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Schedule

8:30 AM–8:45 AM **Welcome from FDA**
Dennis Keefe, Director, Office of Food Additive Safety, CFSAN, US FDA, College Park, MD

Welcome from SOT and Speaker Introductions
Norbert E. Kaminski, SOT Past President, Colloquium Chair, Michigan State University, East Lansing, MI

8:45 AM–9:25 AM **Introduction to the Safety Assessment of Foods from Genetically-engineered Plant Varieties**
Jason Dietz, CFSAN US FDA, College Park, MD

9:25 AM–10:05 AM **Factors Influencing the Composition/Metabolic Profile of Food from Plants**
Sherry Flint-Garcia, USDA/ARS, University of Missouri, Columbia, MO

10:05 AM–10:25 AM **Break**

10:25 AM–11:05 AM **Introduction to Metabolic Profiling**
Ann Knolhoff, CFSAN, US FDA, College Park, MD

11:05 AM–11:45 AM **Composition Testing in the Safety Assessment of Foods from Genetically Modified Crops**
Bryan Delaney, Corteva Agriscience™ Agriculture Division of DowDuPont™

11:45 AM–12:45 PM **Roundtable Discussion**
Moderator: Norbert Kaminski, Michigan State University, East Lansing, MI
All speakers
Additional Panelist: Supratim Choudhuri, CFSAN, US FDA, College Park, MD

Welcome from FDA
Dennis Keefe, Director, Office of Food Additive Safety, CFSAN, US FDA, College Park, MD

Good morning and welcome to this colloquium series between FDA and SOT. My name is Dennis Keefe and I am the director of the Office of Food Additive Safety at CFSAN. It is my pleasure to welcome you to the colloquium which will explore the role

and the metabolomics and assessing the safety from genetically-engineered plants. I just want to give you a brief synopsis of the Office of Food Additive Safety. Our role, our mission is to protect and enhance consumer health by ensuring the safety of substances added food and food contact materials. We do this by administering several premarket programs by FDA. The one that is probably most common and well-known is the food additive petition and color additive petition processes. Then there is the Generally Recognized as Safe (GRAS) Substance Notifications and our food contact substance notifications which is what materials that come in contact with foods. So, this would be packaging materials, etc. Most importantly for this group is our biotech consultation process.

This colloquium series is built on a memorandum of understanding between the FDA and the Society of Toxicology. We have been hosting this colloquia series since December 2014. I think Norb and I were talking about this earlier and I thought that this might be a one off maybe for a year and then it will peter out. Since then, we have had several colloquia series, averaging about three per year and they have been highly successful, both for FDA, the Society of Toxicology, and the greater toxicology community globally. I want to thank SOT for partnering for us on this. I want to emphasize the purpose of the colloquia series. As I mentioned, it's a partnership between FDA Center for Food Safety and Nutrition and SOT. It is intended to stimulate a dialogue among leading toxicology experts on future oriented toxicological finds that's relevant to food safety and food ingredient safety. It also provides information for FDA employees and the public with the intent that this will inform science-based discussion on issues that affect society and public health. However, this is not a forum for soliciting regulatory advice, or for discussing food ingredient regulatory issues. We're focusing here merely on the science. This is a forum for discussing the latest toxicological finds in the context of food safety.

I wanted to briefly give you background on the FDA's role in the oversight of foods derived from genetically-engineered plants. The federal government has something referred to as a coordinated frame which lays out the roles of the three important oversight regulatory agencies, the FDA, the US Department of Agriculture, and also the Environmental Protection Agency. You can find more information about this framework online. FDA's role in assessment of foods derived from chemically-engineered plants is co-managed by the Center for Food Safety and Applied Nutrition, and also the Center for Veterinary Medicine. The process, the approach is based on a policy statement we issued in 1992. We have an inventory of consultations that we heavily evaluated with industry submissions for a new variety of plants for food production. You can find information on our program at the website. More recently, the science and technology has evolved and is evolving in terms of how these new varieties of plants are being generated and a particular genome editing. Earlier this year in January, we issued a call for information from the Federal Register on genome editing. Beginning from stakeholders on how we should approach genome-edited plants. Some of the questions we are asking is…it was in the context of science and the relevance of our assessments of transgenetic plants— generated through transgenesis— versus how this might inform our assessments of gene-edited plants.

We also ask the question about genome-edited plants that are categories of genome-edited plants that we could make categorical decisions on the safety or not safeness of that category of modifications. In conclusion, I want to welcome you all. I think we have a very exciting program this morning. I look forward to the very interesting discussion we will have. With that, I will turn it over to Dr. Kaminski.

Welcome from SOT and Speaker Introductions
Norbert E. Kaminski, SOT Past President, Colloquium Chair, Michigan State University, East Lansing, MI

Thank you, Dennis. I just wanted to welcome everybody on behalf of the Society of Toxicology. As Dennis mentioned, we started the series about four years ago and we did think it was going to be a one-off. It has been tremendously successful it has been a great partnership for SOT as well as FDA. I am really happy to be here today, and as a little bit of background about SOT, the mission of SOT is to create a healthier and safer world by advancing the science and increasing the impact of toxicology. We did do a strategic plan several years ago. And among some of our strategic priorities, which I think fit very well in terms of this colloquium, one of which was to strengthen the relevance and impact of toxicology. Also to develop and support toxicologists to capitalize on future opportunities, as well as expand outreach and impact globally. It is amazing how many participants we have joining virtually from all over the world. We are really excited that we are able to participate in this. As I have mentioned, we have been doing this for four years and tried to do four of these each year. We have done a variety of topics. Those that are listening or here in the audience, we would love to hear your suggestions on future topics. By having served on this Organizing Committee for quite a few years, we appreciate the input that we've gotten from the audience and would love to hear your recommendations for future topics. As we typically do, we have over 300 registrants for today, most of which are webcasting in. I also wanted to let everyone know that the talks are available through the SOT website. We record these and provide the slides. You can always come back if you miss something during the actual presentation.

We have four colloquia scheduled for 2018-19 and the topics we have selected…and these are being put together now. In October, we will have a colloquium on food tolerance allergenicity, in December, bio-printing as a tool for testing, in February, foods and flavor modifiers. The last one for this year will probably be in April or May timeframe and preliminarily the title for this one is, has the time passed for separate cancer and non-cancer risk assessment? I think these are going to be really interesting topics. I also really want to acknowledge a few people that have been instrumental in organizing these. I want to acknowledge Allen Rudman because he has done a fantastic job over the years in serving on this committee and has really been a driving force in getting these together. Suzy Fitzpatrick is one of the initial people who contacted SOT to start the series. Then Dr. Keefe who has been tremendously supportive. Bryan Delaney, who is the current chair of the Organizing Committee, I definitely want to acknowledge Bryan, Specifically for this colloquium the driving force has been Jason Dietz and he has done a remarkable job in terms of bringing this

together and helping identify the best speakers we could for the topic. I really want to thank those folks in particular. With that, this probably leads us in. Jason is the co-chair. I will just introduce Jason and we will go through the topics. We basically have four presentations. We will have a roundtable discussion at the end. I think we will have a little bit of time for questions at the end of each talk. If you can, you might want to save the questions for the panel discussion. With that, let me introduce our first speaker and co-chair. Jason Dietz is our first speaker. Jason is part of CFSAN and coordinates crosscutting biotechnology-related activities in FDA CFSAN. He regularly provides technical policy influence on CFSAN and its interagency partners with food biology technology issues related to food safety and regulation to labeling, genome editing, as well as media scientific communication. Jason has been the driving force behind this colloquium and he will be presenting the first talk. It will be an introduction to the safety assessment of food and genetically-engineered plant variety. With that, I'm going to turn this over to you.

Introduction to the Safety Assessment of Foods from Genetically-Engineered Plant Varieties
Jason Dietz, CFSAN US FDA, College Park, MD

Thank you, Dr. Kaminski. I think you will be impressed with the depth and breadth of the speakers today and we have an exciting lineup. I will echo what Dr. Kaminski said. There is a tremendous amount of behind the scenes work that goes on to put on the colloquia. I would like to acknowledge those individuals as well, Betty Eidemiller from SOT and Allen Rudman from FDA. My job is to give you an introduction and to set the tone for our colloquium. For conflict of interest I'm a federal employee, as many of you in the room are. We're screened very carefully for conflicts of interest or even the appearance of a conflict of interest. I certainly have none to declare. What I want to talk about is just to give you some background about genetically-engineered plants and their place in our food supply today. I will talk about how genetically-engineered plants are produced, and then I want to think about food safety. And think about whole foods as very complex mixtures. Our last colloquium was on mixtures and I think whole foods may be every bit as complex a mixture and a dynamic mixture as that.

I will talk about some of the basic components of safety assessment. Not just in the United States, but around the world, I think that will help you write a framework for some of the follow-up discussion and panel discussion later. First, let me say that in the United States, genetically-engineered foods are commonplace in our diet. These are the commercialized varieties that are available today. Anything from corn, soy, sugar beets, papaya, potatoes, apples, squash, and alfalfa. It is very likely of between a product that is produced through a genetically-engineered food, whether it be through something with a starch in it, cooking oil, or a protein isolate, or a summer squash that you happened to buy. I will emphasize some of these crops are highly adopted by farmers. This is a graph by the USDA economic research service and you can see that as of 2017, about 90 percent of the corn, soy, cotton acres have been planted to genetically-engineered varieties. This technology has been very highly adopted by farmers in the United States. You will also notice in the graph that we are

going all the way back to 1996. We now have more than 20 years of experience with just these particular varieties. And we also want to point out that genetically-engineered sugar beets also very highly adopted.

Let's talk about how we would go about producing a genetically-engineered plant variety. To do that I think it is important to have context that recognizes that man has been producing new plant varieties for a very long time. This is an excellent timeline from current opinion in biotechnology. You can see that as far back as 8000 BC, man was beginning to produce new plant varieties with desirable agricultural qualities via domestication of what you see here to something that becomes more along the lines of what we would recognize as corn. We've got millennia of experience associated with plant domestication. In the 1860s we have the work of Gregor Mendel where we can begin to apply genetics in a scientific way to plant breeding. In the 1920s, we have the use of radiation, and also eventually, chemicals, to treat seeds to produce mutations that may have desirable agricultural properties. Not until 1994 do we see a genetically-engineered plant variety, the first of which being the flavor saver tomato. And here in 2012, many of you have read about CRISPR cas 9. 2012 would be the introduction of that technology. Maybe not so much for commercialization, but at least for research usage. I think what is important to take away from this is that while the genetic variety of may have started in the 90s, research back into the 80s, but even before that, we had a wealth of experience producing new plant varieties and developing varieties that sustained human populations.

If I were to take a soil bacterium here, and I wanted to introduce the gene from the soil bacterium into a corn variety, my first step would be to isolate the genomic DNA from the soil bacterium. When I was in graduate school, we would have cloned out the gene of interest. Now you might just synthesize it based on sequence information. But you would in some way isolate the gene you're interested in inserting. You would probably isolate it and express the plant in the way you would desire. And then you would put into a vector that would have desirable promoters and terminators. And then you would insert or introduce the vector into plants grown in cell culture. There are two ways to do this. You could do it via a method called --- which you may have heard of referred to as a gene gun, or bacterium ---. Either technique is workable for some variety. For some plants you have to use one method versus the other. Nonetheless, they have the ability to introduce DNA into these cells. And then here in step four, you would be selecting the plants that have taken up the newly inserted DNA. You may do a selectable marker that has herbicide pollen associated.

Then the real work begins. You may have tens or hundreds or thousands of transformants. And you somehow need to evaluate them to find the ones that are most useful for their agronomic properties and food production properties. That is really what step five is. That would be crossing the events that you have inserted into the new variety, into other lines to produce hybrid or inbred for further analysis. Often times, I think we don't talk about steps 4 and 5 as much as we talk about steps one through three. 4 and 5 are important. They are not unique to genetic engineering. But they are a general principle in plant breeding. The notion of selecting or eliminating varieties

that have undesirable characteristics to wean down to just the few that are most desirable for commercialization. These are diagrams for my recent article in crop science. On the left it does a nice job explaining that when you begin doing selection, and in this particular case it was for corn, you may have as many as 150,000 potential products. You may be growing them at 10 sites. Each year you eliminate the varieties that do not perform to your standards. You can see as we go from year-to-year we get down from 150,000, all the way down to 100. Far less than even .1 percent make it through the selection process. As developers are going through the process, they are looking for a number of different criteria. Plants that grow well agronomically, plants that produce desirable foods, plants that are sufficiently resistant to disease. You can see that on the right, you will be looking at different qualities and life stages of the plant. While this is for corn, I think it is important to remember that this general process applies to plant breeding in general, whether it is corn, soy, or some other crop. And that this is a basic foundation of producing new varieties that perform in the field and also produce safe food for us.

Let's talk about safety assessment. Our last colloquium was about mixtures. Thinking about this colloquium, it dawned on me that whole foods is a real mixture. It is a complex one. You are talking about a mixture of thousands of chemicals having structural compounds…
compounds for energy use and storage, compounds for defense against pests and drought resistant or flood tolerant. Those compounds can vary with the growth stage of plants and the environment in which it is growing and the weather condition and genetic background. A good example is in the picture of the carrots. You can see that they vary in color and a little bit in size or shape. But they are all still carrots. Over millennia, humans have identified the plants that are safe to eat, and those that are not. I think it is actually hard to identify a plant variety that was withdrawn for the market in recent history because of safety concerns. If you search the literature, it is hard to find them. I can only find very few examples. It is remarkable that you do not find examples of those. While there may have been some variety changes in crops, I think it is important to recognize that the variety changes come from the small changes in the mixtures and generally have not resulted in food safety or nutritional concerns. A great example of apples. If you go to the grocery store today you will see red apples and green apples. Some apples were made for fresh consumption and some made for cooking. We also know them as apples. They have characteristics as we know for being safe as used for apples. That is not to say that there aren’t some substances in food that scientists and plant breeders need to be attuned to because if they are raised to certain levels, they may cause some degree of harm. They may be alkaloids and potatoes from soybeans. Erucic acid and canola. We would expect canola would have low levels of erucic acid. And squash. I also want to point out, to put into a plant breeder’s perspective, while some of the chemicals may be of interest from a food safety perspective, some of these may also be important for the natural growth of the plant. They need service defense mechanisms for the plant against pests in the field. There may be a point where you balance the amount of these substances in a plant and get the desired disease resistance, but you also get the food safety that you are looking for. It is also important to point out that while these chemicals exist in food, that

plant breeders and food processing techniques have adapted to mitigate the effects of these in our daily food consumption. For instance, plant breeders screen potatoes for alkaline levels. Lima beans are soaked before cooking. Where there are chemicals that may pose a question, generally, plant breeders are aware of this and taking it into account.

Food processing can also play a role as well. Thinking about the millennia of experience that we have with new plant varieties; how do we apply that to new genetically-engineered plant varieties? We have a wealth of experience and it is great if we can experience that. The way we do that is through a process. We compare safety aspects of the new plant variety with those that we have historically safely consumed. OECD pointed out in ‘99 that a long history of use is a reassuring a practical starting point for evaluating the safety. You will see that we have over 20 years of experience using the approach and it has proven to be sufficiently flexible and robust to make sure that the products in the market that mentioned at the beginning of my talk are safe for us to consume.

Another important point about whole foods that make them a little bit different from small molecules is that they are not easily amenable to animal feeding studies. They are not a small molecule that does not contribute much into the diet and can be fed to animals in large magnitudes of what a human may be exposed to. For whole foods, think about a fruit or tomato. They have a lot of bulk and are very complex mixtures. Incorporating them into a diet at a high level may make it difficult to balance the diet nutritionally. It may make it difficult to make the diet palatable. And once you conduct the study, there may be questions of is the study sufficiently sensitive to detect changes that might be important? And if you see a difference, how do you attribute the differences? What do you attribute them to? To the fact that you are creating an animal, giving an animal a high amount of food that they would not normally consume or is it a treatment effect?

I would like to think of two examples to put this into a useful context. Imagine if you had a very spicy chili pepper and you were going to try to incorporate that into an animal diet at a high level. With the 33 percent. You would likely make your diet completely unpalatable to the animal. The effects that you see would be hard to determine if it is an adaptation, or what is causing it. If you can get the animal to even eat the diet. Another example that I like to use is fruits. Many fruits have sugar alcohols such as sorbitol. Into many of you know that if you eat too much sorbitol it can have a laxative effect. Can you imagine trying to do a whole foods feeding study with animals eating a high amount of fruit? A lot of sorbitol and a lot of laxation. There would be a lot of compounding factors.

I think the important thing to recognize is while small molecules may be very amendable to traditional toxicological studies, the complex mixtures that are bulky may not be amenable. That is what makes the comparative approach so useful. This comparative approach is very widely accepted. In 2003, Codex Alimentarius adopted a guideline that was based on the comparative approach for safety assessment and

generally around the world, this is the gold standard. This is the approach used. There are modifications around it from country to country but generally the comparative approach has proven to be quite useful. I mentioned that what you would be looking for would be not comparing every single aspect of the food, but the aspects that are relevant to food safety and nutrition to aspects of the conventional counterpart, are the variety that has a history of safe use to the aspects relevant to food safety and nutrition. Depending upon how your comparison falls out, it may lead to additional studies, or it may not. The end result of the comparative approach, which I think is important, is that you are looking for whether the new variety is as safe and nutritious as what has been historically consumed. We are asking the question if food from the new variety is as safe as the foods that we historically have consumed without much concern. We recognize that no food is absolutely safe. For instance, there are certain foods that individuals are sensitive to in terms of allergens. The answer here is not looking for absolute safety, but the safest foods that we have historically safely consumed or know how to process and consume safely.

I would like to switch to talk about general kinds of data that people look at when assessing safety. I will generally be talking about a molecular characterization that has been introduced into the plant from a DNA perspective. The safety of any newly expressed substances and the levels of important key nutrients. Anti-nutrients or toxins from a plant. The assessment of keen nutrients, anti-nutrients and toxicants gets the notion that I'm sure you may have read about in the literature or maybe the popular press. The notion of unintended effects relevant to food safety and nutrition. I think it is important for us as scientists to understand and recognize that even through traditional plant breeding, there may be effects that are not intended. But that certainly does not mean they pose a safety risk. The notion that something was occurring unexpected is really independent from whether it represents a safety concern but as scientists, we would have to consider whether it indicates a safety concern. That is one of the things we would consider as part of this component of the assessment. In terms of molecular characterization, I like to think of this as defining the plant of what you are looking at. We generally look for information about the newly inserted DNA and the characterization of it. The kinds of questions we would ask are what actually has been inserted into the plant? One of the sources is the DNA. What are the other identities and what is the function of the specific elements? And of course, what products are expressed from the inserted DNA? That will lead us into our next step of the assessment and evaluated the newly expressed substances. It is also important to have a sense that the assessment being performed will be relevant to future varieties. One of the questions we would ask, generally is the inserted DNA stable. Is there some rationale to say the assessment performed today will be valid for future varieties? We would look at the molecular organizations across generations and also the DNA inherited in the predictable fashion, I would say from historical perspectives we generally have not had issues with this, but it is important to sort of establish that what we are looking at today would be predictive of what would be expected in the future. Certainly, any molecular characterization can help us target subsequent steps in the assessment process. When we think about safety of the newly expressed

substances, we are talking about if you have introduced a new protein, sampled an enzyme. We would want to know if the enzyme is a toxin or does it have any allergenicity concerns associated with it? We would also consider any reaction products produced from the enzyme and their safety.

Finally, we think about key nutrients and anti-nutrients and toxicants in the foods being evaluated. It is very important to recognize that this particular aspect is case-by-case based on what you are assessing. As scientists we recognize that corn is not an orange, which is not a tomato. We think about key nutrients, anti-nutrients, and toxicants that are important to consider the food you are evaluating because the key nutrients and anti-nutrients and toxicants in corn will be different from those in an orange. We would look at the levels of these and see whether they are outside the range of what we have historically consumed, or even if they are, whether they would present a food safety or nutritional issue.

I want to make an important point when we talk about composition. When we perform plant breeding, the intention is to produce a new trait. To make the plant different in some way to plants that have come before. For past resistance or consumer preferences, or some other reason. When you think about positions, there may be a difference in compositions, but the difference may not be important in terms of food safety and nutrition. Simply being different in and of itself is not a safety concern. It is up to us to evaluate whether the change is it meaningful. I think that is an important point. Often times I hear people talk about substantially equivalent. We are not looking for it to be the same. We are looking at whether it is as safe as, which does not mean equal. We expect there will be differences and it is our job to interpret the differences and decipher those that are important to safety, and those that are not important to safety. As any good risk assessors comment we would also consider any other information that was relevant to the food, based on the nature of the change in the food and the nature of how it would be used. Lastly, I wanted to point out that this is a high-level overview. There may be some differences country to country or regulatory agencies and based on the kinds of things that they are looking at, but I think this gives you a nice set up for our discussion today.

What brings us to today's topic of metabolite mix and what role they could have been for genetically engineered plants is the notion that there are new chemistry-based techniques and there have been suggestions that they could become components of the safety assessment. What makes these techniques so interesting is that they are able to simultaneously measure many compounds all at once. Metabolite mix is one of the words that we use to describe the. As any term in science we know that there are probably several other terms the folks may feel is a better descriptor. Some of the questions we may ponder through our discussions today are what these differences make for food safety assessment and what considerations would be important considering the use and the data derived from them. Would they routinely make our decision better? Our next speakers will provide excellent talks that will help enrich the discussion around this topic. One of the next speakers will talk about how much plants vary naturally in terms of composition. For example, I mentioned about these being

complex mixtures that are dynamic. Dr. Flint-Garcia is an expert in corn and corn genetics and I think she will be able to provide us some thoughts and perspective on just how much variability there may be. We will talk a little about metabolomics. Which is, how it can be used, and what are important factors to consider if you are thinking about applying metabolomics to food safety assessment from new safely engineered varieties. And then there is the question that is really the toxicological crux of this. How would a toxicologist use metabolomics for food safety assessment as the process already performed? How would such data be used? What kind of value would add? If you are looking for additional reading on the information I have presented, there are references that include references to some graphics that I have used as well as the Codex guidelines. With that I think that sets you up for our next talk. I am very glad to see all of you here today. I look forward to a great panel discussion.

Kaminski: Do we have a quick question for Jason? Maybe they are saving it for the panel discussion.

Factors Influencing the Composition/Metabolic Profile of Food from Plants
Sherry Flint-Garcia, USDA/ARS, University of Missouri, Columbia, MO

Kaminski: While they are exchanging microphones, let me introduce our next speaker. Sherry Flint-Garcia. Sherry is a research geneticist. She has been with the USDA agricultural research service since 2006. She is also an adjunct associate professor in the division of planned sciences at the University of Missouri. Her area of expertise revolves around research around the analysis – specifically an analysis of kernel and maize. Her talk is on factors affecting the metabolic profile of food implants. I will turn over to you.

Flint-Garcia: I am testing the microphone. Thank you. I work for the USDA. I am a research geneticist. I do not work on toxicology and I do not work on GMO's. I'm strictly interested in linking genes to phenotypes. I am not used to saying this, but I also have no conflicts of interest to declare. Today we are talking about domestication broadly. I will be talking about more recent plant breeding biotechnology and using maize as a case study. Looking at the genetics of kernel composition and touch on surveys that I have participated in composition and cetabolites. I have an appendix right at the end of the presentation if you're interested in more overview of the genetics I will briefly touch on.

Domestication is really an adaptation of plants and humans who evolve into work and live together. I like the idea that plants and people have evolved together. This domestication is manifested in something we called the domestication syndrome. It is different for every plant species. In general, the domesticate have a more robust plant structure. There is a synchrony of the male and female flowering primarily driven by the harvest is the yield one can piece from the new relative to the ancestor. I want to point out the larger size of the domesticate on the inside of the circle as compared to wild ancestors of the outside. We can all see the larger seed size as a result of domestication. I want to give you a couple of vignettes of crops other than maize to

show it is not just maize. Each crop is a different step of evolutionary forces and have incubated themselves in domestication. There is a complex evolutionary history. Due to polyploidy and we have a number of different wild ancestors. The *Aegilops* and *Triticum* species that have contributed genomes to making the new species. For example, there is the A, B, and D genomes resulting in different species that have been subject to plant breeding to provide different market classes for leeks out there. Tomato is a beautiful example of domestication. Domestication details are not very clear of the initial domestication that may have happened in Ecuador or Peru, or possibly in Mexico. Here you can see the wild ancestor has a very tiny fruit that is smaller than a marble. Pre-domestication are something on the order of what we know today as cherry tomatoes. Now we have all of this wonderful diversity in shapes, colors, sizes, and flavors in the domesticated tomatoes. This has continued to expand since Columbus brought tomatoes to Europe and it has been a model species for people to study the genetics of domestication.

The second topic is plant breeding and biotechnology. Plant breeding is taught with the art and science of plant improvements. Biotechnology is just an extension of the art and science to use various living assistance to develop new and useful products. The first of plant breeders were thousands of years ago. It was likely women who were busy doing the first plant domestication of selecting plants they could make it to food while their husbands were off hunting the buffalo. A hundred years ago, especially in the case of maize, and the settlement of the Americas, there were farmers that had their favorite heirloom varieties and would take them to state fairs and show their favorite varieties, have these family varieties or heirlooms. In 1926 the first company Pioneer Hi-Bred was founded. They continued the high-tech breeding that we know today.

The plant breeding is really just the selection Jason mentioned earlier plants with a desirable trait. When we talk about traits and selections there is often no understanding of the genes underlying the trait. We are just selecting the trait values. Of course, traits are governed by genes. There are genes underlying and these are different variants. As plant breeders when we select a trait, we are selecting to try to increase the favorable alleles and decrease the unfavorable various stresses. The process Jason mentioned has been working for over 100 years. There are two various flavors of plant breeding out there depending upon what kind of system you work in. The overall idea's that you want to change the mean of your population from what you had before to what you want to achieve. If we do not make a change in the phenotype we are unsuccessful plant breeders. If we are doing population improvements on the left side of the screen, maybe we have a population with variations for the trait centered around an average of 200. If we select the best individuals in the population, we will hopefully increase the need of the population to for example, 218. On the other hand, there may be materials that you have in your breeding population that have various characteristics that are desirable. A low yielding line that has a resistance and a high-yielding line that is susceptible to disease or insect. You hope to cross the two together using a pedigree system to bring the traits together in a population with the genomes shuffled between the two parents. Then you will ultimately select and

develop all of the characteristics that you want for your product. Those are the two types of processes we use in plant breeding. Biotechnology is really just an extension of that, where we now have knowledge of the gene that is underlying with the trait in many cases. Jason gave a great overview of how the process works. I do not do this myself. It is cartoonish for me. The gene is excised from the donor species, whatever it may be. It is put into a construct that is introduced into the species. The plant species that you are working on in your breeding program. And the transgene is then crossed into whatever line of interest, many times to recover as much of the recurrent parent as possible. In a traditional breeding, if we do or do not know the gene of interest, we are shuffling the chromosomes together into the new variety will be a combination of the new and the old variety, to some degree. Where in biotechnology only the gene of interest is introduced into a commercial variety, it is much more precise in terms of the number of genes that are transferred.

Now we are going into what I know and love, maize. We will talk about what we can learn from diversity with breeding and genetics and genomics. I want to point out that maize is an important crop. I feel very proud that I work on maize. We will talk about domestication and diversity. The inbred light research that I worked on. We will talk about the wild ancestor that I have worked on. My research is funded by a grant that is composed of a number of different researchers headed by me and Ed Butler from Ithaca, New York. All of the people in the green boxes are researchers. We have researchers with different expertise across the US that have different specialties. It is a wonderful group of people to work with.

The first point I want to talk about is maize is that maize is an incredibly diverse species. If you take any two inbred lines and look at their sequence diversity, they are more different from each other, than humans are chimpanzees. This is a very high-level diversity. You think that you are different than a chimpanzee. You cannot call two inbred lines the same. It is not just corn. The maize was domesticated from teosinte about 9000 years ago in Southwest Mexico. From a wild plant called teosinte and it was part of landraces. It is a very different plant architecture. You can see there is not a single corn stuck here. It is a bushy plant. It does not make the kernel or ears of corn that we know today. We have hundreds of tiny ear structures of seeds that are on top of each other. The seed is small. Approximately 10 percent of maize and enclosed in a hard fruit case. It is very difficult to open without fingernail clippers. I do not know how they did it 9000 years ago. Regardless, the people in Mexico identified some part of the population that was different and started to treat it differently than the regular seed. Overtime, they made primitive trimming. It has become more useful and people carry their corn with them across the Americas. Anytime primitive trimming, it encountered a new environment it adapted to the environment. Get a hot and dry plateau or a rain forest with a lot of disease pressure. The locally adapted landraces are what we know if we only talk about diverse
maize around the world. A lot of great colors come outside of… and shapes.

Back approximately about 300 years ago or so, farmers began to settle the US and interacted more with Native Americans, and obtained the corn already growing in the

region and began proactive corn breeding. In the corn breeding world, we have to develop inbred lines by self-pollinating. Basically, purebreds like the dogs that we all know. Everyone knows a poodle, and everyone knows a Chihuahua. They are different. Then in the two inbred lines are crossed together. I would like to talk very briefly about the him. Just like the product we have a him product. It is called 373, from Iowa. Maize has 10 chromosomes. It is a whole genome of chromosomes, approximately 5 to 12,000, based on the genome product they were 30,000 predicted. Various studies have shown the studies higher as a species were closer to 40,000. At approximately 85 percent of the genome is what we call retro elements. Only a very small proportion of the genome actually makes maize. In our teosinte diversity product, we have done a lot of different studies overtime to look at diversity. One of the first questions we had was how diverse is maize? The picture I showed of the chimpanzee versus humans, we had no idea what the answer was before we started the research. By doing these fierce panels of inbred lines of landraces, we are now over the course of a path product identifying 83 million stamps. A single nucleotide polymorphism that characterized the diversity of maize. Just to give you an example, the two most historically inbred lines in corn breeding represent the male and female of industry breeding. They differ by more than 20 million snips we know of today. As we search more, we will probably find more snips. I will come back to this towards the end of the talk. In addition to the sequence diversity of the different. We have something called non-collinearity. If we look at the chimpanzees and humans we would expect the team order along chromosomes would be similar, or possibly the same. In the case of maize there is a lot of diversity at the level where entire genes are not present in one line versus the other. You may be missing a gene that I have.

One of the other early questions we had with how domestication really happens. Doing a lot of research there are several genes that are controlling some of the big scale changes in the plant morphology that have been identified. Teosinte branch gene controls the architecture in the bushing of the plant. Teosinte bloom architecture and aging that controls the hard case that surrounds the seed. Those are two very important genes. We can see where people have selected making fewer places to look for years. Doing sequence space analyses, we have been able to identify that there are approximately 1000 genes that have played a role in domestication. This is not just a simple select a couple of plants that were different and make corn. This was a long process. My argument, my job as a research geneticist is to try to study what all of this diversity is about. The impact of selection on the diversity overtime. This is a cartoon that is a little colorful and hard to ingest. If we look at sequence diversity of some sort in a pool of teosinte and landraces and inbred lines. We have our different gene variants we considered variations the levels of diversity change over time depending upon domestication and plant breeding. You can see perhaps during domestication because these were samples. Perhaps we lost the purple allele. Certain lines were chosen for plant breeding and perhaps another was lost.

For the vast majority of genes, we have wonderful genetic diversity we can use and plant breeding using inbred lines. The story is different at domestication and plant breeding where we have an intent selection on certain genes in the process. There is a

mutation that opened up the coat and naturally everyone selected that one. When you look at landraces of all the corn around the world you did not see seed coats. Now we have all of the landraces and inbred lines. Likewise, a comment during plant breeding, there was a bottleneck that was stronger selection. Certain allele with plant sensitivity. We are planting corn at a high density today. There's been a selection on genes from tolerating your neighbors. We have about 1000 genes that I mentioned before that happened involved in domestication or plant breeding. I think those are the most important to study because they are the ones that are responsible for making corn a corn. There is a little bit of a black box because commercial breeders have a very restricted germ plasma. They have trade secret that they keep for their business models. They do not share their germ knowledge. Plant breeders and the public sectors do not know how much diversity is present in commercial germ plasmas. We have ideas. Which genes in which traits. Which germplasm has been selected and used. My suspicion is that a lot of this, wherever it happened in the model, the bottleneck was tight because there is limited genetic diversity in commercial germ classes.

I will talk about the research that I do setting inbred lines. It is a very large field six to eight acres of corn. It is comprised of a number of inbred lines from around the world. My job as a geneticist is to link genotype to phenotype and we have multiple methods to do it. I'm going to spare you the detail. There are two general classifications. We talk about a QTL. A quantitative trait locus. A trait that has continuous variation. Not controlled by one gene. We will have identified the region in the chromosome where the gene would be. A QTL is a broad chunk and we do not know what gene it is. We can say with statistical certainty in the region of the chromosome there is a QTL that controls. For example, corn oil. We can test certain genes for their involvement in the trait we can get down to pointing of a specific gene thing it is involved. My first job was to make an association panel with three in-bred lines of corn. We crossed it to make the line back in the day it was as SR markers with the details. We could establish relationships on these lines. All these indecipherable squiggles at the end of the line in the inbred line. We can see how they are related. Two different sides of the pedigree for corn breeding in the United States are called non-stocks. When you cross the two groups together you make a nice hybrid. There is breeding that has been separate because genome involved. Popcorn is an example. This is the source of a lot of the variation. Instead of working with 300 lines and detailed levels we decided to choose 25. Based solely on genetic diversity we told 25 lines. B 73 is a genome line that was already on our list. We toast different inbred lines from around the world to maximize our diversity. We then took all of the 25 diverse lines ranging from Iowa, all the way down to Nigeria. We crossed them to our genome lines and for each cross we made a population so that we could do QTL mapping. The diversity present across the pipeline is much more like an associated method study. We get to do the mundane statistic of power and resolution of the two methods to do cool genetics.

The genetics have been used to study many traits. My group focused on composition. It was a post document lab and while we would love to study every team composition in the green we had to limit ourselves to something that was easy to measure. It is

protein an oil on the 5000 lines going at several locations. A very large study. We are down to a couple of slides. Here are the QTL the control Kernel. composition in the diversity panel and redline. We can see that we have starch and blue, approaching red oil and green and all of the bumps along the chromosomes one through 10 are regions of the chromosomes that control the trait. We will not bother with those parts of the details here. For example, this large green chromosome 6, somewhere in there is a gene that controls oil. We know that the population is made of 25 different populations flexibly put together for each of the 25 lines. We can try to create from swapping out from B73 to the other parent. That is shown on the side. Many do not change oil considerably, but we have very strong. Some of the wheels can change oil by 0.2 percent. If we change both alleles to B73 we can change both oil by three percent or four percent. Out of eight, 2.5 percent of a very large change. One gene can control a lot of variation. Also wants to point out the messiness of the lot. There are QTL for composition everywhere. If you are making a transgenic line and you ruined my genes. There is a good chance he could land in my gene and change competition. Very complex and the lines that we have we always found between 23 and 27 QTL for each of the traits, it is only the tip of the iceberg. It is complicated. In order to try to get down to the team level resolution, we did some association analysis where we took 1.6 million snaps across the genome and did our statistical testing to get out the question, is the gene involved or not. The green boxes highlight the original QTL. I showed you before. The dots that you see are the snaps within that have a relationship with oil. Several of those show up in my favorite genome camp, chromosome 6. We are getting closer to say that this gene is involved in oil. But we know the gene, and all of these others we do not know much about them yet. There are a lot of unknowns about which genes control which traits.

Now I want to talk about expanding diversity even more. My research program has been developed following a teosinte progression line where we took B73 and we crossed it with took species from Mexico and you can see there is a difference in seed sides. The F1 hybrid, the plant is animated in branching us. The ears are small. If we pushed on the ear a little bit, the ear will break and half easily. It does not have the COB structure. Any seed the seed can squeeze out of the top and when you pull away the seed coat looks like a little snowman. Is able to escape. We took the 10 different F1 hybrids back with his crossed corn and back crossed the corn four times to end up with a line that is mostly corned but will have little bits of sunscreen. Here are the key four proteins, starch, and oil. Hopefully you can see that this is very different than what we thought earlier with an inbred line. There are fewer regions and I want to point out the chromosome 3. The changing of the effect of starch. Some alleles scanned and chart increase starch and decrease starch. If we doubled the silver, we start by 5 percent. Is a very large change for once genome region. If we compare the NAM population from around the world to materials we can see that a lot of the QTLs overlap. My favorite is involved a chromosome 6. It is not present bread lines around the world. This is what I Mike Turzai was a genius selection in its quarter domestication when we had fixed the variation they were all the same red dot that went through the pipeline. Maybe that gene was involved with making it better. Because starch and seed ways are intimately related. My interest in corn breeding is to reintroduce variation of

teosinte into corn.

The biological hypothesis is a loss of genetic results in a loss of phenotypic variation. If we can introduce it, we can make better freshener or more. Whatever you want. A case of starch pathway in Greece three of the of the six genomes show signs of slowing. It takes some of these genes to introduce them back into corn. Spot market which is of the traits that are relevant nine doesn't go when 1000 years ago, according to hand the species in Americas, or 100 years ago when pioneers began. The traits may not to be important to us today. The traits are controlled by genes. Therefore, the LDL that were selected 9000 years ago or 1000 years ago, or 100 years ago may not be the right one. But that is what we have in our breeding closet. We want to rethink what germ we want and were to go shopping for genetic diversity. To conclude this part of the talk, looking at the impact of selection and Metabolomics their selection on the genome over thousands of years. Over 100 years of plant breeding and over 20 years of biotechnology. Leaders make selection. During this we have been able to modify key compositions to return additional breeding, like what I do in biotechnology. I do not work in those areas. I am not aware of any died until the toxic corn. There are strange corns out there and people eat them.

I want to briefly touch on metabolism next. I teamed up with a number of groups in the past to look at diverse examples for a lot of different traits. We have our NAM inbred parents shown in blue from around the world. In 27 different landraces from the Americas we have crossed the hybrid because farmers grow hybrid. Where will have sown these hybrids in a number of locations and gathered the grain here. This is a picture of hybrid ears pick they look at like corn. We know genetically how all of the lines are related to each other and the color coding will come into play later. The first thing, looking at compositional analysis. This is composition of other international traits. When one does an analysis, one can see a distinct grouping of the different materials, based on their composition. The color codes are related back to genetic diversity and how lines are related. You can see it in the NAM population and landraces. The different groupings are very strong and related to compositional traits. Looking at a number of the tools in the inverse to provide you can see that there is a wide lane of approximate total carbohydrates. Wide range and the existing and redline. Ranging from fat of 3.702744. Total coverage is also quite…I rate when we get down to the sub molecules, the steady access, Menelik and a wake-up. We can see that for both of these traits there are twofold differences for the fatty acids. This is the inbred line panel. If you are looking for small changes I can show you large changes that do not have anything to do is transfer the diversity is a breeder and geneticist will say. When we compare landraces to inbred lines, we can see nearly 6 fold different between the inbred lines and landraces, and within the. Likewise, for beta-carotene, what of the vitamin precursors. A huge range in variation in both inbred lines and landraces. This is to make sure. In the second study I teamed up with UC Davis. The table profiles of this. And the part of the study we identified 675 metabolites. Only a subset made the QC check at about 174 of them were annotated so they know what it was to give it a name. NMR was also used to look at metabolites and they were able to identify 33 of them. Mostly amino acids are an organic only triggers a few others.

Similar types of analysis can be used. This is a partially discriminated analysis which is a multivariate analysis for grouping things. They were able to group together the germplasm based on their relationships. Of the lines of a certain group tenderly characterized by higher or lower levels of various metabolites. Metabolite drives relationships among lines. Not only of the complicated enough looking at any given metabolites or any given compositional traits, or genetics, we also have environments that contribute to things. We also have genotype buyer environment attractions. Just where pollen comes from to make the kernel that you are studying has a big impact. Here we are starting something called the Gene Allele effect of the source of the pollen on variation for kernel traits. Here is an example of a yellow ear of corn that was planted there something that makes purple kernels. When the pollen drifts and we can have a change in color. You may say it is not a big deal. You can see it changes white to yellow and have something. Sweet corn has wrinkled kernels because of the sugars and starches that are not developed. The kernels are shrunken in sweet corn and when you have field court pollinating the corn you get normal kernels. Now you're messing with flavor and texture. Ornamental corn that is there today. We capitalize be fun and beautiful color combinations. If we did not have Xenia it would have an ear that was white and yellow, and we would not have these great colors. Looking more in-depth, we have a new study we are trying to find funding for. We are collecting preliminary data for our grant. We collect grain and we have been operating on the assumption we have 5000 plants to get the grain to make sure it is poor for the analyses. That is what we have done up to this point. On this grant I preferred not to do all of the work. We start the study to look at the Xenia will affect. If we separated Kernel. into the embryo and run a profile of protein you can see the endosperm major profiles. One can use HPLC to divide and quantify the different genomes. We look at the profile and can see the primary family and all of these is the alpha family. They would be a way for us to we could put these amino acids in our grams. There causes the ground to become opaque because the starch and protein do not pack record. When we compare to B73 with the low levels of lysine and tryptophan to the opaque which now lacks the xenia family we have higher levels in the opaque two of these amino acids. If we take the 73 years upon it with opaque to pollen, we do not see much change in lysine as expected. It is the mother plant. But if we take opaque two that has highlights and we use the B73 pollen to make the seed, we see very different levels of lysine and tryptophan. The pollen grain can affect amino acids. In my study, we have to pollinate all of these layers. I also want to point out that the B73 are genetically the same there are still significant differences between the two. Matters which direction the cross is made. There is a lot of variability that shows up in the experimental enzyme.

To summarize my talk on the whole, maize is a very diverse species both on the phenotypic levels in the plant level. Outlooks and all the way down to the metabolite level. The gene, the environment, and the by environment attributed to the variation in composition, which I study. Many genes contributed to the variation and even the pollen grain source continues to the variation. To summarize, for transgenesis and breeders we want to understand genes and traits in the link between them. We want as much diversity as possible. If I would run a study and I could not find the diversity

that I wanted to in the inbred line, I would go looking for more variation. I am very surprised when I do not find variation in maize, but I would go just to find the variation. The more the better. Every seed the farmer puts in the ground is the same. They are different by 20 million snaps. They are each the purebred poodle and the chihuahua. When we cross…if we had 1000 feet of one and 1000's of the other, they are the same to each other. When we cross those together to make 1 million seeds planted on 30 acres, every single one of the highbred seeds are the same that the farmer puts in the ground. When the F1 hybrids are going to reproduction the genomes are shuffled, just like what breeders do. If you have 20 million, they are different between the two and you are shuffling those at a random event every single time, every one of the million years with 500 on them. Every seed will come back different. If you take two seeds, they will probably differ for anything that you want to analyze. Height, composition, and metabolite. Variations are out there, and they are real. Very complicated. I have listed a number of references if you would like to read up on anything. I have provided the appendix slides to look at if you need more background. With this, I will take questions.

Audience Question: Christina from Biotechnologies Regulatory Services. You mentioned about the inbred line being fit stock and non-fit stock. Does that mean you have to get variations of one of the inbred lines to be one of the other? That what you are searching for when you are breeding and looking for more diversity?

Flint-Garcia: The question is fit stock versus non-fit stock. That is in order to get maximum yield in a commercial setting. The best combinations are basically fit stock and non-fit stock. You can cross raisins and get good production. To maximize the yield for a commercial setup you can actually take two fit stocks and cross them together. You can take to variations of B73 across them. They are different flavors of the B73 depending on where they came from. We actually cross one with the other. Variation happens.

Kaminski: Does anyone have any other questions?

Audience Question: I have two more. How difficult was it to cross with the domesticated inbred line? That is my next question. And the third one is, you said you have never seen or known of any corn or maize that was toxic. While keep looking at corn for toxicity. I guess this is an FDA question. When will we stop looking at corn for toxicity?

Flint-Garcia: The first question of how hard it was to cross species with mates is very easy. In one direction. When we pony corn, we have to prevent unwanted kernels. The male has a test on the top and the ear in the middle of the plant. When it comes out there is a little girl and what we have to do is cover up the ear before they come out. On maize we put a bag on the ear and we dump the pollen in there. The other direction where we have hundreds of tiny ears and each one only makes five kernels, but is not impossible, but very challenging to make it across the direction from a genetic standpoint. I will refer your other question about the need for questioning the toxicology to the FDA.

Audience Question: I have a few questions related to something that you said. 85 percent of the genomes transposed. And you called them junk.

Flint-Garcia: That is what we used to call it. Now there are these other and regulatory, gene regulation elements that are embedded.

Audience Question: Do some plant species have no transversal elements?

Flint-Garcia: I am not an expert. Many, the vast majority of plant species have a large majority.

Audience Question: Corn has more than anything we know if?

Flint-Garcia: Not anything else but the vast majority.

Audience Question: Of the 85 percent, are they all jumping?

Flint-Garcia: No. Most of them are not. They are just sitting there. They are retro. They jumped in and became inactive. Something jumped in and it is a big mass. They're retro because…

Audience Question: Does maize have more diversity because of the transposable elements?

Flint-Garcia: That is one of the mechanisms they are proposing. Is a certain transposable element that can actually pick up genes and lift them into a tornado somewhere else in the genome. It gives me chills thinking about the Wizard of Oz.

Audience Question: You mentioned several times that genetic variation is actually a good thing. The question that I have as we move more to bioengineering, are we actually defeating what is really important mechanism, so to speak, trying to propagate variation?

Flint-Garcia: Correct. There are various people and groups out there. Public versus private and other divisions of wealth that will argue about how much diversity is necessary. And various models of how to make the yielding corn and some people would argue that you do not need to cross two lines together. You just have to find the right combination of one in 1 trillion, or whatever it may be a plant to have the right combinations of genes to make it powerful. Is a public per person I would say more diversity is better because you can always find new cool things? I am starting a new breeding program for food corn. My husband is from Mexico. When you go to little villages you tasted the flavors of the corn. It is not what we have in the US. No fault of anyone. I'm trying to ask the question of what propositional traits and gives the aroma, flavor, and texture to make a good food corn. Have we lost the domestication in plant breeding? I think different kinds of corn for animals versus humans and anything else

will have a different formula that makes the best thing and diversity will differ.

Kaminski: Okay, I think we will go ahead and get started.

So, it's my pleasure to introduce my next speaker and she is a chemist by training, and she is with FDA, also from Food Safety and Applied Nutrition. And her primary role as a research chemist is to develop non-targeted screening approaches using liquid, photography and high-resolution mass, and the title of her presentation if I can find it here, the Introduction to Metabolic Profiling. So, with that I will turn it over to you.

Introduction to Metabolic Profiling
Ann Knolhoff, CFSAN, US FDA, College Park, MD

All right, can everybody hear me okay? Great. So, thank you for the opportunity to speak today. So, a little bit of my background, so my PhD work, part of it was focused on metabolite but with neurochemistry and specific cell types, and when I came here, I switched to food, at the common thing that links those two things together under is the type of instrument I use and also how we process the data. And so, what is metabolomics? So, this is viewed as the complete small molecule characterization, and so this would be a snapshot in time of a particular physiological state, the system that you are trying to study. And often these studies are, often these characterizations are similar to…they complement other homing technologies that you have probably heard of. And metabolomics is the downstream products of how these different technologies complement one another. And often metabolites made are used to link chemical content. It's similar to the work that Sherry was talking about as well.

And so, I've included some of the structures here, so you can get an idea of the size and the elements that are present. The structures, there's polarity differences, so this can be challenging to characterize every single one of these metabolites that may be present in a particular sample. So, this can include things like amino acids, lipids, steroids, small peptides, carbohydrates, and also other things, sort of like for example if you take a drug, and this includes many others as well, but these are probably compound classes that you are familiar with. And so, there is a difference between what is considered metabolite profiling and metabolomics. Metabolite profiling is an approach where you are interested in the listed screening. It will be the list of current compounds that you're interested in. However, if you have compounds that are present that may be toxic, you would miss the compounds that are not on this list. And so, this is where metabolomics will be really helpful. You are trying to detect everything that is present, and so it's similar more to like a needle in a haystack type of approach. So, if you're trying to find a biomarker for a particular type of disease you will be detecting everything in haystack and then trying to find that little nugget of something that might be an indicator of that state. And so, metabolomics has really picked up the last 15 years. So, I wanted to include a figure where I searched for publications with metabolite make in the title. So, on the X axis you have the publication date and the y-axis is that number of metabolism in the title. So, it's really booming right now. And the ranges of application in this area are varied. As I already hinted at before, a lot of this

is looking at disease state, injuries, disorders, looking at model organisms, trying to link chemical content dysfunction. Also, gene modification. Where you might want to look at differences you have when you take a certain type of drug or if you are exposed to a particular type of toxin. How does your body respond? And many of the studies are comparative. I'd also like to point out because the metabolite is so large, but all of that is weighed larger when you get to metabolism, because it's a downstream end product. So, if you think of yourself, each organ metabolizes things differently. And so, that metabolism in your body may be large much larger than what you have in your brain. So, a lot of people are doing metabolite make biology already. And because plants have bigger genomes they also have bigger metabolisms.

So, it's predicted that greater than 200,000 metabolites can be present in a plant sample. We are looking at the plant growth and development, stress responses, so, stress that may be due to the environment such as drought, or the biology such as pathogens. And there are a lot of drugs derived from plants, like aspirin. Some people try to look to the next big drug that may be present in plants. So, the most common use of techniques tends to be mass spectrometry area and where this is most useful is for structure. You can also analyze the sample with other techniques and sample preparation is fairly minimal. The data acquisition to minimize the sample is fairly fast. So, it tends to be complementary to these things, so, that means things you get from the mass paternity tree, but it's also better on the identification side. You also get better limited detection, so you can detect very low-level compounds in the sample, but you can also have a large dynamic range, so you can detect compounds that have less amounts. And so, then you can relate this back to the molecular weight of a compound. Often, chemical separation is done prior to mass spectrometry. Especially with a compound sample. So, I've listed here, I think this is orange juice. But with each peak has a mass spectrum associated with it, you can imagine there are thousands peaks present. So, when compounds go through the stationary phase, depending on how they interact with that column, they will either go faster or slower. And this can be reproducible between experiments so that you can link these things together. And this is typically done with volatile compounds. And there are a number of retrospect then which can be used to identify. I typically use liquid chromatography where they interact with it stationary phase but don't need to be volatile compounds. And you can separate hydrophilic and hydrophobic compounds from each other. And so, this is really useful for polar plants.

High Resolution Mass Spectrometry is a technology that is available and the main advantage to this is that you can get accurate maps. I included a clot just to give you an idea of the number compounds that exist within a particular molecular range. So, for example between 300 and 350 Daltons we have more than 157 compounds within that range. If we look at the even higher range of 300 to 301 Daltons, you have 300,000 compounds within that range. So, the better mass accuracy we can get, the better we can do at narrowing down the molecular formula. So, here I listed a mass spectrum. And so, this is a master charge ratio of amoxicillin, and so, typically I report, and you can generate a molecular formula. The probability of generating a molecular formula is high when the isotopic ratio is low. So, all you need to know is that it's within a very

tight range. And these instruments can do this. The isotopic ratio is just measuring the natural abundance of the isotopic that we know are present in different elements. So, this can give us information with the molecular formula is as well. The other advantage of using high-resolution optometry is that it can have a larger mass of charge ratio. So, again this is amoxicillin. And so, the blue on the outside is the lowest resolving power that I've chose to show which is 17 1/2. And when we go up, the highest is 140,000, so, you can see how much more narrow that becomes. And this is important if you have others that are present in the same range. So, if you look at this isotope, you can see the future of the sulfur. You can see these ions start to separate out. So, this can really help us in terms of determining the chemical content that is there. I'd also like to mention mass spectrometry imaging. This is a technique where you can actually get some idea of localization of work compounds are in your sample if this is important. So, what happens is the instrument will scan the sample, and the sample will be moved, and you collecting mess spectrum at each point. So, each pixel contains a mass spectrum. So, if you are interested any particular type of metabolite, let's say glutamic acid, let's say we can look for everything, and you can have this nice pretty image and see where the different metabolites are localized. And you can compare to the optical image that was taken before it was introduced into the instrument you can also see colocalization in metabolites that are present as well. And so, I color-coded each of those different so, you can see how these compounds relate to one another.

But for the purposes of this I just want to focus more on the LC/MS, because it is more frequently used for chemical characterization. So, the sample preparation for this, there are a number of things we need to take into account. The first is sampling. So, Sherry pointed out, even within a corn crop, you might have different kernels of corn that are different from one another. So, how do you represent this? So, the result you get that if you checked the entire corn plant, versus the leaves, it's all going to be different. Do you get all the corn if you extract all the corn together, that might be a different result than individual kernels where you would have more kernel variability that you might be able to see? So, depending on what result you were trying to get, you would sample this differently. Sample sizes are also important, so you might choose a different sample technique over another. You also have to get the metabolism. So, if there's certain things happening you want to get them at a certain point in time, replicants are also important. The biggest one for me, this one is incredibly useful for my data processing, to make sure that changes I see are real. It brings confidence to your platform, but it's also useful in trying to capture that variability. If you didn't do a good enough job sampling the first part, you can see how much variability there is. Also, if you are interested in particular compounds of interest, so, if you are just interested in amino acids, the sample preparation can be very targeted to those molecular species. But if you are trying to look for everything like any metabolism make type Experian, then it tends to be dirtier, and can cause deterioration for your data quality.

So, it's a balance between cleaning your sample enough to be able to accurately detect and identify compounds of interest, but also not removing features out that might be important. You also want to choose something where the sample and analyze

stability is good. But also analyze and extract the link. But this is a sample that is treated identically but does not have the sample of interest in it. So, what you would do with this is because this technique can detect a lot of different types of compounds, and it can also detect compounds from your sample strategy. So, compounds that come from your tips, or your solvents that you use. Matrix sites are also useful. What this is, is just putting in a specific compound or mixture of, sounds into your sample to say how good is my extraction, how well is my data processing working.

So, you look for specific compounds and see if they are found in your data or not. So, for metabolite profiling, again,0 this is for specified analysis. So, it shows amoxicillin as an example. It gets to a peak and then you can integrate into this area which corresponds to the abundance. So, if you have amino acid picture, you can analyze this at different concentrations, and then if you have the sample you can relate this back to the quantitative level by comparing it to the standards that you analyze. And this can be done with a fairly large compound. For metabolite, it's much more challenging. Because it's much easier for software to say I'm looking for this thing and I want to get the data out for this thing. But, if you are looking for any types of different compounds that are present, sometimes the software might not pick out the same feature in the sample even if it's present. Because it's having to dig through thousands of features that are present in that data set. There are also many unknown analytes, which could be challenging to identify. You also need to make sure that you remove those background samples that we talked about. And there are also many different iron types. Just because you ionize the particular kind compound, there might be another type of iron, but it's related. So, clustering the ions into one feature is useful. And again, as I mentioned earlier, because the metabolomic experiment is all-encompassing, it is reproducibly extracting features from the data.

So, I thought I would just put up a workflow of what is typically done. For kind of this non-targeting approach. So, here we have the data processing software we have, and send that into a list. If you have multiple lists you can try to put them together so, you can compare different files. It will interpret these ions in terms of master charge, and then based on this large list of compounds that you have detected, you can use different identification strategies to figure out what that compound is. So, you can generate a molecular formula, you can generate a molecular formula against a molecular database. There are many out there, I listed a few of them here. And then you can also use MS/MS approaches where you break apart the molecule and based on each fragment this will help give you an idea of what the identity compound is. And then once you have the potential identification then you can confirm it with the analytical standard. But because that is as of compounds may be present in one sample, it's often advantageous to try to whittle this list down. What compound in this sample matters? So, often people use statistical analyses. This is most commonly used when comparing two states. So, control versus alter-state. So, this is much easier to determine molecular defenses rather than just giving a sampling thing. Is there anything wrong with it? Identifying sample outliers can be done using these tools as well. It can also look for compounds that are responsible for any molecular differences between sample sites. And with any kind of workflow like this, quality control such as

matrix sites can be used to make sure your platform is working well.

So, briefly I want to talk about data factors that can influence output and quality. So, first is the complexity part. If your sample is really complex, software has a harder time trying to figure out what features are actually there. But even with that, you can have other effects that happen such as iron compression, so, if you have a highly abundant heat that is ionized sometimes that can compress lower level abundance. You can also matrix effects or you can have mass accuracy errors, if you have a compound that is close. You want to make sure that you are appropriately detecting compounds in that concentration range that you are interested in. So, are you looking over a broad range, or do you only care about low-level analytes that may be present. You want to make sure that you are resolving compounds both in the graphic timescale but also in terms of that mess spectrum as well. And the data analysis software that you use can also greatly influence what your output is. And so, a lot of software is gender specific, so, it might be tailored to that specific instrument even though they're multiple high-resolution instruments. But ideally, we would like to use something that anybody can use, so, that this would be extremely accurate approach.

So, as I already mentioned earlier, reducing the number of features is critical. And so, a feature as I'm defining it here is one detected compound with all the associated ions that might be related to it. And because thousands of features can be detected in a single sample, we want to make sure we are focusing on ones that really matter. So, just to give you an idea of why this would be the case, I searched these different small molecule databases for particular molecular formulas that I listed here, For example, there can be 4500 compounds found in this particular molecular formula, so, I customized with that particular compound. But the nice thing is that you might not care about identifying anything. So, if you can get it down to a reasonable number, it's actually not too hard. And a molecular formula can also be generated for a single feature. So, here I just searched one, but other molecular formulas might also be generated for the same. So, identifications can be done, and so, what this does so, if you are interested in identifying ion here you can choose that ion and break it apart. You can then relate it back to the structure and look at those parts. But you can also break these parts down even further. And so, ion is being further broken apart. And so, MS/MS methods are used for both target methods, and there are MS/MS libraries available where this can help aid in your identification process.

So, my recent work that we've been doing was actually looking at some tests for some of our non-targets that we are developing here. We are looking at rolled oats in different oat products. So, this comparison was done with a particular lot of rolled oats that was spiked with the nine-compound standard mixture, and we compared it to other samples that we collected of the same brand. We also used that same sample and compared it to different brands that we collected to see what the diversity would look like and so, when we do this comparison, the features that we find of all samples are greater than 3000. 3000 was just looking at the same brand, but there are almost 5000 features that are found to encompass all the different brands together. But when we compare the spiked versus the non-spiked samples, we can reduce the number of

features that are different down to 13. That's pretty amazing. Given the number of features we have, that is much more manageable to try to identify that number than 3000. We also wanted to look at how many. We can also use other statistical methods to try to get an idea of variability, so, this is a principal opponent analysis. Essentially what you need to look at here, each dot represents a sample. And closer together the dots are to one another the more closely related they are. The further apart they are the more different they are. So, these are just for brands we collected three times throughout 2017. So, these two collections, these were three replicas done, so, this one collection is more similar to this other data sent down here. And you can also see these green samples from, I think that's brand too, these are actually the results of the Irish oats. The other example was produced here in the U.S. So, you would expect there to be some diversity we were also interested in what the data would look like if we analyze different types of oats that were also collected three times in the past year and see how they relate to one another. So, they were all processed similarly, they were all milled at the same consistency and process the same way. And yet we still see differences between the different oat types. So, you will see over here, the rolled oats, the old-fashioned, and the cluster separately from one another. And these are different brands being included in each of the bubbles. But we can also see some brands cluster together. So, even though these are three different types of oats here, they are all fairly close in proximity, but that's not always the case though. For brand three, these two are actually from the same brand, but these are rolled oats, and these are steel cut oats. So, there are still some variations that we can see.

So, I briefly just want to focus, just bring you back on terms of what the nontargeted data analysis workflow is, just to give you an idea of each part that goes into it. This can be challenging. But anything that we can do to try to limit down the feature list is really important. So major points and as I see it, the biggest challenge in the metabolism is the data mining part because it is fairly fast to prepare your sample and put it on the instrument. It's another thing to dig through these large data sets and try to make sense of it and determine what is important and what is not. But even with these challenges, there are a number of advantages to metabolomics. And that's one of the reasons why this area is booming so much. And that's just because we have the information data sets we can collect so we can get a lot of information about a particular data site. And you can determine these molecular differences between controls and altered states. And if you were to do that, you can also identify molecular targets. And if you do that and you say I will focus on these particular ones that might be changing based on a gene modification, then you can develop strategies where you have the target approach anywhere just looking for these compounds of interest and that is much more straightforward and using the accurate method.

So, here I just wanted to list some publications that I found in terms of studies that are already being done. A lot of these are kind of comparative analyses as well. For example, looking at similarities and trying to figure out what the downstream products of the genomic changes are, and what might be occurring. So, for the references I used in the publications, you can look the slides up later, and look these references up later if you're interested.

I'd also like to acknowledge a few people, Tim Cooley brought me on at the FDA and started me with the nontargeted framework I am doing now. Christine Fisher works with me and is working with me on some of the oat data I presented today. Clark Ridge works with me and does MNR, he's the MNR expert if you have any questions about that. And Cecil. So, if you have any questions on that type of realm you can go to him. And with that, I will answer any questions you might have.

Audience Question: Thanks, and great talk. But linking it back to sequence, genetic sequence data. Has that been done on a big scale?

Knolhoff: So, a lot of people are working on that right now where they tried to do these big scale projects, so the work I did in grad school was linking metabolism and data, and to see what disturbances there might be in there was a model that we were looking at. But people are doing this as well and trying to combine large data sets, so that is being done.

Audience Question: Alright, thank you. This is Patrick Conway from Food and Safety, and I was interested in the table that showed that you are able to whittle down the differences in the oats with just a few different components. But when you do that principal component analysis looks like there was a lot of richness of their, so I wanted to know how you narrowed that down to a few components.

Knolhoff: Right, so I don't know how familiar you are with PCA, but you usually get a plot associated with this, and it's called a loading plot. Each plot represents a compound that has been detected. And based on where that is on the plot you can see what is contributing to the differentiation. So, the main advantage to using PCA is that it can detect is that it can help you find small changes that might be present that are contributing to sample variability. If we look at the table, our criteria where much stricter than that. So, we are not looking for small change, we are looking for bigger changes. So here, redo an area cut off, we also look at and adjusted P value to compare against, to scope the differences. But also looking for compounds that are larger than a twofold difference. So that is contributing to a smaller number of features that we find.

Kaminski: Alright, our last presentation is by Dr. Bryan Delaney. He is a research fellow and toxicologist who has been with DuPont Pioneer since 2003. With the recent merger of DuPont and Dow, the new name now as Bryan told me is Corteva Agriscience and he is with the agriculture division. Bryan specializes in the safety assessment of food and feed ingredients with special emphasis on agricultural biotechnology and the title of his presentation this morning is “Composition Testing in the Safety Assessment of Foods from Genetically Modified Plants.”

Composition Testing in the Safety Assessment of Foods from Genetically Modified Crops

Bryan Delaney, Corteva Agriscience™ Agriculture Division of DowDuPont™, Johnston, IA

Thank you, Norm. Thank you everybody for coming today. For clarification, it is actually pronounced (Cortah-va). We are a company that makes genetically modified products. To keep within the scope of a toxicologists, we work with individual chemicals. If you open a toxicology journal, you will see a panoply of studies that were conducted with each individual chemical. Within that world, it is important to understand the type of testing that we do. This is what we will talk about today. We will talk about the type of composition study that we do. A little about the methods and results. Feeding studies and then placing it within the context of metabolic profiling. Let's start with the scope. I want to emphasize that today is not about toxicology but is about chemistry. What I am trying to propose here is that I am trying to stay away from regulatory regulations. The overall question that I want to ask you after we discussed the composition testing is do you think profiling would improve the safety of GMO plants? We will talk about that after we talk about the scope. We will not talk about regulatory testing. Not every agency will require all the same things at the same level. But we will put together the same battery of tests and make sure we get the appropriate of studies to the appropriate agencies for approval. One of our fellow front row people here is asking why do you do composition testing? The reason we do it is because it is not possible to demonstrate that any food is absolutely safe. Nevertheless, what this has developed into is a comparison-based approach based largely on composition analysis between the GMO crop and near isoline. This will be the closest genetic comparator is that that does not have any modification. The issue that we are looking to investigate is if there are any changes to the products.

Which components do you measure? How do you account for natural variability? What about the spontaneous or introduced changes to DNA from traditional breeding practices? This is more of a term that we use in our company. What we do with these studies, we call them CEA studies. C is for composition, E is for expression, and A for agronomics. When you think about it, this is a type of reporting involved. The way we do this, for a typical composition file, AGMO or non-GMO comparator from the same field, you go to an additional non-GMO commercially available reference line. 6 to 8 different crops, then you have to do eight different geographic locations. We're talking about a lot of locations and a lot of samples. We do grain for animal feeding studies and forage. These are all compositionally tested using validated analytical methods. You can see that the CEA trial is a very large trial and it is very complicated. We conducted these typically with stacks that contain more than one. When we say stacks it just means more than one event. We connect the central theme that we start with. Foods are complex mixtures, but we know the identities a lot of the components. We also know the composition ranges in which these are measured. So, with the analytical methods and databases with historic ranges, it accounts for about 95% of the bio maps. There could be thousands of other substances there.

When we do the compositional testing, these are the components we test. I will go over them sort of as a laundry list. We look at nutrients, anti-nutrients, secondary metabolites, toxicants when available and in some cases, we do testing of allergens. So, start with the nutrients. What we look at is when you look at a standard

composition performance trial, what you will see is the nutritional measure. We also look at fiber. These are some of the primary nutrients that we look at. In addition to this, these are some of the minerals that we look at in corn. You can see that there are eight additional things that we did not have in that list of nutrients. We also look at individual amino acids. I am sticking with corn here. It would also apply for soybean, cotton, wheat, whatever you are working with. We look to amino acids. Fatty acids as well. These are the ones we look at. We look at vitamins. We look at anti-nutrients. In corn, we have these anti-nutrients here. We look at secondary metabolites in corn. Again, we are looking at all these different samples. I want to emphasize that while we are talking today a lot about metabolite profiling, as a component of understanding the composition of your GMO product. We will come back to the essential theme of will it really make the product safer. Let's talk about toxicants. This is based on what we know from published documents. We really did not identify any soybeans or corn. Potatoes are some examples and canola and tomatoes and wheat. Finally, we look for endogenous allergens. Again, not really falling into my specialty for allergy. I don't know that it changes the allergens of a crop if you are allergic to soy beans changing the concentration, I don't know if it will make it more or less allergenic. We have certain regulatory requirements and we complete them as necessary.

We have a lot of groups and the primary comparison we have in these as outlined here is the composition, the component of the GMO is compared to the non-GMO crop. If you have that many samples that many locations, you will anticipate that there will be some statistical differences. I can guarantee it. You will construct a tolerance interval from the reference line. You wonder why we have more references than we have controlling comparisons? This is why. Because if you see a come comparison difference you see where it falls in the range. Doing a comparison for online databases that have crop data and it is a complicated statistical difference. It is significantly different from the GMO crop. You will have to do follow-up to explain what those differences are. You've got thousands of additional substances that you don't measure now, where they would fall in this paradigm would be probably additional hundreds of statistical differences, that you would have to explain. The point here is that a lot of this will be for future reference. If you want to know where these documents are available, it is available online and open access journals.

The results, and again I am not going to show a lot of specific results here, show the composition within the context of these components. The largest impact is the environment. When you compare the impact to biotechnology to the impact of an environment, it’s not even close. If you have a non-GMO and GMO comparison here, this is for amino acids. The green is the GMO and the blue is a non-GMO. You can see just how tight it is, the comparison. This is pretty standard for the differences you will see among these crops. This is what we have found so far. Really, when you come down to the end of it, I want to consider the composition test and we do animal feeding studies. The primary one you will see published will be the 90-day rat study. Some people criticize it. It could be longer, it could have more animals. They always criticize something. It can be complicated to do an animal feeding trial with the corn or a soybean because you can put in a guide of how many concentrations, but it does not

work that way. Corn is about 35% of the rat diet that we use. We will formulate diet with 35% of the GMO crop or non-GMO crop. What we found in those studies is that no adverse effects were detected. In addition to those studies, we often see boiler chicken studies. These animals are exposed to different concentrations. You mix it into the diet and then you follow their life span for the next six weeks. You look at various indicators of nutritional performance such as bodyweight. We started think then of the testing that we do now, do we believe it is adequate to evaluate the safety? That is the question. I am not going to answer it, but it is there for you to consider. This is what some of the data looks like. This is an example of a feeding study that was conducted with a broiler chicken. It started out life here with about 50 grams and we weigh them about every seven days. We submitted a paper years ago to a journal to discuss the nutritional performance study and used a line graph. They sent it back and said it was wrong. It was, as it was just one line. But this is the variable that you normally would measure in a chicken. The growth and the performance. This is one that talked about no adverse of adverse nutritional effects from consumption of feed fractions from GMO crops. Point of emphasis, we have used this for a long time if not seeing anything.

Consideration from metabolomic profiling. We know they are there, but we don't know how much of them are there, the standard ranges. We start to see differences, it starts to create an issue. Metabolomic profiling has been conducted on GMO crops. When we consider the metabolomic profiling in the context of safety assessment, it is typically going to measure substances that we analyze already. We do not necessarily know that any of them really have any biologic relevance on any of the animals. We know that the overall impact of the composition was lower in biotechnology. Can it increase your knowledge about composition of foods from GMO crops? Yes, it can. Does that increase knowledge, helping to establish the safety? The answer is yes, no, or maybe. I don't know. In some cases, if you have a reason to go looking for certain components, you may need to go find what that is. There is still uncertainty about whether it belongs to the components of analytical data. We continue to do composition studies. It is expected of us. We have not found anything significant with regard to compositional differences so far.

I will talk about be analytic comparison we do. We support substantial equivalence and we found that environmental genotype has a smaller impact. The question then becomes is more data better? I don't want to tell you I have the answers. Do we have the technology? We do. We use it for research. We have very good methods and instrumentation. There are some real uncertainties about the future and it may not be necessary to present my opinion to figure out what it might be. I will tell you, when we do a composition, you're probably looking at 12 to 1500 pages of data. It is expensive. You combine that with the animal feeding trials. Just for to studies that we do, you are talking about thousands and thousands of additional data. We have never found anything and now we are talking about doing additional tests. Just keep an open mind what it is we are really asking.

I want to acknowledge the input from my coworkers, and again these are just a few of the people that are involved. They are the people who have been working with me and

they are terrific scientists. I included some references here. Thank you for listening.

Roundtable Discussion
Moderator: Norbert Kaminski
All speakers
Additional Panelist: Supratim Choudhuri, CFSAN, US FDA, College Park, MD

Moderator: I'm sorry, I was not using the microphone. Maybe the chair of the panel discussion…I'll ask the first question. You mentioned something that I was thinking about even before coming to today's workshop. That is the role of environments and how that is going to affect plants in general. Often, we think about where they are grown and what is in the ground. I worry a lot about the stress that the plants are under. I recall years ago some work being done by the Dow Chemical Company where they were looking at insects in the grass where these plants were grown and saw many of the gene of toxic products that these plants are making to defend themselves. I am just curious in the assessment that you were talking about, do some of them also involve that kind of stress?

Delaney: Yes and no. Stress definitely impacts the composition. Can you hear me? Stress will definitely impact the composition. What we have seen in the case of, for example, like insect protected crops, they oftentimes, if you look at the concentration of mycotoxins, they have lower concentrations because the corn is better protected from the insect damage. If the corn kernels are damaged in the field by stress it is more susceptible to toxins. Those are the kind of components that we test. I will tell you that stress can come in many forms. Weather and insect or from drought class will respond to their environment. We have some of that information, but for regulatory packages, it is often not included.

Audience Question: Hello, I'm Jeff Patton from the FDA. I think I understood that you could take, for example, and orange juice sample and run that and then adulterate it or alter it and do a subtractive comparison?

Knolhoff: I don't know if I would define it as subtractive. Yes, you can do a comparative analysis to try to figure out what the adulterated compounds are.

Audience Question: Bob Mercker, FDA. I think part of what I have heard today is at least from Bryan, is that we have basically been doing the same experiments hundreds if not thousands of times. Then what I heard from Sherry is that corn is safe, and it has a lot of inherent variation. Why are we doing the same experiments hundreds of thousands of times?

Delaney: Because we have to. Honestly, I don't want to be the industry person appear on the panel talk about what tests we don't want to do. Typically, oftentimes, we do not have to do extensive testing. If you do not have a composition trial, your product may not get approved and certain geographies. We watched the temptation to incorporate additional requirements, whether in your competition testing we look at it with concern.

Choudhuri: I would like to add what Bryan addressed in response to the question about stress to plants. There are a couple of nice reviews published of various plants, rice, wheat, potato, tomato, soybean. The conclusion of almost all of them is the same. Genetic modification creates as much variability as the growing composition its self. There is no difference. So, stress is no different between GE and non-GE counterparts. So, it is basically a safety point of view. That is one way a plant's response to survival.

Audience Question: Good morning, I am Ron Lorentzen. I am a former FDA toxicologist and risk assessor. I will say that I go back to the days when they use to feed crunched up tomatoes to rats. Toxicology is not a science that studies food. You are never going to find a killer tomato or a killer corn. What you are changing is so small from the rest of the tomato. And tomato itself is grass. All food is grass. We have to assume that unless we are going to be studying food as part of our task, and we are not. What you are looking for, I think, and maybe the genetics people can wise me up a little bit, but you are looking for change in a very small part of the tomato. The real task is to find that needle in a haystack. It is not to look at the whole haystack. The haystack is grass. Am I crazy? You need to find out what part has changed. It seems to me is getting easier. We should be able to isolate that and deal with that separately.

Delaney: We do that. Will he test and insecticidal protein, it is subjected to that and I did not discuss today. But normally you consider the source. Does it take grade and digest fluid? All of the different analyses we do it to just determine whether the protein itself represents a hazard. To your point, that is the needle that we do test. We test the haystack just to see the process used to make the plant that makes that protein and that is why we do all the composition testing in the feeding study.

Audience Question: Is that rational to do that?

Delaney: 20 years ago, it was.

Audience Question: Genetic engineering by shotgun, that is not the case now.

Delaney: I don't know if the regulatory requirements. You don't have to convince me. I'm with you.

Audience Question: The geneticists tell me that if you can isolate things…can you be confident that the rest of the tomato is unaffected?

Response: Everybody thinks it is and I do not think it is we have to have a different paradigm. We cannot study food with toxicology. I know people do it, but isolating it seems to be the real task.

Audience Question: This is a question for Sherry. First of all, I enjoyed your presentation, especially on the evolution of teosinte. You talk about domestication of plants or food production. As a former plant biologist, I am very keenly aware of the

secondary metabolite, the chemical factories that plants really are. How they adapt to their environment. I wonder when you think about the domestication of plants, is there something in the step there of domestication that has minimized or further reduced the likelihood of unexpected toxins in food production? Is there anyone who was done research in this area or who has thought about this, an additional screening in terms of the safety of these compounds?

Flint-Garcia: In the context of domestication, whether anyone has looked at toxic chemicals as they happen during domestication? Just imagine 9000 years ago, people hungry and roaming and looking for food, how does anyone know what is safe? And so, trial and error. If something happens, you don't eat that plant. I don't know of anybody in particular who has looked at domestication. Obviously, potatoes and tomatoes have a different story. But I am not an expert in that area.

Audience Question: When these paradigms were set up, I was still a graduate student in the lab. There were questions about position affects and questions about if we do this and we interrupt a gene, but we do an insertion and things get scrambled, you can have an issue. At that time, we had a lot less knowledge about the genomes of plants. So, I am wondering, given the increased knowledge, the tools that we have now allow us to really do a thorough characterization and the question about position effects seems like we have a lot of information with the history here. So, what you think…how do we use the information that we know from all the basic studies that have been done to help us and in a targeted analysis?

Dietz: I think part of it goes back to the slide where we talk about the kinds of things that plant breeders typically breed out through an elimination process and variety selection. That would capture a lot of those things. They would ultimately show up as a phenotype. One thing we need to recognize is that generally it is the phenotype that we assess for safety. That is what you would be analyzing in most cases. You may not know the exact nature of the genetic change, but you could still perform a safety assessment to get to a point of saying that this product would be safe. Even and light of what we know about the genome, it is important to realize that it is the phenotype ultimately that is probably the most important aspect.

Flint-Garcia: To add to that, just because we have a genome sequence of all the major crop species, we do not know what all the genes doing. The vast majority are hypothetical protein, it does something. So even if your favorite transgene lands into my gene, there is no way to predict what the effect might be.

Choudhuri: I was going to add one example. Even though plant is more plastic and redundant, I would assume that effect would be visible. That might be weeded out during selection.

Audience Question: This is Jeff Patton, FDA. I'm a toxicologist. I have not thought this through completely. In our work, when we have a low level of toxicity, we are often most concerned with carcinogenicity and developmental toxicity. We don't have good

correlate of cancer implants that I am aware of. I would think that plant embryos would be selected and maybe perhaps for corn, you would look at corncobs and seeds and see how many are unviable? Is anyone aware of any attempts to look at the success rate of embryos from genetic engineering? Has anyone taken that kind of approach?

Delaney: I don't know the answer.

Dietz: I do think that sort of goes back to the phenotype point as well. If you can't have a viable phenotype, that variety is not going to make it past first round.

Audience Question: I'm sorry, I should have prefaced that. It wasn't really a safety question in my mind regarding food. It might be, at least of academic interest, to be able to go in and look at the seeds and see why they failed. Was it a mutational problem or was it an altered protein, altered enzymes? All those stress response genes, they start out as embryonic developmental expressed genes.

Dietz: Your question would be then; do we test the ones that failed after transformation for why they failed? I am going to say no we don't. I think it was common back in the days. That you would generate thousands of and if they grew, they grew. If they didn't, they didn't.

Audience Question: I had a question for Bryan. I want to thank you for saying what goes into the testing. One thing that seems to be missing, for example, in the corn, where the transgenic insertion to make that plant express a toxin that hasn’t been expressed before, it looks like what I looked for in the compositional testing. I was curious why looking at the prevalence of those weren’t included?

Delaney: We do that. You might call it a composition expression. That's when you look for the expression of the protein. Technically, it is part of the composition, but I did not go over it. That could be a lecture in and of itself. This is a very narrow spectrum of testing that we do. To get your point, we do that.

Dietz: In fact, that particular type of protein would be evaluated by EPA and they have a very extensive assessment. Be assured that that is being taken care of.

Audience: It was interesting to hear about the environment, what it plays in the shift in expression. I was wondering if long-term environmental change could come. Have you guys thought about any novel stress responses from plants? I read about nutrients decreasing in a higher carbon dioxide environment. I was wondering if you could comment on that.

Delaney: I will share with you that we are looking at different stress proteins. I don't want to get into specifics. Whether it is drought or salt, the most easily observed impact is decreased yields. That is probably the primary thing you are looking for. If you can fix that, then you would also look at the composition. If you cannot adjust for the yield, then probably it is not going to be marketable. One of the things that I believe

in, to your point about stressors, plants respond differently to different stressors. The ideal plant stress would appear only when something is necessary and will go away when it is not necessary anymore. I don't know if we are there yet with that type of technology. We are certainly investigating it. Those are more complicated issues maybe Sherry may have some thoughts about that.

Flint-Garcia: Given that I don't work on stresses, things like that, yes, we do field out the ultimate output of stressors and things like that. I don't think I really have anything to comment about this.

Dietz: In one of my slides, I talked about the number of locations at field trials. Should our environment be changing so that those field trials are occurring in that change some ways you are controlling simply by doing field trials. The one diagram had as many as 100 locations. You could imagine that it could be everywhere from Puerto Rico to Hawaii to Maine. A wide variety of environments for things like corn that could grow. A pretty large geographic region.

Audience Question: Hello, Allison Edwards. Do you think, given the range of composition with different environments, the range of composition but maybe changes over time, do you think metabolomics could identify where historical ranges are maybe not accurate before a certain point or where perhaps the existing body of data does not adequately capture natural variability in a crop?

Knolhoff: Are you asking can current technologies supplements past pathological data or nutritional data and see of those matches? Is that the question?

Audience Question: Could [indistinct] be used as a way to add what way of adding to…do we have the natural variability captured adequately?

Knolhoff: I forgot to say this during my talk. My background is not with plants, so I cannot comment too much on that. In terms of the technology that is available, because you can detect so many different things, and once you identify those things you can quantify them, and it could add to the current knowledge that is already available. You could do quantitative studies as well to see if a particular modification is within that range, I don't know how much the specified ranges in terms of the known variability that is already present, but from a scientific standpoint, I would be interested in how those levels change to historical data.

Flint-Garcia: I think I would add that it's a different story depending on the crop species where you have a huge industry present in developing the materials, like in corn, me, I would probably never develop a line where someone would grow in the field voluntarily. We don’t know exactly how much diversity there is out there. When you extended beyond into the world, I certainly do not know what the germ has some for China, for Thailand, for South America, Brazil, anywhere in the world, how much the diversity that I talk about is in the germplasm that they use. Or is it all just using corporate germplasm and they just tweak it slightly. The question about getting back to

your question about what's the right range to use for values, I think that is related to this black box.

Audience Question: Listening to Sherry talk about the vast numbers of alleles variance you heard and talk about the vast amount of data and then Bryan about the large number of studies. Is there a role for big data and cloud computing in this to put this together in a way so that you don't have to complete the same experiment over and over again indefinitely, but rather can identify…one approach to be used is to identify safe spaces? So, if you're in this data range of say space, you probably don't need as much data. Any comments?

Delaney: You would not have any trouble finding people at my company who would say why do we have to keep having to do the same composition testing if we never found anything. I certainly understand that. I think as long as each new event is considered a new chemical, the thought that we could somehow reduce repetitive testing would be something that our company would like to do because are expensive and complicated.

Audience Question: My thought would be to not just minimize testing but actually to identify real issues. Will issues that may have occurred rather than beating the same thing without knowing what you are looking for.

Knolhoff: I did not talk too much about the types of methods that I try to develop, but for me I try to develop nontargeted screening methods. Typically, where this would be used if some kind of adverse event and none of our targeted methods were able to identify what features were present. A lot of my time is spent looking at the data and how to treat the data and can we find ways to elucidate the compounds that are there. Absolutely, cloud computing, things like this can help. That is part of the reason why we started to look at trying to figure out what kind of variability that there might be. I could see where company, if they know there is a particular type of sample that they are producing routinely, that they could develop their molecular database that we know this food is safe there is no adverse effects we can measure the chemical content. You could do screening afterward to say does that fit within the compounds that are present but that is hard because there are lots of different types of food if there was one particular type of matrix, that would be easier. But it is hard to test everything.

Dietz: I would like to take the opportunity to maybe clear what is a misperception. We talk about key nutrient intoxicants…that does not mean we are testing every one. That means we are focused on the ones that if they were changed will result in a nutritional issue or toxicological issue. From that standpoint, if there is some rationale to focusing on specific substances and not looking at the single substance in the plant. That is very important to emphasize. It's also important to emphasize that while many of these foods are used for humans, they are also used for animal feed so there are some animal feed concerns that go beyond human food concerns. Corn, for example, being a major component of animal's diet. It really comes into play when you're talking about some of these minor crops that are not a major commodity that are not used so

much for animal feed.

Delaney: There are certain nutrients in certain crops and some of those components are known not to be there. I am blanking on specifics here that there is a specific nutrient that exists, a vitamin that we know exists in one food source but is not in another food source, we would only measure the things that we would expect to be there. We would have a point of reference.

Audience Question: I know when an event is created, by traditional methods it would be bread into different lines. Different lines are adaptive for growth say for Massachusetts versus Iowa. We know just right there that because you have different effects or all kinds of responses to the environment, the lines have been specifically created to grow in those environments, so you are crossing your event into many different lines. It seems to me that if they did not perform well, the farmer would not be planting them, wherever they are. In a sense, you have the variation test by doing all those crosses.

Delaney: That technical area about how they cross go beyond my comfort level in responding. To your point, the customer is the farmer and the variable is yield. That is what they look for. If it does not perform, then I would assume they probably will not buy your product again or there will be some other type of economic impact. But that is the ultimate variable is crop yield.

Audience Question: The whole idea of talking about what the range of variability is, even if you were to take plants that are grown in different regions of the country and were to do a metabolomic type of analysis, I would presume you would see some kind of differences?

Delaney: I can't guarantee that the individual trait is different from one geography to the next.

Audience Question: I am not talking about the inserted trait, and I'm just talking about the whole.

Delaney: I cannot guarantee that the trait would be expressed in different actual varieties based on region. I do not know if that is accurate.

Audience Question: This is Ron Lorentzen again. Is there any evidence that pre-GMO hybridization all these different genetic crop-style food, is there any indication that with any of these techniques that there is something that is because of a disaster because we have accepted all of these as food and all these changes have happened? We have accepted all of these changes as safe for our food. Is there any indication that we could screw up?

Choudhuri: Based on what we know so far, the short answer is no.

Dietz: I looked in the literature for the hard to find examples of this. The ones that come to mind that were presented in a 2004 national academy study, it was a potato that had higher amounts of solanine living in it. There was also a potato in Sweden, and that also had some issues and was removed from the market. That was not genetically engineered as well. But I have to say I had to look really hard to find those examples those examples are within a 40-year period. To the point that the fact that they occurred is somewhat remarkable in of itself.

Flint-Garcia: As a geneticist and breeder, you can take two very wonderful kinds of corn and cross them together and when you separate the progeny out, you will always get good, bad, and ugly. That is where we get that bell distribution from. There is always something that will come out that is not good. And breeders, when they are walking to their fields, they will say this is not good and they will step on it. There's always something that comes up, but breeders are attuned with their crop

Audience Question: My name is Karen McMann and I'm with the U.S. FDA. I am not a toxicologist but on my first day of training I learned that dose makes a poison. I want to go back to something Bryan said earlier. 95% of the composition is identified in analysis but the remaining 5%. And Ann talked about metabolomic studies…So that 5% that is uncharacterized…when does a toxicologist become concerned what types of compounds in plants at that level do we need to know about?

Delaney: That's a good question. The answer is I don't know. We look at the composition. We compare it to the composition of other plants that are similar in nature. The highs, lows, the mediums, the concentration. It is tempting to speculate that what happens to that 5% is not really significant to the impact. If you start doing metabolite profiling or whatever of that 5% and you start to see minor constituents that goes up by factor of five. Well, I don't know what that means. Until I know what the normal range of that variable is in the crop, it might be significant, it might just be natural variability. The effort that it takes to put together…it took years to develop. If you see a 2x increase or even better, decrease in something in the GMO compared to the non-GMO, I don't know what to do. The chances are pretty good in these substances that you are going to see them.

Knolhoff: I am not a nutritionist, so I can't comment specifically on the range of what is acceptable. You would not run a metabolomic profiling but if you knew the nutrients that you cared about, you can set up methods to look at those and quantify those things. If it falls within a range, fine. Whether in terms of those are hazardous are not are not, those studies would probably need to be done. It was interesting about your 5% comment as well. My PhD work was looking at and analyzing single cells within the brain or in mono-organisms. The impetus for doing that is that if you pull everything together and look at it, you will miss small changes, so you won't detect neurotransmitters, for example. Even if it is in that 5%, it may matter, but it is hard to say whether it will or not. For food screening purposes, we cannot monitor everything. We do not have the budget to do that. Most of the time, most food is safe. Food is safe for the most part. It is a good question, but I don't have a good answer for it.

Delaney: The risk would be if you start seeing differences in substances that are significant biologically, then it could be…I don't know where that information is going to tell you about the safety when all the evidence that you have so far shows through the testing that we have not seen any adverse effects. If we start mining that additional 5% to see changes in substances, I think it is a terrifying prospect.

Audience Question: If we take a step back in time, what percent was compared by companies?

Delaney: I think for purposes of breeding and producing new varieties, they probably did some compositional testing. To my knowledge, there is not any regulatory standard in the world that requires compositional studies for crops produced using traditional breeding methods. Even though we know that that impact has a much greater influence on the composition and bioengineering. It's a good question.

Dietz: Speaking as a non-toxicologist, one thing that hit me as we were discussing this is in this particular area, it may be a unique situation in the standpoint of we already have a lot of history about the substances that we are talking about. It is not a complete novel chemical entity. We have hundreds of years of experience that we can bring to bear on this. That may put the assessment in this particular case in a slightly different place than a novel chemical entity in general. When you get to that 5%, you can say yeah, we may not know exactly what is in that 5%, but we have hundreds of years of experience telling us that that 5% is not harmful.

Knolhoff: Is that 5% consistent? Just in terms of…I think it is probably hard to say.

Delaney: It is hard to say. Could the metabolomic profiling make tools used to gain additional compositional knowledge about any product but is it really going to improve the safety? We already know that the products are safe and that we could be subjected to some additional analytical testing that could result in further toxicological studies as well.

Knolhoff: How is the 5% defined?

Delaney: We measure the 70 or 80 components that we know are there and we know the concentration ranges and they were defined.

Knolhoff: Is that total protein content or specific protein?

Delaney: Each product may have a different…we measure if it is by a protein and we measure the concentration of that protein. There are certain allergens we measure for regulatory requirements.

Audience Question: That pretty much just answers my question. Do you do any global proteomics outside of the protein that you are focusing on? Do you look at any

targets, and if so, to what extent?

Delaney: We have people who proteomics. To my knowledge, we have never submitted anything for a regulatory submission. For investigative purposes, I think these tools are fantastic.

Audience Question: How many plants did not make it through the screening process?

Delaney: It is hard to say exactly. Some of the early methodologies, they were harsh just by treatment. We have something called a gene gun, so it is a pretty harsh and a lot of them did not survive that. I would say, out of 10,000 transformers you apply that to, to the get the one that you wanted. I can't say for certain, but it is somewhere in that range. It's not that the genes themselves are dangerous, but the method that you put in there could be harsh.

Audience Question: Another question, in your experience, how does different geographic environment affect the performance and expression of the newly introduced gene or trait?

Delaney: I could leave it to some of the other folks on the panel to talk about the impact of that on the composition. But the impact on the protein, I don't know specifically that we look for that. I don't know. That we do multiple locations of the field trial. That will contain different geographies. I suspect it would have a huge effect. From a typical field trial, you would have 6 to 8 different locations where the sample is drawn.

Audience Question: Ron Lorentzen again. You said this throughout you talk, but a lot of this reminds us that in safety studies and toxicology, safety is not possible. And other regulatory agencies, they have a line to drop. We are not going to look any harder than this. That is for all food, everything. They say we are not going to look harder than this. There is a size of the study to use, the sensitivity of a study to use. So, you have to know when to stop. I don't think you have defined that. You have to know when to stop looking for absolute safety.

Choudhuri: Even though the question was directed towards Bryan, I think I can answer it. I don't think they do these studies for fun. They know they have to do some regulatory requirements. They do as much as they can, as much information as they can so they can prepare a package tailored towards different countries. I'm sure Bryan agrees with everything you said and many of us do. That is slightly different from what we see, what we have been seeing traditionally.

Delaney: I will supplement that by saying we are living in a time where the general public has access…they could be selective on which they want to believe. We saw earlier this year this G-Twyst study from the European commission. There is no evidence of any adverse effect. There is no reason to do the study, but they ran it anyway and it came back negative. You have to wonder about the resources and the

animals that were used to do that study. Was it really necessary? We knew what the outcome was going to be and that is exactly what happened. It was not driven because it was a scientific reason to investigate it.

Choudhuri: That study is a follow-up on a publication from 2012. That study was questionable and was retracted from the journal.

Audience Question: And the tragedy there, false positives.

Delaney: I agree. I would say what it is. Why we do these studies is if we don't do these studies, someone will step in and do some bad studies. The study that he is referring to was an example of what was considered a study bad enough that it got retracted from the journal. If we don't do good studies, good science, somebody will step in and do bad science.

Audience Question: I have one last statement, and I'm going to paraphrase it from one of our listeners. They were strongly encouraging that summaries of today's colloquium be submitted for peer review publication. That might be something you want might want to think about.

Knolhoff: I have a question for Bryan. Many times, it seems like for what is being edited, it is always for protein expression. Are there any kind of downstream like metabolites that are being focused on? If they are, you might need to do…the reason I ask is you could have breakdown products of that that you might want to do studies on those sorts of things.

Delaney: Absolutely call you are right. I want to emphasize the data that I presented here. That is the standard minimum that we always do. If we have an enzyme or a protein that has activities, then we wouldn't know what the metabolites are or identify what they are. What I'm presenting here is a minimum and if you know that your product leads to the formation of something else, you will also know what that means.

Knolhoff: So that is currently not required?

Delaney: The guidelines are written loosely enough that they would encourage you to look at keep metabolic changes if necessary. I can't imagine regulatory agencies out there who would not have some requirement like that. If you think it might cause compositional changes, what are they? Another example would be when we modify a soybean. We do the standard profile of a composition, but we also do an intensive lipid profile to make sure that the lipids are effective.

Choudhuri: I have a question for Ann specifically. The issue of cost per studies came up so I am wondering if we go back to he would genic sequencing. The first time we did it, it cost $3 billion dollars. Can we see the same trend of cost minimization in metabolomic studies?

Knolhoff: If you are going to do a high-resolution instrument, it could be a quarter million or half $1 million for that instrument. I was talking with somebody recently and they were saying that one of the instruments they had was $100 per sample. I talked to somebody else that I knew who was at an agricultural company and he thought that was cheap. Don't quote me on those, but these are kind of my feeling about it. So, I don't think it's as expensive as genomics was when it first came out. As these technologies become more commonly used, the price gets driven down. There are methods where if you are just profiling specific things, if you know what you are looking for, you may not need the high-resolution instrumentation. There are ways that you can make those, especially the profiling experiments, cheaper.

Audience Question: I have a question that hopefully can tie together a lot of the things we have been talking about. The overriding question is, is there value that metabolomic can add to the safety assessment? To answer that question well, we need to pinpoint what is the importance and what is the purpose of compositional analysis in the first place? The way that these safety evaluations are done now are event specific. Is the subject at hand, what is the hypothesis leading to the hazards we are looking for, and is that an event-specific issue? Then I think, whether the study can improve upon that baseline inquiry.

Delaney: That is a terrific perspective, that question. The answer is that the testing was done and was conducted for unintended changes, to look for unintended changes. We knew what the protein did and its range of activity. So, we are going to rewind back to 1996 when the testing began, and it made perfect sense to do this. We thought, it makes perfect sense why it was done. To your point earlier, is it necessary to do it in every event? Probably not. If regulations change, we will adapt to it. It has been my observation, that when regulations change they do not become less intensive, but they usually become more intensive. I am not really certain that will change anytime soon. A question I would post everybody in this room, will this metabolomic profiling improve the safety assessment or the confidence in the safety of the product developed by technology? I think it is a great research tool and I am glad we have people who do it. I am just not anxious for it to become a regulatory requirement.

Choudhuri: With respect to composition analysis, we have to know what analysis we are going to study. That is where some of the push for metabolomics get a global profile. If you can establish substantial balance, that basically eliminates the need for a toxicity study.

Moderator: If there are no more comments, I would like to thank the panel and all the speakers today. A very thought-provoking colloquium today. Again, to remind you, the colloquium that we have coming up for 2018-2019 and to remind you that the colloquium was recorded, and the slides are available online at no charge along with a captioned text. Again, thank you everybody for your participation, including the audience. Some good questions. Thank you again.