

**The potential of condensed molasses solubles (CMS) to replace
molasses in feedlot diets**

By

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Declaration

I, Gabriel Andries Pienaar, declare that this dissertation, which I hereby submit for the degree MSc (Agric) Animal Science: Animal Nutrition at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution

G.A Pienaar

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Summary

The potential of condensed molasses solubles (CMS) to replace molasses in feedlot diets

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Condensed molasses solubles (CMS) is the syrupy residue left after fermentation of molasses for ethanol production. Although it visually looks similar to molasses, it differs in nutrient composition. Condensed molasses solubles has less energy, a higher ash content especially in sulphur (S) and potassium, has a higher protein and lower dry matter content than molasses. When CMS was used as a replacement for molasses at an inclusion rate of above 5%-10% to ruminant diets, voluntary feed intake and weight gain decreased while feed conversion ratios increased. It was suggested that a possible reason for the poor performance of animals on CMS containing diets is the high level of S in CMS that could reduce (Se) and copper (Cu) absorption, or could lead to the excess production of H₂S in the rumen. Another possibility was the lower energy value of CMS leading to inaccurate feed formulations. The goal of this paper was to investigate possible reasons why ruminants consuming diets

containing CMS, perform poorly in comparison with ruminants on diets containing molasses. Two trials were conducted to identify reason for lower performance of CMS diets. Criteria associated with production performance of CMS diets were measured on an *in vitro* and *in vivo* basis while diets were balanced in protein, energy and moisture.

An *in vitro* trial was conducted to determine the fermentability of diets and gas composition of typical feedlot diets containing different levels of CMS. Four dietary treatments containing 0%, 5%, 10% or 15% CMS were formulated to be iso-nitrogenous and iso-energetic as well as to have similar dry matter values. Diets with different CMS inclusions were used to measure total gas production, using an automatic gas production meter that regulates gas pressure, temperature and simulates rumen motility. Gas production was measured automatically every five minutes over 42 hours. During this period gas production did not differ ($P > 0.05$) at any point of fermentation, and total gas production ranged from 138 mL to 155 mL. The composition of the gas produced supported an effective degradation and indicated no significant difference between treatments. Results indicated that there was no significant difference in hydrogen sulphide gas (H_2S), methane or carbon dioxide concentrations in the gas. The similar H_2S gas production between treatments indicates that, although S was higher in CMS containing diets, H_2S production by sulphur-reducing bacteria was low when pH was regulated above a pH level of 6. Analysis of the four treatment diets indicated that the TMR's contain the same ash content, especially that of S, as in the feed formulation. The variation in composition highlighted the need to establish consistent nutrient parameters for CMS composition in order to formulate diets accurately. From the *in vitro* trial it was concluded that there was no difference in gas production, and therefore fermentability, between diets containing CMS or molasses.

An *in vivo* trial was conducted to determine the effect of CMS inclusion at 0%, 5%, 10% or 15% in feedlot diets of newly weaned bull calves from a mixture of beef breeds. The diets were formulated to be iso-energetic, iso-nitrogenous with a constant dry matter content. One hundred weaners were allocated to the four treatments, and each treatment consisted of five replicates with five bulls per replicate. The weaners were fed for a period of 112 days during which feed intake was measured every week and body weight every second week. Biweekly body weight and feed intake were measured to calculate feed conversion ratio (FCR).

There were no significant differences in the body weight, feed intake or FCR of the treatments at any point during the feeding period. The results indicated a high FCR compared to standard feedlot diets, suggesting that the experimental diets contained lower energy

content than standard feedlot diets. The results indicated that when diets containing CMS were corrected and balanced for energy and protein, CMS have the potential to replace molasses in feedlot total mixed rations. However, high moisture content diluted nutrients, causing a higher FCR, thus reduced the efficiency of nutrient utilization in the diet.

Liver samples of all 100 bulls were collected to determine the effect of S of diets containing CMS on the hepatic Se and Cu concentrations in the cattle. The analysis of trial diets indicated that dietary S did not increase as expected, and therefore results were expected that there was no significant difference in liver Se and Cu concentrations between the treatments, and both Se and Cu concentrations indicated adequate intake of these elements.

The *in vivo* and *in vitro* trials indicated that CMS has potential to replace molasses, but more research is necessary, especially in composition variation and to decrease FCR in more practical feedlot conditions.



Abbreviations

1. ADG: Average daily gain
2. Ca: Calcium
3. CBS: Condensed beet solubles
4. CH₄: Methane
5. Cl: Chloride
6. CMS: Condensed molasses solubles
7. CO₂: Carbon dioxide
8. COCM: Cotton oilcake meal
9. COD: Chemical oxygen demand
10. CP: Crude protein
11. CSL: Corn steep liquor
12. DBG: Dried brewers grain
13. DM: Dry matter
14. dm³: Cubic decimeter
15. EE: Ether extract
16. FACW: Fermented ammoniated condensed whey
17. GHG: Greenhouse gasses
18. H₂S: Hydrogen sulphide gas
19. HS⁻: Hydrogen sulphite
20. K: Potassium
21. L: Litre
22. m: Mol
23. MCP: Mono calcium phosphate
24. mL: Millilitre
25. N: Nitrogen
26. n: Sample size
27. Na: Sodium
28. NaHCO₃: Sodium bicarbonate
29. NFE: Nitrogen free extract
30. NPN: Non-protein nitrogen
31. PEM: Polioencephalomalacia
32. Pr: Pressure



- 33. R: gas production continent
- 34. S: Sulphur
- 35. s^0 : Elemental sulphur
- 36. SD: Standard deviation
- 37. SEM: Standard error of the mean
- 38. SRB: Sulphur-reducing bacteria
- 39. T: Temperature
- 40. TMR: Total mixed ration
- 41. TP: True protein
- 42. V: Volume
- 43. VC: Variation coefficient
- 44. VFA: Volatile fatty acid



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Chapter 1

1 Introduction

In the past decades, energy resources have been dominated by the use of fossil fuels. The consumption of these energy sources, such as petroleum products, has gradually increased as countries developed and population sizes grew. The world crude oil usage has increased from 76 799 barrels of crude oil per day in the year 2000 to 87 356 barrels of crude oil per day in 2011 (EIA 2013). The rise in demand is coupled with the depletion of these non-renewable energy sources, the release of greenhouse gasses into the atmosphere and the escalation in its price. As a result, the search for new sources of energy has been amplified in order to find energy sources that will drive our transportation industry while being renewable, cheaper and more environmentally friendly.

Over the years, the production and use of biofuels have increasingly become a popular alternative for fossil fuels among scientists, companies and the general public. One of the more popular biofuels is bio-ethanol. Bio-ethanol is an alcohol that is produced by the fermentation of plant material (maize, sorghum, sugarcane and sugar beets) or residues of plant-based products (molasses).

In the United States of America the majority of ethanol is produced from maize grain, whereas countries such as Brazil (or other third-world countries) use sugarcane, sugar beet or related by-products of related industries to produce ethanol. The energy and CO₂ balances are positive for using both maize and sugarcane, but sugarcane has a bigger margin in emission reduction and a more positive energy balance. Considering that maize grain is also a main food constituent for animals and humans, of bio-ethanol synthesis from sugarcane it is suggested that the future of bio-ethanol production will shift from maize to sugar plant material (Oliveira *et al.*, 2005).

Another major criterium in favour of these new resources is that it reduces the amount of greenhouse gases (GHG) per liter fuel used. Bio-ethanol is considered a more eco-friendly solution since less CO₂ is released during its combustion in mechanical systems and, since plant material used to produce bio-ethanol can convert CO₂ into biomass, it is a renewable and a

sustainable energy source. The general practice of using bio-ethanol is to mix it with existing petroleum products to produce a fuel source at reduced cost and reduced emissions. The success of bio-ethanol to reduce emissions has been widely debated and conclusions of studies differ. Mixing bio-ethanol with petroleum reduces the CO₂ emission by up to 30% (Lloyd. 2005). These results are supported by Rozakis *et al.* (2013) and Lisboa *et al.* (2011), although they suggested that the efficiency of the agricultural production systems needs to be included in calculations to determine the total effect that bio-ethanol has on GHG emissions.

When considering the success of bio-ethanol use, direct bio-ethanol emissions needs to be considered in combination with emissions spent in the process of ethanol production, effect on the agricultural industry and other economic factors. Rozakis *et al.* (2013) pointed out that the effect that what plant material is used, such as maize grain, for bio-ethanol production has on other agricultural industries as well as the process of ethanol fermentation can also influence total emissions and how environmental friendly bio-ethanol truly is. A review by Oliveira *et al.* (2005) supported the concept that in order for bio-ethanol to be a viable replacement for petroleum products, the effect of bio-ethanol synthesis has on the society, the agricultural industries and the environment needs to be improved. According to their studies the potential for bio-ethanol production has not been met and further studies need to be done to increase the efficiency of the bio-ethanol industry without interfering with other industries. Therefore it is important to maximise the efficiency of all the processes involved.

A key factor that can improve the feasibility of such an enterprise is to use or sell as many as possible of the waste products from the fermentation process. Table 1.1 and Figure 1 1 illustrates the trend in ethanol production indicating an increase in ethanol production in the past seven years. The increase in ethanol production is promoted by an increase in ethanol demand as more countries blend ethanol with petroleum products. As a result of the increased demand for ethanol, more resources are to be used for ethanol production, including distillery products such as molasses.

Molasses is a positive candidate substrate for bio-ethanol production as it is a by-product left after the removal of sugar from sugarcane or sugar beets. The resulting viscous liquid is high in sucrose, which normally is lost to the sugarcane industry but can be fermented into bio-ethanol. Since it is a by-product it will not be as expensive as sugarcane itself and can deliver a good yield of bio-ethanol. Gasmalla *et al.* (2012) tested the composition of different molasses

samples and found that the molasses composition is similar between samples, suggesting that composition of molasses stays more or less constant. By adding the required nutrients, high and constant yields of bio-ethanol can be reached, making molasses a good source to use for bio-ethanol production.

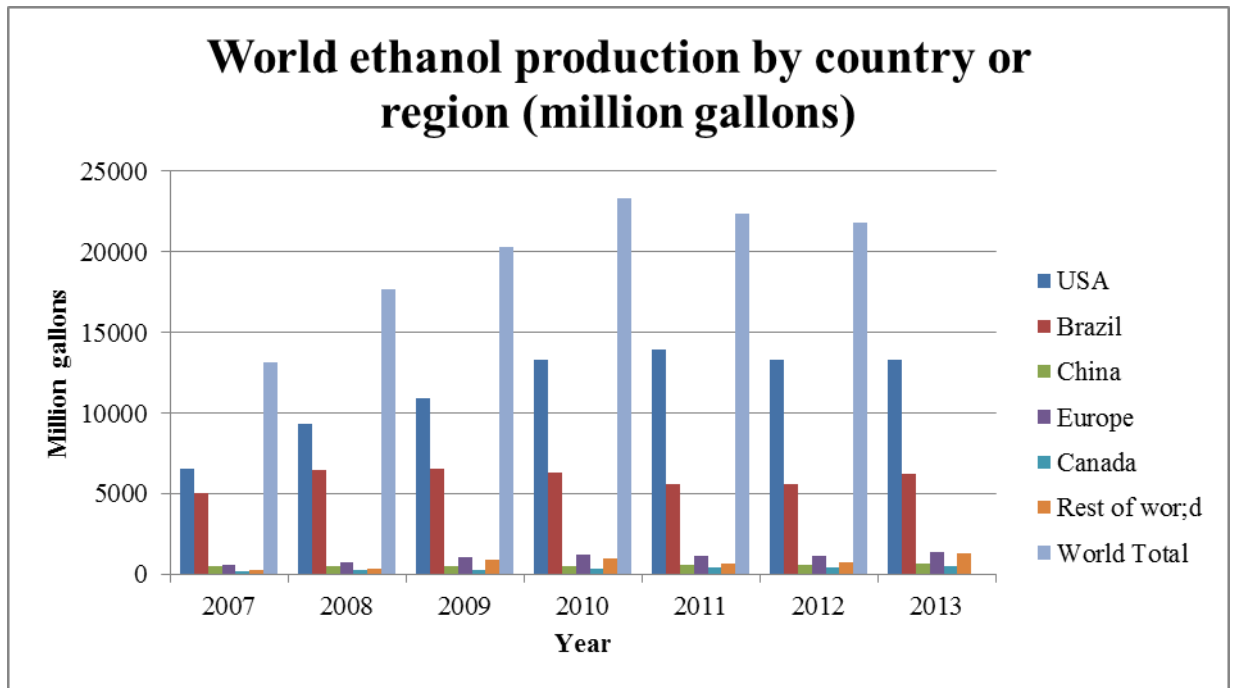


Figure 1.1 Annual ethanol production from 2007 - 2013 in different countries and regions(www.ethanolrfa.org/pages/annual-industry-outlook)

Table 1.1 World ethanol production by country or region (million gallons)¹

Country	2013	2012	2011	2010	2009	2008	2007
USA	13,300	13300	13948	13298	10938	9309	6521
Brazil	6267	5577	5573.2	6291	6578	6472	5019
China	696	555	554.7	541.5	542	502	486
Europe	1371	1179	1167.6	1208.5	1040	734	570
Canada	523	449	462.	356.6	291	238	211
Rest of World	1272	752	698	985	914	389	315
World Total	23429	21812	22404	23311	20303	17644	13123

¹Data Source: F.O. Licht, cited in Renewable Fuels Association, Ethanol Industry Outlook 2008-2013 reports. Available at www.ethanolrfa.org/pages/annual-industry-outlook

Molasses is also commonly used in agricultural industries as a feed ingredient. It is cheap, its viscosity provides binding capabilities in rations and the sugar content improves the palatability for animals to increase feed intake. Therefore it is an important feed ingredient in the agricultural industry, and rises in its use for bio-ethanol production could result in a decrease in its availability and an increase in its price to farmers and animal feed producers. Considering the importance of molasses in processing of animal diets and its inclusion in diets, rising prices would not be favourable and could negatively affect the movement toward bio-ethanol as a renewable energy source. Therefore a compromise needs to be reached.

An advantage of using molasses for bio-ethanol production is that it is already a by-product, making it a cheap and available source for bio-ethanol production. The waste product left after molasses fermentation is called condensed molasses solubles (CMS) and it shares various characteristics with molasses. These similarities provide a possible solution for the accumulation of this waste product when molasses is used for bio-ethanol production, since CMS can be used as a substitute for molasses in ruminant diets.

Chapter 2

2. Introduction

Condensed molasses solubles (known as vinasse in some countries) is a black, syrup-like residue that remains after the fermentation of molasses to produce ethanol, citric acid and yeast products. The process of molasses fermentation involves removal and conversion of sugars. The consequence of removal of sugars is that organic and inorganic substances such as non-protein nitrogen (NPN) and ash, especially potassium (K) and sulphur (S), increase in concentration that can decrease performance of ruminants (Waliszewski & Romero, 1997; Stemme *et al.*, 2004).

Different types of molasses can be used for fermentation such as beetcane-, sugarcane- and citrus molasses and they all produce CMS as a by-product. With CMS being a distillery effluent produced in increasingly large quantities (RFA, 2014) it is critical that CMS be disposed of without polluting the environment (Sheehan & Greenfield, 1980). To achieve this, it is necessary to find a beneficial use for CMS. One such a use of CMS is as a feed ingredient for ruminants.

Benefits of using CMS as a feed ingredient in ruminant diets are driven by two factors. One being that it reduces the cost of disposal of this effluent by selling it as a product instead of just discarding it as a waste product. The selling of CMS will reduce the cost of bio-ethanol production and will improve the viability of bio-ethanol as a fuel source. Secondly, it allows for the use of CMS at a much more affordable cost and better availability than those feed ingredients that could have been used for human consumption such as molasses (Grasser *et al.*, 1995). In large scale production systems such as in feedlots, a slight reduction in cost per diet can make a large difference on feed costs and thus profitability (DiCostanzo *et al.*, 1996).

Condensed molasses solubles shares some physical characteristics with molasses and looks very similar. It has a dark colour and has a texture with a viscosity that enables it to act as a binding agent in total mixed rations (TMR). Characteristics of CMS that are similar to molasses suggest that CMS has the potential to be used as a feed ingredient and a replacement for molasses, but at a much lower cost. Although CMS showed potential in theory, the performance

of ruminants receiving CMS diets was lower than those on diets containing molasses (Chen *et al.*, 1981; Potter *et al.*, 1985a; Fernandez *et al.*, 2009; Lopez *et al.*, 2011).

2.1 Overview of production of condensed molasses solubles (CMS)

The ethanol production process can use various sources of molasses for fermentation. Each substrate for fermentation can produce different amounts of CMS with different chemical compositions. However, the production process of CMS remains similar. Basically the CMS production occurs in four phases; precursor production, harvesting, storage and conversion, but the main focus is placed on the conversion phase because most of the variation in CMS composition occurs at this stage.

The production, harvesting and storage phases consist of the production of the starch crops (e.g. maize), cellulolytic crops (e.g. sorghum) and sugar crops (e.g. sugarcane or beetcane) either for uses in other industries or directly for ethanol production. During these phases the input of nutrients during production and losses during harvesting and storage will influence the composition of CMS slightly.

The conversion phase is critically important and includes the fermentation of substrates to produce ethanol and CMS. During conversion even small fluctuations such as in pH or temperature can have an impact on composition of the CMS. During the initial stages, starch and cellulose crops have to receive some pre-treatment to improve the exposure of the substrates to the yeast in the fermentation stage (Dien & Bothast, 2009). Various pre-treatment procedures can be implemented, including mechanical processing e.g. milling, steam explosion (Bouchard *et al.*, 1990), steam explosion in the presence of acid (Clark & Mackie, 1987; Clark *et al.*, 1989), supercritical explosion by carbon dioxide (Zheng *et al.*, 1998), ammonia freeze explosion and thermal-mechanical processes.

After the pre-treatment is completed, the substrates enter either a continuous or partial continuous fermentation process (Shama, 1984). Both processes require the addition of yeast culture with sulphuric acid to lower the pH (to prevent contamination by other organisms), water and in some cases a nitrogen (N) source to create an optimal environment for only yeast growth and fermentation. The yeast can occupy up to 10% of the fermentation volume and is left to

ferment for two days. The difference between the continuous and partial fermentation methods is the type or origin of the yeast used. During continuous fermentation immobilized yeast (Arasaratnam & Balasubramaniam, 1998) or recycled yeast (Warren *et al.*, 1994) is used and is not collected at the end of the process. The advantage of continuous fermentation is that substrates are allowed to be completely fermented.

The volume of waste produced is inversely proportional to ethanol production and the chemical oxygen demand (COD) of waste (Chamarro, 1979). Therefore by allowing yeast to complete its fermentation fully, waste production is reduced whilst ethanol production per volume substrate is maximised. Therefore, the energy consumption of fermentation is decreased and production capacity is increased (Chamarro, 1979). The disadvantage of this method is the increased susceptibility of contamination by other organisms that produce different end-products rather than ethanol. Larger quantities of contaminating products result in the lower efficiency of fermentation, and therefore less ethanol production. Partial continuous fermentation involves the removal of yeast before distillation, to be used for fermentation in another batch. The reuse of mature yeast reduces the waste/stillage volume, allowing for more ethanol production. The disadvantage of continuous fermentation is that the addition of used yeast increases the COD of the stillage (Shojaosadati *et al.*, 1996).

After the fermentation of substrates is complete, ethanol needs to be separated from the stillage/waste. This is achieved by heating the solution (referred to as “beer”) up to boiling point in a continuous distillation process. Heating can be done in two ways; using steam either directly by injecting the steam into beer in the stripper column, or indirectly through a reboiler heat exchanger. Using the direct method is easier but has a greater effect on the stillage quality. The more water is injected into the beer, the more the stillage volume is increased. Therefore the organic content of the stillage is diluted. Another disadvantage is the increased volume of water required for the heating process. The loss of water requires more replacement water and chemicals involved in processing water (Monteiro, 1975).

Once the boiling point has been reached, ethanol will evaporate into a rectifying column where it will be condensed and collected as 95% ethanol. Some ethanol (0.1% - 0.2%) will be left in the stillage, but if the distillation was not done efficiently it can be higher, thereby decreasing the CMS quality and ethanol production efficiency.

2.2 Characteristics of condensed molasses solubles (CMS)

2.2.1 Animal performance

A major consideration in the successful use of CMS in animal production, and especially in replacing molasses, is that it should not be detrimental to animal health and performance. Feed intake, average daily gain (ADG) and feed digestibility are important parameters to measure and are all correlated with each other in determining animal performance.

During a feeding trial Leontowicz *et al.* (1984) compared the apparent digestibility of the different nutrient fractions between diets containing 27% CBS (condensed beet molasses solubles) and a control diet containing a commercial concentrate. After ensuring that the diets were iso-nitrogenous it was apparent that the digestibility of the fibre fraction in the experimental diets was higher than in the control. Improvement in fibre digestibility was supported by *in vitro* experiments by Hannon & Trenkle (1990) who also recorded higher fibre digestibility when the diets contained CMS as compared to molasses and urea. The cause of the higher fibre digestion was suggested to be owing to the high amount of betaine present in CMS. Betaine is an amino-acid derivative that acts as an osmoprotectant and is known to increase neutral detergent fibre (NDF) digestibility (Weidmeier *et al.*, 1992). This effect of betaine can be increased by increasing pH of the fermentation process (Kulasek *et al.*, 1984) because higher pH levels establish a more favourable environment for cellulolytic bacteria responsible for fibre digestion. An increase in the cellulolytic bacteria population size allows for more attachment and fermentation activity. Although the *in vitro* trials by Hannon & Trenkle (1990) indicated that molasses has the potential to be substituted by CMS, in *in vivo* trials contrasting results were observed. Cattle consuming feed with CMS tended to perform poorly with lower ADG and feed intake. Poor animal performance of CMS in TMR diets has been observed repeatedly once inclusion levels reached 10% or higher (Chen *et al.*, 1981; Potter *et al.*, 1985a; Fernandez *et al.*, 2009; Lopez *et al.*, 2011).

2.2.2 Variations in composition of condensed molasses solubles

The objectives for fermentation process of molasses are focused on maximizing efficiency of bio-ethanol production and not on CMS consistency. Therefore, CMS will differ between different batches of the same type of molasses (Cazetta *et al.*, 2007; Hatano *et al.*, 2009). This variation in composition makes it difficult to categorize the composition and quality of CMS, resulting in the accuracy of its use in diet formulation to be reduced. Table 2.1 contains values of CMS published by Scull *et al.* (2012) to predict the ability of CMS to be used in ruminant diets. It can be seen that the pH is low as a result of the addition of chemicals such as sulphuric acid to decrease pH to reduce bacterial contamination and to stop the fermentation process. The CMS mainly varies in its dry matter (DM), crude protein (CP) and true protein (TP) content, with CP and TP having higher variation coefficients, suggesting that the protein content varies the most between batches of CMS. Differences in nutrients composition can be explained by:

- 1) The original substrate used in distilleries to produce molasses (sugarcane, citrus products or sugarbeet) as well as the quality from production (Eggleston, 2008). Sugar beets and sugar cane vary in their sugar, ash and N contents (Stem & Van der Meer, 1985; Rodushkin *et al.*, 2011) resulting in different amounts of these nutrients being left over after sugar removal and fermentation;

- 2) The differences in fermentation processes of the molasses (Bouallagui *et al.*, 2013). Changing the conditions such as pH, fermentation period and method and temperature can result in varying levels of nutrients left after fermentation of molasses;

- 3) The addition of chemicals and the removal of minerals all influence the composition of the CMS (Nonn, 1993) e.g. the removal of K.

Table 2.1 Physio-chemical characterization of concentrated vinasse (Scull *et al.*, 2012)

Indicator ¹	Sample 1 ²	Statistics ²		Sample 2 ²	Statistics ²	
	(n=5)	SD	CV ³ (%)	(n=5)	SD	CV ³ (%)
pH	4.08	0.07	1.92	3.92	0.008	0.21
Specific weight (%)	1.12	0.004	0.37	1.085	0.008	0.07
Dry matter (%)	29.31	0.02	0.07	21.33	0.20	0.94
OM (%)	77.01	2.71	1.21	76.37	0.19	0.26
Ash (%)	25.00	0.04	0.18	23.63	0.20	0.83
CP (%)	12.39	0.63	5.13	13.26	0.40	3.05
TP (%)	8.89	0.1	1.18	9.29	0.79	8.46
Reducing sugars (%)	4.43	0.9	1.02	4.01	1.10	0.96

¹OM = Organic matter; CP = Crude protein; TP = True protein

²SD = Standard deviation of the mean

³CV = Variation coefficient

The differences between sugarcane CMS and sugar beet CMS are apparent. In Table 2.2 mean composition levels of three analyses of sugarcane- and beet CMS are presented. These values can differ from results from other studies and are used only as an indication of the differences in composition between sugarcane and sugar beet CMS. Ash content in beet CMS is higher because less K has been removed, and constituted one third of the ash content in beet CMS (Stemme *et al.*, 2005). Sugar beet CMS also has a higher concentration of CP, of which betaine and glutamic acid are the major components. Differences in composition reflect the differences in molasses (Stem & Van der Meer, 1885). During diet formulation this variation causes a problem because formulation is based on meeting animal requirements according to the individual contribution of each feedstuff. Since the contributions of CMS cannot be predicted accurately, the diet might not perform as predicted, resulting in lower production and efficiency.

Table 2.2 Comparison between the compositions of condensed molasses solubles (CMS) produced from sugarcane- and sugar beet molasses

Nutrient	Sugar beet	Sugarcane
	CMS ^a (g/kg DM)	CMS ^b (g/kg DM)
Dry matter	668	622
Crude ash	395	335
Crude protein	293	164
Sugar	34.4	32.6

^aMean of 3 analysis of composition of sugar beet CMS (Stemme *et al.*, 2004)

^bMean of 3 analysis of composition of sugarcane CMS (Waliszewski & Romero, 1997)

2.2.3 Composition of condensed molasses solubles (CMS)

2.2.3.1 Dry matter content

As a result of differences in fermentation processes, the composition of CMS vary considerably. The removal of sugars, concentrating of other nutrients and the addition of water are dependent on the fermentation process. As seen in Tables 2.1 and Table 2.2, the moisture content is high in CMS. High moisture content poses a problem with feed intake and feed

conversion ratio (FCR) of ruminants. By increasing moisture in diets, DM intake of the animal decreases, nutrients in the diet diluted and higher feed intakes are required to achieve a required level of nutrient intake (Lahr *et al.*, 1983; Felton & De Vries, 2010). The problem with a low DM content especially in feedlot cattle is that it increases FCR, which can increase the cost of production and therefore reduce cattle performance. Diets with high moisture contents are also prone to mould infestation or growth, therefore exposing animals to mycotoxins and palatability problems which can cause a decrease in feed intake and lower production (Felton & De Vries., 2010).

2.2.3.2 Ash

Condensed molasses solubles are characterized by high ash and NPN levels but is low in sugars. During experiments on pigs, Weigand & Kirchgessner (1980) established that diet containing 30% CMS had almost three times the level of ash in their diet compared to a diet with molasses, with levels ranging between 23.8% and 32.6% DM (Potter *et al.*, 1985).

Condensed molasses solubles has a high ash content and can consist of up to 30% of the total DM (Kulasek *et al.*, 1984a), leading to a high intake of minerals. The two minerals that make up the majority of the minerals in CMS are K and S. Although composition of different CMS varies, K can be up to 104 g/kg DM, and S up to 136 g/kg. (Stemme *et al.*, 2005). It has been suggested that lower digestibilities are due to the effect of these two minerals that are in excess, also possibly reducing the palatability of a diet. Research by Hannon & Trenkle (1990) showed that the decrease in feed intake only starts after a couple of days and that a decrease in digestibility in DM, CP, NDF and acid detergent fibre (ADF) suggests palatability doesn't have an effect on intake. This may reveal that K and S have an effect on performance of the animal by interacting with other minerals or by affecting the health or metabolism of the animal.

2.2.3.2.1 Sulphur in condensed molasses solubles

Sulphur in the diet plays an important role and needs to have a constant intake because S is present in proteins, especially in enzymes and the binding sites of enzymes. According to a review by Goodrich & Garrett (1986) many compounds such as amino acids, hormones and

enzymes and vitamins that are required to maintain ruminant health need adequate amounts of S. In amino acids it is an essential part of S-containing amino acids such as cysteine and methionine. These amino acids contribute mainly to the structure of the proteins due to the free sulphhydryl groups that, through hydrogen binding, form disulphide bonds with other S-containing amino acids. The disulphide bonds of cysteine and methionine play an essential role in the configuration of proteins and function, especially in the active binding sites of enzymes.

Ruminants can use both organic and inorganic S compounds owing to the ability of micro-organisms to reduce S to free HS^- or to incorporate it into microbial compounds and proteins. Inorganic sources of S are reduced and incorporated into organic compounds that will be digested and used by the ruminant (Emery *et al.*, 1957). The most common inorganic S compound is sulphate. It can be metabolized by microorganisms using two pathways; the dissimilatory or assimilatory pathways (Peck, 1962). Sulphur-reducing bacteria utilizing dissimilatory use S to produce energy, or can incorporate S into microbial structures using the assimilatory pathway (Odom & Singleton, 1993). Although S is important for S-containing amino acids, and therefore microbial growth, large concentrations of S can reduce feed intake and ADG (Digesti & Weeth, 1976; Zinn *et al.*, 1997; Wagner *et al.*, 1998; Uwituze *et al.*, 2011). The lower performance of animals due to high S levels is a result of lower oxygen carrying capacity of blood, lesions on the lungs, (Bulgin *et al.*, 1996) and by the formation of the toxic gas, H_2S . The effects of lower energy production can be further aggravated by several health implications. High amounts of H_2S gas produced, can endanger the health of the animal by inducing polioencephalomalacia (PEM) (Gould *et al.*, 1991; Lowe *et al.*, 1996).

2.2.3.2.2 Polioencephalomalacia (PEM)

Polioencephalomalacia is characterized by necrosis of the cerebral cortical portion of the brain and is mostly caused by thiamine deficiencies, but excess S can be a secondary condition to cause PEM. It is possible that high S diets can cause PEM due to its inhibitory effect on thiamine synthesis by either inhibiting the enzyme, thiaminase, production or by reducing thiamine production more directly (Kung J., 2008).

In ruminants, gases such as H_2S and methane are normally removed from the rumen by eructation, therefore regulating gas build up in the rumen. Eructation entails the removal of gas

orally to release pressure built up in the rumen. About 60% of eructated gas released during eructation enters the lungs (Bulgin *et al.*, 1996) which increases the probability of ruminants to develop PEM. In an experiment by Gould *et al.* (1991), they fed animals rations containing below 0.4% S, which is the threshold level for S to cause PEM. Even though the animals should not have developed clinical symptoms of PEM, they still had lesions in the lungs caused by a thiamine deficiency. The results of the study indicated that performance of the animal can be reduced by having subclinical symptoms of PEM. In Table 2.3 is a list of several studies where high S diets were related to PEM symptoms. In many cases the supplementation of thiamine did not always reduce the incidence of PEM. The absence of a response to thiamine is an indication of other factors such as high S level that could have caused these symptoms. By reviewing these studies Kung (2008) strengthened the theory that high S in diets can cause PEM, or at least decrease production due to subclinical symptoms.

The high levels of H₂S can lead to secondary health issues. Hydrogen sulphide gas is very toxic and when it is present in high amounts it can predispose the lungs to viral and bacterial infections (Truong *et al.*, 2006). These infections can cause subclinical symptoms which will reduce performance of the animals as more energy is directed to the immune systems combating the respiratory problems.

Table 2.3 Summary of studies connecting high sulphur levels with incidences of polioencephalomalacia (PEM) (Kung, 2008)

Citation ¹	Symptoms	Diagnosis
Kul <i>et al.</i> (2006)	256 cattle died or slaughtered with signs of PEM	PEM in cattle consuming barley malt sprouts. Total S content in diet was 0.45%
Loneragan <i>et al.</i> (1998)	16 of 150 calves on ranch A and 30 of 4000 calves on ranch B with clinical signs of PEM	PEM in calves consuming feeds with high S: grass hay (0.33% S), Canada thistle (0.9% S), turnips (0.63% S) and rape (0.91% S)
McAllister <i>et al.</i> (1997)	Steers in a feedlot with visual impairment and ataxia. During hot summer months the incidence of PEM was 0.88%	PEM in cattle drinking water containing 2200 mg S/kg to 2800 mg S/kg sulphate corresponding to about 0.67% S intake
Hill & Ebbett (1997)	26 of 99 grazing heifers with signs of ataxia, recumbency and blindness.	PEM in heifers consuming Brassica oleracea that contained 0.85% S
Bulgin <i>et al.</i> (1996)	700 of 2200 ewes with signs of incoordination and abdominal discomfort, death	S toxicity and PEM from field acidified with 35% suspension of S
Low <i>et al.</i> (1996)	21 of 71 lambs with depression, blindness, head pressing and death	PEM Lambs consumed a diet with 0, 43% S for 15-32 days before symptoms appeared.

¹Citation from which data were derived.

2.2.3.2.3 Sulphur and mineral interactions

Dietary S interacts with a number of mineral, which might influence production. The most important interactions are with copper (Cu) and selenium (Se). Selenium plays an important role in the immune system with its synergistic relationship with vitamin E. In a report Weiss *et al.* (1990) studied a commercial dairy herd and their response to Se supplementation. An increase in blood Se and vitamin E (due to the synergistic relationship between Se and vitamin E) resulted in a decrease in the incidence of clinical mastitis. This improvement in the occurrence of mastitis in the herd is a result of the increase in the neutrophil killing ability that was influenced by Se (Grasso *et al.*, 1990; Hogan *et al.*, 1990). Selenium, furthermore, improved the health of the animal by acting with vitamin E as an antioxidant to reduce free radicals in the body. The S and Se interaction are still not very clear but some of it can theoretically be explained by the minerals having similar orbital structures that cause them to be structural analogues of each other (Meyer, 1976). Selenium consequently has the capability to bind to S in cysteine and methionine to form selenoamino-acids. Binding to these amino acids, which often makes part of the binding sites of enzymes, reduces Se and reduces the enzymes ability to bind to substrates (Shrift, 1958). These minerals therefore have an antagonistic relationship. With an increase in dietary S, the Se absorption decreased, resulting in a lower plasma concentration of Se and higher faecal excretion (Ivancic & Weiss, 2001)

It was previously mentioned that with an increase in dietary S intake, DM intake (DMI) decreases and consequently animal performance. This affect is further aggravated by the interaction between S and Se. According to a study by Ivancic & Weiss (2001) high S diets decreased DMI without Se supplementation but DMI decreased even further with Se supplementation. This can be partially explained by the binding of Se to S containing amino acids in enzymes. The decrease in enzymatic activity reduced digestion and flow of digesta. Production in those animals decreased as a result of the lower DMI.

Sulphur interacts with Cu by reducing its absorption. The mode of action of this interaction involves the binding of thiomolybdate (a combination between sulphur and molybdenum) with copper to form Cu-thiomolybdate. The result from this compound is that it reduces the absorption of Cu and increases its faecal losses. Although Cu is mainly stored in the liver, a chronic deficiency of dietary Cu leads to a gradual decrease in concentrations of Cu in

the liver and its transport protein, caeruloplasmin (Arthington, 1996b). Arthington (2006) theorized in a review that low caeruloplasmin concentrations can cause chronic inflammation. Caeruloplasmin is an acute-phase protein that is part of the negative feedback system in inflammatory responses. If there is any alteration in the negative feedback system, such as lower caeruloplasmin, it could prevent the release of anti-inflammatory mechanisms and cause chronic inflammation (Baumann & Gauldie, 1994). Therefore, low Cu levels can decrease the immune functions and result in lower production of livestock due to the increased susceptibility through a decreased immune system.

2.2.3.2.4 Potassium

Potassium is one of the major essential nutrients in muscles and intracellular fluid of ruminants, and is the third most abundant mineral in the body, only being surpassed by calcium (Ca) and phosphorus (P). Found everywhere in the body, K makes up about 5% of the ruminant body. It is required in adequate amounts to maintain water balance, osmotic pressure and acid-base balance, to activate enzymes, to help metabolise carbohydrates and proteins, helping to regulate neuromuscular activity and to regulate heat. Having such important functions, acceptable amounts in the diets are essential. Then again, K is found in most feedstuff and is ingested in sufficient amounts through most diets. According to the NRC (2001) the requirement for dairy cows is 0.9% - 1% of DM, although a review by Stumpff & Martens (2007) summarized that ruminants must be able to handle K variations between 3.8 mmol K/kg per day (Scott, 1969) and 20 mmol K/kg per day (Rabinowitz *et al.*, 1984). Normal grazing practices usually adhere to these ranges, but in the cases of fertilizing forages with K, or even high concentrate feeds, an excess of K can be consumed.

Table 2.4 Chemical composition of condensed molasses solubles (vinasse) derived from sugar beets and sugarcane (Robertiello, 1982; Espana-Gamboa *et al.*, 2012)

	Vinasse ¹	
	Sugarcane	Beet
pH	3.9	5.1
Potassium	2.06	1
Sulphate	0.71	0.62
Magnesium	0.247	0.12

¹Every parameter except pH is measured in g/kg

2.2.3.2.5 Depotasification

After fermentation of molasses, CMS is condensed to be used as a dietary by-product, and causes an increase in mineral concentration in the diet, specifically that of K and S (Stemme *et al.*, 2004). Condensed molasses solubles can vary in mineral content due to different molasses sources or different fermentation processes being used, and can contain up to 30% minerals (Kulasek *et al.*, 1984a). In Table 2.4 from a review by Christofolletti *et al.* (2013) it can be seen that K levels can be up to 2.06 g K/kg. Therefore, including CMS in the diet at high inclusion rates can increase K in the diet beyond the maximum allowed level of 0.7% K of total DM in the diet (NRC, 2005). A possible solution to the high K in CMS is to remove K by depotasification, as seen in beet CMS (Table 2.4). The removal of K may involve different methods. Most of these methods involve the addition of ammonium sulphate. The addition of S removes K by forming potassium sulphate crystals, but some S can be left in CMS and that will increase the S concentration in CMS (Zhang *et al.*, 2012).

In a study, Krzeminski *et al.* (1984a) compared the use of depotasified condensed beet molasses solubles (CBS) and technical CBS (CMS made from sugarbeet molasses) in terms of the effect on volatile fatty acid (VFA), pH and microbial load. By depotasifying the CBS, K concentration was reduced by up to 70% on a DM basis. When these two diets were fed to sheep the technical CBS resulted in a pH of 6.62 and the depotasiated CBS in a higher rumen pH of 6.95, which can be beneficial for fibre digestion and prevention of acidosis. Although the technical CBS resulted in a higher concentration of total VFAs of 87.52 mmol/L compared to

depotassiated CBS with 75.5 mmol/L, the propionate was higher for depotassiated CBS. Higher propionate provides the advantage of more glucose being produced to provide energy with better efficiency of utilization. When the microbial load in the rumen was observed no significant difference was seen, although the different species of microbes were not classified. It is interesting to note that Kulasek *et al.* (1984a) also performed an electrocardiographic examination during an intra-ruminal drench of sheep. Results of the examination revealed an increase in the amplitude of the t-wave characteristic, which is an indication of hyperpotassaemia. Thus, care needs to be taken when including CMS in animal diets to prevent K toxicity.

2.2.3.2.6 Potassium regulation in the body

The body has mechanisms to regulate blood K concentrations. Bodily reactions to high K levels involve increasing urinary excretion of K (Anderson & Pickering, 1962) and the using of the rumen as a distribution reservoir (Stumpff & Martens, 2007). As result of K regulation in the urine, severe diuresis occurs to rid the body of excess K. Although urinary excretion of K is an effective regulatory mechanism, absolute urinary excretion of K does not occur (Sellers & Roepke, 1951). The increased urinary output is coupled with increased losses of Na, Ca and Cl. Therefore, large amounts of other minerals are lost, possibly exposing animals to deficiencies even if the diet provides adequate quantities.

An alternative method to regulate K levels is by using the rumen as a dispersal reservoir which could lead to levels of about 31% (Belyea *et al.*, 1978) of the total body K being found in the rumen. It is well established that when K levels increase, the permeability of the rumen wall for K increases (Warner & Stacy, 1972; Greene *et al.*, 1983; Stumpff & Martens, 2007). Literature indicates that the permeability of the rumen epithealia for K absorption is low. Therefore, K is absorbed mainly from the small intestines (Grace *et al.*, 1974; Khorasani *et al.*, 1997), but as K levels increase K permeability also increases, leading to higher K absorption in the rumen (Warner & Stacy, 1972; Greene *et al.*, 1983; Stumpff & Martens, 2007). The change in K absorption results in equal amounts of K being absorbed in the rumen and small intestines (Green *et al.*, 1983). The mechanism to change rumen K permeability is explained by Stumpff & Martens (2007), viz. that Mg absorption changes. With an increase in K levels in the diet,

depolarization of the apical membrane occurs. The depolarization reduces Mg absorption (Newton *et al.*, 1972; Martens & Rayssiguier, 1980) resulting in the non-selective cation channels to open and increase Na absorption and rumen Na to decrease. The low rumen Na and high blood K levels cause the reduction of the electrochemical gradient and results in an efflux of K into the rumen, in order to maintain normal ruminal osmolality (Sellers & Dobson, 1960). During the rise in blood Na, urinary excretion of K to increases. Therefore, when the influx of K occurs into the rumen, less K will return to the rumen than the amount that was absorbed. Using the rumen as a redistribution reservoir is driven by the Na/K-ATPase, which uses energy. If the K levels continue to be high or increase further, energy available will become less and eventually this regulation of K by urinary excretion and ruminal redistribution can be exhausted. Once the regulatory mechanisms are exceeded toxicities can occur.

2.2.3.2.7 Potassium mineral interactions

As mentioned above, high concentrations of K in diets reduce Mg absorption and could lead to Mg deficiencies. Large amounts of K can induce the disease, grass tetany, more commonly known as hypomagnesaemia (Martens & Rayssiguier, 1980). De Groot (1962) reviewed studies and concluded that even though there are conflicting results whether high K levels induce grass tetany, hypomagnesaemia is definitely associated with high blood K levels. Although the body stores Mg in intracellular spaces, if the Mg absorption is depressed to a high extent or for too long, these stores can become depleted. Even though K may not directly cause hypomagnesaemia it may be a secondary inducing factor. In some cases, if ruminants are gradually exposed to increasing K levels, the kidneys and rumen can slowly accommodate the higher K levels in order to prevent hypomagnesaemia (Pickering, 1965).

There are ways to bring relief to the effects of high K on Mg metabolism. Absorption of Mg never stops absolutely. Some Mg absorption still occurs when there is an excess of K in the diet. Increasing the Mg levels in the diet will result in more Mg being available for absorption. Increasing Mg reliefs grass tetany by counteracting the inhibitory actions of potassium and also provides more Mg to be absorbed (Schonewille *et al.*, 1997).

2.2.3.3 Protein and amino-acids

Protein is one of the most limiting feed constituents in animal feeds and also one of the most expensive. The price of feed ingredients high in protein has driven the industry to make use of resources that provides the most efficient source of protein.

As indicated by the Table 2.1, CP levels in CMS can range between 12% - 15% CP (Scull *et al.*, 2012). Nevertheless as with various other components of CMS, its CP content can vary considerably due to differences in different sources of molasses and fermentation procedures. In Table 2.2 the CP concentration of CMS is illustrated. Sugar beet CMS can have up to 293 g CP/kg DM (Stemme *et al.*, 2004) while sugarcane CMS can contain up to 163.71 g CP/kg DM (Waliszewski & Romero, 1997). According to Weigand & Kirchgessner (1981) sugarbeet CMS has a higher betaine and glutamic acid levels which contributes up to 20% to N digestibility of the CP fraction than sugarcane CMS. This results in a higher apparent digestibility of the N in sugarbeet CMS than in sugarcane CMS. Although sugarcane CMS has a lower CP level than sugar beet CMS, it is superior in its amino acid profile (Waliszewski *et al.*, 1997), increasing its biological value.

A considerable part of the N fraction of CMS consists of amino acids and their derivatives, such as betaine. However, NPN such as ammonia can make up a considerable part of the CP as well. Condensed molasses solubles consists of 29.6 g N/kg DM of which ammonia N, betaine N and amino acids making up 3.5%, 9.1% and 28.6% of the total N, respectively. Of the 28.6% amino acids, 40% consists of glutamic acid (Weigand & Kirchgessner, 1980).

Microbial protein serves as a good source of protein to ruminants especially of essential amino acids. The larger the microbial protein flow, the less of the total protein requirement of the animal have to be met by rumen undegradable protein. The effect of the high NPN fraction of CMS in comparison with urea and molasses was studied by Hannon & Trenkle (1990). The study used and compared CMS as an N source in comparison to urea with molasses on an *in vitro* and *in vivo* basis. During the *in vitro* studie Hannon & Trenkle (1990) compared microbial digestion (and thus microbial synthesis) between urea and CMS in concentrate and roughage based diets. In a concentrate diet, both urea and CMS results were similar. Surprisingly, when adding cellulose at increasing levels, CMS resulted in higher microbial digestion and amino acid production. *In vitro* digestion results therefore suggest that CMS serves as a better N source than

urea, especially with high roughage diets. During an *in vivo* trial, diets consisting of cob corn grain containing 0%, 2.5% and 5% CMS (made iso-nitrogenous by addition of urea) were fed to cattle and the results was contrasting to that of the *in vitro* study.

Apparent digestibility of the OM and CP was not significantly different when adding CMS to ruminant diets instead of molasses (Karalazos & Swan, 1977; Stemme *et al.*, 2005) but above 5% inclusion lowered digestibility was observed. The results of Hannon & Trenkle (1990) was supported by Potter *et al.* (1985) who indicated that more than 5% inclusion during the first 56 days decreased animal performance, and at 15% inclusion of CMS, cattle performed so badly they had to be given normal control diets. Hannon & Trenkle (1990) illustrated that CMS has the potential to be an excellent source of NPN, especially on diets high in cellulose such as diets with a higher roughage content, but is somehow limited in performance in practical diets at high inclusion rates. These results are supported by Wagner *et al.* (1983) who compared iso-nitrogenous diets between CMS, corn steep liquor and fermented ammoniated condensed whey for protein retention. When the mentioned three feedstuffs were given to cattle, CMS showed the highest weight loss, suggesting that the protein in CMS was not utilized as well as in the other feedstuffs.

2.2.3.4 Volatile fatty acids

Volatile fatty acids are the main source of glucose substrates in the ruminant animal. An indication of the three main VFAs, acetate, propionate and butyrate, can be an indication of how efficiently a diet or feedstuff provides energy or how production can vary between diets. Propionate is a more efficient energy provider (being a glucose producing substrate using less energy for gluconeogenesis) (Moran, 2005). Therefore diets that produce less acetate and more propionate are more efficient in energy utilization.

Butyrate is an unique VFA in its ability to stimulate rumen epithelia to grow. The growth is owing to butyrate being used for epithelia metabolism, increasing cell proliferation (Sakata & Tamate, 1978). An increase of the epithelia and their health, absorption of nutrients can be improved.

Acetate can also be used for gluconeogenesis, but requires more energy to be converted to glucose. Therefore, it can be expected that energy provided for growth, products and maintenance will be lower when comparing diets that produce a larger proportion of propionate. With an increase in the acetate : propionate ratio, feed required per unit gain is increased as a result of the decrease in propionate (Chalupa, 1980). When used in lactating cows, the higher butyrate and acetate would increase milk fat, but decrease total milk yield. The higher acetate concentration further benefits production by improving carcass quality due to the increase in dressing percentage (Potter *et al.*, 1985).

Condensed molasses soluble inclusion in diets can change the ratios of VFAs, resulting in a change in the energy dynamics. With an increase in CMS the acetate : propionate ratio (a : p) increases (Potter *et al.*, 1985), resulting in an increase in acetate and butyrate (Karalazos & Swan, 1977). A series of studies supported Karalazos & Swan (1977) that indicated that acetate increased and propionate decreased when CBS was included in a diet (Krzeminski *et al.*, 1984; Kulasek *et al.*, 1984; Leontowicz *et al.*, 1984).

A shift in the VFA ratio is a characteristic of diets high in fibre or forage. When diets high in forage is ingested, the pH in the rumen increases and microorganisms favouring fibrolytic activity increases. The major VFA product of fibrolytic bacteria is acetate.

2.3 Conclusion

The importance of using CMS successfully in animal diets stems from the need to improve the efficiency of ethanol production and replacing its substrate, molasses, in ruminant TMR's. Since the objective of the fermentation process of molasses is the production of ethanol production and not CMS production, factors vary and consequently the composition of CMS. The variation in composition thus complicates formulating balanced diets, using CMS.

Due to molasses being the precursor for CMS, the two feedstuff share similar characteristics such as colour, binding capability and palatability. As a residue left after fermentation of sugars in molasses, CMS has lower energy, higher CP, a lower DM and higher ash content than molasses, and vary considerably in composition between batches. Considering the differences and similarities, attempts were made to substitute molasses with CMS.

In vitro results indicated that CMS can substitute molasses without compromising performance. Once used during *in vivo* trials, contrasting results were observed. Once CMS is included at more than 10%, ADG, feed intake start to increase and FCR to increase. Possible causes to the lower performance have been identified as the high S content, high moisture content and energy differences once CMS is included above 5% of total DM.

The objective of this investigation was to evaluate the nutritive value of CMS produced by Voermol (Maidstone Village, Tongaat, 4380, KwaZulu-Natal). It was assumed that cattle on diets containing CMS would perform poorly in comparison with those on diets containing molasses. To achieve the goal an *in vitro* and *in vivo* study were conducted and compared.

It is postulated that a high S content in CMS would suppress cattle performance but that the performance of cattle would not be affected if the experimental diets, containing different levels of CMS and molasses, are balanced to contain equal concentrations of protein, moisture and energy.

Chapter 3

Materials and Methods

Introduction

To determine the limitations of CMS as a feed ingredient to cattle, *in vitro* and *in vivo* studies were conducted on CMS when included in feedlot diets for cattle. A limited amount of research has been done on CMS in *in vitro* rumen fermentation studies. Using *in vitro* fermentation studies would allow for faster, regular and easier trials to determine the effect of CMS on rumen fermentation characteristics without any animal factors interfering. *In vitro* studies could therefore highlight potential reasons for the lower performance of cattle receiving CMS containing diets compared to diets containing molasses.

3.1 *In vitro* study

3.1.1 Total gas production

The aim of measuring total gas production was to simulate, under controlled conditions, the ruminal fermentation process of feedlot diets containing different levels of CMS. The technique allows for measuring gas production as indication of degree of ruminal fermentation taking place on the specific substrates and thus the production of the major source of energy to the ruminant in the form of VFA. The technique also allows for the measuring of the composition of the gas produced, especially gases other than VFAs such as S-containing gases, that might have ill-effects on the health of the animal.

3.1.1.1 Experimental design

The gas production study was performed on the *in vivo* TMR trial diets. The aim was to make all four trial TMRs iso-energetic and iso-nitrogenous to prevent energy and protein differences from affecting the results. The trial TMRs were formulated and mixed by Voermol (Voermol feeds, Maidstone Village, Maidstone, KwaZulu-Natal, South-Africa).

Four diets were used, varying in the amount of CMS included. Table 3.1 presents the ingredient composition of the TMR diets. The diets contained 0%, 5%, 10% and 15% CMS. Each treatment consists of two replicates during each run of gas production, with a total of four runs of gas production. During each run a blank was added to correct for gases produced by the buffer-rumen fluid mixture, bringing the total modules to 10 per run. Before the collection of rumen fluid, 1.0 g of each sample was weighed and put into a 250 mL glass bottle which was used during the gas production study.

Table 3.1 Ingredient composition of total mixed rations containing different levels of condensed molasses solubles (CMS) on an “as is” basis

Ingredient	Treatment (g/kg) ¹			
	Control	Treatment 1	Treatment 2	Treatment 3
CMS	0	50	100	150
Molasses	150	100	50	0
Bagasse	155	145	130	120
Maize meal	545	570	600	620
COCM*	120	105	90	80
Limestone	15	15	15	15
Urea	10	10	10	10
Salt	5	5	5	5

Nutrient	Treatment (% of DM) ¹			
	Control	Treatment 1	Treatment 2	Treatment 3
Dry matter	87.6	86.1	84.7	83.2
Crude protein	14.78	14.69	14.65	14.70
TDN**	79.5	79.4	79.6	79.4
Crude fibre	11.44	10.98	10.27	9.83
Sulphur	0.20	0.25	0.30	0.35
Potassium	1.12	1.19	1.27	1.35
Calcium	0.83	0.85	0.87	0.9
Phosphorus	0.33	0.32	0.32	0.32

¹Treatment diets composition on as is basis. Control: Diet containing 0% CMS; Treatment 1: Diets containing 5% CMS; Treatment 2: Diets containing 10% CMS; Treatment 3: Diets containing 15% CMS

*COCM= Cotton oilcake meal

**TDN= Total digestible nutrients.

3.1.1.2 Buffer media preparation

The buffer solution was prepared in large quantities and utilized as needed, as described in Goering & Van Soest (1970). The Goering & Van Soest buffer solution (Appendix 1) was prepared and used according to the ANKOM gas production method. A buffer solution was mixed fresh on the morning of the experiment. Water, trypticase peptone, micro-mineral solution, macro-mineral solution, buffer solution, resazurin and reducing solution were mixed as set out by Goering & Van Soest (1970) before rumen fluid collection in the ratios shown in Table 3.2. Resazurin is a reducing agent to remove oxygen to ensure anaerobic conditions. The initially buffer mixture is dark purple in colour. In order to remove oxygen from the buffer mixture, CO₂ was bubbled through the buffer mixture. As the oxygen is being removed from the mixture, it changes colour from dark purple, to pink, ending with a colourless appearance once it is reduced completely (Figure 3.1).



Figure 3.1 Illustration of the reduction process of buffer solution put under CO₂ stream.

Table 3.2 Composition of buffer solution used during *in vitro* gas production studies (Goering & Van Soest, 1970)

Component	Volume ¹
Distilled water (mL)	800
Trypticase peptone (g)	4
Micro-mineral solution (mL)	0.2
Buffer solution (mL)	400
Macro-mineral solution (mL)	400
Resazurin (mL)	2
Reducing solution (mL)	80

¹All values except for trypticase peptone measured in mL. Trypticase peptone measured in grams.

Once reduced, 100 mL of buffer solution was added to each bottle and purged with CO₂. Samples were then placed in the water bath at 39 °C before rumen fluid was collected from cannulated sheep. This allows the buffer solution and CO₂ to equilibrate. Before rumen fluid collection, the ANKOM gas production meter modules were activated. Gas measurements before and after rumen fluid addition were used to determine the zero concentration value of gas production profiles.

3.1.1.3 Animals and rumen fluid collection

The rumen fluid was collected before morning feeding from three ruminally cannulated Merino wethers. The sheep were fed *ad libitum* on lucerne hay, and rumen fluid collection occurred before morning feeding. Approximately 200 mL rumen fluid was collected from each donor animal. Rumen fluid was mixed to reduce variation in microbes, then strained through four layers of cheesecloth and transferred to pre-heated thermos flasks. In the laboratory, the flasks were emptied into an industrial blender while being purged with CO₂ to maintain anaerobic conditions (Grant & Mertens, 1992). Once blending was completed the rumen fluid was transferred to a large glass beaker inside a 39 °C water bath whilst being continuously purged with CO₂ and continuously stirred, as recommended by Goering & Van Soest (1970). Once

purged, 25 mL of rumen fluid was added to each 250 mL bottle, purged again with CO₂ and sealed tightly to prevent gas from escaping.

3.1.1.4 Gas production

An ANKOM RFS Gas Production Measurement System (Ankom 2008) was used to automatically measure gas production in modules containing samples (Figure 3.2). Each ANKOM module uses a gas production module that screws tightly to close the 250 mL bottles and measures gas build up in pounds per square inch (Psi). Values were recorded every five minutes and measured as a cumulative value over 48 hours. Inoculated samples containing sample of diets, buffer mixture and rumen fluid were placed in a water bath at 39 °C. The ideal length of fermentation would have been 48 hours but the condition of the battery units used in the ANKOM gas production modules only allowed a maximum of 40 hours between all the modules. During the fermentation period modules were set to measure gas production in Psi every 5 minutes. Once gas pressure reached 1 Psi, the ANKOM modules were set to open valves to release pressure. Gas was automatically measured cumulatively over the 40 hour period.



Figure 3.2 ANKOM gas production modules used to measure gas production in Psi during fermentation

3.1.1.5 Calculation of total gas volume

Once gas production has been terminated, recorded gas production readings were converted to volume using the following equation

BASIC:

$$PV = mRT \dots\dots\dots (1)$$

$$M = PV/RT \dots\dots\dots (2)$$

BUT P = Psi units and are converted to kPa

$$P \text{ (kPa)} = 6.894757 * \text{Psi}$$

$$V = 0.125 \text{ L (headspace in bottle, 0.250 L-0.1 L buffer-0.025L rumen fluid)}$$

$$R = \text{constant} = 8.314472 \text{ L.kPa/K/mol}$$

$$T = 312 \text{ K (273+39)}$$

Thus $m = PV/RT$

$$= 6.894757 \text{ Psi} * 0.125 / (8.314472 * 312) \dots\dots\dots (3)$$

After calculating m it is converted to mL gas by using the following:

$$V = 25.6 * m * 1000 \text{ (1 mol gas produced 25.6 mL gas at 312 K)} \quad (4)$$

K= Kelvin

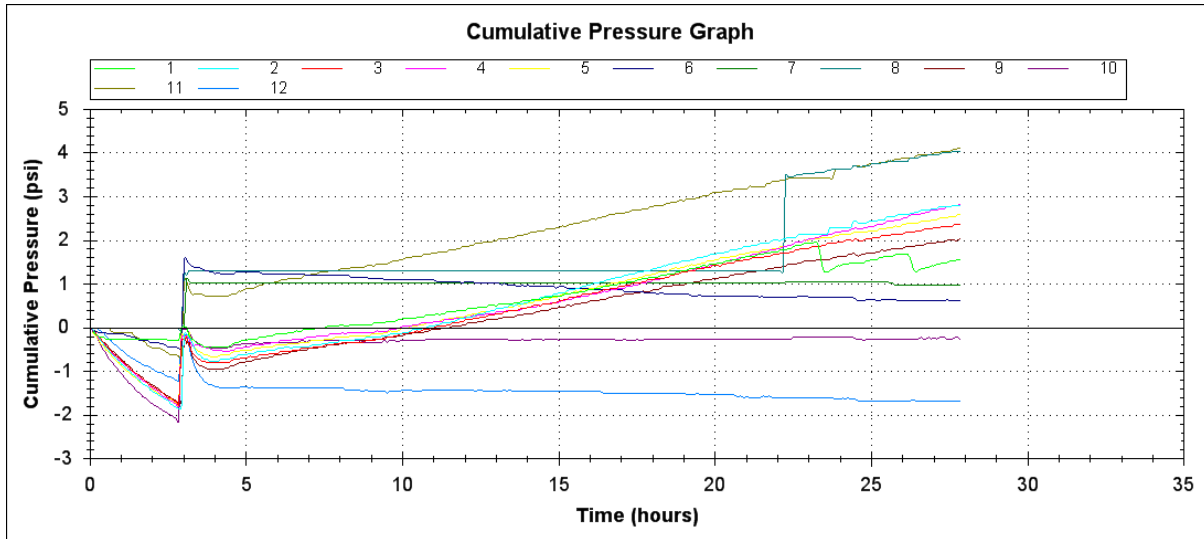


Figure 3.3 Example of a gas production profile, indicating the initial spike and drop of pressure

3.1.1.6 Value correction

Zero hour values are defined to accurately measure the gas production over time. To achieve the zero hour, the gas production profile is used. Figure 3.3 is an example of a gas production profile. At the three hour mark, values have a spike, followed by a slight drop in pressure. The spike and drop occur as a result of CO₂ being poured over the sample and buffer solution before rumen fluid was collected. When rumen fluid is injected, the modules have to be opened and some gas escapes, resulting in the slight drop in cumulative pressure that build up before the addition of rumen fluid. The drop is followed by a gradual rise in gas production and marks the zero hour of gas production. The starting value is subtracted from every value of the gas profile to get an accurate value for gas production. Gas values achieved after correction provide better comparisons between samples since they resemble only gas produced from samples fermentation.

3.1.1.7 Statistical analysis

Repeated measures analysis on gas production was used. Linear mixed model analysis, also known as REML analysis (Payne *et al.*, 2012), was applied to the gas production to model the correlation over 42 hours in a repeated measurements analysis (Payne *et al.*, 2012). The fixed effects were specified as hour, diet and hour by diet interaction, while the random effect was specified as the subject by hour interaction. A power model of order 1 (correlation decreases as time between measurements increase for unequally spaced measurements) and modelling for variances that change over time, were found to model the correlation best over time.

3.1.2 *In vitro* gas composition measurement

It is well established that the composition of CMS varies between batches and substrate sources, but its ash content is consistently high. A mineral in CMS ash to consider is S, since excessive concentrations could be detrimental to animal health. The effect of S is especially dangerous when it is converted into H₂S gas. The aim of the gas composition trials is to simulate gas production within the rumen and to compare H₂S production between samples. Carbon dioxide was also measured to indicate buffering activity, especially the buffering of propionate.

3.1.2.1 Experimental design

Gas production was compared between the TMR trial diets. The aim was to make all four experimental TMR treatments iso-energetic and iso-nitrogenous to prevent energy and protein differences from affecting the results. Trial TMRs were formulated and mixed by Voermol (Voermol feeds, Maidstone Village, Maidstone, KwaZulu-Natal, South-Africa).

Four treatment samples were used, each containing a different level of added CMS. In Table 3.1 the ingredient composition of the TMR samples is presented. Sample diets contained 0%, 5%, 10% and 15% CMS. Each treatment consists of two replicates during each run of gas production, with a total of five runs per treatment. During each run a blank was added to correct

for gases produced by the buffer-rumen fluid mixture, bringing the total batch to 10 per run. Before the collection of rumen fluid, 400 mg of each sample was weighed and put into a 120 mL glass bottle used for measuring the gas production.

3.1.2.2 Buffer solution preparation

Buffer solution is prepared as mentioned above in total gas production using the Goering & Van Soest (1970) buffering solution.

3.1.2.3 Gas composition analysis.

A sample of approximately 400 mg of the respective feed sample was weighed into a 120 mL serum bottle. Then 40 mL of rumen fluid and buffer medium was added under a stream of CO₂ to each serum bottle. Bottles were closed with rubber stoppers and crimp seal caps.

A needle was inserted through a rubber stopper of each serum bottles for about 5 seconds to release small amount of gas that might have built up, allowing samples to have an equal starting point for incubation. All serum bottles were returned to the incubator set at a temperature of 39 °C and the rotary shaker was turned on 120 rpm.

3.1.2.4 Gas composition determination

To quantify the gas produced from the fermentation of the samples, the gas produced from only the buffer solution and rumen fluid was determined by adding a blank and subtracted from the total gas produced. There were five replicates of four different treatments. Samples were inoculated for 24 hours after which they were removed from the incubator and placed into ice water to stop fermentation. The cool box was sealed to maintain cool temperature for transport. The samples were transported to NECSA (South African Nuclear Energy Corporation SOC Limited, Pelindaba, North-West) where the composition of the gas in the bottles was determined using calibrated gas chromatography.

3.1.2.5 Statistical analysis

Repeated measures analysis on gas production was used. Linear mixed model analysis, also known as REML analysis (Payne *et al.*, 2012), was applied to the gas production to model the correlation over 24 hours in a repeated measurements analysis (Payne *et al.*, 2012). The fixed effects were specified as hour, diet and hour by diet interaction, while the random effect was specified as the subject by hour interaction. A power model of order 1 (correlation decreases as time between measurements increase for unequally spaced measurements) and modelling for variances that change over time, were found to model the correlation best over time.

3.1.2.6 Feed sample chemical analysis

Samples of the TMRs used during the *in vitro* trial were formulated, mixed and analysed by Voermol. The following nutrients was analysed for: CP (CP (Leco Trumac N determinator, model FP-428, Leco corporation, St Joseph, MI) used to determine N. Crude protein was calculated using Dumas method, (AOAC, procedure 968.06)), ether extract (EE) (AOAC, procedure 920.39), DM (Sartorius moisture analyser, model mark 3, Sartorius Corporation, Goettingen, Germany), crude fibre (CF) (Ankom 2000 fibre analyser, Ankom Technologies, Macedon, NY, USA), K (AOAC, procedure 927.02) and S (AOAC, process 955.48).

3.2 Feedlot feeding trial

The objective of the *in vivo* trial was to measure the performance of feedlot cattle diets containing different levels of CMS while the DM, energy and CP levels were formulated to be similar. Feed intake and weight gain were measured regularly to indicate any trends or differences during the experimental period. After the cattle were slaughtered, liver samples were collected and used to measure liver Se and Cu concentrations to evaluate the effect of S in CMS on the Se and Cu metabolism in the animals.

3.2.1 Feedlot trial

Ethics approval for the study was granted by the Animal Ethics Committee (AEC) of the University of Pretoria (Ref: EC112-13).

3.2.1.1 Location

The study was conducted in the experimental feedlot facility on the UP Experimental Farm of the University of Pretoria (S25° 45' 10" E28° 14' 46")

3.2.1.2 Duration

The feedlot trial was conducted over a period of 100 days. Animals arrived on 14 February 2014. The trial started 06 March 2014 and continued until 06 June 2014.

3.2.1.3 Animals and allocation to treatments

Hundred and eleven weaned bulls were acquired for the trial. The bulls consisted out of a variety of breeds, though were not stratified according to breed but only to weight. Upon arrival bulls were weighed (Figure 3.7) and blocked into groups according to weight (Table 3.3). Within blocks the animals were allocated randomly to the four experimental treatments with 25 animals per treatment. Eleven outliers on weight were left out of the study. The experimental animals were identified with eartags on the right ear. Tags were colour coated red, green, blue or yellow according to treatment, 1, 2, 3 and 4, respectively (Figure 3.4).



Figure 3.4 Illustration of the colour tag system used to identify and organise cattle

Table 3.3 Body weight (kg) distribution of bull calves between treatments at onset of study, blocked according to body weight

Blocks	Control ²	Treatment 1 ²	Treatment 2 ²	Treatment 3 ²
1	168	188	172	200
2	238	221	236	218
3	260	253	248	264
4	276	281	290	286
5	303	293	286	283
Block average ¹	249	247.	246.	250.
6	197	200	186	198
7	217	-	245	238
8	268	254	247	230
9	272	294	277	285
10	307	313	307	308
Block average ¹	252	265	252	252
11	199	209	208	201
12	243	232	235	250
13	264	260	262	256
14	307	287	280	280
15	279	306	314	292
Block average ¹	258	259	260	256
16	209	212	216	205
17	232	236	248	225
18	281	267	268	262
19	283	285	272	276
20	297	302	313	321
Block average ¹	260	260	263	258
21	218	218	217	231
22	253	231	248	240
23	275	263	273	263
24	310	314	284	293
25	322	336	321	324
Block average ¹	272	272	269	270

¹All block averages include the weight of five animals per group and compares weights between corresponding groups in other treatment diets. Weights in corresponding block averages should be within ± 5 kg range of each other.

3.2.1.4 Pens

There were 22 feeding pens available and 20 were used for the study. The remaining two were used to house surplus or sick animals. Each pen was 4 m wide and 9 m in length. A roof above the feeding area provides shade with a 4 m feeding trough beneath it. The water trough was 3 m from the gate on the opposite side of the feeding trough to prevent contamination with feed and crowding at water troughs during eating (Figure 3.5). Five bulls were in each pen, which allowed for social interaction.



Figure 3.5 Illustration of one of the pens used to house the cattle

3.2.1.5 Feeding

The bulls were fed twice a day, at 8:00 and 16:00. The TMR consisted of concentrates and forages that were formulated to supply in the cattle's needs, with CMS inclusions at 0%, 5%, 10% and 15%. The diets were iso-nitrogenous, iso-energetic and the moisture content adjusted

to be equal in all treatments and thus to provide the same amounts of nutrients per kg on an “as is” basis. The diets were formulated and supplied by Voermol (Ltd) according to NRC (2001) guidelines. The diets contained monensin at 33 g active monensin/ton of DM and a mould inhibitor.

Table 3.4 Ingredient composition (in g/kg feed) of the four treatment diets fed to feedlot cattle on an “as is” basis

Ingredient	Control	Treatment 1	Treatment 2	Treatment 3
CMS ¹	0	5	10	15
Molasses ¹	150	100	50	0
Water ⁶	60	41	15	0
Bagasse	120	120	120	85
Maize meal	479	525	561	521
COCM ²	0	0	0	24
DBG ³	155	134	126	200
Limestone	14	12	11	12
Urea	10	11	10	3
Salt	5	3	3	3
MCP ⁵	3	3	3	1
Amm.sulphate ⁴	3	0	0	0
Premix	1	1	1	1

¹ Diets differ in the amount of condensed molasses solubles (CMS) and molasses included; Control: 0% CMS; Treatment 1: 5% CMS; Treatment 2: 10% CMS; Treatment 3: 15% CMS.

² COCM= cotton seed oilcake meal;

³DBG= dried brewers grain

⁴Amm. sulphate= ammonium sulphate;

⁵MCP= mono calcium phosphate.

⁶Water was added to equalize the moisture content.



Figure 3.6 Illustration of the feeding trough used. Feeding trough allowed adequate space for five cattle to feed at the same time

3.2.1.6 Experimental procedure

The experimental animals were blocked according to weight and assigned to treatments within each block. Table 3.4 presents the composition of the treatment diets. In the control group the TMR contained molasses and no CMS. In the control 15% molasses was included, treatment 1 5% of CMS was added with 10% molasses, in. Treatment 2 10% CMS was included with 5% molasses and in Treatment 3 15% CMS.

The bulls were put on an adaptation programme for 19 days to ensure a good transition onto TMRs. At arrival the bulls were fed a 100% roughage diet for one week to become accustomed to the environment and to reduce stress. After the 1st week, roughage was slowly

replaced by a designated TMR treatment. After the initial seven days of feeding only roughage, 25% of roughage intake was replaced with TMR for a period of four days. After day four, 50% of intake was replaced with TMR for four days. After four days, 75% of intake was replaced with TMR for four days after which only the TMR was given for the experimental period (Figure 3.6).

Feed intake of bulls per pen was measured on a weekly basis. Individual body weights were measured every second week. At the end of the trial, total feed intake and weight gain per pen over the experimental period were used to calculate FCR per pen.



Figure 3.7 Scale used to weigh cattle every second week

3.2.1.7 Statistical analysis

3.2.1.7.1 Feed intake

Data were analysed as a one-way analysis of variance using the GLM model (SAS, 2013) to calculate the average effects of the systems. Means and standard error were calculated and significance of differences ($P < 0.05$) between means were determined using the Fischer's test (Samuels, 1989).

The linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where:

Y_{ij} = variable studied during period

μ : overall mean of the population

T_i = effect of the i th treatment

e_{ij} : error associated with each Y

3.2.1.7.2 Weight gain

Repeated Measures of Variance with the GLM model (SAS, 2013) was used for repeated period measures. Bodyweight at day 0, as a covariant within age group for body weight, were

included in the model. Means and standard error of mean for the different treatments were calculated and significance of difference ($P < 0.05$) between means were determined by the Fischer's test (Samuels, 1989). Weight gains were analysed, comparing regressions of the gains over the experimental period. Morris (1999) pointed out that when analysing repeated measurements such as weight gains over a period of time, comparing of regressions is the preferred method of analysing the data.

3.2.1.7.3 Feed analysis

Samples of the treatment diets were collected every week and pooled for analysis. The samples were milled (SWC hammer mill, 1 mm sieve) and analysed at the UP-Nutrilab (Department of Animal and Wildlife Science, University of Pretoria, Pretoria). Samples were analysed for the following: DM (AOAC, 2000, procedure 934.01), ash (AOAC, 2000, procedure 942.05), CP (Leco Trumac N determinator, model FP-428, Leco corporation, St Joseph, MI) used to determine N. CP calculated from $N \times 6.25$ (AOAC, 2000, procedure 968.06) and crude fat (AOAC, 2000, procedure 920.39).

3.2.1.7.4 Crisis management

During the feeding period, problems with the freshness and supply of feed were experienced. As a result of the high moisture content of the feed, mould growth started to occur in the TMRs. As is common practise, mould inhibitors were included in the TMRs, but in this study, were apparently not corrected for the higher than normal moisture content. Within 3-4 weeks of delivery, mouldy growth was observed in all the treatment diets. To prevent the moulding from influencing the trial, feed bags were observed and managed in such a way to provide as little mould as possible to the cattle. As a result many bags of feed were discarded. Furthermore, samples of moulded feed were sent to test for mycotoxin analysis to indicate if dangerous concentrations of mycotoxins occurred. As a further preventative method, any piece of mouldy feed found during feeding, was removed and a mould inhibitor was manually added to

the feed. This procedure was applied until new fresh feed with corrected mould inhibitors, was delivered.

At 8 weeks in the feeding period, a feed supply crisis occurred. During this period a labour strike at the feed company, Voermol (providing the feed) occurred. As a result, normal delivery of feed could not be made and feed supplies became depleted. To feed the cattle, all treatment groups were placed on lucerne diets for five days. Once the first delivery of feed arrived, it only provided the control diet (with no CMS) and the 5% CMS treatment. Therefore, only the control groups received the 0% diet and Treatments 1, 2 and 3 received the 5% CMS diet for 7 days. Treatments 2 and 3 were given the 5% CMS diet to ensure that the cattle still received some CMS in their diets. At the next feed delivery, the experimental diets were correctly supplied.

At the end of the feeding trial, feed intake and weight change over time were observed and indicated that the mouldy feed did not influence feed intake, but the feed supply problems compromised the feedlot trial and therefore the feed supply problems should be considered when viewing the results (See Chapter 5).

3.3 Selenium and copper concentrations in liver samples Processing of livers

At slaughter a liver sample was collected from each bull and stored in a cool room. The samples were dried and ground to determine their Se and Cu concentrations on a DM basis. Since the bulls were fed in groups and feed intake was measured per pen, the liver samples from each pen were pooled, giving five replications per experimental treatment.

Dry matter was determined for each individual liver sample before being pooled. On average, a 2 g liver sample was taken and cut up into small pieces of ± 1 cm in size. The wet liver sample was placed on a foil plate and weighed. The weighted samples were placed in an industrial drying oven for of 48 hours. After the initial 24 hours of drying, the samples were turned to ensure equal drying throughout the sample and left for the last 24 hours. After 48 hours the dry samples were weighed and DM% was calculated as follows:

$$DM\% = \frac{\text{Weight of wet sample} - \text{weight of foil plate}}{\text{Weight of dried sample} - \text{weight of foil plate}} * 100$$

The dried liver samples were pooled according to pen and ground into a powder in preparation for the Se and Cu analyses.

3.3.2 Selenium analysis

To determine the Se concentration the continuous hydride generation atomic absorption method (AOAC, 2000, process 935.13) was used. Samples were read using a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer at an absorbency of 196 nm and lamp energy of 16 mA. Bovine liver was used as a control (National Institute of Standards and Technology standard reference material 1577b. US Department of Commerce, Gaithersburg, MD 20899) and included in each batch of analyses.

Wet digestion requires 0.1 g of liver samples to be placed in test tubes and the addition of 5 mL of digestive acid solution (consisting out of 65% HNO₃ and 72% HClO₄). After the addition of digestive acid solution, sample tubes were placed in a digestion block for a total of 16 hours of digestion with the following programme:

- 1) Four hours at room temperature
- 2) One hour, with temperature increasing from room temperature to 100 °C
- 3) One hour at 100 °C
- 4) One hour with temperature increasing from 100 °C to 180 °C
- 5) Six hours at 180 °C
- 6) Two hours, decreasing temperature from 180 °C to 130 °C
- 7) One hour at 130 °C

During the 16 hour digestion period, Se is gradually converted from Se IV to Se VI.

Tubes were removed from the digestion block and left to cool for 10 minutes. Once cooled, 2.5 mL of a 20% HCL was added to each tube and allowed to digest for another 40 minutes in the digestion rack. This process allows for the conversion of Se VI to Se IV. After the 40 minute conversion period, relevant acid (20% HCL for standard and 10% HCL for samples) was used to increase all test tubes to the 20 mL mark (approximately 15.5 mL added).

Samples were put through a hydride generator (Vapor Generation Accessory VGA-77) using 20% HCL as an oxidizing agent and sodium borohydride (NaBH₄) in a 0.5% sodium hydroxide (NaOH) solution as a reducing agent. Samples were read by a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer at an absorbency of 196 nm and lamp energy of 16 mA.

All readings of Se were recorded and included in the following equation to determine the Se concentration in ng Se/g dry liver:

$$\frac{\text{ng}}{\text{g}} \text{ Se} = \frac{(\text{Reading (ng)}) * \text{Dilution}(20)}{\text{Mass (g)}}$$

3.3.3 Copper analysis

Copper concentration was measured using the wet ash method (AOAC, 2000, procedure 935.13). Samples were read using a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer at an absorbency of 196 nm and lamp energy of 16 mA. Bovine liver was used as a control (National Institute of Standards and Technology standard reference material 1577b. US Department of Commerce, Gaithersburg, MD 20899) and included in each batch of analyses.

After drying, 0.5 g of each sample was transferred to a digestion tube and the mass was recorded followed by adding 10 mL HNO₃ to predigest for 15 minutes. Following pre-digestion, the tubes were closed and loaded a turntable and installed into a digestion block. The correct programme for Cu digestion was chosen and allowed to digest.

At the completion of the programme the tubes were removed and allowed to cool. Once cooled the tubes were open carefully to release pressure build up in the tubes. When sufficient pressure was released, 2.5 mL HClO₄ was added to each tube and rinsed with a small volume of deionized H₂O. When samples were completely cooled they were transferred quantitatively into

50 mL volumetric flasks and volume was filled with deionized H₂O and shaken well. Samples were transferred into clean 50 mL medicine bottles.

Two drops of methyl-red were added to indicate pH change. The solution was acidified by adding NH₄OH drop-wise until it reached a pH of 5.6, indicated by an orange-brown colour. Once two drops of HCL were added, the colour of the solution would turn to pink, indicating a pH of 2.5 – 3.0. The solution was diluted to 150 mL with deionized H₂O, brought to boil and whilst stirring 10 mL hot saturated solution of (NH₄)₂C₂O₃ was added. The solution was allowed to stand overnight for the precipitant to settle.

Once the solution settled, the supernatant was filtered through filter paper and the precipitant was washed thoroughly with NH₄OH. The precipitant was placed into a 150 mL beaker and a mixture of 125 mL H₂O and 5 mL of H₂SO₄ was added. The solution was heated to 70 °C and titrated with 0,05 N KMnO₄ until the solution turns slight pink. To increase the accuracy of the reading the blank was corrected and Cu% was calculated.

3.3.4 Statistical analysis

One-way unblocked ANOVA was applied to the Se and Cu data to test for differences between diet effects (Payne *et al.*, 2012). ANOVA assumptions were satisfied and significance testing was done at the 5% level.

Chapter 4

Results and discussion

4.1 Chemical analysis of condensed molasses solubles (CMS) compared to molasses

Table 4.1 Chemical analysis (g/kg DM) of condensed molasses solubles (CMS) and molasses

	Crude protein	Ash	Potassium	Sulphur
CMS source 1	56	120	73	5.7
CMS source 2	56	132	65	5.9
Molasses	43	111	69	5.0

4.2 *In vitro* gas production study

4.2.1 Chemical analysis of experimental diets

The chemical composition of samples of the TMR diets used during the *in vitro* trials is presented in Table 4.2. The respective energy and protein levels between treatments were formulated to be iso-nitrogenous and iso-energetic to prevent any influence of these nutrients on experimental results between the trial diets. Diets only vary in the CMS and molasses content included.

Table 4.2 Chemical analysis of the total mixed rations used during the *in vitro* trials

Nutrient ¹	Experimental diets containing condensed molasses solubles at:			
	0%	5%	10%	15%
DM (g/kg)	899.2	883.8	865.7	845.7
ME(MJ/kg DM) ³	13.1	13.03	12.0	12.2
CP (g/kg DM)	142.4	137.9	140.8	134.7
CF (g/kg DM)	83.5	93.6	61.6	72.4
Fat (g/kg DM)	31.3	23.9	27.9	20.9
Ash (g/kg DM)	93.3	104.5	114.5	93.9
Ca (g/kg DM)	11.9	11.4	12.9	11.7
P (g/kg DM)	4.2	3.8	4	3.1
K (g/kg DM)	10.8	10.9	12.29	12.9
S (g/kg DM)	3.1	3.1	3.86	6.93

¹DM= Dry matter, ME= Metabolisable Energy, CP=Crude protein, CF= Crude fibre, Ca= Calcium, P= Phosphorus, K= Potassium, S= Sulphur

²ME (MJ/kg DM) = (15.2×CP)+(34.2×EE)+(12.8×CF)+(15.9×NFE)/1000 (Kaustell *et al.*, 1997).

As seen in Table 4.2, iso-nitrogenous, iso-energy and similar moisture levels between the trial diets were achieved. A consequence of adding water to get the moisture levels similar between diets, the ME for the diets appear to be low for traditional TMRs (Table 4.2). A high difference in the ash content was expected, especially due to K and S content. Although S and K levels were slightly higher with higher CMS inclusions, the overall ash content remained similar between CMS and molasses diets. As a result of the indifference between experimental diets in S, it is expected that S would not influence H₂S production significantly.

4.2.2 Total gas production

The total gas produced was measured using an ANKOM^{RF} gas production measuring system. The pressure measured, was converted to mL at 4, 8, 16, 24, 30 and 42 hours of fermentation and are illustrated in Table 4.3 and Figure 4.1.

Table 4.3 Cumulative gas production (mL) during 4, 8, 16, 24, 30 and 42 hours of *in vitro* fermentation of treatments diets containing different levels of condensed molasses solubles (CMS) (n=16).

Hour	Experimental diets containing condensed molasses solubles at: ¹				
	0%	5%	10%	15%	SEM ²
04	18	16	17	13	2
08	48	46 ^a	50	46	5
16	108	106	115	114	8
24	125	122	135	134	9
30	132	130	143	145	9
42	140	138	151	155	9

¹Treatments vary in the amount of CMS in the diet. Control: Contains no CMS and 15% molasses; Treatment 1: Contain 5% CMS and 10% molasses; Treatment 2: Contain 10% CMS and 5% molasses; Treatment 3: Contain 15% CMS and no molasses.

²Standard error of the mean

Differences between rows were not statistically significant ($P > 0.05$).

Initial gas production and measurements over the measured time period between the diets did not differ ($P > 0.05$) or have any tendencies to differ. The results are unexpected since other *in vivo* studies indicated that ruminants receiving diets containing up to 5% CMS or 10% CMS showed a decreased digestibility, feed intake and growth (Chen *et al.*, 1981; Potter *et al.*, 1985a; 5b; Fernandez *et al.*, 2009; Lopez *et al.*, 2011). During initial gas production (during the lag phase), 15% CMS diets had an insignificantly lower gas production than the 0% CMS diet. As fermentation continuous, the gas production by CMS containing diets become progressively more than diets with lower CMS inclusion rates. Although at the end of fermentation there is a tendency towards higher gas production for 15% CMS samples than 0% CMS samples, the difference remained insignificant.

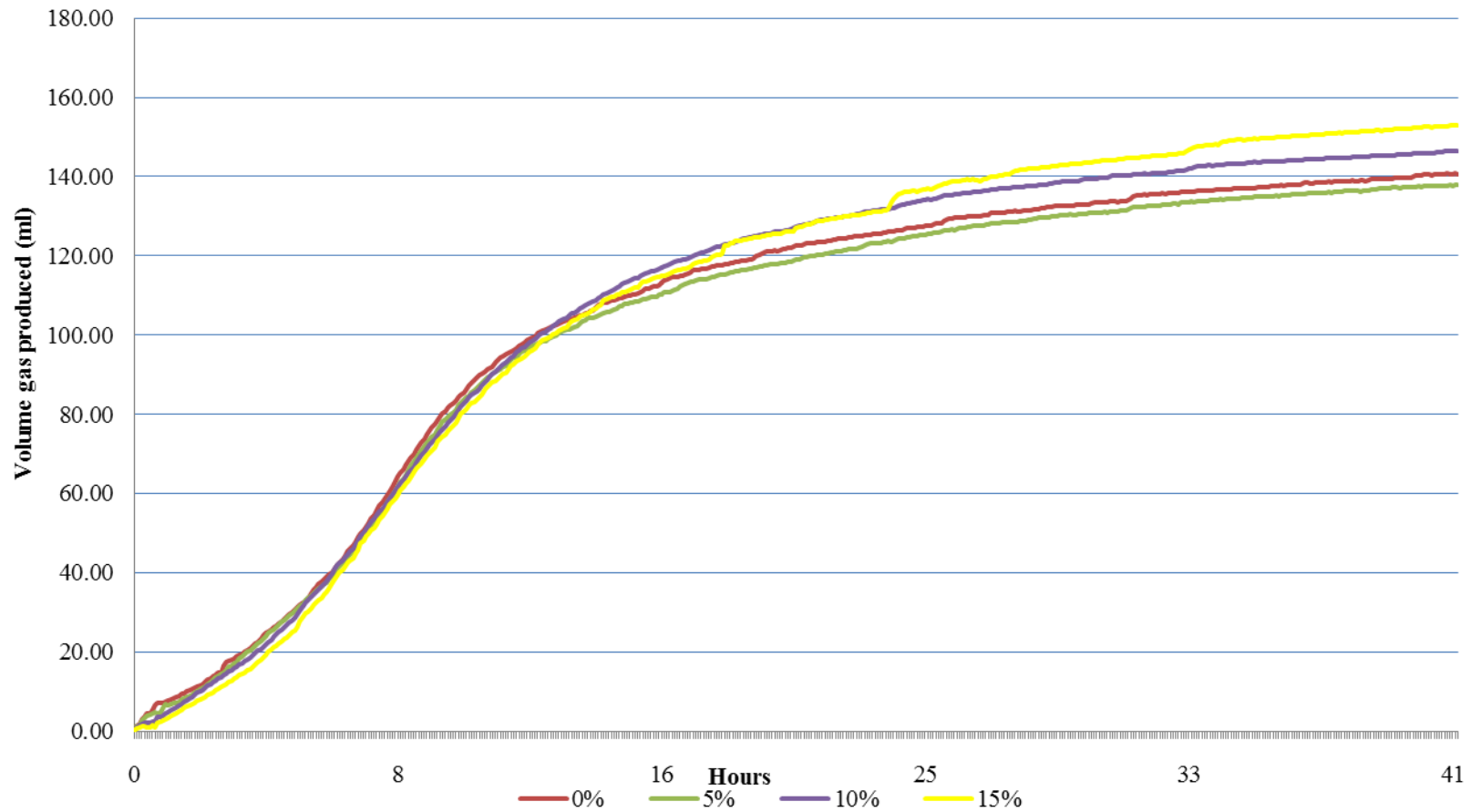


Figure 4.1 Cumulative gas production of trial diets over a 42 hour fermentation period (n=16). Control: Contains no CMS and 15% molasses; Treatment 1: Contains 5% CMS and 10% molasses; Treatment 2: Contains 10% CMS and 5% molasses; Treatment 3: Contains 15% CMS and no molasses
Differences not significant ($P>0.05$)

Only a few *in vitro* studies have been conducted with CMS where an inoculant from the rumen was used to simulate fermentation. Most research on CMS has been done involving *in vivo* studies. It is therefore difficult to compare the results from the current *in vitro* study with other *in vitro* studies. As a result the results from the current *in vitro* study were compared to results from *in vivo* trials. In an *in vitro* study, Hannon & Trenkle (1990) compared microbial digestion between diets containing as N sources, either urea with molasses or CMS. The study was done on both concentrate and roughage diets. Microbial protein was measured as indication of N use and fermentation capability of the diets. Hannon & Trenkle (1990) found that on a roughage diet there were no significant differences ($P > 0.05$) in microbial digestion between N sources. However, when CMS and urea were compared in the concentrate diet, performance tended to be better on the CMS compared to the urea and molasses diet (Hannon & Trenkl, 1990). Results indicated that on an *in vitro* base CMS had the potential to replace molasses and urea as ingredients in TMR diets without sacrificing fermentation activity.

During the current study, gas production during the fermentation period was used as a measure of microbial digestion of the TMRs into nutrients. Gas production by micro-organisms in the rumen is mainly due to the fermentation of carbohydrates with the synthesis of the VFAs, acetate, propionate and butyrate, while gas produced from protein fermentation is relatively low, and from fat, negligible (Wolin, 1960). By using the significant correlation ($P < 0.05$) between volume of VFA production and volume of gas production (Beuvink & Spoelstra, 1992; Blummel & Ørskov, 1993; Van Soest, 1994), gas volume can be used as an indication of VFA production and thus how efficient the TMR is fermented.

Considering the total gas production and its correlation with VFA production, it can be suggested that an increase/decrease in gas production is accompanied by corresponding increase/decrease in VFA production. Based on this suggestion the current study did not indicate any significant differences in gas production or VFA production between CMS treatments. It is concluded that the inclusion of CMS at different levels in TMR diets did not affect fermentation differently from that of molasses

Table 4.4 Degradation rate (mL/hour) and effective degradation of *in vitro* gas production between treatment containing 0%, 5%, 10% and 15% CMS respectively over 42 hour fermentation period (mL) (n=16)

Hour	Experimental diets containing condensed molasses solubles at: ¹			
	0%	5%	10%	15%
0	-11	-14	-18	-15
2	10	7	4	8
4	27	26	23	29
6	43	42	40	47
8	57	57	55	62
10	69	69	68	76
12	79	80	80	87
16	97	99	99	107
20	110	113	114	121
24	120	125	126	132
30	131	137	139	144
36	139	146	148	152
42	143	152	155	157
Eff degr²	137	146	149	149

¹Treatments vary in the amount of CMS in the diet. Control: Contains no CMS and 15% molasses; Treatment 1: Contains 5% CMS and 10% molasses; Treatment 2: Contains 10% CMS and 5% molasses; Treatment 3: Contains 15% CMS and no molasses.

²Effective degradation

Results of the current study are supported indirectly by several studies that showed a general trend that at an inclusion of up to 10% CMS, there was no difference in ruminal total VFA concentrations between CMS and molasses containing diets (Krzeminski *et al.*, 1984; Leontowicz *et al.*, 1985; Potter *et al.*, 1985).

Leontowicz *et al.* (1984b) compared diets containing 27% CBS (condensed beet molasses solubles) with a diet containing molasses and no CBS. Determination of VFA concentration in the rumen before feeding in the morning showed that CBS diets resulted in a higher ($P < 0.05$) total VFA concentration of 43.3 mmol/dm³ compared to 35.2 mmol/dm³ for the molasses diet. Two hours after morning feeding the fermentation results differed, with no difference ($P < 0.05$)

between CBS and molasses diets. These results illustrated that initial (short term) differences in VFA production (and thus gas production) may occur between CMS and non-CMS diets, but over time (long term) these differences are minimized and no difference occurred. Considering the results of the above mentioned studies and the current gas production study, CMS seems to result in similar ruminal VFA production and fermentation efficiency.

As mentioned before there is a good correlation between gas produced and VFA concentration in the rumen. Therefore, substrates that produced a higher volume of gas suggest increased fermentation efficiency and digestibility of feed than substrates with a lower volume gas. This is not always the case. Gas produced can be divided into either gas produced as a direct result of fermentation and production of VFAs or indirectly from buffering of low pH caused by VFA. Direct gas production is accompanied mainly by the production of acetate and butyrate, whereas direct gas production from propionate is relatively unsubstantial. Propionate production mainly contributes indirectly to gas production due to the production of carbon dioxide as a result of buffering (of low pH caused by propionate synthesis) using sodium bicarbonate (NaHCO_3) (Wolin, 1960). As a review by Getachew *et al.* (1998) indicated, samples used in gas production studies with higher gas production volumes had a higher acetate : propionate ratio than samples with lower gas production. These results suggested that an increased gas and VFA production did not necessarily lead to an increased efficiency of fermentation of the TMR.

During the current trial 15% CMS TMR had a slightly higher gas production and degradation rate than molasses diets, although this difference was statistically insignificant (Table 4.3 and Table 4.4). According to the mentioned research earlier, this slight difference of 15% CMS diet with 155 mL in comparison to the 140 mL of the control might suggested an increased efficiency in fermentation due to higher VFA volume production, but could also be accompanied by an increased acetate : propionate ratio. During a study with cattle fed 27% CBS diet, Leontowicz *et al.* (1985) indicated that, although after morning feeding VFA production was similar between CBS and molasses diets, CBS diets resulted in higher concentrations of ruminal acetate. Potter *et al.* (1985b) measured VFA production in finishing cattle fed up to 15% CMS in their diets for 56 days. At the end of the experimental period the acetate concentration has an increase production relatively to propionate, therefore increasing the acetate : propionate ratio. The suggestion of higher acetate in CMS treatments is supported by *in vivo* reviews by other researchers who recorded higher acetate production in CMS diets than in control diets

containing molasses or no molasses (Krzeminski *et al.*, 1984). Although the molasses and CMS diets indicated equal fermentability (by measuring gas production) during the current trial, it is still unclear what the real VFA volume and acetate : propionate ratio was. What sets the current study apart from previous studies is that the energy, protein and moisture levels were formulated to be similar between the respective diets. The results therefore show that if the energy, protein and moisture are corrected for in TMRs, detrimental effects of CMS can be avoided. In order to validate the findings of the gas production research, corresponding VFA determination needs to be done with gas production studies to indicate the acetate : propionate ratios in the total gas produced. Furthermore, *in vitro* studies need to be done in tandem with *in vivo* to provide a more practical indication of VFA production from CMS.

4.2.3 Composition of gas

Composition of the gas was measured to establish if the fermentations of the different diets resulted in variations in gas composition, and specifically if the concentrations of H₂S differed. The different diet samples were incubated for 24 hours, resembling more realistic incubation times of feedlot TMR's. To acquire the composition (volume) of gasses at 24 hours of fermentation, the percentage of gases measured was used with the total gas production values measured in the gas production study. Production of CO₂, H₂S, N and methane (CH₄) measured in mL volume is illustrated in Table 4.5 and Figure 4.2

Table 4.5 Volume (mL) of carbon dioxide, hydrogen sulphide, nitrogen and methane (mL) between treatments fermented over a 24 hour period (n = 16)

Gas ¹ (mL)	Experimental diets containing condensed molasses				SEM ²
	0%	5%	10%	15%	
CO ₂	99	95	106	105	0.24
H ₂ S	2	3	3	3	0.05
N	3	4	4	3	0.51
CH ₄	20	20	22	22	0.18

¹Gas composition volume (in mL) calculated by multiplying the percentage gas as measured with the total gas production at 24 hours for the respective treatment using the value obtained from total gas production trial. CO₂=Carbon dioxide; H₂S= Hydrogen sulphide; N= Nitrogen; CH₄= Methane

²Standard error of the mean.

Production of CO₂, H₂S, N and CH₄ did not differ significantly ($P>0.05$) between treatments. The results were not expected as treatment diets vary in mineral content, and slight differences which could have caused differences in gas production.

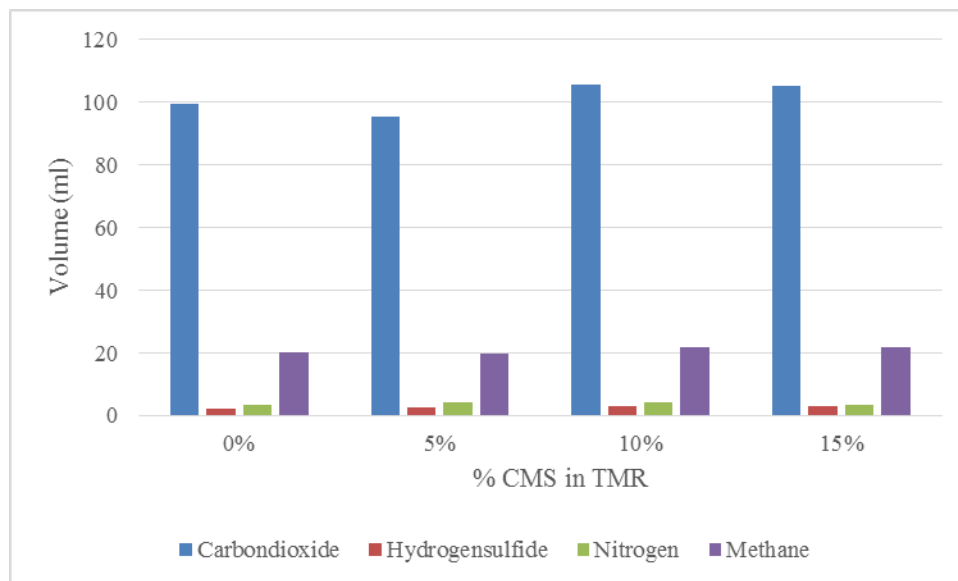


Figure 4.2 Carbon dioxide, hydrogen sulphide and methane gas measurement (measured in mL) between treatments over a fermentation period of 24 hours. Treatments were equal in energy and protein with 0%, 5%, 10% and 15% CMS in control, Treatments 1, Treatments 2 and Treatments 3 respectively (n=20).

4.2.3.1 Carbon dioxide

Carbon dioxide production was measured as a percentage and converted into volume using the total gas production profiles determined in the current study. The production of CO₂ did not differ between treatments ($P > 0.05$). Results are as expected due to the total gas production being the same between treatments.

Throughout fermentation, gas is produced due to the fermentation of feed and the synthesis of VFA. The majority of this gas is produced directly from acetic acid production while propionic acid contributes indirectly in the form of CO₂ as a result of buffering in the rumen (Wolin, 1960). During ruminal fermentation the more soluble and fermentable fraction such as starch is fermented first, producing largely propionate (Doane *et al.*, 1997). The increase in propionic acid is followed by a decrease in pH which is naturally buffered in animals with sodium bicarbonate (NaHCO₃) (Kohn & Dunlap, 1998). Bearing in mind the correlation between gas, CO₂ and propionate production, diets producing more propionate should in theory produce a higher volume of CO₂. Therefore the equal volumes of CO₂ levels in the current study suggest that there was no difference in the amount of propionic acid produced within 24 hours of fermentation.

Using the established relationship between ruminal CO₂ and propionate production (Beuvink & Spoelstra, 1992; Blummel & Ørskov, 1993; Van Soest, 1994), the CO₂ production in the current study can be used as an indirect indication of propionate production of the gas production profiles, even though VFAs were not measured directly. The CO₂ : propionate relationship also allows as an indicator (not a measured indicator) of the effect of CMS on ruminal VFA production in the current study with *in vivo* results of other studies.

Fernandez *et al.* (2009) reported similar the results in the current study by studying\ the effect of CMS on ruminal VFA production when diets of sugar beet pulp with or without CMS were fed to sheep. Volatile fatty acid measurements were taken before feeding, three hours- and nine hours after feeding. Although CMS has less energy than molasses, results indicated that there were no differences in propionate concentration between diets fed 130 g CMS and diets fed no CMS at any period after feeding. The similar propionate production could have been caused by the sugar-rich beet pulp diet that compensated for the energy differences between the diets

with CMS and without CMS. The effect of CMS and its low energy value was illustrated by Leontowicz *et al.* (1984). During the trial Leontowicz *et al.* (1984) fed cattle diets containing no CMS or diets containing 27% CMS without compensating for the lower energy value of CMS. Testing for ruminal VFA concentration directly after feeding indicated lower propionate in the CMS treatments than in the control, but after 2 hours of feeding the difference diminished between CMS and control diets. Results from Leontowicz *et al.* (1984) suggested that initial fermentation of CMS diets lacks the amount of soluble or readily fermentable carbohydrates, but a characteristic of CMS enables compensatory fermentation over time. Therefore the initial propionate and CO₂ production may be lower, but the total amount of propionate produced from CMS diets was equal at later stages of fermentation.

Potter *et al.* (1985b) found contrasting results when feeding diets that are iso-nitrogenous but not equal in energy. Diets containing 5% CMS diets resulted in a decreased concentration of propionate in the rumen from 44.3 mol/100 mol to 38.3 mol/100 mol. The study by Potter *et al.* (1985b) indicated that if available energy is not corrected for during formulation of diets containing CMS, significant differences in propionate production would occur.

From the previous studies it is clear that when substituting molasses with CMS, the energy content needs to be increased or significant differences in CO₂ and propionate production would occur. In the current study the diets were iso-energetic and iso-nitrogenous. Although CMS has a lower energy value than molasses, the energy was made up by other energy sources due to the formulation of the diets as seen in Table 4.2. With energy values of the diets being similar, the amount of CO₂ (and therefore propionate) was expected to be similar. Another possible contributor to the similar CO₂ (and propionate) production between treatments and control is the ability of CMS to improve NDF and fibre digestion (Chen *et al.*, 1981). Increasing the breakdown of NDF increases exposure of the more fermentable content of grains to fermentation and can slightly compensate for lower energy values of CMS.

The current study therefore indicates that, to maintain similar VFA production (indicated by CO₂ production), energy values of the whole diet need to be similar by correcting for the lower energy value of CMS. This is indicated by the similar CO₂ production between CMS and molasses TMR's. The results of similar CO₂ production are mirrored by the total gas production that indicates the need to correct for energy in CMS.

4.2.3.2. Hydrogen sulphide gas

Hydrogen sulphide gas was measured to determine the effect of S in CMS diets on producing high amounts of toxic H₂S. Results of H₂S gas production are illustrated in Table 4.5 and Figure 4.2. There was no significant difference ($P > 0.05$) between the treatments. Considering the slight differences in S in the diet formulations (Table 4.2), differences in H₂S were expected. But as seen in the TMR composition analysis for S concentration, the current results could have been expected due to the actual low differences in S in the TMRs. The results possibly indicate that the variation and inconsistency in CMS composition could cause difficulties in determining the effect of CMS ash content.

Few studies have been conducted in ruminants consuming CMS where the H₂S production was measured. The majority of research has been done on diets containing no CMS but were high in S. In *in vivo* trials it has been observed that where diets high in S have been used, H₂S production increased, indicating the potential of high S diets to produce H₂S (Kung, 2008).

Quinn *et al.* (2009) conducted a study during which H₂S gas was measured between diets without or with added S equivalent to 0.42% of the dietary DM. Quinn *et al.* (2009) recorded a significant increase ($P < 0.05$) in H₂S, from 2.35 $\mu\text{mol/g}$ of fermentable DM to 8.21 $\mu\text{mol/g}$, when diets contained added S. Kung *et al.* (2000) conducted an *in vitro* fermentation trial to measure gas production between a control containing 29 g S/kg DM and a diet containing 109 g S/kg DM. The H₂S gas concentration increased with higher S levels. Additionally Kung *et al.* (2000) reviewed the literature and concluded that many researchers supported the concept of increased H₂S production with higher dietary S levels (Bulgin *et al.* 1996; Low *et al.*, 1996; Hill & Ebbett, 1997; McAllister *et al.*, 1997; Loneragan *et al.*, 1998; Kul *et al.*, 2006).

The established relationship between H₂S and increasing dietary S does not resemble the same relationship within the current study, but can be explained by the actual amount of S in TMRs, the interaction between S compounds, pH and pKa values and interactions with other nutrients. During the formulation of the sample TMRs containing different levels of CMS it was expected that S would increase with higher CMS inclusions (Table 3.1). After the chemical analysis of the TMRs, the actual S concentrations in the TMRs differed from the levels in the

formulation (Table 3.1). Due to the differences in formulation and actual S in the TMRs, difference in H₂S could not be well established between increasing levels of CMS.

Odom & Singleton (1993) suggested that any increase in concentration of H₂S in the gas is mainly due to the increased activity of sulphur-reducing bacteria (SRB) that use SO₄ and other S compounds for energy. However, SRB are sensitive for pH levels due to the pK_a values of S compound being between 7.0 and 7.2. Therefore, at a decreasing pH, more S (mostly in the form of SO₄) is converted into H₂S (Beauchamp *et al.*, 1984). The effect of pH results in a negative correlation (Morine *et al.*, 2014) between H₂S production and pH. During the current study pH was regulated by the addition of a buffer solution to maintain a standardized pH at 6.8 and to prevent pH from decreasing and influencing fermentation. At a high pH the activity of SRB can be reduced to such a state that even though CMS diets contained more S, the H₂S production between treatments would be the same. The result is that there is no difference in H₂S production between the treatments.

The effect that CMS has on NDF digestibility further emphasizes the negative relationship between H₂S and pH. It is well established that a higher NDF content or digestibility increases the pH (Morine *et al.*, 2014) resulting in the amount of H₂S in the rumen to decrease. The ability of CMS to increase NDF digestibility has been illustrated by Fernandez *et al.* (2009). When sheep were fed a control diet with no CMS and a diet containing 130 g CMS/kg DM the digestibility of NDF increased in the CMS diets. The increased NDF digestibility was supported by Chen *et al.* (1981), Leontowicz *et al.* (1984b) and Chen *et al.* (2011a). It was suggested that the increased NDF digestibility of CMS is due to its high betaine content (Rink *et al.*, 2011). Betaine is a trimethyl derivative of the amino acid glycine and is able to increase microbial fermentation of NDF (Weidmeier *et al.*, 1992; Rink *et al.*, 2011) thereby indirectly regulating pH. The betaine content of CMS decreases H₂S production further by its ability to bind S and be converted into S containing amino-acids. By binding the S, the betaine can actively reduce the amount of S-substrate available to be converted into H₂S.

4.2.4 Conclusion

The importance of conducting *in vitro* trials is to establish any differences in ruminal fermentation between treatment diets containing 5%, 10% or 15% CMS or a control without CMS without any animal factors (such as palatability or rumen environment) playing a factor. As a result an *in vitro* study can indicate the potential of CMS in TMR's.

During the total gas production measurement, no significant differences were observed between the control and TMR diets at 5%, 10% or 15% CMS inclusion rates at any point during 42 hours of fermentation. Due to the high correlation between ruminal VFA and gas production, the similar gas production indicates equal fermentation of TMRs irrespective of CMS inclusion rate. Gas production profiles were supported by the effective degradation rate that showed no significant difference ($P>0.05$) between treatments. Although gas production was similar between the control and CMS TMRs, and thus the total VFA production, the composition of the VFA needs to be considered to determine how efficient fermentation was. In theory, if acetate would have differed, gas production at later stages of *in vitro* fermentation would have been higher with more acetate production. After combining the gas production and composition measurements at 24 hours of fermentation, CO₂ production suggested similar results to the total gas production with propionate levels that did not differ between CMS and control diets. These results from an *in vitro* study suggested that CMS can successfully replace molasses without compromising performance.

Additionally, the H₂S gas was measured to determine the effect of S in CMS on producing high amounts of the toxic gas. After chemical analysis of the diets, there were only small differences in the S concentrations between the diets containing CMS and molasses. The chemical analysis did not resemble the formulation (Table 3.1) that indicated larger differences in S between CMS diets and the control, further indicating the variation in CMS composition. Even though S differences between diets were small, small differences in H₂S concentration were expected. But there were no differences between the treatment and the control, indicating low activity of SBR in all treatments. A possible reason that H₂S concentrations did not differ between treatments is that pH conditions during the *in vitro* fermentation were regulated above a

pH where SRB activity would produce significant amounts of H₂S. The result can indicate a possible solution towards effectively handling the S levels is CMS.

4.3 *In vivo* trial under feedlot conditions

4.3.1 Feed analysis

During the feedlot trial four treatment diets were formulated; control, treatment 1, treatment 2 and treatment 3 each containing 0%, 5%, 10% and 15% CMS, respectively. To determine differences in performance owing to CMS content, diets were made iso-energetic and iso-nitrogenous. Condensed molasses solubles has a high moisture content and contributes to a lower DM content of the treatment diets. Therefore water was added to diets to try and make the DM content as similar as possible between diets. As a result, nutrients were diluted causing lower nutrients per kilogram. Results of diet analysis are illustrated in Table 4.6. As seen in Table 4.6 iso-nitrogenous, iso-energetic levels were obtained between the samples. Although an attempt was made to balance moisture there was a slight drop in moisture for 15% CMS inclusion. This might be a result of moisture that evaporated from the feed samples. What is interesting to note is that the ash content remains similar between all inclusion levels of CMS when it was expected to increase due to the high ash content of CMS (Weigand & Kirchgessner, 1980; Stemme *et al.*, 2005). With higher CMS inclusion rates there was a slight increase in S between the diets, but not as big a difference as would have been expected, and further indicates the variation of CMS composition and the effect on diet formulations.

Table 4.6 Chemical composition of treatment diets. Each treatment was formulated to be equal in energy and protein, only varying in CMS content

Nutrient ¹	Experimental diets containing CMS at:			
	0%	5%	10%	15%
DM (g/kg)	853.25	885.22	850.14	805.61
ME(MJ/kg DM) ²	12.55	12.32	12.22	12.05
CP (g/kg DM)	138	152	134	138
CF (g/kg DM)	93.6	109	82.7	102
Fat (g/kg DM)	29	26.8	28.2	14
Ash (g/kg DM)	94.3	101	116	110
K (g/kg DM)	12.7	14.6	12.8	15.2
S (g/kg DM)	2.7	3.22	5.54	4.67

¹DM= Dry matter; ME= Metabolisable energy; CP= Crude protein; CF= Crude fibre; Ca= Calcium; P= Phosphorus; K= Potassium; S= Sulphur

³ME calculated ME= ((15.21*CP) + (12.8*CF) +(34.2*EE) + (15.9*NFE)) (MAFF, 1975; Kaustell *et al.*, 1997)).

4.3.2 Weight gain

Body weights were measured every second week over a period of 112 days, namely at 0, 14, 28, 42, 56, 70, 84, 91, 98 and 112 days. Treatment groups were compared to determine differences and patterns of growth. Body weights over the different days are illustrated in Table 4.7 and Figure 4.3.

Table 4.7 Mean body weight (kg) of feedlot cattle over a period of 112 days (n=100)

Day	Experimental diets containing CMS ¹ at:				SEM ²
	0%	5%	10%	15%	
0	251	261	258	257	1.8
14	286	288	282	285	2.6
28	310	314	313	310	2.3
42	338	328	337	338	3.4
56	369	369	372	371	3.2
70	408	404	401	408	3.2
91	440	435	442	442	3.1
98	456	453	455	456	3.6
112	477	473	476	476	3.5
Total weight gained (kg)	218	212	217	219	

¹ CMS: Condensed molasses solubles

² Standard error of the mean.

Differences between diets over 112 days were not significant ($P > 0.05$)

Weight gain (kg) did not differ ($P > 0.05$) between treatments at any period during the feedlot trial and indicated no difference in the pattern of growth. Tests for the interaction between days and diet were insignificant ($P > 0.05$), illustrating that weights did not differ between treatments over time. Results are unexpected as with results from other studies, it was expected that weight gain would decrease at high levels of CMS inclusion (Chen *et al.*, 1981; Potter *et al.*, 1985a; Potter *et al.*, 1985b; Fernandez *et al.*, 2009; Lopez *et al.*, 2011).

Results from the current study are supported by Tillman & Kidwell (1951) who conducted two studies with diets containing no CMS, a 25%-75% CMS-molasses mixture or a 50%-50% CMS-molasses mixture. However, although Tillman & Kidwell (1951) formulated diets to be iso-energetic and iso-nitrogenous in this study, the diets containing CMS were not fed *ad libitum* and no differences in growth between treatments were observed.

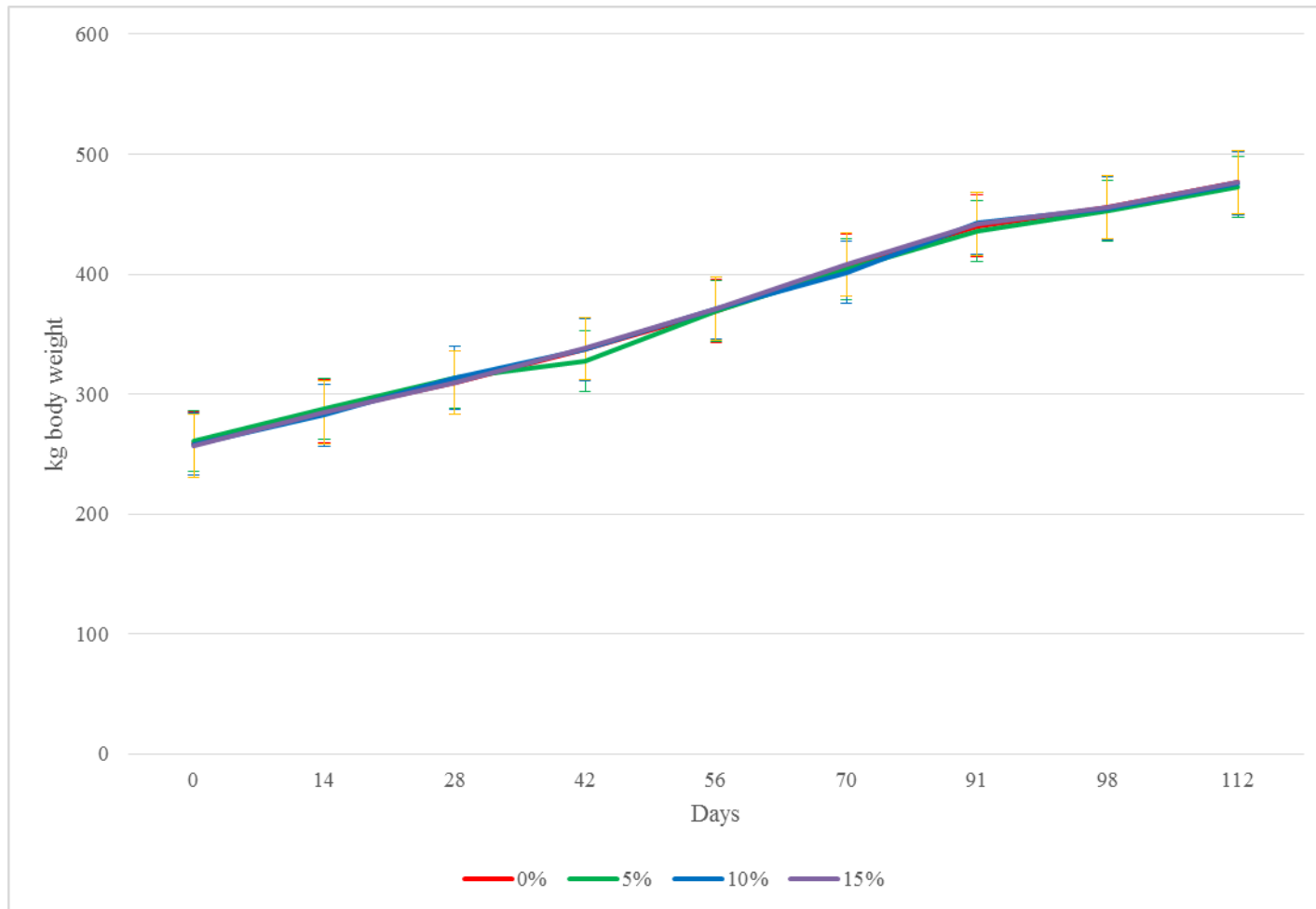


Figure 4.3 Cumulative body weights of feedlot cattle over a period of 112 days (n=100). Cattle were fed treatment diets made iso-energetic and iso-nitrogenous and vary in CMS content. Cattle were fed diets were formulated to vary in CMS concentration with Control, treatment 1, treatment 2 and treatment 3 containing 0%, 5%, 10% and 15% CMS respectively. Error bars represent the SEM

A general trend in trials measuring weight gain differences between diets with or without CMS was that protein levels were constant between trial diets, but differences in available energy were not corrected for. Chen *et al.* (1981) fed citrus CMS at 0%, 7%, 14% and 20% inclusion rates in trials with cattle and sheep. No adverse effects on growth were observed in cattle or sheep up to 14% CMS, but lower weight gains were observed above an inclusion of 14% CMS.

Various studies have been conducted with diets including CMS during which the diets were not iso-energetic. Potter *et al.* (1985a) conducted two studies with feedlot cattle received sugarcane CMS in their diets, namely diets containing 0%, 5%, 10% and 15% CMS and equal levels of protein. However, no information was provided whether energy levels were balanced between the treatments. Over the 56 days feeding period, at inclusion rate of above 5% CMS, weight gains decreased ($P > 0.05$) with ADGs of 0.83, 0.58, 0.37 and 0.35 kg/day for the diets containing 0%, 5%, 10% and 15% CMS, respectively. During the 56 days cattle fed 15% CMS diets performed so poorly that they had to be placed on basal diets, where they experience compensatory growth, indicating the poor performance of cattle on the 15% CMS diet. However, when one of the studies was extended to 86 days the difference in weight gain diminished, possibly indicating the improved growth of cattle on high CMS diets over longer periods. Potter *et al.* (1985b) studied the effect of CMS fed to cattle at 5% inclusion rates with or without molasses and found no difference in growth. A follow-up study with higher CMS inclusion rates resulted in a decrease ($P < 0.05$) in the ADG when cattle were fed diets containing 10% CMS. Similarly, decreased weight gains were illustrated by Lopez *et al.* (2010) when cattle were fed CMS diets and concluded that the probable cause of lower performance was due to the lower energy available when substituting molasses directly with CMS without correcting for energy differences. As seen from the mentioned studies, results showed a general trend when CMS was included beyond 5% where energy levels were not balanced, which resulted in lower growth rates than on diets containing molasses and no CMS. During the mentioned studies the effect of the DM content of CMS on performance was not considered important or was ignored.

During the current study no differences between treatments were observed in weight gain. Considering that the diets were made iso-energetic and iso-nitrogenous (and therefore formulated to compensate for CMS lower energy value in comparison to molasses), it could be assumed that the lower energy content of CMS could have contributed to lower growth rates in

diets where the energy contents of the treatments were not balanced. Average daily gains in South African feedlots vary, starting from 1.5 kg/day (Agri benchmark, 2012) and with about 15% of cattle gaining 2 kg per day (Meissner *et al.*, 1995). At mean ADGs of 1.97 kg, 1.91 kg, 1.99 kg and 1.95 kg for the control, 5%, 10% and 15% CMS containing TMR's, respectively, growth rates were acceptable for feedlot cattle considering the lower than normal DM contents of the diets. Although the ADG were acceptable, the efficiency in which this growth has been achieved needs to be considered to determine if this growth was economically acceptable. Furthermore the breed composition of cattle varied. Although an attempt to negate the breed variety with grouping of cattle according to weight, any differences in weight gain would have been clearer if the feedlot cattle consisted of a single breed (and of the same frame size).

4.3.3 Feed intake

Feed intake was measured every week, but only measurements corresponding with weight measurements are illustrated in Table 4.8 and Figure 4.4. Feed intake was measured twice a week over the 112 days in order to detect if factors such as palatability influenced feed intake.

Feed intake remained the same ($P > 0.05$) between treatments throughout the study. Initial feed intake illustrates that there was no differences in feed intake between the diets containing different levels of CMS. With no difference in initial feed intake, it is suggested that palatability of CMS diets did not influence feed intake of the animal. Diet and day interactions were insignificant ($P > 0.05$), indicating that over time no difference in feed intake was recorded between the treatments. The results are as expected since the energy levels did not differ between diets with or without CMS. It was expected that initial feed intake might differ due to the palatability problems or due to the high ash content of CMS.

Table 4.8 Mean daily feed intake of feedlot cattle over periods during the 112 day experiment on as fed basis. (n=20/treatment).

Day	Experimental diets containing CMS ² at:				SEM ¹
	0%	5%	10%	15%	
14	10.7	11.7	11.4	11.8	0.5
28	12.0	13.1	13.1	13.9	0.2
42	13.4	13.3	12.2	12.9	0.2
56	13.6	13.6	12.8	12.7	0.3
70	15.1	14.4	14.2	13.4	0.3
91	14.7	14.8	14.7	14.3	0.4
98	14.1	13.6	13.	13.4	0.2
112	13.1	15.5	15.3	13.6	0.4

Feed intake was measured as a group average and calculated as kg feed per animal per day

¹Standard error of the mean.

²CMS: condensed molasses solubles.

Differences were not significant ($P>0.05$).

Feed intake is dependent on the digestibility and palatability of a feed. Higher digestibility allows feed to be digested/fermented faster and that would tend to increase the passage rate of the feed (Blaxter & Wilson, 1962; Hungate, 1966). Therefore when the passage rate increases rumen fill decreases, allowing space for more feed to be ingested (Balch & Campling, 1962; Ellis, 1978).

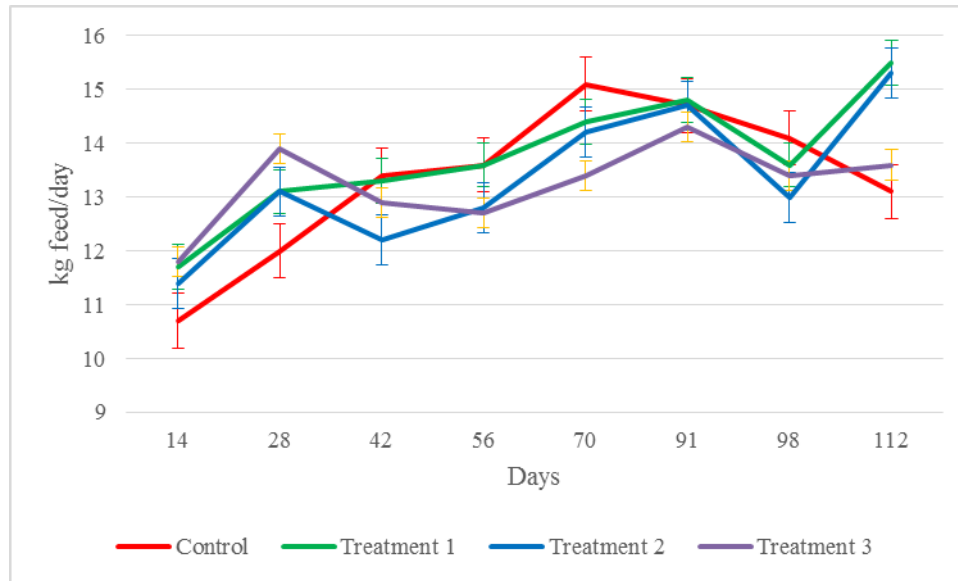


Figure 4.4 Feed intake of feedlot cattle fed control, treatment 1, treatment 2 or treatment 3 TMR diets containing 0%, 5%, 10% or 15% CMS respectively (n=20). Measurements were made over 112 days and measured as an average of the group in a pen. Error bars represent the SEM of measurements.

Fernandez *et al.* (2009) conducted a study to determine the preference of sheep for CMS containing diets as well as the voluntary intake and digestibility of CMS diets. During the study, results indicated a preference for CMS diets, but with no increase in feed intake. When measuring diet digestibility there was an increase in NDF and CP digestibility, but the higher digestibility was not reflected in the feed intake and total tract digestibility between diets with or without CMS. Similar trends were observed in studies where CMS was included above 5% of the diets which resulted in increased NDF digestibility, but with no increase in feed intake (Tillman & Kidwell, 1951; Leontowica *et al.*, 1984)

Condensed molasses solubles is lower in energy and higher in ash than molasses. When CMS substitutes molasses directly without correcting for nutrient differences, lower voluntary feed intake could result. Lopez *et al.* (2010) recorded lower feed intakes when CMS substituted molasses without correcting for energy or protein. Due to the lower energy concentration in CMS and high ash content in comparison with molasses, it can be suggested that differences in feed intake were due to differences in nutrient concentrations between CMS and molasses. The result was partially supported by Potter *et al.* (1985a) when only protein was corrected for in

diets containing CMS. Although the ADG differed with increasing CMS levels of 0%, 5%, 10% or 15% CMS, feed intake remained the same. In a following study Potter *et al.* (1985b) indicated that when CMS is included at 5% or 10% no differences in feed intake were observed in cattle. The general trend is that if nutrients are correct for (balanced for lower energy in CMS) when substituting molasses with CMS no difference in feed intake was observed (Chen *et al.*, 1981)

During the current study diets were formulated to be iso-energetic and iso-nitrogenous to determine the effect of CMS on feed intake. As indicated in Table 4.8 no differences in feed intake were observed at any point. Concerning the initial feed intake at 14 days it can be suggested that palatability of CMS did not influence the current study, as supported by Fernandez *et al.* (2009). Because feed intake between different CMS diets was the same ($P>0.05$) results can suggest that total tract digestibility of TMRs containing 0%, 5%, 10% or 15% CMS remained the same. Additionally, because the day and diet interactions were the same between diets varying in CMS ($P>0.05$), feed intake will not vary if the feeding period was extended. Results of feed intake during the *in vivo* study supported the *in vitro* gas production study where gas production (and therefore fermentation of diets) did not differ between treatments with the corresponding CMS inclusions. Considering that it has been reported that CMS in diets increased NDF digestion (Leontiwicz *et al.*, 1984; Fernandez *et al.*, 2009) probably due to the betaine content of CMS, it is strange that feed intake did not increase. Although increased NDF digestion did not influence feed intake, VFA production could have been influenced. Therefore FCR needs to be considered to explain cases where feed intake remains equal, but ADG was reduced with high CMS inclusion rates.

4.3.4 Feed conversion ratio

Feed conversion ratio was calculated by using the corresponding weights and feed intakes over the 112 days. Differences in feed intake and weight gain need to be compared to the FCR to determine how efficient growth and feed intake was. Results of FCR measurements are illustrated in kg feed per kg weight gain by Table 4.9 and Figure 4.5.

Table 4.9 Feed conversion (kg feed/kg weight gain) ratio over a period of 112 days of treatments diets differing in condensed molasses solubles (CMS) content (n=20)

Day	Experimental diets containing CMS at:1				SEM ²
	0%	5%	10%	15%	
42	6.98	7.06	6.52	7.1	0.17
56	6.59	6.94	5.98	6.38	0.1
70	6.23	6.58	6.16	6.04	0.09
91	6.27	7.28	6.52	6.62	0.1
98	6.86	7.08	6.54	6.62	0.1
112	7.04	6.78	6.32	6.34	0.083

¹Control: Contains no CMS and 15% molasses; Treatment 1: Contains 5% CMS and 10% molasses; Treatment 2: Contains 10% CMS and 5% molasses; Treatment 3: Contains 15% CMS and no molasses.

²Standard error of the mean.

Although diets with increasing CMS content seemed to have lower FCRs over time, there were no significant differences ($P>0.05$) between the treatments. The FCR of the diets are relatively high as a result of the low DM of diets. Due to the low DM, the diets were diluted and required higher feed intake per kg body weight gain.

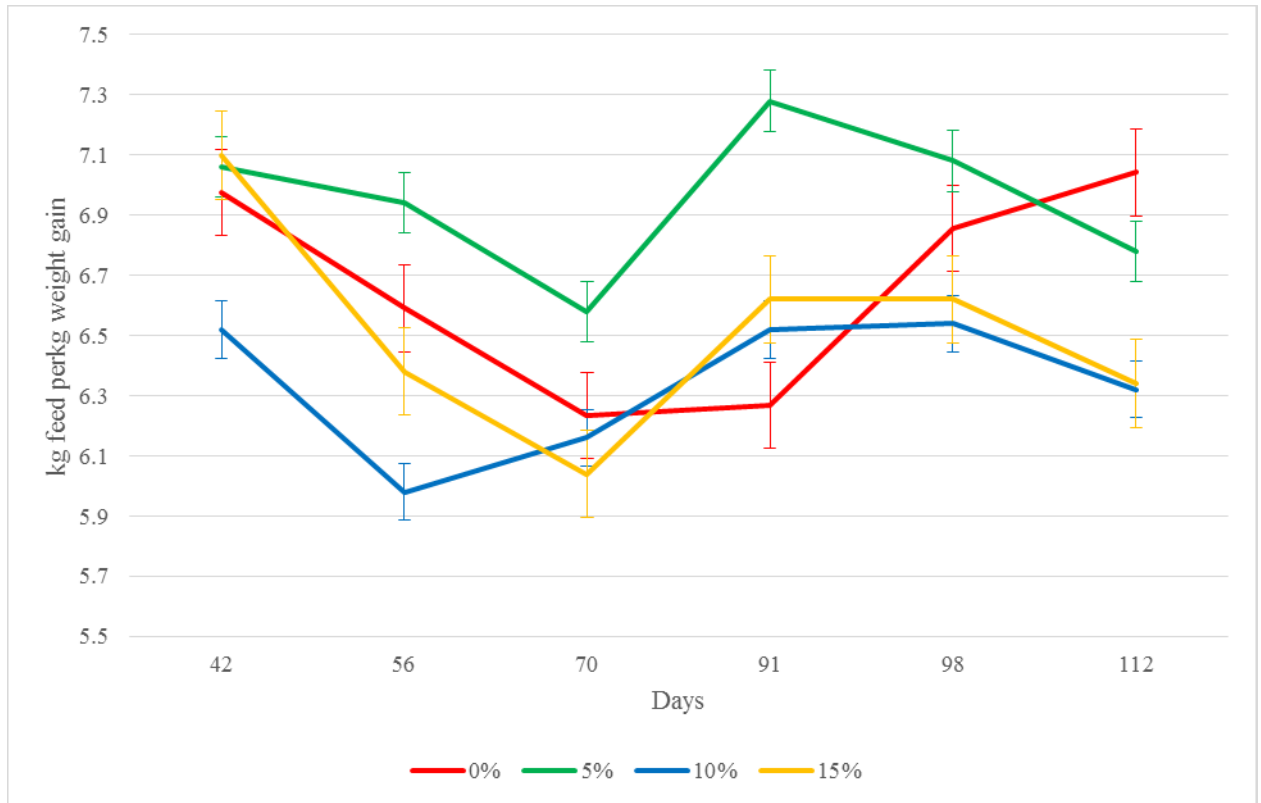


Figure 4.5 Difference in FCR (kg feed/ kg weight gain) of feedlot cattle between treatments over 112 days (n=100). Treatments varied in CMS (condensed molasses soluble) content; Control: contain 0% CMS and 15% molasses; Treatment 1: Contain 5% CMS and 10% molasses; Treatment 2 : Contain 10% CMS and 5% molasses; Treatment 3: Contain 15% CMS and no molasses.

Most studies done on ruminants with CMS diets measured weight gain, feed intake and FCR to determine the efficiency of CMS diets and the results of those studies contrasted the current study. Potter *et al.* (1985a) conducted two trials during a study over different lengths of time. During 56 days of feeding, feed intake remained the same among treatments of 0%, 5%, 10% or 15% CMS. Average daily gain was lower and the corresponding FCR was higher for diets with CMS with FCR values of 10.7, 14.0, 21.6 and 20.2 dry feed/gain for 0%, 5%, 10% and 15% CMS diets, respectively. When the trial was extended to 86 days, ADG became equal for all treatments, but the difference in FCR remained. The difference in FCR correspond with Potter *et al.* (1985b) who tested the VFA ratios in the rumen among treatments varying in CMS levels. Although acetic acid remained the same between treatments, propionic acid decreased resulting in a higher acetate : propionate ratio. Similar results were reported by Leontowics *et al.* (1984)

who gave 15% CMS to sheep and found a decrease in propionate and constant acetate concentration, resulting in a higher FCR. It was reported that lower performance of animals with higher CMS diets was due to lower amount of glycogenic substances resulting in higher acetate : propionate ratios and FCR (Kulasek *et al.*, 1984; Lopez *et al.*, 2009).

During the current study there was no significant difference in FCR between the treatment diets. Therefore the FCR of diets did not decrease with CMS inclusion as was the case with previous studies mentioned above. The result is possibly caused by the fact that the diets were formulated to be iso-nitrogenous and iso-energetic. The result of the FCR measurements indicate that if nutrients are corrected for (formulated to be balance in energy and protein), similar efficiencies could be expected between molasses or CMS diets. It should be noted that the FCR is high, an indication that the diets were not very efficiently utilized. This could be as a result of the higher moisture content of the trial diets (due to CMS and addition of water).

Although higher CMS diets showed no statistical difference between treatments ($P>0.05$), it should be noted that statistical analysis of the interaction between diet and time indicated that if the study continued longer, significant differences might have occurred with lower FCR for CMS diets. Even though CMS diets did not differ in FCR with molasses diets, diets should be undiluted in future studies to indicate if the moisture content of CMS could be a possible detrimental factor in a TMR.

4.3.5 Liver selenium and copper concentrations

Concentrations of Se and Cu were measured in the liver to determine if the S concentrations in CMS diets influenced absorbance of Se and Cu. Results are illustrated in Table 4.10.

There was no significant difference ($P> 0.05$) between the liver Se or Cu concentrations between any of the treatments. The results were not anticipated since it was expected that Se and Cu concentrations would have been reduced with more CMS and thus higher S concentrations in the diets (Table 4.10). The results demonstrates again that, in the present study, the inclusion of CMS in the diets did not increase S concentrations substantially in the diets containing CMS, as

expected. The variation in CMS composition and the need to establish nutrient parameters in CMS for accurate feed formulations are once again highlighted.

Table 4.10 Concentrations of selenium and copper in the liver on a DM basis (n=20)

%CMS ¹	Selenium		Copper	
	mg/kg	SEM ²	mg/kg	SEM ²
0%	4.08	0.21	394	22.66
5%	3.94	0.12	365	35.78
10%	3.96	0.19	358	28.22
15%	3.5	0.14	330	39.61

¹0%: Contains no CMS and 15% molasses; 5%: Contains 5% CMS and 10% molasses; 10%: Contains 10% CMS and 5% molasses; 15%: Contains 15% CMS and no molasses.

²Standard error of the mean.

There was no statistical differences ($P>0.05$) between the liver Se and Cu between different inclusion levels of CMS in the diet.

There has been no previous study with CMS that recorded liver Se and Cu concentrations, making comparisons with previous research difficult. It has been well documented that if ruminants are fed diets containing high concentrations of S, the bioavailability of Se (Ivancic & Weiss, 2001; Arthington & Plate 2002) and Cu (Underwood & Scuttle, 1999) is reduced. In the present study the concentrations of both Se and Cu were well above the marginal ranges where deficiencies might occur, *viz.* 0.38 - 0.75 mg Se/kg and 15 - 75 mg/kg (as is basis) (Puls, 1994), suggesting that the bulls received sufficient Se and Cu in their diets. Therefore, even if S affected the bioavailability of these two elements, it is unlikely that S would have precipitated deficiencies in the bulls.

According to literature, trials conducted with high S diets led to lower Se and Cu absorption. During the current study the dietary S concentrations increased with increased CMS in the diet, although not as much as anticipated (Table 4.6). Even so, it was expected that Se and Cu concentrations, as measured in the liver, would decrease due to interactions with S. Although there was a slight decrease in Se and Cu levels (as seen in Table 4.10) with higher CMS levels in diets, there was no significant differences between the diets.

Chapter 5

Conclusion and general discussion

Condensed molasses solubles (CMS) is a waste product in the production of ethanol from molasses. To overcome the problem of this waste product accumulating and polluting the environment, solutions are sought by trying to utilize it. This investigation focused on the possibility of using CMS as a feed ingredient in ruminant diets.

In the current study *in vitro* and *in vivo* trials were done with diets containing CMS to study the performance of CMS on a simulating ruminal level (fermentability) and animal performance level (animal factors). Diets in both trials were formulated to be balanced in energy, protein and moisture between different CMS inclusion levels to try and indicate if CMS can be a valid molasses replacement. Emphasis was placed on balancing diets as any diet in practice should always adhere to certain levels of nutrients. It was difficult to achieve these balanced diets because of the high moisture of CMS and how much it contributed to the total moisture of the TMR's. To achieve the balance, water was added in diets containing molasses to reach the same DM level as diets with CMS and to be able to compare results of different diets with different CMS inclusions. After chemical analysis, balanced TMR's were achieved but as a result of the dilution, all results need to be considered in context to the fact that diets with lower CMS inclusions (more molasses) had water added to try and balance for moisture content. As a result, lower CMS diets could have had different performance results in both *in vitro* and *in vivo* studies without the added moisture (diets with less CMS would have had less moisture without added water). After analysis of the TMRs, ash levels, specifically that of S, were not as expected it to be. Sulphur was similar between all diets where it was expected to be higher with CMS inclusion. The lower than expected S levels were an indication of the variation in CMS composition. This is most likely a result of the different variations in the method to produce ethanol and CMS. For future studies and even the practical use of CMS in the industry, proper characterization and measurement of CMS composition over a range of samples are required to establish a stable CMS composition.

During the *in vitro* study, gas production was measured as an indication as the fermentability of CMS diets at different inclusion levels. Gas production is a direct result of fermentation of TMRs by micro-organisms and total gas measurements served as a good indication of fermentability of TMRs over a time period. Results in the current study indicated that similar amounts of gas were produced and it was concluded that there was no difference in the fermentability of diets with or without CMS. The total gas production was supported by gas composition measurement studies during which no differences occurred in gas composition. Although total gas measurement is an excellent way to measure real time gas production (due to the high frequency of measurements) there is no indication of the VFA composition after fermentation periods. The VFA would have indicated how much gas was contributed by which VFA synthesis, giving further insight into the efficiency of CMS use. Furthermore, to improve the *in vitro* research on CMS, trial setup can be changed a bit to be more indicative. Similar trials can be repeated but each containing a different batch of CMS to allow to identify fermentation efficiency between different sources of CMS. Using an automatic gas measurement procedure takes 48 hours, it is crucial that adequate training is provided in standardizing the procedure to ensure repeatability of the automatic gas production system. If a set of fermentations are started and a slight mistake in setup is made, the failed fermentation simulation of one unit out of the batch can cause the whole batch to be scrapped, usually only noticed after half or the full fermentation period. With a practiced procedure, using automatic gas production measurement can provide a variety of fermentation results that can be very indicative of the fermentability of CMS diets on an *in vitro* level and should be exploited more.

During the *in vivo* feedlot trial there were no differences in feed intake, weight gain or FCR between diets with varying CMS inclusion levels. This is promising as it is an indication that CMS can be a possible substitute for molasses. But although the diets in the study was balanced for nutrients (protein and energy), they were diluted as a result of the high moisture content (either added or from CMS in). Therefore FCR usually reached in feedlots was not achieved in this trial. Only if similar performance parameters of a feedlot can be reached with CMS can it truly be classified as a molasses replacement. But the current results currently look promising. Furthermore, the trial itself and its results should be observed with an objective mind as various problems occurred during the trial that could have influenced the results. The cattle used in the trial consisted of more than one breed (and therefore mature frame sizes) and could

have attributed to variation, and in future studies a single breed should rather be used. Feed difficulties were experienced during the trial where TMRs became mouldy. This is as a direct result of the high moisture content of the feed. The immediate observation is that appropriate consideration needs to be put into the moisture content attributed by CMS when formulating diets and when considering the shelf life of TMRs. Further feeding problems were experienced when the respective TMRs were not delivered on time and cattle were fed hay. This occurrence was managed as best possible, feeding all groups hay until the feed arrived, therefore exposing all groups to the same conditions. Even though attempts were made to still reach a good result for this trial, the period without trial TMRs could have influenced final performance parameters. As a result of these difficulties the results of the trial need to be considered with the difficulties in mind as it could have influenced the results.

To conclude the study, CMS has been identified as a possible replacement for molasses. Before that point is reached though, more research has to be done with CMS as an ingredient on an *in vitro* and *in vivo* basis. Additionally these trials will have to focus on the composition of CMS and ensuring that all nutrients are balanced for in trial diets. Furthermore the efficiency of these diets needs to be measured by measuring the FCR.

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Appendix A Van Soest Buffer

Resaruzin 0.1% (w/v) solution

Dissolve 0.1 g resaruzin into 100 ml H₂O

In vitro buffer solution

NH ₄ HCO ₃	4 g
NaHCO ₃	35 g
Bring volume to 1 L using Distilled	

In vitro micromineral solution

CaCl ₂ ·2 H ₂ O	13.2 g
MnCl ₂ ·4H ₂ O	10.0 g
FeCl ₃ ·6 H ₂ O	8.0 g
Bring volume to 100 ml using Distilled Water	

In vitro macromineral solution

Na ₂ HPO ₄ anhydrous	5.7 g
KH ₂ PO ₄ anhydrous	6.2 g
MgSO ₄ ·7 H ₂ O	0.6 g

Reducing solution

Cysteine·HCl	625.0 mg
1N NaOH	4.0 ml
Na ₂ S·9H ₂ O	625.0 mg
Bring volume to 100 ml using Distilled Water	