Metabolomic analysis of GM and non-GM maize and its preference by cattle.

By

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SUPERVISOR: Prof. G. Prinsloo (UNISA)

STUDENT DECLARATION

I, Joseph Payne, with student number: 3969 454 2 declare that:

1. The dissertation's research is based on my work and studies at the University of South Africa's environmental sciences and agriculture college. In addition, the analysis of metabolomics was carried out by the CSIR and Biometry unit.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain data, figures, or writing, unless specifically acknowledged, or copied from other researchers.

Signed at…PRETORIA……. on the……day of…… 2023.

DECLARATION BY SUPERVISOR

I declare that I was the supervisor of the student for the duration of his studies:

Student's Full Name: Joseph Payne
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Metabolomic analysis of GM and non-GM maize and its preference by cattle

During the investigation, I regularly consulted with the student about the project and provided him with the necessary advice and guidance. I also approved the final documentation for submission to the university's designated examination authorities.

SUPERVISOR: Prof Gerhard Prinsloo

Prof G. Prinsloo……Date…….....

COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCES

DECLARATION 1 – PLAGIARISM:

I, Joseph Payne, student number, 39694542, declare that "**Metabolomic analysis of GM and non-GM maize and its preference by cattle".** My own work is the subject of this declaration, and all the references I used have been acknowledged and indicated. Furthermore, I have not submitted any part of this work for any other qualification or examination at any other institution or University of South Africa.

Signed

Joseph Payne……..……

Ethics

This study complies with all ethical requirements of the University of South Africa which have been obtained with the following reference number: 2019/CAES_HREC 133. This study is guided by integrity, accountability, and rigorous research.

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Abstract

GM organisms have started to infiltrate South Africa's agricultural landscape, altering how farmers deal with weeds and insects. Adopted from the United States of America, it is a technology that the commercial farming sector cannot do without anymore and the uptake was enormous in the last 10 years. The technology involves altering the DNA of an organism to provide a specific benefit using genetic engineering techniques.

But all new technologies and inventions bring the responsibility of stewardship and accountability with it, and it is impossible to predict all possible outcomes of such an invention to every possible scenario in terms of environmental stewardship. The question and occurrence of cattle and wild animals seemingly choosing non-GM containing maize plants have raised the question whether this is really the case of the presence of the GM or maybe it is just due to the differences in plant genetic makeup. Quicker deterioration of short, seasoned cultivars opposed to the longer seasoned cultivar that is known to be more resistant to diseases and plant pathogens, might therefore not be as easily affected by mainly pathogens and stay much tastier for longer.

This study used beef cattle of the Drakensberger indigenous breed to determine the taste preference with the use of a trial that is planted with the different types of maize cultivars that includes GM and non-GM maize and short and longer seasoned cultivars. The trial was performed on a commercial farm near Standerton, Mpumalanga in the high rainfall area of South Africa. The results clearly indicated that cattle could not differentiate the GM maize from the non-GM maize, but that they preferred the longer season cultivar.

The chemical profiles of different materials, including those made from GM and non-GM, were analyzed using NMR spectroscopy and OPLS-DA and pre-processing techniques. The results of the metabolomic study revealed that the former had slightly different metabolomics profiles than the latter. The differences were mainly concentrated in the aliphatic and sugar regions. On the other hand, the long and short-season GM had similar metabolomic profiles.

The study therefore clearly indicates that cattle could not differentiate GM from non-GM material on taste and that any material can be provided to cattle. The slight difference in the metabolomics profiles however raise a concern for differential metabolomic pathways and should be investigated further.

Theories and concepts

Genetically modified organism (GMO): An animal or plant that has undergone genetic transformations that are not naturally occurring is referred to as an altered species (Gouse et al., 2016).

Maize cultivar or variety: A cultivar or variety is a hybrid of two plants of the same species and is the basic grouping or taxon for cultivated varieties.

Technology stewardship: The requirements necessary to ensure the long-term safe use and viability of a certain new technology to maintain all ethical and safe usage thereof.

Bt maize: A genetically modified maize cultivar contains several proteins from a bacterium known as Bacillus thuringiensis. This produces a toxin that kills certain pests, such as corn stalk borers. The modified maize also triggers the development of a pore in the intestine of an insect after it consumes the toxin (Pigott & Ellar, 2007).

Roundup-Ready maize**:** Maize or other crops such as Soybeans that carry the gene, also derived from an *Agrobacterium* species, this enzyme is designed to carry out a function that's useful when it comes to protecting against the effects of glyphosate, which is a widely used herbicide. (Barry et al., 1997).

Maturity class definition in maize: The number of days it takes for a maize cultivar to develop from emergence to tasseling is regarded as the classification standard. This is also referred to as the Cumulative relative maturity or CRM of the cultivar. Maize cultivars are classified in the following classes from the quickest to the slowest: short season, medium season and long season growth classes (Oluwaranti et al., 2015).

^{1}H-NMR spectroscopy metabolomics: This method can be utilized to determine and quantify the various chemicals found in complex mixture (Tyagi & Malik, 2010), and describe metabolites as the small chemical components in every cell using NMR analysis. The traits that contribute to the quality of food and taste are evaluated using metabolomics.

Chapter 1: General background and introduction

Commercial farmers have started using genetically modified crops to increase their yields of maize (Fischer et al., 2015). The first genetically modified Zea mays seed was brought to South Africa in 1998. There are currently two kinds of GM traits used in South Africa's maize plants: the Bt and the Roundup Ready variety.

All over the world, the growers of maize face the same challenge which is the attack of the plants by mainly the Lepidopteran larvae. Conventional methods in combatting this pest mainly involve the use of pesticides. However, this was always done as a corrective measure and the need was born to develop technology that was able to eliminate the pest preventatively. Most maize producers in the world have been adopting a combination of cultural, biological or chemical (insecticides) methods to protect their crop (Hutchison et al., 2010), including the use of GM technology. The Bt trait, which is engineered into seed varieties such as MON 810, BT 11 or MON 89 maize contains a protein that controls maize stalk borers which include *Busseola fusca* and *Chilo partellus* which falls under the Lepidopteran class. The most common types of worms that attack a maize plant are the ones that are known to occur during the October to February growing season in South Africa. (Campagne-Ibarcq et al., 2013).

Questions are however often raised about what the Bt toxin is, how it affects crops and what additional effects it might have. The type of bacteria known as Bacillus thuringiensis produces a protein that can be toxic or fatal to certain pests. This protein is known as Cry1A. This is also referred to as natural insecticides as they differ from most conventional insecticides because of their small range of target organisms. The function of this specific protein in the maize plant is to employ specific pH levels, enzymes and midgut receptors to activate and bind the Cry toxin to midgut cells, which lead to pore formation in the insect's intestine. It is believed that the protein, which is very precise, can be used to describe a lock and key approach to killing an insect. This method only works if the protein and the midgut receptor are matched (Flagel et al., 2018).

There exist several proteins developed over the years which are categorized as Cry proteins. They are Cry1, Cry2 which targets Lepidopteran species and Cry3 which targets Coleoptera (Pigott & Ellar, 2007). It should be noted that due to the convenience of the genetically engineered seed containing "in the bag" protection because of the Bt gene incorporated into the seed, the popularity of this seed is increasing and expected to increase. Growers are attracted to the overall yield and plant protection and improved grain quality associated with Bt maize. Additionally, due to the introduction of Bt maize, farmers spend less time in applying toxic insecticides which are healthier for the environment (Hutchison et al., 2010).

The emergence of the Bt gene has been widely considered to have a positive impact on the quality and yield protection of maize crops. It has been reported that the population of corn borers has been suppressed in Europe (Figure 1) (Hutchison et al., 2010).

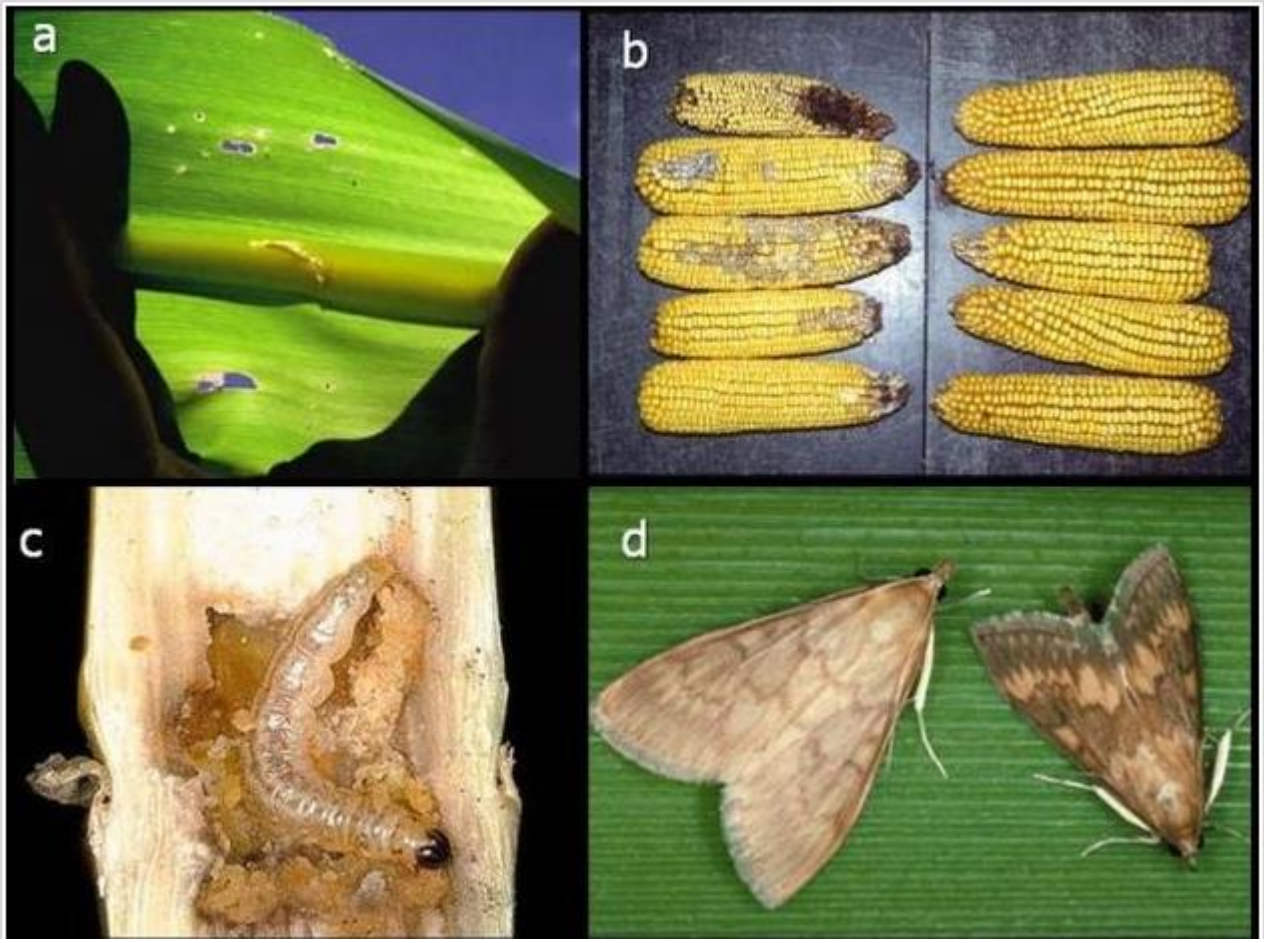

Figure 1: European corn borer. Shot holes and tunnel in leaf midrib (a), damage and fungal infection in non-Bt maize (left) and Bt maize (right) (b), stalk tunneling (c), and adult female (left) and male (right) (d) (Hutchison et al., 2010).

The development of genetically modified crops that are resistant to commonly used herbicides has greatly improved the efficiency of their control over weeds. One of the most important factors that prevented the development of these crops was the broad-spectrum herbicide known as glyphosate. Roundup-Ready crop lines contain a gene derived from an *Agrobacterium* sp. strain CP4, encoding a glyphosate-tolerant enzyme, the so-called CP4 EPSP synthase (Barry et al., 1997). Glyphosate works by preventing plants from being able to make the proteins they need to survive. Since virtually all plants make these essential proteins the same way, glyphosate affects nearly all plants (Duke & Powles, 2009).

Roundup-Ready crops is a trademark of the American based company Monsanto. Monsanto also produces the herbicide Roundup which is a broad-spectrum herbicide. Today the German based company, Bayer, acquired Monsanto as a whole. The main concern is the development of resistance over time as the plants cross pollinates with wild plant of the same families, and therefore their offspring could contain the herbicide tolerance trait and give birth to seeds that has a tolerance to the herbicide (Gutterson, 2020).

In South Africa, the use of the herbicide Glyphosate is considered to be the most prevalent. In 2012, approximately 23.253 million liters of this chemical was purchased (Figure 2) (Kotey et al., 2016).

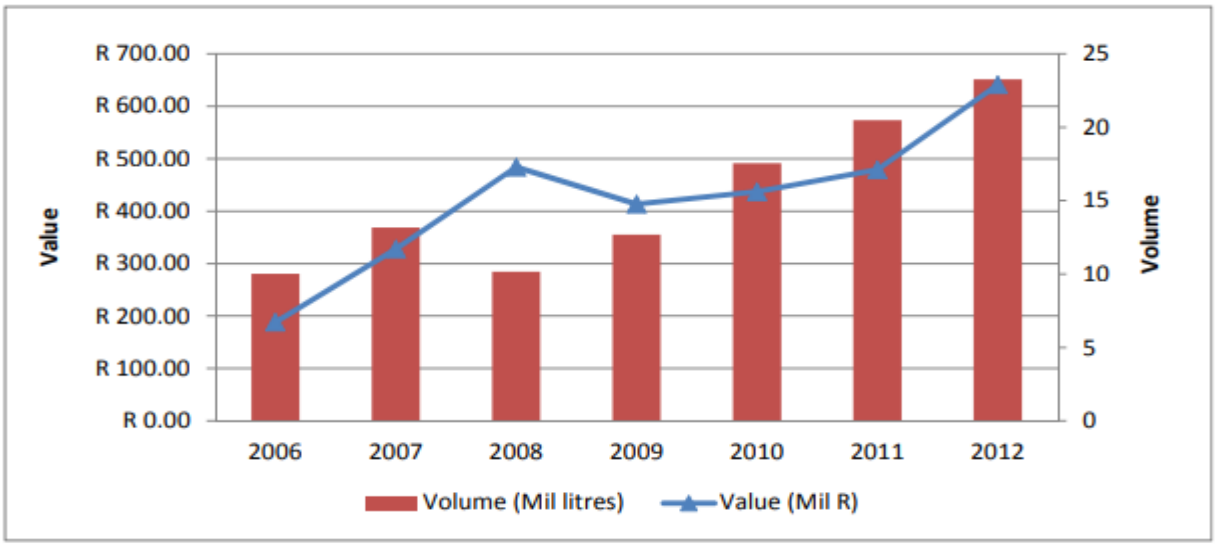

Figure 2: Volume and value of Glyphosate sales in South Africa since 2006 (Gouse, 2014).

The use of Glyphosate on maize has been leading with wheat the second highest application and shows the importance of this single herbicide in maize production in South Africa in 2012 (Table 1).

Table 1 Volume and value of Glyphosate sales in South Africa 2012 (Gouse, 2014).

Rank	Crop	Volume (1000 litres)	Percentage of total	Total 2012 crop area in hectares
1	Maize	10 590	46%	2 699 200
2	Wheat	2 928	13%	511 200
3	Industrial	1 946	8%	-
4	Soybeans	1 311	6%	516 500
5	Citrus	1 196	5%	62 000
6	Forestry	1 000	4%	1 270 000
7	Wine grapes	995	4%	100 093
8	Table grapes	611	3%	25 872
9	Sugarcane	515	2%	264 409
10	Sorghum	398	2%	48 550
11	Pome fruit	395	2%	33 866
12	Sunflower seed	266	1%	504 700
13	Barley	187	1%	84 940
14	Pastures	167	1%	n a
15	Nuts	148	1%	>25 000
16	Stone fruit	135	1%	11 876
17	Groundnuts	125	1%	51 000
18	All other	341	1%	
	Total	**23 253**		

Source: ADI, SAGIS, SA CANEGROWERS, BFAP, Hortgro, SAMAC

Since 1996, animals have been consuming feed that contains genetically modified (GM) ingredients. In 2012, a study revealed that around 17 million farmers worldwide cultivated GM crops, and over 70% of these were consumed by food-producing livestock (van Eenennaam, 2013). With all the controversy over GM containing animal feed it is important to note that over 100 regulatory submissions have showed that GM containing animal feeds composition have showed to be equivalent to the GM free or conventional counterparts (van Eenennaam, 2013). It is therefore now accepted that GM containing animal feed is just as safe to feed to animals as normal conventional feeds.

The use of metabolomics in plant analysis is inevitable in plant research. Especially when doing experiments on GM containing plants. Through plant metabolomics, scientists were able to improve crops by monitoring changes in their chemical composition. This method also allows them to understand the mechanisms that underlie cellular functions (Sakurai, 2022).

1.2 Problem statement & research justification

In South Africa, the commercial cultivation of genetically modified (GM) maize has been carried out since 1998. This is a widely accepted and high-yielding crop. Many farmers are concerned that their cattle may be able to differentiate between genetically modified (GM) and non-GM maize. Whether the Bt or Roundup-Ready gene that was introduced into the plant genome have affected the preference of cattle seemingly choosing non-GM maize is currently not known. Therefore, this study investigated whether cattle has a preference or if it can be defined in the use of different maturity groups of maize. Due to the growth speed, differences in short and long seasoned maize cultivars can result in a difference in taste because the quicker cultivars are prone to faster deterioration after reaching physiological maturity than the later maturing cultivars. The study made use of identical cultivars, only differing in the two genes (Bt and Roundup-Ready) which were introduced into the genome. A short season and longer season cultivar with or without the introduced gene were planted and fed to cattle in a feeding trial. Cattle had an option on which material to feed on, and the preference to different cultivars (GM or non-GM) was determined. A quantitative analysis of the chemical profiles of different plant materials was performed using a 600 MHz NMR spectrometer and a direct extraction method. The goal of the study was to determine the changes in the composition of the GM maize that could affect the cattle's preference for this type of plant.

1.3 Aim

To determine if cattle have a preference to different maize cultivars and the presence or the absence of introduced genes (GM) and determining significant metabolic changes in the different cultivars. The main question is whether cattle have a taste preference over non-GM containing maize cultivars opposed to GM containing maize cultivars.

1.4 Objectives

-To provide the same feed options to cattle with both non-genetically modified and genetically modified (GM) materials grown under the same conditions.
- Determining cattle preference to the different cultivars by weighing of feed at the end of the feeding sessions.
- To identify the differences between the different chemical profiles of different cultivars, a metabolomic analysis is performed.
- Annotate compounds differentiating the GM and non-GM maize cultivars.

1.5 Hypothesis

Cattle is not able to identify GM maize, as they are not able to detect the protein that the plant contains in its DNA and therefore cannot choose GM free cultivars opposed to GM containing plants.

Dissertation layout

The dissertation consists of six chapters, an addendum and a separate reference list which is provided at the end of the dissertation.

The first chapter presents an overview of the research, including the scope, goals, theories, hypothesis, justification, and problems. This chapter aligns the scope of the study with the actual execution thereof.

Chapter 2 contains a comprehensive literature study on genetically modified organisms, genetically modified maize and metabolomics. The literature review especially focusses on the maize cultivars used in the research which consists of 2 gene types namely Bt and Roundup-Ready containing maize cultivars. The basics of metabolomics, the application and importance are provided to support the use of this tool in determining metabolic changes in maize.

Chapter 3 covers the feeding trails, including the layout of the field planting. The chapter provides the layout, statistical analysis, results and the conclusions drawn from the data obtained in the trials.

The fourth chapter of this report contains the results of a 1H NMR-based study on the chemical composition of maize plant materials. It also provides information on the differences between the metabolite profiles of non-GM and GM materials.

Chapter 5 covers the compound annotation based on the metabolomics analysis. The chapter also discusses the role and responsibilities, and the potential role specifically in GM plants of the annotated compounds.

Chapter 6 provides the general discussion, conclusion and recommendations.

Addendum. The addendum provides the original data and additional statistical analyses.

The full list of references is provided after the addendum.

Chapter 2: Literature review

2.1 GM crops and the adoption thereof

Due to the positive effects of genetically modified crops on the environment and the economy, many small-scale farmers are expanding their use of these crops (ISAA 2018). In Africa, the introduction of new genetically modified (GM) crops and the increasing adoption of stacked traits have shown progress. This is very important because the development of biotech crops can help address the various challenges that the world faces, such as food security, climate change, and sustainability. During the initial stages of development, it was envisioned that GMs could be used for a wide range of applications, such as producing vaccines against diseases like Hepatitis B (Kumar et al., 2008), Various factors have led to the development of faster and more nutritious food, such as the use of metabolically-modified fish that can grow faster, nut trees and fruit that yield earlier, and plants that can create new biodegradable plastics (Lackner, 2015). The rapid development in molecular techniques quickly proved the wide and universal application of genetic engineering in various fields.

In 2018, 25 nations cultivated approximately 192 million hectares of genetically modified (GM) crops. The US, Canada, India, Brazil, and Argentina were responsible for most of this area. Since the first GM soybean was planted in 1996, the crops have been used commercially for almost three decades. In 2018, 25 nations cultivated approximately 192 million hectares of genetically modified crops. The majority of this area, which is over 90%, was occupied by India, Brazil, Canada, and the USA (ISAAA, 2018). In addition to these nations, 44 are also importing genetically modified (GM) crops. In Argentina, 18.2 million hectares were planted with GM soybeans in 2018, while 5.5 million hectares were planted with GM maize and 0.37 million were planted with cotton. The adoption rate for these crops was 99% for GM soybean herbicide tolerant, 97% for GM maize insect resistant, and 93% for GM cotton resistant. In the US, almost all the corn, soybeans, and cotton were grown with genetically modified technology that has been able to resist various pests and herbicides. There is a striking lack of GM crops being grown in Africa or Europe, even though they are being imported by these regions.

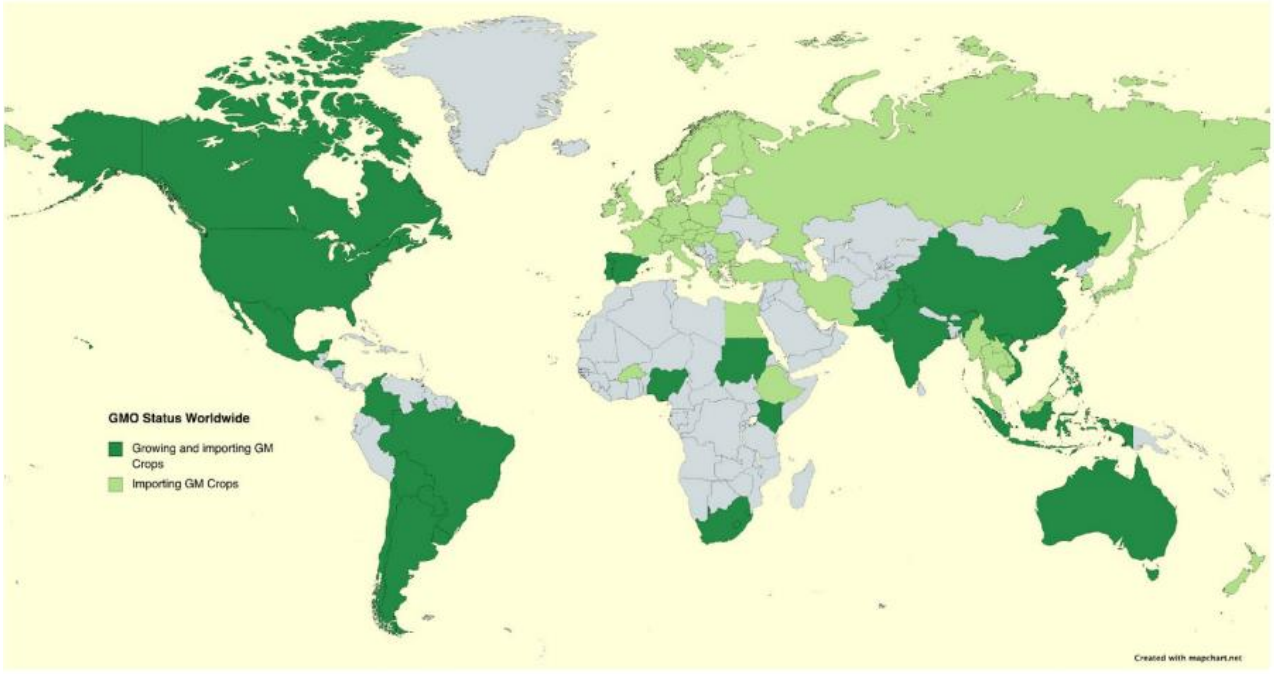

Figure 3: Status of GM crop commercial deployment worldwide. Dark green countries growing and importing GM crops, light green countries importing GM crops (ISAAA, 2018).

The US was able to produce over 73.1 million hectares of genetically modified crops in 2010. South Africa was able to plant 2.7 million hectares of GM crops, making it one of the nations that planted the most. The number of farmers cultivating biotech crops has increased significantly over the past two decades, reaching 190 million globally. From 1998 to 2016, the income generated from South Africa's GM crops was valued at over $2 billion. Around 90% of this came from the cultivation of genetically modified maize varieties.

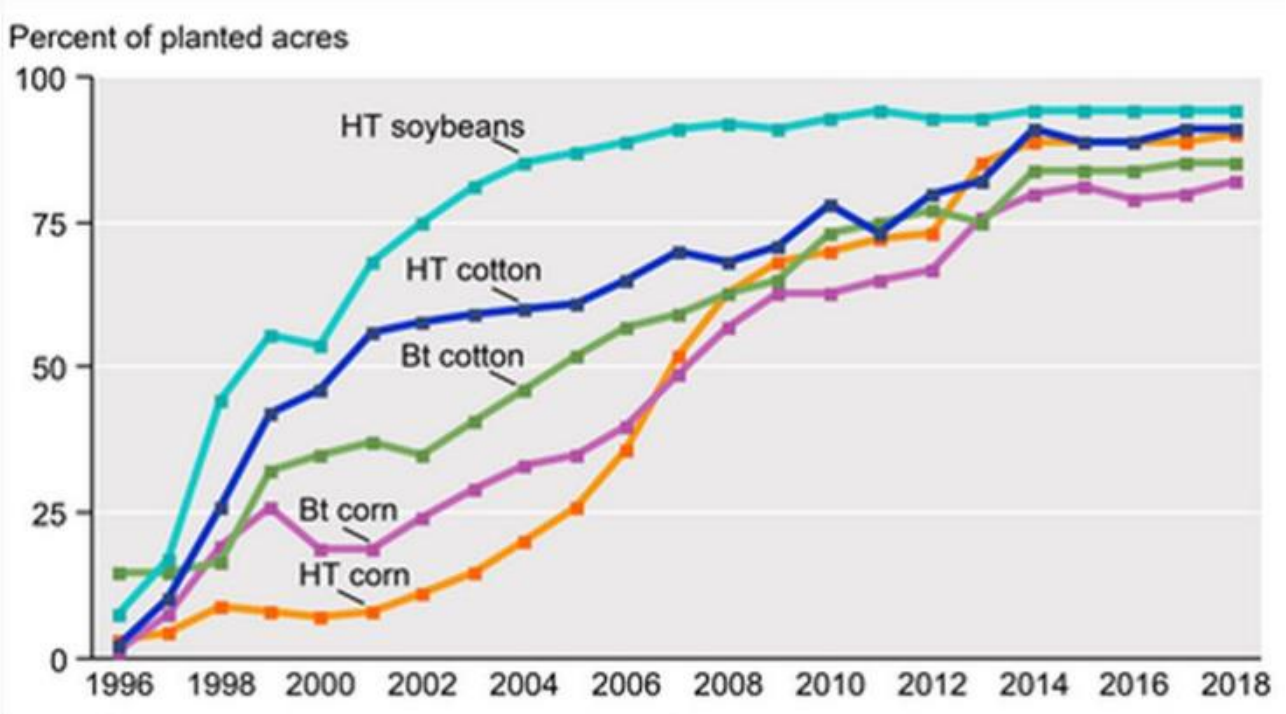

Figure 4: Adoption of GM crops in the USA 1996-2018 (Brookes & Barfoot, 2020).

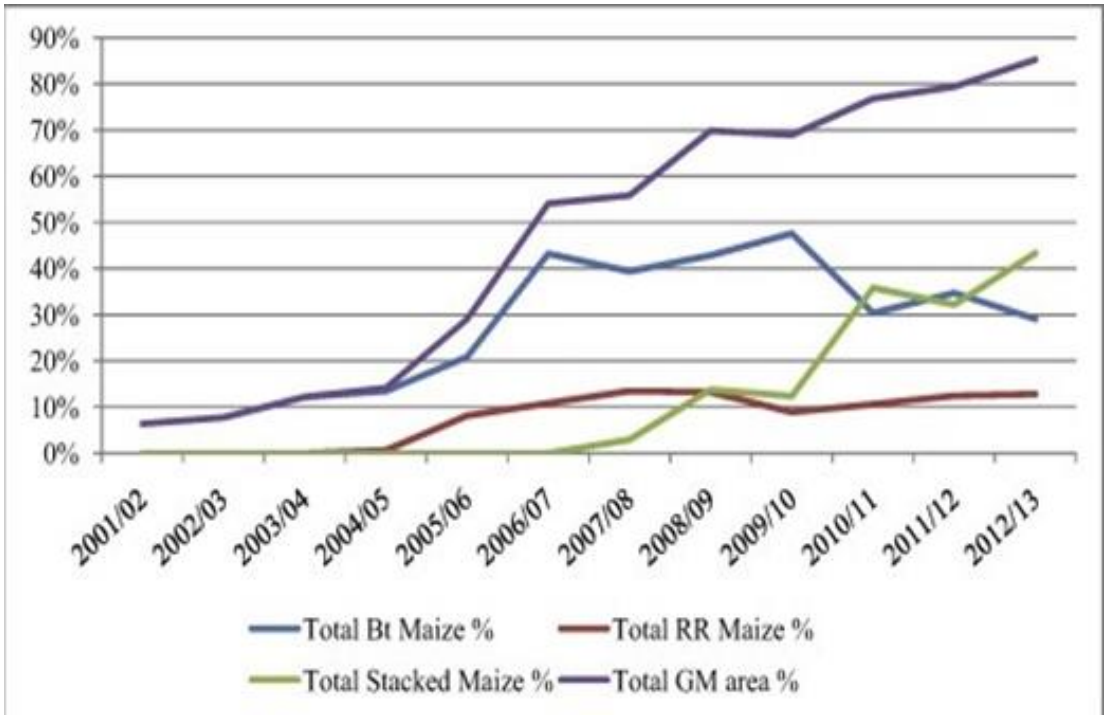

Figure 5: Total GM maize planted in South Africa 2001-2013 (Gouse et al., 2016)

Although the term "GM" is often associated with negativity, it is still widely used several years after its inception. While cotton is the most studied genetically modified crop in the world, maize is also following the social implications of these innovations. Most of the studies that were carried out on the field for the development of genetically modified crops were concentrated in the North American, European, and Asian regions. About 90% of the studies were carried out in the US, with the rest being done in Illinois, Nebraska, and Iowa. In nine European countries, studies were carried out on the field. Some of these included France, Germany, Spain, Italy, the UK, Hungary, Slovakia, and the Czech Republic. In South America, these studies were conducted in Chile, Brazil, and Argentina. The yield response of hybrid maize was mainly based on the observations of single event and double, triple, and quadruple stack varieties (Pellegrino et al., 2018).

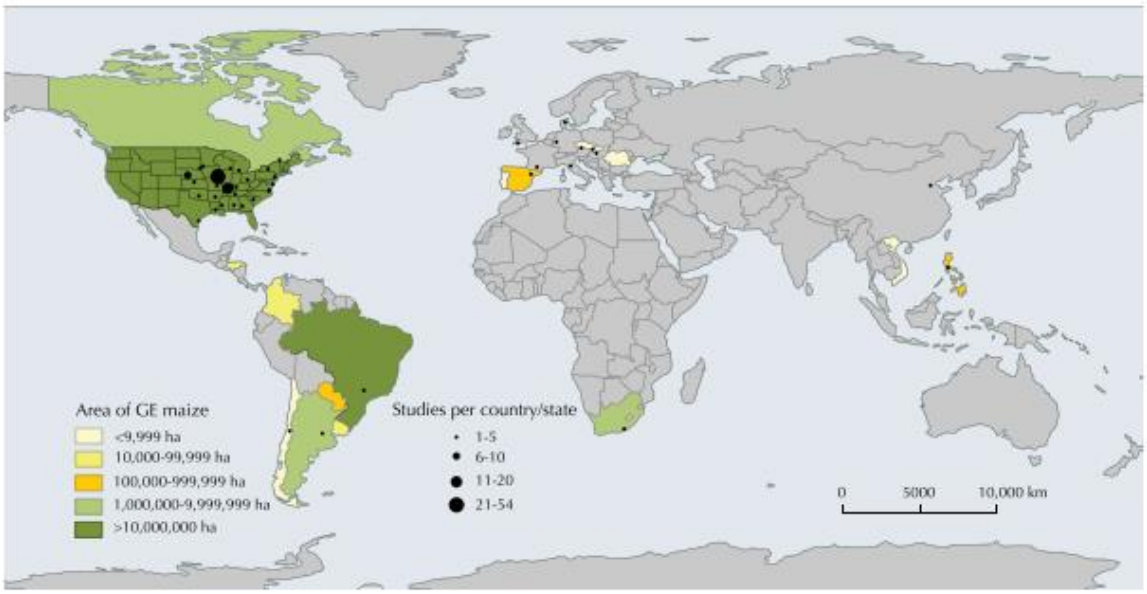

Figure 6: Worldwide distribution of field studies of GM crops (Pellegrino et al., 2018)

2.2 Global GM maize production

South Africa and many other African countries rely on maize meal, which is a staple food. In the Americas, maize is an indigenous plant, and the U.S. is the biggest producer of this crop. China is close behind with around 92 million tons annually (Ala-Kokko et al., 2021). South Africa is the largest producer of maize in Africa, with an annual production of around 10 million tons. However, this is mainly dependent on rainfall, which can vary from 3 to 14 million tons in a year. Maize meal is produced by the dry milling industry, which processes 3.5 million tons annually. The quality of a maize plant is influenced by various factors such as the soil, mechanical conditions, and harvest condition. These can also affect the selection and quality of the plant. In 2020, approximately a third of the world's farms are expected to have cultivated maize.

Currently, maize is on the trajectory to overtake wheat to be the most traded grain commodity (Stein & Santini, 2022). This shows the active global maize trading taking place and the consumption thereof may be worlds apart. The countries that are known to be the biggest exporters of maize are the US, Brazil, Argentina, Romania, and Ukraine. Each of these countries is expected to export around 5 to 54 million tonnes annually (Erenstein et al., 2021). The main global staple food shows similar price trends over the period, with maize being the lowest in the price ranges (Figure 7) (Erenstein et al., 2022).

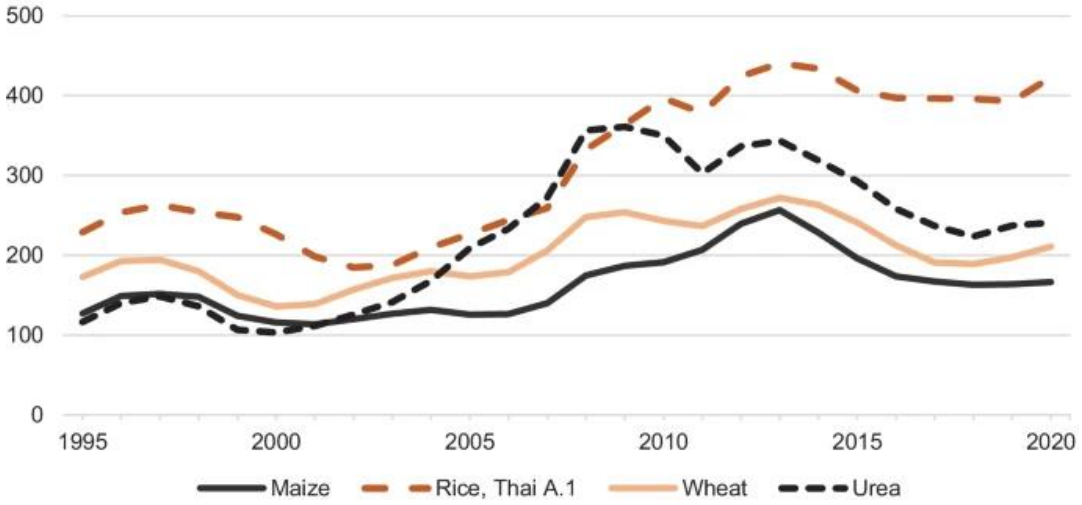

Figure 7: Selected cereal and urea prices in real US$ per ton 1995-2020 (Erenstein et al., 2022).

2.3 GM Maize production in sub-Sahara Africa

Maize has been successfully cultivated on more than 40 million hectares in Sub-Saharan Africa. It is regarded as one of the region's most important crops, and it is also one of the most common grains. From 1961 to 2011, the production of this plant has increased from 205 to 1145 million tons (Cairns et al., 2021). To satisfy the ever-increasing need of consumers maize production needs to increase 2.2% per year (Cairns et al., 2013; Erenstein et al., 2022). It was estimated that the global production of maize increased by around 1.7 to 1.8% from 1981 to 2008 (Cairns et al., 2021).For example, in Southern Africa, the past decade (2010–2020) had six years below average rainfall with a severe El Nino-induced drought in 2016 (Cairns et al., 2021), resulting in a under production in Malawi, Mozambique and Zambia. Should there be no maize yield increase the area of maize production should increase with 184% by 2050 to meet future food security needs (Kenea et al., 2021). Better crop genetics and fertilizer optimization were key components of the Green Revolution and have been widely advocated to increase maize production. Recent investments in maize genetics in SSA are estimated to have benefited over 53 million people (Cairns et al., 2021), but more is needed and the slow speed of the introduction of new cultivars, i.e., the tendency for old cultivars to dominate seeds sales despite the availability of newer and better performing cultivars, has become an issue of concern and discussion (Cairns et al., 2021). Technological advancements have allowed the establishment of genomic selection as a standard component of the breeding process. This method is commonly used for complex traits, such as drought tolerance. It has a shorter breeding cycle time and improved stability (Cairns et al., 2013). The development of the breeding program

has led to a significant increase in the genetic yield of maize. From 2000 to 2010, the yield gained under drought stress was 35 kg/ha/year (Eriksson et al., 2018).

2.4 Benefits of GM crops

With the ability to improve crops' nutritional quality and efficiency, biotech crops can help farmers save money on their agricultural inputs. This technology can also help them avoid using harmful chemicals such as pesticides and fertilizers. (Visser & van den Berg, 2021).

The increasing presence of diseases and pests has led to the development of lower and inferior yields. These factors are usually caused by factors such as weather conditions, transboundary movement, and pathogen drift (Muzhinji & Ntuli, 2020). Pesticides can result in the development of resistance to pesticides, which can negatively affect the management of crop diseases. According to literature, the use of genetically modified (GM) technology alongside other strategies can help address this issue.

Economists who deal with the use of genetically modified (GM) crops see different perspectives when it comes to technology. According to a survey conducted by the National Survey Council, the economic gain from the use of GM crops is distributed among various groups, such as farmers, seed companies, and consumers. The adoption rates of GM crops in the US and other countries are also considered to be evidence of their positive effects on farmers (Klümper and Qaim 2014) An average increase of 68% in farmer profits as a result of GM crop adoption was found, while the total production costs are raised by merely 3%. This result is the complex effect of both yield increase and decrease in other costs, especially pesticide costs. Farmers using GM crops enjoy other benefits, which are harder to estimate in currency value. For example, lowering yield instability and reduced adverse health effects by noxious pesticides have been observed in China and South Africa (Xu et al., 2017).

South Africa is one of the first countries to introduce genetically modified (GM) crops. In 2018, it was ranked as one of the top ten producers of GMs globally. The country planted over 2.7 million hectares of these crops. The emergence of new technologies and the availability of bioinformatic tools has created new opportunities for breeders and scientists (Xu et al., 2017). The challenges of improving crop productivity have led to the development of new farming methods and the search for new crop cultivars that are disease-resistant, nutritious, and resistant to harsh environmental conditions (Thrall et al., 2010).The safety and regulations to ensure that material on farms are for instance not harmful to catis, is however of utmost importance and highlights the purpose of this study.

2.5 GM – background, regulation and safety

Food that is genetically modified can help alleviate the world's hunger and malnutrition and contribute to the preservation of the environment. It can also increase yields and reduce reliance on harmful chemicals. GM crops that have been developed to resist pests have led to a reduction of over 775.4 million kilograms of pesticides usage since 1996 (Brookes & Barfoot, 2020). Despite the advantages of genetically modified crops, there are still many challenges that remain to be overcome in the field of biotechnology. These include safety testing, food labeling, and the regulation of genetically modified products (Pandey et al., 2010). The use of GM maize also brings stewardship of the technology as a non-negotiable to the user. When planting Bt-maize, planting a refuge area is a requirement by law, and if refuge areas (area of non Bt containing maize) are not correctly planted by the farmer, the farmer can be denied using this technology further (Kotey et al., 2016).

The testing of GM crops and products is carried out before they are marketed to the public. This ensures that the food and feed produced from these innovations are safe (Okazaki et al., 2019).

Due to the enhanced genetic characteristics of GM maize, it is protected by the Plant Breeders' Rights Act of 1976 (Ministry of Agriculture, 2015) and regulated by the genetically modified organisms act of 1997. Thus, it is important to note that, end users, which include farmers, are required to sign an agreement license with the breeding company to make sure they comply with the statutory laws applicable to it (Iversen et al., 2014). A good example of this is where farmers who purchase seed with the Bt trait are required to plant the so called “refuge area” where this allows maize which contain the Bt-gene to be separated by an area of maize not containing the Bt-gene in the same field. This is specifically important in commercial farming practices where bigger areas are planted and thus it will delay the resistance evolution of resistant pests (Visser & van den Berg, 2021).

Regulatory requirements are often not followed, and after a study that was done in regards with compliance in planting the correct refuge area using Bt maize, it was found that the compliance rate was alarmingly low. The study involved 105 of South Africa’s commercial farmers covering an area of 87 778 hectares of maize. A survey was carried out to determine the number of people who are following the requirements of the Act when it comes to the establishment of refuge areas. It revealed that the number of individuals who planted refuge areas is low. Farmers were also very busy using insecticides to prevent the spread of stem borer larvae in the refuge area. Many farmers reported high levels of borer infestation on Bt maize. The presence of this resistance has been confirmed in the country, with around 5% and 93% of farmers in all districts using insecticides to limit the damage caused by the pests. A very high number of farmers applied insecticides as prevention on Bt maize in the refuge

area irrespective of stem borer infestation levels. A large proportion of farmers reported significant borer infestation levels on Bt maize and between 5% and 93% farmers in all districts applied insecticides to Bt maize to limit borer damage, indicating that the occurrence of resistance is more widespread in the country than previously thought (Kruger et al., 2012).

Considering the various challenges faced by farmers in Southern Africa, it is important that the agricultural systems are designed to address the needs of the region's food security and sustainability. This can be done through the development of high-yielding crop varieties, the use of modern biotechnology, and conservation agriculture (Adenle, 2011) Until now, there has been no single approach that can effectively address the various challenges faced by farmers in Southern Africa. Instead, different approaches have been used in different countries to address the issues. This will allow for the transformation of agricultural systems and the establishment of food security (Muzhinji & Ntuli, 2020) and China (Xu et al., 2017) where different manifestations of agriculture have been in use with great success (Velten et al., 2015). In various countries, the use of scientific innovations has allowed for the development of new agricultural systems that have delivered substantial benefits to the consumers and the farmers (ISAAA, 2018).

2.6 Bt-maize

In agriculture, Bt maize is commonly used to kill insects. It is produced by a bacterium that can cause endotoxin, which can be found in a crystalline form. Other proteins can also enter a host and disrupt its immune system (Murall et al., 2017). This *B. thuringiensis* bacterium is derived from the soil and has been used since the 1950's as a biological insect control method. The endotoxins which are produced by this bacterium, affect the gut of the insect and inhibits it from eating resulting in death. This phenomenon only happens in insects with the special receptors to the endotoxins and not in any other organism (Schnepf et al., 1998).

Insects, bacteria and fungi are the greatest in threatening the yields and viability of the crop apart from weed pressure. In South Africa the Lepidopteran species which contributes the most in causing damage to crops are *Busseola fusca*, *Chilo partellus* and *Sesamia calamistis* with *Busseola fusca* (corn stalk borer) being the biggest in causing damage to maize. Bt crops can provide numerous benefits to farmers, such as reducing their use of pesticides and improving the effectiveness of their pest management (Brookes & Barfoot 2018).

In South Africa, the use of genetically modified Bt-maize maize to combat the *Busseola fusca* was very successful until 2006. According to farmers, one of the main reasons why they are more likely to adopt this technology is due to the ease of management (Kruger et al., 2012). Nevertheless, Bt-resistant *Busseola fusca* populations have been reported throughout the maize production region of South Africa (Kruger et al., 2012). However, the resistance shown to GM maize was not as recessive as previously believed. This means that it will be very challenging to manage this pest in African farming systems. (Kotey et al., 2016). Additionally, implementation of an insect resistance management system is of utmost importance to ensure that resistance does not develop in the target pests towards the technology or Bt trait itself. This is obtained by the high dose/refuge strategy (Iversen et al., 2014). Therefore, the stewardship of using this technology entails that cultivar planted which express a high dose of insecticidal proteins (Bt trait) are planted among plants or cultivars which does not express any protein that inhibits the target pest.

Due to the increasing number of countries in Africa that are considering allowing the use of genetically modified crops, the pressure is on for the approval of this technology. In 2019, three new countries joined the growing field of GM crops: South Africa, Swaziland, and Sudan. In the year 2019, several African countries, such as Nigeria, Kenya, and Malawi, were also able to grant the necessary approval to the commercial production of genetically modified cotton (table 2). In Nigeria, the country's authorities allowed the commercial production of genetically modified cotton in 2019. The country's government also allowed the release of a new type of maize known as "TELA maize." This Bt crop is tolerant to insects and drought (Zambrano et al., 2022).

Table 2: The current area of land under cultivation of genetically modified crops in Sub-Saharan Africa (Zambrano et al., 2022).

Country	GM crop/trait	Area planted (hectares)	Year
Eswatini	Insect-resistant cotton	403	2019
Ethiopia	Insect-resistant cotton	311	2019
Kenya	Insect-resistant cotton	n.a.	2020
Malawi	Insect-resistant cotton	6,000	2019
Nigeria	Insect-resistant cotton	700	2019
	Insect-resistant cowpea	n.a.	2021
South Africa	Insect-resistant, herbicide-tolerant maize	2,134,000	2020
	Herbicide-tolerant soybean	785,745	2020
	Insect-resistant cotton	16,176	2020
Sudan	Insect-resistant cotton	236,200	2019

2.7 Round-up

Glyphosate, which is the active ingredient in the herbicide Round-up, inhibits a plant enzyme, 5-enolpyrvul-3-phosphoshikimc acid synthetase (EPSPS). EPSPS is present in plants, bacteria and fungi but not in animals (Osemwegie et al., 2020). This enzyme is responsible for the biosynthesis of aromatic amino acids, vitamins and other secondary plant metabolites in plants and other micro-organisms (Blanchard et al., 2017). A plant that would be resistant to the application of Glyphosate, was therefore especially attractive in managing weed infestation. The inhibition of the EPSP enzyme results in the depletion of the amino acids phenylalanine, tryptophan, and tyrosine, which are required for protein synthesis. A new generation of crops that are resistant to glyphosate has been developed that can prevent this from happening (Hummel et al., 2018).

Should the safety of genetically modified crops be questioned? The added yield capabilities of such crops have been demonstrated to be beneficial. For example, young maize seedlings are very vulnerable to weed competition especially when they reach the V5 or 5 leaf stage. This stage is also referred to as the switching point of the young developing plant and weed pressure in this stage can lead to yield drag or losses of up to 75% or 3 metric tons per hectare. Introducing the Roundup-Ready trait resulting in Glyphosate resistant maize was developed exactly for this scenario where the farmer cannot apply Glyphosate at this early development stage of the plants to eliminate the competition effect (Nielsen, 2006).

2.8 Allergenicity studies

Apart from safety studies, it is also important to know if these GM foods would have an allergic effect on humans. In vitro tests can be used to establish this. For instance, RAST or Immunoblotting can be performed with a sera sample from individuals who were sensitized to the crop (Bawa & Anilakumar, 2013). This was demonstrated in GM soybeans expressing the brasil nut 2S proteins (Ulie et al., 1996) or in GM potatoes expressing cod protein genes (Bawa & Anilakumar, 2013). It is also relatively easy to assess whether genetic engineering affected the potency of endogenous allergens (Bawa & Anilakumar, 2013). Workers who were exposed to the Bt pesticide developed IgE antibodies and skin sensitization. It is also possible to test for the Bt toxin's allergenicity in GM crops (Bawa & Anilakumar, 2013). It is important to note that Cry1Ac is a potent Bt toxin that can be used as an adjuvant and oral/nasal antigen (Pérez et al., 2013).

It was previously believed that allergenic proteins were more resistant to GI enzymes than non-allergenic ones due to their increased exposure to the gut (Herman & Roper, 2021). It was following international regulations and guidance that indicated that introducing new genetically modified crops that have been designed to resist digestion could cause allergic reactions (Herman et al., 2022). The EPA rejected the approval of a modified version of the Cry9c protein found in StarLinkTM maize due to concerns it could cause allergic reactions (Bawa & Anilakumar, 2013).

2.9 Risk and controversy

There are various opinions surrounding the safety of genetically modified food. Some of these include whether it should be labeled, the use of biotechnology in agriculture, and the need to address the world's hunger (Zhang et al., 2022). Food security is all about the physical, social and economic availability to food to give adequate nourishment and maintain healthy life. (Saint Ville et al., 2019). The use of biotechnology in agriculture has allowed for the rapid development of new crops that have high nutritional quality and are resistant to pests and diseases. This technology can also help farmers reduce their use of pesticides and fertilizers (Aliber & Hall, 2012).

The CPB is a supplement to the CBD that provides a framework for addressing the various issues related to the use of biotechnology. It acknowledges its potential to help improve the environment and food security. It also provides a precautionary principle that protects the rights of farmers and breeders (Godfrey, 2013). GM supporters, such as the US and South Africa, contribute to the relief efforts of drought-stricken countries by providing aid in the form of food (Zerbe, 2004). South Africa is a major supplier of genetically modified (GM) maize and soybean seeds to its neighbors in the Southern African Development Community (SADC) and other countries (Muzhinji & Ntuli, 2020).

Over the years, the use of genetically modified crops has been allowed to continue. There have not been any reports of food safety issues or feed contamination resulting from the technology (Resnik, 2015).

The safety of foods consumed on a large basis worldwide has been a widely debated topic including multiple food sources such as tomatoes, maize, soybeans, rice and peas. Prior to considering the use of genetically modified food products, one must first understand its disadvantages and advantages. It is feared that these may have detrimental impacts on the human body (Bawa, 2013). It is widely believed that the use of genetically modified food products could result in the development of diseases that are resistant to antibiotics. There are also various cultural and religious concerns about this technology (Bawa & Anilakumar, 2013). This not only applies to human food, but also GM material used as feed for animals, as will be investigated in this study.

In addition to improving the safety of food, metabolomics can also help the consumer accept genetically modified products more easily. (Zilberman et al., 2018).

2.10 Metabolomics

The concept of metabolomics refers to the systematic analysis of the various metabolites of an organism or biological substance (Idle & Gonzalez, 2007). The use of mass spectrometry and nuclear magnetic resonance spectroscopy has allowed scientists to simultaneously determine thousands of chemical entities. This has led to the development of new studies on small molecules in plants, animals, and bacteria. The development of analytical platforms that can provide a high resolution and simultaneous analysis of these small molecules will allow for the continued development of metabolomics. (Idle & Gonzalez, 2007).

The main components of metabolomics are the acquisition of data and the interpretation and processing of this information. Mass spectrometry and NMR are regarded as the most suitable instruments for this type of analysis (Jahangir et al., 2018)(Figure 8). NMR profiling has enabled scientists to accumulate spectra of more than 1000 metabolites, 20 000 plant extracts and various other organisms (Harrigan et al., 2016).

Metabolomics is a process that aims to provide a deeper understanding of the biological system by studying the various metabolic changes that occur in it (Liu et al., 2019). Metabolomics provides a comprehensive view of the various mechanisms and functions of metabolites by mapping their properties on metabolic pathways and biological networks. This process can be performed manually by analyzing the data collected from each of these metabolites (Domingo-Almenara et al., 2018). The traditional method of analyzing data is time-consuming and limited since it focuses on each individual metabolite. Also, it lacks an organized framework for describing an organism's biochemical network. In order to overcome this issue, various computer-based methods have been developed to improve the efficiency of data analysis (Cottret et al., 2018).

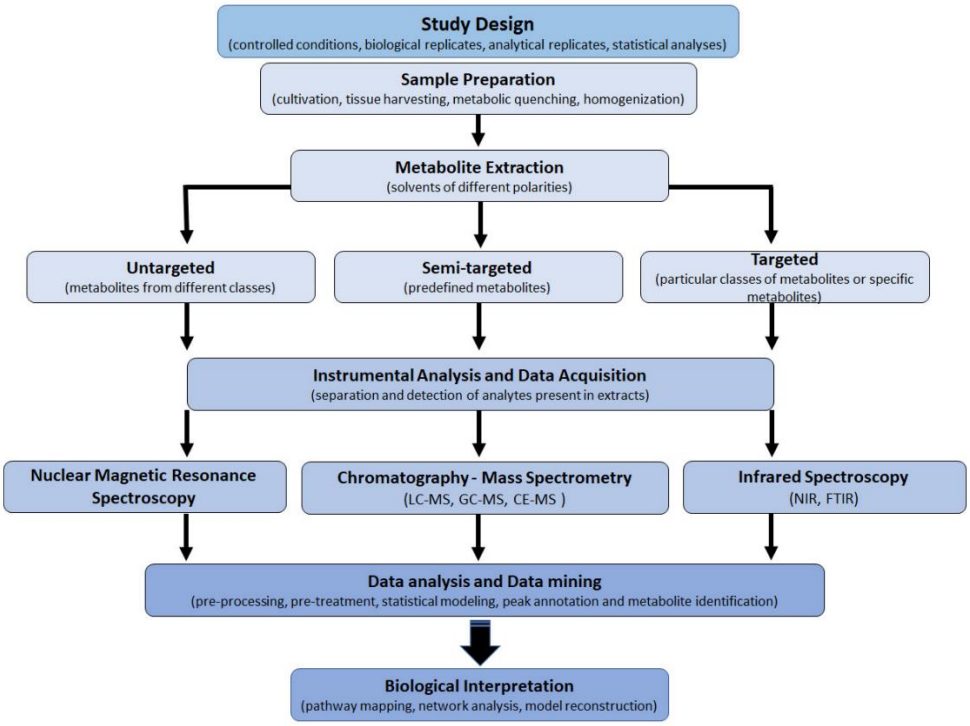

Figure 8: Experimental designs, workflows and analytical platforms used in plant metabolomics (Tugizimana et al., 2013)

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The analysis of the maize metabolome using various methods is like that of other plant metabolomics investigations. These include nuclear magnetic resonance and hyphenated mass spectrometry (Obata & Fernie, 2012). With maize being such a crucial part of South African agriculture and with several examples on how the metabolomic biology of maize plants changed due to environmental issues it is important to examine the plant in this way by conducting intensive metabolomic analysis.

In maize plants, the presence of African cotton leafworm moths can cause various metabolites to change at the site of the infection. The defense-related metabolites in the root vascular exudates and sap increase, while the metabolites in the roots remain relatively unchanged (Erb & Kliebenstein, 2020). A study on the effects of the Ustilago maydis mutation on the metabolism of maize has been carried out (Redkar et al., 2015). A study conducted in maize used the Cry1Ab gene as a model. This was the first time that a PLS-DA statistical method was used for this type of analysis (Gromski et al., 2015) which has since appeared in countless metabolomics publications. Another important population used for the genetic mapping of metabolic traits in maize is the Maize Go panel genetic mapping of metabolic traits consisting of 540 lines (Jie Liu, 2017). Using mGWAS, studies have been able to identify several genes in this population. In some studies, the combination of GWAS and metabolic pathway analysis has been used to determine the effects of these genes on the population. By
Analyzing the data from multiple genes, this approach can provide researchers with new insights into the genetic basis of certain traits. It can also reveal biological insights that are not apparent when focusing solely on a few genes (Liu et al., 2019).

Identifying the metabolites of an organism remains one of the most challenging issues in metabolomics. Several studies have been carried out on the identification of specific metabolites. In some cases, a comparative nature is used to describe the features or peaks of a plant. This approach eliminates the need for identification of metabolites (Baniasadi et al., 2014).

Therefore, metabolomics is used in this study to identify the differences that occur within GM maize and their natural counterparts. It is important to study the plant extracts on the different cultivars of maize on this molecular level to identify the differences and similarities between GM and non-GM maize cultivars. This method of study, apart from the field trial and feeding trial, is necessary to clearly understand the makeup of these maize cultivars used in this study. It is an integral part of understanding the preferences that cattle may show to different maize cultivars.

Chapter 3: Planting and feeding trial.

3.1 Introduction

There are many different views on the effect that GM containing feeds can have on mammalian bodies and some claims have been noted that the organs of cattle can be affected negatively (van Eenennaam & Young, 2014). In some countries around the world GMs are not allowed at all due to beliefs and concerns about the safety of these products.

It has also been noted from seed companies in South Africa that when trials are conducted in the field containing GM and non-GM maize cultivars, that wild animals like warthog and porcupine will not consume the GM containing maize and prefer the conventional or non-GM cultivar (Verbal communication with farmers). The results of the study revealed that the cattle that were subjected to the study preferred the genetically modified maize. This could also suggest that the animals had strong feed preferences (Kyriazakis I & Forbes JM, 1995).

This study was therefore conducted to determine if cattle can distinguish between GM and non-GM maize, as farmers have raised concerns about their cattle feeding mainly on non-GM maize. A trial was planted with GM and non-GM material of a long and short season cultivar under the same experimental conditions. Material was subsequently harvested from all the different types of maize and randomly provided to animals to determine their preference. By determining which plant material is preferred weighing the material left after feeding, a conclusion could be made to whether cattle can indeed distinguish between GM and non-GM maize.

3.2 Materials and Methods

3.2.1 Plant material

The chosen maize plant material for this study was selected according to its suitability for the Val Mpumalanga region, GPS coordinates: -26.7934730, 28.9404540. Four yellow maize cultivars were used to conduct this study which are the following:

- A conventional short season cultivar (BG3292)
- A short season cultivar which contains both the Bt and the Roundup-Ready traits, (stacked genes) (BG3792BR)
- A longer season conventional cultivar (PAN6P-110)
- A longer season cultivar containing both the Bt and the Roundup-Ready traits, (stacked genes) (PAN6Q-710BR)

These 2 groups of cultivars are genetically very different being a long and short season cultivar, but the non-GM plants have the same genetic makeup, only differing in terms of the Bt and Roundup-Ready gene. Thus, the two short season cultivars are genetically the same except the one containing the GM traits. This also applies to the longer maturing cultivar which are also similar in genetic makeup. The longer season cultivar is tropical genetic material which is known to be more resistant to disease than the short season cultivar which comes from the Corn Belt region of the USA and are more susceptible to diseases especially when grown in Africa. The longer season cultivar is also very prolific and tend to develop tillers where the tillers are called active which implies that they also form cobs. The two short season cultivars are bred to be single stemmed plants and produce only one cob per plant.

3.2.2 Field trial

The trial was planted on a farm in Val near Standerton in the Mpumalanga province of South Africa with the following GPS coordinates: -26.7934730, 28.9404540. The four cultivars were planted with 76 cm row width spacing and 12cm in-row spacing resulting in 45 000 plants per hectare. For each type, 9000 plants were planted per replicate. For the three replicates, a total of 27000 plants were planted, therefore providing sufficient material for the feeding trial. The trial was planted on the 13th of October which falls inside the premium planting window for the highveld regions of South Africa. The trial was laid out with three replicates as per trial layout shown in Table 3. In each replicate, the cultivars were randomized. The trials were planted with a commercial tractor and planter. The soil was prepared to obtain a fine seedbed to enhance the seed-soil contact to make sure emergence is even. The soil classification is a deep Hutton soil type with good drainage. Chemical fertilizer was used and applied during planting and as a follow-up application. With planting 350 kg per hectare of 3:2:1 (36) was applied with a follow-up of Ammonium Sulphate of 180 kg per hectare. The trial was rainfed and therefore not irrigated as this is in a high rainfall area. Plant leaf Material was harvested twice. The first harvest was done in December at the V5 stage and the second harvest at the end of the growing season at maturity in June the material was harvested at 9:00 in the morning, weighed and immediately allocated to the batches which were randomly provided to the cattle for feeding.

Table 3: Field trial and randomization planting plan of cultivars in the field. UQ-C = Short season conventional, UQ-BR = Short season, GM, LS-C = Long season conventional, LS-BR = long-season GM.

Replicate 1	**UQ-C**	**LS-BR**	**UQ-BR**	**LS-C**
Replicate 2	**LS-BR**	**UQ-C**	**LS-C**	**UQ-BR**
Replicate 3	**LS-C**	**UQ-BR**	**LS-BR**	**UQ-C**

3.2.3 Animal feeding trial

Six randomly selected Drakensberger beef cattle of the same age and gender were selected for the animal feeding trial (Figure 9). This is an indigenous beef cattle breed which is totally black in color and is adapted to the highveld of South Africa. Six female animals with an average age of 2 years were used as an experimental unit. For each individual cow, four batches of leaves (3 kg) were provided representing the four planted cultivars. The plant material was harvested at two harvesting intervals namely the V5 or 5 leaf stage (3 December) and after maturity (5 June).. For each harvest, the fresh harvested leaves were randomly allocated to the cattle and the consumption of each batch of leaves were determined by weighing and measuring before and after it was fed to the cattle. The cattle were allowed to feed on the material for 30 minutes, after which the material was removed from the cages and weighed. Data was collected on the preference of which cultivar the animals like best. A precise amount of 3 kg of green or wet leaves was given to the animals and the trained observation team, consisting of 5 people, were tasked with the evaluation process (Figure 10). Barriers were used to separate and contain the animals which allowed them to make a choice on the four cultivars available. This experiment was repeated in the same manner after the plants had fully matured and the first frost have fallen. This was regarded as the dry matter leaf test. The trial layout for both harvesting periods is provided in Table 4.

Figure 9: Photo of Drakenberger cattle used in preference test.

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Table 4 Trial layout for the cattle feeding trial indicating the camp number, cattle ID and feed randomization. Yellow bag = UQ-BR (short season GM), Grey bag = LS-BR (long-season GM), White bag = UQ-C (short season conventional), Red bag = LS-C (long-season conventional GM).

Camp number	Cattle ID	Feed randomization (bag color)			
1	E057	Yellow	Grey	White	Red
2	E047	Red	Yellow	Grey	White
3	E009	White	Red	Yellow	Grey
4	E024	Grey	White	Red	Yellow
5	E022	Yellow	Grey	White	Red
6	E003	Red	Yellow	Grey	White

Figure 10: Illustration of the harvesting process of the green maize plant material from the trial (a), collection of the material per cultivar (b), layout of the feeding experiment with the cattle (c) and the randomized cultivars provided to the cattle in each camp (d).

3.2.4 Data collection and analysis

Data collected for analysis consists of weighing the material left of the 3 kg of wet leaves of each of the cultivars which were placed on the color-coded bags. After the elapsed time span of 30 minutes the cattle were allowed to exit, and the remaining leaves were weighed again to determine the material that was consumed by the cattle. This experiment was repeated in the same manner after the plants had fully matured and the first frost had fallen. This is the dry matter leaf test. The data of the first harvest was submitted to the ARC (Agricultural Research Council) Biometric unit, Pretoria for statistical analysis. The data was analyzed by using the Annova method, Genstat 64-bit Release 18.2 (PC/Windows 8) 20/2/2020 10:31:36 Copyright 2016, VSN International Ltd. Registered to: ARC as per attached addendum on page 66.

3.2.5 Results

First harvest

The results are based on the 6 heads of cattle given the 3 kg green plant material of each of the 4 different types of cultivars that were planted in trial and measured after 30 minutes. The amount of material left after feeding is provided in Table 5 and the material consumed is provided in Figure 11, indicating the statistically significant differences as different letters.

Table 5: Green maize plant material weigh-in after 30-minute elapsed time exposure to cattle. UQ-C = Short season conventional, UQ-BR = Short season containing Bt and Roundup-Ready. LS-BR = Longer season containing Bt and Roundup-Ready and LS-C = Longer season conventional.

Green plant material left after 30 minutes feeding time (kg)							
Cattle ID	**E 057**	**E 047**	**E 009**	**E 024**	**E 022**	**E 003**	**Avg/ animal (kg)**
UQ-C	1,37	1,41	1,25	0,91	0,98	1,2	1,19
UQ-BR	1,2	1,25	1,5	1	1,1	0,91	1,16
LS-BR	0,62	0,5	0,52	0,32	0,43	0,67	0,68
LS-C	0,52	0,43	0,69	1,2	0,32	0,2	0,71

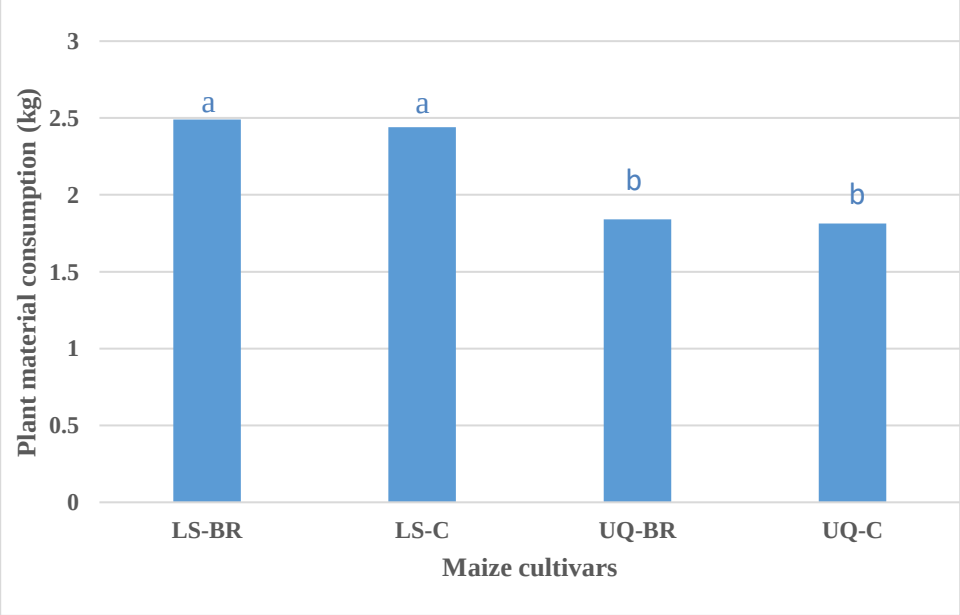


Figure 11: Plant consumption per head of cattle after 30 minutes in kg. UQ-C = Short season conventional, UQ-BR = Short season containing Bt and Roundup-Ready. LS-BR = Longer

season containing Bt and Roundup-Ready and LS-C = Longer season conventional. Different letters indicate statistically significant differences.

Second harvest

Although a dry material test was performed to obtain results in terms of the preference in dry leaf material, the test was inconclusive. Because of the dry harsh weather conditions during winter months on the highveld of South Africa, these cattle have been provided with extra supplemental nutrition. Therefore, it can be concluded that the cattle showed no interest in the tasteless dry material because of the supplementary feed they receive for the duration of the winter months.

3.2.6 Discussion

Planting of GM maize is often controversial and if concerns are raised by farmers, regarding feeding patterns of cattle when supplied with GM and non-GM material, it warrants further investigation.

The feeding experiment was therefore conducted by using green maize plant material, consisting of long season and short season cultivars of the same genetic material, only differing in the presence of the Bt and Roundup-Ready genes. The experimental conditions were kept he same throughout the field and growing season to ensure that no differences could be introduced in the plants material. An exact amount of 3kg of each of the four types of material were randomly provided to the cattle and by weighing the material after feeding, it was observed that the cattle did not have a preference for non-GM as was anticipated. However, the cattle did show a preference for the longer season cultivars, by consuming larger amounts of the GM and non-GM long season material.

Another study was conducted on 16 lactating Holstein cattle to analyze the effects of different maize cultivars on their growth and development. The study was carried out at the University of Nebraska. It utilized four different treatments: non-Bt early maturing, Bt containing early maturing, non-Bt late maturing and Bt late maturing (Clark & Ipharraguerre, 2001). In this study it was found that that the composition of the milk produced by the cows was not affected by feeding on any of the different treatments. The authors concluded that the feeding value for the Bt and non-Bt silage were equal and no difference could be established (Clark & Ipharraguerre, 2001).

A third study analyzed the effects of the consumption of non-Bt and Bt corn silage on the daily gain, abdominal fat, dressing percentage, and hot carcass weight of Holstein bulls. The results of the analysis revealed that the various parameters of these animals were not different

(Clark & Ipharraguerre, 2001). The analysis of the corn silage showed no differences in its composition and nutrional values (Table 6).

Table 6 Composition of non-Bt and Bt corn silages, feed intake and perfomace of Holstein bulls fed corn silage (Clark & Ipharraguerre, 2001).

	Corn silages	
Item	Non-Bt (Cesar)	Bt
Silage composition		
DM, %	33.7	32.1
OM, %	95.5	95.8
CP, %	8.4	8.7
Crude fat, %	2.9	2.8
Crude fiber, %	18.6	19.1
Metabolizable energy, MJ/kg	10.95	10.91
Feed intake		
Concentrate, kg/d	1.78	1.80
Corn silage, kg/d	18.8	18.7
DMI, kg/d	8.00[a]	7.78[b]
Protein intake, g/d	1102	1110
Metabolizable energy intake, MJ/d	91.2[a]	88.6[b]
Steer performance		
Final BW, kg	537.0	534.5
ADG, g/d	1487	1482
Metabolizable energy/BW gain, MJ/kg	61.5	60.1
Hot carcass weight, kg	281.3	282.0
Dressing, %	52.4	52.8
Abdominal fat, kg	49.6	48.7

These studies support the findings of a study that found that the presence of genetically modified (GM) maize does not affect the animal's feeding preference. They also indicate that the preference for longer season cultivars is not affected by the presence of Roundup-Ready genes or Bt.

3.2.7 Conclusion

By providing cattle with two types of maize, each containing a non-GM and GM variety, it could be determined if cattle shows preference to GM or non-GM maize. The material was all grown under the same conditions and harvested at the same time, therefore ensuring no differences in the material provided as feed. The trial clearly showed that cattle does not have a preference for non-GM maize cultivars as was expected, but rather distinguised clearly between the longer and shorter season maize cultivars. It can therefore be concluded that there is not a preference present in cattle to choose non-GM cultivars opposed to GM cultivars as demonstrated in the feeding trial experiment.

Chapter 4: Metabolomics

Metabolomics, mainly using data from NMR spectroscopy and mass spectrometry with multivariate data analysis, affords the opportunity to determine and compare many chemical compounds in an organism. This therefore provide insight and expansion of small molecule biochemistry studies in living organisms, especially plants, bacteria and mammals (Jahangir et al., 2018).

In this chapter, metabolomics was used to further understand the chemical composition and difference in chemical profile of the plant material that was used in the feeding trial (Chapter 3). Since only one the Bt and Roundup-Ready genes were inserted in the GM plant, it is expected that the metabolism would not be affected, and the chemical profiles therefore be identical. This assumption would also support the findings in the feeding trial, where the animals could not distinguish between GM and non-GM maize plant material, therefore indicative of a similar chemical profile. Metabolomic analysis however, showed generally similar profiles, although some distinct differences in the GM and non-GM maize plants were observed, although the importance of these differences in the plants were not yet determined. Metabolomics therefore assisted in finding similarities and differences in the maize plants, although considering the findings in Chapter 3, indicating that the differences observed were probably not impacting on the major metabolic pathways of the plants, and could be indicating differences in growth and development of the plants.

4.1 Materials and methods

4.2 Plant material

Samples of the four types of plant material that was used in Chapter 3 for the feeding trial, was prepared for metabolomic analysis. From the three replicates, two combined samples were used in the analysis of each type of material as this was envisaged as a pilot study and large sample numbers were therefore not required. The material was dried at room temperature protected from direct sunlight and stored at room temperature until analysis at the CSIR in Pretoria.

4.3 Sample preparation for NMR – based metabolomics

Fifty mg of powdered leaf material, that was grinded down by hand, was weighed and stored in 2 mL Eppendorf tubes and extracted following an established direct extraction method. Maize plant leaves were grinded by hand. Plant material was extracted with 0.75 mL deuterated methanol (CD_3OD) and 0.75 mL, deuterium water (D_2O) (pH 6.0), with potassium dihydrogen phosphate (KH_2PO_4) and 0.1 % (w/w) TSP (Trimethylsilyl propionic

acid sodium salt) added as a standard. The samples were vortexed for 1 minute at room temperature, extracted with ultra-sonification (Branson 2800, USA) for 15 minutes, and then centrifuged for 20 minutes to separate the supernatant from the pellet. Six hundred µl of the supernatant was then transferred to a standard 5-mm NMR tube (Norell, SiGMa-Aldrich) for NMR analysis. Gradient shimming was used to improve magnetic field homogeneity prior to all acquisitions with 32 scans recorded.

4.4 Multivariate data analysis

NMR analyses were conducted on a Varian 600 MHz spectrometer, operating at a proton NMR frequency of 600.13 MHz. The ^{1}H NMR spectra were reduced to ASCII files using Mestrenova 9.0. (Mestrelab research, Spain). All spectra were baseline-corrected, referenced, normalized and pareto scaled before statistical analysis (Jahangir et al., 2018). The region of 0.00-10.00 ppm was bucketed into bins 0.04 ppm in width. The region ranging from 3.28-3.36 ppm (residual MeOH) and 4.60-5.00 ppm (residual water) were removed prior to statistical analyses. The ASCII files generated were then imported into Microsoft Excel 2010 for secondary variable labeling after which the files have been imported into Simca version 13.0 (Umetrics Umea, Sweden) for multivariate data analysis. Two powerful statistical tools were utilized to analyze the variations in the samples. These tools are PCA and OPLS-DA.

4.5 Results

The PCA showed partial clustering of the samples based on the type of material used, especially for the long season cultivars (Figure 12). Other analysis was carried out using the supervised OPLS-DA model to obtain clear groupings between the four types of material used. Even though a small number of samples were used, the variation was well described for the PCA (R2X = 0.619) and the OPLS-DA (R2X =0.776 and R2Y =0.327) although the predictability score was low (Q2 = 0.014).

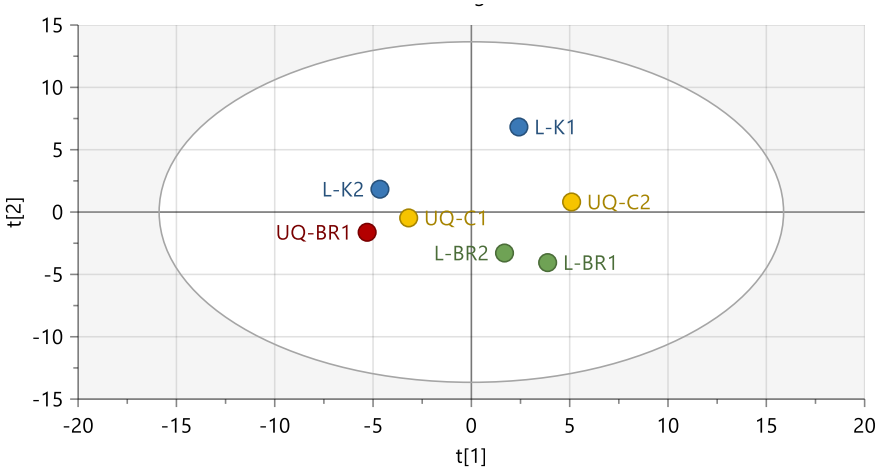


Figure 12: PCA scatter plot showing UQ-C = Short season conventional (yellow), UQ-BR =Short season GM (red), L-K = Longer season conventional (blue) and L-BR = Longer season GM (green) samples.

The OPLS-DA scatter plot shows clear separation of the four different types of maize plant samples (Figure 13). The short season conventional (UQ-C, yellow dots) separated from the short season Bt and Roundup-Ready containing samples (UQ-BR, red dot), which was also observed with the long season conventional (L-K, blue dots) and long season Bt and Roundup-Ready containing samples (L-BR, green dots). Figure 13 therefore indicates that the chemical profile of the GM maize samples are different from the non-GM maize samples for both the longer season and short season samples, even though it was expected that they would group together.

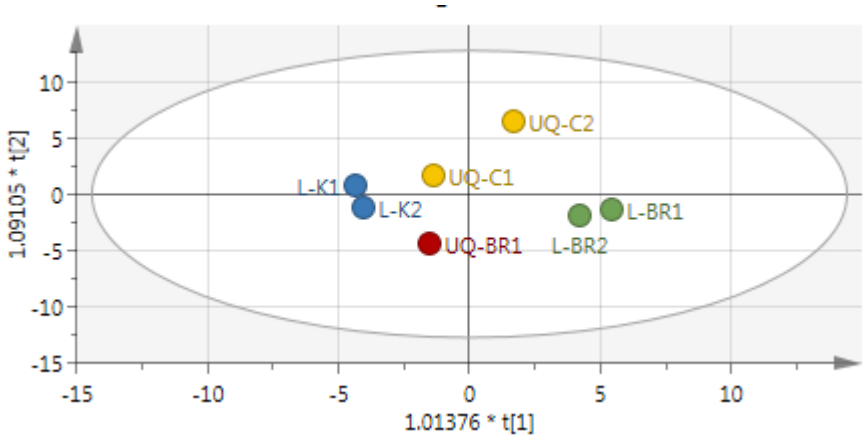


Figure 13: OPLS-DA score scatter plot of the four different plant samples. UQ-C = Short season conventional, UQ-BR =Short season GM, L-K = Longer season conventional and L-BR = Longer season GM. R2X =0.776 and R2Y =0.327 and Q2 = 0.014

The contribution plot of the long season conventional vs the long season GM plant samples is shown in Figure 14. The contribution plot clearly shows different NMR spectral regions for the GM and non-GM samples, which resulted in the separation of the samples.

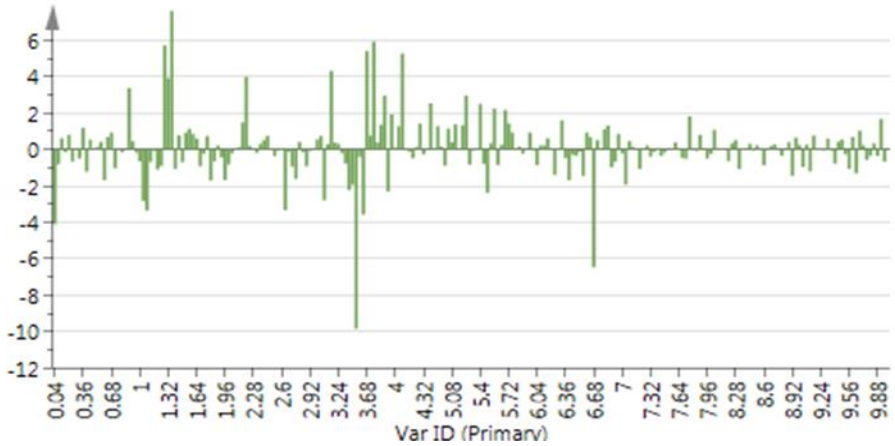


Figure 14: Contribution plot showing the NMR regions positively associated with the long season GM samples (bars above the line).

Since there was separation of the GM samples from the non-GM samples from the same genetic material, it was important to compare the NMR profiles to determine the differences in the samples. Figure 15 shows the overlay of the NMR analysis of the long season GM samples (red line) with the non-GM samples (blue line). It can be observed that there are several differences in the profiles, supporting the OPLS-DA clustering and the contribution plot. Additionally, the height of the peaks is indicative of the concentration of the compounds, and a higher peak therefore indicates a compound that is present in a higher concentration by comparison.

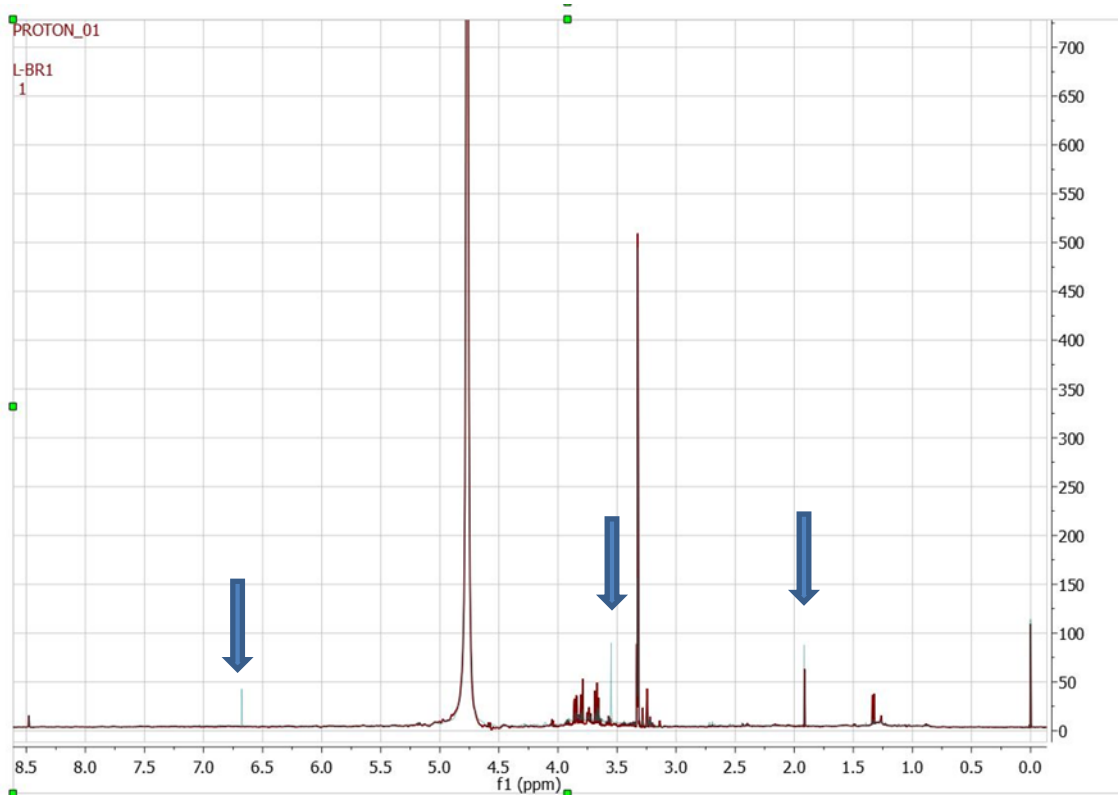


Figure 15: Comparison of long season GM samples (red line) with the non-GM samples (blue line) showing differences in the peaks and peak height as indicated by the arrows.

By inspecting the various NMR regions, several differences were observed in the concentration of peaks (indicated by height) in the profile for the long season variety (Figures 16 A-D) with non-GM (blue) and GM (red). Clear differences quantitatively and qualitatively can be observed in the aliphatic region (Figures 16 A and B) as well as in the sugar region (Figures 16C and D).

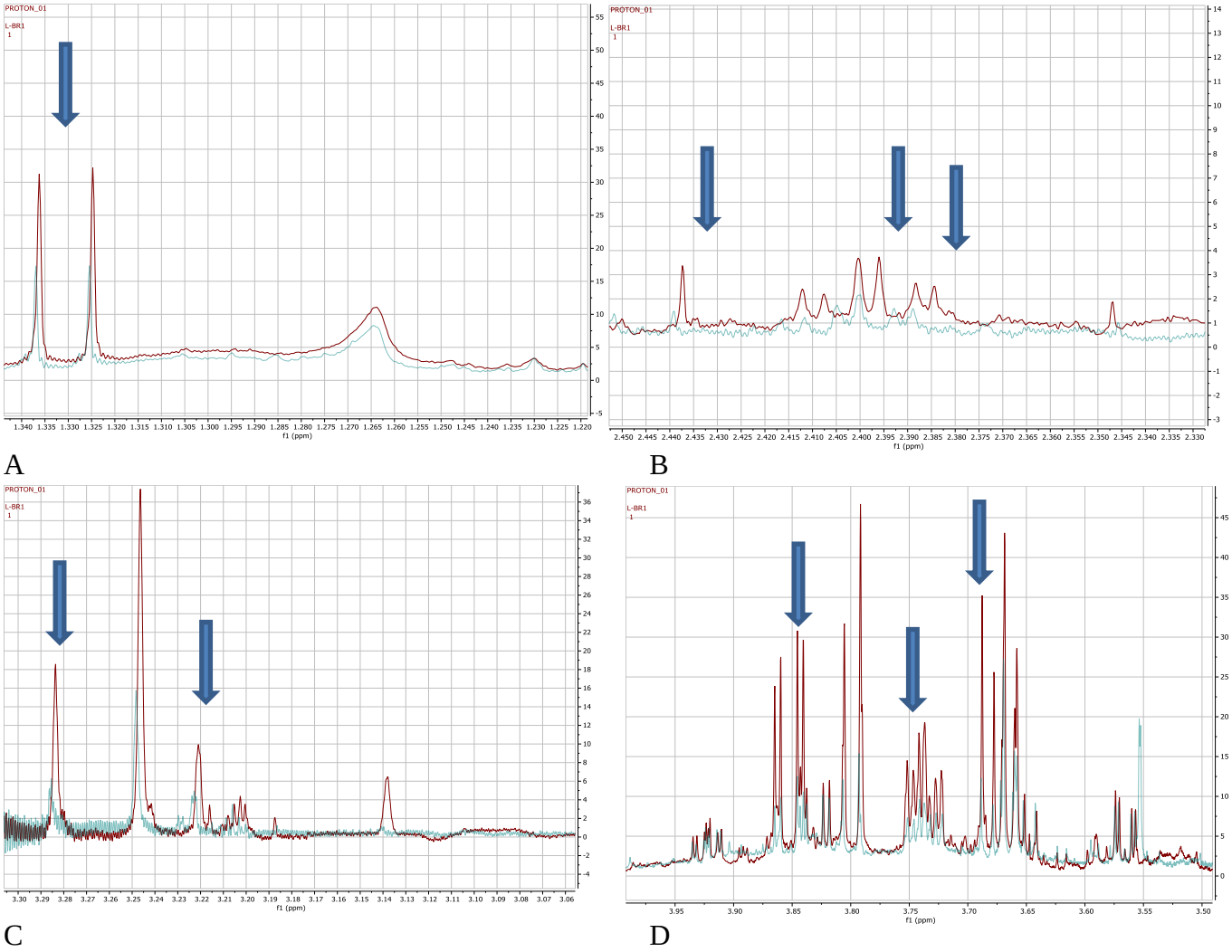


Figure 16: A-D showing enlarged NMR regions of the long season samples that indicate the differences in peaks in all the regions. A = 1.2-1.3 ppm, B = 2.3-2.45 ppm, C = 3.06-3.3 ppm and D = 3.5-4.0 ppm. Red = GM and blue = non-GM.

Figure 17 shows the overlay of the NMR analysis of the short season GM samples (red line) with the non-GM samples (blue line). It can be observed that there are also several differences in the profiles, supporting the OPLS-DA clustering observed in Figure 13.

Figures 18 A-C clearly shows the differences in concentration of compounds with the red line above the blue line, indicating an increased concentration for the compounds in the GM samples. Figure 18 D shows variable concentrations for the sugars with an increased concentration in the GM samples for example the peaks at 3.85, 3.8 and 3.68 ppm. Similar or even higher peaks for the non-GM samples can be observed at 3.93, 3.57 and 3.55 ppm.

The same was observed when the sugar region of the long season samples were compared to the short season samples.

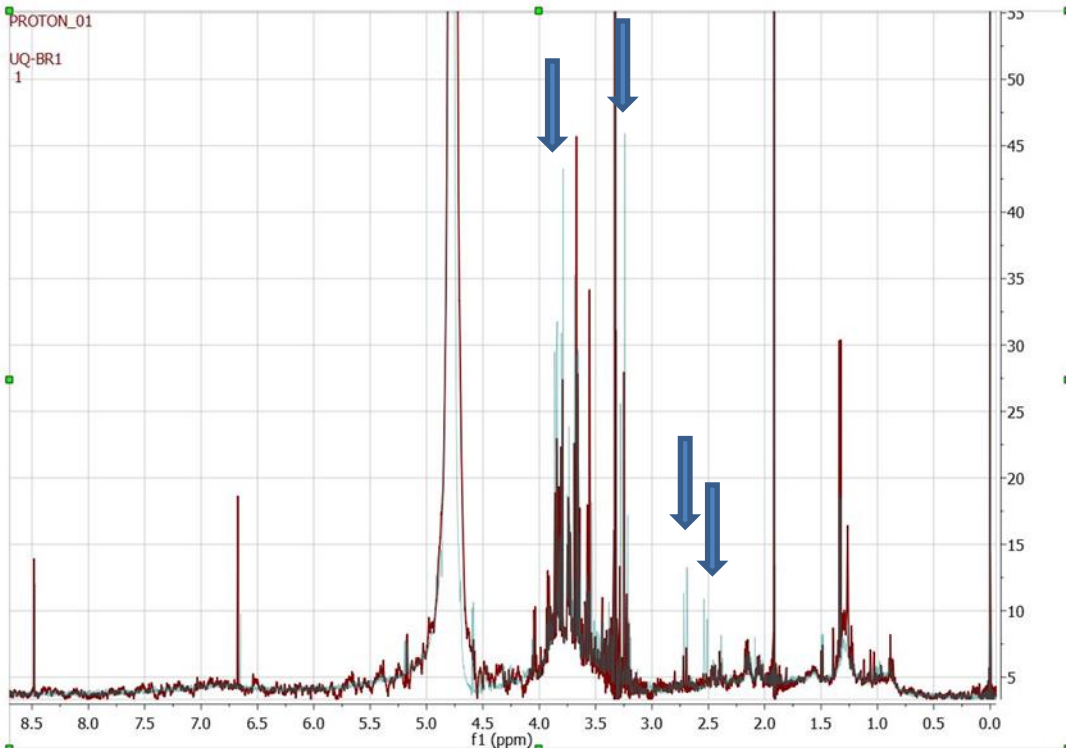
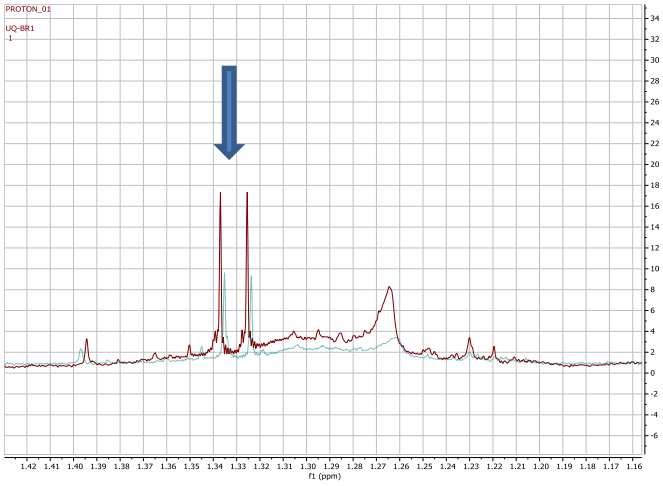
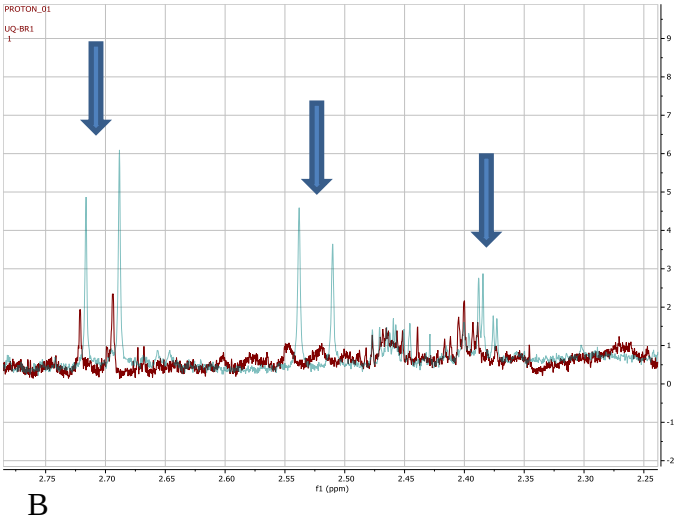


Figure 17: Comparison of the short season GM samples (red line) with the non-GM samples (blue line) showing differences in the peaks and peak height.

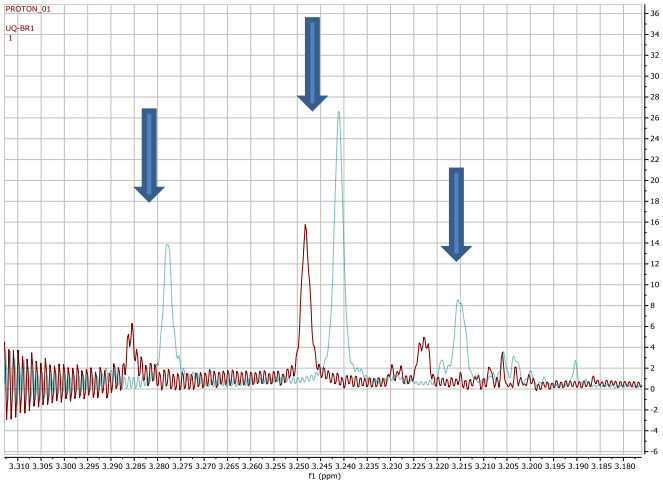
By enlarging the NMR profiles, several clear differences could be observed in many of the NMR regions for the short season varieties. The differences are shown in Figures 18 A-D and can be observed in the aliphatic (A and B) and the sugar region (C and D).



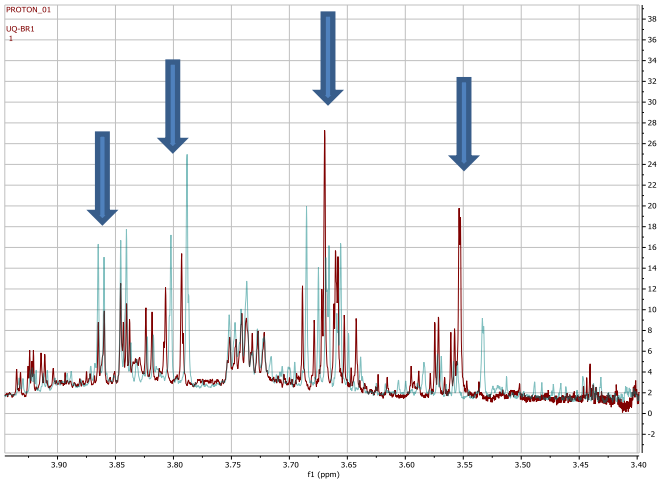
A



B



C



D

Figure 18: A-D showing enlarged NMR regions to indicate the differences in peaks in all the regions for the short season samples. A = 1.16-1.42 ppm, B = 2.25-2.75 ppm, C = 3.18-3.3 and D = 3.4-5.0 ppm. Red = GM and blue = non-GM.

Figures 18 A shows the differences in concentration of compounds with the red line above the blue line, indicating an increased concentration for the compounds in the GM samples. Figures 18 B and 18C show a higher concentration of the compounds in the non-GM samples when compared to the GM samples (red line) which is different for the long season

varieties. However, the sugar region again shows variable concentrations for the peaks in the sugar region with an increased concentration in the GM samples for example the peaks at 3.95, 3.68 and 3.55 ppm. Similar or higher peaks for the non-GM samples can be observed at 3.85, 3.80 and 3.53 ppm.

4.6 Discussion

Recent studies have shown that the varying levels of metabolites produced by different cultivars can be influenced by their growing conditions and environments. Despite the various studies that have been carried out on the effects of genetically modified crops, there are still a lot of questions that remain unanswered. One of these is the possibility that the traits introduced in these crops could lead to unintended differences in the food that consumers consume (Rischer et al., 2006).

Metabolomics research has shown in the past and supported the findings that GM trait insertion does not have any meaningful effect on crop metabolite makeup or profile (Harrigan et al., 2016). Studies on the metabolomic properties of maize have revealed that the growing location and conventional germplasm have the most significant impact on the development of metabolite (Harrigan et al., 2016). Therefore, no studies in the past have been able to show that the insertion of GM traits, whether the Roundup-Ready or Bt genes have altered the crop in a negative way.

The goal of this study was to analyze how the metabolomic traits of maize cultivars grown with and without a GM trait compare to those of their counterparts. The design of the study allowed researchers to thoroughly study the effects of GM on the cultivars' chemical composition. The maize cultivars used in this experiment are therefore the same in terms of their genotypic make-up, except for the insertion of the GM traits which consists of both the Bt and Roundup-Ready genes. Therefore, the expectation was that the chemical profile of the GM and non-GM for the two groups should be identical. However, as was reported by Harrigan et al., (2016), it would be expected that GM material would show some differences as is also expected with conventional breeding. The assumption is however, that GM would not introduce many changes as is the case with conventional breeding as only a single gene is inserted. This is often highlighted as a benefit of GM technology as opposed to conventional breeding where the genetic material of two individuals is crossed.

The metabolomic analysis in this study (Figure 13) clearly shows on the scatter plot that there is indeed a significant difference in the chemical and or metabolomic make-up of the GM and non-GM cultivars as the four types of material showed distinct clusters. The clustering of the samples is based on the similarities of the chemical profile, where samples

that are clustered close together resemble similar profiles. Furthermore Figures 15-18 also show that there are distinct differences within the chemical profiles of the groups of cultivars used in this experiment.

Figures 16 A-D showed enlarged regions of the NMR profile which highlights the differences in the peaks for the long season samples, especially peak heights in the GM samples when compared to the non-GM samples. In Figures 16 A-C the peak heights, which are indicative of the concentration of the compounds, are higher in the GM samples when compared to the non-GM samples. In Figure 16 D, there are some peaks higher for the non-GM and others higher for the GM samples in the sugar region, showing that various sugars have been affected by GM insertion, although not all affected in the same level.

Figures 18 A-D showed enlarged regions of the NMR profile which highlights the differences in the peaks for the short season samples. Again, the peak heights in the GM samples when compared to the non-GM samples are indicative of concentration differences of the compounds in the samples.

GMGMAlthough supporting literature could not be identified in explaining the inner working of sugars in GM maize it was however interesting to note that literature showed that sugars from GM and non-GM sugar beet were found to be without any differences, especially for the sugar fructose (Bawa & Anilakumar, 2013b).

It was however important to determine the compounds responsible for the peak differences to establish if these changes are insignificant as reported in previous studies. The chemical differences found in the two groups of cultivars were further investigated in Chapter 5.

4.7 Conclusion

The study analyzed the effects of residual genetic variation and the insertion of genetically modified traits on the maize metabolome. Overall, the study was characterized by significant differences, and it was evident that GM trait insertion influenced the maize leaf metabolome. Although residual genetic variation was a contributing factor to variation, it was not as significant as the effects of GM. This suggests that the presence of these compounds could be a source of differences between the two types of comparators. Some differences were also observed between the two GM hybrids. It is not yet clear if these compounds are insignificant changes or if they are caused by gene insertion.

Chapter 5: Compound annotation and discussion

The identification of metabolites and peaks in untargeted metabolomics remains an area of concern. This process usually begins by identifying the relevant peaks using a combination of peak-picking techniques and databases (Tugizimana et al., 2013). Databases such as the Human Metabolome Database (HMDB) and software programmes such as Chenomx are widely used to assist in compound identification within samples.

In this study the compound peaks were matched with peaks of possible compounds in the databases. Compounds such as lactate, caprylate and arabinitol were annotated in the samples and found to be higher in the GM cultivars of both the long and short season samples, whereas mannitol was variable between GM and non-GM samples. The role and importance of these chemical compounds were further investigated to determine the possible effects that changes in these metabolite levels will have on the functioning of the plants.

5.1 Materials and methods

In chapter 3 section 2.2 the trial plan and plant materials are explained that were used in preparation for both the animal feeding trial and metabolomic study. This chapter elaborates on the metabolomic analysis to match the peaks to possible compounds which were observed in Chapter 4.

5.2 Compound annotation

The contribution plots were constructed from the OPLS-DA score plots (Figure 14), and used to determine the important NMR spectral regions, differentiating the four types of samples from each other. Upon identifying the NMR spectral regions of interest, the peak patterns were compared to potential compounds by using software programmes such as Chenomx and databases such as the Human Metabolome Database (HMDB). The peak patterns of the possible compounds were then further compared to published literature, to confirm the annotation of the compounds.

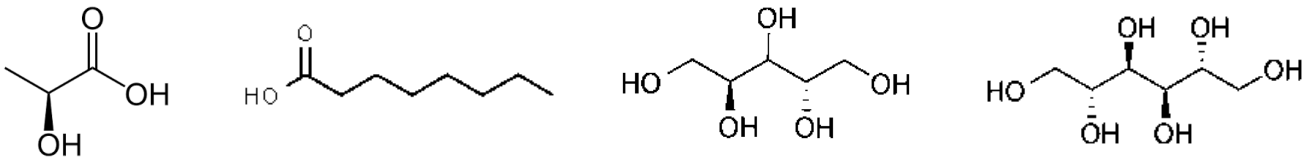
5.3 Results

Chapter 4 indicated that there were distinct differences in the chemical profiles of the GM and non-GM samples, for both the long season and short season cultivars. It was therefore important to investigate these profiles further as it is important to annotate the compounds to determine the effect or possible role of these compounds in the plant.

NMR regions identified in the contribution plots were matched with possible compounds by comparing the spectral regions. The list of annotated compounds is provided in Table 7, with the NMR regions as obtained from the samples, Chenomx, and the HMDB. The chemical structures of the annotated compounds are provided in Figure 19.

Table 7: Annotated compounds with the NMR regions obtained from the samples, Chenomx and the HMDB

Compounds	**^{1}H-NMR Chemical shifts (ppm) from samples**	**Chenomx (ppm)**	**HMDB (ppm)**	**Sample annotation**
Lactate	1.33 4.11	1.33 4.1	1.32 4.10	Higher concentration in long and short season GM maize.
Caprylate	0.89 1.27 2.17	0.86 1.27 1.53 2.16	0.85 1.27 1.53 2.16	Higher concentration in long and short season GM maize.
Arabinitol	3.55-3.57 3.65 3.67-3.68 3.72-3.75 3.82 3.90-3.95	3.56-3.58 3.64 3.66-3.68 3.73-3.76 3.82-3.84 3.91-3.94	3.62-3.65 3.69-3.71 3.73 3.81-3.84 3.92-3.93	Higher concentration in long and short season GM maize.
Mannitol	3.65-3.7 3.72-3.75 3.79 3.84 3.87	3.65-3.69 3.72-3.77 3.79 3.85 3.87	3.62-3.66 3.68-3.71 3.81 3.84 3.87	Higher in GM long season maize and non-GM short season maize.



A B C D

Figure 19: Chemical structures of annotated compounds found in metabolomic analysis of GM and non-GM maize. A = lactate, B = caprylate, C = arabinitol and D = mannitol.

5.4 Discussion

In Chapter 4, distinct clustering was observed for the four types of samples analyzed. It was therefore important to determine what these compounds are as it might have an influence on the metabolism, growth and general development of the plant. The following compounds were found to be higher in the GM containing maize cultivars than their non-GM counterparts namely: arabinitol, caprylate and lactate. Therefore, it is important to have a better understanding of what these compounds are and their contribution or role within the plants. The only compound that differed in both the cultivar groups in the metabolomic analysis was mannitol, found to be higher in GM long season and non-GM short season maize.

Xylitol production is carried out using hydrolysates containing arabinose. This produces arabinitol, which has similar chemical properties to xylitol. It can also be turned into a yellow product by sodium periodate. The physical properties of both compounds are similar, including the boiling point, polarity, and solubility. But when arabinitol is used, the product value of Xylitol decreases. Various fruits and vegetables, such as mushrooms, oats, and berries, as well as fibers from plant stalks and corncobs, are known to contain xylitol, which is a type of sugar alcohol. It can be used as a natural or low-calorie sweetener (Zhang et al., 2016).

The term caprylate refers to the salts and esters of the compound octanoic acid. It can also be called caprylates or octanoates (Longardner & Haubenberger, 2022). Oxidation of C8 aldehyde produces octanoic acid, a widely used industrial chemical. It is a heptane that has a carboxy group, and it can be found in various plant oils, such as palm kernel oil and coconut oil. It can also be used as an antimicrobial agent (Berger, 2003). In plants it is a membrane stabilizer, energy source, energy storage and nutrient (Berger, 2003).

Additionally, studies and literature provided evidence that caprylate or octanoic acid can act as a herbicide due to the herbicidal effects shown against four major weeds namely *Calotropis gigantea* (R.Br.), *Parthenium hysterophorus* (L.), *Datura metel* (L.) and *Tridax procumbens* (L.) (Rajasekharreddy & Nagaiah, 2011). All weed plants treated with (2n-octylcycloprop-1-enyl)-octanoic acid and the deformities were observed at 24, 48 and 72 h after foliar application. Chlorosis followed by necrosis, occurred in all treated plants at different concentrations of 60, 40, 30 and 35 mg/L against the four plants respectively. The first apparent symptom after application was a downward twisting of leaves and stems.

Lactic acid has been shown to promote plant growth. Lactic acid bacteria is a widespread bacterial group in nature in niches of dairy, meat and vegetable origin. It also occurs in human and animal gastrointestinal and urogenital tracts (Ruiz Rodríguez et al., 2019). Dry weight of maize was more than doubled when plants were grown in media containing the dimer of lactic acid, lactoyllactic acid. Higher polymers were equally effective at increasing plant biomass (Kinnersley et al., 1990). Plant with a higher concentration of lactate will therefore perform better, as is observed with the GM maize cultivars.

A type of carbohydrate known as maltitol is not found in most plants. It is a polyol or sugar alcohol, and it can be used as a photosynthetic component in over a hundred species (Reidel et al., 2009). Brown algae, mushrooms, tree bark, and other fruits and vegetables can contain mannitol, which can be found in various forms of confectionary products such as chocolate coatings and chewing gum. Global health authorities have confirmed that this substance is safe to consume (Bawa & Anilakumar, 2013).

Its catabolic enzyme's regulation is intricate. This complex process is affected by simple sugars and salts, among other factors (Meena et al., 2015). Because of its growth-repressive properties, manmannitol can be utilized as a potential study material for growth-regulating events (Stoop et al., 1996). In fungi, mannitol plays a role in metabolism and a role in pathogenesis. In response to pathogen invasion, plants produce ROS in the extracellular space or apoplast for defense (Bolwell & Wojtaszek, 1997). Hydroxyl radicals may be responsible for oxidative damage during drought or chilling stress. It has been shown that the presence of mannitol in chloroplasts can protect plants against oxidative damage by

hydroxyl radicals. Mannitol is known as a hydroxyl radical scavenger *in vitro* and *in vivo* (Shen et al., 1997).

As the world moves toward a more sustainable food supply, it is important that the various aspects of the food chain are considered when it comes to the development of new crop varieties. The safety assessments conducted on new biotechnology-based traits are carried out according to internationally recognized standards (Dupendant, 2016). This process involves the detailed molecular analysis of a new genetically modified variety to ensure that it incorporates the intended DNA sequence and the measurement of its levels. One of the main principles of assessments is the substantial equivalence of the new product's nutrient and phenotypic characteristics to that of a conventional variety. Conventional comparators typically share the same genetic background as the new product, but they do not exhibit the new biotechnology trait. These assessments have shown that the new genetically modified crops are generally comparable to their conventional counterparts. They also noted that the introduction of new traits does not affect the pre-existing characteristics of crops (Harrigan et al., 2016).

In this study, for both the long and shot season cultivars a GM and non-GM variety of the same genetic material was used. Although other studies have showed minimal differences in GM and conventional cultivars this study shows that on the metabolomic profiles obtained there is indeed differences detected. Metabolomics has the potential to provide new dimensions to GM analysis, allowing detection of the effects that might take place because of genetic engineering application whether it was intended or not.

It is important to differentiate between primary and secondary metabolites in plants. When using metabolomics, the findings are mainly categorized in primary and secondary metabolites. Primary metabolites, such as amino acids, organic acids and carbohydrates are essential in all plants (Lee et al., 2013) and are essential for life and exist in all plants. Secondary metabolites are not directly involved in the normal life cycle but help the plant adjust to the surroundings (Erb & Kliebenstein, 2020). Secondary metabolites are species-specific and usually exhibit ecological functions.

Of the compounds annotated in the study, caprylate functions as a secondary metabolite, showing protective properties with the use as for example a fungicide, herbicide and virucide. The increase in caprylate will therefore provide a benefit to the survival and growth of the GM crop. Similarly, lactic acid has proven to improve growth, although the compound has not been regarded as a secondary metabolite, and probably improve the general growth conditions of the plant internally.

In the leaves of several plants, Arabinitol can act as a strong inhibitor of Rubico. When exposed to oxygen, Rubisco forms a single molecule of 2-phosphoglycollate and another of 3-phospolycolate. The presence of light triggers the release of an inhibitor compound, such that 2-carboxyarabinol-1-phosphate, which is bound to the active site. These inhibitors prevent the enzyme from performing its function (Raghavendra et al., 2016).

5.5 Conclusion

The compounds annotated in this study all play their different roles in plant physiology. It is therefore important to understand the roles they play in plant health and the effects it can have on plants and especially maize plant as was studied in this experiment.

Caprylate, lactate and arabinitol were found to be present in higher concentration in the short and long season GM maize cultivars. The only chemical compound that showed a different result was mannitol which was in higher concentration in the GM long season and non-GM short season cultivars.

Caprylate, lactate and arabinitol does however show to all have a positive effect and or working inside the plants as it occurs in and contributes greatly to plant response to the environment and to maintain general plant health.

Chapter 6: General Discussion, conclusion and recommendations.

The use of GM's has been on the rise in South Africa since 1998 and in 2016 a total of 2.7 million hectares were planted under GM maize, cotton and soybeans (Masehela et al., 2016). It has been reported in several studies that GM does not change the metabolome of the plant, however farmers claim that cattle prefer non-GM material. There is therefore a continuous concern that GM containing plants might not be identical to the original or conventional plants as the metabolome might be altered, in excess of the gene or genes that are introduced. In this study it was determined if cattle can distinguish between GM and non-GM maize plants. To serve as an internal control, short season and long season maize containing the Bt gene and Roundup-Ready genes, and the conventional maize with the same genetic material were used in this study.

In this experiment where identical groups of maize cultivars were used to determine if cattle do prefer maize plants without the inserted GM traits namely Roundup-Ready and Bt, it can be confirmed that cattle could not differentiate between the GM and identical non-GM maize. Although the cattle were expected to show a preference for non-GM plant material it was surprising that they showed more interest in in the longer season cultivars opposed to the short season cultivars irrespective of the GM traits it possessed. It could therefore be concluded that the GM and non-GM maize material did not differ in taste as cattle consumed equal amounts of both groups of GMs. The preference to long season material can be due to their inherent resistance to pathogen infections which can influence their palatability and slight changes in the metabolome, this can also be an indicator that these cultivars must resist the pathogens on the leaves to a much larger extent than the short season cultivar which is known to be more prone specially to leaf diseases such as Northern corn leaf blight and Grey leaf spot. These diseases will lead to plant secretions on the leaf which should influence the taste and smell of the plants making the longer seasoned cultivar much more preferable. It can also be recommended to promote certain longer seasoned cultivars keeping in mind that cattle would prefer to overwinter on them as opposed to the short-seasoned cultivars. In the event of a farmer not having cattle to overwinter on the maize material. this will have no influence and any cultivar whether it is a short season or longer season and can be planted and should therefore rather be based on the cultivar's adaptability to the immediate environment. These possibilities, however, warrant further research into the specific reasons for the preference of longer season cultivars.

Furthermore, another objective of this study was to determine if there were any chemical differences in GM maize when compared to non-GM maize, using an NMR-based metabolomic approach. As the genetic material of GM and non-GM material only differ in the addition of the Bt and Roundup-Ready genes, no differentiation between the chemical profiles is expected (Simó et al., 2014). In this study it clearly showed that there were indeed a chemical composition difference of the GM and non-GM cultivars as explained in Chapter 5. The GM and non-GM material separated in the score plots, indicating a significant difference in the metabolites of the plant samples.

After the metabolomic analysis was done, certain NMR regions were identified that differed in terms of the presence or absence and height (concentration). By using software programmes and databases such as Chenomx and HMDB, and literature, four compounds were annotated in the plant samples that were linked to the NMR regions that differentiated the different types and the presence or absence of the introduced genes. Lactate, caprylate and arabinitol/arabitol was found to be present in higher concentration in the short and long season GM maize cultivars. The only chemical compound that showed a different result was mannitol which was in a higher concentration in the GM long season and non-GM short season cultivars, therefore not specific to GM or non-GM material or long or short season cultivars.

Known as a plant compound, lactate is a major source of accumulation in plants. It was found that the glyoxalase pathway is responsible for the detoxification of methylglyoxal. MG is a type of toxic metabolite that can be accumulated under stressful conditions (Anaya-Sanchez Id et al., 2021). Lactate additionally promote plant growth where it was found that dry weight of maize was more than doubled when plants were grown in media containing the dimer lactate, lactoyllactic acid. Lactate, therefore, is possibly playing an important role in promoting plant growth, supporting the superior growth of GM maize when compared to non-GM maize.

Despite its wide distribution, the plant scientists do not usually pay much attention to the six-carbon sugar alcohol known as mannitol. Recent studies have shown that plants that are capable of converting mannitol have various advantages over those that only translocate sugars. One of the main advantages of mannitol is its ability to increase a plant's tolerance to both osmotic and salt-induced stress. It is also believed that its metabolism can play a role in the plant's response to pathogens (Stoop et al., 1996). Mannitol has additionally a radical scavenging effect (hydroxyl radical scavenger) in plants which can promote plant health. This compound was present in both GM and non-GM material, indicating that this compound is present in maize to mitigate plant stress and prevent ROS in general in maize plants.

This study answered and exceeded expectations in terms of what was initially anticipated. Cattle was expected to be able to distinguish between GM and non-GM plant material, although the study could not find any evidence of cattle preferring non-GM material as feed. Where one would also expect the chemical profile to be similar in the two different groups of cultivars used this was also surprising where compounds showed differences in the chemical profiling. This would not only be expected as the cattle could not differentiate between them, but also the reports in literature stating that GM material is similar to the conventional material. The changes that were observed could possibly be attributed to the additional or secondary benefits of the protection of the introduced genes. The gene products such as Bt toxin, provides protection to plant attack, thereby ensuring a more enabling growth environment for the plant, even though not directly from the gene product. The gene product therefore provides indirect additional benefits than merely producing a toxin. A similar situation would probably be created for the Roundup-Ready GM material. As the gene product ensures a weed free environment, thereby also a more enabling environment to growth and to grow well. Since the plant is in a more enabling environment, growth is enhanced which also supports the superior growth and yield of GM maize.

It can be noted that this study has reached and surpassed its research objectives as stated earlier in the aim and objectives statements. The hypothesis is supported by the results of the study, as there is no indication that the cattle could distinguish between GM and non-GM material, and therefore preferred the long season cultivar irrespective of GM. As the study made use of an internal control with both short season and long season maize containing the Bt gene and Roundup-Ready genes, and the corresponding conventional maize, it allowed careful examination of the factors that influence cattle feed preferences. Metabolomics analysis also supported the feeding trial results as there were no major changes in the plant metabolome, and therefore all objectives could be achieved without any bias.

Recommendations

This study clearly shows metabolomic differences in GM and non-GM containing maize cultivars. It is therefore strongly recommended that further studies are done to determine more detailed analysis of the differentiating compounds, their contribution and probable effect on the plant in terms of stress tolerance. The differences in this study indicated minor changes in the metabolome, although it was not an extensive study to investigate all differences between GM and non-GM maize. Only a small sample was used for the study, to see if there were any differences. A more detailed analysis using a larger sample dataset, probably also more types and cultivars should be used, additionally employing other analytical techniques such as LC-MS to further identify compounds that differentiate GM and non-GM maize material. This will provide a more holistic view of the changes that are introduced, even if not important in the complex metabolism of plants.

Especially in an arid country like South Africa, recommendations can be based on the chemical profiling of new genetic material to determine their risk factor to stress and therefore be prescribed in accordance with the specific climatological region where these cultivars are being planted. Knowledge obtained in further studies may also help plant breeders to further understand the collaboration of selecting the appropriate genetics for certain regions by considering the contribution of the metabolomic profiles.

In addition to this, understanding the chemical compounds in maize cultivars may help in the development of further technology regarding new GM traits in the future.

An in-depth study is therefore strongly advised to broaden the scope of this research and to assist the agricultural sector to better understand the complex chemical interactions and networks of cash crops like maize and the significance that the chemical compounds in the cultivars themselves can or cannot contribute to obtaining higher yields and to help feed an ever-growing world population.

8 Addendum:

Annova model for green plant material consumption by the cattle. Source: ARC

file name is green plant material weights after 30 min exposure to cattle.gen
Green plant material weights and intake after 30 min exposure to cattle

Message: You have input sufficient data, READ terminated.

Identifier	Minimum	Mean	Maximum	Values	Missing
Wt30min	0.2000	0.8542	1.500	24	0

Identifier	Values	Missing	Levels
Animal	24	0	6
Cult	24	0	4

Analysis of variance

Variate: Wt30min

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Animal stratum	5	0.23658	0.04732	0.79	
Animal. *Units* stratum					
Cult	3	2.45445	0.81815	13.64	<.001
Residual	15	0.89995	0.06000		
Total	23	3.59098			

Tables of means

Variate: Wt 30min

Grand mean 0.854

Cult	LS-BR	LS-C	UQ-BR	UQ-C
	0.510	0.560	1.160	1.187

Standard errors of means

Table	Cult
rep.	6
d.f.	15
e.s.e.	0.1000

Least significant differences of means (5% level)

Table	Cult
rep.	6
d.f.	15
l.s.d.	0.3014

Stratum standard errors and coefficients of variation

Variate: Wt30min

Stratum	d.f.	s.e.	cv%
Animal	5	0.1088	12.7
Animal. *Units*	15	0.2449	28.7

Fisher's protected least significant difference test

Cult

	Mean	
UQ-C	1.1867	a
UQ-BR	1.1600	a
LS-C	0.5600	b
LS-BR	0.5100	b

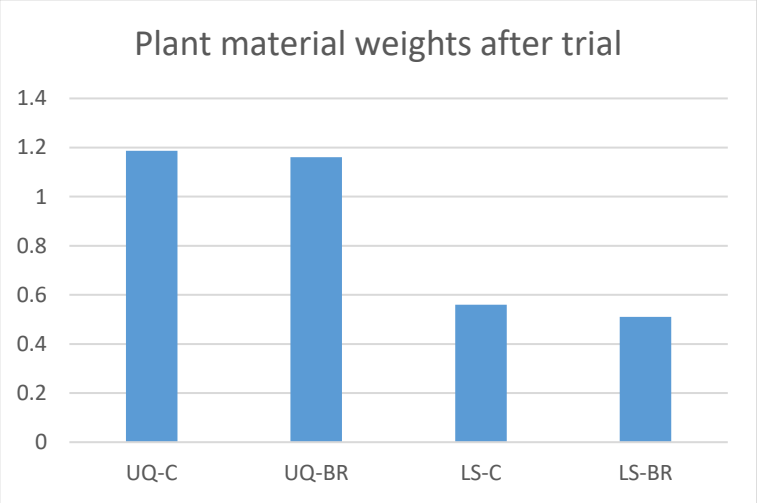


Figure 20: Remaining plant material after preference test with cattle.

========== Summary of original data =========

Nobservd	Mean	Variance	s.d.	
Cult				
LS-BR	6	0.5100	0.01608	0.1268
LS-C	6	0.5600	0.12644	0.3556
UQ-BR	6	1.1600	0.04340	0.2083
UQ-C	6	1.1867	0.04139	0.2034
Margin	24	0.8542	0.15613	0.3951

Animal	Cult	Wt30min	FITTED	RESIDUAL
E057	UQ-C	1.3700	1.2600	0.1100
E057	UQ-BR	1.2000	1.2333	-0.0333
E057	LS-BR	0.6200	0.5833	0.0367
E057	LS-C	0.5200	0.6333	-0.1133
E047	UQ-C	1.4100	1.2300	0.1800

E047	UQ-BR	1.2500	1.2033	0.0467
E047	LS-BR	0.5000	0.5533	-0.0533
E047	LS-C	0.4300	0.6033	-0.1733
E009	UQ-C	1.2500	1.3225	-0.0725
E009	UQ-BR	1.5000	1.2958	0.2042
E009	LS-BR	0.5200	0.6458	-0.1258
E009	LS-C	0.6900	0.6958	-0.0058
E024	UQ-C	0.9100	1.1900	-0.2800
E024	UQ-BR	1.0000	1.1633	-0.1633
E024	LS-BR	0.3200	0.5133	-0.1933
E024	LS-C	1.2000	0.5633	0.6367
E022	UQ-C	0.9800	1.0400	-0.0600
E022	UQ-BR	1.1000	1.0133	0.0867
E022	LS-BR	0.4300	0.3633	0.0667
E022	LS-C	0.3200	0.4133	-0.0933
E003	UQ-C	1.2000	1.0775	0.1225
E003	UQ-BR	0.9100	1.0508	-0.1408
E003	LS-BR	0.6700	0.4008	0.2692
E003	LS-C	0.2000	0.4508	-0.2508

Analysis of variance

Variate: Intake

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Animal stratum	5	0.23658	0.04732	0.79	
Animal. *Units* stratum					
Cult	3	2.45445	0.81815	13.64	<.001
Residual	15	0.89995	0.06000		
Total	23	3.59098			

Tables of means

Variate: Intake

Grand mean 2.146

Cult	LS-BR	LS-C	UQ-BR	UQ-C
	2.490	2.440	1.840	1.813

Standard errors of means

Table	Cult
rep.	6
d.f.	15
e.s.e.	0.1000

Least significant differences of means (5% level)

Table	Cult
rep.	6
d.f.	15
l.s.d.	0.3014

Stratum standard errors and coefficients of variation

Variate: Intake

Stratum	d.f.	s.e.	cv%
Animal	5	0.1088	5.1
Animal. *Units*	15	0.2449	11.4

Fisher's protected least significant difference test

Cult	
Mean	
LS-BR 2.490	a
LS-C 2.440	a
UQ-BR 1.840	b
UQ-C 1.813	b

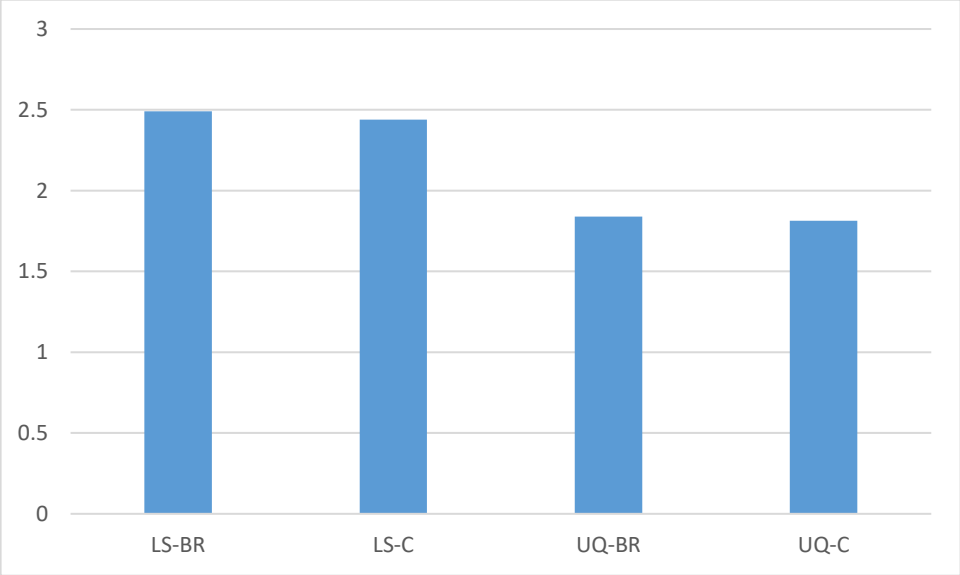


Figure 21: Remaining plant material after the feeding trial was conducted.

========== Summary of original data =========

	Nobservd	Mean	Variance	s.d.
Cult				
LS-BR	6	2.490	0.01608	0.1268
LS-C	6	2.440	0.12644	0.3556
UQ-BR	6	1.840	0.04340	0.2083
UQ-C	6	1.813	0.04139	0.2034
Margin	24	2.146	0.15613	0.3951

Animal	Cult	Intake	FITTED	RESIDUAL
E057	UQ-C	1.630	1.740	-0.1100
E057	UQ-BR	1.800	1.767	0.0333
E057	LS-BR	2.380	2.417	-0.0367
E057	LS-C	2.480	2.367	0.1133
E047	UQ-C	1.590	1.770	-0.1800
E047	UQ-BR	1.750	1.797	-0.0467
E047	LS-BR	2.500	2.447	0.0533
E047	LS-C	2.570	2.397	0.1733
E009	UQ-C	1.750	1.677	0.0725
E009	UQ-BR	1.500	1.704	-0.2042
E009	LS-BR	2.480	2.354	0.1258
E009	LS-C	2.310	2.304	0.0058
E024	UQ-C	2.090	1.810	0.2800
E024	UQ-BR	2.000	1.837	0.1633
E024	LS-BR	2.680	2.487	0.1933
E024	LS-C	1.800	2.437	-0.6367
E022	UQ-C	2.020	1.960	0.0600
E022	UQ-BR	1.900	1.987	-0.0867
E022	LS-BR	2.570	2.637	-0.0667
E022	LS-C	2.680	2.587	0.0933
E003	UQ-C	1.800	1.922	-0.1225
E003	UQ-BR	2.090	1.949	0.1408
E003	LS-BR	2.330	2.599	-0.2692
E003	LS-C	2.800	2.549	0.2508

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Genstat 64-bit Release 18.2 (PC/Windows 8) 20/2/2020 10:31:36

Registered to: ARC

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