SRDC Research Project Final Report

Sugarcane compositional analysis to enable food safety assessment of modified varieties

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TABLE OF CONTENTS

	Page no.
Executive summary	**3**
Background	**4**
Objectives	**6**
Methodology	**6**
1. Understanding the requirements for the baseline data	**6**
2. Selection of samples for analysis	**6**
3. Sample consistency	**7**
4. Analytical methods	**8**
5. Role of the consultative panel in guiding the project and evaluating its success	**9**
Outputs	**10**
1. Analysis of a broad set of samples completed	**10**
2. Range of compositions in Australian varieties	**10**
3. Sources of variation in composition	**15**
Intellectual Property and Confidentiality	**17**
Environmental and Social Impacts	**17**
Expected Outcomes	**17**
Future Research Needs	**18**
Recommendations	**18**
References cited in this report	**19**
List of Publications	**19**
List of Appendices	**20**

Executive Summary:

In Australia and internationally, genetically modified (GM) varieties of sugarcane are being developed in research institutions and biotechnology companies. Before these varieties can be grown commercially in Australia or the products used, they will need approval from regulatory authorities. This approval will also be critical for the export of products from Australian sugarcane varieties to other countries.

The science-based regulatory assessment in Australia relies on good baseline data on the biology of conventional varieties for comparison with the new modified varieties to identify any potential changes. Projects supported by the SRDC are working towards providing the baseline data needed by the Office of the Gene Technology Regulator (OGTR) for assessment of the environmental impacts. The need addressed by project CPI020 was to provide good quality baseline data to enable food safety assessment by Food Standards Australia and New Zealand (FSANZ).

An important component of demonstrating that the products of GM sugarcane varieties are substantially equivalent to conventional varieties is whether the nutritional composition falls within the range of compositions that are currently found in production. For sugarcane, the food safety assessment will focus on the stalk, as the plant part that provides the food product, and the nutritional components that will be assessed are the proximates (comprising moisture content, crude fibre, protein, fat, ash and N-free extractives) and the soluble sugars. For feed purposes, information on the proportions of neutral detergent fibre (NDF) and acid detergent fibre (ADF) will also be required. Although some components such as sugars are routinely measured in sugarcane stalks, there was a lack of information on most of the nutritional components. The aim of this project was to determine the range of nutritional compositions found in Australian sugarcane varieties under normal agronomic practices.

The project was guided by a consultative panel which included representatives of the major end users of the data, the regulatory authority and the technology developers. The panel reviewed plans and results annually and their recommendations were incorporated into the project on an ongoing basis. Interaction with the panel members also helped to disseminate the project outputs to the industry.

The first stage of the project analysed likely sources of variation in composition by examining published reports and a preliminary set of samples. Six major sources of variation were identified: (i) genotype, (ii) geographical region, (iii) crop type, (iv) soil nutrition, (iv) crop age and (vi) season-to-season differences. A sampling strategy was then designed to capture a range of conditions and also to allow an analysis of the relative influence of each factor on the composition. The nutritional composition of 239 sugarcane samples has been analysed in total. These samples comprise 159 samples of stalk from conventional varieties, including 59 samples from a single widely-grown variety, Q208. The remaining samples were from outside current genotypes of agronomic practices but may become relevant in future cropping systems. These included early-harvested conventional varieties, high-fibre lines from an experimental population and tops from conventional varieties. The proximate analysis was performed by outsourcing to a NATA-accredited analytical laboratory while the analysis of sugars was performed in the CSIRO laboratory.

The project successfully produced ranges for the proximates, sugars and forage values. This is the most comprehensive set of compositional data for sugarcane available. It extends the known ranges published in previous documents, including the OECD Consensus Document, and provides information on a component (N-free extractives) that was not previously reported. When the data were analysed according to the major sources of variation, the genotype and crop/ratoon cycle appeared to have the least influence on composition, while growth region, soil composition and climatic conditions appeared to have a larger influence. Since genetic background is not a critical factor in composition, the results produced in this study should be relevant for baseline comparisons with varieties released into the future.

A publication is being prepared for an international journal so that the results will be peer-reviewed and made widely-available. Once this is completed, the results will be used as an input to assessment by FSANZ and by proponents of applications for approval of GM varieties in the near future. The outputs will also be incorporated into updates of the OECD Consensus Document for Sugarcane which will benefit the assessment of Australian sugarcane varieties and their products internationally. The major outcomes of the work will be economic benefits in speeding up the approval process for GM varieties.

Background:

As in many other crop species, genetically modified (GM) varieties of sugarcane are forecast to improve profitability by reducing input costs, increasing product yield or introducing novel products. GM sugarcane varieties are under development in Australia and work is in progress to facilitate commercial deployment of GM varieties. Before these cultivars can be grown commercially and the products derived from them used, they will need approval by regulatory authorities. As most of the sugar from the Australian sugarcane crop is exported, approvals for growing GM sugarcane and approval for the sugar manufactured from it to be used as food will be required in export markets as well as in Australia.

In Australia, the regulatory functions for GM crops are spread across several agencies, depending upon the modification (Mitchell, 2011). Depending on the gene modifications involved, approvals will have to be granted by the Office of the Gene Technology Regulator (OGTR), Food Standards Australia and New Zealand (FSANZ) and Australian Pesticides and Veterinary Medicines Authority (APVMA).There is a need for research to underpin sound, science-based decision-making by the Australian regulatory authorities. Recent projects supported by the SRDC have investigated the sexual reproductive biology of sugarcane to support the environmental assessment of GM sugarcane by the OGTR (Bonnett *et al.*, 2007, 2010). Data to assist food safety assessment by FSANZ is a step in this process which had not previously been addressed.

Assessments of the safety of foods and food components derived through modern biotechnology are based on establishing “substantial equivalence” of the new food product to its conventional counterpart (OECD, 1993; FAO/WHO, 2000). The concept of “substantial equivalence” implies that, if a new food is found to be substantially equivalent to an existing conventional food, it can be concluded to be as safe as the conventional food. If unintended changes have occurred, it would then be necessary to demonstrate that no harmful consequences would result from the modification.

One key indicator of substantial equivalence is that the nutritional composition of the food derived from a GM cultivar should fall within the range of compositions found in unmodified varieties. To assist this comparison and to ensure consistency between countries, the OECD's Task Force for the Safety of Novel Foods and Feeds produces consensus documents on compositional characteristics of crops used as food and/or feed. The consensus documents include information on macro- and micro-nutrients, reflecting the range across different plant varieties and geographical zones. Data need to be produced by reliable methods and ideally published in an international peer-reviewed journal.

At the start of this project, consensus documents had been published for 17 species. A consensus document on sugarcane composition was in preparation, lead by FSANZ representatives at the OECD. However, the available information on comparative compositions of sugarcane varieties was limited. The information in published papers was derived from international cultivars and was up to 20 years old, and therefore was not representative of current and near-to-release Australian cultivars. Much more extensive information from Australian varieties and environments would be required for regulatory decisions in Australia. In the absence of good data, an applicant submitting a proposal to FSANZ would have to also provide extensive data on the composition of conventional varieties to allow a comparison.

In Australia, food safety regulation by FSANZ aligns with the OECD recommendations using substantial equivalence as a guide. However, the regulatory system has specific requirements for consideration of highly refined products from GM plants, such as oil or sugar. For these products, the assessment focuses on the part of the plant that the food is derived from. In the case of sugarcane, FSANZ considers that analysis of the refined sugar is not sufficient for two reasons:

(i) The composition of refined sugar is close to 100% sucrose and any variation in the impurities is more likely to reflect differences in the refining process rather than differences derived from the modified plant tissue.

(ii) When food safety approval is granted, the approval covers all products and potential products from the modified plant. Although unprocessed sugarcane is not widespread as a food source in Australia, there are niche markets for juice, and juice and pulp are routinely consumed in other countries. Therefore, food safety assessment needs to consider the potential use of other parts of the sugarcane plant.

For these reasons, the assessment would be performed on the sugarcane stalk, as this is more likely to reflect the composition of the whole plant. The draft Consensus Document also made recommendations about which components should be analysed in the stalks (Table 1)

Table 1. Components required for compositional analysis of a GM sugarcane stalk.

Analysis	Components
Food	Moisture, crude fibre (% insoluble dry matter), crude protein, fat (as ether extract), ash, sucrose, total sugars*
Feed[+]	Neutral detergent fibre (NDF) and acid detergent fibre (ADF)

[*]free monosaccharides and disaccharides. For sugarcane, these primarily consist of sucrose plus lesser amounts of reducing sugars (glucose and fructose). [+] Additional measurements for feed.

The intention of the current project was to improve the information available for assessment by providing knowledge of the range of compositions in current Australian varieties. The outcome of this work should be a set of data that will be useful in testing the substantial equivalence of GM and conventional varieties.

Objectives:

The aim of this project was to provide information that will assist Australian regulatory approval of GM sugarcane varieties and products from these varieties. The project aimed to provide the necessary reference data on the range of compositions that are found in current sugarcane varieties grown in Australia to enable food safety assessment.

The planned output was a reference set of compositional data for Australian sugarcane varieties which will be published in an international peer-reviewed journal and will be available for use by both applicants and regulators for food safety assessments.

The objective of the project was fully achieved. We have produced a set of compositional data that represents the range found in current Australian sugarcane varieties and growing conditions. The results have been reviewed by a consultative panel including technology developers and regulators. A publication is now being prepared so that the results will be subjected to peer review and will be available for use more widely.

Methodology:

1. Understanding the requirements for the baseline data

At the commencement of the project, several teleconferences with staff from Food Standards Australia and New Zealand (FSANZ) were held to gain an understanding of the food safety regulatory process in Australia. Both Anne Rae and Graham Bonnett also visited the FSANZ office in Canberra for discussions.

These discussions identified the stalk as the primary focus of the analysis (as described in Background). The recommendation was to select samples that would give the best possible estimate of the normal ranges encountered in Australian sugarcane growing conditions and practices.

2. Selection of samples for analysis

A suitable reference is required for substantial equivalence comparisons. If the baseline constructed is too narrow, assessed samples may frequently fall out of the range, for reasons not associated with the genetic change, consequently raising potential safety concerns that would require further investigation. We proposed to construct a range for the major compositional components that would capture the major sources of variation likely to be encountered.

An effective strategy for sampling sugarcane varieties to capture the range of compositions in commercial production was worked out by using data from two sources:

(i) Published reports on routinely measured components of sugarcane (e.g. CCS, moisture content and fibre) from a variety of genetic, geographic and agronomic backgrounds were used to determine the factors that have most influence on the composition. This strategy was described in a publication: Rae AL and Bonnett GD (2011). Sugarcane nutritional analysis to enable food safety assessment of GM cultivars – approaches to establishing a baseline. *Proceedings of the 33rd Annual Conference of the Australian Society for Sugar Cane Technologists*, Mackay, Qld., May 4-6 (See Appendix 7)

(ii) Chemical analysis was performed on a preliminary set of samples from a selection of genotypes, locations and crop ages.

The results defined six major influences on composition: (i) genotype, (ii) geographical region, (iii) crop type, (iv) soil nutrition, (iv) crop age and (vi) season-to-season variation. The results also suggested that environmental and management influences would have a greater influence than genetic background on the range of compositions.

The following samples were collected to capture a range of compositions and to allow an analysis of factors that influence composition:

Stalk samples:

(i) Four varieties grown at a single site in the Southern region in 2010 (3 replicates each of Q151, Q155, Q232, KQ228).

(ii) Four varieties grown at a single site in the Burdekin in 2011 (3 replicates each of Q183, Q200, Q208, KQ228).

(iii) A second season of sampling from the same plots in the Burdekin in 2012 (3 replicates each of Q183, Q200, Q208, KQ228).

(v) Samples of a single variety (Q208) from plant, 1st ratoon and 2nd ratoon crops (3 or 4 replicates of each) grown at sites in four regions (Meringa, Burdekin, Southern and Harwood-NSW).

(vi) Six varieties grown at a site in the Southern region with two rates of nitrogen application, a high nitrogen treatment of 200 kg N/ha or a low N treatment of 20 kg N/ha (3 replicates for each treatment of Q190, Q200, Q208, Q218, KQ228, Q232).

(vii) Samples from an early harvest (April 2012) and a later harvest (November 2012) of 5 varieties (3 replicates each of Q200, Q208, Q241, MQ239 and KQ228) from 3 regions (Herbert, Burdekin and Central).

(viii) Samples from experimental lines with high fibre content (3 replicates each of 4 lines from high and low fibre tails of *Saccharum spontaneum* BC1 population) grown in the Burdekin region.

Leaf samples:

Tops from 7-month old crops of varieties Q200, Q208 and KQ228 (3 replicates of each) grown at Meringa.

3. Sample consistency

Two types of sampling for stalk material were tested: whole millable stalk and fibrated millable stalk. The fibrated samples were prepared in a similar way to the material used for SpectraCane analysis. Each replicate therefore represents a number of stalks from the same plot passed through the cutter-grinder to produce a sample of approximately 1 kg in weight. The fibrated samples were placed in a sealable plastic bag, labelled and frozen at -20^{o}C then transferred to Brisbane on dry ice. Preliminary analyses showed that the results obtained from whole stalks were extremely variable compared to the results from fibrated stalks. The analyses of whole stalks also reported low moisture contents, compared to parallel fresh weight/dry weight measurements performed by drying samples in the lab. The reasons for the discrepancy appear to be loss of moisture during transport

and loss of juice during processing of whole stalks at the analytical facility. Following this trial, the decision was made to use fibrated millable stalk.

Samples of tops were sliced into small pieces with a knife before being frozen at -20^{o}C.

4. Analytical methods

The primary nutritional components of a food substance, referred to as "proximate analysis" include moisture, protein, fat, carbohydrates, total dietary fibre and ash. Forage value is measured by the analysis of acid detergent fibre (ADF) and neutral detergent fibre (NDF). The analytical methods for measurement of proximates and forage values are defined by international standards. Analyses for these components in the sugarcane samples were conducted by SGS Agriculture and Food Australia.

A flow chart summarising the measurement of proximates is shown in Figure 1. Moisture content is determined gravimetrically after oven drying of the sample. The remaining material, the dry matter, is the starting material for all other assays. Ash is determined gravimetrically after heating a sample of the dry matter to 550^{o}C and consists of the mineral content of the material. A separate sample of the dry matter is used for determination of nitrogen content which is then expressed as protein content by the use of a standard conversion factor. The fat content is determined by extraction of the dry matter with ether. The ether-extracted material is then treated by boiling in acid solution followed by boiling in alkaline solution. The remaining material is termed crude fibre, which is considered to be indigestible. It is clear from this protocol that dry matter and crude fibre are not equivalent. The content of N-free extractives is calculated by subtracting the amounts of fat, ash, protein and crude fibre from the amount of dry matter. The N-free extractives include soluble sugars, starch, pectins, organic acids, pigments and some polysaccharides. The moisture content is expressed as % of fresh weight while all other components are expressed as % of dry weight.

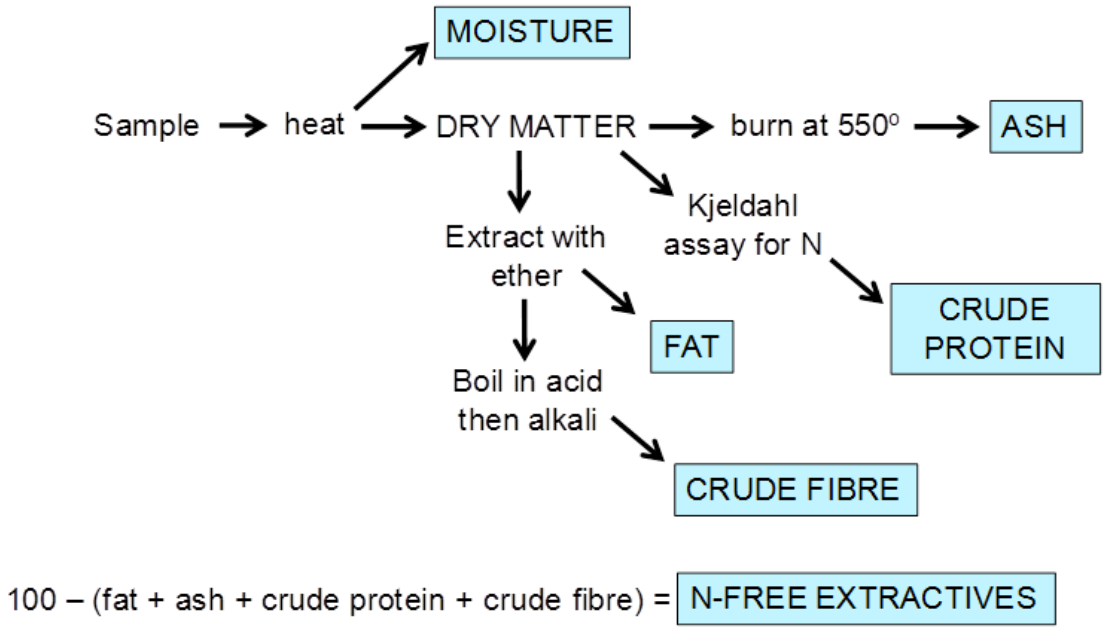


Figure 1. Flow diagram for the determination of proximates in a sample.

For sugarcane, analysis of soluble sugars will also be required for the assessment of compositional changes. Sugar contents were analysed in the CSIRO laboratory using an HPLC with HPAE-PAD detector (Glassop et al. 2009).

A flow chart summarising the measurement of forage values is shown in Figure 2. Neutral detergent fibre (NDF) is determined by boiling the sample in a detergent solution at neutral pH, which removes sugars, pectin, protein and fat. NDF thus consists of cell wall material including cellulose, hemicelluloses and lignin. This residue is then boiled in a detergent solution at acid pH, which removes the hemicellulose fraction. The resulting residue, termed acid detergent fibre (ADF) consists of cellulose and lignin. For forages, low NDF and ADF values are desirable. NDF and ADF are expressed as % of dry weight.

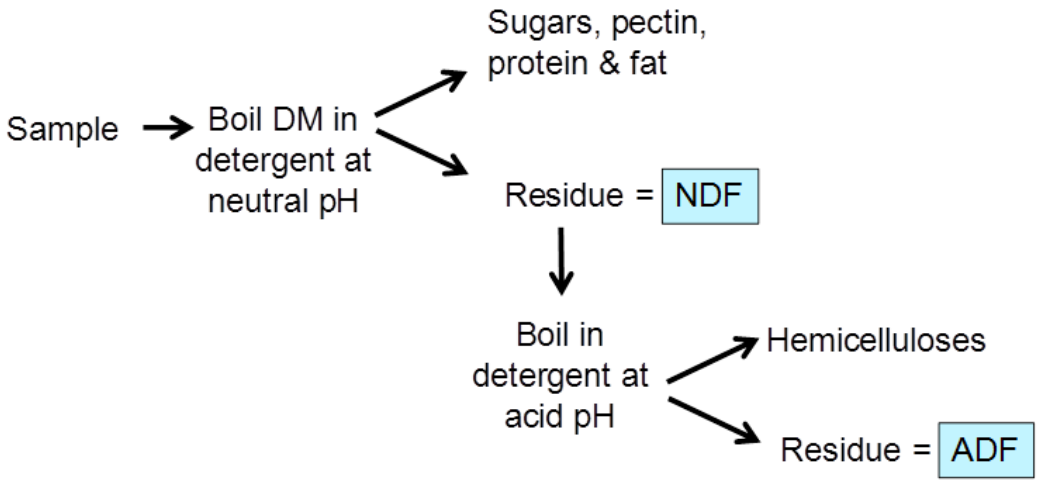


Figure 2. Flow diagram for the determination of forage value.

5. Role of the consultative panel in guiding the project and evaluating its success

A consultative panel has been valuable in providing feedback to previous projects and this method was also adopted for the current project. The consultative group comprised representatives of the sugar industry and regulatory authorities who have an interest in the outcomes of the project. Through regular meetings with the project team during the course of the project, the role of the panel members was to:

(i) Ensure that project outputs meet the future needs of the sugar industry, regulatory authorities and the community by:

- discussing proposed strategies
- critically reviewing results

(ii) Help disseminate information to end users

The members of the consultative panel were:

- Warren Males (Formerly the Alliances and International Affairs Adviser, Australian Sugar Industry Alliance, now at Canegrowers)
- Sophie O'Neill (GM Regulatory Officer, BSES Ltd)
- Bianca Cairns (Investment Manager, Sugar Research and Development Corporation)
- Lynda Graf (Senior Scientist, Risk Assessment Branch, FSANZ)

The first meeting of the panel took place on Tuesday 30 November 2010. At this meeting the panel members were introduced to each other and the role of the panel was agreed. The proposed list of components to be analysed was discussed and work on assessing the major sources of variation was presented. At subsequent annual meetings (28 April 2011 and 23 April 2012), the results from the preceding year's analyses were presented and the plans for the coming year discussed. Feedback from the panel was incorporated into the sampling plans. The last meeting of the panel took place on 21 May 2013, when the final results were presented. Good feedback was received from the panel members:

(i) The panel confirmed that the study was now completed and no major gaps were identified.

(ii) The panel strongly supported publishing the results as a journal paper so that the results can be peer-reviewed and be made available internationally. The results can then be used to update the OECD Consensus Document (OECD, 2011).

Outputs:

1. Analysis of a broad set of samples completed

The major output of the project is the range of nutritional compositions of sugarcane grown under commercial conditions. This comprises a set of data tables that define the compositions found in conventional Australian varieties of sugarcane. A total of 158 samples of mature stalk from commercial varieties have been analysed. Of these samples, 59 were derived from a single widely-grown variety, Q208. In addition, a number of samples from crops that are outside normal agronomic practices have been analysed. These include 45 samples of early-harvested commercial varieties, 24 samples from an experimental population of a variety backcrossed to *Saccharum spontaneum* and 12 samples of tops from commercial varieties. The breadth of sample types allows calculation of the range of compositions as well as an analysis of the variation according to genotype, geographical region, crop type, soil nutrition, crop age and season.

2. Range of compositions in Australian varieties

2.1 Proximates

The range of proximates is shown in Table 2 as minimum and maximum value found amongst the samples. For most components, the range found amongst the Q208 stalks alone falls inside the total range, although the range for a single variety grown across a range of locations is broadly similar to the total range. This is partly explained by the large number of Q208 samples in the total (approximately one-third), but the result also agrees with the preliminary analysis of published results, which suggested that environmental factors were responsible for the majority of the variation in composition.

Table 2. Range of proximates in Australian sugarcane varieties.

	n	Moisture %FW	Crude fibre %DW	Protein %DW	Fat %DW	Ash %DW	N-free extractives %DW
All stalks	158	63.2 - 72.6	16.6 - 33.7	0.8 - 5.3	0 - 3.3	0.7 - 3.3	62.9 - 79.3
All Q208	59	63.2 – 72.6	18.8 – 32.5	1.3 – 2.9	0 – 1.2	0.8 – 3.3	63.3 – 76.1

Moisture contents have been measured previously in sugarcane. A range of 61-72% moisture was found in stalks from 40 trials in 2009 in the northern region (Berding and Marston 2010), consistent with the present study. As shown above in the Methodology section, crude fibre and N-free extractives represent new data as they are not equivalent to measurements made in the sugar industry. There are few previous reports of the protein, fat and ash contents of sugarcane stalks. A study by Keating et al. (1999) reported a range of 0.63-3.75% protein in stalks from eight trial sites between Grafton and Ingham. A study by Banda and Valdez (1976) reported fat contents of 0.81-1.10%. These values are also consistent with the results of our study.

The results from Australian varieties can also be compared with the results published in the Consensus Document for Sugarcane (OECD, 2011) which are derived from a range of older international sources. Table 3 shows that results of the present study extend the ranges of composition for most components. Concentrations of N-free extractives had not previously been published so no data was available for inclusion in the OECD document.

Table 3. Range of proximates in Australian sugarcane varieties compared to the range published in the OECD Consensus Document for Sugarcane. NR = not reported

	Moisture	Crude fibre	Protein	Fat	Ash	N-free extractives
All stalks	63.2 - 72.6	16.6 - 33.7	0.8 - 5.3	0 - 3.3	0.7 - 3.3	62.9 - 79.3
Consensus	67.5 - 80.9	22.7 - 35.9	1.8 - 4.1	0.8 - 1.3	1.2 - 2.0	NR

Comparison with other crops also confirms that the concentrations and values of the proximates in sugarcane are reasonable. Table 4 shows the ranges of protein, fat and ash found in rice straw and barley straw, the equivalent plant parts to sugarcane stalk. Although rice and barley have higher ash contents than sugarcane, the concentrations and ranges of protein and fat are broadly similar.

Table 4. Range of contents of the minor components of dry matter in cereal crop stalks. Data for sugarcane is from the present study. Data for rice and barley are from the OECD Consensus Documents for these crops (OECD, 2004a, 2004b).

	Protein	Fat	Ash
Sugarcane	0.8 - 5.3	0 - 3.3	0.7 - 3.3
Rice straw	1.2 -7.5	0.8 -2.1	12.2-21.4
Barley straw	3.8 - 4.4	1.7-1.9	6.4-7.5

2.2 Soluble sugars

The range of sucrose, glucose and fructose concentrations is shown in Table 5 as minimum and maximum value found amongst the samples. The range of sucrose concentrations included some surprisingly low values, well below the range considered acceptable for milling. However, these probably represent outliers as the mean values were 7.69 and 10.71 for all stalks and Q208 stalks respectively. Analysis of the most recent set of stalk samples has not yet been completed due to equipment breakdown, and hence the number of samples included in this table is lower than the total number analysed for proximates. However, the numbers are large enough to generate a reasonable range and they include variation for all the factors noted above.

Table 5. Range of sucrose, glucose and fructose concentrations in Australian sugarcane varieties expressed as % FW.

	n	Sucrose	Glucose	Fructose
All stalks	120	1.4 – 17.31	0.65 – 4.72	0.59 – 2.54
All Q208	45	3.80 – 17.31	0.65 – 2.32	0.59 – 2.25

2.3 Forage value

The range of forage values is shown in Table 6. Since NDF and ADF represent the residues of sequential extractions, ADF would be expected to be lower than NDF.As shown for the proximate, the measurements for Q208 encompass most or all of the range measured for all varieties.

Table 6. Range of NDF and ADF values in Australian sugarcane varieties.

	n	NDF (%DW)	ADF (%DW)
All stalks	158	36.8-51	23.3-36.7
All Q208	59	36.8-51	24.4-34.3

2.4 Antinutrients

The OECD Consensus Documents also identify potential anti-nutrients in foods. A possible antinutrient in sugarcane was identified by the OECD's Task Force for the Safety of Novel Foods and Feeds and was listed in the final Consensus Document for Sugarcane (OECD, 2011). Dhurrin, a cyanogenic glycoside, is present in the leaves of some varieties of sorghum and some reports in the literature have described dhurrin in sugarcane. Although the Consensus Document considers dhurrin toxicity to be a low risk, further data would be valuable to confirm this.

Dhurrin content is measured as the concentration of cyanide released from the plant material a spectrophotometric assay. Tops from mature plants of Q174 and Q208 grown in the Burdekin were submitted to SGS Ltd. for analysis of cyanide content. The results shown in Table 7 showed an extremely low content of cyanogenic compounds, close to the limit of detection for the assay (0.025 mg/g DW). For comparison, the concentration of cyanide generated from a sorghum leaf blade is 1.4 – 2.8 mg/g DW.

Table 7. Cyanide content of tops from two sugarcane varieties

Variety	Q174	Q208
HCN content (mg/gDW)	0.045	0.028

2.5 Composition of samples outside the current agronomic practice:

In addition to producing a range of compositions that reflect commercial varieties grown in normal agronomic practice, the study analysed some samples that are relevant to possible future cropping systems:

(i) Early-harvested commercial varieties. In the future, the harvesting season may be extended to make better use of mills or to harvest more frequently, for example in a biomass production system.

(ii) High-fibre varieties. If the value of ligno-cellulosic biomass increases in the future, for example in a biofuel production system, genotypes with higher fibre may become part of the harvested cane pool. The highest fibre tails of a population resulting from a backcross to *S.spontaneum* were analysed.

(iii) Tops. Currently, tops are used for forage and may become part of the harvested cane pool if whole-crop harvesting becomes more widespread.

Figure 3 shows the results from these sample types compared to the results shown above for all commercial varieties and for all Q208 samples. Each table shows the highest, lowest and mean value for each component of the proximate and forage analysis. Below each table, the results are shown as a graph where the line represents the range with the dot marking the mean. A number of conclusions can be drawn:

(i) The early harvested samples had a higher moisture content although the range overlapped with conventional varieties. The content of N-free extractives was correspondingly low, reflecting the low sugar content of the immature cane stalk.

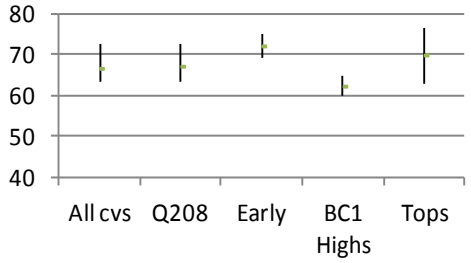
(ii) The high-fibre lines had a lower moisture content as expected, reflecting a high dry matter content. The content of crude fibre was relatively high and the content of N-free extractives was relatively low, although the ranges overlapped with conventional varieties.

(iii) The composition of tops was the most different from conventional varieties and the range of some components was wholly outside the range found in stalks of conventional varieties. The crude fibre content of the tops was lower than any of the conventional stalk samples and the content of N-free extractives was below the range found in stalks. The protein content was relatively high although the range overlapped with conventional varieties.

Figure 3. Comparison of proximate and forage values for a range of sample types

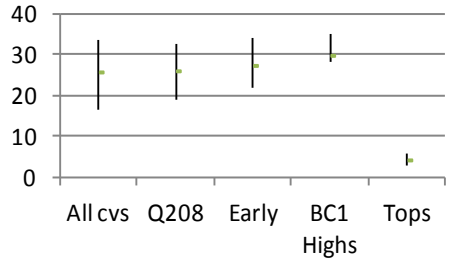
Moisture content

	All cvs	Q208	Early	BC1 Highs	Tops
High	72.6	72.6	74.9	64.8	76.6
Low	63.2	63.2	69.3	59.9	62.8
Mean	67.0	67.6	72.5	62.7	70.3



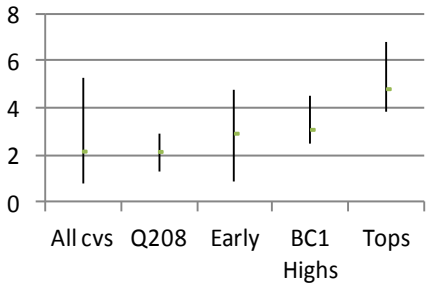
Crude fibre

	All cvs	Q208	Early	BC1 Highs	Tops
High	33.7	32.5	34.20	34.9	5.8
Low	16.6	18.8	22.10	28.2	2.9
Mean	26.1	26.4	27.8	30.2	4.6



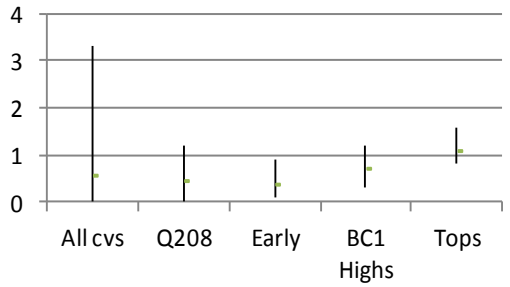
Protein

	All cvs	Q208	Early	BC1 Highs	Tops
High	5.3	2.9	4.8	4.5	6.8
Low	0.8	1.3	0.9	2.5	3.8
Mean	2.2	2.2	2.9	3.1	4.8



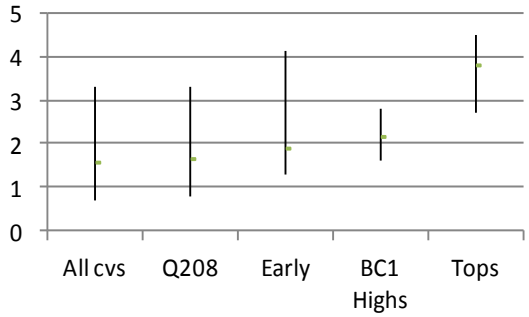
Fat

	All cvs	Q208	Early	BC1 Highs	Tops
High	5.3	2.9	4.8	4.5	6.8
Low	0.8	1.3	0.9	2.5	3.8
Mean	2.2	2.2	2.9	3.1	4.8



Ash

	All cvs	Q208	Early	BC1 Highs	Tops
High	3.3	3.3	4.1	2.8	4.5
Low	0.7	0.8	1.3	1.6	2.7
Mean	1.6	1.7	1.9	2.2	3.8



N-Free Extractives

	All cvs	Q208	Early	BC1 Highs	Tops
High	79.3	76.1	72.6	66.4	88.3
Low	62.9	63.3	59.6	58.4	83
Mean	69.5	69.3	67.0	63.8	85.7

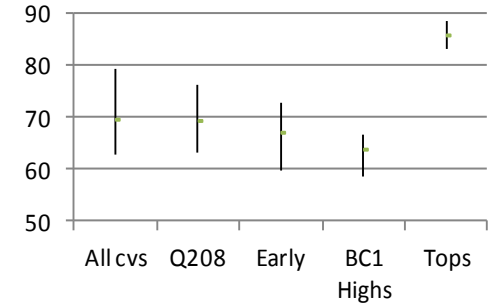


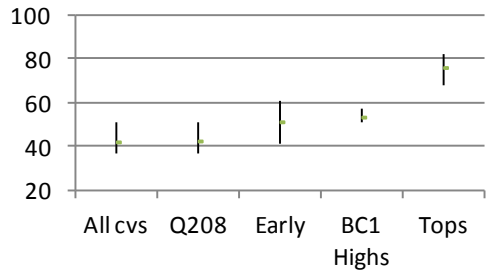
Figure 3 continued. Comparison of proximate and forage values for a range of sample types

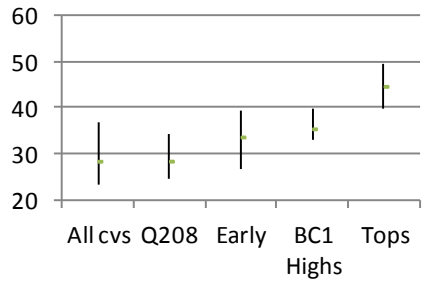
NDF

	All cvs	Q208	Early	BC1 Highs	Tops
High	51	51	60.50	57.1	82.1
Low	36.8	36.8	41.30	50.8	67.7
Mean	42.5	42.9	51.7	53.9	76.6

ADF

	All cvs	Q208	Early	BC1 Highs	Tops
High	36.7	34.3	39.4	39.6	49.4
Low	23.3	24.4	26.5	33.0	39.5
Mean	28.6	28.6	33.9	35.6	44.8





3. Sources of variation in composition

A number of potential sources of variation in composition were identified. The results of compositional analysis have been examined to test for the influence of these factors. Because the tables and graphs are quite large, the full set of data has been included as appendices at the end of this report and summaries are discussed here.

3.1 Variation due to genotype

To test the influence of genotype, four varieties (Q151, Q155, Q232, KQ228) grown at a single site in the Southern region in 2011 were sampled. In response to a recommendation from the consultative panel, the experiment was repeated by sampling four varieties grown at a second site (Burdekin) in 2012. The results shown in Appendix 1 and Appendix 2 showed that there was little variation due to genetic background when varieties were grown at a single site.

3.2 Variation due to geographical region

A single variety (Q208) was grown at four sites in different regions. The results shown in Appendix 3 represent combined samples from each region, regardless of crop type (i.e. plant, 1st ratoon or 2nd ratoon). Although the changes are small, a wider range of compositions was found when a single variety was grown in four sites, compared to the results from four varieties grown at single sites.

3.3 Variation due to crop type

A single variety (Q208) was harvested from plant and ratoon crops across four regions (Meringa, Burdekin, Southern and Harwood districts). Results shown in Appendix 4 are the composition of Q208 stalks from plant, 1st ratoon and 2nd ratoon crops, regardless of growth region (i.e. samples of each crop type from each region have been pooled). When the data were analysed according to crop type, the range of compositions found appeared to be lower than the range found when the same variety was grown in different sites. The same result was observed for crop types from a single site

or when crop types were grouped across sites (although the sample sets for these analyses were small).

3.4 Variation due to rate of nitrogen application

Six varieties (Q190, Q200, Q208, Q218, KQ228, Q232) were grown at a site in the Southern region with two rates of nitrogen application (200 kg N/ha and 20 kg N/ha). The results are shown in Appendix 5 as means for each variety under each nitrogen treatment. In this case the results from different varieties were not pooled as they appeared to respond differently to the treatments. In some varieties, relatively large changes in composition were observed, particularly in protein concentration. The highest recorded protein contents in the total compositional range were obtained from samples in this experiment.

3.5 Variation due to crop age

Five varieties (3 replicates each of Q200, Q208, Q241, MQ239 and KQ228) from 3 regions (Herbert, Burdekin and Central) were harvested early (April 2012) and later in the same season (November 2012). The results from this experiment have been discussed above in section 2.5.

3.6 Season-to-season variation.

Three varieties (Q183, Q200, Q208 and KQ228) were harvested from the same plots in the Burdekin in consecutive years (2011 and 2012). The results in Appendix 6 show that differences in the average composition were observed from year to year. For example, the 2012 average was higher in both moisture and N-free extractives and lower in crude fibre than the 2011 average. Season-to-season differences in water availability are likely to be responsible for this change.

3.7 Factors most likely to influence composition

A number of observations were made:

(i) When varieties grown together in a single site were compared, only small differences were found. This result was found early in the project with a set of varieties from a site in the Southern region and was confirmed with a set of varieties from a different location (Burdekin). The results suggest that genotype has only a small influence on variation in composition.

(ii) The varieties in this dataset span 18 years of variety selection, from the earliest seedling date of 1981 to the latest date of 1998. The observation that composition does not vary greatly amongst this set probably reflects consistent selection for composition in the breeding program and gives confidence that the results will be applicable for varieties developed into the future.

(iii) For a single variety (Q208), only small differences in composition were detected when comparing plant, 1st ratoon and 2nd ratoon crops. This suggests that, at least for this variety, crop type is not a major cause of variation in composition.

(iv) Growth region appeared to influence composition to a greater extent than genotype. When a single variety was grown in four different sites, a greater range of ash contents was detected, compared to the ash contents of a number of varieties at the same site.

(v) Nitrogen availability also influenced composition to a greater extent than genotype. When varieties were grown with two rates of fertiliser application, broader ranges of protein and total carbohydrate contents were detected.

(vi) Season-to-season variation affected the moisture content and therefore the content of soluble sugars and the proportion of fibre.

In summary, genotype and crop/ratoon cycle appear to have the least influence on composition, while growth region, soil composition and climatic conditions appear to have a larger influence. Although the compositional range determined by this study takes some of these factors into account, it is possible that different growing conditions might produce compositions which fall outside this range.

Intellectual Property and Confidentiality:

The results of this project are not confidential. The aim of the project was to generate information which will be made public so that it can be used as widely as possible. Thus the results have been discussed openly during the course of the project at a range of conferences and other meetings:

(i) A talk was presented at the ComBio2011 conference in Cairns in September 2011. Title: Sugarcane nutritional analysis to enable food safety assessment of GM cultivars. Authors: A.L. Rae, J.M. Perroux, S.R. Hermann, S. O'Neill and G.D. Bonnett.

(ii) A poster was presented at the ASSCT Conference in Cairns in May 2012. Title: Sugarcane compositional analysis to enable food safety assessment of GM cultivars: influence of variety and crop management. Authors: Anne Rae, Jai Perroux, Scott Hermann, Sophie O'Neill and Graham Bonnett. Reference: Proc. Aust. Soc. Sugar Cane Technol. Vol 34, p.66.

(iii) The project aims and results were presented by Anne Rae and Graham Bonnett at a series of SRDC Regional Expo presentations in Maryborough, Claredale, Ingham, Tully and Mossman between 9 - 15 May 2012. Updates were also presented at meetings of the Sugarcane Gene Technology Group.

One paper has been published (see below) and the intention is to publish the final results in an international journal.

Environmental and Social Impacts:

There are no environmental and social impacts from conducting this project. Samples have been obtained from existing trial sites. Although the information can be used for assessment of the products of genetically modified varieties in the future, the project did not involve the use of any modified varieties.

Expected Outcomes:

At the start of this project, the information available on the nutritional composition of sugarcane was very limited and was not representative of modern Australian varieties. This project has now delivered a comprehensive set of data describing the range of compositions found in Australian non-GM sugarcane varieties and growing conditions.

The outcome of this work is that the Australian regulatory authority, FSANZ, will recognise and accept the outputs of the project and will use these as a baseline against which to assess GM

varieties in the near future, thus enabling good decisions, based on sound science. The outputs will also be incorporated into updates of the OECD Consensus Document which will benefit the assessment of Australian sugar exports by other countries.

Since the outputs of the study will be publically available, a further economic outcome will be that that the data can be used by every GM application proponent will not have to repeat these analyses, thus saving time and resources. The major outcomes of the work will be economic benefits in speeding up the approval process for GM varieties.

Future Research Needs:

The objective of this study was to provide data which could be used by both technology developers and regulators in the assessment of products from GM sugarcane varieties. The study is complete for the current needs and we expect the data to remain relevant for many years. However, if there are changes to the regulatory system either in Australia or in export countries, further studies may needed to provide new data.

Confirmation of low dhurrin content

Although the preliminary analysis of sugarcane tops for cyanogenic compounds did not find significant levels of dhurrin, a larger study which could be published in an international journal would be useful for disseminating these results. Examination of the published data for sorghum suggests that the content of dhurrin is higher in young sorghum seedlings or in water-stressed plants. Samples of sugarcane seedlings and tops from water-stressed plants should be collected and analysed. Another useful approach would be to search the sugarcane genome and transcript sequences for homologues of the genes which are known to be involved in dhurrin synthesis in sorghum. The absence of these genes would confirm that sugarcane does not synthesise dhurrin.

Recommendations:

As described above, the results need to be published in an international journal to increase their utility and make the information widely available. A journal paper is currently being prepared and will be submitted to a journal during 2013.

Other alternatives for increasing public access to the information will also be sought. The ILSI Crop Composition Database is a comprehensive public database that provides information on the natural variability in composition of conventionally bred crops (Ridley et al, 2004; Alba et al. 2010). The aim is to make the data available to scientists from academia, government agencies, and industry, and to the general public. Currently, the database contains more than 113,000 data points representing 94 compositional components in corn, soybean and cotton. The intention is to expand the database with data for other crops and the database manager has expressed an interest in including the data from sugarcane. This possibility will be explored, as it could potentially increase the uptake of the results.

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Ridley, W.P., Shillito, R.D., Coats, I., Steiner, H-Y, Shawgo, M., Phillips, A., Dussold, P., Kurtyka, L, 2004. Development of the International Life Sciences Institute Crop Composition Database. Journal of Food Composition and Analysis 17, 423-438.

List of Publications:

Rae AL and Bonnett GD (2011). Sugarcane nutritional analysis to enable food safety assessment of GM cultivars – approaches to establishing a baseline. *Proceedings of the 33rd Annual Conference of the Australian Society for Sugar Cane Technologists*, Mackay, Qld., May 4-6

List of Appendices:

Appendix 1. Influence of genotype – region 1

Appendix 2. Influence of genotype – region 2

Appendix 3. Influence of geographical region

Appendix 4.Variation due to crop type

Appendix 5. Influence of N fertiliser on composition

Appendix 6. Season-to season variation

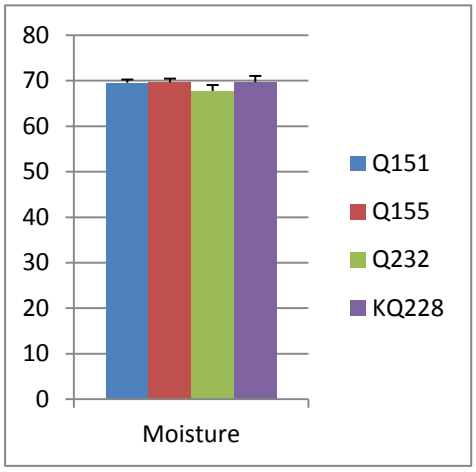
Appendix 7

Rae AL and Bonnett GD (2011). Sugarcane nutritional analysis to enable food safety assessment of GM cultivars – approaches to establishing a baseline. *Proceedings of the 33rd Annual Conference of the Australian Society for Sugar Cane Technologists*, Mackay, Qld., May 4-6

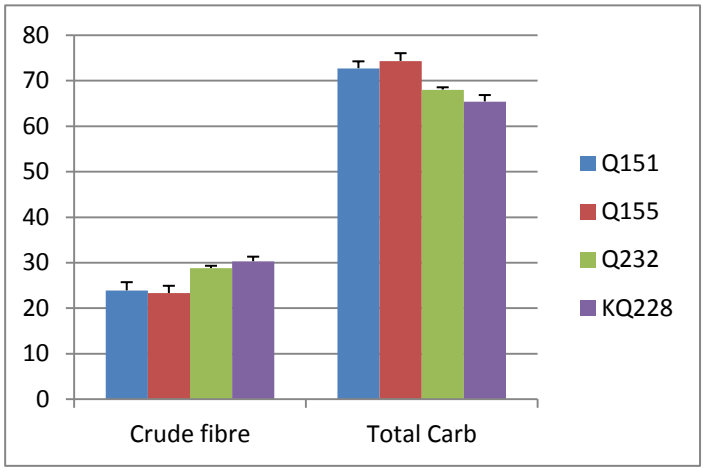
APPENDIX 1. Influence of genotype – region 1

Four varieties were grown at a single site in the Southern region in 2011. Results are shown as means ± standard deviation of the components for each variety.

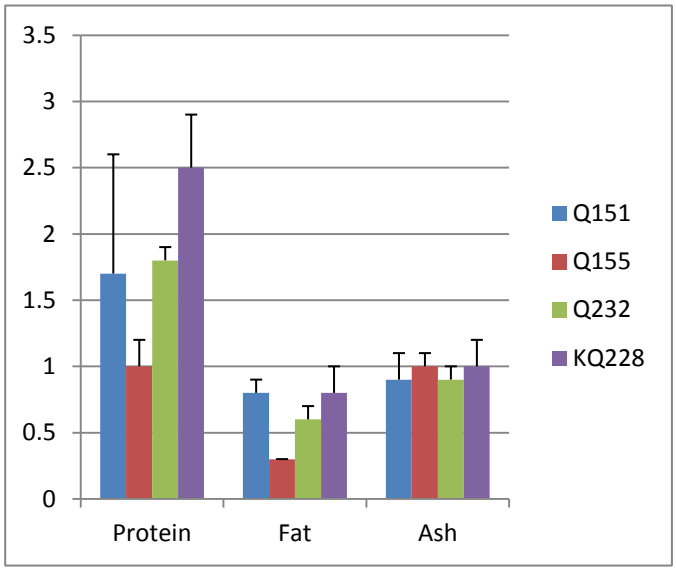
	Q151	Q155	Q232	KQ228
ADF	26.25 ± 0.57	27.95 ± 1.84	30.20 ± 0.80	27.93 ± 2.19
NDF	39.4 ± 0.85	40.55 ± 0.52	43.65 ± 1.05	40.73 ± 1.21
Moisture	69.53 ± 0.67	69.63 ± 0.79	67.68 ± 1.26	69.63 ± 1.36
Protein	1.68 ± 0.90	1.00 ± 0.18	1.80 ± 0.14	2.45 ± 0.37
Fat	0.80 ± 0.08	0.33 ± 0.05	0.58 ± 0.10	0.78 ± 0.15
Ash	0.85 ± 0.24	1.03 ± 0.05	0.88 ± 0.13	1.03 ± 0.21
Crude fibre	23.93 ± 1.77	23.33 ± 1.62	28.75 ± 0.52	30.30 ± 1.01
Total carbohydrates	72.73 ± 2.55	74.30 ± 1.71	68.00 ± 0.49	65.43 ± 1.38



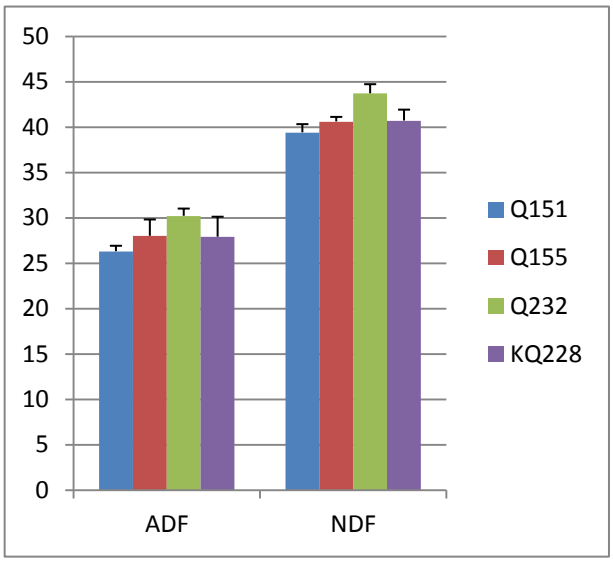
Moisture content as % fresh weight.



Major components of dry matter: crude fibre and N-free extractives (equivalent to total carbohydrates).



Minor components of dry matter: protein, fat and ash as % of dry weight.

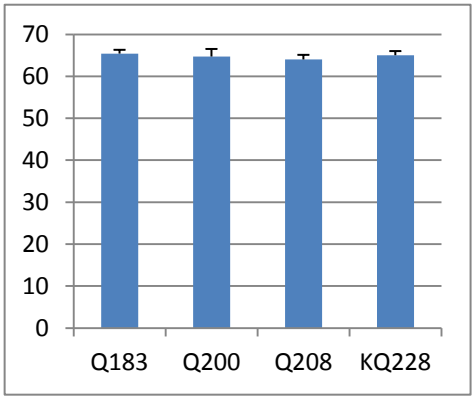


Forage components ADF and NDF.

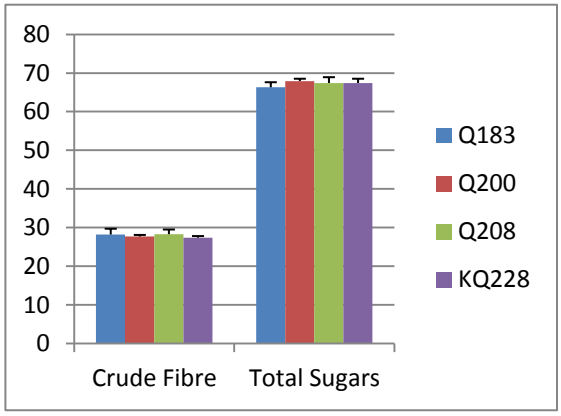
APPENDIX 2. Influence of genotype – region 2

Four varieties were grown at a single site in the Burdekin region in 2012. Results are shown as means ± standard deviation of the components for each variety.

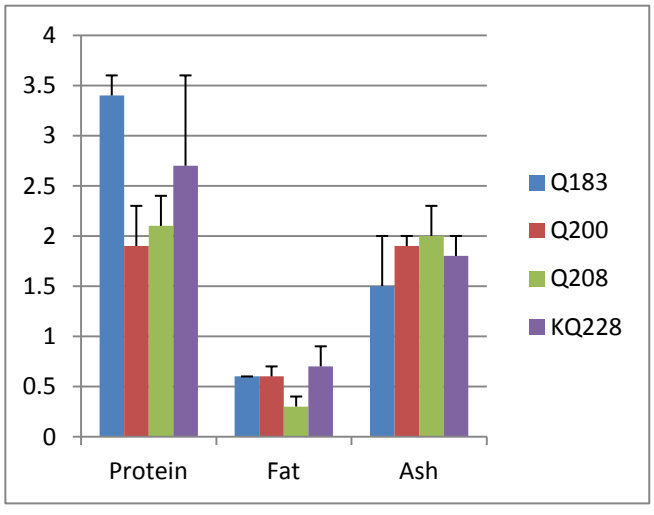
	Q183	Q200	Q208	KQ228
ADF	28.73 ± 1.31	28.87 ± 1.07	30.63 ± 0.4	28.97 ± 1.14
NDF	44.20 ± 1.80	42.27 ± 1.16	44.23 ± 2.00	43.83 1±.46
Moisture	65.40 ± 0.92	64.73 ± 1.81	63.97 ± 1.08	65.00 ± 0.98
Protein	3.37 ± 0.15	1.90 ± 0.36	2.07 ± 0.31	2.73 ± 0.90
Fat	0.69 ± 0.00	0.57 ± 0.06	0.33 ± 0.06	0.67 ± 0.15
Ash	1.47 ± 0.49	1.93 ± 0.12	1.97 ± 0.31	1.80 ± 0.17
Crude fibre	28.23 ± 1.53	27.73 ± 0.35	28.27 ± 1.17	27.37 ± 0.40
N-Free extractives	66.30 ± 1.25	67.87 ± 0.55	67.40 ± 1.54	67.37 ± 1.06



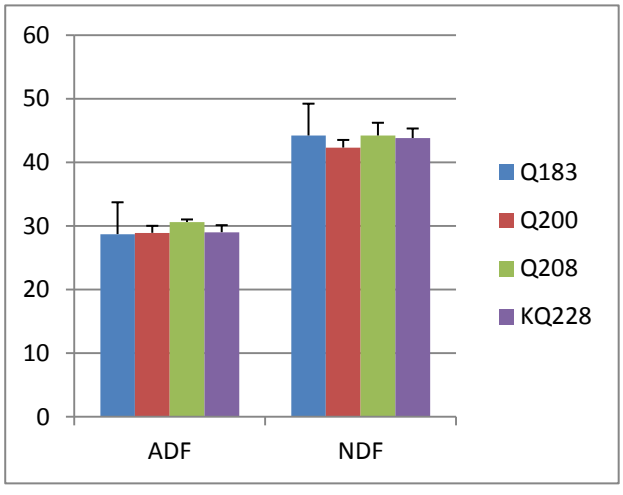
Moisture content as % fresh weight.



Major components of dry matter: crude fibre and N-free extractives (equivalent to total sugars).



Minor components of dry matter: protein, fat and ash as % of dry weight.

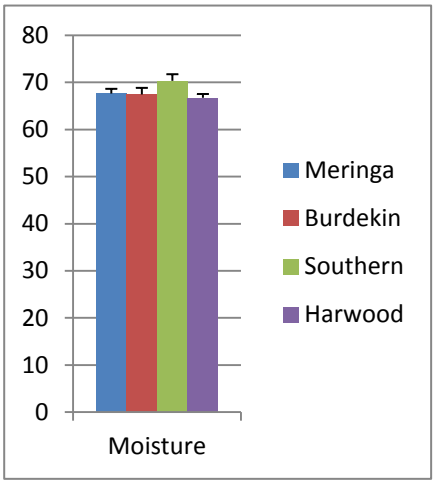


Forage components ADF and NDF.

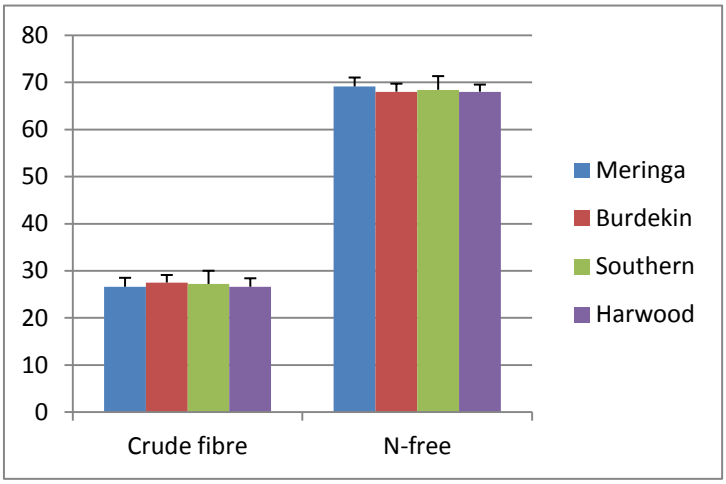
APPENDIX 3. Influence of geographical region

A single variety (Q208) was grown at four sites in different regions. Results represent combined samples from each region, regardless of crop type (i.e. plant, 1st ratoon or 2nd ratoon), presented as mean ± standard deviation.

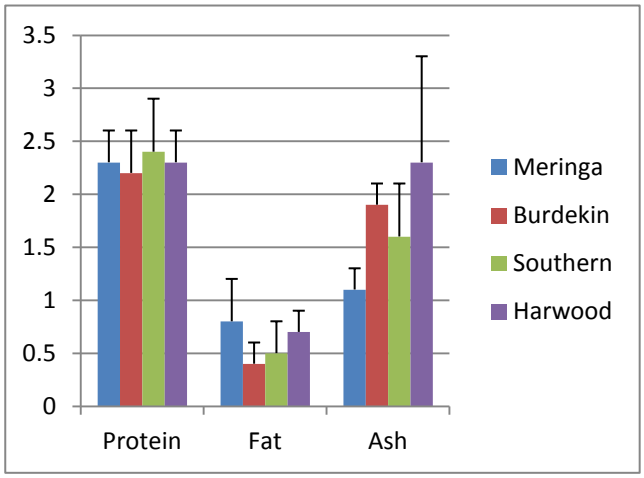
	Meringa	Burdekin	Southern	Harwood
ADF	28.7 ± 2.4	29.7 ± 1.3	27.2 ± 1.9	28.4 ± 2.2
NDF	40.4 ± 2.5	43.6 ± 2.6	44.1 ± 1.6	47.6 ± 3.1
Moisture	67.6 ± 1.0	67.4 ± 1.4	70.3 ± 1.4	66.7 ± 0.8
Protein	2.3 ± 0.3	2.2 ± 0.4	2.4 ± 0.5	2.4 ± 0.3
Fat	0.8 ± 0.4	0.4 ± 0.2	0.5 ± 0.3	0.7 ± 0.2
Ash	1.1 ± 0.2	1.9 ± 0.2	1.6 ± 0.5	2.3 ± 1.0
Crude fibre	26.6 ± 1.9	27.5 ± 1.6	27.2 ± 2.8	26.6 ± 1.8
Total carbohydrates	69.1 ± 1.9	68.0 ± 1.7	68.4 ± 2.9	68.0 ± 1.5



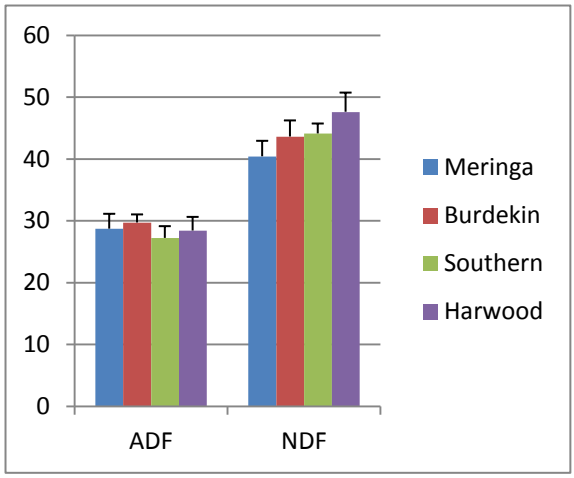
Moisture content as % fresh weight.



Major components of dry matter: crude fibre and N-free extractives.



Minor components of dry matter: protein, fat and ash as % of dry weight.

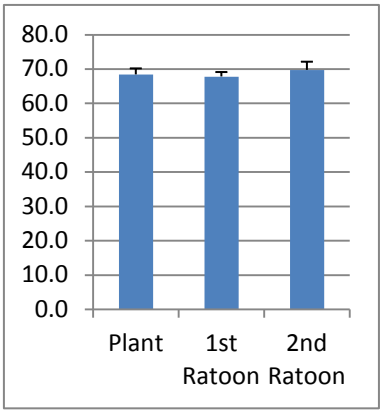


Forage components ADF and NDF.

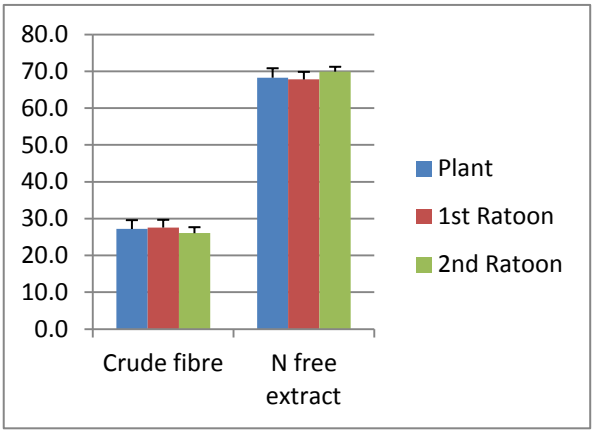
APPENDIX 4. Variation due to crop type

A single variety (Q208) was harvested from plant and ratoon crops across four regions (Meringa, Burdekin, Southern and Harwood districts). Results shown are the composition of Q208 from plant, 1st ratoon and 2nd ratoon crops, regardless of growth region (i.e. samples of each crop type from each region have been pooled). Results are shown as means ± standard deviation.

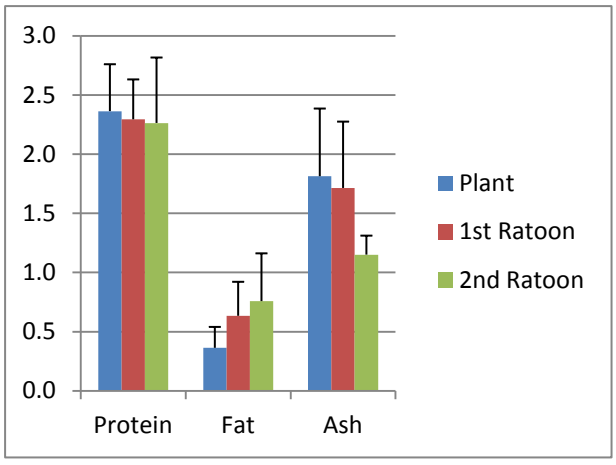
	Plant	1st Ratoon	2nd Ratoon
ADF	28.1 ± 2.0	29.3 ± 1.8	28.1 ± 2.2
NDF	42.4 ± 2.5	44.0 ± 3.0	42.6 ± 3.6
Moisture	68.4 ± 1.7	67.7 ± 1.3	69.7 ± 2.4
Protein	2.4 ± 0.4	2.3 ± 0.3	2.3 ± 0.6
Fat	0.4 ± 0.2	0.6 ± 0.3	0.8 ± 0.4
Ash	1.8 ± 0.6	1.7 ± 0.6	1.2 ± 0.2
Crude fibre	27.2 ± 2.4	27.6 ± 2.1	26.0 ± 1.6
N-Free extractives	68.3 ± 2.6	67.8 ± 2.1	69.9 ± 1.3



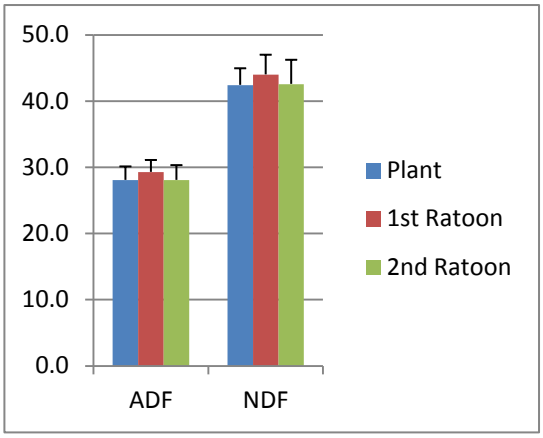
Moisture content as % fresh weight.



Major components of dry matter: crude fibre and N-free extractives.



Minor components of dry matter: protein, fat and ash as % of dry weight.



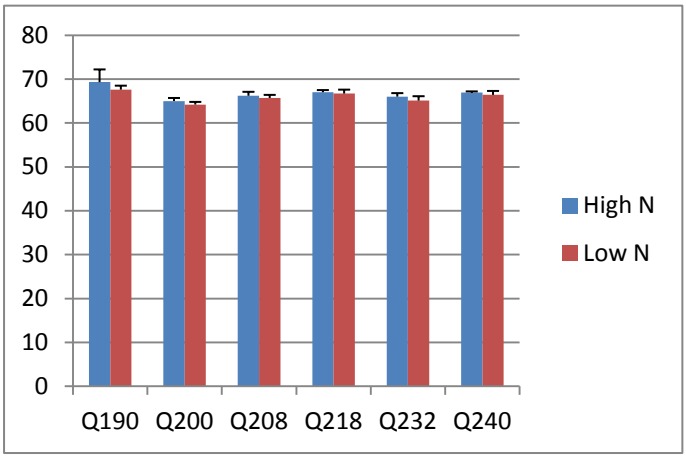
Forage components ADF and NDF.

APPENDIX 5. Influence of N fertiliser on composition

Six varieties were grown with two fertiliser rates. Results are means ± standard deviations derived from 3 replicate samples.

1. Moisture content as % fresh weight.

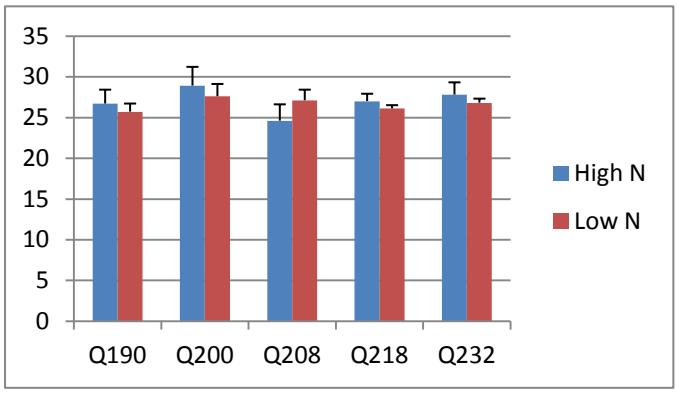
Variety	High N	Low N
Q190	69.3 ± 2.9	67.6 ± 0.9
Q200	65.0 ± 0.7	64.2 ± 0.6
Q208	66.2 ± 0.9	65.7 ± 0.7
Q218	67.0 ± 0.5	66.7 ± 0.9
Q232	66.0 ± 0.8	65.1 ± 1.0
Q240	66.9 ± 0.3	66.4 ± 0.9



2. Major components of dry matter: crude fibre and N-free extractives as % dry matter.

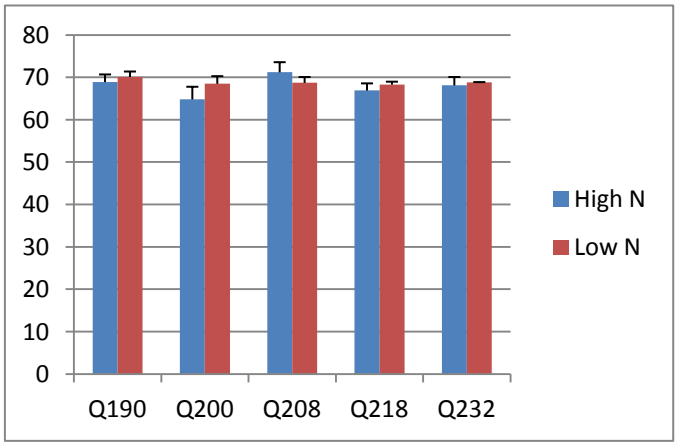
(i) Crude fibre

Variety	High N	Low N
Q190	26.7 ± 1.7	25.7 ± 1.0
Q200	28.9 ± 2.3	27.6 ± 1.5
Q208	24.6 ± 2.0	27.1 ± 1.3
Q218	27.0 ± 0.9	26.1 ± 0.4
Q232	27.8 ± 1.5	26.8 ± 0.5



(ii) N-free extractives

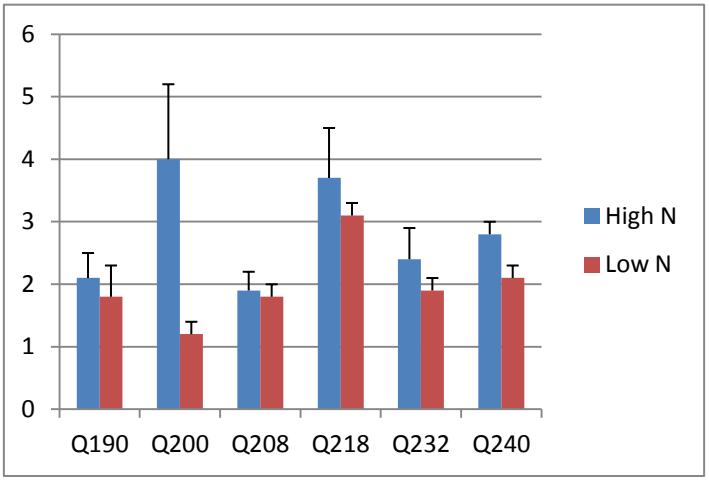
Variety	High N	Low N
Q190	68.9 ± 1.8	70.1 ± 1.3
Q200	64.8 ± 3.0	68.5 ± 1.8
Q208	71.2 ± 2.4	68.7 ± 0.7
Q218	66.9 ± 1.7	68.3 ± 0.7
Q232	68.1 ± 2.0	68.8 ± 0.1



3. Minor components of dry matter: protein, fat and ash as % dry matter.

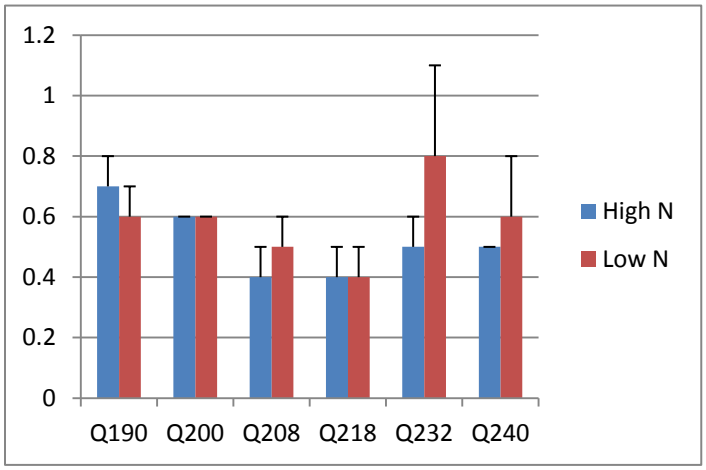
(i) Protein

Variety	High N	Low N
Q190	2.1 ± 0.4	1.8 ± 0.5
Q200	4.0 ± 1.2	1.2 ± 0.2
Q208	1.9 ± 0.3	1.8 ± 0.2
Q218	3.7 ± 0.8	3.1 ± 0.2
Q232	2.4 ± 0.5	1.9 ± 0.2
Q240	2.8 ± 0.2	2.1 ± 0.2



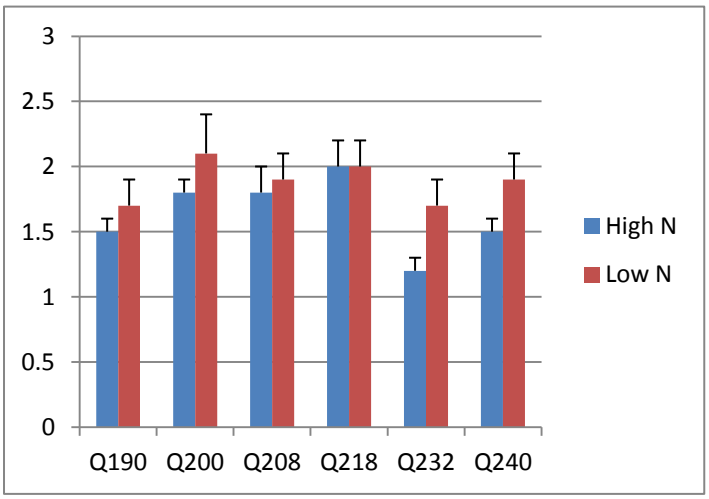
(ii) Fat

Variety	High N	Low N
Q190	0.7 ± 0.1	0,6 ± 0.1
Q200	0.6 ± 0.0	0.6 ± 0.0
Q208	0.4 ± 0.1	0.5 ± 0.1
Q218	0.4 ± 0.1	0.4 ± 0.1
Q232	0.5 ± 0.1	0.8 ± 0.3
Q240	0.5 ± 0.0	0.6 ± 0.2



(iii) Ash

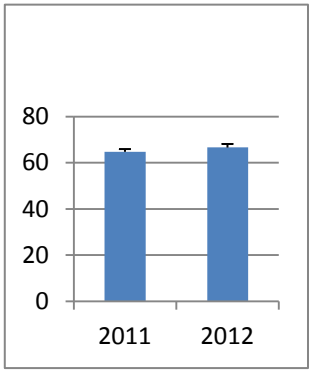
Variety	High N	Low N
Q190	1.5 ± 0.1	1.7 ± 0.2
Q200	1.8 ± 0.1	2.1 ± 0.3
Q208	1.8 ± 0.2	1.9 ± 0.2
Q218	2.0 ± 0.2	2.0 ± 0.2
Q232	1.2 ± 0.1	1.7 ± 0.2
Q240	1.5 ± 0.1	1.9 ± 0.2



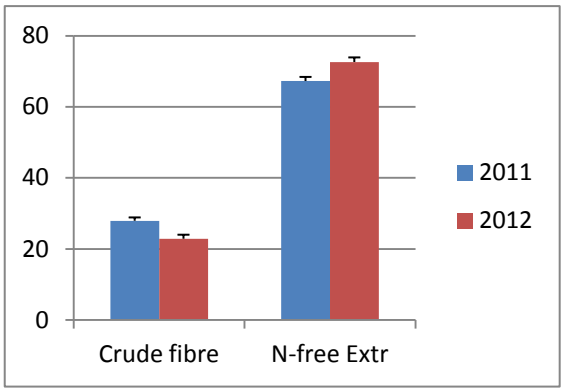
APPENDIX 6. Season-to season variation

Three varieties (Q183, Q200, Q208 and KQ228) were harvested from the same plots in the Burdekin in consecutive years (2011 and 2012). Results shown are the average composition in 2011 compared to the average in 2012. Results are shown as means ± standard deviation.

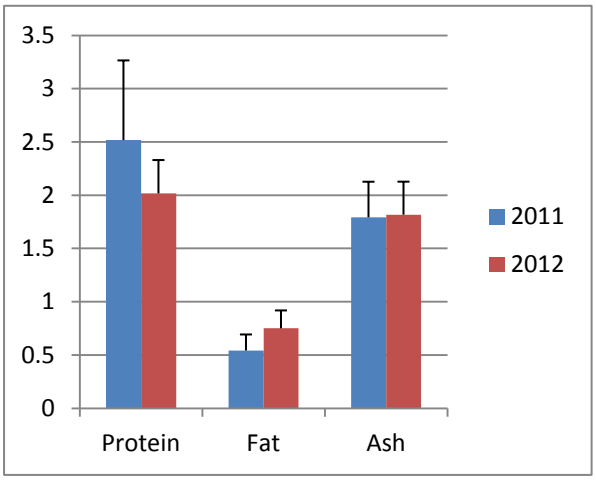
	2011	2012
ADF	29.3 ± 1.2	31.0 ± 1.4
NDF	43.6 ±1.6	43.0 ± 1.5
Moisture	64.8 ± 1.2	66.6 ± 1.6
Protein	2.5 ± 0.8	2.0 ± 0.3
Fat	0.5 ± 0.2	0.8 ± 0.2
Ash	1.8 ± 0.3	1.8 ± 0.3
Crude fibre	27.9 ± 0.9	22.8 ± 1.1
N-Free extractives	67.2 ± 1.2	72.6 ± 1.3



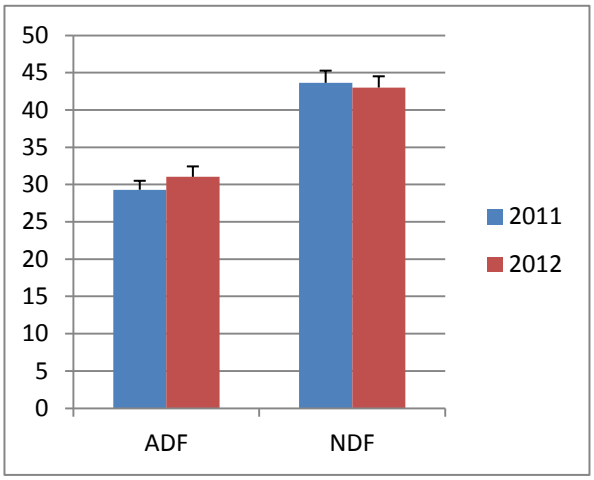
Moisture content as % fresh weight.



Major components of dry matter: crude fibre and N-free extractives.



Minor components of dry matter: protein, fat and ash as % of dry weight.



Forage components ADF and NDF.

APPENDIX 7.

Ag 8 SUGARCANE NUTRITIONAL ANALYSIS TO ENABLE FOOD SAFETY ASSESSMENT OF GM CULTIVARS – APPROACHES TO ESTABLISHING A BASELINE

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KEYWORDS: Genetic Modification, Regulation, Composition of Sugarcane

Abstract

Genetically modified (GM) sugarcane cultivars are under development. Before these cultivars can be grown commercially and the products derived from them used, they will need approval by regulatory authorities. The pathway for regulation of the food derived from genetically modified sugarcane is discussed, in particular the likely requirements for the compositional analysis of sugarcane. Some of the components required for analysis are not normally measured. However, using data from some of the components of the plant that are frequently measured (sucrose, ash, nitrogen, fibre) it is possible to determine how factors such as genetics, season, geography, management (e.g. nutrition) and time of year may affect them. From this, a strategy for sampling crops and experiments to determine the range of values, particularly for the components not routinely measured such as fats and protein, can be defined. This strategy will facilitate sampling and measurement to document the range of compositions that could be expected from existing production systems. A set of baseline measurements that can be used for comparing the composition of GM plants to existing cultivars during the assessment process will be the end result.

Introduction

There has been increased activity around the world in the development of GM sugarcane (Bonnett *et al.*, 2010). As the potential to commercialise GM sugarcane gets closer, increased emphasis is being placed on the regulatory processes that need to be fulfilled in order to get approval to grow GM cultivars and export the sugar and other products derived from them. In Australia, the regulatory functions for GM crops are spread across several agencies, depending upon the modification (Mitchell, 2011). Approvals for GM crops are granted for a specific genetic "event", defined as the insertion site of introduced DNA into the genome. The insertion site of the introduced DNA will be in different positions in the genome in each individual cell line that is regenerated into a GM plant. As most of the sugar from the Australian sugarcane crop is exported, approvals for growing GM sugarcane and approval for the sugar manufactured from it to be used as food will be required in export markets as well as in Australia. For example, GM sugar beet has been approved for commercial release in the USA, Canada and Japan but, in addition, at least one event has also been approved for use as food in 11 administrative territories (Table 1, James, 2009).

Table 1 – Countries where GM sugar beet has been approved for growing and/or use as food/feed (Data extracted from James, 2009)

Jurisdiction	Approval for growing		Approval for food/feed	
	Number of events	Year first event approved	Number of events	Year first event approved
Australia			2	2002
Canada	2	2001	2	2000
EU*			1	2007
Japan	1	2007	3	2001
Mexico			1	2006
New Zealand			2	2002

Philippines			2	2004
Russian Federation			2	2001
Singapore			1	2007
South Korea			1	2006
USA	3	1998	3	1998

* comprising 27 member states

The potential in the near future for the commercialisation of GM sugarcane has also stimulated regulatory agencies around the world to prepare for the evaluation of GM sugarcane. This is apparent from the formation of Organisation for Economic Co-operation and Development (OECD) working groups to prepare both "Consensus Document on the Biology of Sugarcane" and "Consensus Document on Compositional Considerations for New Varieties of Sugarcane: Key Nutritional and Feed Nutrients and Anti-nutrients" documents. These documents will support the assessment of environmental impacts and the safety of food/feed respectively. Australia is leading the development of these documents through the Office of the Gene Technology Regulator (OGTR) and Food Standards Australia and New Zealand (FSANZ), respectively.

Recent investigations in Australia have resulted in increased understanding of the sexual reproductive biology of sugarcane which will support the environmental assessment of GM sugarcane (Bonnett *et al.*, 2007, 2010). Whilst some activity in this area remains to be completed, we have started the research necessary to create baseline information to support the assessment of food uses of GM sugarcane. This paper describes the approach taken.

General concepts of food safety assessment

Food safety is defined as "providing assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use" (FAO/WHO, 1996). The assessment of food derived from biotechnology considers both intended changes caused by the modification and any unintended consequences, if they are present. For example, a GM plant variety that includes a gene conferring herbicide tolerance would be expected to produce the enzyme active in herbicide detoxification but would not be expected to show changes in any other traits. The accepted approach to satisfying this requirement is to demonstrate "substantial equivalence" of the modified variety to a similar but unmodified variety with a history of safe use for food production (OECD, 1993; FAO/WHO, 2000). The concept of "substantial equivalence" implies that, if a new food is found to be substantially equivalent to an existing conventional food, it can be concluded to be as safe as the conventional food. If unintended changes have occurred, it would then be necessary to demonstrate that no harmful consequences would result from the modification.

The primary case that there are no unintended consequences is constructed by a weight of evidence approach using three "pillars of regulation": (i) molecular characterisation of the genetic modification, (ii) phenotypic and morphological characterisation, (iii) compositional analysis.

Molecular characterisation

The intent of the molecular characterisation is to demonstrate that changes to the genetic code are limited only to the intended changes. The analysis required often includes the number and position of the changed elements, and the sequence context of the insertions. From this, the potential for creating new coding sequences that lead to the production of toxic or allergenic proteins can be assessed.

Phenotypic and morphological characterisation

Plant growth parameters can be used as indicators of developmental and physiological processes. The analysis would normally be performed on samples of the GM variety grown in the field together with conventional varieties as comparators. The measurements required comprise many of those that are normally made as agronomic performance is being assessed.

Compositional analysis

The nutritional components of the food derived from a GM cultivar should be substantially equivalent to its conventional counterpart, focussing on key nutrients, anti-nutrients and/or toxicants. To assist this comparison and to ensure consistency between countries, the OECD's Task Force for the Safety of Novel Foods and Feeds produces consensus documents on compositional characteristics of crops used as food and/or feed. The consensus documents include information on the use of plants for food and feed and the composition of food/feed products. The compositional analyses include macronutrients and micronutrients, as well as toxins and allergens specific to that crop species. Data ranges are normally included and these reflect the natural variability of the constituents in different plant varieties and in a range of geographical and climatic zones. Data need to be produced by reliable methods and ideally published in an international peer-reviewed journal. Consensus documents have been published for 17 species and more are in preparation including one for sugarcane. Those published are available at http://www.oecd.org/document/9/0,3343,en_2649_34391_1812041_1_1_1_1,00.html.

An example is provided by the document for maize (OECD, 2002a). In this document, the typical concentrations of a broad range of compounds were considered, together with the relevance of these compounds to the products of maize consumed by humans and animals. The primary nutritional components of a food substance, referred to as "proximate analysis" include protein, fat, carbohydrates, total dietary fibre and ash. In addition, the document considered other nutrients:

- minerals (Na, K, Ca, P, Mg, Fe, Cu, Se and Zn)
- vitamins (Vit. A, Vit. B1 (thiamin), Vit. B2 (riboflavin), Vit. B6 (pyridoxine), Vit. C (ascorbic acid), Vit. E, folate and niacin)
- individual amino acids
- fatty acids (palmitic, stearic, oleic, linoleic and linolenic).

The anti-nutrients considered were phytic acid (myo-inositol hexa-phosphate), which reduces the bioavailability of phosphorus, DIMBOA (2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) which is a natural pesticide and raffinose, a non-digestible oligosaccharide which can cause abdominal discomfort. Although they are neither nutrients nor anti-nutrients, the secondary metabolites, furfural, ferulic acid and *p*-coumaric acid were also considered because they are characteristic metabolites in maize and may act as useful comparators for determining equivalence.

The consensus document makes recommendations on the components to be analysed for assessment of a food product that originates from a GM maize cultivar. For assessment of whole kernel, comparison of proximates, minerals, vitamins, amino acids, fatty acids, phytic acid, raffinose, furfural, ferulic acid and *p*-coumaric acid is recommended. For assessment of specific products derived from maize kernels, the document recommends analysis of a limited range of components. For example, analysis of fatty acids is considered to be the key factor for assessment of maize oil, while analysis of proximates is suggested as appropriate for assessment of maize starch.

Similar analyses are suggested for crops such as rice, barley, sorghum and wheat, where whole grain is consumed. For crops such as sugar beet, where the products are refined sugar or molasses, and whole plant tissue is less likely to be consumed by humans, the OECD document recognises that the products are unlikely to contain high levels of modified proteins or DNA. For

sugar beet, the document recommends that a modified variety be considered substantially equivalent if the composition of the root falls within the published range of concentrations of the key nutrients: ash (5.0-8.1% of dry matter (DM)), crude protein 4.7-6.8% DM), crude fibre (4.9-6.3% DM), sucrose (64.7-70.0% DM) and phosphorus (1.4-2.2% DM) (OECD, 2002b).

Regulation by Food Standards Australia and New Zealand (FSANZ)

In Australia, food safety regulation by FSANZ aligns with the OECD recommendations using substantial equivalence as a guide. However, the regulatory system has specific requirements for consideration of highly refined products from GM plants, such as oil or sugar. For these products, the assessment focuses on the part of the plant that the food is derived from. In the case of sugarcane, FSANZ considers that analysis of the refined sugar is not sufficient for two reasons:
(i) The composition of refined sugar is close to 100% sucrose and any variation in the impurities is more likely to reflect differences in the refining process rather than differences derived from the modified plant tissue.
(ii) When food safety approval is granted, the approval covers all products and potential products from the modified plant. Although unprocessed sugarcane is not widespread as a food source in Australia, there are niche markets for juice, and juice and pulp are routinely consumed in other countries. Therefore, food safety assessment needs to consider the potential use of other parts of the sugarcane plant.

For these reasons, the assessment is likely to be performed on the sugarcane stalk, as this is more likely to reflect the composition of the whole plant. In a food safety assessment, FSANZ uses information on the composition of conventional varieties from a variety of sources including the OECD consensus document, data submitted by the proponents and published information. The key constituents that are likely to be included in the assessment of sugarcane are shown in Table 2.

Table 2 – Components, food and feed that are likely to be required for compositional analysis of a GM sugarcane stalk.

Analysis	Components
Food	Moisture, crude fibre (% insoluble dry matter), crude protein, fat (as ether extract), ash, sucrose, total sugars*
Feed^{+}	Neutral detergent fibre (NDF) and acid detergent fibre (ADF)

*free monosaccharides and disaccharides. For sugarcane, these primarily consist of sucrose plus lesser amounts of reducing sugars (glucose and fructose). $^{+}$ Additional measurements for feed.

How should a baseline be defined for substantial equivalence?

A suitable reference is required for substantial equivalence comparisons. If the baseline constructed is too narrow, assessed samples may frequently fall out of the range, for reasons not associated with the genetic change, consequently raising potential safety concerns that would require further investigation. Baker *et al.* (2006) compared the amino acid composition of wheat that had been transformed to alter seed protein composition and noted that, while there were differences found through direct comparison to the untransformed lines in some sites, it was less than the range of the controls across the sites tested. Similarly, Moller and Bak (2005) have highlighted that any comparisons made using substantial equivalence need to place the results in the context of the other sources of variation affecting the components under study. Consequently, we propose to construct a range for the major compositional components that would capture the major sources of variation likely to be encountered.

It is impractical to construct the baseline by analysing the composition of all commercial sugarcane varieties under all possible growing conditions. Therefore we propose to select samples that will give the best possible estimate of the normal ranges encountered in the Australian environment. To guide the selection of these samples, we have examined the existing records of

sugarcane composition to determine the factors that have most influence on the composition, such as plant variety, time of harvest, growing region and management practices.

Some of the proposed components (e.g. crude protein and fat) are rarely measured in experiments designed to evaluate the performance of potential cultivars, or the effects of agronomic treatments on yield and sugar content. Fortunately, many of the other components are routinely measured. Analysis of the likely ranges of these components and the sources of variation that give rise to the largest differences can be used to inform a sampling strategy that encompasses the range of these constituents likely to be found across a range of sugarcane growing environments and practices.

Sources of variation

The three major components of sugarcane stalks are moisture, sucrose and fibre. The remaining components are often referred to in an industry context as impurities and they mostly remain in molasses along with the sucrose that cannot be extracted.

There are many factors that affect the sucrose content of sugarcane stalks. CCS, the industry estimate of sucrose, can vary with season (e.g. the seasonal average for Mulgrave from 1903-1996 varied from about 11.8 – 15.7; Pope, 1997); mill region (the 1994 seasonal average of all individual mills across Queensland was 11.99-15.77; BSES, 1995); and time of the year (8-17 measured on whole stalks from May to September; Cox *et al.*, 1998). Measurements of CCS made at the mill are not made on just stalk material and so underestimate stalk CCS. While CCS will also vary with many other factors, these are all probably captured within the ranges quoted above. The data from Cox *et al*. (1998) include samples taken outside the normal harvested season in May. However, the range of 8-17 probably encompasses the range seen at most Australian sugar mills. Cane samples with CCS values below 7 would not be accepted by mills because they indicate such low sucrose content that it becomes uneconomical to process the cane and values above 17, though desirable, would be rarely seen (Inman-Bamber *et al.*, 2011).

Increased sucrose content is normally at the expense of reducing sugars and moisture (Bonnett *et al.*, 2006). Moisture in sugarcane stalks will also be highly variable. Berding and Marston (2010) showed in measurements of whole stalks that moisture content from 40 trials in 2009 in the northern region, varied between 61 and 72%. In the same samples, fibre varied between 9 and 18%. In a range of cultivars across the Mackay district sampled in September 1988, fibre ranged from 10.5 to 13.9% (Ivin and Doyle, 1989), well within the range generated by Berding and Marston (2010).

Therefore, sampling stalks early and later in the season is likely to cover the range of sugar contents, moisture and fibre contents found in cane presented to mills. However, the real value of this sampling strategy will be an attempt to cover the range of other, less well studied components. Below, we discuss what is known about the variation and its causes in these lesser-studied components.

***Protein*:** The protein content of food products is frequently estimated from the total nitrogen content which is measured by the Kjeldahl assay or a similar method. The average nitrogen content of proteins is assumed to be approximately 16%, resulting in a conversion factor of [N] x 6.25 (FAO, 2003). Some indication of the likely variation in protein content of the sugarcane stalk can be derived from studies of nitrogen content.

Keating *et al.* (1999) studied variation in the content and chemical form of nitrogen in cane samples that were supplied with different amounts of N fertiliser from eight trial sites between Grafton and Ingham. The aim of the work was to design production systems that optimise profitability by minimising input costs and by changing the composition of the sugarcane stalk towards more profitable components. The total N content of whole cane stalks for the trials sampled was 0.1-0.6% on a dry matter basis (Table 3), equivalent to 0.63-3.75% protein using the above conversion factor.

The study used the amino acid N content of juice (amino N) to dissect the influence of variables such as fertiliser treatment, crop age and water stress. Although the proportion of total stalk N found in juice can be as low as 30%, in most cases 50-70% of the total stalk N was recovered in the juice, suggesting that juice N was a reasonable indicator of stalk N content. The amino N ranged from 100-900 μg N/mL juice (Table 3), with the higher contents found in trials receiving high fertiliser inputs or trials where cane yield was limited by environmental factors such as water stress. The crop cycle was a significant influence on amino N in juice, as cane from plant crops had amino N levels that were 40-50% higher than cane from ratoon crops. Within the range of N contents found, no clear influence of variety was found although some regional effects were apparent. Overall, the results suggest that environmental and management factors will be the greatest source of variation in protein content of the sugarcane stalk.

***Ash*:** Nitrogen also affects the level of ash, measured as conductivity of juice. For example, the addition of 280 kg N/ ha reduced the conductivity/impurity fraction by 20% compared to a control treatment (Jackson *et al.,* 2008). In the same study, conductivity/impurity was increased by about 30% when fertiliser potassium was increased from 0 to 260 kg K/ha (Table 3).

Table 3 – Some effects of different sources of variation on the ash and nitrogen content of sugarcane stalks and juice

Source of variation	Component	
	Nitrogen (as total N or amino N)	Ash
Nitrogen fertiliser	100-900 μg amino N/mL juice[1]	Decrease by 20%[2]
Potassium fertiliser		Increase of 30%[2]
Region/Variety	0.1-0.6% total N in dry stalk[1]	0.55-0.72% of first expressed juice[3]
Crop cycle	40-50% higher amino N in juice in plant crop	

[1]Keating *et al.* (1999); [2]Jackson *et al.* (2008); [3] Kingston and Kirby (1979);

Different geographic regions of the Australian sugarcane industry are spread along a large range of latitudes, consequently experiencing considerable variation in rainfall, temperature and other climatic factors. In addition, soil types vary both within and between regions. A good example of how this affects sugarcane composition is demonstrated by the levels of ash. This can vary within and between regions based on several factors such as water quality. Kingston and Kirby (1979) found that in cane delivered to the Qunaba mill east of Bundaberg, ash levels in first expressed juice varied between 0.55 and 0.72% when samples were grouped on the basis of water quality used for irrigation (Table 3). This variation would be expected to be reflected in the ash content of whole stems.

***Fat*:** There are very few measures of fat (ether extract) of sugarcane stalks. One study reported 0.81% fat in dry matter of mature cane (16 months old) and 1.10% in immature cane (Banda and Valdez, 1976). The paucity of studies means that there are no clues as to how environmental or management conditions may affect the fat composition of sugarcane.

***Total sugars*:** The measurement of total sugars is highly correlated with Brix, which is often measured. The study of Berding and Marston (2010) showed a range of Brix between 18 and 27%. It is anticipated that sampling to encompass the range of sucrose content will also mean we will capture a large range of total sugars.

***Neutral detergent fibre (NDF) and acid detergent fibre (ADF)*:** In some countries, such as Brazil, sugarcane is an important source of feed for ruminant animals, especially during the dry season. When biomass for feed is analysed by sequential chemical extraction, the fraction remaining after neutral detergent extraction (NDF) comprises most of the fibre (lignin, hemicellulose and cellulose but not pectin), while further extraction with acid detergent results in a

fraction (ADF) comprising only the cellulose and lignin components of the fibre. NDF is used as a measure for bulk forage volume, while ADF is related to the energy value of the forage, since digestibility decreases as ADF increases. The nutritional value of different sugarcane varieties as feed has been compared and several studies have been published in Brazilian scientific journals. Although it is not always possible to isolate stalk composition from these studies, they can give some indication on the likely sources of variation in composition in whole plants. In a study by de Souza Franca *et al.* (2005), ten varieties that were grown on the same site showed significant variation in content of crude protein (although the total amount was low) and in NDF content. Similar studies by Azevedo *et al.* (2003a) and Rodrigues (1997) found significant differences in NDF. The influence of environment and management practices on nutritional composition has been less studied. Azevedo *et al.* (2003b) and Fernandes *et al.* (2003) showed that the time of harvest of sugarcane affected the content of crude protein and NDF. Although we were not able to find similar work in sugarcane, the effect of irrigation on the nutritional value of two forage grasses, *Panicum maximum* and *Pennisetum purpureum* has been analysed (Palieraqui *et al.,* 2006). The results showed that irrigation did not affect the content of crude protein, ash, NDF or ADF. The lack of information available for sugarcane suggests that a study to investigate the effects of environment on nutritional content would be worthwhile.

Strategy for sampling to capture sources of variation

Some interesting insights into the major sources of variation in composition have been gained by examining the records of frequently analysed components. Although genetic background would seem, intuitively, to be a major factor, the results show that environmental conditions and management practices are actually more important. For the commonly analysed components of CCS, water and fibre, the time of harvest had the greatest influence. Although fewer records were available for the smaller components, protein and ash, the major influences appeared to be the quality of soil and water, including fertiliser applications.

In the light of these results, samples from a small number of genotypes grown across a wide variety of regions will be collected. Sampling at the start and middle of the harvesting season will probably capture a large range of compositions for total sugars, sucrose, moisture and fibre. However, for some components such as protein and ash, a more targeted sampling of crops that have had high levels of N applied and treatments that increase ash content may be required to capture the range. When preliminary analyses are complete, these can be used to see what further sampling, if any, would be required to extend the analyses of fat, NDF and ADF values to capture the likely range in sugarcane stalks grown across the Australian industry.

Conclusions

A significant component of the assessment of the food safety of proposed GM sugarcane releases will be the compositional analysis of sugarcane stalks. The number of components to be analysed is likely to be much less than for grains and other less refined food products. It is important that a baseline for comparison encompasses the likely range of compositions encountered in Australian growing conditions. Examining records of sugarcane composition suggests that environmentally induced variations are generally greater than variation among commercial cultivars. A two-stage strategy for sampling is proposed to capture this variation: (i) initially sampling a small number of varieties from different regions at the start and middle of the harvest season, and (ii) targeting sampling of crops in particular environments to supplement the range if necessary. The outcome of this work should be a set of data that will be useful in testing the substantial equivalence of GM and conventional varieties.

Acknowledgements

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