Anaerobic Co-Digestion of Fruit Juice Industry Wastes with Lignocellulosic Biomass

by

Carissa Jordan Kayla Kell

Thesis presented in partial fulfilment

of the requirements for the Degree

of

MASTER OF ENGINEERING

(CHEMICAL ENGINEERING)

in the Faculty of Engineering

at Stellenbosch University

Supervisor

Prof. Johann F. Görgens

Co-Supervisors

Dr. T.M. Louw and Dr. L.D. Gottumukkala

April 2019

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Abstract

The fruit juice industry in South Africa forms an important part of the South African economy, however it generates large quantities of liquid and solid organic wastes. Landfilling is typically used to dispose of these wastes, resulting in uncontrolled greenhouse gas emissions (GHG). Anaerobic digestion (AD) offers an alternative waste disposal method and produces two valuable by-products: biogas (a renewable energy source) and a liquid fertiliser. The high sugar content of fruit waste alone often results in AD failure due to acidification, resulting in poor quality biogas. Consequently, there is relatively little information available on the AD of apple fruit juice process wastes (FJPW). Identification of substrate combinations that improve the energy value of the resultant biogas may mitigate GHG emissions and generate valuable by-products which provide additional revenue streams to industry. This study thus aimed to identify optimal substrate combinations to aid in waste disposal of FJPW and energy value of biogas from fruit juice industry waste based on seasonal availability of waste streams.

Five waste streams: manure, food waste, retentate, pomace and waste apples were incorporated into a five-factor mixture design to assess food waste and manure as co-substrates of FJPW. This design was carried out in a series of biomethane potential (BMP) tests performed in 100 mL serum bottles. A second mixture design was performed using BMP tests in 100 mL bottles to evaluate lignocellulosic biomass (LCB) as a potential co-substrate of FJPW. A biogas and methane optimisation substrate mixture (50% manure, 30% LCB, 20% Retentate) and a manure minimisation mixture (30% manure, 30% LCB, 30% retentate, 10%waste apples) were selected and scaled up in 50 L CSTR reactors in batch process for 32 days with intermittent mixing. Two substrate combinations based on biogas optimisation and manure minimisation were scaled-up in 50 L reactors in semi-continuous process and fed increasing organic loading rates (OLRs) from 1-4 gVS/L/day over the course of 32 days to identify the maximum OLR that can be stably operated for each point.

The results indicated food waste was highly variable and behaved similarly to FJPW when digested, thus food waste was deemed unsuitable as a co-substrate for FJPW. An ANOVA was performed on the results of the LCB mixture design revealing both biogas and methane production to be significant ($p < 0.05$). The standardised effect estimates of all five feedstocks revealed manure, LCB and retentate to have a significant ($p<0.05$) effect on biogas and methane production. LCB addition was found to significantly improve biogas production and prevent acid crash, however it mainly did so when compensating for the fruit waste fraction rather than the manure fraction except for two mixtures: 20% manure, 30% LCB, 30% pomace and 20% retentate and 20% manure; 30% LCB, 30% waste apples and 20% retentate. The highest yields obtained from the LCB supplementation experiment were 410.01 mL.gVS^{-1} $_{fed}$ biogas and 167.10 mL.gVS^{-1} $_{fed}$ methane for the fruit-juice producing season and 325.69 mL.gVS^{-1} $_{fed}$ and 131.95 mL.gVS^{-1} $_{fed}$ for the non- juice producing season. The improved biogas and methane yields in the batch experiment compared to lab-scale were as a result of slow intermittent mixing at 125 rpm for 5-10 minutes twice daily. The biogas optimisation point gave the highest yields at an OLR of 4 gVS/L/day. The manure minimisation point demonstrated the highest biogas and methane production at an OLR of 3.5 gVS/L/day, with the system showing signs of organic overloading at a higher OLR.

To conclude, this study found a 30% LCB addition to improve digestibility of fruit process waste mixture for certain combinations of pomace and retentate, and waste apples and retentate with 20% manure. As this study only investigated 0%, 20% and 30% LCB supplementation, future research should focus on a broader array of supplementation levels in order to further maximise fruit waste disposal via AD.

Acknowledgments

This thesis is dedicated to my incredible, beautiful and multi-talented sister Gabrielle Jenna Kell. I could not and would not have begun this journey without your encouragement. My only regret is that you are no longer here to see me finish it. Your zest for life, your husky laugh and your drive to constantly improve yourself and to help and encourage those around you has shaped me into the person I am today and will stay with me for the rest of my life. I would also like to acknowledge my Aunty June and my amazing Gran who also passed away during this process. Even when I desperately wanted to leave and travel home in those last moments, you both insisted I stay and finish what I started. I would not be here today without either of you and I miss you all beyond words.

I would also like to thank my wonderful partner Anthony Clark for the much needed emotional, mental and physical support over the last seven years, but especially throughout the last three. I would like to also thank my wonderful family, especially my mom, for all the over-the-phone motivational pep talks and emotional support during times when you yourself were barely holding it together. Thank you all for being such strong role-models in my life.

To the amazing friends I've made along the way: Lorinda, Jacqui, Gerard, Lia, Lukas, Julia and Kwame I cannot express my gratitude for your expertise and assistance with regards to my work and for taking the time to teach me new techniques. Those Friday afternoon symposiums saved me in more ways than you know. I would also like to thank Marli for the several afternoon's she spent shoulders deep in manure with me, you deserve more than just an acknowledgment.

In addition, I would of course like to thank my supervisors: Prof. J.F. Görgens, Dr. T.M. Louw and Dr. L.D. Gottumuluka for this opportunity and for all their understanding, encouragement, expertise and guidance throughout this process. Furthermore, I would like to thank Levine Simmers for her tireless HPLC efforts.

I would also like to acknowledge CSIR, NRF, DST Waste Roadmap as well as the CRSES for their invaluable financial contributions toward the project. Additionally, I would like to thank SAB and EFJ for the provision of substrates and inoculum. Quantum Laboratories and CAF should also be acknowledged for any chemical composition analyses performed.

Lastly, I would also like add a special mention to both the ancient Greeks and Friedlieb Ferdinand Runge for their invaluable contributions to both science and society as a whole. Without which this would not have been possible.

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AD: Anaerobic Digestion

ATP: Adenosine Triphosphate

BMP: Biomethane Potential

CHP: Combined Heat and Power

COD: Chemical Oxygen Demand

CSTR: Continuously-Stirred Tank Reactor

DM: Dry Matter

EFJ: Elgin Fruit Juices

FVW: Fruit and Vegetable Waste

FJPW: Fruit Juice Process Waste

GC: Gas Chromatography

GHG: Greenhouse Gas

HPLC: High-Performance Liquid Chromatography

HRT: Hydraulic Retention Time

IPEEC: International Partnership for Energy Efficiency Cooperation

LCB: Lignocellulosic Biomass

LPG: Liquid Petroleum Gas

OLR: Organic Loading Rate

RPM: Revolutions per Minute

RSM: Response Surface Methodology

SA: South Africa

SAB: South African Breweries

TS: Total Solids

VFA: Volatile Fatty Acid

VS: Volatile Solids

Chapter 1: Introduction

1.1 Background and Motivation

Since the beginning of the Industrial Revolution, humanity has relied heavily on fossil fuels as a primary source of energy. Due to the finite nature of fossil fuels, prices have steadily risen with the demand as the global population has increased. As a result of the increasing costs and the added pressures of climate change, the International Partnership for Energy Efficiency Cooperation (IPEEC) is encouraging all nations to consider alternative, more cost-effective and ultimately sustainable forms of energy production (*IPEEC*, 2018).

As of 2016, it was estimated that South Africa produced approximately 53,425 tonnes of municipal solid waste per day. This value is predicted to rise to about 72,146 tonnes per day by 2025 due to climbing population growth (*World Bank*, 2016) . Of the 53,425 tonnes MSW produced per day, approximately 24,750 tonnes will be organic waste - due to losses from wasted food both in the household and the waste products formed during and after food manufacturing and processing (Oelofse and Nahman, 2012). Currently, South Africa's primary waste management technique is broadly considered to be landfilling. Although countries such as Canada, Germany and Sweden (DEA, 2012) have banned the landfilling of organic waste, South Africa has not yet done so. As a result, landfilling in South Africa is calculated to account for approximately 4.3% of South Africa's total greenhouse gas emissions due to the uncontrolled production of methane (Oelofse *et al.*, 2012).

In short, there are multiple incentives for South Africa to make the switch to alternative energy sources and waste management methods. Anaerobic digestion (AD) is one such method which offers two valuable by-products namely biogas and digestate, in addition to acting as a waste disposal system. The biogas produced typically consists of methane (50-70%), which represents the energy rich fraction of the gas, and carbon dioxide (30-50%). Biogas can be upgraded and used as an alternative to natural gas and the liquid by-product, known as digestate, can be used as a liquid fertiliser depending on the quality - pathogen load, chemical composition and nutrient profile (Makdi, Tomcsik and Orosz, 2012).

Seeing as though the fruit juice industry produces substantial amounts of organic waste (Allobergenova, 2006), it makes sense that anaerobic digestion is an attractive alternative to landfilling of fruit waste as it potentially offers decreased reliance on grid-supplied electricity and possibly provides another income stream in addition to mitigating waste disposal costs. Despite these incentives, there is relatively little information on the optimisation of using fruit waste, especially apple waste, as the sole substrate for anaerobic digestion due to the common problem of acid crash due to simple sugar degradation and the resultant VFA accumulation (Edwiges *et al.*, 2018).

VFAs are precursors to methane during the process of anaerobic digestion and are also valuable compounds used mainly in the food and beverage, chemical fabrication and pharmaceutical industries (Chen *et al.*, 2017). To the author's knowledge, no studies to date have investigated the possibility of producing large quantities of VFAs via the manipulation of substrate combinations for anaerobic digestion without sacrificing biogas and methane yields. This research may prove academically valuable to the development of co-production of biogas and VFA technologies in future.

This study aimed to characterise the effects of individual fruit juice industry waste streams on biogas yields and quality, as well as identify the most energy rich substrate combinations and those with the highest waste disposal value. This was achieved through Biomethane Potential (BMP) tests in 100 mL serum bottles in accordance with a five-factor mixture design taking into account seasonal availability of feedstocks. In addition, co-digestion with lignocellulosic biomass (LCB) was investigated for its effectiveness as a fruit waste co-substrate in terms of biogas production and quality through a series of BMP tests. In addition, all lab-scale experiments and subsequent substrate combinations were assessed for VFA production and used to identify any points of value for the future development of co-production of biogas and VFAs bio-refinery approach. Finally, two selected points identified in the lab-scale experiments were selected based on biogas maximisation and on minimisation of the manure fraction of the substrate mixture and were scaled-up in 50 L CSTR reactors in both batch and semi-continuous process. In addition, increasing organic loading rates (OLRs) were tested (1-4 gVS/L/day) in order to identify the highest possible OLR for stable process operation for both points, in order to maximise the amount of fruit waste reduced.

Chapter 2: Literature Review

2.1 Introduction

Among the most substantial organic waste producers, are food providers such as restaurants, catering companies, food stores and fruit juice companies (Oelofse and Nahman, 2012). As can be seen in Figure 2.1, the food commodity group which contributes the largest amount to total food waste is the fruit and vegetable group. Fruit and vegetable processing, especially in fruit juice production, generates large amounts of both solid and liquid wastes. The liquid wastes are formed predominantly from the many wash steps that occur during processing, whereas the solid wastes mainly consist of skins, rinds, pulp, seeds and can also include pre-processing wastes such as stems, stalks and rotten fruits (Allobergenova, 2006).
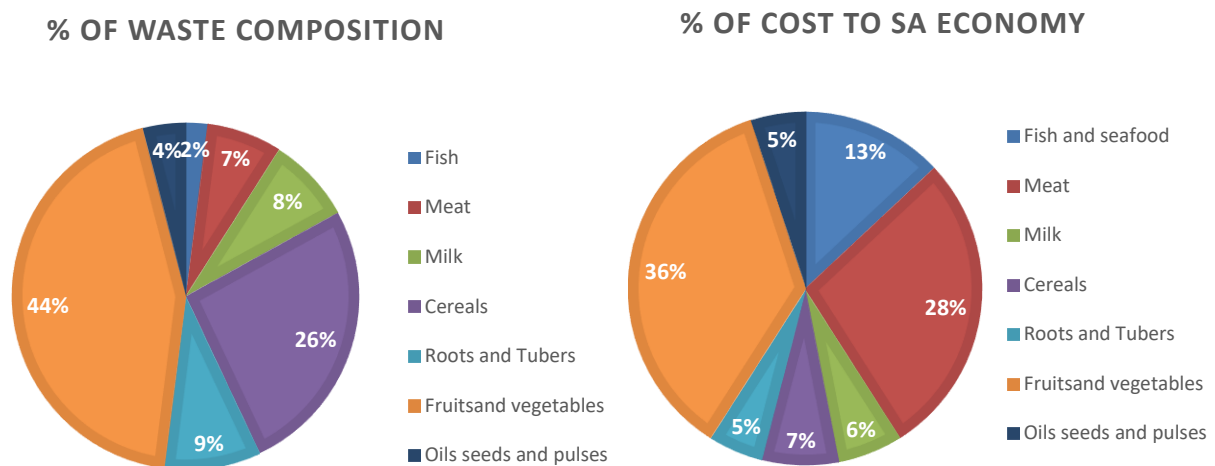
Figure 2. 1: The relative contribution of each food commodity group to total food wastage in South Africa. (Adapted from Oelofse, 2013).

The current waste management methods employed by most companies within the fruit juice industry in South Africa not only have negative environmental implications but are also cost ineffective as many potentially valuable products are discarded with the waste. Specifically, the fruit waste generated during fruit juice production can be used in the production of many commercially valuable products including the production of enzymes and biofuels. The major fruit crops produced in South Africa are citrus, apples and grapes (Khan *et al.*, 2015). As a result, much of the fruit waste generated in South Africa consists of citrus, apple or grape processing waste. Table 2.1 summarises the potential commercial applications of these different fruit wastes.

Table 2. 1 Reported applications of Citrus, Apple and Grape processing waste

Reported application	Type of fruit waste			Reference
	Citrus	Apple	Grape	
Enzyme production	✓	✓	✓	(Daroit *et al.*, 2007)(Dhillon *et al.*, 2013)(Mamma *et al.*, 2007)
Biofuel production	✓	✓	✓	(Pourbafrani *et al.*, 2010)(Vendruscolo *et al.*, 2008)(Fernandez *et al.*, 2010)
Animal feed	✓	✓	✓	(Gchol, 1978)(Dhillon *et al.*, 2013)(Sphangero *et al.*, 2009)
Polyphenolic/phenolic compounds (Antioxidants)	✓	✓	✓	(Deng *et al.*, 2012)(Vendruscolo *et al.*, 2008)(Chamorro *et al.*, 2012)(Zheng *et al.*, 2012)
Citric acid production	✓	✓	–	(Rivas *et al.*, 2008)(Gullon *et al.*, 2006)
Lactic acid production	–	✓	✓	(Gullon *et al.*, 2006)(Riviera *et al.*, 2007)
Composting	✓	✓	✓	(van Heerden *et al.*, 2002)(Burg *et al.*, 2011)
Ethylene production	✓	–	–	(Chalutz *et al.*, 1983)
Substrate (single cell protein)	✓	–	–	(Scerra *et al.* 1983)
Limonene and Pectin source	✓	–	–	(Pourbafrani *et al.*, 2010)
Immobilisation carrier (solid state fermentation)	✓	–	–	(Orzua *et al.*, 2009)
Xanthum gum	✓	–	–	(Bilanovic *et al.*, 1994)
Mushroom production	✓	✓	✓	(Labaneiah *et al.*, 1979)(Park *et al.*, 2012)(Pardo *et al.*, 2007)
Formulation of resin	–	–	✓	(Ping *et al.*, 2011)
Pullulan production	–	–	✓	(Sugumaran *et al.*, 2012)
Production of biosurfactants	–	–	✓	(Riviera *et al.*, 2007)
Pigments and aroma compounds	–	✓	–	(Vendruscolo *et al.*, 2008)
Incorporation into food products	–	✓	–	(Min *et al.*, 2010)
Substrate (production of biopolymers)	–	✓	–	(Dhillon *et al.*, 2013)
Cream of tatar	–	–	✓	(Brenn-O-Kern)
Calcium tartrate	–	–	✓	(Brenn-O-Kern)
Grape seed extract	–	–	✓	(Brenn-O-Kern)

Where "✓" indicates the substrate is suitable for the application in question and "-"indicates that either the substrate is unsuitable or that there is currently no literature to support the use of the substrate for that particular application.

As can be seen, many valuable products can be produced from specific types of fruit processing waste; however, the implementation of technologies to extract/ produce these products may incur substantial costs. For example, all three types of fruit wastes described above have potential for use in enzyme production, however at an industrial level; the expense of culture media is greater than the cost of equipment or operating costs, making the process cost inefficient (Khan *et al.*, 2015). For many of the products listed in Table 1, the development of cost-effective processes for production at an industry level has yet to be achieved. However, certain other value-added products including bioethanol and biogas may be more readily applied to industry due to already established technologies and relatively low operational costs.

This review aims to highlight the characteristics of   fruit waste streams, identify reported conditions for optimal biogas production from fruit waste and to compare the effects of various co-substrates with fruit processing waste on biogas and methane yields.

2.2 Fruit Juice Processing

2.2.1 Fruit juice manufacturing process

As described by Bates, Morris and Crandall (2001) many unit operations are required to produce fruit juice from whole fruits. The main unit operations and resultant wastes are illustrated in Figure 2.2.
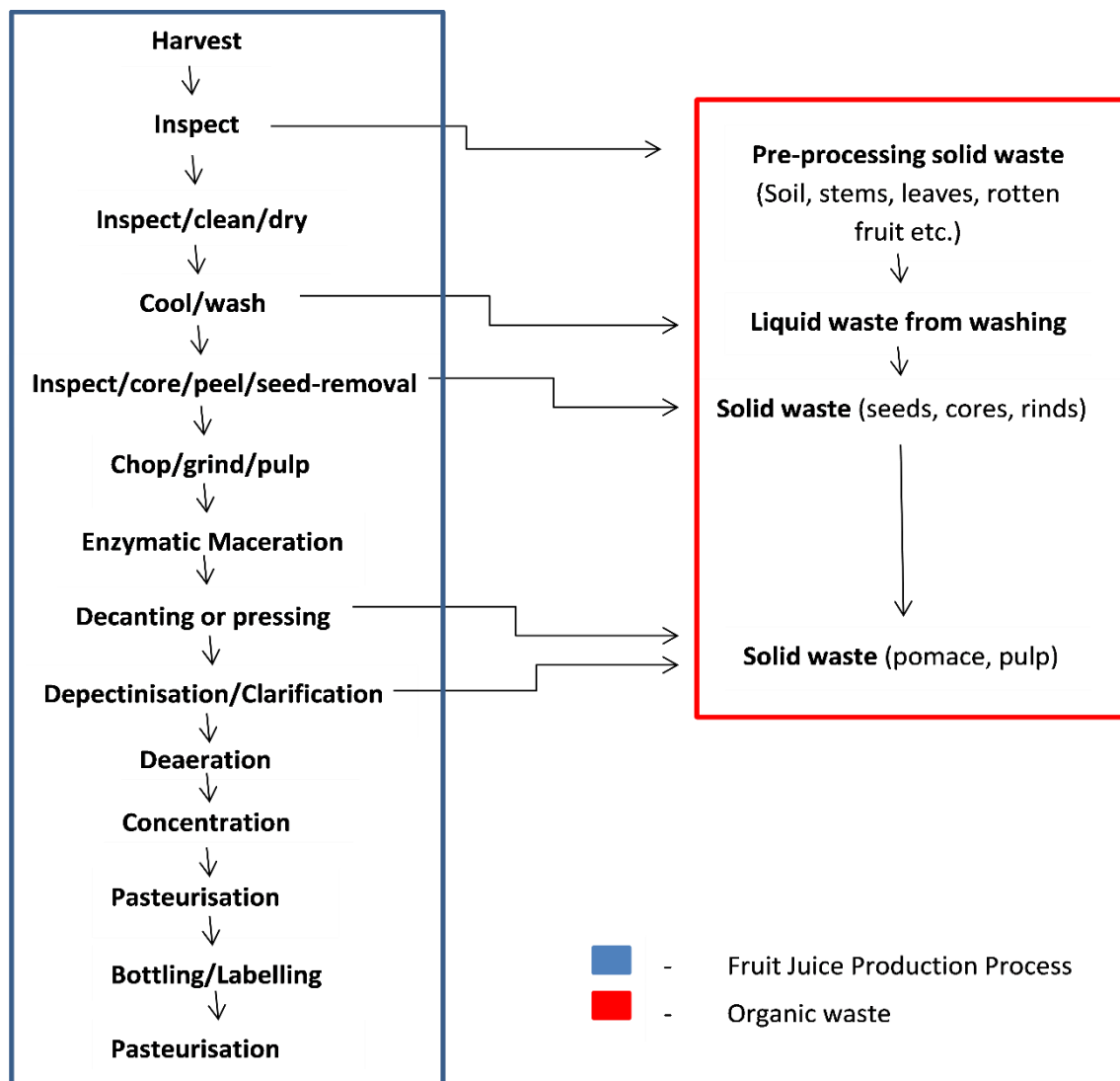
Figure 2.2: Flow chart illustrating the processing steps in fruit juice production as well as the resultant wastes from each processing step. Adapted from "Anaerobic fermentation of organic waste from juice plant in Uzbekistan" Allobergenova, 2006.

Inspect

Harvested fruits intended for processing are initially examined for visible defects as well as for foreign materials before undergoing analysis to determine any pesticide residues, pathogens, sugar content, acidity, microbial load, flavour compounds and colour. Any foreign materials or fruits which are considered to be unsanitary or poor quality are discarded during this step and form part of the pre-processing organic waste.

Inspect/Clean/ Dry/Cool

Further inspection occurs at this stage and any poor-quality fruits are once again discarded and occasionally fruit size is also examined. Should water be scarce or unsanitary, dry pre-cleaning steps and water recycling systems may be necessary (Allobergenova, 2006). Cooling and cleaning may be performed physically using either brushes or air jet separation to remove surface debris before being washed with water.

Inspect/Core/Peel/Seed-removal

Once again fruits are examined for imperfections and at this stage this examination may occur manually with employees performing the task or automatically with the use of computer operated sensors which detect fruits with undesirable shapes, colours or sizes.

Chop/Grind/Pulp

A cone screw or paddle pulper fitted with suitable screens are used for separating particulate matter and juice from soft fruits. Brush paddles may be used in place of metal bars when skin or seed crushing is problematic.

Enzymatic Maceration

Enzymes, specifically pectinases, are commonly added after pulping, as they disrupt the cell walls of fruit cells and therefore increase juice yields.

Decanting/Pressing

After undergoing pulping, the raw material is transferred to either a presser or a decanter. Decanters consist of a horizontal, cylinder-shaped screen lined with press cloth material which contains an inflatable tube. As the centre tube is inflated, the raw material is pressed against the screen and the whole component is rotated. The extracted juice falls into a catch trough. Pressures exerted on the tube can reach a maximum of approximately 600 kPa (Bates *et al.* 2001). Occasionally, pulp can accumulate and stick onto the press cloth, at the flow of juice. In such cases, press aids may be required. Solid waste discharged from the end of the decanter may be treated enzymatically for additional juice withdrawal.

Depectinisation and Clarification

For certain juices where cloudiness or turbidity is undesirable, the principal extracted juice must be processed further. For juices where cloudiness is desired, centrifugation alone is sufficient. When cloudiness is undesirable, clarity can be achieved through centrifugation and filtration. When cloudiness is unable to be removed through centrifugation and filtration, the addition of pectinases may be required as the cloudiness

is likely due to the association of pectin with other plant polymers and cellular debris. In such cases, the freshly extracted juice is transported to a stirred holding tank where pectinases such as Pectinase 444L, Macer8 FJ or Pectinase 62L may be added and incubated at 40°C -50°C (Biocatalysts Limited 2016).

De-aeration

Once the juice has been clarified, de-aeration is performed by either using a vacuum chamber or saturating the juice using an inert gas such as nitrogen or carbon dioxide. This is done by bubbling the inert gas through the juice and then storing it under inert atmosphere, thus for all consequent processing steps the juice needs to be protected from the atmosphere.

Concentration

The concentration of the fruit juice usually involves four stages. In the first stage, the juice is evaporated at 90°C to between 20-25°Brix, where fractional distillation is used to capture the concentrate. In the second stage, the captured concentrate is brought to approximately 40-45°Brix at 100°C. In the third stage; the juice is concentrated to approximately 50-60°Brix at about 45°C. Finally the juice concentrate is further concentrated to about 71°Brix before cooling to 4-5°C and being standardised to 70°Brix (Allobergenova, 2006). Subsequently bottling may occur.

Pasteurisation

Flash pasteurisation is often used as a form of preservation. This involves heating the juice to close to boiling point (higher than 88°C) for approximately 25 to 30 seconds (Allobergenova, 2006). This is done by passing the juice between heated plates or tubes. This process ensures all microorganisms present in the juice are destroyed and that the juice is preserved.

2.2.2 Properties of fruit juice processing waste

As mentioned previously, the fruit juice production process generates both liquid and solid wastes. The discarded portion of certain fruits can be quite large leading to difficulties regarding waste disposal. Other characteristics of fruit waste include having a low heating value (0.004MJ/kg) (Lohr, 1991) as well as high moisture contents (62%-88%) (Rynk *et al.*, 1992). In addition, fruit waste can contain hazardous by-products such as fertiliser or pesticide residues, harmful chemicals from cleaning and bleaching processes and occasionally heavy metals in processing or fruit cannery waste (Allobergenova, 2006). It is therefore important to consider these characteristics when selecting a suitable waste management method.

2.3 Waste management methods for fruit processing waste

Due to anaerobic digestion being able to process biomass sources with high moisture contents (less than 40% dry matter), these sources may be processed without pre-treatment which is contrary to many other waste conversion methods (Ward *et al.*, 2008). For example, the incineration of waste is only energy efficient if the water content is below 60% (Table 2.2) and in such cases, the majority of the produced energy is used for the

evaporation of water. Therefore, many of these technologies require a pre-drying step for wastes with high moisture contents (Lohr, 1991).

In comparison to other waste disposal technologies, anaerobic digestion produces two commercially useful products: biogas and digestate. Furthermore, 99% of volatile compounds are completely oxidised during combustion of biogas as seen in several studies (Smet, Van Langenhove and De Bo, 1999), this is contrary to other technologies such as incinerators which require thorough flue gas purification as they can emit hazardous compounds such as dioxins. In addition to biogas, a nitrogen-rich slurry (digestate) is also produced which may be used as a fertiliser or soil amendment (Tambone *et al.*, 2009). Alternatively, the digestate may be converted into biochar which can be used to enhance soil or as an adsorbent in the purification of flue gas or wastewater (Inyang *et al.*, 2010). Anaerobic digestion offers several other advantages over other waste disposal technologies such as the ability for it to be successfully implemented on both small and large scales and in contrast to other methods, for example incineration, does not face a negative public opinion (Appels *et al.*, 2011). The many other advantages and disadvantages for each of the different waste disposal methods are summarised in Table 2.2.

Table 2. 2: Summary of the advantages and disadvantages of different waste disposal methods.

Waste Disposal Method	Advantages	Disadvantages
Incineration	• Waste volume reduce by up to 90% • Weight reduce by 70% • Majority of calorific value of waste is converted into usable energy • Reduced demand for landfills • Stabilises putrescible waste (reducing leachate and gas production in landfills) • More effective energy recovery than anaerobic digestion and landfills • Hygienic	• Gas and liquid pollutants may be released into the atmosphere (wet scrubbing systems) • Fly ash is produced • Dust and odour issues during waste storage • Negative public opinions • Calorific value changes of waste can cause changes in operational costs • Mainly using incineration as a waste disposal method may limit waste minimisation and recycling • Not suitable for wastes with high water contents
Animal feed	• Rich nutrient content	• Often requires nutrient/protein supplementation • Not suitable for wastes with high moisture contents • Fruit juice waste can contain cleansing and bleaching agent, salts, pesticide residues or heavy metals and other compounds • Non-protein nitrogen (in apple pomace) can cause weight loss, birth defects and reproductive problems in cattle • Waste analysed for nutrient, protein and energetic value per unit before being used for feed (labour intense, cost-ineffective compared to other methods)

Direct land spreading	• **Relatively inexpensive** • **Effective way to recycle wastewater solids** • **Enhances conditions for vegetative growth**	• **Waste is required to first be analysed for content of organic waste and pH which can occur additional costs and requires additional time** • **Requires the use of specialised equipment not commonly available on most farms** • **Labour intensive** • **Land application limited to specific times of year (weather plays a large role) – requires waste storage facilities.** • **Potential negative public opinion** • **Possible eutrophication – surplus nutrients can be washed into ground and surface water** • **Potential environmental/public health issues**
Land filling	• **Used as a restoration method for mineral extraction sites** • **Lowest cost**	• **Putrescible waste produces landfill gas and leachate** • **Potential dust odour and vermin problems** • **Can take about 50 years for a landfill site to be stabilised** • **Opposition to location of sites** • **Negative effects on landscape and local amenities** • **Landfill tax liabilities** • **Inhibits waste minimisation and recycling as a preferred method** • **Uncontrolled gas production contributes to global warming**
Composting	• Improves soil condition • Saves landfill space (therefore reduces leachate in landfills) • Useful way of recycling nutrients (which requires less energy than the use of virgin materials) • Controls pathogens from waste and wastewater • Saves resources by using plant nutrients and water as liquid fertiliser • Waste volume reduction	• Expensive • Not suitable for high moisture organic wastes • Requires separation and screening • Requires controlled conditions and careful management • Some organic waste can be unsuitable due to persistent contamination • Odour and leachate problems if not contained • Health and safety issues need to be addressed • High emissions (ammonia, methane, nitrous oxide, hydrogen sulphate)
Anaerobic digestion	• Generates biogas- source of renewable energy • Comparatively low running costs • No odour problems • Little space requirements • Possibility of using remaining material as a fill or soil conditioner • High degree of automation • Prevents rotting waste from being landfilled reducing leachate into groundwater and uncontrolled methane production Waste volume reduction • Can aid fruit juice processing industry in developing a net zero emissions approach	• Careful screening to remove contaminants, specifically metals • Controlled conditions and careful management for optimisation of biogas production • Produces a residue that if found to contain hazardous materials may require landfilling • Gas may require clean-up prior to use • Solid residues may require landfilling if markets aren't available • Fruit waste contains high sugar content – pH drops quickly

References: (Lohr 1991; Waste Management Plan 2016; Land Application of Municipal Sludge-advantages and Concerns 1996; The Art and Science of Composting, A resource for farmers and compost producers 2002; Eberle 1997)

2.4 Anaerobic digestion process

Anaerobic digestion or anaerobic fermentation describes a sequence of biological processes performed by microorganisms in the absence of oxygen to produce biogas (Costa *et al.*, 2015). The process of anaerobic digestion produces both a renewable energy source known as biogas as an end-product; as well as an effluent that can be used as a soil conditioner. There are four main phases of anaerobic digestion namely the hydrolysis phase, the acidogenesis phase, the acetogenesis phase and the methanogenesis phase (Figure 2.3).
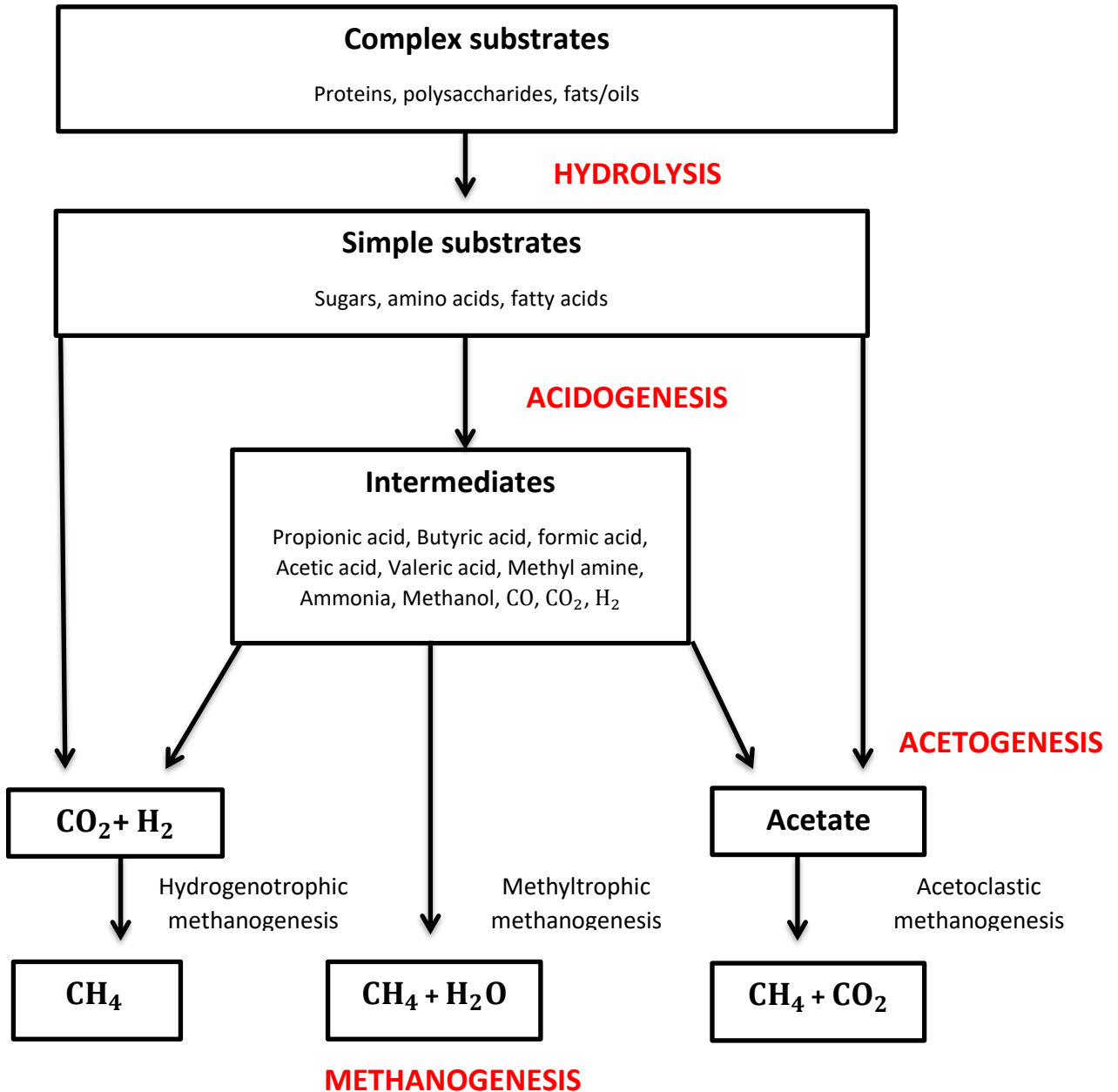
Figure 2.3: The Four Phases of Anaerobic Digestion. "The Microbiology of Anaerobic Digesters" Gerardi (2003).

In the hydrolysis stage, organic compounds are broken down into amino acids, sugars and fatty acids (Parawira *et al.*, 2008) as is illustrated in the following reaction:

Biomass + H_2O → monomers + H_2 [1]

This is accomplished by extracellular hydrolytic enzymes which cleave the covalent bonds in the polymers, using water (Parawira *et al.*, 2008). Complex structures such as lignocelluloses may require weeks for hydrolysis to occur. Even then, the degradation is frequently not complete (Bayer, Henrissat and Lamed, 2008). For these substrates, hydrolysis is the rate-limiting step, whereas for easily degradable substrates methanogenesis is the rate-limiting step (Vavilin *et al.*, 2008).

The second phase is acidogenesis. The products produced after hydrolysis are then metabolised further by fermentative bacteria to produce short-chain organic acids typically consisting of two to six carbon atoms (Figure 2.4) (Brody, 1999; Clifford, 2018). In this phase alcohols, ammonia, hydrogen and carbon dioxide are also produced. The main reactions which occur during acidogenesis are represented in reactions 2-3:

$C_6H_{12}O_6$ + $2H_2$ → $2CH_3CH_2COOH$ + $2H_2O$ [2]

$C_6H_{12}O_6$ → $2CH_3CH_2OH$ + $2CO_2$ [3]

Under stable conditions with low partial pressure of hydrogen, acetate, hydrogen and carbon dioxide are the primary products. Conversely, under conditions where the partial pressure of hydrogen is elevated, more VFA's and alcohols are produced (Schink, 1997).

Certain products formed during acidogenesis may be used directly by methanogenic microorganisms. However certain compounds are degraded further into acetic acid, carbon dioxide and hydrogen during the acetogenesis phase namely: fatty acids with more than two carbons, alcohols with more than one carbon as well as aromatic and branched chain fatty acids (Teghammar, 2013). Reactions 4-7 illustrate the main reactions which occur during the acetogenesis phase (Clifford, 2018):

$CH_3CH_2COO^-$ + $3H_2O$ → CH_3COO^- + H^+ + HCO_3^- + $3H_2$ [4]

$C_6H_{12}O_6$ + $2H_2O$ → $2CH_3COOH$ + $2CO_2$ + $4H_2$ [5]

CH_3CH_2OH + $2H_2O$ → CH_3COO^- + $2H_2$ + H^+ [6]

$2HCO_3^-$ + $4H_2$ + H^+ → CH_3COO^- + $4H_2O$ [7]
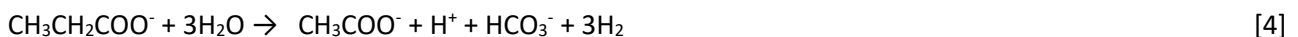
The principal bacteria involved in acetogenesis are obligatory H_2 producers and are therefore found living symbiotically with H_2 consumers (methanogens) which facilitate their growth by maintaining a low hydrogen partial pressure (Gerardi, 2003). However, as the concentration of hydrogen increases, the concentration of organic acids in the digester concomitantly increases, causing the pH to drop. This pH drop results in a toxic environment for the methanogenic microorganisms. As acetic acid is produced from the short-chained

organic acids, homoacetogenic bacteria reduce carbon dioxide and hydrogen to produce more acetic acid (Gerardi, 2003).

The final stage in the anaerobic digestion process is methanogenesis. The principal organisms in this stage are the methanogenic archaea, which are highly oxygen sensitive as well as sensitive to environmental stressors such as heavy metals or unfavourable pH values (Chen, Cheng and Creamer, 2008; Liu & Whitman, 2008). In addition, methanogens have the longest doubling time of all the microorganisms in the bioreactor. Hence, for easily-degraded substrates, methanogenesis becomes rate-limiting. Although acetate produced in the acetogenesis stage is the principal organic acid used by methanogens to produce methane and carbon dioxide (acetoclastic methanogenesis) during the methanogenic phase; methane may be produced through two other processes namely, hydrogenotrophic methanogenesis and methyltrophic methanogenesis (Figure 2). Hydrogenotrophic methanogenesis uses hydrogen and carbon dioxide to produce only methane and the methyltrophic methanogenesis uses methanol to produce methane and water (Gerardi, 2003). Approximately 70% of the produced methane is from acetoclastic methanogenesis and 30% is from hydrogenotrophic methanogenesis. Reactions 8-13 represent the main reactions which occur during the methanogenesis phase (Clifford, 2018):

$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COOH + CH_4 \tag{8}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \tag{9}$$

$$CH_3OH \rightarrow CH_4 + H_2O \tag{10}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \tag{11}$$

$$CH_3COO^- + SO_4^{2-} + H^+ \rightarrow 2HCO_3 + H_2S \tag{12}$$

$$CH_3COO^- + NO^- + H_2O + H^+ \rightarrow 2HCO_3 + NH_4^+ \tag{13}$$

2.4.1 Anaerobic digestion of fruit processing waste: Process Parameters

A number of factors influence biogas production, such as different operational variables namely temperature, organic loading rate (OLR), alkalinity, hydraulic retention time (HRT) and pH as well as the variety of feedstock that is used.

2.4.1.1 Particle size and mixing

The particle size of the substrate has been seen to have a substantial effect on methane production. By reducing the particle size of the substrate, the surface area is increased allowing for greater exposure of the substrate to microbial activities. A study conducted by Izumi *et al.*, (2010) investigated the effects of the reduction of particle size on solubilisation and the production of biogas from food waste. The study concluded that as the particle size decreased, solubilisation of the food waste and VFA production increased. This

resulted in improved biogas production of up to 28%, however when the particle size was excessively reduced (0.393mm or smaller), VFA accumulation occurred leading to a deterioration in methane production (Izumi *et al.*, 2010). This study posits that particle size may be optimised and suggests that this optimisation, together with the optimisation of microbial growth, can greatly improve methane yields.

In addition to particle size, mixing digester content has been seen to be highly advantageous to the process of anaerobic digestion as it aids in the even dispersion of nutrients, bacteria and substrate as well as temperature (Gerardi, 2003). Additionally, any toxic materials are also dispersed, meaning toxicity is also minimised. Methanogens are highly sensitive to rapid mixing. Mild, slow mixing ensures that acetogenic and methanogenic bacteria are in close proximity. This allows the methanogens more immediate access to the products produced by the acetogens. In addition, mixing allows for efficient hydrolysis of substrates and the generation of products by acid-producing bacteria. Gerardi (2003) reported that prevention of clumping of insoluble starches through mixing, which allows a larger surface area of the starches to be exposed to the hydrolytic bacteria leading to faster hydrolysis. Another benefit of mixing is that it prevents grit from settling and reduces scum build-up. Over time, the accumulation of solids can lead to a decrease in digester performance as the digester hydraulics become more restricted. Satisfactory volatile solids destruction can be achieved through routine periods of mixing per day as an alternative to continuous mixing, which is costly and requires specialised facilities.

2.4.1.2 Alkalinity and pH

One of the most influential parameters on the process of anaerobic digestion is pH as it can affect the equilibrium between most chemical species. The anaerobic digester contains a consortium of microorganisms with different optimal pH ranges. Specifically, the acid-producers favour a pH range of 5.0-8.5, whereas methanogens prefer a pH range of 6.5-8.0. Optimally, anaerobic digesters are run within a pH range of 7.0-8.5, outside this range imbalances can occur (Boe 2006; Schnürer and Jarvis 2009). In addition, methane production is reported to cease once the pH drops below 6.0 (Gerardi, 2003).

In order to maintain a stable pH within the digester, it is vital that the alkalinity is kept high and steady. Alkalinity can be considered the quantity of basic compounds within the bioreactor. At high alkalinity values, the buffering capacity is higher thus contributing to the stabilisation of the pH (Teghammar, 2013). Alkalinity is predominantly based upon carbonate (CO_3^{2-}) in equilibrium with dissolved carbon dioxide (CO_2). Substrates which are protein rich may also contribute to the alkalinity as ammonia is released as the proteins are broken down (Gerardi, 2003; Schnürer and Jarvis, 2009). Specifically, carbon dioxide produced during anaerobic digestion solubilises, due to the partial pressure of gas within the digester, and reacts with water reversibly to form carbonic acid (Bischofsberger et al. 2005; Tchobanoglous et al., 2003):

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \hspace{8cm} [14]$$
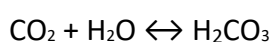
Sufficient alkalinity is thus required to buffer the drop in pH due to carbonic acid formation as well as Volatile fatty acid (VFA) formation during the anaerobic digestion process. Alkalinity is therefore used as a measure of the buffering capacity and is expressed in terms of calcium carbonate in mg/L. For anaerobic digesters operating within the acceptable pH range, pH is regulated mainly by the bicarbonate buffering system. Bicarbonate alkalinity is generated via the degradation of nitrogen-containing material and the reaction of the resultant ammonia-nitrogen with carbon dioxide (Grady et al., 1999). The subsequent equation represents the formation of alkalinity during anaerobic conditions as a result of the conversion of protein containing organic matter (Tchobanoglous et al., 2003):

$$NH_3 + H_2O + CO_2 \rightarrow NH_4^+ + HCO_3^- \hspace{4cm} [15]$$

As can be seen in Figure2.4, the bicarbonate alkalinity concentration in solution is related to the carbon dioxide content of the gas in the headspace of the digester as well as the digester pH. When VFAs begin to accumulate during the AD process, the bicarbonate alkalinity neutralises them as is shown in the following reaction equation for acetic acid (HAc):

$$HCO_3^- + HAc \leftrightarrow H_2O + CO_2 + Ac^- \hspace{4cm} [16]$$

During unstable digester operation, VFAs react with bicarbonate alkalinity thus reducing the bicarbonate alkalinity concentration and producing carbon dioxide, which increases the carbon dioxide content in the headspace of the digester. Hence, digester stability is usually achieved via the maintenance of a high bicarbonate alkalinity concentration so that VFA formation can be endured without drastically decreasing the digester pH (Grady et al., 1999). It should, however, be noted that the primary consumer of alkalinity within the digester is not VFAs as is commonly believed, but rather carbon dioxide (Tchobanoglous et al., 2003).

Digesters fed with animal manure usually demonstrate high bicarbonate buffering capacity and high ammonia contents, which contribute toward pH stability between 7.5-8.0, allowing the system to tolerate higher VFA concentrations before pH drop occurs (Boe, 2006).
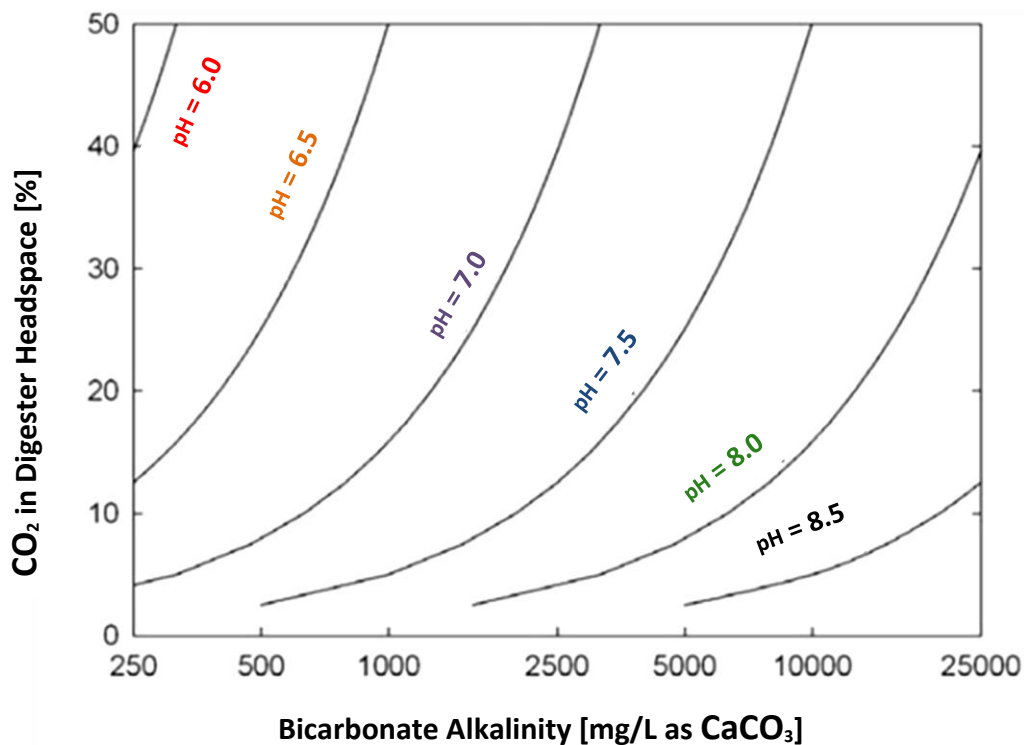
Figure 2. 4: The effect of pH on the bicarbonate alkalinity in the aqueous phase and the carbon dioxide content in the headspace during anaerobic digestion at T=35°C (based on calculations provided in Grady et al., 1999; Tchobanoglous et al., 2003). Adapted from "Numerical Modelling of Anaerobic Digestion Processes in Agricultural Biogas plants". 2009. M. Schön.

The VFA:alkalinity ratio is one criterion which may be used to judge the stability of the digester, of which there are three threshold values (Zickerfoose & Hayes 1976; Switzenbaum *et al.* 1990). Digesters with a VFA:alkalinity ratio less than 0.4 should be stable, however between 0.4 and 0.8 some instability is likely to occur. At VFA:alkalinity values higher than or equal to 0.8, significant instability is likely to occur. Lane (1984) proposes that the alkalinity should be more than 1500 mg $CaCO_3$/l and Volatile fatty acids (VFA):alkalinity ratio should be less than 0.7 in order to maintain stability in a reactor operated using fruit and vegetable wastes.

2.4.1.3 Temperature

As with pH, the different microorganisms have different optimal temperatures for growth. Most commonly digesters either function within a mesophillic temperature range (at approximately 35°C) or a thermophillic range (between 50°C-57°C). Anaerobic digesters that are operated at thermophillic temperatures are known to result in higher methane yields however the thermophillic microorganisms exhibit greater sensitivity to temperature changes or toxic compounds (Duran and Speece, 2016). In contrast, digesters operated under mesophillic conditions are more stable and less at risk for ammonia-toxicity but result in lower methane yields (Schnürer and Jarvis 2009). The stability of mesophillic digestions compared with thermophillic systems is likely due to the greater variety of mesophiles compared with thermophiles (Leven, Eriksson & Schnürer 2007).

2.4.1.4 Organic loading rate and Hydraulic retention time

Organic loading rate (OLR) can be defined as the quantity of substrate added per digester volume and time. For solid wastes OLRs are typically measured based on volatile solids (VS) added per unit of time, however, for liquid wastes chemical oxygen demand (COD) per unit of time is generally used (Sawyer, McCarty and Parkin, 2003). Hence, for anaerobic digestion of solid wastes methane productivity is measured predominantly in terms of VS fed or VS removed. VS removed; as residence time nears infinity, is known as the ultimate methane yield (B_o) (Moller, Sommer & Ahring 2004). For all substrates, this value is lower than the theoretical methane yield (based on COD or VS) due to losses associated with the presence of non-degradable matter and organic materials used for microbial growth (Atandi & Rahman, 2012). During process start-up, a lower OLR is required while established systems can manage higher OLRs. Typically, mesophillic systems work at lower volatile solids (VS) loadings of approximately 2-3 kg VS/m^3/day whereas for thermophillic systems the ORL is higher around 4-5 kg VS/m^3/day (Schnürer and Jarvis 2009). If substrates which are easily degraded are added at a high OLR, VFA accumulation may occur resulting in inhibition of the process (Fang, 2010).

The amount of time that the sludge or wastewater remains in the reactor is known as the hydraulic retention time (HRT) (Gerardi, 2003). Often not all the material is broken down as the OLR is usually higher than the methane and carbon dioxide production. Anaerobic digesters usually have an HRT of 10-25 days or more (Schnürer and Jarvis 2009). Materials high in cellulose which are degraded at a slower rate often require a longer HRT than materials high in fermentable sugars which are quickly degraded. With higher organic loading rates, a higher HRT is usually required (Teghammar, 2013).

2.4.1.5 Chemical Oxygen Demand (COD)

COD is typically used as an indication of the strength (in terms of concentration of pollutants) of a sample of sludge or wastewater (Gerardi, 2003). It can be defined as the total oxygen necessary to oxidise all organic material into carbon dioxide and water and the oxidation of inorganic chemicals such as ammonia and nitrate. Therefore COD can be considered a measure of the total amount of organic matter in a particular substance (Watershed Protection Plan Development Guidebook, 2001). The amount of substrate or COD of the digester feed sludge may be used to determine the quantity of nitrogen and phosphorus that is necessary for optimal digester performance. Although nutrient requirements differ according to the organic loading rates, COD: N: P ratios of 1000:7:1 and 350:7:1 are typically used for high-strength wastes and low loading rates respectively (Gerardi, 2003). When using either of the COD: N: P ratios, the assumption is made that 12% of the dry weight of bacterial cells consist of nitrogen and 2% of phosphorus. With this knowledge, along with the assumption that approximately 10% of the COD within the digester is used for bacterial growth, the required amounts of nitrogen and phosphorus for optimal growth and functioning may be calculated

(Gerardi, 2003). In this way, the nutrient requirements of anaerobic digesters may be determined by providing a minimum quantity of a nutrient as a percentage of the COD loading within the digester.

2.4.1.6 Substrates

The variety of substrate used directly influences both the biogas yield and quality. For example, organic matter rich in fats/lipids have a higher biomethane potential than those rich in carbohydrates or proteins due to the extensive oxidation required to break down fats compared to carbohydrates or proteins (Neves, Oliveira and Alves, 2009a). An assortment of organic materials may be used in anaerobic digestion for the production of biogas namely sewage sludge, animal manure, energy crops, slaughterhouse wastes, wastewater and food wastes to name a few (Deublein and Steinhauser, 2008). In order for biogas production to be optimised, the microorganisms involved must achieve an adequate level of growth. In the bioreactor, microorganisms utilise fats, proteins and carbohydrates as an energy source with CO_2 as the electron acceptor. Energy is produced through the oxidation of the energy source, with electrons/protons being transferred through a variety of intermediates before finally being accepted by CO_2 (Schnürer and Jarvis 2009). In addition to an energy source, many macro- and micronutrients are required for microbial growth and optimal functioning. Important macronutrients for growth include carbon, potassium, hydrogen, nitrogen, sulphur and phosphorous (Kayhanian & Rich 1995). Micronutrients such as cobalt, selenium, tungsten, copper, iron, molybdenum, zinc and nickel and also vitamins are also required (Kayhanian & Rich 1995).

Apart from the organic content, the carbon to nitrogen ratio (C/N) is considered important for biogas production. Ideally, the C/N ratio should be between 10-30, however ratios between 25-30 (Liu & Whitman 2008; Yadvika, Santosh, Sreekrishnan, Kohli & Rana 2004) are considered optimal for digester functioning. Lower C/N ratios are problematic as ammonia inhibition may occur, creating unfavourable conditions for methanogens. As a result, volatile fatty acids can accumulate causing a pH drop and leading to digester failure. Equally undesirable are high C:N ratios which are at risk of having lower methane yields due to a lack of nitrogen for cell growth (Alvarez and Lidén, 2008).

Common co-substrates of fruit processing waste

Fruit waste as a single substrate can lead to a rapid decrease in pH due to the high sugar content, ultimately leading to digester failure (Edwiges *et al.*, 2018). In addition, fruit waste alone does not provide all the necessary vitamins and micro-nutrients necessary to sustain the growth of important microorganisms involved in methane production. For example, fruit and vegetables have low phosphorous and nitrogen contents. As a result, fruit and vegetable waste (FVW) alone has limited potential for biogas production.

One option for improving biogas yields from FVW is through the addition of co-substrates. Co-digestion refers to the simultaneous fermentation of a homogenous blend of two or more substrates. Typically co-digestion involves a primary substrate that is mixed with lesser amounts of one or several secondary substrates (Braun, Holm-nielsen and Seadi, 2002). The improved biogas yields from co-digestion are due to the establishment of beneficial synergisms in the digestive medium as well as the provision of missing nutrients (Mata-Alvarez, Macé and Llabrés, 2000).

Many studies have been conducted using FVW in combination with a variety of different co-substrates such as livestock manure, slaughterhouse wastes and food wastes. By evaluating the biogas yields and the quality of biogas produced from the different co-substrates digested together with FVW, more insight may be gained as to the optimal conditions for the production of high quality biogas using FVW as a primary substrate.

2.4.1.6.1 Livestock Manure

Livestock manure is abundant and when left untreated can be a major source of soil, water and air pollution. Nutrient leaching and GHG emissions are among the greatest environmental threats. However, the anaerobic digestion of livestock manure not only prevents these threats from being realised but also reduces pathogens, improves fertilizer value and reduces odour. Manure is favourable as a co-substrate as it is rich in nitrogen, is a source of microorganisms and provides buffering capacity. However, not all livestock manure has the same properties. The benefits and complications of co-digestion with the different varieties of livestock wastes are discussed below.

i.   Cattle Manure

Cattle manure as a co-substrate for anaerobic digestion is widely used due to the number of advantages it offers. For example, the use of manure in co-digestion assists in the mitigation of uncontrolled GHG emissions from manure left in the environment, improves the fertiliser value of the digestate, increases biogas production and saves costs related to waste treatment (Braun, Holm-nielsen and Seadi, 2002; Holm-Nielsen, Al Seadi and Oleskowicz-Popiel, 2009). In addition, cattle manure itself is often used as a primary substrate in anaerobic digestion due to its abundance, as well as characteristics such as its high water content and buffering capacity. Cattle manure also contains almost all essential nutrients as well as trace elements important for microbial growth (Li *et al.*, 2009), thus when used in combination with FVW, digester failure due to a deficiency in micronutrients is unlikely to occur.

Properties of cattle manure depend on factors such as the fibre and protein contents of the feed source, animal age, digestibility and environment (Hubbard & Lowrance 2001). Biogas plants which use dairy manure as a sole substrate are infamous for low biogas yields per unit mass of manure added and are therefore associated with a low return of investment (Tafdrup, 1995; Atandi & Rahman, 2012). As a result, cattle manure is considered to be uneconomical as a sole substrate for anaerobic digestion. However, methane

yield and therefore economic value is seen to be improved through the addition of co-substrates. Good candidates for co-digestion with cattle manure are substrates rich in lipids and/or carbohydrates that have a high VS content (Labatut *et al.* 2011; Cuetos, Gómez & Otero 2008).

As seen in Table 2.3, in comparison to the other studies which co-digested fruit waste with cattle manure, Callaghan *et al.* (2002) produced the highest methane yield. When different ratios of FVW to cattle manure were tested it was discovered that when the FVW portion was increased from 20% to 50% the methane yield increased from 0.23 to 0.45 m^3 CH_4/kg VS. Thus Callaghan *et al.* (2002) found the optimal ratio of FVW to cattle manure to be 1:1. Despite these increased yields however, Callaghan *et al.* (2002) also discovered that as the FVW proportion was increased to 30% and above, the VFA: alkalinity ratio entered the range of 0.4-0.8 where digester instability is likely to occur. A similar study was conducted by Prakash & Singh (2013) which tested fruit and vegetable wastes separately with cattle manure, which found that for vegetable waste and cattle manure gas production was maximised (0.245 m^3/kg VS) at ratios of 1:1. This is in contrast to the combination of cattle manure and fruit waste where the optimal gas production of 0.230 m^3/kg VS was seen at ratios of 2:1. It should be noted, however, that the methane yields from particular fruit and vegetable residues vary considerably based on differing amounts of carbohydrates, lipids and proteins (Nallathambi Gunaseelan, 1997). Therefore, although these ratios of FVW to cattle manure may be used as a guideline, optimal conditions may change based on the composition of the FVW used.

Table 2. 3 Comparative methane yields of cattle manure as a co-substrate of FVW

Reference	Description	Reactor type/Volume	CH_4 yield (m^3/kg VS)	Notes
(Callaghan *et al.*, 2002)	Cattle manure + FVW	CSTR, 18L	0.450	Co-digested with FVW 50:50 (wet weight)
(Callaghan *et al.*, 1999)	Cattle manure + FVW	Batch, 1L	0.255	70% Cattle manure, 20% FVW and 10% inoculum
(Lantz *et al.*, 2007)	Cattle manure + FVW	N/A	0.240	N/A
(Prakash and Singh, 2013)	Cattle manure + FVW	Batch, 1.5 L	0.245	Ratio of 2:1 of manure to fruit waste

It should be noted that there are currently a limited number of studies involving cattle manure and FVW, with most studies involving both these substrates including a third substrate such as an additional type of manure or food waste. Even so, despite the lack of research on methane production from solely FVW and cattle manure, it can be seen that it is possible to improve methane yields through the co-digestion of these two substrates.

ii. Swine and Poultry Manure

In comparison to cattle manure, both poultry and swine manure frequently exhibit total ammonia concentrations higher than 4 g-N/L (Hansen, Angelidaki and Ahring, 1998). Ammonia concentrations of 4 gN/L or greater have been found to be inhibitory to the anaerobic digestion process (Angelidaki & Ahring 1994). Ammonia inhibition is therefore more likely to occur when swine or poultry manure are used as co-substrates rather than when cattle manure is used. Furthermore, anaerobic fermentation of swine manure alone is considered unattractive as it produces a low methane yield due to the high water content and fibres within the material (Molinuevo, 2002). Many complications also exist for using poultry manure as a sole substrate for anaerobic digestion. For example, seeing as poultry do not have a urinary tract, poultry excretions have a total solids content of approximately 25% (Digesting poultry litter, 2016). Furthermore, due to natural drying which occurs on belts within the poultry houses, the waste is often required to be diluted to a solids content of 7-10%. In order to achieve this, 5 to 8 times the volume of water is required which presents the additional problem of waste water disposal (Digesting poultry litter, 2016). However, as with cattle manure, many of the shortcomings associated with the mono-digestion of swine or poultry manure may be overcome through co-digestion with a carbon-rich substrate.

As seen with cattle manure, a limited amount of studies have been performed using only swine or poultry manure in combination with FVW for biogas production as listed in Table 2.4. The average methane yields for swine and poultry manure with FVW based on these studies are much greater than those seen with cattle manure (Tables 2.3 & 2.4). This is likely due to the higher nitrogen concentrations in these wastes, which is often seen to contribute to ammonia toxicity. However, if used in the correct ratio with FVW, poultry or swine manure may lead to improved biogas and more specifically methane yields.

Table 2. 4 Comparative methane yields of poultry and swine manure as co-substrates of FVW.

Reference	Description	Reactor type/Volume	CH_4 yield (m^3/kg VS)	Notes
(Molinuevo, 2002)	Poultry manure + FVW	BMP, 500 ml	0.233	More than 25% FVW resulted in inhibition of biogas production
(Lane, 1984)	Apple press cake + dried poultry manure	Horizontal plug flow digester, 4L	0.357	Mainly apple waste (5 days a week), chicken manure fed once per week
(Molineuvo 2002)	Swine manure + FVW (mainly peas)	BMP, 500 ml	0.386	75% pm:25% FVW highest yield
(Knol, Van Der Most and De Waart, 1978)	Waste apples + Swine manure	CSTR, 1L	0.324-0.338	Ratio of 1:1 apple waste: pig manure.
(Ferreira 2012)	Swine manure + FVW	CSTR, 11L	0.378	15% FVW, 85% swine manure

As can be seen in Table 2.4, Lane (1984) obtained the highest methane yield (0.357 m^3/kg VS) of the two studies which used poultry manure, by means of a horizontal plug flow digester. An HRT of 48 days was used with an OLR of 2.20 g VS/L/day. The apple press cake was fed into the digester 5 days per week, with a weekly addition of dried poultry litter. This study's findings were contradictory to that of both Molinuevo (2002) and Callaghan *et al.* (2002) which both describe inhibition of biogas production when poultry manure was used as a co-substrate. Moulinuevo (2002) found that once the FVW portion was increased to 25%, process inhibition occurred most likely due to a decline in pH as a result of excessive VFA accumulation. Callaghan *et al.* (2002) found that as the quantity of poultry manure was increased, and the organic loading increased, volatile solids (VS) reduction declined and the methane yield decreased - which was likely due to the presence of free (unionised) ammonia.

In contrast to the poultry manure, both studies using swine manure in co-digestion with FVW produced very similar methane yields (Table 2.4), with Molineuvo (2002) producing the highest. It was discovered that a ratio of 3:1 of swine manure to FVW produced the highest yield. Ferreira (2012) found that at the ratio of 15% FVW to 85% pig manure the highest methane yields were produced (0.378 m^3/kg VS) compared to FVW alone (0.101 m^3/kg VS). Therefore, all of the studies mentioned which used swine manure in combination with FVW found that using between 15%-50% FVW was effective in improving methane yields.

2.4.1.6.2 Multi-substrate studies

As can be seen in Table 2.5, many studies have been conducted involving more than two substrates. This is owing to the success of co-digestion in terms of digester stability and biogas production. The methane yields obtained in the studies using multiple substrates reflect this, as they are the highest reported in this review (Table 2.5). This is most likely because the greater variety of substrates provides a greater variety of nutrients, in addition to improving alkalinity. A few of the studies described in Table 2.5 are combinations of the substrates discussed previously, however also discussed are studies involving food waste which are of particular interest as they often result in high methane yields. Although it is often treated as a single substrate, food waste can be considered a multi-substrate feedstock as it may consist of a combination of slaughterhouse waste, FVW, and other edible organic wastes which are each considered individual substrates. In addition to its availability as well as varied nutrient content, food waste often has high lipid and nitrogen contents which improve methane yields. This is another possible explanation for the high methane yields seen in Table 2.5. However, with the high nitrogen content comes the risk of ammonia inhibition, therefore the ratio of substrates used is important. It should also be noted that food waste composition may differ drastically, which makes studies involving food waste difficult to compare. Therefore, the compositional analyses of each of the wastes must be taken into account when comparing methane yields from each study.

Table 2. 5: **Comparative methane yields of mixed wastes.**

Reference	Description	Reactor type/Size	CH$_4$ yield (m^3/kg VS)	Notes
(Satyanarayan, Murkute and Ramakant, 2008)	Cattle manure + FVW + Maize	CSTR, Pilot, 380 L	0.380	Thermophillic digestion
(Maranon *et al.*, 2012)	Cattle manure + FVW+ sewage sludge	CSTR, 5L	0.603	A mixture of 70% cattle manure, 20% FVW and 10% sewage sludge
(Alvarez and Lidén, 2008)	Slaughterhouse waste + FVW + manure	Semi-continuous, 2L	0.350	4% slaughterhouse waste, 4% manure 23% FVW
(El-Mashad and Zhang, 2010)	Food waste + cattle manure	Batch, 1L	0.531	48% Food waste, 52% cattle manure
(Neves, Oliveira and Alves, 2009b)	Food waste + cattle manure+ fish oils	CSTR, 5L	0.900*	1:1 Food waste to cattle manure + 4.8 gCOD/L oil
(Murto, Björnsson and Mattiasson, 2004)	Pig manure, slaughterhouse + industrial waste + Food waste	CSTR, 3L	0.682	17% industrial waste, 66% pig manure, 12% slaughterhouse waste + 5% catering waste
(Mata-Alvarez *et al.*, 1992)	Food waste (FVW+ seafood waste)	CSTR, 3L	0.478	6:5:7 ratio of fruit to vegetables to seafood waste

*Unit of measurement is gCOD-CH4/gVS.

A trend which is evident in Table 2.5 is that the studies which obtained the highest methane yields used feedstocks with a substrate composition of 50% manure or greater. This is likely due to the provision of trace elements, as well as the improved alkalinity, the provision of nitrogen and additional microorganisms from manure. Among the highest methane yields described in Table 2.5 is that obtained in the study conducted by Murto *et al.* (2004). The industrial waste used in this study consisted predominantly of grease trap residues (87%) but also contained small amounts of dairy wastes, bakery wastes, confectionery waste and mill wastes. Therefore, the high methane yield obtained in this study is likely attributed to the varied nutrient composition and increased buffer-capacity within the system as well as the high nitrogen and lipid contents of the feedstock. This is in line with the findings of Neves *et al.* (2009b) which observed that with the addition of oily wastes to equal proportions of food waste and cattle manure, the methane yield increased from 0.280 gCOD-CH4/gVS to 0.900 gCOD-CH4/gVS. However, a threshold value of 12 gCODoil/lreactor was reported. At oily waste inputs greater than 12 gCODoil/lreactor, persistent process inhibition occurred due to VFA accumulation. This idea also supports the findings of El-Mushad & Zhang (2010) which found that the methane yield increased with increasing proportions of food waste (consisting mainly of catering waste) which often has a high lipid content. Hence, it should be noted that when selecting multiple substrates for anaerobic digestion, having a wide variety of substrates and high lipid and nitrogen content is important for improved methane yields. In addition, the combination of food waste with manure is likely to provide good methane yields however this is largely dependent on the composition of food waste and the ratios of substrate used.

2.1.1.6.3 Lignocellulosic Biomass (LCB)

A potential co-substrate for FVW is lignocellulosic biomass, as it contains complex carbohydrates which can aid in buffering the drastic pH drop typically associated with the anaerobic digestion of FVW alone. A third

(nitrogen-rich) substrate such as manure would need to be included however, as both LCB and FVW are rich carbon-sources. Therefore, the co-digestion of FVW and LCB alone would result in unfavourable C/N ratios.

LCB generally refers to the fibrous, wood-like and usually inedible fraction of plant matter (Bash, 2006). Lignocellulose characteristically resists degradation and provides hydrolytic stability and structural robustness to the cell walls of plants through the crosslinking of cellulose and hemicellulose to lignin by means of ester and ether bonds (Bash, 2006). LCB can be used as a co-substrate for anaerobic digestion as it is a rich source of carbon. However, raw LCB is not often preferred as a co-substrate owing to the inaccessibility of the cellulose due to lignin which makes biodegradation difficult and results in decreased biogas and methane yields (Zheng *et al.*, 2014; Yang *et al.*, 2015). Furthermore, it has been reported that anaerobic digestion of LCB with lignin contents greater than 100 g/kg VS result in especially low methane yields (Triolo *et al.*, 2012).

As a result of the recalcitrance to degradation, crops with high lignocellulose contents usually require pretreatments prior to anaerobic digestion to free cellulose from lignin, thus making it available for degradation (Taherzadeh and Karimi, 2008). Unit operations such as mechanical milling, washing with hot water, steam explosion, ammonia fibre expansion and alkali pretreatments are often used for this purpose. This facilitates the conversion of biomass to bioenergy products through the removal of hemicellulose and/or lignin, reduction of the crystallinity of the biomass structure and by increasing the porosity (Agbor *et al.*, 2011; Monlau *et al.*, 2013). Yet, many of these pretreatments incur substantial environmental and economic costs due to the generation of solid and liquid waste streams (Shrestha *et al.*, 2008; Kumar *et al.*, 2009; Alvira *et al.*, 2010; Agbor *et al.*, 2011; Monlau *et al.*, 2013). Due to the success of using pretreated LCB as a co-substrate for enhanced biogas yields, much research has been conducted with the aim of improving these processes and minimising the associated costs. However, one approach that has yet to be explored is the use of young (immature) LCB as a co-substrate. In theory, due to the lower lignin contents found in immature plants, this method would avoid the pretreatment step but share the benefits of using LCB as a co-substrate, thus proving more cost-effective for industry.

In addition to lignin content, the nutritive value of the plant changes according to maturity (Azim *et al.*, 1989). In a study by Azim *et al.* (1989), it was reported that the crude protein content of the whole plant decreased by 30% at two months of age, and by 66% at 3 months of age. Conversely, the crude fibre content was seen to increase with plant maturity. Factors other than plant maturity seen to influence lignin content and overall plant composition include geographical location, agricultural practises (such as the quantity of water, fertiliser etc.) and plant species (Amon *et al.*, 2007). This is evident in Table 2.6 where the lignin contents can be seen to differ substantially between the different species of LCB. Of the species listed below, sorghum straw (7.52%) and maize stover (8.40%) were among the lowest reported lignin contents.

Table 2. 6 Composition of Lignocellulosic Biomass.

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	C/N ratio	Reference
Maize stover	37.50	30.0	8.40	59	(Li, Li, Zheng, Fu, & Lar, 2009)
Wheat straw	38.20	21.20	18.0-23.4	60	(Wang *et al.*, 2009; Brown, Shi and Li, 2012)
Switch grass	31.00-45.00	20.00-31.00	12.00-18.00	90	(Brown, Shi and Li, 2012; Karthikeyan and Visvanathan, 2013)
Bagasse	38.20	27.10	20.20	118	(Brown, Shi and Li, 2012; Karthikeyan and Visvanathan, 2013)
Sugarcane	25.00	117.00	12.00	N/A	(Brown, Shi and Li, 2012; Karthikeyan and Visvanathan, 2013)
Rice straw	32.00	24.00	13.00	47	(Brown, Shi and Li, 2012; Karthikeyan and Visvanathan, 2013)
Oat straw	41.65	N/A	13.00	46	(Stallcup, 1958)
Biomass sorghum	22.20	19.4	21.40	N/A	(Monlau *et al.*, 2012)
Barley straw	37.50	25.3	26.10	N/A	(Florian Monlau *et al.*, 2013)
Rye Straw	38.00	36.9	17.60	20	(Nizami, Korres and Murphy, 2009; Monlau *et al.*, 2013)
Sorghum straw	35.87	26.04	7.52	29	(Cardoso *et al.*, 2013)
Sunflower stalk	31.00	15.6	29.20	N/A	(Monlau *et al.*, 2012)

Adapted from "Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities" Sawatdeenarunat *et al.* (2015).

In a study conducted by Amon *et al.* (2007), the effects of harvesting time with biogas production from maize was investigated. The authors concluded that the highest methane yield per hectare was obtained after 122 days of vegetation at the wax ripeness phase (in the vegetation stage, milk to wax-ripeness). At this age, the plant consisted of between 35-39% dry matter. No significant increases in methane yield were observed after 155 days; this is most likely due to the increased lignin content. This information suggests that either maize or sorghum residues would be promising substrates in the investigation of the effect of younger LCB as a co-substrate of fruit juice process wastes on methane yields, due to their much lower reported lignin contents.

2.5 Biogas composition

Biogas predominantly consists of methane (50-70%) and carbon dioxide (30-40%), however it can also contain other gases such as H_2, O_2, N_2, H_2S and water vapour. Of the inorganic acids produced in the digester hydrogen sulphide (H_2S) is the most detrimental as large quantities will damage the digester equipment. Excess hydrogen sulphide is usually the result of the digestion of large amounts of sulphur-containing waste such as proteinaceous compounds. Hydrogen sulphide can be scrubbed from biogas, however this is expensive and likely cost-prohibitive for small treatment plants (Gerardi, 2003).

Methane represents the most valuable fraction of biogas, with pure methane having a heating value of approximately 37 MJ/m^3. The heating value of biogas however, is somewhat lower at approximately $18-22$

MJ/m^3, due to dilution with carbon dioxide (Gerardi, 2003). As the carbon dioxide portion of biogas increases, the heating value decreases. Should the carbon dioxide portion of biogas become too large, the biogas will need to be supplemented as it will not produce a self-sustained burn on its own. If the carbon dioxide portion exceeds 30%, the concentration of acid in the sludge increases and causes the pH to drop below 7. At pH levels below 7, substantial acid fermentation occurs.

2.6 Conclusion

Fruit and vegetable waste has potential as a precursor for the production of many valuable products. Among the best characterised and most economical at an industrial scale is the use of FVW for biogas production. Methane yields from anaerobic digestion of FVW are improved through co-digestion with other wastes, especially multiple substrates due to improved C/N ratios, nutrient supplementation and better alkalinity. Manure and food waste as co-substrates for FVW may especially improve methane yields compared with other co-substrates. However, food waste composition is variable and the improved methane yields associated with it are likely due to balanced nutrient composition and high lipid content. The ratios of particular substrates in multi-substrate feedstocks may be optimised using statistical methods to evaluate methane potential. In addition, lignocellulosic biomass (LCB) has potential as one co-substrate of FVW as it contains complex carbohydrates which would buffer the rapid decline in pH typically seen with FVW digestion. Currently, few studies have investigated the effects of LCB as a co-substrate for anaerobic digestion. Due to the improved nutrient content and lower lignin content in younger LCB compared with mature LCB, expensive chemical and heat pretreatments may be avoided thus minimising costs associated with LCB for anaerobic digestion. Using plant species which have particularly low lignin contents, such as maize, is likely to further improve biogas yields. Therefore, a study investigating the effects of younger LCB as a co-substrate for anaerobic digestion would be both economically and academically valuable.

Chapter 3: Research Questions and Objectives

The aim of this research was to identify substrate combinations from five fruit juice processing wastes which resulted in the highest biogas and methane yields and to identify combinations which specifically minimise manure addition and maximise fruit waste disposal through anaerobic digestion. This was achieved by meeting the following objectives:

- Determine the methane potential and the effect of the addition of individual substrates through Biomethane Potential (BMP) tests
- Identify substrate combinations with high methane potential and biogas yields as well as substrate combinations which minimise manure supplementation and maximise waste disposal value using BMP tests
- Determine the effect of varying substrate combinations on pre- and post-digestion VFA concentrations
- Test the viability and stability of two selected points in 50L reactors in both batch and semi-continuous processes.
- Determine the maximum OLR, within the tested range (1-4 gVS/L/Day), that can be used in semi-continuous process for two selected points in 50L reactors

The following research questions were identified in this study:

- What are the effects of each of the individual substrates namely pomace, retentate, waste apples manure and food waste on both biogas and methane yields?

- Which substrate combinations incorporate the greatest quantity of fruit wastes streams (i.e. have the highest waste disposal value) without displaying decreased biogas quality of less than 40% methane?

- Does co-digestion of fruit juice waste streams with LCB improve biogas and methane yields and does LCB addition compensate for and thus allow minimisation of the manure fraction in the substrate mix?

- Do any of the tested conditions result in high VFA concentrations without severely compromising biogas production or producing low quality biogas (<40% methane)?

- Can the optimum substrate combinations determined at lab-scale produce comparable results and maintain process stability when tested at a larger scale in batch process?

- What is the maximum OLR, within the tested range, that can be stably operated under semi-continuous conditions in 50 L reactors?

Chapter 4: Materials and Methods

4.1 Feedstock preparation

All feedstocks with the exception of the lignocellulosic biomass were supplied by Elgin Fruit Juices (EFJ), namely fresh apples, apple pomace, retentate, food waste and cow manure. The pomace, retentate and waste apples are all waste products generated by EFJ, whereas the initial sources of the food waste and manure feedstocks are Cape Town Market and Interfoods (Woolworths suppliers) and a nearby dairy farm, respectively. Each of the feedstocks was mixed using the cone and quarter sampling method to ensure homogenisation and thus obtain more representative samples. After feedstock collection from EFJ, the feedstocks were macerated using a bowl cutter. The macerated feedstocks were then stored at -20 °C in 1 kg aliquots until use. The lignocellulosic biomass (maize) used in this study was grown at Agricultural Research Council (ARC) facilities and was harvested after approximately 142 days of growth. After harvesting, the LCB was dried in a greenhouse over a period of 4 weeks. Once dry, the LCB was milled using a hammer mill fitted with a 2 mm screen. After milling, aliquots (of approximately 1 kg) of the LCB were vacuum sealed and stored at room temperature until use.

4.2 Inoculum preparation

Fresh inoculum was obtained from the anaerobic digesters currently in use at South African Breweries (SAB) and New Horizons Energy (NHE) for bench scale experiments. Inoculum used for the scale-up experiments in the 50 L stirred reactors was obtained directly from the EFJ anaerobic digester due to complications with the previous suppliers. In each case, the obtained inoculum subsequently underwent degassing (pre-incubation) in a CSTR bioreactor (TF Design (Pty) Ltd) with intermittent mixing so as to ensure the digestion of any residual substrate in the inoculum, which may otherwise have led to inaccuracies in methane potential determination. This pre-incubation was conducted at the same temperature as the process temperature (37°C). The amount of time required for degassing is dependent on the quantity of residual substrate in the inoculum; however, the required time is typically between 2-5 days. In cases where the inoculum is obtained from a reactor fed with wastes containing high lipid contents, the amount of incubation time required may be longer.

4.3 Substrate characterisation

Waste characterisation involved proximate analysis as well as the determination of the total solids (TS), volatile solids (VS) and C/N ratios for each type of waste (both single and multi-substrates). For fruit-derived feedstocks, pectin content was determined. For lignocellulosic materials cellulose, hemicellulose and lignin contents was also measured.

4.3.1 Moisture content

Proximate analysis of the different wastes involved determination of the moisture and ash contents, crude lipid and crude protein as well as the amount of digestible carbohydrates present in the feedstocks. The moisture content was determined by weighing the samples before and after convection oven drying at 105°C overnight, until a constant weight was achieved (APHA 2540 B method). The mass difference before and after drying represents the quantity of evapourated water, however this can often be overestimated when determining the moisture contents of acidic compounds as VFAs may be evapourated along with water. The moisture content (%) was then calculated as follows:

$$\% \text{ Moisture} = \frac{(\text{Weight}_{\text{Dish}} + \text{Weight}_{\text{Wet sample}}) - (\text{Weight}_{\text{Dish}} + \text{Weight}_{\text{Dry sample}})}{(\text{Weight}_{\text{Dish}} + \text{Weight}_{\text{Wet sample}})} \text{ X } 100 \qquad [1]$$

4.3.2 Total solids analysis

Total solids are typically determined by the same method mentioned used for analysis of the moisture content. Similarly, VS may be underestimated due to VFA losses during TS determination. Thus, the pH of the waste should first be increased in order to decrease the volatility of VFAs, before the determination of TS. For extremely volatile samples, TS determination should be conducted at a maximum temperature of 90°C rather than 105°C until constant weight. Using this method, the total solids were then calculated according to the following equation:

$$\% \text{ Total Solids} = \frac{(\text{Weight}_{\text{Dish}} + \text{Weight}_{\text{Dry sample}})}{(\text{Weight}_{\text{Dish}} + \text{Weight}_{\text{Wet sample}})} \text{ X } 100 \qquad [2]$$

4.3.3 Volatile solids analysis

The residue produced from the APHA 2540 B method was then transferred to a muffle furnace at 550°C until a constant weight, in order to determine the volatile solids and ash contents (APHA 2540 E method). Volatile solids may then be calculated using the following formula:

$$\% \text{ Volatile Solids} = \frac{(\text{weight of dried sample} - \text{Ash})}{(\text{Weight of dried sample})} \text{ X } 100 \qquad [3]$$

4.3.4 Macronutrient analysis

 Samples were sent to Quantum Analytical Services (Malmesbury, Western Cape, South Africa) for crude fibre, crude protein, lipid content, carbohydrates by difference and ammonia quantification.

For crude protein the Dumas method of protein determination was used, whereby the sample was combusted at 900°C in the presence of pure oxygen to nitrogen, carbon dioxide and water. The nitrogen was then measured by a thermal conductivity detector after selective removal of the carbon dioxide and water.

Crude fat was determined using the ANKOM XT15 extraction system and measuring the loss in mass after extraction with petroleum ether of fat or oil from the sample encapsulated in a filter bag.

 For crude fibre analysis protein, fat, starch and other digestible carbohydrates were removed from the sample by hydrolysis with hot acid and alkali. The residue was dried, and the ash content was determined to calculate the loss on ignition of the crude fibre.

Lastly, total carbohydrates by difference were determined by subtracting crude fats, crude protein, ash and moisture from the total sample weight and therefore represents the sum of both digestible and non-digestible carbohydrates. Total available carbohydrates were then determined through the subtraction of the total crude fibre from the total carbohydrates.

4.3.5 Elemental analysis

Samples were sent to Central Analytical Facility (CAF) for elemental analysis. Carbon, oxygen, nitrogen, sulphur and hydrogen concentrations were measured using an Elementar Vario EL Cube Elemental Analyzer (Elementar, Langenselbold, Germany). Between 1 mg and 1 g of liquid sample was transferred in tin vessels and loaded into the integrated carousel. Each sample was then flushed with helium to remove atmospheric nitrogen. The catalytic combustion was then carried out at a fixed temperature of 1200°C. The reduction of the combustion gases on hot copper occurred in a second furnace so that the analysis gases remain in the carrier gas system. The gas mixture was then separated into individual components via three separate columns and was consequently measured by a thermal conductivity detector (TCD). The elemental concentrations within each of the samples were then calculated by the vario EL cube firmware based on the detector signal and sample weight based on stored calibration curves.

4.3.6 Chemical Oxygen demand (COD) determination

The chemical oxygen demand was determined bi-weekly for samples from 50 L batch assays using the Spectroquant® COD Cell Test kit (Merk, Darmstadt, Germany) which follows the closed reflux method (APHA 5220 D). Samples were first homogenised using a vortex and subsequently 1 ml of the homogenised sample was transferred to the reaction cell containing premeasured quantities of predominantly potassium dichromate, silver sulphate and chloride masked with mercury sulphate. The cell was then heated at 148°C for 120 minutes and consequently allowed to cool for 10 minutes before swirling the cell and allowing it to cool for a further 30 min. The COD may then be determined photometrically by measuring the concentration of chromium ions produced as a result of the potassium dichromate reacting with oxidisable substances in the sample. To improve accuracy samples were tested in duplicate and were measured against a reaction cell blank with COD-free water.

4.4 Biomethane Potential Tests (BMP)

4.4.1 Experimental set-up

The ultimate biogas potential of each of the individual substrates as well as for the combinations of feedstocks was quantified using the protocol defined by Angelidaki et al. (2009).

Each of the serum bottles used had a total volume of 100 ml. A working volume of 70 ml was used in order to leave a headspace of 30 ml to accommodate for gas production. In each case a pure substrate or substrate mixture was transferred to the bottle. Subsequently, degassed inoculum was added to make up 10% of the total solids loading. During the pure substrate screening experiments, no buffer was used in order to gain a better understanding of how the individual substrates perform without interference or supplementation. Instead, water was used to obtain the desired solids loading of 10%. For all subsequent BMP experiments, namely the mixed substrate interaction study and the LCB supplementation study, 1% calcium carbonate was added together with water to provide buffering capacity and to obtain the desired solids loading of 3%. In each case, the pH of the mixture was measured and, when necessary, was adjusted using 1 M sulphuric acid and 1 M potassium hydroxide to obtain the desired pH of 7. Throughout preparation, the substrate mixture was stirred thoroughly to ensure homogeneity. The neutral mixture was then transferred to a serum bottle where it was sealed using a butyl stopper and an aluminium crimp. To recreate anaerobic conditions, each bottle was sparged using nitrogen gas for a minimum of 5 minutes. Sparging with nitrogen gas was performed using a hollow needle connected to the nitrogen gas pipeline, which was then used to puncture the butyl stopper, while a second hollow needle was pricked into the stopper to create a passage for the gas to exit. After 5 minutes of sparging, the needles were removed, and the bottles were stored in an incubator at 37°C for approximately 30-32 days.

4.4.2   Parameters

For all experimental runs conducted in serum bottles, certain parameters were held constant and are listed as follows:

- 1 M sulphuric acid and 1 M potassium hydroxide was used to adjust the pH in the serum bottles prior to incubation, while 0.05 M sulphuric acid and 1 M potassium hydroxide was used to adjust the pH during the VFAs titration procedure.
- All bottles were incubated at a temperature of 37°C and thus were maintained within a mesophilic range.

4.4.3 Control assays

Despite degassing the inoculum before use, inoculum blanks were still required to account for any gas produced as a result of any remaining residual substrate. These blanks consisted of only inoculum and buffer medium with no substrate. The gas produced by these blanks was deducted from the gas produced by the assay. In addition to the inoculum control, substrate control assays were also conducted. All BMP experiments, with the exception of the mixture design experiments, were conducted in triplicate so as to ensure the reproducibility of the obtained results. In the case of the mixture design experiments, due to the larger number of runs, multiple centre-points were used as a measure of reproducibility rather than triplicates of each run.

4.4.4 Analytical methods

4.4.4.1   VFA's estimation

In order to determine the total quantity of VFAs present at the end of the incubation period for screening experiments, Kapp's titration method was followed (Drosg, 2013). Initially, the material was removed from each serum bottle and the final pH of each mixture was measured. As per the requirements of Kapp's method, each sample is required to be free of suspended solids prior to titration. This was achieved by centrifuging the sample at 8000 r.p.m for 20 minutes. Subsequently, the pH of the sample liquid was then recorded. A volume of 20 ml of sample is required for titration according to Kapp's method. In those cases where an insufficient amount of sample liquid was obtained after centrifugation, dilutions with deionised water were made. The dilution factor was taken into account after the final VFAs concentration had been determined. For each 20 ml sample, 0.05 M sulphuric acid was used for titration of the sample to reach the desired pH values of 5, 4.3 and 4. The volume of acid required to reach each end point was recorded. Equation 4 was then used to estimate the final concentration of VFAs in each sample.

$$\text{Total VFAs} = [131{,}340 * (V_{pH4.0} - V_{pH5.0}) * NH_2SO_4 / V_{sample}] - [3.08 * V_{pH4.3} * NH_2SO_4 / V_{sample} * 1{,}000] - 10.9 \qquad [4]$$

$V_{pH4.0}$: Volume of added acid until pH= 4.0 in mL
$V_{pH4.3}$: Volume of added acid until pH= 4.3 in mL
$V_{pH5.0}$: Volume of added acid until pH= 5.0 in mL
V_{sample}: Volume of titration sample (recommended 20 mL, see Buchauer, 1997)
NH_2SO_4: Normality of used acid (0.1 for 0.05 mol L-1 sulphuric acid)

For all later experiments, individual VFA concentrations were determined using high performance liquid chromatography (HPLC). The specifications and settings for VFA analysis are described in Table 4.1.

Table 4. 1: HPLC Instruments specifications and settings for VFA analysis

Instrument 1	TSP (Thermo Separations Product) HPLC UV detector
Instrument 2	Dionex UltiMate 3000 HPLC UV detector
UV detector wavelength	210 nm
Column information	Biorad HPX-87H column, 250 x 7.8mm with guard cartridge
Column temperature	65 °C
Mobile phase	0.005M sulphuric acid
Flow rate	0.6 mL/min.

Thereafter, the totals of the individual concentrations of acetic, propionic, butyric, caproic and valeric acids were summed to provide an estimation of total VFAs present in the sample.

4.4.4.2   pH

The pH value provides an estimation of the stability of the anaerobic digestion process. Seeing as methanogens are highly pH sensitive (optimal pH range of 6.5-8.0), it is important to monitor the pH value as methane production is optimal within a specific pH range. For scale-up tests conducted in the 50 L digesters, pH was measured by taking effluent samples twice a week and using electrometric methods.

4.4.5 Gas quality and volume measurements

4.4.5.1 Gas measurement

For bench-scale (serum bottle) experiments, the volume of biogas that was produced was measured using a needle and syringe. A needle attached to the syringe was used to puncture the butyl stopper. The resultant pressure from the released gas pushes up the butyl stopper. Once the butyl stopper stopped moving, the corresponding amount of gas in millilitres could be recorded as millilitres of gas produced. These measurements were performed every 1-2 days depending on the amount of biogas formed. Total gas production at a reactor level was tracked automatically as the biogas digesters have a built-in gas production measuring device. This device is based on a manometer tube design and contains two valves for releasing gas and a sensor calibrated to read water displaced in the manometer tube.

4.4.5.2 Gas chromatography

The biogas was analysed using a CompactGC4.0 Gas Chromatograph (GC) in order to determine the gas composition. Specifications of this GC are provided in Table 4.2.

Table 4. 2: CompactGC4.0 specifications and settings

Carrier gases	Helium & Argon
Carrier gas flow rates	5.00 ml/min
Reference gas flow rate	1.00 ml/min
Thermal conductivity detector temperature	110 °C
Filament temperature	210 °C
Oven temperature	50.00 °C (detector 2); 65.00°C (detector 3)
Injector temperature	60.00 °C

The GC had two Thermal Conductivity Detectors (TCD). The second detector (Channel 2) functioned to identify the carbon dioxide composition. The third detector (Channel 3) identified the relative amounts of methane, nitrogen and oxygen present within the gas sample.

The calibration curves for carbon dioxide, methane, nitrogen and oxygen were used to determine the composition of these gases within each sample. When the sum of the gas compositions did not add up to 100%, the error was assumed to be due to other components in the sample or to variations in the sample injection pressure. Calibration of the CompactGC4.0 Gas Chromatograph equipment was performed every 6 months by heating the columns 50°C higher than normal temperature settings overnight in order to get rid of any residues within the columns. Subsequently, under the usual settings described in Table 4.2, gas samples of known purity were injected into the GC and the calibration curves were adjusted accordingly.

For lab-scale experiments, GC analysis was only performed on those samples which had enough gas pressure within the headspace of the serum bottle resulting in at least 10 ml of gas entering a syringe. For samples producing more than 10 ml of gas, duplicate measurements were taken. The average of the measurements in this case was then used. Those serum bottles having insufficient gas pressure were not analysed using GC. On a large scale, it is only possible to use the biogas being expelled due to a build-up of pressure and not the biogas within the headspace of the digester. Therefore, the same principle was applied at lab-scale. In the case of the scale-up experiments, gas samples were collected from the digesters using tedlar bags (1 L capacity). Due to the larger volume of gas produced in the scale up experiments, all gas samples were analysed in triplicate.

4.5 Experimental Design

4.5.1 Individual substrate BMP tests

All five EFJ feedstocks namely, fresh apples, apple pomace, retentate, food waste and cow manure were first evaluated on an individual basis for biomethane potential in serum bottles. These preliminary BMP tests

were conducted with a solids loading of 1% (w/v) and with an inoculum loading of 10% of the solids loading, without the addition of buffer, in order to understand how each of the substrates behaved without intervention. All BMP tests were conducted in triplicate, including the substrate control assays.

4.5.2 Mixed Substrate Study (including food waste)

The second set of preliminary experiments aimed to provide information as to the relationship between the concentrations of each of the five feedstocks examined and biogas and VFA production. From this information, the ranges of feedstock addition which favour biogas production could be identified and optimised for three scenarios. These three scenarios are designed to best reflect EFJ's feedstock availability throughout the year and are as follows:

1. Assuming waste apples and fruit processing waste (pomace and retentate) are available in excess (January-June: when fruit waste is the primary substrate used in the EFJ anaerobic digester) Cow manure will also be used in this scenario to provide nitrogen and thus to favourably adjust C:N ratios

2. Assuming no apple pomace or retentate is available (only waste apples, cattle manure and food waste). (July-December: fruit juice production season has ended, wastes that are available all year round are mainly used in the EFJ anaerobic digester)

3. Assuming all feedstocks are available (waste apples, manure, food waste, apple pomace and retentate) (January-June: all feedstocks are available)

It should be noted that although Scenarios 1 and 3 occur during the same season (January-June), Scenario 1 aims to identify the largest quantity of fruit waste that may be used in the digester, whereas Scenario 3 aims solely to identify which ratios of each of the feedstocks result in the greatest biogas production. More specifically, Scenario 1 aims to address a situation where pomace and retentate are available in abundance, and therefore to identify the maximum amount of fruit waste that can be added to the digester (i.e. using fruit waste as a primary substrate) without causing VFA accumulation and whilst still producing adequate quantities of biogas. In addition, Scenario 1 will help us to understand whether or not it is possible to produce biogas using fruit industry waste alone supplemented with LCB and if so, to identify the maximum amount of biogas that is able to be produced. Scenario 3, however, will provide an indication of the quantities of each of the available feedstocks which should be added for maximum biogas production (optimised ratio).

A five-level, five factor design was developed using Statistica 13.2 (StatSoft, Tulsa, OK, USA) and was used to investigate the effects of the five independent variables namely fruit waste, retentate, pomace, cow manure and food waste on the responses of net VFA's and total biogas produced. Experiments were conducted in 100 mL serum bottles and performed according to the BMP protocol described previously. Due to the large number

of runs, six replicates of the centre-point were used as a measure of reproducibility. The parameters for the experiment are described in Appendix A.

4.5.3 LCB Supplementation Study

Based on the results of the five factor five-level mixed feedstock experiment it was evident that the points including food waste displayed poor reproducibility. Seeing as manure was the only nitrogen source, food waste was initially selected as a feedstock as a second nitrogen source, in order to create more favourable C/N ratios when co-digested with fruit waste, which is carbon-rich. Due to reasons further elucidated in Chapter 5, the decision was made to exclude food waste from the following mixture design. It should be noted that with the exclusion of food waste, Scenario 1 and Scenario 3 become synonymous. Therefore, from this design onwards there are only two scenarios, namely:

Scenario 1: Waste apples, retentate, and pomace and manure (representative of juice producing season in the first half of the year)

Scenario 2: Waste apples and manure (feedstocks which are available all year)

Based on ranges identified for the remaining four feedstocks in the previous experiment, a five factor constrained mixture design (Appendix A) incorporating LCB as the fifth feedstock was developed in order to better understand the effects of LCB as a co-substrate of fruit waste and to determine whether the addition of manure could be minimised through the addition of LCB (seeing as manure is the most limited feedstock in terms of availability and is the only nitrogen source). Trends identified in the initial five factor design were taken into consideration when designing the new five factor constrained mixture design, specifically:

a. No individual fruit waste can exceed more than 30% w/w of the total substrate mixture (so as no fruit waste combinations exceed 60% and lead to acid crash)
b. Manure is added at a minimum of 20% (as a nitrogen source)
c. LCB addition does not exceed 30% of the substrate mixture
d. No combination of manure and LCB can exceed 80% of the substrate mixture

Experiments were conducted in 100 mL serum bottles and performed according to the BMP protocol described previously. Due to the large number of runs, only the centre-point was conducted in triplicate, along with two other randomly selected points and the inoculum controls.

4.5.4 Batch scale-up of two selected points in 50 L reactors

Two substrate combinations were selected to be performed in scale-up in 50 L reactors. The highest producing substrate combination in terms of biogas and methane yields (referred to as the biogas optimisation point) was selected as well as a substrate combination which aimed to minimise the manure fraction and maximise the fruit waste fraction within the mixture, whilst incorporating LCB and producing a

biogas quality of above 40% methane (referred to as manure minimisation point). Both substrate mixtures were tested in duplicate in a scale-up batch experiment. Scale up batch experiments were conducted in a 50 L tank reactor at 37°C with intermittent mixing at a low stirring rate of 150 rpm for a minimum of 5 minutes per day, twice a day, for a total of 32 days. The reactor has a working volume of 35 L (70% of the total digester volume). Gas was measured by a manometer-based online gas measurement system, with the volume of gas produced determined by water displacement in the manometer and a built-in sensor. The manometers connected to the 50L digesters were calibrated before used by the manufacturers. This calibration was performed by attaching a 1 L capacity Tedlar bag to the gas release valve on each digester and adding 14 mL of water to each manometer tube and keeping track of the number of times the sensor registered gas production (clicked) during the time taken to produce 1 L of gas. The total amount of gas produced (1 L) was then divided by the total number of clicks in order to determine the amount of gas produced 'per click' that was able to displace the 14 mL of water high enough to activate the sensor and this value was input into the PLC for online measurement. Tedlar bags (1 kg capacity) were then also used to collect gas samples from the upper part of the digester every 7 days in order to analyse gas composition. In addition, liquid samples were collected twice a week in order to monitor changes in COD (sampled from the aqueous phase), VS, pH and VFA production over time.

4.5.5 Semi-continuous 50 L reactor runs of selected points with increasing OLRs

The same two optimised points for biogas yield and quality as well as for manure minimisation were tested in duplicate in a semi-continuous process. The experiment was also conducted in 50 L tank reactors at 37°C with intermittent mixing at a low stirring rate of 150 r.p.m. for a minimum of 5 minutes per day, twice a day including after feeding, for a total of 32 days. The reactors were fed daily, starting with an organic loading of 1 gVS/L/day and increasing every 4-5 days by increments of 0.5 gVS/L/day until an organic loading of 4 gVS/L/day was reached. For daily feed mixtures, pH was corrected to a pH value of 7 and 1% (w/w) calcium carbonate was added to the feed mixture prior to feeding. As performed in the batch experiment, gas was measured by the same manometer-based online gas measurement system and tedlar bags (1 Kg capacity) were used to collect gas samples from the upper part of the digester approximately every 7 days in order to analyse gas composition. As was with the previous batch experiment, liquid samples were collected twice a week in order to monitor changes in COD, VS, pH and VFA production over time.

Chapter 5: Results and Discussion

5.1 Substrate Characterisation

The results obtained from both proximate analysis and nutrient profiling of the 6 individual feedstocks are detailed in Table 5.1.

Table 5. 1**: Characteristics of Individual Substrates**

Analysis	Substrate					
	Manure	**LCB (Maize stover)**	**Waste Apples**	**Pomace**	**Retentate**	**Food waste**
1. TS (% w/w)	9.83 ± 1.03	91.06 ± 0.30	13.11 ± 2.67	18.13 ± 3.00	5.35 ± 3.28	30.57 ± 0.47
2. VS (% of TS)	84.54 ± 1.74	91.73 ± 0.20	98.52 ± 0.04	98.23 ± 0.23	89.73 ± 0.16	89.46 ± 0.16
3. VS (% w/w)	1.52 ± 1.74	83.53 ± 0.20	11.05 ± 0.04	19.89 ± 0.23	2.72 ± 0.16	27.65 ±0.16
4. Ash (%TS)	15.51 ± 0.30	8.27 ± 0.20	1.48 ± 0.04	1.78 ± 0.23	10.27 ± 0.17	10.54 ±0.16
5. Moisture (% w/w)	90.17 ± 1.03	8.95 ± 0.30	86.89 ± 2.67	81.88 ± 3.00	94.64 ± 3.28	69.44 ± 0.47
6. Crude protein (%TS)	9.88 ± 0.45	11.47 ± 0.34	4.54 ± 0.14	10.59 ± 0.76	14.49 ± 0.57	16.06 ±0.13
7. Crude fats (% TS)	5.33 ± 0.34	0.68 ± 0.15	3.63 ± 0.04	8.11 ± 0.52	2.83 ± 0.74	26.90 ± 1.19
8. Carbohydrates (%TS)	33.23 ± 3.21	52.08 ± 1.68	77.65 ± 1.32	41.27 ± 0.76	45.05 ± 1.36	40.53 ± 2.29
9. Total Crude Fibre (%TS)	29.19± 1.58	27.51 ± 1.29	12.71 ± 1.25	38.26 ± 1.82	27.38 ± 1.70	5.97 ± 0.82
9.1 Cellulose (% fibre)	19.53 ± 0.13	31.74 ± 0.13	19.02 ± 0.33	29.18 ± 0.68	N/A	N/A
9.2 Hemicellulose (%fibre)	21.73 ± 0.33	11.40 ± 0.16	14.34 ± 0.12	12.40 ± 0.67	N/A	N/A
9.3 Lignin (%fibre)	32.59 ± 0.2	20.73 ± 0.02	29.11 ± 0.23	26.35 ± 0.67	N/A	N/A
10. Pectin (% TS)	N/A	N/A	4.33 ± 0.29	3.00 ± 0.5	2.94 ± 0.22	N/A
11. C:N	28.88 ± 1.17	23.45± 0.25	101.41 ± 22.64	29.01 ± 0.46	30.14 ± 2.06	16.90 ± 0.48
12. pH [-]	7.17	5.75	3.94	3.37	3.43	4.04

A wide range of dairy cow manures were analysed by Pettygrove (2009) who obtained minimum and maximum C/N values of 9.3 and 33.4. The C/N ratio of manure samples obtained in the study (Table 5.1) falls within this reported range (Pettygrove, 2009). Pettygrove (2009) described a median pH value of 7.8 for dairy cow manure, which is comparable the pH of 7.17 obtained in this study. It should be mentioned that the wide ranges of nutrient properties accounted by Pettygrove (2010) are typical, as manure composition is known to be highly dependent on the diet of the animal. In addition, the age of the manure when obtained and storage conditions of the manure will also play a role in overall composition (Hubbard & Lowrance 2001).

Li et al. (2017) performed a proximate analysis of sun-dried maize stover and obtained similar moisture, dry matter, volatile solids and ash contents of 9.2 ± 1.0, 90.8 ± 1.0, 88.7 ± 0.8 and 3.5 ± 0.6 respectively. Both this study and the study by Li et al. (2017) reported similar initial pH values of around 5, with Li et al. (2017) obtaining an initial pH of 5.4 (Table 4.1). A lignin content of 7.5 ± 0.8 (%w/w) was reported by Li et al., this value is slightly higher than the 5.19 (% w/w) lignin content found in this study. In addition, the cellulose and hemicellulose contents obtained in the study by Li et al. (2017) were much higher compared to the findings

of this study, however composition between maize cultivars and between the same cultivar at different stages of vegetative growth are known to significantly differ (Firdous and Gilani, 1999).

Wikandri (2014) reported a moisture content of 88%, a total solids content of 12% and a VS content of 98 (% TS) for fresh apples. These findings are consistent with the outcomes of this study having obtained a moisture content of 86.89 ± 2.67%, a solids content of 13.11 ± 2.67% and a VS content of 98.52 ± 0.04%. The values Wikandri (2014) reported for carbohydrate, protein and fat contents of apples namely 91 (% TS, including fibre), 4.17 (% TS) and 2.66 (% TS) respectively, are also highly comparable to the results listed in Table 5.1.

Dhillon et al. (2013), reported a moisture content of between 70-75% for apple pomace, which is slightly lower but still comparable to the 81.88 % obtained in this study (Table 5.1). Carbohydrate yields were slightly low compared to the study by Dhillon et al. (2013) which described a carbohydrate content of 48-83 (% TS). In contrast, apple pomace was reported as having a protein content of 2.9-5.7 (% TS), much lower than the 10.59% obtained in this study. This translated to a difference in C/N ratios, with 18.8 being described by Dhillon et al. (2013) and a C/N result of 29.01 ± 0.46 obtained in this study. The lipid content of apple pomace obtained in this study was more than twice the reported range of 1.2-3.9 (% TS). Crude fibre obtained in this study was within the reported range of 4.7-51.10 (% TS) (Dhillon, Kaur and Brar, 2013). Pectin yield was within the range of 3.5-14.32 (% TS) as described by Dhillon et al. (2013). Hemicellulose and cellulose values were also within the reported ranges, however the lignin content of apple pomace described in this study was 5% lower than the lowest reported value described by Dhillon et al. (2013). Lastly, the initial pH of apple pomace in this study was comparable to the pH of 3.5 reported in the study by Dhillon et al. (2013), any other compositional differences are most likely due to naturally-occurring compositional differences between apple cultivars and levels of decay between apple samples.

Despite the abundance of fruit juice processing plants, there is relatively little compositional data on apple retentate. One study found the initial pH value of apple retentate to be 3.3 which is comparable to the pH of 3.43 observed in this study (Dhillon, Kaur and Brar, 2013). The moisture content of retentate in this study was slightly higher than the approximate moisture content of 87.5% obtained in the study by Dhillon et al. (2013). This is most likely due to the addition of apple-washing waste water to the retentate in this study as compared with the retentate in the study by Dhillon et al. (2013). Protein and fat contents were lower than reported values by Dhillon et al (2013), however carbohydrates were within the described range (Table 5.1). As a result of the difference in protein contents the C/N value of 30.14 ± 2.06 obtained in this study was much higher than the C/N ratio of approximately 18.86 reported by Dhillon et al. (2013).

The nutrient profiles between food wastes are highly variable as restaurant wastes differ considerably according to seasonal availability of foods, growth climates within the country as well as due to cultural and personal food preferences. Balazs (1971) tested food waste samples taken from multiple locations within the same region and found protein contents ranged between 15-27 %, crude fibre ranged from 4.3-7.4% and that

total solids ranged from 8.7 to 16.8%. Although no two food waste samples are alike, the fibre and protein contents are comparable to the results of 5.97% and 16.06% obtained in this study (Table5.1). In addition, the reported fat contents ranged from 22.20 % to 54.40% (Balazs, Hugh and Brooks, 1971). This is also in line with the results of this study which obtained a fat content of 26.90% for food waste. Lastly, the pH values obtained in both studies were similar, having both attained a pH of around 4 for food waste.

Proximate analysis of all six substrates showed that retentate had the lowest solids content and LCB had the highest solids content (Table 5.1). This is understandable as retentate is a liquid, having been mixed with waste water and the LCB having had been dried in a greenhouse, was in straw form. All substrates appeared to have high volatile solids contents, with the lowest being 84.54 (%TS) belonging to manure and the highest 98.52% belonging to waste apples (Table 4.1). Materials high in volatile solids are thought to have a greater methane production capacity during anaerobic digestion. Manure had the highest ash content 15.51(% TS) of all the feedstocks followed by food waste and retentate.

Upon examination of the nutrient profiles of the six substrates, food waste was seen to have the highest protein content, followed by retentate, LCB, pomace and then manure, with waste apples exhibiting the lowest (Table 5.1). It is unexpected that retentate should have the second highest protein content and that manure should have the second lowest. However, the retentate is the membrane fouling layer remaining after juice clarification via ultrafiltration and the primary components are pectin, proteins, fibre, phenolics and starch which may explain the higher protein levels compared to other fruit wastes (Riedl, 1996). The lower protein content in manure might be explained by a lower crude protein content in the dairy cattle feed, however the amount of time the manure was left out in the open before sample collection might also have contributed to an underestimation of manure protein content. This is as a direct result of ammonia volatilisation, which is a known problem with leaving the manure exposed to the environment as ammonia is an atmospheric pollutant, and volatilisation is exacerbated by warmer weather conditions (Huijsmans, 2003). This could explain the lower nitrogen content in the manure sample as the manure samples obtained in this study were stored outside for several weeks and the resultant loss of ammonia due to volatilisation would reflect as a lower nitrogen concentration in the manure sample.

Waste apples displayed the highest levels of available carbohydrates (77 %TS) of all the substrates, followed by LCB and retentate (Table 5.1). Manure had the lowest available carbohydrate content, likely due to having already undergone ruminant digestion. The highest fibre content belonged to pomace, followed by manure and LCB. Of the fibre fractions, manure displayed the highest lignin content followed by waste apples, pomace and then LCB (Table 5.1). The high lignin content of manure is explicable, as the manure consists of all the indigestible compounds that could not be degraded by the cattle. The lower lignin content of LCB is likely due to the relatively immature nature of the maize when harvested. Of all substrates, LCB contained the highest cellulose content and manure had the highest hemicellulose content. Of the fruit wastes, waste apples contained the most pectin.

All substrates except for waste apples, food waste and LCB fell into the optimum C/N range of 25-30. LCB almost fell within the optimum range with a C/N value of 23.45 (%TS). Although C/N is a factor which greatly influences anaerobic digestion, it is not the only factor responsible for optimal biogas and methane yields. Substrates also high in easily reducible sugars, will not be prevented from causing digester acidification by favourable C/N alone. In addition, the C/N ratios mentioned in this study accounted for both available and unavailable carbon, therefore the reported C/N ratios will not reflect the ratio of available carbon to nitrogen ratio, which is a more accurate indicator for digester performance.

Lastly, all the substrates with the exception of manure, fell within the acidic pH range. Seeing as though optimum pH range for anaerobic digestion is typically reported as above 7 (Gerardi, 2003), fruit wastes and food waste would need to be pH corrected and likely would also require a buffering agent for mono-digestion. Based on the observed pH ranges, it can be seen how manure as a co-substrate of fruit juice processing wastes could improve biogas and methane yields, by raising the pH and improving buffering capacity - seeing as manure is rich in nitrogenous compounds which are subsequently degraded into ammonia which then counteracts the effect of the pH drop due to VFA production, thus demonstrating a buffering effect (Li, Chen and Li, 2010).

In conclusion, all of the substrates used in this study demonstrated similar compositions to those reported in literature to have been used in anaerobic digestion previously, and thus were considered suitable for anaerobic digestion. In addition, the highest C/N ratio of 101.41 ± 22.64 belonging to the fresh waste apples, indicated that waste apples would acidify quicker due to rapid VFA production as a result of the greater quantity of available carbohydrates (Table 5.1) compared to the other substrates, and therefore would require the greatest degree of supplementation with a nitrogen-rich substrate in order to prevent acid crash. Therefore, of all tested substrates, waste apples were predicted to pose the biggest obstacle in mono-digestion and thus display the poorest methane yields, however this assumption required experimental evidence to support this theory.

5.2 Individual substrate BMP test results

BMP tests of individual substrates were conducted using a solids loading of 10% (w/v), without buffer, in order to determine the viability of using each substrate in mono-digestion for biogas, methane and VFA production, thus determining the need for co-digestion of fruit juice process waste with additional substrates. The results are illustrated in Figures 5.1 and 5.2 below.
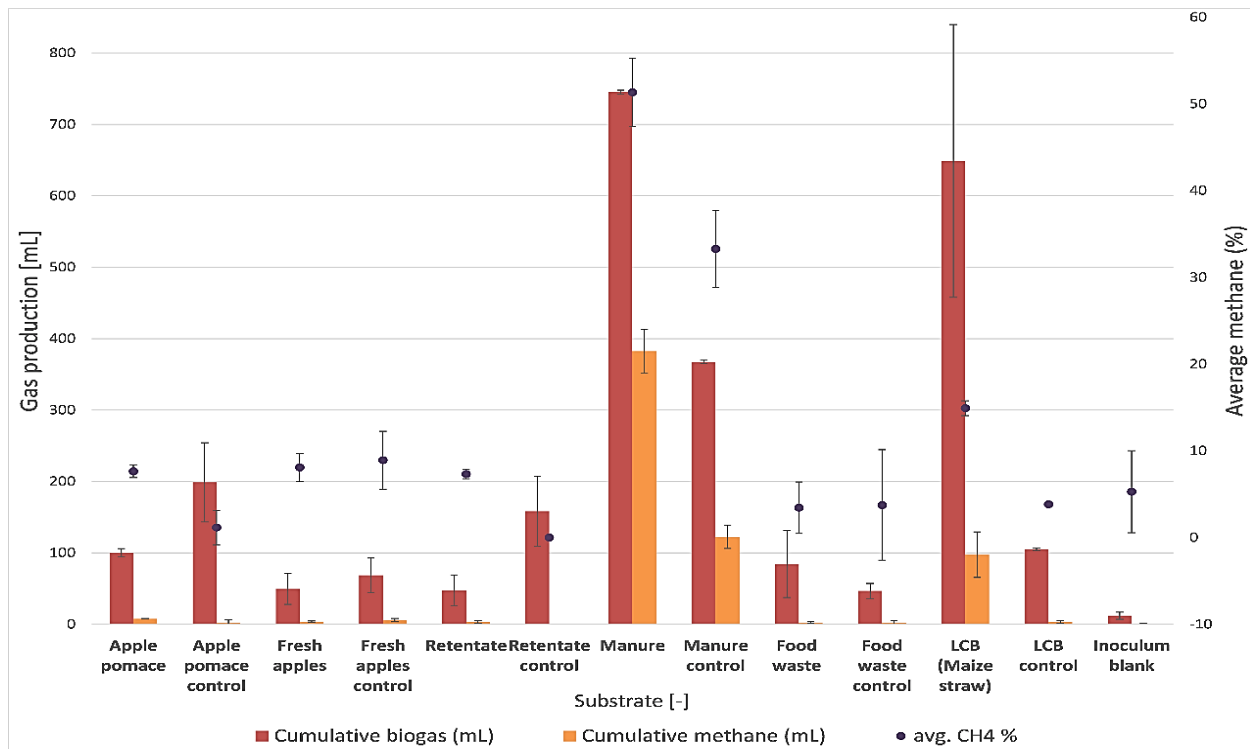
Figure 5. 1: Biomethane Potential (BMP) test results for individual substrates and substrate and inoculum controls without buffer displaying total biogas (mL) and methane (mL) produced as well as the average

As is illustrated in Figure 5.1, all fruit processing wastes produced poor biogas and methane yields, with the highest biogas and methane yields produced by pomace and shown to be 100.33 ± 5.57 mL and 7.65 ± 0.43 mL, respectively. This indicated pomace was slightly more stable than the other two fruit wastes during mono-digestion. The subsequent relatively improved biogas and methane yields from pomace compared to waste apples and retentate are as a result of pomace having the highest volatile solids (% w/w) of the three fruit process wastes and having lower quantities of degradable sugars than the other fruit wastes, preventing VFA accumulation to a greater degree than in the cases of waste apples and retentate (see Table 5.1). The slightly higher post-digestion pH value of 3.8 obtained from pomace further supports this theory, as retentate and waste apples yielded even lower final pH values of 3.52 and 3.14 respectively. These lower pH values are explained by the higher VFA concentrations produced by retentate and waste apples (10.29 ± 1.66 and 8.98 ± 0.88 g/L) than pomace (6.20 ± 0.44 g/L) as seen in Figure 4.2 and explain why waste apples and retentate both produced the lowest biogas and methane yields overall. Retentate produced the greatest amount of VFAs over the course of

the digestion with a net VFA concentration of 8.15 g/L. The apples produced the second highest net VFA concentration of 7.23 g/L VFAs produced and pomace the third highest at 3.33 g/L VFAs produced. This increased VFA production post-digestion for all three fruit wastes can be explained by the higher VS content of the fruit wastes and suggests a greater quantity of easily reducible sugars present than in manure or food waste (Table 5.1). This would likely lead to VFAs being produced faster than they could be converted to biogas. This theory explains the higher VFA yields post digestion and subsequent low methane yields seen in Figures 5.1 and 5.2. These low pH values and high VFA concentrations were indicative of acid crash, seeing as all three fruit wastes displayed very low post-digestion pH values of below 3.8, and given that this experiment did not include any additional compounds to provide buffering capacity, to help prevent rapid acidification.

As is evident in Figure 5.1, all three fruit waste substrate controls produced higher biogas yields than their BMP assay counterparts which included inoculum, however they did not display higher methane yields. The predominant gas in all three cases was found to be carbon dioxide with lesser amounts of hydrogen and trace amounts of ethylene and methane for all three fruit waste controls. This is most likely due to fermentation of the fruit wastes which occurs due to the naturally occurring microorganisms present on the fruit. One study investigated the different microorganisms present on the surface of apples before and after different storage methods, and found that the predominant microorganisms present before storage were microscopic fungi, specifically moulds and yeasts (Juhneviča, Skudra and Skudra, 2011). The predominant microorganism found on apples after storage in an oxygen-limited environment were found to be yeasts (Juhneviča, Skudra and Skudra, 2011). This is understandable as yeasts are known facultative anaerobes which can switch to anaerobic respiration in the absence of oxygen and are the primary organisms responsible for fermentation. This could explain the increased biogas, predominantly consisting of hydrogen and carbon dioxide, in the fruit waste controls compared to their BMP test counterparts with inoculum.

This is not to say the fungi does not also exist in the BMP test counterparts as well as in the substrate controls, but rather that synergisms exist between the fungi and methanogenic organisms present in the inoculum which result in differences in metabolic end products. For example, several studies have reported synergistic relationships between anaerobic fungi and methanogens involving hydrogen-transfer between species resulting in methane production, as well as improved efficiency in regenerating oxidised nucleotides (NAD^{+}, NADP^{+}) (Orpin and Joblin, 1997; Cheng et al., 2009). This transfer of hydrogen also affects fungal catabolic pathways causing a shift from the formation of oxidised products such as ethanol and lactate to reduced end products, such as formate and acetate, which are then used as growth substrates by methanogens (Nakashimada et al., 2000; Cheng et al., 2009; Guebitz et al., 2015).
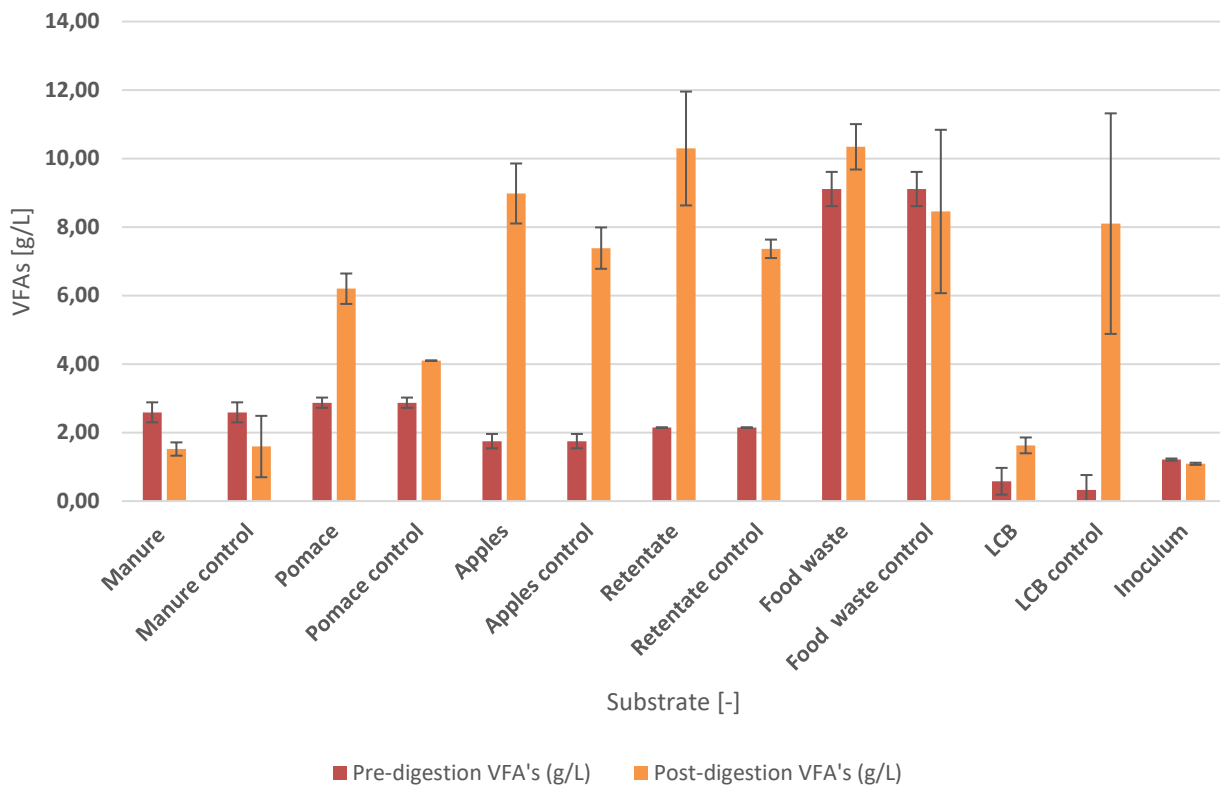
Figure 5. 2: Volatile fatty acid (VFA) production pre- and post-digestion for individual substrates including substrate controls.

Similarly, to the fruit waste mono-digestions, the food waste assay also demonstrated poor biogas and methane yields, producing lower yields (84.5 ± 47.01 mL biogas and 2.40 ± 1.55 methane) than pomace (100.33 ± 5.57 mL biogas and 7.65 ± 0.43 mL methane) (Figure 5.1), resulting in the lowest quantity of methane produced out of all substrate BMPs. Food waste was initially selected as a fruit waste co-substrate for a number of reasons. The food waste consisted of restaurant wastes but more specifically: vegetable wastes, meat products and oils. As mentioned in Chapter 2, vegetable wastes improve the buffering capacity when co-digested with fruit waste compared to fruit waste mono-digestion (Knol, Van Der Most and De Waart, 1978). The meat fraction of the food waste should have contributed additional nitrogen, decreasing the manure requirement in order to obtain favourable C/N ratios when combined with fruit wastes, however in this study the C/N ratio was one of the least favourable for mono-digestion (Table 5.1). Finally, the food waste that was chosen was rich in fats and oils which have a high energy value and have seen to improve biogas and methane yields (Neves, Oliveira and Alves, 2009a). Moreover, the more variety of feedstocks, the greater the chance of covering any nutrient gaps and preventing any digester crashes due to nutrient deficiencies. Despite the aforementioned benefits described in literature, food waste displayed some of the lowest biogas yields among all substrates. The most likely explanation is due to acid crash from the high quantity of VFAs in the digestate. As can be seen in Table 5.2, the food waste assay and controls contained both the highest amount of initial VFAs at 9.11 g/L and the highest post-digestion VFA concentration at

10.34 g/L. This explains the unexpectedly low pH value seen in Table 5.1. It also suggests very little conversion of VFAs to biogas seeing as though the biogas yields for food waste were very low. The high initial pH of food waste shown in Table 5.1 is due to the high quantity of pre-digest VFAs shown in Figure 5.2. Despite correcting the pre-digestion pH to 7, the post-digestion pH decreased to 4.43. This suggests the conversion of VFAs to methane became rate limiting, hence the higher post-digestion VFA concentration of 10.34 g/L compared to the pre-digestion VFA concentration and the subsequent low biogas and methane yields. In contrast, the food waste substrate control produced higher pre-digest VFA concentrations than post-digest VFA Concentrations by 0.65 g/L (Figure 5.2). This, combined with the low biogas production from the food waste substrate control (Figure 5.1), also implies residual microbial activity due to pre-existing microorganisms. Given that food waste was stored outside for several weeks before use, some microbial degradation had already occurred hence the high initial VFA concentration seen in Figure 5.2, and given that degradation of organic matter in the environment is primarily conducted by fungi (Berg and McClaugherty, 2008), it is evident that some endogenous species were present and respired to produce small amounts of biogas from VFAs in the controls.

The LCB assay produced the second highest quantity of biogas: 648.67 ± 190.93 mL and the third highest methane yield (97.54 ± 31.82 mL) next to the manure and the manure substrate control (Figure 5.1). This is likely due to the higher buffering capacity as a result of the higher protein content and complex carbohydrate content of maize as compared with the fruit wastes and food waste. As can be seen in Figure 5.2, LCB produced the lowest pre- and post-digestion VFA concentrations with the highest being 1.63 ± 0.23 g/L post-digestion. This slight increase in post-digestion VFA concentration together with a final pH of 5.80, suggests a more stable process during LCB ono-digestion compared to fruit and food wastes. In contrast to the LCB BMP assay, the LCB control which produced much higher VFA concentrations of 8.10 ± 3.22 post-digestion, produced substantially less biogas (543.67 mL less) than the LCB BMP assay (Figures 5.1 and 5.2). This indicates that more VFAs were converted to biogas in the LCB BMP assay, whereas acidogenesis due to residual microbial activity from naturally occurring bacteria from maize was nevertheless occurring in the LCB control without methanogenesis, leading to a slow accumulation of VFAs. Moreover, it should be added that the inoculum used in this study was taken from an established AD plant which was seeded by cow manure. One study characterised the diversity of anaerobic fungi present in cow manure and proposed that these fungi aid in the hydrolysis of lignocellulosic biomass within the ruminant (Fliegerová et al., 2010). Anaerobic fungi are unique from other fungi in that they contain cellulosomes which are multi-enzyme complexes made up of cellulolytic and hemi-cellulolytic enzymes (Guebitz et al., 2015). It is through these use of these enzyme complexes along with the ability of anaerobic fungi to physically break up plant matter via rhizoid growth, allowing for greater surface area exposure for microbial activity, that anaerobic fungi have been seen to enhance biogas yields from LCB (Guebitz et al., 2015). This is likely also a reason for the higher biogas yields seen in the LCB BMP assay.

Lastly, as can be seen in Figure 5.1, manure produced both the highest total biogas and methane yields (745 ± 2.83mL and 382.45 ± 30.57 mL). This is expected, given that manure contains many necessary trace elements and has increased buffering capacity. Manure is also a natural source of microorganisms required for the production of biogas (Tufaner and Avsar, 2015). This also explains why the manure controls performed better than the other BMP test assays and substrate controls. The manure controls produced 377.67 mL less biogas than the manure BMP test assays. This is likely as a result of the increased number of microorganisms present in the test assays, due to the inoculum addition as compared to the manure controls. Seeing as manure has a greater buffering capacity and less available carbohydrates when compared to fruit wastes (see Table 5.1), acid build up does not occur to the same extent. As can be seen in Figure 5.2, the manure and manure control assays produced the lowest post-digestion VFA concentrations of 1.52 and 1.59 g/L, respectively. Both the manure BMP assay and the manure substrate control showed a decreased VFA concentration post-digest as opposed to the pre-digestion VFA concentration, this loss is likely explained by the conversion of VFAs to biogas during the course of the run. Unlike the fruit wastes digestions, VFA formation did not exceed the rate of VFA conversion to biogas, resulting in a lower post-digestion VFA concentration. In addition, final pH values of both the manure BMP assay and the manure substrate control increased from 7 to 7.94 and 7.92 respectively over the course of the run, due to the accumulation of ammonia. Based on the stability of the manure mono-digestion process and the increase in pH which will counteract pH decrease due to VFA accumulation observed in fruit waste mono-digestions, it is evident that manure is a suitable co-substrate for fruit process wastes.

From this study it was evident that fruit processing wastes cannot be digested alone for methane production (Figure 5.1) and without additional buffering capacity, rapid acidification due to VFA accumulation will occur (Figure 5.2). In addition, it was found that despite the variety of nutrients and the high nitrogen content, food waste behaved more like the fruit wastes in mono-digestion than a nitrogen-rich source such as manure, due to its low initial pH and low biogas yields (Table 5.1 and Figure 5.1). It was thus concluded that for optimum biogas and methane yields co-digestion of fruit wastes and food waste with a nitrogen-rich substrate would benefit biogas and methane production by improving both buffering capacity and C/N. From this experiment, it was also apparent that due to the rapid acidification seen in the fruit waste assays, it would be necessary to include a buffer in future assays.

5.3 Mixed substrate interaction study

The primary aim of the mixed interaction study was to identify the substrate mixtures which gave the highest biogas and methane yields for each of three scenarios based on season availability and secondly, to identify substrate combinations with the highest waste disposal value (i.e. with the least manure and largest quantity of waste products) and a minimum average methane concentration of 40%. The results of the mixed substrate study are summarised in Tables 5.2 – 5.6 below.

5.3.1 Statistical Analysis

The data from the mixed substrate study mixture design was analysed using response surface methodology (RSM), fitting both biogas and methane yields as a function of the substrate ratios. An ANOVA was performed on the above mixture design using both total biogas (mL) and total methane (mL) as outcome variables. Although the linear model was shown to be significant with a p-value<0.05 for both outcome variables, the overall lack of fit of the model was also shown to be highly significant with a p-value < 0.001 (Appendix A). Due to the lack of fit of the model, the results were analysed qualitatively. Any observed trends were taken into account and used to develop parameters for the following mixture design experiment.

5.3.2 Biogas and methane production

100% Supplementation

The 100% supplementation level is comparable to the initial BMP tests conducted with individual substrates and differs only in the addition of a calcium carbonate buffer (1% w/w) as well as using a lower solids loading of 6%. Despite the differences, the resultant trends were similar - with only the pure manure assay yielded an average methane quality of above 40%, and producing the highest overall biogas and methane yields of 114.65 mL.gVS$^{-1}_{fed}$ and 65.78 mL.gVS$^{-1}_{fed}$, respectively (Table 5.2). As was seen in the first BMP experiment (Figure 5.1), pomace produced the most methane (9.83 mL.gVS$^{-1}_{fed}$) out of the fruit and food wastes. However, ultimately no individual feedstock other than manure could be mono-digested and still produce good quality biogas of more than 40% methane, despite the addition of the buffer.

Table 5. 2: Results of the mixed substrate interaction study.

	ID	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Food waste (% w/w)	Manure (% w/w)	Total Biogas (mL)	Average Methane (%)	Average total methane (mL)	Biogas (mL.gVS$^{-1}_{fed}$)	Methane (mL.gVS$^{-1}_{fed}$)	C/N
100 %	1	0	100	0	0	0	282.00	12.59	35.50	78.11	9.83	29.01
	2	0	0	0	0	100	437.00	57.37	250.71	114.65	65.78	28.87
	3	0	0	100	0	0	125.00	0.00	0.00	31.62	0.00	30.09
	4	100	0	0	0	0	307.00	2.85	8.75	86.11	2.45	101.41
	5	0	0	0	100	0	99.00	9.84	9.74	32.89	3.24	16.90

20% and 25% supplementation

Substrate mixture 1 in Table 5.3, represents the centre-point of the substrate mixture design experiment with 20% supplementation of all feedstocks. The combination of all five feedstocks performed poorly overall, yielding low quantities of both biogas (18.92 mL.gVS$^{-1}_{fed}$) and methane (5.50 mL.gVS$^{-1}_{fed}$). This is likely because of the large quantity of acidic feedstocks compared to the relatively low level of manure

supplementation. The quantity of manure was evidently not enough to buffer the acidity of the substrate mixture. The large standard deviation between the eight replicate runs was due to the heterogeneity of the food waste fraction seeing as it was the feedstock with the most varied composition. This variation is also the likeliest cause of the lack of fit of the model being significant ($p < 0.05$) and will be discussed in further detail in the next section.

	ID	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Food waste (% w/w)	Manure (% w/w)	Total Biogas (mL)	Average Methane (%)	Average total methane (mL)	Biogas (mL.gVS^{-1} fed)	Methane (mL.gVS^{-1} fed)	C/N
							Table 5. 3: Results of the mixed substrate interaction study.					
20%	1	20	20	20	20	20	148.78 ±157.02	16.70 ±13.40	43.26 ± 94.51	18.92 ± 19.67	5.50	28.55
25%	2	0	25	25	25	25	171.00	7.71	13.18	47.55	3.67	24.62
	3	25	0	25	25	25	369.00	12.71	46.90	102.93	13.08	28.43
	4	25	25	25	0	25	292.00	16.67	48.68	78.18	13.03	35.02
	5	25	25	25	25	0	172.5	28.26	48.75	48.80	13.79	28.47
	6	25	25	0	25	25	285.00	15.56	44.35	81.44	12.67	28.21

As can be seen in Table 5.3, the highest biogas (102.93 mL.gVS^{-1} fed) and second highest methane (13.08 mL.gVS^{-1} fed) yields from the 25% supplementation level were obtained when all substrates except pomace were included. The lowest biogas and methane yields were obtained from the pomace, retentate, food waste and manure substrate mixture. The highest methane yield (13.79 mL.gVS^{-1} fed) at the 25% supplementation level was achieved by the substrate combination excluding manure, however this combination also resulted in the second lowest biogas yield (Table 5.2).

No substrate mixtures at a 25% supplementation level produced an average methane percentage of above 40%. This suggests that a manure supplementation of 25% and an acidic fraction of 75% of the total mixture was not ideal for good biogas and methane yields.

33% Supplementation

Three substrate combinations produced above 40% average methane and showed the highest methane and biogas yields within the 33% supplementation level (Table 5.4). These results were obtained when each of the individual fruit wastes were used in combination with food waste and manure, with the combination of pomace, food waste and manure resulting in the highest overall methane (94.19 mL.gVS^{-1} fed) and biogas (182.19 mL.gVS^{-1} fed) yields (Table 5.4).

The second highest yields were achieved by retentate, food waste and manure with biogas and methane yields of 164.75 mL.gVS^{-1} fed and 76.89 mL.gVS^{-1} fed, respectively (Table5.4). Of the three fruit wastes, waste

apples performed the least well with biogas and methane yields of 151.85 mL.gVS^{-1} $_{fed}$ and 67.91 mL.gVS^{-1} $_{fed}$, respectively. Substrate mixtures without manure showed the lowest biogas and methane yields within the 33% supplementation level.

Fruit waste combinations with manure performed better than their corresponding fruit waste combinations with food waste (Table 5.4). No fruit waste combination with manure alone was able to produce an average methane concentration of above 40%, suggesting that manure would need to be supplemented to a greater degree when co-digested with fruit waste alone.

Table 5. 4: Results of the mixed substrate interaction study.

	ID	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Food waste (% w/w)	Manure (% w/w)	Total Biogas (mL)	Average Methane (%)	Average total methane (mL)	Biogas (mL.gVS^{-1} $_{fed}$)	Methane (mL.gVS^{-1} $_{fed}$)	C/N
33%	7	33.3	33.3	0	0	33.3	228	24.96	56.91	62.70	15.65	36.95
	8	33.3	0	33.3	33.3	0	119.5	20.86	24.93	34.30	7.15	28.28
	9	33.3	0	33.3	0	33.3	438.00	32.57	142.66	116.81	38.05	37.96
	10	0	33.3	33.3	0	33.3	215.00	28.12	60.46	57.11	16.06	29.29
	11	0	33.3	0	33.3	33.3	629.00	51.70	325.19	182.19	94.19	23.33
	12	33.3	33.3	0	33.3	0	189.00	11.97	22.62	56.07	6.71	28.00
	13	0	33.3	33.3	33.3	0	37.50	14.30	5.36	10.72	1.53	23.49
	14	0	0	33.3	33.3	33.3	587.50	46.67	274.19	164.75	76.89	23.36
	15	33.3	33.3	33.3	0	0	289.00	22.91	66.21	78.47	17.98	37.77
	16	33.3	0	0	33.3	33.3	522.00	44.72	233.44	151.85	67.91	27.93

50% Supplementation

As can be seen in Table 5.5, all four acidic feedstocks produced a methane concentration of above 40% when supplemented at 50% with manure. The substrate combination which produced the highest biogas and methane yields across all supplementation levels was equal parts retentate and manure which gave yields of 190.35 mL.gVS^{-1} $_{fed}$ and 120.46 mL.gVS^{-1} $_{fed}$, respectively. The second highest yields were obtained by 50% food waste and manure with a biogas yield of 172.83 mL.gVS^{-1} $_{fed}$ and a methane yield of 79.47 mL.gVS^{-1} $_{fed}$ followed by waste apples and manure (151.96 mL.gVS^{-1} $_{fed}$; 79.47 mL.gVS^{-1} $_{fed}$) and lastly by pomace and manure (107.79 mL.gVS^{-1} $_{fed}$; 45.52mL.gVS^{-1} $_{fed}$ respectively). Combinations of any two fruit wastes either together or in combination with food waste resulted in poor quality biogas of less than 40% and low overall biogas yields.

Table 5. 5: Results of the mixed substrate interaction study.

	ID	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Food waste (% w/w)	Manure (% w/w)	Total Biogas (mL)	Average Methane (%)	Average total methane (mL)	Biogas (mL.gVS^{-1} fed)	Methane (mL.gVS^{-1} fed)	C/N
50%	17	50	0	0	0	50	560.50	46.68	261.64	151.96	70.94	43.61
	18	0	50	50	0	0	38.00	18.31	6.96	10.05	1.84	29.50
	19	50	0	50	0	0	273.00	4.16	11.36	72.62	3.02	45.68
	20	0	50	0	0	50	400.00	42.23	168.92	107.79	45.52	28.94
	21	50	50	0	0	0	196.00	3.51	6.88	54.63	1.92	43.03
	22	50	0	0	50	0	142.50	5.80	8.27	43.34	2.51	27.48
	23	0	50	0	50	0	277.50	12.15	33.72	83.83	10.19	21.39
	24	0	0	0	50	50	589.50	45.98	271.05	172.83	79.47	21.17
	25	0	0	50	50	0	295.00	30.32	89.44	10.05	1.84	21.33
	26	0	0	50	0	50	739.00	63.28	467.64	190.35	120.46	29.45

5.3.3 Optimum substrate combinations from interaction study for biogas quality

The substrate combinations which produced the highest quality biogas (above 40% methane) and the highest overall biogas and methane yields from the interaction study for each of the three scenarios are highlighted in Table 5.6 and discussed below.

Table 5. 6: Summary of substrate combinations which produced above 40% methane for each of the three scenarios

	Substrate mixture*	Carbohydrates (%TS)	Protein (%TS)	Fats (%TS)	Fibre (%TS)	C/N	Biogas yield (mL.gVS^{-1} fed)	Methane yield (mL.gVS^{-1} fed)
Scenario 1	50% R & M	39.14	12.18	4.08	28.29	29.45	190.35	120.46
	50% FA & M	55.44	7.21	4.48	20.95	43.61	151.96	70.94
	50% P & M	37.25	10.24	6.72	33.73	28.94	107.79	45.52
Scenario 2	50% FW & M	36.88	12.97	16.12	17.58	21.17	172.83	79.47
	33.33% FA, FW & M	49.97	10.06	13.31	15.80	27.93	151.85	67.91
Scenario 3	33.33% P, FW & M	37.96	12.06	13.31	24.23	23.33	182.19	94.19
	33.33% R, FW & M	39.21	13.34	11.57	20.64	23.36	164.75	76.89
	100% M	33.23	9.88	5.33	29.19	28.87	114.65	65.78

*Where R = retentate, M =manure, FA = fresh waste apples, P= pomace and FW = food waste.

The substrate combination with the highest biogas (190.35 mL.gVS^{-1} fed) and methane (120.46 mL.gVS^{-1} fed) yields in the mixed interaction study consisted of equal parts retentate and manure and also represents the top performing point in the fruit juice producing season (Scenario 1) (Table 5.6). The mixture with the second

highest yields (182.19 mL.gVS^{-1} $_{fed}$ biogas and 94.19 mL.gVS^{-1} $_{fed}$ methane) was comprised of 33.33% pomace, 33.33% food waste and 33.33% manure and fell under Scenario 3. The third highest biogas and methane yields were obtained by 50% food waste and 50% manure and represent the highest performing point in Scenario 2 (Table 4.6). All three substrate mixtures had similar carbohydrate and protein contents within the ranges of 36.88 – 39.14% TS and 12.06-12.97%, respectively (Table 5.6). However, the fat and fibre contents of the three points varied to a greater degree, demonstrating wider ranges of 4.08 -16.12% TS for fats and 17.58-28.29% TS for fibre (Table 5.6). The top performing substrate mixture overall (50% retentate and manure) was the only point within the optimum C/N range of 25-30, the other two points had lower C/N ratios of 21.17 and 23.33 (Table 5.6). In addition, the other two points representing the top performing points in Scenarios 2 and 3 had slightly lower available carbohydrate contents and slightly lower fibre contents (Table 5.6). The higher fibre content seen in the 50% retentate and manure mixture evidently extended the degradation time for the total substrate mixture due to the slower hydrolysis of cellulose and hemicellulose, thus compensating for the higher available carbohydrate content with faster degradation times -hence, improving process stability (Table 5.6). The higher available carbohydrate content compared to the other two points was clearly beneficial as, together with the higher fibre content, improved the C/N ratio compared to other two points. In addition, it was evident that the maximum amount of fruit waste that could be digested whilst still producing an average methane (%) above 40, was 50% for any given fruit process waste (Table 5.6). However, the maximum amount of acidic wastes (fruit and food waste) that could be co-digested with manure and still produce good quality biogas was 66.66% (Table 5.6). Furthermore, it was observed that 2 of the 3 substrate mixtures which gave the highest yields contained food waste (Table 5.6).

5.3.4 Food waste as an additional nitrogen source

Food waste was initially selected as an additional feedstock to the fruit wastes in order to provide an additional source of nitrogen, thereby decreasing reliance on manure as the main nitrogen supplier seeing as manure is the most limited feedstock at Elgin Fruit Juices. Secondly, foods with a high fat content were incorporated in the food waste in an attempt to further improve biogas quality. As can be seen in Figure 5.3 below, food waste could not completely replace manure as a nitrogen source without dramatically decreasing biogas yields. The maximum amount of biogas obtained from manure in combination with fruit wastes is greater than 600 mL whereas food waste in combination with fruit wastes produced a maximum of around 240 mL of biogas (Figure 5.3).
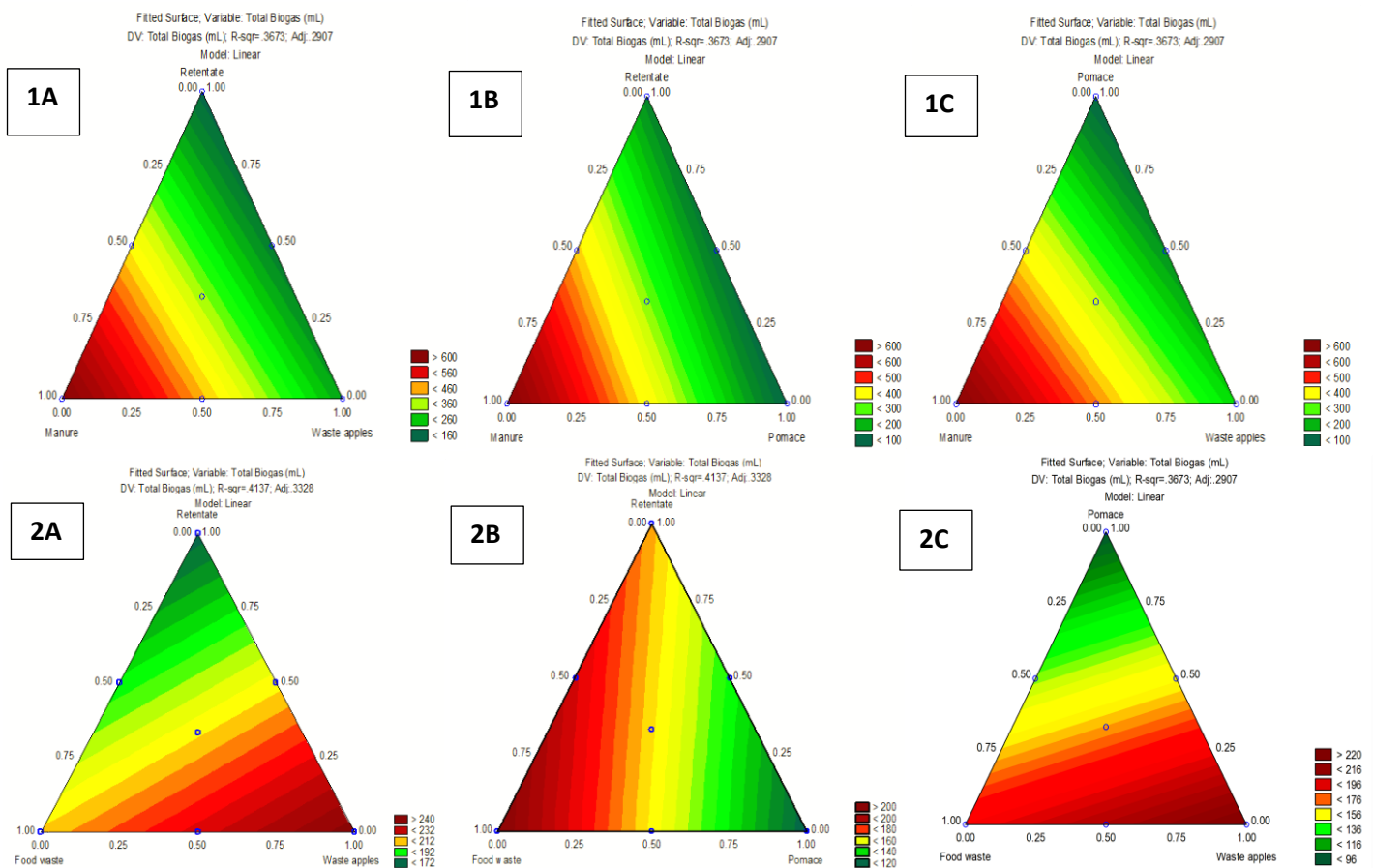
Figure 5. 3: Contour plots comparing manure and food waste as nitrogen sources when co-digested with fruit wastes for biogas production (mL). Figures (1A-1C) show the effect of manure and multiple fruit wastes on total biogas yield (mL), whereas figures (2A-2C) demonstrate the effect of food waste in co-digestion with multiple fruit wastes on biogas yields (mL).

Similarly, the methane yields neither improved nor equalled the yields obtained from manure as a nitrogen source with fruit wastes (Figure 5.4). As is illustrated in Figure 5.4, the approximate maximum amount of methane produced from food waste in co-digestion with fruit wastes was 52 mL as opposed to the 360 mL from manure-fruit waste co-digestion. These results suggest that food waste is not able to completely replace manure as the primary nitrogen provider (Figure 5.3 and 5.4). This being said, food waste showed promise as a third co-substrate when supplemented at 33% with both manure and fruit waste (Tables 5.3 and 5.6). It can therefore be assumed that food waste can, to some extent, decrease the manure requirement and still result in adequate biogas and methane yields provided that the food waste fraction is supplemented at a relatively low level. Evidently, food waste can be added up to 50% when supplemented with manure and produce high methane yields (Table 5.6) however, if supplemented with another acidic waste or in quantities greater than 50%, the buffering capacity of the substrate mixture is insufficient and VFA accumulation occurs resulting in methanogen death and inhibited methane production.
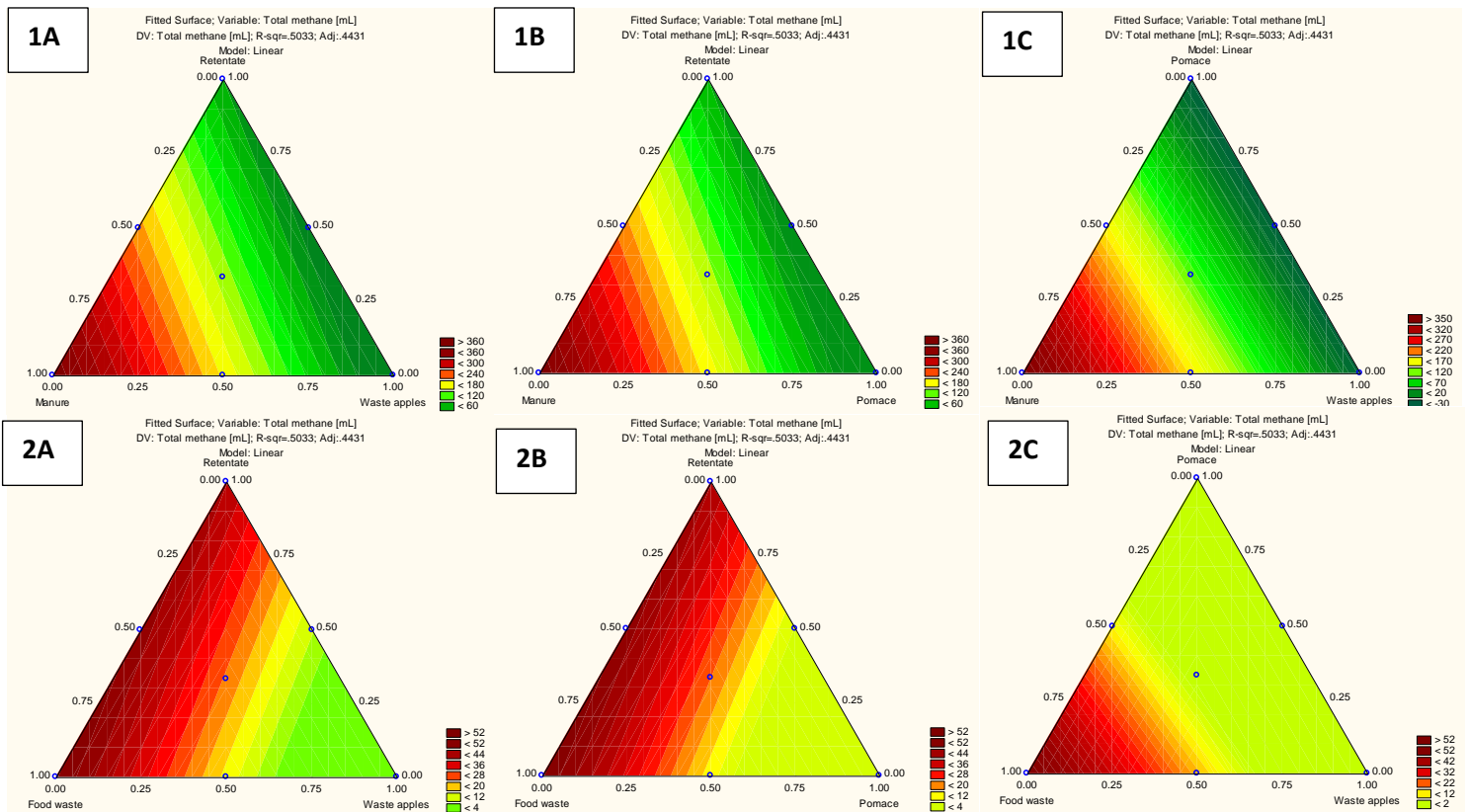
Figure 5. 4: Contour plots comparing manure and food waste as nitrogen sources when co-digested with fruit wastes for methane production (mL). Figures (1A-1C) show the effect of manure and multiple fruit wastes on total biogas yield (mL), whereas figures (2A-2C) demonstrate the effect of food waste in co-digestion with multiple fruit wastes on methane yields (mL).

Despite the benefits observed during low level co-digestion with food waste, there still remained the issue of high variability between centre-point replicates as well as the large standard deviations observed during food waste characterisation (Tables 5.1, 5.3). The bowl cutter used to macerate the food waste sample was incapable of macerating the food waste components to a comparable particle size. Due to the different sizes, textures, high moisture and high lipid content of the food waste, it was not possible to sieve the food waste to improve particle size homogeneity. Although the cone and quatering sampling method may improve homogeneity on a larger scale, it did not appear to be adequate at a bench scale owing to large particles and low solids loading, as the effects of variations in particle size is more evident between replicates. The EFJ AD plant eventually excluded food waste as a co-feed as it was not substantially benefitting their biogas yields. Seeing as the project was designed, in part, to optimise the feed ratios for Elgin fruit juices and to minimise manure and maximise the fruit waste disposal, it was decided to exclude food waste from further optimisation experiments.

5.4 LCB Supplementation Study

The primary aims of this study was to investigate whether supplementation with LCB could improve biogas and methane yields and whether or not manure addition could be minimised by compensation with LCB. Seeing as though food waste supplementation was able to reduce the manure requirements, and that food waste was excluded from the study, another substrate was required to help compensate for lower manure supplementation levels without drastically decreasing biogas yields. LCB was chosen due to the possible increased buffering capacity it might provide when co-digested with fruit waste due to its complex carbohydrate content.

5.4.1 Statistical Analysis

The mixture design was analysed using an ANOVA for both methane (mL.gVS) and biogas (mL.gVS) as outcome variables. The results of the statistical analyses can be found in Appendix B. Biogas (mL.gVS) was found to be highly significant with a p-value of p=0.0027. Methane (mL.gVS) yielded a p-value less than 0.05 (p=0.033) and thus was also significant. The lack of fit of the model was found to be insignificant for both outcome variables. The total biogas variable yielded a low R^2 value (R^2 =0.44) meaning that less than half the observed variation is accounted for by the model. Variations in particle size of the LCB could in part explain the greater variability as smaller particle sizes are hydrolysed faster than larger ones due to an increased surface area. Different ratios of mixed particle sizes can therefore be added despite adding the same quantity, resulting in greater variation between similar samples. Another possible explanation is the presence of stems, seeds, sand and stones present in the waste apples, pomace and manure. These indigestible materials account for a portion of the total solids added at BMP level and can be present in differing amounts between runs. With low solids loadings, this can result in an overestimation of volatile solids added and can cause larger deviations between samples than at a larger scale.

As can be seen in Figure 5.5A, based on the standardised effect estimate all substrates except for pomace and fruit waste (waste apples) significantly impacted biogas production. As was expected, manure had the greatest influence, followed by LCB. Manure also significantly affected methane production along with retentate and LCB (Figure 5.5B). Retentate had a significant effect on both biogas and methane most likely owing to its higher quantities of slower degrading sugars compared to waste apples and pomace, higher protein content and optimal C/N ratio. It is likely that the effect of the addition of pomace and waste apples on biogas and methane production was calculated as insignificant as a result of the variability due to the presence of stems and seeds in these substrates. Seeing as substrates were added on a total solids basis and that differing amounts of stems and seeds (indigestible materials) will be present in each sample, it is reasonable to assume that the lack of trend and therefore the lack of significance of the addition of these two substrates is as a result of this variability. This would explain the significance of retentate, as it lacks the stems and seeds and other indigestible materials present in the other fruit waste streams and therefore displays a more direct relationship between its addition and biogas and methane yields.
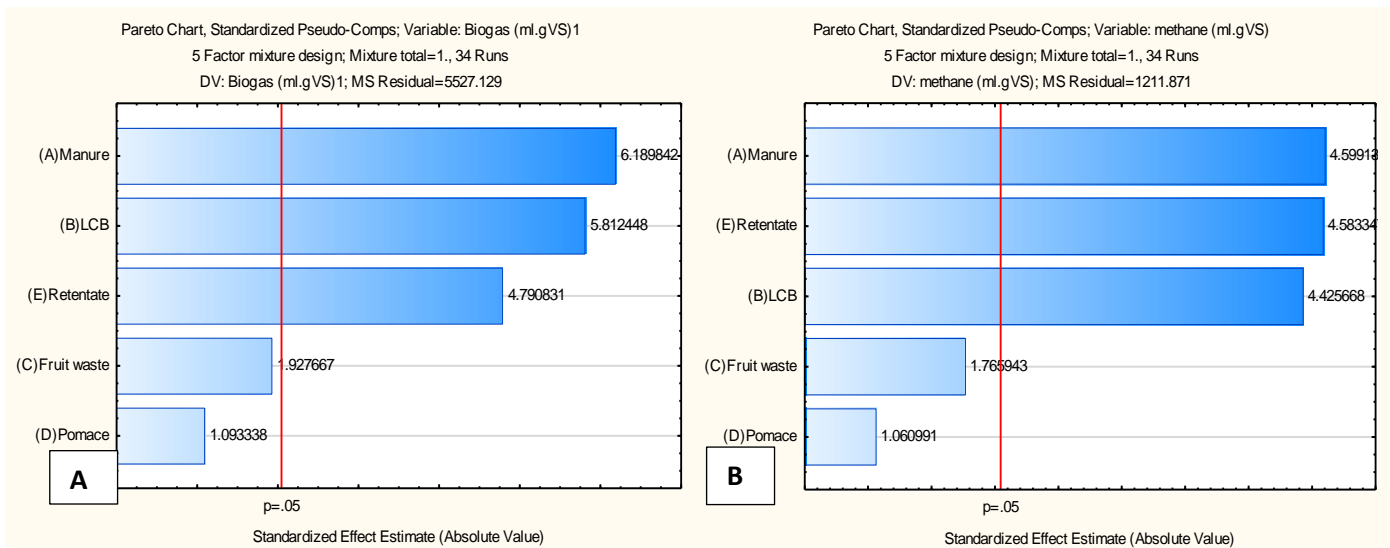
Figure 5. 5: Pareto charts to show significant substrates on biogas (A) and methane (B) production

5.4.2 Biogas and methane production

20% manure without LCB supplementation

Table 5.7 lists the results of 20% manure supplementation level without the addition of LCB. This level represents the runs with the highest fruit waste component and therefore have the highest waste disposal value without LCB supplementation. The maximum biogas and methane yields obtained at this level were achieved when the waste apple fraction was minimised and the pomace and retentate fractions were maximised. Assays 1 and 2 obtained some of the lowest biogas and methane yields in the study. It is possible that the higher loading of waste apples (30% of substrate mixture), caused the mixture to acidify too rapidly due to the amount of easily reducible sugars supplied by the waste apples fraction. This would explain why when added at a 20% supplementation level (Assay 3) the mixture performance was enhanced, as the quantities of pomace, retentate and manure were high enough to compensate for the large amounts of reducible sugars from waste apples due to the higher quantities of ash and complex carbohydrates in these wastes therefore improving substrate mixture stability.

Assay 3 not only produced the highest biogas and methane yields of the 20% manure supplementation level, but this result is also higher than the highest biogas and methane yields obtained in the previous study including food waste, which yielded a maximum of 190.35 mL.gVS^{-1} $_{fed}$ of biogas and 120.46 mL.gVS^{-1} $_{fed}$ methane.

Table 5. 7: BMP results for combined manure and LCB fraction of 20%

Assay	Combined Man. &LCB ratio	Manure	LCB	Waste apples	Pomace	Retentate	Total biogas (mL)	Total CH$_4$ (mL)	Avg. CH$_4$ %	Biogas (mL.gVS^{-1} $_{fed}$)	methane (mL.gVS^{-1} $_{fed}$)	C/N
1	20.00	0.20	0.00	0.30	0.30	0.20	235.50	105.42	49.18	127.10	56.89	31.34
2	20.00	0.20	0.00	0.30	0.20	0.30	406.00	151.95	48.49	217.12	81.26	32.13
3	20.00	0.20	0.00	0.20	0.30	0.30	565.00	231.97	50.55	301.79	123.90	31.19

40% combined manure and LCB supplementation

As can be seen in Table 5.8, Assays 1 and 4 obtained the lowest yields in the entire LCB supplementation study. This is likely due to the high concentration of waste apples in the substrate mix as was also observed at the 20% manure supplementation level. In spite of the higher quantities of manure and LCB, the amount of easily reducible sugars was likely still too high and thus the lower methane and biogas yields are probably due to microbial death as a result of acid crash. Apart from the low biogas and methane yield, the difference of 40% manure supplementation in Assay 1 compared to 20% manure and 20% LCB supplementation in Assay 4, did not drastically affect the methane yields, however more biogas was produced in Assay 1 with increased manure and 0% LCB supplementation.

Assays 3 and 6 produced the highest yields at the 40% combined manure and LCB level (316.92 mL.gVS^{-1} $_{fed}$ biogas, 117.70 mL.gVS^{-1} $_{fed}$ methane and 287.27 mL.gVS^{-1} $_{fed}$ biogas and 117.42 mL.gVS^{-1} $_{fed}$ methane, respectively). Once again, a compensation of the manure fraction with 20% LCB produced comparable methane yields with a small reduction in biogas yields. Assays 5, containing pomace and retentate, displayed higher biogas and methane yields with 20% LCB supplementation compared to Assay 2 with no LCB supplementation. These findings suggest that LCB compensation of 20% of the manure fraction can improve biogas yields but only when used in combination with pomace and retentate. This phenomenon is most likely explained by the addition of volatile solids via LCB supplementation compared with manure alone. All four substrates in the substrate mix are more stable and less prone to acidification compared to waste apples and contain adequate levels of protein. As can be seen in Table 4.8, the C/N ratio of the point with both pomace and retentate was decreased into a more favourable range with the minimisation of manure and the addition of LCB. However, it should be noted that the reported C/N ratios take into account all carbon and nitrogen, including fixed carbon, and therefore do not only represent the available carbon to nitrogen ratio, which is a better indicator of performance. Thus, the available C/N ratio will be lower than the reported values in this study.

Table 5. 8: BMP results for combined manure and LCB fraction of 40%

Assay	Combined Man. &LCB ratio	Manure	LCB	Waste apples	Pomace	Retentate	Total biogas (mL)	Total CH$_4$ (mL)	Avg. CH$_4$ %	Biogas (mL.gVS^{-1} $_{fed}$)	methane (mL.gVS^{-1} $_{fed}$)	C/N
1	40.00	0.40	0.00	0.30	0.30	0.00	217.33	48.13	24.49 ± 2.73	118.20	26.18	36.95
2	40.00	0.40	0.00	0.00	0.30	0.30	455.50	160.69	42.55	240.14	84.72	36.23
3	40.00	0.40	0.00	0.30	0.00	0.30	599.00	222.46	45.27	316.92	117.70	40.35
4	40.00	0.20	0.20	0.30	0.30	0.00	144.00	41.65	38.84	78.61	22.74	31.34
5	40.00	0.20	0.20	0.00	0.30	0.30	495.00	212.42	50.89	261.90	112.39	30.91
6	40.00	0.20	0.20	0.30	0.00	0.30	541.00	221.13	51.12	287.27	117.42	33.75

50% combined manure and LCB Supplementation

As can be seen in Table 5.9, the average biogas and methane yields were higher at the 50% manure and LCB supplementation level than at the 20% and 40% levels. Assays 6 produced the highest biogas and methane yields, with 364.04 mL.gVS^{-1} $_{fed}$ of biogas and 150.84 mL.gVS^{-1} $_{fed}$ of methane produced, however the corresponding point with 30% LCB supplementation (Assay 12), produced much lower biogas and methane yields of 221 mL.gVS^{-1} $_{fed}$ and 86.63 mL.gVS^{-1} $_{fed}$, respectively (see Table 5.9). This same trend is observed in Assay 3, also containing waste apples and pomace. Assay 3 produced high yields when supplemented with 50% manure and relatively low yields when supplemented with 20% manure and 30% LCB (Assay 9). This suggests that the combination of waste apples and pomace require higher levels of buffering from the manure fraction to prevent acid crash.

Table 5. 9: BMP results for combined manure and LCB fraction of 50%

Assay	Combined Man. &LCB ratio	Manure	LCB	Waste apples	Pomace	Retentate	Total biogas (mL)	Total CH$_4$ (mL)	Avg. CH$_4$ %	Biogas (mL.gVS^{-1} $_{fed}$)	methane (mL.gVS^{-1} $_{fed}$)	C/N
1	50.00	0.50	0.00	0.20	0.00	0.30	536.00	211.41	47.07	281.75	111.13	44.40
2	50.00	0.50	0.00	0.00	0.20	0.30	513.00	211.93	53.39	269.03	111.14	40.97
3	50.00	0.50	0.00	0.20	0.30	0.00	595.00	240.18	50.82	321.45	129.76	40.37
4	50.00	0.50	0.00	0.30	0.00	0.20	673.00	266.89	47.69	357.41	141.74	44.81
5	50.00	0.50	0.00	0.00	0.30	0.20	467.00	272.39	49.71	247.13	144.14	39.75
6	50.00	0.50	0.00	0.30	0.20	0.00	673.00	278.86	49.18	364.04	150.84	42.00
7	50.00	0.20	0.30	0.20	0.00	0.30	505.00	201.20	50.20	266.89	106.33	33.55
8	50.00	0.20	0.30	0.00	0.20	0.30	506.00	195.64	50.80	266.79	103.15	31.64
9	50.00	0.20	0.30	0.20	0.30	0.00	463.00	166.50	41.14	251.53	90.45	31.19
10	50.00	0.20	0.30	0.30	0.00	0.20	580.00	241.28	50.31	309.70	128.83	33.73
11	50.00	0.20	0.30	0.00	0.30	0.20	656.00	281.21	49.40	349.03	149.62	30.91
12	50.00	0.20	0.30	0.30	0.20	0.00	407.00	159.27	49.35	221.38	86.63	32.11
13	52.00*	0.36	0.16	0.16	0.16	0.16	459.25 ± 72.84	172.45 ± 27.70	44.81 ± 1.37	244.82 ± 44.84	104.41 ± 21.18	36,66

*Centre point

As seen in Table 5.9, Assay 11 produced the second highest biogas and methane yields with 349.03 mL.gVS^{-1} $_{fed}$ and 150.84 mL.gVS^{-1} $_{fed}$ produced, respectively. This was slightly higher than the yields obtained from the corresponding substrate mixture with higher manure and no LCB compensation (Assay 5) (Table 5.9). Similarly, the other assay consisting of pomace and retentate in co-digestion (Assay 2), obtained comparable yields to the corresponding assay with LCB supplementation (Assay 8), with a slight sacrifice in biogas and methane yields observed in Assay 8. Of the two combinations of pomace and retentate, the point with a higher pomace concentration (Assays 5 and 11) gave higher yields than the point with a greater quantity of retentate (Assays 2 and 8). This suggests that LCB may be able to compensate for 30% of the manure fraction when used in combination with equal quantities of pomace and retentate, only if the amount of manure saved justifies the small sacrifice in biogas and methane yields.

A similar trend was observed when waste apples and retentate were co-digested as when pomace and retentate were co-digested. Assay 4, which contains more waste apples than retentate, performed slightly better than the corresponding point which contained more retentate (Assay 1), however both showed slight decreases in biogas and methane yields when supplementing the manure fraction with 30% LCB (Assays 10 and 7, respectively) (Table 4.9). Once again, this implies that LCB may also be able to compensate for 30% of the manure fraction when used in combination with waste apples and retentate, only if the small decrease in biogas and methane yields is justified by the quantity of manure saved.

70% and 80% combined manure and LCB supplementation

Seeing as the previous mixture design indicated that a minimum of 50% of manure in the substrate mixture is required for good biogas and methane yields, the 70% and 80% manure and LCB supplementation levels represent biogas maximisation rather than manure minimisation as did the 40% and 50% levels. In essence, the larger quantities of manure and LCB present at these higher levels result in a minimisation of fruit waste supplementation and instead focus on higher overall biogas and methane yields. As is evident in Table 5.10, within the 70% supplementation level, for combinations containing 40% manure and 30% LCB, waste apples proved to be the best co-substrate, producing 325.69 mL.gVS^{-1} $_{fed}$ of biogas and 131.95 mL.gVS^{-1} $_{fed}$ of methane (Assay 3). However, when the manure fraction was increased to 50% and the LCB fraction decreased to 20%, co-digestion with apples produced the lowest yields (Assay 6).

Table 5. 10: BMP results for a combined manure and LCB supplementation level of 70%.

Assay	Combined Man. &LCB ratio	Manure	LCB	Waste apples	Pomace	Retentate	Total biogas (mL)	Total CH$_4$ (mL)	Avg. CH$_4$ %	Biogas (mL.gVS^{-1} fed)	methane (mL.gVS^{-1} fed)	C/N
1	70.00	0.40	0.30	0.00	0.30	0.00	412.00	146.73	48.53	220.87	78.66	36.11
2	70.00	0.40	0.30	0.00	0.00	0.30	573.00	189.24	40.30	298.94	98.73	39.23
3	70.00	0.40	0.30	0.30	0.00	0.00	605.33	245.25	41.79	325.69	131.95	40.10
4	70.00	0.50	0.20	0.00	0.30	0.00	772.00	307.24	47.44	413.11	164.41	39.60
5	70.00	0.50	0.20	0.00	0.00	0.30	592.00	214.75	45.39	308.30	111.84	43.37
6	70.00	0.50	0.20	0.30	0.00	0.00	446.00	152.50	46.21	239.52	81.90	44.54
7	80.00	0.50	0.30	0.00	0.20	0.00	679.00	232.93	43.13	362.05	124.20	40.71
8	80.00	0.50	0.30	0.00	0.00	0.20	783.00	319.10	46.40	410.01	167.10	43.26
9	80.00	0.50	0.30	0.20	0.00	0.00	916.00	154.92	43.77	489.60	82.80	44.01

When the LCB fraction was increased further and the waste apple fraction decreased at the 80% level (Assay 9), methane yields obtained were comparable to Assay 6, however biogas yields more than doubled. This suggests that co-digestion with LCB could be beneficial with higher concentrations of waste apples. Seeing as waste apples have the highest C/N ratio of all the tested substrates in this study, it is likely that the addition of the LCB, which has a higher nitrogen content helped to balance the C/N ratio of the substrate

mixture. This would explain why LCB was more beneficial than manure as a co-feed, as manure had a lower nitrogen concentration than LCB (see Table 5.1) and therefore would not be as effective at decreasing the C/N ratio of the whole substrate mixture, as it wold contribute more carbon per nitrogen. Even though the reported C/N ratios are still higher than the optimum range (25-30) and that the true (available) C/N ratio is likely lower, the trend of increased C/N ratio with manure addition can be observed in Table 5.10.

In contrast, pomace as a co-substrate produced the highest yields at the 70% level with higher manure supplementation (Assay 4) and produced drastically less biogas and methane when 40% manure and 30% LCB was used (Assay 1) despite having a lower, more favourable C/N ratio with lesser manure addition (Table 5.10). Furthermore, the pomace fraction was decreased to 20% as the LCB fraction was increased at the 80% level, the biogas and methane yields from pomace decreased (Assay 7). This is likely as a result of a reduced amount of easily reducible sugars in the pomace as compared to waste apples. The pomace consists of more complex carbohydrates left behind after juice extraction and consequently, higher concentrations of pomace means slightly more easily hydrolysed carbohydrates, which results in higher biogas and methane yields provided there is adequate alkalinity.

At the 80% level, retentate proved to be the best co-substrate, producing biogas and methane yields of 410.01 mL.gVS^{-1} $_{fed}$ and 167.10 mL.gVS^{-1} $_{fed}$, respectively (Table 5.10). These yields represent the highest observed yields in the study. As can be seen in Table 4.10, the biogas and methane yields from retentate increased with greater manure and LCB supplementation. The yields produced from retentate in co-digestion with 40% manure and 30% LCB (Assay 2), despite being slightly lower, were still comparable to the yields produced from 50% manure and 30% LCB with retentate (Assay 5) within the 70% combined supplementation level (Table 5.10).

5.4.3 Substrate combinations which produced the highest biogas and methane yields in LCB supplementation study

All points listed in Table 5.11 produced higher biogas and methane yields than the top performing assay in the first mixture experiment with food waste. OF the 11 top performing substrate combinations, 5 did not contain LCB. LCB was only able to minimise the manure fraction and improve biogas and methane yields for two substrate mixtures namely Assays 4 and 9 in Table 5.11. For the majority of cases with LCB, LCB addition was able to improve yields providing it compensated for the fruit waste fraction of the substrate mixture rather than the manure fraction, with the exception of pomace which gave higher yields with 30% pomace and less LCB addition. Finally, Assay 11 represents the substrate mixture with the highest waste disposal value without LCB addition as it consists of 80% fruit wastes. Lastly, it should be noted that only one of the substrate mixtures listed above fell under scenario 2 (feedstocks available in non-juice production seasons), namely Assay 7 (Table 5.11).

Table 5. 11: Highest biogas and methane yields obtained in the LCB supplementation study.

Assay	Combined Man. &LCB ratio	Manure	LCB	Waste apples	Pomace	Retentate	Total biogas (mL)	Total CH$_4$ (mL)	Avg. CH$_4$ %	Biogas (mL.gVS^{-1} fed)	Methane (mL.gVS^{-1} fed)
1	80.00	0.50	0.30	0.00	0.00	0.20	783.00	319.10	46.40	410.01	167.10
2	70.00	0.50	0.20	0.00	0.30	0.00	772.00	307.24	47.44	413.11	164.41
3	50.00	0.50	0.00	0.30	0.20	0.00	673.00	278.86	49.18	364.04	150.84
4	50.00	0.20	0.30	0.00	0.30	0.20	656.00	281.21	49.40	349.03	149.62
5	50.00	0.50	0.00	0.00	0.30	0.20	467.00	272.39	53.39	247.13	144.14
6	50.00	0.50	0.00	0.30	0.00	0.20	673.00	266.89	47.69	357.41	141.74
7	70.00	0.40	0.30	0.30	0.00	0.00	605.33	245.25	51.35 ± 0.76	325.69	131.95
8	50.00	0.50	0.00	0.20	0.30	0.00	595.00	240.18	50.82	321.45	129.76
9	50.00	0.20	0.30	0.30	0.00	0.20	580.00	241.28	50.31	309.70	128.83
10	80.00	0.50	0.30	0.00	0.20	0.00	679.00	232.93	43.13	362.05	124.20
11	20.00	0.20	0.00	0.20	0.30	0.30	565.00	231.97	50.55	301.79	123.90

Based on the LCB supplementation study results response desirability profiling was used to identify two points for scale up in 50 L tank reactors (Appendix B). One point was chosen to optimise biogas and methane yields (biogas optimisation point) whereas the other point focused on minimising the manure fraction and maximising the fruit waste proportion of the mixture while still producing good quality biogas (above 40% methane) and high methane yields (referred to as the manure minimisation point). Seeing as though most substrate combinations fell under Scenario 1 (the juice producing season) and due to equipment availability and time constraints both points were scaled up for Scenario 1 only. The predicted substrate combinations and corresponding predicted outcome variable values for both selected points are listed in Table 5.12.

Table 5. 12: Points to be scaled up in 50 L reactors based on desirability profiling.

ID	Function	Manure	LCB	Waste apples	Pomace	Retentate	Total biogas (mL)	Total ch4 (mL)	Avg. CH4 %	Biogas (mL.gVS^{-1} fed)	methane (mL.gVS^{-1} fed)
1	Biogas maximisation	0.50	0.30	0.00	0.00	0.20	783.00	319.10	50.95	410.01	167.10
2	Waste disposal	0.30	0.30	0.10	0.00	0.30	613.60	227.94	48.00	322.78	119.89

5.5 VFA Production: Mixture Designs (Lab Scale)

All substrate combinations for both the Mixed Substrate Interaction study and the LCB Supplementation Study were analysed for pre- and post-digestion VFA concentrations in order to identify any points with adequate biogas yields and relatively high VFA concentrations. Table 5.13 lists the substrate mixtures which produced the highest post-digestion VFA concentrations for the first mixture design including food waste. The first mixture design with food waste had the highest post-digestion VFA concentrations of the whole study, however no points with high VFA concentrations also produced good biogas yields. Conversely, all points which produced good biogas yields, subsequently had very low post-digest VFA concentrations (Appendix C). Of the ten assays listed in Table 5.13, seven of them contained food waste. The highest concentration of VFAs post-digestion was produced by 1:1 food waste and retentate co-digestion. This is in line with the first VFA screening experiment from mono-digestion of each substrate where food waste produced the greatest amount of VFAs followed by retentate (Figure 5.2). It therefore stands to reason that the co-digestion of both these wastes would result in the highest post-digest VFA yields.

Table 5. 13: Top 10 highest post-digest VFA concentrations from mixed substrate interaction study

Assay	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Food waste (% w/w)	Manure (% w/w)	Biogas (mL.gVS$^{-1}_{fed}$)	Methane (mL.gVS$^{-1}_{fed}$)	Pre-digest VFAs (g/L)	Post-digest VFAs (g/L)
1	0	0	50	50	0	10.05	1.84	10.27	40.40
2	0	0	0	100	0	32.89	3.24	3.48	29.53
3	33.33	33.33	0	33.33	0	56.07	6.71	9.34	28.33
4	50	0	50	0	0	72.62	3.02	7.73	28.00
5	33.33	0	33.33	33.33	0	34.30	7.15	5.12	27.92
6	25	0	25	25	25	102.93	13.08	10.70	27.88
7	50	50	0	0	0	54.63	1.92	0.31	27.31
8	0	25	25	25	25	47.55	3.67	2.67	27.26
9	0	50	50	0	0	10.05	1.84	3.50	27.14
10	0	33.33	33.33	33.33	0	10.72	1.53	8.59	26.37

Table 5.14 lists the top 3 highest post-digest VFA concentrations produced during the LCB supplementation study. Seeing as LCB was initially added to provide greater buffering capacity to the mixture when co-digested with fruit waste, it stands to reason that less VFAs were produced post-digestion in this study. The higher biogas yields produced during the LCB mixture design as opposed to the mixture design including food waste, combined with lower VFA yields suggests more efficient conversion of VFAs to biogas and methane. Of the three highest concentrations of VFAs produced during the LCB supplementation experiment, two of the assays produced low biogas and methane yields, however, Assay 1 produced the highest post-digestion VFA concentration and moderate biogas and methane yields.

Table 5. 14: Top 3 highest post-digest VFA concentrations from LCB mixture design

ID	Manure (% w/w)	LCB (% w/w)	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Biogas (mL.gVS^{-1} $_{fed}$)	Methane (mL.gVS^{-1} $_{fed}$)	Pre-digest VFAs (g/L)	Post-digest VFAs (g/L)
1	50	20	0	0	30	303.30	111.84	0.23	17.34
2	20	20	30	30	0	78.61	22.74	0.26	16.599
3	40	0	30	30	0	118.20	26.18	0.16	16.01

5.6 Batch process scale-up of selected points in 50 L reactors

5.6.1 Comparison of lab scale BMP test and 50 L reactor scale up of selected points

The two substrate combinations that were selected based on desirability profiling from the LCB Supplementation Study to optimise both biogas and methane yields as well as manure minimisation and fruit waste disposal for Scenario 1 were scaled up in 50 L tank reactors, in batch process, in order to test the validity of the predicted results at a larger scale. Table 5.15 compares the results of the bench scale and scale-up experimental results for both selected points in terms of biogas quality and yields:

	Table 5. 15: Comparison of results between two selected points at lab scale and 50 L reactor level in batch process				
Mixture	Composition	Function	Measurements	BMP*	Batch
1	50 % Manure 30 % LCB 20 % Retentate	Biogas maximisation	Biogas yield (NL.KgVS^{-1} $_{fed}$)	410.01	692.43 ± 154.75
			Methane yield (NL.KgVS^{-1} $_{fed}$)	167.10	214.46 ± 97.65
			Average methane (%)	50.95	53.10 ± 0.04
			Final methane (%)	N/A	63.32 ± 1.62
2	30 % Manure 30% LCB 30% Retentate 10 % Apples	Manure minimisation	Biogas yield (NL.KgVS^{-1} $_{fed}$)	322.78	351.51 ± 70.04
			Methane yield (NL.KgVS^{-1} $_{fed}$)	119.89	153.69 ± 28.89
			Average methane (%)	48.00	41.45 ± 3.64
			Final methane (%)	N/A	50.48 ± 5.73

*As predicted by response surface methodology.

As can be seen in Table 5.15, the biogas maximisation point performed significantly better than what was predicted for all outcome variables. Furthermore, the scale-up batch process results for the manure minimisation point were more comparable to the predicted bench scale results than the biogas maximisation point, however it also yielded slightly higher values than was predicted for all outcome

variables except average methane percentage (Table 5.15). The increased yields for both points obtained from the batch scale-up experiment are likely due to three main factors, specifically the addition of mixing, differences in inoculum sources and variances in temperature due to different heat transfer mechanisms.

For the BMP experiments conducted in 100 mL serum bottles, regular mixing was not possible. Instead, bottles were lightly shaken once or twice a week before gas chromatography. This is not ideal as regular mixing is known to aid the distribution of nutrients amongst the microbial communities and to homogenise the added feed within the digester, as well as to assist in the removal of metabolic end products and regulate the temperature of the digestate (Forster *et al.*, 1982).

 Multiple studies have demonstrated the benefits of mixing on biogas and methane yields (Stroot *et al.*, 2001; Ong, Greenfield and Pullammanappallil, 2002; Karim *et al.*, 2005; Vavilin *et al.*, 2007). One such study by Lin and Pearce (2001), revealed an increased methane yield with intermittent mixing as opposed to performing the same process without mixing. In addition to the benefits from mixing as opposed to stagnation, both the stirring rate and mode of mixing (either continuous or intermittent) have been shown to greatly affect biogas yields (Lindmark *et al.*, 2014). Several studies have also found intermittent mixing to be the most beneficial mode of mixing in improving overall biogas yields and moreover, when used with short mixing intervals (Stroot *et al.*, 2001; Kaparaju *et al.*, 2008; Sulaiman *et al.*, 2009). Interestingly, several studies found that gas release increased by up to 70% during mixing periods in intermittently mixed digesters (Sung, no date; Mills, 1979; Ong, Greenfield and Pullammanappallil, 2002). This suggests that the release of gas is hampered when the digestate is unmixed and that mixing improves the mass transfer from the liquid to the gas phase (Lindmark *et al.*, 2014). Furthermore, low stirring rates are supposed to be preferable for biogas production as higher stirring rates can exacerbate VFA production and lead to process instability as well as disrupt methanogenic floc formations leading to decreased methane yields (Whitmore *et al.*, 1987; Stroot *et al.*, 2001; Kim, Ahn and Speece, 2002; Sulaiman *et al.*, 2009). The slower, intermittent stirring therefore allows for floc formation and longer periods of time for certain microbial species to be juxtaposed, which is important for syntrophic interactions to occur. One such essential interaction is the transfer of hydrogen between acetogens and methanogens. This helps keep the partial hydrogen pressure low, which would otherwise negatively affect the AD process (Conrad, Phelps and Zeikus3, 1985; Dolfing, 1992; Gerardi, 2003). The conclusions drawn by these studies with regard to intermittent mixing at low stirring rates for short mixing periods on improving biogas yields gives credit to the theory that the introduction of intermittent mixing could be a plausible reason for the higher yields observed in the batch process as this study, which made use of intermittent mixing, twice daily for shorter mixing periods of ten minutes at a low stirring rate (125 rpm).

Furthermore, differences in inoculum sources can also have a large effect on biogas yields (Lopes, Leite and Prasad, 2004; Xu *et al.*, 2013). For the lab scale experiments in serum bottles, inoculum was obtained from the SAB AD plant. Due to circumstances beyond our control, the SAB inoculum was unavailable by the time

the scale-up experiments were performed and so the inoculum required for the scale-up experiments was attained from the EFJ anaerobic digester. Seeing as the SAB AD plant is adapted to brewery grains, which are a source of lignocellulose, are more likely to contain a higher proportion of hydrolytic and specifically cellulose-degrading bacteria (Malakhova *et al.*, 2015; Sun, 2015), it is reasonable to assume there would be a difference in biogas yields compared with the EFJ anaerobic digester which is already adapted to fruit waste and would most likely have different dominant microbial communities.

Lastly, another factor possibly contributing to the difference in yields between the 50 L batch experiments compared to the lab scale experiments is the method of heat transfer used. At the BMP level, serum bottles are placed in an incubator at 37°C where the temperature of the substrate mixture is assumed to be that of the ambient temperature of the incubator; whereas in the 50 L reactors heat is applied more directly in the form of a water jacket with a temperature probe to track digestate temperature and, together with mixing, provides a more accurate and even temperature distribution. In addition, one study found that in leaving the incubator open for 3 minutes, the time of recovery for the incubator to reach the desired temperature range was between 10 – 60 minutes (depending on the type of incubator and the required temperature) and that the temperature varied between certain areas and levels within the incubator (Hulme Knezek, Dorn and Fleming, 1983). Seeing as the incubator was opened at minimum once a day in order to measure gas production, this could have a negative effect on biogas yields seeing as though the AD process is temperature sensitive and will not typically result in optimum yields at lower temperatures (Gerardi, 2003).

Seeing as one of the main benefits of mixing is the distribution of materials amongst the microbial community in the digestate, which can facilitate hydrolysis of organic matter, it stands to reason that out of the two points the biogas optimisation point would benefit the most, as it contains a greater amount of complex carbohydrates (see Table 5.1). The manure minimisation point, however, is comprised of more easily reducible sugars than the biogas maximisation point and thus contains less complex organic materials (Table 5.1). As a result, all simple substrates may be converted directly to biogas by surrounding microbes, without mixing, to a greater degree than the point with more complex substrates which are more reliant on interactions with hydrolytic organisms in order to form simple substrates in order to generate biogas. This would explain why the manure minimisation point performed better in scale-up than lab-scale, but still similarly, whilst the biogas optimisation point produced much higher yields than at lab-scale (see Table 5.15). The mixing therefore improves the chances of complex substrates encountering hydrolytic organisms resulting in a more complete breakdown of organic matter and a higher conversion of organic matter into biogas.

As can be seen in Table 5.16, the VS reduction was slightly lower for the biogas optimisation point than the manure minimisation point, but the COD reduction was higher. This suggests that the retentate component (which is mixed with waste water and contains more easily reducible sugars than the LCB or manure components (Table 5.1)) is primarily converted to biogas and that the more complex components, which

make up the majority of the mixture, have not completely been converted to soluble COD and still remain as un-hydrolysed VS content in the mixture. This would stand to reason as substrates rich in lignocellulosic materials are known to have longer retention times of up to 45 days, and would explain the incomplete VS reduction after only 32 days (Gerardi, 2003). This would also explain the higher VS reduction in the manure minimisation point with more fruit waste, as well as the slightly lower COD reduction. Due to there being a larger quantity of simple substrates in the manure minimisation mixture as a result of a larger fruit waste component, there is a greater initial COD in the substrate mixture, meaning more COD would need to be reduced in order to achieve the same COD reduction (%).

Table 5. 16: Characteristics of 50 L batch process runs for both substrate mixtures.

Mixture #	Composition	VS fed (g)	VS Reduction (%)	COD Reduction (%)	C/N
1	50 % Manure 30 % LCB 20 % Retentate	958.29	68.59	83.48 ± 0.60	28.49 ± 0.13
2	30 % Manure 30% LCB 10 % Apples 30% Retentate.	955.27	70.38	64.53 ± 10.97	27.93 ± 1.00

The idea of incomplete hydrolysis and therefore a suboptimal HRT for the batch process run is further supported by Figure 5.6. As can be seen, cumulative biogas did not reach a plateau phase for either substrate mixture, indicating that there was residual organic matter in the mixture and alluding to the idea that a longer HRT would result in even higher biogas yields (Figures 5.6 (1A & 1B)).
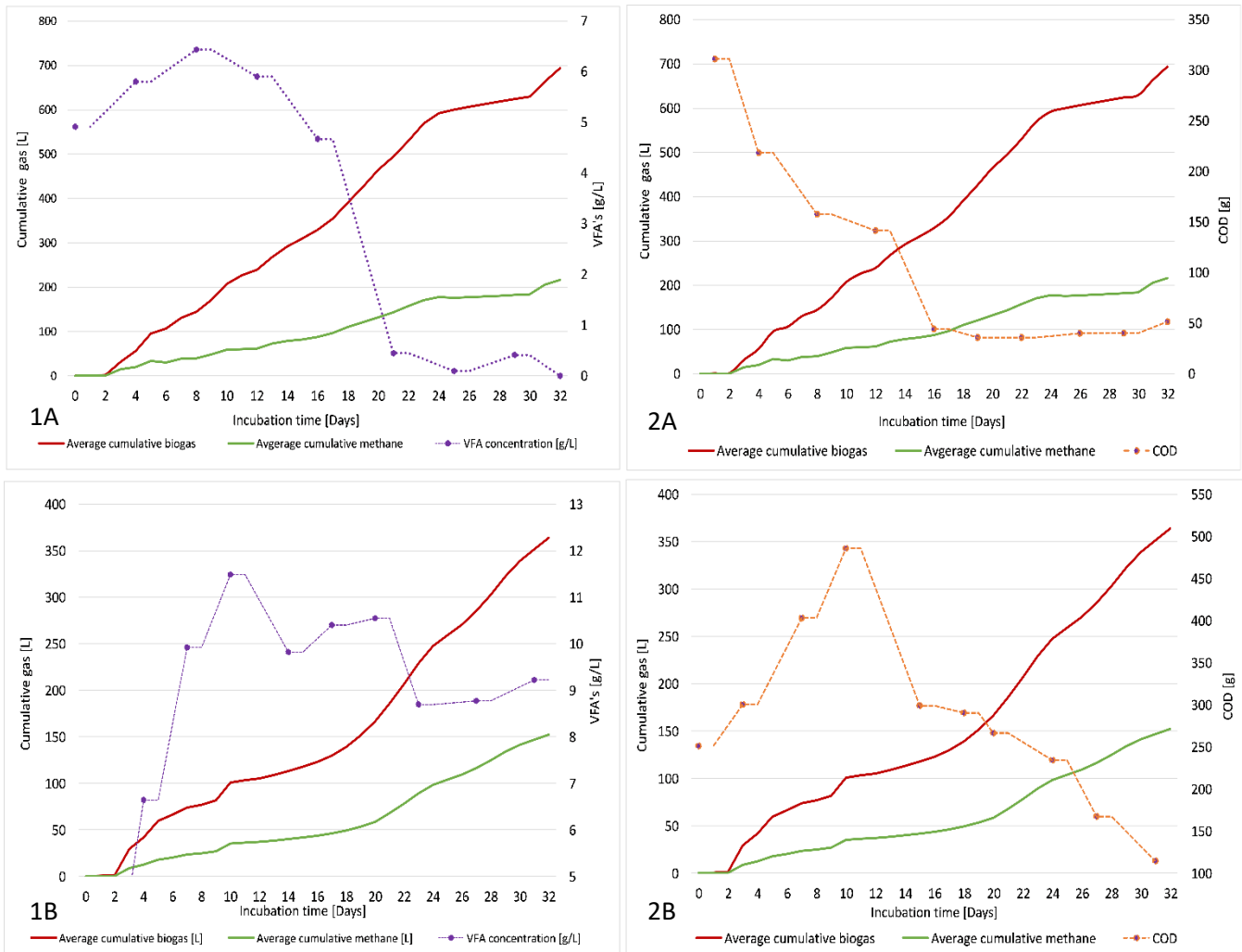
Figure 5. 6: Relationship between (1) VFA concentration and gas production and between (2) COD and gas production over 32 days for both the (A) biogas maximisation and (B) manure minimisation substrate mixtures.

As can be seen in Figure 5.6 (2A), COD gradually decreased from 311.24 g to 35.79 g over the first 17 days. Although COD steadily decreased, there was a slight increase in COD from 35.79 g on day 17 to 51.54 on day 32. The initial drop in COD corresponding with the steady increase in biogas production is likely due to the rapid conversion of simple substrates to biogas. The decrease in the rate of COD reduction evident on days 12-13 (Figure 2A) is as a result of the complete hydrolysis of the simple substrates and the longer hydrolysis time of the more complex substrates and thus represents the breakdown of solid material into the aqueous phase. Furthermore, methane production demonstrates the steepest incline from 120.89 L of methane on

day 19 on up until 177.69 L of methane on day 24, indicating faster conversion of intermediates to biogas and specifically methane (Figure 5.6 (2A)).

 Conversely, the manure minimisation point shows an increase in soluble COD from 251.04 g on day 1 up until 448.35 g on day 10, demonstrating the conversion of a greater quantity of more readily reducible substances and the subsequent consumption thereof (Figure 5.6 (2B)). This consumption of COD resulted in a steady, steep increase in biogas and methane production - from 100.77 L on day 10 to 247.45 L on day 24 for biogas and 35.13 L to 98.20 L for methane. This increase in biogas and methane yields directly corresponds to a decrease in VFA levels from day 10 to day 14 (Figure 5.6 1B). Although the VFA levels began to increase from day 14 to 22, the biogas and methane levels concomitantly increased and the COD decreased from 298.99 g to 114.41 g - demonstrating a stable conversion of COD and therefore VFAs to biogas and methane (Figures 4.6 (1B, 2B)). In contrast to the manure minimisation point, the biogas optimisation point showed the bulk of VFA production in the first 8 days, and then a subsequent gradual decrease in VFA levels from day 8 onwards as the VFAs were converted to biogas and methane (Figure 5.6 (1A)). The plateau in biogas and methane production reached on day 24 corresponds to a depletion of total VFAs in the reactor, however the small increase in VFAs from 0 g/L on day 26 to 0.41 g/L on day 29, is likely due to the slow hydrolysis of complex materials in the background and is met with a subsequent increase in biogas and methane yields from day 30 onward (Figure 5.6 (1A)). The relationship between VFAs and COD levels in the reactor for each substrate mixture is illustrated more clearly in Figure 5.7 below.

Figure 5. 7: Relationship between COD and VFA concentration over time for both the biogas maximisation mixture (A) and the manure minimisation mixture (B). Axes were selected to best illustrate the effect of COD and VFAs over time for each substrate mixture.

Figures 5.7 (A & B) show that a relationship exists between COD and VFA levels, and more specifically that VFAs make up a large portion of the COD present in the digester. This relationship is initially not as clear for the biogas optimisation point, as VFA levels remain high while COD steadily decreased from 311.24 g to 51.45 g (Figure 5.7A) whereas the manure minimisation point indicated a rise in COD with VFA production (Figure 5.7B). This is likely due to a faster conversion rate of organic materials in the biogas maximisation

point to biogas, as is reflected by the subsequent greater biogas and methane yields as compared to the manure minimisation point. One reasonable explanation is due to the better buffering capacity of the biogas optimisation point as opposed to the manure minimisation point. This theory is further supported by Figure 5.8 which shows how the pH decreased by a greater degree into the unfavourable range in the manure minimisation point (7.34-6.16) compared with the biogas maximisation point (7.13 – 8.13).
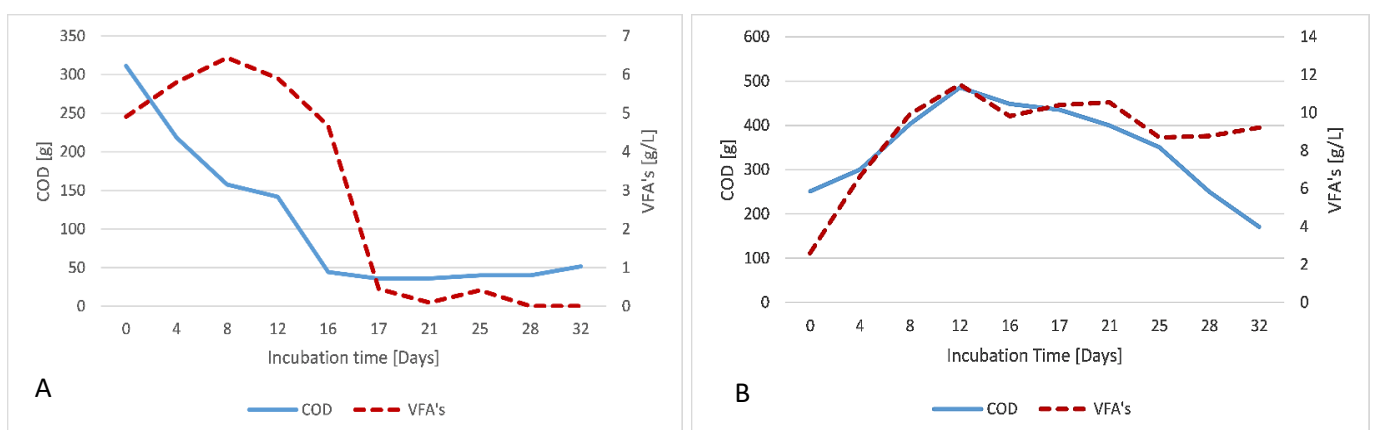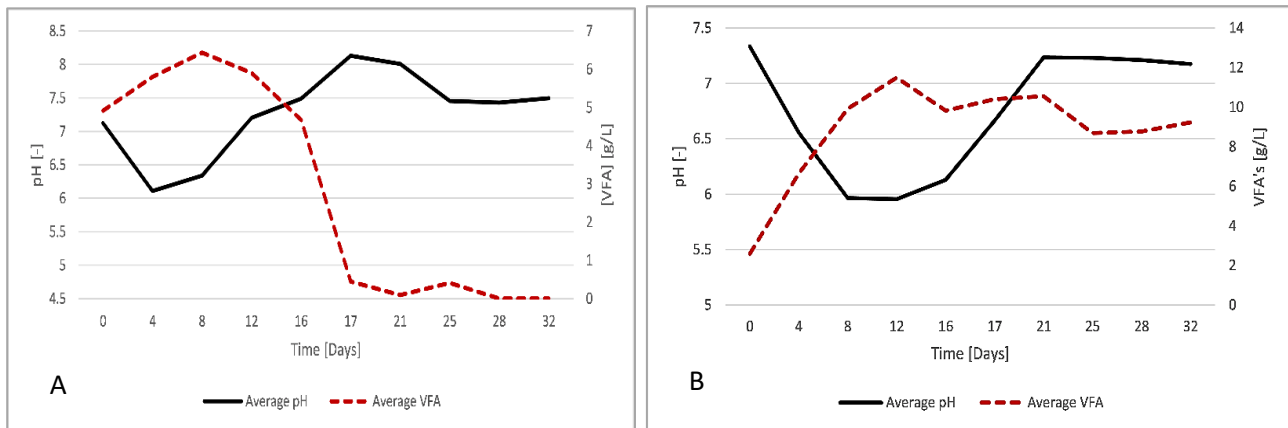

Figure 5. 8: Relationship between pH and VFA concentration over time for both the biogas maximisation (A) and manure minimisation (B) mixtures. Axes were selected to best illustrate the effect of pH and VFAs over time for each substrate mixture.

As can be seen in Figure 5.8 B, the pH of the digestate decreased to 6.16 between days 8-12 for the manure minimisation point. A pH value below 6.2 is considered toxic to methanogens but beneficial to acidogens (Chandra, Takeuchi and Hasegawa, 2012). This is a two-fold phenomenon whereby an increase in VFA concentration results in a decrease in pH and the decrease in pH results in further VFA production in a system that is not well-buffered, hence the corresponding increase in VFA production. This could also explain why the biogas optimisation point gave better yields than the manure minimisation point. However, despite this, the system recovered, and the pH was shown to increase as the VFAs were consumed. Vavilan and Angelidaki (2005) reported that less regular mixing can result in the formation of initiation zones whereby acetogens and methanogens occupy distinct spatial zones, thus allowing the methanogens to be protected from increased acid production from acidogens during the start-up phase. This could explain in part why the system was able to recover despite venturing into toxic pH zones.

 The same inverse relationship between pH and VFAs observed for the manure minimisation point is also seen for the biogas maximisation point, which is to say that as VFAs increased the pH decreased and as the VFAs were consumed, the pH increased (Figure 5.8A). Both Figures 5.8A and 5.8B, demonstrate stable processes, with pH levels beginning and ending in the favourable (neutral) pH range after the initial hydrolysis phase. This is likely also a large contributing factor as to the high biogas and methane yields observed for both substrate mixtures during the 50 L batch scale-up experiment (Table 5.15).

5.7 Selected Points in Semi-continuous process

The two selected substrate combinations that were chosen to optimise both biogas and methane yields as well as waste disposal value for Scenario 1 were scaled up in 50 L tank reactors, in semi-continuous process. For mixtures co-digested with lignocellulosic materials, an OLR within the range of 1.5-3.5 gVS/L/day is typically used (Ziganshin *et al.*, 2013; Lebuhn *et al.*, 2014; Lucas *et al.*, 2015). OLRs within this range are also used at the EFJ plant during continuous operation. In this study, values slightly outside this range were tested as the OLR was increased from 1 - 4 gVS/L/Day over the course of 32 days in order to determine the maximum OLR that can be used for each substrate mixture, as well as to identify the OLR that gives the best biogas and methane yields. It should be noted that this experiment is only representative of a partial process optimisation primarily aimed at substrate optimisation, in order to test the viability of each substrate combination within the typical range employed by EFJ and is therefore not indicative of long-term stability of the reactor. The subsequent average biogas and methane yields from different OLRs are reported in Table 5.17 and discussed below.

Table 5. 17: Comparison of different OLRs and resultant yields for the biogas maximisation substrate mixture (50% M, 30% L, 20% R) in 50 L reactors in semi-continuous process

OLR (gVS/L/day)	Days fed	Avg. VS Reduction* (%)	Avg. Daily Biogas yield (NL.KgVS^{-1} $_{fed.}$Day^{-1})	Avg. Daily Methane yield (NL.KgVS^{-1} $_{fed.}$ Day^{-1})		Total Biogas [L/Day]	Total Methane [L/Day]	Methane (%)	C/N
1.0	5	86.12 ± 4.75	35.21 ± 4.45	9.97 ± 1.26	6.16 ± 0.78	1.75 ± 0.22	28.33		
1.5	4	83.56 ± 5.91	69.17 ± 1.73	24.50 ± 0.79	18.16 ± 0.45	5.15 ± 0.17	35.97		
2.0	5	79.41 ± 8.71	91.85 ± 19.79	30.30 ± 5.84	25.72 ± 5.54	10.61 ± 2.04	36.93		
2.5	5	74.27 ± 6.04	71.59 ± 6.97	28.54 ± 5.14	25.06 ± 2.44	12.49 ± 2.25	41.10	25.94 ± 2.88
3.0	5	66.14 ± 5.05	73.64 ± 0.27	28.15 ± 0.11	38.66 ± 0.15	14.78 ± 0.06	38.23		
3.5	4	68.26 ± 8.88	76.58 ± 0.00	22.72 ± 0.00	37.52 ± 0.00	11.13 ± 0.00	39.53		
4.0	4	61.38± 5.03	74.70 ± 3.64	37.93 ± 1.85	52.29 ± 2.55	26.55 ± 1.29	50.77		

**After 24 hours

As can be seen in Table 5.17, the total biogas and methane yields increased with increasing OLR, thus for the point directed at biogas maximisation, the highest OLR tested (4 gVS/L/day) produced the highest average methane yield of 37.93 ± 1.85 NL.KgVS^{-1} $_{fed.}$Day^{-1} and the highest total biogas and total methane yields of 52.29 ± 2.55 L/Day and 26.55 ± 1.29 L/Day, respectively. The highest methane percentage for the biogas optimisation point was also obtained at this OLR (50.77 %) (Table 5.17). The trend of increasing biogas and methane yields with OLR is better illustrated in Figure 5.9. From Figure 5.9B, it is evident that the COD decreased within the first 9 days. This is likely because of the low OLRs and the high concentration of

microbes in the effluent. This results in a lower substrate to microbe ratio, and also provides an explanation for the highest VS reduction seen at the lowest OLRs (Table 5.17). As the OLR was increased to 2 gVS/L/Day, there was an increase in COD and a brief dip in biogas and methane production. This is likely as a result of a brief adaptation of the microbes to the higher organic loading rate and would explain why the biogas and methane production then increases after a day. The same phenomenon is observed as the OLR was increased from 2 gVS/L/Day to 2.5 gVS/L/Day. From an OLR of 2.5 gVS/L/Day to 3 gVS/L/Day, there is only a slight decrease in biogas and methane and the COD remains relatively constant, increasing only slightly. However, as the OLR was increased from 3 gVS/L/Day to 3.5 gVS/L/Day, there was a drastic dip in biogas production, and a subsequent increase in COD and VFA levels (Figures 5.9 (A & B)).
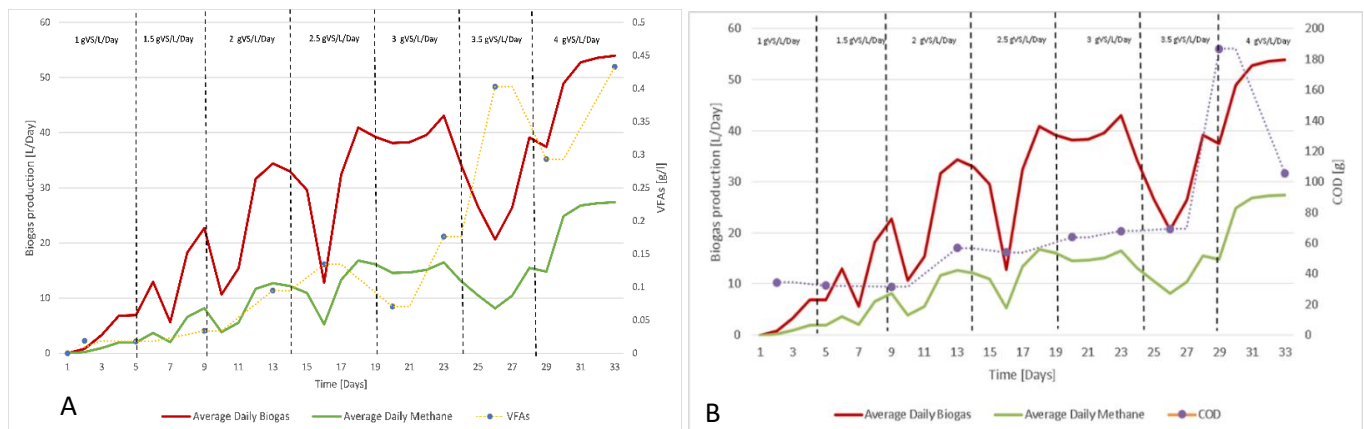
Figure 5.9: Biogas and methane production over time at different OLRs against (A) VFAs and (B) COD for the 50%M, 30%L, 20R mixture (Biogas maximisation point).

 Given that the system recovered, and biogas and methane production increased again after 2 days, it is reasonable to assume that it was another adaptation phase to the higher OLR. Seeing as the COD slowly increased in the digestate with increasing OLR up until an OLR of 3.5 gVS/L/Day, after which there was a drastic increase in COD levels, it is likely that the accumulation of residual digestible organic content coupled with the higher amounts of VS added was too great and resulted in a briefly shocked the system.

This increase in organic content is reflected in the VS reduction, as the VS reduction slowly decreased with the increased OLR up until the OLR of 3.5 gVS/L/day where VS reduction improves and there is a subsequent increase in COD and VFA production (Table 5.17). VS reduction then decreases again at the highest loading rate, due to a higher content of residual organic material in the digestate as a result of the higher OLR. This also explains the high VFA concentrations at the same time point (Figure 5.9A). After the adaptation phase observed from days 24 to 28, there was only a small dip in biogas and methane production as the OLR was increased to 4 gVS/L/Day, thereafter the highest methane and biogas yields were observed, and the COD levels concomitantly decreased (Figure 5.9B). The VFA concentrations toward the end of the experiment remained high and seeing as though the OLR of 4 gVS/L/Day was only fed for a total of 4 days, there is no way of knowing whether the process would have remained stable or if VFA accumulation and acid crash would

have occurred. This warrants further investigation, as operating at higher OLRs offers greater waste disposal value.

For the manure minimisation point, the same trend in increasing biogas and methane yields with increasing OLR is seen, however only up until an OLR of 3.5 gVS/L/Day (Table 5.18). This OLR resulted in the highest biogas and methane yields per VS fed, that is 100.66 ± 21.91 N L.Kg VS^{-1} $_{fed.}$Day^{-1} and 52.89 ± 11.51 NL.KgVS^{-1} $_{fed.}$Day^{-1} respectively (Table 5.18). At an OLR of 4 gVS/L/Day, the biogas and methane production per VS fed decreased, and produced only slightly better yields than at the 1.5 gVS/L/Day OLR. However, the total amount of biogas per day increased, with a slight drop in daily methane production. These results are indicative of organic overloading.

Table 5. 18: Comparison of different OLRs and resultant yields for the manure minimisation substrate mixture (30% M, 30% L, 30% R, 10% FA) in 50 L reactors in semi-continuous process

OLR (gVS/L/day)	Days fed	Average VS Reduction* (%)	Avg. Daily Biogas yield (NL.KgVS^{-1} $_{fed.}$Day^{-1})	Avg. Daily Methane yield (NL.KgVS^{-1} $_{fed.}$ Day^{-1})	Total Biogas [L/Day]	Total Methane [L/Day]	Methane (%)	C/N
1.0	5	80.21 ± 1.87	32.50 ± 1.67	10.29 ± 0.53	5.69 ± 0.29	1.80 ± 0.09	31.67	
1.5	4	75.92 ± 1.70	69.95 ± 1.67	31.12 ± 0.74	14.69 ± 0.35	6.53 ± 0.16	44.49	
2.0	5	73.47 ± 4.74	80.04 ± 3.34	40.50 ± 1.50	28.02 ± 1.17	14.17 ± 0.53	51.07	
2.5	5	71.23 ± 3.78	82.93 ± 8.99	45.31 ± 4.99	36.28 ± 3.93	19.82 ± 2.18	55.21	29.71 ± 0.89
3.0	5	63.48 ± 8.67	83.71 ± 2.03	43.90 ± 1.07	43.95 ± 1.06	23.05 ± 0.56	52.41	
3.5	4	68.15 ± 7.75	100.66 ± 21.91	52.89 ± 11.51	49.33 ± 10.73	25.92 ± 5.64	52.55	
4.0	4	49.21 ± 9.52	73.58 ± 6.57	32.88 ± 2.94	51.50 ± 4.60	23.02 ± 2.06	44.69	

*After 24 hours

As can be seen in Figure 5.10B, the COD drastically increased during the 4 gVS/L/Day OLR. In addition, the VS reduction was lowest at the highest OLR, indicating an accumulation of organic material which suggests that the rate of conversion to biogas was slower than the rate of hydrolysis of organic material.
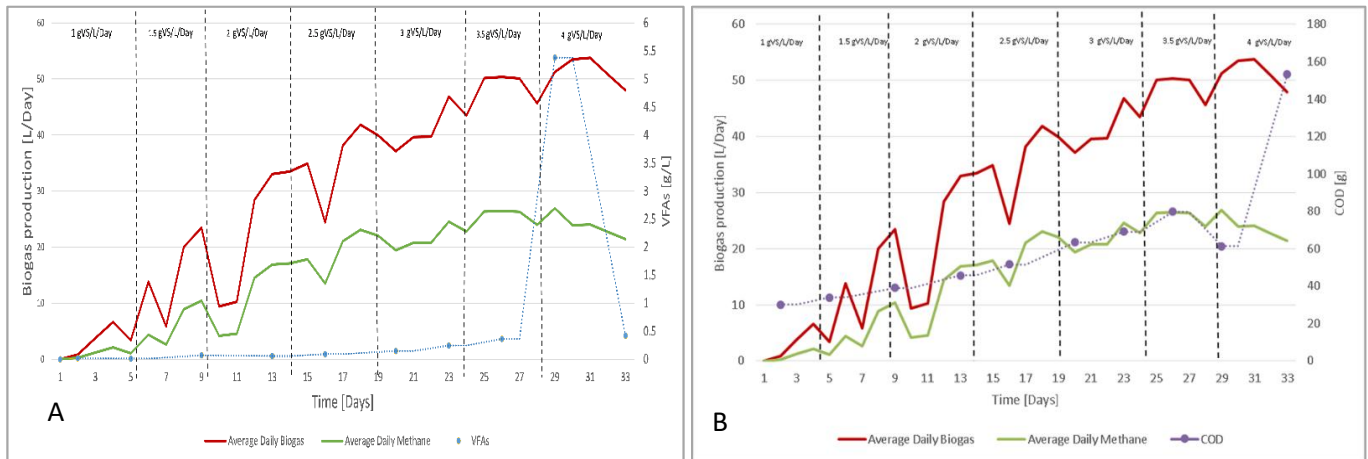
Figure 5. 9: Biogas and methane production over time at different OLRs against VFAs [A] and COD [B] for the 30%M, 30%L, 30%R,10%FA (manure minimisation) mixture (B).

As is evident in Figure 5.10A, the total VFA concentration increased drastically when the OLR increased from 3.5 to 4 gVS/L/Day OLR, although the VFA concentration dropped by the final day of the experiment. This together with the increased COD and lower methane yields indicates process instability and that the upper bounds of the OLR have been reached. Another contributing factor to the decrease in methane production at an OLR of 4 gVS/L/Day could be due to an increase in substrate to inoculum ratio. Seeing as though each OLR was only fed for a period of 4-5 days and seeing as equal quantities of digestate are fed as are removed per day, it is possible that hydraulic overload occurred. Hydraulic overloading or digester washout is known to occur when methanogenic bacteria are removed at a faster rate than they can reproduce (Gerardi, 2003). Seeing as methanogens have a doubling time between 3- 30 days depending on the species, it is possible that there was a lower concentration of methanogenic organisms during the 4 gVS/L/day OLR than at lower OLRs (Gerardi, 2003). Therefore, in order to truly confirm whether or not it is possible to stably operate at an OLR of 4 gVS/L/day for the manure minimisation point, further experimentation looking into longer feeding times for a minimum of one month would need to be conducted.

Interestingly, the manure minimisation point ended up producing higher biogas and methane yields compared with the biogas maximisation point in semi-continuous process (Tables 5.17, 5.18). The highest yields attained by the manure minimisation point were 100.66 ± 21.91 NL.KgVS^{-1} $_{fed.}$Day^{-1} of biogas and 52.89 ± 11.51 NL.KgVS^{-1} $_{fed.}$Day^{-1} of methane at an OLR of 3.5 gVS/L/Day. The biogas maximisation point produced maximum values of 74.70 ± 3.64 NL.KgVS^{-1} $_{fed.}$Day^{-1} of biogas and 37.93 ± 1.85 NL.KgVS^{-1} $_{fed.}$Day^{-1} of methane at an OLR of 4 gVS/L/Day. The reason for the overall high yields as well as for the manure minimisation point out performing the biogas maximisation point in semi-continuous process but not in batch process, is most likely explained by the incremental adaptation of the inoculum to the feed mixture over time.

Digesters equipped with a well-adapted microbial community have been shown to have improved stability during the start-up phase and with increasing loading rates (Angelidaki *et al.*, 2006). The slow incremental

increase of OLR allows time for the biocoenosis to adapt to both the type of feed and quantity of feed by fluctuating the abundance and ratios of certain microbial species and thus adapting the synergetic relationships toward biogas production over time. This explains the better tolerance and improved yields at higher OLR's observed in this study.

The most likely explanation for the manure minimisation point performing better than the biogas maximisation point in semi-continuous process but not batch process is that the pH remained relatively stable (Figure 5.11B) during the semi-continuous process compared to the batch process as a result of correcting the pH of the feed mixture to a neutral pH range around 7 with daily feeding, and the addition of small amounts of calcium carbonate incorporated into the daily feed mixture. This prevented the pH

dropping below the toxic pH of 6.2 for methanogens, unlike in the batch process (Figure 5.9B). As can be seen in Figure 5.11B the pH of the system ranged between 7.2-7.6 over the course of the experiment for the manure minimisation point, which corresponds with the optimum pH range for certain commonly occurring methanogenic species of 6.6-7.8 (Gerardi, 2003). This pH range of 7.2-7.6 is much narrower and more stable than the pH range of 5.93-7.40 observed for the manure minimisation point in batch process (Figure 5.8B).
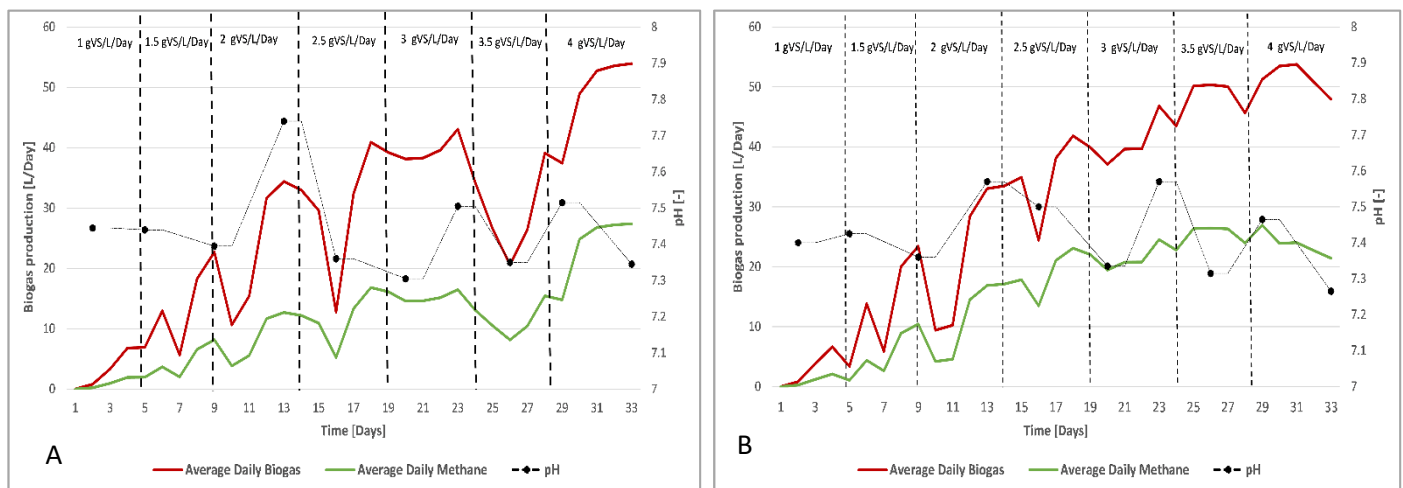
Figure 5.10: Biogas and methane production over time at different OLRs against pH for both biogas maximisation point (A) and the manure minimisation point (B).

Although the pH of the biogas optimisation point was also maintained within the optimal range of 6.1-7.8 (Figure 5.11A), the reason mixtures with higher fruit waste concentrations, such as the manure minimisation point, usually fail is due to decreases in pH below 6.2 which are toxic to methanogenic bacteria (Gerardi, 2003; Chandra, Takeuchi and Hasegawa, 2012). Therefore, it stands to reason that by correcting the pH and with the addition of calcium carbonate (1% w/w) daily, the alkalinity and therefore stability of a normally volatile system is improved and thus results in improved biogas and methane yields.

5.8 Summary

There is a paucity of research into the optimization of co-substrates for the anaerobic digestion (AD) of apple-based fruit juice processing waste. AD is a viable and environmentally responsible method of fruit waste disposal that includes additional benefits to industry, such as providing a renewable energy source and generating digestate as a valuable by-product which can be sold as a liquid fertilizer for additional revenue. This study aimed to identify optimal substrate combinations of five fruit juice industry waste streams resulting in the highest biogas and methane yields based on seasonal availability of feedstocks. In addition, the study aimed to test the process stability and viability of these points at a larger scale in 50 L stirred reactors, in both batch and semi-continuous process. The five waste streams namely manure, food waste, retentate, pomace and waste apples were incorporated into a five-factor mixture design in order to assess the methane potential of the various substrate combinations at lab scale in 100 mL serum bottles.

The substrate combinations and ratios which gave the highest yields for each scenario were the following: S1: 50% manure and 50% retentate; S2: 50% food waste and 50% manure; S3: 33.33% pomace, 33.33% manure and 33.33% food waste. The results of the mixture design with food waste displayed high variability and statistical analyses revealed the lack of fit of the model to be significant for both total methane (mL) and total biogas (mL) as outcome variables. In addition to high variability, the results showed that food waste could not be used in quantities greater than 33% in co-digestion with fruit waste and manure without impeding biogas and methane production. This was due to the high initial VFA concentrations in the food waste. It was found that food waste could not be digested alone or with acidic wastes and that co-digestion in lower quantities with manure and fruit waste appeared to benefit biogas and methane production, likely due to a dilution effect and improved buffering capacity due to manure co-digestion. Since food waste was highly variable and did not improve the waste disposal value of the fruit waste, it was excluded from further study.

A second mixture design incorporating a slower degrading substrate (LCB) was conducted to aid in the waste disposal of the fruit waste fraction, which is prone to instability as a result of VFA accumulation. The results of the second mixture design showed both biogas and methane to be significant (p< 0.05) and the lack of fit of the model to be insignificant. The standardised effect estimates of all five feedstocks revealed manure, LCB and retentate to have a significant (p<0.05) effect on biogas and methane production. The LCB supplementation study produced much higher biogas and methane yields when co-digested with fruit juice industry wastes than the initial mixture design with food waste. LCB was found to compensate for the manure fraction and improve biogas and methane yields for two substrate mixtures (20% manure 30% LCB 30% pomace 20% retentate and 20% manure, 30% LCB, 30% waste apples and 20% retentate). For the majority of cases with LCB, LCB addition was able to improve yields providing it compensated for the fruit waste fraction of the substrate mixture rather than the manure fraction, with the exception of pomace which gave higher yields with 30% pomace and less LCB addition. The highest biogas and methane yields

obtained from the LCB supplementation experiment were 410.01 mL.gVS^{-1} $_{fed}$ and 167.10 mL.gVS^{-1} $_{fed}$ for the fruit-juice producing season (S1) from a substrate combination of 50% manure, 30% LCB and 20% retentate. The highest yields obtained for the non-fruit juice producing season (S2) were 325.69 mL.gVS^{-1} $_{fed}$ and 131.95 mL.gVS^{-1} $_{fed}$ from a mixture of 40% manure, 30% LCB and 30% waste apples.

A tertiary aim of the study was to determine whether any of the tested substrate combinations at lab-scale resulted in higher levels of VFAs without severely impeding biogas and methane yields. HPLC analysis of the two mixture designs revealed that the highest total post-digestion VFA yields were produced during the first mixture design using food waste, with the highest observed yields ranging from 26.37-40.40 g/L of VFAs. All of the points observed to have high VFA concentrations in the first mixture design also displayed poor biogas and methane yields. In contrast, the mixture design with LCB displayed much lower VFA yields overall with improved biogas and methane yields. One substrate mixture (50% manure, 20% LCB and 30% retentate) observed in the LCB supplementation mixture experiment produced a moderately high VFA yield of 17. 34 g/L and moderate yields of 303.30 mL.gVS^{-1} $_{fed}$ of biogas and 111.84 mL.gVS^{-1} $_{fed}$ of methane.

Two optimal points were chosen for a scale-up in 50 L reactors for Scenario 1 only. The two selected points for biogas optimisation (50% manure, 30% LCB, 20% Retentate) and for manure minimisation (30% manure, 30% LCB, 30% retentate and 10%waste apples) were scaled up in 50 L CSTR reactors in batch process for 32 days with intermittent mixing in order to test the validity of the predicted results from the lab scale experiments. The scale-up of the manure minimisation point in batch process revealed comparable yet improved results compared to the lab-scale predictions, and produced a biogas yield of 351.51 ± 70.04 NL.KgVS^{-1} $_{fed}$ and a methane yield of 153.69 ± 28.89 NL.KgVS^{-1} $_{fed.}$ The batch scale-up of the biogas optimisation substrate mixture showed a substantial increase in biogas and methane yields compared to the lab-scale results, producing 692.43 ± 154.75 NL.KgVS^{-1} $_{fed}$ and 214 ± 97.65 NL.KgVS^{-1} $_{fed}$ of methane. The improved biogas and methane yields in the batch experiment were as a result of slow intermittent mixing at 125 rpm for 5-10 minutes twice daily, which has been shown to improve mass transfer and aid VS reduction.

The same two points for biogas optimisation and manure minimisation were scaled-up in 50 L reactors in semi-continuous process and fed increasing OLRs from 1-4 gVS/L/day over the course of 32 days in order to identify the maximum OLR that can be stably operated for each point. The biogas optimisation point gave the highest yields at an OLR of 4 gVS/L/day. The manure minimisation point demonstrated highest biogas and methane yields at an OLR of 3.5 gVS/L/day, with the system showing signs of organic overloading at higher OLRs. The manure minimisation point ended up producing higher biogas and methane yields than the biogas optimisation point in semi-continuous process owing to the improved stability and alkalinity of the pH throughout the process. This was as a direct result of daily pH adjustment to a pH of 7 and addition of calcium carbonate (1% w/w) to the feed mixture.

5.9 Concluding Remarks

In conclusion, this study found that fruit wastes could not be digested without a minimum of 20% supplementation with manure, with the exception of waste apples which required greater supplementation. Moreover, it was concluded that food waste was unsuitable as a co-substrate of fruit industry waste at concentrations greater than 33.33% of the total substrate mixture. This is primarily as a result of high VFA concentrations and low initial pH of the food waste. Of the fruit wastes, waste apples required the greatest amount of supplementation due to its high easily degradable carbohydrate content and high C/N ratio. Buffering capacity of mixtures was improved by the addition of LCB due to its increased complex carbohydrate content. However, although LCB addition was found to significantly improve biogas production and prevent acid crash, it mainly did so when compensating for the fruit waste fraction rather than the manure fraction with the exception of two substrate combinations. It was found that 30% LCB addition to improved digestibility and stability of fruit process wastes for certain ratios of pomace and retentate and waste apples and retentate with 20% manure. However, this study only investigated 0, 20% and 30% LCB supplementation, therefore future research should focus on a broader array of supplementation levels within these substrate combinations in order to further maximise fruit waste disposal via AD. Future studies should also investigate the viability of other low-lignin co-substrates such as sorghum that will improve the C/N and buffering capacity of the mixture when co-digested with fruit wastes, in order to maximise fruit waste disposal. Furthermore, one substrate combination (50% manure 20% LCB 30% retentate) in this study was identified which produced relatively high VFA concentrations without producing low quality biogas (<40% methane) or drastically diminishing total biogas and methane yields. Future studies investigating the bio-refinery techniques involving the extraction of VFAs for co-production of biogas and VFAs on this substrate mixture would be of particular value to this area of research. Lastly, this study showed it was possible to operate the two manure, LCB and fruit waste co-feed mixtures at higher OLRs of 3.5-4 gVS/L/day (within the range typically used at EFJ) however, due to time constraints the stability of the process at these higher OLRS could not be elucidated over longer feeding times and thus do not provide information as to the stability of the tested OLRs during long-term operation of the digester. Therefore, future studies should investigate the long-term effects of feeding the respective optimal OLRS determined in this study for each substrate mixture on overall process stability.

5.10 Limitations and Recommendations

The limitations that were encountered in this study are listed below, along with (where possible) proposed solutions for future experiments as well as suggestions for future studies.

- The mixed particle size of LCB at lab-scale causes large variations in biogas yields. Initially, the decision to include mixed particle size was made, as this was thought to better reflect industrial conditions. However, in future, bags of LCB should be thoroughly mixed before use to prevent powder settling at the bottom and to obtain more representative samples of the mixture. In addition, BMPs should be conducted in larger bottles to further minimise variations in biogas production.

- The presence of stalks, stems and seeds in pomace and waste apples samples caused an underestimation in VFAs fed in lab scale experiments, as well as the presence of sand and stones in manure samples. In future, BMPs should be performed in larger bottles of at least 1 L capacity and should be performed with more replicates to obtain more accurate representations of yields for substrates containing indigestible materials.

- Power outages occurred multiple times during the scale-up experiments, resulting in an underestimation of total biogas production. The automated biogas measurement system as part of the reactor set-up was not able to track biogas production during power outages. Due to gas release valves closing, the power outages caused gas build-ups which pushed all water out of the manometer, causing gas production to be unaccounted for during the time up until the manometer to be filled again.

- In addition, the method and accuracy of tracking total methane produced from the 50 L reactors could be greatly improved by the attachment of gas chromatography equipment to the automated biogas tracking system. This would allow gas composition to be tracked in real time instead of only analysed twice weekly and would therefore be able to provide an accurate reflection of how gas composition changes over time or in response to feeding and thus provide more valuable results. The current method of analysing methane (%) over time is by taking 5 GC measurements over the course of the run and obtaining an average methane (%). Due to fluctuations in methane (%) over time this method can negatively impact reproducibility of the experiments, hence final methane (%) is typically also included as this gives a better indication of the methane (%) of the gas during stable operation.

- Due to the large quantities of feedstocks required for the study, it was not possible to freeze and prepare all required feedstocks during one sample collection. As a result, there could have been deviations in chemical compositions between the manure samples used in the lab-scale experiment compared to the manure used in the scale-up experiments.

- Due to time constraints, optimum points for the non-fruit juice producing season as well as the fruit waste maximisation point without LCB addition (80% fruit wastes, 20% manure) could not be scaled-up to reactor level experiments. In future, optimisation of waste combinations and ratios for non-fruit juice wastes should be optimised to provide the greatest energy value to the plant over the off season. Furthermore, the viability of the fruit waste maximisation point in scale-up should be tested to fully maximise waste disposal of fruit wastes at EFJ via AD. In addition, future studies should analyse the nutrient profiles and pathogen loads of the resulting digester effluents from all optimised points determined in this study, in order to assess their suitability to be used as liquid fertiliser and sold for additional revenue.

- Ideally, semi-continuous experiments with longer adaptation times to increasing OLRs as well as higher OLRs would have been conducted, however due to time constraints this was not possible. Thus, the results of the semi-continuous experiments are not reflective of long-term operation of the reactor. In order to test the stability of each OLR for each substrate combination longer feeding times should be studied.

- Another limitation is that VS reduction samples should have been larger, however the available equipment only allowed for small sample sizes to be tested, which is not as accurate as testing larger volumes given that very little solids remained after drying due to the high moisture content. In addition, VS reduction for each OLR was only tested after the first day of feeding any given OLR. In future VS reduction should be conducted in larger volumes and samples should be taken several times over the course of feeding each OLR, the average of which should be taken as the true VS reduction value.

- Ideally, inoculum from the same source should be used and preferably also from a source which is fed consistently using a homogenous substrate in order to minimise error due to differences in inoculum microbial compositions, however, in this study this was not possible due to circumstances beyond our control.

- Lastly, due to circumstances beyond our control, maize was harvested at approximately 142 days of growth. Since physiological maturity of maize is reached at 112-119 days of growth, the maize in this study could not be considered physiologically immature. In future, studies should investigate the effect of younger LCB with less than 112 days of growth on biogas production and as a co-substrate of fruit juice process wastes.

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Appendix A: Statistical Designs

Table A. 1: Mixture Interaction Study Statistical Design according to scenarios reflecting seasonal availability of feedstocks.

	Substrate concentration				
	Waste apples (% w/w)	Pomace (% w/w)	Retentate (% w/w)	Food waste (% w/w)	Manure (% w/w)
scenario 1	50	50	0	0	0
	0	100	0	0	0
	25	25	25	0	25
	0	0	100	0	0
	0	50	0	0	50
	100	0	0	0	0
	33.3	0	33.3	0	33.3
	0	50	50	0	0
	50	0	50	0	0
	0	33.3	33.3	0	33.3
	50	0	0	0	50
	0	0	50	0	50
	33.3	33.3	33.3	0	0
1 & 2	100	0	0	0	0
scenario 2	0	0	0	50	50
	50	0	0	50	0
	33.3	0	0	33.3	33.3
	0	0	0	100	0
2 & 3	50	0	0	50	0
	0	0	0	50	50
scenario 3	20	20	20	20	20
	0	33.3	0	33.3	33.3
	0	0	33.3	33.3	33.3
	33.3	33.3	0	33.3	0
	0	33.3	33.3	33.3	0
	0	0	0	0	100
	0	25	25	25	25
	0	0	0	100	0
	25	0	25	25	25
	0	50	0	50	0
	0	0	33.3	33.3	33.3
	0	0	50	50	0
	25	25	0	25	25
All	0	0	0	0	100

Table A. 2: LCB Supplementation Mixture Design according to scenario.

Scenario		Run order [-]	Manure concentration (% w/w)	LCB concentration (% w/w)	Waste apples concentration (% w/w)	Pomace concentration (% w/w)	Retentate concentration (% w/w)
					Substrates		
	2	1	50.00	30.00	20.00	0.00	0.00
		6	50.00	20.00	30.00	0.00	0.00
		9	40.00	30.00	30.00	0.00	0.00
	1	4	50.00	0.00	30.00	20.00	0.00
		19	50.00	20.00	0.00	0.00	30.00
		13	20.00	30.00	0.00	30.00	20.00
		22	40.00	30.00	0.00	0.00	30.00
		24	20.00	30.00	0.00	20.00	30.00
		16	20.00	0.00	30.00	30.00	20.00
		15	20.00	30.00	20.00	30.00	0.00
		7	20.00	30.00	30.00	20.00	0.00
		5	50.00	0.00	30.00	0.00	20.00
		23	20.00	30.00	20.00	0.00	30.00
		12	50.00	0.00	20.00	30.00	0.00
		21	50.00	0.00	0.00	20.00	30.00
		3	50.00	30.00	0.00	0.00	20.00
		26	20.00	20.00	30.00	0.00	30.00
		29	20.00	20.00	0.00	30.00	30.00
		10	50.00	0.00	0.00	30.00	20.00
		2	50.00	30.00	0.00	20.00	0.00
		18	20.00	20.00	30.00	30.00	0.00
		31 C(4)	36.00	16.00	16.00	16.00	16.00
		30	20.00	0.00	20.00	30.00	30.00
		25	40.00	0.00	30.00	0.00	30.00
		28	40.00	0.00	0.00	30.00	30.00
		14	40.00	30.00	0.00	30.00	0.00
		17	40.00	0.00	30.00	30.00	0.00
		20	50.00	0.00	20.00	0.00	30.00
		8	20.00	30.00	30.00	0.00	20.00
		11	50.00	20.00	0.00	30.00	0.00
		27	20.00	0.00	30.00	20.00	30.00

Appendix B: Statistical analyses for mixture designs

Table B. 1: Interaction Study (BMP mixture design) ANOVA results with total biogas (mL) as response variable:

Model	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p	R-Sqr	R-S Adju
ANOVA; Var.:Total Biogas (mL) (Responses.sta) 5 Factor mixture design; Mixture total=100., 38 Runs Sequential fit of models of increasing complexity										
Linear	462825	4	115706.2	797082.4	33	24154.0	4.790353	0.003699	0.367348	0.2
Total Adjusted	1259907	37	34051.5							

Source	SS	df	MS	F	p
Overall Fit of Model; Var.: Total Biogas (mL) (Responses.sta) 5 Factor mixture design; Mixture total=100., 38 Runs					
Model	462825	4	115706.2	4.7904	0.003699
Total Error	797082	33	24154.0		
Lack of Fit	795670	26	30602.7	151.7266	0.000000
Pure Error	1412	7	201.7		
Total Adjusted	1259907	37	34051.5		

Table B. 2: Interaction study (BMP mixture design) ANOVA results using total methane (mL) as the outcome variable:

Model	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p	R-Sqr	R-Sqr Adjusted
ANOVA; Var.:Total methane [mL] (Responses.sta) 5 Factor mixture design; Mixture total=100., 38 Runs Sequential fit of models of increasing complexity										
Linear	242611.8	4	60652.94	239466.6	33	7256.565	8.358354	0.000090	0.503262	0.443051
Total Adjusted	482078.4	37	13029.15							

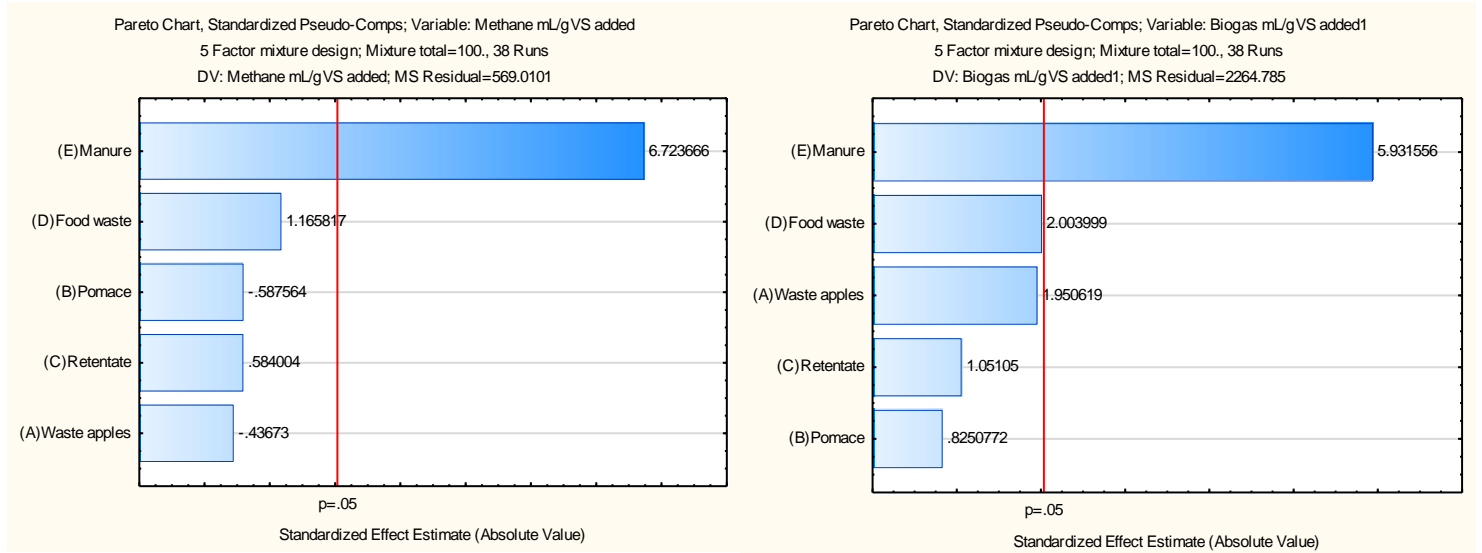| Source	SS	df	MS	F	p
Overall Fit of Model; Var.: Total methane [mL] (Responses.sta) 5 Factor mixture design; Mixture total=100., 38 Runs					
Model	242611.8	4	60652.94	8.358	0.000090
Total Error	239466.6	33	7256.57		
Lack of Fit	239437.7	26	9209.14	2230.086	0.000000
Pure Error	28.9	7	4.13		
Total Adjusted	482078.4	37	13029.15		

Figure B. 1: Pareto chart illustrating the standardised effect estimates for all feedstocks in the mixture design including food waste

Table B. 3: ANOVA results for LCB supplementation study with total biogas as the outcome variable.

	Overall Fit of Model; Var.: Total biogas (5 Factor Constrained Mixture (BMP 6).sta) 5 Factor mixture design; Mixture total=1., 34 Runs				
Source	SS	df	MS	F	p
Model	436369.7	4	109092.4	5.731154	0.001589
Total Error	552014.5	29	19035.0		
Lack of Fit	482393.1	22	21927.0	2.204619	0.142940
Pure Error	69621.4	7	9945.9		
Total Adjusted	988384.2	33	29951.0		

	ANOVA; Var.:Total biogas (5 Factor Constrained Mixture (BMP 6).sta) 5 Factor mixture design; Mixture total=1., 34 Runs Sequential fit of models of increasing complexity									
Model	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p	R-Sqr	R-Sqr Adjusted
Linear	436369.7	4	109092.4	552014.5	29	19034.98	5.731154	0.001589	0.441498	0.364463
Total Adjusted	988384.2	33	29951.0							

Table B. 4: ANOVA results for LCB supplementation study with total methane as the outcome variable.

| Model | ANOVA; Var.:Total CH4 (mL) (5 Factor Constrained Mixture (BMP 6).sta) 5 Factor mixture design; Mixture total=1., 38 Runs Sequential fit of models of increasing complexity | | | | | | | | | |
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p	R-Sqr	R-Sqr Adjusted
Linear	58927.3	4	14731.82	137741.1	33	4173.973	3.529447	0.016672	0.299628	0.214734
Total Adjusted	196668.4	37	5315.36							

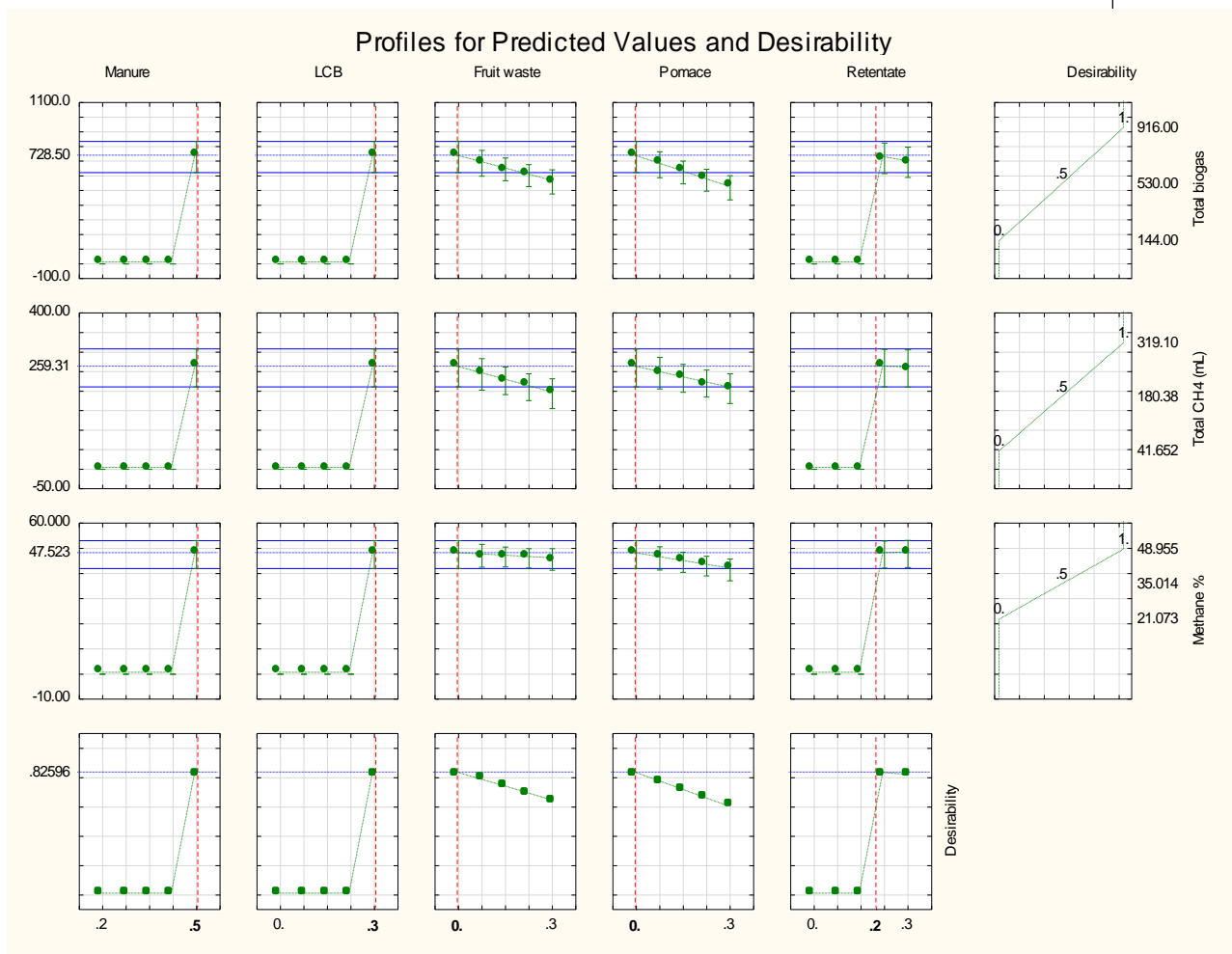| Source | Overall Fit of Model; Var.: Total CH4 (mL) (5 Factor Constrained Mixture (BMP 6).sta) 5 Factor mixture design; Mixture total=1., 38 Runs | | | | |
	SS	df	MS	F	p
Model	58927.3	4	14731.82	3.529447	0.016672
Total Error	137741.1	33	4173.97		
Lack of Fit	121104.8	26	4657.88	1.959877	0.181626
Pure Error	16636.3	7	2376.62		
Total Adjusted	196668.4	37	5315.36		

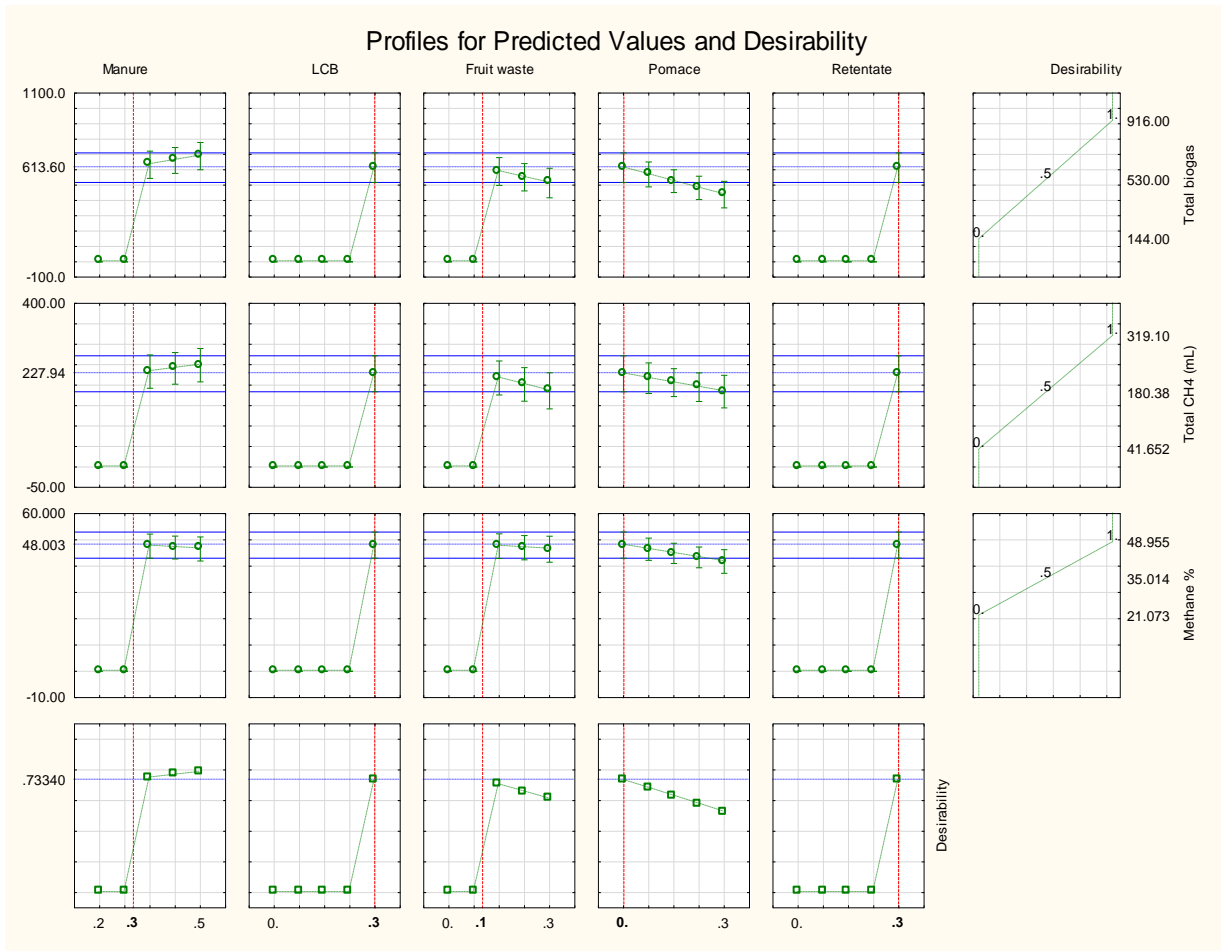Figure B. 2: Response desirability results for the biogas optimisation point

Figure B. 3: Response desirability results for manure minimisation point.

Appendix C: Sample Calculations Bmp Bottle Make-Up

BMP bottle make-up example:

Assuming $\rho_{water} = 1\frac{g}{ml}$

Dry solids for a bottle volume of 70ml and 10% solids loading:

Dry solids = $\frac{10}{100}$ x 70 ml $= 7$ g

Dry manure for a 100% manure concentration:

Dry manure = $\frac{100}{100}$ x 7g $= 7$ g

Dry inoculum for 10% of solids loading:

Dry inoculum = $\frac{10}{100}$ x 7g $= 0.7$ g

Wet manure for a moisture content of 84.46%:

Wet M= $\frac{7 \text{ g}}{\frac{100-84.46}{100}}$ $= 45.05$ g

Water content of M = 45.05 g – 7 g = 38.05 g

Wet inoculum for a moisture content of 90.35%:

Wet I= $\frac{0.7 \text{ g}}{\frac{100-90.35}{100}}$ $= 7.26$ g

Water content of I = 7.26 g–0.7 g = 6.56 g

Additional water added: 70 ml – 38.05 ml – 6.56 ml = 25.39 ml

Appendix D: GC Analysis
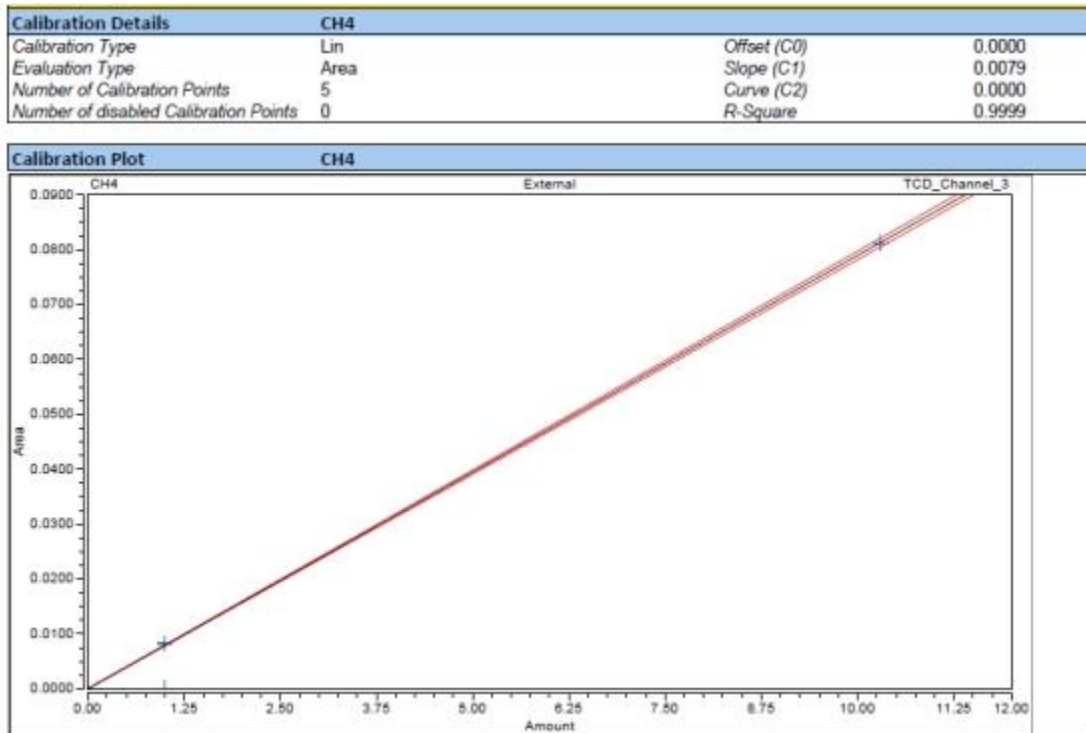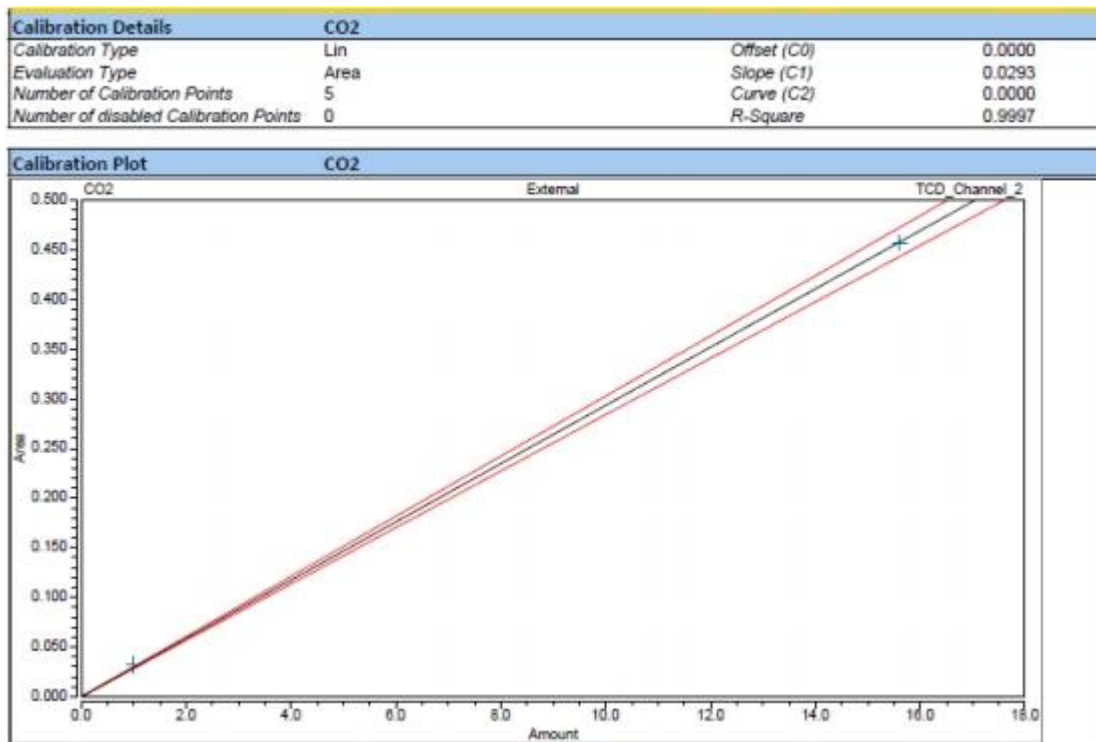
D. 1   GC Calibration Curves

Calibration Details	CH4		Offset (C0)	0.0000
Calibration Type	Lin		Offset (C0)	0.0000
Evaluation Type	Area		Slope (C1)	0.0079
Number of Calibration Points	5		Curve (C2)	0.0000
Number of disabled Calibration Points	0		R-Square	0.9999

Figure D. 1: Methane calibration curve

Calibration Details	CO2		Offset (C0)	0.0000
Calibration Type	Lin		Offset (C0)	0.0000
Evaluation Type	Area		Slope (C1)	0.0293
Number of Calibration Points	5		Curve (C2)	0.0000
Number of disabled Calibration Points	0		R-Square	0.9997

Figure D. 2: Carbon dioxide calibration curve

Calibration Details	N2		
Calibration Type	Lin	Offset (C0)	0.0000
Evaluation Type	Area	Slope (C1)	0.0031
Number of Calibration Points	8	Curve (C2)	0.0000
Number of disabled Calibration Points	0	R-Square	0.9998

Calibration Plot	N2
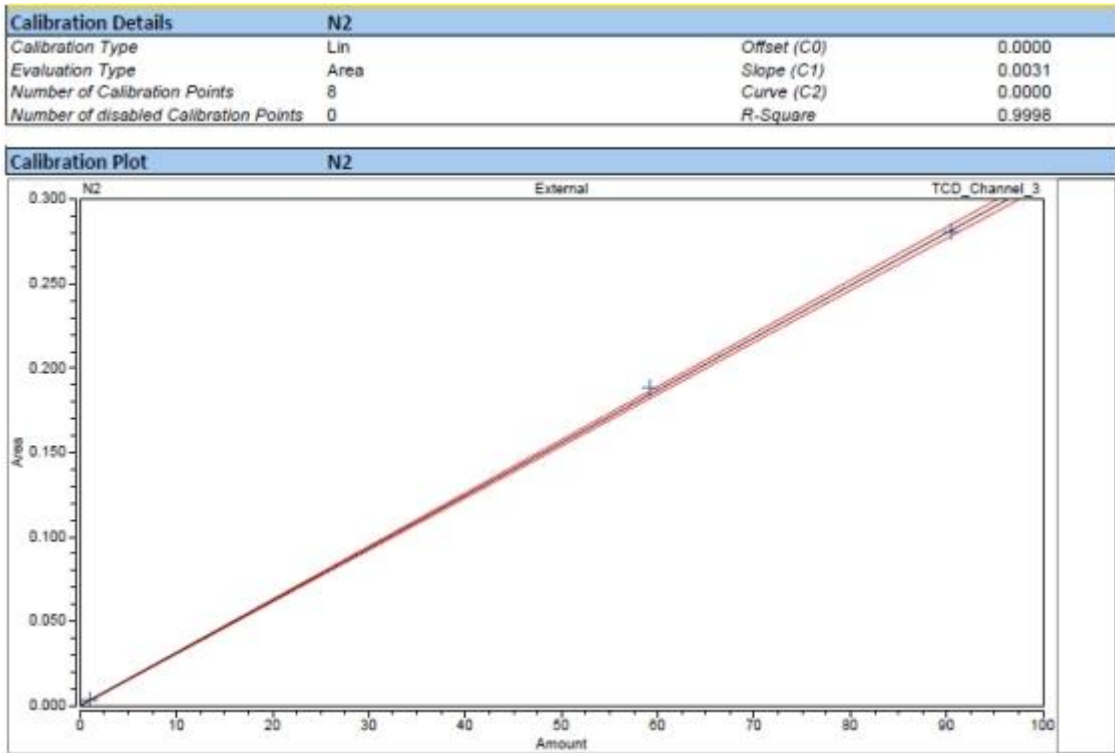
Figure D. 3: Nitrogen calibration curve

Calibration Details	Oxygen		
Calibration Type	Lin	Offset (C0)	0.0000
Evaluation Type	Area	Slope (C1)	0.0037
Number of Calibration Points	3	Curve (C2)	0.0000
Number of disabled Calibration Points	0	R-Square	0.0000

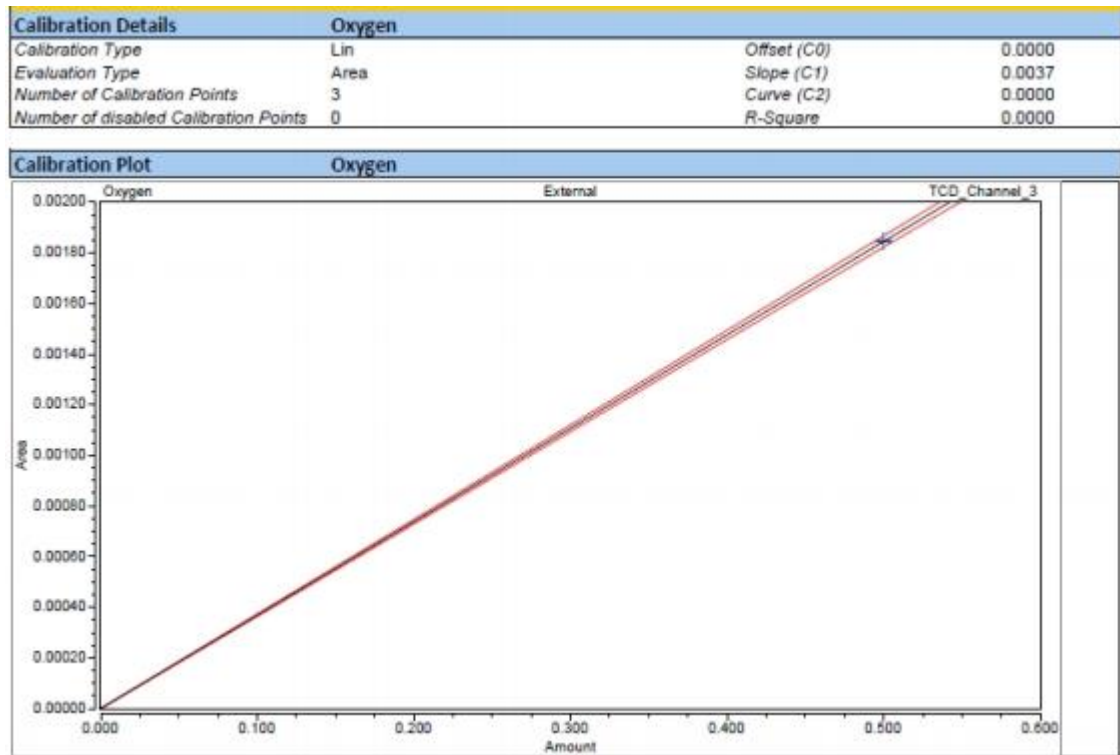| Calibration Plot	Oxygen

Figure D. 4: Oxygen calibration curve

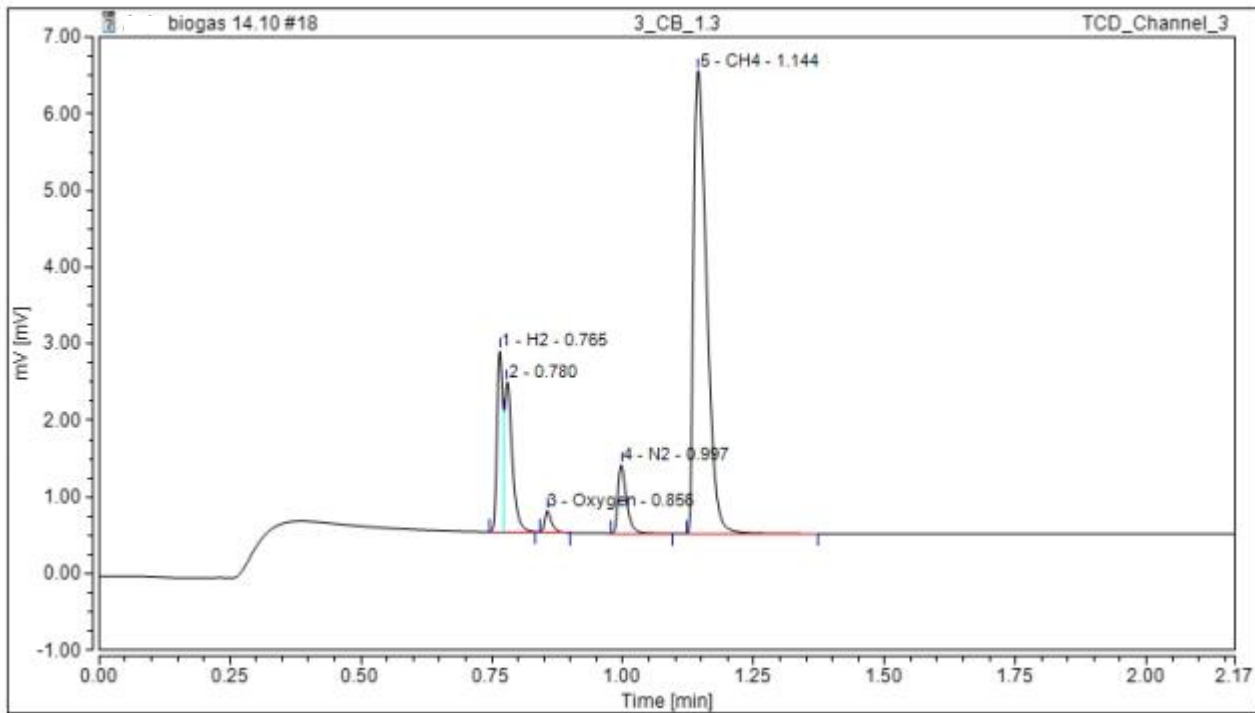## D. 2   Example of a duplicate GC measurement

Figure D.5: Chromatogram Example 1

Table D. 1: Integration Results for Measurement 1

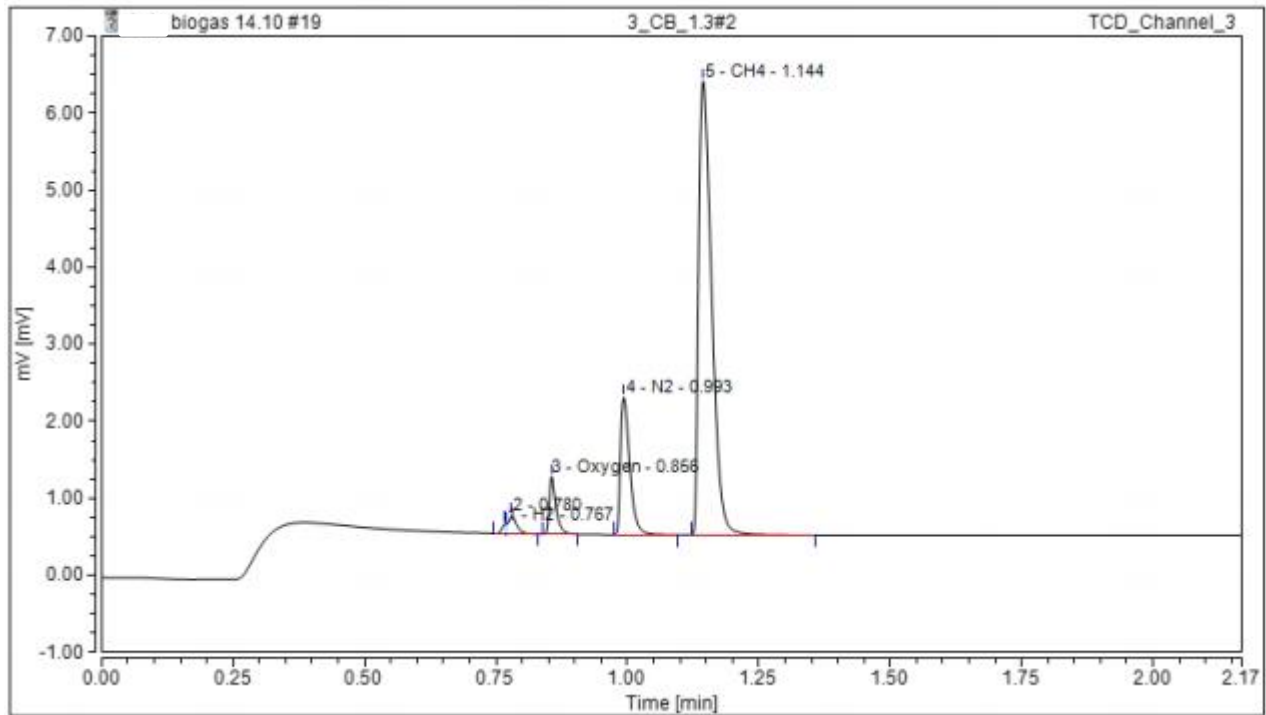| No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount %
1	H2	0.765	0.03	2.36	11.76	20.47	0.69
3	Oxygen	0.856	0.00	0.29	1.59	2.47	1.12
4	N2	0.997	0.02	0.89	6.52	7.70	5.44
5	CH4	1.144	0.18	6.04	67.51	52.37	22.19
Total:					100.00	100.00	

Figure D. 6: Chromatogram Example 2

Table D. 2: Integration Results for Measurement 2

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount %
1	H2	0.77	0.00	0.12	0.56	1.41	0.03
3	Oxygen	0.86	0.01	0.75	4.95	8.51	2.98
4	N2	0.99	0.04	1.78	16.51	20.30	11.80
5	CH4	1.14	0.17	5.90	76.09	67.12	21.44
Total:					100.00	100.00	

Average methane content per duplicate per run

$$\% \, CH_4 = \frac{22.20 + 21.43}{2} = 21.81\%$$

$$SD = 0.53$$

$$RSD = \frac{100S}{\bar{x}} = 100 \times \frac{0.53}{21.81} = 2.44\%$$