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Profiling Milk from Grass – Biochemical and Sensory Analysis of Dairy Commodities

Thesis presented by

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for the degree of

Doctor of Philosophy

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Agriculture and Food Development Authority

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Dedication

Declaration

This declaration is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signature:



Date: 14 December 2021

Holly J. Clarke

Student Number: 117221603

Dedication

Dedication

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Contributions

Chapter 3: Ms. Gloria Ho carried out free fatty acid analysis on the milk powder samples. Mr. William P. Mc Carthy and Mr. Nicolas Perrigault assisted with the sensory analysis. Mr. William P. Mc Carthy also assisted with the microbial analysis.

Chapter 4: Ms. Carol Griffin carried out the full descriptive sensory analysis.

Chapter 5: Ms. Diana Apopei carried out free fatty acid analysis on the milk samples, Ms. Carol Griffin carried out the full descriptive sensory analysis and Mr. Johnathan Magan assisted with the collection of the milk samples. Ms. Joanne Hayes and Ms. Natasha Leeuwendaal assisted with the microbial analysis.

Chapter 6: Ms. Ellen Fitzpatrick kindly collected the feed samples and Mr. Mark Timlin kindly collected the milk samples.

Conference Presentations

Peer Reviewed Publications

1. **Clarke**, H. J., D. T. Mannion, M. G. O'Sullivan, J. P. Kerry, and K. N. Kilcawley. **2019**. Development of a headspace solid-phase microextraction gas chromatography mass spectrometry method for the quantification of volatiles associated with lipid oxidation in whole milk powder using response surface methodology. *Food Chemistry* 292:75-80. 10.1016/j.foodchem.2019.04.027.

2. **Clarke**, H. J., C. Griffin, D. K. Rai, T. F. O'Callaghan, M. G. O'Sullivan, J. P. Kerry, and K. N. Kilcawley. **2020**. Dietary compounds influencing the sensorial, volatile and phytochemical properties of bovine milk. *Molecules* 25, 26. doi.org/10.3390/molecules25010026.

3. **Clarke**, H. J., M. G. O'Sullivan, J. P. Kerry and K. N. Kilcawley. **2020**. Correlating Volatile Lipid Oxidation Compounds with Consumer Sensory Data in Dairy Based Powders during Storage. *Antioxidants* 9(4):338. doi.org/10.3390/antiox9040338.

4. **Clarke**, H. J., C. Griffin, D. Hennessy, T. F. O'Callaghan, M. G. O'Sullivan, J. P. Kerry, and K. N. Kilcawley. **2021**. Effect of bovine feeding system (pasture or concentrate) on the oxidative and sensory shelf life of whole milk powder. *Journal of Dairy Science* 104(10): 10654-10668. 10.3168/jds.2021-20299.

5. **Clarke**, H. J., W. P. Mc Carthy, M. G. O'Sullivan, J. P. Kerry, and K. N. Kilcawley. **2021.** Oxidative Quality of Dairy Powders: Influencing Factors and Analysis. *Foods* 10(10): 2315. doi.org/10.3390/foods10102315.

6. **Clarke**, H. J., E. Fitzpatrick, D. Hennessy, M G. O'Sullivan, J. P. Kerry, and K. N. Kilcawley. **2022**. The Influence of Pasture and Non-pasture-Based Feeding Systems on the Aroma of Raw Bovine Milk. *Frontiers in Nutrition* 9(841454). doi: 10.3389/fnut.2022.841454.

7. Kilcawley, K. N., H. Faulkner, H. J. **Clarke**, M. G. O'Sullivan, and J. P. Kerry. **2018**. Factors Influencing the Flavour of Bovine Milk and Cheese from Grass Based versus Non-Grass Based Milk Production Systems. *Foods* 7(3):37. 10.3390/foods7030037.

Awards

Conference Presentations

Oral

1. Holly J. **Clarke**, David T. Mannion, Maurice G. O'Sullivan, Joseph P. Kerry and Kieran N. Kilcawley. *''Validation of a HS-SPME GCMS Method for the Quantification of Volatiles Associated with Lipid Oxidation in Whole Milk Powder using Response Surface Methodology''*. Irish Mass Spectrometry Society Meeting (IMSS), Dublin, Ireland, May 9th **2018.**

2. Holly J. **Clarke**, David T. Mannion, Maurice G. O'Sullivan, Joseph P. Kerry and Kieran N. Kilcawley. *''Development of a HS-SPME GCMS Method for the Quantification of Volatiles Associated with Lipid Oxidation in Whole Milk Powder''*. EuroFed Lipid Conference, Belfast, Northern Ireland, September 16th – 19th **2018.**

3. Holly J. **Clarke**, Carol Griffin, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley. *''The Effect of Bovine Feeding System on the Oxidative and Sensory Shelf Life of Whole Milk Powder''.* EuroSense 2020. Virtual online. December 13th – 16th **2020.**

4. Holly J. **Clarke**, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley. ''*Pasture and Non-pasture-based Feeding Systems Influence Aroma-active Compounds in Raw Bovine Milk''.* 49th Annual Food Science and Technology Conference hosted by Technological University of Dublin (TUD) and the IFSTI. Virtual online. December 15th **2020.**

Poster

1. Holly J. **Clarke**, Carol Griffin, Dilip Rai, Kathy Ridgeway, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley. *''Dietary Volatile Compounds Influencing the Sensory Properties of Bovine Milk''.* Grass-fed Dairy Conference, Osprey hotel, Naas, Co. Kildare, Ireland, October 25th **2018.**

2. Holly J. **Clarke**, Carol Griffin, Dilip Rai, Kathy Ridgeway, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley. ***''****Dietary Volatile Compounds Influencing the Sensory Properties of Bovine Milk''*. 47th Annual Food Science and Technology Conference hosted by University College Cork (UCC) and the Institute of Food Science and Technology of Ireland (IFSTI), December 6-7th **2018.**

Awards

3. Holly J. **Clarke**, Carol Griffin, Dilip Rai, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley. ***''Dietary Volatile Compounds Influencing the Sensory Properties of Bovine Milk''.*** Pangborn 2019, Edinburgh International Conference Centre, Edinburgh, United Kingdom, July 28th – August 01st **2019.**

4. Holly J. **Clarke**, Carol Griffin, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley.
''Correlating Consumer Sensory Data with the Volatile Profile of Dairy Based Powders during Storage''. 48th Annual Food Science and Technology Conference hosted by University of Limerick (UL) and the IFSTI, December 16th **2019.**

5. Holly J. **Clarke**, Maurice G. O' Sullivan, Joe P. Kerry and Kieran N. Kilcawley. *''HiSorb Centri-GCMS/Olfactometry Analysis of Aroma Compounds in Raw Bovine Milk''.* Irish Mass Spectrometry Society Conference. Virtual online, May 11th and 12th **2021**.

6. Hope Faulkner, Tom F. O'Callaghan, Stephen McAuliffe, Deirdre Hennessy, Catherine Stanton, Holly J. **Clarke**, Maurice G. O'Sullivan, Joseph P. Kerry and Kieran .N. Kilcawley. ***'' Effect of Different Forage Types on the Volatile and Sensory Properties of Bovine Milk''.*** European Grassland Federation (EGF), Cork, Ireland, June 17th – 21st **2018.** Reference: Faulkner, H., T. F. O'Callaghan, S. McAuliffe, D. Hennessy, C. Stanton, M. G. O'Sullivan, J. P. Kerry, and K. N. Kilcawley. 2018. *Effect of different forage types on the volatile and sensory properties of bovine milk.* J Dairy Sci. 101(2):1034-1047.

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List of Abbreviations

%	Percentage
°C	Degrees Celsius
µL	Microlitre
α	Alpha
ANOVA	Analysis of variance
AEDA	Aroma Extraction Dilution Analysis
bn	Billion
C	Carbon
CAS	Chemical Abstracts Service
CCD	Composite Rotatable Design
CLV	Clover
cm	Centimetre
Co	Cobalt
Cu	Copper
dH_2O	Distilled Water
DI	Direct Immersive
DM	Dry Matter
FA	Fatty Acid
FFA	Free Fatty Acid
g	Gram
GC-O	Gas-Chromatography Olfactometry
GCMS	Gas Chromatography Mass Spectrometry
GRS	Grass
h	hour
HS-SPME	Headspace Solid Phase Microextraction
I	Iodine
IMF	Infant Milk Formula
IS	Internal Standard
KAA	Kannamycin Aescilin Azide
kg	Kilogram
kV	Kilavolt
L	Litre
LO	Lipid Oxidation

List of Abbreviations

LOD	Limits of Detection
LOQ	Limits of Quantification
LRI	Linear Retention Index
m	meta
M	Molar
Mg	Magnesium
Mg	Milligram
min	Minute
mL	Millilitre
mmol	Millimoles
Mn	Manganese
mol	Mole
MS	Mass Spectrometry
No.	Number
MPCA	Plate Count Skim Milk Agar
Na	Sodium
OI	Odour Intensity
p	Pasteruised
P	Phosphorous
PCA	Principal Component Analysis
pH	Potential Hydrogen
PLS	Partial Least Squares
r	Raw
RB	Rumen blended
rcf	Relative Centrifugal Force
RF	Rumen fluid
RSD	Relative Standard Deviation
RSM	Response Surface Methodology
S/N	Signal-to-noise
Se	Selenium
SIM	Single Ion Monitoring
SMP	Skim Milk Powder
SPE	Solid-Phase Extraction
SPSS	Statistical Package for the Social Sciences
TMR	Total Mixed Ration

List of Abbreviations

UK	United Kingdom
UNC	Unidentified
US	United States
V	Volt
VOC	Volatile Organic Compound
VRBA	Violet Bile Blood Agar
W/v or wt/vol	Weight per Volume
WMP	Whole Milk Powder
Zn	Zinc

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Figure 3.5: Principal component analysis (PCA) biplot demonstrating the correlations between the sensory perception and volatile compounds for infant milk formula (IMF) samples at T4, T8, T12 and T16 of storage. AM: ambient; 21 °C, ACC: accelerated; 37 °C.

Figure 4.1: (A) Principal component analysis (PCA) biplot, representing the correlation structure of the variables (sensory attributes identified by full descriptive analysis and volatile compounds identified by headspace solid-phase microextraction gas chromatography mass spectrometry) and the relationship between the whole milk powder samples derived from grass (GRS) from times (T) 0–6 mo. (B) PCA biplot analysis of the variables and the milk powder samples derived from perennial ryegrass and white clover (CLV) from T0–T6. (C) PCA biplot analysis of the variables and the milk powder samples derived from TMR from T0–T6. Colour gradient: low = white, mid = blue, high = red; midpoint set at 1.0. Flav = flavour; AE = aftereffect. Dim = dimension.

Figure 4.2: Graphs illustrating the increase in painty flavour and hexanal and the decrease in buttery/toffee flavour and aroma in whole milk powders derived from (A) grass (GRS) stored at 21°C and (B) GRS 37°C; (C) clover (CLV) 21°C and (D) CLV 37°C; and (E) TMR 21°C and (F) TMR 37°C during 6 mo of storage (T0–T6). Normalized data were used for the purpose of these graphs.

Figure 4.3: Graphs illustrating the concentrations (mg/kg) of the 13 selected volatile compounds in (A) grass (GRS) whole milk powder (WMP) stored at 21°C during 6 mo of storage (T0–T6); (B) clover (CLV) WMP 21°C; (C) TMR WMP 21°C; (D) GRS WMP 37°C; (E) CLV WMP 37°C; and (F) TMR WMP 37°C.

Figure 5.1: (a) Principal component analysis **(**PCA) biplot showing the correlations between the isoflavones (apigenin, daidzein, formononetin, and genistein) identified in feed samples (grass, grass/clover and TMR) samples and the corresponding raw (r) and pasteurised (p) (GRS, CLV and TMR) milk samples as determined by liquid chromatography tandem mass spectrometry (LC-MSMS); **(b)** PCA biplot showing the correlation of the isoflavones (apigenin, daidzein, formononetin, and genistein) to raw (r) and pasteurised (p) GRS, CLV and TMR milk samples as determined by LC-MSMS.

Figure 5.2: (a) 3D plot demonstrating the effect of storage time (days) on the volatile profile of the rGRS, rCLV and rTMR milk samples; pink - day 3, light blue - day 9 and dark blue - day 14; **(b)** 3D plot demonstrating the effect of feeding system (GRS, CLV and TMR) on the volatile profile of the rGRS, rCLV and rTMR milk samples; pink - GRS, light blue - TMR and dark blue – CLV; **(c)** 3D plot demonstrating the effect of pasteurisation on the volatile profile of the r and p GRS, CLV and TMR milk samples. Grey – rGRS grass, pink – pGRS, orange – rCLV, dark blue – pCLV, light blue – pasteurised TMR and green – raw TMR. p = pasteurised, r = raw. Figure 5.3: PCA Biplot of raw (r) and pasteurised (p) GRS, CLV and TMR milk samples and the top 60 volatile compounds contributing to the differences between the samples, identified by HS-SPME GCMS. Colour gradient; low = white, mid = blue and high = red, midpoint set at 1.0.

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Figure 5.6: Principal component analysis (PCA) Biplot of pasteurised GRS, CLV and TMR milk samples showing correlations between the sensory attributes and the volatile organic compounds.

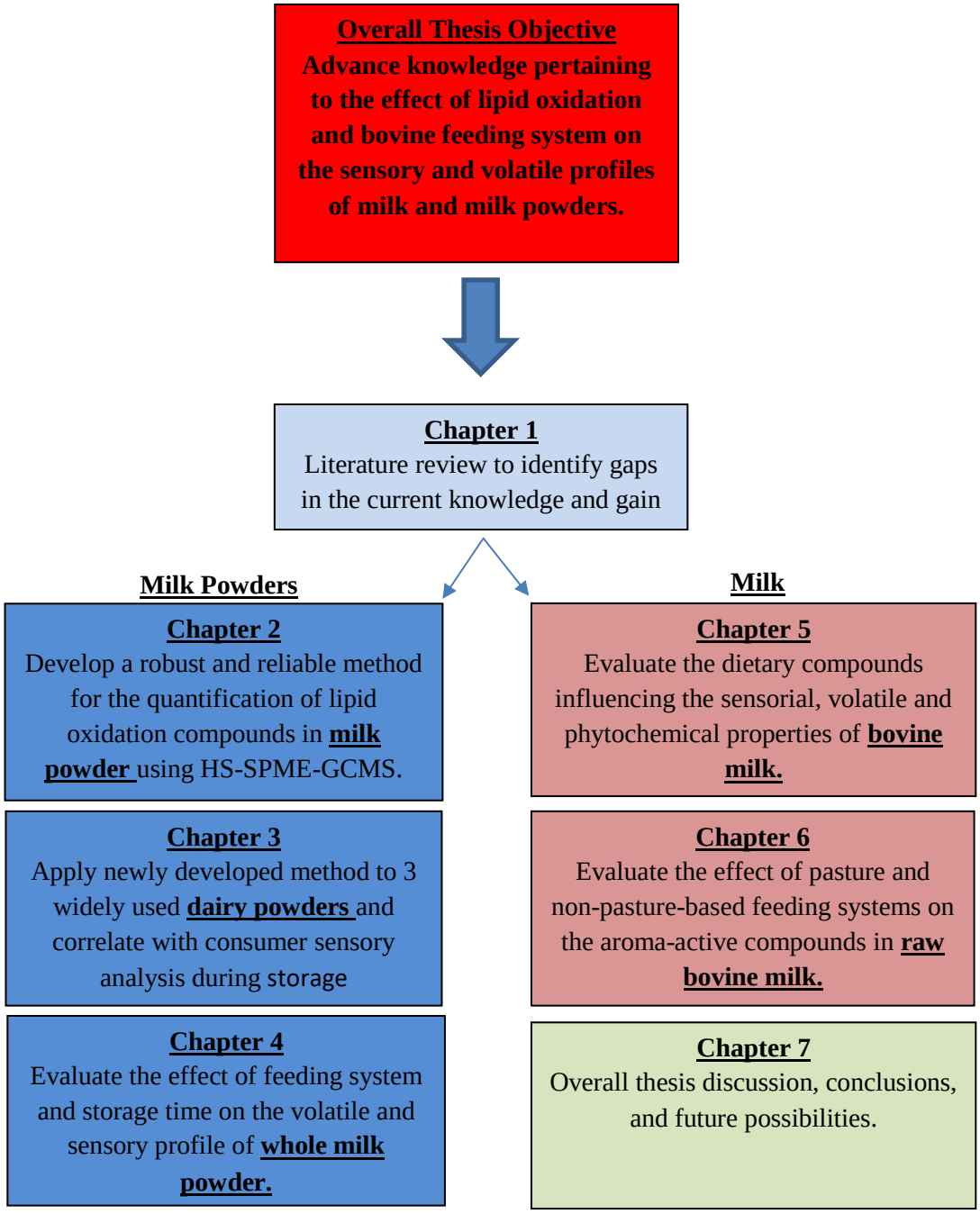
Figure 6.1: Principal component Biplot analysis of the odour descriptors perceived by the 5 panellists based on odour intensity values for raw GRS and TMR milk. Colour gradient: low = white, mid = blue, high = red, midpoint set at 1.0.

Figure 6.2: Principal component analysis (PCA) of the odour intensities perceived by panellists based on odour intensity values for raw GRS and TMR milk. **A**: replicate data (n=10); **B**: averaged data for each panellist (n=5).

Figure 6.3: Bar Chart illustrating the aroma extraction dilution analysis factor dilution values for the 44 volatile organic compounds identified via gas-chromatography olfactometry in raw grass milk (GRS) and raw total mixed ration (TMR) milk; range: 0-100. The higher the FD, the more odour intense the compound.

Thesis Objectives

The relationships between the objectives of individual chapters and the overall objective of this thesis are presented in the figure below.



Abstract

The presented thesis investigates the effect of pasture (cows outdoors on perennial ryegrass [GRS] and cows outdoors on perennial ryegrass/white clover mix [CLV]) and non-pasture based (cow indoors on total mixed ration [TMR]) bovine feeding systems on the sensory and volatile profiles of raw milk, pasteurised milk, and whole milk powder (WMP) produced at Teagasc, Moorepark, Fermoy, Co. Cork. The impact of these different bovine feeding systems on lipid oxidation (LO) was also investigated on WMP, and in comparison to commercially available WMP, skim milk powder (SMP), and infant milk formula (IMF).

This thesis has highlighted the possibility of generating valuable volatile organic compound (VOC) and aroma profiles of milk and milk powders through the use of sophisticated extraction, separation, and identification gas chromatography mass spectrometry techniques (GCMS). This in-depth profiling provides information on the sensory characteristics and shelf-life of the dairy products, following processing or storage. The work in this thesis primarily employed headspace solid phase microextraction GCMS (HS-SPME-GCMS) for VOC profiling, but also the use of a new technique, high capacity sorptive extraction (HiSorb) with polydimethylsiloxane (PDMS) probes and thermal desorption (TD) for gas-chromatography olfactrometry (GC-O) applications.

Chapter 1 provides an updated review of the influencing factors and analysis of LO in dairy powders, focusing on the combination of instrumental and sensory techniques which provides a more complete profile of the dairy products sensorial and volatile characteristics.

A HS-SPME GCMS method was validated in **Chapter 2** to quantify 13 VOC associated with LO which was employed to monitor the stability of commercially available WMP, SMP, and IMF under controlled storage conditions in tandem with sensory analysis in **Chapter 3.** Additional analysis included powder composition, colour, and fatty acid (FA) analysis. Trained assessors (n = 18) carried out acceptance testing (hedonics) and Optimised Descriptive Profiling (ODP) every month for 4 months. The WMP and SMP samples remained stable for up to 4 months, but the IMF had concentrations of LO VOC above their odour thresholds immediately after manufacture, which continued to increase during storage. Hexanal, heptanal and pentanal were correlated with painty, oxidised, cooked, and caramelised odours in all samples. The main

parameters affecting LO of dairy powders in this research were the addition of poly-unsaturated fatty acids (PUFA) prior to thermal processing, and storage conditions.

In **Chapter 4**, 13 VOC originating from LO were again used to track the VOC profile of WMP derived from GRS, CLV, and TMR feeding systems during storage at 25°C and 37°C. Significant variations in the concentrations of 14 FA were observed in WMP based on feeding system. Similar trends in sensory attributes were observed with an increase in painty attributes, corresponding to an increase in hexanal. Buttery/toffee attributes were found to be more closely correlated with TMR WMP. Whole milk powder derived from GRS were more susceptible to LO, particularly in relation to aldehyde development, which is likely due to increased concentrations of conjugated linoleic acid and α-linolenic acid in these samples. WMP from pasture diets was more susceptible to LO based on its volatile profile, as it contained higher concentrations of specific unsaturated FA which were oxidised to aldehydes known to adversely influence aroma perception. Bovine feeding system was shown to impact both LO stability and sensory properties of WMP. WMP from pasture were more closely associated with a dairy sweet sensory attribute. WMP produced from cows fed TMR was whiter than that from cows maintained on pasture diets (GRS and CLV). Buttery/toffee attributes were closely correlated with WMP from TMR, although these eventually became masked over storage as the concentration of LO VOC increased.

Significant differences were also observed in the phytochemical profile of the milk samples derived from pasture and non-pasture-based feeding systems in **Chapter 5**. Pasteurisation significantly altered the VOC profile of all milks from different feeding systems, and resulted in modifying the isoflavone content. Formonoetin was found to be significantly higher in CLV feed samples while daidzein, genistein and apigenin were highly correlated to raw CLV milk samples, likely present as metabolites of other phytochemical compounds. Milk from cows fed TMR scored significantly higher for hay-like flavour and white colour while GRS and CLV milk scored significantly higher for creamy colour due to the β-carotene content of pasture derived dairy products. Overall, milk from both pasture and TMR feeding systems were easily distinguishable by sensory panellists and by their volatile profile. Even though most of the VOC present in the milk were similar regardless of feeding system, their abundances differed significantly.

Chapter 6 demonstrated that the odour active VOC influencing sensory perception were quite different between pasture and non-pasture feeding systems, with

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most VOC influencing the perception of raw milk likely present from direct transfer from feed. Heretofore this was not anticipated, as it was assumed that rumen metabolism was by far the biggest factor influencing the VOC profile of raw milk. Thirty-four and thirty-six aroma-active compounds were identified in raw milk from GRS and TMR, respectively. Many of the key dietary-derived-odour-active VOC in raw TMR milk, likely arose during the production of the TMR as most were either derived from Maillard reactions or impacted by heat. Aromas such as fishy and solvent were observed for certain compounds but attributes such as painty and metallic that are commonly associated with LO were largely absent. Overall, sweet, caramel and toffee notes were more prevalent in raw GRS milk while barnyard, animal, roasted and smokey notes dominated the aroma of raw TMR milk. Identification of odour active VOC in raw milk could be an important factor when milk is being chosen for further processing. This study showed that while the flavour of raw milk is subtle, it is possible to differentiate the aroma of GRS and TMR milks with both analytical techniques and human panellists. The VOC profile of raw milk is impacted by diet and this is a factor that should be taken into consideration post processing as this profile can influence sensory characteristics of products produced from it, especially dairy powders.

This study has conclusively demonstrated the impact of cow diet on the volatile and sensory character of milk but also how this can subsequently impact dairy products such as WMP made from these milks. It also clearly demonstrates significant unique differences in milk and dairy products produced with pasture-based feeding systems as applied in Ireland, as opposed to concentrate-based feeding systems (such as TMR), more widely practiced globally.

The outcomes of this thesis provide insights into: (1) the effect of feeding system (pasture versus non-pasture) on the oxidative stability and sensory perception of milk and milk powders, (2) the aromas associated with certain VOC found in raw milk associated with diet, (3) the effect of LO on the sensory perception of milk powders during storage, and (4) future possibilities and considerations.

Thesis Introduction

The presented thesis investigates the effect bovine feeding system on the composition, sensory, volatile and LO profiles of raw and pasteurised milk, WMP, IMF, and SMP.

Lipid oxidation (LO) is recognised as the main factor in the development of undesirable flavours in dairy products and is responsible for the formation of primary and secondary oxidation products including aldehydes, ketones and alcohols, which impact adversely on sensory perception (Boroski et al. 2012). Factors that contribute to the oxidative stability of dairy powders include the quality of the raw milk, FA composition, bovine diet, processing parameters, powder composition (especially water activity), presence of pro- and anti-oxidants (natural or added during processing), packaging materials, storage and transport conditions. LO is a free radical chain reaction consisting of three stages; initiation, propagation, and termination. Free radicals and peroxides (tasteless, flavourless compounds) (Kochhar, 1996) are generated during the initiation phase when molecular oxygen reacts with unsaturated FA (Kolanowski et al., 2007). The resultant termination products (secondary LO products) are generally quite stable, however, it is these secondary LO products) that actually contribute to off-flavour development (Lloyd et al., 2009a, Li and Wang, 2016). The presence and increase of numerous secondary LO products in dairy powders during processing and storage is well documented (Hall et al., 1985, Lloyd et al., 2009a, Hougaard et al., 2011). However, there is a lack of knowledge linking the quantification of volatiles associated with LO to descriptive sensory attributes in dairy products in general (Kilcawley et al., 2018). This thesis seeks to address this issue by identifying and quantifying secondary LO compounds as molecular-level indicators of oxidised flavours in dairy products instrumentally and to utilise sensory analysis where appropriate in order to obtain better information on the identity and concentration of specific volatiles and problematic sensory attributes.

Whole milk powder (WMP) is an important dairy commodity that is largely produced in countries with an abundant supply of fresh milk and exported to be reconstituted and consumed directly as a nutritious beverage or used in soups and sauces, or in baking and confectionary (Early, 2012). Spray drying enables milk to be easily transported and stored as WMP for extended periods of time. However, the spray drying process facilitates oxidative changes as the high fat content is exposed to elevated temperatures, resulting in potential reduced shelf life due to off-flavour development.

Moreover, WMP can also be subjected to extreme temperature fluctuations during transport and storage, further affecting oxidative stability.

LO is an even greater concern in IMF as this product undergoes extreme processing (Lopez et al., 2015) as its fortified with PUFA, such as linoleic acid (C18:2 n6), α-linolenic (C18:3 n3), arachidonic (C20:4 n6) and docosahexaenoic acid (C22:6 n3) (Tvrzicka et al., 2011), with the aim of simulating human breast milk as closely as possible. These PUFA have very low oxidative stability and are readily degraded to primary and secondary oxidation products (Kilcawley et al., 2018), a process that can be initiated and exacerbated by the high inlet temperatures required for spray drying (120 – 180 °C), and by contact with oxygen (Cesa et al., 2015). Hydroperoxides are the initial products formed by the LO cascade process and are unstable and reactive, eventually forming compounds that are known to cause off-flavours in dairy powders, such as specific aldehydes and ketones (Kilcawley et al., 2018). Thus, having more information on volatile products of LO is important regarding the stability of products throughout their shelf-life.

The effect of bovine feeding system on the composition, volatile, and flavour profile of dairy products is well documented (Chilliard and Ferlay, 2004, O'Callaghan et al., 2016, Faulkner et al., 2018, Wang et al., 2018). However, conflicting results exist on the effect of feeding system on the flavour and abundance of VOC and their impact on the sensory perception of milk and dairy products. Studies suggest that certain VOC in dairy products could prove to be useful metabolic markers in tracing animal diets (Coppa et al., 2011, Kilcawley et al., 2018). Alterations to feeding system have been shown to have effects on milk fat composition, protein content, urea, citrate and soluble calcium (list not exhaustive), which can subsequently influence the oxidative stability and flavour of the milk (Palmquist et al., 1993). VOC in bovine milk consist of a range of different chemical classes including; aldehydes, ketones, lactones, esters, alcohols, acids, terpenes, furans, hydrocarbons, pyrazines, phenolic and sulphur compounds (Bugaud et al., 2001, O'Callaghan et al., 2016, Faulkner et al., 2018, Clarke et al., 2020a). However, their potential impact on sensory perception depends upon their relative concentration and odour activity. Previous studies have reported direct transfer of VOC from bovine feed to milk (Addis et al., 2006, Kilcawley et al., 2018), and that many compounds such phytochemicals in feed may be metabolised in the rumen to contribute to even more volatile odour active compounds in the milk (Bugaud et al., 2001). Evaluating raw milk enables those VOC originating from the bovine feeding system to be more easily

evaluated, as other VOC arising from milk heat treatment or formed during shelf life by microbial activity are not present. Information on the aroma perceptions and intensities of individual VOC in raw milk from different feeding systems may also prove important when selecting raw milk for future applications. For example; further processing to generate commodity dairy products may positively or negatively alter and/or exacerbate specific odours that may impact consumer preference.

Chapter 1: Literature review - Lipid Oxidation of Dairy Powders: Influencing Factors and Analysis

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Review

Oxidative Quality of Dairy Powders: Influencing Factors and Analysis

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Abstract

Lipid oxidation (LO) is a primary cause of quality deterioration in fat-containing dairy powders and is often used as an estimation of a products shelf-life and consumer acceptability. The LO process produces numerous volatile organic compounds (VOC) including aldehydes, ketones and alcohols, which are known to contribute to the development of off-flavours in dairy powders. The main factors influencing the oxidative state of dairy powders and the various analytical techniques used to detect VOC as indicators of LO in dairy powders are outlined. As the ability to identify and quantify specific VOC associated with LO improves this review highlights how these techniques can be used in conjunction with olfactory and sensory analysis to better understand product specific LO processes with the aim of maximizing shelf-life without compromising quality.

Keywords: lipid oxidation; dairy powder; sensory

1.1 Introduction

Oxidation of bovine milk fat is recognised as the main factor in the development of undesirable flavours in products such as whole milk powder (WMP) and infant milk formula (IMF). Lipid oxidation (LO) is responsible for the formation of primary and secondary oxidation products including aldehydes, ketones and alcohols, which can impact on nutritional and sensory properties of dairy powders (Boroski et al. 2012). Factors that can contribute to the oxidative stability of dairy powders include the quality of the raw milk, (fatty acid (FA) composition, bovine diet, and storage conditions), processing parameters, powder composition (especially water activity), presence of pro- and anti-oxidants (natural or added during processing), packaging materials, storage and transport conditions. LO is a free radical chain reaction consisting of three stages; initiation, propagation, and termination. Free radicals and peroxides (tasteless, flavourless compounds) (Kochhar, 1996) are generated during the initiation phase when molecular oxygen reacts with unsaturated FA (Kolanowski et al., 2007). The rate of the propagation cycle is directly proportional to the degree of lipid unsaturation (Kubow, 1992). The resultant termination products (secondary LO products) are generally quite stable, however, it is these secondary LO products (mainly aldehydes, ketones, alcohols, and hydrocarbons) that actually contribute to off-flavour development and have been described as grassy, soapy, cardboard-like, painty, tallowy and/or fishy (Lloyd et al., 2009a, Li and Wang, 2016). Secondary LO compounds can be monitored and quantified instrumentally as molecular-level indicators of oxidised flavours in dairy products. This measurement can be used in place or in combination with sensory analysis to provide an overall profile of the flavour stability of a dairy powder (Romeu-Nadal et al., 2007, Lloyd et al., 2009a). The presence and increase of numerous secondary LO products in dairy powders during processing and storage is well documented (Hall et al., 1985, Lloyd et al., 2009a, Hougaard et al., 2011). However, there is a lack of knowledge linking the quantification of volatiles associated with LO to descriptive sensory attributes in dairy products in general (Kilcawley et al., 2018). The aims of this review are as follows: (1) to summarize the main factors influencing the oxidation of dairy powders, (2) to summarise the various analytical techniques used to detect and quantify VOC as indicators of LO, and (3) to highlight the use of combined analytical and sensory approaches to better understand the LO process in dairy powders.

1.2. Bovine Milk Lipids

Bovine milk fat is one of the most complex fats found in nature and varies widely from animal to animal due to factors including dietary composition (O'Callaghan et al., 2016), breed (Roesch et al., 2005), seasonality (Heck et al., 2009, Larsen et al., 2012), and stage of lactation (Craninx et al., 2008, Palladino et al., 2010). Milk fat contains over 400 different FA (Jensen and Clark, 1988) which originate from diet (Lindmark Månsson, 2008), microbial activity in the rumen (and transported to the secretory cells via the blood and lymph), or from synthesis in the secretory cells. The main milk lipids are triglycerides comprised of a glycerol backbone with three esterified FA. The FA are composed of a hydrocarbon chain and a carboxyl group. The major FA found in milk are: C14:0—myristic (11% *w*/*v*), C16:0—palmitic (26% *w*/*v*), C18:0—stearic (10% *w*/*v*), C18:1—oleic (20% *w*/*v*, and short chain FA (11% *w*/*v*): C4:0—butyric, C6:0—caproic, C8:0—caprylic, and C10:0—capric (Birdi, 2009). A milk fat globule membrane (MFGM) is a surface-active membrane with a phospholipid structure that comprises of a polar lipid bilayer, proteins, enzymes, neutral lipids, and trace components, and envelops each fat globule (Danthine et al., 2000). Approximately 25% of the FA in milk are mono-unsaturated FA (MUFA) while 2.3% are poly-unsaturated with an omega-6/omega-3 ratio of around 2:3. Trans-FA comprise approximately 2.7% of total milk FA. The amount of poly-unsaturated FA (PUFA) consumed by ruminants is an important factor in the rate at which LO progresses because they are generally dehydrogenated in the rumen by microbial action and this impacts on subsequent levels in the milk. The most abundant FA in bovine milk is α-linolenic acid (C18:3) (Markiewicz-Kęszycka et al., 2013). MUFA are not oxidised as readily as PUFA, however, the most abundant MUFA (oleic acid) in bovine milk is the source of important secondary oxidation products (Kilcawley et al., 2018). Saturated FA in milk are generally stable compounds that are not easily oxidised and thus are not major LO contributors in dairy powders. The abundance of individual FA in milk and milk products is important (Romeu-Nadal et al., 2004) as they can dictate the rate at which LO progresses, but also which specific oxidation products are formed.

1.3 Mechanism of Lipid Oxidation

It is generally accepted that oxygen reacts naturally with many organic substrates resulting in the formation of primary oxidation products; hydroperoxides and other oxygenated compounds. There are three known types of LO that can affect dairy products; auto-oxidation, photo-oxidation, and metal induced oxidation (Gutierrez, 2014).

The mechanism of the auto-oxidation of PUFA as a radical chain reaction was established more than half a century ago. The process of LO can be broken into three distinct, but partially overlapping phases of radical reactions; initiation, propagation and termination (Shahidi and Zhong, 2005) (Figures 1.1–1.3). Free radicals and peroxides, both of which are highly reactive, are generated during the initiation phase when molecular oxygen reacts with unsaturated FA. In addition to oxygen, oxidative initiators such as chemical oxidisers, transition metals (e.g., copper and iron), and enzymes (e.g., lipoxygenases) contribute to the rate of the initiation phase (Kolanowski et al., 2007). Heat and light also exacerbate the rate of the initiation phase and the other phases of LO (Frankel, 1998). The rate of auto-oxidation is increased by increasing unsaturation of the alkyl chain (Frankel, 1998), and the matrix also plays a role in the susceptibility of a product to oxidation (Miyashita, 2002). FA alkyl chains are susceptible to oxidation at alkene bonds and neighbouring allylic carbons.

Photo-oxidation and free-radical reactions at allylic carbons are responsible for the breakdown of unsaturated lipids (Rawls and Van Santen, 1970, Frankel, 1998, Shahidi and Zhong, 2005). These reactions produce hydroperoxides in these allylic bonds, and cause changes in the position and geometry of double bonds. Auto-oxidation and photo-oxidation are associated with different hydroperoxide reaction products, indicating that different reaction mechanisms are involved (Simkovsky and Ecker, 1998). Photo-oxidation of milk has been well documented (Wishner, 1964, Gutierrez, 2014), exposure to light, either natural or artificial, can cause development of off-flavours in milk within 15 min (Brothersen et al., 2016). The subsequent aromas have been characterised as burnt protein, cabbage-like and plastic (Gutierrez, 2014), however, their intensity can decrease the longer the milk is exposed to light, allowing newly activated off-flavours to dominate. These off-flavours have been described as cardboard-like, metallic and rancid (Stull, 1953, Jung et al., 1998, Hedegaard et al., 2006). Exposure to ultra-violet (UV) light can enable the oxidation of fat to volatile aldehyde compounds and has also been found to cause the degradation of sulfur–containing compounds, both of which are major contributors to off-flavours in milk (Brothersen et al., 2016). A study by Silcock et al. (2014) reported good correlation between negative sensory perceptions and VOC formation for milk stored in light-exposed containers, these include photo-oxidation and auto-oxidation compounds such as dimethyl disulfide, and aldehydes such as heptanal, pentanal and hexanal. For milk stored in containers protected from light, no correlation between the sensory attributes and VOC was documented.

Furthermore, the type of light the product is exposed too can also have an impact on the levels of oxidation. A study by Brothersen et al. (2016) demonstrated that exposure of milk

to fluorescent light (commonly used in the retail of dairy products) resulted in greater changes in LO levels, compared with exposure to white light-emitting diodes (LED). This study demonstrated that even high quality milk is susceptible to photo-oxidation at the point of sale dependent upon the type of lighting.

There are two mechanisms by which photo-oxidation may occur; (1) a radical cascade reaction initiated by the removal of a hydrogen or electron from an unsaturated allylic FA system, or (2) when oxygen is converted to its excited singlet state and reacts rapidly with an olefinic bond producing hydroperoxides on one of the original olefinic carbons and shifting of the cis bond to a trans configuration (Figure 1.4).

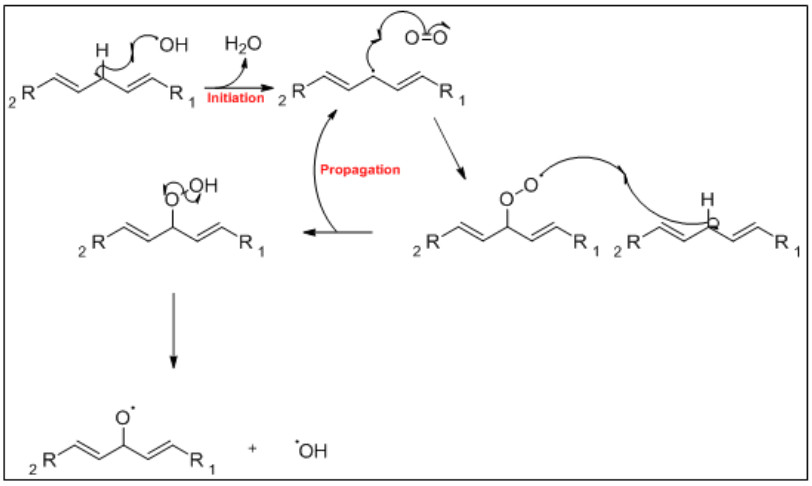


Figure 1.1: The mechanism of the first two phases of the lipid oxidation process; initiation and propagation.

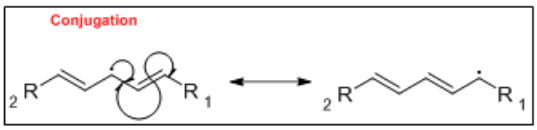


Figure 1.2: The mechanism of conjugation of the lipid oxidation process.

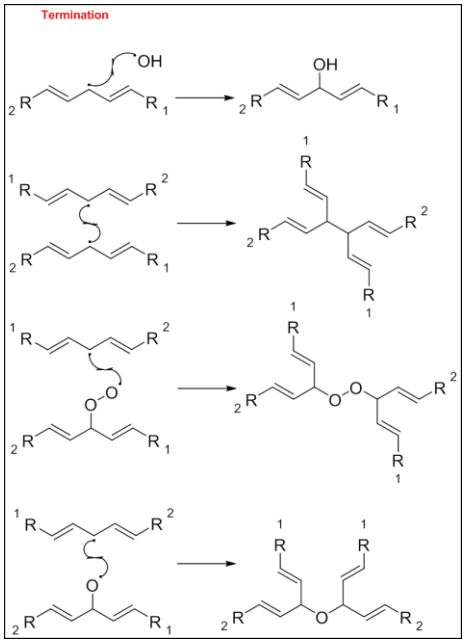


Figure 1.3: Common termination products of the lipid oxidation process.

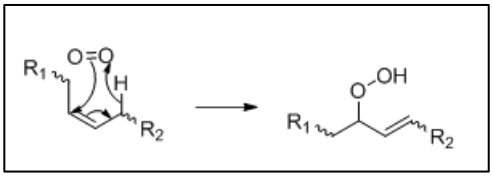


Figure 1.4: Ene reaction of an olefinic bond with a singlet oxygen. The formation of the hydroperoxide can happen at either of the olefinic sp2 hybridised carbons.

1.4 Secondary Reactions Associated with Lipid Oxidation

Various FA within milk are broken down via oxidation to primary and secondary oxidation products. The formation of a hydroxyperoxide through the oxidative mechanisms discussed earlier, breaks down to form an alkoxyl radical which splits by homolytic β-scission each side of the carbon bonded to the oxygen radical. The major FA in milk and some of their associated breakdown products are outlined in Figure 1.5a–e.

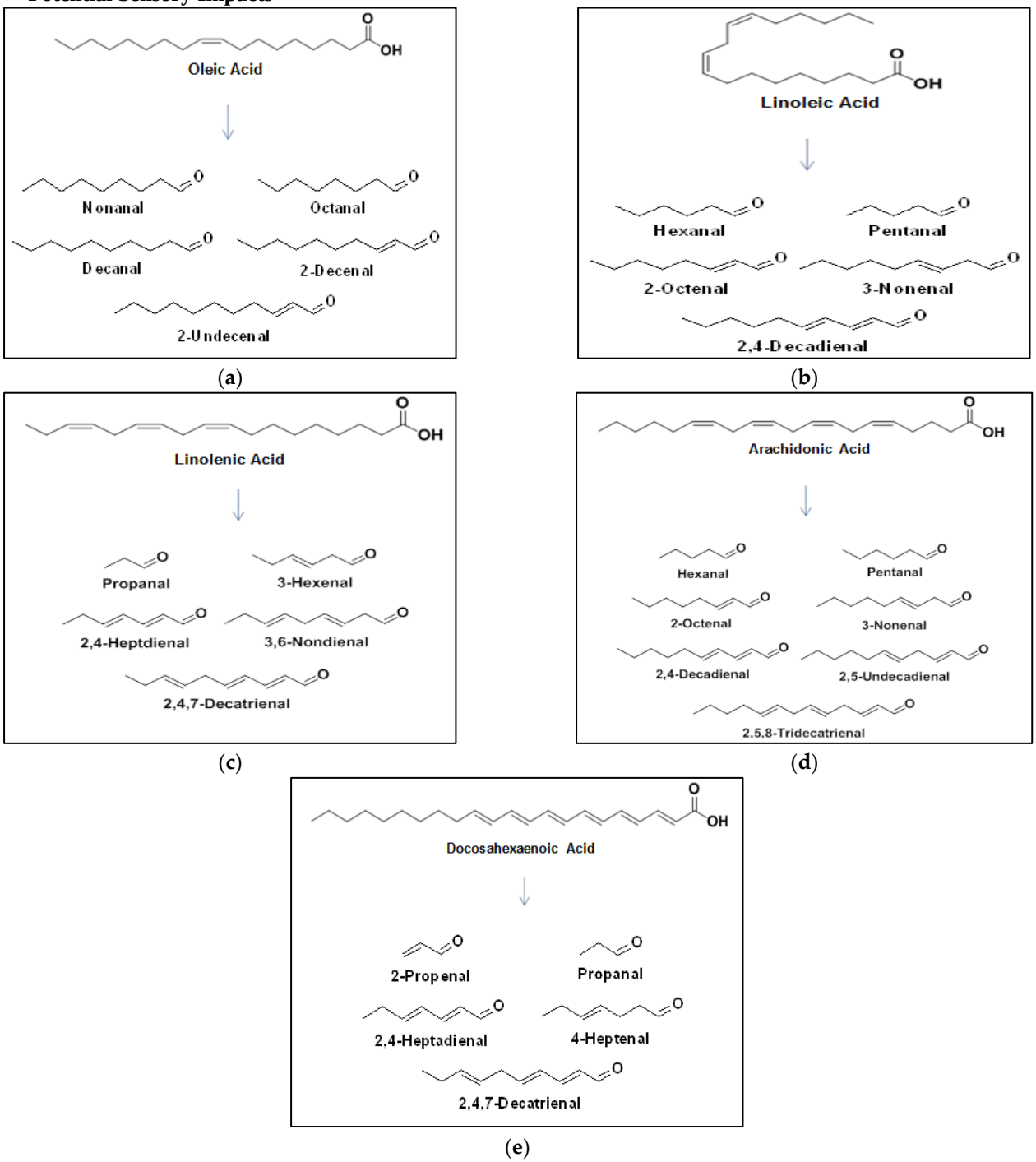


Figure 1.5: Some major fatty acids found in milk and some of their associated breakdown products; (a) oleic acid, (b) linoleic acid, (c) linolenic acid, (d) arachidonic acid, and (e) docosahexaenoic acid.

1.4.1. The Maillard Reaction

Along with LO, the Maillard reaction is an important chemical reaction that occurs in numerous foods, and both reactions have been shown to influence each other (Zamora and Hidalgo, 2005). The Maillard reaction is a well-documented, non-enzymatic browning

reaction between the amine groups of free amino acids, peptides or proteins and reactive carbonyl groups of reducing sugars under thermal processing and/or storage conditions (Chen and Kitts, 2012). This reaction can occur at room temperature, but is optimal at much higher temperatures (140–165 °C). The Maillard reaction has been identified as a main factor in quality deterioration of IMF (Nunes et al., 2019). However, in whey protein concentrate (WPC) and whey protein isolate (WPI), Maillard reaction products contribute to a lesser extent to flavour formation than LO (Whetstine et al., 2005, Tunick et al., 2016). The moisture content must be below 3% *w*/*w* for the Maillard reaction to conclude, a value that is not reached in most dried dairy products (Sienkiewicz and Riedel, 1990). The Maillard reaction mechanism is outlined in Figure 1.6.

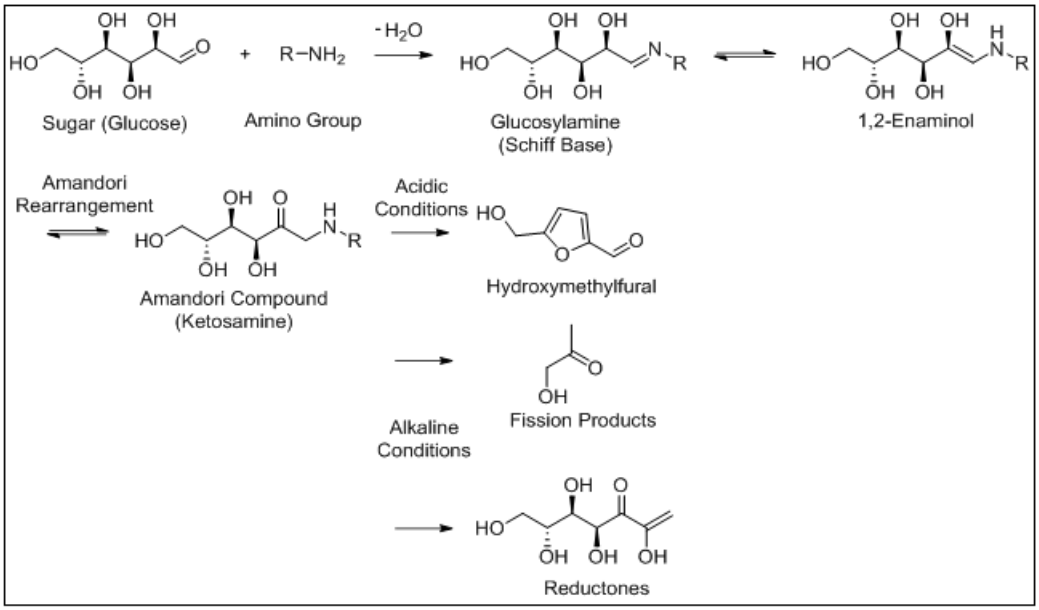


Figure 1.6: Schematic of the Maillard reaction of glucose with a generic amino group RNH2. The carbonyl functional group on the sugar undergoes a substitution reaction with the amino group of a protein or amino acid to form an N-substituted glycosylamine. This undergoes isomerisation by undergoing an Amandori rearrangement forming a ketosamine. This can undergo a number of reactions to produce a range of compounds which can undergo further reactions.

1.4.2. The Strecker Reaction

Similar to the Maillard reaction, the Strecker reaction mechanism is also linked to LO. Aldehydes are readily converted to secondary alcohols or acids and are therefore known as transitory volatile compounds with some known to be a result of Strecker reactions (Atasoy et al., 2013, Kondyli et al., 2013). The degradation of amino acids during the Strecker reaction is one of the primary mechanisms resulting in the final aroma compounds of the Maillard reaction. The process involves the oxidative deamination and decarboxylation of the amino

acid in the presence of α-dicarbonyl compounds formed in the Maillard reaction and the formation of the corresponding Strecker aldehyde (Mottram, 1998, Estévez et al., 2011). Each amino acid produces a specific Strecker aldehyde which comprises one carbon atom less than the amino acid from which it is formed. Strecker aldehydes such as 3-methylbutanal (malty flavour) (Zhou et al., 2002) and phenylacetaldehyde (honey-like flavour) are derived from leucine and phenylalanine, respectively, and are commonly reported as aroma contributors in dairy products (Delgado et al., 2010). LO and Maillard reactions interact in complex food systems and can share common chemical mechanisms and intermediate compounds (Zamora and Hidalgo, 2005). Moreover, certain carbonyls derived from LO such as alkadienals and ketodienes have been shown to promote the oxidative degradation of amino acids to produce the corresponding Strecker aldehydes via Strecker-type reactions (Zamora et al., 2007, 2008). The Strecker reaction is outlined in Figure 1.7.

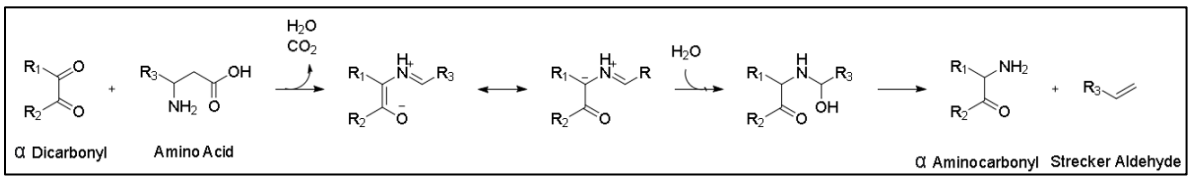


Figure 1.7: Schematic of the Strecker degradation mechanism, where an α-dicarbonyl compound and an amino acid undergo a decarboxylation reaction to form an α-aminocarbonyl compound and Strecker aldehyde product.

1.5. Lipid Oxidation in Dairy Powders

1.5.1. Whole Milk Powder

Due to its high fat content (26–42% *w*/*w*) and significant amount of exposed fat on its surface, WMP (Lloyd et al., 2009a) is highly susceptible to LO during processing, transport and storage, which can adversely impact its sensory and nutritional properties. Some odour-active compounds have already been identified in WMP (Whetstine and Drake, 2007), with the most important off-flavour compounds resulting from LO such as, hexanal, other aldehydes and ketones. Determining the cause of undesirable LO changes in WMP flavour is complex owing to the fact that many of the aroma-active compounds are produced by two or more mechanisms (Whetstine and Drake, 2007, Cadwallader and Singh, 2009, Lloyd et al., 2009a). Moreover, differences in the FA profile of WMP influences its susceptibility to LO (Clarke et al., 2020b, Clarke et al., 2021). Bovine feeding system is one of the major factors affecting the FA profile of milk and milk powders (Palmquist et al., 1993, O'Callaghan et al.,

2016). Maintaining quality and potentially increasing the storage stability and shelf-life of WMP is of great importance to manufacturers. Whetstine and Drake (2007) documented changes in the flavour of WMP at ambient temperatures and found that the formation of off-flavours occurred after 3 months of storage, primarily as a result of LO. It is also important to note that off-flavours in dairy powders can carry over into product applications (Whetstine and Drake, 2007), therefore having information in relation to the LO status of the starting powder is very important not only for the oxidative stability of the powder itself but also for future applications.

1.5.2. Skim Milk Powder

Few studies have focused on the impact of LO on the quality and stability of SMP, likely due to its low fat content (0.6–1.25% *w*/*w*). SMP should have a flavour similar to that of fluid milk (Caudle et al., 2005); however, differences in manufacturing processes (Banavara et al., 2003, Wolf et al., 2013) and milk composition (Abdalla et al., 2017) can result in the formation of different flavour characteristics and intensities. Shiratsuchi et al. (1994) was one of the first studies to profile the VOC content and flavour of SMP. The compounds identified included aldehydes, ketones, alcohols, esters, furans and, phenolic compounds, and was also one of the first studies to identify monoterpene and sesquiterpene hydrocarbons in milk. Methyl ketones were abundant in SMP, but were below their flavour thresholds. Alcohols were also found to have little influence on SMP flavour. The primary contributors to the flavour of SMP were free FA, comprising approximately 79% of the total VOC profile, however lactones were also present at high concentrations.

Abdalla et al. (2017) evaluated the sensory characteristics of nonfat dry milk (NFDM) and SMP, and found that the intensity of the heat treatment used during production influenced the flavour of the final product, with medium heat powders having a cooked flavour, and low heat powders having oxidised and metallic flavours. Heat treatment can exacerbate the intensities of undesirable flavours in SMP. Whetstine and Drake (2007) found that the impact of LO on the flavour of SMP was much more variable than with WMP. The authors also found that some SMP developed off-flavours immediately upon storage, while the flavour of others remained stable throughout storage. These results are somewhat surprising as it was anticipated that SMP should be less susceptible to LO than WMP due to its much lower fat content. However, the fat in WMP and in other high-fat dairy powders may act as a solvent for secondary oxidation products, especially non-polar molecules, impacting their transition to the gaseous phase. Thus, the low fat content in SMP may result in non-polar oxidative

products being more easily perceived (Frøst and Janhøj, 2007), as in theory they should be more easily transferred from the fat phase to the gaseous phase (or to the aqueous phase when hydrated).

1.5.3. Infant Milk Formula

The fat content of IMF is approximately 28% *w*/*w* and is designed to contain a FA composition similar to that of human milk. This is generally attained through the addition of fish, soya and/or vegetable oils (Lopez et al., 2015). Increased levels of PUFA from these sources may however result in an unstable product that is highly susceptible to LO (Kilcawley et al., 2018). For this reason, understanding the modifications of PUFA in IMF is important with regard to the stability and safety of IMF throughout its proposed shelf-life. A review by Saphier and Silberstein (2014) focused on the storage conditions of IMF and the levels of LO. The study concluded that IMF comprising more unsaturated FA was more susceptible to LO, and that exposing IMF to known LO contributors (oxygen and elevated temperatures of >37 °C) increased the rate of LO.

A study by Romeu-Nadal et al. (2007) focused on the oxidative stability of milk formulas (packed in sealed aluminium foil bags flushed with N_2) that had been supplemented with various FA and stored at 25 °C and 37 °C. The study employed the use of headspace solid phase micro-extraction (HS-SPME) gas chromatography mass spectrometry (GC-MS), and sensory analysis to track important volatile markers of LO over 15 months of storage. Propanal was used to monitor oxidative changes in *n*–3 PUFA, with hexanal and pentanal used to monitor changes in powders fortified with *n*–6 PUFA. Samples stored at 37 °C were found to be less stable than those stored at 25 °C, confirming that storage temperature effects the rate of LO in IMF. Rancid off-flavour was not detected in samples stored at 25 °C until after 15 months. The combination of sensory and volatile analysis provided beneficial information on the oxidative stability of the formulations and concluded that the shelf-life of IMF is dependent on the PUFA content, storage temperature and time.

A study by Clarke et al. (2020b) found that LO aldehydes and ketones were excessively high in IMF in comparison to WMP and SMP. Overall, painty, oxidised, and rancid attributes were more associated with IMF regardless of storage temperature (21 °C or 37 °C). However, to date very few studies have been undertaken on the impact of LO on the volatile and sensory profiles of IMF. Cesa et al. (2015) investigated the effect of storage conditions (20, 28, 40 and 55 °C) on the levels of malondialdehyde (MDA) in IMF and found that the PUFA enriched IMF samples demonstrated good stability at; 20 °C for up to 1 year, 40 °C for up to 3 months,

and 55 °C for up to two weeks.. A study by Jia et al. (2019) investigated the stability of milk based IMF supplemented with PUFA stored at 42 °C and 50 °C for 90 days. The authors found significant differences in colour, possibly due to the Maillard reaction in addition to significant differences in the VOC profile, peroxide value (POV) and headspace oxygen over the storage period.

Most IMF LO studies have only monitored the concentrations of MDA or the POV, with very little research focused on individual VOC associated with LO. Investigating the levels of primary and secondary oxidation products (aldehydes, ketones and alcohols) in IMF in conjunction with sensory analysis will provide more information on the impact of supplementation with PUFA on the rate of LO during storage, the specific VOC involved, and in theory the concentration at which individual VOC begin to adversely influence sensory perception.

1.5.4. Whey Protein Concentrate and Whey Protein Isolate

Whey is used as an ingredient in many food products and generally fractionated to yield products with different compositions and functionalities (Morr and Ha, 1993). WPC has a low fat content of between 3–6.6% *w*/*w*, with negligible amounts of saturated FA, PUFA, and MUFA (USAID, 2016). As such, LO is not considered a major issue and therefore only limited LO studies of WPC exist.

Tomaino et al. (2004) suggested that the starter culture used during cheese production can initiate the oxidation process, which influences the flavour and oxidative stability of liquid whey, ultimately, affecting the characteristics of whey powder. Compared with the control, starter cultures were found to have contributed to the production of acetaldehyde, ethanol, diacetyl, 1-propanol, and 2-propanol. Further results suggested that LO was initiated during the production of the liquid whey and was accelerated during 14 days of refrigerated storage. (USAID, 2016).

Jensen et al. (2012) studied oxidation in WPC (6.5% *w*/*w* fat) and in whey fat concentrate (WFC) for 12 months at 20 °C. WFC is the remaining fraction of WPC after WPI is removed and has a fat content of 13.5–21.5% *w*/*w*. The study evaluated the primary oxidation products for hydroperoxides, electron spin resonance (ESR) for radicals, secondary volatile LO products by HS-SPME GC-MS, and some protein oxidation products (dimethyl disulfide, benzaldehyde, and dityrosine) by reverse-phase high performance liquid chromatography. WFC was found to be more susceptible to oxidation than WPC, as dimethyl disulfide,

benzaldehyde, dityrosine, heptanal, nonenal, hexanal, and radicals were significantly higher, likely due to the higher fat content of WFC.

WPI is produced from WPC and contains minimal fat. Berton-Carabin et al. (2016) investigated LO in conjunction with protein oxidation as this is seen as a more prevalent issue in WPI owing to its very high protein content (≥90% *w*/*w*). The authors used controlled levels of LO and protein oxidation to investigate the impacts on the viscoelasticity of whey protein layers at the oil-water interface. Results demonstrated that both protein oxidation and LO led to a decrease in interfacial elasticity when compared to the samples that were not oxidised. LO induced the formation of surface active compounds, which were thought to have formed segregated domains at the interface. Limited studies of LO in WPI suggest that it is not a major issue.

Wright et al. (2009) investigated the sensory and volatile stability of WPC80 and WPI stored in polyethylene lidded bins at 21 °C, at 50% relative humidity for 18 months. Sensory properties were evaluated using the descriptive spectrum method while HS-SPME GC-MS was employed to extract and characterise VOC. Differences in the sensory profiles were documented between WPC80 and WPI, in agreement with previous studies on the topic (Whetstine et al., 2005, Russell et al., 2006). Sixteen VOC were quantified in WPC80 and WPI. The authors chose these VOC as they represented a range of Maillard reaction, LO, or fermentation derived volatiles that were consistently detected in 3 or more whey products. Hexanal was found to be the most abundant compound identified in fresh WPC80, followed by trans-2-nonenal. The study concluded that the flavour of both WPC80 and WPI changed during storage, with increases in the abundance of VOC. The optimum shelf-life for non-agglomerated WPC80 and WPI stored at 21 °C was 12 to 15 months, and 8 to 12 months for steam-agglomerated or lecithin-agglomerated WPC80 and WPI.

1.6 Main Factors Influencing Lipid Oxidation in Dairy Powders

Numerous studies have evaluated the effect of feeding system on the flavour and abundance of VOC in dairy products and bovine diet has been proven to be one of the most significant influencers of VOC and FA profiles of dairy products (Coppa et al., 2011, O'Callaghan et al., 2016, O'Callaghan et al., 2017, Cheng et al., 2020, Clarke et al., 2020a). Milk fat composition can be readily modified by changing a cows' feeding regimen, but this alteration impacts the protein, urea, citrate and the soluble calcium present in the milk (list not exhaustive). Only a limited number of studies have focused on the effect of feeding system on the sensory, flavour and flavour stability of dairy powders (Jia et al., 2019, Clarke et al.,

2020b). Bovine dietary changes that affect the FA profile of milk are also likely to influence its oxidative stability and flavour (Palmquist et al., 1993), but also the stability of resultant powders. Moreover, milk quality and composition are important aspects to consider when producing products such as WMP, SMP and IMF.

The review by Chilliard et al. (2001) summarised the effects of bovine forage on milk fat secretion and composition. The study highlighted the need to evaluate how different feeding systems impact on aspects of milk fat quality, such as flavour, oxidative stability and manufacturing value.

Studies investigating the composition of milk produced from many supplemented and altered diets including supplementation with: flaxseed (Caroprese et al., 2017), lipid complex (grapeseed oil with synthesised conjugated linoleic acid (CLA) and Atlantic mackerel oil enriched with *n*-3 FA) (Bodkowski et al., 2016), iodine (Schöne et al., 2017) marine algae (Glover et al., 2012), oregano and caraway essential oils (Lejonklev et al., 2016), hull-less barley (Yang et al., 2017) and sunflower/fish oil (AbuGhazaleh and Holmes, 2007) have been undertaken. These studies focused mainly on production performance, milk composition, milk yield, FA composition and to a lesser extent, on flavour and sensory characteristics of the raw, pasteurised or homogenised milk. Volatile analysis was included in most studies but very few combined this with sensory analysis or attempted to correlate both data streams.

Villeneuve et al. (2013) investigated 3 types of feeding system (timothy hay, pasture, and silage) and found that untrained sensory panel members could not distinguish a flavour difference between the milk produced from the cows fed hay and the cows fed silage. However, a significant number of panellists could detect a difference between milk from hay-fed cows in comparison to milk from pasture-fed cows. A study by Faulkner et al. (2018) demonstrated that feeding system can influence the sensory properties of bovine milk. The flavour compounds from forage can be transferred to milk from the cow through two pathways; by inhalation or digestion, and also through the rumen gases (Toso et al., 2002). VOC can also be ingested by the animal and encapsulated in the fat or protein portion of the milk. Pasture based feeding has been attributed to increased herbaceous flavours in milk (Faulkner et al., 2018).

A study by Vanbergue et al. (2017) investigated the effect of breed (Holstein and Normande), feeding system (high and low energy), and stage of lactation (early, mid and late) on milk fat characteristics in dairy cows. No significant interaction was observed between breed and feeding system. Milk yields were higher for Holstein cows compared with Normande cows throughout lactation and were significantly higher in cows that consumed the

high-energy diet. In general, fat content was higher for Holstein cows, but saturated FA were higher in Normande cows and MUFA were higher for Holstein cows. Feeding system had no significant effect on saturated FA content except during early lactation where levels were higher in milk from cows fed the high-energy diet. MUFA and PUFA contents were higher in milk from cows fed grass silage (low energy) vs. corn silage (high energy) during early lactation The study clearly demonstrated the effect of cow breed and feeding system on milk fat characteristics.

It is clear that more comprehensive research is required to establish definitive links between bovine diet and the sensory attributes of subsequent milk and dairy powders, where noticeable changes in VOC and FA profile occur. As well as feeding regime, many other factors influence the sensory attributes of milk and dairy products.

1.7 Impact of Processing Conditions on Dairy Powders

There are several factors involved in milk processing which can affect the stability of the resulting dairy powder and its subsequent sensory characteristics, including preheat treatment (Baldwin and Ackland, 1991, Stapelfeldt et al., 1997, Oldfield et al., 2005, Li et al., 2012), and the distribution of fat in the dried powder particles (Park and Drake, 2014).

Baldwin and Ackland (1991) studied the effect of 4 preheat treatments (85, 95, 110, and 125 °C) each in combination with 4 holding times (10, 20, 60, and 240 s) on the aroma and flavour characteristics of WMP stored in an air atmosphere at 30 °C for 18 months. Nine sensory characteristics incorporating flavour, aroma, and texture were evaluated by 16 trained panellists throughout storage. Some of the primary sensory attributes associated with WMP were significantly affected by preheat temperature and holding time. Cooked flavour was significantly increased by longer preheat holding time and a higher preheat temperature from 85 °C to 125 °C. Sweetness was higher when longer holding times and low preheat temperature were applied, and at shorter holding times at high preheat temperatures. Oxidised flavour was significantly affected by preheat temperature and holding time. WMP manufactured using short holding times and low temperatures exhibited weak oxidised flavour compared to WMP produced using high heat treatments and longer holding times. Oxidised flavour correlated well with oxidised aroma and was perceivable by panellists after 9 months of storage. The study concluded that preheat temperatures of 95 °C or greater and holding times of 20 s or greater are considered effective in inducing stability against oxidative deterioration. This finding was in agreement with that of Abdalla et al. (2017) for NFDM and SMP.

Li et al. (2012) evaluated the oxidative stability of milk powders throughout 3 and 6 months of storage at 20 ± 1 °C. Milk powders were stored in plastic bags and stored in a dry, air-tight container. In addition to HS-SPME, POV was used to evaluate the oxidative stability of the milk powders. Milk powders and concentrated milks had higher POV than raw and heated milks. The study also found increased levels of aldehydes and ketones in stored milk powders when the concentration temperature was 40 °C as opposed to 50 °C. Results also demonstrated that aldehyde and ketone levels in fluid milk, both raw and heated, were lower compared to levels found in concentrated milk and milk powders. The increased number of processing steps involved in milk powder production, such as preheating and spray drying were thought to be the cause of this increase. The temperature of the preheat treatment is important for regulating the technological characteristics of the final product (Oldfield et al., 2005).

Stapelfeldt et al. (1997) demonstrated that the shelf-life of WMP depends on the preheat treatment of the milk, the temperature at which the WMP is stored and the water activity of the powder. The study compared the storage stability of low-heat, medium-heat, and high-heat milk powders at three water activity values (0.11 a_w, 0.23/0.17 a_w, and 0.33/0.31 a_w), and at two storage temperatures (25 °C and 45 °C). The freshly manufactured milk powder was packed in 400 g cans under a 70% N_2, 30% CO_2 gas mixture. The low-heat milk powder (milk pasteurised at 73 °C for 20 s followed by a preheat treatment of 72 °C) had the lowest storage stability as it was subject to severe oxidative changes and non-enzymatic-browning. However, during accelerated storage at 45 °C, the medium-heat (milk pasteurised at 80 °C for 20 s followed by preheat treatment of 72 °C), and high-heat powders (milk pasteurised 88 °C for 20 s followed by preheat treatment of 72 °C) were less susceptible to oxidative changes and enzymatic browning. In the study, thiobarbituric acid reactive substances (TBARS) was used as a measure of sensory quality and values increased to a greater extent in powders stored at 45 °C than at 25 °C.

Park et al. (2016) explored the effect of homogenisation pressure on the flavour, and flavour stability of WMP. The sensory properties of the powders were evaluated at 0, 3 and 6 months of storage at 21 °C by descriptive analysis using the spectrum method (Drake et al., 2003). The study reported the flavour profiles of WMP produced by various homogenisation treatments were distinct, and that improper or inadequate homogenisation adversely affected shelf-life and flavour stability.

Monitoring the temperature and conditions during the drying of milk is crucial to the overall quality and sensory stability of the end product. Other parameters such as particle size and microbiological stability must also be considered (Gharsallaoui et al., 2007).

1.8 Volatile Organic Compounds Associated with Lipid Oxidation in Milk

VOC are a diverse group of carbon-based chemicals with boiling points ranging from 50 to 260 °C (WHO, 1989). VOC including aldehydes, ketones, alcohols, and ethyl esters represent the primary aromatic constituents of milk. VOC are significant as their quantitative differences can explain the different odours that characterise milk and dairy powders (Moio et al., 1993a, Cadwallader and Singh, 2009). The concentrations of individual VOC in fluid milk are known to affect its sensory properties (Faulkner et al., 2018). GC is capable of identifying >109 molecules of an odour in 1 mL of air, but the human nose has been found to be 10–100 times more sensitive (Ross, 2009). Therefore, VOC analysis in combination with GC-olfactometry (GC-O) can provide more useful information about which VOC influence sensory perception and the degree of their influence. Although GC-O has some limitations it remains a very useful technique; (1) it can be difficult to identify every odour, as some remain below the limits of detection of the GC-MS, (2) co-elution makes it more difficult to obtain dependable data on those VOC, (3) odours created through interactive effects of two or more VOC cannot be taken into account as the VOC are largely detected as individual compounds, and (4) it is very time consuming and requires extensive panellist training. In addition, some VOC found in dairy products have more than one odour descriptor that may also be dependent upon their concentration as well as the composition of the product (Kilcawley et al., 2018). Although the human nose is very sensitive it also has limitations, such as the 'opinion factor' of panellists, lack of standards and reproducibility due to differences in capability either related to physical, genetic or health issues (Tunick, 2014). A review by Kilcawley et al. (2018) summarises the potentially important compounds in bovine milk and their associated aroma descriptors (Table 1.1).

In addition to LO, Maillard, and Strecker reaction products, other documented sources of off-flavours in dairy products include the presence of microbial-derived terpenoid compounds, such as endo-borneol, 2-methylisoborneol and α-terpineol (Potts and Peterson, 2018), sulfur compounds present as a result of heat treatment (Vazquez-Landaverde et al., 2006, Al-Attabi et al., 2014), or direct transfer from feed and possibly isoflavone metabolism in the rumen leading to the formation of aromatic phenolic compounds (Kilic and Lindsay, 2005, Clarke et al., 2020a). Thus, incorporating GC-O analysis when attempting to identify the source of off-flavours in milk can be extremely beneficial.

Table 1.1. Some potentially important volatiles and their associated aroma descriptors found in dairy products (derived from lipid oxidation).

Compound	Associated Aroma Descriptors	LRI	Odour Reference	LRI Reference
Aldehyde				
Pentanal	Fermented, bready, fruity	735	*	(Clarke et al., 2020a)
Propanal	Alcohol, earthy	506	*	
Hexanal	Cardboard like, metallic off flavour, green	837	*	
(E)-2-Nonenal	Green, fatty	1160	*	(Marsili, 2001)
Heptanal	Fatty, oily, green, woody	901	*	(Clarke et al., 2020a)
(Z)-4-Heptenal	Oily, fatty, green, milky, dairy	901	*	(Zhao et al., 2015)
2,4- Decadienal	Fatty, oily, green, chicken skin-like, fried	1300	*	(Wang et al., 2020)
Undecanal	Soapy, aldehydic, waxy, floral	1311	*	(Xu et al., 2017a)
Ketone				
Acetone	Earthy, strong fruity, wood pulp, hay	532	(Fox et al., 2017)	(Clarke et al., 2020a)
2-Nonanone	Malty, fruity, hot milk, smoked cheese	1092		(Rouseff and Cadwallader, 2001)
2-Heptanone	Blue cheese, spicy, Roquefort cheese	890		
2-Pentanone	Orange peel, sweet, fruity	727		(Clarke et al., 2020a)
3-Octen-2-one	Earth, oily, ketonic, sweet, hay, mushroom-like	1096	*	(Clarke et al., 2020b)
2,3-Octanedione	Dill, herbal, buttery	981	*	(Serrano et al., 2011)
1-Octen-3-one	Metallic, mushroom-like	1294	*	(Rothe, 1997)
3,5-Octadien-2-one	Mushroom-like, fatty	1030	*	(Clarke et al., 2020a)
Alcohol				
1-Heptanol	Sweet, green, woody	972	*	(Clarke et al., 2020a)
1-Octanol	Waxy, green, citrus, floral, sweet, fatty, coconut	1116	*	
1-Pentanol	Fermented, sweet, balsam, yeasty, solvent-like	794	*	
1-Hexanol	Green, herbal, alcohol, sweet	894	*	

LRI: Linear retention indices on a DB5 column; * Odour reference from The Good Scents Company (The Good Scents Company Information System).

1.9 Qualitative and Quantitative Measurement of Lipid Oxidation Compounds in Dairy Products

There are various techniques and strategies used to measure LO in dairy products. Some commonly used, relatively simple, and practical methods to assess LO are POV, TBARS, and the KREIS test. Their widespread use is mainly due to ease of use and low cost, although they are more qualitative rather than quantitative.

Several analytical methods have been optimised for detecting off-flavours associated with LO in dairy products, such as solvent-assisted flavour evaporation (SAFE), GC-MS (Havemose et al., 2007), and GC-O (Zellner et al., 2008). GC-flame ionization detection (FID) or GC-MS have become the methods of choice for quantitative VOC analysis. These approaches are undertaken in combination with a specific method to extract and concentrate the VOC using either static or dynamic headspace techniques, sorption-based techniques, liquid based extraction or solvent assisted techniques. As previously mentioned, care must be taken not to increase VOC associated with LO during the analytical technique, as previous studies have demonstrated that certain LO VOC can increase between 37 °C and 60 °C (Panseri et al., 2011), therefore, including appropriate controls is necessary to prevent false positives.

1.9.1. Peroxide Value

POV is still widely used by the food industry as a qualitative indicator of oxidative stability in dairy products as it is inexpensive and relatively easy to use. The titrimetric method is described in the AOAC standard (Hortwitz, 2002), and the spectrophotometric method is described by Østdal et al. (2000). The basis of the assay is a solvent separation, followed by a reaction and absorbance reading at 470–500 nm. However, its accuracy and usefulness is questionable as it only considers the first stage of the LO reaction i.e., the initiation phase (Smet et al., 2008). Thus, in theory sensory properties can deteriorate further (due to hydroperoxides breaking down to form odour active oxidation products such as aldehydes, ketones and alcohols) without any increase in POV values. It is also not possible to make a judgement on the sensory characteristics of a product using the POV as hydroperoxides are generally tasteless and flavourless (Saxby, 2012). In addition, the breakdown of hydroperoxides can occur at a faster rate than their formation, therefore it is still possible to

have sensory issues at low POV levels. However, the POV can be used as a tentative non-specific, non-quantitative indicator of quality deterioration over time (Decker et al., 2010).

1.9.2. Thiobarbituric Acid Reactive Substances

TBARS methodology is a relatively simple spectrophotometric assay and remains widely used in the food industry. Some widely acknowledged limitations include a lack of specificity as the method uses the formation of MDA to represent the overall formation of aldehydes, thus providing no information on any individual LO volatiles. In addition, the TBARS reaction is not specific to MDA; the presence of any sugar can react with the thiobarbituric acid and yield a colour change, leading to an overestimation of the extent of LO (Ross and Smith, 2006). The method also fails to account for the numerous aldehydes, ketones and alcohols resulting from LO that are responsible for off-flavours associated with LO in dairy products. Another disadvantage of this method is the requirement for solvents and the associated risk assessments (Devasagayam et al., 2003). Studies report the levels of MDA in milk to be between 0.028–0.036 ppm (O'Sullivan et al., 2014), and higher in milk powders (0.3 ppm) (Fenaille et al., 2001) and IMF (0.1–1.2 ppm) (Cesa, 2004).

1.9.3. KREIS Test

The KREIS test was one of the earliest methods used to determine the oxidative deterioration of vegetable oils and is similar to TBARS in that solvents, a colour change and spectrophotometric measurements are involved. The primary reagent in the KREIS test (phloroglucinol) reacts with aldehydes and ketones to develop a pink colour i.e., a positive result. The KREIS test does not appear to be as widely used as TBARS or POV. A study investigating rancidity in edible oils (Narasimhan et al., 1999) has linked certain odour-active aldehydes identified using the KREIS test with deterioration in quality and has promoted the use of the KREIS test for the early detection of scission products of FA (Gray, 1978). The presence of some aldehyde compounds that are not associated with rancidity have been shown to give false positive results and other compounds such as vanillin were shown to interfere with results (Kerr and Sorber, 1923). Overall, the KREIS test is not considered a reliable LO indicator (Mehlenbacher, 1960, Pignitter and Somoza, 2012).

1.9.4. Physical Evaluation Methods

The application of NMR spectroscopy monitors the change in FA profile in oils by calculating the ratio of aliphatic to olefinic protons. NMR profiling has previously been applied to evaluate variations in the milk metabolite profile (Klein et al., 2010, Sundekilde et al., 2011) and more recently has been employed as a tool to identify and determine the quality of the lipid fraction of organic and conventionally produced bovine milk with emphasis on metabolites with potential health benefits (Tsiafoulis et al., 2019). Other physical evaluation methods have been employed to determine the oxidative stability of dairy products, including ESR spectroscopy (Hedegaard et al., 2006) and evaluating the presence of conjugated dienes present as a result of PUFA oxidation by UV absorption at 234 nm. However, this method was not considered useful for early detection of VOC that cause sensory defects in powders (Siefarth et al., 2014). It is also possible to qualitatively assess the conjugation of PUFA dienes by refractometry (Arya et al., 1969) and infrared spectroscopy (Ahlers and McTaggart, 1954).

1.9.5. Gas Chromatography Olfactometry

As previously mentioned, the advantage of including GC-O analysis is that it allows trained human assessors to identify VOC that are aroma-active and thus contributing to sensory perception in real time (Lawless and Heymann, 2010). This enables VOC that are contributing to the overall flavour to be identified and even their potential sensory influence in terms of intensity and character to be defined. The integration of GC-O and GC-MS and/or GC-FID techniques also makes it possible to establish direct relationships between a compound present in a food sample and any correlated odour. However, it is important to note that odours are extremely complex mixtures often consisting of numerous VOC which vary in concentration. VOC can interact synergistically or additively to produce the overall odour of a product (Brattoli et al., 2013). Therefore, while it is beneficial to know which VOC in a sample are odour active, the overall odour of a product can differ from that of each individual VOC. Friedrich and Acree (1998) and Rychlik and Bosset (2001) provided good descriptors of VOC present in dairy products as detected by GC-O. Kobayashi and Nishimura (2014) employed thirteen panellists to compare WMP samples from different regions using GC-O analysis, and concluded that the differences between the WMP based on region was caused by differences in the balance of the aroma-active VOC present.

A limited amount of studies have included GC-O analysis for the VOC in SMP samples. Karagül-Yüceer et al. (2002) undertook GC-O analysis on six NFDM powders, a product that is consumed directly as well as being used as an ingredient in other preparations. The study

identified a wide range of aldehydes, ketones and free FA which were found to be responsible for the generation of flavours in NFDM over storage. Samples were analysed by GC-MS, GC-O, and sensory analysis. Methional, a Strecker degradation product of methionine, was identified as an off-flavour compound with its corresponding aroma being characterised as boiled potato-like (Ballance, 1961, Tressl et al., 1989). These methods ensured a comprehensive evaluation of the products sensory characteristics in terms of the main source VOC responsible. This study also verifies the importance of raw milk quality even as an ingredient in end product applications.

1.9.6. Analysis of Volatile Organic Compounds by Gas Chromatography

Some of the most commonly utilised volatile extraction methods used in combination with GC to assess LO are outlined below, namely HS-SPME, thermal desorption (TD), SAFE, and sorptive extraction (SE).

HS-SPME has become a standard approach for the volatile profiling of food samples (Qualley and Dudareva, 2009). The basic principle of HS-SPME is that the sample of interest is placed in a sealed vial and heated under controlled conditions so that an equilibrium of the VOC is formed in the headspace which is representative of the sample. However as with all HS techniques, the nature of the sample matrix as well as the chemical properties of the individual VOC have a significant influence on the release of VOC. HS-SPME has become the most widely used volatile extraction technique as it requires minimal sample preparation, is solventless, fully automatable, easy to use, very versatile due to the wide range of fibre phases available, reproducible, and is relatively inexpensive (Heaven and Nash, 2012). Once the VOC equilibrium is formed, a polymer phase coated fiber is exposed to the headspace under controlled conditions (time, temperature and agitation). The VOC interact with the phase(s) and the fiber is retracted and subsequently desorbed for 2–3 min in a heated GC injector port (typically between 250–270 °C). The desorbed VOC are transferred onto the GC column in an inert gas flow and separated by their interaction with a GC column phase on heating in a column oven, and subsequently detected and/or quantified either by FID or MS. Thus far, HS-SPME GC-MS techniques have been applied to a variety of dairy products such as raw and pasteurised milk (Mounchili et al., 2005, Vazquez-Landaverde et al., 2005b), dairy powders (Wang et al., 2016, Chen et al., 2018, Clarke et al., 2019), and liquid or powdered IMF (García-Llatas et al., 2007, Nie et al., 2013). A review by Merkle et al. (2015) summarised the recent developments and applications of HS-SPME for analysis of complex food matrices. The study mentioned the occurrence of the matrix effect i.e., the binding of analytes to the

matrix resulting in low concentrations of the analytes in the headspace. Thus, the matrix effect may be an issue when developing a HS-SPME method for the extraction of volatiles from dairy products with increased fat contents. In an effort to off-set the matrix effect, response surface methodology has been used to determine the most useful HS-SPME extraction parameters for the quantification of VOC associated with LO in dairy powders (Clarke et al., 2019).

TD also works on the bases of heating samples to allow VOC reach the gaseous phase. As with HS-SPME, TD is used as an extraction and pre-concentration step prior to analysis by GC. VOC and some semi-VOC are extracted by this technique onto suitable phases packed into TD tubes, with many different phases available that can target individual VOC or chemical classes, or for more generic untargeted approaches. Removal of the trapped compounds from the phase(s) onto the GC column involves heating of the TD tube in a gas flow, and sometimes further concentration is possible using an in-line focusing trap. TD has been applied to a number of dairy products (Valero et al., 1997, Faulkner et al., 2018), milk powder (Francesca et al., 2015), and IMF (Cheng et al., 2017). A number of application notes are also available on the use of TD (Esteban et al., 1997, Hoffmann and Heiden, 2000, Roberts et al., 2016). Its widespread use in dairy products may be limited as moisture management can be problematic.

SAFE is a useful method for the isolation of volatiles from complex food matrices. Engel et al. (1999) reported that the application of SAFE to model solutions containing a range of aroma compounds resulted in increased yields from both solvent extracts and fatty matrices (50% fat) when compared with high vacuum transfer. SAFE could be particularly useful for longer chain, fat soluble compounds that have difficulty reaching the gaseous phase. SAFE has been used to prepare volatile extracts from dairy products for GC-O evaluation; Bendall (Bendall, 2001) used SAFE to extract volatiles from milk to be analysed via GC-O, 71 different aroma-active compounds were isolated from the milk, 66 of which were identified. However, SAFE has limitations, such as tedious sample preparation, the requirement for solvents, requirement of expensive specialist glassware, reproducibility issues, manual sample manipulations, and may require further concentration steps prior to introduction to the GC (High et al., 2019).

Stir bar sorptive extraction (SBSE) is a solventless technique with simple sample preparation that has been used for flavour research of dairy products including dried dairy ingredients, and milk. Traditionally this technique employed glass-encapsulated magnetic bars with a sorbent coating to extract volatiles. Stir bars can be immersed within a liquid

sample or suspended in the headspace of a solid, liquid, or gaseous sample during the extraction process. Volatiles are typically thermally desorbed followed by a cryofocusing step and GC-MS (Baltussen et al., 1999, McGorrin, 2007, Prieto et al., 2010). Baltussen et al. (1999) was one of the first to describe this technique, and employed a polydimethylsiloxane (PDMS) phase to extract and concentrate the VOC. Park and Drake (2016a) used SBSE for the extraction of flavour compounds from NFDM concentrated by reverse osmosis or evaporation and found that the volatile profiles were consistent with the descriptive sensory results.

Faulkner et al. (2018) achieved good results for milk samples (up to 65 volatile compounds from a range of chemical classes) using a new high capacity SE technique called HiSorb followed by GC-MS analysis. Currently PDMS-coated stir-bars are the only phase commercially available for these techniques, which somewhat reduces the applicability of SBSE to the extraction of non-polar compounds due to the poor extractability of more polar analytes (Prieto et al., 2010). However, Ochiai et al. (2016) demonstrated that solvent-assisted SBSE improves peak resolution and extraction efficiency of polar and non-polar compounds. Moreover, Schiano et al. (2019) concluded that solvent-assisted SBSE provided the most consistent detection of selected compounds in commercial milks, although the levels of compounds detected were not significantly ($p > 0.05$) higher compared to conventional SBSE or SPME extraction methods. Some of the most common techniques used for the extraction of volatiles from dairy powders are outlined in Table 1.2.

Table 1.2. Summary of the primary methodologies used for volatile extraction and analysis of dairy powders.

Method	Advantages	Limitations	Applications	Reference
		Extraction Methodology		
Headspace solid-phase microextraction (HS-SPME)	• Minimal sample preparation • Does not require organic solvents • Simple to use • 'Clean' method in comparison to LC • High sample throughput • Reproducibility • Large selection of phases	• Fiber saturation • Low phase capacity • Possible carryover of compounds	• Wide range of volatiles in food products • Raw and pasteurised milk • Liquid and powdered infant formulas • Milk powders	(Qualley and Dudareva, 2009) (Heaven and Nash, 2012) (Vazquez-Landaverde et al., 2005b) (García-Llatas et al., 2007) (Wang et al., 2016, Clarke et al., 2019)
In-tube extraction (ITEX)	• Does not require the use of solvents • Dynamic extraction • Well matched to the analysis of trace organic compounds	• Repeatability issues • Possible issues with moisture and needle blockage	• Volatile organic hydrocarbons from aqueous samples	(Jochmann et al., 2008)
Thermal desorption (TD)	• Good sample throughput • Minimal sample preparation • Does not require organic solvents • Large selection of phases available • Sample collection and enrichment capabilities	• Tedious if not automated • Moisture control	• Bovine milk • Milk and cheese • Milk powder	(Faulkner et al., 2018) (Valero et al., 1997) (Francesca et al., 2015) (Esteban et al., 1997, Hoffmann and Heiden, 2000, Roberts et al., 2016)
Solvent-assisted flavour evaporation (SAFE)	• Simple method • Capable of rapid and in situ identification of volatile compounds	• Requirement for solvents • Expensive glassware • Requirement for risk assessment	• Milk • Skim milk powder (SMP)	(Bendall, 2001)
Stir bar sorptive extraction (SBSE)	• High effectiveness for the extraction of non-polar and medium-polarity compounds • Large amount of phase • Good sensitivity and recovery • Automated systems under development	• Manual removal and washing of stir bar required if not automated	• Liquid samples or liquid extracts • Dairy products • Can be used for headspace analysis	(Ochiai et al., 2016, Bader, 2018) (Hoffmann and Heiden, 2000, Park and Drake, 2016a)
HiSorb extraction	• Effective for the extraction of volatile and semi-volatile compounds • Large amount of phase • Possible to perform immersive and headspace extraction • Automated systems available	• Extended extraction times • One phase currently available	• Liquid samples or liquid extracts • Can be used for headspace analysis	(Markes International, 2016)

		Identification Methodology		
Mass Spectrometry (MS)	• Powerful compound identification abilities • Can compare spectra to libraries • Useful for unknown analysis • Qualitative analysis • Versatility	• Reproducibility for quantification purposes	• Various volatile and semi-volatile dairy and food products	(Toso et al., 2002, Clarke et al., 2020a, Clarke et al., 2020b)
Flame Ionised Detector (FID)	• Reproducibility • Sensitivity • Reliability • Quantification abilities	• Requires standards for identification • No identification ability	• Various volatile and semi-volatile dairy and food products	(Mannion et al., 2016a)
Gas chromatography olfactometry (GC-O)	• Ability to link volatile organic compounds to odour descriptors • Provides good odour descriptors • Allows for odour thresholds to be determined	• Time consuming • Ongoing requirement for panel members • Must be coupled with the correct extraction method—possible method development required	• SMP • Any food sample with odour above threshold level	(Karagül-Yüceer et al., 2002)
GCxCG-ToF-MS (Time of Flight-MS)	• Good for the separation of complex mixtures • Generation of 3D plots • Good sensitivity • Enhanced resolution • Ability to separate co-eluting peaks in the second dimension • Ability to reduce or enhance elements of the chromatogram in the second dimension	• Complexity of the data generated	• Milk lipids	(Tranchida et al., 2013)

1.10 Sensory Analysis

Regardless of the processing dairy products undergo, consumer acceptance remains primarily based on appearance and flavour (Adhikari et al., 2010). As milk has a naturally subtle and somewhat bland flavour, any development of off-flavours is relatively easily perceived by the consumer (Shiratsuchi et al., 1994). The impact of different feeding systems, production regions, cultural differences and storage conditions have been identified as the motivating factors behind dairy product purchase (Coppa et al., 2011, Faulkner et al., 2018, Cheng et al., 2020). Consumers have a desire to know more about where their milk and milk products are coming from and how they are produced (DSM, 2016). Previous sensory studies have employed between 25 and 100 panellists for consumer testing and this technique has been widely applied to dairy products (Santos et al., 2003, Gandy et al., 2008, Potts et al., 2017). Full descriptive sensory analysis requires fewer panellists as they are trained on how to specifically assess the product using pre-defined sensory attributes (Clarke et al., 2021). Panellists are not asked about their own liking or preferences toward the product, but rather they are employed as calibrated analytical instruments to give results on the intensity of particular descriptors known to be characteristic to that product. When recruiting a panel for sensory analysis, screening tests are performed to ensure each panellist provides an accurate and reliable result. Selection factors include; continued availability, health status, ability to perceive flavours, familiarity of the product under evaluation, previous experience, allergies, and medication. However, even with a stringent selection protocol, the 'opinion factor' continues to play a role in sensory analysis due to genetic and cultural differences between panellists (Ferdenzi et al., 2017).

When implementing quantitative descriptive sensory analysis, efforts should be made to ensure the sampling technique is consistent across the panel and each panellist understands how the odours and flavours are to be interpreted and described. Selecting the product descriptors (lexicons/attributes) in the final evaluation is generally a consensus process, decided upon in a focus group, conducted prior to sensory evaluation (Murray et al., 2001). The final descriptors must comprehensively describe the sensory attributes and their intensities.

Combining sensory analysis with any of the aforementioned GC techniques provides more detailed information on numerous aspects of dairy products including consumer acceptability, levels of rancidity, extent of LO, and intensities of compounds with known odour descriptors. Moreover, the concentration of LO compounds perceived as

unacceptable can be determined. The review by Kilcawley et al. (2018) summarises recent studies that included sensory analysis of dairy products and the various sensory methodologies used.

Table 1.1 summarises the aromas associated with some important volatile compounds in milk. LO generally results in off-aromas and flavours in dairy powders including painty (hexanal, nonanal) (Lloyd et al., 2009a), cardboard-like *(*hexanal, pentanal) (Hall et al., 1985, Lloyd et al., 2009a), metallic (hexanal, pentanal, vinyl ketones) (Forss, 1964), and fishy (carbonyl compounds, 2,4-unsaturated aldehydes, trimethylamine) (Forss, 1964). Some studies have suggested that producing dairy powders below 4% *w*/*w* moisture can delay the development of fishy and tallow off-flavours (Brown and Thurston, 1940), however it is most likely also dependent upon the range of factors that are known to influence the concentration of the various VOC responsible. Sulfur compounds can be problematic as they have very low odour thresholds and are as associated with cooked flavours in ultra-heat-treated milk (Zabbia et al., 2012).

Jo et al. (2019) documented an interaction between milk proteins and sulfur compounds in milk, affected by serum proteins associated with casein during heat treatment. The study confirmed that hydrogen sulfide and carbon disulfide contributed to eggy and sulfur/burnt flavours in heat-treated milk, respectively. Interestingly, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide, and methional were found not to be associated with sulfur/burnt and eggy flavours in heat-treated milk.

Various studies have investigated the impact of oxidation on the flavour and stability of dairy products (Cadwallader and Singh, 2009, Lloyd et al., 2009a, Lloyd et al., 2009b, Wright et al., 2009, Park et al., 2016). A review by Su et al. (2020) summarises the sensory lexicons used for the evaluation of dairy products and established the connection between off-aroma lexicons and volatile formation pathways existing in dairy ingredients. This review concluded that many off-aromas are as a result of protein, fat, and sugar breakdown products from lipid degradation and Maillard reaction pathways. The review suggested that to minimise off-aromas developing in dairy products, in particular high protein formulations, a high quality starting material is required, and processing parameters should be monitored and adjusted accordingly to decrease the rate of flavour degradation.

1.11 Conclusions

There are numerous factors that influence the rate of LO in dairy products; such as cow breed and diet, stage of lactation, levels of PUFA and unsaturated FA, storage and processing conditions (exposure to heat, oxygen and/or light). Processing conditions require monitoring to ensure the quality of the end product is consistent and free from any undesirable flavours. The quality of the raw milk used for the production of dairy powders is very important, but also the manner in which the milk is handled, processed and stored has a significant impact on the extent to which the milk fat is oxidised throughout its shelf-life. LO has been shown to impact on the quality, nutritional and sensory properties of various dairy powders resulting in undesirable flavours and reduced shelf-life. Many analytical techniques are available for the qualitative and quantitative analysis of LO in dairy powders, many of which can be used in combination to elucidate more detailed information. This is especially the case where sophisticated techniques such as GC-MS can be used to identify and quantify individual VOC associated with LO, but in combination with GC-O and/or sensory analysis can provide a much more in-depth understanding of the whole LO process pertaining to a specific product. Therefore, using this approach to determine the concentrations of VOC associated with LO that adversely impact sensory perception, can provide insights into the production parameters that could maximise shelf-life and product quality of dairy powders.

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Chapter 1 Highlights

- LO continues to play a role in dairy powder quality and sensory deteroriation.
- IMF fortified with vegetable and fish oils is very susceptible to LO.
- Processing conditions is one of the main factors influencing the rate of LO.
- Many odour active VOC are associated with LO in dairy powders.
- Combining sensory and analytical techniques provides more detailed information regarding the oxidative state of dairy powders.
- High quality starting material is required for powder production, and processing parameters should be monitored and adjusted accordingly to decrease the rate of flavour degradation.

Chapter 2: Development of a HS-SPME GCMS Method for the Quantification of Volatiles Associated with Lipid Oxidation in Whole Milk Powder using Response Surface Methodology

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Development of a headspace solid-phase microextraction gas chromatography mass spectrometry method for the quantification of volatiles associated with lipid oxidation in whole milk powder using response surface methodology

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Abstract

Lipid oxidation is a major contributor to the deterioration of the sensory quality of fat-containing dairy powders. Hydroperoxides are the primary oxidation products from unsaturated fatty-acids that readily yield a complex mixture of volatile organic compounds that can adversely impact product quality and shelf life. Headspace Solid-Phase Microextraction Gas-Chromatography Mass-Spectrometry (HS-SPME GC-MS) was chosen to quantify thirteen lipid oxidation compounds in whole milk powder encompassing a range of volatilities and chemical classes. A central composite rotatable design (CCD, α=1.1) based on a 2^3 factorial table was used with response surface methodology to optimize the HS-SPME parameters; determined at; 45 min extraction time and 43°C extraction temperature. The significant model terms were found to be extraction temperature (p <0.05) and the interaction between time and temperature (p

<0.05). Precision, accuracy, LOD and LOQ were determined and the method was validated for whole milk powder.

Keywords: Lipid oxidation, whole milk powder

Chemical compounds studied in this article

2-pentanone (PubChem CID: 7895); Pentanal (PubChem CID: 8063); 1-heptanol (PubChem CID: 8129); hexanal (PubChem CID: 6184); 2-heptanone (PubChem CID: 8051); heptanal (PubChem CID: 8130); octanal (PubChem CID: 454); 3-octen-2-one (PubChem CID: 5363229); 2-nonanone (PubChem CID: 13187); (E)-2-nonenal (PubChem CID: 5283335)

2.1 Introduction

Lipid oxidation (LO) is a major contributor to the deterioration of food-quality, resulting in the formation of undesirable compounds, odours and flavours (Frankel, 1991, Fritsch, 1994, Jacobsen, 1999, Amamcharla and Metzger, 2014). This can limit the shelf life of fat containing dairy powders through deterioration of flavour and product stability/quality (Park and Goins, 1992, Fenaille et al., 2003). Hydroperoxides are the primary oxidation products of unsaturated fatty acids. They are highly reactive compounds that break-down readily, yielding aldehydes, hydrocarbons, alcohols, ketones and other non-volatile compounds. Thus, reliable and robust methodology for the indicators of LO in fat-containing dairy powders is of great interest.

Flavour is a primary factor influencing consumer acceptance of milk powder products (Nursten, 1997, Hough et al., 2002, Drake, 2004). Due to its high fat content (between 26-40%) and large exposed surface area; whole milk powder (WMP) is vulnerable to the formation of odour-active compounds during processing and storage resulting in alterations to its sensory properties (Lloyd et al., 2009a). While a certain amount of these compounds form naturally and contribute to the desirable aroma characteristics of the product, their increased intensity can lead to adverse sensory perceptions. The sensory perception and composition of milk powders is heavily dependent on the milk from which it was derived (Sharma et al., 2012, Murphy et al., 2016) and how it is processed (Abdalla et al., 2017). Processing parameters such as temperature, drying time, packaging material and storage conditions have been found to be critical factors affecting the flavour of WMP (Biolatto et al., 2007, Cadwallader and Singh, 2009, Lloyd et al., 2009a) More than 60 aroma-active compounds have been

identified in WMP (Whetstine and Drake, 2007, Cadwallader and Singh, 2009, Lloyd et al., 2009a) with the most important off-flavour causing compounds found to be a result of LO (in particular, hexanal, other aldehydes and ketones). However, determining the cause of undesirable changes in WMP flavour is complex owing to the fact that many of the aroma-active compounds are produced by more than one mechanism (Whetstine and Drake, 2007, Cadwallader and Singh, 2009, Lloyd et al., 2009a).

Headspace solid-phase microextraction gas chromatography mass spectrometry (HS-SPME GC-MS) analysis has become a standard approach for volatile profiling of dairy products (Verzera et al., 2004, Vazquez-Landaverde et al., 2005a, García-Llatas et al., 2007, Panseri et al., 2011, Dursun et al., 2017, Sandoval-Copado et al., 2018). The basic principle of HS-SPME is that the headspace contains all of the volatiles that account for the odour perception of a food sample. A distinct advantage of HS-SPME is that it is a simple, easy, automated and reproducible extraction technique requiring minimal sample preparation without the requirement for solvents. The development of a HS-SPME method capable of detecting volatiles below 0.05 mg/L is imperative as certain aldehyde compounds associated with LO have odour thresholds below these levels in milk. The odour thresholds of ketones are generally greater (~0.14-2.3 mg/L) (He et al., 2013). To date no published optimized validated HS-SPME GC-MS method exists to quantify LO volatiles in WMP. In addition this method includes analytical performance characteristics of linearity, precision, accuracy, limits of detection (LOD) and limits of quantification (LOQ) for thirteen key LO volatiles in WMP.

2.2 Materials and Methods

2.2.1 Powder Samples

Fresh commercial WMP samples were obtained from local suppliers in sealed 25kg bags. The following are approximate values; 34% protein, 26% fat, 3.5% moisture, 6% ash, 162mL/100g bulk density, <50ppm nitrates and <2ppm nitrites.

2.2.2 Standard Solutions

All standards were obtained from Merck Ireland (Arklow, Co. Wicklow, Ireland) and stored at room temperature unless otherwise stated. All standard solutions were prepared at 0.1% (w/v) in methanol and stored at -18°C until required for analysis, but for no longer than 4 months. Standard mixtures were sonicated using a Decon FSI100b ultrasonic bath (Decon Ultrasonics Ltd., UK) at room temperature for 10 min to ensure

all the compounds were dissolved prior to analysis. For all calibration curves or when preparing standards for accuracy and precision studies the external standard mixture was prepared at 0.004% (w/v) (4 mL in 100 mL distilled water (dH_2O)) and the internal standard (IS) mixture (2-methyl-3-heptanone, 4-methyl-2-pentanol and isovaleraldehyde) was prepared at 0.001% (w/v) (1 mL in 100 mL dH_2O). For the preparation of calibration curves, varying levels of the standard mixture were prepared in 10 mL volumetric flasks in dH_2O.

2.2.3 Sample Preparation

WMP samples were stored at room temperature in light omitting aluminium foil, sealed bags. The required amount of powder was weighed out directly into the 20 mL amber head-space vials (Apex Scientific Ltd, Maynooth Co.Kildare, Ireland) ready for analysis. 2.50g of dH_2O and 250μL 0.001 % (w/v) IS was added to each sample. A calibration curve was also prepared by spiking a set of the hydrated WMP samples with varying levels of the external standard mixture. Matrix (control) samples (WMP sample + dH_2O only) were also included in each run. Six replicates were analysed for each set of parameters outlined in Table 2.1, three contained WMP sample + 250μL 4 mg/L external standard mixture + 250μL 10 mg/L IS + 250g dH_2O, and three contained WMP sample + 250μL 10 mg/L IS + 2.50g dH_2O.

2.3 Analysis of Volatile Compounds by HS-SPME-GC-MS

2.3.1 GC Parameters

All of the incubation, extraction and injection processes were implemented using a Bruker CombiPal autosampler (Elementec Ltd, Maynooth, Co.Kildare Ireland). Extraction time and temperature ranged from 6-75 min and 30-80°C, respectively. A mid-polar DB – 624 UI column (60m x 0.32mm x 1.80μm) (Agilent Technologies Ireland Ltd, Little Island, Cork, Ireland) was used. A 2cm, 50/30μm, DVB/Carboxen/PDMS Stableflex SPME fiber (Agilent Technologies Ltd, Ireland) was selected for this study by means of literature reviews and has been shown to be suitable for the extraction of volatile compounds from dairy products (Tunick et al., 2013, Salum et al., 2017). Following extraction, the SPME fiber was retracted and injected into the split/splitless 1177 GC inlet for 5 min at 250°C in split mode at a ratio of 10:1 using a Scion 456-GC (Elementec, Ltd, Ireland). The column oven was held at 65°C for 10 min, then increased to 240°C at a rate of 10°C/min and held for 5 min. Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min.

2.3.2 HS-SPME Conditions

The injector was fitted with a Merlin Microseal septum (Merck, Ireland) and an inert glass SPME inlet liner (Agilent Technologies Ltd, Ireland). Amber headspace vials (20mL) with magnetic caps and Silicone/Polytetrafluoroethylene 1.3mm 45° Shore A septa were used throughout the study (Apex Scientific Ltd, Ireland). Depending on the run, samples were incubated and extracted at a temperature according to table 3.1, extraction temperature *(x_1).* Each sample was subjected to a 10 minute pre-extraction incubation time with pulsed agitation of 5s at 500rpm, automated by the CombiPal agitator/heater. The fiber was conditioned at 270°C for 5 min using a CombiPal bakeout station between each sample to ensure no carryover of samples occurred.

2.3.3 MS Parameters and Chromatogram Analysis

All MS analysis was carried out on a Scion 456-GC-TQ (Elementec Ltd, Ireland) in single quadrupole mode using single ion monitoring (SIM) with the method set up to include one quantifier and two qualifier ions for each compound. The MS mode was electronic ionisation (70v). The transfer line temperature was set to 260°C and the ion source to 280°C. A full scan was included in each run to provide a full profile of each sample. Autotunes were carried out on the MS system monthly while air and water reports were carried out daily. The same concentration of the external standard mixture (40 mg/L) and the IS mixture (10 mg/L) were included in each run to evaluate the performance of the GC column, SPME fiber and the MS. Each total ion chromatogram generated was analysed using Bruker MS Workstation version 8 (Elementec Ltd, Ireland). Quantification of each compound was carried out using calibration curves and the relevant internal standard values.

2.3.4 Optimization of HS-SPME Extraction Parameters

RSM was employed to assess the effects of extraction conditions on the isolation of the selected volatile compounds in WMP. The current literature suggests that extraction temperature (x_1, *T)* and extraction time *(x_2, t)* have the most significant effect on extraction efficiency of volatile organic compounds (VOCs) from dairy products using HS-SPME-GCMS analysis (Panseri et al., 2011, Tunick et al., 2013, Bezerra et al., 2016) Tunick et al. (2013) evaluated the effect of equilibrium temperature (21 and 40°C), holding time (0, 30 and 60 min) and extraction time (30 and 60 min) on organic and conventional milk samples. The same study evaluated the effect of holding time (10 and 25 min) and holding temperature (40 and 60°C) on the extraction efficiency of whey

protein concentrate (WPC). Bezerra et al. (2016) evaluated the effect of extraction temperature (20, 30, 45, 60, and 70°C) and equilibrium time (3, 10, 20, 30, and 37 min) before fibre exposure and extraction time (6, 20, 40, 60, and 74 min) on the extraction efficiency of Coalho cheese. Panseri et al. (2011) investigated the effect of varying extraction times (60, 120, 180 and 240 min) on the extraction efficiency of hexanal as a marker of LO in equilibrated (60 min at 4°C) butter samples. The third variable, sample amount *($x_{3,}$ S),* was selected based on the study by Lee et al. (2003) on Parmesan cheese suggesting that sample amount can have an effect on the concentration of VOCs in the headspace of a sample due to the ratio of sample to headspace volume. However, few other studies have investigated the effect of sample amount on HS-SPME extraction efficiency (Altaki et al., 2007). Thirteen volatile compounds (2, 4-decadienal, heptanal, hexanal, (E)-2-nonenal, pentanal, undecanal, octanal, 2-heptanone, 2-nonanone, 2-pentanone, 3-octen-2-one, acetone and 1-heptanol) all of which are known to contribute to the sensory perception of dairy products (Faulkner et al., 2018, Kilcawley et al., 2018) were selected to demonstrate the overall effectiveness of the varying HS-SPME parameters. The thirteen target compounds encompassed a range of molecular weights and chemical classes. Optimization was performed to obtain the maximum extraction efficiency i.e. identifying the extraction parameters that result in the greatest area value for each target compound. A central composite rotatable design (CCD, α=1.1) based on a 2^3 factorial table constructed using Design Expert version 10.0.0 (Stat-Ease, Inc., USA) was used to optimize the chosen HS-SPME conditions. The design consisted of 20 trials comprising eight factorial points, five axial points and six replicates of the central point.

Table 2.1: Central composite rotatable design (2^3) showing the levels of each independent variable (temperature (x_1, *T*), extraction time (x_2, *t*), amount of sample (x_3, *S*)) constructed using Design Expert (Stat-Ease, Inc., USA) for the HS-SPME extraction of volatiles in WMP.

		Independent variables		
run	**std**	**Extraction temperature (°C, x_1)**	**Extraction time (min, x_2)**	**Sample amount(g, x_3)**
1	12	55(0)	78.45(+α)	2.75(0)
2	17	55(0)	40.5(0)	2.75(0)
3	8	80(+)	75(+)	5(+)
4	9	27.5(-α)	40.5(0)	2.75(0)
5	2	80(+)	6(-)	0.5(-)
6	4	80(+)	75(+)	0.5(-)
7	11	55(0)	2.55(-α)	2.75(0)
8	1	80(+)	6(-)	0.5(-)
9	6	80(+)	6(-)	5(+)
10	19	55(0)	40.5(0)	2.75(0)
11	5	30(-)	6(-)	5(+)
12	10	82.5(+α)	40.5(0)	2.75(0)
13	14	55(0)	40.5(0)	5.225(+α)
14	7	30(-)	75(+)	5(+)
15	13	55(0)	40.5(0)	0.275(-α)
16	3	30(-)	75(+)	0.5(-)
17	20	55(0)	40.5(0)	2.75(0)
18	16	55(0)	40.5(0)	2.75(0)
19	15	55(0)	40.5(0)	2.75(0)
20	18	55(0)	40.5(0)	2.75(0)

2.4 Method Development and Validation

2.4.1 Calibration, Linearity, Limits of Detection (LOD) and Limits of Quantification (LOQ)

2-Methyl-3-heptanone, 4-methyl-2-pentanol and isovaleraldehyde (3-methyl butanal) were used as internal standards (IS) for the quantification of ketones, alcohols and aldehyde compounds, respectively. Calibration curves were created using a set of WMP samples spiked with increasing concentration levels of the 0.004% (w/v) external standard mixture. The amount of IS in each sample remained constant at 0.001% (w/v). Linearity was determined using external standard calibration curves with five concentration levels for each compound in addition to evaluating regression curves (ratio of the standard peak areas to the relevant IS peak area versus the concentration) and results were expressed by the squared determination coefficient (R^2). Detection limits were estimated as the concentration of compounds that generated a signal of three times the signal-to-noise (S/N = 3). For LOQ measurements, each compound was identifiable,

discrete and reproducible with precision of 20%. A standard curve was constructed and the lowest point on the curve yielding reproducible results was accepted as the LOQ for each compound.

2.4.2 Precision Study

Repeatability is expressed as the relative standard deviation (RSD) of relevant peak areas of a number of replicates. The intra- and inter-day precision was estimated by analysing six replicates of three different QC levels (0.8, 2 and 4 mg/L). The intra-day precision of the method was estimated by calculating the relative standard deviation (RSD) for the analysis of QC samples in six replicates and inter-day precision was determined by the analysis of six replicates of QC samples over three consecutive days.

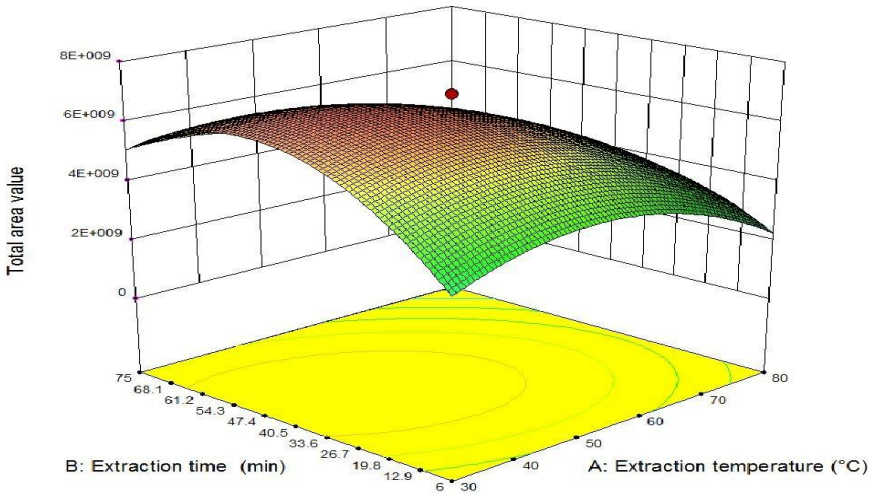
2.4.3 Statistical Analysis

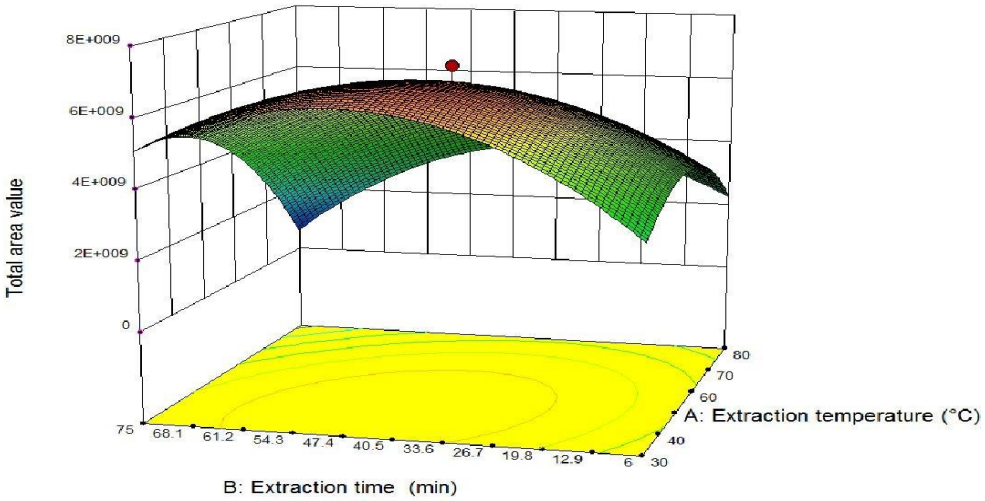
As previously mentioned, RSM was employed to create a predictive model for the optimum extraction method. One-way ANOVA was used to determine if there were any significant differences between interactive effects between the three independent variables and also to determine if the model itself was significant and capable of being a beneficial predictive model.

2.5 Results and Discussion

Response surface graphs (figure 2.1) were constructed with the aim of localizing the optimal region and thus, verifying the independent variable values that produce the best area response for volatiles in WMP. Based on figure 2.1, it was observed that the region producing the best responses for the compounds of interest is located between 50 and 75 min extraction time and between 30°C and 50°C extraction temperature. Software confirmed that the optimized conditions for extraction time and temperature were 45 min and 43°C, respectively and that 2.40g of sample was found to be the optimum amount for the best response, based on the desirability factor. The resulting R^2 value for the model was 0.9394 which according to the F test, demonstrated significant regression i.e. good correlation between the experimental data and the fitted model. Moreover, the lack of fit F-value of 3.14 implied that lack of fit is not significant relative to the pure error. Significant model terms, as determined by statistical analysis (one-way ANOVA) were found to be; the overall model (p <0.0001), extraction temperature (p 0.0003), the interaction between extraction time and temperature (p 0.0076). The predicted R-squared

value of 0.8210 is in reasonable agreement with the adjusted R-squared value of 0.9119 i.e. the difference is <0.2.





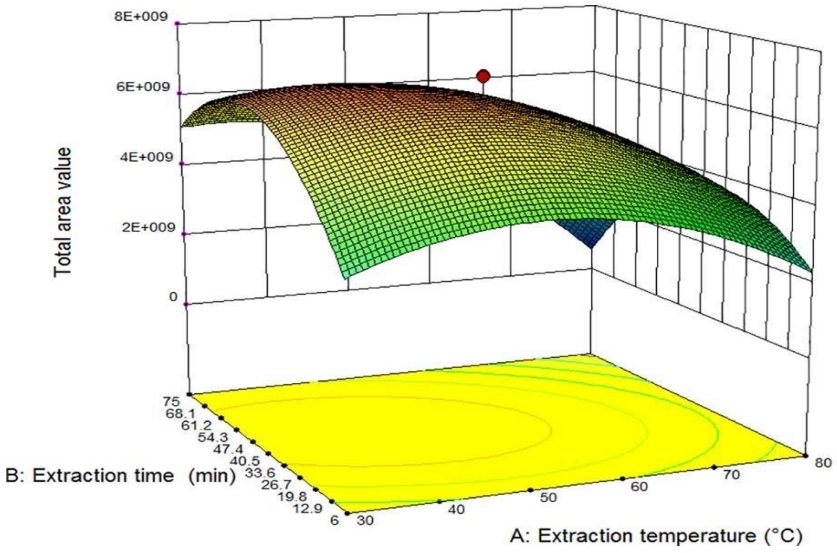


Figure 2.1: Response Surface graphs for the dependent variable (total area value) vs. extraction time (min) and extraction temperature (°C) showing the optimum extraction conditions for the target compounds from whole milk powder.

2.5.1 Method Development and Validation

2.5.1.1 Linearity, Limits of Detection (LOD), Limits of Quantification (LOQ) and Precision

Linearity was determined using external standard calibration curves with five concentration levels for each of the thirteen compounds in addition to evaluating regression curves (the ratio of the standard peak areas to the relevant IS peak area versus the concentration) and results were expressed by the squared determination coefficient (R^2). The calibration curve equations, the determination coefficient (R^2), LOD, LOQ and precision data for the compounds of interest can be found in table 2.2.

Table 2.2: Validation parameters for the optimized method: limit of detection (LOD), limit of quantification (LOQ), standard calibration line, R^2 value and validation range, precision and accuracy data.

Compound	**LOD (mg/L)**	**LOQ (mg/L)**	**Standard calibration line at validation range**	**R^2 value**	**Linear range (mg/L)**	**Validation range (mg/L)**	**Concentration of external standard mixture (mg/L)**	**Intra-day repeatability**		**Inter-day repeatability**
Aldehyde								**Peak area RSD (%)**	**Retention time RSD (%)**	**Peak area RSD (%)**
2,4-decadienal	0.002	0.066	y = 8E-08x - 0.1763	0.9494	0.066 – 4	0.066 – 4	4	4.6	0.12	9.9
							2	2.2	0.12	8.8
							0.8	1.3	0.12	9.9
Heptanal	0.002	0.066	y = 2E-09x - 0.043	0.9751	0.066 – 4	0.066 – 4	4	2.3	0.28	8.1
							2	2.4	0.28	7.8
							0.8	3.3	0.28	7.8
Hexanal	0.005	0.066	y = 6E-09x - 0.0464	0.9751	0.066 – 4	0.066 – 4	4	3.0	0.05	3.6
							2	1.6	0.05	7.3
							0.8	3.4	0.05	6.4
(E)-2-nonenal	0.004	0.05	y = 2E-09x - 0.01	0.9036	0.05 – 4	0.05 – 4	4	6.3	0.03	6.5
							2	2.6	0.03	5.1
							0.8	2.6	0.03	10
Pentanal	0.005	0.066		0.976	0.066 – 4	0.066 – 4	4	2.8	0.07	2.8

			y = 4E-09x - 0.0979				2	3.8	0.07	7.7
							0.8	4.2	0.07	8
Undecanal	0.002	0.05	y = 2E-09x - 0.0091	0.9243	0.05 – 4	0.05 – 4	4	8.0	0.05	8.1
							2	4.9	0.05	8.8
							0.8	6.9	0.05	10
Octanal	0.004	0.066	y = 2E-09x - 0.0381	0.9754	0.066 – 4	0.066 – 4	4	4.6	0.22	3.8
							2	3.9	0.22	9.7
							0.8	5.0	0.22	4.9
Ketone										
2-heptanone	0.002	0.066	y = 5E-10x - 0.0413	0.9815	0.066 – 4	0.066 – 4	4	2.2	0.04	9.8
							2	1.6	0.04	9
							0.8	1.9	0.04	6.5
2-nonanone	0.002	0.066	y = 8E-10x - 0.0493	0.9824	0.066 – 4	0.066 – 4	4	5.2	0.13	10
							2	3.4	0.13	4.9
							0.8	3.0	0.13	8.6
2-pentanone	0.004	0.066		0.9793	0.066 – 4	0.066 – 4	4	1.7	0.08	5.6

			y = 2E-09x - 0.1159				2	1.5	0.08	9.7
							0.8	5.6	0.08	9.2
3-octen-2-one	0.004	0.066	y = 1E-09x - 0.0393	0.9831	0.066 – 4	0.066 – 4	4	3.3	0.04	9
							2	1.8	0.04	4.4
							0.8	2.2	0.04	5.6
Acetone	0.006	0.066	y = 3E-08x - 0.2044	0.9842	0.066 – 4	0.066 – 4	4	2.8	0.25	7
							2	2.2	0.25	9.9
							0.8	7.5	0.25	7.5
Alcohol										
1-heptanol	0.0044	0.066	y = 2E-08x - 0.0032	0.9624	0.066 – 4	0.66 – 4	4	3.6	0.20	8.9
							0.2	3.4	0.20	5.2
							0.08	4.0	0.20	5.8

2.5.1.2 Method Application

The validated method was applied to fresh WMP samples to assess the levels of the selected LO compounds and to ensure the robustness of the method when applied to real samples. The validated method has the ability to detect and quantify some or all of the targeted compounds (if present) in commercial WMP samples at low levels. The validation range (0.066–4 mg/L) was significantly lower than the levels of most target compounds found in commercial WMP (~0.4-0.6 mg/L) as determined by calibration curves and by the ratio of the standard peak areas to the relevant IS peak area versus the concentration. The RSD for a triplicate analysis of WMP ranged from 1.3–12% for the targeted compounds. The heating and processing conditions required to produce WMP has been shown to cause the development of some off-flavour compounds associated with LO (Li et al., 2012, Li et al., 2013). Thus, some of the compounds of interest in this study are often present in fresh WMP at low levels; therefore, it is important that analytical methods are capable of detecting and quantifying these compounds at low levels. Furthermore, this can offer insight into the effects of processing conditions on the products sensory stability and shelf life.

2.5.1.3 Matrix Effect

The matrix effect observed in this study is likely caused by binding of certain VOCs to the sample matrix and this reduced the response of some of the target compounds, in spiked samples, particular the longer chain aldehydes (E)-2-nonenal, 2,4-decadienal and undecanal (figure 2.2, peak 11, 12 and 13, respectively). These compounds can be observed as well-defined, sharp peaks yielding a good response in figure 2.2 (A) but show a reduced response in figure 2.1 (B). The low concentration of these particular aldehyde compounds in the headspace of spiked samples could be due to the longer chain aldehydes being more fat soluble and thus having greater affinity to the fat portion of the WMP. It is generally accepted that aldehydes and ketones with fewer than six carbon atoms are water soluble because they contain a highly polar carbonyl group (-CO), that forms reasonably strong bonds with water molecules, but solubility decreases with chain length (Covarrubias-Cervantes et al., 2005). Preparing samples in methanol instead of dH_2O could help overcome this issue as most aldehydes are readily soluble in organic solvents.

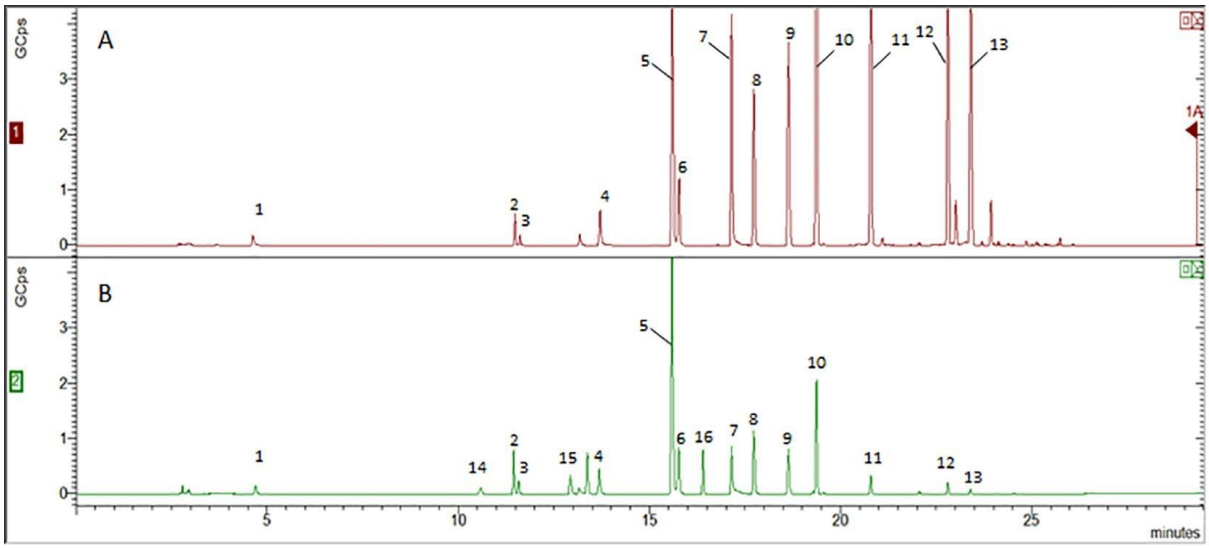


Figure 2.2: (A) A typical SIM chromatogram obtained from 250 µL of the external standard mixture prepared at 0.0004% (w/v); **(B)** A typical SIM chromatogram obtained from a whole milk powder (WMP) sample (2.40 g) spiked with 250 µL external standard mixture prepared at 0.0004% (w/v), 250 µL internal standard mixture prepared at 0.001% (w/v) and 3.50 g dH2O. Peak identification: (1) acetone, (2) 2-pentanone, (3) pentanal, (4) hexanal, (5) 2-heptanone, (6) heptanal, (7) 1-heptanol, (8) octanal, (9) 3-octen-2-one, (10) 2-nonanone, (11) (E)-2-nonenal, (12) undecanal, (13) 2,4-decadienal, (14) isovaleraldehyde (IS), (15) 4-methyl-2-pentanol (IS), (16) 2-methyl-3-heptanone (IS).

For the vast majority of the compounds, the LODs varied between 0.002 and 0.006 mg/L, with LOQs between 0.05 and 0.066 mg/L. The selected validation range ensured a consistent result for each sample. Regression curves for each compound in this range were acceptable, with R^2 values ranging from 0.9036 - 0.9842, demonstrating good linearity. The responses obtained with the extraction parameters used in this method for each compound varied, but the method has been shown to be the most efficient for the range of targeted compound classes evaluated. For example, a method focused solely on extracting longer chain, fat-soluble compounds may require a higher extraction temperature. This may provide more energy for the less volatile compounds to overcome barriers that bind them to the matrix, by increasing the vapor pressure for the mass transfer process (Zhang and Pawliszyn, 1993), thus facilitating the release of volatile compounds into the headspace (Ma et al., 2013). (Ma et al., 2013). For a method focused on extracting alcohol compounds, the extraction time could have a significant impact as some studies suggest that compounds that bind to the fiber early in the extraction process (usually the most volatile), such as alcohol compounds, may be replaced by compounds that reach the

gaseous phase later, but have a greater affinity for the fiber polymer (Mondello et al., 2005).

Extraction time and temperature are generally accepted to have significant effects on extraction efficiency using HS-SPME analysis (Pawliszyn, 2000, Nongonierma et al., 2006, Januszkiewicz et al., 2008, Bezerra et al., 2016). In this study, significant model terms were extraction temperature, and the interaction between extraction time and temperature. Sample amount was not significant to the overall extraction efficiency of the model (*p* 0.9365). It has been reported that sample amount will not have an effect on the extraction efficiency of polar compounds, whereas the opposite has been observed for non-polar compounds (Penton, 1999). Perhaps this is why sample amount did not have a significant effect here, as the polarities of the target compounds varied.

2.6 Conclusions

The study presents an optimized validated HS-SPME GC-MS method for the simultaneous detection and quantification of varying classes of volatile compounds derived from LO in WMP. The optimal conditions for the HS-SPME extraction of volatile compounds known to be important in the sensory perception of WMP were 45 min extraction time combined with an extraction temperature of 43°C. The optimum sample amount was found to be 2.40g. Optimization of the design model was confirmed using the desirability factor (0.924). LOD and LOQ values were sufficiently sensitive to identify and quantify the target compounds; moreover, the lowest level of the calibration curve chosen for method validation (0.05 mg/L) was significantly lower than that found in a typical commercial WMP sample (~0.4-0.6 mg/L).

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Conflict of interest: The authors declare no conflict of interest.

Chapter 2 Highlights

- Thirteen compounds derived from LO were quantified by HS-SPME GC–MS.
- A central composite rotatable design was used to optimize HS-SPME parameters.
- Response surface methodology assessed the effects of extraction parameters.
- Method validation included LOD, LOQ, and precision.

Chapter 3: Correlating Consumer Sensory Data with the Volatile Profile of Dairy Powders during Storage

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Article

Correlating Volatile Lipid Oxidation Compounds with Consumer Sensory Data in Dairy Based Powders during Storage

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Abstract

Lipid oxidation (LO) is a recognised problem in dairy powders due to the formation of volatile odour compounds that can negatively impact on sensory perception. Three commercial dairy powders; fat-filled whole milk powder (FFWMP), skim milk powder (SMP) and infant milk formula (IMF), stored under different conditions (21 °C, 37 °C or 25 °C with 50% humidity) were evaluated by consumer acceptance studies, ranked descriptive sensory analysis and by LO volatile profiling using headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME GCMS) over 16 weeks. Significant (P = 0.001) differences in the concentration of LO compounds and sensory perception were

evident between sample types at the different storage conditions. The sensory acceptance scores for FFWMP and SMP remained stable throughout storage, but the IMF sample was perceived negatively from the start of storage. Increases in hexanal, heptanal and pentanal were correlated with 'painty', 'oxidised', 'cooked' and 'caramelised' attributes in the FFWMP, IMF and SMP powders. Levels of some LO volatiles in the IMF sample were far in excess of FFWMP and SMP and also above their odour thresholds, presumably due to the addition of polyunsaturated fatty acids (PUFA) in the formulation.

Keywords: dairy powder, infant milk formula, lipid oxidation, fatty acid, sensory, flavour, volatile profile

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3.1 Introduction

Lipid oxidation (LO) is a well-documented cause of quality deterioration in dairy powders (Park and Drake, 2014). Oxidation of unsaturated fatty acids (FA) results in the formation of a complex mixture of primary and secondary compounds including aldehydes, ketones and alcohols that impart off-flavours and limit shelf-life and storage stability (Frankel, 2014). Sensory evaluation coupled with instrumental techniques such as headspace solid-phase microextraction gas chromatography mass spectrometry (HS-SPME GCMS) have been employed for the detection and quantification of volatile aromatic compounds in various dairy products (Croissant et al., 2007, Aprea et al., 2016). However, fewer studies exist that link volatile aromatic compounds to their corresponding sensory attribute(s) and/or changes in consumer perception over shelf life (Lloyd et al., 2009a, Chen et al., 2018). Some studies have been carried out investigating the effects of exposing whole milk powder (WMP) to accelerated storage temperatures (45 °C) on the products oxidative stability (Stapelfeldt et al., 1997), LO of WMP (Thomsen et al., 2005), and flavour and shelf-life of WMP (Lloyd et al., 2009a). However, less information is available on the effect of storage conditions on skim milk powder (SMP) (Ford et al., 1983, Fitzpatrick et al., 2004) and infant milk formula (IMF) (Angulo et al., 1998, Cesa et al., 2015, Jia et al., 2019). IMF undergoes extreme processing with the aim of simulating human breast milk from bovine milk as closely as possible (Lopez et al., 2015). However, these additional processing steps along with the addition of

polyunsaturated fatty acids (PUFA) can increase LO susceptibility (MacLean Jr et al., 2010). IMF is usually fortified with PUFA such as linoleic acid (C18:2 n6), α-linolenic (C18:3 n3), arachidonic (C20:4 n6) and docosahexaenoic acid (C22:6 n3) (Tvrzicka et al., 2011), prior to thermal processing i.e. spray drying for potential health benefits. Due to their unsaturated nature and low oxidative stability, PUFA are readily degraded to primary and secondary oxidation products (Kilcawley et al., 2018), a process that can be initiated by the high inlet temperatures required for spray drying (120 – 180 °C) and contact with oxygen (Cesa et al., 2015). Hydroperoxides are the initial products formed by the LO cascade process and are unstable and reactive, eventually forming compounds that are known to cause off-flavours in dairy powders, such as aldehydes and ketones (Kilcawley et al., 2018). Thus, having more information on volatile products of LO is important regarding the stability of products throughout their shelf-life. LO products impart specific off-flavours on milk powders; some of the most documented include 'painty', 'metallic', 'fishy' and 'grassy'. Optimised descriptive profiling (ODP) has previously been used to assess consumer acceptability of dairy products (Chizoti et al., 2018, Faulkner et al., 2018). The present study investigates the concentrations of 13 volatile LO products, in three types of dairy powders (fat-filled (FF) WMP, SMP and IMF) by a validated HS-SPME GCMS method (Clarke et al., 2019) in combination with hedonic and ODP assessment over a controlled 16 week period at different storage conditions.

3.2 Materials and Methods

3.2.1 Powder Samples

Three batches of FFWMP and SMP were obtained from local suppliers as commercial products, while three batches of IMF were purchased from local retailers. FFWMP, SMP and IMF samples were manufactured on the following dates: December 2017, May 2018 and October 2017, respectively, each with a 24-month shelf-life. The FFWMP, SMP and IMF were 11, 3, and 10 months into their shelf-life, respectively, at the beginning of the study. For each sample type (FFWMP, SMP and IMF), the three batches were mixed together thoroughly to remove batch effect and to create a bulk sample. The bulk sample was subsequently separated into four 1.5kg lots and placed in light-omitting sealed bags at the beginning of the study (T0). One bag of each sample was stored at one of the four storage treatments; -18 °C

(control), 21 °C (ambient), 37 °C (accelerated) and 25 °C with humidity controlled at 50% (humidity) resulting in 12 samples in total. Samples were stored at -18 °C (freezer) and 21 °C and 37 °C (incubation rooms) at the Teagasc Food Research Centre (Fermoy, Cork, Ireland) and samples kept at 25 °C with humidity controlled at 50% were stored in a Binder KBF P Series Humidity Test Chamber (Binder GmbH, Tuttlingen, Germany) located at University College Cork (Cork, Ireland). Ambient is denoted as **AM**, control as **CON**, accelerated as **ACC** and humidity as **HUM** throughout the study, unless otherwise stated. Volatile data was undertaken at T0, T2, T4, T6, T8, T10, T12, T14 and T16 which represent the number of weeks of storage. Sensory analysis was carried out at T4, T8, T12 and T16 for practical purposes and sensory and volatile data were correlated at T4, T8, T12 and T16. An additional six IMF samples were purchased from local retail units and immediately analysed for LO volatiles (in triplicate) for comparative purposes as it was necessary to get a better understanding of the potential LO range in these products as little or no data was available.

3.2.2 Compound Selection

Thirteen volatile aromatic compounds including seven aldehydes; hexanal, pentanal, heptanal, octanal, (E)-2-nonenal 2,4-decadienal, and undecanal, four ketones; 2-heptanone, 2-nonanone, 2-pentanone and 3-octen-2-one and two alcohols; 1-heptanol and 1-pentanol, known to be important to the sensory perception of dairy products were selected for quantification based on current literature (Van Aardt et al., 2005, Faulkner et al., 2018, Kilcawley et al., 2018). Authentic standards for each of the target compounds and internal standard compounds (isovaleraldehyde, 2-methyl-3-heptanone and 4-methyl-2-pentanol) were purchased from Merck (Arklow, Wicklow, Ireland).

3.2.3 Powder Composition

Each sample was analysed for fat, protein, lactose, true protein and casein content using a Bentley DairySpec FT (Technopath Distribution, Co. Tipperary, Ireland). Samples were reconstituted to 10% total solids for SMP and 13% for FFWMP and IMF, as per the manufacturer's instructions using distilled water (dH_2O) 24 h prior to analysis. Samples were heated to ~40 °C immediately before sampling. Results were expressed as the averages of 2 replicates.

3.2.4 Microbial Analysis

The pour plate method was used to estimate the total bacterial count of the reconstituted milk powder samples prior to each sensory evaluation session. Serial dilutions from 10^0-10^4 were prepared in 9 mL maximum recover diluent (Thermo Fisher Scientific Oxoid CM0733, Basingstoke, UK). 1 mL of each dilution was pipetted onto sterile petri dishes and covered with warm (45 ± 2 °C), sterile milk plate count agar (15 mL) (MPCA; Thermo Fisher Scientific Oxoid CM0681, Basingstoke, UK). The mixture was allowed to cool and solidify, and plates were incubated for 72 h at 30 °C. Analysis was performed in duplicate.

3.2.5 Milk Powder Colour Measurements

Colour measurements were performed on each of the 12 milk powder samples according to the CIE Lab system (CIE, 1978, L* is a measure of lightness; a* is a measure of green-to-red colour on a negative to positive scale, respectively and b* is a measure of blue-to-yellow colour on a negative to positive scale, respectively), using a Minolta Colourimeter (Minolta Camera, Osaka, Japan). Samples were reconstituted in dH_2O 24 h prior to analysis and chilled at 4 °C. Approximately 2 mL of sample was placed in a spectrophotometric cuvette 1 h prior to analysis. Results were expressed as the average of triplicate measurements of each liquid sample (Faulkner et al., 2018).

3.2.6 Fatty acid analysis

Lipid extraction and methyl ester derivatisation of triglycerides were carried out as per De Jong and Badings (1990) and O'Callaghan et al. (2019). All milk powder samples were reconstituted to 12% total solids 1 h prior to analysis and 10 mL of reconstituted milk powder was used for analysis. Chromatographic conditions are also as outlined by O'Callaghan et al. (2019). Briefly, analysis was performed on an Agilent 7890A gas chromatograph, equipped with an Agilent 7693 autosampler (Agilent Technologies Ltd, Cork, Ireland) and flame ionised detector (FID). The column was a Select FAME capillary column (100 m × 250 µm Internal diameter (I.D.), 0.25 µm phase thickness, part number: CP7420) (Agilent Technologies Ltd). The injector was held at 250 °C for the entire run and was operated in split mode using a split ratio of 1:10. The inlet liner was a split gooseneck liner (Part no.: 8004–

0164, Agilent Technologies Ltd). The column oven was held at 80 °C for 8 min and raised to 200 °C at 8.5 °C/min, and held for 55 min. The total runtime was 77.12 min. The FID was operated at 300 °C. The carrier gas was hydrogen and was held at a constant flow of 1.0 mL/min. Results were processed using OpenLab CDS Chemstation edition software version Rev.C.01.04 (35) (Agilent Technologies Ltd).

A 37 component fatty acid methyl ester (FAME) reference mix containing C4:0 to C24:0 (Part number 35077) (Thames Restek Ltd., Buckinghamshire, UK) was analysed as an in-run quality control sample, with the FAMEs present at 60–180 ppm concentration. This was used to ensure accurate quantitation was achieved throughout sample analysis. The FAME mix was analysed once every five samples in the sequence. Accuracy was monitored by comparing the measured concentration of the FAME mix against its true concentration.

3.2.7 Volatile Analysis

3.2.7.1 HS-SPME Conditions

Headspace solid-phase microextraction (HS-SPME) analysis was carried out using a HS-SPME method optimized for the detection and quantification of LO compounds in WMP as per Clarke et al. (2019). Briefly, 2.40g of each powder sample was weighed out directly into La-Pha-Pack headspace vials (20 mL) with magnetic screw caps and Silicone/Polytetrafluoroethylene 1.3mm 45° Shore A septa (Apex Scientific Ltd, Maynooth Co.Kildare, Ireland). To each sample, 250 µL of the internal standard mixture (2-methyl-3-heptanone, 4-methyl-2-pentanol and isovaleraldehyde) prepared at 0.001% (w/v) in dH_2O was added along with 3.5 mL dH_2O. A calibration curve was prepared by spiking a set of the hydrated FFWMP samples with varying levels of the external standard mixture (13 compounds of interest prepared at 0.004% (w/v) in dH_2O). Matrix (control) samples (FFWMP sample + dH_2O only) were also included in each run. Samples were extracted for 45 minutes at a temperature of 43 °C. Each sample was subjected to a 10-minute pre-extraction incubation time at 43 °C with pulsed agitation of 5s at 500rpm, automated by a Bruker CombiPal autosampler (Elementec Ltd, Maynooth, Co. Kildare, Ireland). Each sample was analysed in triplicate every two weeks during the 16-week storage period.

3.2.7.2 GC Conditions

Incubation, extraction and injection processes were implemented using a Bruker CombiPal autosampler (Elementec Ltd, Ireland). A mid-polar DB 624 UI column (60m x 0.32mm x 1.80µm) (Agilent Technologies Ltd, Ireland) and a 2cm, 50/30µm, DVB/Carboxen/PDMS Stableflex SPME fiber (Agilent Technologies Ltd, Ireland) were used for the duration of the study. Following extraction, the SPME fiber was retracted and injected into the split/splitless 1177 GC inlet for 5 min at 250 °C in split mode at a ratio of 10:1 followed by 2 min at 270 °C in a bake-out station to minimise carry-over of compounds. The column oven was held at 65 °C for 10 min, then increased to 240 °C at a rate of 10 °C/min and held for 4 min. Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min.

3.2.8 Sensory Analysis

Powder samples were reconstituted in dH_2O based on the fat and protein content as per the equation outlined by the IDF (IDF, 1997), to a total volume of 1.5 L 24 h prior to scoring and chilled 4 °C. Samples were allowed to equilibrate to room temperature before sampling commenced. Acceptance testing (hedonics) and ODP (dos Santos Navarro et al., 2012, dos Santos Navarro et al., 2013) was undertaken with panels in Ireland. Assessors were trained in University College Cork, Ireland (n = 18). These assessors were presented with all samples simultaneously but with randomised order to prevent first order and carry-over effects (MacFie et al., 1989). Assessors used the consensus list of sensory descriptors which were measured on a 10 cm line scale with the term “none” used as the anchor point for the 0 cm end of the scale and “extreme” for the 10 cm end of the scale. For this study training and the use of a consensus sensory lexicon were used as described by (Cheng et al., 2020). Sensory terms, which were the main sensory dimensions, were pre-selected from the sample set using an expert sensory panel (n = 10). Assessors evaluated the intensity of each attribute for each sample on the scales. Attributes were presented along with the table describing the sensory terms (Cheng et al., 2020). All milk powder samples were labelled with random three-digit codes and presented in triplicate. Panellits were given as much time as they required to complete the scoring and were provided with unlimited water.

3.2.9 Statistical Analysis

Statistical analysis for data relating to colour, composition and microbial analysis was carried out using one-way ANOVA with post-hoc Tukey tests using Statistical Package for the Social Sciences (SPSS) software, version 24 (IBM Statistics Inc., Armonk, NY). Pearson correlation analysis was carried out on the sensory and volatile data using SPSS. Principal component analysis (PCA) biplots of the volatile vs. sensory data were used to demonstrate correlations between the volatile compounds and the sensory attributes. These were constructed using the 'factoextra' and 'FactoMinoR' packages in R (v 3.4.1) (R Core Team, 2013).

3.3 Results and Discussion

3.3.1 General Powder Characteristics

Compositional results for the 12 reconstituted milk powder samples (10% total solids for SMP; 13% for FFWMP and IMF) are outlined in Table S3.1. The fat, protein, true protein and casein content varied significantly ($P \leq 0.05$) between the powders. As expected, the FFWMP contained the highest fat content (3.77%) and the SMP the lowest (0.02%). The IMF contained the highest level of lactose across all the storage conditions (7.53-7.89%). True protein and casein values were higher in SMP (3.64 and 2.89, respectively) than FFWMP and IMF.

No significant differences were observed between the total bacterial counts for the 12 reconstituted milk powder samples (4x FFWMP, 4x SMP and 4x IMF) over the 16-week storage period (data not shown). Total bacteria counts were below the limit for powdered milk and milk based products intended for human consumption within Europe at all-time points (European Commission, 2001).

3.3.2 Milk Powder Colour Analysis

The L* (lightness), a* (green-to-red colour) and b* (blue-to-yellow colour) values were statistically different ($P \leq 0.001$) between the samples (Table S3.2). The L* and b* values were significantly ($P \leq 0.001$) higher in FFWMP samples than in SMP and IMF samples regardless of the storage treatments. The a* values were negative across all samples with significantly ($P \leq 0.001$) higher values observed for SMP than FFWMP and IMF samples across all treatments with the highest a* value observed in the SMP stored at 37 °C (-5.83). As expected, significantly ($P \leq 0.001$)

higher b* (yellowness) values were observed in FFWMP samples, due to the increased fat content (Pugliese et al., 2017). Increased b* values (blue-to-yellow colour) in dairy products as observed in the FFWMP powder samples have previously been associated with β-carotene content (Faulkner et al., 2018) which has been linked with dairy products produced from pasture (Martin et al., 2005). The significantly ($P \leq 0.001$) higher a* value (green-to-red colour) observed in SMP samples analysed in this study could be due to The Tyndall effect; the scattering of light as it passes through a colloid. The higher casein content in SMP (2.79% compared with 2.31% and 1.07% for FFWMP and IMF, respectively) scatters more blue light than red. Also, β-carotene is lost when milk is skimmed, removing the source of the yellow colour observed in FFWMP and IMF samples (Helmenstine, 2019). The L* (lightness) values were also significantly ($P \leq 0.001$) different between the samples. Several other factors such as the modification of particle size, Maillard reactions forming brownish pigments during heat treatment (Walstra et al., 2005), storage time and temperature could all have contributed to the differences in colour.

3.3.3 Fatty Acid Analysis

Significant differences were observed for 19 of the 27 FA analysed based on sample type (C4:0, C6:0, C10:0, C12:0, C13:0, C14:0, C14:1 c9, C15:0, C16:0, C17:0, C18:1 n9c, C18:2 n6c, C18:2 n6t, C18:3 n3, C20:0, C20:1, C24:1 n9, C20:5 and CLA C18:2 c9t11), as shown in Table 3.1.

Table 3.1: Fatty acid (FA) composition (g/100g of FA ± SD; n=3) of fat-filled whole milk powder (FFWMP), skim milk powder (SMP) and infant milk formula (IMF). Different superscripts within a row indicate significant differences between the samples (P = 0.05).

Fatty Acids	FFWMP	SMP	IMF	*P* - Value
Butyric acid C4:0	0.09 ± 0.02^{b}	21.03 ± 5.93^{a}	1.03 ± 0.07^{b}	0.014
Caproic acid C6:0	0.04 ± 0.01^{b}	6.64 ± 2.02^{a}	0.41 ± 0.01^{b}	0.018
Octanoic acid C8:0	0.04 ± 0.01	1.32 ± 1.86	0.44 ± 0.01	0.549
Decanoic acid C10:0	0.04 ± 0.01^{b}	3.98 ± 0.28^{a}	0.65 ± 0.03^{b}	<0.001
Undecanoic acid C11:0	ND	ND	0.01 ± 0.01	0.465
Lauric Acid C12:0	0.28 ± 0.02^{b}	4.40 ± 0.83^{a}	3.03 ± 0.15^{ac}	0.008
Tridecanoic acid C13:0	ND^{b}	ND^{b}	0.02 ± 0.01^{a}	<0.001
Myristic acid C14:0	0.49 ± 0.29^{b}	ND^{b}	2.43 ± 0.08^{a}	0.002
Myristoleic acid C14:1 c9	0.01 ± 0.01^{b}	ND^{b}	0.13 ± 0.01^{a}	0.001
Pentadecanoic acid C15:0	0.06 ± 0.01^{b}	ND^{c}	0.23 ± 0.01^{a}	<0.001
Palmitic acid C16:0	32.9 ± 3.71^{a}	17.18 ± 3.71^{bc}	20.06 ± 0.21^{b}	0.027
Palmitoleic acid C16:1 c9	0.13 ± 0.01	0.88 ±0.37	0.26 ± 0.01	0.075
Heptadecanoic acid C17:0	0.08 ± 0.01^{b}	ND^{c}	0.14 ± 0.01^{a}	0.001
Stearic acid C18:0	3.36 ± 0.37	1.22 ± 1.72	4.31 ± 0.07	0.114
Oleic acid C18:1 n9c	29.22 ± 2.89^{a}	10.59 ± 6.74^{b}	26.77 ± 0.45^{ab}	0.040
Elaidic acid C18:1 n9t	2.59 ± 0.36	2.01 ± 2.85	2.92 ± 0.03	0.863
Linoleic acid C18:2 n6c	8.40 ± 3.19^{b}	30.76 ± 2.99^{a}	17.32 ± 0.24^{bc}	0.007
trans-9,12-octadecadienoate C18:2 n6t	21.60 ± 3.25^{a}	ND^{b}	15.91 ± 0.28^{a}	0.003
α-Linolenic acid C18:3 n3	0.17 ± 0.02^{b}	ND^{c}	1.79 ± 0.04^{a}	<0.001
Gamma Linolenic Acid c18:3 n6	0.01 ± 0.01	ND	0.07 ± 0.01	0.151
Eicosanoic acid C20:0	0.24 ± 0.03^{a}	ND^{b}	0.24 ± 0.01^{a}	0.001
cis-11-Eicosenoic acid C20:1	0.10 ± 0.01^{b}	ND^{c}	0.24 ± 0.01^{a}	<0.001
Eicosenoic acid C20:2	ND	ND	0.01 ± 0.02	0.465
Eicosadienoic acid C20:3 n6	ND	ND	0.01 ± 0.02	0.465
Nervonic acid C24:1 n9	ND^{b}	ND^{b}	0.28 ± 0.05^{a}	0.004
Eicosapentaenoic acid C20:5	ND^{b}	ND^{b}	0.05 ± 0.05a	<0.001
CLA C18:2 c9t11	0.14 ± 0.03^{b}	ND^{c}	1.28 ± 0.01a	<0.001

CLA: conjugated linoleic acid; ND: not detected; c: *cis*; t: *trans*.

The FA composition of milk is generally derived from two primary sources, *via* uptake of existing FA or through de novo synthesis (Lock and Bauman, 2004). C4:0-C14:0 and some C16:0 are synthesised de novo by the cows mammary gland which uses acetate and ß-hydroxybutyrate as substrates (Lock and Bauman, 2004). The remaining C16:0 and the long chain FA originate from dietary lipids and from lipolysis of adipose tissue triacylglycerols (Parodi, 2004). Bovine diet has been shown to significantly impact the FA profile of milk (Tripathi, 2014, O'Callaghan et al., 2016), and subsequently milk powder. The FA with an odd number of carbons such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are synthesised by microflora in the rumen (German and Dillard, 2006). The FA profile of the FFWMP and the IMF varied more than the SMP due to the higher fat content of these powders. Less FA was detected in SMP due to the low-fat content. FFWMP contained significantly ($P \leq 0.05$) higher proportions of palmitic acid (C16:0) than the SMP and IMF samples. The IMF sample contained higher proportions of linoleic acid (C18:2 n6c) and α-linolenic acid (C18:3 n3) than the FFWMP, likely due to fortification. The FA C20:2, C20:3 n6, C24:1 n9 and C20:5 were identified only in the IMF sample. Some of these FA are found in fish oils (DURMUŞ, 2018) and vegetable oils (Aung et al., 2018, Castro et al., 2019), and thus are the likely sources in the IMF sample and are added during processing. The probable FA sources for the 13 compounds of interest are outlined in Table 3.2.

Table 3.2: The probable fatty acid sources for the 13 volatile compounds of interest.

Class	Compound	Fatty acid source	Reference
Aldehyde	Hexanal	Oleic and linoleic acid	Kilcawley et al. (2018)
	Pentanal	Arachidonic and linoleic acid	Kilcawley et al. (2018)
	Heptanal	Possibly linoleic and oleic acid	Lee and Min (2009)
	Octanal	Oleic acid	Decker et al. (2010)
	(E)-2-Nonenal	Linoleic and possibly palmitoleic acid	Decker et al. (2010)
	2,4-Decadienal	Linoleic acid	Decker et al. (2010)
	Undecanal	Possibly oleic acid	-
Ketone	2-Nonanone	Decanoic acid	Charalambous (2012)
	2-Heptanone	Octanoic acid	Charalambous (2012)
	2-Pentanone	Hexanoic acid	Charalambous (2012)
	3-Octen-2-one	Arachidonic and linoleic acid	Kilcawley et al. (2018)
Alcohol	1-Heptanol	Possibly lipid oxidation of heptanal	-
	1-Pentanol	Lipid oxidation of pentanal	Kilcawley et al. (2018)

3.3.4 HS-SPME-GCMS Volatile Analysis

3.3.4.1 FFWMP

Volatile analysis was carried out on each of the milk powder samples by HS-SPME GCMS every two weeks (T0, T2, T4, T6, T8, T10, T12, T14, and T16) over the 16-week storage period. The 13 selected volatile aromatic compounds were quantified at each time point. As expected, the highest levels of primary oxidation products were observed in FFWMP at T16 for ACC. Significant differences were observed in the concentrations of ten of the LO compounds in samples stored at ACC, nine stored at CON, seven stored at AM and ten stored at HUM.

3.3.4.2 SMP

Much less variability in the levels of aldehyde and ketone compounds were observed in the SMP samples. However, the alcohol compounds, 1-heptanol and 1-pentanol were more unstable in the SMP samples. As with the FFWMP samples, more compounds (ten) varied significantly in the SMP samples stored at ACC than samples stored at CON (two), AM (three) and HUM (five).

3.3.4.3 IMF

When compared to FFWMP and SMP, increased levels of some oxidation products were observed in IMF sample at T0 and throughout the study at all-time points and storage conditions (CON, AM, ACC and HUM). Hexanal, pentanal, heptanal, octanal, 2,4-decadienal and 2-nonanone were present at 5986, 1209, 861, 784, 154 and 5170 ppm, respectively at T0. The highest levels LO volatiles were observed at T12 and T16 in IMF samples stored at ACC (similar to the trend for the FFWMP samples). The concentrations of 11 target compounds varied significantly during ACC storage, 11 compounds varied significantly in CON samples, seven in AM samples and 11 in HUM samples. Concentration (mg/kg of powder) results for each volatile compound are outlined in Table 3.3.

Table 3.3: Concentrations (mg/kg of powder; n=3) of the 13 volatile compounds in the fat-filled whole milk powder (FFWMP), skim milk powder (SMP) and infant milk formula (IMF) samples at each time point (T0, T4, T8, T12 and T16). CON: control; -18 °C, AM: ambient; 21 °C, ACC: accelerated; 37 °C, HUM: humidity; 25 °C and 50% humidity. * $P = 0.05$, *** $P = 0.001$.

Compound	LRI	T0 FFWMP	T4 FFWMP (CON)	T8 FFWMP (CON)	T12 FFWMP (CON)	T16 FFWMP (CON)	*P*-value	T4 FFWMP (AM)	T8 FFWMP (AM)	T12 FFWMP (AM)	T16 FFWMP (AM)	*P*-value	T4 FFWMP (ACC)	T8 FFWMP (ACC)	T12 FFWMP (ACC)	T16 FFWMP (ACC)	*P*-value	T4 FFWMP (HUM)	T8 FFWMP (HUM)	T12 FFWMP (HUM)	T16 FFWMP (HUM)	*P*-Value
Hexanal	840	0	277	197	133	82	*	193	145	132	118	***	259	189	324	382	***	217	207	174	124	*
Pentanal	735	0	11	9	13	6	*	10	8	14	10	***	13	13	56	41	***	9	12	18	16	*
Heptanal	944	15	42	17	15	20	*	35	22	19	23	NS	31	20	40	50	***	24	20	26	25	*
Octanal	1047	22	33	17	18	21	*	36	15	21	12	NS	28	12	30	37	***	30	25	23	28	*
(E)-2-Nonenal	1151	14	5	9	10	9	*	6	11	16	12	NS	4	4	6	5	***	12	9	9	11	*
2,4-Decadienal	1399	37	2	15	20	17	*	6	33	61	54	NS	8	15	20	22	***	6	53	31	29	*
Undecanal	1359	7	3	5	3	2	*	4	7	13	9	***	4	4	32	17	***	19	34	17	14	*
2-Nonanone	1140	0	17	10	82	66	*	21	12	3	2	***	5	1	30	109	***	5	4	36	92	NS
2-Heptanone	935	10	9	6	5	6	NS	28	7	7	6	NS	19	6	16	19	***	22	6	8	8	NS
2-Pentanone	730	2	1	3	3	1	NS	2	1	1	0	***	1	2	2	10	NS	2	1	1	5	*
3-Octen-2-one	1096	20	21	10	4	19	NS	24	9	5	7	***	6	2	7	9	***	6	6	3	3	*
1-Heptanol	1016	0	0	41	19	31	*	0	36	50	32	***	0	29	51	46	NS	0	55	48	38	*
1-Pentanol	815	70	60	329	136	189	NS	54	274	311	17	NS	48	365	39	48	NS	74	35	124	1793	NS
Compound	**LRI**	**T0 SMP**	**T4 SMP (CON)**	**T8 SMP (CON)**	**T12 SMP (CON)**	**T16 SMP (CON)**	***P*-value**	**T4 SMP (AM)**	**T8 SMP (AM)**	**T12 SMP (AM)**	**T16 SMP (AM)**	***P*-value**	**T4 SMP (ACC)**	**T8 SMP (ACC)**	**T12 SMP (ACC)**	**T16 SMP (ACC)**	***P*-value**	**T4 SMP (HUM)**	**T8 SMP (HUM)**	**T12 SMP (HUM)**	**T16 SMP (HUM)**	***P*-Value**
Hexanal	840	0	14	42	28	20	NS	2	4	29	11	*	14	48	29	10	*	8	42	19	8	*
Pentanal	735	3	2	1	2	1	NS	7	1	2	2	NS	2	4	1	1	*	3	3	1	1	NS
Heptanal	944	16	10	16	5	7	NS	16	13	6	8	NS	8	4	5	9	*	16	15	1	3	*

Octanal	1047	18	12	6	13	17	*	17	7	9	4	NS	10	5	7	7	*	10	9	10	12	NS
(E)-2-Nonenal	1151	20	2	4	2	2	NS	2	5	4	3	NS	2	2	1	2	*	3	2	2	3	NS
2,4-Decadienal	1399	15	1	6	5	5	NS	1	8	8	7	NS	1	4	4	4	*	1	8	5	6	NS
Undecanal	1359	5	1	2	1	2	NS	1	4	3	2	NS	1	2	1	1	*	2	2	2	1	NS
2-Nonanone	1140	7	6	5	3	3	*	7	5	4	3	*	4	4	6	8	*	4	3	4	4	*
2-Heptanone	935	9	6	7	3	3	NS	7	5	6	4	NS	4	5	7	13	*	5	5	7	9	NS
2-Pentanone	730	1	1	0	0	0	NS	1	0	0	0	NS	1	0	0	0	NS	1	0	0	0	NS
3-Octen-2-one	1096	4	4	2	1	1	NS	5	3	0	2	NS	2	1	0	1	*	1	6	1	1	*
1-Heptanol	1016	37	0	55	61	49	NS	0	61	16	22	NS	0	46	35	24	NS	0	41	43	53	*
1-Pentanol	815	0	61	83	4710	82	NS	63	32	123	47	*	62	1014	82	50	NS	56	105	47	51	NS

Compound	**LRI**	**T0 IMF**	**T4 IMF (CON)**	**T8 IMF (CON)**	**T12 IMF (CON)**	**T16 IMF (CON)**	***P*-value**	**T4 IMF (AM)**	**T8 IMF (AM)**	**T12 IMF (AM)**	**T16 IMF (AM)**	***P*-value**	**T4 IMF (ACC)**	**T8 IMF (ACC)**	**T12 IMF (ACC)**	**T16 IMF (ACC)**	***P*-value**	**T4 IMF (HUM)**	**T8 IMF (HUM)**	**T12 IMF (HUM)**	**T16 IMF (HUM)**	***P*-Value**
Hexanal	840	5986	10674	13408	11700	13408	*	14364	17047	14581	17047	*	15628	19874	20741	19874	*	12550	18947	14353	15659	*
Pentanal	735	1209	1200	1111	1520	1111	*	1367	1530	2006	1530	*	3934	3621	3999	3621	*	1348	1401	1628	1641	*
Heptanal	944	861	915	1080	915	1080	*	1310	1313	1143	1313	*	1322	1410	1588	1410	*	1095	1399	1055	1295	*
Octanal	1047	784	608	864	803	864	*	910	1095	1039	1095	NS	133	146	141	146	*	799	1370	841	1090	NS
(E)-2-Nonenal	1151	64	36	53	60	53	*	46	79	93	79	*	48	74	110	74	*	55	86	132	97	*
2,4-Decadienal	1399	154	25	33	60	33	*	17	82	120	82	NS	39	72	136	72	*	37	82	148	122	*
Undecanal	1359	97	3	6	3	6	NS	6	18	11	18	NS	69	7	68	7	*	32	5	16	5	*
2-Nonanone	1140	5170	31	30	23	30	*	53	44	29	44	*	29	32	30	32	*	7064	7048	3871	3066	*
2-Heptanone	935	55	49	44	48	44	*	53	66	57	66	NS	51	69	81	69	*	49	49	48	57	*
2-Pentanone	730	8	10	9	9	9	*	7	13	11	13	*	11	15	16	15	*	8	9	14	15	*
3-Octen-2-one	1096	38	76	67	155	67	*	171	166	32	127	NS	174	147	218	147	NS	25	26	96	95	*
1-Heptanol	1016	0	0	1485	1309	1485	NS	0	2341	888	2341	NS	0	1731	1419	1191	NS	0	2201	1656	1116	NS

1-Pentanol	815	62	534	68	85	68	*	685	112	69	112	*	848	102	129	102	*	1250	107	85	85	*

LRI: Linear retention index. NS: not significant.

The concentrations of the aldehydes hexanal, pentanal and heptanal increased throughout storage in the both the FFWMP and IMF samples stored at ACC (3.77 and 2.91% fat, respectively). The sensory attributes associated with these compounds have been described as painty, cardboard-like, and grassy (Faulkner et al., 2018, Kilcawley et al., 2018). Hexanal and 2-heptanone have been found to be good predictors of 'grassy flavour' in previous studies while hexanal, octanal, and 3-octen-2-one have been found to be good predictors of 'painty flavour' in WMP (Lloyd et al., 2009a). Hexanal has also been identified as the main contributor to 'oxidised flavour' often observed in dairy powders as LO progresses (Li et al., 2012). Increased concentrations of octanal and 3-octen-2-one were observed in IMF samples compared to FFWMP and SMP samples. Li et al. (2012) reported higher levels of octanal and 3-octen-2-one in concentrated milk and milk powders than in raw and heated milk, suggesting that these compounds are likely thermally induced LO products. Park and Drake (2016b) also reported increases in octanal in liquid condensed milk after 24 h storage, indicative of LO. However, in the present study the levels of octanal fluctuated throughout storage in the IMF samples and the lowest levels were observed in ACC samples (142 ppm ± 5.32). (E)-2-Nonenal, 2,4-decadienal, and 1-heptanol were found to be higher in IMF samples, particularly in the samples stored at ACC. The concentrations of some other volatile compounds fluctuated and/or decreased during storage, possibly due to conversion and breakdown to other compounds that are not quantified in this study.

3.3.5 Sensory Evaluation

No significant differences were observed between the FFWMP or SMP samples for hedonic scoring over the storage period. Significant differences were found between liking of flavour and overall acceptability for the IMF samples. The IMF samples scored lowest for overall acceptability across all time points and storage treatments. At T4, significant differences were observed between the FFWMP, SMP and IMF samples for liking of appearance, liking of aroma, liking of flavour, overall acceptability, colour, creamy aroma, oxidised aroma, painty aroma, powdery texture, oxidised flavour and off-flavour. Again at T8, differences between the samples were observed for liking of appearance, liking of aroma, liking of flavour and overall acceptability, with IMF samples scoring the lowest for liking of aroma, liking of flavour and overall acceptability and highest for liking of appearance. SMP samples

scored highest for liking of aroma, liking of flavour and overall acceptability. In the ODP, differences were again observed for oxidised aroma', painty aroma, powdery texture' and 'off-flavour' in addition to 'rancid butter flavour' and 'painty flavour', all of which were more correlated with IMF samples except for 'powdery texture' which was more correlated with FFWMP samples. Finally, at T16 significant differences were observed for 'creamy aroma', 'oxidised aroma', 'painty aroma', 'painty flavour' and 'off-flavour' (Figure 3.1). This study demonstrated the ability of panellists to identify and rate the intensity of 'painty' and 'oxidised' attributes in powders with high levels of LO volatiles. When the samples were compared based on treatment type, fewer differences were observed suggesting the differences were based on sample type regardless of the storage conditions.

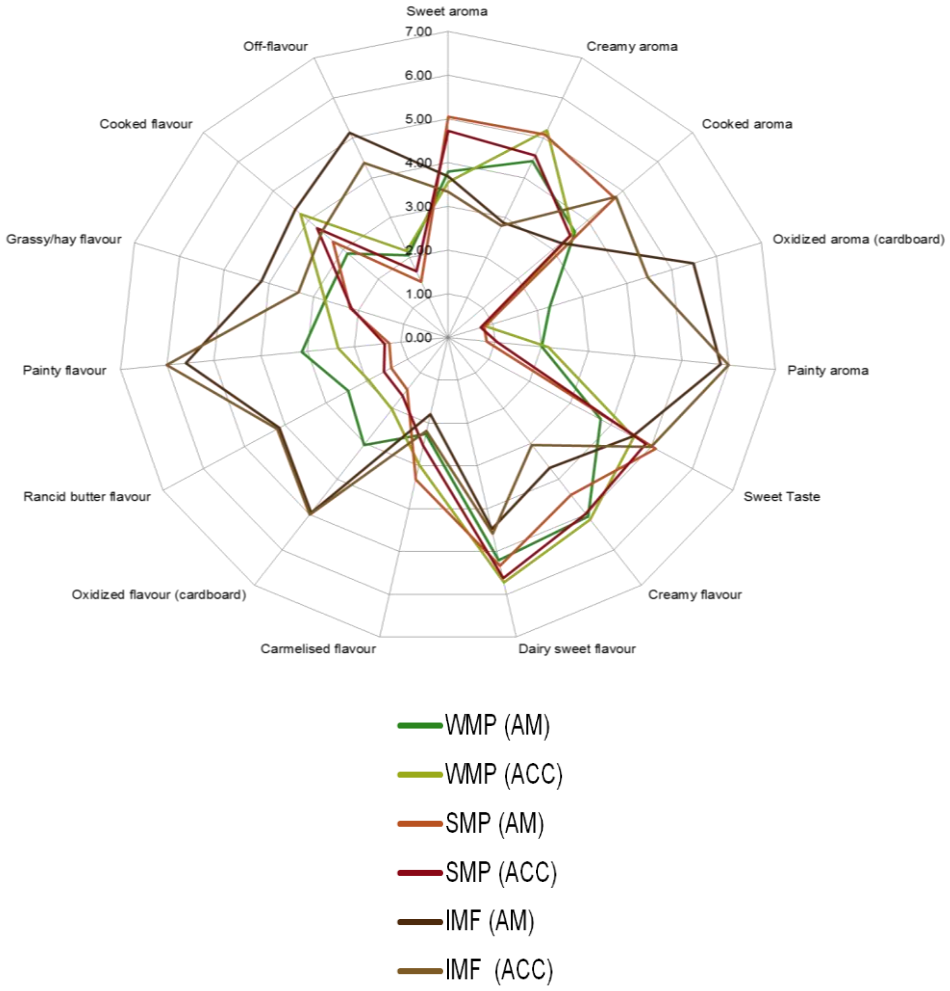


Figure 3.1: Radar plot illustrating the optimised descriptive profiling (ODP) scores for the sensory attributes evaluated at T16 for samples stored at 21°C (AM) and 37 °C (ACC).

The odour threshold of many volatile compounds is greater in oil and fat matrices when compared with water and air, generally due to the complexity of the matrix and possible matrix binding (Chambers and Koppel, 2013). In the present study, FFWMP samples contained hexanal at 380 ppm and pentanal at 56 ppm, but remained acceptable to the sensory panellists. Decker et al. (2010) reported an odour threshold (ppm) in oil of 320, 240, 55, and 10 for hexanal, pentanal, octanal, 2,4–decadienal, respectively. The increased levels of numerous compounds above their odour thresholds in IMF samples compared to FFWMP and SMP samples is likely responsible for the unacceptable scores of panellists for these IMF samples. Additionally, certain compounds such as (E)-2-octenal (linoleic acid degradation), (Z)-2-heptenal (linoleic acid degradation), (E)-2-hexenal (linolenic acid degradation), 2,4-heptadienal (linolenic acid degradation), 4-pentenal and (E,E)- 3,5-octadien-2-one (arachidonic and linoleic acid degradation) were identified only in IMF samples. 2,4–Decadienal, an oxidation product of linoleic acid has been described as having a 'frying' or 'fried' odour and is reported to have a pleasant association with high quality fried foods, it is only when the concentrations are excessive that a product becomes unacceptable to the consumer (Decker et al., 2010), as the odour changes to a more rancid off-note. 2,4-Decadienal was found to be correlated with 'rancid butter flavour' in FFWMP samples from T8 to T16, where the concentrations ranged from 15 – 61 ppm. However, 2,4–decadienal was also found to be correlated with 'painty aroma' and 'painty flavour'. Karahadian and Lindsay (1989) reported that 2,4–decadienal can cause 'painty flavours' in fish oils and this may also apply for dairy powders once the concentrations reach a certain level. Furthermore, similar volatiles could have the same descriptors in different products. For example, 2,4-decadienal and undecanal could both be descriptors of 'oxidised flavour' in FFWMP and IMF, respectively. 3-Octen-2-one was again highest in IMF samples followed by FFWMP and SMP samples. PCA analysis showed 3-octen-2-one was correlated with 'caramelised flavour' and 'sweet taste' in FFWMP, and 'oxidised flavour' and 'painty flavour' in IMF. Overall, relative humidity did not significantly affect the levels of any LO volatile compound as samples stored at HUM were comparable to those stored at AM. Most differences were observed between samples stored at AM and ACC, thus for clarity we have only focussed on AM and ACC samples in figures 3.2-3.5.

Many significant differences were observed for the hedonic and ODP scores, particularly between SMP and IMF samples, but also within SMP and IMF samples stored at different conditions at different time points. FFWMP remained stable and acceptable throughout the 16-week storage period despite increases in LO volatile compounds. This suggests that although these compounds were present at levels above their odour thresholds, and thus potentially perceivable, they did not adversely impact on sensory perception, presumably because they were not concentrated enough in the FFWMP matrix. SMP samples remained acceptable throughout storage, also scoring highest for 'overall acceptability' across the storage treatments. The IMF samples were found to be unacceptable at each time point.

Levels of the oxidation products hexanal, pentanal, heptanal were present at 5986, 1209 and 861 ppm, respectively in IMF samples at T0 with significant increases over storage. As previously mentioned, the level of sensory acceptability for IMF samples remained the same from T4 to T16 which suggests that once LO products reach certain levels above their odour thresholds panellists deemed the product as 'unsatisfactory'. Many of the descriptors commonly used to describe off-flavours associated with LO in dairy products were most correlated with IMF samples (Figure 3.2). Correlations between sensory data and volatile profiles for FFWMP, SMP and IMF are displayed in Figures 3.3, 3.4 and 3.5, respectively. 'Painty', 'oxidised', 'rancid butter' and 'off-flavours' were most correlated with the IMF samples at T12 and T16, corresponding with increases in hexanal, heptanal, pentanal (Figure 3.5).

The ability of the sensory panel to perceive differences in many sensory attributes in SMP was unexpected as it is typically less susceptible to LO due to its low-fat content. However, the lipid phase of WMP and other high-fat dairy powders could act as a solvent for LO compounds (Chevance and Farmer, 1999), thus, the lack of fat in SMP could mean that any oxidation products are more readily released and therefore are more easily perceived. It is difficult to compare the sensory perception of IMF to that of FFWMP and SMP for a number of reasons; (i) the differences in manufacturing processes and the addition of PUFA to IMF samples, (ii) the adult panellists employed in the study are not familiar with the consumption of IMF and (iii) it is impossible to gather information from the proposed consumers of IMF (infants and babies) on sensory perception. However, it is not unusual to see higher levels of LO products in IMF when compared to conventional milk powders, Cesa et al. (2015) reported the levels of malondialdehyde (MDA), a common indicator of the

LO process, up to five times higher in IMF compared to bovine milk samples. Furthermore, Jia et al. (2019) conducted an accelerated stability study on milk based IMF stored at 42 and 50 °C for 90 days. Results demonstrated little change in the FA profile of the IMF during storage except for docosahexaenoic acid (C22:0). However, differences in the volatile profiles were observed and an unpleasant 'oxidised flavour' was observed in IMF samples stored at 50 °C. Samples stored at 50 °C were found to have increased peroxide values and decreased headspace oxygen after 90 days of storage. As little or no changes in the FA profile were evident, it suggests that the susceptibility of IMF to LO depends mainly on the FA composition directly after manufacture and subsequently on the rate at which the FA oxidise.

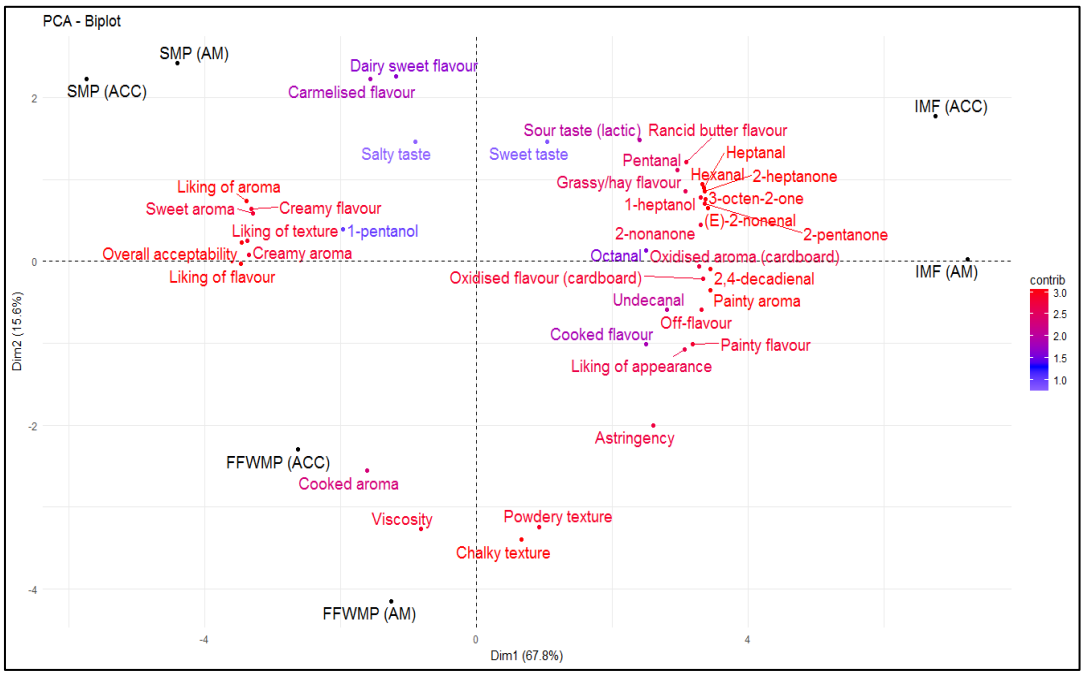


Figure 3.2: Principal component analysis (PCA) biplot of fat-filled whole milk powder (FFWMP), skim milk powder (SMP) and infant milk formula (IMF) samples at T8 demonstrating the trends observed throughout the study for the optimised descriptive profiling (ODP) and volatile analyses. AM: ambient; 21 °C, ACC: accelerated; 37 °C.

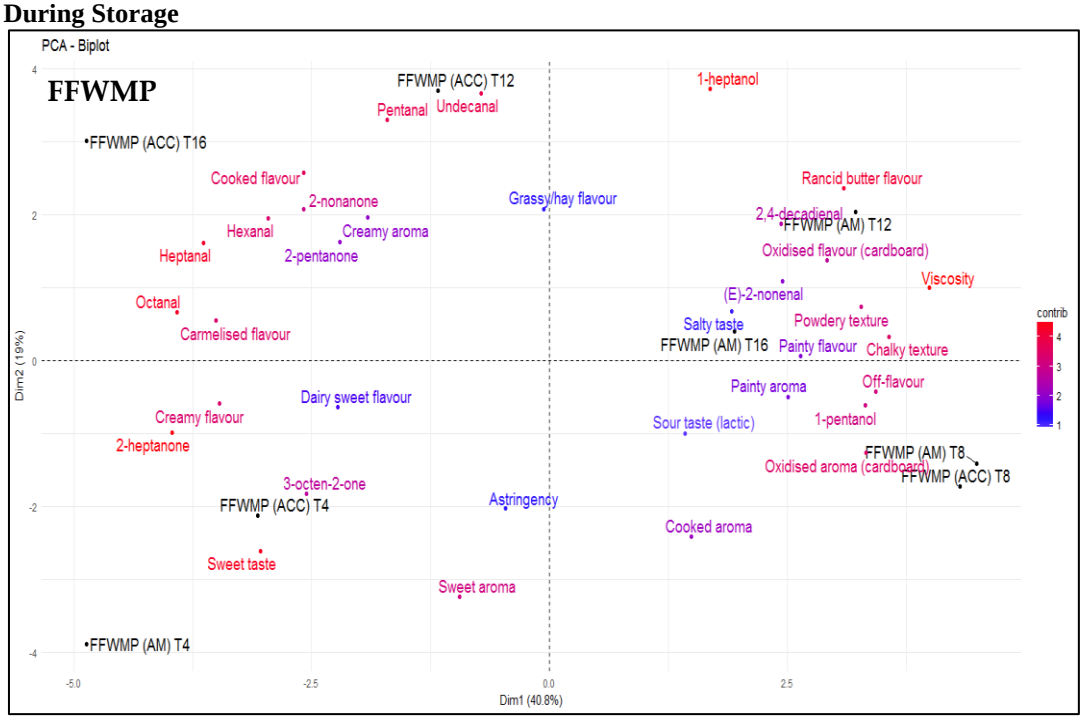


Figure 3.3: Principal component analysis (PCA) biplot demonstrating the correlations between the sensory perception and volatile compounds for fat-filled whole milk powder (FFWMP) at T4, T8, T12 and T16 of storage. AM: ambient; 21 °C, ACC: accelerated; 37 °C.

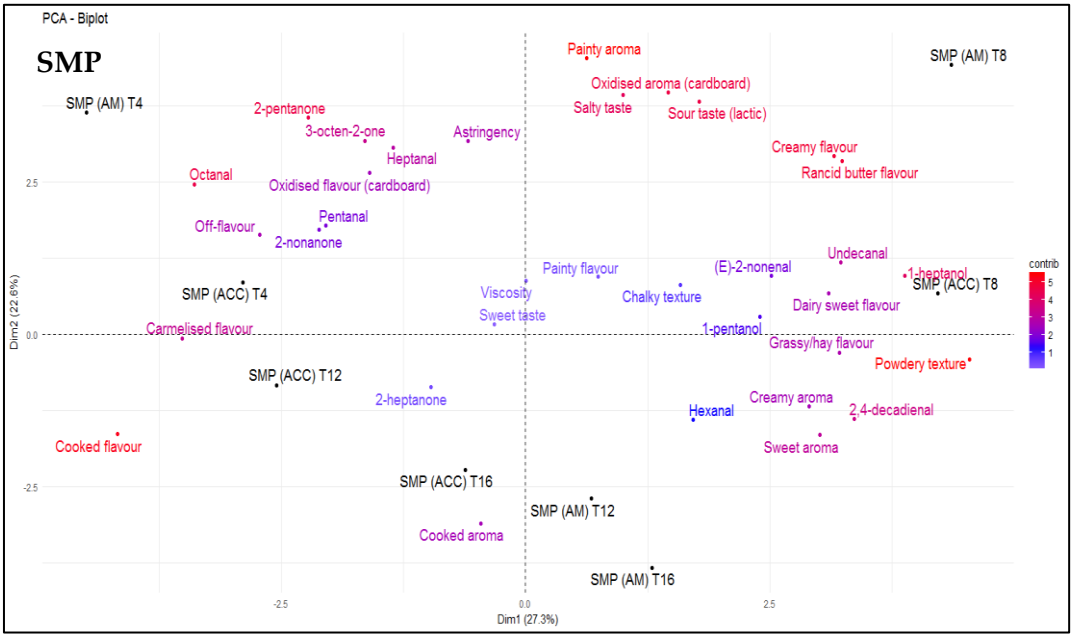


Figure 3.4: PCA biplot demonstrating the correlations between the sensory perception and volatile compounds for skim milk powder (SMP) at T4, T8, T12 and T16 of storage. AM: ambient; 21 °C, ACC: accelerated; 37 °C.

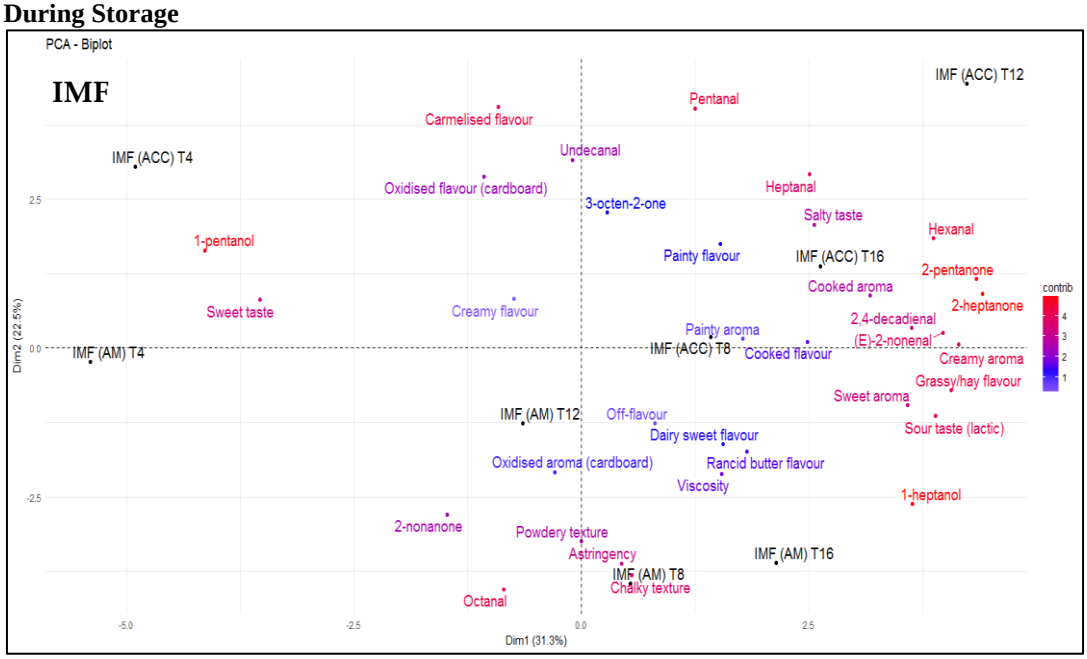


Figure 3.5: Principal component analysis (PCA) biplot demonstrating the correlations between the sensory perception and volatile compounds for infant milk formula (IMF) samples at T4, T8, T12 and T16 of storage. AM: ambient; 21 °C, ACC: accelerated; 37 °C.

Correlation relationships were observed between volatile compounds and sensory perception, and between the concentrations of individual LO volatile compounds. The main correlation relationships observed in FFWMP samples are outlined in Table 3.4. Furthermore, 'sweet taste' decreased as 'rancid butter flavour' increased in the AM FFWMP samples throughout storage. Less correlation was evident in SMP samples, however, 'cooked flavour' was negatively correlated with undecanal (-0.83). In the IMF samples, 'grassy/hay flavour' was positively correlated with 1-heptanol (r = 0.74) and 1-pentanol (r = 0.74), and 'off-flavours' were positively correlated with (E)-2-nonenal. 'Overall acceptance' was negatively correlated with 'oxidised aroma' (-0.53) in IMF samples and there was no correlation between 'overall acceptability' and sensory attributes in the FFWMP and SMP samples. Lloyd et al. (2009a) reported similar correlations for hexanal and heptanal and 'painty flavour' and 'grassy flavours' in WMP. The same study concluded that the optimum shelf life of WMP was approximately 12 weeks from a sensory standpoint.

Table 3.4: Correlation relationships between volatile organic compounds (VOCs) and sensory attributes observed in fat-filled whole milk powder (FFWMP). Positive and negative r values indicate significant ($P \leq 0.001$) positive and negative correlations between the VOCs and sensory attributes, respectively.

Volatile organic compound	Hexanal	Rancid butter flavour	Grassy/hay flavour	Painty flavour	Painty aroma	Oxidised flavour	Oxidised aroma	Creamy flavour	Creamy aroma
Hexanal	-	0.87	0.82	0.89	0.92	0.92	0.88	- 0.80	- 0.81
Pentanal	0.91	-	-	0.81	0.80	0.83	-	-	-
Heptanal	0.99	0.87	0.81	0.89	0.93	0.92	0.89	- 0.80	- 0.83
(E)-2-Nonenal	0.93	0.83	0.85	-	0.91	0.90	0.84	- 0.84	-
Octanal	-	-	-	-	0.80	-	-	-	-
2,4-Decadienal	-	-	-	-		0.80	-	-	-
2-Heptanone	-	0.82	0.80	-	0.90	0.88	-	-	- 0.81
2-Pentanone	-	0.84	0.84	-	0.91	0.88	-	-	-
1-Heptanol	-	-	0.81	-	-	-	-	-	-

In the present study, freshly opened WMP and SMP remained acceptable to the sensory panel after 16 weeks of storage despite levels of primary oxidation products being present above their odour thresholds. Storing samples at ACC versus AM accelerated the formation of LO compounds which contradicts the results by Cesa et al. (2015) which stated that 40 °C was too low a temperature to perform accelerated oxidation studies and that the levels of MDA in samples stored at 40 °C was comparable to those stored at 20 °C after 12 weeks of storage. The study by Cesa et al. (2015) recommended a temperature of 55 °C for performing acceleration studies. However, in the present study differences in the volatile profiles were evident in FFWMP and SMP samples stored at AM and ACC. MDA quantification also has limitations (Grotto et al., 2009, Papastergiadis et al., 2012), as other compounds can interfere with the assay and also that it is specific for only one LO chemical class, therefore accurate quantification of LO volatile compounds by HS-SPME GCMS as preformed in this study is much more reliable.

3.3.6 Range of Lipid Oxidation in Six Retail Infant Milk Formula Products

The amount of total fat as outlined by the manufacturers did not vary significantly between the IMF retail brands (Table 3.5), however, the fatty acid profile, including the levels of PUFA did (Table S3.3).

Levels of the aldehydes hexanal, pentanal, heptanal and octanal were significantly ($P \leq 0.001$) higher in brand 1 compared to the other 4 brands of IMF powdered samples (brand 2-5), the same was observed for the alcohol compounds 1-heptanol and 1-pentanol. Trans-2-nonenal and 2,4–decadienal were significantly higher in brand 3, 4 and 5 when compared to brand 1 and 2. 2-Heptanone was significantly higher in brand 2 compared with brand 1, 3, 4 and 5. Levels of undecanal, 2-nonanone, 2-pentanone and 3-octen-2-one did not vary significantly between the samples (Table 3.6). The UHT ready-made IMF (brand 6) contained increased levels of pentanal, hexanal, heptanal and 2-heptanone present at 4549, 317, 269 and 174 ppm, respectively. The other nine LO compounds of interest (octanal, undecanal, (E)-2-nonenal, 2,4-decadienal, 2-penanone, 2-nonanone, 3-octen-2-one, 1-heptanol, 1-pentanol) were not detected. A total of 25 FA were quantified in the IMF samples, 20 of which varied significantly.

Brand 5 contained the highest percentage of palmitic acid (C16:0) (24.36 ± 1.72) and oleic acid (C18:1) n9c (30.36 ± 1.52). Brand 2, 3 and 4 contained significantly higher proportions of lauric acid (C12:0) compared to brand 1 and 5. Total FA contents of IMF brand 1-5 are available in Table S3.3.

Table 3.5: Amount (g)/ 100 mL of prepared infant milk formula as stated on the product packaging on brand 1-6.

Typical values (g)	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Protein	1.6	1.25	1.3	1.3	1.25	1.3
Fat	3.3	3.6	3.4	3.4	3.5	3.4
of which saturates	1.1	1.5	1.5	1.5	1.2	1.5
of which unsaturates	1.8	2.1	1.9	1.9	0.7	1.9
LCPs (not specified)	0.028	-	0.015	0.024	0.02	0.024
Linoleic acid	-	0.55	-	-	0.6	-
α-linolenic acid	-	0.067	-	-	0.07	-
Arachidonic acid (AA)	0.012	0.0084	0.006	0.011	0.012	0.011
Docosahexaenoic acid (DHA)	0.011	0.0084	0.006	0.01	0.007	0.01
Vegetable oils	Y	Y	Y	Y	Y	Y
Fish oils	Y	Y	Y	Y	Y	Y

LCP: Long chain polyunsaturated fatty acid. Y: yes.

Table 3.6: Concentrations (mg/kg of powder; n=3) of the 13 volatile compounds quantified in the five infant milk formula (IMF) powder samples (brand 1-5) purchased from local retail units. Different superscripts within a row indicate statistical differences when $P = 0.001$.

Class	Volatile organic Compound	CAS no.	LRI	IMF Brand 1	IMF Brand 2	IMF Brand 3	IMF Brand 4	IMF Brand 5	*P* - Value
Aldehyde	**Hexanal**	66-25-1	840	17935^{a}	488^{b}	175^{b}	285^{b}	849^{b}	*
	Pentanal	110-62-3	735	2013^{a}	10^{b}	15^{b}	28^{b}	51^{b}	*
	Heptanal	111-71-7	944	1401^{a}	73^{b}	53^{b}	52^{b}	109^{b}	*
	Octanal	124-13-0	1047	1815^{a}	99^{b}	162^{b}	142^{b}	212^{b}	*
	(E)-2-Nonenal	18829-56-6	1151	662^{ad}	100^{bc}	1304^{cd}	1017^{c}	1119^{d}	*
	2,4-Decadienal	2363-88-4	1399	651^{a}	1987^{a}	14869^{b}	13685^{b}	16276^{b}	*
	Undecanal	112-44-7	1359	1078^{a}	938^{a}	1229^{a}	984^{a}	405^{a}	NS
Ketone	**2-Nonanone**	821-55-6	1140	112^{a}	17^{a}	100^{a}	109^{a}	132^{a}	NS
	2-Heptanone	110-43-0	935	83^{b}	2395^{a}	22^{b}	37^{b}	41^{b}	*
	2-Pentanone	107-87-9	730	18^{a}	10^{a}	4^{a}	40^{a}	33^{a}	NS
	3-Octen-2-one	1669-44-9	1096	1004^{a}	712^{a}	414^{a}	554^{a}	169^{a}	NS
Alcohol	**1-Heptanol**	111-70-6	1016	1177^{a}	104^{b}	176^{b}	164^{b}	271^{b}	*
	1-Pentanol	71-41-0	815	2402^{a}	538^{b}	59^{b}	93^{b}	284^{b}	*

CAS no.: Chemical Abstracts Service number. LRI: Linear retention index.

The increased concentrations of certain volatile compounds observed in the six retail IMF samples likely relate to the significant differences in FA profile. The FA potentially originating from the addition of fish and vegetable oils (C20:2, C20:3 n6, C24:1 n9 and C20:5) are likely some of the main contributors to the observed oxidative state of the IMF powders. The significantly higher levels of primary aldehyde and secondary alcohol compounds in brand 1 compared to the other brands indicates issues in relation to the fat component of this product, that have resulted in more LO products present immediately after manufacture. The level of long-chain PUFA was slightly higher in brand 1, however, unlikely to be responsible for the significantly higher levels of short and medium chain aldehydes. The compounds (E,E)–2,4-heptadienal, (E,E)–2,4-nonadienal (linolenic and linoleic acid degradation) and 2-pentylfuran (linoleic acid degradation) were only identified in brand 1. These compounds have been shown to be high-impact flavour compounds in edible oils with relative odour activity values ≥ 1 (Xu et al., 2017b). Compounds with relative odour activity values ≥ 1 significantly contribute to aroma and are

considered key volatile components. Brand 6, the UHT ready-made IMF contained less oxidation compounds that the powdered products (brand 1-5). UHT is used in the dairy industry as a means of preparing milk for long term storage without the need for refrigeration. Ajmal et al. (2018) found that the level of short, medium and long chain FA decreased in UHT milk compared to raw milk and the FA profile of UHT milk remained stable during 30 days of storage. However, studies have reported Maillard reaction products present in milk post UHT processing (Clare et al., 2005) and sulfury flavours in milk have also been reported as a result of UHT processing (Deeth, 2010). Dimethyl disulfide and dimethyl trisulfide were also identified in brand 6 in this study.

The antioxidant capacity of dairy powders is mainly determined by the levels of sulfur containing amino acids such as cysteine, but also levels of phosphate, carotenoids, zinc, selenium, and vitamins A and E (Khan et al., 2019). Depending on their concentration and polarity, the natural occurrence of these antioxidants in milk as well as supplementation during processing plays a role in the rate at which LO progresses. Antioxidants work by scavenging free radicals and donating hydrogen, potentially slowing the LO cascade mechanism (Choe et al., 2005). However, the levels of antioxidants were comparable across all brands of IMF which provides further evidence that processing conditions are the main factor behind the rate of LO observed, especially evident for brand 1. IMF is generally produced by wet mixing, dry blending or a combination of both (McSweeney, 2008). Studies have shown that the oxidative stability of IMF is influenced by the quality of the blended emulsion prior to spray drying and that un-homogenised emulsions can result in higher levels of free fat in the final product (Vignolles et al., 2009). In addition to antioxidant addition, encapsulation of PUFA has been employed as a secondary approach to protect them against oxidation in IMF (Barrow et al., 2013). Some of the materials available for carriers of PUFA and other lipids are plant polysaccharides, proteins and peptides (Kaushik et al., 2015). The quality and source of the milk from which the IMF is produced is also likely to impact on LO susceptibility of the final product as bovine diet has also been proven to impact on the milk fatty acid profile (Tripathi, 2014, O'Callaghan et al., 2016).

3.4 Conclusions

This study evaluated and compared the FA content, volatile profile and sensory perception of three dairy based powders (FFWMP, SMP and IMF) throughout 16 weeks of storage. The FA profile varied significantly between the samples and some long chain FA were identified in IMF only (C20:2, C20:3 n6, C24:1 n9 and C20:5). The likely sources for these FA in the IMF is fortification with vegetable and fish oils during manufacture followed by thermal processing which can initiate the LO process; this resulted in the IMF being more susceptible to LO throughout storage. The volatile profile of the three powders also varied significantly. This is due to the differences in FA profile, the rate at which LO progresses and possibly due to the origin of the milk i.e. bovine diet. Overall, increases in the concentrations of hexanal, heptanal and pentanal were good indicators of LO occurring in FFWMP. Fewer significant increases in LO compounds were evident in SMP; however, changes in concentrations of hexanal, 1-heptanol and 1-pentanol were evident. Regarding IMF, significant increases in specific aldehydes hexanal, pentanal, heptanal and octanal were good indicators of LO. The concentrations of (E)-2-nonenal and 2,4-decadienal were also higher in IMF samples compared to FFWMP and SMP. The sensory acceptance scores for FFWMP and SMP remained stable throughout storage, despite some LO compounds being perceived by the panellists. The IMF sample was perceived negatively from the start of storage due to high levels of numerous LO compounds present. ‘Oxidised’ and ‘painty’ attributes were correlated with increased concentrations of hexanal and heptanal and were particularly evident in IMF samples. In addition to the main experiment, the FA content and volatile profile of six widely available IMF brands (five powdered and one ready-to-use) were evaluated. The proportions of 20 of the 25 FA identified varied significantly between the powdered IMF brands while the concentrations of 9 of the 13 volatile compounds varied significantly. Similar to the main experiment, the concentrations of volatile compounds, in particular aldehydes, were higher in the six IMF brands when compared to the FFWMP and SMP evaluated in the main experiment.

Supplementary Materials Chapter 3

Table S3.1: Composition analysis for Fat-filled whole milk powder (FFWMP), skim milk powder (SMP) and infant milk formula (IMF). Each result is the average of 2 replicates.; **Table S3.2**: Results of One-way ANOVA followed by post hoc Tukey test for the colour of the 12 reconstituted milk powders after 4 months in storage. Data are expressed as mean ± standard deviation (n=3). $^{a-k}$ values within a column with different superscripts are statistically different at $P < 0.001$.; **Table S3.3**: Fatty acid (FA) composition (g/100g of FA ± SD; n=3) of infant milk formula (IMF) powder samples (brand 1-5).

Table S3.1: Composition analysis for Fat-filled whole milk powder (FFWMP), skim milk powder (SMP) and infant milk formula (IMF). Each result is the average of 2 replicates.

Sample	Fat %	Protein %	Lactose %	Total solids %	True protein %	Casein %
FFWMP (AM)	3.6	3.4	5.1	13.2	3.2	2.3
SMP (AM)	0.03	3.7	5.6	10	3.5	2.8
IMF (AM)	2.8	1.7	7.5	12.8	1.5	1.1
FFWMP (CON)	3.7	3.4	5.2	12.8	3.2	2.4
SMP (CON)	0.03	3.8	5.6	10.2	3.6	2.9
IMF (CON)	2.8	1.7	7.9	12.8	1.5	1.1
FFWMP (ACC)	3.8	3.5	5.3	13.2	3.3	2.4
SMP (ACC)	0.02	3.7	5.6	10	3.6	2.8
IMF (ACC)	2.9	1.7	7.8	12.8	1.5	1.1
FFWMP (HUM)	3.6	3.4	5.1	12.6	3.2	2.3
SMP (HUM)	0.03	3.8	5.7	10.2	3.6	2.9
IMF (HUM)	2.8	1.6	7.8	12.7	1.5	1.1

ACC = accelerated; AM = ambient; CON = control; HUM = humidity

Table S3.2: Results of One-way ANOVA followed by post hoc Tukey test for the colour of the 12 reconstituted milk powders after 4 months in storage. Data are expressed as mean ± standard deviation (n=3). Different superscripts within a column indicate significant differences ($P = 0.001$).

Sample	L	a (-)	b*	*P-value*
FFWMP (AM)	87.62 ± 0.01a	2.89 ± 0.49a	6.31 ± 0.07a	***
SMP (AM)	77.09 ± 0.11b	5.72 ± 0.44b	2.48 ± 0.68b	***
IMF (AM)	84.88 ± 0.02c	3.10 ± 0.92c	4.51 ± 0.10c	***
FFWMP (CON)	86.03 ± 0.01d	2.68 ± 0.63d	6.05 ± 0.08d	***
SMP (CON)	77.52 ± 0.01e	5.77 ± 0.37b	2.44 ± 0.33e	***
IMF (CON)	83.43 ± 0.12f	3.08 ± 1.07c	4.20 ± 0.30f	***
FFWMP (ACC)	87.30 ± 0.01g	2.89 ± 0.43a	6.63 ± 0.00g	***
SMP (ACC)	77.64 ± 0.03e	5.83 ± 0.08b	2.65 ± 0.31h	***
IMF (ACC)	84.89 ± 0.01c	3.07 ± 0.31c	4.75 ± 0.31i	***
FFWMP (HUM)	90.30 ± 0.01h	2.79 ± 0.34i	6.26 ± 0.08j	***
SMP (HUM)	77.46 ± 0.03e	5.71 ± 0.44b	2.62 ± 0.48h	***
IMF (HUM)	84.42 ± 0.01c	3.14 ± 1.05c	4.15 ± 0.34k	***

ACC = accelerated; AM = ambient; CON = control; HUM = humidity. L* = lightness, a* = green-to-red colour; b* blue-to-yellow colour.

Table S3.3: Fatty acid (FA) composition (g/100g of FA ± SD; n=3) of infant milk formula (IMF) powder samples (brand 1-5).

Fatty Acids	IMF Brand 1	IMF Brand 2	IMF Brand 3	IMF Brand 4	IMF Brand 5	*P* - Value
Butyric acid C4:0	0.82 ± 0.08	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	<0.001
Caproic acid C6:0	0.32 ± 0.01	0.20 ± 0.01	0.26 ± 0.02	0.24 ± 0.02	0.02 ± 0.01	<0.001
Octanoic acidC8:0	0.26 ± 0.01	1.74 ± 0.07	2.36 ± 0.13	2.18 ± 0.19	0.03 ± 0.01	0.03
Decanoic acid C10:0	0.44 ± 0.01	1.33 ± 0.01	1.75 ± 0.01	1.63 ± 0.13	0.04 ± 0.01	<0.001
Lauric Acid C12:0	1.35 ± 0.01	9.51 ± 0.66	12.38 ± 0.26	11.66 ± 0.89	0.21 ± 0.01	<0.001
Tridecanoic acid C13:0	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	ND	<0.001
Myristic acid C14:0	1.83 ± 0.01	3.93 ± 0.20	4.78 ± 0.05	4.58 ± 0.30	0.70 ± 0.07	<0.001
Myristoleic acid C14:1 c9	0.15 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	ND	<0.001
Pentadecanoic acid C15:0	0.25 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	<0.001
Palmitic acid C16:0	18.97 ± 0.38	17.28 ± 0.17	14.92 ± 0.09	14.77 ± 0.58	24.36 ± 1.72	<0.001
Palmitoleic acid C16:1 c9	0.26 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	<0.001
Heptadecanoic acid C17:0	0.14 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.001
Stearic acid C18:0	4.15 ± 0.23	2.56 ± 0.10	2.19 ± 0.05	2.20 ± 0.02	3.30 ± 0.11	<0.001
Oleic acid C18:1 n9c	27.54 ± 0.75	28.02 ± 0.80	27.61 ± 0.47	27.54 ± 0.52	30.36 ± 1.52	0.092
Elaidic acid C18:1 n9t	3.18 ± 0.18	1.59 ± 0.01	1.48 ± 0.01	1.58 ± 0.21	2.23 ± 0.42	0.003
Linoleic acid C18:2 n6c	17.54 ± 0.56	13.12 ± 0.32	9.97 ± 0.15	10.18 ± 0.17	15.45 ± 0.82	<0.001
trans-9,12-octadecadienoate C18:2 n6t	18.47 ± 1.74	16.87 ± 0.22	17.91 ± 0.13	18.97 ± 2.53	19.78 ± 3.78	0.736
α-Linolenic acid C18:3 n3	1.94 ± 0.06	1.49 ± 0.05	1.74 ± 0.01	1.83 ± 0.04	1.52 ± 0.07	0.001
Gamma Linolenic Acid c18:3 n6	0.02 ± 0.01	0.10 ± 0.01	0.17 ± 0.03	0.16 ± 0.04	0.08 ± 0.02	0.009
Eicosanoic acid C20:0	0.24 ± 0.01	0.22 ± 0.02	0.21 ± 0.01	0.21 ± 0.01	0.27 ± 0.01	0.011
cis-11-Eicosenoic acid C20:1	0.24 ± 0.01	0.26 ± 0.02	0.27 ± 0.01	0.28 ± 0.01	ND	<0.001
Eicosenoic acid C20:2	0.03 ± 0.01	0.01 ± 0.01	ND	ND	ND	0.044
Nervonic acid C24:1 n9	0.26 ± 0.02	0.21 ± 0.09	0.15 ± 0.09	0.16 ± 0.01	0.20 ± 0.09	0.541
Eicosapentaenoic acid C20:5	0.05 ± 0.01	0.04 ± 0.06	ND	ND	ND	0.247
CLA C18:2 c9t11	1.55 ± 0.14	1.12 ± 0.06	1.32 ± 0.01	1.48 ± 0.20	1.15 ± 0.23	0.111

CLA = conjugated linoleic acid; SD = standard deviation.

Chapter 3 Highlights

- The FA profile varied significantly between FFWMP, SMP, and IMF.
- C20:2, C20:3 n6, C24:1 n9, and C20:5 were identified in IMF only, likely due to fortification with vegetable and fish oils during manufacture followed by thermal processing.
- Increases in the concentrations of hexanal, heptanal, and pentanal were good indicators of LO occurring in FFWMP.
- Increases in hexanal, pentanal, heptanal, and octanal were good indicators of LO in IMF.
- Oxidised and painty attributes were correlated with increased concentrations of hexanal and heptanal and were particularly evident in IMF samples.

Chapter 4: Effect of Bovine Feeding System (Pasture or Concentrate) on the Oxidative and Sensory Shelf Life of Whole Milk Powder

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Effect of bovine feeding system (pasture or concentrate) on the oxidative and sensory shelf life of whole milk powder

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Abstract

Correlating volatile compounds with the sensory attributes of whole milk powder (WMP) is fundamental for appreciating the effect of lipid oxidation (LO) on sensory perception. LO compounds can adversely affect the sensory perception of WMP by imparting rancid, metallic, and painty notes. Whole milk powders derived from milk produced by cows maintained on a pasture diet (grass and grass-clover mix) versus a non-pasture diet [total mixed ration (TMR); concentrates and silage] were stored at room temperature 21°C (ambient storage) and 37°C (accelerated storage) and analysed for volatile compounds and sensory attributes every 2 months for a total of 6 mo. Thirteen volatile compounds originating from LO were chosen to track the volatile profile of the WMP during storage. Colour, composition, total fatty

acid, and free fatty acid profiling were also carried out. Significant variations in the concentrations of 14 fatty acids were observed in WMP based on diet. Concentrations of free fatty acids increased in all sample types during storage. Similar trends in sensory attributes were observed with an increase in painty attributes, corresponding to an increase in hexanal. Buttery/toffee attributes were found to be more closely correlated with TMR WMP. Those WMP derived from pasture diets were found to be more susceptible to LO from a volatile perspective, particularly in relation to aldehyde development, which is likely due to increased concentrations of conjugated linoleic acid and α-linolenic acid found in these samples.

Keywords: pasture, total mixed ration, sensory, volatile, whole milk powder

4.1 Introduction

Whole milk powder (WMP) is an important dairy commodity that is largely produced in countries with an abundant supply of fresh milk and exported to be reconstituted and consumed directly as a nutritious beverage or used in soups and sauces, or in baking and confectionary (Early, 2012). Spray drying enables milk to be easily transported and stored as WMP for extended periods of time. However, the spray drying process can also facilitate oxidative changes as the high fat content is exposed to elevated temperatures, resulting in reduced shelf life due to off-flavour development. Moreover, WMP can also be subjected to extreme temperature fluctuations during transport and storage, further affecting oxidative stability. Lipid oxidation is a major cause of quality deterioration in fat-containing foodstuffs, which results in alterations to taste and odour through the creation of oxidation compounds, such as aldehydes, ketones, and alcohols. Dairy products containing increased levels of specific PUFA may be more susceptible to lipid oxidation (LO; Hedegaard et al. (2006)). Bovine diet is known to influence many aspects of milk composition, but especially the fatty acid (FA) profile (Liu et al., 2016, O'Callaghan et al., 2019). Quantifying both total fatty acids (TFA) and free fatty acids (FFA) in dairy products is important (Mannion et al., 2016a, 2019), to understand the potential susceptibility of dairy products to LO and to know the abundance of specific FFA that can directly contribute to flavour. Bovine milk also contains various natural oxidants and antioxidants that can also be affected by feeding system, but to date very little research has been published on the susceptibility of milk or WMP to LO dependent

on bovine feeding system. Headspace solid phase-microextraction (HS-SPME) GC-MS is a widely used technique for the identification and quantification of volatile compounds in dairy products (Tunick et al., 2013). A validated HS-SPME GC-MS method (Clarke et al., 2019) was used to quantify 13 compounds from 3 chemical classes (aldehydes, ketones, and alcohols) associated with LO. Quantitative descriptive analysis has previously been applied to a variety of dairy products (Stone and Sidel, 1998), and is based on the ability of trained panellists to measure specific attributes of a product (Chapman et al., 2001). This technique was employed to track the development of taste and odour attributes in WMP as LO progressed at 2 storage temperatures (21°C and 37°C) in opened lidded cans. Panellists (n = 9) were trained to rate the WMP samples based on selected relevant sensory attributes (Drake et al., 2003, Lloyd et al., 2009a, Lloyd et al., 2009b, Park et al., 2016), which were defined and agreed upon by all panellists before final scoring. Analyses of TFA, FFA content, colour, composition, sensory attributes, and volatile profile of the WMP were undertaken at 3 time points over a predefined period of 6 mo.

4.2 Materials and Methods

4.2.2 Milk Production

Fifty-four lactating Friesian cows were divided into 3 groups, namely, grass-only cows (GRS), grass-clover cows (CLV), and TMR cows (n = 18). The GRS cows were maintained outdoors on perennial ryegrass (*Lolium perenne L.*) and received approximately 2 kg of concentrate and 15 kg DM of grass per cow; CLV cows were also maintained outdoors on perennial ryegrass and white clover mix (*Trifolium repens L.*) and received 2 kg of concentrate and 15 kg DM of grass-clover per cow; TMR cows were housed indoors and received 9 kg DM of maize silage + 4.5 kg DM of grass silage + 8.5 kg DM of concentrate throughout the study. Cows within the TMR group were fed daily in electronically controlled Griffith Elder Mealmaster individual feed bins (Griffith Elder and Company Ltd.), and feed was available ad libitum. The CLV sward contained ~20% white clover, as outlined by O'Callaghan *et al*. (2016). Cows in the GRS and CLV groups received a mineral supplement in the form of a liquid mineral preparation injected into the water supply (Terra Liquid Minerals), giving a mean intake (mg/cow per day) of Na, Mg, Zn, Cu, Se, and Co of 5.0, 1.2, 219, 106, 3.8, and 3.0, respectively. The concentrate portion of the TMR feed was supplemented with a commercial mineral balancer (Dairy Hi-Phos;

McDonnell Bros. Agricultural Suppliers Ltd.) to give added Ca, Na, P, Zn, Cu, Mn, I, Co, and Se of 3,340, 2000, 1200, 140, 100, 70, 10, 2, and 0.8 mg/kg, respectively (Gulati et al., 2018).

4.2.3 Manufacture of WMP

Medium-heat WMP was produced in triplicate from milk from each group (n = 18) at Moorepark Technology Limited BioFunctional Food Engineering pilot plant (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland) over a 3-wk period in May 2019. Raw whole milk was collected both morning and evening from each group (GRS, CLV, and TMR) for 3 consecutive days, and each batch (~1,000 kg) was preheated to 50°C and pasteurised at 90°C for 30 s in an APV plate heat-exchanger (SPX Flow Technology), before homogenization using an APV-Gaulin 2-stage homogenizer at first- and second-stage pressures of 150 and 50 bar, respectively. The homogenized milk was evaporated to ~40% TS in a single-effect recirculating evaporator (Scheffers). The concentrate was preheated to 65°C in a plate heat-exchanger and transferred to an Anhydro 3-stage spray dryer (SPX Flow Technology Denmark A/S; air inlet temperature 170°C; air outlet temperature 65°C). First and second fluid bed temperatures were set at 65°C and 25°C, respectively. Fines were returned to the top of the spray dryer from the second fluid bed and the cyclone, yielding an agglomerated WMP of approximately 97% TS. Production of WMP from each feeding system was carried out in triplicate from 3 independent raw milk collections outlined previously (Magan *et al*., 2019). All equipment was cleaned thoroughly between batches. All WMP were packed in 400-g aluminum cans, flushed with N2, and sealed immediately. The WMP produced from grass-only, grass-clover, and TMR milk were denoted as GRS, CLV, and TMR, respectively. All results reported are the averages of triplicate analysis of the 3 production batches for GRS, CLV, and TMR powders (n = 9) unless otherwise stated.

4.2.4 Shelf Life Study Design

The fresh WMP produced from each feeding system were split into 2 storage groups, 21°C and 37°C; this resulted in 6 samples in total, denoted GRS 21°C, GRS 37°C, CLV 21°C, CLV 37°C, TMR 21°C, and TMR 37°C. All sample cans were opened at T0, and initial sensory and volatile measurements were taken. The opened cans were used for all subsequent analysis throughout the study, and plastic lids were

placed on the open cans (closed but not sealed) while in storage (Cesa *et al*., 2015), to best reflect typical use in a domestic environment. The time points chosen for analysis of WMP colour, volatile profile, and sensory perception were 0 (T0), 2 (T2), 4 (T4), and 6 (T6) mo. The FFA content was assessed at T0, T4, and T6, whereas TFA content and WMP composition were assessed at T0 only.

4.2.5 Colour Measurements

Colour measurements were performed on the GRS, CLV, and TMR WMP stored at 21°C and 37°C at each time point according to the CIE Lab system (CIE, 1978; L* is a measure of lightness; a* is a measure of green-to-red colour on a negative-to-positive scale, respectively, and b* is a measure of blue-to-yellow colour on a negative-to-positive scale, respectively), using a Minolta Colourimeter (Minolta Camera). All WMP were reconstituted to 12% TS using distilled water (dH2O) 24 h before analysis and chilled at 4°C. Approximately 2 mL of sample was placed in a spectrophotometric cuvette and allowed to stabilize at room temperature for 30 min before analysis, as per Faulkner et al. (2018). Results were expressed as the average of triplicate measurements of each liquid sample.

4.2.6 Powder Composition

Each WMP was analysed for fat, protein, lactose, true protein, and casein content directly after manufacture (T0) using a Bentley DairySpec FT (Technopath Distribution). The WMP samples were reconstituted to 3.5% fat using dH2O, as per the equation outlined by the International Dairy Federation (IDF, 1997) 24 h before analysis. Results were expressed as the averages of 2 replicates.

4.2.7 Fatty Acid Profiling

Free Fatty Acid Profiling. Analysis of FFA was carried out on the GRS, CLV, and TMR WMP at T0, T4, and T6 months of storage at 21°C and 37°C. Lipid extraction, butyl ester derivatization of triglycerides, solid-phase extraction and gas chromatography instrument conditions were performed as per Mannion et al. (2019). Each powder (4 g) was analysed in duplicate, and the extracts were pooled for solid-phase extraction. Total Fatty Acid Profiling. Profiling of TFA was carried out on the GRS, CLV, and TMR WMP at T0. Lipid extraction and methyl ester derivatization of triglycerides were carried out as per De Jong and Badings (1990) and O'Callaghan

et al. (2019). The WMP were reconstituted to 12% TS using dH2O 1 h before analysis, and 10 mL of reconstitute was used for analysis. The GC conditions were also outlined by O'Callaghan et al. (2019). Briefly, analysis was performed on an Agilent 7890A GC, equipped with an Agilent 7693 autosampler (Agilent Technologies Ltd.) and flame ionization detector. The column was a Select FAME capillary column (100 m × 250-µm internal diameter, 0.25-µm phase thickness; part number CP7420; Agilent Technologies Ltd.). The injector was held at 250°C for the entire run and was operated in split mode using a split ratio of 1:10. The column oven was held at 80°C for 8 min, raised to 200°C at 8.5°C/min, and held for 55 min. The total runtime was 77.12 min. The flame ionization detector was operated at 300°C. Results were processed using OpenLab CDS Chemstation edition software version Rev.C.01.04 (35) (Agilent Technologies Ltd.). A 37-component FAME reference mix containing C4:0 to C24:0 (part number 35077; Thames Restek UK Ltd.) was analysed as an in-run quality control sample, with the FAME present at concentrations of 60 to 180 mg/kg. This was used to ensure that accurate quantification was being achieved throughout sample analysis. The FAME mix was analysed once every 5 samples in the sequence. Accuracy was monitored by comparing the measured concentration of this FAME mix against its true concentration.

4.2.8 Sensory Evaluation

Quantitative descriptive analysis was carried out on the WMP in Teagasc Ashtown (Dublin, Ireland). An external trained sensory panel consisting of 9 members was recruited, based on their ability to perceive a wide variety of attributes and their continued availability. Panellists had between three and four years' experience working as descriptive panellists on a weekly basis. Panel training for WMP evaluation consisted of 2 attribute generation sessions (3 h duration each), wherein the panellists evaluated a variety of volatile compounds on cotton wool (Supplemental Table S4.1, http://hdl.handle.net/ 11019/2424) in addition to the use of 12 Sniffing Sticks (cardboard, rancid, butter, soapy, musty/cellar, cheesy/ sweaty, mushroom, earthy, malty, cabbage, animal/stable, and fishy; Dohler GmbH) designed specifically for this study. A further 4 sessions of panel training were carried out using a variety of product standards to create aroma, texture, flavour, and aftereffect scales for each sensory descriptor that was subsequently applied to the GRS, CLV,

and TMR WMP (Supplemental Table S4.2, http://hdl.handle.net/11019/2424). Panellists used the consensus list of descriptors for scoring, measured on a scale of 1 to 10 with 1 to 3 considered low intensity, 4 to 7 considered medium intensity, and 8 to 10 considered high intensity. Triangle tests were used before the final scoring to ensure that panellists were performing within expectations. The WMP were reconstituted 24 h before scoring based on the fat and protein content (IDF, 1997) using dH2O and were stored at 2°C until approximately 1 h before each training and scoring session. Samples were allowed to reach 11 to 12°C before serving. The reconstituted WMP were gently stirred and poured into 20-mL clear plastic cups, which were labelled with random 3-digit codes. Panellists were given water and plain crackers or green apples to cleanse the palate between samples. The project was set up as a complete block design using Compusense 5.6 (sensory data capture package, https://compusense.com/). The WMP were scored in triplicate for each trial replicate (GRS, CLV, and TMR), for each descriptor, and the results were averaged (n = 9). Analysis of colour was also carried out by the panellists on each WMP.

4.2.9 Volatile Analysis

Thirteen volatile aromatic compounds including 7 aldehydes [hexanal, pentanal, heptanal, octanal, (E)- 2-nonenal, 2,4-decadienal, undecanal], 4 ketones (2-heptanone, 2-nonanone, 2-pentanone, 3-octen-2-one), and 2 alcohols (1-heptanol and 1-pentanol) known to be important to the sensory perception of dairy products were selected for quantification based on current literature (Van Aardt et al., 2005, Faulkner et al., 2018, Kilcawley et al., 2018). Authentic standards for each of the compounds were obtained from Merck Ireland and stored at room temperature. All standard solutions for HS-SPME GCMS analysis were prepared at 0.1% (w/v) in methanol and stored at −18°C until required for analysis, but for no longer than 6 mo. For all calibration curves the external standard mixture was prepared at 0.004% (wt/vol; 4 mL in 100 mL of dH2O) and the internal standard mixture (2-methyl-3-heptanone, 4-methyl-2-pentanol, and isovaleraldehyde) was prepared at 0.001% (wt/vol; 1 mL in 100 mL of dH2O). For the preparation of calibration curves, varying levels of the standard mixture were prepared in 10-mL volumetric flasks with dH2O. The HS-SPME GCMS analysis was carried out as described by Clarke *et al*. (2019) at T0, T2, T4, and T6 sampling points. Briefly, WMP (2.40 g) was weighed out directly into an amber La-Pha-Pack headspace vials (20 mL) with magnetic caps and

silicone/ polytetrafluoroethylene 1.3-mm 45° Shore A septa (Apex Scientific Ltd.). Then dH2O (2.50 g) and 250 µL of 0.001% (wt/vol) internal standard were added to each sample. A calibration curve was also prepared by spiking a set of the reconstituted WMP samples with varying levels of the external standard mixture. Matrix (control) samples (WMP sample + dH2O only) were also included in each run. Samples were incubated for 45 min at a temperature of 43°C using a CombiPal agitator/heater module (Elementec Ltd.), followed by a 10-min pre-extraction incubation time with pulsed agitation of 5 s at 500 rpm. Each sample was analysed in triplicate at each time point.

4.2.10 Statistical Analysis

Statistical analysis for data relating to colour and composition was carried out using one-way ANOVA with post-hoc Tukey tests using SPSS software, version 24 (IBM Corp.). Pearson correlation analysis was carried out on the sensory and volatile data using SPSS. Principal component analysis biplots of the volatile versus sensory data were constructed using the factoextra and FactoMinoR packages within R (R Core Team, 2013)(v. 3.4.1; R Core Team, 2013). All sensory and volatile data were averaged before analysis.

4.3 Results and Discussion

4.3.1 Milk Colour

The TMR WMP scored significantly ($P < 0.05$) lower for a* and b* values compared with the GRS and CLV WMP at each time point (Table 4.1). The TMR WMP scored significantly ($P < 0.05$) higher for L* values at T0 and T2, and, although not statistically different at T4 and T6, L* values remained higher than GRS and CLV WMP. These results were in agreement with previous studies on dairy products produced from pasture versus concentrate-based feeding systems (Faulkner et al., 2018, Clarke et al., 2020a). The significant differences in colour between pasture-derived dairy products and those derived from concentrate has previously been attributed to the abundance of β-carotene in pasture forages, which results in a more yellow or creamy colour (Martin et al., 2005). This study corroborates these results with TMR samples scoring significantly ($P < 0.05$) higher for white colour, whereas pasture-derived products (GRS and CLV) were scored higher for creamy colour by the sensory panel. The TMR WMP also scored significantly ($P < 0.05$) lower for a*

and b* values compared with the GRS and CLV powders at each time point. Lightness also varied significantly ($P < 0.05$) between the 3 WMP at T0 and T2. Although lightness was not statistically significant at T4 and T6, similar trends in lightness were observed between the different types of WMP, with slight increases observed. The b* values (blue-to-yellow) also increased slightly in all 3 WMP from T0 to T6, possibly due to Maillard browning reactions occurring during storage (Bastos et al., 2012). The rate of Maillard browning is known to be affected by several factors, including the chemical nature of the reactants (type of amine and carbonyl groups), water activity, pH, temperature, heating time, and protein-to-sugar ratio (Labuza, 1992, Rozycki et al., 2007).

Table 4.1: Results of colour measurements (n = 3) taken of the 3 reconstituted whole milk powders derived from milk from cows fed perennial ryegrass (GRS), perennial ryegrass and white clover (CLV), or TMR at times (T) 0, 2, 4, and 6 months of storage at 21°C and 37°C[1].

Sample	L*[1]	a* (-)[2]	b*[3]
GRS T0	80.97[c]	3.24[a]	7.24[a]
CLV T0	82.16[b]	3.24[a]	6.96[a]
TMR T0	83.26[a]	2.84[b]	4.86[b]
GRS 21°C T2	86.02[b]	3.51[a]	8.11[a]
CLV 21°C T2	85.94[b]	3.32[a]	7.75[a]
TMR 21°C T2	86.56[a]	2.86[b]	6.37[b]
GRS 37°C T2	85.40[b]	3.51[a]	8.02[a]
CLV 37°C T2	85.88[b]	3.49[a]	7.84[a]
TMR 37°C T2	86.14[a]	2.91[b]	5.62[b]
GRS 21°C T4	85.92	3.39[a]	8.04[a]
CLV 21°C T4	85.58	3.35[a]	7.38[a]
TMR 21°C T4	86.02	2.59[b]	5.28[b]
GRS 37°C T4	85.72	3.41[a]	8.01[a]
CLV 37°C T4	82.64	3.35[a]	7.73[a]
TMR 37°C T4	85.67	2.58[b]	5.38[b]
GRS 21°C T6	85.49	3.40[a]	7.77[a]
CLV 21°C T6	82.52	3.28[a]	7.55[a]
TMR 21°C T6	85.87	2.72[b]	5.41[b]
GRS 37°C T6	84.90	3.52[a]	7.55[a]
CLV 37°C T6	84.97	3.43[a]	7.51[a]
TMR 37°C T6	85.06	2.78[b]	5.21[b]

[a–c] Mean values in the same column (analysed by time point: T0, T2, T4, T6, representing 0, 2, 4, and 6 months) with different superscripts differ ($P < 0.05$) based on feeding system. 1 Each result is the average of triplicate analysis of WMP derived the from 3 production trials for GRS-, CLV-, and TMR-based feeding systems (n = 9). L* is a measure of lightness; a* is a measure of green-to red colour on a negative-to-positive scale, respectively; b* is a measure of blue-to-yellow colour on a negative-to-positive scale, respectively. All a* values are negative, indicated by (−).

4.3.2 Milk Powder Composition

No significant differences were observed between the fat, protein, lactose, true protein, and casein contents of the GRS, CLV, and TMR WMP analysed directly after manufacture at T0 (Supplemental Table S4.3).

4.3.3 Fatty Acid Profiling

4.3.3.1 Free Fatty Acid Profiling

Levels of FFA in the WMP were quantified at T0, T4, and T6. Results showed that C16:0 (palmitic acid) was the most abundant FFA in all sample types at T0 (62–67 mg/kg), followed by C18:0 (stearic acid; 33–38 mg/kg), and C18:1 (oleic acid; 26–32 mg/kg; Table 4.2). The concentrations of FFA in the GRS, CLV, and TMR WMP did not vary significantly ($P < 0.05$) at T0. However, increases in C16:0, C18:0, and C18:1 were observed in all 3 WMP from T0 to T6. Significant ($P < 0.001$) increases in the concentrations of C18:0 were observed in CLV and TMR WMP from T4 to T6. Increases in the total FFA content were also observed in all WMP from T0 to T6. The levels of individual FFA in the GRS, CLV, and TMR WMP were comparable to those reported by Páez et al. (2006). Certain FA are directly responsible for off-flavours such as rancid, astringent, and butyric (Deeth, 2006), but, perhaps more importantly from a sensory standpoint, FA are precursors of oxidation reactions resulting in the production of aldehydes and ketones, which are responsible for oxidized, metallic, and tallowy off-flavours often observed in milk powders (Muir, 1996, Páez et al., 2006). It has been speculated that powders derived from pasture may be more susceptible to LO due to the increased presence of PUFA such as arachidonic acid and docosahexaenoic acid (Kilcawley et al., 2018); however, it has been noted that the presence or concentration of natural antioxidants and pro-oxidants are also major factors (Romeu-Nadal et al., 2004).

4.3.3.2 Total Fatty Acid Profiling

A total of 29 FA were quantified (g/100 g of milk fat) in the WMP. It was evident that feeding system had a significant ($P = 0.05$) effect on the concentrations of 14 of the 29 FA quantified in the WMP, which is in agreement with previous studies on milk and WMP (Semeniuc et al., 2008, O'Callaghan et al., 2016). The 14 FA that varied significantly ($P < 0.05$) based on feeding system were undecanoic acid (C11:0), tridecanoic acid (C13:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), palmitic acid (C16:0), trans-9,12-octadecadienoate (C18:2 n6t), α-linolenic acid (C18:3 n3), γ-linolenic acid (c18:3 n6), eicosanoic acid (C20:0), eicosenoic acid (C20:2), eicosapentaenoic acid (C20:5), heneicosanoic acid (C21:0), CLA (c10t12), and CLA (c9t11; Table 4.3). As previously reported in milk (O'Callaghan *et al.*,

2016), palmitic acid (C16:0) and oleic acid (C18:1 n9c) were found to be the most abundant FA in all WMP analysed. Average palmitic acid content was highest in TMR powders (29.94 ± 2.98), significantly ($P < 0.05$) higher than in GRS (24.96 ± 0.90) and CLV (25.06 ± 0.28) WMP. The concentration of oleic acid (C18:1 n9c) did not vary significantly between the WMP in this study at T0. Higher proportions of CLA have previously been reported in milk from cows consuming significant quantities of grazed grass than cows whose diet primarily consists of conserved forages and concentrates (Kelly et al., 1998). The significantly ($P < 0.05$) higher proportions of CLA observed in pasture-derived WMP (GRS and CLV) agrees with studies on milk and cheese from the same feeding systems used in this study (O'Callaghan et al., 2016, O'Callaghan et al., 2017).

Table 4.2: Concentrations (mg/kg) of the individual free fatty acids (FFA: C4, C6, C8, C10, C12, C14, C16, C18, C18:1, C18:2, and C18:3, ± SD) quantified in whole milk powders (WMP) derived from milk from cows fed perennial ryegrass (GRS), perennial ryegrass and white clover (CLV), or TMR at times (T) 0, 4, and 6 months of storage at 37°C[1].

Fatty Acid	GRS T0	CLV T0	TMR T0	GRS T4	CLV T4	TMR T4	GRS T6	CLV T6	TMR T6	*P*-value
Butyric acid C4:0	16 ± 7.11	10 ± 7.09	10 ± 7.64	13 ± 3.34	18 ± 18.42	16 ± 2.03	16 ± 8.53	13 ± 3.05	9 ± 1.59	NS 0.71
Caproic acid C6:0	14 ± 7.43	9 ± 7.68	8 ± 7.65	9 ± 0.13	9 ± 9.34	9 ± 1.11	10 ± 2.80	10 ± 1.38	8 ± 1.06	NS 0.95
Octanoic acid C8:0	14 ± 7.83	8 ± 8.14	8 ± 7.92	6 ± 0.57	7 ± 0.43	6 ± 1.22	6 ± 1.81	6 ± 1.22	4 ± 1.21	NS 0.75
Decanoic acid C10:0	18 ± 9.21	12 ± 9.50	11 ± 9.20	8 ± 1.39	10 ± 0.55	10 ± 3.01	11 ± 3.07	11 ± 1.36	9 ± 2.64	NS 0.84
Lauric acid C12:0	22 ± 11.07	14 ± 11.81	14 ± 10.84	12 ± 4.20	21 ± 0.15	17 ± 6.96	18 ± 8.38	16 ± 2.80	12 ± 3.40	NS 0.88
Myristic acid C14:0	28 ± 10.52	21 ± 11.74	21 ± 10.54	19 ± 4.57	27 ± 2.84	24 ± 10.36	34 ± 6.84	36 ± 4.20	30 ± 7.54	NS 0.42
Palmitic acid C16:0	67 ± 3.07	62 ± 7.64	67 ± 12.20	64 ± 17.13	88 ± 18.38	88 ± 37.90	113 ± 16.46	121 ± 8.05	117 ± 24.58	* 0.02
Stearic acid C18:0	38 ± 6.46	33 ± 7.31	33 ± 6.86	15 ± 10.86	9 ± 1.12	8 ± 2.60	36 ± 17.66	55 ± 3.97	52 ± 7.22	* <0.001
Oleic acid C18:1	32 ± 5.11	26 ± 7.09	27 ± 8.28	29 ± 11.91	45 ± 14.21	42 ± 22.68	57 ± 11.62	54 ± 12.77	46 ± 10.22	NS 0.11
Linoleic acid C18:2	13 ± 8.86	6 ± 8.46	6 ± 8.86	2 ± 1.37	0 ± 0.00	0 ± 0.00	3 ± 2.23	5 ± 0.67	7 ± 1.40	NS 0.37
α-Linolenic acid C18:3	10 ± 7.35	5 ± 7.09	5 ± 6.75	1 ± 1.93	0 ± 0.00	2 ± 2.72	0 ± 0.00	1 ± 2.02	0 ± 0.00	NS 0.31
Total FFA	272 ± 30.93	205 ± 45.42	210 41.21	179 ± 20.72	235 ± 17.18	222 ± 38.38	305 ± 13.36	329 ± 8.28	294 ± 19.16	NS 0.338

[1]Each result is the average of duplicate analysis of WMP derived the from 3 production trials for GRS-, CLV-, and TMR-based feeding systems (n = 6). NS = not significant. *Significant differences in FFA composition when P = 0.05.

4.3.4 Sensory Evaluation

White colour was more closely associated with TMR WMP, whereas creamy colour was associated with pasture-derived (GRS and CLV) WMP. At T0, significant ($P < 0.001$) differences were observed for colour, dairy sweet aroma, buttery/toffee aroma, and buttery/toffee flavour between the different WMP samples. The attribute dairy sweet aroma was higher in GRS and CLV WMP, with buttery/toffee aroma and flavour higher in TMR WMP. The white colour association with the TMR WMP observed by panellists is in agreement with the instrumental colour analysis. At T2, the differences between the WMP were more apparent for both storage temperatures (21°C and 37°C). At T2 cooked milk aroma, dairy sweet flavour, cooked milk flavour, and dairy sweet aftereffect varied significantly ($P < 0.05$) between the different WMP. For the pasture-derived WMP (GRS and CLV), creamy flavour and dairy sweet flavour dominated at T0. However, a barnyard/cowy aroma and flavour, cooked milk aroma, and hay-like flavour and aroma were to the fore at T2. An increase in painty flavour and astringency were observed in GRS and CLV WMP stored at 37°C at T4 and T6, which corresponded with an increase the concentration of all volatile compounds. The increases in painty flavour and painty flavour aftereffect were more pronounced in CLV WMP compared with GRS WMP samples (Figure 4.1A and 4.1B). In TMR WMP, the pleasant attributes that are commonly associated with fresh reconstituted WMP (creamy and dairy sweet) were dominant at T0. At T2, buttery/toffee and hay-like flavours became more pronounced in both TMR 21°C and TMR 37°C. At T4, dairy sweet aroma remained, but metallic off-flavours began to be perceived. At T6, painty flavour and solvent-like aroma dominated in TMR WMP stored at 37°C, whereas metallic flavour and cooked milk aroma were to the fore in TMR WMP stored at 21°C (Figure 4.1C). When comparing the 3 WMP (GRS, CLV, and TMR) dairy sweet flavour, creamy flavour, creaminess, and viscosity were associated with all 3 WMP samples at T0. Barnyard/cowy aroma, hay-like aroma, and hay-like flavour were more closely correlated with the GRS and CLV WMP at T2, whereas painty aroma, painty flavour, and painty flavour aftereffect were more correlated with TMR samples stored at 37°C at T6. The ability of the sensory panellists to identify and rate the intensity of a painty flavour and aroma in some WMP suggests that the levels of volatile aromatic compounds responsible for this attribute had increased above their odour thresholds over the 6-

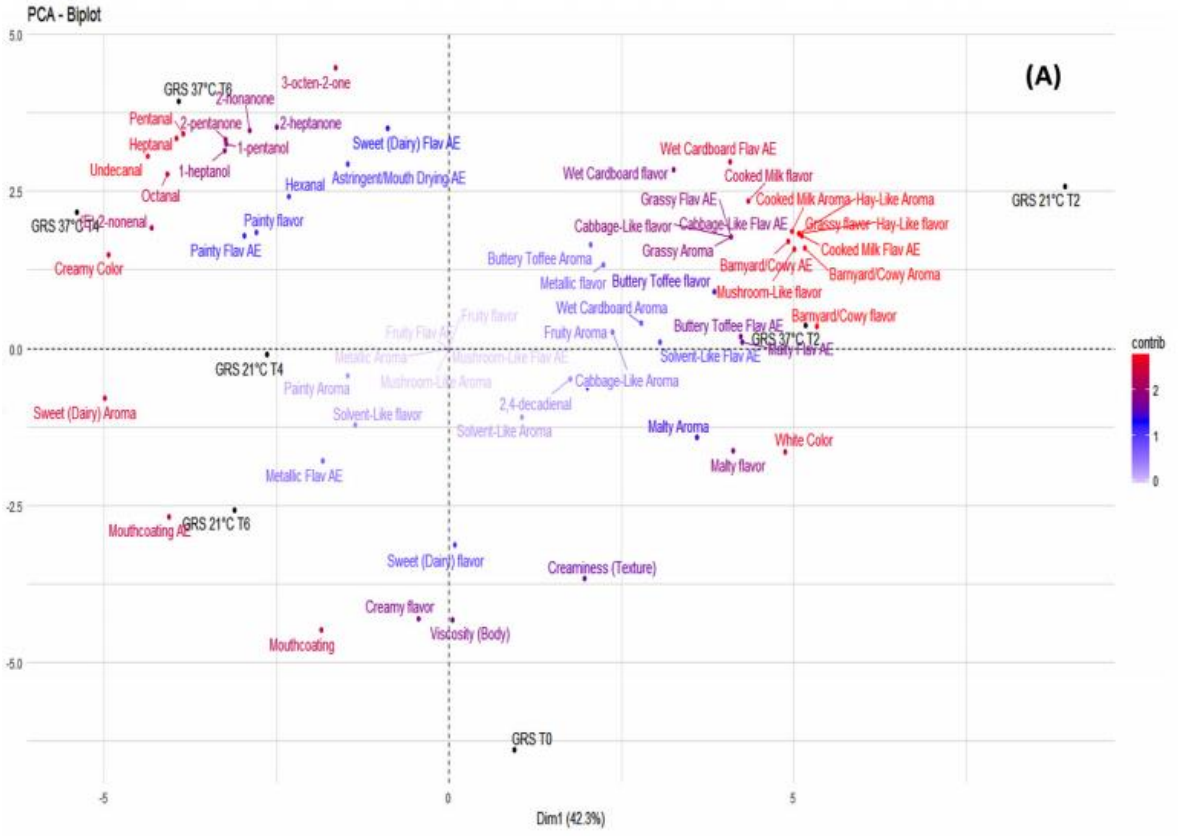
mo storage period. For example, hexanal, derived primarily from linoleic acid, has been shown to be responsible for a painty off-flavour in milk powders (Lloyd et al., 2009a, Clarke et al., 2020b) and was present in GRS, CLV, and TMR WMP at highest concentrations (1,957, 2,092, and 1,791 mg/ kg, respectively) at T6 stored at 37°C. Concentrations of pentanal and heptanal were also significantly ($P < 0.05$) higher in the GRS and CLV WMP compared with the TMR WMP, possibly due to the greater amount of linoleic acid present in the GRS and CLV WMP samples. The odour threshold (mg/kg) in an oil matrix for hexanal, pentanal, and heptanal has been reported as 320, 240, and 3,200 mg/kg, respectively (Decker et al., 2010). Thus, hexanal and pentanal were present at >4 times their odour threshold in all WMP stored at 37°C at T6, which may likely explain the increase in painty attributes. Heptanal increased above its odour threshold in CLV WMP stored at 37°C at T4 and T6 (4,325 and 4,409, respectively). Levels of heptanal were also more abundant in GRS WMP stored at 37°C at T4 and T6 (2,937 and 2,994, respectively) but likely below its odour threshold. Panellists identified a dominant buttery/toffee note in all WMP samples, which decreased over time, as a painty aroma and flavour became more dominant (Figure 4.2A–F). This trend was particularly evident in TMR WMP, where the increase in the painty aroma and flavour was strongly correlated with increases in the concentrations of hexanal (Figure 4.2E and 4.2F). Hexanal, heptanal, and octanal have previously been found to be good predictors of painty and grassy flavours in WMP (Lloyd et al., 2009a). Hexanal reached similar concentrations across all 3 WMP, but the associated painty flavour was more readily identifiable in TMR WMP at T6, as the buttery/toffee notes declined. Thus, it appears that other volatile compounds must also contribute to painty flavour or possibly are masking painty flavour. Maillard and caramelisation reactions have been shown to produce caramel-like and toffee-like flavours in dairy products (Patton, 1955), likely enhanced by the spray drying process. The strong association of the buttery/toffee attribute with the TMR WMP may be the result of more Maillard or oxidative browning, which was subsequently masked by the production of LO products over storage.

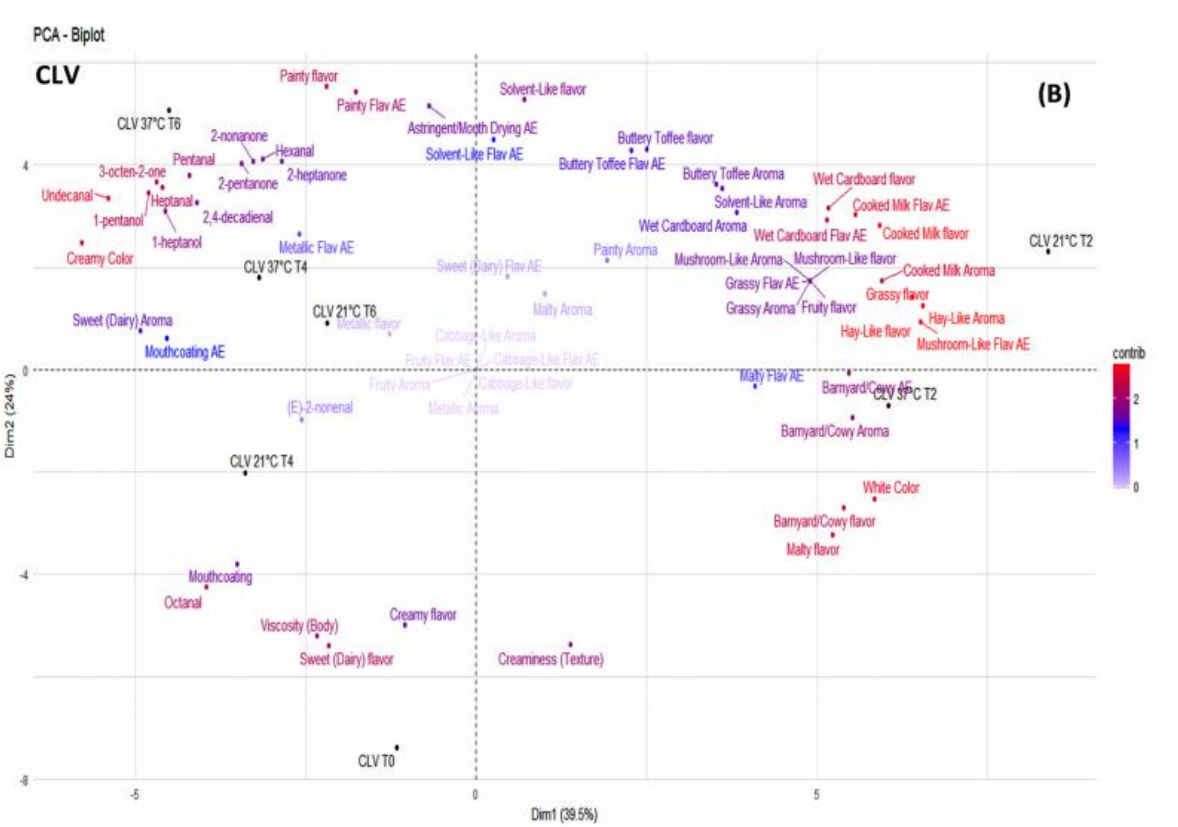
Table 4.3: Mean fatty acid composition of whole milk powders (g/100 g of milk fat ± SD) produced from milk from cows fed perennial ryegrass (GRS), perennial ryegrass and white clover (CLV), or TMR[1].

Fatty Acid	GRS	CLV	TMR	*P*-value	
Butyric acid C4:0	6.02 ± 0.34	5.81 ± 0.25	5.20 ± 0.55	NS	0.24
Caproic acid C6:0	2.62 ± 0.10	2.55 ± 0.10	2.28 ± 0.20	NS	0.16
Octanoic acid C8:0	1.53 ± 0.06	1.50 ± 0.06	1.41 ± 0.05	NS	0.17
Decanoic acid C10:0	3.70 ± 0.18	3.69 ± 0.30	3.25 ± 0.24	NS	0.132
Undecanoic acid C11:0	0.12 ± 0.02[a]	0.13 ± 0.03[a]	0.08 ± 0.00[b]	*	0.002
Lauric acid C12:0	4.15 ± 0.18	4.25 ± 0.37	3.80 ± 0.27	NS	0.246
Tridecanoic acid C13:0	0.14 ± 0.02[a]	0.16 ± 0.03[a]	0.10 ± 0.00[b]	*	0.002
Myristic acid C14:0	10.98 ± 0.09	11.40 ± 0.30	10.45 ± 0.89	NS	0.363
Myristoleic acid C14:1 c9	0.84 ± 0.01[ab]	0.95 ± 0.06[a]	0.76 ± 0.04[b]	*	0.006
Pentadecanoic acid C15:0	1.77 ± 0.07a	1.98 ± 0.08[a]	1.44 ± 0.13[b]	*	<0.001
Palmitic acid C16:0	24.96 ± 0.90[b]	25.06 ± 0.28[b]	29.94 ± 2.98[a]	*	0.003
Palmitoleic acid C16:1 c9	1.26 ± 0.06	1.20 ± 0.08	1.24 ± 0.07	NS	0.561
Heptadecanoic acid C17:0	0.52 ± 0.01	0.52 ± 0.01	0.50 ± 0.02	NS	0.561
Stearic acid C18:0	7.71 ± 0.46	7.52 ± 0.29	7.91 ± 0.50	NS	0.477
Oleic acid C18:1 n9c	13.35 ± 0.41	13.03 ± 0.28	13.13 ± 0.11	NS	0.657
Elaidic acid C18:1 n9t	6.55 ± 0.16	6.48 ± 0.15	6.62 ± 0.48	NS	0.943
Linoleic acid C18:2 n6c	0.84 ± 0.02[b]	0.85 ± 0.02[b]	1.29 ± 0.08[a]	*	<0.001
trans-9,12-octadecadienoate C18:2 n6t	10.54 ± 0.68	10.35 ± 0.95	9.43 ± 1.05	NS	0.407
α-Linolenic acid C18:3 n3	0.50 ± 0.02[a]	0.52 ± 0.03[a]	0.26 ± 0.02[b]	*	<0.001
Gamma Linolenic acid c18:3 n6	0.05 ± 0.00[b]	0.05 ± 0.00[ab]	0.06 ± 0.00[a]	*	0.013
Eicosanoic acid C20:0	0.07 ± 0.00[b]	0.07 ± 0.00[b]	0.12 ± 0.00[a]	*	<0.001
cis-11-Eicosenoic acid C20:1	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	NS	0.79
Eicosenoic acid C20:2	0.01 ± 0.01[b]	0.01 ± 0.01[b]	0.03 ± 0.00[a]	*	0.02
Eicosadienoic acid C20:3 n6	0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.01	NS	0.18
Eicosapentaenoic acid C20:5	0.05 ± 0.00[b]	0.06 ± 0.00[b]	0.00 ± 0.00[a]	*	<0.001
Heneicosanoic acid C21:0	0.05 ± 0.01[a]	0.06 ± 0.02[a]	0.01 ± 0.00[b]	*	<0.001
Tricosanoic acid C23:0	0.01 ± 0.01	0.01 ± 0.02	0.00 ± 0.00	NS	0.28
CLA (c10t12)	1.02 ± 0.06[a]	1.15 ± 0.13[a]	0.25 ± 0.11[b]	*	<0.001
CLA (c9t11)	0.54 ± 0.04[a]	0.56 ± 0.06[a]	0.33 ± 0.03[b]	*	<0.001

[a,b] Mean values in the same row with different superscripts differ (P = 0.05) based on feeding system.

[1]Each result is the average of duplicate analysis of WMP derived the from 3 production trials for GRS-, CLV-, and TMR-based feeding systems (n = 6). Statistical analysis was carried out by one-way ANOVA. 2 c = cis; t = trans. 3 NS = not significant. *Significant differences in fatty acid composition when P = 0.05.


PCA - Biplot
(A)
GRS 37°C T6
3-octen-2-one
2-nonanone
Pentanal
2-pentanone
2-heptanone
Heptanal
1-pentanol
Sweet (Dairy) Flav AE
Undecanal
1-heptanol
Astringent/Mouth Drying AE
Hexanal
Octanal
GRS 37°C T4
(E)-2-nonenal
Painty flavor
Painty Flav AE
Creamy Color
Wet Cardboard Flav AE
Wet Cardboard flavor
Cooked Milk flavor
Grassy Flav AE
Cooked Milk Aroma
Hay-Like Aroma
GRS 21°C T2
Cabbage-Like flavor
Cabbage-Like Flav AE
Grassy flavor
Hay-Like flavor
Grassy Aroma
Cooked Milk Flav AE
Buttery Toffee Aroma
Barnyard/Cowy AE
Barnyard/Cowy Aroma
Metallic flavor
Buttery Toffee flavor
Mushroom-Like flavor
Wet Cardboard Aroma
Barnyard/Cowy flavor
Fruity flavor
Buttery Toffee Flav AE
Fruity Flav AE
Fruity Aroma
GRS 37°C T2
Solvent-Like Flav AE
Malty Flav AE
Metallic Aroma
Mushroom-Like Flav AE
GRS 21°C T4
Painty Aroma
Mushroom-Like Aroma
Cabbage-Like Aroma
2,4-decadienal
Sweet (Dairy) Aroma
Solvent-Like flavor
Solvent-Like Aroma
Malty Aroma
White Color
Malty flavor
Metallic Flav AE
Mouthcoating AE
GRS 21°C T6
Sweet (Dairy) flavor
Creaminess (Texture)
Creamy flavor
Viscosity (Body)
Mouthcoating
GRS T0
Dim1 (42.3%)
contrib


PCA - Biplot
CLV
(B)
Painty flavor
Painty Flav AE
Solvent-Like flavor
CLV 37°C T6
Astringent/Mouth Drying AE
2-nonanone
Hexanal
Buttery Toffee flavor
Pentanal
Solvent-Like Flav AE
Buttery Toffee Flav AE
3-octen-2-one
2-pentanone
2-heptanone
Buttery Toffee Aroma
Wet Cardboard flavor
Undecanal
Heptanal
Solvent-Like Aroma
Cooked Milk Flav AE
2,4-decadienal
1-pentanol
Wet Cardboard Aroma
Wet Cardboard Flav AE
Cooked Milk flavor
1-heptanol
CLV 21°C T2
Metallic Flav AE
Painty Aroma
Creamy Color
CLV 37°C T4
Sweet (Dairy) Flav AE
Mushroom-Like Aroma
Mushroom-Like flavor
Cooked Milk Aroma
Grassy Flav AE
Grassy flavor
Malty Aroma
CLV 21°C T6
Fruity flavor
Sweet (Dairy) Aroma
Metallic flavor
Grassy Aroma
Hay-Like Aroma
Hay-Like flavor
Cabbage-Like Aroma
Mouthcoating AE
Mushroom-Like Flav AE
Fruity Flav AE
Cabbage-Like Flav AE
Malty Flav AE
Fruity Aroma
Barnyard/Cowy AE
Cabbage-Like flavor
CLV 37°C T2
(E)-2-nonenal
Metallic Aroma
Barnyard/Cowy Aroma
CLV 21°C T4
White Color
Barnyard/Cowy flavor
Malty flavor
Mouthcoating
Octanal
Creamy flavor
Viscosity (Body)
Sweet (Dairy) flavor
Creaminess (Texture)
CLV T0
Dim1 (39.5%)
Dim2 (24%)
contrib

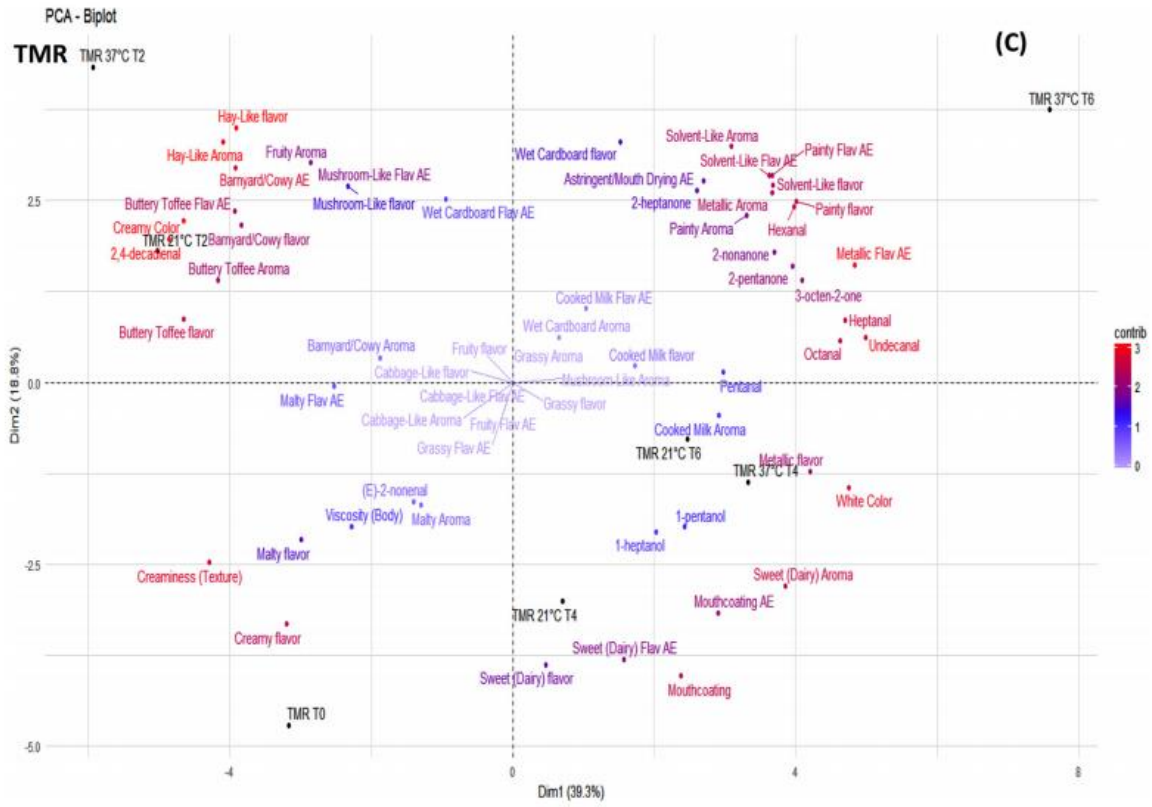


Figure 4.1: (A) Principal component analysis (PCA) biplot, representing the correlation structure of the variables (sensory attributes identified by full descriptive analysis and volatile compounds identified by headspace solid-phase microextraction gas chromatography mass spectrometry) and the relationship between the whole milk powder samples derived from grass (GRS) from times (T) 0–6 mo. **(B)** PCA biplot analysis of the variables and the milk powder samples derived from perennial ryegrass and white clover (CLV) from T0–T6. **(C)** PCA biplot analysis of the variables and the milk powder samples derived from TMR from T0–T6. Colour gradient: low = white, mid = blue, high = red; midpoint set at 1.0. Flav = flavour; AE = aftereffect. Dim = dimension.

Pearson correlation relationships indicated that the sensory attributes hay-like aroma and flavour, grassy aroma and flavour, and barnyard/cowy aroma and flavour can be grouped together, and therefore an increase in one attribute indicates that all increase. However, this could also indicate that all 3 attributes may have had similar identification criteria by the sensory panel. Additionally, buttery/toffee aroma and flavour were consistently correlated with dairy sweet attributes. Another group of attributes that were correlated were painty, solvent-like, and metallic aromas, flavours, and aftereffects. Specific individual volatile aldehydes, ketones, and alcohols are good indicators of how their overall chemical classes behave during the LO process. Therefore, an increase of one compound within a chemical class can indicate that others within that class derived from the same process, such as LO, will

also increase. The increased concentrations of the alcohol compounds 1-heptanol and 1-pentanol observed in GRS and CLV WMP were very likely directly associated with the levels of precursors heptanal and pentanal, respectively (Kilcawley *et al*., 2018).

4.3.5 Volatile Analysis

The highest concentrations of the aldehyde compounds hexanal, pentanal, and heptanal were observed in GRS and CLV WMP stored at 37°C at T4 and T6 (>1,500 mg/kg; Figure 4.3B and 54.3D). These compounds were consistently higher in GRS and CLV WMP compared with TMR WMP. Aldehydes (≥8 carbons) and branched-chain aldehydes were best correlated with CLV WMP. The ketone compounds 2-nonanone, 2-heptanone, 2-pentanone, and 3-octen-2-one were consistently higher in GRS and TMR WMP samples stored at 37°C throughout the study, whereas the alcohol compounds 1-heptanol and 1-pentanol were highest in GRS and CLV WMP stored at 37°C. One-way ANOVA analysis with post hoc Tukey's test showed significant ($P < 0.001$) increases in the concentrations of hexanal, heptanal, 2-nonanone, 2-heptanone, 2-pentanone, 3-octen-2-one, and 1-pentanol in all the WMP from T0 to T6. Increases in the concentrations of hexanal, pentanal, heptanal, octanal, 2,4-decadienal, undecanal, and 2-pentanone were observed in the GRS WMP stored at 21°C from T0 to T4. After T4, hexanal was the only compound that continued to increase; all the other compounds either remained at the same concentration or decreased slightly (Figure 4.3A and Supplemental Table S4.4A, http://hdl.handle.net/11019/2424), a trend that has been observed previously in WMP after 10 months of storage (Lloyd et al., 2009b) and in whey protein concentrate after 4 months of storage (Javidipour and Qian, 2008). The decrease in certain volatile compounds after they have peaked has previously been attributed to their degradation or catabolism (Neilson et al., 2006, Wright et al., 2009). Other studies have speculated that proteins, flavonoids, and some enzymes such as superoxide dismutase can inhibit LO by antioxidant activity (Eriksson, 1982). The same study stated that LO inhibitors induced by thermal processing, such as Maillard reaction products and native protein hydrolysates, may also be factors in inhibiting the LO mechanism. In addition to flavonoids, β-carotene has the potential to protect dairy products against oxidation (Havemose et al., 2006). As previously mentioned, β-carotene is known to be higher in dairy products produced from pasture and is responsible for their greater yellow colour compared with those derived from TMR systems. Strecker-type degradation

of amino acids has also been shown to be produced by some LO products (Hidalgo and Zamora, 2004), which may indicate the consumption of some LO end products in these reactions. A decrease in water activity within WMP during storage may also inhibit LO reaction rates. In GRS WMP stored at 37°C, 4 aldehydes, namely hexanal, pentanal, heptanal, and undecanal, continued to increase from T4 to T6. The other 8 volatile compounds reached their highest concentrations at T4 and decreased by T6, except for 2,4-decadienal and (E)-2-nonenal, which were highest at T0 (Figure 4.3B and Supplemental Table S4B, http://hdl.handle.net/ 11019/2424). In CLV WMP stored at 21°C, similar trends to those of the GRS WMP stored at 21°C were observed. Concentrations of hexanal, pentanal, heptanal, octanal, (E)-2-nonenal, 2,4-decadienal, undecanal, 2-nonanone, 3-octen-2-one, 1-heptanol, and 1-pentanol increased from T0 to T4 and decreased thereafter, apart from hexanal, which continued to increase to T6 (Figure 4.3C and Supplemental Table S4.4C, http://hdl.handle.net/ 11019/2424). For CLV WMP stored at 37°C, the aldehydes hexanal, pentanal, and heptanal reached much higher concentrations than in CLV WMP stored at 21°C.

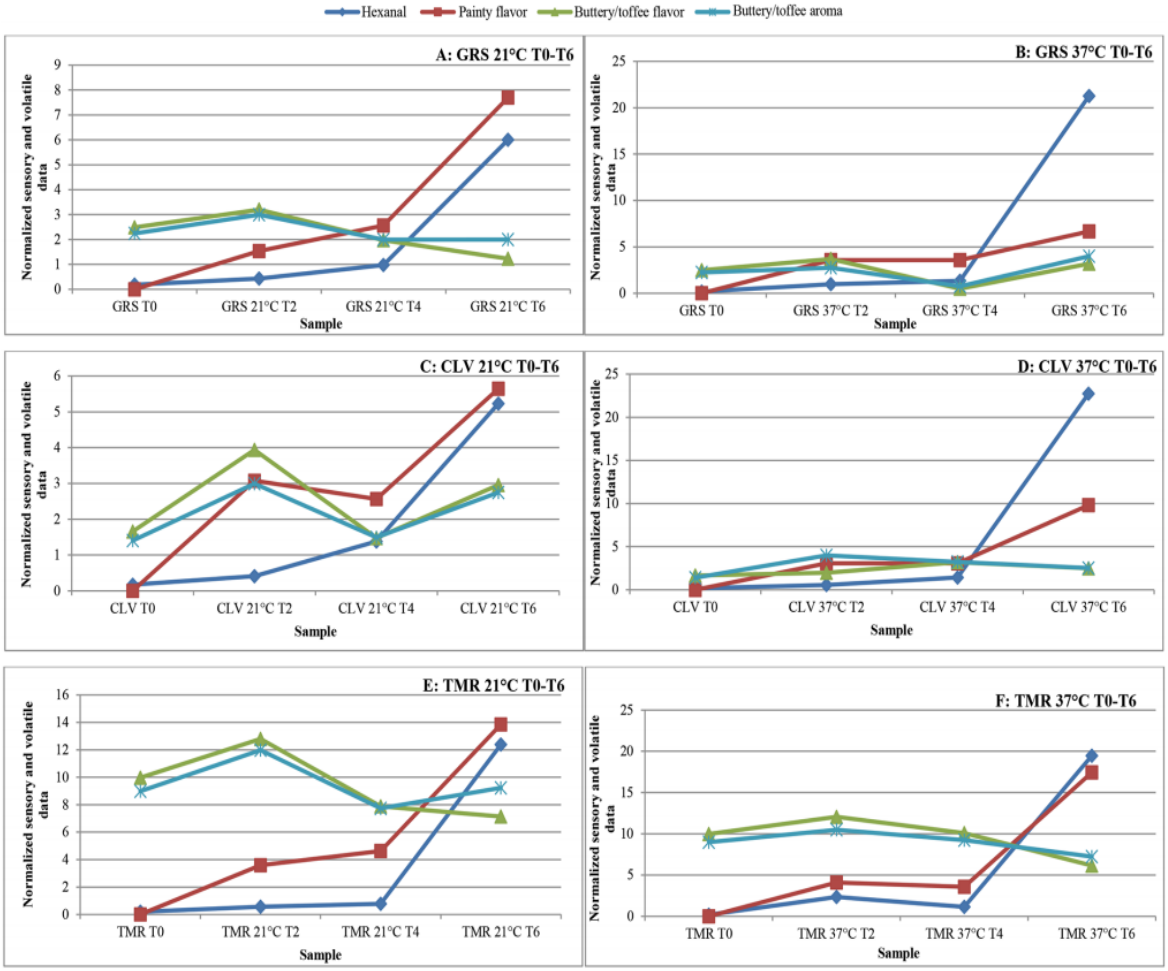


Figure 4.2: Graphs illustrating the increase in painty flavour and hexanal and the decrease in buttery/toffee flavour and aroma in whole milk powders derived from (A) grass (GRS) stored at 21°C and (B) GRS 37°C; (C) clover (CLV) 21°C and (D) CLV 37°C; and (E) TMR 21°C and (F) TMR 37°C during 6 mo of storage (T0–T6). Normalized data were used for the purpose of these graphs.

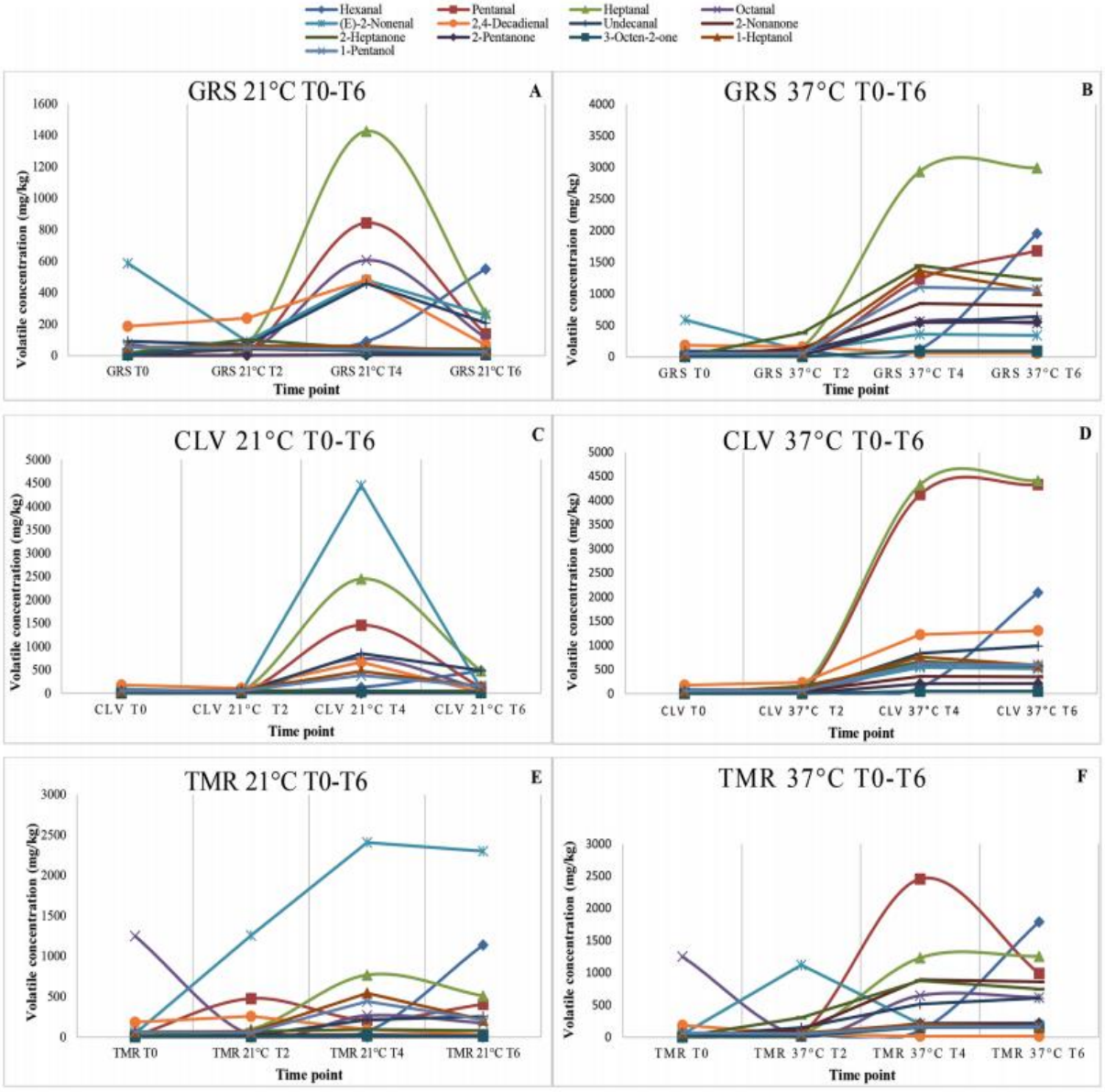


Figure 4.3: Graphs illustrating the concentrations (mg/kg) of the 13 selected volatile compounds in (A) grass (GRS) whole milk powder (WMP) stored at 21°C during 6 mo of storage (T0–T6); (B) clover (CLV) WMP 21°C; (C) TMR WMP 21°C; (D) GRS WMP 37°C; (E) CLV WMP 37°C; and (F) TMR WMP 37°C.

Similar to GRS WMP stored at 37°C, hexanal, pentanal, heptanal, and 2,4-decadienal continued to increase after T4 in the CLV WMP stored at 37°C. The other 9 volatile compounds remained the same or decreased by T6 [octanal, (E)-2-nonenal, undecanal, 2-nonanone, 2-heptanone, 2-pentanone, 3-octen-2-one, 1-heptanol, and 1-pentanol; Figure 3D and Supplemental Table S4D, http://hdl.handle.net/11019/2424]. In TMR WMP, (E)-2-nonenal was higher in samples stored at 21°C than 37°C; it increased gradually from T0 to T4 and then

decreased by T6. In TMR WMP stored at 21°C (E)-2-nonenal was the most abundant compound at a concentration of 2,407 mg/kg at T4. In the 37°C samples it reached its highest concentration at T2 (1,122 mg/kg) but decreased thereafter. For TMR WMP stored at 21°C, hexanal, undecanal, and 2-pentanone increased gradually from T0 to T6; the remaining 10 compounds either decreased [heptanal, (E)- 2-nonenal, 2-nonanone, 2-heptanone, 3-octen-2-one, 1-heptanol, and 1-pentanol] from T4 to T6 or fluctuated throughout storage (pentanal, octanal, and 2,4-decadienal; Figure 4.3E and Supplemental Table S4E, http:// hdl.handle.net/11019/2424). In TMR WMP stored at 37°C hexanal, heptanal, undecanal, and 2-pentanone increased from T0 to T6. The remaining 8 volatile compounds either decreased (pentanal, 2-heptanone, 1-heptanol, and 1-pentanol) from T4 to T6, fluctuated throughout storage (octanal), plateaued (3-octen2-one), or reached their highest concentration early in storage and began to decrease thereafter [2,4-decadienal and (E)-2-nonenal; Figure 4.3F and Supplemental Table S4.4F, http://hdl.handle.net/11019/2424].

As previously mentioned, compounds that begin to decrease in storage are likely degraded or catabolized, in many cases to other volatile compounds. Thus, for most volatile LO compounds in this study, the rate of formation was exceeded by degradation after 4-mo storage, presumably due to a lack of available FA substrate or other factors inhibiting the primary LO process. Maximum levels of some compounds were reached more rapidly in the WMP stored at 37°C due to the increased temperature.

4.3.6 Correlations between Volatile Components and Sensory Attributes

Pearson correlation analysis was carried out on the sensory and volatile data using SPSS. Numerous positive correlations were evident between volatile components and sensory attributes, but only strong correlations ≥0.7 are reported here. Hay-like aroma was significantly ($P < 0.001$) correlated with barnyard/ cowy aroma (0.794), grassy aroma (0.734), cooked milk flavour (0.777), grassy flavour (0.956), hay-like flavour (0.930), and barnyard/cowy flavour (0.792). Buttery/ toffee aroma was correlated with dairy sweet aroma (0.809), dairy sweet flavour (0.969), and buttery/toffee flavour (0.969). Painty aroma was correlated with painty flavour (0.791), painty flavour aftereffect (0.812), and metallic flavour aftereffect (0.754). Metallic aroma was correlated with increases in solvent-like flavour (0.828). Creamy

flavour was correlated with viscosity (0.897), creaminess (0.870), and astringency (−0.776). Painty flavour was correlated with solvent-like flavour (0.716) and increasing concentrations of hexanal (0.793). Painty flavour aftereffect was also correlated with hexanal (0.749). Increases in concentrations of pentanal were correlated with increases in heptanal (0.911), 2,4-decadienal (0.750), and undecanal (0.843). Increases in heptanal were also correlated with increases in undecanal (0.784), 1-heptanol (0.784), and 1-pentanol (0.794). 2-Nonanone was significantly correlated with increases in levels of 2-heptanone (0.924), 2-pentanone (0.874), and 3-octen-2-one (0.923). As observed with heptanal, 2-heptanone and 2-pentanone were correlated with increases in the alcohol compounds 1-heptanol (0.750 and 0.858, respectively) and 1-pentanol (0.756 and 0.880, respectively). 2-Heptanone was also correlated with 3-octen-2-one (0.743), and 1-heptanol was correlated with 1-pentanol (0.991). The key factor influencing the perception of LO compounds is their odour thresholds (the lowest concentration of a compound perceivable by the human nose), which vary considerably and also depend upon the matrix effect (the binding of compounds to components of the sample affecting their release). The highest concentrations of the aldehydes hexanal, pentanal, heptanal were observed in GRS and CLV WMP stored at 37°C at T4 and T6 (>1,500 mg/kg; Figure 4.2B and 4.2D). These compounds were consistently higher in GRS and CLV WMP compared with TMR WMP. Conversely, painty attributes, which are commonly associated with increases in hexanal were correlated more with TMR WMP toward the latter stages of storage. Although the results of this study show correlations between painty attributes and concentrations of hexanal, it is unlikely that one single compound is responsible for specific attributes when numerous other odour-active compounds are present (Kobayashi and Nishimura, 2014). However, increases in the volatile compounds evaluated in this study are good indicators of LO state, but perhaps other approaches such as olfactometry are required to absolutely associate the concentration of specific volatiles with specific aroma descriptors. Overall, aldehydes (≥8 carbons) and branched-chain aldehydes were most correlated with CLV WMP. The ketone compounds 2-nonanone, 2-heptanone, 2-pentanone, and 3-octen-2-one were consistently higher in GRS and TMR WMP samples stored at 37°C throughout the study, whereas the alcohol compounds 1-heptanol and 1-pentanol were highest in GRS and CLV stored at 37°C. Concentrations of the FA (C4:0–C12:0) were higher overall in GRS and CLV WMP compared with TMR WMP (Table 4.2), which may

have influenced LO susceptibility, particularly in relation to aldehyde formation. The concentrations of oleic acid were not significantly different between the WMP samples, but it is thought to be a major precursor for hexanal formation. Linoleic acid was significantly ($P < 0.05$) higher in TMR WMP and is also a precursor of hexanal, in addition to pentanal and 3-octen-2-one. Linoleic acid likely influenced the higher levels of 3-octen-2-one in TMR WMP, but because both hexanal and pentanal were higher in pasture-derived WMP, other sources or factors must be influencing their formation, such as the presence of natural pro- and antioxidants. α-Linolenic acid (C18:3 n3) has been shown to produce hexanal by Tawfik *et al*. (2017) and was significantly higher ($P < 0.05$) in pasture-derived WMP (CLV > GRS). Eicosanoic acid (C20:0) and heneicosanoic acid (C21:0), α-linolenic acid (CLA; c10t12), and CLA (c9t11) were also significantly ($P < 0.05$) different based on diet and likely also affected aldehyde formation. In addition, CLA has been shown to oxidize more rapidly than linoleic acid, supporting evidence that the conjugated double bond is more susceptible to oxidation than a nonconjugated double bond, thus facilitating volatile compound formation and release (Moon et al., 2008).

4.4 Conclusions

The main finding from this study was that the bovine feeding system pasture (GRS and CLV) versus non-pasture (TMR) significantly affected the TFA, FFA, volatile profile, and sensory attributes of WMP. Pasture derived WMP (GRS and CLV) were best correlated with creamy colour, dairy sweet aroma, and hay-like attributes, whereas non-pasture-derived WMP (TMR) was best correlated with white colour and buttery/toffee and painty attributes. Buttery/toffee attributes were found to be more closely correlated with TMR WMP. Increases in many of the volatiles studied were evident during storage at both 21°C and 37°C, with some compounds peaking at T4 and then plateauing or decreasing slightly by T6, likely due to degradation exceeding formation. Pasture-derived WMP (GRS and CLV) were found to be more susceptible to LO from a volatile perspective, particularly in relation to aldehyde development, possibly due to increased concentrations of CLA and α-linolenic acid. Pleasant attributes, possibly associated with Maillard reaction products were perceivable in the WMP at the beginning of the study but became masked by LO compounds with off-flavours by T4. Correlations were made between concentrations of hexanal and painty attributes, but it is unlikely that a single

compound was responsible for these attributes. Regardless of this, however, the recommended shelf life for WMP once opened was <4 months from a sensory perspective.

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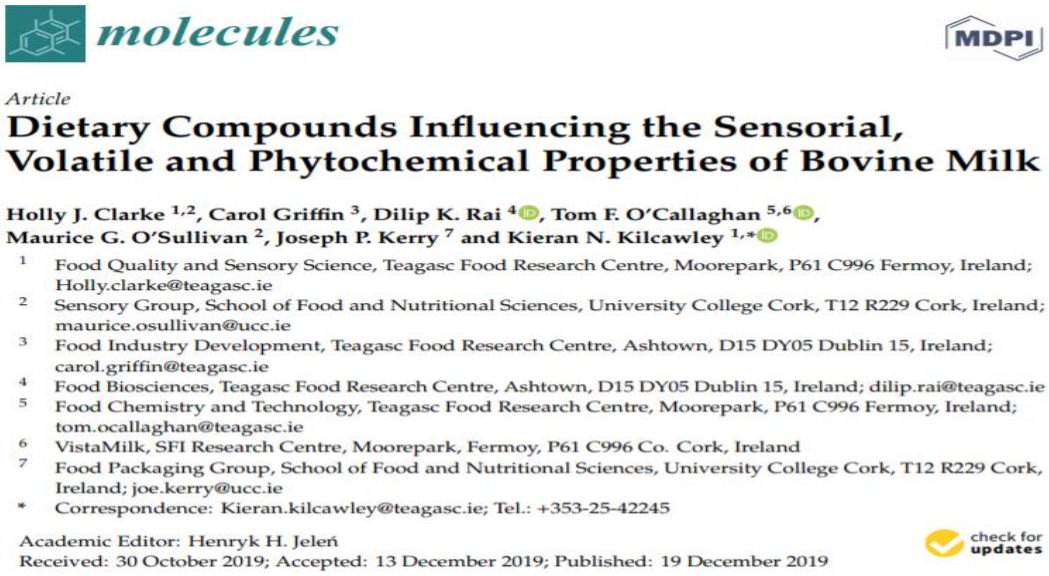
Chapter 4 Highlights

- Pasture (GRS and CLV) versus non-pasture (TMR) feeding systems significantly affected the TFA, FFA, volatile profile, and sensory attributes of WMP.
- Increases in many of the volatiles studied were evident during storage at both 21°C and 37°C, with some compounds peaking at T4 and then plateauing or decreasing slightly by T6, likely due to degradation exceeding formation.
- Pasture-derived WMP (GRS and CLV) were found to be more susceptible to LO from a volatile perspective, particularly in relation to aldehyde development, possibly due to increased concentrations of CLA and α-linolenic acid.

Chapter 5: Dietary Compounds Influencing the Sensorial, Volatile and Phytochemical Properties of Bovine Milk

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Dietary Compounds Influencing the Sensorial, Volatile and Phytochemical Properties of Bovine Milk

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Abstract

The main aim of this study was to evaluate the volatile profile, sensory perception and phytochemical content of milk produced from cows fed three distinct feeding systems (grass [GRS], grass/clover [CLV], and total mixed ration [TMR]). Previous studies have highlighted that compounds from feed can potentially impact milk flavour directly as aromatic compounds or indirectly by acting as precursors to other volatile aromatic compounds. In the present study, significant differences were observed in the phytochemical profile of the feed and milk samples. Formonoetin was found to be significantly higher in CLV feed samples while daidzein, genistein and apigenin were highly correlated to raw CLV milk samples, likely present as metabolites of other phytochemical compounds. Milk samples were easily distinguishable based on the effect of storage time, feeding system and pasteurisation

on the volatile profile. TMR milk scored significantly higher for hay-like flavour and white colour while GRS and CLV milk scored significantly higher for creamy colour due to the β-carotene content of pasture derived dairy products. The difference in creaminess is due to the higher fat content of CLV milk samples. Evaluating the isoflavone content and volatile profiles of the milk in combination with sensory analysis proved to be useful in the identification of important odour-active compounds in milk. This study also supports evidence suggesting that potential biomarkers for authenticity of pasture-derived products exist and have the ability to impact on milk flavour. Further research is required to fully ascertain the phytochemical breakdown pathways in the rumen and the potential impact on the sensory perception of milk.

Keywords: dairy; feeding system; volatile organic compounds (VOCs); sensory; isoflavones

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5.1 Introduction

The effect of bovine diet on the composition and flavour profile of milk is well documented (Chilliard and Ferlay, 2004, Faulkner et al., 2018, Wang et al., 2018); however, conflicting results exist on the effect of feeding system on the flavour and abundance of volatile organic compounds (VOCs) in dairy products and their impact on the sensory perception of milk. Studies suggest that certain VOCs in milk could prove to be useful metabolic markers in tracing animal diets (Coppa et al., 2011, Kilcawley et al., 2018). Alterations to feeding system have been shown to have effects on milk fat composition, protein content, urea, citrate and soluble calcium (list not exhaustive), which can subsequently influence the oxidative stability and flavour of the milk (Palmquist et al., 1993). The review by Chilliard et al. (2001) summarized the effects of forage type on milk fat and milk composition and highlighted the need to evaluate the impact of feeding systems on other aspects of milk fat quality, such as flavour and oxidative stability. Milk produced from many supplemented and altered diets have been investigated including supplementation with flaxseed (Caroprese et al., 2017), lipid complex (Bodkowski et al., 2016), crude protein (Danes et al., 2013), iodine (Schöne et al., 2017) marine algae (Glover et al., 2012), oregano and caraway essential oils (Lejonklev et al., 2016), hull-less barley (Yang et al., 2017)

and sunflower/fish oil (AbuGhazaleh and Holmes, 2007). These studies focused mainly on animal production performance, milk composition, milk yield, milk fatty acid composition and to a lesser extent, the flavour and sensory characteristics of milk. The study by O'Callaghan et al. (2019) investigated the influence of four supplemental feed choices for pasture-based cows on the fatty acid and volatile profile of milk. Some studies have also evaluated the effect of storage conditions on the microbiological quality of milk (Liao et al., 2018, Paludetti et al., 2018). In the present study, the volatile profile and free fatty acid (FFA) content of the milk samples were evaluated over a 14 day storage period at 4 °C in order to ascertain the level of lipid oxidation occurring within the milk and to track volatile compounds forming or changing during refrigerated storage. Free fatty acids (FFAs) in milk are produced by two mechanisms; incomplete esterification in the mammary gland before lipid excretion (Marsili, 2016) or lipid hydrolysis after milking and during storage (Santos et al., 2003). The FFAs influence product quality, flavour, nutrition and texture, thus, accurate quantification is important for quality control, research and development purposes (Mannion et al., 2016b). FFA levels >1.5 mmol/L are unacceptable to most consumers (IDF, 1987). A number of factors including individual animals, feeding system, stage of lactation, farm practices, bacterial contamination and storage quality influence the level of FFA in milk (Hanuš et al., 2008). Increased levels of unsaturated fatty acids bound in the lipid molecules (triacylglycerol or phospholipids) or as FFA have been shown to increase the susceptibility of milk to lipid oxidation (Jacobsen, 2019) thus impacting negatively on quality and sensory properties. Increased levels of short and medium chain FFA in particular have been shown to be responsible for off-flavours described as rancid, butyric and astringent (Deeth, 2006). Increased levels of ethyl esters of short-chain fatty acids, particularly, ethyl butanoate and ethyl hexanoate imparts a fruity off-flavour in milk (Liu et al., 2004).

Furthermore, four important isoflavones (apigenin, daidzein, formononetin and genistein) with potentially important sensory implications were also investigated. Isoflavones are a group of phytoestrogens with estrogenic or hormone-like properties and are known to have positive effects on various diseases including atherosclerosis, osteoporosis and some cancers (Skaanild and Nielsen, 2010), but also may act as substrates for biomarkers of pasture feeding and influence sensory properties through degradation to odour active compounds (Faulkner et al., 2018, Kilcawley et al.,

2018). Isoflavones in bovine milk are likely present as a result of direct transfer from feeds including leguminous plants such as clover and soybean, which are naturally rich in phytoestrogens. Dairy produce from pasture-based farming systems is considered to be more natural by consumers from an animal welfare and environmental standpoint (Verkerk, 2003). Feeding TMR and housing cows indoors year-round is a widely implemented farming practice in the United States and many parts of Europe. Such systems have been linked with increased lameness, reduced comfort and increases in mastitis, all of which affect animal performance (Haskell et al., 2006, Fregonesi et al., 2007). Therefore, the main aim of this study was to investigate the effect of three widely implemented feeding regimes; outdoors on perennial ryegrass (*Lolium perenne* L.), outdoors on perennial ryegrass/white clover (*Trifolium repens* L.) and indoors on total mixed ration (TMR) on the phytochemical, volatile and descriptive sensory profiles of bovine milk. To the authors' best knowledge, no published study has investigated the impact of feeding system on the phytochemical, volatile and descriptive sensory profiles of bovine milk.

5.2 Results and discussion

5.2.1 Microbial Analyses

Each raw and pasteurised milk sample was tested for the presence of coliforms and enterococci in addition to the total bacteria count. Results are presented in Table S5.1. As expected, there was a significant decrease in microbial activity post pasteurisation and no coliforms were detected.

5.2.2 Pasteurised Milk Compositions

The fat, protein, lactose, true protein and casein contents for the milk samples taken at mid and late lactation are available in Table S5.2. Significant differences were observed between the levels of fat, protein, lactose, true protein and casein at $P = 0.001$ based on stage of lactation, in agreement with the study by O'Callaghan et al. (2016) who reported significant differences between fat, protein and casein but not lactose over an entire lactation.

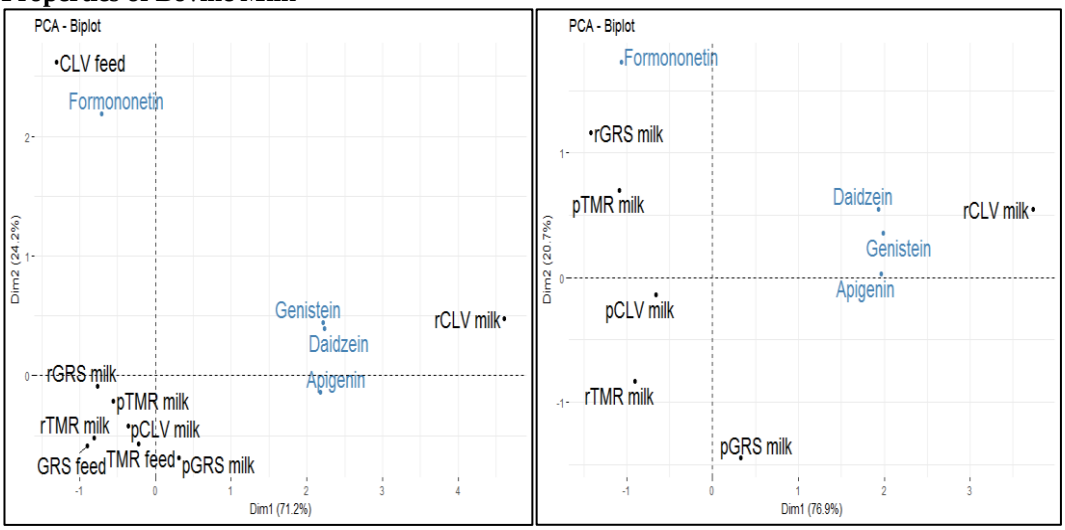
5.2.3 Free Fatty Acid Analyses

Results showed a significant increase in C18:1 in grass (GRS) milk samples from day 3 to 14. In the grass/clover (CLV) samples, significant differences were

observed across all the 11 FFAs and finally, significant increases were observed in the levels of C6, C8, C14, C16, C18 and C18:1 in the TMR samples from day 3 to 14 (Table S5.3 and S5.4). Variations in FFA content of milk in the present study are in agreement with the study conducted by Villeneuve, *et al*. (Villeneuve et al., 2013) whereby levels of FFA with chain length of 4 (butanoic acid) to 16 (tetradecanoic acid) were found to be higher in milk from cows fed pasture than milk from cows fed silage produced from timothy grass swards. Similarly, levels of C18:1 were higher in milk from pasture compared with milk from silage. (TMR day 9 milk was omitted from results processing due to possible microbial contamination). The levels of FFA across all samples, particularly the short chain FFA were low and so were unlikely to cause any objectionable off-flavours associated with FFA described above, this is also indicative of good quality milk.

5.2.4 Phytochemical Analyses

Isoflavones are important as they have the ability to be directly transferred from feed to milk and subsequently be reduced to compounds that can potentially impact the sensory properties of milk and other dairy products. In particular, formononetin, which has been linked to the production of p-cresol (Kilic and Lindsay, 2005). p-Cresol was not detected in the milk samples at day 3 but was detected in all samples at day 9 and 14 of storage. It is possible that it was present in the milk samples at day 3 in sulfonated form or below levels of detection and was subsequently released by enzymatic action, specifically by arylsulfatase during the storage period (Stressler et al., 2016). The concentrations of formononetin was found to be significantly correlated to white clover (CLV) feed samples (Figure 5.1a). Levels of apigenin, daidzein and genistein were found to be significantly different between the raw (r) and pasteurised (p) GRS, CLV and TMR milk samples. Daidzein and genistein were highly correlated to rCLV milk and formononetin was more closely correlated with rGRS milk (Figure 5.1b).



(a) **(b)**

Figure 5.1: (a) Principal component analysis **(**PCA) biplot showing the correlations between the isoflavones (apigenin, daidzein, formononetin, and genistein) identified in feed samples (grass, grass/clover and TMR) samples and the corresponding raw (r) and pasteurised (p) (GRS, CLV and TMR) milk samples as determined by liquid chromatography tandem mass spectrometry (LC-MSMS); **(b)** PCA biplot showing the correlation of the isoflavones (apigenin, daidzein, formononetin, and genistein) to raw (r) and pasteurised (p) GRS, CLV and TMR milk samples as determined by LC-MSMS.

The concentration of formononetin was found to be highest in rGRS milk, as previously mentioned, formononetin is likely degraded to p-cresol, a compound that has been associated with barnyard aroma in dairy products. Both r and p GRS milk had the highest levels of p-cresol at day 9 and 14, pGRS milk was also more correlated with barnyard aroma than the pCLV and pTMR. The significant correlation of formononetin to CLV feed samples (Figure 5.1) is expected as leguminous plants such as clover are naturally rich in phytoestrogens (Steinshamn et al., 2008). The difference in levels between r and p milks suggests an effect of pasteurisation on the compounds but it is possible that some or all of the formononetin present in the samples was demethylated to daidzein, which is highest in rCLV milk and not detected in the corresponding CLV feed samples (Figure S5.1). It is also possible that daidzein was further reduced via hydrogenation and ring scission to equol (a microbial metabolite of isoflavone with high estrogenic activity) (Daems et al., 2016) or metabolized to O-desmethylangolensin (Mace et al., 2019). The composition of the individual bovine gut microflora impacts largely on the metabolism of daidzein and subsequently on the rate of equol excretion (Steinshamn

et al., 2008). Although formononetin is closely correlated to rGRS milk, the TMR feeding system implemented in this study is partly soya based in addition to containing grass silage and maize silage which could explain its proximity to pTMR milk (Figure 5.1). The other three isoflavones (daidzein, genistein and apigenin) are significantly correlated to rCLV milk. Numerous isoflavones are readily reduced or converted to other phytoestrogens. As previously mentioned, formononetin can be converted to other isoflavones such as daidzein and subsequently equol (Bone et al., 2012). Genistein and daidzein both require degradation to the active compound by gut microflora in order to become bioavailable; S(–)-equol is the active metabolite of daidzein. Genistein, a metabolite of biochanin A, is generally metabolized to glucuronides and sulfate conjugates (Smit et al., 2014). Genistein can also be degraded to the higher homolog of p-cresol; 4-ethyl-phenol, by gut microflora (Sakakibara et al., 2004). 4-Ethyl-phenol is an inactive metabolite with no estrogenic activity. p-Cresol-sulfate has been shown to be a gut-mediated metabolite of genistein. Apigenin has been reported to be metabolized to luteolin, mediated by the enzyme cytochrome P450 (Breinholt et al., 2002). Isoflavone metabolites are also known to be excreted in the urine of ruminants (Jones et al., 1994) and so losses occur. Moreover, Turner (Turner, 1958) postulated that the epithelial cells in the mammary gland may only be semi-permeable to estrogenic compounds resulting in limited transfer from the blood to the milk. Thus, it seems likely that any isoflavones that are present through ingestion can be metabolized to odour-active compounds that potentially impact on the sensory properties of bovine milk.

5.2.5 Volatile Analyses (Feed, Raw and Pasteurised Milk)

Volatile profile analysis by HS-SPME GCMS was performed on the GRS, CLV and TMR feed samples and on the r and p GRS, CLV and TMR milk samples on days 3, 9 and 14 of refrigerated storage. The transfer of VOCs from feed to bovine milk is well documented, studies have shown that volatile compounds in forage and feed can enter milk by two mechanisms; absorption via the digestive tract (rumen or intestine) before diffusing into the blood and subsequently the mammary gland, and/or through the pulmonary system where volatiles present in the air are inhaled, absorbed through the lungs, enter the blood steam and diffuse into the mammary gland (Vialloninsta et al., 2000, Faulkner et al., 2018). 90 and 104 compounds were identified in GRS and CLV feed samples, respectively, consisting mainly of

aldehydes, ketones, esters, alcohols and, hydrocarbons. 94 compounds were identified in TMR samples consisting mainly of aldehydes, ketones, esters, alcohols, acids and hydrocarbons, (Figures S5.2 and S5.3).

Eleven aldehydes, 10 ketones, 30 esters, 10 alcohols, 7 acids, 2 fatty acid esters, 1 terpene, 4 furans, 5 hydrocarbons, 2 phenols, 2 sulphur compounds, 2 lactones, 4 pyrazines and 1 ether compound varied significantly ($P < 0.001$) between the feed types (Table 5.1). As well as direct transfer from feed, alterations in VOCs in milk can occur during pasteurisation (thermal) or during storage (oxidative, microbial and enzymatic). Of the 32 volatile compounds identified in the feed samples and the corresponding raw milk samples, 20 were identified across all samples (decanal, heptanal, hexanal, nonanal, 2-heptanone, 2-pentanone, acetone, acetophenone, 2-methyl-1-butanol, 3-methyl-1-butanol, cumene, mesitylene, 2,4-dimethylfuran, 1,3-bis(1,1-dimethylethyl)-benzene, 2,4-dimethyl-benzaldehyde, p-xylene, tert-butylbenzene, toluene, dimethyl sulphide and vinylisopentyl ether). It is probable that some of these compounds were transferred directly from the feed to the milk. Decanal (swe*et al*dehydic), heptanal (green, fatty, herbal), hexanal (green, fatty), nonanal (waxy, orange-peel, fatty), octanal (aldehydic, waxy, fatty) and pentanal (fermented, cardboard-like, bready, nutty) are lipid oxidation products resulting from fatty acid degradation (Romeu-Nadal et al., 2004, Villeneuve et al., 2013) and 2-heptanone (fruity, spicy, sweet), 2-pentanone (sweet, fruity, ethereal) are secondary oxidation products. Acetone (hay, earthy, wood pulp) has previously been reported to originate from the cows diet (Marsili, 2016), acetophenone (floral) is a product of phenylalanine metabolism (Fox, 2013) and is also a product of the Maillard reaction, which has been attributed to the heated or sterilized flavour of ultra-heat treated milk (Griffiths, 2010). 2-Methyl-1-butanol (roasted, wine, onion, fruity) and 3-methyl-1-butanol (fermented) may have originated from the degradation of isoleucine and leucine, respectively, by *Saccharomyces cerevisiae*, as well as other yeasts (Bigelis et al., 1983). They may have also been produced from their corresponding methylketones by reductase activity (Dan et al., 2017). 1-Pentanol (fermented, bready, yeasty) is derived from the primary aldehyde pentanal by oxidation (Faulkner et al., 2018). α-Pinene (herbal) is most likely derived from plant diet and was highest in GRS feed and rGRS and pGRS milk samples. Cumene (gasoline like), also a plant derived alkylbenzene (Howard et al., 1990) was highest in rCLV and pCLV milk samples. Mesitylene (sweet) is a benzene derivative that is structurally related to

toluene (nutty, bitter, almond, plastic), m-xylene (plastic) and ethylbenzene (gasoline-like) and was found to be highest in CLV feed samples. It is possible that mesitylene is formed through carotenoid degradation, which is observed for other benzene compounds but it has also been found unchanged in the blood and urine of human patients as a result of air exposure and so could be introduced through the inhalation pathway (Janasik et al., 2008). It is possible that the furan 2,4-dimethylfuran (odour unknown) in the milk samples is due to the thermal degradation of certain amino acids including serine and cysteine (Vranova and Ciesarova, 2009). 1,3-Bis(1,1-dimethylethyl)-benzene (odour unknown), 2,4-dimethyl-benzaldehyde (naphthyl) and tert-butylbenzene (phenolic) could have entered the milk through inhalation or ingestion and were possibly partially degraded to phenol (phenolic) (Wardhan, 2016), however, some benzene compounds are thought to be products of the Strecker reaction (Yue et al., 2015). p-Xylene (sweet) may be present as a result of β-carotene degradation in the rumen (Zepka et al., 2014) or from direct transfer from feed (Buchin et al., 1998). Toluene, a product of β-carotene degradation (Faulkner et al., 2018) is also derived from plant diet and is highest in CLV feed samples but was higher in r and p GRS milk samples. Dimethyl sulphide (sulphurous, onion, cabbage) has been shown to be transferred from the rumen to milk (Shipe et al., 1962) and two possible precursors, originating in plant materials may account for this, namely dimethyl-fl-propiothetin and methylmetbioninesulphonium salt (Day et al., 1964). Dimethylsulfoniopropionate can also undergo degradation via cleavage to dimethyl sulphide or demethylation and demethiolation to methanethiol (Yoch, 2002) which was detected in all feed samples but only in rCLV milk samples at day 14 of storage. Dimethyl sulfone (sulphurous, burnt) is also a product of methionine degradation and a product of plant diet (Vazquez-Landaverde et al., 2005b, Villeneuve et al., 2013), thus, the higher levels identified in GRS feed, and in the corresponding r and p GRS milk samples, possibly from the higher concentrations of digestible proteins are in agreement with previous studies (Toso et al., 2002, Faulkner et al., 2018). 2-Hexanone (fruity, meaty, buttery), and methyl isobutyl ketone (green, fruity) are likely lipid oxidation products (Faulkner et al., 2018, Thomsen et al., 2018) and were identified in all milk samples. Acetyl valeryl (2,3-heptanedione) (buttery) was only identified in CLV feed samples. Concentrations of acetyl valeryl in cheese products has been previously associated with the presence of certain *Lactococcus Lactic* strains, milk storage temperatures before cheese making (Centeno et al., 2004)

and seasonal variations (Fernandez-Garcia et al., 2002), suggesting that it could be dependent on feed composition. The levels of acetyl valeryl increased in r and p CLV milk samples during storage at 4 °C. 2-Butanone (buttery, sour milk, ethereal) derived from carbohydrate metabolism, was only detected in TMR milk samples, and has previously been reported to originate from the cows diet and from carbohydrate metabolism (Marsili, 2016). Ethylbenzene, likely a product of carotenoid degradation, and 1-hexanol, derived from the aldehyde hexanal (Kilcawley et al., 2018) were also detected in rTMR milk only.

Table 5.1: Concentrations of the volatile compounds identified by headspace solid-phase microextraction (HS-SPME GCMS) analysis of the feed samples (grass (GRS), grass/clover (CLV) and TMR); values indicate average area values of triplicate analysis for each compound. CAS no. = Chemical Abstracts Service number. One-way ANOVA statistical analysis $P < 0.001$.

Compound	LRI[1]	Ref LRI[2]	CAS No.	Grass	Grass/Clover	TMR	*p*-Value
Aldehyde							
2-Methyl butanal	700	700	96-17-3	6.25×10^8	2.03×10^8	6.29×10^8	NS 0.067
3-Methyl butanal	690	692	590-86-3	5.69×10^8	7.97×10^8	2.28×10^9	***<0.001
Acetaldehyde	449	452	75-07-0	1.19×10^8	2.73×10^8	4.38×10^7	***0.002
Butanal	627	622	123-72-8	8.78×10^7	1.81×10^8	0.00×00	***<0.001
Decanal	1251	1256	112-31-2	4.39×10^9	4.90×10^9	2.08×10^9	NS 0.315
Furfural	870	899	98-01-1	1.09×10^8	4.31×10^7	2.20×10^7	***0.017
Heptanal	941	943	111-71-7	1.83×10^9	2.75×10^9	4.26×10^7	***0.004
Hexanal	837	839	66-25-1	1.69×10^{10}	3.59×10^{10}	1.15×10^9	***0.001
Nonanal	1147	1150	124-19-6	2.60×10^9	5.02×10^9	2.43×10^8	***0.001
Octanal	1044	1047	124-13-0	1.47×10^9	2.44×10^9	0.00×00	***0.001
Pentanal	735	733	110-62-3	1.49×10^9	2.07×10^9	0.00×00	***0.002
Propanal	526	523	123-38-6	1.83×10^8	4.92×10^8	0.00×00	***<0.001
Methacrolein	570	574	78-85-3	0.00×00	6.23×10^6	0.00×00	***0.006
Ketone							
1-Hydroxy-2-propanone	734	734	116-09-6	0.00×00	2.80×10^8	4.22×10^7	***0.001
2-Butanone	638	639	78-93-3	0.00×00	0.00×00	1.08×10^8	NS 0.080
2-Heptanone	932	936	110-43-0	6.22×10^8	1.16×10^9	2.60×10^8	***0.011
2-Hexanone	831	834	591-78-6	1.10×10^8	1.94×10^8	0.00×00	***0.017
2-Nonanone	1137	1140	821-55-6	1.18×10^9	1.61×10^9	0.00×00	***0.010
2-Pentanone	728	730	107-87-9	5.36×10^8	6.40×10^8	4.79×10^7	***0.027
4-Hydroxy-4-methyl-2-pentanone	913	913	123-42-2	4.00×10^9	3.50×10^9	1.43×10^8	***0.027
6-Methyl-5-hepten-2-one	1031	1034	110-93-0	7.89×10^8	2.84×10^9	1.07×10^9	***<0.001
Acetoin	778	778	513-86-0	1.33×10^9	1.62×10^9	2.74×10^8	NS 0.053
Acetone	532	533	67-64-1	3.31×10^8	2.85×10^9	5.43×10^7	***<0.001
Acetophenone	1141	1030	98-86-2	1.08×10^8	1.77×10^8	9.55×10^7	NS 0.188
Acetyl valeryl (2,3-heptanedione)	875	-	96-04-8	0.00×00	4.32×10^8	0.00×00	***<0.001
Cyclohexanone	958	957	108-94-1	1.28×10^9	1.33×10^9	0.00×00	***0.017
Methyl Isobutyl Ketone	781	784	108-10-1	1.80×10^8	0.00×00	0.00×00	***<0.001
Ester							
2-Methylbutyl acetate	906	906	624-41-9	0.00×00	0.00×00	1.44×10^8	NS 0.076
2-Methylbutyl butanoate	1080	-	51115-64-1	0.00×00	8.43×10^6	9.70×10^8	***0.005
Amyl isobutyrate (or isomer)	1121	-	2445-72-9	0.00×00	2.73×10^9	2.60×10^{10}	***<0.001
Amyl propionate	992	-	105-68-0	2.59×10^7	3.19×10^7	6.81×10^8	***<0.001
Butyl acetate	842	842	123-86-4	0.00×00	0.00×00	9.12×10^8	***<0.001
B-Phenylethyl acetate	1339	-	103-45-7	0.00×00	3.75×10^7	2.21×10^8	***0.009
Dimethyl succinate	1081	1082	106-65-0	2.91×10^7	2.87×10^7	0.00×00	***0.019

Ethyl heptanoate	1121	-	106-30-9	6.69×10^{7}	1.13×10^{9}	6.42×10^{9}	***<0.001
Ethyl acetate	641	642	141-78-6	4.27×10^{8}	1.06×10^{9}	1.10×10^{9}	***0.002
Ethyl benzoate	1229	-	93-89-0	7.81×10^{7}	7.40×10^{7}	1.37×10^{7}	NS 0.083
Ethyl butanoate	823	826	105-54-4	0.00 × 00	2.68×10^{9}	3.02×10^{10}	***<0.001
Ethyl decanoate	1420	1422	110-38-3	0.00 × 00	2.22×10^{8}	1.45×10^{8}	***<0.001
Ethyl dodecanoate	1622	1621	106-33-2	0.00 × 00	1.93×10^{8}	3.05×10^{8}	***<0.001
Ethyl hexanoate	1021	1024	123-66-0	2.33×10^{9}	8.03×10^{9}	5.44×10^{10}	***<0.001
Ethyl lactate	861	862	97-64-3	0.00 × 00	1.62×10^{8}	2.60×10^{9}	***<0.001
Ethyl nonanoate	1319	-	123-29-5	0.00 × 00	6.26×10^{8}	4.45×10^{8}	NS 0.104
Ethyl octanoate	1220	-	106-32-1	4.51×10^{8}	1.80×10^{9}	4.76×10^{9}	***<0.001
Ethyl pentanoate	923	924	539-82-2	2.25×10^{8}	1.22×10^{9}	1.34×10^{10}	***<0.001
Ethyl propanoate	735	737	105-37-3	2.65×10^{8}	7.16×10^{8}	1.26×10^{9}	***<0.001
Hexyl acetate	1038	-	142-92-7	1.83×10^{8}	3.43×10^{8}	2.09×10^{9}	***<0.001
Isoamyl acetate	902	902	123-92-2	2.48×10^{8}	3.21×10^{8}	5.19×10^{8}	***0.029
Isoamyl isobutanoate	1038	-	2050-01-3	3.07×10^{7}	8.29×10^{6}	2.96×10^{9}	***<0.001
Isobutyl butyrate	978	-	539-90-2	0.00 × 00	0.00 × 00	7.09×10^{8}	***<0.001
Isopentyl hexanoate	1276	-	2198-61-0	0.00 × 00	2.15×10^{7}	6.22×10^{8}	***<0.001
Methyl butanoate	748	-	623-42-7	9.77×10^{7}	9.73×10^{8}	3.04×10^{9}	***<0.001
Methyl decanoate	1351	-	110-42-9	0.00 × 00	2.91×10^{8}	0.00 × 00	***<0.001
Methyl dodecanoate	1550	-	111-82-0	0.00 × 00	1.19×10^{8}	6.07×10^{7}	***<0.001
Methyl hexanoate	949	-	106-70-7	5.52×10^{8}	7.42×10^{9}	1.13×10^{10}	***<0.001
Methyl propionate	657	-	554-12-1	1.95×10^{7}	1.63×10^{8}	1.08×10^{8}	***<0.001
n-Propyl acetate	739	-	109-60-4	1.60×10^{9}	1.97×10^{9}	3.43×10^{9}	***0.008
Pentyl acetate	901	-	628-63-7	6.38×10^{8}	1.72×10^{8}	7.74×10^{8}	***0.012
Propyl 2-methylbutanoate	969	-	37064-20-3	3.67×10^{7}	5.63×10^{7}	0.00 × 00	NS 0.112
Propyl butyrate	921	-	644-49-5	2.57×10^{9}	3.56×10^{9}	3.28×10^{10}	***<0.001
Propyl hexanoate	1118	-	626-77-7	1.53×10^{9}	3.07×10^{9}	3.57×10^{10}	***<0.001
Alcohol							***<0.001
1-Hexanol	903	916	111-27-3	7.07×10^{8}	1.95×10^{9}	8.42×10^{8}	NS 0.502
1-Octanol	1112	1118	111-87-5	1.56×10^{9}	2.39×10^{9}	0.00 × 00	***0.008
1-Pentanol	816	815	71-41-0	1.02×10^{9}	1.30×10^{9}	0.00 × 00	***0.018
1-Propanol	612	612	71-23-8	2.10×10^{9}	3.68×10^{9}	1.06×10^{9}	***0.007
1-Methoxy-2-propanol	713	713	107-98-2	4.91×10^{8}	4.80×10^{8}	1.49×10^{7}	***0.043
2-Methyl-1-butanol	783	789	137-32-6	2.75×10^{8}	3.89×10^{8}	2.77×10^{8}	NS 0.418
2-Methyl-1-propanol	678	678	78-83-1	4.48×10^{7}	8.06×10^{7}	7.45×10^{6}	***0.005
2-Methyl propanol	609	-	78-84-2	6.69×10^{7}	7.84×10^{7}	0.00 × 00	NS 0.112
2-Butanol	648	648	78-92-2	4.15×10^{8}	6.63×10^{8}	1.28×10^{8}	***0.005
3-Methyl-1-butanol	783	784	123-51-3	2.41×10^{8}	1.54×10^{8}	2.45×10^{7}	***0.043
Ethanol	506	506	64-17-5	1.23×10^{9}	4.60×10^{9}	1.33×10^{9}	***<0.001
Isopropyl Alcohol	543	-	67-63-0	6.64×10^{7}	1.23×10^{8}	1.80×10^{7}	***0.011
Phenylethyl Alcohol	1199	-	60-12-8	0.00 × 00	9.02×10^{7}	8.73×10^{8}	***<0.001
Acid							
2,2-Dimethyl-propanoic acid	837	869	75-98-9	0.00 × 00	2.13×10^{8}	0.00 × 00	***0.004
3-Methyl-butanoic acid	918	-	503-74-2	0.00 × 00	0.00 × 00	1.04×10^{10}	***<0.001
Acetic acid	690	690	64-19-7	8.94×10^{9}	1.02×10^{10}	9.85×10^{10}	***<0.001

Butanoic acid	864	864	107-92-6	8.80×10^{9}	9.17×10^{9}	1.04×10^{11}	***<0.001
Pentanoic acid	995	-	109-52-4	0.00×00	0.00×00	2.51×10^{10}	***<0.001
Propanoic acid	778	802	79-09-4	1.51×10^{9}	1.09×10^{9}	2.02×10^{10}	***<0.001
Hexanoic acid	1052	1052	142-62-1	3.23×10^{9}	2.90×10^{9}	2.49×10^{10}	***<0.001
Fatty acid esters							***<0.001
Propanoic acid, butyl ester	931	-	590-01-2	1.79×10^{8}	0.00×00	9.44×10^{8}	***<0.001
Butanoic acid, butyl ester	978	-	109-21-7	0.00×00	0.00×00	6.04×10^{9}	***<0.001
Terpene							
3-Carene	1035	1027	13466-78-9	5.45×10^{7}	2.41×10^{7}	0.00×00	NS 0.084
α-Pinene	954	951	80-56-8	1.34×10^{7}	0.00×00	$8.17 \times 1\ 0^{6}$	NS 0.270
Cumene	991	-	98-82-8	6.13×10^{7}	1.11×10^{8}	5.04×10^{7}	NS 0.166
Mesitylene	1029	-	108-67-8	8.64×10^{8}	2.03×10^{9}	1.11×10^{7}	*** 0.044
Furan							
2-Ethyl furan	717	720	3208-16-0	4.50×10^{8}	1.25×10^{9}	1.89×10^{7}	***<0.001
2-Methyl furan	615	615	534-22-5	1.64×10^{7}	4.55×10^{7}	0.00×00	***0.005
2-Pentyl furan	1010	1012	3777-69-3	1.12×10^{9}	2.52×10^{9}	5.17×10^{8}	***0.004
2-n-Butyl furan	917	-	4466-24-4	0.00×00	6.00×10^{7}	0.00×00	NS 0.079
2,4-Dimethyl furan	733	-	3710-43-8	3.27×10^{7}	3.11×10^{7}	0.00×00	***0.019
Hydrocarbon							
1,3-bis(1,1-dimethylethyl)-benzene	1284	-	1014-60-4	1.31×10^{11}	1.73×10^{11}	5.81×10^{10}	NS 0.060
2,4-Dimethyl-benzaldehyde	1305	-	15764-16-6	7.46×10^{8}	9.18×10^{8}	2.93×10^{8}	***0.019
Benzaldehyde	1027	1032	100-52-7	3.41×10^{9}	3.44×10^{9}	2.05×10^{9}	NS 0.466
Benzothiazole	1320	-	95-16-9	4.06×10^{8}	6.04×10^{8}	5.28×10^{8}	NS 0.156
Ethylbenzene	897	890	100-41-4	0.00×00	0.00×00	6.02×10^{7}	***<0.001
Mesitylene	1028	-	108-67-8	8.64×10^{8}	2.03×10^{9}	1.11×10^{7}	***0.044
o-Cymene	1056	-	527-84-4	1.72×10^{7}	4.73×10^{6}	0.00×00	NS 0.055
o-xylene	925	916	95-47-6	1.09×10^{8}	9.63×10^{8}	0.00×00	NS 0.108
p-Xylene	895	895	106-42-3	7.52×10^{8}	1.32×10^{9}	5.47×10^{8}	NS 0.146
p-Cresol	1193	-	106-44-5	4.52×10^{8}	2.89×10^{8}	4.40×10^{8}	NS 0.113
Styrene	927	929	100-42-5	1.21×10^{8}	2.37×10^{8}	1.07×10^{8}	NS 0.078
tert-Butylbenzene	1024	-	98-06-6	4.74×10^{8}	6.60×10^{8}	1.53×10^{8}	***0.037
Toluene	792	794	108-88-3	8.30×10^{7}	1.31×10^{8}	3.62×10^{7}	***0.040
Phenolic							
Phenol	1096	1112	108-95-2	0.00×00	0.00×00	5.87×10^{8}	***<0.001
2-Methoxy-4-vinylphenol	1150	-	7786-61-0	0.00×00	0.00×00	3.98×10^{7}	***<0.001
Sulfur							
Dimethyl sulfide	537	538	75-18-3	1.78×10^{8}	2.90×10^{8}	6.17×10^{7}	***0.004
Dimethyl sulfone	1054	1055	67-71-0	2.21×10^{8}	6.32×10^{7}	0.00×00	***0.014
Methanethiol	459	462	74-93-1	9.29×10^{6}	1.02×10^{7}	8.84×10^{6}	NS 0.855
Ether							
Vinylisopentyl ether	765	-	39782-38-2	1.10×10^{8}	2.89×10^{8}	3.71×10^{7}	***0.033
Lactone							
y-Hexalactone	1166	-	695-06-7	8.51×10^{8}	1.07×10^{9}	4.06×10^{8}	***0.040
y-Nonalactone	1489	-	104-61-0	4.69×10^{8}	6.44×10^{8}	3.75×10^{8}	NS 0.279

Pyrazine							
2,3,5-Trimethyl-6-ethylpyrazine	1190	-	17398-16-2	0.00 × 00	0.00 × 00	1.48×10^8	***<0.001
2,3-Dimethyl-pyrazine	961	-	5910-89-4	1.27×10^8	3.77×10^7	1.33×10^9	***<0.001
3-Ethyl-2,5-dimethyl-pyrazine	1055	-	5910-89-4	0.00 × 00	0.00 × 00	4.71×10^7	***<0.001
Pyrazine	771	-	290-37-9	5.46×10^7	5.55×10^7	4.79×10^7	NS 0.847
Trimethyl-pyrazine	1041	1041	14667-55-1	8.50×10^7	3.89×10^7	9.03×10^8	***<0.001

[1]LRI: Linear retention index. [2]Ref LRI: Linear retention index reference for compounds identified by standards and/or NIST library where available.

Many newly formed compounds were identified in milk samples at day 14 of storage, in particular esters. Moreover, the levels of certain compounds present on day 3 of analysis increased or decreased over storage highlighting that storage time has an effect on the volatile profile of bovine milk (Table 5.2 and S5.5). Rashid et al. (2019) investigated the effect of storage time on the concentrations of volatiles known to cause off-flavours in milk. Results showed the ability of certain compounds to both increase and decrease over time at 4 and 7 °C. Any fluctuations occurring throughout storage are likely due to lipid hydrolysis (Villeneuve et al., 2013), lipid oxidation (Havemose et al., 2006), microbial changes by indigenous or bacterial lipases (Dan et al., 2017) or by enzymatic action (Santos et al., 2003).

Table 5.2: Relationship between cow feeding system (grass, grass/clover and TMR) and the raw (r) milk volatile compounds identified by HS-SPME GC-MS at day 3, 9 and 14 of refrigerated storage; values are expressed as peak area values for each compound. d = day, $P = 0.05$, ND = not detected, NS = not significant, GRS = Grass, CLV = Grass/clover. [1]LRI= Linear retention index.

Compound	CAS No.	LRI[1]	Grass d 3	Grass/ CLV d 3	TMR d 3	Grass d 9	Grass/CL V d 9	TMR day 9	Grass d 14	Grass/CLV d 14	TMR d 14	*p*-Value	*p*-Value (Grass)	*p*-Value (Grass/ CLV)	*p*-Value (TMR)
Aldehyde															
(E)-2-Octenal (or isomer)	2548-87-0	1094	0.00 × 00	0.00 × 00	1.74×10^7	7.29×10^7	2.51×10^8	2.22×10^7	2.85×10^8	1.11×10^9	2.18×10^8	*<0.001	NS 0.051	*0.003	*<0.05
(Z)-2-Heptenal (or isomer)	57266-86-1	1012	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.03×10^7	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	ND	*0.006
Acetaldehyde	75-07-0	449	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.85×10^7	0.00 × 00	2.40×10^6	* 0.029	NS 0.302	ND	NS 0.129
3-Methyl-butanal	590-86-3	690	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	9.93×10^7	7.12×10^8	1.75×10^7	*<0.001	*<0.001	*<0.001	*0.002
Decanal	112-31-2	1250	1.47×10^7	1.29×10^7	3.26×10^6	8.28×10^6	5.23×10^6	4.97×10^6	1.18×10^7	3.73×10^6	3.52×10^6	NS 0.477	NS 0.658	NS 0.515	NS 0.736
Heptanal	111-71-7	941	1.09×10^8	1.08×10^8	1.49×10^8	1.06×10^7	1.72×10^6	7.27×10^8	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	* 0.001	*<0.001	*<0.001
Hexanal	66-25-1	838	3.69×10^8	4.02×10^8	1.72×10^9	0.00 × 00	0.00 × 00	3.56×10^9	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	* 0.000	*<0.001	*<0.001
Nonanal	124-19-6	1147	5.31×10^7	5.02×10^7	6.91×10^7	3.66×10^7	2.81×10^7	1.23×10^8	3.98×10^7	1.90×10^7	3.44×10^7	*<0.001	NS 0.259	*<0.001	*<0.001
Octanal	124-13-0	1044	2.22×10^7	3.11×10^7	4.02×10^7	1.21×10^7	0.00 × 00	8.73×10^7	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	*0.007	*0.009	*<0.001
Pentanal	110-62-3	733	1.39×10^8	1.96×10^8	9.84×10^6	0.00 × 00	0.00 × 00	1.82×10^8	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	*<0.001	*<0.001	*<0.001
Ketone															
2-Butanone	78-93-3	637	3.86×10^7	1.05×10^8	1.49×10^8	5.86×10^7	1.11×10^8	1.48×10^8	2.64×10^7	9.64×10^7	7.42×10^7	*<0.001	NS 0.127	*0.007	*<0.001
2-Heptanone	110-43-0	933	3.37×10^7	3.60×10^7	3.20×10^7	4.37×10^8	8.14×10^8	4.67×10^7	1.52×10^9	3.63×10^9	9.04×10^9	*<0.001	*<0.001	*<0.001	*<0.001
2-Hexanone	591-78-6	831	1.77×10^7	9.14×10^6	8.93×10^6	2.33×10^7	2.96×10^7	2.35×10^6	2.63×10^7	6.70×10^7	8.27×10^7	*<0.001	NS 0.603	*<0.001	*<0.001
2-Nonanone	821-55-6	1137	0.00 × 00	0.00 × 00	0.00 × 00	2.25×10^8	1.49×10^8	0.00 × 00	5.16×10^8	5.69×10^8	2.46×10^9	*<0.001	*0.005	*0.002	*<0.001

2-Octanone	111-13-7	1034	6.82×10^6	1.01×10^7	1.13×10^7	1.04×10^7	2.62×10^7	2.21×10^6	2.31×10^7	5.00×10^7	5.10×10^7	*<0.001	*0.027	*<0.001	*0.001
2-Pentanone	107-87-9	727	5.70×10^7	5.47×10^7	5.36×10^7	1.06×10^8	2.17×10^8	4.00×10^7	1.94×10^8	5.48×10^8	6.91×10^8	*<0.001	*<0.001	*<0.001	*<0.001
2-Undecanone	112-12-9	1353	0.00 × 00	0.00 × 00	0.00 × 00	5.97×10^6	4.63×10^5	0.00 × 00	3.82×10^7	1.52×10^7	2.78×10^8	*<0.001	NS 0.262	*0.047	*<0.001
2,3-Pentanedione	600-14-6	736	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.03×10^8	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	ND	*0.007
3,5-(E,E)-Octadien-2-one (or isomer)	30086-02-3	1130	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.17×10^7	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	ND	*0.015
3-Hexen-2-one	763-93-9	839	6.97×10^6	7.68×10^6	2.76×10^6	1.59×10^7	1.54×10^7	0.00 × 00	1.53×10^7	8.90×10^6	1.58×10^7	NS 0.177	NS 0.557	NS 0.517	NS 0.051
4-Methyl-3-pentene-2-one (tentative)	141-79-7	839	0.00 × 00	0.00 × 00	0.00 × 00	1.15×10^7	5.55×10^6	0.00 × 00	9.92×10^6	1.23×10^7	1.58×10^7	*0.047	NS 0.500	ND 0.065	*0.025
5-Hepten-2-one (tentative)	6714-00-7	921	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.42×10^7	5.23×10^7	*<0.001	ND	*<0.001	*<0.001
Acetone	67-64-1	532	9.16×10^8	8.04×10^8	1.14×10^9	7.81×10^8	7.97×10^8	1.22×10^9	3.00×10^8	7.20×10^8	6.65×10^8	*<0.001	*0.003	NS 0.538	*<0.001
Acetophenone	98-86-2	1030	6.38×10^6	2.05×10^6	1.64×10^6	0.00 × 00	4.23×10^6	3.22×10^6	1.77×10^6	1.06×10^6	0.00 × 00	* 0.044	NS 0.114	NS 0.113	NS 0.251
Cyclohexanone	110-82-7	956	7.99×10^6	0.00 × 00	0.00 × 00	1.37×10^7	1.67×10^6	0.00 × 00	6.52×10^6	1.33×10^6	1.33×10^6	* 0.046	NS 0.728	NS 0.623	NS 0.422
Acetyl valeryl (2,3-heptanedione)	96-04-8	875	3.78×10^5	2.52×10^5	4.29×10^5	3.31×10^6	3.56×10^7	1.77×10^5	0.00 × 00	2.39×10^7	1.31×10^6	*<0.001	NS 0.194	*0.001	NS 0.582
Methyl Isobutyl Ketone	108-10-1	780	2.05×10^8	1.47×10^8	2.00×10^8	2.14×10^8	1.73×10^8	2.46×10^8	1.74×10^8	1.78×10^8	1.75×10^8	*<0.001	*0.035	*0.023	*0.006
Ester															
Ethyl heptanoate	106-30-9	1120	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	4.08×10^7	0.00 × 00	1.35×10^8	*<0.001	*<0.001	ND	*<0.001
Ethyl (Z)-2-butenoate	6776-19-8	875	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	4.12×10^8	2.51×10^6	1.54×10^8	*<0.001	*<0.001	NS 0.422	*<0.001
Ethyl 2-methylbutanoate	7452-79-1	872	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.38×10^7	0.00 × 00	0.00 × 00	*<0.001	*<0.001	ND	ND
Ethyl 3-methylbutanoate	108-64-5	876	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.81×10^8	0.00 × 00	0.00 × 00	*<0.001	*<0.001	ND	ND
Ethyl acetate	141-78-6	639	0.00 × 00	0.00 × 00	0.00 × 00	2.02 × 107	8.47×10^6	0.00 × 00	2.44×10^8	6.08×10^7	1.85×10^8	*<0.001	*<0.001	*<0.001	*<0.001

Ethyl butanoate	105-54-4	823	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	6.10×10^{9}	0.00 × 00	1.02×10^{10}	*<0.001	*<0.001	ND	*<0.001
Ethyl decanoate	110-38-3	1419	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	4.30×10^{8}	9.37×10^{6}	1.59×10^{9}	*<0.001	*0.019	NS 0.155	*<0.001
Ethyl hexanoate	123-66-0	1021	0.00 × 00	0.00 × 00	0.00 × 00	4.18×10^{8}	0.00 × 00	0.00 × 00	6.90×10^{9}	2.80×10^{7}	8.91×10^{9}	*<0.001	*<0.001	*0.005	*<0.001
Ethyl octanoate	106-32-1	1220	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	7.85×10^{8}	3.72×10^{6}	3.07×10^{9}	*<0.001	*<0.001	NS 0.105	*<0.001
Ethyl pentanoate	539-82-2	923	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	5.50×10^{7}	0.00 × 00	8.08×10^{7}	*<0.001	*0.001	ND	*<0.001
Ethyl propanoate	105-37-3	735	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	4.73×10^{6}	0.00 × 00	2.51×10^{7}	*<0.001	NS 0.465	ND	*<0.001
Methyl butanoate	623-42-7	747	0.00 × 00	0.00 × 00	0.00 × 00	2.25×10^{6}	4.90×10^{6}	0.00 × 00	9.84×10^{5}	2.78×10^{7}	1.47×10^{7}	*<0.001	NS 0.590	*<0.001	*<0.001
Methyl decanoate	110-42-9	1350	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.52×10^{6}	NS 0.084	ND	ND	NS 0.143
Methyl hexanoate	106-70-7	949	0.00 × 00	0.00 × 00	0.00 × 00	1.34×10^{6}	0.00 × 00	0.00 × 00	3.65×10^{6}	0.00 × 00	3.00×10^{7}	*<0.001	NS 0.244	ND	*<0.001
Methyl methacrylate	80-62-6	736	7.47×10^{6}	2.22×10^{6}	3.88×10^{6}	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.002	*<0.001	NS 0.422	*<0.001
Alcohol															
1-Butanol	71-36-3	715	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	4.08×10^{6}	7.33×10^{5}	0.00 × 00	0.00 × 00	0.00 × 00	*0.040	ND	NS 0.080	NS 0.422
2-Methyl-1-butanol	137-32-6	765	2.27×10^{7}	2.15×10^{7}	1.97×10^{7}	2.23×10^{7}	2.02×10^{7}	1.95×10^{7}	0.00 × 00	0.00 × 00	1.18×10^{8}	*<0.001	*0.003	*0.003	*<0.001
3-Methyl-1-butanol	123-51-3	767	5.73×10^{7}	6.92×10^{7}	1.11×10^{8}	5.48×10^{7}	3.01×10^{7}	5.04×10^{7}	1.05×10^{9}	2.56×10^{9}	3.33×10^{8}	*<0.001	*0.011	*<0.001	*<0.001
1-Hexanol	111-27-3	894	0.00 × 00	0.00 × 00	5.83×10^{7}	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	NS 0.080	*0.004
2-Ethyl-1-hexanol	104-76-7	1075	3.69×10^{7}	3.34×10^{7}	1.96×10^{7}	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	*<0.001	*0.001	NS 0.108
1-Octanol	111-87-5	1116	0.00 × 00	0.00 × 00	2.06×10^{7}	0.00 × 00	0.00 × 00	2.22×10^{7}	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	ND	*0.012
1-Pentanol	71-41-0	794	6.10×10^{7}	9.15×10^{7}	1.57×10^{7}	2.95×10^{7}	5.39×10^{7}	8.62×10^{7}	0.00 × 00	2.52×10^{6}	2.52×10^{7}	*<0.001	NS 0.075	*<0.001	*<0.001
Ethanol	64-17-5	505	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.35×10^{7}	0.00 × 00	2.10×10^{8}	4.36×10^{8}	1.14×10^{9}	* 0.000	*0.005	NS 0.070	*0.003

Isopropyl Alcohol	67-63-0	541	0.00 × 00	0.00 × 00	0.00 × 00	2.54×10^7	2.33×10^7	0.00 × 00	4.29×10^7	2.82×10^7	6.11×10^7	*<0.001	NS 0.194	NS 0.146	*<0.001
Acid															
Butanoic acid	107-92-6	863	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	9.61×10^8	NS 0.526	ND	ND	NS0.385
Hexanoic acid	142-62-1	1052	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.70×10^8	0.00 × 00	6.72×10^9	NS 0.371	NS 0.259	ND	NS 0.306
Octanoic acid	124-07-2	1245	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.67×10^9	NS 0.471	ND	ND	NS 0.358
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl	77-68-9	1460	6.78×10^7	5.62×10^7	3.46×10^7	1.62×10^7	2.71×10^7	1.01×10^7	3.01×10^7	5.71×10^6	7.09 10^5	*0.003	NS 0.026	*0.015	NS 0.181
Terpene															
3-Carene	13466-78-9	1035	0.00 × 00	0.00 × 00	1.34×10^7	0.00 × 00	9.50 10^5	7.02×10^6	0.00 × 00	0.00 × 00	0.00 × 00	NS 0.144	ND	NS 0.144	NS 0.285
α-Pinene	80-56-8	953	7.81×10^6	6.03×10^6	4.56×10^6	7.50×10^7	6.39×10^7	4.88×10^7	1.62×10^7	2.73×10^7	1.10×10^7	*<0.001	*0.001	*0.012	*0.003
Cumene	98-82-8	990	2.26×10^6	2.80×10^6	3.47×10^6	5.58×10^6	7.86×10^6	3.13×10^6	3.71×10^6	1.06×10^7	1.64×10^7	*0.035	NS 0.323	NS 0.220	*0.037
D-Limonene	5989-27-5	1055	0.00 × 00	2.78×10^7	1.61×10^7	1.26×10^6	3.05 10^5	1.77×10^7	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	NS 0.465	*<0.001	0.013
Mesitylene	108-67-8	1028	4.47×10^7	3.71×10^7	5.38×10^7	5.14×10^7	4.54×10^7	2.98×10^7	7.23×10^7	6.79×10^7	8.22×10^7	*0.017	NS 0.104	NS 0.070	*0.042
trans-β-Ocimene (or isomer)	3779-61-1	1035	0.00 × 00	1.74×10^7	1.34×10^7	0.00 × 00	0.00 × 00	1.77×10^7	0.00 × 00	0.00 × 00	0.00 × 00	*0.020	ND	NS 0.088	NS 0.067
Furan															
2,4-Dimethylfuran	3710-43-8	732	8.90×10^6	3.64×10^6	7.27×10^6	1.11×10^7	1.36×10^7	0.00 × 00	8.34×10^6	1.56×10^7	1.17×10^7	*<0.001	NS 0.357	*<0.001	*<0.001
2,5-Dimethylfuran	625-86-5	734	8.90×10^6	3.64×10^6	7.27×10^6	1.11×10^7	1.36×10^7	0.00 × 00	8.34×10^6	1.56×10^7	1.17×10^7	*<0.001	NS 0.357	*0.002	*<0.001
2-Ethylfuran	3208-16-0	717	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	3.04×10^6	0.00 × 00	0.00 × 00	0.00 × 00	NS 0.090	ND	NS 0.422	NS 0.172
Hydrocarbon															
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	14035-34-8	1684	1.13×10^7	1.88×10^7	1.35×10^7	2.41×10^7	3.89×10^7	2.39×10^7	5.18×10^6	1.63×10^7	2.83×10^7	*0.012	NS 0.347	*0.008	NS 0.171

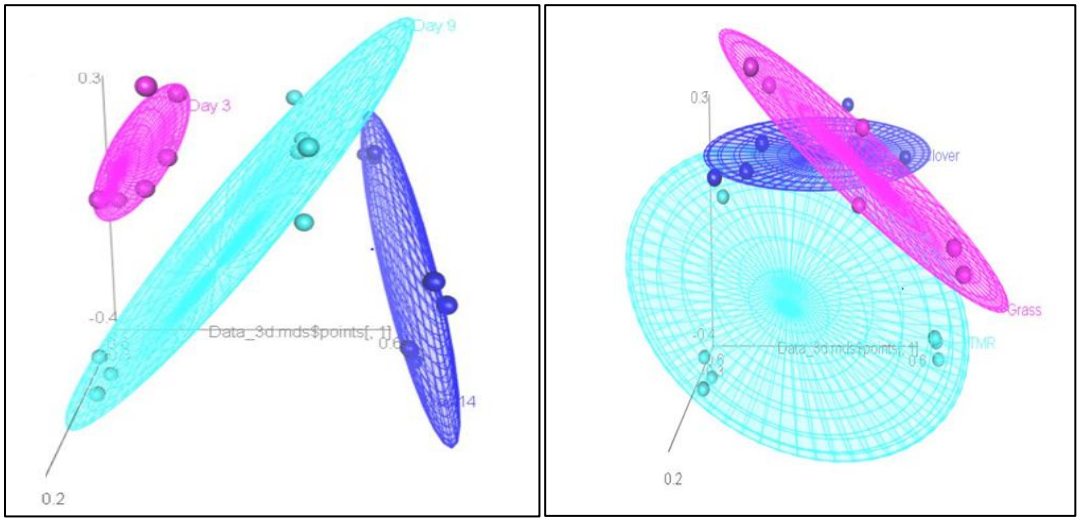
2,4-Dimethyl-benzaldehyde	15764-16-6	1305	6.81×10^{6}	2.24×10^{6}	2.48×10^{6}	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	*<0.001	*<0.001	*0.028	*0.028
Benzene	71-43-2	684	2.55×10^{6}	2.57×10^{6}	4.69×10^{6}	3.63×10^{6}	8.95×10^{7}	1.98×10^{5}	3.21×10^{6}	4.13×10^{7}	2.80×10^{6}	*<0.001	NS 0.943	* 0.017	NS 0.232
1,2,3-Trimethyl-benzene	526-73-8	1028	4.47×10^{7}	3.71×10^{7}	5.38×10^{7}	5.14×10^{7}	4.54×10^{7}	2.98×10^{7}	7.23×10^{7}	6.79×10^{7}	8.22×10^{7}	*0.017	NS 0.104	NS 0.070	*0.042
1,3-Bis(1,1-dimethylethyl)-benzene	1014-60-4	1284	3.49×10^{8}	3.08×10^{8}	2.33×10^{8}	3.67×10^{8}	3.10×10^{8}	2.01×10^{8}	4.52×10^{8}	4.41×10^{8}	6.52×10^{8}	*<0.001	NS 0.185	*0.007	*<0.001
Ethylbenzene	100-41-4	897	1.01×10^{8}	8.28×10^{7}	9.66×10^{7}	1.69×10^{8}	2.08×10^{8}	7.05×10^{7}	2.07×10^{8}	3.05×10^{8}	3.32×10^{8}	*<0.001	*0.004	*<0.001	*<0.001
o-Cymene	527-84-4	1055	0.00×00	0.00×00	0.00×00	5.76×10^{6}	3.30×10^{6}	0.00×00	0.00×00	0.00×00	0.00×00	*<0.001	*<0.001	NS 0.128	ND
p-Cresol	106-44-5	1182	0.00×00	0.00×00	0.00×00	8.49×10^{8}	3.62×10^{8}	2.48×10^{8}	6.35×10^{7}	5.22×10^{7}	5.46×10^{7}	*<0.001	*0.003	*<0.001	*<0.001
p-Xylene	106-42-3	895	1.01×10^{8}	8.28×10^{7}	9.66×10^{7}	1.69×10^{8}	2.08×10^{8}	7.05×10^{7}	2.07×10^{8}	3.05×108	3.32×10^{8}	*<0.001	*0.004	*<0.001	*<0.001
Styrene	100-42-5	927	0.00×00	0.00×00	0.00×00	6.15×10^{6}	0.00×00	2.92×10^{6}	4.11×10^{6}	0.00×00	8.62×10^{6}	NS 0.223	NS 0.631	ND	NS 0.214
tert-Butylbenzene	98-06-6	1024	9.63×10^{6}	8.24×10^{6}	6.27×10^{6}	8.48×10^{6}	6.51×10^{6}	3.21×10^{6}	8.80×10^{6}	1.23×10^{7}	1.32×10^{7}	NS 0.072	NS 0.815	NS 0.223	*0.044
Toluene	108-88-3	792	1.94×10^{9}	1.27×10^{9}	4.98×10^{7}	1.97×10^{9}	1.41×10^{9}	4.51×10^{7}	1.59×10^{9}	1.22×10^{9}	4.97×10^{7}	*<0.001	NS 0.075	*0.008	NS 0.695
Phenolic															
Phenol	108-95-2	1093	0.00×00	0.00×00	0.00×00	4.65×10^{6}	0.00×00	5.29×10^{6}	9.74×10^{6}	0.00×00	5.77×10^{6}	*0.021	NS 0.205	ND	NS 0.220
2,4-Di-tert-butylphenol	96-76-4	1595	0.00×00	0.00×00	0.00×00	5.79×10^{7}	1.39×10^{7}	1.38×10^{7}	2.37×10^{7}	2.00×10^{6}	1.01×10^{7}	*0.043	NS 0.363	NS 0.065	NS 0.130
Sulfur															
Dimethyl sulfide	75-18-3	536	1.11×10^{7}	1.05×10^{7}	6.30×10^{6}	2.39×10^{7}	1.75×10^{7}	0.00×00	1.66×10^{8}	1.38×10^{8}	5.51×10^{7}	*<0.001	*<0.001	*<0.001	*<0.001
Dimethyl sulfone	67-71-0	1052	3.96×10^{7}	2.94×10^{7}	0.00×00	3.88×10^{7}	2.80×10^{7}	0.00×00	3.60×10^{7}	2.41×10^{7}	0.00×00	*<0.001	NS 0.961	NS 0.675	ND
Dimethyl disulfide	624-92-0	776	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	*0.033	ND	NS 0.093	NS 0.422
Methanethiol	74-93-1	459	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	0.00×00	4.79×10^{7}	0.00×00	*<0.001	ND	*0.013	ND
Ether															

Ethyl ether	60-29-7	514	1.86×10^{7}	1.09×10^{7}	6.58×10^{6}	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.006	NS 0.013	NS 0.086	NS 0.232
Vinylisopentyl ether	39782-38-2	767	5.73×10^{7}	7.61×10^{7}	7.42×10^{7}	4.76×10^{7}	3.01×10^{7}	2.71×10^{7}	0.00 × 00	0.00 × 00	1.35×10^{8}	NS 0.377	NS 0.645	NS 0.234	NS 0.337

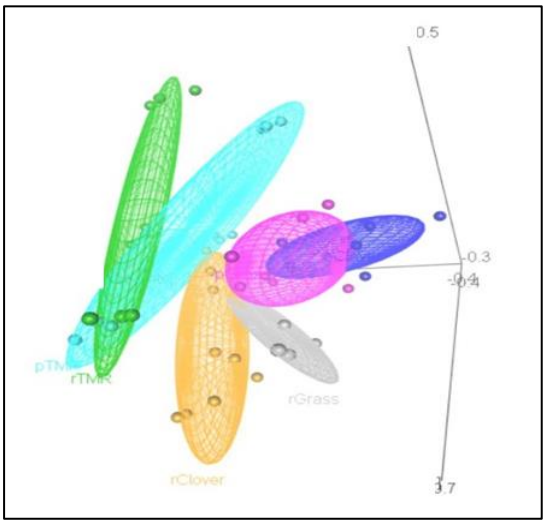
Esters and aldehydes were more closely correlated with TMR milk samples. Esters of short chain fatty acids (C4-C10) are important aroma active compounds (Liu et al., 2004) that are responsible for fruity off-flavours in milk (Moio et al., 1993b, Friedrich and Acree, 1998). It is possible for esters to be formed through esterification reactions (the formation of esters from alcohols and carboxylic acids) or alcoholysis (the production of esters from alcohols and acylglycerols or from alcohols and fatty acyl-CoAs derived from metabolism of fatty acids, amino acids and/or carbohydrates) (Liu et al., 2004). Esters in pasteurised milk are occasionally present as a result of post-pasteurisation microbial contamination and microbial activity (Wellnitz-Ruen et al., 1982, Whitfield et al., 2000). Ethyl butanoate (fruity) was identified in rGRS and rTMR samples at day 14 of storage and ethyl hexanoate (fruity, malty pineapple, waxy) was identified in r and p GRS milk at day 9 and increased at day 14. It was also identified in rCLV and rTMR samples at day 14 only. Ethyl butanoate was identified in rGRS and rTMR milk at day 14 of storage but was not identified in any p milk samples. The contribution of esters to the flavour of milk is concentration dependent, at low levels, esters contribute positively to the overall flavour balance; but at high concentrations they can cause a fruity defect as mentioned previously (Liu et al., 2004). Hydrocarbons and sulphur compounds were more closely associated with GRS and CLV milk samples. Al-Attabi et al. (2014) reported that sulfur compounds such as hydrogen sulfide, methanthiol, dimethyl sulfide and dimethyl trisulfide were present in commercial ultra-heat treated milk samples at levels above their documented odour thresholds. The same study identified carbon disulfide, dimethyl sulfide, dimethyl sulfoxide and dimethyl disulfide in pasteurised milk samples, although below their reported threshold values. Sulphur compounds are thought to be important contributors to cooked flavour in milk.

Significant differences were observed between the GRS, CLV and TMR milk samples based on storage time, feeding system and pasteurisation (Figure 5.2 a, b and c, respectively). Differences between the rGRS, rCLV and rTMR milk samples at day 3 were dominated by alcohols (3) and aldehydes (2). Differences between the p milk samples based on feeding system at day 3 were dominated by aldehydes (3), alcohols (2) and hydrocarbons (2). Raw milk samples at day 9 were dominated by aldehydes (6), alcohols (3) and ketones (3). Pasteurised milk samples at day 9 were dominated by esters (6), ketones (6) and alcohols (4). r milk samples were significantly ($P < 0.05$)

dominated by esters (9), ketones (7), alcohols (4), aldehydes (3) and hydrocarbons (4) after 14 days of storage while p milk samples contained higher levels of aldehydes (7), ketones (6) and hydrocarbons (5) at day 14. All results for the concentrations of volatile organic compounds identified in r and p milk samples are outlined in Table 5.2 and S5.5, respectively.



(a) **(b)**



(c)

Figure 5.2: (a) 3D plot demonstrating the effect of storage time (days) on the volatile profile of the rGRS, rCLV and rTMR milk samples; pink - day 3, light blue - day 9 and dark blue - day 14; **(b)** 3D plot demonstrating the effect of feeding system (GRS, CLV and TMR) on the volatile profile of the rGRS, rCLV and rTMR milk samples; pink - GRS, light blue - TMR and dark blue – CLV; **(c)** 3D plot demonstrating the effect of pasteurisation on the volatile profile of the r and p GRS, CLV and TMR

milk samples. Grey – rGRS grass, pink – pGRS, orange – rCLV, dark blue – pCLV, light blue – pasteurised TMR and green – raw TMR. p = pasteurised, r = raw.

Twenty eight compounds identified in the grass GRS feed samples were identified in the corresponding r milk samples (decanal, heptanal, hexanal, nonanal, octanal, pentanal, 2-heptanone, 2-hexanone, 2-pentanone, acetone, acetophenone, cyclohexanone, methyl isobutyl ketone, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol, α-pinene, cumene, mesitylene, 2,4-Dimethylfuran, 2,4-Dimethyl-benzaldehyde, 1,3-Bis(1,1-dimethylethyl)-benzene, p-xylene, tert-Butylbenzene, toluene, dimethyl sulphide, dimethyl sulfone and vinylisopentyl ether). The same compounds were present in the CLV feed samples and the corresponding r milk samples excluding cyclohexanone, α-pinene and methyl isobutyl ketone. Acetyl valeryl was the only compound present in CLV feed and corresponding r milks that was not in GRS samples. The majority of the same compounds were present in TMR feed and corresponding r milk excluding octanal, pentanal, 2-hexanone, cyclohexanone, 1-pentanol and dimethyl sulfone. The following three compounds; 2-butanone, 1-hexanol and ethylbenzene were identified in TMR feed samples, but not in GRS or CLV feed samples. Figure 5.3 demonstrates the correlation of the volatile compounds to the r and p milk samples.

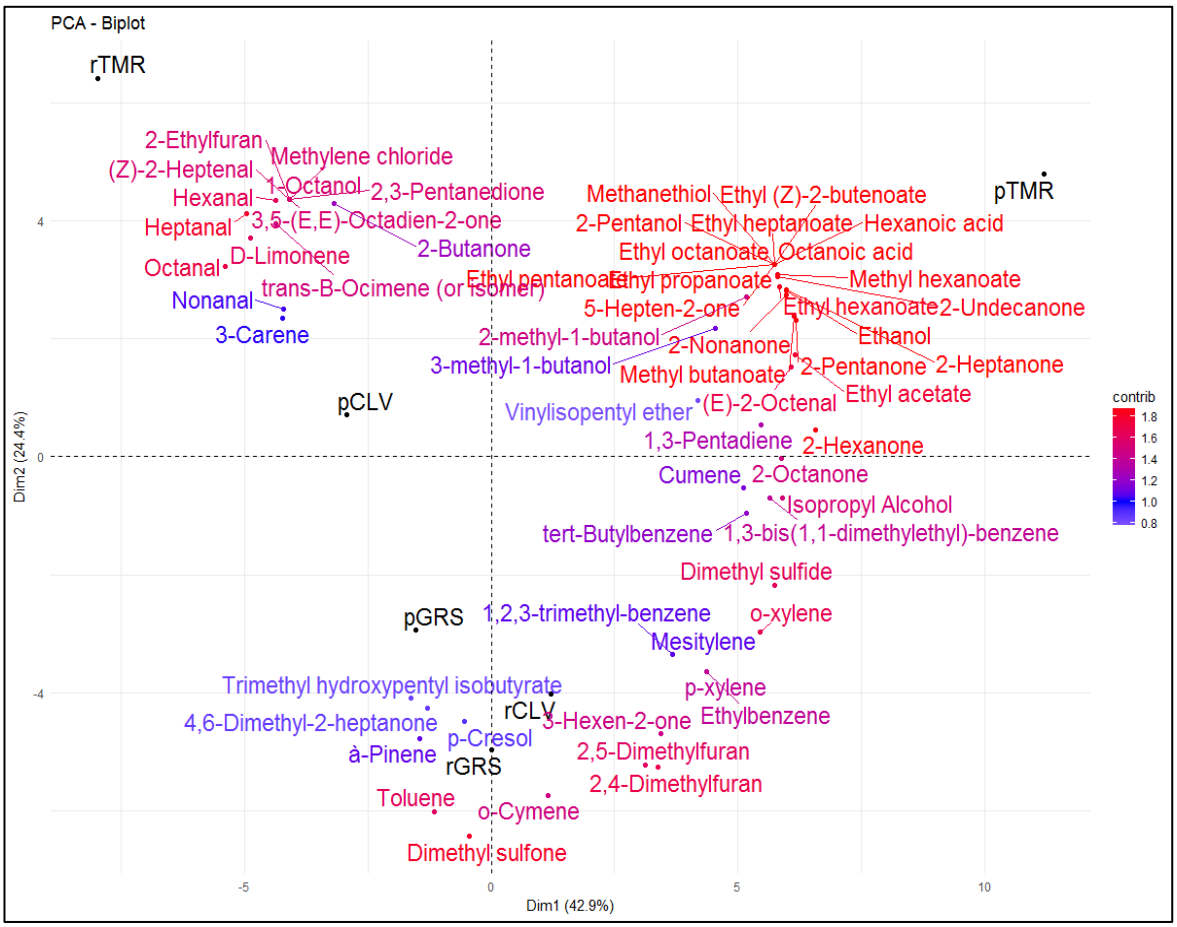


Figure 5.3: PCA Biplot of raw (r) and pasteurised (p) GRS, CLV and TMR milk samples and the top 60 volatile compounds contributing to the differences between the samples, identified by HS-SPME GCMS. Colour gradient; low = white, mid = blue and high = red, midpoint set at 1.0.

Fifty five compounds were identified in the p milk samples at day 3, nine of which varied significantly. 2-Methyl-1-butanol was significantly higher in pTMR samples, and has been linked to a malty, microbial-induced off-flavour related to the poor refrigeration of milk (Marsili, 2016). 1-Pentanol (fermented, bready, yeasty, fusel) was significantly correlated with pCLV samples, as previously mentioned, 1-pentanol is derived from pentanal (Faulkner et al., 2018), and its concentrations were linked to this aldehyde which was also greater in CLV>GRS>TMR. 2-Butanone has previously been reported to originate from the cows' feeding system (Marsili, 2016), specifically from carbohydrate metabolism which could explain why levels of this compound were highest in pTMR samples. 3-Hexen-2-one (nutty, blue-cheese, plastic) was higher in GRS and CLV milk samples compared to TMR milk samples. It is likely that 3-hexen-2-one is derived from aerobic oxidation of linoleic or linolenic acid (C18:2 and c18:3)

(Moreno and Peinado, 2012). Dimethyl sulfone was highest in the pGRS milk samples. Heptanal and nonanal were significantly correlated with r and p TMR milk samples and their presence has previously been reported in milk (Brothersen et al., 2016). Both compounds are transferred from feed but are also products of lipid oxidation (Faulkner et al., 2018). 3-Hydroxy-2,2,4-trimethylpentyl-ester-2-methyl-propanoic acid (sour, bitter, herb) was more closely correlated with GRS milk samples. It has been identified in numerous plant species (Cho et al., 2013, de Oliveira et al., 2015, Wright et al., 2017) and as an odourant of some hardwood species (Liu et al., 2018b). 2-Methyl-propanoic acid (Isobutyric acid) has previously been reported in TMR milk (Faulkner et al., 2018), it has a characteristic sweet-like odour and is a plant metabolite produced from the intermediary hepatic and microbial metabolism of the amino acids valine and leucine (Menahan and Schultz, 1964, Clayton and Clayton, 1981). However, conflicting results exist on whether 2-methyl-propanoic acid is transferred from feed to milk as Bingham et al. (2001) reported that no carryover was evident in the milk of cows supplemented with 170 mg/kg/day of the acid for 10 days due to the rapid metabolism of dairy cattle. It is also possible that the compound entered the milk through the inhalation pathway. Tert-butylbenzene is possibly derived from carotenoid degradation as observed with other benzene compounds and was highest in CLV milk samples. Toluene is a product of β-carotene degradation and has been identified as a potential biomarker for dairy products produced from pasture GRS>CLV>TMR (Kilcawley et al., 2018), but is not very odour active (Kilcawley et al., 2018). Seventy four compounds were identified in the p milk samples at day 9. Twenty nine volatiles varied significantly; (E)-2-octenal (fatty, green, cucumber) has previously been detected in milk fermented with *S. thermophiles* and was found to be an important contributor to the flavour of the milk (Dan et al., 2018). 2-Heptanone (cheesy, fruity, woody, herbal), 2-hexanone, 2-nonanone (fruity, sweet, green, earthy), 2-pentanone (fruity, wine, banana, ethereal), 2-undecanone (fruity, waxy, creamy, floral) and 5-hepten-2-one (citrus, green, apple lemongrass) are all ketone compounds commonly identified in milk and some have been identified as thermally derived off-flavours linked to the level of fat in the milk (Vazquez-Landaverde et al., 2005b). All ketone compounds were highest in pTMR samples at day 9. Cumene is derived from benzene and its abundance was similar to that of benzene, in the order of; TMR>GRS>CLV. Cumene has previously been identified in grass and plant material

(Howard et al., 1990) and thus could be transferred directly from the feed. Dimethyl sulphide was highest in TMR samples and dimethyl sulfone, was higher in GRS and CLV samples possibly due to the presence of more digestible proteins (Faulkner et al., 2018). The ester compounds; ethyl (Z)-2-butenoate (fermented, chemical, caramel), ethyl acetate (ethereal, fruity, sweet), ethyl hexanoate, ethyl octanoate (wax, sweet, apple), ethyl pentanoate (fruity, acidic, green), methyl butanoate (fruity, apple, fusel) and methyl hexanoate (fruity, pineapple, ether) were all significantly higher in pTMR samples; possibly due to the amount of ethanol available to form ethyl esters, and methanol to form methyl esters, a reaction that can occur spontaneously or be catalysed by esterases or lipases produced by lactic acid bacteria (Holland et al., 2005, Zhang et al., 2016). Heptanal was more closely correlated with pGRS milk samples at day 9. Hexanal, a primary product of lipid oxidation (oleic and linoleic acid) is a well-known contributor to off-flavours in dairy products (Li et al., 2012, Clarke et al., 2019) and was found to be higher in pGRS milk at day 9. Nonanal and octanal were significantly correlated with pGRS samples at day 9, both compounds have previously been identified as thermally derived off-flavours in milk (Vazquez-Landaverde et al., 2005b) and products of light-induced oxidation (Brothersen et al., 2016). Pentanal was greatest in pCLV samples. Methanethiol (sulfurous, cabbage, garlic), is derived from the Strecker degradation of methinonine and also from riboflavin (Al-Attabi et al., 2014) and was only detected in pTMR samples. Methanethiol can also be easily oxidized to form dimethyl disulfide (Weimer, 2007). Styrene (balsamic, woody) is produced from the degradation of cinnamic acid and as a by-product of fungal and microbial metabolism (Shirai and Hisatsuka, 1979, Shimada et al., 1992) and was only detected in pGRS samples. Toluene concentrations were higher in GRS samples followed by CLV then TMR. Seventy eight compounds were identified in the milk samples at day 14, 15 of which varied significantly between the milk types; (Z)-2-heptenal (green, fatty); 1-octanol (waxy, green, mushroom) is a fatty alcohol that could be derived from octanal; 1-Pentanol increased in pGRS samples at day 14; 2-Butanone remained correlated with pTMR samples at day 14; 3,5-(E,E)-Octadien-2-one (grassy, fruity, green) is a product of linolenic acid degradation (Im et al., 2004) and was significantly higher in pTMR samples, which may explain the perceived hay-like flavour in the pTMR samples; 4-Methyl-3-penten-2-one (honey, vegetable, earthy) was identified in pGRS and pCLV samples only, and was found to be more closely

correlated with the pCLV samples; acetone, as previously mentioned is thought to originate from the cows' diet (Marsili, 2016) was also higher in pTMR sample; butanal (chocolate, pungent, musty), a primary aldehyde product of lipid oxidation (Repetto et al., 2012) was detected in the pGRS and pTMR samples only, being most abundant in pTMR samples; heptanal, hexanal, nonanal and octanal were all more closely correlated with TMR samples at day 14, as these are all products of lipid oxidation (Repetto et al., 2012) their increased concentrations in pTMR samples could indicate quality deterioration and an increase in off-flavours; pentanal remained correlated with pCLV samples after 14 days of storage; p-cresol (barnyard, cowy, phenolic) is derived from the metabolism of β-carotene and aromatic amino acids (mainly tyrosine) in the rumen and may be a potential biomarker for dairy products derived from pasture (Moio et al., 1993a, Khanal et al., 2005, Kilcawley et al., 2018). p-Cresol was strongly correlated with pGRS milk. Tyrosine has been shown to be a pre-curser for the production of both p-cresol and phenol (Mathus et al., 1995), both compounds follow the same trend across all p milk samples with the exception of TMR samples at day 9. p-Cresol may also be present from the metabolism of isoflavones in the feed (Kilic and Lindsay, 2005). Toluene was also found to be significantly correlated with the pGRS samples. In addition to the number of compounds increasing in the p milk samples throughout the storage period, the levels of numerous VOCs decreased, possibly due to degradation and/or formation of secondary compounds. It is well known that pasteurisation has an effect on certain volatile compounds in milk and can lead to losses or changes (Faulkner et al., 2018). This can be seen as some compounds that are present in the r milk samples are absent in the corresponding p milk samples or vice versa. This is very evident for esters which could have been formed by heat-catalyzed esterification reactions (Vazquez-Landaverde et al., 2005b, Faulkner et al., 2018), some aldehydes (possibly from the activation of lipid oxidation after heat treatment, auto-oxidation or light induced oxidation) and some ketones (Calvo and de la Hoz, 1992). It has been noted that enzymatic and metabolic reactions that occur in raw milk during storage may also lead to loss of compounds post pasteurisation (Calvo and de la Hoz, 1992, Contarini et al., 1997). Storage time was also shown to have an effect on the volatile profile; this could be due to enzymatic reactions from microbes.

5.2.6 Sensory Analyses of Pasteurised Milk Samples

It can be observed from Figure 5.4 and 5.5 that there is considerable discrimination between the three milk samples. Three significant differences ($P < 0.05$) were observed between the three milk types; creaminess, colour and hay-like flavour. Post hoc Tukey's test showed that the difference in creaminess exists between GRS and CLV milk, the difference in colour exists between TMR and the other two milks, with TMR milk scoring highest for white colour and GRS and CLV milks scoring highest for creamy colour. The significant difference in creaminess is likely to be linked to the higher level of fat in the CLV milk, as creaminess is linked to milk fat globules in dairy products (Frøst and Janhøj, 2007). Fat takes the form of emulsified globules in liquid dairy products which are perceived as smooth and creamy (Mela, 1988). The fatty acid profile of milk also has an impact on texture, the ratio of oleic acid (C18:1; low melting point) to palmitic acid (C16:0; high melting point) has been used as a measure of hardness in cheese and butter (Martin et al., 2005). Faulkner et al. (2018) reported that milk samples produced from pasture scored significantly higher for viscosity, possibly due to the lower ratio of oleic acid to palmitic acid. In this study, CLV samples scored significantly higher for creaminess and contained a lower ratio of oleic acid to palmitic acid followed by GRS and TMR samples, which is in agreement with the previous studies (Martin et al., 2005). Free FA profile also impacts on surface tension and foaming capacity of milk which contribute to texture (Mannion et al., 2016b). β-Carotene content is responsible for the difference in colour with the study by Martin et al. (2005) concluding that dairy products produced from cows fed pasture have a higher yellow intensity.

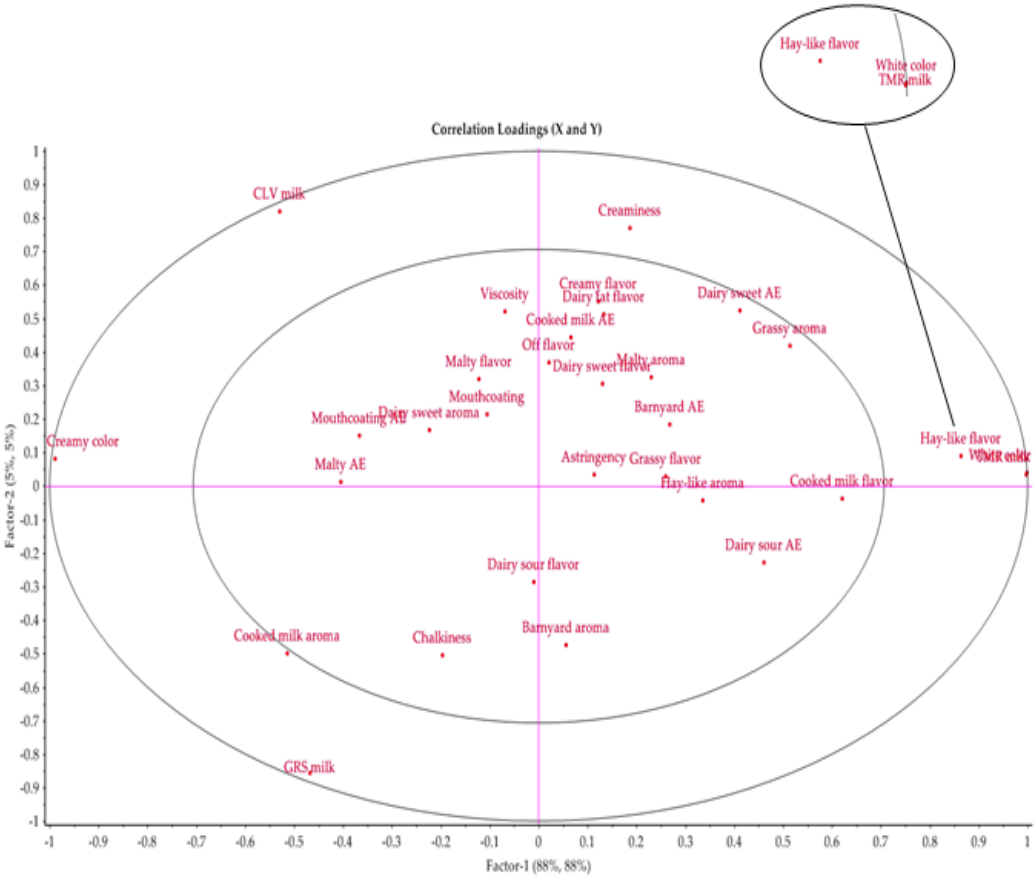


Figure 5.4: Multivariate data analysis partial least squares (PLS) regression plot of sensory descriptors for pasteurised milk samples. AE denotes after effect. $P = 0.05\%$.

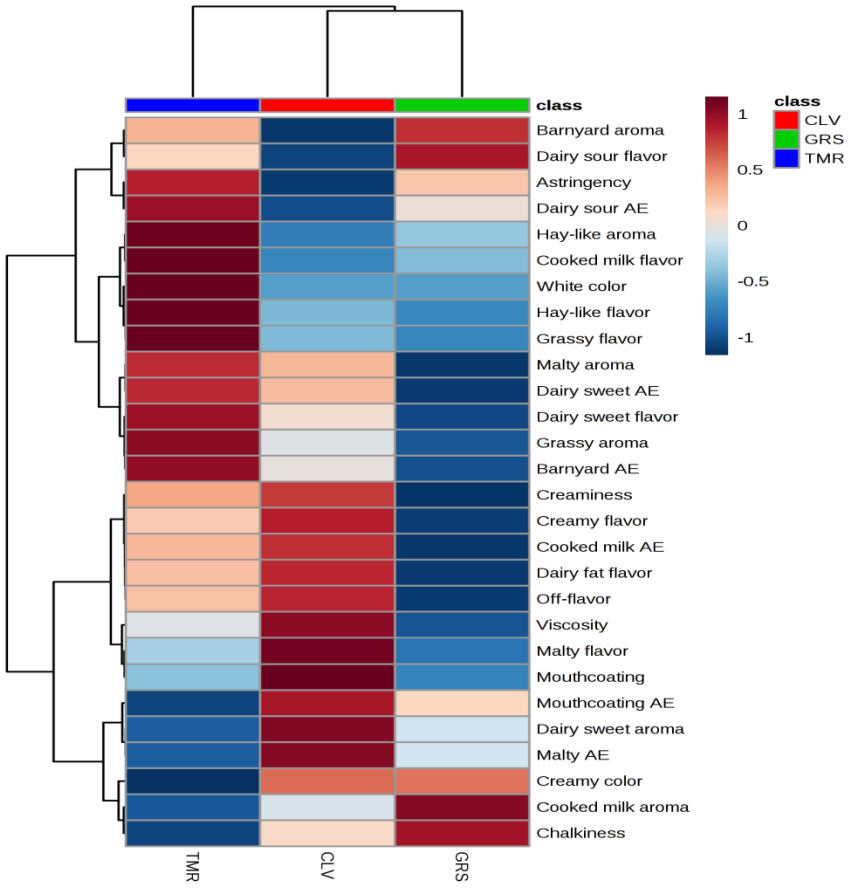


Figure 5.5: Hierarchal clustering analysis (Heatmap) of the average values for each sensory descriptor applied to the 3 pasteurised milk samples (GRS, CLV and TMR) as determined by full descriptive sensory analysis (n=7). Positive and negative correlations between diet treatment and sensory descriptors is denoted by +1 (red) and -1 (blue). AE: aftereffect.

A difference in hay-like flavour was found between TMR milk and the other two milks, being significantly higher in the TMR samples. Previous studies have found that the oxidation of unsaturated fatty acids, yielding a complex mixture of volatile compounds can be involved in the formation of a hay-like flavour in food products (Sapers et al., 1973, Murray et al., 1976). Masanetz and Grosch (1998) speculated that the compound 3-methyl-2,4-nonanedione could be responsible for a hay-like off-flavour in dried parsley. Interestingly, 3-methyl-2,4-nonanedione has been found to be the main contributor to the light-induced off-flavour of butter and butter oil (Grosch et al., 1992). While this specific compound was not identified in the present study, the compound 3,5-(E,E)-octadien-2-one has been described as having a grassy aroma and present at higher levels in TMR samples, this may be contributing to the hay-like flavour. Other compounds have also previously been linked to a hay-like sensory note, including, hexanal (Murray et al., 1976), 1-hexanol and trans-2-hexen-1-ol (Spanier et

al., 2001). However, 1-hexanol was present in rTMR samples but not pTMR samples. Thermal oxidation of vitamin A palmitate has also previously been attributed to hay-like off-flavour in non-fat milk powder (Suyama et al., 1983). 2,3-Pentanedione (buttery, sweet, nutty) may have been formed from 3-methyl-2,4-nonanedione through photo-oxidation (Sigrist et al., 2003) and was only detected in TMR milk samples at day 9. It is also possible that the hay-like off-flavour is being caused by a complex mixture of compounds rather than a single compound. The correlations between the sensory attributes and the VOCs are presented in Figure 5.6. Further variations in the volatile and sensorial profiles might be observed or accentuated in whole milk powders produced from the GRS, CLV and TMR feeding systems as the milk undergoes processing.

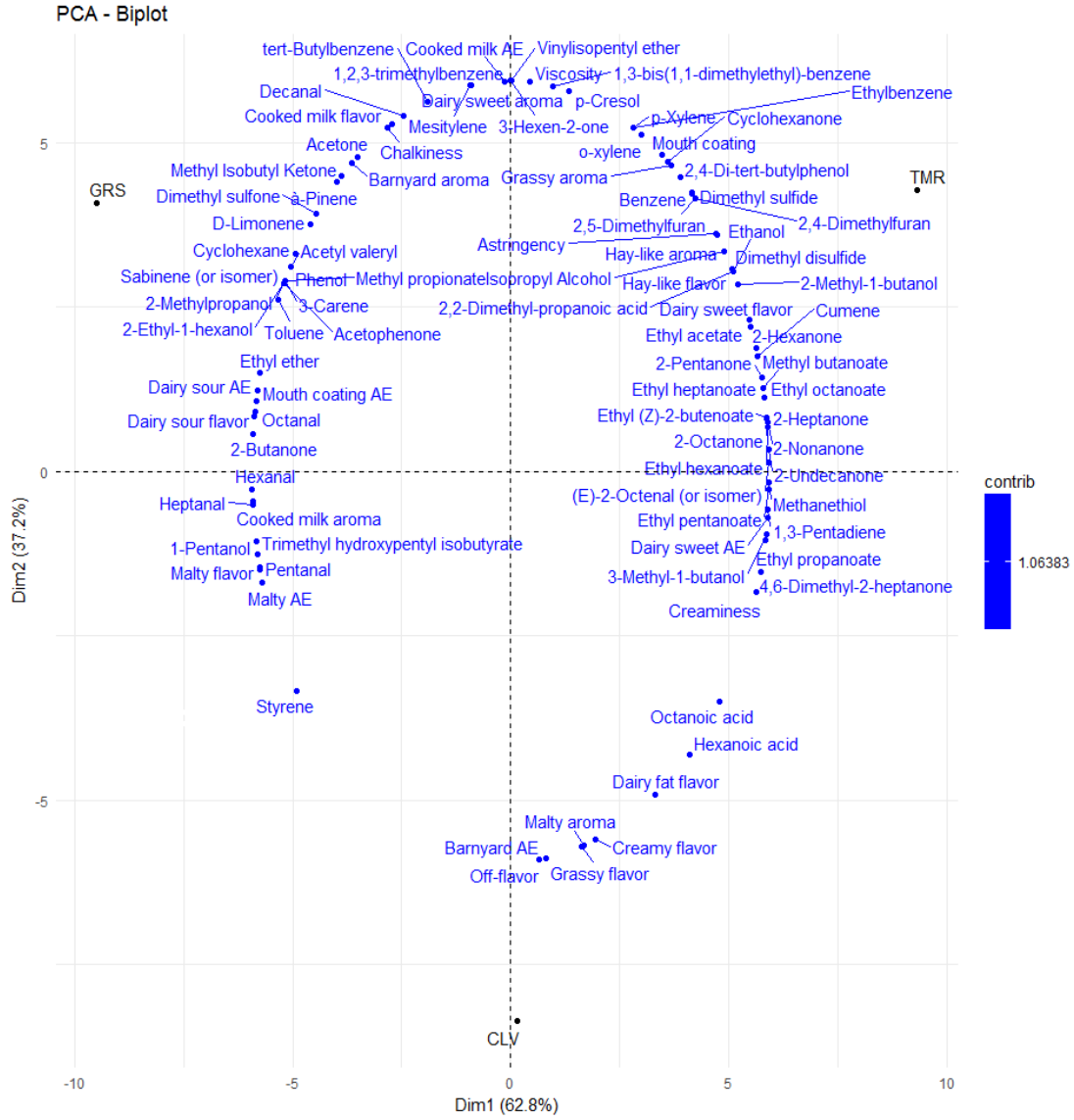


Figure 5.6: Principal component analysis (PCA) Biplot of pasteurised GRS, CLV and TMR milk samples showing correlations between the sensory attributes and the volatile organic compounds.

5.3 Materials and Methods

5.3.1 Feed Samples

The perennial ryegrass and perennial ryegrass/white clover samples were acquired using grass clippers cutting just above the root and were collected at 2 m intervals on a diagonal transect across each representative paddock and pooled together for each sample. Representative total mixed rations samples (mixture of grass silage, maize silage and concentrates) were taken from the cows' feeders. Grass samples were denoted as 'GRS feed', grass/clover samples denoted as 'CLV feed' and TMR samples as 'TMR feed'. Samples were taken at time points corresponding to the

milk collections and the results were averaged. Grass-only cows (GRS) received 2 kg concentrate and 15 kg DM Grass/cow, Grass-clover (CLV) cows received 2 kg concentrate and 15 kg DM Grass-Clover/cow and TMR cows received 9 kg DM maize silage + 4.5 kg DM grass silage + 8.5 kg DM concentrate throughout the study. Cows within the TMR system were fed at 08:30 h daily into electronically controlled Griffith Elder Mealmaster individual feed bins (Griffith Elder and Company Ltd, Suffolk, England) and feed was available ad-libitum. The CLV sward contained ~20 % white clover as outlined by O'Callaghan et al. (2016). Cows on pasture received a mineral supplement in the form of a liquid mineral preparation injected into the water supply (Terra Liquid Minerals, Moone Lodge, Moone, Athy, Co. Kildare, Ireland), giving a mean intake (mg/cow per d) of Na, Mg, Zn, Cu, Se, and Co of 5.0, 1.2, 219, 106, 3.8, and 3.0, respectively. The concentrate portion of the TMR feed was supplemented with a commercial mineral balancer, Dairy Hi-Phos (McDonnell Bros. Agricultural Suppliers Ltd., Fermoy, Co. Cork, Ireland) to give added Ca, Na, P, Zn, Cu, Mn, I, Co, and Se of 3,340, 2,000, 1,200, 140, 100, 70, 10, 2, and 0.8 mg/kg, respectively (Gulati et al., 2018).

5.3.2 Milk Samples and Processing

Raw milk was collected in duplicate from fifty-four spring-calving Friesian cows allocated to three experimental feeding groups (n = 18) based at the Teagasc Moorepark dairy farm (Fermoy, Co. Cork, Ireland) as outlined by O'Callaghan et al. (2016) at two stages of lactation (mid and late). Briefly, the milk from the cows in each of the three feeding systems; perennial ryegrass only, perennial ryegrass/white clover and TMR were separated into designated 5,000-L refrigerated tanks. The evening milk was stored at 4 °C overnight, to which the morning milk was then added and agitated before collection. Late lactation pasteurised milk was used to train the sensory panel on the descriptors used for the final scoring and for the focus groups. Mid lactation milk from each diet was used for the final scoring. Each milk sample was homogenized [GEA Niro Soavi S.p.A. Type: NS2006H (non-aseptic)] using 2-stage homogenization at 5,000 to 150,000 kPa. The milk was pasteurised using a Microthermics (UHT/HTST Electric Model 25HV Hybrid, Liquid Technologies, Wexford, Ireland) unit heated to 72 °C and held for 15 s, then cooled to 4 °C. Each milk sample was transferred at 4 °C to the sterile product outlet and aseptically packed into sterile 1-L glass bottles (Faulkner et al., 2018). Pasteurisation was performed

within 3 h of collection, microbial analysis was performed immediately after pasteurisation and sensory analysis within one week. Samples were frozen and stored at -18 °C prior to any analysis that was not performed immediately. The volatile profile of the raw and pasteurised milk samples were analysed at day 3, 9 and 14 of refrigerated storage in addition to FFA analysis storage period at 4 °C in order to ascertain the level of lipid oxidation occurring within the milk and to track volatile compounds forming or changing during storage. For the purpose of this study, grass milk samples are denoted as 'GRS', grass/clover samples as 'CLV and total mixed ration as 'TMR'. Where necessary, the prefix r is used to denote raw milk and p for pasteurised milk.

5.3.3 Microbial Analyses

Microbial analysis was performed as described by (Faulkner et al., 2018) with the following modifications. Each of the raw and pasteurised milk samples was plated out on three agar types; plate count skim milk agar (MPCA) to obtain the total bacteria plate count, violet red bile blood agar (VRBA) to test for the presence of coliforms and kannamycin aescilin azide agar base (KAA) to test for the presence of enterococci species. The VRBA plates were incubated at 30 °C for 24 h and the KAA plates at 37 °C for 24 h. Following incubation, all colonies that had developed were counted and the number of microorganisms per mL of milk sample was calculated.

5.3.4 Raw and Pasteurised Milk Compositions

Each milk sample was analysed for fat, protein, lactose, true protein and casein using a Bentley DairySpec FT (Technopath Distribution, Co. Tipperary, Ireland). Samples were heated to ~40 °C in 50 mL plastic tubes (Sarstedt Ltd., Wexford, Ireland) before analysis. Results were expressed as the average of 2 replicates.

5.3.5 Free Fatty Acid Analyses

Free FA analysis was carried out on the p milk samples 3, 9 and 14 days post pasteurisation. The samples were stored at 4 °C throughout analysis. Lipid extraction, methyl ester derivatization of triglycerides, solid-phase extraction (SPE) and GC instrument conditions were performed as per Mannion, *et al*. (Mannion et al., 2019). 10mL of each milk sample was analysed in duplicate and the extracts were pooled for SPE.

5.3.6 Phytochemical Extraction and Analyses

Milk and feed samples from the three experimental diets (GRS, CLV and TMR) taken at two time points were pooled together for each diet, milk samples were frozen at -18 °C and feed samples were freeze dried using a Labconco stoppering tray dryer (VWR International Ltd., Dublin, Ireland). Freeze dried feed samples were milled at 10,000 rpm through a 0.5mm mill using a Retsch Ultra Centrifugal mill ZM 200 (Lab Unlimited, Dublin, Ireland) and stored in sterile containers in a cool, dry place until required for analysis.

The extraction procedure for milk was adapted from Antignac, *et al*. (Antignac et al., 2004); 10mL of milk sample was mixed with 2 mL acetate buffer (pH 5.0; 2.0 mol/L) and 8 mL acetone for the removal of fat and protein and vortexed for 1 min and left for 16 h. The mixture was centrifuged at 435 rcf for 15 min using a Sorvall legend RT (Aquilant Scientific, Dublin, Ireland). The acetone phase was evaporated off at 45 ± 5 °C under reduced pressure to a 2-fold reduced volume using a Buchi Rotavapor R-210 (Mason Technology Ltd, Dublin, Ireland). The residue was incubated with 8 mg of a mixture of purified B-glucuronidase and sulfatase type H2 (Sigma-aldrich, Wicklow, Ireland) for 3-4 hr allowing hydrolysis of the conjugated phase II metabolites followed by centrifugation at 435 rcf for 15 min. The clear supernatant was collected and applied onto C18 SPE cartridges (50 mg solid phase; Agilent Technologies, Ireland), previously activated with 6 mL methanol and 6 mL water. Following a washing step with 6 mL water, analytes were eluted with 6 ml methanol. The extract was evaporated to dryness at 45 °C under reduced pressure and reconstituted in 250 µL methanol and 250 µL 0.1 M acetate buffer (50:50, v:v) and the extracts were transferred to 1.5 mL amber vials capped with PTFE/WS 9 mm caps (Agilent technologies) ready for analysis.

The extraction procedure for feed samples was adapted from (Steinshamn et al., 2008); 0.1 g of the milled feed sample was added to a mixture of methanol (3.5 ml) and 0.1 mol/L acetate buffer, pH 5.0 (1.5 ml), vortexed and left for 3-4 hr. The mixture was centrifuged at 344 rcf for 15 min. The clear supernatant was evaporated to dryness at 40 ± 5 °C under reduced pressure. The residue was dissolved in 3 mL of

0.1 mol/L acetate buffer, pH 5.0 and incubated with 20 mg of cellulase and 8 mg B-glucuronidase (Sigma-aldrich, Ireland) for 16 hr at room temperature (~21 °C). Following a centrifugation at 455 rcf for 15 min, the extracts were transferred to 1.5 mL amber vials capped with PTFE/WS 9 mm caps.

Mass spectrometry profiling of the phytochemicals in the extracts was carried out on an Alliance 2695 high performance liquid chromatography unit coupled to a quadrupole time of flight mass spectrometry (HPLC-Q-Tof, Waters Corp. Milford, USA). Separation of the analytes was achieved on an Atlantis T3 column 2.1 x 100 mm, 3 µm, (Waters Corp., USA) using a binary solvent gradient of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B). The stepwise gradient consisted of: 10 % B (0-1min), 40 % B (1-6 min), 50 % B (6-8 min), 70 % B (8-14 min), 80 % B (14-18 min) and finally back to initial gradient of 10 % B at 20-25 min with flow rate of 300 µL/min. Mass spectral data were acquired in electrospray ionisation mode using the following parameters: capillary voltage at 2.5 kV, cone voltage at 39 V, source temperature at 150 °C and the desolvation temperature at 300 °C with the desolvation gas flow at 1200 L/h and mass scan range for m/z 100-1000. Accurate mass measurements of the analytes were determined using a lock mass reference leucine solv (monoisoptic mass, 555.2693 Da) following the external calibration of the mass analysers using sodium formate solutions.

Quantification of the isoflavanoids was carried out on an Acquity ultra-high performance liquid chromatography-tandem quadrupole mass spectrometer (UPLC-TQD, Waters Corp. Milford, USA) through multiple reaction monitoring (MRM) method. The MRM transitions of each of the four standards (apigenin, formononetin, genistein and naringenin) were generated using the Waters Intellistart® software, daidzein was detected through MRM transitions. Separation of the analytes was achieved on an Acquity UPLC HSS T3 column (2.1 X 100 mm, 1.8 µm) using a binary solvent gradient of solvent A (water + 0.1 % formic acid) and solvent B (acetonitrile + 0.1 % formic acid). The solvent gradient totaling 5 min as follows: 2 % B (0-0.5 min), 10 % B (0.5-1.25 min), 15 % B (1.25- 3 min), 35 %B (3.0-3.7 min), 98% B (3.7-4.7 min) and back to initial gradient of 2 % B to 5 min at the flow rate of 500 µL/min was used. Data was acquired both on positive (for apigenin) and negative (for all other isoflavanoids) electrospray ionisation modes with the following settings: capillary

voltage at 3 kV, cone voltage at 42 V, source temperature at 150 °C and the desolvation temperature at 350 °C with the desolvation gas flow at 1200 L/h.

5.3.7 Volatile Analyses

HS-SPME GCMS is a widely used analytical method for volatile profiling of dairy products. Volatile analyses was performed using a method developed in Teagasc Moorepark (Fermoy, Co. Cork) for the volatile profiling of bovine milk on a Bruker Scion 456-GC-TQ (Elementec Ltd, Maynooth, Co.Kildare Ireland). All the incubation, extraction and injection processes were implemented using a Bruker CombiPal autosampler (Elementec Ltd, Ireland). A mid-polar DB 624 UI column (60m x 0.32mm x 1.80µm) (Agilent Technologies Ireland Ltd, Little Island, Cork, Ireland) was used. A 2cm, 50/30µm, DVB/Carboxen/PDMS Stableflex SPME fiber (Agilent Technologies Ltd, Ireland) was selected for this study as a result of literature reviews and shown to be suitable for the extraction of volatile compounds from dairy products (Tunick et al., 2013, Salum et al., 2017). Raw and pasteurised milk samples were stored at 4 °C and analysed in triplicate on days 3, 9 and 14. Milk (2 g) was aliquoted into amber La-Pha-Pack headspace vials (20mL) with magnetic caps and Silicone/Polytetrafluoroethylene 1.3mm 45° Shore A septa (Apex Scientific Ltd, Ireland). Each sample was incubated at 40 °C with pulsed agitation for 10 min. The SPME fiber was then exposed to the headspace of the milk for 20 min while the sample was agitated. Following extraction, the SPME fiber was retracted and injected into the split/splitless 1177 GC inlet for 3 min at 250 °C in split mode at a ratio of 10:1. The column oven was held at 35 °C for 2 min, then ramped to 230 °C at a rate of 6.5 °C/min and held for 2 min and finally ramped to 260 °C at a rate of 15 °C/min and held for 5 min, yielding a total run time of 41 min. Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. Compounds were identified using an in-house library based on mass spectra obtained from NIST MS searching (v.2.3) and authentic standards where available. Results were processed with AMDIS software (v.2.73). Identification of compounds was based on target and qualifier ions and linear retention indices (LRI) (Van den Dool, 1963). An auto-tune of the GCMS system was performed regularly in order to ensure optimal GCMS performance. Air and water reports were performed prior to each run.

5.3.8 Sensory Analyses

Full descriptive sensory analysis was carried out on the three pasteurised milk samples (pGRS, pCLV and pTMR) in Teagasc Ashtown Food Research Centre (Dublin, Ireland). A 12 member external, trained descriptive sensory panel was used to assess the milk samples, using the average of seven panellist judgements per sample. The panel had been recruited based on their ability to perceive certain attributes and their continued availability. Panellists had previously received 60 hours of training and had between two and three years' experience of working as descriptive panellists on a weekly basis. Training of the panel on the three milk samples consisted of two attribute generation sessions (of three hours duration each). A further four sessions of panel training took place using a variety of product standards to create a aroma / texture / flavour / after effect scales for each sensory descriptor that was subsequently applied to the pGRS, pCLV and pTMR milk samples. Panel performance assessments were carried out prior to final scoring of the three milk types. The milks were stored at 2 – 4 °C until approximately an hour before each training and scoring session and were allowed to reach 11 – 12 °C before serving. The milks were gently stirred and poured into 20 mL clear plastic cups which were labelled with random three digit codes. Panellists were given water and plain crackers or green apples to cleanse the palate between samples. The project was set up as a complete block design using Compusense 5.6 (sensory data capture package). All samples were scored in triplicate for each descriptor. Descriptors are outlined in Table S5.6 and the results are expressed as averages. Analysis of colour was also carried out by the panellists on each sample.

5.3.9 Statistical Analyses

Statistical analysis relating to the sensory, phytochemical and volatile data were examined using Statistical Package for the Social Sciences (SPSS) software, version 24 (IBM Statistics Inc., Armonk, NY). A between- and within-subjects ANOVA with post hoc Tukey's test were used to compare volatile compounds and sensory attribute scores of milks from herds on different feeding systems (GRS, CLV and TMR). Feeding system was the factor (independent variable) and the scores for each sensory attribute were the dependent variables. For the volatile data, feeding system was again the factor (independent variable) and the peak area responses for each volatile compound were the dependent variables. Partial least squares regression

plots for the sensory results were constructed by Unscrambler Software, version 10.3 (CAMO ASA, Trondheim, Norway). The X and Y matrix was designed so that X was the sample name(s) and Y was the experimental data. Proximity of the data to the diet type (GRS, CLV, and TMR) indicated correlation between the sample and the data. The level of significance for correlation was set at $P < 0.05$ for all statistical tests unless otherwise stated. PCA biplots of the phytochemical data and the volatile vs. sensory data were constructed using the 'factoextra' and 'FactoMinoR' packages within R (v 3.4.1) (R Core Team, 2013).

5.4 Conclusions

This study evaluated the effect of three widely implemented bovine feeding systems on various milk quality indicators. Significant differences were observed in volatile profile, isoflavone content and sensory perception of milk based on the feeding system. Isoflavone content was evaluated with focus on the possible breakdown products and subsequent potential effect on sensory perception. Formononetin was found to be significantly correlated to white clover feed samples and levels of apigenin, daidzein and genistein were found to be significantly different between the r and p milk samples. Daidzein, genistein and apigenin were highly correlated to rCLV milk, likely present as metabolism products from other isoflavone compounds. Formononetin was more closely correlated with rGRS milk, despite levels of this isoflavone being higher in CLV feed .It is possible that the formonoetin in CLV feed was present in a more readily metabolised form when compared to the formonoetin content in GRS feed. p-Cresol is likely derived from the metabolism of formononetin and has been reported to be responsible for a barnyard aroma associated with milk derived from pasture. Both r and p GRS milk had the highest levels of p-cresol at day 9 and 14 of storage and pGRS milk was found to be more correlated with barnyard aroma than the pCLV and pTMR milk samples. Volatile profiling proved to be a useful tool for the identification of important odour-active compounds in addition to biomarkers demonstrating the authenticity of pasture-derived products. Dimethyl sulfone was identified in GRS and CLV feed and milk samples but not in TMR feed or the corresponding TMR milk samples. Most benzene compounds increased in GRS and CLV milks after pasteurisation but not in TMR samples. Toluene was significantly higher in both r and p GRS and CLV milk samples throughout storage. Overall, GRS and CLV feed samples contained higher levels of alcohol compounds than TMR feed,

however, this trend was not evident in the r or p milk samples suggesting the breakdown or conversion of alcohol compounds through metabolism and or pasteurisation. Acid compounds were higher in TMR feed than GRS and CLV feeds. TMR feed and r and p TMR milk contained higher levels of ethyl and methyl esters, likely due to the presence of more available carbohydrate in the TMR diet combined with the presence of alcohol compounds. Full descriptive sensory analysis provided a reliable insight into the differences of the milks based on feeding system, with TMR milk having greater white colour and the GRS and CLV milk scoring higher for creamy colour. Creaminess and hay-like flavour were also found to be significantly different between the p milk samples. Only the GRS and CLV milks were significantly different for creaminess, while TMR milk scored significantly highest for hay-like flavour. Results demonstrate the ability of volatile profiling and sensory techniques to distinguish milk produced from pasture versus indoor TMR feeding systems. Further research is required to ascertain the complex breakdown pathways of isoflavone compounds derived from feed and their effect on the sensory perception of bovine milk.

Supplementary Materials Chapter 5

Table S5.1: Microbial results for the raw and pasteurised milk samples (GRS, CLV and TMR). VRB: Violet Red Bile, KAA: Kanamycin Aesculin Azide, MPCA: Milk Plate Count Agar, **Table S5.2:** Composition analysis results for pasteurised grass (GRS), clover (CLV) and TMR milk samples in early, mid and late lactation. Each result is the average of 2 replicates, **Table S5.3:** Individual Free Fatty Acid Content mg/kg or ppm (relative standard deviation of the results between replicates as a percent in brackets) for each of the p milk samples (GRS, CLV and TMR) at day 3, 9 and 14 of refrigerated storage. P = 0.05, d = day, **Table S5.4:** Individual Free fatty acid content mg/kg (relative standard deviation of the results between replicates as a percent in brackets) for each of the p milk samples (GRS, CLV and TMR) at day 3, 9 and 14 of refrigerated storage and the significance between the fatty acids analysed for each sample (GRS, CLV and TMR). P = 0.05%, d = day, NS = not significant, **Figure S5.1:** Bar chart showing the levels of important isoflavones in feed samples (grass, Grass/clover and TMR) and the corresponding raw (r) and pasteurised (p) milk samples. Grass [GRS], grass/clover [CLV]; **Figure S5.2:** Hierarchal clustering analysis (Heatmap) of the average values for the top 65 volatile organic compounds contributing to the differences between grass, grass/clover and TMR feed samples, as determined by headspace solid-phase microextraction GC-MS analysis. Positive and negative correlations between feeding system (grass, grass/clover and TMR) and volatile organic compounds is denoted by +1 (red) and -1 (blue), **Figure S5.3:** Bar charts showing the percentage of each chemical class (aldehydes, ketones, alcohols, acids, fatty acid esters, terpenes, furans, hydrocarbons, sulphurs, lactones, pyrazines, ether and phenol) identified in each feed type (grass, grass/clover and TMR). 90, 104 and 94 compounds were identified in grass, grass/clover and TMR feeds, respectively. **Table S5.5:** Relationship between cow diet (Grass, Clover and TMR) and the pasteurised milk (p) volatile compounds identified by HS-SPME GC-MS at day 3, 9 and 14 of refrigerated storage; values are expressed as peak area values for each compound.; values are expressed as peak area values for each compound. d = day, P = 0.05 ND = not detected, NS = not significant, **Table S5.6:** The 26 sensory descriptors used for the evaluation of the 3 pasteurised milk samples (GRS, CLV and TMR) by full descriptive sensory analysis.

Table S5.1: Microbial results for the raw and pasteurised milk samples (grass (GRS), clover (CLV) and total mixed ration (TMR)). VRB: Violet Red Bile, KAA: Kanamycin Aesculin Azide, MPCA: Milk Plate Count Agar.

			Raw Milk			Pasteurised Milk		
		Agar Type	**GRS**	**CLV**	**TMR**	**GRS**	**CLV**	**TMR**
Early lactation	Bacteria count (Log_{10})	VRB agar	0.0	1.5	0.0	0.0	0.0	0.0
		KAA agar	1.0	0.7	1.9	0.7	0.7	1.0
		MPCA	0.0	0.0	0.0	0.0	0.0	0.0
Mid lactation	Bacteria count (Log_{10})	VRB agar	0.0	0.0	1.4	0.0	0.0	0.0
		KAA agar	1.0	1.0	1.7	0.0	0.0	0.0
		MPCA	2.0	0.0	2.3	0.0	0.0	0.0
Late lactation	Bacteria count (Log_{10})	VRB agar	1.0	1.5	3.8	0.0	0.0	0.0
		KAA agar	0.0	1.7	2.4	0.0	0.0	0.0
		MPCA	0.0	0.0	0.0	0.0	0.0	0.0

Table S5.2: Monthly averages of composition analysis for pasteurised milk samples from cows on different feeding systems; perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) during mid and late lactation.

Component	Diet	Mid Lactation		Late Lactation		
		May	June	September	October	*p*-Value
Fat %	GRS	3.4	3.7	2.7	2.8	<0.001
	CLV	3.4	3.4	5.1	4.7	
	TMR	4.2	3.9	2.2	4.4	
Protein %	GRS	3.5	3.6	4.0	3.6	<0.001
	CLV	3.6	3.6	4.1	3.8	
	TMR	3.2	3.4	3.9	3.7	
Lactose %	GRS	4.8	4.7	4.8	4.3	<0.001
	CLV	5.0	4.7	4.8	4.6	
	TMR	4.8	4.7	5.0	4.7	
True protein %	GRS	3.3	3.4	3.8	3.4	<0.001
	CLV	3.4	3.5	3.9	3.6	
	TMR	3.1	3.2	3.7	3.5	
Casein %	GRS	2.7	2.7	3.1	2.7	<0.001
	CLV	2.7	2.8	3.2	2.9	
	TMR	2.4	3.2	3.7	2.9	

Table S5.3: Individual Free Fatty Acid Content mg/kg or ppm (relative standard deviation of the results between replicates as a percent in brackets) for each of the p milk samples (grass (GRS) grass/clover (CLV) and total mixed ration (TMR)) at day 3, 9 and 14 of refrigerated storage. *p = 0.05, d = day.

Fatty Acid	Grass d 3	Grass/Clover d 3	TMR d 3	Grass d 9	Grass/Clover d 9	Grass d 14	Grass/Clover d 14	TMR d 14	p-Value
C4	3.0 (41.3)	0.0 (0)	0.0 (0)	3.9 (11.9)	8.5 (11)	0.0 (0)	0.0 (0)	0.0 (0)	*
C6	4.4 (4.7)	2.4 (17.1)	2.3 (2.8)	5.3 (2.9)	8.2 (5.2)	6.9 (32.9)	6.4 (14.4)	5.6 (17.3)	*
C8	4.2 (4.9)	2.3 (18.5)	2.5 (4.8)	6.0 (0.1)	8.9 (4)	7.6 (32.2)	6.8 (14.3)	6.1 (18.4)	*
C10	7.5 (7)	4.3 (15.3)	5.1 (22.2)	11.3 (0.2)	16.7 (3.7)	14.7 (31.7)	12.9 (14.9)	12.0 (17.9)	*
C12	8.7 (4.1)	4.9 (15.2)	6.9 (19.5)	12.9 (0.7)	18.7 (2.7)	16.4 (26.2)	13.6 (9.1)	15.0 (16.9)	*
C14	15.9 (7.4)	10.2 (16)	10.6 (14.3)	24.3 (0.3)	38.1 (2.8)	33.3 (25.5)	28.1 (6.1)	29.2 (14.8)	*
C16	67.4 (5.5)	48.9 (13.8)	51.1 (4.1)	88.2 (2.5)	130.8 (2.6)	122.8 (19.6)	91.6 (2.5)	111.8 (8.5)	*
C18	38.7 (6.3)	26.7 (22.3)	30.0 (1.8)	45.5 (3.1)	56.8 (2.7)	58.7 (14.9)	42.6 (3.4)	47.6 (4.6)	*
C18:1	27.1 (4.2)	20.1 (36.4)	14.4 (3.1)	63.9 (1)	92.8 (0.5)	99.9 (24.3)	78.4 (10.6)	87.0 (10.2)	*
C18:2	3.2 (5.7)	2.8 (55.1)	3.6 (29.9)	5.8 (3.8)	8.2 (12.4)	8.8 (27.9)	8.2 (12.2)	10.1 (24.4)	*
C18:3	3.7 (1.3)	1.5 (44.7)	4.1 (20.8)	6.2 (6.7)	7.2 (4.3)	5.0 (22.1)	6.0 (2.4)	2.2 (24.3)	*
Result Total	183.7 (4.7)	124.1 (21.1)	130.6 (6.1)	273.3 (1.5)	394.8 (2)	374 (22.1)	294.5 (6.8)	326.7 (10.6)	*

Table S5.4: Individual Free fatty acid content mg/kg (relative standard deviation of the results between replicates as a percent in brackets) for each of the p milk samples (grass (GRS), grass/clover (CLV) and total mixed ration (TMR)) at day 3, 9 and 14 of refrigerated storage and the significance between the fatty acids analysed for each sample (GRS, CLV and TMR). p = 0.05%, d = day, NS = not significant.

Fatty Acid	Grass d 3	Grass d 9	Grass d 14	*p*-Value	Grass/Clover d 3	Grass/Clover d 9	Grass/Clover d 14	*p*-Value	TMR d 3	TMR d 14	*p*-Value
C4	3.0 (41.3)	3.9 (11.9)	0.0 (0)	*	0.0 (0)	8.5 (11)	0.0 (0)	*	0.0 (0)	0.0 (0)	NS
C6	4.4 (4.7)	5.3 (2.9)	6.9 (32.9)	NS	2.4 (17.1)	8.2 (5.2)	6.4 (14.4)	*	2.3 (2.8)	5.6 (17.3)	*
C8	4.2 (4.9)	6.0 (0.1)	7.6 (32.2)	NS	2.3 (18.5)	8.9 (4)	6.8 (14.3)	*	2.5 (4.8)	6.1 (18.4)	*
C10	7.5 (7)	11.3 (0.2)	14.7 (31.7)	NS	4.3 (15.3)	16.7 (3.7)	12.9 (14.9)	*	5.1 (22.2)	12.0 (17.9)	NS
C12	8.7 (4.1)	12.9 (0.7)	16.4 (26.2)	NS	4.9 (15.2)	18.7 (2.7)	13.6 (9.1)	*	6.9 (19.5)	15.0 (16.9)	NS
C14	15.9 (7.4)	24.3 (0.3)	33.3 (25.5)	NS	10.2 (16)	38.1 (2.8)	28.1 (6.1)	*	10.6 (14.3)	29.2 (14.8)	*
C16	67.4 (5.5)	88.2 (2.5)	122.8 (19.6)	NS	48.9 (13.8)	130.8 (2.6)	91.6 (2.5)	*	51.1 (4.1)	111.8 (8.5)	*
C18	38.7 (6.3)	45.5 (3.1)	58.7 (14.9)	NS	26.7 (22.3)	56.8 (2.7)	42.6 (3.4)	*	30.0 (1.8)	47.6 (4.6)	*
C18:1	27.1 (4.2)	63.9 (1)	99.9 (24.3)	*	20.1 (36.4)	92.8 (0.5)	78.4 (10.6)	*	14.4 (3.1)	87.0 (10.2)	*
C18:2	3.2 (5.7)	5.8 (3.8)	8.8 (27.9)	NS	2.8 (55.1)	8.2 (12.4)	8.2 (12.2)	*	3.6 (29.9)	10.1 (24.4)	NS
C18:3	3.7 (1.3)	6.2 (6.7)	5.0 (22.1)	NS	1.5 (44.7)	7.2 (4.3)	6.0 (2.4)	*	4.1 (20.8)	2.2 (24.3)	NS

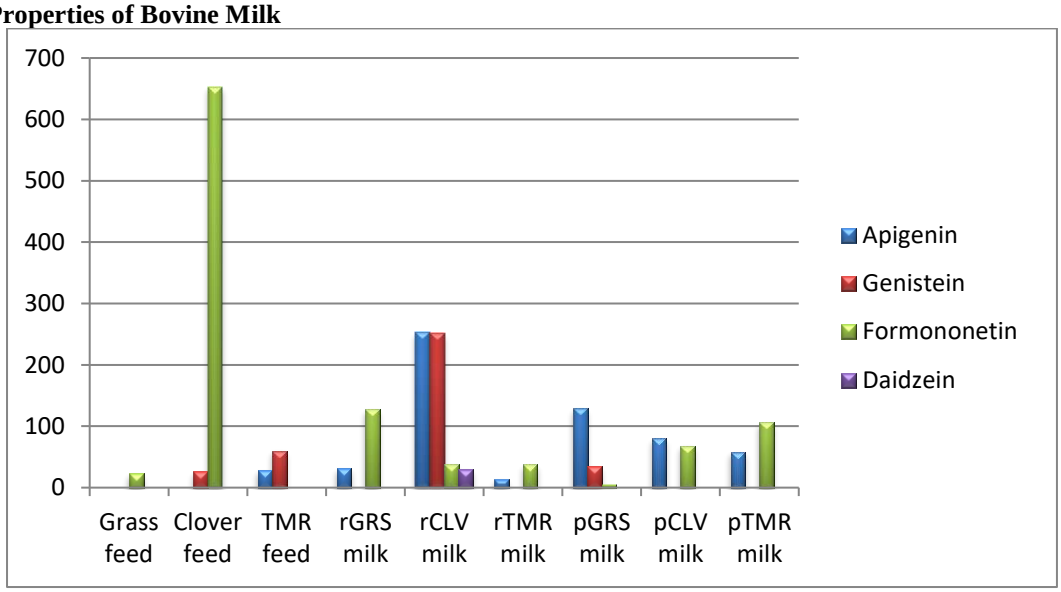


Figure S5.1: Bar chart showing the levels of important isoflavones in feed samples (grass, clover and total mixed ration (TMR)) and the corresponding raw (r) and pasteurised (p) grass (GRS), clover (CLV) and total mixed ration (TMR) milk samples.

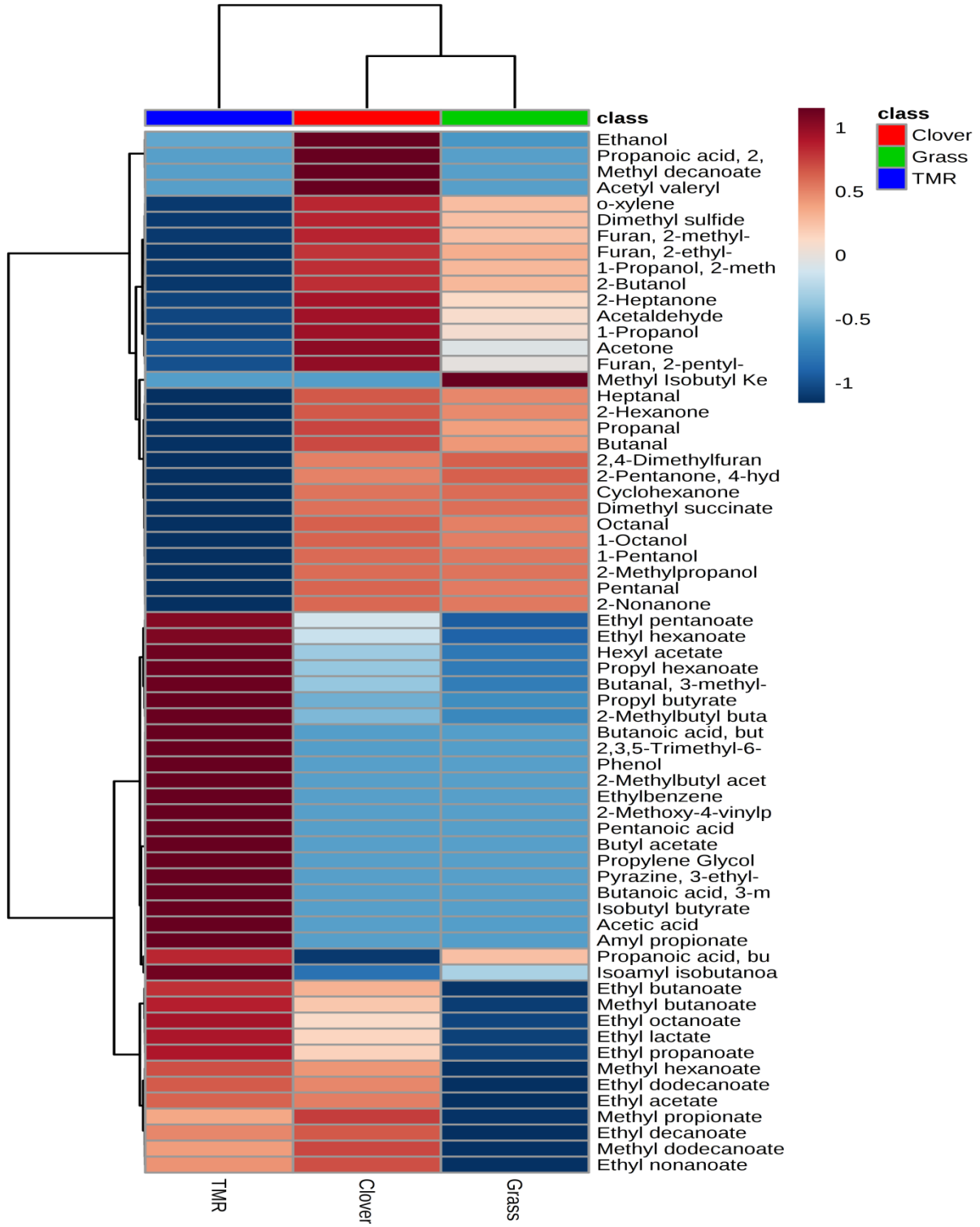


Figure S5.2: Hierarchal clustering analysis (Heatmap) of the average values for the top 65 volatile organic compounds contributing to the differences between grass, grass/clover and total mixed ration (TMR) feed samples, as determined by headspace solid-phase microextraction gas-chromatography mass spectrometry (HS-SPME GC-MS). Positive and negative correlations between feeding system (grass, grass/clover and TMR) and volatile organic compounds is denoted by +1 (red) and −1 (blue).

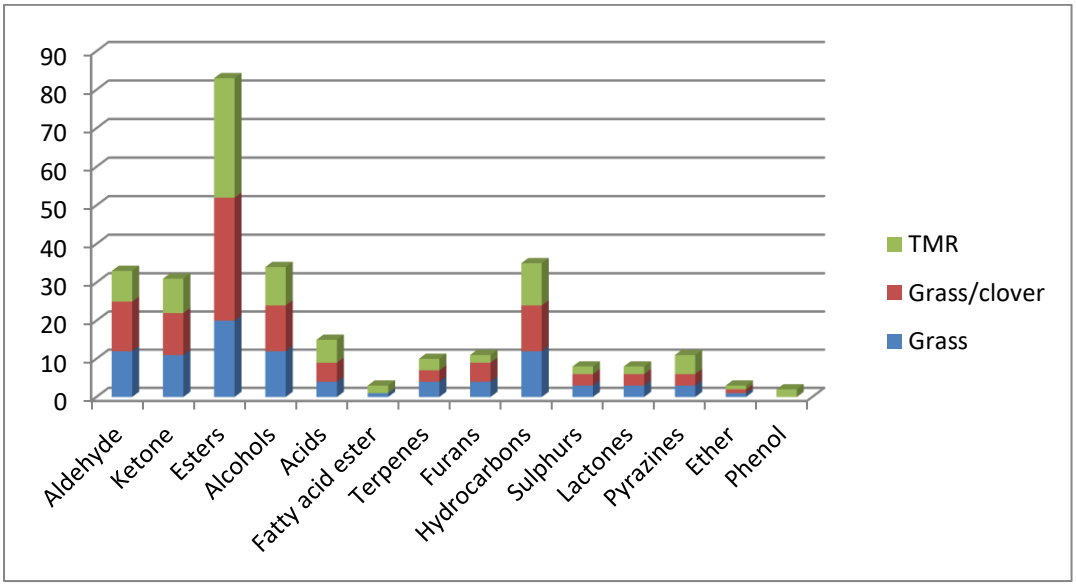


Figure S5.3: Bar charts showing the percentage of each chemical class (aldehydes, ketones, alcohols, acids, fatty acid esters, terpenes, furans, hydrocarbons, sulphurs, lactones, pyrazines, ether and phenol) identified in each feed type (grass, grass/clover and total mixed ration (TMR). 90, 104 and 94 compounds were identified in grass, grass/clover and TMR feeds, respectively.

Table S5.5: Relationship between cow diet (grass, grass/clover and total mixed ration (TMR)) and the pasteurised (p) milk volatile compounds identified by headspace solid-phase microextraction gas-chromatography mass spectrometry (HS-SPME GC-MS) at day 3, 9 and 14 of refrigerated storage; values are expressed as peak area values for each compound.; values are expressed as peak area values for each compound. d = day, $p = 0.05$ ND = not detected, NS = not significant. LRI = Linear retention index.

Compound	CAS No.	LRI	Grass d 3	Grass/Clover d 3	TMR d 3	Grass d 9	Grass/Clover d 9	TMR d 9	Grass Day 14	Grass/Clover d 14	TMR d 14	*p*-Value	*p*-Value (Grass)	*p*-Value (Grass/Clover)	*p*-Value (TMR)
Aldehyde															
(E)-2-Octenal (or isomer)	2548-87-0	1094	0.00 × 00	0.00 × 00	0.00 × 00	3.80×10^7	0.00 × 00	5.05×10^8	1.67×10^8	0.00 × 00	2.20×10^7	*<0.001	NS 0.499	ND	*<0.001
(Z)-2-Heptenal (or isomer)	57266-86-1	1012	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.26×10^7	*<0.001	ND	ND	*<0.001
Butanal	123-72-8	627	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.31×10^6	0.00 × 00	1.08×10^7	*<0.001	NS 0.422	ND	*0.001
3-Methyl-butanal	590-86-3	690	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	NS 0.469	NS 0.422	ND	ND
Decanal	112-31-2	1250	8.17×10^6	4.70×10^6	4.45×10^6	1.46×10^7	2.56×10^6	6.46×10^6	1.06×10^7	4.68×10^6	6.58×10^6	NS 0.100	NS 0.470	NS 0.211	NS 0.825
Heptanal	111-71-7	941	2.64×10^8	2.74×10^8	1.33×10^8	1.98×10^8	1.81×10^8	0.00 × 00	6.35×10^8	2.52×10^8	1.00×10^9	*<0.001	*<0.001	* 0.009	*<0.001
Hexanal	66-25-1	838	3.91×10^8	3.65×10^8	5.35 × 108	2.74 × 108	2.50×10^8	0.00 × 00	6.33×10^8	2.91×10^8	4.45×10^9	*<0.001	*0.018	*0.031	*<0.001
Nonanal	124-19-6	1147	1.49×10^8	2.31×10^8	1.83×10^8	1.28×10^8	9.04×10^7	4.55×10^7	8.62×10^7	8.08×10^7	1.42×10^8	*<0.001	NS 0.120	*0.009	*<0.001
Octanal	124-13-0	1044	4.97×10^7	6.24×10^7	4.56×10^7	4.54×10^7	3.49×10^7	0.00 × 00	4.38×10^7	5.88×10^7	1.09×10^8	*<0.001	NS 0.867	*0.012	*<0.001
Pentanal	110-62-3	733	7.14×10^8	7.90×10^8	6.47×10^7	5.25×10^8	7.04×10^8	0.00 × 00	5.87×10^8	6.29×10^8	2.46×10^8	*<0.001	*0.014	*0.025	*0.018

Ketone															
2-Butanone	78-93-3	637	6.30×10^7	1.04×10^8	1.53×10^8	7.61×10^7	9.97×10^7	8.76×10^7	7.26×10^7	9.84 × 107	1.43 × 108	*0.001	NS 0.868	NS 0.892	*0.001
2-Heptanone	110-43-0	933	3.73×10^7	3.80×10^7	3.18×10^7	1.40×10^8	4.27 × 107	7.14×10^9	6.77×10^8	6.23 × 107	5.85 × 107	*<0.001	NS 0.416	* 0.011	*<0.001
2-Hexanone	591-78-6	831	1.10×10^7	6.07×10^6	4.99×10^6	1.51×10^7	1.15 × 107	6.02×10^7	2.74 × 107	2.49 × 107	1.66 × 107	*<0.001	NS 0.281	* 0.003	*<0.001
2-Nonanone	821-55-6	1137	0.00 × 00	0.00 × 00	0.00 × 00	8.11×10^7	0.00 × 00	2.15×10^9	1.97 × 108	0.00 × 00	0.00 × 00	*0.001	NS 0.556	ND	*<0.001
2-Octanone	111-13-7	1034	5.44×10^6	5.16×10^6	3.37×10^6	1.27×10^7	1.89×10^7	3.45×10^7	2.69 × 107	1.87 × 107	1.43 × 107	NS 0.071	NS 0.330	NS 0.226	*0.011
2-Pentanone	107-87-9	727	7.97×10^7	6.78×10^7	6.73×10^7	1.06×10^8	5.82×10^7	7.97×10^8	1.40×10^8	6.48 × 107	6.08 × 107	*<0.001	NS 0.726	NS 0.235	*<0.001
2-Undecanone	112-12-9	1353	0.00 × 00	0.00 × 00	0.00 × 00	8.09×10^6	0.00 × 00	1.50×10^8	1.86×10^7	0.00 × 00	0.00 × 00	*0.001	NS 0.516	ND	*<0.001
3-Hexen-2-one	763-93-9	839	1.20×10^7	1.70×10^6	0.00 × 00	5.93×10^6	9.68×10^6	1.05×10^7	3.80×10^6	0.00 × 00	0.00 × 00	NS 0.065	NS 0.244	NS 0.129	NS 0.111
3,5-(E,E)-Octadien-2-one (or isomer)	30086-02-3	1130	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.39×10^7	0.00 × 00	2.86×10^7	*<0.001	*0.002	ND	* 0.001
4-Methyl-3-pentene-2-one (tentative)	141-79-7	839	0.00 × 00	0.00 × 00	0.00 × 00	1.03×10^7	1.37×10^7	9.34×10^6	1.65×10^7	1.79×10^7	0.00 × 00	*0.001	*0.025	*<0.001	NS 0.211
4,6-Dimethyl-2-heptanone	19549-80-5		7.33×10^6	9.45×10^6	8.92×10^6	4.66×10^6	3.16×10^6	0.00 × 00	9.63×10^6	1.22×10^7	1.91×10^7	NS 0.187	NS 0.766	NS 0.071	NS 0.117
5-Hepten-2-one (tentative)	6714-00-7	921	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	7.28 × 107	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	NS	*<0.001
Acetone	67-64-1	532	1.23×10^9	9.62×10^8	1.20×10^9	1.24×10^9	5.67×10^8	7.51 × 108	1.15×10^9	6.00×10^8	1.20 × 109	*0.041	NS 0.959	NS 0.218	*<0.001
Acetophenone	98-86-2	1030	3.90×10^6	2.18×10^6	3.56×10^6	4.80×10^6	0.00 × 00	2.70 × 106	1.57×10^6	5.82×10^5	8.14×10^5	*0.048	NS 0.260	NS 0.170	NS 0.224

Cyclohexanone	108-94-1	956	8.34×10^5	8.05×10^6	5.36×10^6	1.74×10^6	9.14×10^5	0.00 × 00	0.00 × 00	1.93×10^6	0.00 × 00	NS 0.338	NS 0.574	NS 0.054	NS 0.117
Acetyl valeryl (2,3-heptanedione)	96-04-8	875	3.25×10^6	1.46×10^6	3.68×10^6	5.53×10^6	2.14×10^6	7.10×10^6	2.38×10^6	6.73×10^6	1.80×10^6	NS 0.680	NS 0.701	NS 0.364	NS 0.347
Methyl Isobutyl Ketone	108-10-1	780	3.08×10^8	1.84×10^8	2.41×10^8	3.12×10^8	1.67×10^8	1.80×10^8	3.25×10^8	1.70×10^8	2.45×10^8	*0.045	NS 0.982	0.512	*0.027
Ester															
Ethyl heptanoate	106-30-9	1120	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	3.77×10^6	8.69×10^6	0.00 × 00	0.00 × 00	NS 0.421	NS 0.422	ND	*<0.001
Ethyl (Z)-2-butenoate	6776-19-8	875	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	3.01×10^7	1.16×10^8	0.00 × 00	0.00 × 00	NS 0.468	NS 0.422	ND	*<0.001
Ethyl acetate	141-78-6	639	0.00 × 00	0.00 × 00	0.00 × 00	7.91×10^6	0.00 × 00	6.48×10^7	9.66×10^7	0.00 × 00	0.00 × 00	NS 0.334	NS 0.449	ND	*<0.001
Ethyl decanoate	110-38-3	1419	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.24 × 108	0.00 × 00	0.00 × 00	NS 0.051	NS 0.163	ND	ND
Ethyl hexanoate	123-66-0	1021	0.00 × 00	0.00 × 00	0.00 × 00	1.34×10^8	0.00 × 00	3.79×10^9	2.37×10^9	0.00 × 00	0.00 × 00	0.021	NS 0.436	ND	*<0.001
Ethyl octanoate	106-32-1	1220	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	3.01×10^7	2.65×10^8	0.00 × 00	0.00 × 00	NS 0.445	NS 0.405	ND	*0.002
Ethyl pentanoate	539-82-2	923	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	3.07×10^7	2.13×10^7	0.00 × 00	0.00 × 00	*0.045	NS 0.422	ND	*<0.001
Ethyl propanoate	105-37-3	735	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	1.65×10^7	5.24×10^6	0.00 × 00	0.00 × 00	*0.039	NS 0.422	ND	NS 0.095
Methyl butanoate	105-54-4	747	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.16×10^7	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	ND	*<0.001
Methyl hexanoate	123-66-0	949	0.00 × 00	0.00 × 00	0.00 × 00	6.18 × 105	0.00 × 00	2.74×10^7	6.60×10^5	0.00 × 00	0.00 × 00	*<0.001	NS 0.629	ND	*0.004

Methyl methacrylate	80-62-6	736	5.44×10^6	9.65×10^6	6.33×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.007	NS 0.087	NS 0.079	*<0.001
Alcohol															
2-Methyl-1-butanol	137-32-6	715	5.50×10^7	8.54×10^7	8.58×10^7	5.30×10^7	5.28×10^7	1.09×10^8	4.92×10^7	4.69×10^7	3.56×10^7	*<0.001	NS 0.851	*0.002	*0.003
3-Methyl-1-butanol	123-51-3	765	7.22×10^7	1.92×10^7	2.30×10^8	2.13×10^8	1.22×10^8	3.05×10^8	7.19×10^8	1.32×10^8	1.81×10^8	NS 0.321	NS 0.356	NS 0.323	NS 0.404
3-Dimethyl-2-butanol (tentative)	594-60-5	773	2.46 × 106	2.61×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	4.55×10^6	2.19×10^6	2.83×10^6	NS 0.054	NS 0.118	NS 0.959	*<0.001
Ethanol	64-17-5	505	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	7.39×10^7	7.00×10^8	0.00 × 00	0.00 × 00	NS 0.481	NS 0.422	NS	NS 0.082
1-Hexanol	111-27-3	894	1.63×10^6	7.06×10^5	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.001	*<0.001	NS 0.422	ND
2-Ethyl-1-hexanol	104-76-7	1075	4.60×10^7	5.01×10^7	3.65×10^7	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	*<0.001	*0.001	*<0.001
1-Octanol	111-87-5	1116	0.00 × 00	0.00 × 00	2.56×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	2.20×10^7	*<0.001	ND	ND	*<0.001
1-Pentanol	71-41-0	794	1.11×10^9	1.20×10^9	1.01×10^8	6.45×10^8	7.05×10^8	3.48×10^7	4.18×10^8	3.05×10^8	4.67×10^7	*<0.001	*<0.001	*<0.001	*0.040
Isopropyl Alcohol	67-63-0	451	0.00 × 00	0.00 × 00	0.00 × 00	3.31×10^6	0.00 × 00	3.93×10^7	2.58×10^7	0.00 × 00	0.00 × 00	NS 0.147	*<0.001	ND	NS 0.083
Acid															
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	77-68-9	1460	3.82×10^7	1.38×10^7	0.00 × 00	6.86×10^6	5.48×10^6	0.00 × 00	3.74×10^6	2.31×10^6	0.00 × 00	*0.023	NS 0.121	NS 0.340	ND
Terpene															
3-Carene	13466-78-9	1035	0.00 × 00	0.00 × 00	0.00 × 00	5.14×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	NS 0.192	NS 0.276	NS	ND

α-Pinene	80-56-8	953	1.01×10^7	8.21×10^6	3.97×10^6	8.04×10^7	2.80×10^7	3.25×10^7	1.56×10^7	7.54×10^6	3.19×10^6	*<0.001	*0.023	*0.004	*0.020
Cumene	98-82-8	990	5.37×10^5	2.26×10^6	1.07×10^6	3.59×10^6	2.03×105	9.07×10^6	5.11×10^6	3.59×10^6	1.31×10^6	*<0.001	NS 0.093	NS 0.130	*0.002
D-Limonene	5989-27-5	1055	2.15×10^7	1.34×10^7	1.08×10^7	6.08×10^6	2.63×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.001	*0.003	NS 0.148	*0.001
Mesitylene	108-67-8	1028	8.50 × 107	5.33 × 107	5.03 × 107	5.68 × 107	2.55 × 107	5.06 × 107	5.13 × 107	3.32 × 107	3.18 × 107	NS 0.151	NS 0.522	*0.025	NS 0.172
trans-β-Ocimene (or isomer)	3779-61-1	1035	0.00 × 00	0.00 × 00	1.08×10^7	4.38×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.002	NS 0.422	ND	*0.001
Furan															
2,4-Dimethylfuran	3710-43-8	732	1.38×10^7	1.26×10^7	6.20×10^6	1.27×10^7	4.19×10^6	9.01×10^6	9.23×10^6	1.22×10^7	1.54×10^6	NS 0.265	NS 0.820	NS 0.183	*0.020
2,5-Dimethylfuran	625-86-5	734	1.55×10^7	1.03×10^7	3.76×10^6	1.42×10^7	4.19×10^6	9.01×10^6	1.96×10^8	1.17×10^8	7.36×10^7	NS 0.452	NS 0.648	NS 0.618	*0.049
2-Ethylfuran	3208-16-0	717	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	7.87×10^6	1.12×10^7	1.54×10^6	NS 0.114	NS 0.326	NS 0.422	NS 0.124
Hydrocarbon															
2,4-Di-tert-butylphenol	96-76-4	1595	0.00 × 00	0.00 × 00	0.00 × 00	1.30×10^7	1.81×10^6	9.30×10^6	3.94×10^6	0.00 × 00	0.00 × 00	*0.021	NS 0.108	NS 0.422	NS 0.089
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol (tentative)	14035-34-8	1684	2.29×10^7	1.12×10^7	8.54×10^6	2.82×10^7	1.49×10^7	2.18×10^7	1.81×10^7	2.89×10^7	2.55×10^7	NS 0.463	NS 0.801	*0.048	NS 0.163
2,4-Dimethyl-benzaldehyde	15764-16-6	1305	3.50×10^6	5.16×10^6	3.50×10^6	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	*0.001	*0.008	*0.025	*0.048
Benzene	71-43-2	684	7.00 × 106	5.78×10^6	4.10×10^6	4.71×10^6	2.68×10^6	6.17×10^6	2.55×10^6	2.32×10^6	4.06×10^6	NS 0.542	NS 0.154	NS 0.331	NS 0.756

1,2,3-Trimethyl-benzene	526-73-8	1028	8.50×10^7	5.33×10^7	5.03×10^7	5.68×10^7	2.55×10^7	5.06×10^7	5.13×10^7	3.32×10^7	3.18×10^7	NS 0.151	NS 0.522	*0.025	NS 0.172
1,3-bis(1,1-dimethylethyl)-benzene	1014-60-4	1284	5.04×10^8	6.01×10^8	4.70×10^8	4.46×10^8	3.33×10^8	5.35×10^8	5.30×10^8	4.51×10^8	3.09×10^8	NS 0.247	NS 0.902	*0.003	*<0.001
Ethylbenzene	100-41-4	897	9.72×10^7	7.10×10^7	5.62×10^7	1.37×10^8	6.50×10^7	1.71×10^8	1.96×10^8	1.17×10^8	7.36×10^7	*0.032	NS 0.427	*0.003	*<0.001
o-Cymene	527-84-4	1055	1.22×10^6	4.97×10^5	0.00 × 00	3.10×10^6	4.71×10^5	1.25×10^6	0.00 × 00	0.00 × 00	0.00 × 00	*0.016	*0.015	NS 0.629	NS 0.422
o-xylene	95-47-6	897	7.42×10^7	5.95×10^7	5.60×10^7	8.02×10^7	3.98×10^7	1.08×10^8	1.04×10^8	5.92×10^7	5.09×10^7	NS 0.185	NS 0.768	*0.010	*0.010
p-Cresol	106-44-5	1182	0.00 × 00	0.00 × 00	0.00 × 00	3.58×10^8	1.06×10^8	1.51×10^8	5.93×10^7	2.03×10^7	1.88×10^7	*0.013	NS 0.116	*<0.001	*<0.001
p-Xylene	106-42-3	895	9.72×10^7	7.10×10^7	5.62×10^7	1.37×10^8	6.50×10^7	1.71×10^8	1.96×10^8	1.17×10^8	7.36×10^7	*0.032	NS 0.427	* 0.003	*<0.001
Styrene	100-42-5	927	4.87×10^6	4.63×10^6	0.00 × 00	0.00 × 00	1.12×10^7	0.00 × 00	7.15×10^6	2.14×10^6	5.53×10^6	NS 0.105	NS 0.241	NS 0.233	NS 0.090
tert-Butylbenzene	98-06-6	1024	1.39×10^7	1.71×10^7	7.51×10^6	1.17×10^7	8.23×10^6	1.33×10^7	1.17×10^7	1.29×10^7	6.30×10^6	NS 0.324	NS 0.889	NS 0.121	NS 0.287
Toluene	108-88-3	792	2.42×10^9	1.30×10^9	6.28×10^7	2.41×10^9	1.23×10^9	4.91×10^7	2.35×10^9	1.15×10^9	4.73×10^7	*<0.001	NS 0.996	NS 0.281	*0.035
Phenolic															
Phenol	108-95-2	1093	0.00 × 00	0.00 × 00	0.00 × 00	7.60×10^6	1.23×10^6	0.00 × 00	4.96×10^6	1.05 × 106	7.23×10^5	NS 0.074	NS 0.343	NS 0.626	NS 0.422
Sulfur															
Dimethyl sulfide	75-18-3	536	9.61×10^6	5.73×10^6	2.29×10^6	1.40×10^7	2.36×10^6	2.89×10^7	6.23×10^7	1.16×10^6	0.00 × 00	NS 0.402	NS 0.501	NS 0.409	*<0.001
Dimethyl sulfone	67-71-0	1052	1.77×10^7	3.34×10^6	0.00 × 00	2.60×10^7	9.68×10^6	0.00 × 00	1.11×10^7	1.34×10^6	0.00 × 00	*0.001	NS 0.277	NS 0.200	ND
Methanethiol	74-93-1	459	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	0.00 × 00	9.25×10^6	0.00 × 00	0.00 × 00	0.00 × 00	*<0.001	ND	ND	*<0.001

Ether															
Ethyl ether	60-29-7	514	8.30×10^6	1.01×10^7	9.61×10^6	7.75×10^6	7.17×10^6	2.51×10^6	5.50×10^6	4.57×10^6	4.81×10^6	NS 0.886	NS 0.865	NS 0.661	NS 0.399
Vinylisopentyl ether	39782-38-2	767	3.91×10^7	2.04×10^8	2.96×10^8	1.84×10^8	6.42×10^7	1.97×10^8	1.21×10^8	6.23×10^7	5.10×10^7	NS 0.111	NS 0.126	NS 0.397	NS 0.094

Table S5.6: The 26 sensory descriptors applied to the three pasteurised milk samples (grass (GRS), clover (CLV) and total mixed ration (TMR)) by full descriptive sensory analysis.

Section	Descriptor
Aroma	
	1. Dairy sweet aroma
	2. Cooked milk
	3. Barnyard aroma
	4. Grassy aroma
	5. Hay like aroma
	6. Malty aroma
Flavour	
	7. Dairy sweet flavour
	8. Cooked milk flavour
	9. Dairy fat flavour
	10. Malty flavour
	11. Creamy flavour
	12. Hay like flavour
	13. Grassy flavour
	14. Dairy sour flavour
	15. Off flavour
Mouth feel	
	16. Viscosity
	17. Creaminess
	18. Mouth coating
	19. Chalkiness
After effect (AE)	
	20. Astringency
	21. Mouth coating AE
	22. Dairy sweet AE
	23. Cooked milk AE
	24. Dairy sour AE
	25. Malty AE
	26. Barnyard AE

Chapter 5 Highlights

- Significant differences were observed in volatile profile, isoflavone content and sensory perception of milk based on the feeding system.
- Formononetin was found to be significantly correlated to CLV feed samples and levels of apigenin.
- Concentrations of daidzein and genistein were found to be significantly different between the r and p milk samples.
- Most benzene compounds increased in GRS and CLV milk after pasteurisation but not in TMR samples. Toluene was significantly higher in both r and p GRS and CLV milk samples throughout storage.
- Full descriptive sensory analysis provided a reliable insight into the differences of the milks based on feeding system.
- Further research is required to ascertain the complex breakdown pathways of isoflavone compounds derived from feed and their effect on the sensory perception of bovine milk.

Chapter 6: The influence of Pasture and Non-pasture-based Feeding Systems on the Aroma of Raw Bovine Milk

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The Influence of Pasture and Non-pasture-Based Feeding Systems on the Aroma of Raw Bovine Milk

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Abstract

Aroma-active compounds in raw bovine milk produced from cows fed perennial ryegrass (GRS) or total mixed ration (TMR) consisting of grass silage, maize silage and concentrates were identified by direct immersion sorptive extraction (DI Hi-Sorb) coupled with gas-chromatography-mass spectrometry and olfactometry using odour intensity and aroma extraction dilution analysis (AEDA). Ninety nine volatile organic compounds (VOC) were identified in these raw GRS and TMR milk samples 33 of which were also present in the feed and rumen samples from these diets. Only the abundance of 13 VOC varied significantly based on diet. However, the odours of both raw milks were quite distinct as aroma perception is not only influenced by abundance alone, but also by the odour activity of each VOC. Approximately 30% of the VOC influenced aroma perception of these raw milks. This study clearly highlighted the significant impact of VOC transferring from the diet that influenced the perception of both raw GRS and TMR milk. The aroma of the raw TMR milk was more complex than that of the raw GRS milk, and many of the key dietary derived odour active VOC, likely arose during the production of the TMR feed as most were either derived from Malliard reactions or impacted by heat. Seventeen of the 44 odour

activities detected differed between both sample types. This study has clearly demonstrated the impact of diet on the aroma perception of raw bovine milk.

Keywords: aroma, pasture, raw bovine milk, volatile, GC-O, HiSorb-GC-MS

6.1 Introduction

Previous studies have demonstrated a significant effect of feeding system on the composition of raw milk, and particularly its impact on the fatty acid content (O'Callaghan et al., 2016). It is also established that diet has a significant impact on the volatile profile of bovine milk (O'Callaghan et al., 2016, Salum et al., 2017, Faccia, 2020) and it may even be possible to use volatile organic compounds (VOC) to authenticate pasture-based dairy products (Kilcawley et al., 2018, Clarke et al., 2020a). VOC in bovine milk consist of a range of different chemical classes including; aldehydes, ketones, lactones, esters, alcohols, acids, terpenes, furans, hydrocarbons, pyrazines, phenolic and sulphur compounds (Bugaud et al., 2001, O'Callaghan et al., 2016, Faulkner et al., 2018, Clarke et al., 2020a). However, their potential impact on sensory perception depends upon their relative concentration and odour activity. Previous studies have reported direct transfer of VOC from bovine feed to milk (Addis et al., 2006, Kilcawley et al., 2018), and that compounds such phytochemicals in feed may be metabolised in the rumen to more volatile odour active compounds in the milk (Bugaud et al., 2001). Evaluating raw milk enables those VOC originating from the bovine feeding system to be more easily assessed, as other VOC arising from milk heat treatment or formed during shelf life by microbial activity are not present. Information on the aroma perceptions and intensities of individual VOC in raw milk from different feeding systems may also prove important when selecting raw milk for future applications. For example; further processing to generate commodity dairy products may positively or negatively alter and/or exacerbate specific odours that may impact consumer preference.

Gas-chromatography-olfactometry (GC-O) is a very useful approach to identify odour active VOC in food products (Delahunty et al., 2006). GC-O refers to the use of human assessors to detect aroma active VOC extracted and separated using GC in tandem with mass spectrometry and/or flame ionization detection. Friedrich and Acree (1998) reviewed GC-O studies on dairy products including those on raw and pasteurised bovine milk and highlighted the significance of esters in raw milk and the creation of other VOC during pasteurisation.

At that time only 2 studies had been undertaken (Moio et al., 1993b, Moio et al., 1994) and both used vacuum distillation as the extraction process. Solvent-assisted flavour evaporation (SAFE) was subsequently used to extract VOC in pasteurised bovine milk (Bendall, 2001) from cows on two distinct diets (pasture and total mixed ration). These authors found 66 odour active VOC in bovine milk, and that diet only influenced the abundance of these VOC, rather than creating unique VOC, except for γ-12:2 lactone (γ-dodec-cis-6, cis-9-dienolactone) which was absent in the milk derived from a pasture diet. Other authors (Mounchili et al., 2005) have used headspace solid phase micro-extraction (HS-SPME) to identify aroma active VOC in bovine milk. These authors found 75 odour active VOC in bovine milk, but only found differences in abundances of individual VOC, not any distinct VOC associated with diet, despite the fact that the milks were selected as either good quality milk or deemed to be tainted with a 'feed' off-flavour as determined by certified expert sensory graders. A recent study of raw bovine milk (Ai et al., 2015) also used HS-SPME, but only identified 9 aroma active VOC consisting of 7 acids, 1 aldehyde and 1 ketone. Although HS-SPME is widely applied as a volatile extraction technique, it has some well-known limitations, particularly relating to the low volume of sorbent phase which can result in VOC competition and migration during the equilibration phase (Mondello et al., 2005), and by a propensity to preferentially extract very volatile low molecular weight VOC (Salum et al., 2017). Although SAFE is a well-established extraction technique it is time consuming, requires solvents, complex glass apparatus (Bertuzzi et al., 2018) and has poor reproducibility (Thomsen et al., 2014).

One of the potential reasons why so few GC-O studies have been undertaken on either raw or heat-treated bovine milk may be due to its subtle flavour, making it difficult to discern aroma characteristics. Thus it is imperative that VOC are concentrated sufficiently prior to separation by GC in order to be more easily perceived by olfactometry panellists. More green or environmentally friendly automated or semi-automated extraction techniques such as stir bar sorptive extraction (SBSE) are used to identify favour compounds, due to their ease of use, good reproducibility and high sorption capability (Wang et al., 2020). These appear to be more effective as direct immersive procedures rather than as headspace extraction procedures (High et al., 2019). A new version of sorptive extraction called high-capacity sorptive extraction (HiSorb) was successfully utilised by Faulkner et al. (2018) to profile VOC from bovine milk produced by different diets, and out preformed HS-SPME in a study

by Cheng et al. (Cheng et al., 2021) to profile VOC in whole milk powder. Thus, HiSorb as a direct immersion technique (DI-HiSorb) utilising polydimethylsiloxane (PDMS) as the sorbent appears ideally suited for GC-O analysis of bovine milk.

This study is the first study to determine if any differences exist in relation to odour active VOC in milk from different diets (pasture vs. non-pasture), using DI-HiSorb (with a PDMS sorbent phase) in tandem with GC-O and GC-MS.

6.2 Materials and Methods

6.2.1 Experimental Design and Milk Collection

Fifty-four spring-calving Friesian cows based at the Teagasc Moorepark dairy farm (Fermoy, Co. Cork, Ireland) were allocated to experimental feeding groups (n=18) namely; outdoors on perennial ryegrass (*Lolium perenne L.)*, indoors on total mixed ration which comprised a mixture of grass silage, maize silage, and concentrates, or on pasture mixed ration (50:50 pasture:TMR) whereby the cows were outdoors by day and indoors by night. The TMR diet consisted of, on a DM basis, 7.15 kg of grass silage, 7.15 kg of maize silage, and 8.3 kg of concentrates. Each cow received TMR ad libitum. The pasture based cows consumed ~18 kg of dry matter per day measured by pre- and postgrazing sward heights using the rising plate meter (Jenquip, Feilding, New Zealand), whereas pregrazing herbage mass was measured with an Etesia mower (Etesia UK Ltd., Warwick, UK). The full composition including chemical composition. The full composition including chemical composition of the diets was outlined by O'Callaghan et al. (2016). A Latin Square design was employed whereby each group of 18 cows received either GRS, TMR, or pasture mixed ration for 16 days and were then transferred to one of the other two diets for another 16 days which resulted in each group of cows receiving each diet treatment over a 48-day period. Days 1-14 were used to acclimatise the cows to the feeding regimens and samples were collected on days 15 and 16. In this instance, because large amounts of milk were required for other processing, the morning milk from day 14 was included in this study.

Only milk from the cows that received the just grass or just the total mixed ration diets were considered for this study and are denoted as GRS and TMR, respectively. Morning milk from the GRS and TMR diets was collected at 07:30 and stored at 4°C in designated 5000-L refrigerated tanks until the evening milk (15:30) was added and the tank was stirred. Milk samples from five separate milkings over 3 days (3 morning and 2 evening) were taken and

pooled together during the final three days of each 16-day feeding period. Samples were stored in sterile plastic containers at refrigeration temperature, prior to GC-MS and GC-O analysis, which occurred less than 24 h later.

6.2.1.1 Feed Sampling

Feed samples for GRS and TMR were taken from pasture paddocks on the corresponding milk collection day (day 16). Pasture samples were cut just above the root from various sections across the paddocks. TMR samples were taken directly from the cow feeders. One sample was taken from each feeder (n=18) and pooled together. Samples were frozen at -18°C until required for analysis. Sixty grams of GRS and TMR samples were each blended with 150 mL dH_2O until homogeneous and analysed immediately.

6.2.1.2 Rumen Sampling

Rumen samples were taken from cannulated cows (n=3 per feeding system) in the morning and evening on day 15 and 16 of the 16-day feeding period. The rumen fluid was separated from the solid portion via cheese cloth filtration and is denoted as RF. The solid portion was blended until homogenous and is denoted as RB. Samples were frozen at -18°C until required for analysis. Directly prior to analysis, the three morning and three evening RF and RB samples from day 15 and 16 were pooled together for GRS (n=12) and TMR (n=12).

6.2.2 Volatile Compound Analysis by HiSorb Gas-Chromatography Mass-Spectrometry

The extraction of compounds from the feed, rumen and raw milk samples was carried using conditioned (50°C for 10 min followed by 300°C for 30 min) HiSorb PDMS probes (Product Code: H1-AXAAC; Markes International Ltd., Llantrisant, UK). Milk samples (10 mL), feed samples (15 g), and RF and RB samples (15 g) were placed in 20 mL crimp top, round bottomed clear vials (Product Code: C-VCC20; Markes International Ltd., Llantrisant, UK) and capped with HiSorb-P1 crimp caps and HiSorb septa (Product Code: C-HSPCCS; Markes International Ltd., Llantrisant, UK). NaCl (2.5 g) (Merck Ireland; Arklow, Co. Wicklow, Ireland) and 100 µl internal standard (4-methyl-2-pentanol; 500µl of 1000ppm stock solution in 10 mL dH_2O) were added to each milk sample prior to extraction. HiSorb probes were fully immersed in the feed, rumen and milk samples for 1 hr at 40°C with

agitation at 400rpm using a HiSorb agitator (Part no: U-HSAG-20; Markes International Ltd., Llantrisant, UK). After extraction, the probes were removed and rinsed with dH_2O and dried with lint free paper. The probes were placed inside empty thermal desorption (TD) tubes (Product Code: C0-AXXX-0000; Markes International Ltd., Llantrisant, UK) and capped with brass storage caps fitted with one-piece PTFE ferrules (Product Code: C-CF020; Markes International Ltd., Llantrisant, UK) until analysis. All extractions were carried out at the same time for each sample type. The brass caps were replaced by inert coated stainless steel DiffLok caps (Product Code: C-DLS10; Markes International Ltd., Llantrisant, UK) immediately prior to analysis. The GC-MS conditions for the HiSorb desorption analysis were performed as described by Vilar et al. (2020b). All samples were analysed in triplicate. The system check standards used were 1-butanol, dimethyl disulfide, butyl acetate, cyclohexanone, benzaldehyde, and 2-phenyl-D5-ethanol.

6.2.3 Gas-Chromatography Olfactometry Odour Intensity Analysis

The DI-HiSorb extractions of the raw milks for GC-O evaluation were carried out as described in Section 2.2. Desorption of the HiSorb probes was automated by a Markes Centri system (Markes International Ltd., Llantrisant, UK). Probes were desorbed for 10 min at 280°C onto the material emissions cold trap (Part No: U-T12ME-2S) which was held at 30°C. Prior to desorption of the trap, a 1 min pre-purge step of nitrogen gas was carried out at with a 1:50 split. Trap desorption was performed by heating the trap to 300°C and holding for 5 min. GC conditions were performed as described by Vilar et al. (2020a). All samples were analysed in splitless mode and were evaluated by each panellist (n=5) in duplicate.

6.2.3.2 GC-O Analytical Standards

Olfactory training standards were of analytical grade; ethyl butyrate, octanal, p-cresol, and dimethyl disulphide and heptanal of ≥99% and ≥95% purity respectively (Merck Ireland, Arklow, Co. Wicklow, Ireland), were prepared at 0.3% (w/v) in methanol and stored at −18 °C until required. For each GC-O training session, a stock solution was diluted to 0.03% (w/v) in distilled water to allow the odours to be of adequate potency.

Five experienced sensorial assessors evaluated the odour perceptions of the VOC in the raw GRS and TMR milks. Sniffing time was approximately 29 min and each assessor carried out one session per day. Prior to sample analysis, the panellists were exposed to a

standard stock solution (as described in Section 2.3.1), designed for GC-O training, comprised of 5 compounds; dimethyl disulphide ('sulphur','decomposing'), ethyl butyrate ('fruity', 'pineapple'), heptanal ('fatty', 'green'), octanal ('orange', 'fruity'), and ρ-cresol ('barnyard'). This step allowed panellists to familiarise themselves with the GC-O process and software, as well as the range of odours they could potentially encounter during the GC-O analysis of these raw milk samples. Panelists did not receive formal training on all compounds identified in raw milk as it was deemed impractical due to time constraints. Similar to the study by Vilar et al. (2020a), the panellists were asked to rate (i) the intensity of the eluted aroma using a four-point category scale (1 = weak, hardly recognisable odour; 2 = clear but not intense odour; 3 = intense odour; 4 = very intense odour), recorded by a Gerstel OID Interface/ODP-Recorder (Anatune Ltd, Cambridge, UK), and (ii) the odour perceived, by voice recording. Significant odourants were those that were perceived by at least three of the five assessors. Compound identifications were carried out as described by Vilar et al. (2020a). Odour intensities (OI) for each compound were determined by averaging the panellists' intensity ratings, thus all values have an OI range between 1 and 4.

6.2.4 Gas-Chromatography Oflactometry Aroma Extraction Dilution Analysis (AEDA)

AEDA was carried out on the GRS and TMR milks as described by Garvey et al. (2020). Briefly, the technique was carried out by manipulation of the desorption split ratio (Feng et al., 2015). The split ratio was adjusted to 1:1, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, and 1:150, allowing for adequate dilution to determine the most odour active compounds. Undertaking AEDA using the split approach removed any potential matrix effects that can occur if the sample was diluted. The assessor who demonstrated the highest olfactory perception in the previous analysis was chosen for the AEDA study. The last split ratio at which a compound could be perceived was referred to as the factor dilution (FD) for that compound. AEDA analysis was carried out in duplicate for each sample.

6.2.5 Statistical Analysis

Statistical analysis relating to the volatile compounds identified in raw milk samples was carried out using the Independent Samples t-Test in Statistical Package for the Social Sciences (SPSS) (IBM corp., Armonk, NY, USA). Principal component analysis biplots of the volatile and odour descriptor data were constructed using the factoextra and FactoMinoR

packages within R (v. 3.4.1; R Core Team, 2013). All sensory and volatile data were averaged before analysis. Figure 6.2 was created using MetaboAnalyst v. 5.0.

6.3 Results and Discussion

The VOC identified in raw GRS and TMR bovine milk are provided in Table 6.1. The volatile compounds identified in the feed, RF, RB, and raw milk samples by DI-HiSorb GC-MS are outlined in Table S6.1. Thirty three VOC consisting of 5 acids, 9 alcohols, 6 aldehydes, 1 furan, 5 hydrocarbons, 4 ketones, 1 lactone, 1 pyridine, and 1 other were present across all sample types (feed, RB, RF, and raw milk) (Table 6.2).

Table 6.1: Volatile compounds identified in raw bovine milk from cows fed grass (GRS) or total mixed ration (TMR) by HiSorb-GC-MS.

Compound	CAS no	RI	IM	GRS	TMR	*p*-value
Acids						
Formic acid	64-18-6	605.8	MS	$8.53x10^4$	$6.58x10^4$	NS
Acetic acid	64-19-7	662.8	MS, IHL, LRI	$1.96x10^6$	$2.57x10^6$	NS
Propanoic acid	79-09-4	786	MS, IHL, LRI	$2.49x10^5$	$2.64x10^5$	NS
2-Methylpropanoic acid	79-31-2	841.8	MS, IHL, LRI	$1.92x10^4$	$1.44x10^4$	NS
2-Methyl-2-propenoic acid	79-41-4	882.2	MS	$3.78x10^4$	$5.37x10^4$	NS
Butanoic acid	107-92-6	882.5	MS, IHL	$7.93x10^5$	$1.10x10^6$	NS
3-Methylbutanoic acid	503-74-2	932.8	MS, IHL	ND	$4.35x10^4$	NS
2-Methylbutanoic acid	116-53-0	937.1	MS, IHL	$2.07x10^4$	$1.13x10^5$	*
1-Methylpropyl ester butanoic acid	819-97-6	961.9	MS	$6.56x10^3$	ND	NS
Pentanoic acid	109-52-4	973.9	MS	$1.60x10^5$	$3.77x10^5$	NS
Hexanoic acid	142-62-1	1069.7	MS, IHL, LRI	$7.81x10^5$	$1.67x10^6$	*
Heptanoic acid	111-14-8	1164.1	MS, IHL	$1.82x10^5$	$3.53x10^5$	NS
Octanoic acid	124-07-2	1261.9	MS	$1.41x10^6$	$3.45x10^6$	*
Benzoic acid	65-85-0	1285.2	MS	$2.35x10^5$	$3.40x10^4$	NS
Nonanoic acid	112-05-0	1353.8	MS, IHL	$3.98x10^5$	$6.88x10^5$	NS
Decanoic acid	334-48-5	1452.3	MS, IHL	$2.97x10^6$	$6.71x10^6$	*
Hydrocinnamic acid	501-52-0	1460	MS	$8.27x10^4$	$2.89x10^4$	NS
Undecanoic acid	112-53-8	1544	MS, LRI	$8.26x10^4$	$1.45x10^5$	NS
Dodecanoic acid	143-07-7	1640.2	MS	$2.02x10^6$	$1.94x10^6$	NS
Tetradecanoic acid	544-63-8	1839.7	MS	$4.39x10^5$	$2.91x10^5$	NS
Alcohols						
Ethanol	64-17-5	504.3	MS, IHL, LRI	$3.51x10^6$	$2.74x10^6$	NS
1-Butanol	71-36-3	688.9	MS, IHL	$9.70x10^3$	ND	NS
3-Methylbutanol	123-51-3	774.5	MS, IHL	$2.20x10^5$	$1.19x10^4$	NS
4-Methyl-2-pentanol	108-11-2	796.4	MS, IHL, LRI	$7.85x10^3$	$3.54x10^3$	NS
1-Pentanol	71-41-0	810.6	MS, IHL, LRI	$1.07x10^5$	$1.95x10^4$	*
3-Furanmethanol	4412-91-3	868.3	MS	$1.81x10^5$	$2.33x10^5$	NS
1-Hexanol	111-27-3	917.2	MS, IHL, LRI	$1.88x10^4$	$3.75x10^3$	NS
2-Furanmethanol	98-00-0	929.5	MS, LRI	$5.21x10^6$	$6.80x10^6$	NS
2-Butoxyethanol	111-76-2	952	MS, IHL, LRI	$1.87x10^4$	$2.38x10^3$	NS
3-Methyl-1-hexyn-3-ol	4339-05-3	1046.4	MS	$9.68x10^2$	$6.44x10^2$	NS
Dihydroxyacetone	96-26-4	1046.5	MS	$2.76x10^4$	$1.35x10^4$	NS
2-Ethylhexanol	104-76-7	1078.1	MS, IHL, LRI	$9.46x10^4$	$7.65x10^4$	NS
Phenylethyl Alcohol	60-12-8	1193	MS, IHL, LRI	$1.09x10^4$	$1.42x10^4$	NS
1-Octanol	111-87-5	1120.1	MS, IHL	$7.39x10^4$	$5.37x10^4$	NS
2-Phenoxyethanol	122-99-6	1320.4	MS, IHL, LRI	$2.94x10^5$	$1.55x10^5$	NS
1-Dodecanol	112-53-8	1523.3	MS, LRI	$1.35x10^5$	$1.05x10^5$	NS
Tetradecanol	112-72-1	1724.2	MS	$1.49x10^5$	$1.78x10^5$	NS
Aldehydes						
Acetaldehyde	75-07-0	449.5	MS, IHL, LRI	$5.50x10^6$	$4.97x10^6$	NS
2-Methylpropanal	78-84-2	582.5	MS, IHL, LRI	ND	$4.93x10^5$	*
3-Methylbutanal	590-86-3	650.4	MS, IHL, LRI	$2.43x10^4$	$2.24x10^4$	NS
Hexanal	66-25-1	828.9	MS, IHL	$2.21x10^5$	$1.85x10^5$	NS
Furfural	98-01-1	891.7	MS, IHL, LRI	$1.09x10^6$	$1.31x10^6$	NS
(E)-2-Hexenal	6728-26-3	901.2	MS	$1.35x10^4$	$5.05x10^3$	NS
Heptanal	111-71-7	938.7	MS, IHL, LRI	$2.13x10^5$	$1.60x10^5$	NS
Benzaldehyde	100-52-7	1019.3	MS, IHL	$2.19x10^5$	$2.03x10^5$	NS
5-methyl furfural	620-02-0	1032.2	MS	$1.73x10^5$	$2.07x10^5$	NS
Octanal	124-13-0	1043.6	MS, IHL	$3.07x10^5$	$2.27x10^5$	NS
(E,E)-2,4-Heptadienal	4313-03-5	1074.2	MS, LRI	$2.56x10^4$	$1.39x10^3$	*
Benzeneacetaldehyde	122-78-1	1108.5	MS, IHL, LRI	$2.76x10^4$	$3.94x10^4$	NS

Nonanal	124-19-6	1147.6	MS, IHL, LRI	$1.11x10^6$	$7.50x10^5$	NS
Decanal	112-31-2	1252.6	MS, IHL, LRI	$4.16x10^5$	$3.02x10^5$	NS
Dodecanal	112-54-9	1457.2	MS, IHL	$5.68x10^4$	ND	NS
Tridecanal	10486-19-8	1558.7	MS, LRI	$2.28x10^4$	$8.22x10^3$	NS
Esters and Ethers						
Butyl acetate	123-86-4	834.2	MS, IHL, LRI	$7.36x10^3$	$3.51x10^3$	NS
Ethyl pentanoate	539-82-2	923.3	MS, IHL, LRI	$1.05x10^5$	$5.34x10^3$	NS
Butyl butanoate	100-52-7	1021.4	MS, IHL, LRI	$8.17x10^3$	ND	NS
Ethyl hexanoate	123-66-0	1024.9	MS, IHL, LRI	$1.28x10^5$	$1.41x10^4$	NS
Dimethyl succinate	106-65-0	1085.3	MS	$2.49x10^3$	$1.26x10^3$	NS
Methyl-2-furoate	611-13-2	1170	MS	$4.18x10^5$	$4.88x10^5$	NS
Ethyl octanoate	106-32-1	1225.7	MS, IHL, LRI	$8.64x10^4$	$2.02x10^4$	NS
Ethyl decanoate	110-38-3	1422.5	MS, IHL, LRI	$9.29x10^4$	$3.18x10^4$	NS
Ethyl dodecanoate	106-33-2	1620	MS, IHL, LRI	$1.13x10^4$	$7.60x10^3$	NS
Furans						
2-Methylfuran	79-09-4	793.9	MS	$1.36x10^4$	$2.25x10^4$	NS
2-Pentylfuran	3777-69-3	1008.9	MS, IHL, LRI	$7.76x10^3$	$1.31x10^4$	NS
Isomaltol	3420-59-5	1040	MS, LRI	$1.22x10^6$	$1.10x10^6$	NS
Hydrocarbons and Benzenes						
Toluene	108-88-3	773.5	MS, IHL	$1.14x10^5$	$1.53x10^4$	*
p-Xylene	106-42-3	888.2	MS, IHL, LRI	$2.25x10^4$	$1.16x10^4$	NS
Phenol	108-95-2	1104.5	MS, IHL, LRI	$1.00x10^5$	$8.47x10^4$	NS
p-Cresol	106-44-5	1195	MS, IHL, LRI	$5.82x10^5$	$2.90x10^5$	NS
Benzothiazole	95-16-9	1296.2	MS, IHL, LRI	$7.36x10^4$	$3.39x10^4$	*
2-Methoxy-4-vinylphenol	7786-61-0	1411.9	MS, IHL	$3.63x10^5$	$4.19x10^4$	NS
Indole	110-38-3	1430.8	MS, IHL, LRI	$1.49x10^4$	$1.61x10^4$	NS
Ketones						
Acetone	67-64-1	491.9	MS, IHL, LRI	$2.40x10^5$	$3.94x10^5$	NS
2,3-Butanedione (Diacetyl)	431-03-8	574.6	MS, IHL, LRI	$1.12x10^5$	$1.65x10^5$	NS
2-Pentanone	107-87-9	704.2	MS, IHL, LRI	$4.38x10^4$	$3.61x10^4$	NS
1-Hydroxy-2-propanone	116-09-6	709.8	MS, IHL	$6.83x10^5$	$9.41x10^5$	NS
Methyl Isobutyl Ketone	108-10-1	764.5	MS, IHL	$2.34x10^4$	$1.34x10^4$	NS
2-Heptanone	110-43-0	931.4	MS, IHL, LRI	$1.37x10^5$	$1.97x10^4$	NS
Dihydroxyacetone	96-26-4	1046.5	MS	$2.76x10^4$	$1.35x10^4$	NS
Acetophenone	98-86-2	1132.5	MS, IHL, LRI	$6.74x10^4$	$5.18x10^4$	NS
2-Undecanone	112-12-9	1343.3	MS, IHL	$1.02x10^5$	$7.08x10^4$	NS
2-Tridecanone	593-08-8	1546.2	MS	$2.60x10^5$	$1.79x10^5$	NS
Lactones						
γ-Butyrolactone	96-48-0	1021.1	MS, IHL, LRI	$2.85x10^4$	$6.91x10^4$	NS
2(5H)-Furanone	497-23-4	1026.3	MS, LRI	$7.72x10^5$	$1.08x10^6$	NS
γ-Hexalactone	695-06-7	1163	MS, IHL, LRI	$1.95x10^4$	$8.67x10^4$	NS
2(3H)-Furanone, dihydro-4-hydroxy-	5469-16-9	1382	MS	$1.43x10^6$	$1.71x10^6$	NS
γ-Nonalactone	104-61-0	1485	MS, IHL, LRI	$3.34x10^4$	$1.84x10^5$	*
Pyrazines and Pyridines						
Pyrazine	290-37-9	753	MS	$1.59x10^4$	$2.86x10^4$	*
Pyridine	110-86-1	775.8	MS, IHL	$6.22x10^3$	$6.74x10^3$	NS
2,5-Dimethylpyrazine	123-32-0	950	MS, IHL, LRI	ND	$7.27x10^4$	NS
2,3-Dimethylpyrazine	5910-89-4	959	MS, IHL, LRI	$3.77x10^3$	$5.71x10^4$	NS
Sulphurs						
Methanethiol	90500-11-1	460.1	MS, IHL, LRI	$4.55x10^5$	$4.79x10^5$	NS
Dimethyl disulfide	624-92-0	754.6	MS	$3.74x10^4$	$7.44x10^4$	NS
Dimethyl sulfone	67-71-0	1056	MS, IHL, LRI	$2.49x10^3$	$2.08x10^4$	*
Other						
2-Methyl-1H-pyrrole	636-41-9	918.1	MS, IHL, LRI	$4.94x10^3$	$3.94x10^3$	NS
3-Methyl-2,5-furandione	110-00-9	1050.8	MS	$4.96x10^5$	$8.05x10^5$	NS
1H-Pyrrole-2,5-dione	541-59-3	1102.9	MS	$8.09x10^3$	$9.32x10^3$	NS
Maltol	118-71-8	1193	MS, LRI	$1.28x10^6$	$2.39x10^6$	*

2-Pyrrolidone	88-12-0	1196	MS	$2.28x10^5$	$9.37x10^4$	NS

Levels of volatile compounds are expressed as abundances (mean values from 3 extractions from each raw milk sample (GRS and TMR).

LRI: retention index on a DB-624 UI column; IM: identification method; MS: spectra comparison using NIST mass spectral database; IHL: in-house library created using authentic compounds with target and qualifier ions and linear RI for each compound; LRI: RI agree with literature values.

ND: not detected; NS: not significant; *: $p < 0.05$; significance of raw milk samples based on diet according to the Independent Samples t-Test.

Table 6.2: Volatile organic compounds present in all samples [feed (grass (GRS) and total mixed ration (TMR), rumen fluid (RF), rumen blended (RB) and raw grass (GRS) and total mixed ration (TMR) milk].

Number	Compound	CAS no.	LRI[a]	Grass feed	TMR feed	Grass RF	TMR RF	Grass RB	TMR RB	Raw GRS milk	Raw TMR milk	Occurrence
Acids												
1	Acetic acid	64-19-7	662.8	$3.72x10^6$	$2.29x10^7$	$2.61x10^7$	$4.58x10^7$	$7.11x10^7$	$5.26x10^7$	$1.96x10^6$	$2.57x10^6$	AO
2	Propanoic acid	79-09-4	786	$1.69x10^6$	$1.16x10^7$	$1.19x10^7$	$2.72x10^7$	$3.16x10^7$	$2.70x10^7$	$2.49x10^5$	$2.64x10^5$	AO
3	Butanoic acid	107-92-6	882.5	$3.68x10^6$	$9.57x10^7$	$3.03x10^7$	$8.33x10^7$	$1.03x10^8$	$1.79x10^8$	$7.93x10^5$	$1.10x10^6$	AO
4	Dodecanoic acid	143-07-7	1640.2	$6.15x10^5$	$7.63x10^6$	$3.36x10^6$	$1.35x10^7$	$2.39x10^6$	$5.12x10^6$	$2.02x10^6$	$1.94x10^6$	AO
5	Hexanoic acid	142-62-1	1069.7	$1.01x10^6$	$8.67x10^7$	$9.27x10^6$	$1.14x10^8$	$2.05x10^7$	$1.57x10^8$	$7.81x10^5$	$1.67x10^6$	A
Alcohols												
6	Ethanol	64-17-5	504.3	$8.61x10^5$	$5.18x10^6$	$3.79x10^6$	$3.52x10^6$	$2.21x10^6$	$3.30x10^6$	$3.51x10^6$	$2.74x10^6$	A
7	1-Butanol	71-36-3	688.9	$1.10x10^5$	$5.37x10^6$	$2.40x10^6$	$5.90x10^6$	$5.69x10^6$	$9.15x10^6$	$9.70x10^3$	ND	A
8	1-Pentanol	71-41-0	810.6	$9.06x10^5$	$1.53x10^7$	$2.16x10^6$	$6.53x10^6$	$2.98x10^6$	$8.94x10^6$	$1.07x10^5$	$1.95x10^4$	AO
9	1-Hexanol	111-27-3	917.2	$7.57x10^6$	$7.47x10^6$	$2.44x10^6$	$1.95x10^7$	$1.52x10^6$	$1.93x10^7$	$1.88x10^4$	$3.75x10^3$	A
10	2-Ethylhexanol	104-76-7	1078.1	$1.61x10^6$	$4.84x10^5$	$1.40x10^6$	$7.40x10^5$	$2.19x10^5$	$2.62x10^5$	$9.46x10^4$	$7.65x10^4$	AO
11	1-Octanol	111-87-5	1120.1	$2.44x10^5$	$2.85x10^6$	$1.14x10^5$	$2.72x10^5$	$1.92x10^5$	$9.23x10^5$	$7.39x10^4$	$5.37x10^4$	AO
12	Phenylethyl Alcohol	60-12-8	1193	$1.31x10^6$	$2.76x10^7$	$2.92x10^6$	$6.44x10^6$	$1.80x10^6$	$6.88x10^6$	$1.09x10^4$	$1.42x10^4$	AO
13	1-Dodecanol	112-53-8	1523.3	$2.69x10^5$	$1.02x10^5$	$8.39x10^5$	$1.44x10^6$	$1.99x10^5$	$4.43x10^5$	$1.35x10^5$	$1.05x10^5$	A
14	Tetradecanol	112-72-1	1724.2	$2.36x10^4$	ND	$3.60x10^4$	$6.73x10^4$	ND	ND	$1.49x10^5$	$1.78x10^5$	AO
Aldehydes												
15	Acetaldehyde	75-07-0	449.5	$1.06x10^6$	$1.63x10^6$	$2.97x10^6$	$2.94x10^6$	$3.30x10^6$	$2.75x10^6$	$5.50x10^6$	$4.97x10^6$	A
16	3-Methylbutanal	590-86-3	650.4	$1.15x10^6$	$8.85x10^5$	$2.16x10^5$	$2.27x10^5$	$5.16x10^5$	$3.43x10^5$	$2.43x10^4$	$2.24x10^4$	A
17	Benzaldehyde	100-52-7	1019.3	$2.02x10^6$	$3.67x10^6$	$3.84x10^6$	$3.11x10^6$	$2.10x10^6$	$1.66x10^6$	$2.19x10^5$	$2.03x10^5$	AO
18	Heptanal	111-71-7	938.7	$8.52x10^5$	$7.74x10^5$	$6.81x10^5$	$7.17x10^5$	$3.01x10^5$	$4.02x10^5$	$2.13x10^5$	$1.60x10^5$	AO
19	Decanal	112-31-2	1252.6	$1.12x10^6$	$9.88x10^5$	$1.41x10^6$	$1.12x10^6$	$6.27x10^5$	$5.75x10^5$	$4.16x10^5$	$3.02x10^5$	AO
20	Octanal	124-13-0	1043.6	$5.92x10^5$	$7.53x10^5$	$9.14x10^5$	$4.52x10^5$	$3.06x10^5$	$4.69x10^5$	$3.07x10^5$	$2.27x10^5$	A
Furan												
21	2-Pentylfuran	3777-69-3	1008.9	$4.94x10^5$	$1.25x10^6$	$1.35x10^6$	$1.25x10^6$	$4.47x10^5$	$5.72x10^5$	$7.76x10^3$	$1.31x10^4$	AO
Hydrocarbons and Benzenes												
22	Toluene	108-88-3	773.5	$5.71x10^5$	$5.06x10^5$	$3.20x10^7$	$1.97x10^6$	$7.03x10^6$	$1.04x10^6$	$1.14x10^5$	$1.53x10^4$	AO
23	p-Xylene	106-42-3	888.2	$7.52x10^5$	$1.13x10^5$	$6.36x10^5$	$1.61x10^5$	$2.36x10^5$	$3.56x10^5$	$2.25x10^4$	$1.16x10^4$	A
24	Phenol	108-95-2	1104.5	$5.95x10^5$	$1.85x10^6$	$3.23x10^6$	$5.08x10^6$	$4.86x10^6$	$6.17x10^6$	$1.00x10^5$	$8.47x10^4$	A
25	p-Cresol	106-44-5	1195	$9.80x10^5$	$1.51x10^6$	$2.25x10^8$	$1.93x10^8$	$2.06x10^8$	$1.93x10^8$	$5.82x10^5$	$2.90x10^5$	AO
26	2-Methoxy-4-vinylphenol	7786-61-0	1411.9	$1.01x10^5$	$1.16x10^7$	$3.99x10^5$	$3.14x10^5$	$1.27x10^6$	$5.93x10^5$	$3.63x10^5$	$4.19x10^4$	AO
Ketones												
27	Acetone	67-64-1	491.9	$9.47x10^5$	$1.81x10^6$	$4.67x10^6$	$2.72x10^6$	$2.79x10^6$	$1.87x10^6$	$2.40x10^5$	$3.94x10^5$	A
28	2,3-Butanedione	431-03-8	574.6	$3.03x10^5$	$1.08x10^6$	$1.46x10^5$	$4.79x10^5$	$4.95x10^5$	$6.61x10^5$	$1.12x10^5$	$1.65x10^5$	AO
29	2-Pentanone	116-09-6	704.2	$5.74x10^4$	$4.55x10^5$	$1.24x10^5$	$1.63x10^5$	$6.37x10^5$	$2.06x10^5$	$4.38x10^4$	$3.61x10^4$	A

30	2-Heptanone	110-43-0	931.4	$3.27x10^5$	$5.94x10^5$	$9.14x10^5$	$1.64x10^6$	$1.53x10^5$	$3.66x10^5$	$1.37x10^5$	$1.97x10^4$	AO
Lactones												
31	γ -Nonalactone	104-61-0	1485	$2.68x10^5$	$2.16x10^7$	$1.50x10^7$	$1.20x10^7$	$5.81x10^6$	$6.84x10^6$	$3.34x10^4$	$1.84x10^5$	AO
Pyrazines and Pyridines												
32	Pyridine	110-86-1	775.8	$1.04x10^4$	$5.52x10^4$	$6.43x10^4$	$1.28x10^5$	$1.29x10^4$	$6.90x10^4$	$6.22x10^3$	$6.74x10^3$	A
Other												
33	2-Methyl-1H-pyrrole	636-41-9	918.1	$9.44x10^3$	$3.71x10^4$	$3.14x10^4$	$5.40x10^4$	$1.70x10^5$	$6.99x10^4$	$4.94x10^3$	$3.94x10^3$	A

6.3.1 Volatiles identified in Raw GRS and TMR Milk

Ninety nine VOC were identified in raw milk by DI-HiSorb-GC-MS which is significantly more than previous studies (Moio et al., 1993b, Moio et al., 1994, Friedrich and Acree, 1998, Bendall, 2001, Mounchili et al., 2005, Ai et al., 2015) and highlights the capability of the DI-HiSorb extraction technique (Table 6.1). Thirteen VOC varied significantly ($p = 0.05$) based on diet. Four of these VOC (1-pentanol, (E,E)-2,4-heptadienal, toluene and benzothiazole) were significantly higher in raw GRS milk, and ten (2-methyl butanoic acid, hexanoic acid, octanoic acid, decanoic acid, 2-methylpropanal, 3-/ 4-ethylphenol (tentative identification), γ-nonalactone, pyrazine, dimethyl sulfone and maltol) were significantly higher in raw TMR milk. Only 1-methylpropyl ester butanoic acid, 1-butanol, dodecanal, and butyl butanoate were identified in raw GRS milk and not in raw TMR milk. Only 3-methylbutanoic acid, 2-methylpropanal, and 2,5-dimethylpyrazine were identified in raw TMR milk and not in raw GRS milk. These results generally concur with previous studies which highlight no major differences in individual VOC due to diet in bovine milk, but show significant differences in abundances due to diet (Bendall, 2001, Mounchili et al., 2005, Croissant et al., 2007, Villeneuve et al., 2013).

6.3.1.2 Most Abundant Volatiles in Raw GRS Milk

The VOC that were statistically more abundant in the raw GRS milk diet consisted mainly of products of lipid oxidation (1-pentanol and (E,E)-2,4-heptadienal), metabolism of β-carotene (toluene) and/or derived from Maillard reactions (benzothiazole). Comparable to other studies, 1-pentanol, and toluene were significantly higher in milk produced from cows fed pasture (Villeneuve et al., 2013, Faulkner et al., 2018, Clarke et al., 2020a). 1-Pentanol is a major product of lipid oxidation, and likely relates to differences in the abundance of specific unsaturated fatty acids due to the different bovine diets (Kilcawley et al., 2018). However it is also likely that it was directly transferred from the diet, as it was present in all feed and rumen samples (Table S6.1). Toluene was present in all feed and rumen samples with greatest abundances in GRS RF (Table S6.1). Toluene is not particularly odour active, but has been suggested as a potential biomarker for pasture fed bovine milk and associated dairy products, as it is a product of β-carotene metabolism in the rumen (Faulkner et al., 2018, Kilcawley et al., 2018). (E,E)-2,4-Heptadienal is another product of lipid oxidation, and was significantly higher in raw GRS milk and also likely related to higher levels of

linolenic and linoleic acid due to the GRS diet, and is known to be quite odour active (Clarke et al., 2020b). However, few studies have previously identified this VOC in milk. Benzothiazole was significantly higher in raw GRS milk and was present in only GRS feed and not in any rumen samples (Table S6.1). Benzothiazole is thought to originate from phenylalanine through Maillard reactions (Li and Wang, 2016) and has been described as having a burning rubber smell in milk, the odour of which increases with increasing heat treatment (Morgan et al., 1966, Moio et al., 1994, Coppa et al., 2011). Benzothiazole has also previously been found to have greater aroma impact in milk derived from pasture than TMR, and has a medium odour threshold (Bendall, 2001, O'Callaghan et al., 2016).

6.3.1.3 Most Abundant Volatiles in Raw TMR Milk

The range of VOC present at statistically (p = 0.05) higher abundances in the raw TMR milk encompassed numerous chemical classes and reflects a more complex VOC profile than in raw GRS milk. Both 2-methylbutanoic acid (a branched-chain fatty acid) and 2-methylpropanal (a branched-chain aldehyde) are products of Strecker degradation from isoleucine and leucine, respectively. 2-Methylpropanal has a characteristic musty aroma and a very low odour threshold (Bendall, 2001), and 2-methylbutanoic acid has a fruity, sweaty, rancid, burnt, sour aroma (Mounchili et al., 2005). A previous study identified 2-methylbutanoic acid in TMR feed, but not in pasture feed (grass [GRS] or grass/clover [CLV]), however it was not detected in milk derived from these feeding systems (Kilcawley et al., 2018). 2-Methylpropanal was absent in raw GRS milk, but present at a relatively high abundance in raw TMR milk (Table S6.1). These results generally contradict other studies which have found that herbage based diets tend to result in more products of amino acid metabolism in the resultant milk, as they typically have a high protein to digestible carbohydrate ratio than a concentrate diet, such as TMR (Croissant et al., 2007, Ueda, 2018). However, it is appears that 2-methylpropanal is also directly transferred from the diet as it was present in each feed and rumen sample (Table S6.1). Dimethyl sulfone is derived from the oxidation of dimethyl sulphide, which may be formed via the metabolism of methionine and/or cysteine (Garvey et al., 2020), or from heat-induced oxidation of methionine (Vazquez-Landaverde et al., 2006). It may also be transferred directly from plant based diets (Faccia, 2020), but this was not evident in this study (Table S6.1). Similar to the branched acids and aldehydes, numerous studies have found higher abundances of dimethyl sulfone in bovine milk derived from a pasture feeding system than from a concentrate feeding system

(Croissant et al., 2007, Kilcawley et al., 2018, Manousi and Zachariadis, 2019, Faccia, 2020), suggesting it is likely related to the increased availability of more digestible protein (methionine) in the pasture based feeding system, which again contradicts the findings of this study. Faulkner et al. (2018) found lower abundances of dimethyl sulfone in milk after pasteurisation, yet Moio et al (Moio et al., 1994) only found a reduction in the abundance of dimethyl sulfone after UHT treatment. Therefore heat treatment of milk or volatile extraction conditions appear to also influence its abundance. Vazquez-Landaverde et al. (Vazquez-Landaverde et al., 2006) suggested that as the odour threshold of dimethyl sulfone is quite high it is therefore unlikely to be a key aroma active compound. Its odour had been described as sulphurous, hot milk, burnt, leather, and sweat-like (Moio et al., 1994, Vazquez-Landaverde et al., 2006).

The short chain fatty acids, hexanoic, octanoic, and decanoic were statistically (p = 0.05) higher in TMR milk and have the following aromas; hexanoic acid (unpleasant, chemical, caramel-like), octanoic acid (intense, burnt milk or pudding), and decanoic acid (burnt, persistent, phenolic) (Mounchili et al., 2005). Short chain fatty acids are primarily produced by *de novo* synthesis in the mammary gland which is impacted by diet, but can also be directly transferred from the diet in free form (O'Callaghan et al., 2016). The evidence in this study also highlights the potential for direct transfer, as each of these acids were found in each feed and rumen sample (Table S6.1). Each of these acids were also in greatest abundance in TMR feed and TMR RF. These acids are also produced by lipolytic activity from lipoprotein lipase or by esterases from psychrotrophic bacteria (Beuvier and Buchin, 2004), however as the milks in this study were treated in the same manner, it is more likely that the differences in short chain fatty acids were either directly or indirectly a result of diet. However, these results are in conflict with previous studies which found no dietary impact on the abundance of free short chain fatty acids or, that more free short chain fatty acids were associated with pasture feeding (Coppa et al., 2011, Villeneuve et al., 2013, Faulkner et al., 2018).

Pyrazines can be formed via the Strecker reaction driven by heat treatment (Manousi and Zachariadis, 2019), via Maillard browning or via microbial metabolism (Carpino et al., 2004). Clarke et al (Clarke et al., 2020a) did not find any statistical difference in pyrazine between different bovine diets (GRS, CLV, and TMR, but did find statistical differences in the abundance of other pyrazines related to those diets (2,3,5-trimethyl-6-ethylpyrazine, 2,3-

dimethyl-pyrazine, 3-ethyl-2,5-dimethyl-pyrazine, and trimethyl-pyrazine). No pyrazines were identified in the resultant raw milks in that same study. Pyrazine was not identified in any feed or rumen sample in this study (Table S6.1). Maltol is a known Maillard end product from lactose and/or lysine residues (Hodge and Moser, 1961, Van Boekel, 1998). Maltol has previously been identified in heat-treated milks and has been described as a potent sweet aromatic compound (Jo et al., 2018). Maltol is derived from the metabolism of dietary sugars such as maltose, and was not identified in any feed or rumen sample in this study (Table S6.1), and as the milk did not undergo thermal treatment it is difficult to discern the source of maltol, but it may have been created by microbial activity in the raw milk. 3-/ 4-Ethylphenol (tentative identification) are phenolic compounds that has been previously been identified in bovine milk (Kilic and Lindsay, 2005) and are likely a result of isoflavone (formononetin, biochanin A, and genistein) or amino acid metabolism in the rumen (Batterham et al., 1965, Braden et al., 1967) but also potentially in the milk. A previous study by Faulkner et al. (2018) found higher levels 4-ethylphenol in TMR feed than in pasture, but it was not detected in the milk from these feeding systems which is in agreement with the results of this study whereby 3/4-ethylphenol were detected in both GRS and TMR feed (higher in TMR), but not detected in the rumen or milk samples (Table S6.1). 4-Ethylphenol has a very characteristic horse stable-like, faecal, and medicinal aroma, while 3-ethylphenol has a leather-like and ink-like aroma (Czerny et al., 2011).

Only one lactone γ-nonalactone was significantly different in these milks and at higher abundances in TMR milk. γ-Nonalactone has been described as having a coconut, peach-like aroma and is very odour active (Kilcawley, 2019). Lactones such as γ-nonalactone are fat derived aroma compounds and are generally formed through thermal degradation of hydroxyacids (Calvo and de la Hoz, 1992), or via β-oxidation of hydroxy acids followed by cyclisation Villeneuve et al., (2013), or by one-step non-enzymatic reactions (Alewijn et al., 2007). Ueda (2018) proposed that diets which included grains, meals, and oats, resulted in greater abundances of some lactones (γ-dodecalactone and δ-dodecalactone) in the resultant milks, as these diets induced propionate (a hydroxycarboxylic acid) metabolism in the rumen. Villeneuve et al., (2013) found higher levels of some γ-lactones in milk from hay fed cows, than in cows fed silage or pasture. They also suggested that unsaturated fatty acids may be transformed by hydration to intermediate hydroxyl acids in the rumen and subsequently create lactones through oxidation and cyclization that end up

in animal tissue and milk. γ-Nonalactone was present in all feed and rumen samples in this study (Table S6.1), suggesting its potential direct transfer from diet, and abundances were much higher in TMR feed than GRS feed (but abundances were similar in the rumen samples). Bendall (2001) also found an odour active lactone (γ-dodec-cis-6,cis-9-dienolactone) was present in milk derived from a TMR diet but absent in cows on a pasture diet. However, it appears that the extraction method can also have a significant impact on the recovery of many lactones (High et al., 2019, Cheng et al., 2021), and that HS-SPME is not very suitable for the extraction of lactones (dependent upon fibre type), an extraction technique widely used for VOC analysis in bovine milk (Croissant et al., 2007, Coppa et al., 2011, Villeneuve et al., 2013, Jo et al., 2018).

6.3.2 Key Aroma Active Volatiles Identified by Odour Intensity Analysis

In total, 44 distinct odour activities were perceived by the panellists in raw bovine milks (Table 6.3), most were from individual VOC, but eight consisted of two or three co-eluted VOC and 6 remain unidentified (UNC), which is not uncommon when a VOC is present at a concentration above its odour threshold, but below limit of MS detection. The total OI values for GRS and TMR milk were 61.2 and 66.2, respectively. The higher OI of TMR milk likely reflects the greater number of odour active VOC, and likely influenced by the more diverse composition of the TMR diet. Only 27 of the 44 odour activities were present in both sample types, highlighting the diversity of aromas between both samples derived from the GRS and TMR diets. In summary, 34 VOC were perceived in raw GRS milk compared to 36 VOC in raw TMR milk. For the purpose of this study only those odour activities with an average OI of ≥2.0 are discussed in detail (Table 6.3), which corresponds to 20 distinct odour activities between both raw GRS and raw TMR milk.

However, it is worth noting that seven odour activities with OI<2.0 were detected in raw GRS milk, but not in raw TMR milk; ethyl octanoate (OI 1.8), nonanal (OI 1.4), hexanal (OI 1.4), hexanoic acid (OI 1.2), 2-ethylhexanol (OI 1.0), methyl isobutyl ketone (OI 0.9), and hydrocinnamic acid (OI 0.7).

Likewise a further eight odour activities with OI<2.0 were detected in raw TMR milk but not in raw GRS milk: 3/4-ethylphenol (tentative identification)/benzoic acid (OI 1.6), benzothiazole (OI 1.6), 2-phenyoxyethanol (OI 1.6), 2-methylpropanal (OI 1.2),

benzeneacetaldehyde (OI 1.2), 1-octanol (OI 1.2), octanoic acid (OI 1.0), and γ-nonalactone (OI 0.4).

Table.6.3: Aroma-active compounds perceived in raw bovine milk from cows fed grass (GRS) or total mixed ration (TMR) by gas-chromatography Olfactrometry (GC-O).

Retention Index							AEDA FD values		Odour intensity (OI)			
Order of identification	**(RI [a]) DB-624 UI (volatile TD analysis)**	**(RI [b]) DB-624 UI (GC-O analysis)**	**(LRI [c]) LRI (literature)**	**Identification method**	**Compound**	**Aromas perceived by panellists**	**Raw GRS milk**	**Raw TMR milk**	**Raw GRS milk**	**Raw TMR milk**	**Odour threshold (ppb)**	**Odour threshold reference**
1	460	-	458	A, C	Methanethiol	Fishy, cabbage	50	10	1.4	1.6	1	Devos et al. (1990)
2	593	582	629	A, C	2-Methylpropanal	Sweet, fresh	0	2	-	1.2	0.1–2.3	Leffingwell and Associates (2020)
3	631	574	630	A, C	2,3-Butanedione	Fresh, sweet, caramel, butterscotch, biscuity, baked	2	20	2.4	2.2	2.3–6.5	Leffingwell and Associates (2020)
4	686	685	699	A, C	Acetic acid	Vinegar	5	5	2.2	2.1	480–1000	New Jersey Department of Health (2016)
5	779	755.4	785	A, C	Dimethyl disulfide	Musty, cardboard, sulphur, fishy	10	10	1.4	1.3	0.16-12	Leffingwell and Associates (2020)
6	781	764	784	A, C	Methyl Isobutyl Ketone	Sweet	1	0	0.9	-	-	-
7	794 815	774 810	796 812	A, C A, C	Toluene 1-Pentanol	Musty, damp, earthy, plastic	20	10	1.9	1.4	4680 4000	Leonardos et al. (1974) Leffingwell and Associates (2020)
8	-	830	837	A, C	Hexanal	Roasted, fresh, floral, herbal, vegetable	10	0	1.4	-	4.5-5.0	Leffingwell and Associates (2020)

9	866	841	868	B, C	2-Methylpropanoic acid	Fruity, citrus, fatty, roast chicken, cheesy	5	5	1.5	1.5	8100	Leffingwell and Associates (2020)
10	862	878	859	A, C	Butanoic acid	Cheesy, dairy, buttery	20	5	2.3	2.5	240	Leffingwell and Associates (2020)
11	899	892	901	A, C	Furfural	Cheesy, sour, sour milk, dairy, nutty, bready, baked, roasted	50	50	2.2	2.4	16000	Franco et al. (2004)
12	927 936	929 931	926.6 931.5	B, C A, C	2-Furanmethanol 2-Heptanone	Barnyard, animal, musty, bready, cheesy	10	5	1.5	2.2	8000 14-3000	U.S. National Library of Medicine and National Center for Biotechnology Information (2016)
13	914 922 945	932 937 938	917 935 939	A, C A, C A, C	3-Methylbutanoic acid 2-Methylbutanoic acid Heptanal	Buttery, animal, barnyard, nutty, bready	10	5	1.8	2.8	120-170 3-360	Leffingwell and Associates (2020) Burdock (2001)
14	- -	950 959	952 963	A, C A, C	2,5-Dimethylpyrazine 2,3-Dimethylpyrazine	Smokey, barnyard, animal, roasted, toasted, cooked potato	20	50	2.0	2.6	800-1800 2500-35000	Leffingwell and Associates (2020)

15	-	1009	1014	A, C	2-Pentylfuran	Roasted, toasted, bready, potato, popcorn	20	50	2.7	2.5	6	Leffingwell and Associates (2020)
16	-	-	-	C	Unidentified 1	Cooked potato, roasty, musty	1	1	2.3	1.9	-	-
17	-	1010	-	C	Unidentified 2	Fishy, salty, stale, sulphur, chemical, woody, cabbage	10	10	1.6	1.7	-	-
18	1034 1031 1028	1020 1025 1027	1032 1027 1024	A, C A, C A, C	Benzaldehyde Butyrolactone Ethyl hexanoate	Sweet, caramel, herbal, fruity, cherry	50	50	1.9	2.5	350-3500 1000 30	Leffingwell and Associates (2020) Poisson and Schieberle (2008) Guo et al. (2019)
19	-	1028	1023.7	B, C	1-Octen-3-ol (tentative)	Green, fresh, earthy, mushroom	50	50	1.7	2.0	1	Leffingwell and Associates (2020)
20	-	1044	-	C	Unidentified 3	Green, floral, fresh, grassy, earthy	20	10	1.9	2.1	-	-
21	1040	-	1037.5	A, C	Isomaltol	Sweet, cotton candy, fruity, aniseed, medicinal	20	10	1.3	1.6	0.002	Cliff et al. (2011)

22	1051	1096	1052	A, C	Hexanoic acid	Cheesy, smokey, bready, roasted	1	0	1.2	-	3000	Leffingwell and Associates (2020)
23	1077	1079	1079	A, C	2-Ethylhexanol	Sweet, solvent	1	0	1.0	-	270000	Leffingwell and Associates (2020)
24	1120	1108	1121	B, C	Benzeneacetaldehyde	Pungent, cleaning agent, musty	0	1	-	1.2	4	Liu et al. (2018a)
25	1117	1120	1124	A, C	1-Octanol	Mushroom, stale, damp	0	1	-	1.2	110-130	Leffingwell and Associates (2020)
26	-	-	-	C	Unidentified 4	Animal, pungent, smokey, burnt, eggy	50	50	2.5	1.2	-	-
27	1151	1148	1151	A, C	Nonanal	Solvent, fresh, artificial, chemical	10	0	1.4	-	1	Leffingwell and Associates (2020)
28	-	1163	1166	A, C	γ-Hexalactone	BBQ, caramel, tobacco, toasted, toffee	20	2	2.3	1.8	1600	Leffingwell and Associates (2020)
29	1182	1170	1171	B, C	Methyl 2-furoate	Toffee, fruity, sweet, caramel	0	20	2.3	2.2	NF	-
30	-	1078	-	C	Unidentified 5	Fresh, herbal, sweet	50	50	2.2	2.4	-	-
31	-	1142	-	C	Unidentified 6	Sweet, floral	50	0	2.5	-	-	-
32	1193	1193.6	1193	A, C	Phenylethyl alcohol	Sweet, herbal, fruity, spicy	50	20	2.3	2.7	65	Leffingwell and Associates (2020)
33	1193	1193	1204	A, C	Maltol	Caramel, sweet, cotton candy	100	10	1.6	2.3	2600	Poisson and Schieberle (2008)

34	1193 1196	1195 -	1183.5 1192	A, C A, C	p-Cresol 2-Pyrrolidone	Barnyard, pungent, animal, solvent	20	50	1.6	2.6	1 NF	Leonardos et al. (1974) -
35	1222	1226	1224	A, C	Ethyl octanoate	Solvent, aldehydic, alcohol	20	0	1.8	-	147	Poisson and Schieberle (2008)
36	1254	1252	1256	A, C	Decanal	Solvent, mushroom, animal, floral, grassy	0	10	-	2.3	0.1	Leffingwell and Associates (2020)
37	1242	1270	1245	A, C	Octanoic acid	Smokey, toasted, animal, burnt milk	0	10	-	1.0	10000	Peinado et al. (2004)
38	 1284	1286 1286 1285	1279 1279 1284	B, C B, C A, C	3-Ethylphenol (tentative) 4-Ethylphenol (tentative) Benzoic acid	Smokey, animal, burnt milk	0	50	-	1.6	1.7-800 21-600	Czerny et al. (2008) Dietz and Traud (1978)
39	1322	1298	1315	A, C	Benzothiazole	Smokey, roasted, caramel	0	10	-	1.6	80	Leffingwell and Associates (2020)
40	1320	1312	-	B, C	2-Phenoxyethanol	Sweet, burnt	0	1	-	1.6	NF	-
41	1345 1411	1343	1352 1408	A, C A, C	2-Undecanone 2-Methoxy-4-vinylphenol	Aniseed, sweet, herbal	2	5	0.8	1.3	7 3	Leffingwell and Associates (2020)
42	1451	1460	1452	A, C	Hydrocinnamic acid	Sweet, floral, creamy	10	0	0.7	-	NF	-
43	-	1485	1489	A, C	γ-Nonalactone	Sweet, caramel, burnt, lactone	0	1	-	0.4	65	Siek et al. (1971)
44	1547 1635 1724	1546 1644 1724	1547 1641 1725	B, C A, C A, C	2-Tridecanone Dodecanoic acid Tetradecanol	Smokey, herbal	2	5	0.7	0.7	NF 10000 NF	- Leffingwell and Associates (2020)

				-
	Total odour intensity	61.2	66.2	

[a] Retention index (RI) calculated from GC-O results on a DB-624 UI column.

[b] Retention index (RI) calculated from Thermal Desorption (TD) results on a DB-624 UI column

[c] Retention index found in the literature (LRI) for a DB-624 UI column.

Identification method (IM): A: identification based on comparison to the NIST mass spectral database, RI values from the literature and an in-house library created using authentic compounds with target and qualifier ions and linear RI for each compound; B: tentative identification using RI values and LRI matching; C: identification with GC-O; NF: Not Found; FD: Factor Dilution.

6.3.2.2 Aroma Active Volatiles in Raw GRS Milk by Odour Intensity Analysis

Thirteen distinct odour activities dominated the aroma of raw GRS milk (OI ≥2.0), four of which were unidentified (UNC 1, UNC 4, UNC 5, and UNC 6). Based on their OI, the order of their potential significance was as follows; 2-pentylfuran (roasted, toasted, bready, potato, popcorn; OI 2.7), UNC 4 (animal, pungent, smokey, burnt, eggy; OI 2.5), UNC 6 (sweet, floral; OI 2.4), 2,3-butanedione (fresh, sweet, butterscotch, biscuity, baked; OI 2.4), butanoic acid (cheesy, dairy, buttery; OI 2.3), UNC 1 (cooked potato, roasty, musty; OI 2.3), γ-hexalactone (barbeque, caramel, tobacco, toasted, toffee; OI 2.3), methyl 2-furoate (toffee, fruity, sweet, caramel; OI 2.3), phenylethyl alcohol (sweet, herbal, fruity, spicy; OI 2.3), acetic acid (vinegar; OI 2.2), furfural (cheesy, sour, sour milk, dairy, nutty, bready, baked, roasted; OI 2.2), UNC 5 (fresh, herbal, sweet; OI 2.2), and 2,5-dimethylpyrazine / 2,3-dimethylpyrazine (smokey, barnyard, animal, roasted, toasted, cooked potato; OI 2.0). Figure 6.1 and Figure 6.2 illustrate the odour descriptors perceived by the 5 panellists for raw GRS and TMR milk.

2-Pentylfuran is likely a Maillard reaction product, that has been associated with caramel odours (Li and Wang, 2016). As it was present in all samples in this study, it is likely derived directly from the diet (Table S6.1). Clarke et al. (2020a) also found 2-pentylfuran in feed but not in the resultant milk. These authors also found it at higher abundances in GRS and GRS/CLV than in TMR feed. However, in another study, 2-pentylfuran was found at greatest abundance in TMR feed, although still present in GRS, GRS/CLV feed, it was only present in raw milk derived from a TMR diet and was not present post pasteurisation (Kilcawley et al., 2018). 2-Pentylfuran was present in both raw milks in this study. 2,3-Butanedione (diacetyl) is quite odour active and described as having a buttery, pastry aroma (Friedrich and Acree, 1998), not that dissimilar to this study (fresh, sweet, butterscotch, biscuity, baked). 2,3-Butanedione is a common VOC in dairy products produced predominately by pyruvate metabolism. Faulkner et al. (2018) found 2,3-butanedione in TMR feed but not in GRS or GRS/CLV feed, and was absent in pasteurised bovine milk derived from these diets. Previous studies have found 2,3-butanedione at greater abundance in raw milk derived from hay than from maize or grass silage (Manousi and Zachariadis, 2019), and in raw milk from TMR and pasture (Moio et al., 1994). In this study 2,3-butanedione was present in all feed and rumen samples (Table S6.1) and therefore is also likely directly transferred from the diet. As mentioned short chain fatty acids such as

butanoic acid are primarily produced by *de novo* synthesis in the mammary gland which is impacted by diet, but can also be directly transferred from the diet in free form (O'Callaghan et al., 2016), or from lipolysis (Beuvier and Buchin, 2004). Butanoic acid was described as cheesy, dairy, buttery and was present in all feed and rumen samples in this study (Table S6.1). It was at very high but similar abundances in both GRS and TMR rumen samples. Other studies have also identified butanoic acid in bovine milk (Bendall, 2001, Mondello et al., 2005, Croissant et al., 2007, Villeneuve et al., 2013, O'Callaghan et al., 2016), but only Bendall (2001) (vomit: feta cheese) and Ai et al. (2015) (green) found it to be odour active. Clarke et al. 2020b and Faulkner et al. 2018 found butanoic acid in GRS, GRS/CLV, and TMR feed, with significantly more in TMR feed. Clarke et al. (2020a) did not find butanoic acid in fresh raw milk produced from these diets, but Faulkner et al. (2018) did, although abundances were not statistically different in raw or pasteurised milk from these diets. The choice of extraction method and GC column used are likely to have a significant effect on the recovery of butanoic acid, as recovery of acids are greatly influenced by the polarity of the sorbent and GC column (Cheng et al., 2021). γ-Hexalactone is a lactone, with a coconut, fruity, sweet aroma and a medium odour threshold (Kilcawley, 2019), but was described in this study as having a barbeque, caramel, tobacco, toasted, toffee aroma (as the odour was so diverse, it is possible that co-elution may have occurred with another odour active VOC that was below the limits of MS detection). As previously mentioned lactones are potentially produced from a number of different routes. Clarke et al. (2020a) found γ-hexalactone in GRS, GRS/CLV and TMR feed (statistically higher abundances in GRS/CLV), but not in raw milk derived from these diets. In this study γ-hexalactone was found in both GRS and TMR feed, but not in any rumen samples.

Methyl 2-furoate is furan product of the Maillard reaction (Stewart et al., 2018) and has also been found in bovine milk derived from crop silage/hay (Riuzzi et al., 2021). It has a sweet, caramel brown sugar musty aroma (Yelliantty et al., 2021) and was described similarly (toffee, fruity, sweet, caramel) in this study. It was not present in any feed or rumen samples in this study (Table S6.1) and therefore unlikely to derive directly from the diet. Phenylethyl alcohol (2-phenylethanol) has been previously found in GRS, GRS/CLV and TMR feed samples, but not in any raw or pasteurised milks derived from these feeds (Faulkner et al., 2018, Clarke et al., 2020a). It was described as having a slightly rose-like aroma and has previously been found in raw milk (Moio et al., 1993b). Its aroma description

was quite different in this study (sweet, herbal, fruity, spicy), and may also indicate co-elution with an odour active VOC that is below the limits of MS detection. It is likely a product of Strecker degradation of phenylalanine (Castellani et al., 2018), and was present in all of the feed and rumen samples in this study (Table S6.1), highlighting potential direct transfer from the diet. Acetic acid has previously been found in milk (Bendall, 2001, Croissant et al., 2007, Villeneuve et al., 2013, Ai et al., 2015, Faulkner et al., 2018). Like other short chain acids, acetic acid is thought to directly transfer from the diet into milk (Kilcawley et al., 2018) and this seems to confirm results in this study where acetic acid was found in all feed and rumen samples, with quite high abundance in the rumen samples (Table S6.1). Faulkner et al., 2018 found highest abundance of acetic acid in TMR feed, but subsequently highest levels in GRS raw milk, but not statically different in pasteurised milk from GRS, GRS/CLV or TMR. Acetic acid has a vinegar aroma, and matches that found in this study, but is actually not that odour active (Bendall, 2001). Again it is worth mentioning like all acids recovery of acetic acid is particularly impacted by the extraction technique. Furfural is commercially produced from lignocellulose biomass (Dutta et al., 2012), so therefore is likely to either be directly present in forage or from metabolised lignin. In this study furfural was present in both GRS and TMR feed (at higher levels in TMR feed) and in both GRS and TMR RF (Table S6.1). Furfural has a distinct barny/brothy aroma (Jo et al., 2018) and was described as cheesy, sour, sour milk, dairy, nutty, bready, baked, roasted in this study (as mentioned earlier the diverse odour descriptors likely indicates co-elution with another odour active VOC that is below the limits of MS detection. Clarke et al., (2020a) found furfural at a higher abundance in GRS than in GRS/CLV or TMR feed, but not in raw milk derived from these diets. 2,5-Dimethylpyrazine / 2,3-Dimethylpyrazine were more odour active in raw GRS milk. Both pyrazines were present in TMR feed only, and 2,3-dimethylpyrazine was not present in any rumen sample but was present in both raw milks (GRS and TMR) (Table S6.1). 2,5-Diemethylpyrazine was present in GRS and TMR RF but at higher levels in TMR RF, however it was absent in raw GRS milk. Clarke et al. (2020a) found 2,3-dimethylpyrazine in GRS, GRS/CLV and TMR feed, with statistically higher levels in TMR feed. These authors did not find any pyrazines in raw or pasteurised milk from these feeds. As stated, pyrazines can be formed via the Strecker reaction enhanced by heat treatment (Manousi and Zachariadis, 2019), via Maillard browning or via microbial metabolism (Carpino et al., 2004). Mounchili et al. (2005) found 2,3-dimethylpyrazine with 2,6-dimethylpyrazine in milk and described them as having an intense, roasted breadcrumbs,

bake house, cooked rice, biscuits, cooked milk aroma. In this study 2,5-dimethylpyrazine and 2,3-dimethylpyrazine were described as having a smokey, barnyard, animal, roasted, toasted, cooked potato aroma.

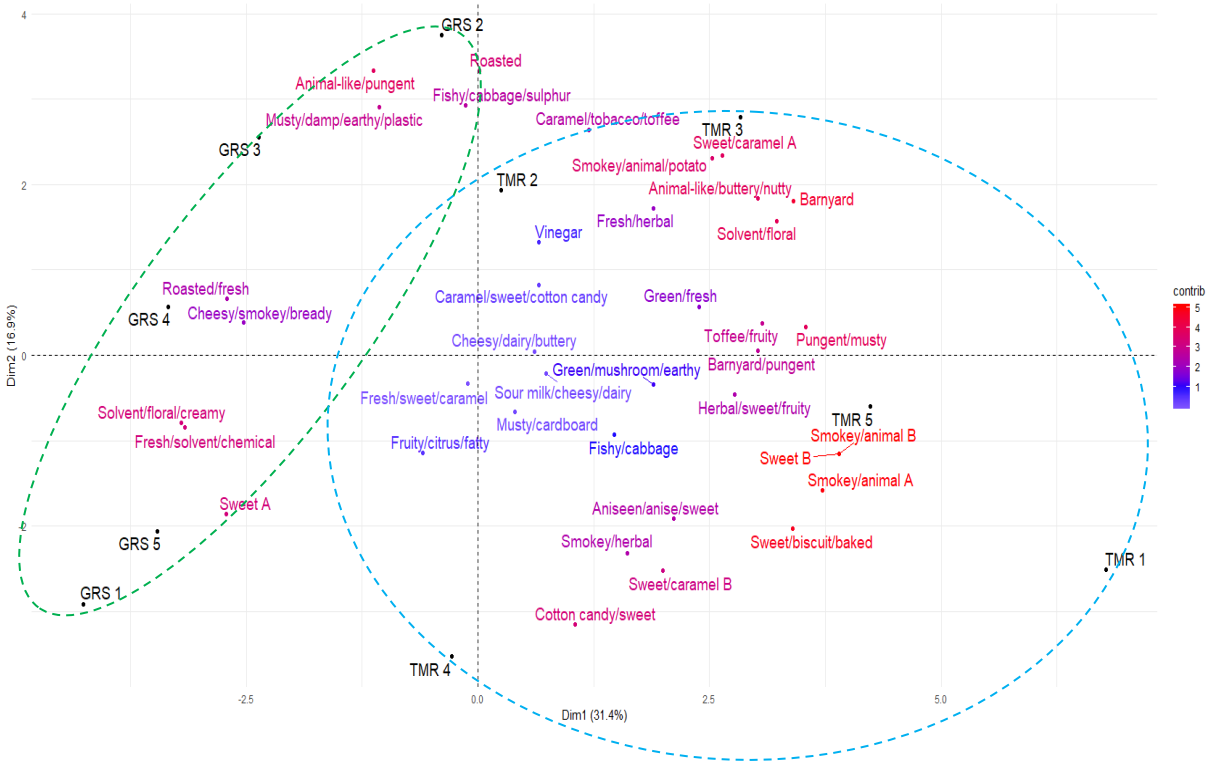


Figure 6.1: Principal component Biplot analysis of the odour descriptors perceived by the 5 panellists based on odour intensity values for raw GRS and TMR milk. Colour gradient: low = white, mid = blue, high = red, midpoint set at 1.0.

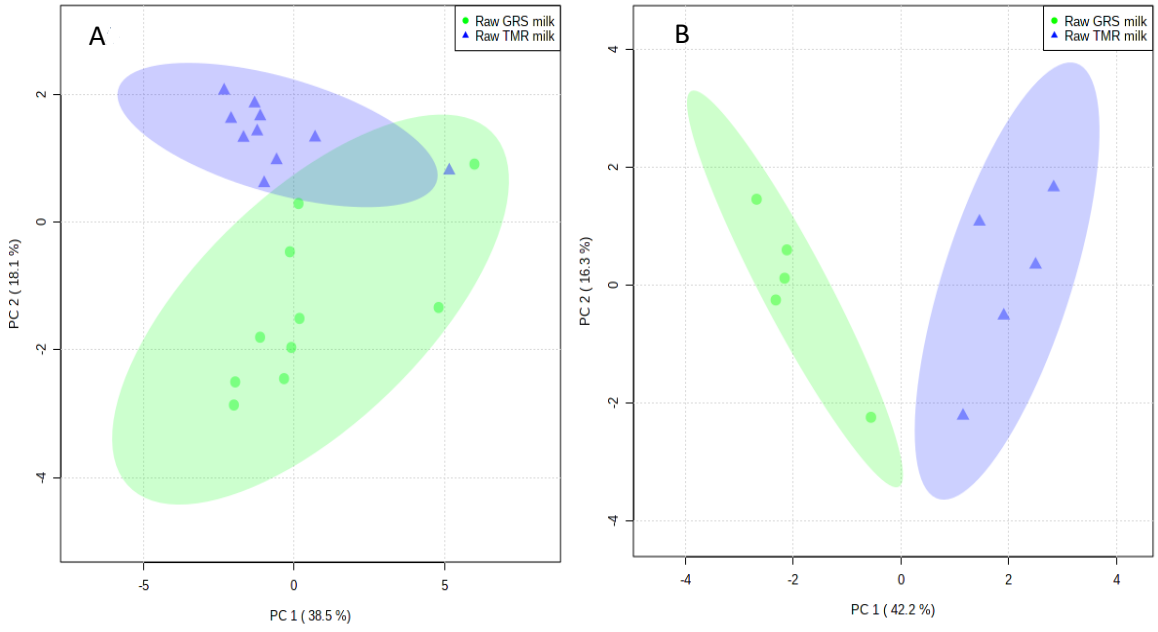


Figure 6.2: Principal component analysis (PCA) of the odour intensities perceived by panellists based on odour intensity values for raw GRS and TMR milk. **A**: replicate data (n=10); **B**: averaged data for each panellist (n=5).

6.3.2.3 Odour Active Volatile in Raw TMR milk by Odour Intensity Analysis

The odour of raw TMR milk was dominated by 17 distinct sets of aromas with OI ≥ 2.0, some of which consisted of co-eluted VOC and two UNC compounds (UNC 3 and UNC 5). The key odours were as follows in order of perceived intensity; 3-methyl butanoic acid / 2-methyl butanoic acid / heptanal_(buttery, animal, barnyard, nutty, bready; OI 2.8), phenylethyl alcohol (sweet, herbal, fruity, spicy; OI 2.7), p-cresol / 2-pyrrolidone (barnyard, pungent, animal, solvent; OI 2.6), 2,5-dimethylpyrazine / 2,3-dimethylpyrazine (smokey, barnyard, animal, roasted, toasted, cooked potato; OI 2.6), 2-pentylfuran (roasted, toasted, bready, potato, popcorn; OI 2.5), benzaldehyde / γ-butyrolactone / ethyl hexanoate (sweet, caramel, herbal, fruity, cherry; OI 2.5), butanoic acid (cheesy, dairy, buttery; OI 2.5), furfural (cheesy, sour, sour milk, dairy, nutty, bready, baked, roasted; OI 2.4), decanal (solvent, mushroom, animal, floral, grassy; OI 2.3), maltol (caramel, sweet, cotton candy; OI 2.3), 2,3-butanedione (fresh, sweet, butterscotch, biscuity, baked; OI 2.2), 2-furanmethanol / 2-heptanone (barnyard, animal, musty, bready, cheesy; OI 2.2), methyl 2-furoate (toffee, fruity, sweet, caramel; OI 2.2), acetic acid (vinegar; OI 2.1), UNC 3 (green, floral, fresh, grassy, earthy; OI 2.1), and 1-octen-3-ol (tentative identification; green, fresh, earthy, mushroom; OI 2.0). Figure 6.1 and Figure 6.2 illustrate the odour descriptors perceived by the 5 panellists for raw GRS and TMR milk.

The most odour active aroma was generated from 3-methyl butanoic acid / 2-methyl butanoic acid / heptanal and described as having a buttery, animal, barnyard, nutty, bready aroma (with co-elution it is difficult to discern which VOC are having the greatest aroma impact). As mentioned previously branched chain acids such 3-methyl butanoic acid and 2-methyl butanoic acid are products of Strecker degradation. 2-Methylbutanoic acid was present at significantly higher levels in raw TMR milk, but was not present in any feed or rumen samples in this study (Table S6.1). However, Faulkner et al. (2018) identified it in TMR feed but not in GRS or GRS/CLV feed, and these same authors did not find it in raw or pasteurised milk from these feeds. It has previously been described as having a fruity, sweaty, rancid, burnt, sour aroma in milk (Mounchili et al., 2005). 3-Methyl butanoic acid

was present in every sample in this study, except in raw milk from GRS or in GRS feed (Table S6.1). Thus, it was not present in feed but was present in GRS RF. It is likely metabolised into corresponding alcohols and esters, which may account for its absence in the raw GRS milk. It has also been found in raw milk and has been described as having a slight grassy, cooked vegetable aroma (Bendall, 2001). Heptanal is a major lipid oxidation product in milk (Clarke et al., 2020a) and has been widely identified in milk described as cheesy caramel (Bendall, 2001) or green sweet (Friedrich and Acree, 1998). Heptanal was present in all samples in this study (Table S6.1) and thus appears to be both directly transferred from diet and derived from lipid oxidation. The next most active aroma; sweet, herbal, fruity, spicy was due to phenylethyl alcohol which has already been identified as an very abundant VOC in raw GRS milk in this study, and likely also derived directly from diet. It is noteworthy that it is impacting on the aroma of both milks, independent of diet. The next most significant aroma; barnyard, pungent, animal, solvent, was generated by co-eluting peaks of p-cresol / 2-pyrrolidone. p-Cresol is a degradation product of β-carotene and isoflavones (Faulkner et al., 2018, Clarke et al., 2020a). In this study p-cresol was identified in all feed, rumen and milk samples, but was one of the most abundant VOC found in rumen samples (Table S6.1). A higher abundance of p-cresol was found in TMR feed, but slightly higher levels were found in raw GRS milk than raw TMR milk. p-Cresol is thought to be one of the main VOC behind the barnyard aroma often perceived in pasture produced dairy products (Faulkner et al., 2018), and therefore it is interesting to note that it appears to have a greater contribution to the aroma of raw TMR milk in this study, although was co-eluting with 2-pyrrolidone. The odour activity of p-cresol is at an intermediate level in comparison to most VOC (Bendall, 2001). 2-Pyrrolidone is a lactam cyclisation product of γ-amino butyric acid (Grewal and Khare, 2017), and thus maybe formed in the rumen but also appears to be present in both GRS and TMR feed (Table S6.1). However, it was only present in the GRS rumen samples, and was at greater abundance in raw GRS milk. Despite the fact that its abundance is lower than in raw GRS milk it appears more easily perceived in raw TMR milk, (although it did co-elute with p-cresol). 2-Pyrrolidone is a VOC that has not been previously identified in bovine milk.

Both 2,5-dimethylpyrazine / 2,3-dimethylpyrazine were described as having the next most intense aroma; smokey, barnyard, animal, roasted, toasted, cooked potato in this study. As mentioned already these VOC were also identified as important odour active compounds

in raw GRS milk, that appear to both come directly from the diet, but also formed by microbial metabolism, Strecker reactions or Maillard browning. It is possible that higher abundances in TMR milk were potentially due to direct transfer rather than other means based on the fact that neither were found in GRS feed in this study (thus not derived directly from GRS feed). The next most significant aroma was attributed to 2-pentylfuran which was described as roasted, toasted, bready, potato, popcorn, somewhat different to caramel (Li et al., 2016) and again may indicate co-elution with another odour active VOC below the limit of MS detection. As mentioned it is a product of the Maillard reaction and also identified as an important VOC in raw GRS milk in this study and appears to be mainly derived directly from the diet.

A sweet, caramel, herbal, fruity, cherry aroma was derived from co-eluting VOC (benzaldehyde / γ-butyrolactone / ethyl hexanoate). Benzaldehyde has previously been described as having an almond-like nutty aroma and was found at low abundances in raw UHT bovine milk (Moio et al., 1993b). Moio et al. (1994) also found benzaldehyde in raw and pasteurised bovine milk, with higher abundances in UHT milk. However, these authors did not find that it contributed to the aroma of milk. Benzaldehyde has also previously been found in raw milk derived from pasture, hay or silage (Villeneuve et al., 2013). Faulkner et al. (2018) also found significantly similar abundances in GRS, GRS/CLV and TMR feed (Faulkner et al., 2018) and in raw milk derived from GRS, but not from GRS/CLV or TMR or in any milk post pasteurisation. Benzaldehyde is thought to derive from the metabolism of phenylalanine (Kilcawley et al., 2018), which likely occurs in the rumen, but also appears to be directly transferred from the diet as all feed, rumen and milk samples in this study contained benzaldehyde (Table S6.1). γ-Butyrolactone is another lactone and has a creamy, oily, subtle fatty aroma (Kilcawley et al., 2018). It was not present in any fed or rumen samples in this study (Table S6.1), which is similar to that found by Faulkner et al. (2018). However, these authors did find it in pasteurised milk produced from TMR, but not in pasteurised milk from GRS or GRS/CLV, or in raw milk produced from any of these feeds. Ethyl hexanoate has a fruity, malty, young cheese, mouldy aroma and is derived from esterification of ethanol and hexanoic acid, but is also directly transferred from feed (Kilcawley et al., 2018). Ethyl hexanoate was present at a high abundance in TMR feed but absent in GRS feed in this study (Table S6.1). Ethyl hexanoate has previously been found to be one of the most abundant esters in raw bovine milk, but absent post pasteurisation (Moio

et al., 1993b, Moio et al., 1994), however more importantly it was thought not to influence the aroma of raw bovine milk (Moio et al., 1994). Ethyl hexanoate was also found to be quite abundant in raw milk from cows fed hay, silage or pasture (Villeneuve et al., 2013) and Clarke et al. (2020a) found ethyl hexanoate in GRS, GRS/CLV and TMR feed, but absent in the subsequent raw and pasteurised milks derived from these diets.

Butanoic acid also contributed to the aroma of raw TMR milk, but as previously mentioned also contributed to the aroma of raw GRS milk.. Butanoic acid appears to be derived directly from the diet, through *de novo* synthesis and lipolysis (Table S6.1) (1, 36). As mentioned furfural, 2,3-butanedione and acetic acid also contributed to the aroma of raw GRS milk and also likely derived from diet. Methyl-2-furoate also contributed to the aroma of raw GRS milk but did not derive from the diet. Decanal is likely a result of lipid oxidation and direct transfer from the diet and has previously been identified in raw bovine milk (Moio et al., 1994, Mounchili et al., 2005, Faulkner et al., 2018, Clarke et al., 2020a). It was described as having solvent, mushroom, animal, floral, grassy aroma in this study and was present in all feed, rumen and raw milk samples (Table S6.1), but only influenced the aroma of raw TMR milk. Maltol did influence the aroma of raw GRS milk (OI 1.6), but was not discussed as levels were below OI 2.0. As mentioned it did not direct derive from diet, but may be a product of microbial activity in the raw milk. 2-Furanmethanol was only identified in milk samples and is a result of Maillard reactions between an amino acid and a sugar, or from oxidation of poly-unsaturated fatty acids (Bugaud et al., 2001). The higher abundance in raw TMR milk and corresponding higher aroma intensity is consistent with findings by Faulkner et al. (2018). 2-Heptanone is a secondary oxidation product commonly found in dairy products (Faulkner et al., 2018, Clarke et al., 2021), and was identified in all feed, RF, RB, and raw milk samples in this study, thus likely derived from the diet. 1-Octen-3-ol (tentative identification based on published odour references and LRI) is a product of lipid oxidation, and likely influenced by the abundances of specific unsaturated fatty acids in the milk due to the different bovine diets (Kilcawley et al., 2018). In this study it was described as having a green, fresh, earthy, mushroom aroma and did contribute to the aroma of both raw GRS and raw TMR milk (greater influence in raw TMR milk by OI). However, 1-octen-3-ol was not identified in either raw GRS or TMR milk by GCMS in this study possibly due to co-elution and / or that the compound was present below its limit of detection by MS (it was only tentatively identified by olfactometry analysis due to low abundance).

Both UNC 3 and UNC 5 influenced the aroma of both raw TMR and GRS milk, with UNC 5 having a slightly greater OI value, and higher in raw TMR than raw GRS milk.

6.3.3 Key Aroma Active Volatiles Identified by Aroma Extraction Dilution Analysis

AEDA can potentially provide more information on the significance of specific aromas than OI alone, as the extract can be diluted extensively in a very controlled manner using the split value in the GC injection port which also negates any sample matrix effect. Therefore aromas that can be perceived at the greatest dilution therefore are likely to be the most significant in terms of aroma and flavour perception.

6.3.3.2 Aroma Active Volatiles in raw GRS milk by Aroma Extraction Dilution Analysis

From the AEDA study based on the FD values (Table 6.3 and Figure 6.3) the primary VOC contributing to the overall aroma of raw GRS milk were maltol (FD100) with each of the following having identical FD values; methanethiol (FD50), furfural (FD50), benzaldehyde/γ-butyrolactone/ethyl hexanoate (FD50), 1-octen-3-ol (FD50), phenylethyl alcohol (FD50), and 3 unidentified VOC; UNC 4 (FD50), UNC 5 (FD50), and UNC 6 (FD 50).

As previously stated Maltol has a sweet odour and is a product of the Maillard reaction (Van Boekel, 1998), and was described as having a having a caramel, sweet, cotton candy aroma in this study and has been previously been identified in bovine milk (Hodge and Moser, 1961, Van Boekel, 1998, Croissant et al., 2007). As stated it appears to arise from dietary sugars as it was not present in any of the feed or rumen samples. Oddly even though it was present at significantly greater abundances in raw TMR milk than in raw GRS milk it appears to have a greater impact on the aroma the raw GRS milk, although from the OI study it was deemed potentially more important to the aroma of raw TMR milk. Methanethiol was described as having a fishy, cabbage aroma although it has previously been described as having an intense potato soup or cooked potato aroma (Dutta et al., 2012), this discrepancy may also be due to co-elution with another odour active VOC that is below the limits of MS detection. It was present in every sample in this study (Table S6.1). It is thought to arise from methionine and influenced by the application of heat (Vazquez-Landaverde et al., 2006), however, it is also readily oxidised to dimethyl disufide and

dimethyl trisulfide (Vazquez-Landaverde et al., 2006). Clarke et al., (2020a) also found methanethiol in GRS, GRS/CLV and TMR feed, but not in raw milk derived from these diets. It was also identified as odour active by OI, but as values were below OI 2.0 it was deemed of less significance, however it is well recognised as a very odour active VOC (Vazquez-Landaverde et al., 2006). Furfural as mentioned was already identified as a key odour active compound in raw GRS milk and raw TMR milk by OI in this study (slightly higher in raw TMR milk by OI), and is likely derived directly from the diet, although may be co-eluting with another odour active VOC not detected by the MS.

A sweet, caramel, herbal, fruity cherry aroma was described for 3 co-eluting VOC, benzaldehyde, γ-butyrolactone and ethyl hexanoate. As stated these VOC have different potential sources from amino acid metabolism (benzaldehyde), diet (benzaldehyde, ethyl hexanoate), lipid oxidation (γ-butyrolactone), and a combination of lipolysis and esterification (ethyl hexanoate) in milk. This same aroma from these co-eluting VOC was also found to be aroma active in both raw milks by OI, but with a greater contribution than in raw GRS milk.

1-Octen-3-ol (tentative identification based on published odour references and LRI) as previously stated is a product of lipid oxidation, with a similar FD value in raw GRS and TMR milk. It was also identified as aroma active by OI (with a slightly higher importance in raw TMR milk). Phenylethyl alcohol had a sweet, herbal, fruity, spicy aroma (but as previously stated maybe co-eluting with an odour active VOC not detected by the MS) and was also identified as aroma active by OI, with a slightly greater influence for raw TMR milk. It appears to derive mainly from the diet, although it is also a product of Strecker degradation and was also like furfural previously identified as an important aroma VOC in raw GRS milk by OI. Three UNC VOC were also found to influence the aroma of raw GRS milk; UNC 4 (animal, pungent, smokey, burnt, eggy), UNC 5 (fresh, herbal, sweet), and UNC 6 (sweet, floral).

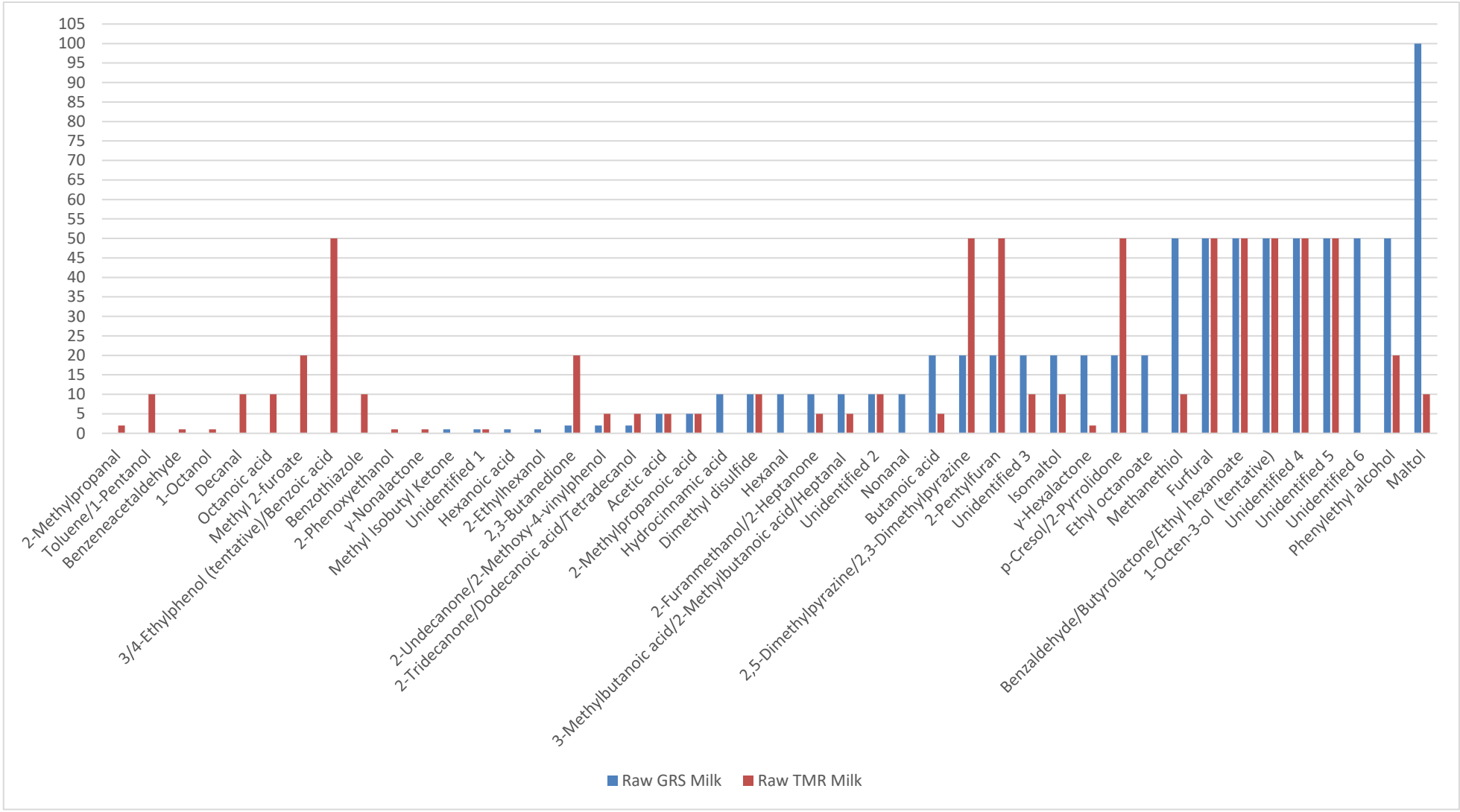


Figure 6.3: Bar Chart illustrating the aroma extraction dilution analysis factor dilution values for the 44 volatile organic compounds identified via gas-chromatography olfactometry in raw grass milk (GRS) and raw total mixed ration (TMR) milk; range: 0-100. The higher the FD, the more odour intense the compound.

6.3.3.3 Aroma Active Volatiles in raw TMR milk by Aroma Extraction Dilution Analysis

Nine aromas were found to influence the perception of raw TMR milk by AEDA, four of which were from co-eluted VOC and one from an unidentified VOC (Table 6.3 and Figure 6.3). All of these VOC were perceived up to FD 50; furfural, 2,5-dimethylpyrazine / 2.3-dimethylpyrazine, 2-pentyl furan, benzaldehyde / γ-butyrolactone / ethyl hexanoate, 1-octen-3-ol (tentative identification based on published odour references and LRI), UNC 4, UNC 5, p-cresol / 2-pyrrolidone, and 3-/ 4-ethylphenol (tentative identification) / benzoic acid. The potential source and aroma of each of these VOC has already been discussed. As mentioned 3-/ 4-ethylphenol are phenolic compounds that has been previously been identified in bovine milk (Kilic and Lindsay, 2005, Faulkner et al., 2018, Faccia, 2020), and appear to be present in both raw GRS and TMR milk in this study. 3-/ 4-Ethylphenol was identified in both feed samples being higher in TMR feed which concurred the results found by Faulkner et al. (2018). This compound was not identified in the rumen or milk samples in this study (Table S6.1) although likely present below its limit of detection. Benzoic acid is known to naturally occur in cultured dairy products from hippuric acid, phenylalanine or from the oxidation of benzaldehyde (Sieber et al., 1995). It was not identified in any feed or rumen sample in this study (Table S6.1). As it is not a common VOC in bovine milk and has a low odour activity, it would appear unlikely to be contributing much to the aroma perceived in this study, therefore 3- / 4-ethylphenol were more likely to be impacting on odour activity of this aroma.

6.3.4 Aromas influenced by diet as detected by OI and AEDA

Eight VOC were perceived by panellists in raw GRS milk by OI and AEDA and not in raw TMR milk (UNC 6 (sweet, floral), ethyl octanoate (solvent, aldehydic, alcohol), hydrocinnamic acid (sweet, floral, creamy), nonanal (solvent, fresh, artificial, chemical), hexanal (roasted, fresh, floral, herbal, vegetable), hexanoic acid (cheesy, smokey, bready, roasted), 2-ethylhexanol (sweet, solvent), and methyl isobutyl ketone (sweet)). Ethyl octanoate has previously been found in GRS, GRS/CLV and TMR feed, but with higher abundances in TMR (Faulkner et al., 2018, Clarke et al., 2020a), but not in fresh raw milk derived from these feeds, although it did appear in raw milk derived from TMR after refrigerated storage (Clarke et al., 2020a). Ethyl octanoate was present in every feed, rumen and milk sample in this study (Table S6.1). Moio et al. (Moio et al., 1994) also found ethyl

octanoate in raw milk derived from hay and it has been described as having an floral aroma (Moio et al., 1993b), somewhat different to the aroma described in this study (solvent, aldehydic, alcohol). As mentioned previously ethyl esters are derived from short chain fatty acids primarily produced by *de novo* synthesis in the mammary gland but may also be derived directly from the diet (O'Callaghan et al., 2016).

Hydrocinnamic acid was identified only in raw milk samples in this study (Table S6.1). It is a metabolite of phenylalanine degradation (Bosset et al., 1990) and thus its higher abundance in GRS milk may be due to the higher protein content in pasture forage (Mackle et al., 1999, Coppa et al., 2011). In this study it was described as having a sweet, floral, creamy aroma. Nonanal has been found in GRS, GRS/CLV and TMR feed (Faulkner et al., 2018, Clarke et al., 2020a) and is commonly found in bovine milk (Moio et al., 1993b, Moio et al., 1994, Toso et al., 2002, Croissant et al., 2007, Villeneuve et al., 2013, Faulkner et al., 2018) as a result of enzymatic breakdown or lipid oxidation. Nonanal was found in every sample in this study (Table S6.1). Clarke et al. (2020a) previously found nonanal in GRS, GRS/CLV and TMR feed, and at higher levels in the pasture diets, and in raw and pasteurised milk from each diet. It has been described as green, grass-like, fatty or tallow with a fatty odour (Moio et al., 1993b, Moio et al., 1994, New Jersey Department of Health, 2016), and as solvent, fresh, artificial, chemical in this study.

Hexanal is commonly found in milk as a result of lipid oxidation of oleic, linoleic, and arachidonic acid (García-Martínez et al., 2009, Tawfik et al., 2017, Faulkner et al., 2018), and differences in these fatty acid contents within the milks are thus likely influencing its abundance. Hexanal was present in both feed samples in this study but was not identified in any rumen samples (Table S6.1). Even though abundances were considerably higher in GRS feed than TMR feed, abundances were similar in both raw milks in this study. Hexanal has also previously been identified in both GRS and TMR feed (Clarke et al., 2020a).Therefore its presence in milk appears to be due to both lipid oxidation and direct transfer from the diet. It was identified in this study as having a cheesy, smokey, bready, roasted aroma, not that dissimilar to that described by Bendall et al. (2001) as cooked. Hexanoic acid was present in every feed, rumen and milk sample in this study (Table S6.1) and always at higher levels in the TMR samples. Faulkner et al. (Faulkner et al., 2018) and Clarke et al. (Clarke et al., 2020a) also found high abundances of hexanoic acid in GRS, GRS/CLV and TMR feed, but only Faulkner et al. (Faulkner et al., 2018) identified it in raw

and pasteurised milks from these diets. Croissant et al. (Croissant et al., 2007) found it in raw milk from both pasture and TMR diets, and Villeneuve et al. (Villeneuve et al., 2013) found it in raw milk from hay, pasture and silage diets, as did Coppa et al., (2011) in milk from hay, rotational grazing and continuous grazing. Previous studies have also found higher levels of hexanoic acid in raw milk produced from TMR (O'Callaghan et al., 2016). In this study it was described as having a cheesy, smokey, bready, roasted aroma. Therefore hexanoic acid is typically present at high abundances in bovine milk, and likely transfers directly from feed, but also indirectly generated by *de novo* synthesis in the mammary gland (Kilcawley et al., 2018). Again as an acid it is likely hugely influenced by the extraction method used.

Moio et al., (1994) found 2-ethylhexanol in raw, pasteurised and UHT milk but did not find that it impacted on odour. However, Mounchili et al. (Mounchili et al., 2005) did find that it contributed to the odour of raw milk, although co-eluted with benzene acealdehyde and was described as honey, vegetable, green, and moist. In this study 2-ethyl-1-hexanol was described as having a sweet, solvent aroma. 2-Ethylhexanol was present in every feed, rumen and milk sample (Table S6.1). It appears 2-ethylhexanol is likely derived directly from the diet as evident in this study. Methyl isobutyl ketone is likely a product of lipid oxidation (Faulkner et al., 2018) and imparted a sweet aroma in GRS milk in this study. Methyl isobutyl ketone was only present in GRS feed although was present in both GRS and TMR RB samples. While not significantly different, abundances were higher in GRS milk, similar to that found previously (Faulkner et al., 2018). Thus, it appears to derive from diet and from lipid oxidation, although not a VOC which has been commonly identified in raw milk to date.

Nine aromas associated with VOC or co-eluting VOC contributed to the aroma of raw TMR milk that did not contribute to the aroma of raw GRS milk (3- /4-ethylphenol (tentative identification) / benzoic acid (smokey, animal, burnt milk), decanal (solvent, mushroom, animal, floral, grassy), benzothiazole (smokey, roasted, caramel), octanoic acid (smokey, toasted, animal, burnt milk), 2-methylpropanal (sweet, fresh), 2-phenoxyethanol (sweet, burnt), benzeneacetaldehyde (pungent, cleaning agent, musty), 1-octenol (mushroom, stale, damp) and γ-nonalactone (sweet, caramel, burnt, lactone)). The source and aroma of 3-/4-ethylphenol / benzoic acid, benzothiazole, and 2-methylpropanal have already been discussed in detail. Octanoic acid was described as having a smokey, toasted,

animal, burnt milk aroma in this study, not that different to that described previously; burnt milk or pudding, intense (Mounchili et al., 2005). As mentioned it was present in all samples in this study (Table S6.1) and was consistently higher in TMR samples, thus the likely reason it was perceived in raw TMR milk. 2-Phenoxyethanol a phenolic compound was identified as having sweet burnt aroma in this study, and was present in every feed, rumen and milk sample in this study (Table S6.1). Levels were not statistically different in raw GRS or TMR milk and therefore it is difficult to understand why it was only perceived in raw TMR milk. It is not a common VOC in bovine milk, although concentrations were previously found to be higher in evening milk compared to morning milk and concentrations were shown to decrease over time possibly due to oxidation (Bergamaschi and Bittante, 2018).

Benzeneacetaldehyde (phenylacetaldehyde) is also a Strecker aldehyde (Hoffmann and Heiden, 2000) produced via phenalanine metabolism and has previously been reported in milk (Mounchili et al., 2005, Coppa et al., 2011). It was not identified in either feed in this study, but was present in most rumen samples and in both raw milks (Table S6.1). It was described as having a pungent, cleaning agent and musty aroma in this study, but is also likely further metabolised to acids, alcohols and esters. Again it is difficult to discern why it was only perceived in the raw TMR milk. 1-Octanol was present in every feed, rumen and raw milk sample in this study (Table S6.1). It was described as having a mushroom, stale damp aroma. Previous studies did not identify it in feed, but did in raw milk derived from CLV (Faulkner et al., 2018). However, other studies found it in both GRS and TMR feed, and raw milk from TMR (Clarke et al., 2020a). It is a product of lipid oxidation but likely also derived from feed. Again it is difficult to discern why it would be perceived in raw TMR milk and not in raw GRS milk. γ-Nonalactone was found to be significantly ($p = 0.05$) higher in TMR milk and was characterised as sweet, caramel, burnt and lactone, but was not perceived in GRS milk. γ-Nonalactone was higher in TMR feed and in TMR RB (Table S6.1). As mentioned lactones are naturally occurring compounds derived from fat, particularly short chain fatty acids in milk (Dimick and Harner, 1968) but can be produced from a range of different sources and apparently also directly transferred from feed, as evident in this study. Lactones are typically important odour VOC as they have relatively low odour thresholds in milk (Patton, 1955).

6.4 Conclusions

Overall this study has confirmed that bovine diet influences the VOC profile of raw milk in relation to their abundance rather than their presence or absence. However, some of the VOC trends in this study did not match those found previously in relation to their abundance and specific diets. Many factors can influence VOC composition in milk through production and analysis, it is difficult compare studies. DI-HiSorb proved to be a very effective VOC extraction method, as evident by the high number of VOC of different chemical classes extracted and identified in all of these samples, (including 99 in the raw milk) and by the fact that panellists could detect so many aromas by GC-O from this extraction method. This study has highlighted that 33 VOC were present in each feed, rumen and milk sample, thus eluding to the fact that these are likely transferred directly from the diet into the raw milk. As previously stated the volatile profile of these raw milks are not that different in terms of content based on diet, but rather some significant differences in abundance are evident due to diet, with five VOC significantly higher ($p < 0.05$) in raw GRS milk and ten significantly higher in raw TMR milk. However, despite the fact that only 13 of 99 VOC were significantly different in terms of abundance, the odours of both milk were quite different based on diet as evaluated by olfactometry using OI and AEDA. This is due to the fact that odour activity of each VOC is based on abundance and odour threshold, and not just abundance alone. The OI of the raw TMR milk (66.2) was greater than that for raw GRS milk (61.2) reflecting the greater abundance of odour activities in the raw TMR milk, likely due to its more complex composition as many odour active VOC appeared to be directly transferred from diet. Seventeen out of 44 odour activities detected differed between both sample types, the main characteristic aromas for raw TMR milk deriving from the increased diversity of the TMR diet that are likely to have been created or enhanced during TMR feed production as many are either derived from Maillard reactions or influenced by heat.

In summary, the following aromas were most associated with both raw GRS and TMR milk: roasted, toasted, bready, potato, popcorn (2-pentyl furan); cheesy, dairy, buttery (butanoic acid); sweet, herbal, fruity, spicey (phenylethyl alcohol); smokey, barnyard, animal, roasted, toasted, cooked potato (2,5-dimethylpyrazine / 2,3-dimethylpyrazine); cheesy, sour, sour milk, dairy, nutty, bready, baked, roasted (furfural); sweet, caramel, herbal, fruity, cherry (benzaldehyde / γ-butyrolactone / ethyl hexanoate); green, fresh, earthy,

mushroom (1-octen-3-ol; tentative identification); apple, pungent, smokey, burnt, eggy (UNC 4) and fresh, herbal, sweet (UNC 5). Therefore these odours and associated VOCs have the greatest influence in relation to the sensory character of the milk independent of the diets used in this study. The overall impact of some of these varied between the raw milks based on diet. A number of aromas: caramel, sweet, cotton candy (maltol); fishy, cabbage (methanethiol), sweet floral (UNC 6), fresh, sweet, caramel, butterscotch, biscuit, baked (2,3-butanedione), cooked potato, roasty, musty (UNC 1), barbeque, caramel, tobacco, toasted, toffee (γ-hexalactone), toffee, fruity, sweet, caramel (methyl-2-furoate) and vinegar (acetic acid), were much more significant for raw GRS milk than raw TMR milk. Likewise for raw TMR milk, the following aromas were of greatest impact: smokey, barnyard, animal, roasted, toasted, cooked potato (2,5-dimethylpyrazine / 2,3-dimethylpyrazine), barnyard, pungent, animal, solvent (p-cresol / 2-pyrrolidone), buttery, animal, barnyard, nutty, bready (3-methylbutanoic acid / 2-methylbutanoic acid / heptanal), and smokey, animal, burnt milk (3 -/ 4-ethylphenol (tentative identification)/ benzoic acid). This is also the first time that so many VOCs potentially coming directly from diet have been shown to influence the aroma of the resultant raw milks. This study clearly highlights the significance of the direct transfer of VOC into raw milk from the diet, the impact of diet and the potential of DI-HiSorb to extract VOC in these feed, rumen and raw milk samples.

Supplementary Materials Chapter 6

Table S6.1: Volatile compounds identified in grass and total mixed ration (TMR) raw milk (n=3), feed (n=3), rumen fluid (n=3) and rumen blended (n=3) samples via HiSorb gas chromatography mass spectrometry.

Compound	CAS no.	RI	IM	Grass feed	TMR feed	Grass RF	TMR RF	Grass RB	TMR RB	Raw GRS milk	Raw TMR milk	Occurrence
Acids												
Formic acid	64-18-6	605.8	MS	ND	ND	ND	ND	ND	ND	$8.53x10^4$	$6.58x10^4$	M
Acetic acid	64-19-7	662.8	MS, IHL, LRI	$3.72x10^6$	$2.29x10^7$	$2.61x10^7$	$4.58x10^7$	$7.11x10^7$	$5.26x10^7$	$1.96x10^6$	$2.57x10^6$	AO
Propanoic acid	79-09-4	786	MS, IHL, LRI	$1.69x10^6$	$1.16x10^7$	$1.19x10^7$	$2.72x10^7$	$3.16x10^7$	$2.70x10^7$	$2.49x10^5$	$2.64x10^5$	AO
2-Methylpropanoic acid	79-31-2	841.8	MS	ND	ND	ND	ND	ND	ND	$1.92x10^4$	$1.44x10^4$	M
2-Methyl-2-propenoic acid	79-41-4	867.1	MS, LRI	ND	ND	ND	ND	ND	ND	$3.78x10^4$	$5.37x10^4$	M
Butanoic acid	107-92-6	914.4	MS, LRI	$3.68x10^6$	$9.57x10^7$	$3.03x10^7$	$8.33x10^7$	$1.03x10^8$	$1.79x10^8$	$7.93x10^5$	$1.10x10^6$	AO
2-Methylbutanoic acid	116-53-0	841.8	MS, IHL, LRI	ND	ND	ND	ND	ND	ND	$2.07x10^4$	$1.13x10^5$	MO
3-Methylbutanoic acid	503-74-2	914.5	MS	ND	$2.61x10^6$	$3.35x10^6$	$1.01x10^7$	$1.24x10^7$	$1.37x10^7$	0.00x00	$4.35x10^4$	-
Pentanoic acid	109-52-4	973.9	MS	$8.40x10^5$	$1.54x10^7$	$8.15x10^6$	$5.50x10^7$	$2.20x10^7$	$8.47x10^7$	$1.60x10^5$	$3.77x10^5$	-
Hexanoic acid	142-62-1	1069.7	MS, IHL, LRI	$1.01x10^6$	$8.67x10^7$	$9.27x10^6$	$1.14x10^8$	$2.05x10^7$	$1.57x10^8$	$7.81x10^5$	$1.67x10^6$	A
Heptanoic acid	111-14-8	1164.1	MS, IHL	ND	ND	ND	ND	ND	ND	$1.82x10^5$	$3.53x10^5$	-
Octanoic acid	124-07-2	1261.9	MS	$7.32x10^5$	$3.28x10^7$	$1.44x10^6$	$1.16x10^7$	$2.05x10^6$	$1.22x10^7$	$1.41x10^6$	$3.45x10^6$	O
Benzoic acid	65-85-0	1285.2	MS	ND	ND	ND	ND	ND	ND	$2.35x10^5$	$3.40x10^4$	MO
Nonanoic acid	112-05-0	1353.8	MS, IHL	ND	ND	ND	ND	ND	ND	$3.98x10^5$	$6.88x10^5$	M
Decanoic acid	334-48-5	1439.0	MS, LRI	$1.77x10^6$	$1.77x10^7$	$4.39x10^6$	$4.65x10^6$	$3.99x10^6$	$6.04x10^6$	$2.97x10^6$	$6.71x10^6$	-
Decanoic acid	334-48-5	1452.3	MS, IHL	$1.77x10^6$	$1.77x10^7$	$4.39x10^6$	$4.65x10^6$	$3.99x10^6$	$6.04x10^6$	$2.97x10^6$	$6.71x10^6$	-
Hydrocinnamic acid	501-52-0	1460	MS	ND	ND	ND	ND	ND	ND	$8.27x10^4$	$2.89x10^4$	MO
Undecanoic acid	112-37-8	1544	MS, LRI	ND	ND	$1.67x10^4$	$5.44x10^5$	ND	ND	$8.26x10^4$	$1.45x10^5$	-
Dodecanoic acid	143-07-7	1640.2	MS	$6.15x10^5$	$7.63x10^6$	$3.36x10^6$	$1.35x10^7$	$2.39x10^6$	$5.12x10^6$	$2.02x10^6$	$1.94x10^6$	AO
Tetradecanoic acid	544-63-8	1839.7	MS	ND	ND	ND	ND	ND	ND	$4.39x10^5$	$2.91x10^5$	-
Alcohols												

Ethanol	64-17-5	504.3	MS, IHL, LRI	$8.61x10^5$	$5.18x10^6$	$3.79x10^6$	$3.52x10^6$	$2.21x10^6$	$3.30x10^6$	$3.51x10^6$	$2.74x10^6$	A
Isopropyl alcohol	67-63-0	542.2	MS	$4.70x10^5$	$6.38x10^5$	$1.17x10^6$	$9.04x10^5$	$5.50x10^5$	$1.02x10^6$	ND	ND	-
Phenylethyl Alcohol	60-12-8	542.2	MS	$1.31x10^6$	$2.76x10^7$	$2.92x10^6$	$6.44x10^6$	$1.80x10^6$	$6.88x10^6$	$1.09x10^4$	$1.42x10^4$	AO
1-Propanol	71-23-8	611.3	MS, LRI	$3.99x10^3$	$8.75x10^5$	$2.19x10^5$	$5.58x10^5$	$1.26x10^6$	$1.79x10^6$	ND	ND	-
2-Butanol	78-92-2	647.4	MS, LRI	$1.87x10^4$	$6.48x10^6$	$7.19x10^5$	$9.02x10^6$	$1.14x10^6$	$1.65x10^7$	ND	ND	-
1-Propanol, 2-methyl-	78-83-1	678.2	MS, LRI	$2.87x10^4$	$8.43x10^5$	$1.87x10^5$	$3.33x10^5$	$2.35x10^5$	$5.23x10^5$	ND	ND	-
1-Butanol	71-36-3	688.9	MS, IHL	$1.10x10^5$	$5.37x10^6$	$2.40x10^6$	$5.90x10^6$	$5.69x10^6$	$9.15x10^6$	$9.70x10^3$	ND	A
2-Methyl-1-butanol	137-32-6	787.1	MS, LRI	$3.01x10^5$	$3.14x10^6$	$6.94x10^5$	$1.80x10^6$	$4.87x10^5$	$1.78x10^6$	$2.20x10^5$	$1.19x10^4$	A
4-Methyl-2-pentanol	108-11-2	796.4	MS, IHL, LRI	ND	ND	ND	ND	ND	ND	$7.85x10^3$	$3.54x10^3$	M
3-Methyl-1-butanol	123-51-3	803.2	MS, LRI	$7.59x10^5$	$1.53x10^7$	$1.84x10^6$	$4.76x10^6$	$2.98x10^6$	$8.94x10^6$	ND	ND	-
1-Pentanol	71-41-0	810.6	MS, IHL, LRI	$9.06x10^5$	$1.53x10^7$	$2.16x10^6$	$6.53x10^6$	$2.98x10^6$	$8.94x10^6$	$1.07x10^5$	$1.95x10^4$	AO
1-Hexanol	111-27-3	915.4	MS, LRI	$7.57x10^6$	$7.47x10^6$	$2.44x10^6$	$1.95x10^7$	$1.52x10^6$	$1.93x10^7$	$1.88x10^4$	$3.75x10^3$	A
2-Furanmethanol	98-00-0	929.5	MS, LRI	ND	ND	ND	ND	ND	ND	$5.21x10^6$	$6.80x10^6$	MO
Ethanol, 2-butoxy-	111-76-2	954.5	MS	$3.47x10^4$	ND	ND	ND	ND	ND	$3.78x10^4$	$9.83x10^4$	-
3-Furanmethanol	4412-91-3	1046.4	MS	ND	ND	ND	ND	ND	ND	$1.81x10^5$	$2.33x10^5$	M
Benzyl alcohol	100-51-6	1122	MS	ND	$4.21x10^6$	ND	ND	$2.72x10^5$	$2.99x10^5$	ND	ND	-
1-Octen-3-ol	3391-86-4	1028	MS, IHL, LRI	ND	ND	ND	ND	ND	ND	$5.18x10^4$	ND	MO
3-Methyl-1-hexyn-3-ol	4339-05-3	1046.4	MS	ND	ND	ND	ND	ND	ND	$9.68x10^2$	$6.44x10^2$	M
Dihydroxyacetone	96-26-4	1040.6	MS, LRI	ND	ND	ND	ND	ND	ND	$2.76x10^4$	$1.35x10^4$	M
Ethanol, 2-(2-ethoxyethoxy)-	111-90-0	1061.2	MS	$4.11x10^4$	ND	$7.33x10^4$	$1.02x10^5$	ND	ND	ND	ND	-
1-Hexanol, 2-ethyl-	104-76-7	1076.4	MS, LRI	$1.61x10^6$	$4.84x10^5$	$1.40x10^6$	$7.40x10^5$	$2.19x10^5$	$2.62x10^5$	$9.46x10^4$	$7.65x10^4$	AO
1-Octanol	111-87-5	1120.1	MS, IHL	$2.44x10^5$	2.85x106	$1.14x10^5$	$2.72x10^5$	$1.92x10^5$	$9.23x10^5$	$7.39x10^4$	$5.37x10^4$	AO
2-Phenoxyethanol	122-99-6	1320.4	MS, IHL, LRI	$3.71x10^5$	ND	$9.99x10^4$	ND	ND	ND	$2.94x10^5$	$1.55x10^5$	MO
1-Dodecanol	112-53-8	1523.3	MS, LRI	$2.69x10^5$	$1.02x10^5$	$8.39x10^5$	$1.44x10^6$	$1.99x10^5$	$4.43x10^5$	$1.35x10^5$	$1.05x10^5$	A
Tetradecanol	112-72-1	1724.2	MS	$2.36x10^4$	ND	$3.60x10^4$	$6.73x10^4$	ND	ND	$1.49x10^5$	$1.78x10^5$	AO
Aldehydes												
Acetaldehyde	75-07-0	451.7	MS	$1.06x10^6$	$1.63x10^6$	$2.97x10^6$	$2.94x10^6$	$3.30x10^6$	$2.75x10^6$	$5.50x10^6$	$4.97x10^6$	A
2-Propenal	107-02-8	524.3	MS	$2.26x10^5$	$2.63x10^5$	$2.24x10^5$	$3.26x10^5$	$2.72x10^5$	$2.44x10^5$	ND	ND	-
Propanal	123-38-6	528.2	MS	$2.34x10^5$	$1.23x10^5$	$7.99x10^4$	$7.19x10^4$	$1.41x10^5$	$1.09x10^5$	ND	ND	-

2-Methyl-propanal	78-84-2	594.4	MS	$2.50x10^5$	$3.62x10^5$	$1.61x10^5$	$1.65x10^5$	$3.20x10^5$	$2.61x10^5$	ND	$4.93x10^5$	O
Butanal	123-72-8	633.4	MS	$8.38x10^5$	$8.50x10^6$	$7.57x10^5$	$9.42x10^5$	$1.02x10^6$	$1.22x10^7$	ND	ND	-
3-Methy-l-butanal	590-86-3	650.4	MS, IHL, LRI	$1.15x10^6$	$8.85x10^5$	$2.16x10^5$	$2.27x10^5$	$5.16x10^5$	$3.43x10^5$	$2.43x10^4$	$2.24x10^4$	A
2-Methy-l-butanal	96-17-3	700.4	MS	$5.83x10^6$	$2.29x10^5$	ND	ND	$6.89x10^5$	$5.44x10^5$	ND	ND	-
Hexanal	66-25-1	828.9	MS, IHL	$2.10x10^6$	$2.15x10^5$	ND	ND	ND	ND	$2.21x10^5$	$1.85x10^5$	O
Furfural	98-01-1	899.3	MS	$1.59x10^5$	$1.30x10^6$	ND	ND	$4.50x10^5$	$6.50x10^5$	$1.09x10^6$	$1.31x10^6$	O
(E)-2-Hexenal	6728-26-3	901.2	MS	ND	ND	ND	ND	ND	ND	$1.35x10^4$	$5.05x10^3$	M
Heptanal	111-71-7	943.1	MS	$8.52x10^5$	$7.74x10^5$	$6.81x10^5$	$7.17x10^5$	$3.01x10^5$	$4.02x10^5$	$2.13x10^5$	$1.60x10^5$	AO
Benzaldehyde	100-52-7	1031.6	MS	$2.02x10^6$	$3.67x10^6$	$3.84x10^6$	$3.11x10^6$	$2.10x10^6$	$1.66x10^6$	$2.19x10^5$	$2.03x10^5$	AO
5-methyl furfural	620-02-0	1040.6	MS	ND	ND	ND	ND	ND	ND	$1.73x10^5$	$2.07x10^5$	M
(E,E)-2,4-Heptadienal	4313-03-5	1074.2	MS, LRI	ND	ND	ND	ND	ND	ND	$2.56x10^4$	$1.39x10^3$	M
Benzeneacetaldehyde	122-78-1	1108.5	MS, IHL, LRI	$3.05x10^6$	ND	$2.92x10^5$	ND	$2.56x10^5$	$9.18x10^3$	$2.76x10^4$	$3.94x10^4$	O
Nonanal	124-19-6	1147.6	MS, IHL, LRI	$3.70x10^6$	$4.10x10^6$	$3.45x10^6$	$3.04x10^6$	$1.81x10^6$	$1.38x10^6$	$1.11x10^6$	$7.50x10^5$	O
Decanal	112-31-2	1252.6	MS, IHL, LRI	$1.12x10^6$	$9.88x10^5$	$1.41x10^6$	$1.12x10^6$	$6.27x10^5$	$5.75x10^5$	$4.16x10^5$	$3.02x10^5$	AO
Dodecanal	112-54-9	1457.2	MS, IHL	$9.39x10^4$	7.11x104	$4.28x10^5$	$1.12x10^6$	$2.55x10^4$	$1.51x10^5$	$5.68x10^4$	0.00x00	-
Tridecanal	10486-19-8	1558.7	MS, LRI	ND	ND	$1.08x10^5$	$2.52x10^5$	ND	ND	$2.28x10^4$	$8.22x10^3$	-
Undecanal	112-44-7	1357.7	MS, LRI	ND	ND	$6.62x10^4$	ND	ND	ND	ND	ND	-
Octanal	124-13-0	1046.9	MS	$5.92x10^5$	$7.53x10^5$	$9.14x10^5$	$4.52x10^5$	$3.06x10^5$	$4.69x10^5$	$3.07x10^5$	$2.27x10^5$	A
Esters and Ethers												
Ethyl ether	60-29-7	514.2	MS	ND	ND	ND	ND	$1.88x10^4$	ND	ND	ND	-
Ethyl acetate	141-78-6	641.1	MS	$3.27x10^5$	$8.59x10^6$	$8.47x10^5$	$6.20x10^6$	$5.33x10^5$	$4.81x10^6$	ND	ND	-
Methyl propionate	922-67-8	657.6	MS	ND	$5.27x10^4$	ND	$7.01x10^3$	$4.82x10^5$	$8.80x10^5$	ND	ND	-
Ethyl propanoate	105-37-3	736.5	MS	ND	$2.39x10^6$	$1.99x10^6$	$1.23x10^7$	$2.25x10^6$	$1.04x10^7$	ND	ND	-
Methyl methacrylate	80-62-6	738.4	MS	$1.53x10^5$	$6.53x10^4$	$6.10x10^4$	$3.89x10^4$	ND	ND	ND	ND	-
n-Propyl acetate	109-60-4	741.7	MS	ND	$1.17x10^6$	ND	$6.73x10^6$	$2.08x10^4$	$1.51x10^6$	ND	ND	-
Methyl butanoate	623-42-7	749.2	MS	$1.81x10^4$	$1.72x10^7$	$3.59x10^4$	$4.77x10^4$	$2.93x10^6$	$1.20x10^7$	ND	ND	-
Isobutyl acetate	110-19-0	800.3	MS	ND	$4.22x10^5$	ND	$1.73x10^5$	ND	ND	ND	ND	-
Ethyl butanoate	903170-13-8	825.1	MS	$3.71x10^5$	$1.74x10^8$	$7.00x10^6$	$9.21x10^7$	$1.93x10^6$	$1.88x10^7$	ND	ND	-
Butyl acetate	123-86-4	834.2	MS, IHL, LRI	ND	$2.54x10^6$	$3.01x10^5$	$9.80x10^6$	ND	ND	$7.36x10^3$	$3.51x10^3$	-

Ethyl 2-methylbutanoate	7452-79-1	874.1	MS	ND	ND	ND	ND	$1.59x10^5$	$1.39x10^7$	ND	ND	-
Ethyl 3-methylbutanoate	108-64-5	877.4	MS	ND	ND	$3.20x10^5$	$3.38x10^6$	$6.60x10^4$	$9.53x10^5$	ND	ND	-
Ethylbenzene	100-41-4	891.4	MS	ND	ND	$1.37x10^5$	$6.84x10^4$	$8.33x10^4$	$2.60x10^4$	ND	ND	-
Ethyl lactate	97-64-3	867.5	MS	ND	$1.68x10^7$	ND	ND	ND	ND	ND	ND	-
Ethyl 2-methylbutanoate	7452-79-1	873.9	MS	$2.34x10^4$	$4.06x10^5$	$2.91 x10^5$	$4.36 x10^7$	ND	ND	ND	ND	-
Isoamyl acetate	123-92-2	902.5	MS	$5.14x10^4$	$5.61x10^6$	$2.57x10^5$	$2.16x10^6$	$4.75x10^4$	$4.49x10^5$	ND	ND	-
2-Methylbutyl acetate	624-41-9	902.7	MS	ND	$6.38x10^6$	$4.90x10^3$	$3.15x10^5$	ND	$2.47x10^5$	ND	ND	-
1-Methylpropyl ester butanoic acid	819-97-6	903	MS	ND	ND	ND	ND	ND	ND	$6.56x10^3$	ND	M
Amyl acetate	628-63-7	915.5	MS	ND	$5.61x10^6$	ND	ND	ND	$1.05x10^6$	ND	ND	-
Propyl butyrate	105-66-8	923.2	MS	$1.09x10^4$	$3.20x10^7$	$4.51x10^6$	$2.12x10^6$	$1.73x10^5$	$2.08x10^5$	ND	ND	-
Ethyl pentanoate	539-82-2	925.2	MS	$2.86x10^5$	$1.31x10^7$	ND	ND	ND	ND	ND	ND	-
Propanoic acid, butyl ester	590-01-2	934	MS	ND	$7.23x10^5$	$3.89x10^6$	$5.03x10^6$	$5.27x10^4$	$5.73x10^5$	ND	ND	-
Pentyl acetate	628-63-7	941.4	MS	ND	ND	ND	$2.19x10^6$	ND	ND	ND	ND	-
Methyl hexanoate	106-70-7	952.9	MS	$2.07x10^4$	$3.61x10^7$	ND	$2.37x10^5$	$1.24x10^6$	$3.15x10^7$	ND	ND	-
Isobutyl butyrate	539-90-2	964.3	MS	ND	$5.09x10^6$	$3.52x10^6$	$1.10x10^7$	ND	ND	ND	ND	-
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	77-68-9	974.2	MS	ND	ND	$1.57x10^5$	$4.69x10^4$	ND	ND	ND	ND	-
Propyl 2-methylbutanoate	37064-20-3	974.2	MS	ND	$3.05x10^4$	$4.96x10^5$	$1.82x10^7$	$1.37x10^4$	$2.20x10^6$	ND	ND	-
Amyl propionate	624-54-4	997.9	MS	ND	$9.48x10^5$	$1.55x10^5$	$9.94x10^5$	ND	$1.13x10^5$	ND	ND	-
Butyl butanoate	100-52-7	1023.6	MS	ND	$4.91x10^7$	$1.87x10^6$	$2.15x10^6$	$1.54x10^4$	$1.34x10^5$	$8.17x10^3$	0.00x00	-
Ethyl hexanoate	123-66-0	1026.2	MS	ND	$1.73x10^8$	$4.81x10^5$	$1.97x10^8$	$1.47x10^5$	$2.65x10^7$	$1.28x10^5$	$1.41x10^4$	O
Hexyl acetate	142-92-7	1042.1	MS	ND	$8.08x10^5$	ND	$1.34x10^6$	ND	ND	ND	ND	-
2-Methylbutyl butanoate	51115-64-1	1082.4	MS	ND	$3.43x10^7$	$1.45x10^5$	$1.43x10^6$	ND	$2.19x10^5$	ND	ND	-
Isoamyl isobutanoate	2050-01-3	1082.4	MS	ND	$3.43x10^7$	$6.09x10^4$	$1.58x10^6$	ND	ND	ND	ND	-
Propyl hexanoate	626-77-7	1120.2	MS	ND	$6.64x10^7$	ND	$2.61x10^7$	ND	$1.94x10^6$	ND	ND	-
Ethyl heptanoate	106-30-9	1122.8	MS	ND	ND	ND	$2.46x10^7$	ND	$2.32x10^6$	ND	ND	-

Isobutyl hexanoate	105-79-3	1156.5	MS	ND	$2.16x10^7$	ND	$8.08x10^6$	ND	ND	ND	ND	-
Methyl-2-furoate	611-13-2	1170	MS	ND	ND	ND	ND	ND	ND	$4.18x10^5$	$4.88x10^5$	MO
Butyl hexanoate	626-82-4	1217.8	MS	ND	$2.08x10^7$	ND	ND	ND	ND	ND	ND	-
Ethyl benzoate	93-89-0	1233	MS	ND	$3.49x10^6$	$1.01x10^5$	$4.90x10^5$	ND	$5.81x10^4$	ND	ND	-
Ethyl octanoate	106-32-1	1225.7	MS, IHL, LRI	ND	$7.76x10^6$	$2.18x10^5$	$2.99x10^6$	$6.39x10^4$	$5.12x10^5$	$8.64x10^4$	$2.02x10^4$	O
Methyl benzeneacetate	101-41-7	1241.3	MS	ND	ND	ND	ND	$4.69x10^5$	$7.98x10^5$	ND	ND	-
Ethyl benzeneacetate	101-97-3	1306.6	MS	ND	$5.66x10^6$	ND	$4.65x10^5$	ND	$1.32x10^5$	ND	ND	-
B-Phenylethyl acetate	103-45-7	1322.1	MS	ND	$6.06x10^6$	ND	ND	ND	ND	ND	ND	-
Butyl ethyl succinate	67233-92-5	1360	MS	ND	$9.23x10^4$	ND	ND	ND	ND	ND	ND	-
Ethyl decanoate	110-38-3	1422.5	MS, IHL, LRI	ND	$6.73x10^5$	ND	$1.29x10^6$	ND	$4.83x10^5$	$9.29x10^4$	$3.18x10^4$	-
Ethyl dodecanoate	106-33-2	1620	MS, IHL, LRI	ND	$1.21x10^5$	ND	ND	ND	$1.69x10^4$	$1.13x10^4$	$7.60x10^3$	-
Ethyl hexadecanoate	628-97-7	2029	MS	ND	$2.85x10^5$	ND	ND	ND	ND	ND	ND	-
Furans												
Furan	110-00-9	518.9	MS	$9.43x10^4$	$9.98x10^4$	$5.03x10^4$	$8.96x10^4$	$7.91x10^4$	$5.25x10^4$	ND	ND	-
2-Ethylfuran	3208-16-0	718.9	MS	$1.22x10^6$	$7.65x10^4$	ND	ND	ND	ND	ND	ND	-
2-Methylfuran	79-09-4	793.9	MS	$8.31x10^4$	$2.11x10^5$	$6.34x10^4$	$7.74x10^4$	$1.29x10^5$	$7.76x10^4$	$1.36x10^4$	$2.25x10^4$	-
2-n-Butyl furan	4466-24-4	912.3	MS	ND	ND	$8.72x10^3$	$3.80x10^3$	ND	ND	ND	ND	-
2-Pentylfuran	3777-69-3	1008.9	MS, IHL, LRI	$4.94x10^5$	$1.25x10^6$	$1.35x10^6$	$1.25x10^6$	$4.47x10^5$	$5.72x10^5$	$7.76x10^3$	$1.31x10^4$	AO
Isomaltol	3420-59-5	1040.6	MS, LRI	ND	ND	ND	ND	ND	ND	$1.22x10^6$	$1.10x10^6$	MO
Diethyl succinate	123-25-1	1227.5		ND	$1.26x10^6$	ND	ND	ND	ND	$2.49x10^3$	$1.26x10^3$	-
Hydrocarbons and Benzenes												
Benzene	71-43-2	686.9	MS	$3.15x10^5$	$6.36x10^5$	$6.57x10^5$	$5.20x10^5$	$1.72x10^5$	$2.88x10^5$	ND	ND	-
Toluene	108-88-3	773.5	MS, IHL	$5.71x10^5$	$5.06x10^5$	$3.20x10^7$	$1.97x10^6$	$7.03x10^6$	$1.04x10^6$	$1.14x10^5$	$1.53x10^4$	AO
p-Xylene	106-42-3	888.2	MS, IHL, LRI	7.52x105	$1.13x10^5$	$6.36x10^5$	$1.61x10^5$	$2.36x10^5$	$3.56x10^5$	$2.25x10^4$	$1.16x10^4$	A
Ethylbenzene	100-41-4	891.4	MS	$7.52x10^5$	$6.53x10^4$	ND	ND	ND	ND	ND	ND	-
o-Xylene	95-47-6	929.7	MS	$5.28x10^5$	$2.60x10^4$	$2.32x10^5$	$2.61x10^5$	ND	ND	ND	ND	-
o-Cymene	527-84-4	1059.4	MS	ND	$8.20x10^4$	$1.94x10^5$	$2.75x10^5$	ND	ND	ND	ND	-
Phenol	108-95-2	1104.5	MS, IHL, LRI	$5.95x10^5$	$1.85x10^6$	$3.23x10^6$	$5.08x10^6$	$4.86x10^6$	$6.17x10^6$	$1.00x10^5$	$8.47x10^4$	A

p-Cresol	106-44-5	1195	MS, IHL, LRI	$9.80x10^5$	$1.51x10^6$	$2.25x10^8$	$1.93x10^8$	$2.06x10^8$	$1.93x10^8$	$5.82x10^5$	$2.90x10^5$	AO
3/4-Ethylphenol	620-17-7/123-07-9	1286	MS	$1.99x10^5$	$5.93x10^5$	ND	ND	ND	ND	ND	ND	O
Benzene, 1,3-bis(1,1-dimethylethyl)-	1014-60-4	1287.7	MS	ND	ND	$8.05x10^4$	$1.93x10^4$	$1.30x10^5$	$8.11x10^4$	ND	ND	-
Benzothiazole	95-16-9	1296.2	MS, IHL, LRI	$1.34x10^5$	ND	ND	ND	ND	ND	$7.36x10^4$	$3.39x10^4$	O
2-Methoxy-4-vinylphenol	7786-61-0	1411.9	MS, IHL	$1.01x10^5$	$1.16x10^7$	$3.99x10^5$	$3.14x10^5$	$1.27x10^6$	$5.93x10^5$	$3.63x10^5$	$4.19x10^4$	AO
Indole	110-38-3	1430.8	MS, IHL, LRI	$2.72x10^5$	$7.09x10^5$	$7.82x10^7$	$4.20x10^5$	$7.49x10^7$	$2.06x10^6$	$1.49x10^4$	$1.61x10^4$	-
Ketones												
Acetone	67-64-1	491.9	MS, IHL, LRI	$9.47x10^5$	$1.81x10^6$	$4.67x10^6$	$2.72x10^6$	$2.79x10^6$	$1.87x10^6$	$2.40x10^5$	$3.94x10^5$	A
2,3-Butanedione (Diacetyl)	431-03-8	574.6	MS, IHL, LRI	$3.03x10^5$	$1.08x10^6$	$1.46x10^5$	$4.79x10^5$	$4.95x10^5$	$6.61x10^5$	1.12x105	$1.65x10^5$	AO
2-Pentanone	116-09-6	704.2	MS, IHL, LRI	$5.74x10^4$	$4.55x10^5$	$1.24x10^5$	$1.63x10^5$	$6.37x10^5$	$2.06x10^5$	$4.38x10^4$	$3.61x10^4$	A
1-Hydroxy-2-propanone	116-09-6	709.8	MS, IHL	ND	ND	ND	ND	ND	ND	$6.83x10^5$	$9.41x10^5$	M
Methyl isobutyl ketone	108-10-1	764.5	MS, IHL	$2.42x10^5$	ND	ND	ND	$4.82x10^4$	$4.20x10^4$	$2.34x10^4$	$1.34x10^4$	O
2-Heptanone	110-43-0	931.4	MS, IHL, LRI	$3.27x10^5$	$5.94x10^5$	$9.14x10^5$	$1.64x10^6$	$1.53x10^5$	$3.66x10^5$	$1.37x10^5$	$1.97x10^4$	AO
2-Hexanone	591-78-6	1132.5	MS, IHL, LRI	$5.45x10^4$	ND	ND	ND	ND	ND	ND	ND	-
Acetophenone	98-86-2	1343.3	MS, IHL	$5.95x10^5$	ND	$1.23x10^6$	ND	$6.80x10^5$	$1.25x10^5$	$6.74x10^4$	$5.18x10^4$	-
2-Undecanone	112-12-9	1546.2	MS	ND	ND	$1.71x10^6$	$3.59x10^6$	$7.26x10^4$	ND	$1.02x10^5$	$7.08x10^4$	O
2-Tridecanone	593-08-8	1547.6	MS	ND	ND	ND	ND	ND	ND	$2.60x10^5$	$1.79x10^5$	MO
2-Butanone	78-93-3	638.7	MS	$3.17x10^5$	$6.64x10^6$	$6.47x10^5$	$7.12x10^6$	$8.59x10^5$	$1.19x10^7$	ND	ND	-
1-Hydroxy-2-propanone	116-09-6	733.3	MS	$2.24x10^6$	$6.00x10^6$	$3.43x10^6$	$3.77x10^6$	$4.05x10^6$	$3.65x10^6$	ND	ND	-
2-Hydroxy-3-pentanone	5704-20-1	867.9	MS	ND	$4.92x10^6$	ND	ND	ND	1.00x105	ND	ND	-
2-Nonanone	821-55-6	1139.7	MS	ND	ND	$2.45x10^6$	$6.25x10^6$	ND	$1.02x10^6$	ND	ND	-
2-Pyrrolidinone	88-12-0	1196	MS	$6.27x10^4$	$7.44x10^5$	$8.21x10^4$	ND	$2.32x10^5$	ND	$2.28x10^5$	$9.37x10^4$	O
Lactones												
2(3H)-Furanone, dihydro-4-hydroxy-	5469-16-9	1382	MS	ND	ND	ND	ND	ND	ND	$1.43x10^6$	$1.71x10^6$	M
γ -Butyrolactone	96-48-0	1021.1	MS, IHL, LRI	$5.08x10^4$	$1.04x10^6$	ND	ND	ND	ND	$2.85x10^4$	$6.91x10^4$	O
2(5H)-Furanone	497-23-4	1026.3	MS, LRI	ND	$8.62x10^5$	ND	ND	ND	ND	7.72x105	$1.08x10^6$	-

γ -Hexalactone	695-06-7	1163	MS, IHL, LRI	$7.49x10^4$	$3.89x10^5$	ND	ND	ND	ND	$1.95x10^4$	$8.67x10^4$	O
γ -Nonalactone	104-61-0	1485	MS, IHL, LRI	$2.68x10^5$	$2.16x10^7$	$1.50x10^7$	$1.20x10^7$	$5.81x10^6$	$6.84x10^6$	$3.34x10^4$	$1.84x10^5$	AO
Pyrazines and Pyradines												
Pyrazine	290-37-9	753	MS	ND	ND	ND	ND	ND	ND	$1.59x10^4$	$2.86x10^4$	M
Pyridine	110-86-1	789.1	MS	$1.04x10^4$	$5.52x10^4$	$6.43x10^4$	$1.28x10^5$	$1.29x10^4$	$6.90x10^4$	$6.22x10^3$	$6.74x10^3$	A
2,5-Dimethylpyrazine	123-32-0	950	MS, IHL, LRI	ND	$5.57x10^5$	$8.52x10^4$	$1.34x10^5$	ND	ND	0.00x00	$7.27x10^4$	O
2,3-Dimethylpyrazine	5910-89-4	959	MS, IHL, LRI	ND	$3.29x10^5$	ND	ND	ND	ND	$3.77x10^3$	$5.71x10^4$	O
Pyrazine, trimethyl-	14667-55-1	1044.2	MS	ND	$5.30x10^5$	ND	ND	ND	ND	ND	ND	-
Pyrazine, 3-ethyl-2,5-dimethyl-	13360-65-1	1115.8	MS	ND	$1.07x10^5$	ND	ND	ND	ND	ND	ND	-
Pyrazine, tetramethyl-	1124-11-4	1123.9	MS	ND	$2.55x10^5$	ND	ND	ND	ND	ND	ND	-
Sulphurs												
Methanethiol	90500-11-1	460.1	MS, IHL, LRI	$1.89x10^4$	$2.71x10^5$	$6.16x10^4$	$2.87x10^5$	$5.07x10^5$	$3.46x10^5$	$4.55x10^5$	$4.79x10^5$	O
Dimethyl sulfide	75-18-3	538	MS	$5.40x10^5$	$7.27x10^4$	$1.39x10^5$	$4.59x10^4$	$9.83x10^6$	$6.76x10^5$	ND	ND	-
Carbon disulfide	75-15-0	546.6	MS	ND	$5.75x10^4$	$1.86x10^5$	$1.32x10^5$	$2.34x10^5$	$1.72x10^5$	ND	ND	-
Disulfide, dimethyl	624-92-0	754.6	MS	$2.43x10^5$	$7.52x10^5$	$9.68x10^5$	$8.26x10^5$	$1.44x10^7$	$3.23x10^6$	$3.74x10^4$	$7.44x10^4$	-
Dimethyl sulfone	67-71-0	1056	MS, IHL, LRI	ND	ND	ND	ND	ND	ND	$2.49x10^3$	$2.08x10^4$	M
Other												
1,3-Pentadiene	1574-41-0	534.5	MS	$2.92x10^5$	ND	ND	ND	ND	ND	ND	ND	-
Methacrolein	78-85-3	615.3	MS	$1.04x10^4$	$5.58x10^3$	ND	ND	ND	ND	ND	ND	-
Trichloromethane	67-66-3	655.1	MS	ND	ND	$6.36x10^3$	ND	ND	ND	ND	ND	-
Mercaptoacetone	24653-75-6	730.3	MS	ND	ND	ND	ND	$4.18x10^4$	ND	ND	ND	-
Acetoin	513-86-0	777.4	MS	ND	$2.22x10^7$	ND	ND	$2.28x10^6$	$1.09x10^5$	ND	ND	-
Propylene glycol	57-55-6	833.1	MS	ND	$6.64x10^5$	ND	ND	ND	ND	ND	ND	-
2,3-Butanediol, [S-(R*,R*)]-	19132-06-0	867.7	MS	ND	$1.28x10^7$	ND	$3.08x10^5$	$7.32x10^4$	$4.87x10^5$	ND	ND	-
2,3-Butanediol	513-85-9	870.0	MS	ND	$1.28x10^7$	ND	$5.82x10^5$	$7.32x10^4$	$5.03x10^5$	ND	ND	-
1H-Pyrrole, 2-methyl-	636-41-9	918.1	MS, IHL, LRI	$9.44x10^3$	$3.71x10^4$	$3.14x10^4$	$5.40x10^4$	$1.70x10^5$	$6.99x10^4$	$4.94x10^3$	$3.94x10^3$	A

1H-Pyrrole, 3-methyl-	2703-17-5	917.6	MS	$9.44x10^3$	3.71x104	ND	ND	$1.70x10^5$	$6.99x10^4$	ND	ND	-
Styrene	100-42-5	929.5	MS	$3.02x10^5$	$3.05x10^5$	$9.50x10^5$	4.12x105	$1.87x10^5$	$1.97x10^5$	ND	ND	-
Cyclohexanone	108-94-1	962.3	MS	$7.30x10^4$	ND	ND	ND	$7.30x10^4$	ND	ND	ND	-
3-Methyl-2,5-furandione	110-00-9	1050.8	MS	ND	ND	ND	ND	ND	ND	$4.96x10^5$	$8.05x10^5$	M
5-Hepten-2-one, 6-methyl-	110-93-0	1036.3	MS	$7.79x10^5$	$4.21x10^5$	$1.55x10^6$	$3.32x10^6$	$1.77x10^5$	$7.15x10^5$	ND	ND	-
2-Furancarboxaldehyde, 5-methyl-	620-02-0	1040.7	MS	$6.33x10^4$	ND	ND	ND	ND	ND	ND	ND	-
3-Carene	13466-78-9	1051.4	MS	ND	ND	$4.50x10^4$	ND	ND	ND	ND	ND	-
1H-Pyrrole-2,5-dione	541-59-3	1102.9	MS	ND	ND	ND	ND	ND	ND	$8.09x10^3$	$9.32x10^3$	M
Linalool	78-70-6	1145.6	MS	$1.69x10^6$	$8.69x10^6$	ND	ND	ND	ND	ND	ND	-
Maltol	88-12-0	1193	MS, LRI	ND	ND	ND	ND	ND	ND	$1.28x10^6$	$2.39x10^6$	MO
Damascenone	23696-85-7	1467.5	MS	ND	$1.06x10^6$	ND	$3.33x10^5$	ND	ND	ND	ND	-

A = compound identified in all sample types; M = compound identified in milk only; O = compound is odour active; ND = not detected.

Levels of volatile compounds are expressed as abundances (mean values from 3 extractions from each sample.

LRI: retention index on a DB-624 UI column; IM: identification method; MS: spectra comparison using NIST mass spectral database; IHL: in-house library created using authentic compounds with target and qualifier ions and linear RI for each compound; LRI: RI agree with literature values.

Consent for Publication

The principal investigator obtained informed consent from each participant to publish the data without breaching confidentiality.

Ethics Statement

Teagasc has both an animal welfare body and animal ethics committee. The animal welfare body is a legal requirement of Article 26 of Directive 2010/63/EU and Regulation 50 of S.I. No. 543 of 2012. The Health Products Regulatory Authority provided project authorisation.

Author Contributions

HJC and KNK designed the research study. HJC carried out the experimental work, data collection and analysis and statistical analysis. HJC drafted the manuscript. KNK, MGOS and JPK reviewed the manuscript. All authors approved the final version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 6 Highlights

- Over 100 VOC were identified via HiSorb-GC-MS.
- Using GC-O, 34 VOC were found to be aroma active in raw GRS milk and 36 in raw TMR milk, therefore ~30% of the VOC in raw milk influence sensory perception.
- While the intensities varied, numerous compounds contributed to the aroma profile of both raw GRS and TMR milk.
- The odour profile of raw GRS milk was dominated cheesy, nutty, sweet, and green aromas which have been attributed to methanethiol, furfural, benzaldehyde, 1-octen-3-ol (tentative identification), phenylethyl alcohol and maltol.
- The odour profile of raw TMR milk was dominated by roasted, smokey, animal, and pungent aromas which have been attributed to furfural, 2,5-dimethylpyrazine/2,3-dimethylpyrazine, 2-pentylfuran, benzaldehyde, 1-octen-3-ol, p-cresol/2-pyrrolidinone and 3/4-ethylphenol.

Chapter 7: General Discussion

7.1 Scientific and Industrial Impact

Dairy powders are important exports for the Irish dairy industry and the prepared foods sector which includes enriched dairy powders was worth €2.32 bn in 2019 (Bord Bia, 2019). Moreover, three of the largest IMF producers are based in Ireland and export to markets in Europe, South East Asia, Middle East, and Canada (Careers Portal, 2018). Ireland has a relatively unique, predominately pasture-based milk production system, in comparison to most other countries where a much higher or total concentrate-based feeding system exists. Numerous reasons including climate, economics, an export driven sector and a predominately spring calving national herd have contributed, but importantly, this type of production system is positively perceived by the consumer as it is considered more traditional, environmentally friendly, organic and animal welfare friendly. A need for innovative concepts and approaches towards marketing Irish dairy and ensuring quality is required now more than ever. This Doctoral thesis undertook necessary and innovative research required to further enhance our understanding of the contribution pasture-based and non-pasture based bovine feeding systems have with respect to volatile aromatic compounds and LO to the sensory quality of milk and selected dairy powder commodity products.

Numerous factors influence the rate of LO in dairy products such as cow diet, antioxidants (naturally present or supplemented), stage of lactation, levels of PUFA and unsaturated FA, storage and processing conditions (exposure to heat, oxygen and/or light). Therefore, understanding the concentrations at which LO VOC become a sensory issue is important for manufacturers in determining the shelf-life of specific powders. Simplistic methods exist such as peroxide values and TBARS but these often fail to accurately capture the extent of LO issues in products and do not provide sufficient information as to the source of adverse odours, or potential LO stability of a product. Although methods for volatile recovery of LO VOC by GCFID or GCMS from dairy products have been outlined, there was an obvious lack of a validated, method targeting the recovery of LO VOC from these product types. The development, optimisation and validation of a volatile extraction method targeted at recovering a representative LO profile from WMP was conducted in **Chapter 2** and is currently applied to industry samples to identify and track LO compound progression.

Method optimisation trials can be cumbersome due to the volume of experiments required prior to drawing a meaningful conclusion. However, with the application of response surface methodology (RSM), a tool consisting of mathematical and statistical concepts, the HS-SPME-GC-MS method was capable of being optimised effectively and efficiently within 20 experimental runs. Fibre type was selected prior to optimisation of the extraction parameters based on previous experience and reference studies. A multi-phase 50/30μm DVB/CAR/PDMS fibre was selected based on the diversity of volatiles extracted. RSM enabled the efficient optimisation and validation of HS-SPME analysis of WMP, with an extraction time of 45 min and an extraction temperature of 43 °C found to be the optimal conditions. This method can also be applied to monitor the changes in LO in other dairy powders including IMF and SMP.

Powder processing conditions require monitoring to ensure the quality of the end product is consistent and free from any undesirable flavours where possible. The quality of the raw milk used for the production of dairy powders is important, but also the manner in which the milk is handled, processed and stored has a significant impact on the extent to which the milk fat is oxidised throughout its shelf-life. LO has been shown to impact on the quality and sensory properties of various dairy powders resulting in undesirable flavours and reduced shelf-life. Sophisticated analytical techniques are available for the qualitative and quantitative analysis of LO in dairy powders, many of which can be used in combination to elucidate much more detailed information. This is especially the case with GC-MS which can be used to identify and quantify individual VOC associated with LO, but in combination with GC-O and/or sensory analysis can provide a much more in-depth understanding of the whole LO process pertaining to a specific product. Therefore, using this approach to determine the concentrations of VOC associated with LO that adversely impact sensory perception, can provide insights into the production parameters that could maximise shelf-life and product quality of dairy powders. Implementing mitigation strategies such as controlled temperatures and holding times during powder production can also aid in slowing the rate of LO.

7.1.1 Milk

The sensory profile of both raw and pasteurised bovine milk has been shown to be significantly impacted by feeding system. Colour, texture and the abundance of various FA and VOC differed based on feeding system. **Chapter 5** and **6** investigated the differences in composition, FA content, volatile and sensory profiles between milks produced from pasture and non-pasture-based feeding systems. Numerous volatile and

sensory differences were observed, however is has been difficult to verify unless the VOC responsible are identified. The work in **Chapter 6** was the first to demonstrate the differences in perception between pasture and non-pasture diets in raw milk samples but also that VOC influencing perception were directly and non-directly transferred from the diet. This also demonstrated that many odour active VOC in milk from TMR must have been generated during the processing of the TMR as these were all heat-derived VOC could not have been generated within the cow or subsequently as the raw milk was not subjected to any heat-treatments.

While LO does play a role in the flavour development of milk, it is not a significant issue in milk compared to milk powders. Full volatile profiling rather than targeted LO profiling was carried out on milk samples for this reason, which again showed significant differences in abundance of various VOCs based in feeding system. The phytochemical profile, likely originating directly from diet or rumen metabolism was also shown to be significantly different based on feeding system. Regardless of the effect of pasteurisation, milk from different feeding systems were always more correlated with one another i.e. raw and pasteurised GRS milk or raw and pasteurised TMR milk.

7.1.2 IMF

With the optimised HS-SPME-GCMS method established, this was subsequently applied to IMF samples over a 6-month storage period to track LO progression in **Chapter 3.** The increased concentrations of certain VOC observed in the commercially available IMF samples relate to the significant differences in FA profile. The FA potentially originating from the addition of fish and vegetable oils (C20:2, C20:3 n6, C24:1 n9 and C20:5) are likely some of the main contributors to the observed oxidative state of the IMF powders. Attention should be given to the source of the FA and their susceptibility to LO prior to manufacture of IMF. This study clearly highlighted significant LO issues in IMF in comparison to WMP and SMP. Encapsulation methodologies have been investigated but are not widely implemented in the powder industry and would require careful development to ascertain at what point the FA are released and if they still provide their intended benefits in the final product.

7.1.3 WMP

Analysis of WMP was also included in **Chapter 3.** The intended shelf-life of fresh WMP is approximately 24 months. Assuming the powder is produced from high quality starting material and produced in a manner cognisant of LO processes, the powder should

remain acceptable during this timeframe. However, once the powder is opened and in contact with oxygen, the findings of the thesis suggest that the shelf-life is reduced to around 4 months, from a sensory acceptability perspective. Results of this thesis showed that WMP remained much more stable and acceptable than IMF throughout storage.

Aside from the impact of cow diet on LO, industry need to be aware of how LO affects the stability and sensory perception of the powder, possibly through the implementation of consumer acceptance testing alongside standard shelf-life testing.

7.1.4 Bovine Feeding System

The effect of bovine feeding system was considered in **Chapters 3**, **4, 5** and **6**. The findings of this thesis demonstrate the ability to differentiate milk and milk powders produced from pasture and non-pasture-based feeding systems through the combination of analytical and sensory methodologies. This will assist in the authentication of grass-fed dairy products and aid in implementing the Grass Fed Standard for Irish dairy initiative launched in 2020 (Bord Bia, 2020). Ensuring the quality of Irish dairy products is of upmost importance to farmers and manufactures and having the ability to unambiguously state that a product is 'grass fed' is a major milestone for the Irish dairy industry and is a unique selling point in such a competitive global market. Currently no standard exists in relation to the use of the term 'grass fed' and therefore false claims could be easily made. Under the Grass Fed Standard for Irish dairy, for a processor to use a Bord Bia verified grass-fed claim on a product, the milk used must average 95% grass-fed on a fresh weight basis (Bord Bia, 2020). Creating libraries of VOC and potential biomarkers known to be present in pasture and non-pasture based milk products could allow for rapid identification and authentication of products and possibly prevent fraudulent 'grass fed' claims.

7.2 Thesis Summary

Chapter 1 provides an updated insight into the analytical techniques that are available for the qualitative and quantitative analysis of LO VOC in dairy powders, with a particular focus on combining techniques to elucidate more detailed information. This is especially the case where sophisticated techniques such as GC-MS can be used to identify and quantify individual VOC associated with LO, but in combination with GC-O and/or sensory analysis can provide a much more in-depth understanding of the entire LO process pertaining to a specific product. Therefore, using this approach to determine the concentrations of VOC associated with LO that adversely impact sensory perception, can provide insights into the production parameters that could maximise shelf-life and quality of dairy powders.

The validated HS-SPME GCMS method outlined in **Chapter 2** is effective at quantifying 13 VOC that originate from LO known to adversely influence the sensory perception of WMP. This method was applied to three dairy powders; WMP, SMP, and IMF in **Chapter 3** over a 6-month storage period at ambient (21 °C) and at accelerated (37 °C) storage. The method was combined with consumer sensory analysis to correlate VOC and sensory attributes and to gain a better understanding of the concentrations of specific VOC perceivable to consumers. Overall, increases in the concentrations of hexanal, heptanal, and pentanal were good indicators of LO occurring in WMP. Fewer significant increases in LO compounds were evident in SMP, however, changes in the concentrations of hexanal, 1-heptanol, and 1-pentanol were evident. Regarding IMF, significant increases in the aldehydes hexanal, pentanal, heptanal, and octanal were good indicators of LO over storage, however concentrations of (E)-2-nonenal and 2,4-decadienal were much higher in IMF than in WMP or SMP. The sensory acceptance scores for WMP and SMP remained stable throughout storage, despite some LO compounds been perceived by the panellists over this period. The IMF was perceived negatively from the start of storage due to high levels of numerous LO compounds at time zero. Oxidised and painty attributes were correlated with increased concentrations of hexanal and heptanal and were particularly evident in IMF. IMF was highlighted as a product that is very susceptible to LO, likely due to high levels of added PUFA (in the form vegetable and fish oils) which are then subjected to thermal processing during the drying process. Careful attention needs be paid to the type, quality, extraction and storage conditions of any oils added to IMF, as these seem to be the main source of additional LO issues with this product as issues found in this study are likely even to occur even

prior to drying. Techniques such as antioxidant addition and encapsulation of PUFA have been suggested as means of mitigating the LO process but either way it is clear that much more research is required in this area.

Following on from **Chapter 3**, medium-heat WMP was produced in triplicate trials from milk from cows maintained on three distinct feeding systems, namely a perennial ryegrass pasture system (GRS), a perennial ryegrass and white clover pasture system (CLV), and an indoor total mixed ration system (TMR) in May 2019. Full descriptive sensory analysis was employed in this chapter (**Chapter 4)** in combination with the validated HS-SPME extraction method outlined in **Chapter 2** and correlations between LO compounds and sensory attributes were evident. Changes were evident for many of the sensory attributes used to describe all three WMP samples over during storage. Buttery/toffee flavour, sweet dairy, creamy flavour and creaminess were the dominant attributes in all WMP samples at time zero. Barnyard/cowy aroma, cooked milk flavour and hay-like flavour were the dominant sensory attributes at 4 months in GRS and CLV WMP samples, while dairy sweet flavour remained perceivable in TMR WMP after 4 months. Solvent-like aroma and flavour, metallic flavour after effect, and painty flavour became the dominant sensory attributes in TMR WMP 6 months. The increase in painty flavour and aroma corresponded with an increase in hexanal, in addition to some ketone compounds. The recommended shelf-life for opened WMP was found to be less than 4 months from a sensory perspective.

A HS-SPME method was also employed in **Chapter 5** for the volatile analysis of raw and pasteurised milk from cows maintained on the same feeding systems as outlined in **Chapter 4**. This analysis was used in combination with full descriptive sensory analysis and was beneficial in distinguishing the milks based on feeding system and provided a full volatile profile of raw and pasteurised milk obtained from the different feeding systems. Creaminess and hay-like flavour were found to be significantly different between the pasteurised milk samples. Only the GRS and CLV milks were significantly different for creaminess, while TMR milk scored significantly higher for hay-like flavour. The isoflavones with the potential to impact sensory perception through degradation and breakdown products were also evaluated in **Chapter 5**. Formononetin was found to be significantly correlated to CLV feed samples and levels of apigenin, while daidzein and genistein were found to be significantly different between the raw and pasteurised milk samples. Daidzein, genistein and apigenin were highly correlated to raw CLV milk, likely present as metabolism products from other isoflavone compounds. Formononetin was

more closely correlated with raw GRS milk, despite levels of this isoflavone being higher in CLV feed. It is possible that the formonoetin in CLV feed was present in a more readily metabolised form when compared to the formonoetin content in GRS feed. Furthermore, p-cresol is likely derived from the metabolism of formononetin and has been reported to be responsible for a 'barnyard aroma' associated with milk derived from pasture (Faulkner et al., 2018). Both raw and pasteursed GRS milk had the highest levels of p-cresol at days nine and fourteen days of refrigerated storage, and pasteurised GRS milk was more correlated with 'barnyard aroma' than the pasteurised CLV or pasteurised TMR milk.

As previously mentioned, certain VOC are known to cause off notes such as painty and metallic-like in dairy products, however, GC-O analysis of raw milk presented in **Chapter 6** demonstrated the potential of aroma-active compounds that are present directly after milking to influence the overall aroma of milk. Thirty-four and thirty-six aroma-active compounds were identified in raw milk from (GRS) and non-grass-fed cows (TMR), respectively. Aromas such as fishy and solvent were observed for certain compounds but attributes such as painty and metallic that are commonly associated with LO were largely absent, which is to be expected in fresh, raw milk. Overall, sweet, caramel and toffee notes were more prevalent in raw GRS milk while barnyard, animal, roasted and smokey notes dominated the aroma of raw TMR milk. Where the compounds that are identified in raw milk could be an important factor is when milk is being chosen for further processing. The compounds could reach high levels during the production process and have a significant effect of the overall flavour of the end product. This study showed that while the flavour of raw milk is subtle, it is possible to differentiate the aroma of GRS and TMR milks with both analytical techniques and human panellists. Finally, when choosing milk for any subsequent processing i.e. powder production, attention should be paid to the aroma-active compounds that are present in the raw milk directly after milking as they will inevitably influence the flavour of the end product.

7.3 Final Conclusions

This thesis has provided scientific information in the form of volatile and sensory data in addition to complementary analysis (microbial, colour, composition, fatty acid profiling, phytochemical content) on a variety of dairy products (raw and pasteurised milk, WMP, IMF, and SMP) produced from pasture and non-pasture bovine feeding systems. The results are therefore of interest to dairy producers, manufacturers, marketers, and consumers alike.

This thesis has provided the following conclusions:

1. Volatile profiling is a useful tool for distinguishing between milk and dairy products derived from different feeding systems.
2. Even though the VOC content of milk is not significantly impacted by different diets, the abundance of several VOC associated with pasture derived dairy products in comparison to non-pasture production systems such as from a total mixed ration feeding systems are different. Further work is required to establish concentrations of these specific VOC that could be used to authenticate dairy products based on diet.
3. Fatty acid profiling can differentiate between dairy products from different feeding systems, most notable pasture from non-pasture derived dairy products. Further work is required using comprehensive gas chromatography to identify unique long chain PUFA associated with pasture based feeding systems for authentication purposes.
4. VOC in milk are derived both indirectly mainly from rumen metabolism but also directly from the diet.
5. The aroma and other sensory properties of milk are influenced by diet, with unique aroma's associated with pasture and non-pasture based feeding systems.
6. The aroma of raw milk derived from a TMR diet, was influenced directly by VOC created during thermal treatements applied in the production of the TMR.
7. Full descriptive sensory analysis using trained panellists provides valuable information on the sensory attributes associated with VOC in both milk and dairy powders.
8. Evaluating the isoflavone content of feed, rumen fluid and milk offers further insight into the degradation products being transferred from feed to milk and the alterations taking place within the rumen.
9. The inclusion of white clover increases isoflavone content in the rumen, which can potentially increase levels of phenolic VOC in milk.

10. GC-O is an excellent technique for identifying aroma-active compounds in milk, and this study has demonstrated that diet impacts VOC content and aroma, through both direct and indirect transfer.

11. The use of higher capacity phase HiSorb probes allows for more effective extraction of aroma-active compounds from milk, which was very beneficial for GC-O evaluation.

12. The current proposed shelf-life of WMP is up to 24 months, however, sensory changes start to occur after 4 months of storage once the product has been exposed to air (oxygen). Processing and storage conditions influence the rate of LO and subsequently the increase in volatiles associated with sensory off-notes. These factors need careful consideration prior to processing.

13. Dietary factors can influence the LO stability of dairy powders, due to differences in PUFA and antioxidants.

14. WMP produced from TMR had more sweet caramel notes that initially masked LO off-notes but after 4 months levels of LO aldehydes responsible for painty odours were predominated.

15. The source and quality of the lipid fractions (fish, vegetable etc.) added to IMF have a significant impact on the products LO susceptibility. Further work is required to create IMF with significantly lower abundances of VOC associated with LO.

7.4 Future Recommendations

Suitable future work expanding on the studies presented in this thesis are:

1. GC-O work on pasteurised milk from cows maintained on the GRS and TMR feeding systems to determine the effects of pasteurisation on the aroma profile of milk.
2. Determine the concentration levels at which the aroma perception of important volatile compounds change within milk.
3. Investigate other extraction methodologies such as SPME-trap or Hi-Sorb with different sorbent phases for the evaluation of milk by GC-O.
4. The use of two-dimensional GC with MS to elucidate more VOC likely influencing aroma perception but currently below LOD.
5. GC-O of WMP and possibly IMF to provide further insight into the compounds causing the numerous off notes observed in **Chapter 3**.

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Appendices

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Review

Oxidative Quality of Dairy Powders: Influencing Factors and Analysis

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Abstract: Lipid oxidation (LO) is a primary cause of quality deterioration in fat-containing dairy powders and is often used as an estimation of a products shelf-life and consumer acceptability. The LO process produces numerous volatile organic compounds (VOC) including aldehydes, ketones and alcohols, which are known to contribute to the development of off-flavours in dairy powders. The main factors influencing the oxidative state of dairy powders and the various analytical techniques used to detect VOC as indicators of LO in dairy powders are outlined. As the ability to identify and quantify specific VOC associated with LO improves this review highlights how these techniques can be used in conjunction with olfactory and sensory analysis to better understand product specific LO processes with the aim of maximizing shelf-life without compromising quality.

Keywords: lipid oxidation; dairy powder; sensory

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1. Introduction

Oxidation of bovine milk fat is recognised as the main factor in the development of undesirable flavours in products such as whole milk powder (WMP) and infant milk formula (IMF). Lipid oxidation (LO) is responsible for the formation of primary and secondary oxidation products including aldehydes, ketones and alcohols, which can impact on nutritional and sensory properties of dairy powders [1]. Factors that can contribute to the oxidative stability of dairy powders include the quality of the raw milk (fatty acid (FA) composition, bovine diet, and storage conditions), processing parameters, powder composition (especially water activity), presence of pro- and anti-oxidants (natural or added during processing), packaging materials, storage and transport conditions. LO is a free radical chain reaction consisting of three stages; initiation, propagation, and termination. Free radicals and peroxides (tasteless, flavourless compounds) [2] are generated during the initiation phase when molecular oxygen reacts with unsaturated FA [3]. The rate of the propagation cycle is directly proportional to the degree of lipid unsaturation [4]. The resultant termination products (secondary LO products) are generally quite stable, however, it is these secondary LO products (mainly aldehydes, ketones, alcohols, and hydrocarbons) that actually contribute to off-flavour development and have been described as grassy, soapy, cardboard-like, painty, tallowy and/or fishy [5,6]. Secondary LO compounds can be monitored and quantified instrumentally as molecular-level indicators of oxidised flavours in dairy products. This measurement can be used in place or in combination with sensory analysis to provide an overall profile of the flavour stability of a dairy powder [6,7].

The presence and increase of numerous secondary LO products in dairy powders during processing and storage is well documented [6,8,9]. However, there is a lack of knowledge linking the quantification of volatiles associated with LO to descriptive sensory attributes in dairy products in general [10]. The aims of this review are as follows: (1) to summarize the main factors influencing the oxidation of dairy powders, (2) to summarise the various analytical techniques used to detect and quantify VOC as indicators of LO, and (3) to highlight the use of combined analytical and sensory approaches to better understand the LO process in dairy powders.

2. Bovine Milk Lipids

Bovine milk fat is one of the most complex fats found in nature and varies widely from animal to animal due to factors including dietary composition [11], breed [12], seasonality [13,14], and stage of lactation [15,16]. Milk fat contains over 400 different FA [17] which originate from diet [18], microbial activity in the rumen (and transported to the secretory cells via the blood and lymph), or from synthesis in the secretory cells. The main milk lipids are triglycerides comprised of a glycerol backbone with three esterified FA. The FA are composed of a hydrocarbon chain and a carboxyl group. The major FA found in milk are: C14:0—myristic (11% w/v), C16:0—palmitic (26% w/v), C18:0—stearic (10% w/v), C18:1—oleic (20% w/v, and short chain FA (11% w/v): C4:0—butyric, C6:0—caproic, C8:0—caprylic, and C10:0—capric [19]. A milk fat globule membrane (MFGM) is a surface-active membrane with a phospholipid structure that comprises of a polar lipid bilayer, proteins, enzymes, neutral lipids, and trace components, and envelops each fat globule [20]. Approximately 25% of the FA in milk are mono-unsaturated FA (MUFA) while 2.3% are poly-unsaturated with an omega-6/omega-3 ratio of around 2:3. Trans-FA comprise approximately 2.7% of total milk FA. The amount of poly-unsaturated FA (PUFA) consumed by ruminants is an important factor in the rate at which LO progresses because they are generally dehydrogenated in the rumen by microbial action and this impacts on subsequent levels in the milk. The most abundant FA in bovine milk is α-linolenic acid (C18:3) [21]. MUFA are not oxidised as readily as PUFA, however, the most abundant MUFA (oleic acid) in bovine milk is the source of important secondary oxidation products [10]. Saturated FA in milk are generally stable compounds that are not easily oxidised and thus are not major LO contributors in dairy powders. The abundance of individual FA in milk and milk products is important [22] as they can dictate the rate at which LO progresses, but also which specific oxidation products are formed.

3. Mechanism of Lipid Oxidation

It is generally accepted that oxygen reacts naturally with many organic substrates resulting in the formation of primary oxidation products; hydroperoxides and other oxygenated compounds. There are three known types of LO that can affect dairy products; auto-oxidation, photo-oxidation, and metal induced oxidation [23].

The mechanism of the auto-oxidation of PUFA as a radical chain reaction was established more than half a century ago. The process of LO can be broken into three distinct, but partially overlapping phases of radical reactions; initiation, propagation and termination [24] (Figures 1–3). Free radicals and peroxides, both of which are highly reactive, are generated during the initiation phase when molecular oxygen reacts with unsaturated FA. In addition to oxygen, oxidative initiators such as chemical oxidisers, transition metals (e.g., copper and iron), and enzymes (e.g., lipoxygenases) contribute to the rate of the initiation phase [3]. Heat and light also exacerbate the rate of the initiation phase and the other phases of LO [25]. The rate of auto-oxidation is increased by increasing unsaturation of the alkyl chain [25], and the matrix also plays a role in the susceptibility of a product to oxidation [26]. FA alkyl chains are susceptible to oxidation at alkene bonds and neighbouring allylic carbons.

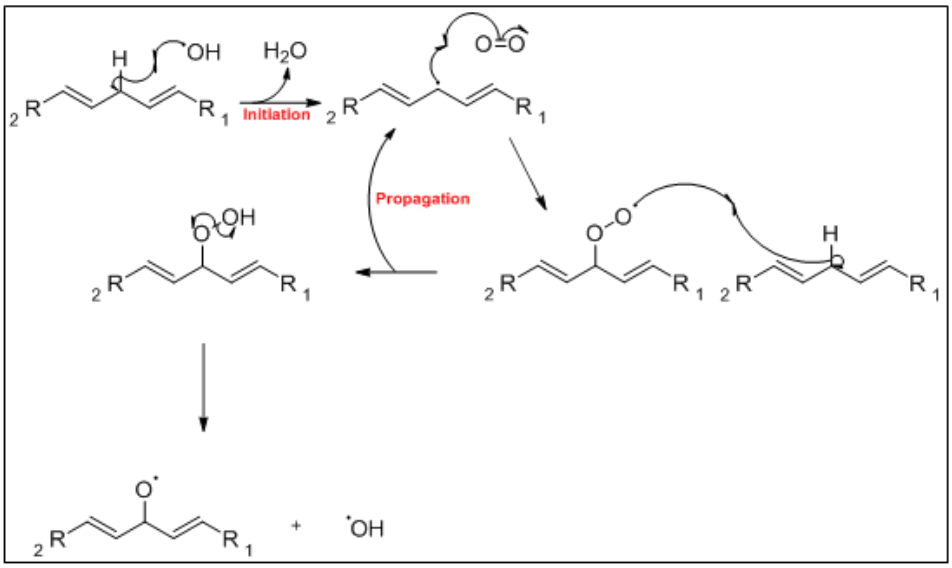


Figure 1. The mechanism of the first two phases of the lipid oxidation process; initiation and propagation.

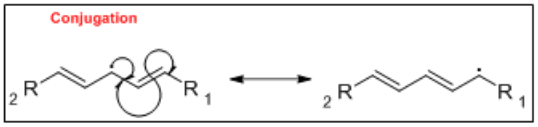


Figure 2. The mechanism of conjugation of the lipid oxidation process.

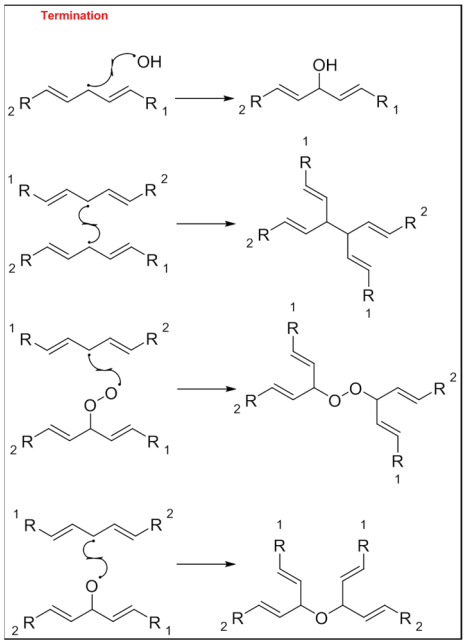


Figure 3. Common termination products of the lipid oxidation process.

Photo-oxidation and free-radical reactions at allylic carbons are responsible for the breakdown of unsaturated lipids [24,25,27]. These reactions produce hydroperoxides in these allylic bonds, and cause changes in the position and geometry of double bonds. Auto-oxidation and photo-oxidation are associated with different hydroperoxide reaction products, indicating that different reaction mechanisms are involved [28]. Photo-oxidation of milk has been well documented [23,29], exposure to light, either natural or artificial, can cause development of off-flavours in milk within 15 min [30]. The subsequent aromas have been characterised as burnt protein, cabbage-like and plastic [23], however, their

intensity can decrease the longer the milk is exposed to light, allowing newly activated off-flavours to dominate. These off-flavours have been described as cardboard-like, metallic and rancid [31–33]. Exposure to ultra-violet (UV) light can enable the oxidation of fat to volatile aldehyde compounds and has also been found to cause the degradation of sulfur–containing compounds, both of which are major contributors to off-flavours in milk [30]. A study by Silcock et al. [34] reported good correlation between negative sensory perceptions and VOC formation for milk stored in light-exposed containers, these include photo-oxidation and auto-oxidation compounds such as dimethyl disulfide, and aldehydes such as heptanal, pentanal and hexanal. For milk stored in containers protected from light, no correlation between the sensory attributes and VOC was documented.

Furthermore, the type of light the product is exposed too can also have an impact on the levels of oxidation. A study by Brothersen et al. [30] demonstrated that exposure of milk to fluorescent light (commonly used in the retail of dairy products) resulted in greater changes in LO levels, compared with exposure to white light-emitting diodes (LED). This study demonstrated that even high quality milk is susceptible to photo-oxidation at the point of sale dependent upon the type of lighting.

There are two mechanisms by which photo-oxidation may occur; (1) a radical cascade reaction initiated by the removal of a hydrogen or electron from an unsaturated allylic FA system, or (2) when oxygen is converted to its excited singlet state and reacts rapidly with an olefinic bond producing hydroperoxides on one of the original olefinic carbons and shifting of the cis bond to a trans configuration (Figure 4).

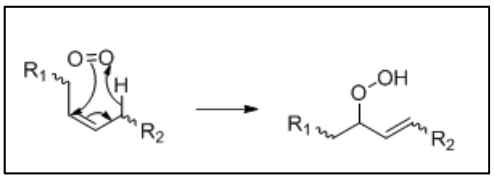


Figure 4. Ene reaction of an olefinic bond with a singlet oxygen. The formation of the hydroperoxide can happen at either of the olefinic sp2 hybridised carbons.

4. Secondary Reactions Associated with Lipid Oxidation

Various FA within milk are broken down via oxidation to primary and secondary oxidation products. The formation of a hydroxyperoxide through the oxidative mechanisms discussed earlier, breaks down to form an alkoxyl radical which splits by homolytic β-scission each side of the carbon bonded to the oxygen radical. The major FA in milk and some of their associated breakdown products are outlined in Figure 5a–e.

4.1. The Maillard Reaction

Along with LO, the Maillard reaction is an important chemical reaction that occurs in numerous foods, and both reactions have been shown to influence each other [35]. The Maillard reaction is a well-documented, non-enzymatic browning reaction between the amine groups of free amino acids, peptides or proteins and reactive carbonyl groups of reducing sugars under thermal processing and/or storage conditions [36]. This reaction can occur at room temperature, but is optimal at much higher temperatures (140–165 °C). The Maillard reaction has been identified as a main factor in quality deterioration of IMF [37]. However, in whey protein concentrate (WPC) and whey protein isolate (WPI), Maillard reaction products contribute to a lesser extent to flavour formation than LO [38,39]. The moisture content must be below 3% *w*/*w* for the Maillard reaction to conclude, a value that is not reached in most dried dairy products [40]. The Maillard reaction mechanism is outlined in Figure 6.

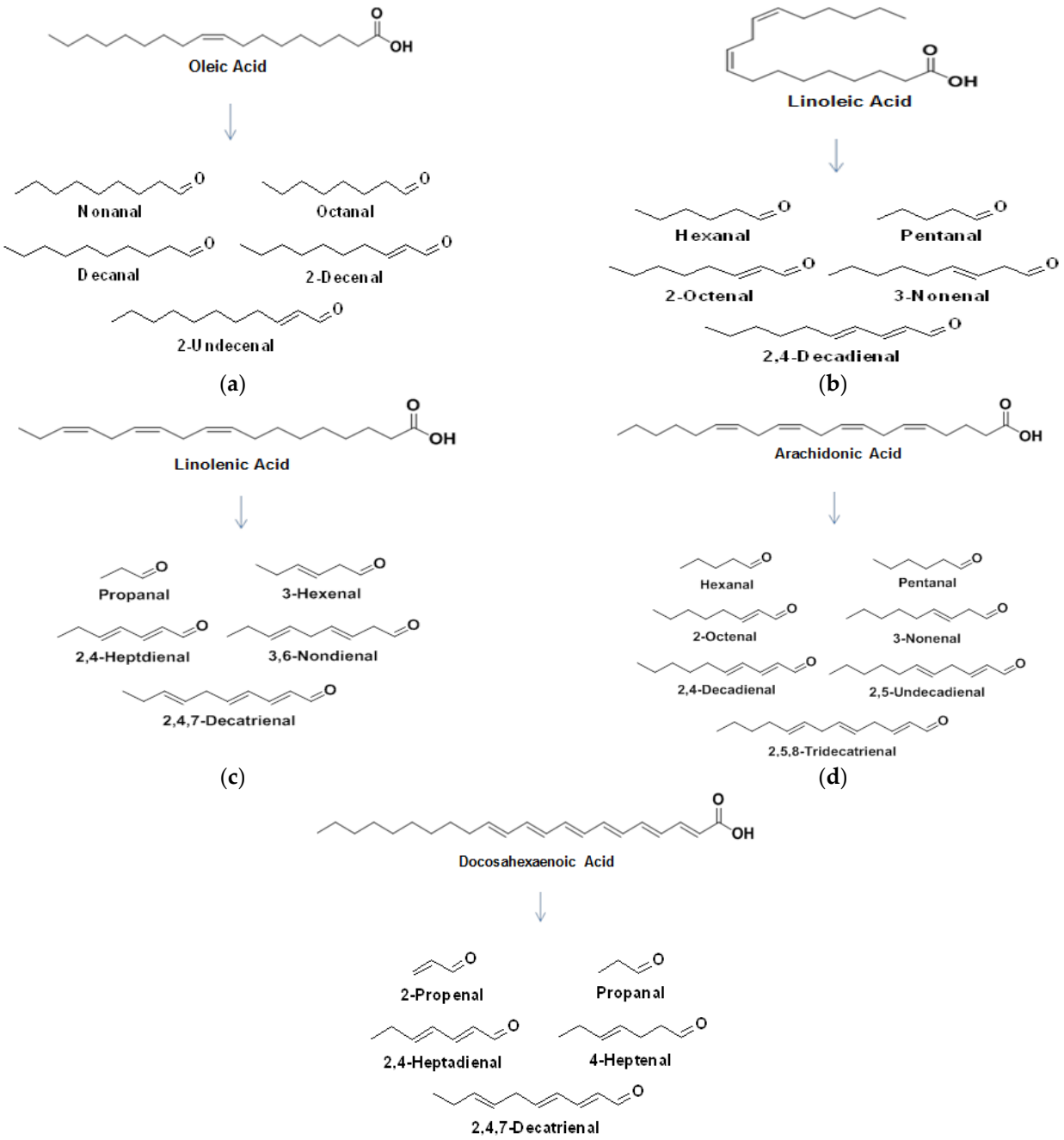


Figure 5. Some major fatty acids found in milk and some of their associated breakdown products; (**a**) oleic acid, (**b**) linoleic acid, (**c**) linolenic acid, (**d**) arachidonic acid, and (**e**) docosahexaenoic acid.

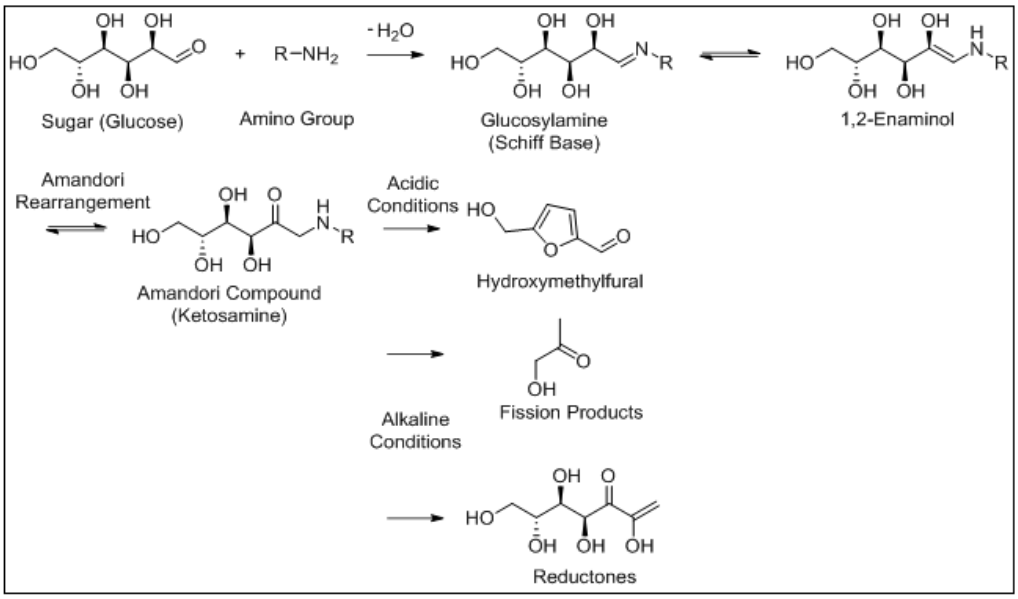


Figure 6. Schematic of the Maillard reaction of glucose with a generic amino group RNH2. The carbonyl functional group on the sugar undergoes a substitution reaction with the amino group of a protein or amino acid to form an N-substituted glycosylamine. This undergoes isomerisation by undergoing an Amandori rearrangement forming a ketosamine. This can undergo a number of reactions to produce a range of compounds which can undergo further reactions.

4.2. The Strecker Reaction

Similar to the Maillard reaction, the Strecker reaction mechanism is also linked to LO. Aldehydes are readily converted to secondary alcohols or acids and are therefore known as transitory volatile compounds with some known to be a result of Strecker reactions [41,42]. The degradation of amino acids during the Strecker reaction is one of the primary mechanisms resulting in the final aroma compounds of the Maillard reaction. The process involves the oxidative deamination and decarboxylation of the amino acid in the presence of α-dicarbonyl compounds formed in the Maillard reaction and the formation of the corresponding Strecker aldehyde [43,44]. Each amino acid produces a specific Strecker aldehyde which comprises one carbon atom less than the amino acid from which it is formed. Strecker aldehydes such as 3-methylbutanal (malty flavour) [45] and phenylacetaldehyde (honey-like flavour) are derived from leucine and phenylalanine, respectively, and are commonly reported as aroma contributors in dairy products [46]. LO and Maillard reactions interact in complex food systems and can share common chemical mechanisms and intermediate compounds [35]. Moreover, certain carbonyls derived from LO such as alkadienals and ketodienes have been shown to promote the oxidative degradation of amino acids to produce the corresponding Strecker aldehydes via Strecker-type reactions [47,48]. The Strecker reaction is outlined in Figure 7.

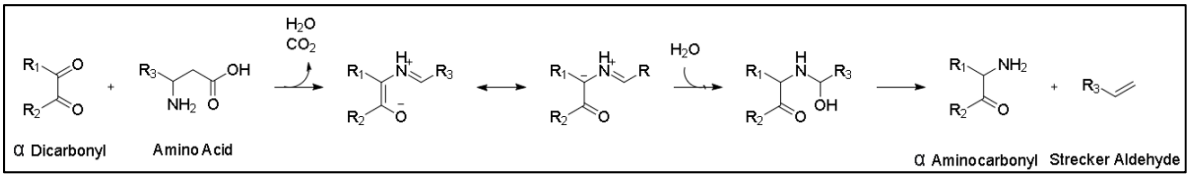


Figure 7. Schematic of the Strecker degradation mechanism, where an α-dicarbonyl compound and an amino acid undergo a decarboxylation reaction to form an α-aminocarbonyl compound and Strecker aldehyde product.

5. Lipid Oxidation in Dairy Powders

5.1. Whole Milk Powder

Due to its high fat content (26–42% w/w) and significant amount of exposed fat on its surface, WMP [6] is highly susceptible to LO during processing, transport and storage, which can adversely impact its sensory and nutritional properties. Some odour-active compounds have already been identified in WMP [49], with the most important off-flavour compounds resulting from LO such as, hexanal, other aldehydes and ketones. Determining the cause of undesirable LO changes in WMP flavour is complex owing to the fact that many of the aroma-active compounds are produced by two or more mechanisms [6,49,50]. Moreover, differences in the FA profile of WMP influences its susceptibility to LO [51,52]. Bovine feeding system is one of the major factors affecting the FA profile of milk and milk powders [11,53]. Maintaining quality and potentially increasing the storage stability and shelf-life of WMP is of great importance to manufacturers. Whetstine and Drake [49] documented changes in the flavour of WMP at ambient temperatures and found that the formation of off-flavours occurred after 3 months of storage, primarily as a result of LO. It is also important to note that off-flavours in dairy powders can carry over into product applications [49], therefore having information in relation to the LO status of the starting powder is very important not only for the oxidative stability of the powder itself but also for future applications.

5.2. Skim Milk Powder

Few studies have focused on the impact of LO on the quality and stability of SMP, likely due to its low fat content (0.6–1.25% w/w). SMP should have a flavour similar to that of fluid milk [54]; however, differences in manufacturing processes [55,56] and milk composition [57] can result in the formation of different flavour characteristics and intensities. Shiratsuchi et al. [58] was one of the first studies to profile the VOC content and flavour of SMP. The compounds identified included aldehydes, ketones, alcohols, esters, furans and, phenolic compounds, and was also one of the first studies to identify monoterpene and sesquiterpene hydrocarbons in milk. Methyl ketones were abundant in SMP, but were below their flavour thresholds. Alcohols were also found to have little influence on SMP flavour. The primary contributors to the flavour of SMP were free FA, comprising approximately 79% of the total VOC profile, however lactones were also present at high concentrations.

Abdalla et al. [57] evaluated the sensory characteristics of nonfat dry milk (NFDM) and SMP, and found that the intensity of the heat treatment used during production influenced the flavour of the final product, with medium heat powders having a cooked flavour, and low heat powders having oxidised and metallic flavours. Heat treatment can exacerbate the intensities of undesirable flavours in SMP. Whetstine and Drake [49] found that the impact of LO on the flavour of SMP was much more variable than with WMP. The authors also found that some SMP developed off-flavours immediately upon storage, while the flavour of others remained stable throughout storage. These results are somewhat surprising as it was anticipated that SMP should be less susceptible to LO than WMP due to its much lower fat content. However, the fat in WMP and in other high-fat dairy powders may act as a solvent for secondary oxidation products, especially non-polar molecules, impacting their transition to the gaseous phase. Thus, the low fat content in SMP may result in non-polar oxidative products being more easily perceived [59], as in theory they should be more easily transferred from the fat phase to the gaseous phase (or to the aqueous phase when hydrated).

5.3. Infant Milk Formula

The fat content of IMF is approximately 28% w/w and is designed to contain a FA composition similar to that of human milk. This is generally attained through the addition of fish, soya and/or vegetable oils [60]. Increased levels of PUFA from these sources may however result in an unstable product that is highly susceptible to LO [10]. For this

reason, understanding the modifications of PUFA in IMF is important with regard to the stability and safety of IMF throughout its proposed shelf-life. A review by Saphier and Silberstein [61] focused on the storage conditions of IMF and the levels of LO. The study concluded that IMF comprising more unsaturated FA was more susceptible to LO, and that exposing IMF to known LO contributors (oxygen and elevated temperatures of >37 °C) increased the rate of LO.

A study by Romeu-Nadal et al. [7] focused on the oxidative stability of milk formulas (packed in sealed aluminium foil bags flushed with N_2) that had been supplemented with various FA and stored at 25 °C and 37 °C. The study employed the use of headspace solid phase micro-extraction (HS-SPME) gas chromatography mass spectrometry (GC-MS), and sensory analysis to track important volatile markers of LO over 15 months of storage. Propanal was used to monitor oxidative changes in n-3 PUFA, with hexanal and pentanal used to monitor changes in powders fortified with n-6 PUFA. Samples stored at 37 °C were found to be less stable than those stored at 25 °C, confirming that storage temperature effects the rate of LO in IMF. Rancid off-flavour was not detected in samples stored at 25 °C until after 15 months. The combination of sensory and volatile analysis provided beneficial information on the oxidative stability of the formulations and concluded that the shelf-life of IMF is dependent on the PUFA content, storage temperature and time.

A study by Clarke et al. [51] found that LO aldehydes and ketones were excessively high in IMF in comparison to WMP and SMP. Overall, painty, oxidised, and rancid attributes were more associated with IMF regardless of storage temperature (21 °C or 37 °C). However, to date very few studies have been undertaken on the impact of LO on the volatile and sensory profiles of IMF. Cesa et al. [62] investigated the effect of storage conditions (20, 28, 40 and 55 °C) on the levels of malondialdehyde (MDA) in IMF and found that the PUFA enriched IMF samples demonstrated good stability at; 20 °C for up to 1 year, 40 °C for up to 3 months, and 55 °C for up to two weeks. A study by Jia et al. [63] investigated the stability of milk based IMF supplemented with PUFA stored at 42 °C and 50 °C for 90 days. The authors found significant differences in colour, possibly due to the Maillard reaction in addition to significant differences in the VOC profile, peroxide value (POV) and headspace oxygen over the storage period.

Most IMF LO studies have only monitored the concentrations of MDA or the POV, with very little research focused on individual VOC associated with LO. Investigating the levels of primary and secondary oxidation products (aldehydes, ketones and alcohols) in IMF in conjunction with sensory analysis will provide more information on the impact of supplementation with PUFA on the rate of LO during storage, the specific VOC involved, and in theory the concentration at which individual VOC begin to adversely influence sensory perception.

5.4. Whey Protein Concentrate and Whey Protein Isolate

Whey is used as an ingredient in many food products and generally fractionated to yield products with different compositions and functionalities [64]. WPC has a low fat content of between 3–6.6% *w*/*w*, with negligible amounts of saturated FA, PUFA, and MUFA [65]. As such, LO is not considered a major issue and therefore only limited LO studies of WPC exist.

Tomaino et al. [66] suggested that the starter culture used during cheese production can initiate the oxidation process, which influences the flavour and oxidative stability of liquid whey, ultimately, affecting the characteristics of whey powder. Compared with the control, starter cultures were found to have contributed to the production of acetaldehyde, ethanol, diacetyl, 1-propanol, and 2-propanol. Further results suggested that LO was initiated during the production of the liquid whey and was accelerated during 14 days of refrigerated storage [65].

Jensen et al. [67] studied oxidation in WPC (6.5% *w*/*w* fat) and in whey fat concentrate (WFC) for 12 months at 20 °C. WFC is the remaining fraction of WPC after WPI is removed and has a fat content of 13.5–21.5% *w*/*w*. The study evaluated the primary oxidation prod-

ucts for hydroperoxides, electron spin resonance (ESR) for radicals, secondary volatile LO products by HS-SPME GC-MS, and some protein oxidation products (dimethyl disulfide, benzaldehyde, and dityrosine) by reverse-phase high performance liquid chromatography. WFC was found to be more susceptible to oxidation than WPC, as dimethyl disulfide, benzaldehyde, dityrosine, heptanal, nonenal, hexanal, and radicals were significantly higher, likely due to the higher fat content of WFC.

WPI is produced from WPC and contains minimal fat. Berton-Carabin et al. [68] investigated LO in conjunction with protein oxidation as this is seen as a more prevalent issue in WPI owing to its very high protein content (≥90% *w*/*w*). The authors used controlled levels of LO and protein oxidation to investigate the impacts on the viscoelasticity of whey protein layers at the oil-water interface. Results demonstrated that both protein oxidation and LO led to a decrease in interfacial elasticity when compared to the samples that were not oxidised. LO induced the formation of surface active compounds, which were thought to have formed segregated domains at the interface. Limited studies of LO in WPI suggest that it is not a major issue.

Wright et al. [69] investigated the sensory and volatile stability of WPC80 and WPI stored in polyethylene lidded bins at 21 °C, at 50% relative humidity for 18 months. Sensory properties were evaluated using the descriptive spectrum method while HS-SPME GC-MS was employed to extract and characterise VOC. Differences in the sensory profiles were documented between WPC80 and WPI, in agreement with previous studies on the topic [38,70]. Sixteen VOC were quantified in WPC80 and WPI. The authors chose these VOC as they represented a range of Maillard reaction, LO, or fermentation derived volatiles that were consistently detected in 3 or more whey products. Hexanal was found to be the most abundant compound identified in fresh WPC80, followed by trans-2-nonenal. The study concluded that the flavour of both WPC80 and WPI changed during storage, with increases in the abundance of VOC. The optimum shelf-life for non-agglomerated WPC80 and WPI stored at 21 °C was 12 to 15 months, and 8 to 12 months for steam-agglomerated or lecithin-agglomerated WPC80 and WPI.

6. Main Factors Influencing Lipid Oxidation in Dairy Powders

Numerous studies have evaluated the effect of feeding system on the flavour and abundance of VOC in dairy products and bovine diet has been proven to be one of the most significant influencers of VOC and FA profiles of dairy products [71–75]. Milk fat composition can be readily modified by changing a cows' feeding regimen, but this alteration impacts the protein, urea, citrate and the soluble calcium present in the milk (list not exhaustive). Only a limited number of studies have focused on the effect of feeding system on the sensory, flavour and flavour stability of dairy powders [51,63]. Bovine dietary changes that affect the FA profile of milk are also likely to influence its oxidative stability and flavour [53], but also the stability of resultant powders. Moreover, milk quality and composition are important aspects to consider when producing products such as WMP, SMP and IMF.

The review by Chilliard et al. [76] summarised the effects of bovine forage on milk fat secretion and composition. The study highlighted the need to evaluate how different feeding systems impact on aspects of milk fat quality, such as flavour, oxidative stability and manufacturing value.

Studies investigating the composition of milk produced from many supplemented and altered diets including supplementation with: flaxseed [77], lipid complex (grapeseed oil with synthesised conjugated linoleic acid (CLA) and Atlantic mackerel oil enriched with n-3 FA) [78], iodine [79] marine algae [80], oregano and caraway essential oils [81], hull-less barley [82] and sunflower/fish oil [83] have been undertaken. These studies focused mainly on production performance, milk composition, milk yield, FA composition and to a lesser extent, on flavour and sensory characteristics of the raw, pasteurised or homogenised milk. Volatile analysis was included in most studies but very few combined this with sensory analysis or attempted to correlate both data streams.

Villeneuve et al. [84] investigated 3 types of feeding system (timothy hay, pasture, and silage) and found that untrained sensory panel members could not distinguish a flavour difference between the milk produced from the cows fed hay and the cows fed silage. However, a significant number of panellists could detect a difference between milk from hay-fed cows in comparison to milk from pasture-fed cows. A study by Faulkner et al. [85] demonstrated that feeding system can influence the sensory properties of bovine milk. The flavour compounds from forage can be transferred to milk from the cow through two pathways; by inhalation or digestion, and also through the rumen gases [86]. VOC can also be ingested by the animal and encapsulated in the fat or protein portion of the milk. Pasture based feeding has been attributed to increased herbaceous flavours in milk [85].

A study by Vanbergue et al. [87] investigated the effect of breed (Holstein and Normande), feeding system (high and low energy), and stage of lactation (early, mid and late) on milk fat characteristics in dairy cows. No significant interaction was observed between breed and feeding system. Milk yields were higher for Holstein cows compared with Normande cows throughout lactation and were significantly higher in cows that consumed the high-energy diet. In general, fat content was higher for Holstein cows, but saturated FA were higher in Normande cows and MUFA were higher for Holstein cows. Feeding system had no significant effect on saturated FA content except during early lactation where levels were higher in milk from cows fed the high-energy diet. MUFA and PUFA contents were higher in milk from cows fed grass silage (low energy) vs. corn silage (high energy) during early lactation The study clearly demonstrated the effect of cow breed and feeding system on milk fat characteristics.

It is clear that more comprehensive research is required to establish definitive links between bovine diet and the sensory attributes of subsequent milk and dairy powders, where noticeable changes in VOC and FA profile occur. As well as feeding regime, many other factors influence the sensory attributes of milk and dairy products.

7. Impact of Processing Conditions on Dairy Powders

There are several factors involved in milk processing which can affect the stability of the resulting dairy powder and its subsequent sensory characteristics, including preheat treatment [88–91], and the distribution of fat in the dried powder particles [92].

Baldwin and Ackland [91] studied the effect of 4 preheat treatments (85, 95, 110, and 125 °C) each in combination with 4 holding times (10, 20, 60, and 240 s) on the aroma and flavour characteristics of WMP stored in an air atmosphere at 30 °C for 18 months. Nine sensory characteristics incorporating flavour, aroma, and texture were evaluated by 16 trained panellists throughout storage. Some of the primary sensory attributes associated with WMP were significantly affected by preheat temperature and holding time. Cooked flavour was significantly increased by longer preheat holding time and a higher preheat temperature from 85 °C to 125 °C. Sweetness was higher when longer holding times and low preheat temperature were applied, and at shorter holding times at high preheat temperatures. Oxidised flavour was significantly affected by preheat temperature and holding time. WMP manufactured using short holding times and low temperatures exhibited weak oxidised flavour compared to WMP produced using high heat treatments and longer holding times. Oxidised flavour correlated well with oxidised aroma and was perceivable by panellists after 9 months of storage. The study concluded that preheat temperatures of 95 °C or greater and holding times of 20 s or greater are considered effective in inducing stability against oxidative deterioration. This finding was in agreement with that of Abdalla et al. [57] for NFDM and SMP.

Li et al. [88] evaluated the oxidative stability of milk powders throughout 3 and 6 months of storage at 20 ± 1 °C. Milk powders were stored in plastic bags and stored in a dry, air-tight container. In addition to HS-SPME, POV was used to evaluate the oxidative stability of the milk powders. Milk powders and concentrated milks had higher POV than raw and heated milks. The study also found increased levels of aldehydes and ketonesin stored milk powders when the concentration temperature was 40 °C as opposed to 50 °C.

Results also demonstrated that aldehyde and ketone levels in fluid milk, both raw and heated, were lower compared to levels found in concentrated milk and milk powders. The increased number of processing steps involved in milk powder production, such as preheating and spray drying were thought to be the cause of this increase. The temperature of the preheat treatment is important for regulating the technological characteristics of the final product [90].

Stapelfeldt et al. [89] demonstrated that the shelf-life of WMP depends on the preheat treatment of the milk, the temperature at which the WMP is stored and the water activity of the powder. The study compared the storage stability of low-heat, medium-heat, and high-heat milk powders at three water activity values (0.11 a_w, 0.23/0.17 a_w, and 0.33/0.31 a_w), and at two storage temperatures (25 °C and 45 °C). The freshly manufactured milk powder was packed in 400 g cans under a 70% N_2, 30% CO_2 gas mixture. The low-heat milk powder (milk pasteurised at 73 °C for 20 s followed by a preheat treatment of 72 °C) had the lowest storage stability as it was subject to severe oxidative changes and non-enzymatic-browning. However, during accelerated storage at 45 °C, the medium-heat (milk pasteurised at 80 °C for 20 s followed by preheat treatment of 72 °C), and high-heat powders (milk pasteurised 88 °C for 20 s followed by preheat treatment of 72 °C) were less susceptible to oxidative changes and enzymatic browning. In the study, thiobarbituric acid reactive substances (TBARS) was used as a measure of sensory quality and values increased to a greater extent in powders stored at 45 °C than at 25 °C.

Park et al. [93] explored the effect of homogenisation pressure on the flavour, and flavour stability of WMP. The sensory properties of the powders were evaluated at 0, 3 and 6 months of storage at 21 °C by descriptive analysis using the spectrum method [94]. The study reported the flavour profiles of WMP produced by various homogenisation treatments were distinct, and that improper or inadequate homogenisation adversely affected shelf-life and flavour stability.

Monitoring the temperature and conditions during the drying of milk is crucial to the overall quality and sensory stability of the end product. Other parameters such as particle size and microbiological stability must also be considered [95].

8. Volatile Organic Compounds Associated with Lipid Oxidation in Milk

VOC are a diverse group of carbon-based chemicals with boiling points ranging from 50 to 260 °C [96]. VOC including aldehydes, ketones, alcohols, and ethyl esters represent the primary aromatic constituents of milk. VOC are significant as their quantitative differences can explain the different odours that characterise milk and dairy powders [50,97]. The concentrations of individual VOC in fluid milk are known to affect its sensory properties [85]. GC is capable of identifying >109 molecules of an odour in 1 mL of air, but the human nose has been found to be 10–100 times more sensitive [98]. Therefore, VOC analysis in combination with GC-olfactometry (GC-O) can provide more useful information about which VOC influence sensory perception and the degree of their influence. Although GC-O has some limitations it remains a very useful technique; (1) it can be difficult to identify every odour, as some remain below the limits of detection of the GC-MS, (2) co-elution makes it more difficult to obtain dependable data on those VOC, (3) odours created through interactive effects of two or more VOC cannot be taken into account as the VOC are largely detected as individual compounds, and (4) it is very time consuming and requires extensive panellist training. In addition, some VOC found in dairy products have more than one odour descriptor that may also be dependent upon their concentration as well as the composition of the product [10]. Although the human nose is very sensitive it also has limitations, such as the 'opinion factor' of panellists, lack of standards and reproducibility due to differences in capability either related to physical, genetic or health issues [99]. A review by Kilcawley et al. [10] summarises the potentially important compounds in bovine milk and their associated aroma descriptors (Table 1).

In addition to LO, Maillard, and Strecker reaction products, other documented sources of off-flavours in dairy products include the presence of microbial-derived terpenoid

compounds, such as endo-borneol, 2-methylisoborneol and α-terpineol [100], sulfur compounds present as a result of heat treatment [101,102], or direct transfer from feed and possibly isoflavone metabolism in the rumen leading to the formation of aromatic phenolic compounds [71,103]. Thus, incorporating GC-O analysis when attempting to identify the source of off-flavours in milk can be extremely beneficial.

Table 1. Some potentially important volatiles and their associated aroma descriptors found in dairy products (derived from lipid oxidation).

Compound	Associated Aroma Descriptors	LRI	Odour Reference	LRI Reference
Aldehyde				
Pentanal	Fermented, bready, fruity	735	*	[71]
Propanal	Alcohol, earthy	506	*	
Hexanal	Cardboard like, metallic off flavour, green	837	*	
(E)-2-Nonenal	Green, fatty	1160	*	[104]
Heptanal	Fatty, oily, green, woody	901	*	[71]
(Z)-4-Heptenal	Oily, fatty, green, milky, dairy	901	*	[105]
2,4-Decadienal	Fatty, oily, green, chicken skin-like, fried	1300	*	[106]
Undecanal	Soapy, aldehydic, waxy, floral	1311	*	[107]
Ketone				
Acetone	Earthy, strong fruity, wood pulp, hay	532	[108]	[71]
2-Nonanone	Malty, fruity, hot milk, smoked cheese	1092		[109]
2-Heptanone	Blue cheese, spicy, Roquefort cheese	890		
2-Pentanone	Orange peel, sweet, fruity	727		[71]
3-Octen-2-one	Earth, oily, ketonic, sweet, hay, mushroom-like	1096	*	[51]
2,3-Octanedione	Dill, herbal, buttery	981	*	[110]
1-Octen-3-one	Metallic, mushroom-like	1294	*	[111]
3,5-Octadien-2-one	Mushroom-like, fatty	1030	*	[71]
Alcohol				
1-Heptanol	Sweet, green, woody	972	*	[71]
1-Octanol	Waxy, green, citrus, floral, sweet, fatty, coconut	1116	*	
1-Pentanol	Fermented, sweet, balsam, yeasty, solvent-like	794	*	
1-Hexanol	Green, herbal, alcohol, sweet	894	*	

LRI: Linear retention indices on a DB5 column; * Odour reference from The Good Scents Company [112].

9. Qualitative and Quantitative Measurement of Lipid Oxidation Compounds in Dairy Products

There are various techniques and strategies used to measure LO in dairy products. Some commonly used, relatively simple, and practical methods to assess LO are POV, TBARS, and the KREIS test. Their widespread use is mainly due to ease of use and low cost, although they are more qualitative rather than quantitative.

Several analytical methods have been optimised for detecting off-flavours associated with LO in dairy products, such as solvent-assisted flavour evaporation (SAFE), GC-MS [113], and GC-O [114]. GC-flame ionization detection (FID) or GC-MS have become the methods of choice for quantitative VOC analysis. These approaches are undertaken in combination with a specific method to extract and concentrate the VOC using either static or dynamic headspace techniques, sorption-based techniques, liquid based extraction or solvent assisted techniques. As previously mentioned, care must be taken not to increase VOC associated with LO during the analytical technique, as previous studies have demonstrated that certain LO VOC can increase between 37 °C and 60 °C [115], therefore, including appropriate controls is necessary to prevent false positives.

9.1. Peroxide Value

POV is still widely used by the food industry as a qualitative indicator of oxidative stability in dairy products as it is inexpensive and relatively easy to use. The titrimetric method is described in the AOAC standard [116], and the spectrophotometric method

is described by Østdal et al. [117]. The basis of the assay is a solvent separation, followed by a reaction and absorbance reading at 470–500 nm. However, its accuracy and usefulness is questionable as it only considers the first stage of the LO reaction i.e., the initiation phase [118]. Thus, in theory sensory properties can deteriorate further (due to hydroperoxides breaking down to form odour active oxidation products such as aldehydes, ketones and alcohols) without any increase in POV values. It is also not possible to make a judgement on the sensory characteristics of a product using the POV as hydroperoxides are generally tasteless and flavourless [119]. In addition, the breakdown of hydroperoxides can occur at a faster rate than their formation, therefore it is still possible to have sensory issues at low POV levels. However, the POV can be used as a tentative non-specific, non-quantitative indicator of quality deterioration over time [120].

9.2. Thiobarbituric Acid Reactive Substances

TBARS methodology is a relatively simple spectrophotometric assay and remains widely used in the food industry. Some widely acknowledged limitations include a lack of specificity as the method uses the formation of MDA to represent the overall formation of aldehydes, thus providing no information on any individual LO volatiles. In addition, the TBARS reaction is not specific to MDA; the presence of any sugar can react with the thiobarbituric acid and yield a colour change, leading to an overestimation of the extent of LO [121]. The method also fails to account for the numerous aldehydes, ketones and alcohols resulting from LO that are responsible for off-flavours associated with LO in dairy products. Another disadvantage of this method is the requirement for solvents and the associated risk assessments [122]. Studies report the levels of MDA in milk to be between 0.028–0.036 ppm [123], and higher in milk powders (0.3 ppm) [124] and IMF (0.1–1.2 ppm) [125].

9.3. KREIS Test

The KREIS test was one of the earliest methods used to determine the oxidative deterioration of vegetable oils and is similar to TBARS in that solvents, a colour change and spectrophotometric measurements are involved. The primary reagent in the KREIS test (phloroglucinol) reacts with aldehydes and ketones to develop a pink colour i.e., a positive result. The KREIS test does not appear to be as widely used as TBARS or POV. A study investigating rancidity in edible oils [126] has linked certain odour-active aldehydes identified using the KREIS test with deterioration in quality and has promoted the use of the KREIS test for the early detection of scission products of FA [127]. The presence of some aldehyde compounds that are not associated with rancidity have been shown to give false positive results and other compounds such as vanillin were shown to interfere with results [128]. Overall, the KREIS test is not considered a reliable LO indicator [129,130].

9.4. Physical Evaluation Methods

The application of NMR spectroscopy monitors the change in FA profile in oils by calculating the ratio of aliphatic to olefinic protons. NMR profiling has previously been applied to evaluate variations in the milk metabolite profile [131,132] and more recently has been employed as a tool to identify and determine the quality of the lipid fraction of organic and conventionally produced bovine milk with emphasis on metabolites with potential health benefits [133]. Other physical evaluation methods have been employed to determine the oxidative stability of dairy products, including ESR spectroscopy [33] and evaluating the presence of conjugated dienes present as a result of PUFA oxidation by UV absorption at 234 nm. However, this method was not considered useful for early detection of VOC that cause sensory defects in powders [134]. It is also possible to qualitatively assess the conjugation of PUFA dienes by refractometry [135] and infrared spectroscopy [136].

9.5. Analysis of Volatile Organic Compounds by Gas Chromatography

Some of the most commonly utilised volatile extraction methods used in combination with GC to assess LO are outlined below, namely HS-SPME, thermal desorption (TD), SAFE, and sorptive extraction (SE).

HS-SPME has become a standard approach for the volatile profiling of food samples [137]. The basic principle of HS-SPME is that the sample of interest is placed in a sealed vial and heated under controlled conditions so that an equilibrium of the VOC is formed in the headspace which is representative of the sample. However as with all HS techniques, the nature of the sample matrix as well as the chemical properties of the individual VOC have a significant influence on the release of VOC. HS-SPME has become the most widely used volatile extraction technique as it requires minimal sample preparation, is solventless, fully automatable, easy to use, very versatile due to the wide range of fibre phases available, reproducible, and is relatively inexpensive [138]. Once the VOC equilibrium is formed, a polymer phase coated fiber is exposed to the headspace under controlled conditions (time, temperature and agitation). The VOC interact with the phase(s) and the fiber is retracted and subsequently desorbed for 2–3 min in a heated GC injector port (typically between 250–270 °C). The desorbed VOC are transferred onto the GC column in an inert gas flow and separated by their interaction with a GC column phase on heating in a column oven, and subsequently detected and/or quantified either by FID or MS. Thus far, HS-SPME GC-MS techniques have been applied to a variety of dairy products such as raw and pasteurised milk [139,140], dairy powders [141–143], and liquid or powdered IMF [144,145]. A review by Merkle et al. [146] summarised the recent developments and applications of HS-SPME for analysis of complex food matrices. The study mentioned the occurrence of the matrix effect i.e., the binding of analytes to the matrix resulting in low concentrations of the analytes in the headspace. Thus, the matrix effect may be an issue when developing a HS-SPME method for the extraction of volatiles from dairy products with increased fat contents. In an effort to off-set the matrix effect, response surface methodology has been used to determine the most useful HS-SPME extraction parameters for the quantification of VOC associated with LO in dairy powders [141].

TD also works on the bases of heating samples to allow VOC reach the gaseous phase. As with HS-SPME, TD is used as an extraction and pre-concentration step prior to analysis by GC. VOC and some semi-VOC are extracted by this technique onto suitable phases packed into TD tubes, with many different phases available that can target individual VOC or chemical classes, or for more generic untargeted approaches. Removal of the trapped compounds from the phase(s) onto the GC column involves heating of the TD tube in a gas flow, and sometimes further concentration is possible using an in-line focusing trap. TD has been applied to a number of dairy products [85,147], milk powder [148], and IMF [149]. A number of application notes are also available on the use of TD [150–152]. Its widespread use in dairy products may be limited as moisture management can be problematic.

SAFE is a useful method for the isolation of volatiles from complex food matrices. Engel et al. [153] reported that the application of SAFE to model solutions containing a range of aroma compounds resulted in increased yields from both solvent extracts and fatty matrices (50% fat) when compared with high vacuum transfer. SAFE could be particularly useful for longer chain, fat soluble compounds that have difficulty reaching the gaseous phase. SAFE has been used to prepare volatile extracts from dairy products for GC-O evaluation; Bendall [154] used SAFE to extract volatiles from milk to be analysed via GC-O, 71 different aroma-active compounds were isolated from the milk, 66 of which were identified. However, SAFE has limitations, such as tedious sample preparation, the requirement for solvents, requirement of expensive specialist glassware, reproducibility issues, manual sample manipulations, and may require further concentration steps prior to introduction to the GC [155].

Stir bar sorptive extraction (SBSE) is a solventless technique with simple sample preparation that has been used for flavour research of dairy products including dried dairy ingredients, and milk. Traditionally this technique employed glass-encapsulated magnetic

bars with a sorbent coating to extract volatiles. Stir bars can be immersed within a liquid sample or suspended in the headspace of a solid, liquid, or gaseous sample during the extraction process. Volatiles are typically thermally desorbed followed by a cryofocusing step and GC-MS [156–158]. Baltussen et al. [156] was one of the first to describe this technique, and employed a polydimethylsiloxane (PDMS) phase to extract and concentrate the VOC. Park and Drake [159] used SBSE for the extraction of flavour compounds from NFDM concentrated by reverse osmosis or evaporation and found that the volatile profiles were consistent with the descriptive sensory results.

Faulkner et al. [85] achieved good results for milk samples (up to 65 volatile compounds from a range of chemical classes) using a new high capacity SE technique called HiSorb followed by GC-MS analysis. Currently PDMS-coated stir-bars are the only phase commercially available for these techniques, which somewhat reduces the applicability of SBSE to the extraction of non-polar compounds due to the poor extractability of more polar analytes [157]. However, Ochiai et al. [160] demonstrated that solvent-assisted SBSE improves peak resolution and extraction efficiency of polar and non-polar compounds. Moreover, Schiano et al. [161] concluded that solvent-assisted SBSE provided the most consistent detection of selected compounds in commercial milks, although the levels of compounds detected were not significantly ($p > 0.05$) higher compared to conventional SBSE or SPME extraction methods. Some of the most common techniques used for the extraction of volatiles from dairy powders are outlined in Table 2.

9.6. Gas Chromatography Olfactometry

As previously mentioned, the advantage of including GC-O analysis is that it allows trained human assessors to identify VOC that are aroma-active and thus contributing to sensory perception in real time [168]. This enables VOC that are contributing to the overall flavour to be identified and even their potential sensory influence in terms of intensity and character to be defined. The integration of GC-O and GC-MS and/or GC-FID techniques also makes it possible to establish direct relationships between a compound present in a food sample and any correlated odour. However, it is important to note that odours are extremely complex mixtures often consisting of numerous VOC which vary in concentration. VOC can interact synergistically or additively to produce the overall odour of a product [169]. Therefore, while it is beneficial to know which VOC in a sample are odour active, the overall odour of a product can differ from that of each individual VOC. Friedrich and Acree [170] and Rychlik and Bosset [171] provided good descriptors of VOC present in dairy products as detected by GC-O. Kobayashi and Nishimura [172] employed thirteen panellists to compare WMP samples from different regions using GC-O analysis, and concluded that the differences between the WMP based on region was caused by differences in the balance of the aroma-active VOC present.

A limited amount of studies have included GC-O analysis for the VOC in SMP samples. Karagül-Yüceer et al. [166] undertook GC-O analysis on six NFDM powders, a product that is consumed directly as well as being used as an ingredient in other preparations. The study identified a wide range of aldehydes, ketones and free FA which were found to be responsible for the generation of flavours in NFDM over storage. Samples were analysed by GC-MS, GC-O, and sensory analysis. Methional, a Strecker degradation product of methionine, was identified as an off-flavour compound with its corresponding aroma being characterised as boiled potato-like [173,174]. These methods ensured a comprehensive evaluation of the products sensory characteristics in terms of the main source VOC responsible. This study also verifies the importance of raw milk quality even as an ingredient in end product applications.

Table 2. Summary of the primary methodologies used for volatile extraction and analysis of dairy powders.

Method	Advantages	Limitations	Applications	Reference
		Extraction Methodology		
Headspace solid-phase microextraction (HS-SPME)	• Minimal sample preparation • Does not require organic solvents • Simple to use • 'Clean' method in comparison to LC • High sample throughput • Reproducibility • Large selection of phases	• Fiber saturation • Low phase capacity • Possible carryover of compounds	• Wide range of volatiles in food products • Raw and pasteruised milk • Liquid and powdered infant formulas • Milk powders	[137–139,141,142,144]
In-tube extraction (ITEX)	• Does not require the use of solvents • Dynamic extraction • Well matched to the analysis of trace organic compounds	• Repeatability issues • Possible issues with moisture and needle blockage	• Volatile organic hydrocarbons from aqueous samples	[162]
Thermal desorption (TD)	• Good sample throughput • Minimal sample preparation • Does not require organic solvents • Large selection of phases available • Sample collection and enrichment capabilities	• Tedious if not automated • Moisture control	• Bovine milk • Milk and cheese • Milk powder	[85,147,148,150–152]
Solvent-assisted flavour evaporation (SAFE)	• Simple method • Capable of rapid and in situ identification of volatile compounds	• Requirement for solvents • Expensive glassware • Requirement for risk assessment	• Milk • Skim milk powder (SMP)	[154]
Stir bar sorptive extraction (SBSE)	• High effectiveness for the extraction of non-polar and medium-polarity compounds • Large amount of phase • Good sensitivity and recovery • Automated systems under development	• Manual removal and washing of stir bar required if not automated	• Liquid samples or liquid extracts • Dairy products • Can be used for headspace analysis	[151,159,160,163]

Table 2. *Cont.*

HiSorb extraction	• Effective for the extraction of volatile and semi-volatile compounds • Large amount of phase • Possible to perform immersive and headspace extraction • Automated systems available	• Extended extraction times • One phase currently available	• Liquid samples or liquid extracts • Can be used for headspace analysis	[164]
Identification Methodology				
Mass Spectrometry (MS)	• Powerful compound identification abilities • Can compare spectra to libraries • Useful for unknown analysis • Qualitative analysis • Versatility	• Reproducibility for quantification purposes	• Various volatile and semi-volatile dairy and food products	[51,71,86]
Flame Ionised Detector (FID)	• Reproducibility • Sensitivity • Reliability • Quantification abilities	• Requires standards for identification • No identification ability	• Various volatile and semi-volatile dairy and food products	[165]
Gas chromatography olfactometry (GC-O)	• Ability to link volatile organic compounds to odour descriptors • Provides good odour descriptors • Allows for odour thresholds to be determined	• Time consuming • Ongoing requirement for panel members • Must be coupled with the correct extraction method—possible method development required	• SMP • Any food sample with odour above threshold level	[166]
GCxCG-ToF-MS (Time of Flight-MS)	• Good for the separation of complex mixtures • Generation of 3D plots • Good sensitivity • Enhanced resolution • Ability to separate co-eluting peaks in the second dimension • Ability to reduce or enhance elements of the chromatogram in the second dimension	• Complexity of the data generated	• Milk lipids	[167]

10. Sensory Analysis

Regardless of the processing dairy products undergo, consumer acceptance remains primarily based on appearance and flavour [175]. As milk has a naturally subtle and somewhat bland flavour, any development of off-flavours is relatively easily perceived by the consumer [58]. The impact of different feeding systems, production regions, cultural differences and storage conditions have been identified as the motivating factors behind dairy product purchase [72,73,85]. Consumers have a desire to know more about where their milk and milk products are coming from and how they are produced [176]. Previous sensory studies have employed between 25 and 100 panellists for consumer testing and this technique has been widely applied to dairy products [177–179]. Full descriptive sensory analysis requires fewer panellists as they are trained on how to specifically assess the product using pre-defined sensory attributes [52]. Panellists are not asked about their own liking or preferences toward the product, but rather they are employed as calibrated analytical instruments to give results on the intensity of particular descriptors known to be characteristic to that product. When recruiting a panel for sensory analysis, screening tests are performed to ensure each panellist provides an accurate and reliable result. Selection factors include; continued availability, health status, ability to perceive flavours, familiarity of the product under evaluation, previous experience, allergies, and medication. However, even with a stringent selection protocol, the 'opinion factor' continues to play a role in sensory analysis due to genetic and cultural differences between panellists [180].

When implementing quantitative descriptive sensory analysis, efforts should be made to ensure the sampling technique is consistent across the panel and each panellist understands how the odours and flavours are to be interpreted and described. Selecting the product descriptors (lexicons/attributes) in the final evaluation is generally a consensus process, decided upon in a focus group, conducted prior to sensory evaluation [181]. The final descriptors must comprehensively describe the sensory attributes and their intensities.

Combining sensory analysis with any of the aforementioned GC techniques provides more detailed information on numerous aspects of dairy products including consumer acceptability, levels of rancidity, extent of LO, and intensities of compounds with known odour descriptors. Moreover, the concentration of LO compounds perceived as unacceptable can be determined. The review by Kilcawley et al. [10] summarises recent studies that included sensory analysis of dairy products and the various sensory methodologies used.

Table 1 summarises the aromas associated with some important volatile compounds in milk. LO generally results in off-aromas and flavours in dairy powders including painty (hexanal, nonanal) [6], cardboard-like (hexanal, pentanal) [6,8], metallic (hexanal, pentanal, vinyl ketones) [182], and fishy (carbonyl compounds, 2,4-unsaturated aldehydes, trimethylamine) [182]. Some studies have suggested that producing dairy powders below 4% *w*/*w* moisture can delay the development of fishy and tallow off-flavours [183], however it is most likely also dependent upon the range of factors that are known to influence the concentration of the various VOC responsible. Sulfur compounds can be problematic as they have very low odour thresholds and are as associated with cooked flavours in ultra-heat-treated milk [184].

Jo et al. [185] documented an interaction between milk proteins and sulfur compounds in milk, affected by serum proteins associated with casein during heat treatment. The study confirmed that hydrogen sulfide and carbon disulfide contributed to eggy and sulfur/burnt flavours in heat-treated milk, respectively. Interestingly, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide, and methional were found not to be associated with sulfur/burnt and eggy flavours in heat-treated milk.

Various studies have investigated the impact of oxidation on the flavour and stability of dairy products [6,50,69,93,186]. A review by Su et al. [187] summarises the sensory lexicons used for the evaluation of dairy products and established the connection between off-aroma lexicons and volatile formation pathways existing in dairy ingredients. This review concluded that many off-aromas are as a result of protein, fat, and sugar breakdown

products from lipid degradation and Maillard reaction pathways. The review suggested that to minimise off-aromas developing in dairy products, in particular high protein formulations, a high quality starting material is required, and processing parameters should be monitored and adjusted accordingly to decrease the rate of flavour degradation.

11. Conclusions

There are numerous factors that influence the rate of LO in dairy products; such as cow breed and diet, stage of lactation, levels of PUFA and unsaturated FA, storage and processing conditions (exposure to heat, oxygen and/or light). Processing conditions require monitoring to ensure the quality of the end product is consistent and free from any undesirable flavours. The quality of the raw milk used for the production of dairy powders is very important, but also the manner in which the milk is handled, processed and stored has a significant impact on the extent to which the milk fat is oxidised throughout its shelf-life. LO has been shown to impact on the quality, nutritional and sensory properties of various dairy powders resulting in undesirable flavours and reduced shelf-life. Many analytical techniques are available for the qualitative and quantitative analysis of LO in dairy powders, many of which can be used in combination to elucidate more detailed information. This is especially the case where sophisticated techniques such as GC-MS can be used to identify and quantify individual VOC associated with LO, but in combination with GC-O and/or sensory analysis can provide a much more in-depth understanding of the whole LO process pertaining to a specific product. Therefore, using this approach to determine the concentrations of VOC associated with LO that adversely impact sensory perception, can provide insights into the production parameters that could maximise shelf-life and product quality of dairy powders.

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