis and guided by specific hypotheses (Codex, 2009; Herman and Ekmay, 2014). Once compositional equivalence has been established there is little value from a safety and nutritional perspective to conduct feeding studies with target species (Bartholomaeus, et al. 2013; Flachowsky, et al. 2005a; ILSI 2004). According to the Council for Agricultural Science and Technology (CAST 2006) *"Feeding studies with target species have been conducted as part of stewardship initiatives, and there is growing evidence to suggest that once compositional equivalence of biotechnology-derived crops has been established, nutritional equivalence has been demonstrated."* Also in agreement, EFSA (EFSA 2008) concluded that *"once compositional equivalence has been established in GM feeds modified for agronomic traits, nutritional equivalence can be assumed and has been demonstrated in many studies with food producing animals."*

Therefore, if compositional equivalence is demonstrated, there is no expectation that differences would be observed if a nutritional feeding study was conducted. This holds true whether the product in question contains a single trait or is a stacked trait product produced by conventional breeding. Compositional equivalence has been demonstrated for many stacked trait products; this includes the highest order stacked trait products as well as the lower-order stack products. In all cases, the grain from the biotechnology-derived single event and lower order sub-stack products were proven to be compositionally equivalent to the conventional controls demonstrating that what was observed in the highest order stacked trait product applies to the lower order sub-stacks as well (Table 2). This also demonstrates that the introduction of the traits has no significant impact on the conventional crop, unless it is intended to modify the composition (e.g., modified oil profiles in soybeans), and any differences observed in composition analysis are due to natural variation and not the presence of the traits (Harrigan et al., 2010; Herman and Price, 2013). Based on the single trait event feeding studies and data that demonstrates compositional equivalence, a feeding study with meal produced from a stacked trait product should not be required for the safety and nutritional evaluation because its primary value is as additional assurance of product safety already established by the comparative assessment of each of the individual events in this stacked product (i.e., stack studies are of limited additional value).

Table 2. Examples of studies where compositional equivalence has been demonstrated for stacked products, different lower order sub-stacks and single trait events when compared to conventional crops.

Product	Compositional equivalence
MON863xMON810xNK603	Ridley et al., 2011
MON863xMON810	Ridley et al., 2011
MON863xNK603	Ridley et al., 2011
MON810xNK603	Ridley et al., 2011
MON 87460 x MON 89034 x MON 88017	Xu et al., 2014
MON 87460 x MON 89034 x NK603	Xu et al., 2014
MON 87460 x NK603	Xu et al., 2014
MON87427 x MON 89034 x NK603	Venkatesh et al., 2014
MON87427	Venkatesh et al., 2014

Various publications exist to corroborate the position that differences will not be observed in nutritional studies if there is compositional equivalence with the conventional grain. In an article by Tufarelli and colleagues (Tufarelli et al., 2015), more than a dozen poultry nutrition (broiler) studies were reviewed and the results from these studies provided further confirmation that when feed containing genetically modified grain is substantially equivalent to its conventional comparator, the grain does not impact the nutritional value in livestock. These studies also demonstrate that the results are the same independent of the stacked trait product in question, demonstrating that the results from the higher order stacked trait product also apply to lower order sub-stacks. Additional studies have been published that also demonstrate this lack of difference in nutritional value (Table 3).

Additionally, a ten-generation feeding study in quails using GM maize found the GM maize did not significantly influence animal health or performance over 10 generations when compared to it's isogenic control (Flachowsky et al., 2005b). A four-generation feeding study using laying hens and cockerels fed diets containing GM maize found the GM diets did not significantly influence animal health or performance over 4 generations when compared to it's isogenic control (Halle and Flachowsky, 2014). These data provide further evidence of the value of the weight of the scientific evidence generated during the substantial equivalence evaluation and call into question the further necessity of animal feeding studies in demonstrating the safety of substantially equivalent crops. This is also the opinion of the FDA (1992) whose policy recognizes that *"feeding studies on whole foods have limited sensitivity".*

Table 3. Published broiler studies using GM feed (*as cited in Tufarelli et al., 2015).

Author	Journal	Year	Transgenic Event
Brake et.al. *	Poult. Sci.	1998	Bt maize
Mireles et. al.*	Poult. Sci.	2000	Bt maize
Sidhu et. al.*	J. Ag. Food Chem	2000	GA21 (Roundup Ready)
Brake et.al. *	Poult. Sci.	2003	Bt 11 maize
Kan et. al.*	Poult. Sci.	2004	Bt Soy
Piva et.al.*	Poult. Sci.	2001	MON810 maize
Taylor et. al. *	Poult. Sci.	2001	MON810 maize
Taylor et. al. *	Poult. Sci.	2001	NK603 maize
Taylor et. al. *	Poult. Sci.	2002	MON810 x NK603 maize
Taylor et. al. *	Poult. Sci.	2002	MON863 maize

Taylor et. al. *	Poult. Sci.	2003	NK603 and MON810 x NK603 maize
Taylor et. al. *	Poult. Sci.	2003	MON810 and MON810 x GA21 maize
Taylor et. al. *	Poult. Sci.	2003	MON863 and MON810 x MON863 maize
Taylor et. al. *	Poult. Sci.	2004	RT73 canola
Taylor et. al.	Poult. Sci.	2005	MON88017 and MON88017 x MON810 maize
Taylor et. al.	Poult. Sci.	2007 a	MON89034 and MON89034 x MON88017 maize
Taylor et. al.	Poult. Sci.	2007 b	MON89034 x NK603 maize
McNaughton et al.	Anim. Feed Sci. Technol.	2007	DAS-59122-7 maize
McNaughton et al.	Appl. Poult. Res.	2011	DAS1507xDAS59122xMON810xNK603 maize
Herman et al.	Reg. Tox Pharm.	2011	DAS-40278-9 maize
Herman et al.	GM Crops	2011	DAS-68416-4 soybean
Papineni et al.	Poult. Sci.	2016	DAS-44406-6 soybean

In conclusion, confirmation of the safety of single events, coupled with a long, safe experience of stacking crop traits by conventional breeding, should provide confidence in the safety of stacked trait products without the need for further safety assessment. In the event that confirmatory studies are considered necessary, compositional analyses represent a robust assessment to confirm that conventional breeding has not resulted in new hazards or unintended effects. Multiple studies have confirmed that the safety of intermediate breeding stacks (sub-stacks) can be inferred from the safety of single events and higher order stacks, without the need for specific studies on each of the sub-stacks.

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Appendix 1

Table A1: Additional examples of higher order stacks where lower order sub-stacks have also been approved in different countries (source www.biotradestatus.com)

Country	**Korea**					
Higher-Order	SYN-BT011 x SYN-IR162 x SYN-IR604 x DAS-01507 x SYN-05307 x MON-00021					
Lower-Order	SYN-BT011	SYN-IR162	SYN-IR604			MON-00021
	SYN-BT011		SYN-IR604	DAS-01507	SYN-05307	MON-00021
	SYN-BT011	SYN-IR162		DAS-01507		MON-00021
	SYN-BT011			DAS-01507		MON-00021
	SYN-BT011	SYN-IR162				MON-00021
	SYN-BT011	SYN-IR162				
	SYN-BT011		SYN-IR604			
	SYN-BT011					MON-00021
			SYN-IR604			MON-00021
Higher-Order	MON-00810 x MON-00863 x MON-00603					
Lower-Order	MON-00810	MON-00863				
	MON-00810		MON-00603			
		MON-00863	MON-00603			
Higher-Order	MON-89034 x DAS-01507 x MON-88017 x DAS-59122 x DAS-40278					
Lower-Order	MON-89034	DAS-01507	MON-88017	DAS-59122		
	MON-89034		MON-88017			
		DAS-01507		DAS-59122		
Higher-Order	DAS-24236 x DAS-21023 x SYN-IR102 x MON-88913					
Lower-Order	DAS-24236	DAS-21023		MON-88913		
	DAS-24236	DAS-21023	SYN-IR102			
	DAS-24236	DAS-21023				
Higher-Order	DAS-01507 x DAS-59122 x MON-00810 x SYN-IR604 x MON-00603					
Lower-Order	DAS-01507	DAS-59122	MON-00810		MON-00603	
	DAS-01507		MON-00810	SYN-IR604	MON-00603	
	DAS-01507	DAS-59122			MON-00603	
	DAS-01507		MON-00810		MON-00603	

	DAS-01507	DAS-59122				
	DAS-01507			SYN-IR604	MON-00603	
	DAS-01507		MON-00810			
	DAS-01507				MON-00603	
		DAS-59122			MON-00603	
			MON-00810		MON-00603	

Table A1- Continued: Additional examples of higher order stacks where lower order sub-stacks have also been approved in different countries (source www.biotradestatus.com)

Country	**Japan**					
Higher-Order	SYN-BT011 x SYN-IR162 x SYN-IR604 x DAS-01507 x SYN-05307 x MON-00021					
Lower-Orders	SYN-BT011		SYN-IR604	DAS-01507	SYN-05307	MON-00021
	SYN-BT011	SYN-IR162	SYN-IR604			MON-00021
	SYN-BT011	SYN-IR162		DAS-01507		MON-00021
	SYN-BT011		SYN-IR604			MON-00021
	SYN-BT011	SYN-IR162				MON-00021
	SYN-BT011		SYN-IR604			
	SYN-BT011					MON-00021
			SYN-IR604			MON-00021
Higher-Order	DAS-01507 x DAS-59122 x MON-00810 x SYN-IR604 x MON-00603					
Lower-Orders	DAS-01507	DAS-59122	MON-00810		MON-00603	
	DAS-01507		MON-00810	SYN-IR604	MON-00603	
	DAS-01507	DAS-59122			MON-00603	
	DAS-01507			SYN-IR604	MON-00603	
	DAS-01507		MON-00810		MON-00603	
	DAS-01507	DAS-59122				
	DAS-01507				MON-00603	
		DAS-59122			MON-00603	
Higher-Order	MON-89034 x DAS-01507 x MON-00603 x DAS-40278					
Lower-Orders	MON-89034	DAS-01507	MON-00603			
		DAS-01507	MON-00603			
			MON-00603	DAS-40278		
	MON-89034		MON-00603			
Higher-Order	MON-00863 x MON-00810 x MON-00603					
Lower-Orders	MON-00863	MON-00810				
		MON-00810	MON-00603			
	MON-00863		MON-00603			
Higher-Order	DAS-24236 x DAS-21023 x SYN-IR102 x MON-88913					
Lower-Orders	DAS-24236	DAS-21023		MON-88913		
	DAS-24236	DAS-21023	SYN-IR102			

	DAS-24236	DAS-21023			

Table A1- Continued: Additional examples of higher order stacks where lower order sub-stacks have also been approved in different countries (source www.biotradestatus.com)

Country	**Taiwan**					
Higher-Order	SYN-BT011 x SYN-IR162 x SYN-IR604 x DAS-01507 x SYN-05307 x MON-00021					
Lower-Orders	SYN-BT011		SYN-IR604	DAS-01507	SYN-05307	MON-00021
	SYN-BT011	SYN-IR162	SYN-IR604			MON-00021
	SYN-BT011	SYN-IR162		DAS-01507		MON-00021
	SYN-BT011		SYN-IR604			MON-00021
	SYN-BT011	SYN-IR162				MON-00021
	SYN-BT011		SYN-IR604			
	SYN-BT011					MON-00021
			SYN-IR604			MON-00021
Higher-Order	DAS-01507 x DAS-59122 x MON-00810 x SYN-IR604 x MON-00603					
Lower-Orders	DAS-01507	DAS-59122	MON-00810		MON-00603	
	DAS-01507		MON-00810	SYN-IR604	MON-00603	
	DAS-01507	DAS-59122			MON-00603	
	DAS-01507			SYN-IR604	MON-00603	
	DAS-01507		MON-00810		MON-00603	
	DAS-01507	DAS-59122				
	DAS-01507				MON-00603	
		DAS-59122			MON-00603	
Higher-Order	MON-89034 x DAS-01507 x MON-00603 x DAS-40278					
Lower-Orders	MON-89034	DAS-01507	MON-00603			
		DAS-01507	MON-00603			
			MON-00603	DAS-40278		
	MON-89034		MON-00603			

Table A1- Continued: Additional examples of higher order stacks where lower order sub-stacks have also been approved in different countries (source www.biotradestatus.com)

Country	**European Union**			
Higher-Order	SYN-BT011 x SYN-IR604 x MON-00021			
Lower-Orders	SYN-BT011	SYN-IR604		
		SYN-IR604	MON-00021	
	SYN-BT011		MON-00021	
Higher-Order	MON-89034 x DAS-01507 x MON-88017 x DAS-59122			
Lower-Orders		DAS-01507		DAS-59122
	MON-89034		MON-88017	
Higher-Order	MON-89034 x DAS-01507 x MON-00603			
Lower-Orders	MON-89034		MON-00603	
		DAS-01507	MON-00603	
	MON-89034		MON-00603	
Higher-Order	DAS-01507 x DAS-59122 x MON-00603			
Lower-Orders	DAS-01507		MON-00603	
		DAS-59122	MON-00603	
	DAS-01507	DAS-59122		