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Effect of intramuscular fat on beef eating quality, flavour generation and flavour release

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Abstract

Meat Standards Australia (MSA) graded beef striploins (n=42) from grass-fed and grain–finished Angus and grass-fed Wagyu, covering a range of nominal marbling levels from low, medium to high, were obtained from Northwest Tasmania in December 2012. The lowest fat beef samples were the AngusGrass (5.2% low, 7.8% medium and 9.9 % high), followed by the AngusGrain (10.2% low, 13.7% medium and 14.9% high fat) and the WagyuGrass (7.8% low, 10.9 % medium and 17.5% high). The concentration of omega-3 fatty acids and conjugated linoleic acid in the intramuscular fat was higher in grass-fed beef compared to grain-fed, in agreement with the literature. Standardised 25 mm steaks were cut and frozen for later sensory evaluation and flavour analysis. A trained panel (*n*=10) was used to develop an appropriate grilled beef vocabulary to measure sensory attributes across the samples. More than 30 consensus attributes were applied to assess beef odour, flavour, taste and texture attributes. Most sensory attributes were directly correlated to the level of marbling (MSA-MB) regardless of the breed or feed type. As a whole, the overall *flavour intensity*, *beef flavour, caramel odour, grassy flavour, oily mouthcoating* and *sweetness* positively increased with marbling. As the level of marbling decreased*, acidity, astringency, hay/grainy* and *liver* flavours became more apparent. More subtle flavour and texture differences were elucidated when breed and feed comparisons were made. Analysis of headspace volatiles by gas chromatography-mass spectrometry indicated that the concentration of most volatile compounds increased with marbling, especially the alkylpyrazines. These compounds are strongly associated with grilled beef flavour. Further analysis of the samples showed that highly marbled beef had unique temporal (time-related) flavour release properties. Key odour-active volatiles were released more rapidly in the mouth at higher levels of marbling leading to more intensely perceived flavour. Similarly, the in-mouth rate of release of non-volatile taste compounds (free amino acids and organic acids) during eating was more rapid in higher marbled samples. Different ratios of sweet and bitter amino acids in the grilled beef corresponded to different taste properties. Overall, beef with high marbling scores > 500 MSA-MB, or a fat content of > 7.5% fat, fed on either grass or grain have quite similar flavour properties. Only the samples with the lowest fat content (< 5%), i.e. the AngusGrass low fat, were characterised by higher *acidic, metallic* and *lingering* aftertaste attributes. Taken as a whole, the data clearly demonstrate the essential role of intramuscular fat in generating beef flavour and on controlling flavour release as well as improving texture attributes.

Executive Summary

Beef with high intramuscular fat (IMF) or high marbling is a premium product enjoyed by consumers for its unique sensory properties. The generally positive influence of IMF on meat palatability (tenderness, juiciness) is well known, however how marbling affects the flavour and other sensory attributes is less certain. In this study, we examined the effects of marbling on the flavour and sensory properties of beef, and also the potential impacts of using different breeds (genotypes) and production (feed) systems. Three distinct sample types were evaluated over a range of marbling levels defined by their Meat Standards Australia marbling score (MSA-MB); Angus grain-fed (AngusGrain) (MSA-MB 500-830), Angus grass-fed (AngusGrass) (MSA-MB 320-730) and Wagyu grass-fed (WagyuGrass) (MSA-MB 620-1110). Samples were grilled to a medium doneness (final internal temperature of 57 ^{o}C) according to the MSA standardised protocol and subjected to descriptive profiling by a trained sensory panel (n=10). In general, after taking into account the effect of IMF, breed and feed-related sensory differences were small. In contrast, most sensory attributes were significantly correlated to the MSA-MB marbling scores regardless of sample type. *Odour Impact, Caramel* and *Grilled Beef* odour increased with marbling score, whereas *Hay/Grainy* odour decreased. *Barnyard* odour was detected only in the lowest fat AngusGrass samples. *Flavour Impact, Grilled Beef, Dairy fat, Bloody* and *Grassy* flavours increased as the level of marbling increased and *Hay/Grain* and *Livery* flavour notes decreased. As the beef marbling increased, the *Saltiness, Sweetness* and *Oily mouthcoating* became more intense and the *Sour/Acidity, Acidic aftertaste*, *Lingering Aftertaste* and *Astringency* decreased. In terms of differences between the sample types, the following general statements applied:

- WagyuGrass compared to AngusGrass (Breed effect). WagyuGrass samples were more *Tender*, more *Juicy* and required a lower *Number of Chews* compared to the AngusGrass samples, after correcting for differences in marbling. WagyuGrass was also *Sweeter*, higher in *Caramel* and *Hay/grain* odour, and *Dairy Fat* and *Grassy* flavour than the AngusGrass.

- AngusGrain compared to AngusGrass (feed effect). AngusGrain had higher *Caramel* odour, higher *Grassy* and *Dairy Fat* flavour and was *Sweeter* compared to the AngusGrass. The AngusGrain was also more *Tender* and *Juicy*.

- After accounting for differences in marbling, there were no odour, flavour or texture differences between the WagyuGrass and AngusGrain samples. The samples differed in taste and aftertaste attributes however; WagyuGrass was less *Acidic,* had less *Acidic Aftertaste* and had lower *Oily Mouthcoating*

Most of the texture-related sensory attributes were positively correlated with Warner-Bratzler shear force measurements as expected. Cooking moisture loss and the amount of liquid lost during resting of grilled meat decreased with increasing marbling. The time to reach the grilling endpoint increased with the level of marbling only for the AngusGrain samples. TBARS, a measure of the oxidative stability of lipids was significantly higher in the WagyuGrass samples, suggesting a different IMF composition compared to the other sample types. The residual meat glycogen content was highest for the AngusGrass and lowest in the WagyuGrass.

As expected, the fat content of the meat increased with MSA-MB. The fat content ranged from an average of 5.2% for the AngusGrass low fat, to an average fat content of 17.5% for the WagyuGrass high fat. The data clearly showed that high levels of IMF can be attained in the Wagyu breeds on a pure grass diet. The concentration of omega-3 fatty acids and conjugated linoleic acid was higher in the grass fed samples compared to the grain-finished, however the grain-fed Angus still contained these “healthy” lipids. In general, as the marbling level (MSA-MB) increased the ratio of saturated to unsaturated lipids in the IMF increased.

A total of 28 odour active volatiles were identified in the headspace of grilled beef samples by olfactometry. The concentration of most volatiles increased with IMF (MSA-MB), especially for 2 and 3-methylbutanal and the alkylpyrazines. Furthermore, differences in rates of volatile production during grilling were assessed in real-time using proton transfer reaction mass spectrometry (PTR-MS). Apart from demonstrating a novel application of this technology, it was clearly demonstrated that the amount of volatiles produced during grilling were strongly correlated to the level of marbling. The PTR-MS was also applied to monitor the release of volatiles in the mouth during eating. The experiments showed that highly marbled beef had unique temporal flavour release properties. Key odour-active volatiles such as 2- and 3-methylbutanal were released more rapidly in the mouth at higher marbling levels leading to more intensely perceived flavour.

The concentration of free amino acids and other non-volatile flavour compounds generally increased in the meat with grilling. The greatest increases occurred in the higher fat samples (AngusGrain and WagyuGrass) compared to the AngusGrass. After grilling, total free amino acids, organic acids and carnosine were highest in WagyuGrass. The concentration of sweet amino acids was higher and the concentration of tryptophan (bitter) was lower in the WagyuGrass and AngusGrass samples compared to the AngusGrain. These differences may also explain the higher *Acidity* and *Astringency* in the AngusGrass samples.

Oral breakdown studies clearly showed that the higher fat samples formed fine particles more quickly than low fat beef. Similarly, the in-mouth rate of release of non-volatile taste compounds such as glutamic acid (umami), methionine (meaty, sweet) and lysine (umami) during eating was more rapid in higher marbled samples, probably leading to more intense perceived flavour. The larger volume of saliva that was produced during consumption of grass-fed compared to grain-fed samples approached significance, suggesting differences in the IMF fatty acid composition may be responsible. Maximum saliva concentrations of serine (sweet), succinic acid (sweet, umami) and aspartic acid (umami) were significantly higher and lactic acid was lower in the Wagyu grass-fed grilled beef, perhaps contributing to the low acidity scores in these samples.

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1 Background

1.1 Research Context

The unique flavour and sensory properties of grilled beef ensures its enduring popularity with consumers around the world. The flavour sensation of grilled beef arises from a complex interaction of aroma volatiles, taste and texture components with olfactory, taste and other mechanosensory receptors during eating. Aroma molecules produced through high temperature grilling and taste-active components released into the warmed meat juice are combined in a unique flavour delivery system comprising a matrix of muscle fibres, collagen and intramuscular fat (IMF). Other factors being equal, it is generally accepted that intramuscular fat or marbling has a positive effect of on the palatability or eating quality in primal beef cuts, although there is no universal consensus (Dikeman 1986). Increases in IMF are generally accompanied by improvements in tenderness, juiciness and mouthfeel (MSA-07, 2010). Marbling describes the small flecks of fat deposited between individual muscle fibres. Marbling scores are taken at either the 10^{th} /11^{th} rib or at the 12^{th} /13^{th} rib on the carcase (MSA-07) on the exposed rib eye or striploin muscle (M. l*ongissimus Dorsi*). Australian and other international meat quality grading systems award a premium for beef with higher marbling scores.

Two systems for rating marbling are used in Australia; the AUS-MEAT system and the Meat Standards Australia (MSA) marbling score system (MSA-MB). The latter is essentially the same as the system developed by the United States Department of Agriculture (USDA). In the AUS-MEAT system, a series of reference standards are used to assign marbling scores from 0 to 10. The MSA-MB system provides a finer scale for measuring marbling; the scoring system ranges from 100 to 1190 in increments of 10. Although highly marbled meat is considered a premium product in many countries, mainly due to the positive effect on eating quality and texture, the relationship between marbling or IMF and beef flavour is less than clear. Many studies show little or no significant affects of IMF on beef flavour scores. In a comprehensive American study (Wheeler, Cundiff & Koch 1994), the relationship between marbling and palatability and flavour was examined in *Bos Taurus* (1,337 animals) and *Bos indicus x Bos Taurus* cross breeds (330 animals). The researchers found small increases in tenderness and juiciness with increased marbling from trace to moderate, but no changes in beef flavour intensity. A recent European study, combining information from 5000 carcasses and 20 breeds in the BIF-BEEF database, found only a weak but significant relationship between IMF and flavour scores (Hocquette *et al.* 2011). A Japanese sensory study comparing high and low fat Wagyu, found no difference in either tenderness or flavour, although the high fat samples were given higher juiciness ratings (Okumura *et. al.* 2007).

In an Australian study, a positive curvilinear relationship between IMF and consumer flavour scores was reported (Thompson 2004). MSA consumer flavour scores increased in a curvilinear relationship with IMF over a range of 0.3% to 15% fat. It was concluded that gains in flavour and juiciness scores plateau between 15 and 20% IMF, with little increase in these sensory attributes with further increases in IMF.

It would be valuable for the Australian beef industry to obtain a clearer understanding of the relationship between IMF and flavour. Better knowledge of this relationship could be applied to better differentiate products on the basis of unique sensory and flavour characteristics for the purposes of marketing and in creating consumer interest in marbled beef. It is

acknowledged within the Australian beef industry, that the importance of flavour in the MSA quality assurance scheme is likely to play a bigger role in the future. The MSA consumer testing system (Polkinghorne *et. al.* 1999) has enabled a very successful quality assurance system; however it allows only a single overall flavour score. This is entirely appropriate for consumer testing. Using a single consumer flavour score allows thousands of meat samples to be assessed around the world every year, adding valuable consumer data to the MSA database. Untrained or naïve consumers however, generally find it difficult to articulate and discuss the nuances of flavour in detail. Detailed information on specific sensory attributes requires the use of trained sensory panels.

1.2 Intramuscular Fat Effects on Meat Sensory Properties – What is Known?

Millar (1994) and others (Drewnowski & Almiron-Roig 2010) have presented various theories about how fat might impact on the sensory qualities of meat (and food in general). Fat may affect the sensory qualities of grilled beef by:

- **Changes in bulk density**
  There is simply less muscle fibre and collagen per unit volume of meat, decreasing the amount of mastication or oral processing required forming a bolus to swallow the meat

- **Decreases in connective tissue toughness**
  The deposition of fat disrupts the network of muscle fibres decreasing toughness. It has been proposed by some researchers that increasing levels of IMF contributes to meat tenderisation by disrupting the organisation of intramuscular connective tissue. Li et al. (2006) found that cook losses, collagen solubility, Warner Bratzler shear force, and perimysial thickness decreased with increasing marbling scores in Wagyu longissimus muscle.

- **Lubrication effect**
  Fats and oils generally facilitate particles of food to slide over each other through its lubricating (slipperiness or oiliness) effect. Fat increases the viscosity (thickness) of saliva and also acts as a binder or glue assisting in formation of a solid bolus in preparation for swallowing

- **Increasing saliva flow**
  There is evidence of the existence of fat-specific receptors, mainly obtained in animal models. The existence of receptors for free fatty acids in humans remains controversial (Cygankiewicz et al. 2013). It has been suggested that fat increases parasympathetic saliva production, perhaps through free fatty acids, increasing perceived juiciness, although extensive scientific evidence for this effect appears to be lacking and inconsistent

- **Changes in mouthfeel**
  Mouthfeel is a wide-ranging sensory term describing the totality of texture interactions that occur in the mouth and how they change during chewing and oral processing. Fat generally makes food softer. The fat in food may produce a coating that remains on

oral surfaces, leading to "mouthcoating" sensations. As many flavour molecules are fat soluble, this may result in greater persistence of fat soluble flavours in the mouth

- **Acting as a substrate for production of odour active volatiles**
  Fats, especially unsaturated fats, and phospholipids undergo complex oxidation reactions, especially under high temperature conditions. Many of the volatile compounds formed have high odour activity and low olfactory thresholds. While low levels of fat derived volatiles are associated with desirable flavour, excess quantities are associated with oxidised and rancid flavours. Reactive intermediates from fat oxidation reactions can react with other flavour pathways such as Strecker degradation of amino acids and other Maillard reaction intermediates.

- **Acting as a solvent for dissolving flavour volatiles**
  Many flavour molecules are highly fat-soluble; especially those that are generated from fat itself. Once formed, fat-soluble flavours tend to partition in the fat globules within the food and saliva affecting flavour release. Less release of odour volatiles may decrease the perceived aroma of a food.

- **Concentrating non fat-soluble flavour molecules in aqueous phase**
  Many flavour compounds are not very fat-soluble. For example salt (Na^+ ions) and many free amino acids do not readily dissolve in fat. By increasing the amount of fat in a food matrix, the water soluble, polar molecules are "pushed" into the aqueous phase increasing their relative concentration. This may increase the perceived intensity of the flavour. Similarly volatiles with low fat solubility are pushed into the headspace in the presence of significant levels of fat.

The important effect of fat as a substrate for the generation of meat aroma (volatile) compounds has been widely documented (Elmore & Mottram 2006); however there are many inconsistencies in the literature past and present. Oxidation of lipids has often associated with rancidity and off "warmed over" flavours in meat and other food systems. This might imply that high-fat beef may have a greater tendency to spoil more rapidly. Common fat derived aldehydes, hexanal, octanal, nonanal, and alcohols, 1-heptanol and 1-octanol have been associated with positive flavour characteristics in cooked meat at certain levels, but with rancidity and off-flavours at higher levels. The effects of lipids on Maillard reaction pathways are complex. Farmer and Mottram (1990) showed that different lipid types, e.g. phospholipids or triacylglycerols, either promote or suppress the formation of different classes of Maillard volatiles in a system with ribose and cysteine. Generally the phospholipid fraction was shown to be more reactive than triacylglycerols. The presence of fat has been shown to affect the rate of volatile release in meat (Carrapiso 2007) and non-meat systems (Frank *et al.* 2011, 2012).

1.3 Feed and Breed Effects on Marbling and Beef Flavour

Much has been written about the influence of different feed systems on meat quality and flavour, beef being no exception (Van Elswyk & McNiell 2014, Duckett *et al.* 2013, Brewer 2013). In a recent review of studies comparing grass and grain feeding systems, it was concluded that grass-fed beef is on average less tender than grain fed beef, but of similar

juiciness (Van Elswyk & McNiell 2014). A number of studies have concluded that overall beef flavour is less intense in grass-fed beef. Ducket *et al*. (2013) compared sensory characteristics of steers finished on either forages or a corn-based concentrate over a three year period. Despite lower average IMF in the pasture-fed animals *vs* grain-fed (US-marbling score 409 vs 657, $p < 0.001$), no differences were found in juiciness or tenderness. Beef flavour intensity was significantly lower in the grass-fed animals compared to grain-fed (3.77 vs 4.79, $p < 0.001$) and "Off-flavour" was higher in grass fed beef (2.71 vs 2.08, $p < 0.001$). The pasture finished samples were also more sour compared to the grain-finished animals ($p = 0.05$). In another recent American study (Maughan *et al.* 2012) grass-fed and grain-fed grilled beef was compared using a consumer and sensory panel. The trained panel found that grass-fed beef was higher in "negative" sensory attributes, such as *Gamey, Barny, Bitter* and *Grassy* and lower in positive sensory attributes such as *Umami, Roast beef, Browned* and *Fatty* flavours. The grass-fed beef was rated as only slightly liked (6.08 on a 9-point scale) compared to grain-fed beef that was moderately liked (7.05 on a 9-point scale) by a consumer panel. The practical significance of such a small difference in consumer-liking scores, i.e. 1 point on a 9-point scale seems questionable; however the difference was statistically significant. Despite some shortcomings in the use of the sensory scale, there was a significant flaw in their experimental design. All of the grass-fed samples used in the study contained on average 3% fat, whereas the grain-fed samples were on average closer to 12%. Such a large difference in the fat content of beef (or any food for that matter) will inevitably lead to large sensory differences. As has been noted in the review by Van Elswyk & McNiell 2014, it is often the case in most published studies, that the pasture-fed samples are lower in IMF than the grain-fed samples. In many cases, the effect of IMF differences has not been specifically accounted for in statistical analyses.

In another consumer study (Garmyn *et al.* 2014, in press), Australian grass-fed and Australian and American grain-fed beef were compared. Grass-fed beef received overall lower consumer scores for tenderness, flavour and overall liking. The researchers did not specify the marbling or fat level in the samples used. If there was a difference in the IMF between the grass- and grain-fed samples that may have been reason enough for the lower overall liking as fat and flavour are likely to be inextricably linked. It is well known that if the texture and juiciness of meat is lower, there is a tendency to rate flavour and overall liking attributes lower as well. It should also be noted that the consumer testing was conducted in Texas, where the majority of consumers would be used to eating grain-fed rather than grass-fed beef. Lack of consumer familiarity with grass-fed flavours may have also been a significant factor in the negative flavour rating.

It is important to realise that consumer tastes and preferences vary from country to country, depending largely on their collective experience and familiarity with a product. American beef consumers are more familiar with grain-fed beef than grass-fed, for example, and may be biased towards what they are familiar with. Even within a population, there is normally a degree of segmentation, with one group of consumers preferring one product and another preferring another. With respect to grain-fed and grass-fed flavour differences, apart from consumer data, there is very little reliable trained sensory panel data to characterise exactly how the sensory and flavour properties of beef produced from these different production systems might vary. Finally, another problem with a single consumer overall flavour score is that it is not possible to discern whether a lower flavour score has been given because there is simply less flavour or because it is less desirable – two quite different propositions.

Consumer preferences are made up of both intrinsic and extrinsic factors. Intrinsic factors of beef include tangible quality attributes, such as tenderness, juiciness, flavour, colour etc., Extrinsic factors affecting consumer perception of beef may include the packaging, price, animal welfare issues, the perceived environmental sustainability and the perceived healthfulness. There are many proponents of the idea that pasture-fed beef has a better nutritional profile — less saturated fat, more unsaturated fatty acids and other healthy fats — and is more "natural" than grain-fed beef (Van Elswyk & McNiell 2014, Daley *et al.* 2010, Scollan *et al.* 2006, Dhiman *et al.* 1999). While we do not seek to investigate these nutritional claims in this current research, there is abundant scientific evidence that there are differences in the composition of the lipids present in the IMF of pasture-fed beef that may have an impact on the sensory characteristics. There are mixed reports that there are discernible desirable and negative sensory and flavour characteristics in beef from pasture-based systems. A strong belief that pasture fed beef may be more nutritious would almost certainly influence ratings of flavour and sensory properties by consumers. Enthusiasts also claim that grass-fed has discernible positive flavour attributes. Application of a trained sensory panel allows an objective assessment of how beef from grain- and grass-fed systems differ. Unique grass and grain-fed flavour profiles, if they exist, could be used to better market and appreciate meat produced from either system. Since the level of fat or marbling is generally poorly controlled in published studies, we have controlled for this in the design and statistical analysis in this study.

Highly marbled beef is almost synonymous with the Wagyu breed (Japanese black). Wagyu is genetically disposed to rapid and high deposition of IMF. Wagyu beef can contain as high as 30% extractable fat (Okumara 2007). While Wagyu is undeniably genetically disposed to high IMF deposition, other breeds such as Angus can also attain high marbling levels, especially on a high nutritional plane (Wheeler *et al.* 1994). Much scientific research effort has been devoted to understanding the genetics of marbling and improving breeding towards high IMF deposition (Johnston & Grasser 2010, Indurain *et al.* 2010, Albrecht *et al.* 2006, Pethick *et al.* 2005).

1.4 Outline of Current Research Rationale

In contrast to consumer testing, comprehensive detailed and objective analysis of flavour requires training a group of professional sensory panellists. Although the initial training is a significant time investment, well-trained human assessors can be used as an objective, accurate and finely calibrated scientific instrument to quantitatively measure defined sensory attributes across samples. The main objective of the current study was to train a sensory panel to characterise and measure the subtle changes that occur in grilled beef flavour with increasing IMF. A further objective was to obtain objective sensory data regarding the possible impacts of animal diet and genetics or breed. Finally, using established and novel flavour chemistry and sensory techniques, we sought to better understand and characterise some of the fundamental changes that occur in beef as the marbling increases.

To achieve these research goals, a comprehensive range of IMF values was obtained in beef striploins from grass-fed Wagyu, grain-fed Angus and grass-fed Angus across low, medium and high marbling ranges. All animals were sourced from the same area of Northwest Tasmania (Cape Grim area), and slaughtered in the same abattoir, to minimise the influence of climatic and abattoir variables.

2 Sample Selection

2.1 Sample collection

The main objective of this research was to comprehensively examine how IMF affects beef eating quality, specifically how it impacts flavour generation (beef aroma) and the release of flavour during eating. Hence it was critical to obtain samples across a broad range of marbling or IMF levels representing typical commercially available beef (**Figure 1**). MSA-MB scores from MSA graded carcasses were used as the selection criterion for IMF. The secondary aim of the study was to objectively assess the possible sensory and flavour differences, brought about by the use of different feeding systems; i.e. pasture or grass-fed compared to grain/feedlot diets. A further secondary objective was to also assess the contribution of genotype or breed to the sensory characteristics of highly marbled beef, i.e. Wagyu vs Angus. Previous published research indicates that genetic or genotype differences generally have the largest effects on the eating quality and flavour of meat. The influence of diet is less consistent. A number of production variables such as climatic variations, animal stress (e.g. from heat, cold or long transport times) and differences in abattoir practices are known to affect meat quality. Hence, it was essential to control for these variables as much as possible in sourcing samples for the study.

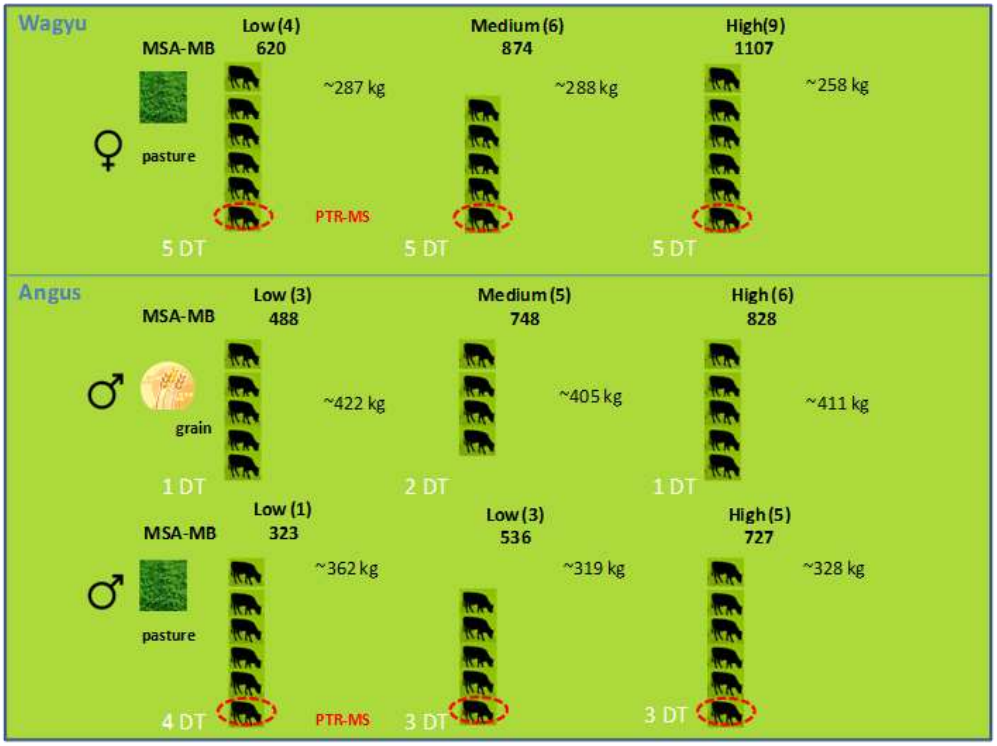

Figure 1: Diagram summarising the experimental design and numbers of animals used. Three feed x breed combinations (WagyuyGrass, AngusGrain & AngusGrass) were selected covering a range of low, medium and high marbling scores (MSA-MB). For the main sensory and flavour experiments 14 animals from each sample type were selected (n=42) were used for the main experiments. Additional animals (circled in red) were used in additional experiments. A total of 48 animals were obtained for the study.

By sourcing all animals from the same part of Tasmania, and using the same abattoir, the effects of these variables were well controlled across sample types. Wagyu grass-fed (WagyuGrass), Angus grass-fed (AngusGrass) and Angus grain-fed (AngusGrain) animals of known genetics were available. In the original balanced design it was hoped that grain-fed

Wagyu beef could also be included in the design; as grain-fed Wagyu was not in production in Tasmania at the time of the study, only the three sample types previously mentioned were used.

Use of genetically identical pure-Angus animals raised on both grass and finished on grain diets allowed for a valid diet comparison. The Wagyu and Angus grass-fed animals were essentially grazing on pasture in the same part of Northwest Tasmania (Cape Grim), ensuring minimal diet variation to allow for true breed effects to be measured if present. In all cases full MSA carcass grading data was available.

Full-blood Japanese Wagyu grass-fed heifers from Robbins Island, Northwest Tasmania (Hammond Farms) and pure Angus grain-finished steers (~200 days) (Tasmanian Feedlot Pty Ltd., Perth, Tasmania) were transported to the Greenham Pty Ltd abattoir (Smithton, Tasmania) and were killed in a normal commercial run, on 12 December 2012. Pure Angus grass-fed yearlings (Muirhead Enterprises, Cape Grim, Tasmania) were killed on the following day (13 December 2012). Carcasses were graded by a Meat Standards Australia inspector the next day after overnight storage in chillers and assigned MSA marbling score (MSA-MB).

After grading, the range of marbling scores available within each sample type was discussed with the grader. CSIRO scientists selected replicate animals according to low, medium and high marbling bands and labelled selected carcasses (all right sides). Carcasses were labelled and tracked into the boning room and full striploins were recovered. The bulk of subcutaneous fat was removed, before whole striploins were packaged under vacuum and chilled. Refrigerated (2 ^{o}C) striploins were transported to CSIRO Animal Food and Health Sciences in Coopers Plains (Brisbane) and aged for 28 days at 2 ^{o}C, before cutting into steaks for sensory and other analyses.

2.2 Carcass characteristics

The final mean carcass characteristics for the three sample types used in the study are summarised in **Table 1**. Five samples from the low and high MSA-MB marbling bands and four from the middle marbling band were obtained for the central sensory experiment. Additional WagyuGrass and AngusGrass samples (circled in red, **Figure 1**) were obtained for additional experiments. Further information is available in the Appendix (**Table 20**). Differences in dentition scores indicated significant differences in animal age; WagyuGrass heifers were older than the AngusGrain steers and the AngusGrass yearlings. In general, it is known that marbling increases with animal age and age differences are inevitable across such a broad marbling range. Other differences in carcass attributes were measured; weight, L* a* b* colour scores, hump and eye muscle area (EMA). Many of these parameters are expected to change with animal age. Of note was the significantly lighter (L*) and redder (a*) colour of the grain-fed samples compared to the grass-fed.

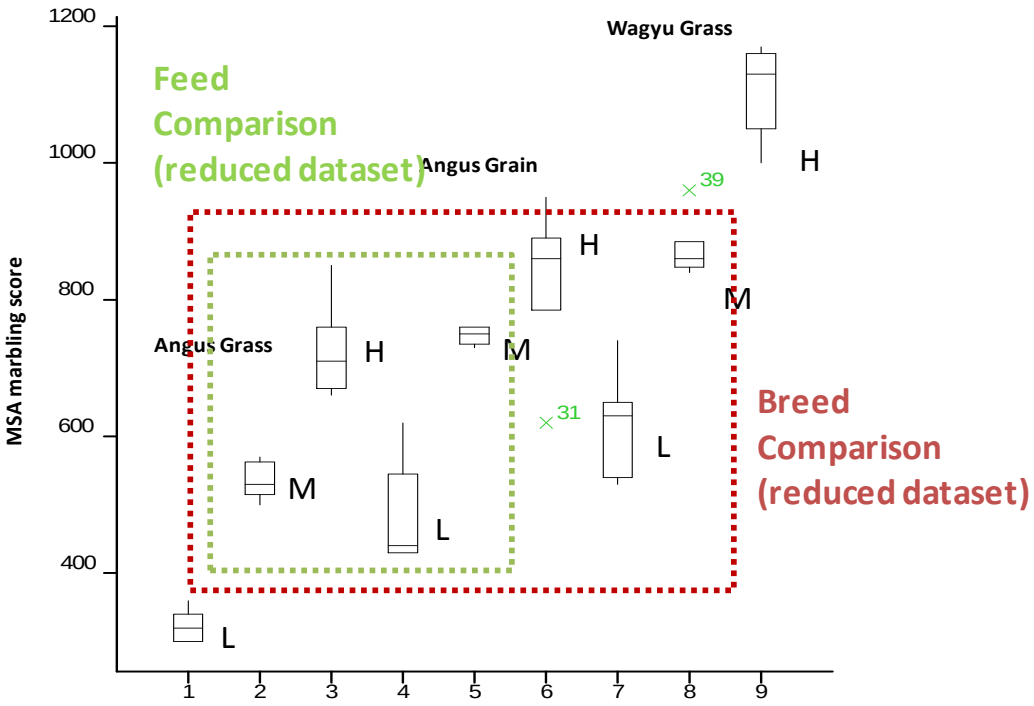

Figure 2: Boxplot summarising the mean and variance in MSA-MB scores of samples obtained for the study. The AngusGrass samples were at the lower end of the range, the WagyuGrass samples were at the high end. The AngusGrain samples were in the middle range. L = low fat, M = medium fat & H = high fat samples.

The final range of marbling scores obtained is summarised graphically in

Figure 2. A good spread of MSA-MB scores was represented by the samples. The AngusGrass yearlings represented the lower end of the MSA-MB scale (300-850). AngusGrain steers occupied the middle range (430-950), whereas the WagyuGrass heifers were at the high end of the MSA-MB marbling scale (530-1170). The average fat content of the AngusGrain and WagyuGrass samples were similar. Both were significantly higher in fat than the AngusGrass samples. The effects of marbling or IMF on sensory and flavour scores were tested across all samples. The effects of diet were assessed by comparing AngusGrass and AngusGrain samples. The effect of breed was assessed by comparing the WagyuGrass and AngusGrass animals. To minimise the effects of extremely low and high IMF samples, reduced datasets were also used for breed and feed comparisons (**Figure 2**).

Table 1: Summary of average carcass characteristics of animals obtained for each sample type and (more detail available in Appendix 1). LSD = least significant difference.

Sample Type	AngusGrass n=17	AngusGrain n=13	WagyuGrass n=17	P Sample Type	LSD
Sex	**M**	**M**	**F**		
Classification	**Yearlings**	**Steers**	**Heifers**		
AUS-MB	2.88	4.62	6.24	<0.001	1.324
MSA-MB	528	703	866	<0.001	138.8
% Fat	7.6	12.9	12.5	<0.001	2.8
EMA	62.6	74.1	69.5	<0.001	5.54
CWT	337.2	411.1	277.2	<0.001	21.88
Dentition	3.06	1.08	4.82	<0.001	0.96
Hump	47.35	48.85	42	0.012	4.62
Oss	158.2	142.3	156.2	0.25	20.28
pH	5.57	5.52	5.54	0.04	0.042
L*	35.2	42.48	36.28	<0.001	2.82
a*	30.8	33.24	30.11	0.01	2.02
b*	23.36	25.77	23.01	0.02	2.02

2.3 Preparation of Standardised Steaks

After aging, striploins were removed from the chiller and transferred to a hygenic meat-processing facility (CSIRO Animal Food and Health Sciences, Coopers Plains, Brisbane). Vacuum bags were opened and bags and striploins were weighed. After removal of meat, the amount of liquid remaining in the packaging and absorbent material was weighed and the dip loss (%) was calculated. An experienced butcher was engaged to cut the striploins into standardised steaks (manually) using a measuring guide. Steaks were cut according to MSA specifications with the following dimensions: 25 mm thick x 25 mm wide and 75 mm long.

Depending on the size of the striploin, either one or two layers of steaks were cut along the muscle according to the diagram in **Figure 3**. Steaks from each section of the muscle were numbered, labelled and packed into bags of five steaks each. Steaks from the thick anterior end of the striploin were used in all sensory panel work. Replicate steaks were randomised across sessions and panellists from different positions from the same anterior end of the striploin muscle. Steaks from the posterior end were used for flavour analyses and other experimental work. A ~10 cm thick meat sample was removed from the posterior end and packaged and frozen for texture and other chemical measurements; TBARS and glycogen.

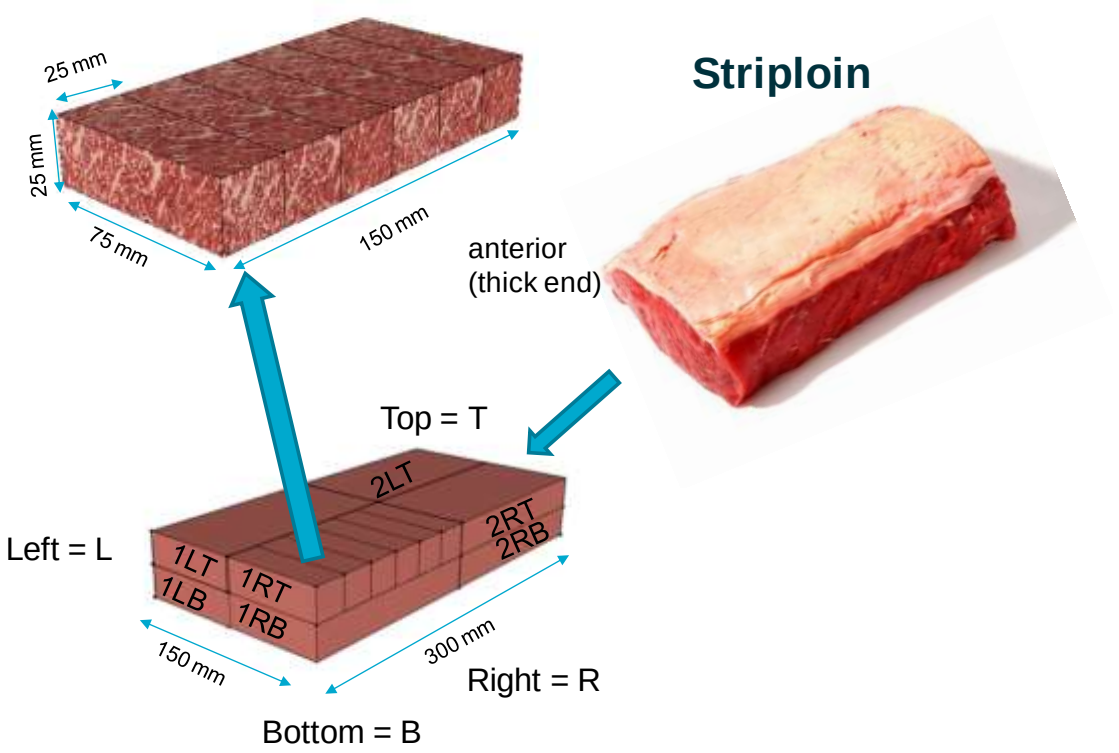

Figure 3: Diagram showing how steaks were cut from the striploin (*M. longissimus dorsi*) muscle for use in sensory assessments and other analyses. Five adjacent steaks were packaged together and frozen for later use.

3 Trained Sensory Panel Assessment of Beef

3.1.1 **Standardised Grilling Protocol**

The same grilling protocol was used throughout this study unless otherwise indicated. Frozen steaks were laid out on aluminium foil on trays according to the randomised presentation order and covered with cling film to defrost overnight in a temperature-controlled chamber (4 °C) prior to cooking. Samples were grilled on a SILEX-grill, which was pre-heated to 220 °C at least 45 min prior to cooking. A hand-held infrared thermometer was used to periodically measure the grill surface temperatures. Both grill surfaces were sprayed with light olive oil. A thermocouple probe was inserted into the middle of the first steak (five steaks of the same type were cooked simultaneously) and the samples were placed in the middle of the grill, the lid was closed and steaks were cooked until a final internal temperature of 57 ºC was reached (Luchak *et al.* 1998). The time to reach the final temperature was recorded using a stopwatch for each set of steaks (in seconds). After grilling, samples were covered loosely with aluminium foil and left to rest for 3 minutes. Moisture loss during cooking of meat was assessed by weighing the sample before and after cooking. Rest loss was measured by

weighing the amount of juice collected in the foil during resting and expressed as the % mass of cooked meat. Samples were immediately cut into four equal pieces. Two pieces were placed into a standard wine glass and covered. Randomly coded samples were then placed on a tray and served to each panellist in the sensory booths.

3.2 Panel Training and Vocabulary

A ten-member beef flavour sensory panel was recruited from the pool of regular, dedicated CSIRO panellists in February 2013. The panel was composed of nine females and one male; average age = 51± 6 years. Panellists were remunerated for their time. All assessors had been screened for sensory acuity and had extensive prior experience in participating in descriptive sensory analysis of products. All samples were prepared and assessed at the sensory facility located in CSIRO Animal Food & Health Sciences in North Ryde, Sydney. Five two-hour training sessions were held to generate and define the sensory vocabulary that best described the differences in odour, flavour, taste, texture, aftertaste and after-feel attributes. Assessors were exposed to samples representing experimental design variables (i.e. WagyuGrass, AngusGrass and AngusGrain samples from each IMF level) during training.

More than 30 consensus attributes were developed for final application to assess beef odour, flavour, taste and texture attributes (**Table 2** & **Table 3**). A number of published studies were used to guide the development of the final vocabulary, during two-hour focus groups conducted over a two-week period (Duckett *et al.* 2013, Maughan *et al.* 2012). Samples were rated using a 100 mm line scale on a computer screen. Panel performance was monitored using Panel Check Software (Nofima Mat, Norway) with regular feedback until every panellist had a clear understanding of attributes.

During panel training, the sensory panel:

- Determined the order of evaluation of sensory modalities (i.e. odour, flavour, texture, taste and aftertaste/after-feel) as well as the order of attributes within each sensory modality.
- Selected reference standards and used selected pure chemical compounds where appropriate to clarify, improve understanding and define sensory attributes.
- Developed the final consensus sensory vocabulary used in evaluations consisted of 29 attributes (9 odour, 9 flavour, 3 taste, 6 texture and 5 aftertaste/after-feel attributes).

The list of attributes, definitions, related terms and reference standards is provided in (**Table 2** & **Table 3**).

3.2.1 Method of assessment

Assessors were served with two pieces of grilled beef weighing approximately 3 g each in a covered wine glass. The assessors were asked to assess odour first, by removing the lid and sniffing the sample (orthonasal evaluation). Assessors were asked to assess *overall odour impact* and other odour attributes. They were allowed to re-sniff the sample headspace as many times they required. For assessment of flavour attributes, panellists were instructed to take a whole piece using a skewer, place in the mouth and evaluate flavour attributes (retronasal evaluation) during eating. Attributes were rated on the computer screen in the order of their perception determined according to the panel consensus.

Prior to assessing texture, taste, aftertaste and after-feel attributes, the assessors were told to cleanse their palate by drinking plain water or eating a piece of cut cucumber. For assessing texture, taste, aftertaste and after-feel attributes, they were instructed to place the second piece in the mouth and start chewing using molars. The texture attributes *Juiciness* and *Tenderness* were assessed after 3 and 10 chews. Panellists were instructed to continue chewing and counting chews until the point of swallow and to rate the amount of *Connective Tissue* just before swallowing and to enter the *Number of Chews* to swallow. Aftertaste and afterfeel attributes were assessed 30 seconds after swallowing the sample.

3.3 Descriptive sensory analysis

Evaluations were carried out in individual sensory booths under white light. Each booth had a computer screen and sensory attributes were rated using a 100 mm line scale and a computer mouse. Samples were presented in a randomised order in wine glasses coded with randomised three-digit code. Samples were presented monadically in covered wine glasses to each sensory booth. The trained sensory panel carried out descriptive sensory analysis in triplicate using the agreed method of assessment and the consensus sensory vocabulary. Sensory evaluation of samples was performed in triplicate over a three-week period, resulting in a total of 30 sensory assessments per sample (n=10 panellists x 3 replicates) x 42 samples x 32 attributes.

For evaluation, a blocked design with 12 samples per session was used. Within blocks, order of sample assessment was randomised. A one-minute inter-stimulus interval was imposed between samples and a five-minute break was imposed after every six samples to reduce assessor fatigue. Plain water and cucumber slices were used as palate cleansers. For each sample, after completing the evaluation of odour and flavour, the assessors were asked to take a drink of water or a bite of cucumber before proceeding to texture, taste, aftertaste and after-feel attributes. Between samples, the assessors were asked to drink water as well as have cucumber slices to cleanse their palate to prevent any carryover.

The samples were blind-coded with random 3-digit codes. The experimental design was produced using the design generation package – CycDesigN. The order of presentation was randomised, with balanced numbers of low, medium and high IMF samples for each sample type included on each day. Attributes were rated on 100 mm unstructured line scales anchored at 5% and 95% for each descriptive attribute. Data were recorded and stored using the Compusense sensory data acquisition software (Version 5.2, 2004; Compusense Inc., Guelph, Ontario, Canada).

3.3.1 **Statistical Analysis**

Unless otherwise indicated, all statistical analyses were performed using GENSTAT 15th Edition (VSN International Ltd, Hemel Hempstead, United Kingdom). Replicate sensory data were subjected to multivariate analysis of variance (MANOVA). Appropriate post-hoc multiple comparison tests (Fisher's least significant difference; LSD) were carried out where significant differences were found.

For assessment of animal effects, replicate sensory data from all samples were assessed using and 'animal x panellist' fixed factor design. Sample type differences were assessed by MANOVA by comparing the three distinct feed x breed combinations; WagyuGrass, AngusGrass and AngusGrain, using "sample type x panellist' as a fixed effect and coding MSA-MB as a covariate term. For the assessment of pure breed effects, data from the AngusGrass and AngusGrain samples were compared by MANOVA using a 'feed type x panellist' fixed factor design; the MSA-MB score was used as a covariate. For breed effects, MANOVA was performed using a 'breed x panellist' fixed factor design, once again with MSA-MB coded as a covariate.

To further characterise relationships between the sensory attributes of beef samples and the IMF level (MSA-MB), Pearson's correlation coefficients were calculated by Genstat and the relationship was subjected to a two-sided test for significance. Relationships were further explored using the Linear Regression with Groups function in Genstat. Sample type, e.g. WagyuGrass, AngusGrass or AngusGrain were used to group samples to better understand potential differences.

Principal component analysis (PCA) was conducted using Genstat to summarise the similarities and differences between the samples and to visualise the relationships between all the samples and the sensory attributes. PCA bi-plots (PC1 and PC2) were generated by Genstat and used without modification in the report.

Table 2: Table summarising the final consensus odour and flavour sensory attributes used to assess the grilled beef samples

ODOUR	Definition	Anchors	Related terms	Reference standard
Overall impact	*Intensity of the overall aroma*	low to high		
Grilled beef	*Odour associated with grilled beef*	low to high	*barbeque, roasted*	
Livery	*Odour associated with grilled liver*	low to high		grilled beef liver
Metallic	*Odour associated with iron*	low to high	*minerals, iron tablets*	iron tablet solution
Bloody	*Odour associated with fresh blood*	low to high		beef blood juice
Caramel	*Sweet odour associated with toffee*	low to high		caramelised sugar solution
Barnyard	*Odour associated with stables*	low to high		p-cresol (1 ppm)
Hay /grainy	*Odour associated with dry hay or unprocessed grains*	low to high	*hay-bale, dried grass*	
Fishy	*Odour associated with oxidised fish oil*	low to high	*fish oil*	fish oil tablets
FLAVOUR	***Definition***	**Anchors**	**Related terms**	**Reference standard**
Overall impact	*Intensity of the overall flavour*	low to high		
Grilled beef	*Flavour associated with grilled meat*	low to high	*barbeque, roasted*	
Livery	*Flavour associated with grilled liver*	low to high		
Metallic	*Flavour associated with iron*	low to high	*minerals, iron tablets*	iron tablet solution
Bloody	*Flavour associated with blood*	low to high		
Dairy Fat	*Flavour associated with milk, butter and other dairy products*	low to high	*milk, butter, cream*	unsalted butter
Grassy	*Flavour associated with freshly cut grass*	low to high	*green, leafy*	hexanal (20 ppm)
Hay/grainy	*Flavour associated with dry hay or unprocessed grains*	low to high		
Fishy	*Flavour associated with fish*	low to high	*fish oil*	fish oil tablets

Table 3: Table summarising the final consensus taste, aftertaste and texture sensory attributes used to assess the grilled beef samples

TASTE	Definition	Anchors	Related terms	Reference standard
Sweet	*The perceived intensity of sweet taste*	low to high	*minerals, iron tablets*	iron tablet solution
Salty	*The perceived intensity of salty taste*	low to high		beef blood juice
Sour /acidic	*The perceived intensity of sour/acidic taste*	low to high		caramelised sugar solution
AFTERTASTE	***Definition***	**Anchors**	**Related terms**	**Reference standard**
Acidic aftertaste	*The residual intensity of acidic taste*	low to high		
Metallic aftertaste	*The residual intensity of iron taste*	low to high		
Astringency	*The dry puckering sensation of the mouth surfaces*	low to high	*mouth drying, dry mouthfeel*	
Oily mouth coating	*Amount of oil left on mouth surfaces*	low to high	*greasy, fatty, tallow*	
	The intensity of the aftertaste after swallowing the sample	low to high		
TEXTURE		**Anchors**	**Related terms**	**Reference standard**
after 3 chews				
Tenderness	*Force required to bite through the sample*	low to high		
Juiciness	*Amount of juice released from the sample*	low to high		
after 10 chews				
Tenderness	*Force required chew between molars*	low to high		
Juiciness	*Amount of juice released from the sample*	low to high		
Connective tissue	*Amount of connective tissue/fibrous present in the sample*	low to high		
Number of chews	*Number of chews required in order to swallow*	low to high		

3.4 Sensory Results

3.4.1 *Individual Animal Effects*

After the sensory evaluation had been completed, and a preliminary analysis of the data evaluated, it was apparent that the sensory data for one of the high fat AngusGrain samples was quite different to the other three samples. After inspection of the remaining frozen meat, it was clear that the sample in question had very low marbling and had been mislabelled. The data from this sample was therefore removed from the dataset prior to further analysis. The effect of assessment day, week or sample replicate were not found to be significant by MANOVA. In the initial statistical analysis, each individual animal (*n*=41) was treated as an experimental unit. Significant sensory differences were found by the panel between animals for every sensory attribute (**Table 2** & **Table 5**), except for *Hay/Grain Odour*. In addition to providing a measure of animal-to-animal variation, these data demonstrated the high acuity, consensus and reproducibility of the trained panel. Sensory variation between individual animals is further discussed in later sections. According to panel mean intensity ratings for odour attributes, in terms of relative intensity, the most important were *Overall Impact* and *Grilled Beef* odour followed by *Bloody* and *Hay/Grain. Metallic, Barnyard* and *Livery* odours played a moderate role, whereas *Fishy* odour was barely detected in any of the samples.

In terms of flavour, *Overall Impact* and *Grilled Beef* were the dominant attributes, followed by *DairyFat* and *Bloody. Hay/Grain, Grassy, Metallic* and *Livery* played a less dominant role. *Fishy* flavour was only detected at a very low level in samples.

Sweet, Sour, Astringent and *Oily mouthcoating* were the dominant taste/aftertaste attributes. All of the texture–related attributes were rated at similar intensities.

3.4.2 *Sample Type Differences*

After adjusting for the effects of IMF, the AngusGrass samples were found to be higher in *Barnyard, Livery* and *Metallic* odour and lower in *Caramel* odour compared to the AngusGrain and WagyuGrass. The AngusGrass samples were on average lower in flavour *Impact* and *Grilled Beef* and *Grassy* Flavour.

The AngusGrain samples were rated as having higher *Dairy Fat* flavour and *Oily mouthcoating* compared to the grass fed samples. The AngusGrass samples had lower *Sweetness*, whereas the WagyuGrass had the lowest *Acidity.* Finally, the AngusGrass was on average less *Tender* and less *Juicy* compared to the other samples. Few sensory differences between the AngusGrain and WagyuGrass samples were measured, except that the AngusGrain was more *Acidic/Sour* and had greater *Acidic aftertaste* and *Oily mouthcoating*.

3.4.3 *Feed Effects*

In the statistical analysis for breed and feed effects, the marbling scores (MSA-MB) were treated as a covariate. For feed comparisons AngusGrain and AngusGrass were initially compared using samples from all marbling levels (low, medium and high). The mean sensory

scores for all attributes are summarised in **Table 4** & **Table 5**. The same comparison was also made after removal of the high IMF Angus grain-fed and low IMF Angus grass-fed samples (reduced dataset, see appendix **Table 20**).

After adjusting for differences in IMF, there were only a few feed-related differences in odour, flavour and taste attributes; the largest and clearest differences were found in texture attributes. *Bloody* and *Caramel* odour was higher ($p < 0.05$) in AngusGrain compared to AngusGrass. These trends were also confirmed in the analysis with the reduced dataset with marbling as a covariate. *Grilled beef* flavour and *Overall impact* were slightly higher in the grain fed beef *(p<0.05), as* were *Dairy Fat* and *Grassy flavour. Oily Mouthcoating* was higher in the AngusGrain and it was rated as slightly *Sweeter* overall; these trends were also repeated in the reduced dataset analysis (**Table 20**). The higher flavour ratings in the AngusGrain compared to the AngusGrass were in agreement with a number of published studies (Duckett *et al.* 2013, Van Eiswyk & 2014). The AngusGrain was higher in *Juiciness* and *Tenderness* scores; the same trends were found in the reduced dataset analysis. The main differences in flavour and texture attributes are summarised in **Table 5**. Of note was the lack of difference found in *Barnyard* and *Fishy* attributes between the grain and grass fed samples. The significantly higher *Grassy* flavour measured in the grain-fed beef was counter-intuitive and in contrast to findings reported by others (Maughan *et al. 2012).*

Barnyard flavours have been associated with the presence of *p*-cresol (4-methylphenol) derived from the breakdown of the amino acid tyrosine. Pasture or grass based diets are generally thought to have higher concentrations of tyrosine compared to grain feeding systems and "pastoral flavours" due to the presence of *p*-cresol have been documented (Watkins *et al.* 2012). Although no significant difference was found between the AngusGrass and AngusGrain samples, a slight *Barnyard* odour was present in the lowest IMF AngusGrass samples; this is discussed in the next section. Higher levels of unsaturation in the fatty acids present in grass-fed beef fat have been linked to fishy and off-odours by some researchers (Duckett *et al*. 2013). The lack of difference in the *Fishy* flavour attributes between grain and grass-fed marbled beef is a significant finding in contrast to some previously published research.

3.4.4 *Breed Effects*

WagyuGrass and AngusGrass beef were compared, using full (**Table 4 & Table 5**) and reduced data sets (**Table 20**). Similar differences were found using both analyses. After correcting for the effects of IMF, very few odour-related differences were measured between the WagyuGrass and AngusGrass steaks. The grass-fed Angus was slightly higher in *Livery, Fishy, Hay/grainy* and *Metallic* odour, whereas the WagyuGrass had slightly more *Caramel* odour. *Fishy* odour was rated higher in the AngusGrass samples compared to the WagyuGrass, although fishy odour or flavour was generally not measured to any extent in any of the samples in the study.

The Wagyu grass-fed had somewhat higher *Flavour impact* and *Grilled beef* flavour as well as relatively more *Dairy fat* and *Grassy* flavour. The only taste or aftertaste difference measured was for greater *Sweetness* in the WagyuGrass. The greatest and most consistent differences were found in texture attributes. Wagyu grass-fed beef was higher in *Juiciness* and *Tenderness* after 3 and 10 chews compared to the AngusGrass samples. The grass-fed Angus had greater remaining *Connective Tissue* and required a greater *Number of Chews* to form a bolus to swallow.

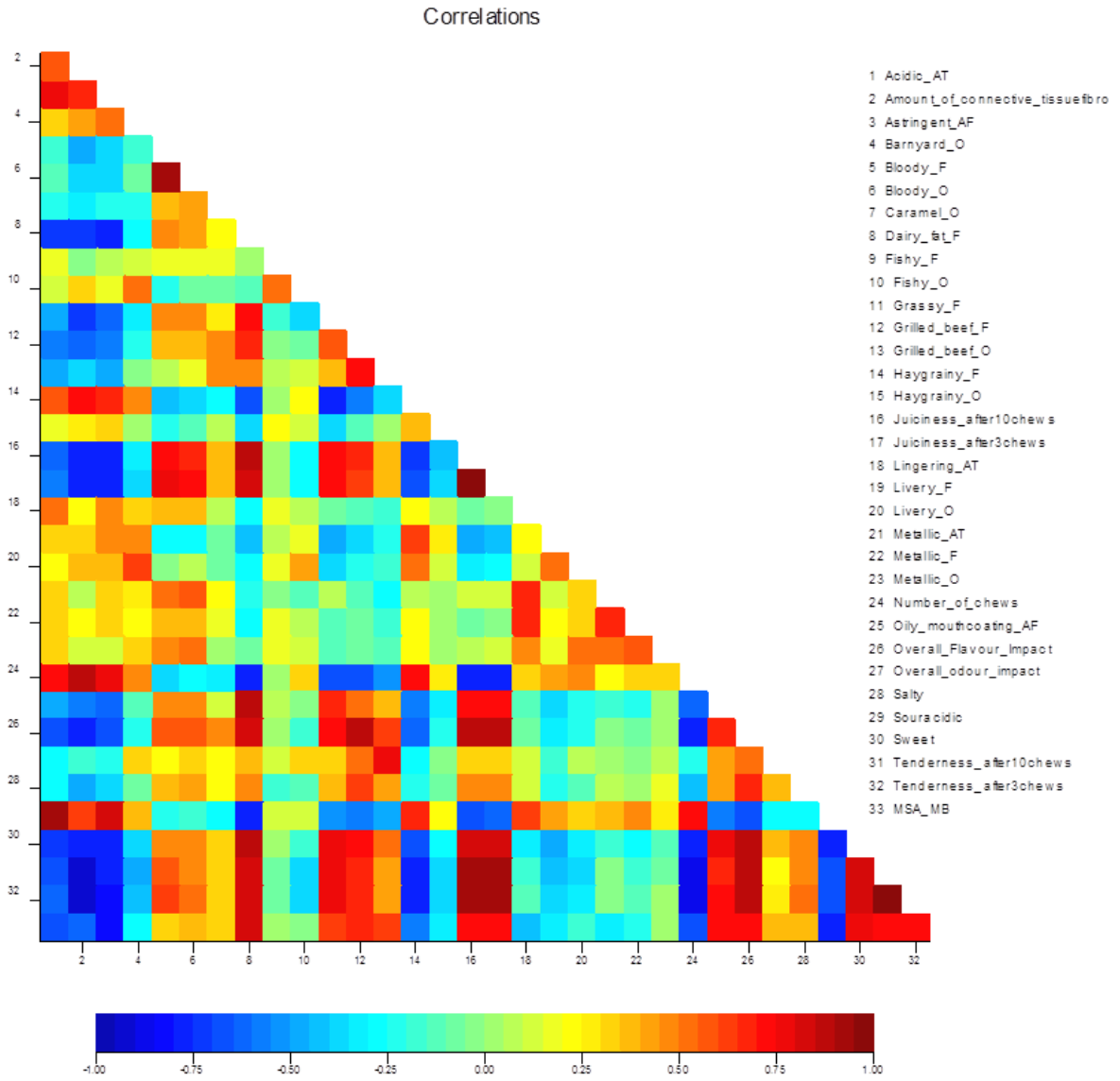

Figure 4: Correlation plot showing the strength of relationships between sensory attributes and MSA-MB (n=41). Dark blue signifies a strong negative correlation and dark red a strong positive correlation.

3.4.5 *Correlations between sensory attributes and between sensory attributes and IMF*

As expected, many sensory attributes were significantly positively or negatively correlated with each other and with the IMF (MSA-MB) across the samples (n=41). The correlation plot in **Figure 4** summarises these relationships. Dark blue signifies a negative correlation and dark red a positive correlation. Weaker correlations are indicated by lighter colours. The strongest overall sensory attributes that increased with IMF were: *Sweetness, Dairy Fat, Grassy,* and *Grilled Beef* flavour, *Grilled Beef* odour*, Overall Flavour Impact, Juiciness* and *Tenderness.* MSA-MB was strongly inversely related to *Number of Chews to Swallow*, *Sour/Acidic, Hay/Grainy Flavour*, *Acidic Aftertaste, Astringent Aftertaste* and the *Amount of Connective Tissue.* Some sensory attributes were either positively or negatively correlated to many other attributes (e.g. *Acidic Aftertaste, Amount of Connective Tissue, Astringent Aftertaste, Number*

of Chews, Dairy Fat flavour, Juiciness etc.), whereas others were more independent (e.g. *Barnyard* odour, *Caramel* odour, *Fishy* odour and flavour, *Metallic* odour etc.). The strong relationship between key sensory attributes and IMF, suggests that the MSA-MB is likely to be a good predictor of the flavour potential of highly marbled beef. This is further explored in the following sections.

Table 4: Mean odour and flavour differences between samples according to animal, sample type feed and breed. MANOVA analysis conducted using MSA-MB score as a covariate. P_{Animal} = P value for comparison between animals, $P_{Sample\ Type}$ = P value for comparison between the three sample types, P_{IMF} = P value of the covariate MSA-MB. P_{Feed} = P value of comparison of AngusGrain to AngusGrass. P_{Breed} = P value of comparison of AngusGrass and WagyuGrass samples.

ODOUR		Sample type						Feed			Breed		
	P Animal	Angus Grain n=390	Angus Grass n=420	Wagyu Grass n=420	LSD	P Sample Type	P IMF	Grain n=390	Grass n=420	P Feed	Angus n=420	Wagyu n=420	P Breed
Overall Impact	***	58.77	59.4	59.5	1.55	—	*	58.6	58.9	—	59.4	59.6	—
Grilled Beef	***	49.75	49.43	51.68	2.02	—	***	49.3	48.7	—	49.8	51.5	—
Livery	***	11.66[b]	12.35[a]	10.31[b]	1.54	*	—	11.8	12.2	—	12.6	10.2	**
Bloody	***	17.69	16.63	16.5	1.52	—	***	17.6	15.8	*	16.6	16.6	—
Fishy	**	4.06	5.18	4.28	1.08	—	—	4.1	5.1	—	5.4	4.1	*
Hay Grain	—	16.79	15.83	17.26	1.37	—	**	17.2	16.2	—	15.8	17.3	*
Barnyard	***	12.13[ab]	13.89[a]	11.6[b]	1.78	*	—	12.7	13.6	—	13.6	11.9	—
Caramel	**	9.58[a]	7.99[a]	10.01[b]	1.28	**	—	**9.5**	7.9	*	8.1	10.0	**
Metallic	*	10.47[ab]	11.06[b]	9.15[a]	1.36	*	—	10.4	10.8	—	11.1	9.2	*

FLAVOUR		Sample type						Feed			Breed		
	P Animal	Angus Grain n=390	Angus Grass n=420	Wagyu Grass n=420	LSD	P Sample Type	P IMF	Grain n=390	Grass n=420	P Feed	Angus n=420	Wagyu n=420	P Breed
Overall Impact	*	59.11[a]	57.57[b]	60.15[a]	1.54	**	***	58.3	56.7	*	57.5	60.4	**
Grilled Beef	***	50.32[a]	48.0[b]	50.45[a]	1.8	*	***	49.3	47.3	*	48.3	50.4	*
Livery	**	13.29	13.6	13.43	1.66	—	*	13.6	14.0	—	13.4	13.7	—
Bloody	***	20.55	19.7	19.57	1.78	—	***	20.1	19.0	—	19.3	20.0	—
Fishy	*	3.6	3.4	4.03	0.98	—	—	3.7	3.4	—	3.6	4.0	—
Hay Grain	**	15.2	15.89	15.1	1.26	—	***	15.6	16.2	—	15.8	15.2	—
Dairy Fat	***	20.6[a]	17.35[b]	19.22[a]	1.86	***	***	18.8	15.0	***	17.4	19.6	*
Grassy	***	15.49[a]	13.17[b]	15.03[a]	1.812	*	***	15.1	12.4	**	13.1	15.1	*
Metallic	*	12.84	13.3	12.06	1.42	—	—	13.0	13.3	—	13.2	12.2	—

P<0.05 = *, P<0.01 = **, p<0.001 ***. Means sharing the same superscript are not significantly different from each other.

Table 5: Mean taste, aftertaste and texture differences between samples according to animal, sample type feed and breed. MANOVA analysis conducted using MSA-MB score used as a covariate. . P_{IMF} = P value of the covariate MSA-MB. P_{Feed} = P value of comparison of AngusGrain to AngusGrass. P_{Breed} = P value of comparison of AngusGrass and WagyuGrass samples

TASTE AFTERTASTE	P Animal	Angus Grain n=390	Angus Grass n=420	Wagyu Grass n=420	LSD	P Sample Type	P IMF	Feed Grain n=390	Feed Grass n=420	P_{Feed}	Breed Angus n=420	Breed Wagyu n=420	P_{Breed}
Salty	***	14.88	14.73	15.13	0.86	—	—	14.8	14.6	—	14.8	15.1	—
Sour/Acidic	***	14.26[a]	13.69[ab]	12.4[b]	1.48	*	***	15.1	14.3	—	13.8	12.2	—
Sweet	***	15.47[a]	13.77[b]	15.52[a]	1.01	***	***	14.9	13.2	***	13.6	15.7	***
Acidic AT	***	13.82[a]	13.1[a]	12.17[b]	1.42	*	***	14.5	13.6	—	13.3	11.9	—
Astringent AT	***	16.84	16.52	16.16	1.61	—	***	17.9	17.6	—	16.5	16.0	—
Lingering AT	***	30.91	31.19	30.14	1.54	—	*	31.5	31.2	—	31.0	30.3	—
Metallic AT	***	13.83	13.94	12.94	1.46	—	—	14.3	13.9	—	13.9	13.0	—
Oily Mouthcoating	***	17.03[a]	15.12[b]	14.19[b]	1.32	***	***	16.1	13.8	***	15.0	14.5	—
TEXTURE	P Animal	Angus Grain n=390	Angus Grass n=420	Wagyu Grass n=420	LSD	P Sample Type	P IMF	Feed Grain n=390	Feed Grass n=420	P_{Feed}	Breed Angus n=420	Breed Wagyu n=420	P_{Breed}
Juiciness 3 chews	***	42.42	40.2	41.84	2.36	—	***	40.4	37.7	*	39.8	42.7	*
Juiciness 10 chews	***	37.1	34.78	36.87	2.2	—	***	35.2	32.6	*	34.4	37.6	**
Tenderness 3 chews	***	53.13[a]	49.92[b]	52.79[a]	2.16	**	***	50.8	47.6	**	49.6	53.4	**
Tenderness 10 chews	***	49.51[a]	46.05[b]	50.06[a]	2.26	***	***	47.4	44.0	**	46.0	50.5	***
Number of chews	***	25.51[b]	26.27[a]	24.14[b]	1.43	*	***	26.3	27.0	—	26.1	24.2	***
Connective Tissue	***	27.55[a]	29.97[b]	26.02[a]	1.96	***	***	28.7	31.0	*	29.8	26.0	***

P<0.05 = *, P<0.01 = **, p<0.001 ***.Means sharing the same superscript are not significantly different from each other.

3.5 Relationship between sensory attributes and IMF

The covariate term MSA-MB — used as a measure of the IMF content in the feed and breed statistical analyses — was often highly significant ($P < 0.001$) for odour, flavour, taste and texture attributes. The effect of IMF on all these attributes was further explored using Pearson's correlations and regression analysis to understand the relationship between marbling scores and sensory attributes for all samples (n=42), as well as for each of the three sample types separately (**Table 6**). Significant correlations were found between most sensory attributes and the MSA-MB score. Linear regression models for selected sensory attributes for each sample type, WagyuGrass (blue symbol), AngusGrain (red symbol) and AngusGrass (green symbol) samples are shown in **Figure 5** to **Figure 8**. In most cases, the relationship between IMF and sensory attributes was similar regardless of sample type; however there were some important exceptions, mainly for the low-fat AngusGrass samples, discussed in later sections. Unlike previous reports based on consumer data, most sensory attributes showed a direct linear relationship with IMF rather than a curvilinear relationship (Thompson 2004).

The weakest overall correlations with IMF were found for odour attributes (**Figure 5**). The direction and strength of the relationships differed depending on the sample type. For example, although an overall significant positive correlation was found between IMF and *Odour Impact* across all the samples (**Table 6** & **Figure 6**) the strength of this trend was different for AngusGrass (practically no relationship) to WagyuGrass (strong positive relationship). Similarly, *Grilled beef* odour increased with increases in IMF for the AngusGrain and WagyuGrass samples, but not for the AngusGrass. Directionally, overall significant positive relationships between MSA-MB and *Bloody and Caramel* odours were measured. An overall negative association between *Hay/grainy* odour and IMF was measured. Although an overall negative relationship between MSA-MB and *Barnyard* odour was measured, the negative relationship applied mainly to the AngusGrass. The mean ratings of *barnyard* odour in the AngusGrass ($p < 0.001$) between nominal fat levels, low (MSA-MB 316), medium (MSA-MB 545) and high (MSA-MB 738); 16.7 > 14.9 > 10.9. Slight increases in *Barnyard odour* were measured as IMF increased the other sample types. Similarly, the *Lingering and Metallic aftertaste* attribute applied mainly to the AngusGrass samples and decreased significantly as the IMF increased from low, medium to high.

Very strong IMF-related effects were found for taste, aftertaste and texture attributes (**Table 6**). As the IMF increased, the *Flavour Intensity, Dairy Fat*, *Grilled Beef* and *Grassy* flavour increased and the *Hay/grainy* flavour decreased. *Livery* flavour decreased with increasing IMF for grass-fed samples but not for the grain-fed samples. Overall it appeared that there was greater consistency in the impact of IMF on flavour attributes compared to odour attributes. With increasing IMF, the perceived *Sweetness* increased and the *Acid/Sour* taste decreased **Table 6**. Similarly the *Astringent* and *Acidic* aftertaste decreased as the *Oily mouth-coating* increased. As expected, strong relationships between IMF and texture were measured; as the fat increased, the *Juiciness* and *Tenderness* increased, whereas the *Number of chews* to swallow and the amount of *Connective tissue* decreased significantly (**Figure 6**). Overall maps of the sensory differences between samples due to breed, feed and IMF are summarised in the PCA score and loadings bi-plots shown in **Figure 9** to **Figure 12**. Each of the sensory modalities — odour, flavour, taste & aftertaste and texture — are plotted separately for clarity.

The nine distinct sample types and marbling bands are colour-coded and bounded by coloured bounding boxes.

Table 6: Pearson's correlation coefficients for the relationship between MSA-MB and mean sensory scores for all samples together (n=41), and separate relationships for sample types, AngusGrass (n=14), WagyuGrass (n=14) and AngusGrain (n=13) samples. Significant relationships denoted by asterisks.

ODOUR	Impact	Barnyard	Bloody	Caramel	Fishy	Liver	Grilled beef	Hay/ Grain	Metallic
All Samples	**0.40****	-0.28*	**0.35****	**0.34****	-0.02	-0.17	**0.62*****	**-0.34****	0.01
Angus Grass	-0.02	**-0.59****	-0.16	0.20	-0.05	-0.18	0.41	**-0.53****	-0.16
Wagyu Grass	**0.67*****	0.46*	**0.64*****	0.01	0.35	0.26	**0.56****	-0.38	0.41
Angus Grain	0.25	0.02	0.28	0.07	-0.08	-0.08	0.29	-0.40	0.34

FLAVOUR	Dairy Fat	Impact	Bloody	Fishy	Grassy	Grilled beef	Hay/ Grain	Liver	Metallic
All Samples	**0.82*****	**0.74*****	0.33**	0.01	**0.60*****	**0.68*****	**-0.57*****	**-0.34****	-0.23
Angus Grass	**0.74*****	0.37	-0.06	-0.27	0.30	**0.62****	**-0.73*****	**-0.58****	-0.24
Wagyu Grass	**0.84*****	**0.77*****	0.41	-0.09	**0.59****	**0.57****	-0.35	-0.28	0.03
Angus Grain	**0.65*****	**0.68*****	0.51*	0.04	0.31	0.45	-0.23	0.06	0.00

TASTE	Salty	Sour/ acidic	Sweet	Lingering	Metallic
All Samples	**0.38*****	**-0.76*****	**0.77*****	**-0.42****	-0.32*
Angus Grass	0.19	**-0.64****	**0.57****	**-0.65****	-0.43
Wagyu Grass	0.44	**-0.60****	**0.67****	0.10	0.19
Angus Grain	-0.03	**-0.73*****	**0.63****	-0.06	-0.19

AFTERTASTE	Oily Mouth-coating	Acidic	Astringent
All Samples	**0.71*****	**-0.70*****	**-0.82*****
Angus Grass	0.48*	**-0.55****	**-0.77*****
Wagyu Grass	**0.81*****	**-0.54****	**-0.76*****
Angus Grain	**0.75*****	**-0.65*****	**-0.62****

TEXTURE	No Chews	Juiciness 3 chews	Juiciness 10 chews	Connective tissue	Tenderness 10 chews	Tenderness 3 chews
All Samples	**-0.68*****	**0.71*****	**0.72*****	**-0.65*****	**0.73*****	**0.75*****
Angus Grass	**-0.59****	0.39	0.43	**-0.50***	**0.50***	**0.54****
Wagyu Grass	**-0.66*****	**0.72*****	**0.71*****	**-0.53****	**0.65*****	**0.70*****
Angus Grain	-0.34	**0.67*****	**0.67*****	-0.40	**0.70****	**0.71*****

P < 0.1 = *, P < 0.05 = **, P < 0.01 = ***, P < 0.001 = ****

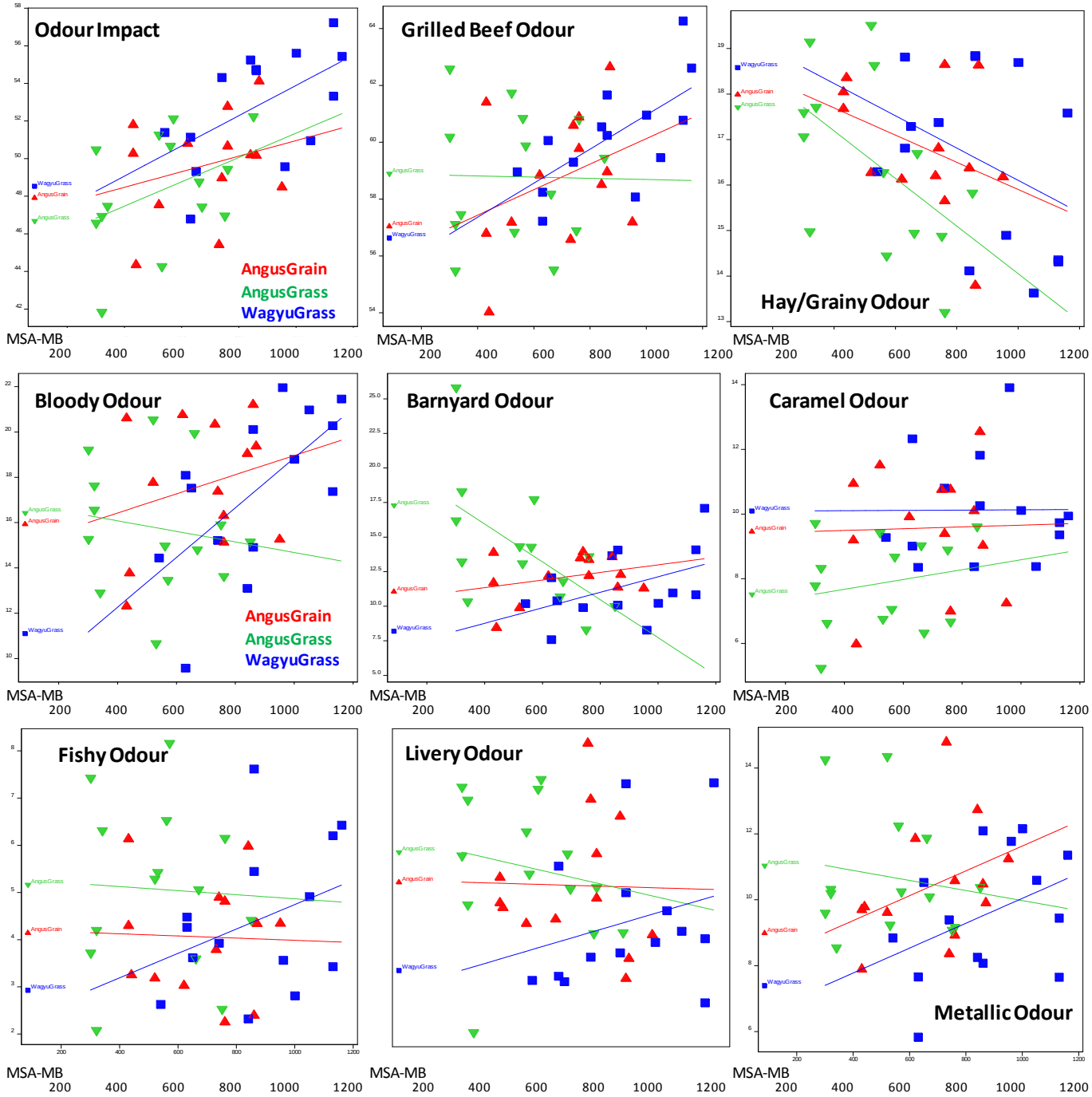

Figure 5: Scatterplot and regression models of the relationship between odour-related sensory attributes and MSA-MB scores. WagyuGrass (blue), AngusGrain (red) and AngusGrass (green).

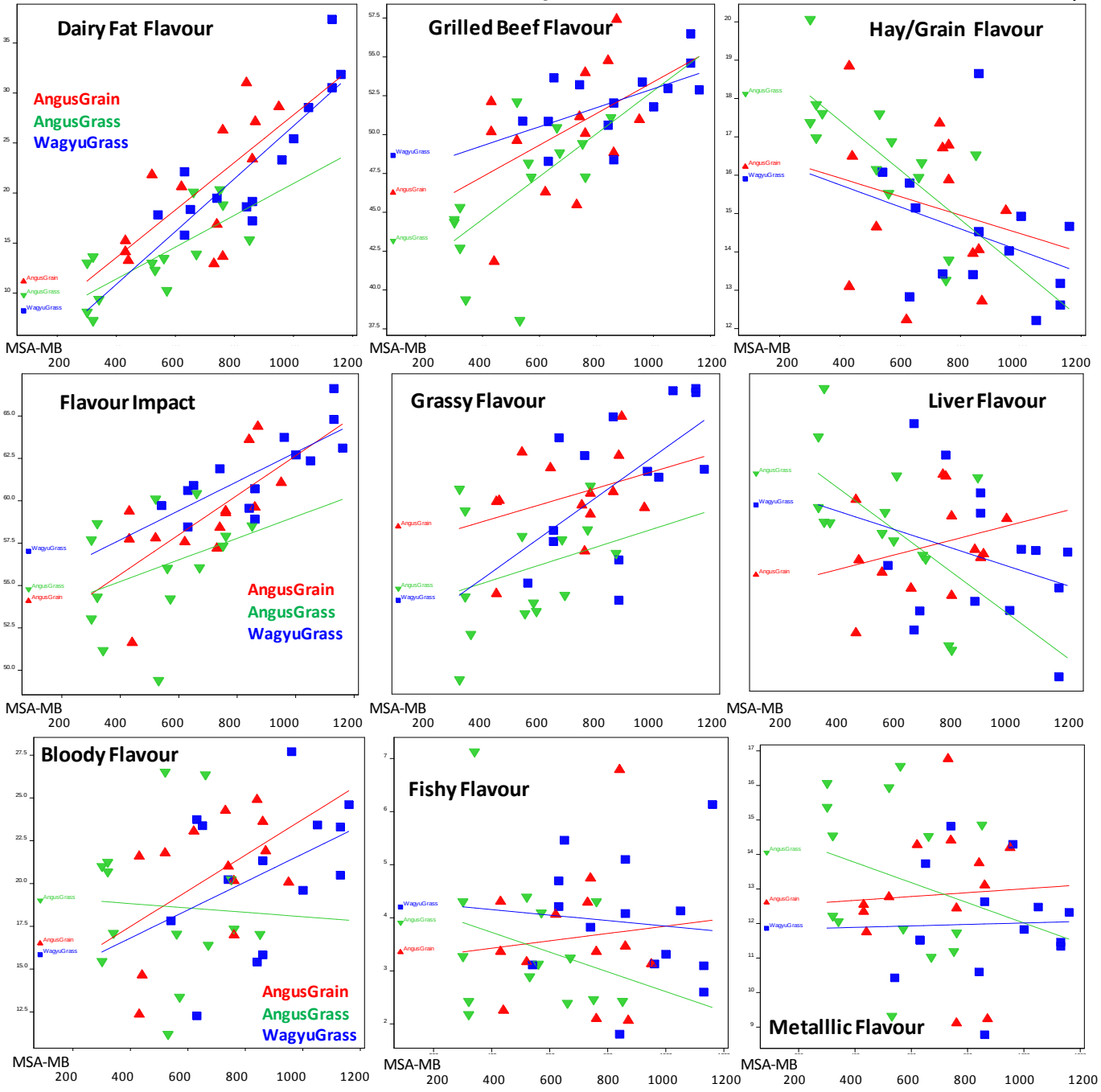

Figure 6: Scatterplot and regression models of the relationship between flavour-related sensory attributes and MSA-MB scores. WagyuGrass (blue), AngusGrain (red) and AngusGrass (green).

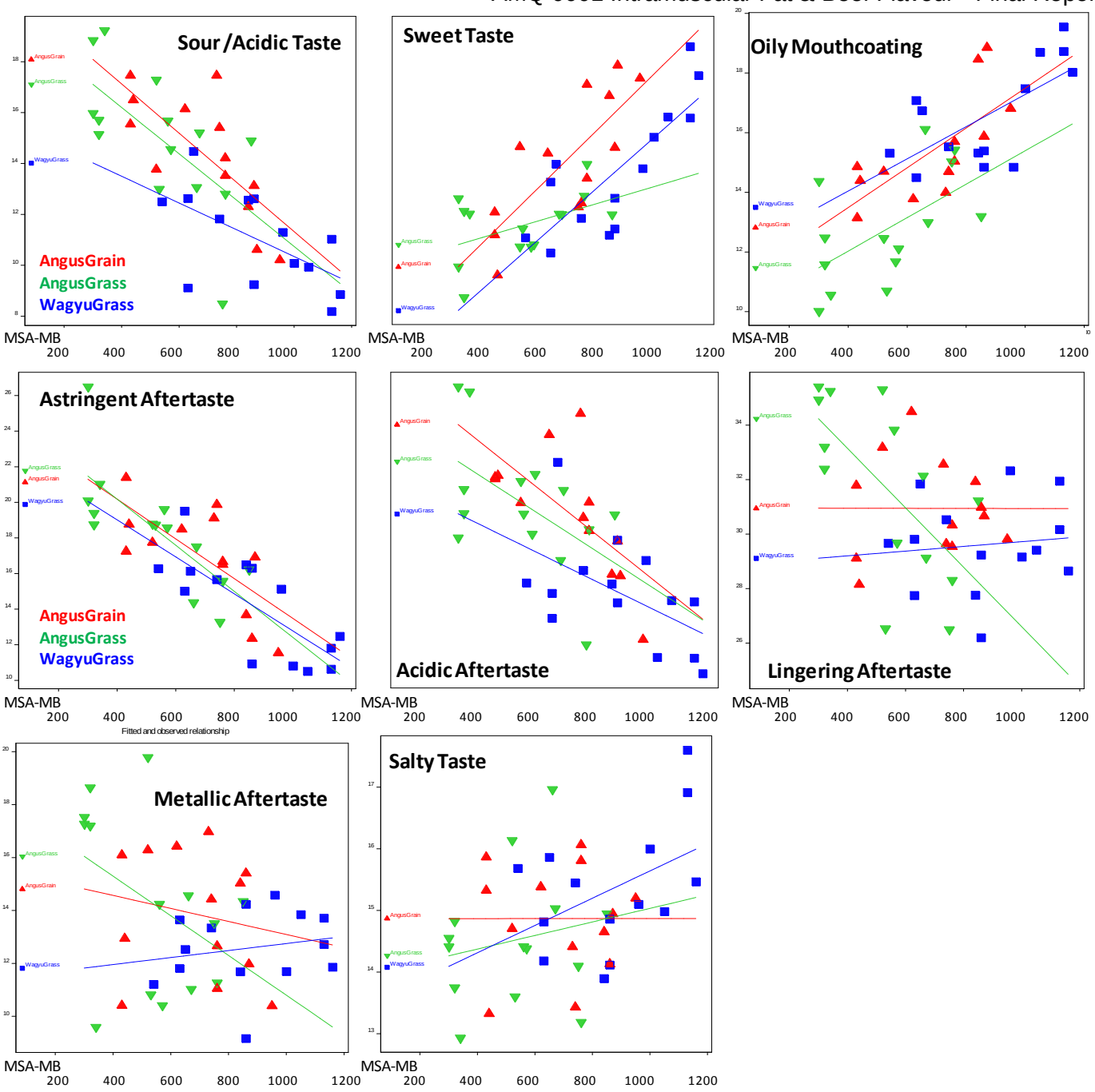

Figure 7: Scatterplot and regression models of the relationship between taste and aftertaste-related sensory attributes and MSA-MB scores. WagyuGrass (blue), AngusGrain (red) and AngusGrass (green).

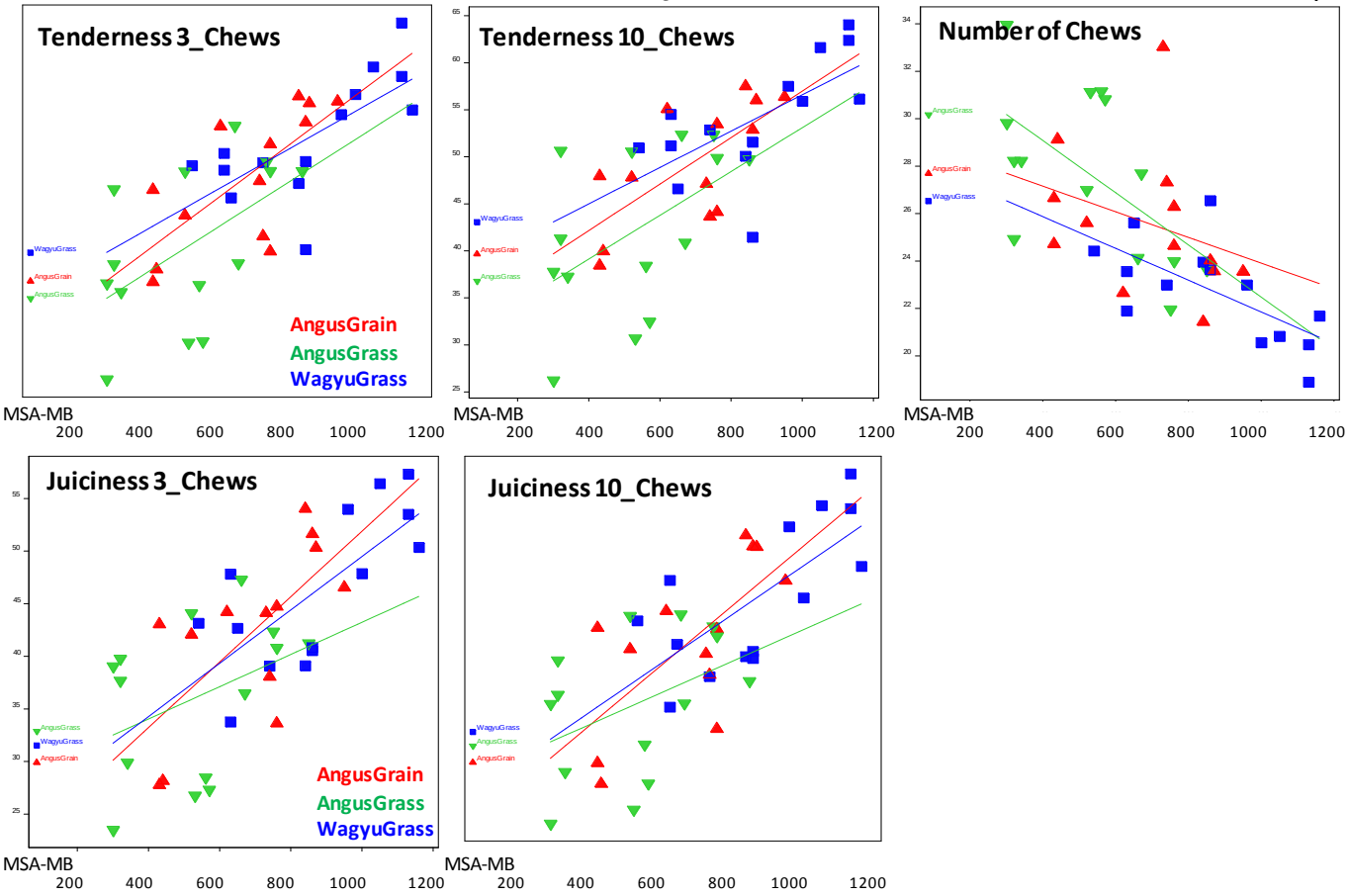

Figure 8: Scatterplot and regression models of the relationship between texture-related sensory attributes and MSA-MB scores. WagyuGrass (blue), AngusGrain (red) and AngusGrass (green).

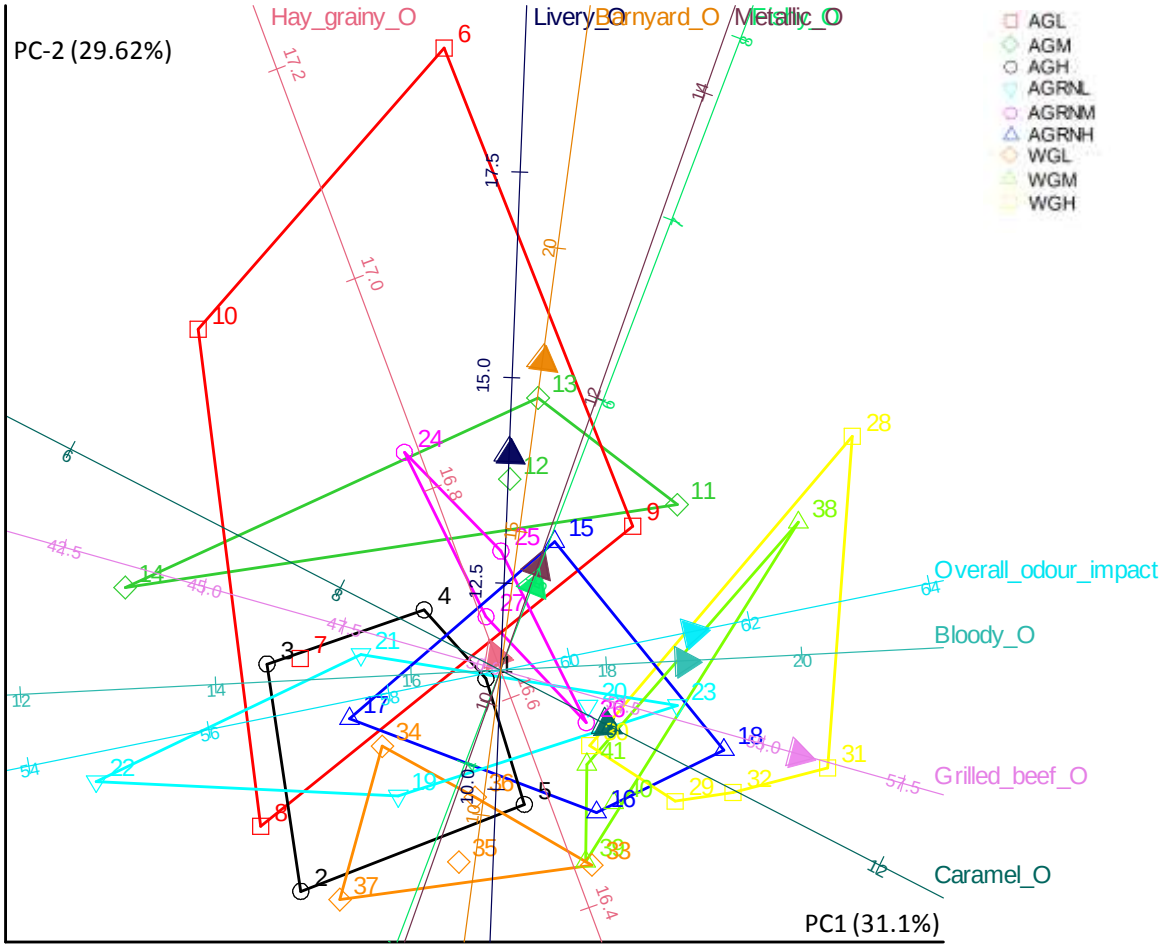

Figure 9: PCA bi-plot showing the relationship between odour attributes and samples (n=41). Sample types are colour coded. The size of the bounding box gives an indication of the sensory variance within a sample type.

3.5.1 *PCA Odour map*

The PCA model or "sensory map" for all odour attributes (**Figure 9**) explains around 60% of the total odour variance across samples. The samples are separated left to right on PC 1 (31.1%), according to increasing IMF (MSA-MB), with the AngusGrass low fat (AGL – coded red) on the furthest left and the highest fat samples, the WagyuGrass high fat (WGH, coded yellow) positioned furthest to the right. As the IMF increased (left-to-right), so too did the *Odour impact*, *Bloody, Grilled Beef* and *Caramel* odour. The odour variability was considerable between some samples within the same type (e.g. AGL) — the size of the bounding box is an indication of the sample variance. The sample odour variability appeared to generally decrease as IMF increased, e.g. the area in the bounding boxes became smaller. PC-2 (29.6 %) separated samples mainly according to *Barnyard, Metallic* and *Livery, Hay/grainy* odour; these attributes were mainly important in the AngusGrass low fat samples. It is important to note that the odour attributes were assessed by sniffing the sample headspace, without taking the samples into the mouth - purely by the orthonasal route.

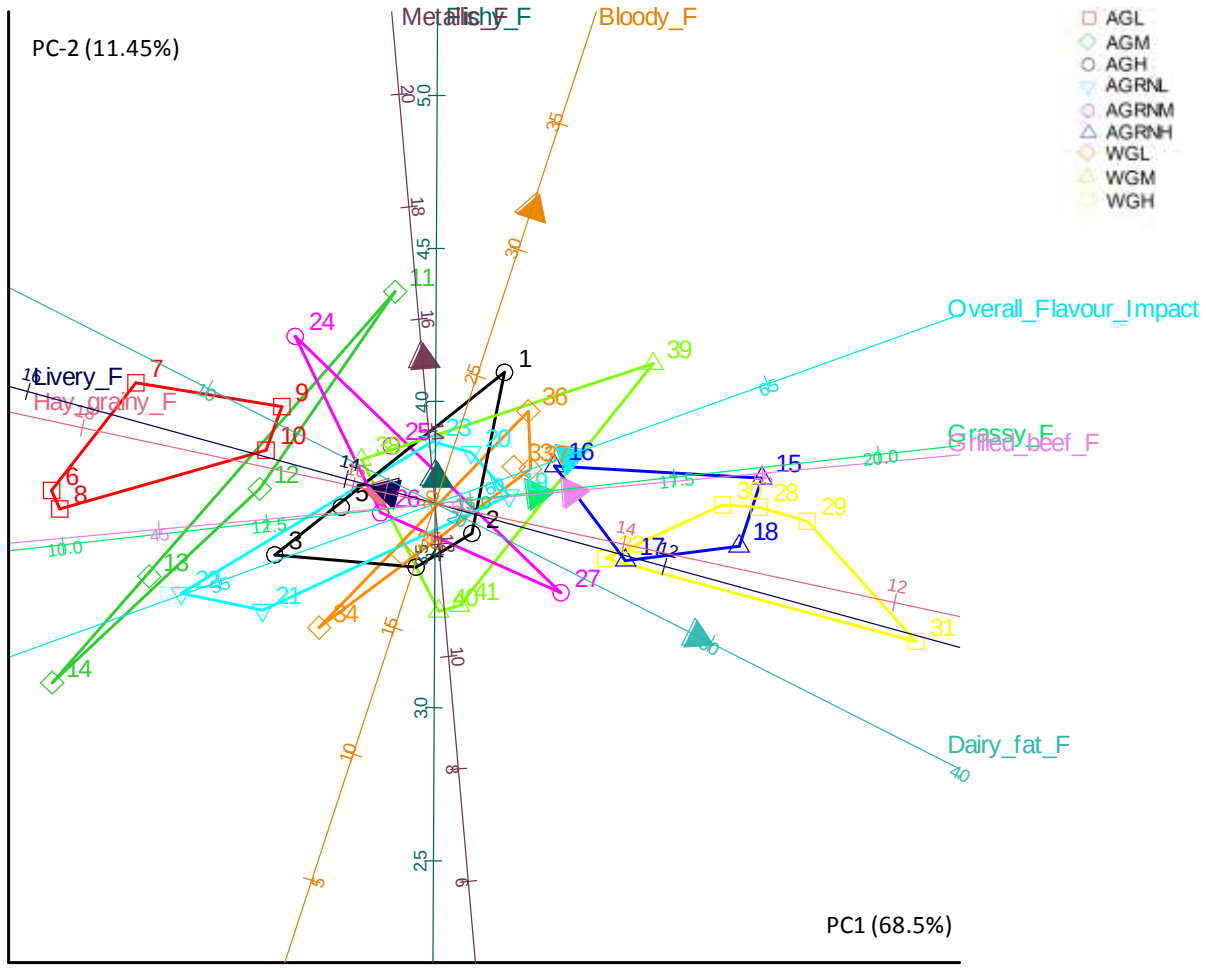

Figure 10: PCA bi-plot showing the relationship between flavour attributes and samples (n=41). Sample types are colour coded. The size of the bounding box gives an indication of the sensory variance within a sample type. AGL-M-H (AngusGrass low-medium-high fat) AGRNL-M-H (AngusGrain low-medium-high fat) & WGL-M-H (WagyuGrass low-medium-high fat).

3.5.2 *PCA Flavour map*

The PCA sensory map for retronasal flavour attributes (**Figure 10**) encompassed almost 80% of the flavour variance across the samples. In general, the relationships between flavour attributes and IMF were much stronger than for odour — see correlations in (**Table 6**). Most of the flavour variance was described by principal component 1 (PC-1) (68.5%) and a relatively small amount by PC-2 (11.45%). The flavour *Impact, Grassy, Grilled beef* and *Dairy fat* flavour increased with increasing IMF (left-to-right). As for odour, *Livery* and *Hay/grainy* flavour generally decreased with increasing IMF, especially for the grass fed samples. The bounding boxes for the same sample types were smaller for flavour attributes compared to odour attributes, suggesting that flavour attributes were either more consistent or easier to rate.

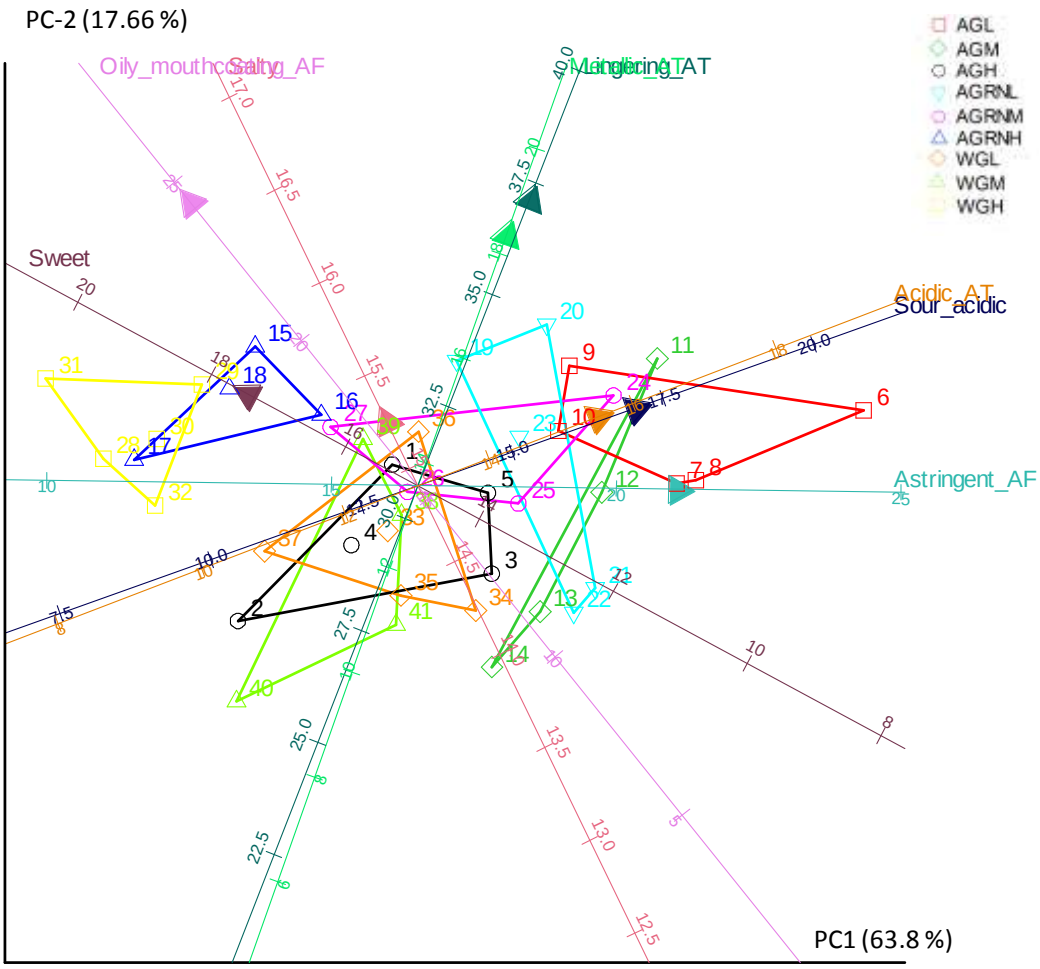

Figure 11: PCA bi-plot showing the relationship between taste and aftertaste attributes and samples (n=41). Sample types are colour coded. The size of the bounding box gives an indication of the sensory variance within a sample type. AGL-M-H (AngusGrass low-medium-high fat) AGRNL-M-H (AngusGrain low-medium-high fat) & WGL-M-H (WagyuGrass low-medium-high fat).

3.5.3 *PCA Taste, Aftertaste and Texture Maps*

The PCA map for taste and aftertaste modalities summarised more than 80% of the variance within the beef sample dataset (**Figure 11**). Most of the sample variance was described by PC-1 (68.5%). The bi-plot clearly shows that *Sweetness* and *Oily Mouthcoating* increased with IMF, and were diametrically opposed to *Sourness, Acidity* and *Astringent Aftertaste* attributes. *Lingering* and *Metallic aftertaste* mainly affected the low-IMF AngusGrass samples.

Finally, the texture-related sensory attributes are summarised in the PCA bi-plot in **Figure 12**. Nearly 100% of the variance in texture attributes is summarised in the model. More than 90 % of the variance was explained by PC-1, which was directly correlated with IMF (increasing left to right). As expected, *Tenderness* and *Juiciness* were strongly positively correlated with IMF, whereas *Number of Chews* and *Connective Tissue* were negatively correlated.

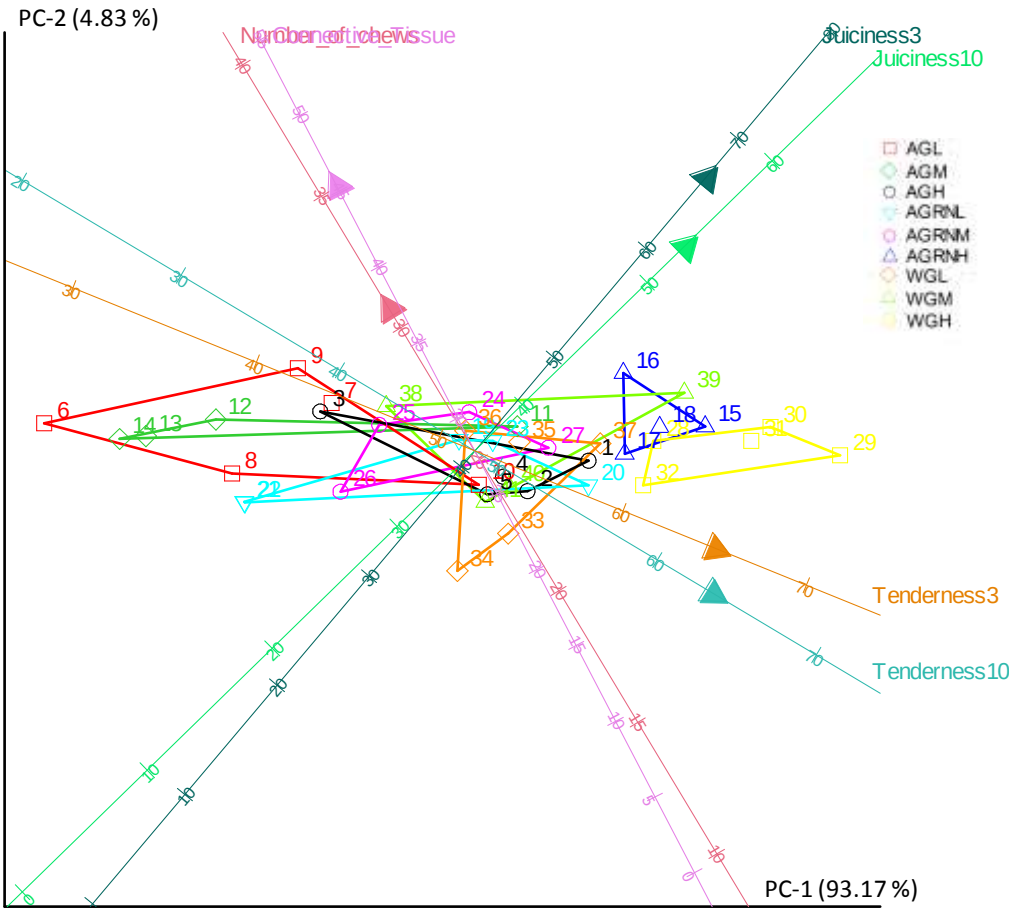

Figure 12: PCA bi-plot showing the relationship between texture attributes and samples (n=41). Sample types are colour coded. The size of the bounding box gives an indication of the sensory variance within a sample type. AGL-M-H (AngusGrass low-medium-high fat) AGRNL-M-H (AngusGrain low-medium-high fat) & WGL-M-H (WagyuGrass low-medium-high fat).

3.6 Correlations between Carcass Measurements and Sensory Attributes

It is clear that MSA-MB is correlated to many of the positive (and negative) sensory attributes in marbled beef; hence MSA-MB is likely to be useful to predict beef flavour scores. It was also of interest to understand whether other carcass measurements showed meaningful associations with sensory scores as well.

3.6.1 *MSA Carcass Measurements and Odour Sensory attributes*

Correlations between sensory attributes and carcass measurements (all samples together), are summarised in **Figure 13**. The additional carcass measurements were generally not strongly associated with any of the odour-related sensory attributes, compared to MSA-MB. Carcass weight (Cwt) was negatively correlated to *Grilled Beef* odour (p = 0.007) odour and *Hay/Grainy* was positively associated with the ossification score (Oss) (p = 0.05). MSA-MB showed the strongest positive relationships with odour-related sensory attributes; e.g. *Caramel, Grilled Beef* and *Odour Impact*.

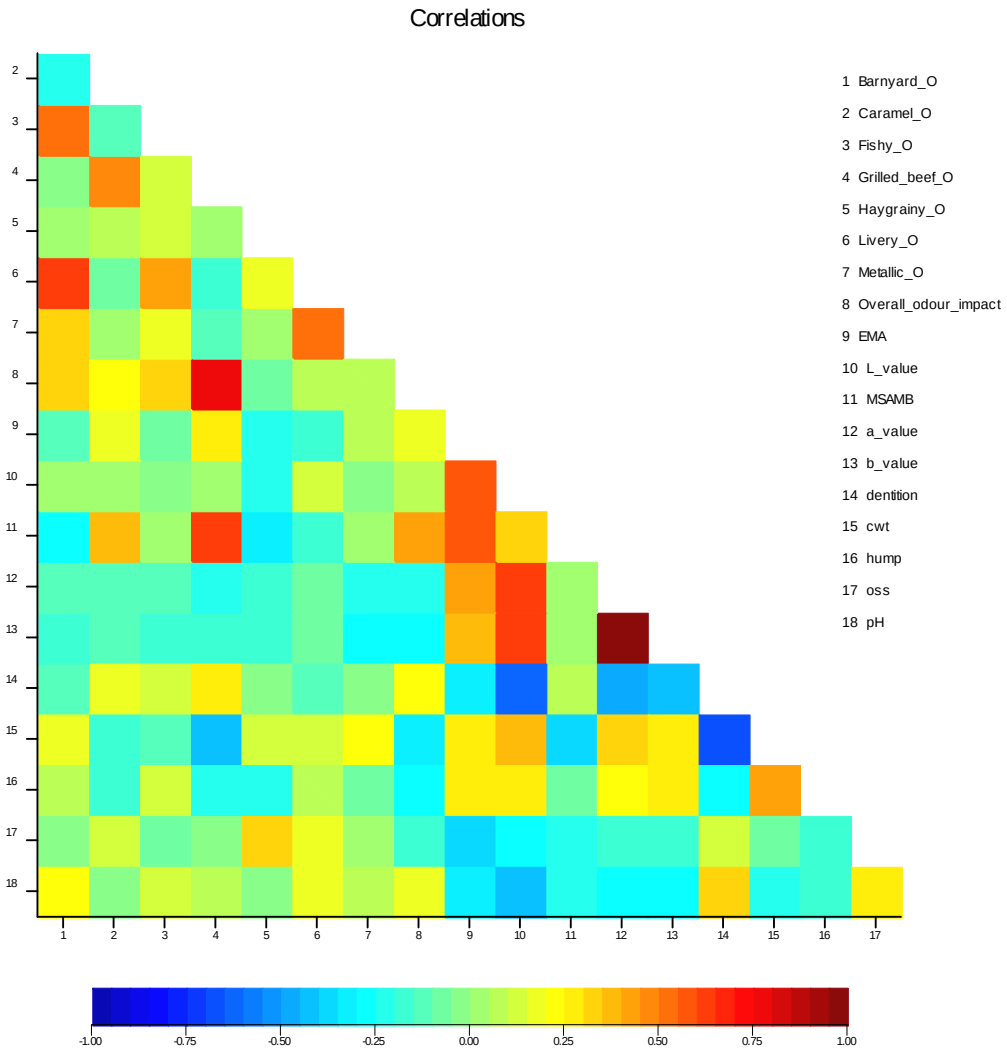

Figure 13: Correlation plot showing the strength of association between odour-related sensory attributes and MSA carcass measurements.

3.6.2 *MSA Carcass Measurements and Flavour Sensory Attributes*

Relationships between flavour-related sensory attributes and carcass measurements (all samples together) are summarised in **Figure 14**. As for the odour attributes, clearly MSA-MB had the strongest relationship to flavour sensory measurements compared to the other carcass parameters. Eye muscle area (ema) showed a similar correlation pattern to MSA-MB, but the correlations were generally weaker. Ossification was negatively correlated to *Dairy Fat* flavour (r = -0.45, p = 0.006) and *Grassy Flavour* (r = -0.48, p = 0.002) and positively correlated to Hay/Grainy flavour (0.43, p = 0.03). Meat pH was positively correlated with *Livery* flavour (r = 0.34, p = 0.03). Dentition was positively correlated to *Bloody* flavour (r = 0.33, 0.03) and *Flavour Impact* (r = 0.32, p = 0.04).

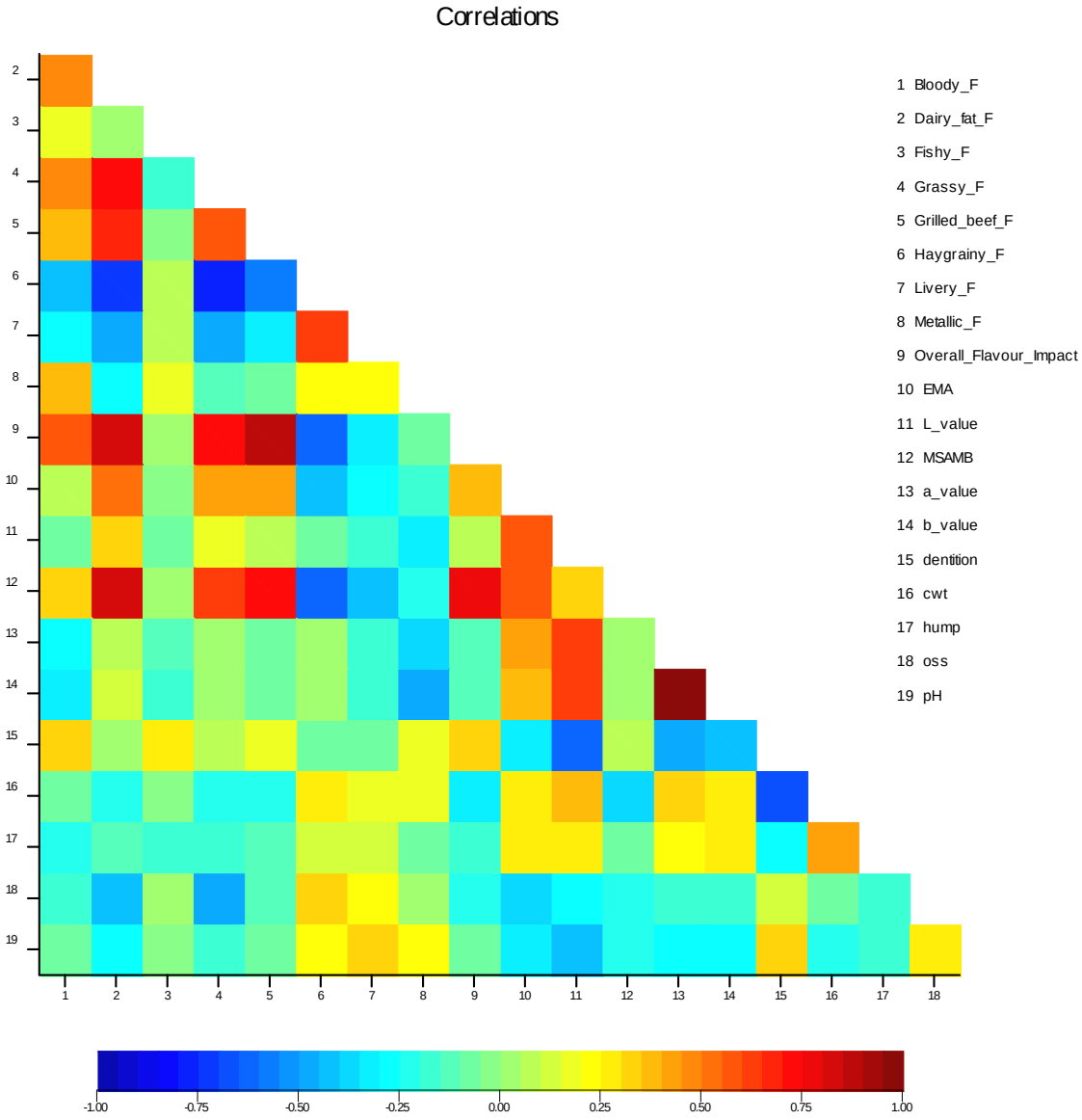

Figure 14: Correlation plot showing the strength of association between flavour-related sensory attributes and MSA carcass measurements

3.6.3 *MSA Carcass Measurements and Taste, Aftertaste and Texture Sensory Attributes*

Relationship between taste, aftertaste and texture attributes and carcass measurements (all samples together) are summarised (**Figure 15**). Once again MSA-MB stands out as the most useful and most strongly correlated carcass parameter. In addition to MSA-MB, carcass weight (cwt) was positively related to *Acidic Aftertaste (0.53, p < 0.001), Astringent Aftertaste (0.44, p = 0.005), Lingering Aftertaste* (r = 0.31, p = 0.05) and *Number of Chews to Swallow (0.35, p = 0.03)*. Metallic Aftertaste was inversely related to the b* value (-0.4, p =0.01) and *Oily Mouthcoating* negatively correlated to ossification (-0.47, p = 0.002). As many of these relationships were different for each sample type (e.g. *Metallic and Lingering Aftertaste* applied mainly to the lowest fat AngusGrass samples – see **Figure 7**) future flavour models may need to account for feed and breed differences.

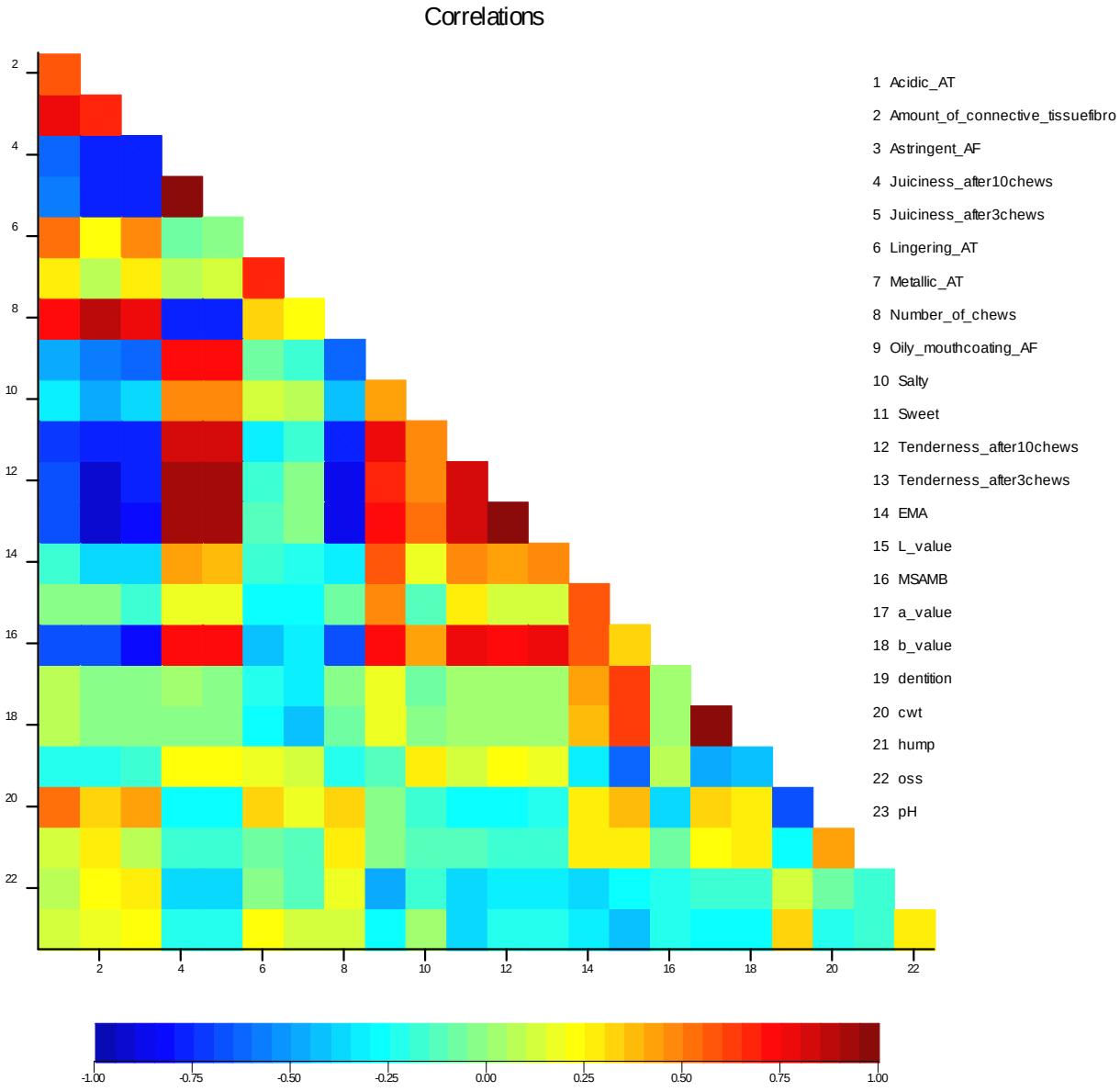

Figure 15: Correlation plot showing the strength of association between taste, aftertaste and texture-related sensory attributes and MSA carcass measurements

3.7 Overview of Findings from Sensory Evaluation

- *Most sensory attributes were significantly correlated with the marbling level (MSA-MB), especially flavour, taste, aftertaste and texture attributes. The MSA-MB score alone would be a good predictor of marbled beef flavour*
- *Although small sensory difference due to breed and feed were measured, the greatest differences were due to the amount of IMF*
- *WagyuGrass compared to AngusGrass (Breed effect). WagyuGrass samples were more Tender, more Juicy and required a lower amount of chews compared to the AngusGrass samples, after correcting for differences in IMF. WagyuGrass was also Sweeter, higher in Caramel and Hay/grain odour, and Dairy Fat and Grassy flavour than the AngusGrass.*
- *AngusGrain compared to AngusGrass (feed effect). AngusGrain had higher Caramel odour, higher Grassy and Dairy fat flavour and was Sweeter compared to the AngusGrass. The AngusGrain was also more Tender and Juicy.*
- *After accounting for differences in IMF, there were no odour, flavour or texture differences between the WagyuGrass and AngusGrain samples, however there were taste differences. The WagyuGrass was less Acidic, had less Acidic Aftertaste and had lower Oily mouthcoating*
- *The relationship between IMF (MSA-MB) and flavour was generally linear rather than curvilinear; i.e. the flavour scores did not plateau after a certain fat level.*
- *Odour-related attributes were least strongly associated with IMF. Variation within sample type was greatest for the odour sensory modality.*
- *Flavour, taste, aftertaste and texture attributes correlated more strongly with MSA-MB*
- *There was little evidence that grass- fed beef was higher in Fishy or Barnyard attributes, compared to grain-fed, especially when the MSA-MB > ~600*
- *The low IMF AngusGrass samples (~ 5.2% fat) were high in Barnyard odour and Liver flavour. These low fat samples also had high Acidity, high Astringency, greater Lingering aftertaste and Metallic aftertaste. As no AngusGrain or WagyuGrass samples with these low marbling scores were used in this experiment, it is not known whether the same attributes would also have been higher at lower IMF levels in these samples.*

4 Chemical and Physical Properties of Beef Samples

4.1.1 *Meat Liquid Loss*

Any loss of liquid from meat - during packaging, storage or cooking - may potentially impact on texture attributes – e.g. decreasing *Juiciness* and *Tenderness*. Loss of liquid, e.g. intra- and extracellular fluids — containing free amino acids and sodium for example — may also potentially affect the flavour intensity. The amount of liquid lost (% w/w) from meat samples during defrosting is referred to as “defrost loss”. The amount of liquid lost from meat during aging in vacuum packaging, “drip loss”, refers to the liquid (% w/w) that remained within the vacuum bag after meat samples were removed. Finally, the amount of water lost from each meat sample during heating in the water bath during the Warner-Bratzler experiments (see following section), is referred to as “cook loss”.

It was of interest to see whether there were differences in these parameters between meat samples types, and also whether a relationship existed between the IMF (measured as MSA-MB) and liquid loss. Scatter plots and fitted regression curves for the relationship between liquid loss parameters and IMF are plotted in **Figure 16**. Sample types are colour coded; WagyuGrass (blue), AngusGrain (red) and AngusGrass (green). A significant overall negative correlation between % cook loss and IMF was measured across all samples combined (r = -0.47, $p < 0.001$); less liquid was lost from steaks as the marbling increased. It should be noted that meat with higher MSA-MB scores naturally has less moisture on a weight basis compared to low fat meat. The correlations for the separate sample types were not significant. No significant relationships between IMF and either % drip loss or % defrost loss were measured. There was, however, a significantly higher % defrost loss for the Wagyu-grass fed samples compared to the Angus grain-fed samples.

Table 7: Estimated means for liquid loss parameters calculated by MANOVA using MSA-MB as a covariate.

Parameter	Angus Grain n=13	Angus Grass n=14	Wagyu Grass n=14	P_{Sample}	LSD	P_{IMF}
% Defrost Loss	1.9	2.46	3.17	0.029	0.91	ns
% Cook Loss	25.2	25.8	25.08	ns	–	0.03
% Drip Loss	2.54	2.51	2.1	ns	–	ns

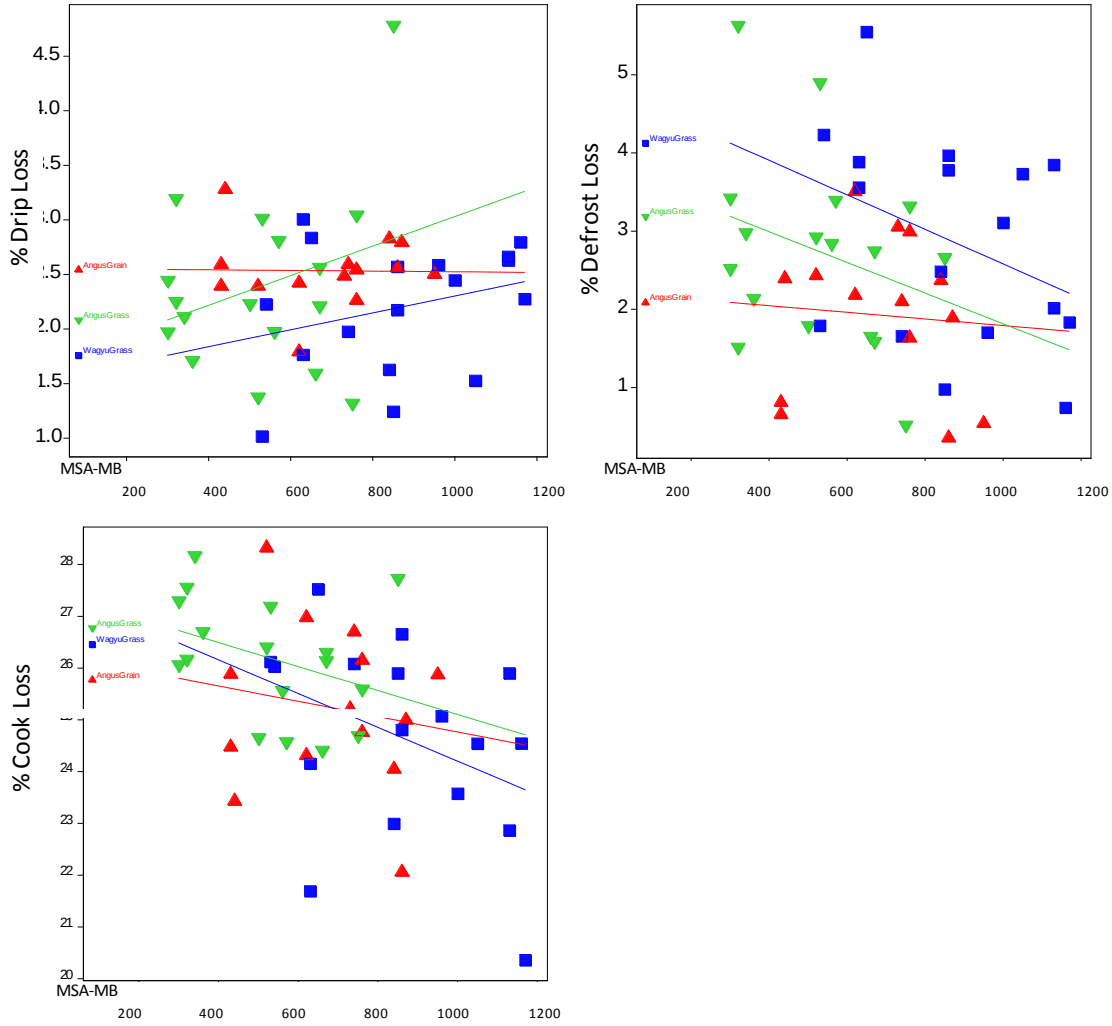

Figure 16: Scatter plots and fitted regression lines for the relationship between IMF (MSA-MB) and % drip loss, % cook loss and % defrost loss.

4.1.2 *Modified Warner-Bratzler Shear Force*

The Warner-Bratzler shear (WBS) force measurement is probably the most widely used objective measure of meat palatability (Guelker *et al.* 2013, Lorenzen *et al.* 2003, Caine *et al.* 2003, Bouton *et al.* 1978). WBS measurements provide an objective instrumental measure of meat tenderness. WBS measurements have also been associated with other beef sensory attributes, such as connective tissue amount and flavour intensity. In a large consumer survey (Lorenzen *et al.* 2003), WBS was negatively correlated with overall liking (-0.18), tenderness (-0.26), juiciness (-0.17), consumer flavour desirability (-0.16) and flavour intensity (-0.16). Although these relationships were all significant, the correlations were quite weak. In a more recent study — based on the U.S. National Beef Tenderness Survey (Guelker *et al.* 2013) — WB measurements were related to consumer sensory scores across different beef cuts. According to their assessment, WB threshold values can be related to consumer tenderness

scores over a range of muscle types: 'Very Tender' (WBS < 31.4 N), 'Tender' (31.4 N < WBS < 38.3 N), 'Intermediate Tender' (38.3 N < WBS < 45.1 N) and 'Tough' (WBS > 45.1 N).

WBS was performed on cooked samples according to previously reported CSIRO protocols. Samples were weighed and suspended in plastic bags in a 70˚C water bath for 60 minutes, cooled in an ice slurry for 20 minutes, patted dry and re-weighed to determine cook loss. Samples were stored over night at 4˚C to set before cutting. The tenderness or toughness of meat samples was determined by using a modification of the Warner-Bratzler shear device (Bratzler, 1932) and a Lloyd Instruments LS 2.5 materials testing machine fitted with a 500N load cell (Lloyd Instruments Ltd., Hampshire, UK). Samples used in the Warner-Bratzler device had a rectangular cross-section 15mm x 6.7mm (1 cm^2 cross-sectional area), and were cut with the fibre orientation parallel to the long axis, and at right angles to the knife blade of the device. The force required to shear through the clamped samples with a triangulated 0.64mm thick blade pulled upward at a speed of 100mm/min was measured.

Data was collected using the Nexygen Plus 3 software (Lloyd Instruments Ltd., Hampshire, UK); the parameters measured from the shear force deformation curves were peak force (PF), initial yield (IY), and peak force minus initial yield (PF-IY). Six determinations were made on each sample and the mean recorded. All analyses were performed at room temperature.

4.1.3 *Warner-Bratzler Results*

Initial yield (IY) is a measure of the myofibril toughness and peak force (PF) is a measure of myofibril and connective tissue toughness. PF is defined as the definitive WBS measure, although both parameters contain useful information. Scatter plots of the relationship between MSA-MB and the mean texture parameters are shown in **Figure 17**. As expected, there was a general trend for both initial yield (IY) and peak force (PF) to decrease with increasing IMF; however significant variance was measured within some sample groups. In general the variability in both IY and PF decreased with increased marbling (MSA-MB).

Using the tenderness thresholds described above, and the PF values measured, most of the samples could be classified as 'Very Tender' to 'Tender'. The low and moderate MSA-MB WagyuGrass samples were mainly categorised as 'Very Tender' (WBS < 31.4). Most of the AngusGrain samples were within the 'Tender' category ((31.4 N < WBS < 38.3 N). The AngusGrass samples, representing the overall lowest MSA-MB scores in the study, were clearly the least tender and most variable samples overall. Some of the AngusGrass samples were unambiguously in the 'Tough' category (WBS > 45) and most were classified as 'Intermediate Tenderness' (38.3 N < WBS < 45.1 N).

4.1.4 *Relationship between WBS and MSA-MB*

MANOVA analysis was conducted comparing the three sample types using MSA-MB as a covariate. After correction for the effect of marbling, there was no difference between the sample types for either initial yield and peak force. In contrast the covariate term (MSA-MB) was highly significant. The relationship between MSA-MB and the WBS texture parameters

was further explored using correlation analysis (**Table 8**). A significant overall negative relationship ($p < 0.001$) was found between MSA-MB and IY and PF across all samples (n=39).

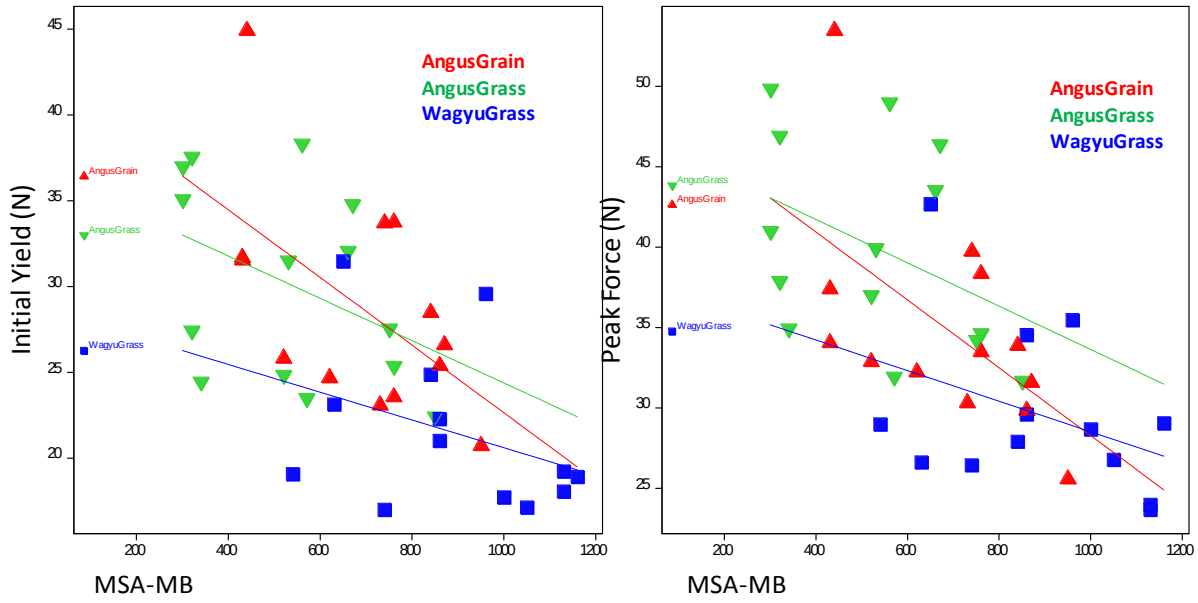

Figure 17: Scatter plots and fitted regression lines for the relationship between IMF (MSA-MB) and Initial Yield and Peak Force (n=42).

Table 8: Correlations between Warner-Bratzler texture attributes and MSA-MB scores

	Initial Yield (IY)		Peak Force (PF)	
	R n=39	P value	R n=39	P value
All Samples	-0.51	<0.001	-0.527	<0.001
Wagyu Grass	-0.57	0.017	-0.58	0.015
Angus Grass	-0.21	ns	-0.18	ns
Angus Grain	-0.56	0.04	-0.55	0.04

4.1.5 *Relationship between WBS and Sensory Attributes*

Significant correlations between WSB measurements and texture related sensory scores were also found (**Error! Not a valid bookmark self-reference.**). As expected, PF correlated more strongly with sensory scores compared to IY. The relationships for the different sample types

are shown in **Figure 18**. For some attributes, such as juiciness, there appeared to be differences for the lowest fat AngusGrass samples. It is of note that these correlations are considerably stronger than many of the published data using consumer data. The quality of trained sensory panel data is generally more reproducible and less noisy than consumer data. Use of a 100 mm line scale for sensory rating, instead of the 10-point scale commonly used in consumer testing may also be relevant.

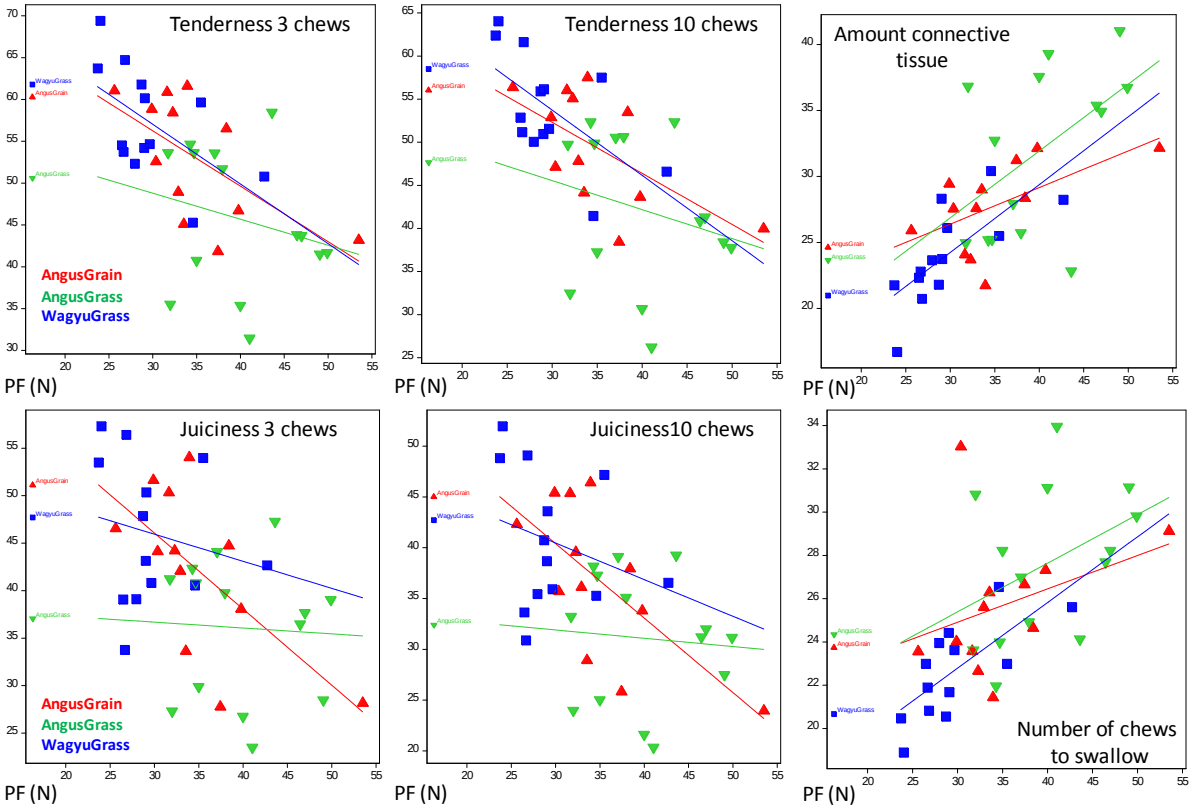

Figure 18: Scatter plots and fitted regression lines for the relationship between WBS peak force (PF) and texture-related sensory attributes (n=39).

Table 9: Pearson's correlations between WBS parameters and texture-related sensory attributes

Sensory Parameter	Peak Force (PF)		Initial Yield (IY)	
	R *n=39*	P value	*R* *n=39*	P value
Connective Tissue	*0.70*	<0.001	*0.65*	<0.001
Juiciness 3 chews	*-0.77*	<0.001	*-0.48*	0.002
Juiciness 10 chews	*-0.78*	<0.001	*-0.5*	<0.001
Tenderness 3 chews	*-0.62*	<0.001	*-0.57*	<0.001
Tenderness 10 chews	*-0.63*	<0.001	*-0.58*	<0.001
Number of chews	*0.63*	<0.001	*0.59*	<0.001

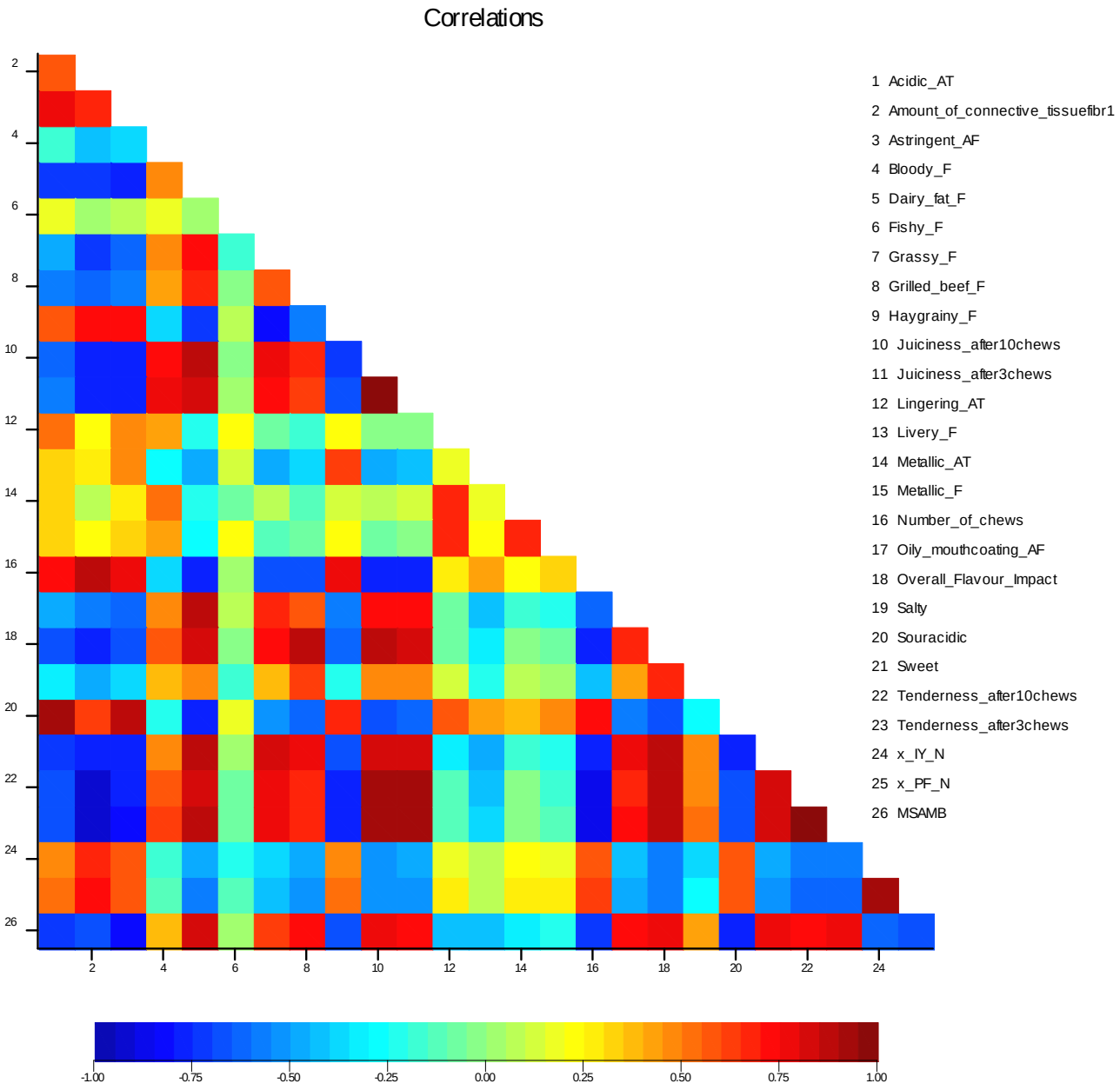

Figure 19: Correlation plot showing the strength of relationship between sensory attributes, MSA-MB and Warner-Bratzler parameters.

As discussed, WBS has been significantly correlated to consumer flavour scores in previous published research. It can be seen that both PF and IY were also correlated with a number of non-texture related sensory scores in the current study (**Figure 19**). WBS PF was positively related to *Sour/Acidic* taste (r = 0.58, p <0.001), *Acidic Aftertaste* (r = 0.5, p=0.001), *Astringent Aftertaste* (r = 0.56, p<0.001), *Hay/Grainy* flavour (r = 0.52, p <0.001). WBS was negatively related to *Overall Flavour Impact* (r = -0.57, p <0.001), *Dairy Fat* flavour (r = -0.58, p <0.001) and *Sweet* taste (r = -0.51, p <0.001).

4.1.6 *Soluble Collagen and Insoluble Collagen*

Tenderness and *Juiciness* were strongly related to the amount of IMF; however other factors such as the amount of collagen and connective tissue are known to affect meat sensory properties (Achile-Conteras *et al.* 2010). The total amount of connective tissue in the muscle was determined by measuring the hydroxyproline content in lyophilised muscle according to the International Standard method (ISO 3496:1994).

The heat soluble amount of connective tissue in the muscle was determined by measuring the hydroxyproline content in defatted lyophilised muscle according to the International Standard method (ISO 3496:1994). The samples were defatted with chloroform /methanol solution before analysis, and the sample hydrolysate and standards were neutralised with a 0.6M NaOH solution prior to performing the assay.

The relationship between IMF and these parameters was different depending on the sample type (**Figure 20**). The total collagen and soluble collagen appeared to be quite variable within a sample type. The variability was greatest for the WagyuGrass samples, especially samples with the highest IMF. Although no overall relationship between IMF and % total collagen was apparent, a significant negative correlation between IMF and % total collagen was measured for the AngusGrain (r = -0.55, p = 0.04) and AngusGrass samples (-0.48, p = 0.05). After correction for the IMF covariate, no differences between total or soluble collagen were found by MANOVA between sample types or for either breed or feed effects.

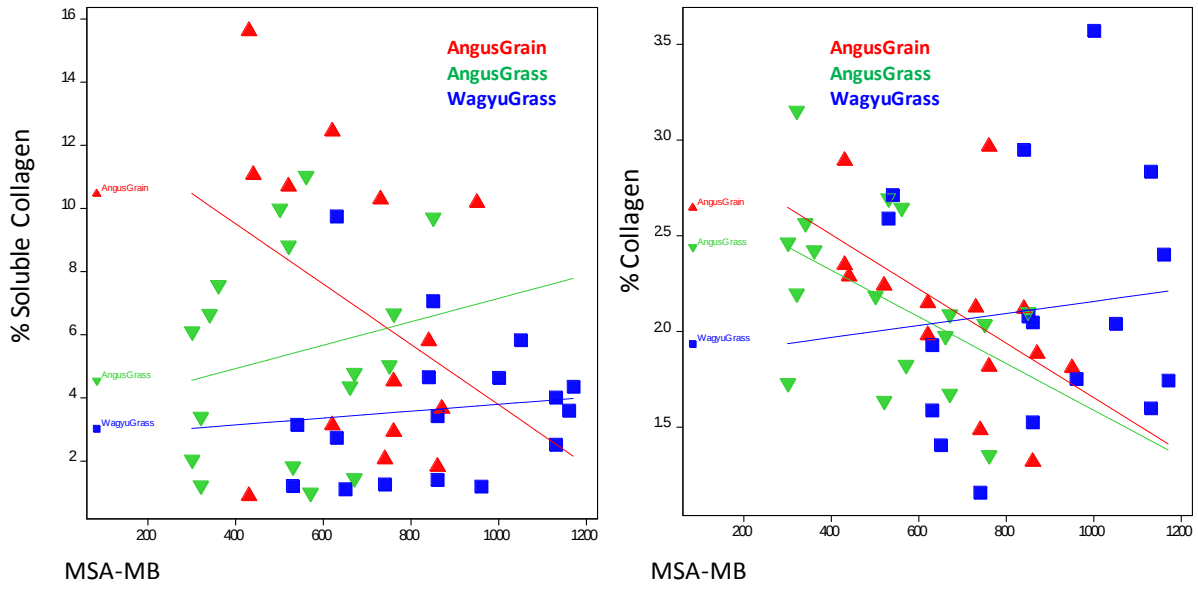

Figure 20: Scatter plots and fitted regression lines for the relationship between IMF (MSA-MB) and Soluble and Total Collagen (n=42).

4.1.7 *Measurement of TBARS, Glycogen & Free Glucose*

The concentration of thiobarbituric reactive species (TBARS) was determined from minced muscle samples (Witte, Krause *et al*. 1970). TBARS is a measure of the oxidative stability of the lipid components in the meat. Duplicate samples were capped and cooked in 75°C water bath for 20 minutes and subsequently cooled for 30 minutes at 5°C prior to extraction. The concentration of malondialdehyde (MDA) equivalents (mg MDA /kg muscle) was calculated from absorbance readings at 530nm, using 1,1,3,3-tetraethoxypropane as a standard.

Glycogen content of the frozen muscle subsamples was measured using the method of (Bergmeyer HU 1974) with rapid assay modification of H_2SO_4 addition. Samples (2g) were homogenised (1:10 w/v) in 30 mM HCl using an Ultra-Turrax 22,000rpm for 2 x 15 second bursts, centrifuged (3,000 rpm, 4°C, 10 minutes) and supernatants containing free glucose and glycogen were frozen at -20 °C until the assay could be performed. Thawed samples were analysed for total glucosyl units by incubating 50 µl (37°C, 90 minutes) with the addition of 500 µL of hydrolysing enzyme amyloglucosidase (1:200 in 40mM acetate buffer pH 4.8). The concentration (µmol/g) of total glucosyl units (considered to be glycogen content) was determined in duplicate using a glucose assay kit (sigma GAGO-20) and glucose as a standard (formula weight 180g/mol). The absorbance of both samples and standards was measured at 540nm.

4.1.8 *TBARS & Glycogen in Beef Samples*

The distributions of TBARS, glycogen and free-glucose values obtained for sample types are shown in **Figure 21**. MANOVA analysis indicated that TBARS was significantly higher in the WagyuGrass samples ($p < 0.001$) compared to the AngusGrain and AngusGrass samples. In the case of the WagyuGrass, TBARS was positively correlated with IMF ($p < 0.001$). The fat from grass-fed animals generally contains a higher amount of unsaturated lipid material compared to grain-fed. The greater TBARS for the WagyuGrass but not the AngusGrass suggests that the lipid profile of the Wagyu samples was quite different.

Glycogen and free glucose are an indication of the nutritional status of the animal prior to slaughter. Average glycogen content for the AngusGrass, AngusGrain and WagyuGrass samples was 16.5 mg/g > 11.9 mg/g < 14.42 mg/g respectively ($p = 0.04$). The muscle glycogen varied considerably within each sample type. There was no overall relationship between glycogen and IMF. Free glucose was positively correlated with IMF ($p < 0.001$), and the amount varied significantly between sample types ($p < 0.001$), with the AngusGrass highest (15.1 mg/g) > AngusGrain (11.3 mg/g) > WagyuGrass (6.9 mg/g). It should be noted that the concentrations of free glucose in the beef are an order of magnitude lower than their taste thresholds in water (~1.6%, Belitz, Grosch & Schieberle 2004). Hence free glucose is not expected to contribute to sweet taste in the samples. Free glucose may also participate as a substrate in the Maillard reaction to form odour volatiles during grilling.

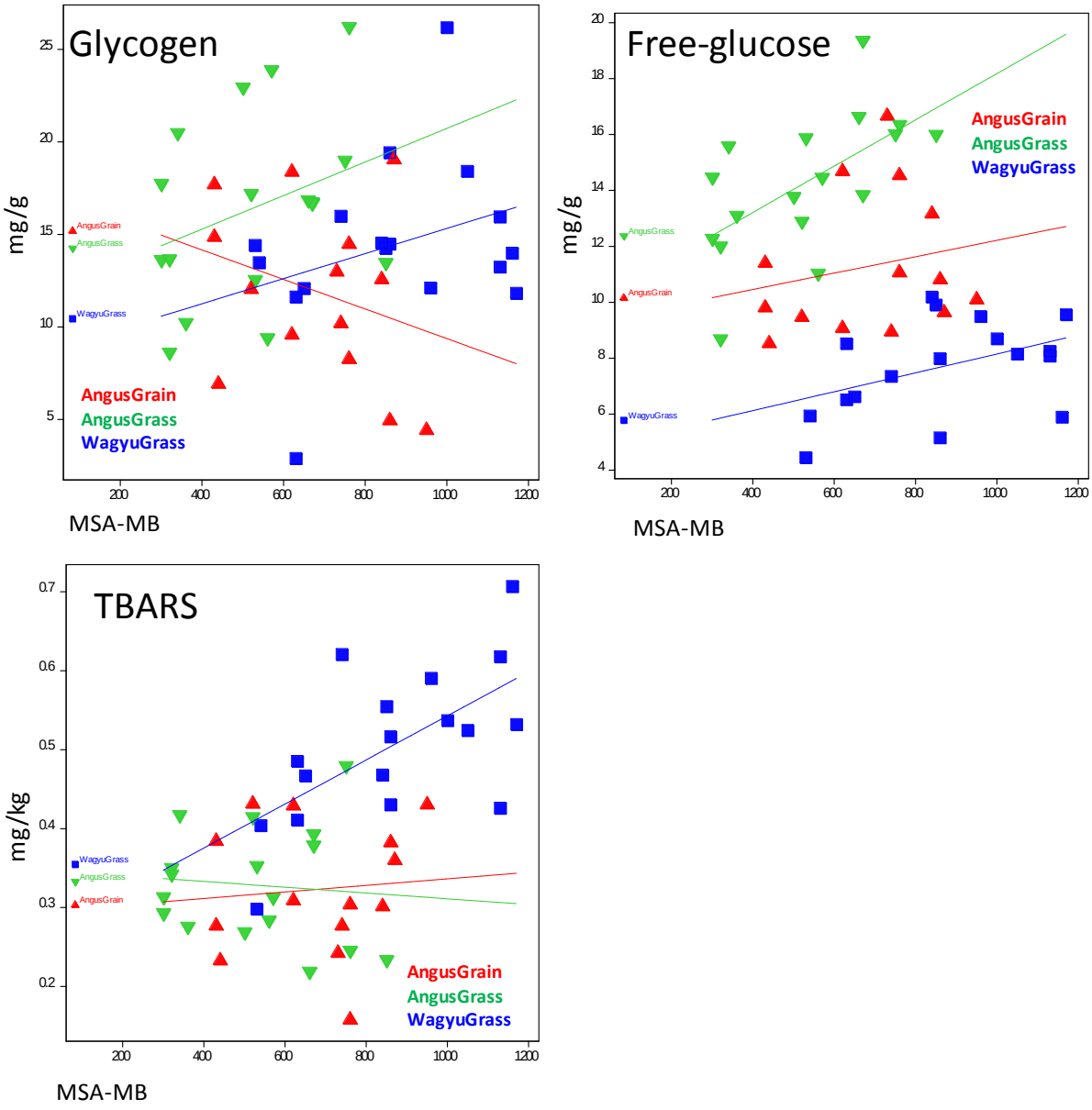

Figure 21: Scatterplot and fitted regression lines showing the relationship between MSA-MB and glycogen, free-glucose and TBARS in the beef samples. The WagyuGrass had higher TBARS than the other samples. Free glucose differed between samples and was correlated with IMF.

4.2 Meat Cooking Parameters

A thermocouple was used to ensure that all beefsteaks were cooked to the same internal temperature (57 ^{o}C) in the Silex grill, before resting under loosely placed foil. This protocol ensured all meat was cooked to the same degree of doneness; e.g. medium doneness with a pink centre. A temperature logger measured the temperature increase at 1 second intervals. The time to reach an internal temperature of 57 ^{o}C, the grill cook loss (% w/w) and rest loss (% w/w) were recorded for each batch of cooked meat. Meat was left to rest under foil for 2 minutes. The amount of cook liquid that came out of the meat during resting was defined as "rest loss" (% w/w). The time to reach an internal temperature of 57 ^{o}C was slightly longer for the AngusGrain samples compared to the WagyuGrass. There were no differences in grilling

cook loss and rest loss between the sample types after correction for the role of IMF. There was no relationship between grilling cook loss and IMF (MSA-MB). In contrast, there was a weak inverse relationship between rest loss and MSA-MB (r = -0.28, p = 0.002), with some differentiation between sample types: Angus Grass (r = -0.35, p=0.02), Angus Grain (-0.15, ns) and Wagyu Grass (r = -0.3, p = 0.06).

Previous research demonstrated that Wagyu beef IMF is naturally higher in monounsaturated fatty acids and has a lower melting point compared to other breeds (Taniguschi *et al.* 2004). The shorter grilling time required for the Wagyu steaks in the current study supports these findings. The distribution and size of the marbling fat may also potentially affect cooking times. Saturated fats have higher melting points than unsaturated fat; feedlot and feed concentrates often increase the ratio of saturated to unsaturated fat in IMF (Scollan *et al.* 2006).

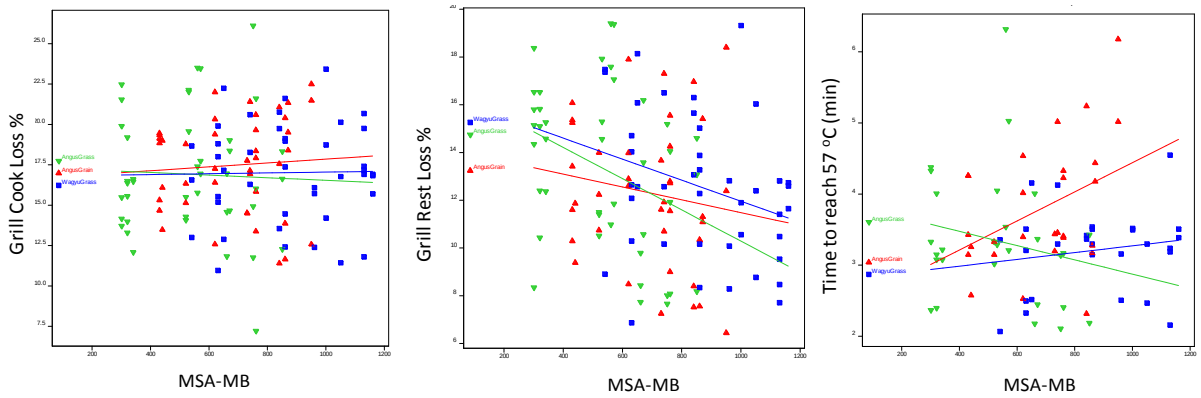

Figure 22: Regression models for the relationship between cook loss, rest loss and time to reach an internal temperature of 57 °C and MSA-MB score.

Table 10: Mean cooking parameters estimated by MANOVA using MSA-MB as a covariate

	Angus Grass	Angus Grain	Wagyu Grass	LSD	P Sample	*P* MSA-MB
Time to 57 °C (m)	3.4	3.8	3.1	0.49	0.017	ns
Rest Loss (%)	12.6	12.3	13.3	—	ns	0.002
Cook Loss (%)	16.9	17.5	17	—	ns	ns

4.3 Summary of Meat Chemical & Physical Properties

- *Cook loss during the Warner-Bratzler sample preparation was negatively correlated with marbling (samples were all cooked the same amount of time)*

- *WagyuGrass samples were categorised mainly as 'Very Tender' (WBS < 31.4). Most of the AngusGrain samples were within the 'Tender' category ((31.4 N < WBS < 38.3 N). The AngusGrass samples were the least tender; some were rated as 'Tough' (WBS > 45) and most were classified as 'Intermediate Tenderness' (38.3 N < WBS < 45.1 N).*

- *Grilling cook loss did not differ according to sample type or marbling level (grilled samples were cooked until an internal temperature of 57 ^{o}C – e.g. cooking times varied).*

- *Glycogen increased with increasing IMF, however the AngusGrass was overall highest and WagyuGrass lowest*

- *TBARS was highest in the WagyuGrass samples and increased with IMF in these samples only. This is likely to be related to differences in the composition the triacylglycerols in the IMF*

- *Residual glycogen was highest in the AngusGrass and lowest in the WagyuGrass. Glycogen is converted to lactic acid in the muscle post mortem. This implies that the concentration of lactic acid in the sample types would follow this pattern; the AngusGrass had the highest Acidity and the WagyuGrass the lowest.*

- *High IMF samples lost significantly less liquid during resting, although the amount of moisture present initially was lower – important non-volatile flavour compounds may be lost in the liquid during resting*

- *The time required to reach an internal temperature of 57 ^{o}C was affected by the level of IMF for the AngusGrain samples only. Grain fed samples required a significantly longer grilling time compared to the grass-fed samples. This may reflect a different fatty acid profile or triacylglycerol composition in the grain-fed IMF, e.g. more saturated fat in grain-fed IMF.*

5 Fatty Acid Composition of Intramuscular

5.1.1 *Relationship between total fat and MSA-MB*

The percent fat contained within the beef samples was determined using the method described in Thornton et al. (1981). Briefly, frozen beef samples were thawed and stored on ice. A ~ 50 g portion was minced using a food processor. Duplicate sub-samples (~15 g) of the minced meat were weighed and dried for 24 hr at 105 °C to constant weight in order to measure the moisture content. The moisture content (% H_2O) was determined using the formula: $\%\,H_2O = \frac{mass_{pre} - mass_{post}}{mass_{pre}}.100\%$, where $mass_{pre}$ and $mass_{post}$ represent the mass of meat prior to, and after, heating at 105 °C for 24 hr. The fat content (% Fat) was calculated, after the validated algorithm published by Thornton *et al* (1981), using the equation: % Fat = 95.6 - %H_2O x 1.24. The relationship between the % fat and the MSA-MB are summarised in **Figure 23**. The amount of fat in the samples ranged from around 3% in the lowest fat sample (AngusGrass low fat) to more than 20% fat in the WagyuGrass high fat samples. ANOVA analysis was conducted using MSA-MB as a covariate; the % fat was significantly higher in the AngusGrain samples compared to the AngusGrass and WagyuGrass; 13.2 %, 10.3% and 9.5% average fat respectively. It can be clearly seen that the % fat in the AngusGrain was higher for a given MSA-MB score compared to the grass-fed samples.

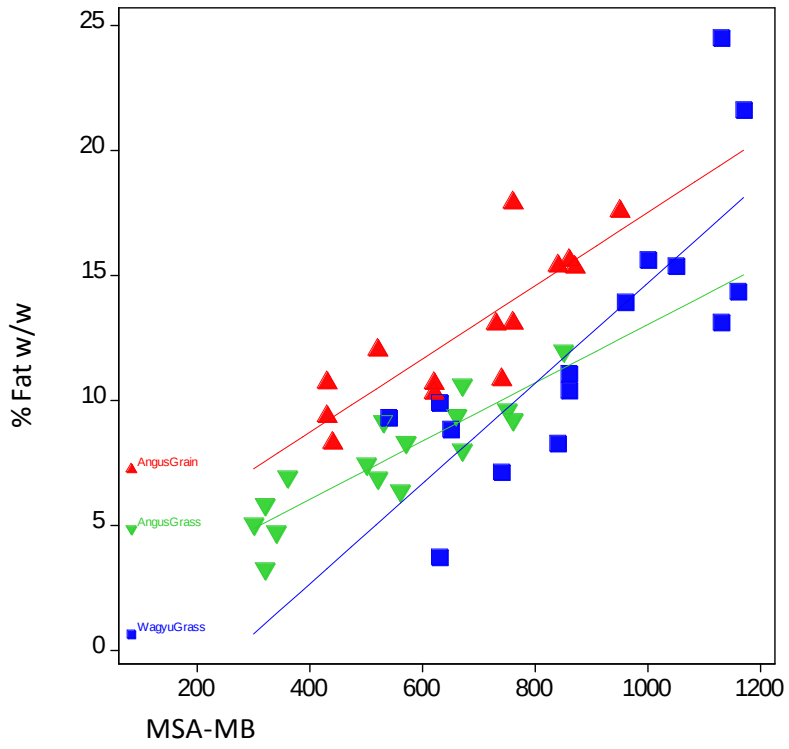

Figure 23: Relationship between MSA-MB and the % fat determined

Table 11: Summary of the fatty acid composition of the intramuscular fat extracted from beef samples

Fatty acid mg/g fat	Common name	Angus Grain n=15	Angus Grass n=16	Wagyu Grass n=15	P Sample Type	P MSA-MB	P Breed	P Feed
C8:0		0.05[b]	0.10[a]	0.09[a]	<0.001	<0.001	ns	<0.001
C10:0		0.21	0.35[a]	0.30[a]	<0.001	<0.001	ns	<0.001
C12:0	*lauric*	0.26[a]	0.33[a]	0.29	0.002	ns	ns	<0.001
C14:0	*myristic*	16.4	18.7	17.6	0.024	0.008	ns	0.002
C15:0		3.1	3.7	3.9	ns	ns	ns	ns
C16:0	*palmitic*	135[b]	154[a]	135[b]	0.002	<0.001	0.02	0.025
C17:0		6.7	5.2	4.2	<0.001	ns	0.04	<0.001
C18:0	*stearic*	73.5	84.2	65.3	0.008	ns	0.01	ns
C20:0	*arachidic*	0.5	0.6	0.5	ns	ns	ns	0.02
C14:1		3.0	2.3	2.1	0.039	ns	ns	ns
C15:1		0.4	0.9	0.7	<0.001	ns	ns	<0.001
C16:1-8*c*		16.4	18.9	20.1	0.02	ns	ns	0.04
C17:1-8*c*		5.0	3.7	3.8	<0.001	ns	ns	<0.001
C18:1-11*t*	*vaccenic*	6.0	9.8	6.8	0.04	ns	ns	ns
C18:1-9c	*oleic*	219	231	232	ns	ns	ns	ns
C18:1-11c		8.8	7.0	8.9	0.012	ns	0.03	<0.001
C18:1-12c		0.44[b]	0.58[a]	0.46[b]	ns	ns	ns	0.02
C18:1-13c		2.51	1.92	2.51	ns	ns	ns	<0.001
C18:1-14c		0.56	1.12	1.10	<0.001	ns	ns	<0.001
C18:2 *t* isomer		0.89	1.24	1.64	<0.001	ns	ns	<0.001
C18:2-9c,12c	*linoleic*	8.17	8.29	9.65	ns	<0.001	ns	ns
C18:2 isomer		0.57	0.43	0.49	0.012	ns	ns	ns
C18:3 (n-3)	*linolenic*	1.18	0.74	1.03	0.01	ns	ns	<0.001
C18:2-9*c*,11*t*	*CLA, rumenic*	2.20	3.18	3.21	ns	ns	ns	0.03
C20:5 (n-3)	*EPA*	1.81[b]	2.82[a]	2.67[a]	0.018	<0.001	ns	0.005
C20:1		1.49	4.55	5.17	<0.001	0.007	ns	<0.001
C22:5 (n-3)	*DPA*	0.14[b]	0.78[a]	0.66[a]	<0.001	<0.001	ns	<0.001
C22:6 (n-3)	*DHA*	0.65[b]	1.83[a]	1.59[a]	<0.001	<0.001	ns	<0.001
Total SFA		235[a]	266[b]	227[a]	0.002	0.03	0.009	0.02
Total MUFA		263	281	283	ns	ns	ns	ns
Total PUFAs		15.61[a]	19.30[b]	20.93[b]	0.008	<0.001	ns	0.007
n-6 Fatty acids		10.81	10.70	12.81	ns	<0.001	ns	ns
n-3 Fatty acids		2.6[b]	5.42[a]	4.91[a]	<0.001	<0.001	ns	<0.001
n-6:n-3 ratio		4.1[a]	1.97[b]	2.60[b]	0.008	<0.001	ns	<0.001
PUFA:SFA		0.066[a]	0.072[a]	0.091[b]	0.02	<0.001	ns	ns

SFA= saturated fatty acid, MUFA= monounsaturated fatty acid, PUFA= polyunsaturated fatty acid, CLA = conjugated linoleic acid (*rumenic acid*), EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid and DHA = docosahexaenoic acid

5.1.2 *Fatty Acid Methyl Ester Analysis*

Muscle samples (~1g) were homogenised with 10 mL chloroform:methanol (2:1) using an Ultraturrax (11000 rpm, 2 x 15 sec) in a 25 mL centrifuge tube. After the addition of 10 mL chloroform:methanol, the tube and its contents were allowed to stand for 2 hr at room temperature. Physiological saline (0.73% NaCl, 1/5th of total volume) was added to the tube and well mixed. The phases were separated using centrifugation (Beckman J2-MC, 1000 rpm for 5 min at 25 °C) and the organic layer was transferred to a pre-weighed scintillation vial. The organic solvent was completely removed using a Speedvac concentrator (room temperature under full vacuum for 16 hr) after which the vial and extracted lipid were weighed.

For methylation, 1 mL THF, 1 mL % H_2SO_4 in methanol and 1 mL of the internal standard (2.00 mg/mL methyl tricosanoate in heptane) was added to the vial containing the lipid, mixed with a Vortex stirrer, and heated at 70 °C for 2 hr. After cooling, 2 mL heptane and 1 mL saturated NaCl solution was added and mixed using a Vortex stirrer (10 s). The organic layer was transferred to another vial, and the aqueous layer was extracted with further heptane (2 mL) using a vortex stirrer (10 s). The organic layers were combined and washed with 1 mL 5% $NaHCO_3$ solution. The methylated lipid material (as fatty acid methyl esters, FAMEs) in the organic layer was ready for analysis and a portion (2 mL) was transferred to a GC vial. The FAMEs (1 µL) were separated using a Supelco SP-2560 column (l = 100 m, i.d. = 0.25 mm, film thickness = 0.2 µm) in an Agilent model 6890 gas chromatograph (GC) with a flame ionisation detector (FID). The GC oven was isothermally heated at 180 °C. Helium was used as the carrier gas (flow rate = 1.2 ml min^{-1}).The injector was heated at 250 °C with split injection (50:1) used for the analysis. The FID was heated at 250 °C and the flow rates for H_2 and air were 45 and 450 mL min^{-1}, respectively. Nitrogen was used as the make-up gas (45 mL min^{-1}) for the FID. FAME identification was made using Supelco GLC-20 FAME mix standard, and comparison with FAME solutions prepared from standard anhydrous milkfat.

The composition of fatty acids within the IMF are summarised in **Table 11**. Each of the three sample types were compared by MANOVA using MSA-MB as a covariate. Breed and feed comparisons were also made. Significant differences were measured for most fatty acids when sample type was compared. Few breed effects were measured. As expected there were many differences when the grass and grain fed Angus were compared. The covariate term, was also significant for a number of lipids. The major saturated fatty acids (SFAs) present in the IMF were palmitic (C16:0) and stearic (C18:0) acid, in agreement with the literature (Daley *et al.* 2010). In the sample type comparison, the AngusGrass samples often had a slightly higher concentration of individual SFAs; total SFAs were highest in the AngusGrass. Although grain-fed cattle generally have a higher concentration of SFAs in their IMF, it is not always the case (Daley 2010, Tume 2014). The grass fed samples were higher in the major monounsaturated fatty acid (MUFA), palmitoleic acid. The unique ruminant *trans*-fatty acid, vaccenic acid, was highest in the AngusGrass samples. The concentrations of the essential fatty acids, linoleic and linolenic acids did not differ largely between sample types, somewhat at odds with published literature, where most studies have found higher levels of both lipids in grass-fed beef (Daley *et al.* 2010). Conjugated linoleic acid (CLA), ascribed with a number of potential health benefits, was higher in both grass-fed samples, in agreement with most published data. All of the polyunsaturated omega-3 fatty acids (PUFAs), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were present at significantly higher concentration in the two grass-fed samples compared to the grain-fed. The n-6/n-3 fatty

acid ratios were significantly higher in the grain-fed samples, in agreement with published data. The covariate term, MSA-MB was significant for a number of lipid classes. In general, the total SFAs increased and the PUFAs decreased in the IMF as MSA-MB increased. The changes in the concentration of selected fatty acids (mg/g IMF) with increasing MSA-MB are shown below in **Figure 24**. It was noteworthy that the concentration of omega-3 fatty acids was highest in the IMF of the lowest fat AngusGrass samples. The elevated concentration of these PUFAs

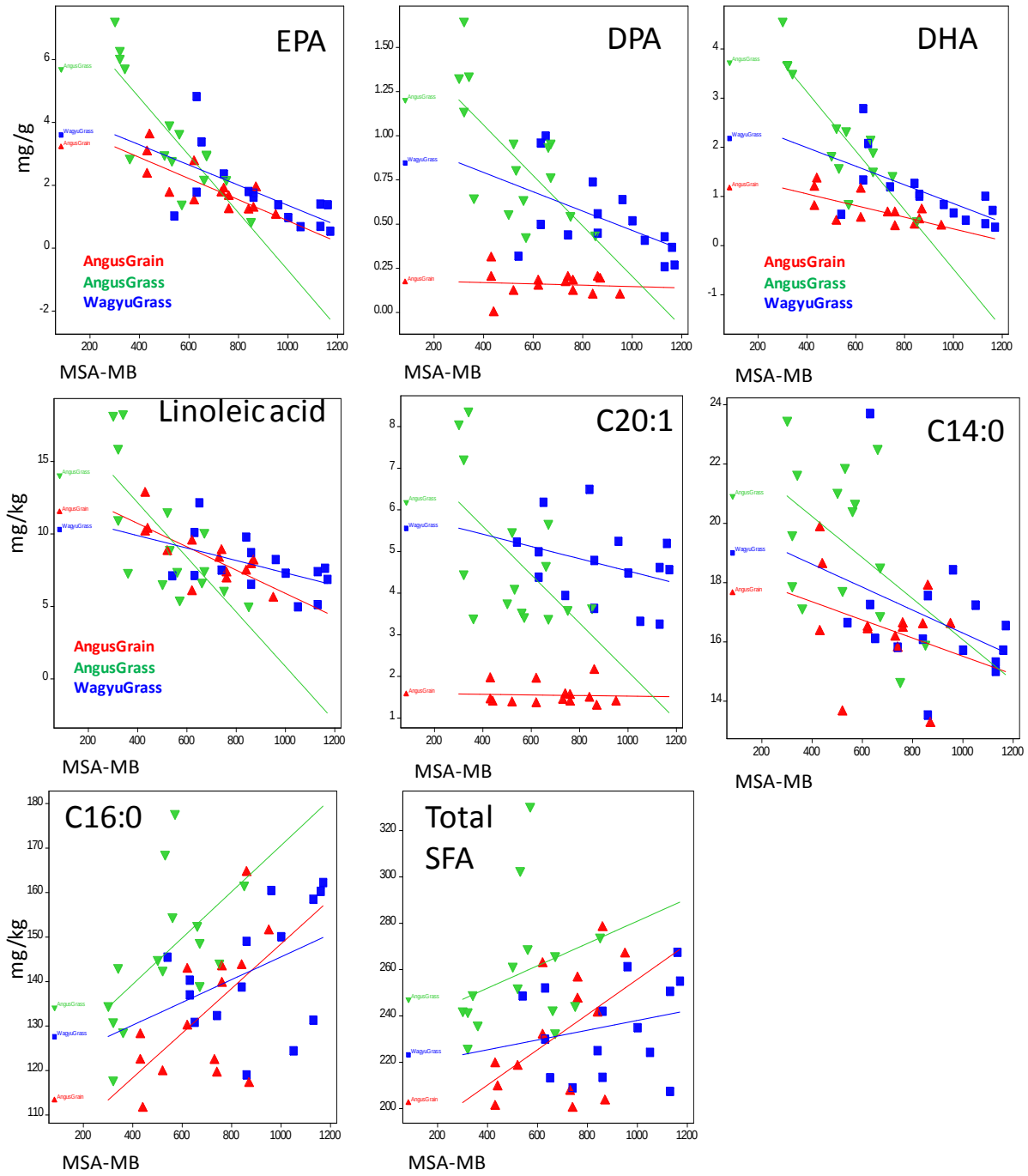

Figure 24: Relationship between increasing marbling (MSA-MB) and the composition of the intramuscular fat for selected fatty acids.

Finally, the average amount of each lipid type (mg) per 100 g serve of beef was estimated (**Table 12**). It can be seen that the concentration of SFA and MUFA increased with marbling level, as did CLA and vaccenic acid.

Table 12: Estimated amounts of fatty acids (mg) consumed per 100g serving of marbled beef.

		AngusGrain			Angus Grass			WagyuGrass		
		Low	Med	High	Low	Med	High	Low	Med	High
% Fat		10	14	15	5	8	10	8	11	17
mg/100g										
C8:0		0.6	0.6	0.8	0.8	0.7	0.9	0.7	0.8	0.9
C10:0		2.1	3.0	3.1	2.2	2.7	3.4	2.3	3.0	4.1
C12:0	*lauric*	2.6	3.9	3.8	1.5	2.7	3.4	2.3	3.0	4.9
C14:0	*myristic*	172	224	240	102	157	175	134	180	278
C15:0		32	47	46	15	28	41	34	45	70
C16:0	*palmitic*	1257	1807	2144	677	1219	1495	1070	1566	2564
C17:0		60	82	109	24	43	49	31	51	78
C18:0	*stearic*	688	966	1165	412	734	756	508	746	1140
C20:0	*arachidic*	5	6	7	3	6	5	4	6	8
C14:1		33	36	47	12	21	23	14	28	30
C15:1		4	6	6	5	8	9	6	8	11
C16:1- 8c	*palmitoleic*	162	236	243	85	138	199	169	213	371
C17:1- 8c		47	64	79	18	28	39	32	41	70
C18:1-11t	*vaccenic*	48	104	103	47	83	109	61	81	100
C18:1-9c	*oleic*	2060	2946	3452	1097	1741	2301	1908	2654	4196
C18:1, c11		87	122	131	37	50	74	77	91	151
C18:1, c13		24	35	40	8	13	22	23	27	47
C18:1, c14		5	7	9	6	8	10	8	13	22
C18:2,	*linoleic*	97	107	112	72	61	70	67	90	112
C18:2, c12		6	7	8	3	3	4	4	6	8
C18:2, t		8	13	14	6	8	13	12	19	34
C18:3a		6	9	12	4	5	8	8	9	14
C18:3 b		13	19	16	4	5	8	9	9	21
C20:1		15	21	24	32	31	42	40	54	73
C18:2-9c,12c	*CLA*	23	37	33	17	28	36	31	28	45
C22:6 (n=3)	*DHA*	9	6	9	15	13	14	11	11	10
C22:5 (n=3)	*DPA*	1.6	2.2	2.2	6.2	5.2	7.2	4.7	6.5	6.3
C20:5 (n=3)	*EPA*	24	22	23	28	22	22	18	18	16
Omega-3		35	30	34	49	41	43	35	35	32
Omega-6		123	148	154	86	79	99	97	127	181
SFA		2219	3140	3718	1238	2192	2528	1787	2601	4146
MUFA		2491	3585	4143	1349	2124	2831	2341	3217	5078
PUFA		181	215	221	151	148	178	162	190	258

6 Volatile Analysis of Grilled Beef Samples

Preparation of representative volatile extracts for analysis by gas chromatography-mass spectrometry (GC-MS) remains a significant analytical challenge. Different extraction/concentrations methods have advantages and disadvantages; a clear understanding of their characteristics and limitations is required. Solid phase microextraction (SPME) is a widely used headspace concentration technique that can easily be automated and requires minimal sample preparation. SPME fibres are, however, sometimes biased towards higher molecular weight volatiles, leading to significant underestimations of the real concentration of important low molecular weight volatiles, such as 2-methylbutanal, 3-methylbutanal and 2,3-butanedione. Dynamic headspace (DHS) purge and trap techniques are considerably more time consuming, less reproducible and less easily automated, but offer greater sensitivity and less bias. Volatile profiles obtained using DHS provide a more balanced "fingerprint" of the composition of the headspace than SPME. DHS allows quantification of potent odour-active volatiles present at very low concentration (parts per trillion), such as methional, 2-acetyl-1-pyrroline and 2-acetyl-2-thiazoline. Examples of typical SPME and Tenax volatile profiles are shown in **Figure 25**. It can be clearly seen that SPME is relatively insensitive to compounds such as 2-methylpropanal and 3-methylbutanal compared to Tenax. Selected samples were analysed by both SPME and DHS to obtain the maximum amount of information from the samples.

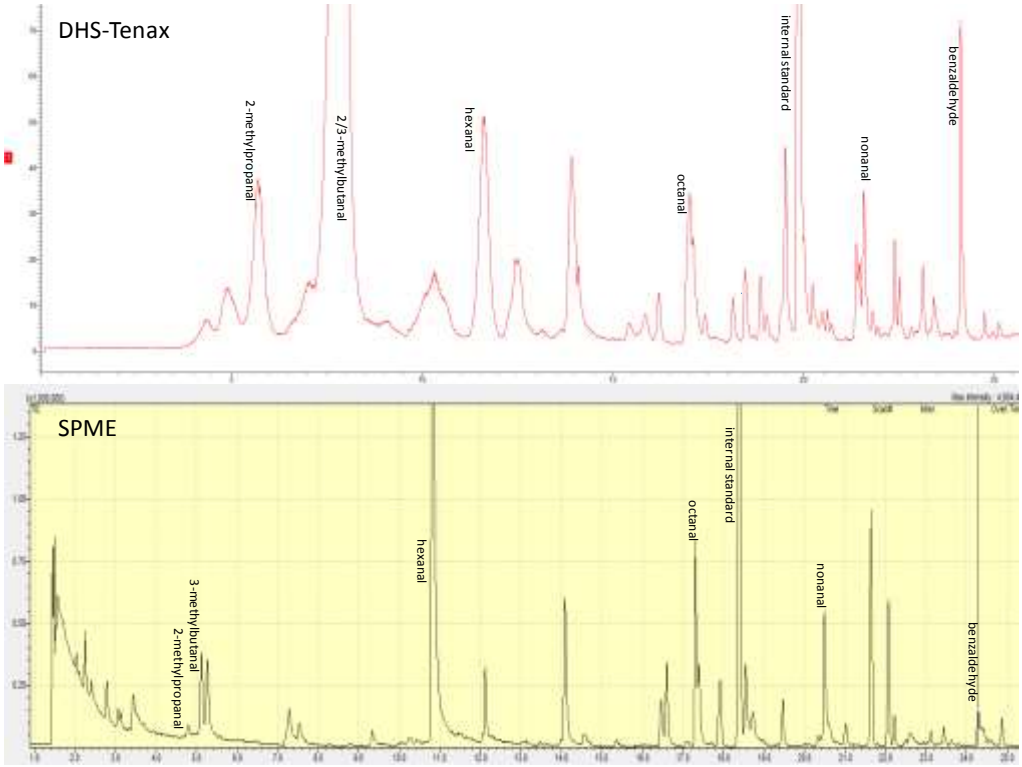

Figure 25: Typical example of the total ion chromatogram obtained using DHS Tenax (top) and SPME (bottom). The internal standard is present at the same concentration in both samples and can be used as a reference point. SPME is relatively insensitive towards low molecular weight volatiles such as 2-methylpropanal and 2 and 3-methylbutanal.

6.1.1 *Background - Beef Volatile Analysis*

Beef samples were grilled according to the standardised method used for sensory analysis. After allowing samples to rest under foil, a middle section of steak was cut and added to Milli-Q water. A ratio of 1 part sample (e.g. 40 g) to 2 parts Milli-Q water (e.g. 80 g at ~ 40 ^{o}C) was immediately macerated in a glass beaker with the hand blender. Replicate samples were made from different steaks. For SPME analyses, three or more replicates were prepared for each sample type. For the more time consuming DHS method, only two replicates per sample type were prepared. For the purposes of volatile analyses, only samples from low and high fat groups were prepared. The final extraction method conditions were selected to reflect the in-mouth conditions of eating. The volatiles produced during grilling are mixed with saliva as the meat structure is broken down into fine particles. Sampling from the headspace of stirred wet slurry, rather than a "dry" piece of meat better reflects *in vivo* release dynamics as meat particles and volatiles are mixed with saliva during eating. Aroma volatiles represent a diverse range of chemical classes and typically differ widely in their fat solubility or octanol/water partitioning behaviour. In foods containing water and lipid phases, compounds partition differentially into either the water or lipid phase depending on their Log*P* values. Log*P* values are widely used measure of relative fat solubility – low values indicate low fat solubility, whereas high values indicate highly fat soluble or lipophilic compounds.

6.1.2 *Solid Phase Microextraction Method*

A 6 g amount of meat slurry, prepared as for DHS, was placed in a headspace vial and a 10 µL aliquot of the internal standard (IS) 4-methylpentanol was added before thoroughly mixing. Sample vials were placed into an AOC-5000 auto-injector (Shimadzu). The headspace volatiles were extracted onto divinylbenzene/Carboxen®/PDMS SPME fibres (23 gauge, Supelco) for 60 minutes at 45 °C with sample agitation. After extraction the volatiles were desorbed into the hot injector (250 °C) in splitless mode and analysed by GC-MS (QP 2010 Plus GC-MS, Shimadzu). Volatiles were separated on a Sol-Gel Wax column (SGE, Australia, 30 m, 0.25 id, 0.25 µm film) using temperature programming; initial temperature 35 ^{o}C (held 5 minutes) and then heated at 5 °C/min to 250 °C. The mass spectrometer was programmed to scan the mass range *m/z* 40-250. Semi-quantitative data were generated using the Shimadzu proprietary software "LabSolutions" (Version 2.53). Integrated area data were normalised to the IS and expressed as a percentage of the IS. Mass spectral matches were conducted with the NIST Mass Spectral Search database.

6.1.3 *Dynamic Headspace Method*

Dynamic headspace (DHS) extraction onto Tenax-TA (poly-2,6-diphenyl-p-phenylene oxide, 100 mg) is generally more sensitive than SPME and is capable of absorbing a greater number of volatiles. This method was optimised to produce volatile extracts for both GC-MS and gas chromatography –olfactometry experiments described in following sections.

A 60 g mass of macerated meat slurry was weighed into a 250 mL Schott bottle with 100 µL of the IS, 4-methylpentanol. The sample bottle was sealed with a gas tight Teflon closure fitted with custom made connecting gas ports. The whole Schott bottle was placed in a water bath

and the internal temperature was equilibrated to 45 ºC. The headspace was purged with 150 mL/min of high purity nitrogen for 30 minutes at 45 °C and volatiles were collected onto Tenax-TR traps (60/80 mesh size, 100 mg). The traps were desorbed using a short path thermal desorption unit (Scientific Instrument Services, New Jersey, USA) directly into the hot GC injector (250 °C). A GC-MS (Varian 4000 ion-trap) and an olfactory port (ODO-II, SGE, Australia) were connected to the GC capillary column via a splitting device; the column effluent was split approximately 1:1 to MS detector and the "sniff-port". Volatile separation was achieved using a Zebron-WAX column (Phenomenex, 30 m, 0.32 i.d., 0.5 µm film) with the following temperature programming; initial temperature 40°C (held for 5 minutes) then increased at 6 °C/ minute to 245 °C (held for 0 minutes)and finally 30°C/min at 260°C.(1 min hold) The transfer line to the MS was held at 260 °C and the ion-trap detector was operated at 200 °C, the emission current set at 10 µAmps for electron impact (EI) mass spectra. In addition to EI mass spectrometry, selected samples were also run in methanol chemical ionisation (CI) mode in order to obtain further information regarding the mass of the $[M+H]^+$ parent ion. Total ion chromatogram data were analysed and integrated by the Varian Star MS-Data Review Software (Vers 6.41). Reference standards were used to confirm the identity of a number of key compounds. Integrated area data were normalised to the IS and expressed as a percentage of the IS. Mass spectral matches were conducted with the NIST Mass Spectral Search database.

6.1.4 *Grilled Beef volatile profiles - Results*

Replicate measures for volatiles were subjected to MANOVA analysis. As for sensory data, breed comparisons were made using only the WagyuGrass and AngusGrass samples. For feed comparisons the AngusGrain and AngusGrass were compared. The MSA-MB scores were used as a covariate in all analyses. Correlations between volatiles and the level of IMF (MSA-MB) were also calculated. Most compounds were significantly correlated with MSA-MB.

A summary of the main volatile compounds identified by both SPME and DHS are listed in the Appendix (**Table 21** & **Table 22**). Volatiles compounds listed are broadly grouped according to class; e.g. alcohols, ketones, pyrazines and other Maillard generated compounds, aldehydes, etc. In some cases volatiles were better detected by one technique compared to the other. For example SPME was not sufficiently sensitive to measure methional and other compounds such as 2-acetyl-1-pyrroline reliably. Both of these compounds were resolved by the DHS method using the ion-trap mass spectrometer. In most cases however, the data obtained by both analytical methods were broadly similar. Selected relationships between the IMF and volatiles are plotted in **Figure 26** (DHS Tenax) and **Figure 27** (SPME). There was a fair degree of variability in the measured concentrations of headspace volatiles by both methods; despite this some significant general trends were apparent. It should be noted that, the sensory data for odour-related attributes was quite variable; hence some variability in volatile profiles may be expected.

In general, the WagyuGrass and AngusGrain samples had higher alcohols in the headspace compared to the AngusGrass. The WagyuGrass samples had on average slightly higher levels of aliphatic alcohols than the AngusGrain (breed effect). The concentration of *p*-cresol (4-methylphenol) did not differ between sample types. Note that an odour corresponding to *p*-cresol, a compound traditionally associated with *Barnyard* odour, was not detected in the

aromagram of beef samples (see next section) indicating that this compound was present at a sub-threshold olfactory concentration.

2-Methylpropanal, 2-methylbutanal and 3-methylbutanal were quantitatively amongst the most dominant volatiles in cooked beef headspace (DHS extracts). The shortcomings of SPME for quantitative analysis of these important volatiles have been discussed. Despite this, both SPME and DHS data showed that both compounds were positively correlated with IMF. The DHS data indicated that the AngusGrain on average had a higher headspace concentration of these compounds. In contrast, more fat soluble odour-active aldehydes, such as octanal, nonanal, (*E)*-2-nonenal were negatively correlated to IMF; i.e. as the MSA-MB increased, the headspace concentration of these fat derived volatiles decreased. Consistent directional trends were measured by SPME and DHS (**Figure 26**, **Figure 27**).

Some small feed and breed differences were apparent; results were somewhat different for each technique. *(Z)*-4-Heptenal, was higher in the grass fed samples and decreased with increasing IMF. In contrast, benzeneacetadehyde, an important odour-active volatile increased with MSA-MB. According to the SPME data methyl butanoate increased with marbling and ethyl butanoate decreased. The water-soluble high impact odour compound, 2,3-butanedione (diacetyl) was not affected by IMF, whereas the related compound, 2,3-pentanedione increased positively. The volatile compound 3-hydroxy-2-butanone was higher in the AngusGrain compared to the AngusGrass samples. The unsaturated lipid breakdown products, (*E,E*)-2,4-hexadienal and *(E,E*)-2,4-nonadienal, both decreased as the IMF increased. The grass fed samples were higher in these compounds compared to the AngusGrain samples, consistent with grass fed meat having a higher amount of unsaturated fat.

In a comprehensive olfactometry study (Resconi *et al.* 2012), grilled meat from grass-fed and grain-feed steers was compared. They found few volatile differences however concluded that a major difference was a higher concentration of methional in the grain-fed samples and higher (*E,E*)-2,4-heptadienal in the grass fed meat. In the current study, this was not the case as methional was higher in the AngusGrain and AngusGrass, compared to the WagyuGrass. In contrast, there was clear evidence that *(E,E)*-2,4-heptadienal and related compounds *(Z)*-4-heptenal, *(E,E)*-2,4-hexadienal and *(E,E)*-2,4-nonadienal were higher in grass fed samples.

The most obvious and consistent volatile difference occurring with increasing marbling, was the increase in all pyrazine compounds and related Maillard reaction products. Both SPME and DHS data confirmed the same trend for these important aroma active molecules. Although the relationship was not overall significant, 2-acetyl-1-pyrroline increased positively with increased fat. Strecker degradation of amino acids can occur purely through interactions with highly reactive lipid degradation oxo-alkanal intermediates (Zamora *et al*. 2013). Interactions between Maillard reaction pathways and lipids have been characterised (Whitfield & Mottram 1992) affecting the rates of formation of volatile classes including alkylpyrazines.

Finally, the concentration of sulphur compounds, including the important odour impact volatile methional, increased with increasing IMF. Although some small breed and feed differences were measured for the sulphur compounds, consistent trends were not apparent.

As a general observation, the release of compounds with the highest lipid solubilities, Log*P* values > 2 (see Log*P* values listed in **Table 13**) e.g. octanal, nonanal, (*E*)-2-nonenal, (*E,E*)-

2,4-nonadienal and (*E,E*)-2,6-nonadienal decreased with increasing IMF or (MSA-MB). In contrast, most of the other volatiles with lower Log*P* values increased with IMF.

6.2 Volatile analysis Summary

- *There was considerable variation in the concentrations of volatiles in the headspace of different replicate steaks prepared from the same sample type. The variability in volatile profiles is consistent with the sensory data for odour-related attributes, which showed the weakest relationship with marbling.*

- *Although small volatile differences due to feed and breed were measured, the most consistent volatile differences were correlated to IMF (MSA-MB)*

- *The Tenax data suggested that the overall amount of volatiles produced in the AngusGrass and AngusGrain were slightly higher than the WagyuGrass*

- *Variability in the concentration of volatiles appeared to increase as fat increased*

- *The headspace concentration of key flavour volatiles increased with increasing IMF; this applied mainly to volatiles with low and intermediate lipophilicities (LogP values < 2). This was especially true for 2-methylbutanal, 3-methylbutanal and alkylpyrazines - key grilled beef flavour volatiles*

- *The headspace concentration of the most fat soluble compounds (LogP >2) decreased with increases in IMF. This is likely due to fat soluble compounds remaining dissolved in the fat phase.*

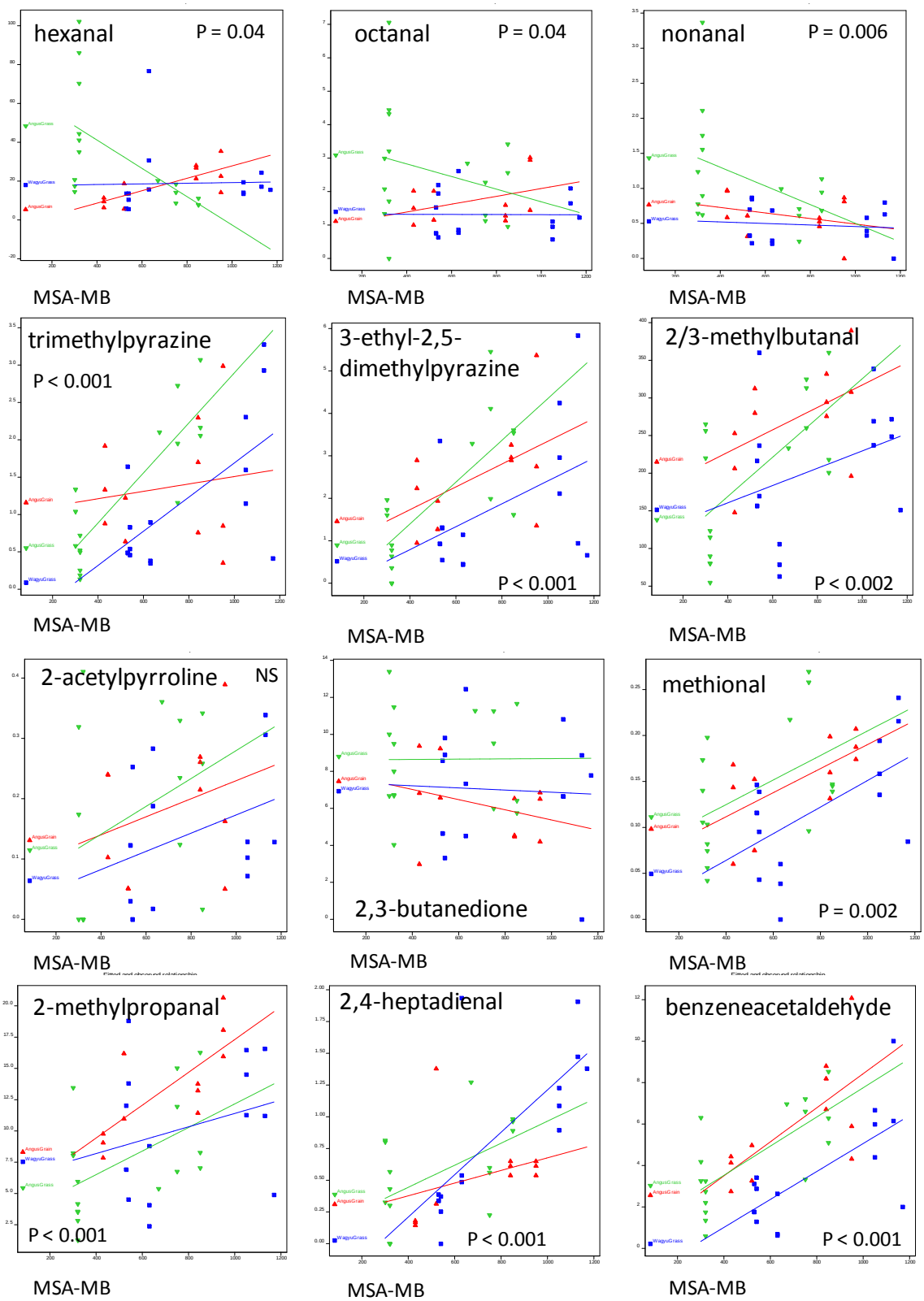

Figure 26: Tenax- Dynamic headspace volatile data. Relationship between selected headspace volatiles and marbling level (MSA-MB).

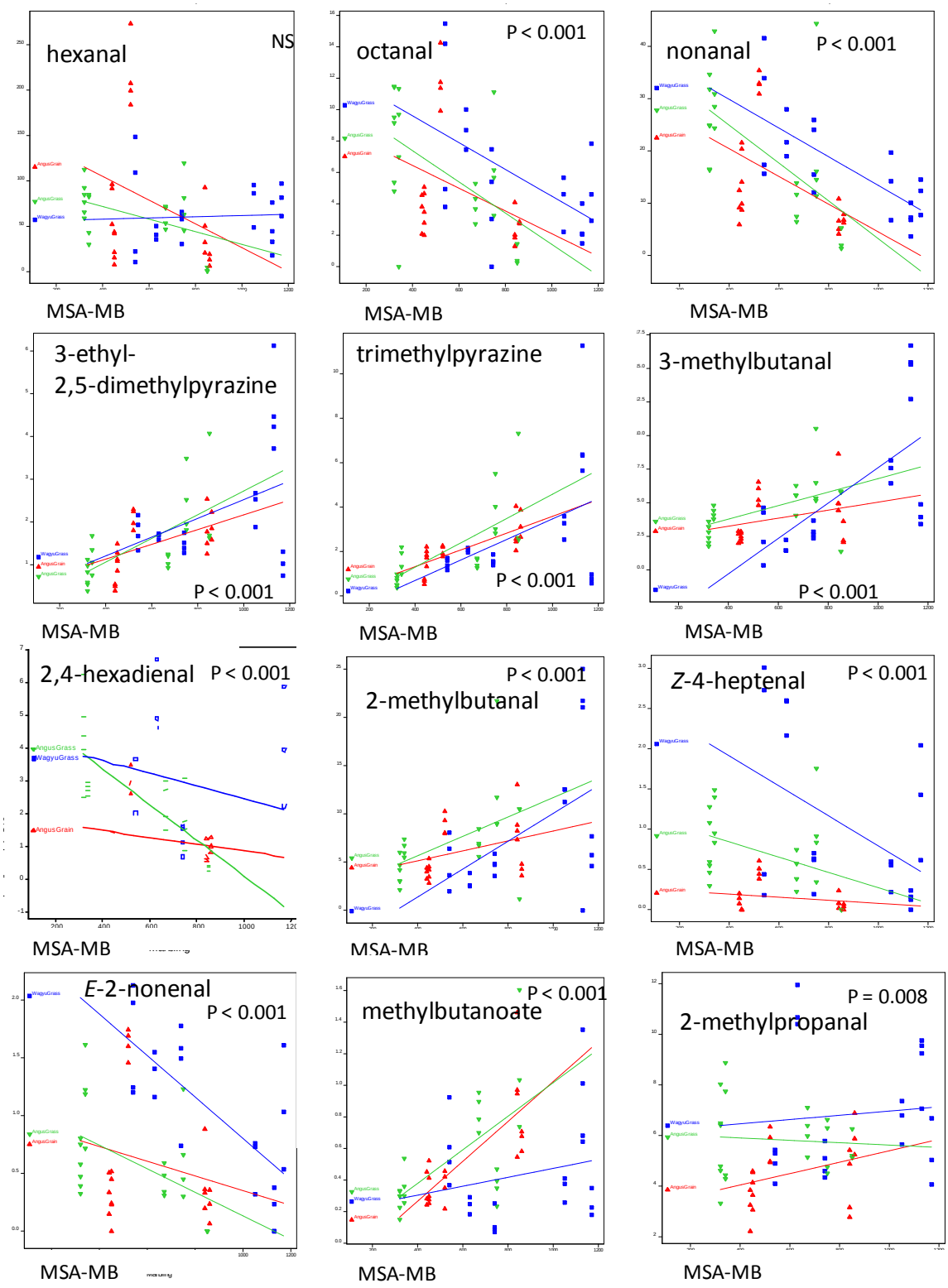

Figure 27: SPME headspace volatile data. Relationship between selected headspace volatiles and marbling level (MSA-MB).

7 Gas Chromatography Olfactometry

In order to elucidate the key odour-active volatile compounds in grilled beef aroma, gas chromatography-olfactometry (GC-O) was performed on headspace samples concentrated by dynamic headspace as described previously. After desorption of samples into the GC-MS (Varian, ion-trap) the effluent was sniffed by a panel of five "sniffers" or trained assessors. The intensity of the odour was recorded using a computer mouse controlled 100 mm line scale using Compusense® data capture software. Time intensity data was captured at a rate of 1 scan per second. Assessors described out loud the quality of the perceived odours, which were recorded onto a digital audio file. A typical example of a total ion chromatogram (TIC) and corresponding individual time intensity profile for grilled beef volatiles is shown in **Figure 28**. The TI data for each individual was annotated with descriptors before further processing. After lining up data across replicate samples, any odours reported by two or less assessors were considered as noise and deleted. In some cases, odour peaks may have corresponded to multiple very closely eluting or co-eluting compounds – in this case only one integrated odour intensity was calculated. The TI responses had both maximum intensity (height) and duration (width). After aligning data, the integrated area for each odour peak was calculated and the average calculated across samples.

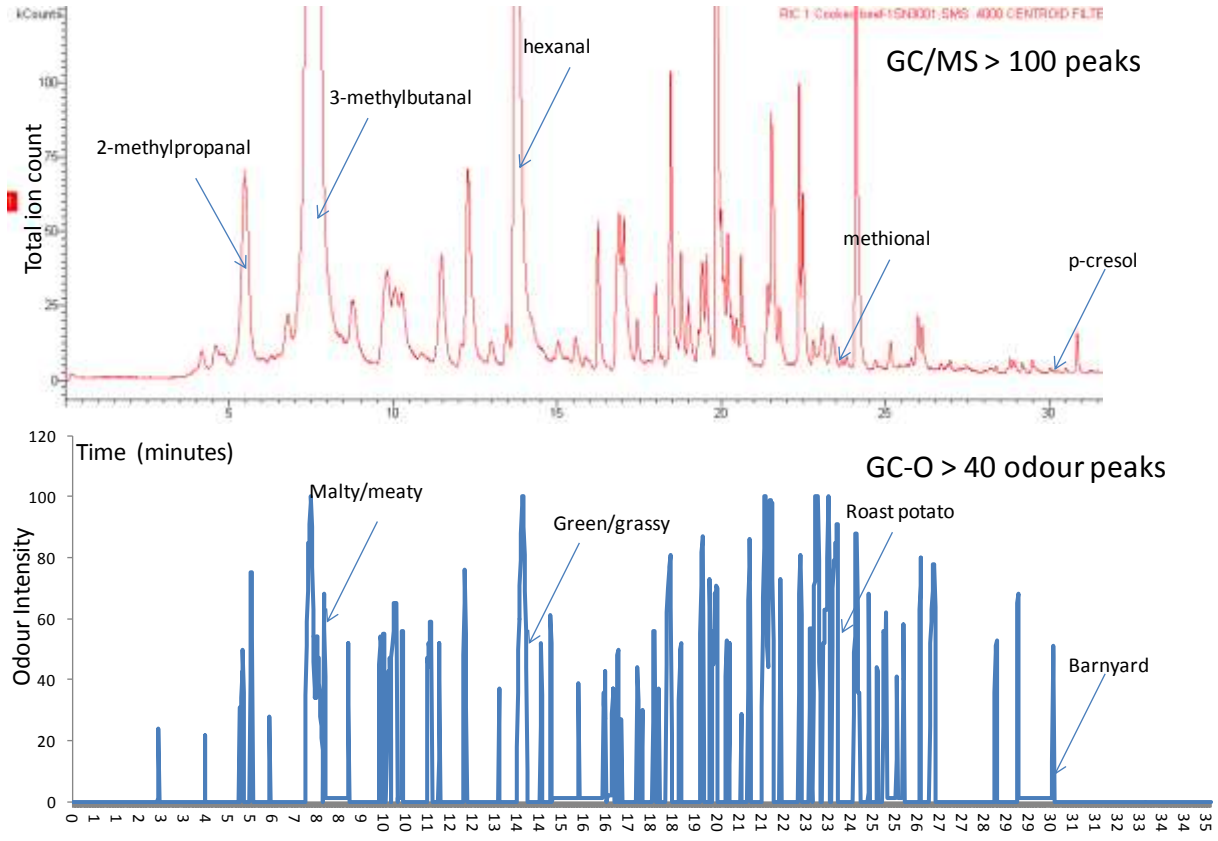

Figure 28: Top- typical GC-MS profile of dynamic headspace extract from grilled beef and corresponding aroma profile obtained using GC-O.

7.1.1 *Olfactometry Results*

Five separate beef samples were grilled for each of the low and high IMF bands. Each sample was sniffed by 5 assessors. Hence 5 x low fat + 5 high fat x 3 samples = 30 samples were sniffed. **Figure 29** shows an average grilled beef aromagram calculated across all beef samples (n=30). The main volatile compound associated with the retention time of the odour peak is given; in many cases there were co-eluting compounds that may also have contributed to the perceived odour (e.g. 2-methylpropanal, 2-butanone and dimethyl sulphide). The most frequent odour descriptors given by assessors at each integrated odour, and co-eluting compounds are summarised in **Table 13.** No odours were unique to any particular sample, but rather differed across samples in terms of reported intensity (indicated by the standard deviation bars in **Figure 29**. The dominant grilled beef odours were from 3- and 2-methylbutanal combined (*malty, yeasty*), closely followed by 2,3-butanedione (*caramel, sweet*). Following these were nonanal/dimethyl trisulphide (*plastic, sweet, metallic, cabbage*), methional (*baked potato, savoury*) and 5-ethyl-2,3-dimethylpyrazine (*roasted beef).* Other important intermediate intensity volatiles were identified as 2,4-(*E,E*)-nonadienal (*savoury, potato*), 2-acetyl-2-thiazoline (*popcorn, roast meat*), hexanal (*fresh, grassy*) and 1-octen-3-one (*mushroom, earthy*). A number of other lower intensity compounds also contributed to the grilled beef aromagram. Further detail of the odour-active compounds identified in the grilled beef headspace extracts is summarised below (**Table 13**).

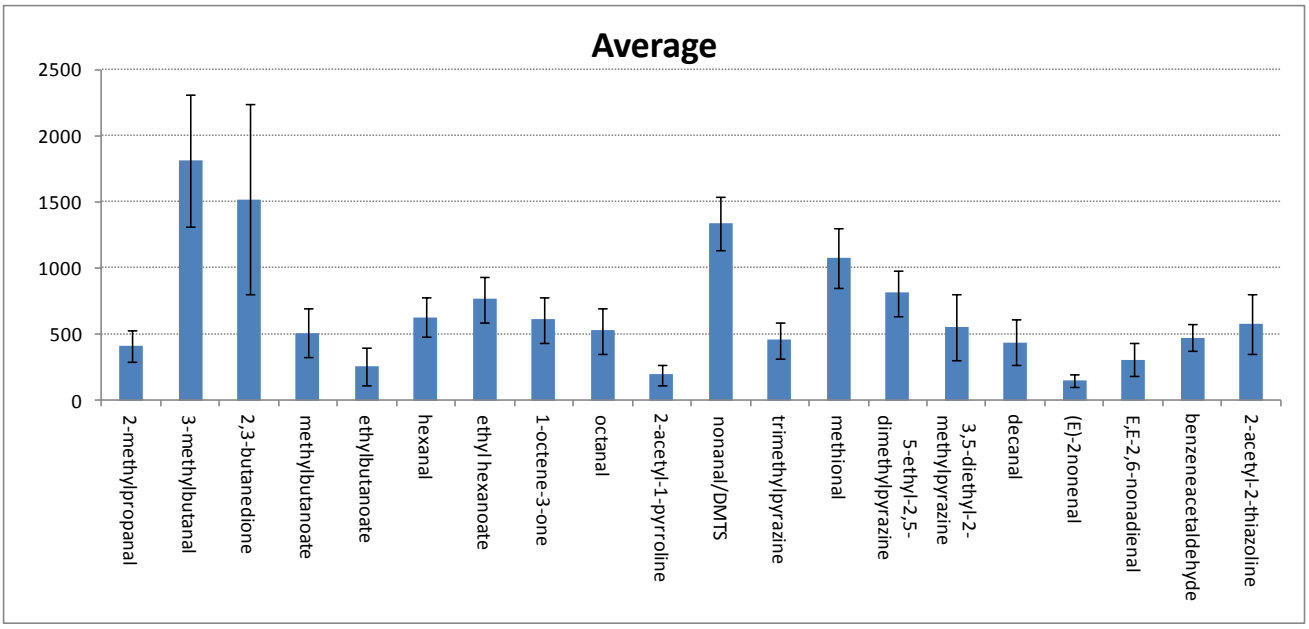

Figure 29: Average grilled beef aromagram obtained over all beef samples (n=30) from DHS-Tenax headspace extracts. The higher the bar the greater the odour-impact, e.g. the odour-impact of 3-methylbutanal was around 18-times higher than (*E*)-2-nonenal. Error bars are a guide to the variability in reported strength of an odour stimulus across samples.

Table 13: Most likely identity of compounds identified by GC-MS corresponding to odour active peaks identified by GC-O. Odour Quality described the most frequent word used to describe the odour. Log*P* = measure of lipid solubility.

Ret Time	Log*P*	Main Compound	Closely or co-eluting compounds (LogP)	Odour Quality Descriptors*
5.8	*0.72*	2-methylpropanal [1,2]	2-butanone (0.37), dimethyl sulphide[2] (0.89)	*Brothy, meaty*
7.9	*1.2*	2/3-methylbutanal [1,2]		*yeasty, malty, sour milk, potato, earthy, raw meaty*
9.7	*-1.3*	2,3-butanedione [1]	pentanal [1], (E,E)-2,4-hexadienal (1.06)	*caramel, sweet*
10.15	*1.05*	methyl butanoate		*burnt, fruity, bubblegum*
12.17	*1.66*	ethyl butanoate		*bubblegum, sweet, vanilla*
14.7	*1.97*	hexanal [1,2]		*fresh, grassy, green*
18.15	*2.9*	*(E,E)*-2,4-nonadienal	ethyl hexanoate[1] (2.8)	*savoury, potato, sweet*
19.43	*2.17*	1-octene-3-one [1,2]		*mushroom, earthy*
19.55	*3.03*	octanal [1,2]		*sweet, citrus*
20.5	*-0.02*	2-acetyl-1-pyrroline [1]	2,6-dimethylpyrazine	*Popcorn, roasted*
21.23	*3.56*	nonanal [1,2]	dimethyl trisulphide[1,2]	*plastic, metallic*
22.32	*1.1*	trimethylpyrazine	2-ethyl-3-methylpyrazine	*chocolate, earthy*
22.38	*0.71*	methional [1,2]		*roast potato, savoury*
22.87	*1.63*	2-ethyl-3,5-dimethylpyrazine[1,2]		
23.22	*1.96*	3,5-diethyl-2-methylpyrazine[1,2]		
23.63	*4.09*	decanal[1]		*fatty, sweet*
24.7	*3.17*	*(E)*-2-nonenal[1,2]		*fatty*
25.73	*2.9*	*(E,E)*-2,6-nonadienal[1]		*sweet, floral*
26.13	*1.78*	benzeneacetaldehyde		*grainy, sweet*
27.13	-0.89	2-acetyl-2-thiazoline[1,2]		*popcorn, roasted, meaty*

LogP = lipophilicity – higher number means more fat soluble. Log*P* values obtained from Chemspider (Royal Society of Chemistry, www.chemspider.com). [1] Previously identified in roast beef by Resconi *et al.* 2012. [2] Previously identified in fried beef patties by Kerler & Grosch 1996.* Most frequent descriptors given by trained assessors.

7.1.2 *Relationship between volatiles and sensory attributes*

The relationship between volatiles (measured by DHS) and sensory attributes (overall significant odour and flavour attributes) was explored using correlation plots and multiple linear regression (MLR) (Genstat 15th Edition). The strength of relationships between volatile compounds and single sensory attributes was first assessed by visual inspection of correlation plots. The strongest positively and negatively correlated volatiles were used as inputs for MLR analysis. The overall regression model and the overall model significance were used to guide inclusion or removal of volatiles. Only volatiles with proven odour activity from the beef aromagram were included in the final models (**Table 14**). *Grilled beef* flavour increased strongly with IMF. Although this attribute was positively correlated to most of the alkylpyrazines, the strongest prediction model was based on the positively correlated

compounds 2-actyl-1-pyrroline and 2/3-methylbutanal and a number of the negatively correlated volatiles such as 2,3-butanedione and nonanal (**Table 14**). Hence, when the headspace is relatively low in fat derived volatiles (e.g. nonanal, 2,3-butanedione) and high in 2/3-methylbutanal and other Maillard products *Grilled Beef* flavour notes dominated and became apparent. Although *DairyFat* flavour was also weakly correlated with most pyrazine compounds, the strongest predictive model was obtained using the Strecker aldehydes (2-methylpropanal and 2/3-methylbutanal). Since *Dairy Fat* flavour was highly correlated to *Oily Mouthcoating*, it is very likely that *Dairy Fat* is a composite attribute, drawing on both mouthfeel and flavour stimuli. *Grassy* flavour was correlated with hexanal and other volatiles; hexanal is most commonly described as resembling fresh cut grass. *Caramel* odour was complex; the MLR model included various aldehydes and pyrazines. *Bloody* odour was strongly associated with a single compound; dimethyl sulphide. Various other sensory attributes were successfully modelled using odour-active volatiles. The odour-active volatiles identified in the current research agree in large part with those reported in Resconi *et al.* (2012), Kerler & Grosch (1996) and Specht & Baltes (1994). It should be noted that only around 26 compounds were identified that had significant odour activity in the latter study.

Table 14: Details of significant multiple linear regression models of sensory attributes using odour-active headspace volatiles

Sensory Attribute	Positive Correlation	Negative correlation	R^2	P value
Grilled Beef Flavour	*2-acetyl-1-pyrroline, 2/3-methylbutanal*	*2,3-butanedione, nonanal, 2,4-(E,E)-nonadienal, (E)-2-nonenal, 1-octen-3-ol*	0.89	0.05
Dairy Fat Flavour	*2-methylpropanal, 2/3-methylbutanal*		0.6	0.01
Grassy Flavour	*dimethyl sulphide, hexanal, guaiacol*	*2,3-butanedione*	0.91	<0.001
Caramel Odour	*2-methylpropanal, 2/3-methylbutanal, trimethylpyrazine, 5-ethyl,-2,3-dimethylpyrazine, dimethylsulphide*		0.75	0.04
Bloody Odour	*dimethyl sulphide*	*octanal*	0.46	0.02
Hay/Grainy Flavour	*nonanal, (E,E)-2,4-nonadienal, octanal, 2,3-butanedione*		0.65	0.02
Livery Flavour	*octanal, hexanal*		0.34	0.08
Fishy Odour	*(E,E)-2,4-nonadienal, 1-pentanol, (E,E)-2,4-decadienal*		0.49	0.03
Barnyard Odour	*(E)-2-nonenal, hexanal*		0.68	0.001

7.2 Summary of Olfactometry Findings

- *No unique odour active peaks were detected between sample types in the grilled beef aromagrams. Differences were found mainly in odour intensity*
- *Around 28 volatile compounds were associated with odour activity in Tenax extracts of the headspace above grilled beef samples*
- *Differences were found between SPME and Tenax DHS data, however overall the results were comparable, with the notable exception of 2-methylpropanal and 2/3-methylbutanal*
- *The odour-active volatiles identified in the current research agree in large part with those reported in Kerler & Grosch (1996) and Resconi et al. 2012 and others*
- *A number of key odour and flavour sensory attributes could be associated with specific volatiles in the beef headspace and successfully modelled using MLR*

8 *In vivo* measurement of volatile release

8.1.1 *Volatile Flavour Release*

Fat plays an important role as a precursor to flavour volatiles. Fat also acts as a solvent or "flavour sink" in food systems solubilising fat soluble volatiles (compounds with high Log*P* values) impeded their release (Frank *et al.* 2011, 2012). In contrast, many important flavour volatiles are only sparingly soluble in fat (low Log*P*). These water-soluble compounds may be repelled by the presence of fat and are released more rapidly when fat is present in the food matrix. Fat also plays a purely mechanical role in food, generally making the matrix softer and easier to chew, which often leads to more rapid oral breakdown of the food, in this case, meat matrix. More rapid breakdown of the food structure effectively increases the particle surface area, leading to greater volatile release as a consequence.

It has also been shown that the overall sensory impression of flavour in high fat foods is attenuated and more "balanced" compared to low fat. The trained panel data indicated that a number of odour and flavour related sensory attributes increased with increasing IMF, such as flavour and odour *Impact, Dairy fat*, *Grassy* and *Grilled beef* flavour.

8.1.2 *In mouth PTR-MS Protocol*

A modified *in vivo* volatile monitoring approach was adopted from a previous study (Frank *et al.* 2012) using a strict chewing and breathing protocol and proton transfer reaction mass spectrometry (PTR-MS). An *in vitro* method was initially used, to ascertain how many volatile compounds could be resolved in the headspace of grilled beef. At least 10 volatiles could be reliably measured in real-time in the headspace above grilled beef samples (see details in later section). A panel of six human subjects were recruited for the *in vivo* volatile release experiments. A computerised animation was presented on a dedicated computer monitor to guide the breath cycles and chewing pattern for each experiment (**Figure 30**). Using this protocol ensured synchronisation of each subject's breathing cycle such that all experiments were linked by breathe cycle and chew number. During the experiments the volatile compounds present in the expired nostril breath were withdrawn through a disposable plastic cannula into the inlet of PTR-MS for real-time measurement.

The panel was trained to rate dynamic sensory changes simultaneously using a push-button time intensity device (validated and published in Frank *et al.* 2011). Warm grilled beef samples (~10 g) were presented to subjects and masticated (chewed) according to the protocol. Replicate samples (n=5) were tested for each sample type for every subject (i.e. 5 x 6 = 30 replicates for each sample type; a total of 30 x 6 sample types = 180 separate experiments). The subjects were instructed to breathe and chew according a strict protocol (

Figure 30). Each blue unit represents one complete breathe cycle (inspiration & expiration) with a total duration of 3 seconds. After five background breathe cycles, the warm grilled beef sample was introduced into the subject's oral cavity. The sample was immediately chewed once for the first breath cycle (one chew per breath). The rate of chewing was effectively slowed down, to allow important differences in release to be resolved; especially for the early chews, thought to be most critical for creating the initial sensory impression. One chew per

breath cycle was continued for the first breathes. For the remaining breath cycles, the subject was instructed to chew twice per breath cycle (a more natural rate). The subject continued to chew the sample until they were ready to swallow; the time of swallow was recorded for each sample by the technician.

Volatile release normally follows a curve similar to that in **Figure 31**. After sample introduction into the oral cavity, the concentration of volatiles in the nostril breath increases during mastication to reach a maximum concentration (I_{max} preswallow) after an amount of time (T_{max} preswallow) before the swallow point. The total area under the curve (AUC preswallow) is a measure of the total amount of volatile released in the preswallow period. Similarly, after swallowing, volatiles are still released as the food bolus passes down the throat. During this phase there may also be a distinctive postswallow I_{max} and T_{max} as well as a postswallow AUC. Differences in any of these parameters can be considered evidence that the food matrix has an effect on the oral processing and flavour release profiles. The accompanying dynamic sensory impression or time intensity (TI) also typically can be explained by the same parameters of $\boldsymbol{I}_{max}$, T_{max} and AUC. Any differences in these parameters can indicate that the temporal perception is different between products.

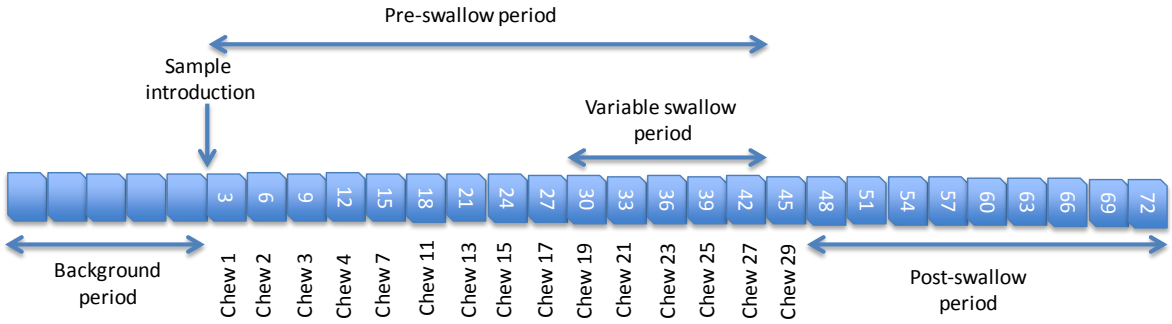

Figure 30: Diagram of the strict timing of the breathing and chewing protocol used in the *in vivo* PTR-MS experiments.

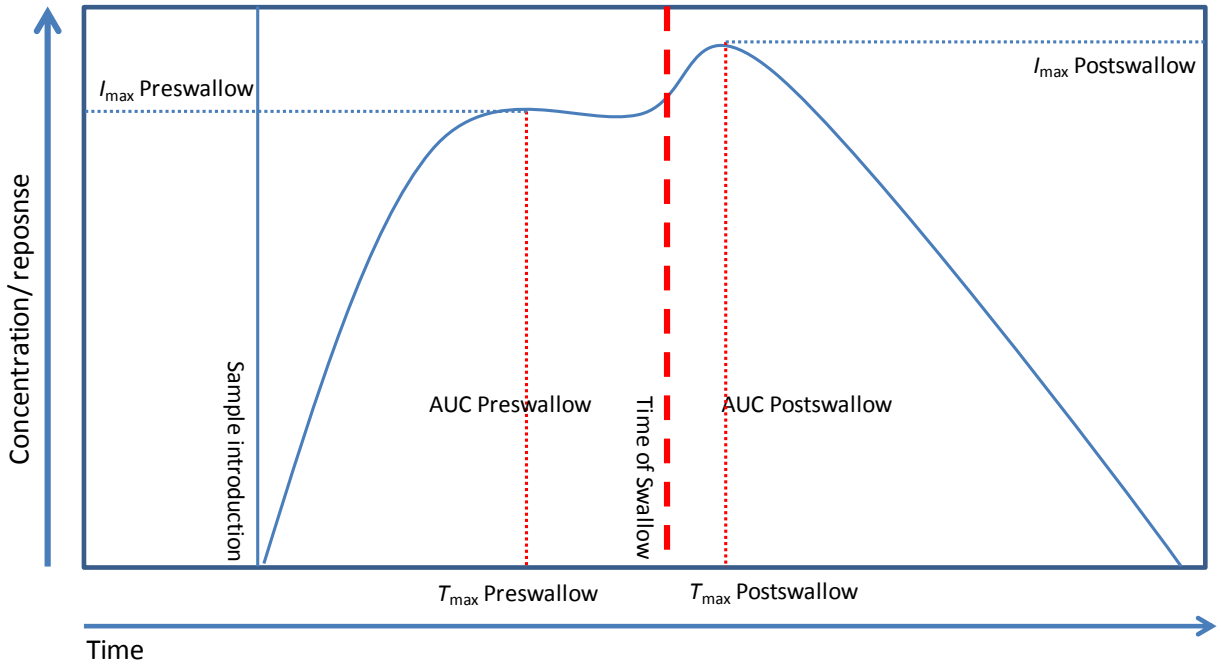

Figure 31: Diagram of idealised flavour release and time intensity curves, showing preswallow and postswallow parameters. I_{max} = maximum concentration/intensity, T_{max} = time of maximum concentration/intensity, AUC = integrated area under the curve.

8.1.3 *In vivo PTR-MS monitoring results*

Although 10 volatiles were monitored during the *in vivo* experiments, sufficient sensitivity was attained only for the two most abundant volatile compounds. Clear signals were measured *in vivo* only for two ions; ions with mass to charge ratio (*m/z)* 73 and *m/z* 87 were resolved sufficiently. The average perceptual time intensity curves (top) and volatile profiles of *m/z* 73 (middle) and *m/z* 87 (bottom) are shown in **Figure 32**. The AngusGrass samples are coded green, the AngusGrain red and the WagyuGrass in blue. The solid line indicates the high fat sample, the dashed line the low fat sample. The corresponding time intensity sensory curves (top) are also shown.

The ion with *m/z* 73 corresponds to the compound(s) 2-methylpropanal/ 2-butanone (MW 72) while the ion at *m/z* 87 corresponds to mainly 2 & 3-methylbutanal (MW 86). It should be noted that different molecular species with the same nominal molecular weight cannot be differentiated. The average panel swallow time for each sample is indicated by the filled circle on the curves.

It can be seen that there was a considerable difference in average swallow time between the high and low fat samples. The effect of sample type and the covariate term MSA-MB on swallow time were both significant ($p < 0.001$). Swallow time decreased with IMF and was negatively correlated with MSA-MB (-0.34, $p < 0.001$). The swallow time data from these experiments was in general agreement with the sensory data, where the *Number of chews to swallow* decreased significantly with increasing IMF (**Table 6**).

The least significant difference (LSD) was calculated for each breath and is represented by the bars in **Figure 32**. The raw curve parameters (Pre and postswallow I_{max}, T_{max} and AUC) from each experimental replicate were subjected to MANOVA analysis, with 'sample type × subject' as fixed factors. The least significant difference (LSD) was calculated for each parameter. Mean data together with statistical parameters are presented in **Table 15**.

Significant differences were found in most parameters for the time intensity (TI) perception data (top) and the volatile data *m/z* 73 (middle) and *m/z* 87 (bottom). Most parameters were significantly correlated to IMF (MSA-MB). For the volatile data pre-, post- and total AUC and pre- and post-swallow I_{max} increased with increasing IMF. In general the perceived intensity increased with increasing IMF, especially for the postswallow period.

T_{max} for the preswallow volatile release was shorter in the high fat samples. It can be seen on the release curves (**Figure 32**) that although the swallow times (and hence preswallow periods) were always shorter for the high fat samples compared to the low fat, the I_{max} on average occurred earlier for the high fat samples compared to low fat. The shorter T_{max} values for high IMF samples are reflected in both the volatile release and perception curves. The rate of decay for the release curves for the lowest fat samples (e.g. AngusGrass) was more rapid compared to the other higher fat samples. Taken as a whole, the data strongly indicate that the rate of release of *m/z* 73 and *m/z* 87 was faster and a greater concentration was reached inside the oral cavity when more fat was present. This was also mirrored in the average TI data. As 2/3-methylbutanal was strongly correlated to *Grilled Beef* flavour (**Table 14**) this is a significant finding.

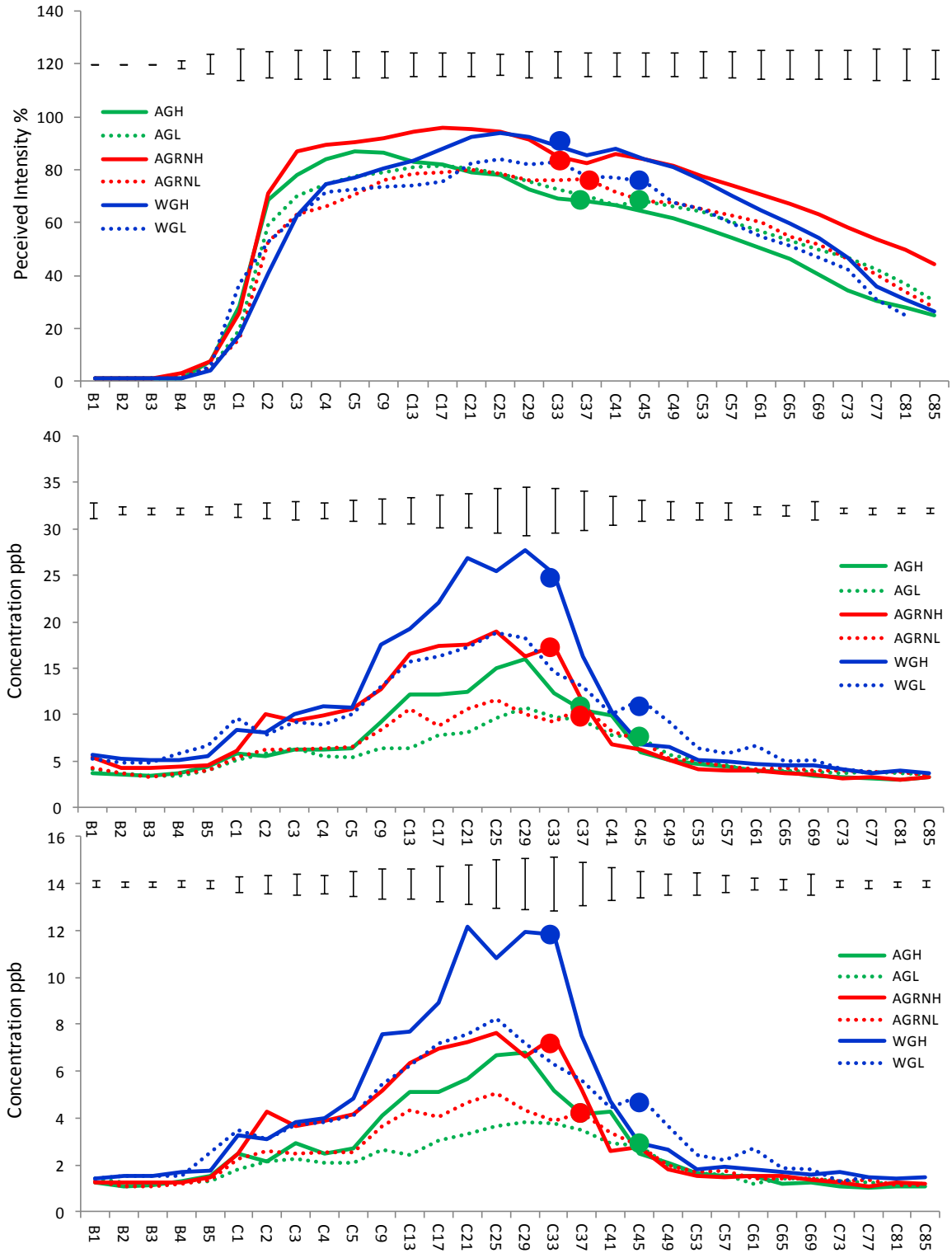

Figure 32: *In vivo* measurement of grilled beef volatiles during eating and swallowing. Each line is the average of 30 replicates. The black bars indicate the least significant difference at a time point. Top - average time intensity profile of overall flavour intensity. Middle – average volatile release profiles at *m/z* 73 (mainly 2-methylpropanal). Bottom - average volatile release profiles at *m/z* 87 (mainly 2/3-methylbutanal). Solid circle represents the average time of swallow. The bars represent the least significant difference (LSD) for a time point. AGH (AngusGrass High Fat), AGL (AngusGrass Low Fat), AGRNH (AngusGrain High Fat), AGRNL (AngusGrain Low Fat), WGH (WagyuGrass High Fat) & WGL (WagyuGrass Low Fat). B= background Breath, C1 = chew 1, C2 = chew 2 etc.

For volatiles that have appreciable fat solubility, it is expected that they will partition into the fat phase and their release will be lower compared to a fat free system (Frank *et al,* 2012). It has also been demonstrated that compounds that are not very fat soluble (lipophobic) are released more rapidly in the presence of fat, compared to a fat free system. Both 2/3-methylbutanal and 2-methylpropanal/2-butanone, have low *LogP* values and are hence considered reasonably lipophobic. Earlier GC-MS volatile data showed that the headspace concentration of both of these compounds increased with IMF. The olfactometry data demonstrated that the combined odour of both 2 & 3-methylbutanal was the most important aroma impact compound in grilled beef aromagrams. The odour associated with 2-methylpropanal was also significant. Quantitatively, the DHS Tenax data showed that these compounds were, by far, the most abundant volatile compounds present in the headspace of freshly grilled beef samples. The PTR-MS release data further confirmed that the concentration of both 2/3-methylbutanal increased with IMF and also showed that the release was also proportional to the degree of marbling *in vivo* during beef consumption. Importantly, significant differences in the timing of release were demonstrated. The volatile were released more rapidly as a “burst’ from the higher fat samples. Not only was the intensity of the sensory stimulus also higher in the high fat samples, it reached maximum intensity more rapidly. Interpreted as a whole, these *in vivo* data provide strong evidence of IMF-related differences in volatile release accompanied by perceivable temporal differences in sensory quality.

Table 15: Summary of mean volatile release curve parameters and statistical significance for time intensity (TI), and volatile ions at *m/z* 73 and *m/z* 87. Significantly higher sample in the low/high fat comparison in shown in bold.

TI	AGL n=30	AGH n=30	AGRNL n=30	AGRNH n=30	WGL n=30	WGH n=30	LSD	P sample	*r* n=6	P IMF n=210
MSA-MB score	**360**	**670**	**430**	**950**	**530**	**1170**				
AUC Pre	1052	961	932	1035	1009	907	104	0.026	-0.40	ns
AUC Post	572	**610**	663	**954**	572	**858**	124	<0.001	0.83	<0.001
AUC Total	1622	1572	1595	**1989**	1581	**1765**	176	<0.001	0.65	0.04
I_{max} Pre	15.8	17	16.2	18.4	18	17.6	1.12	<0.001	0.64	0.03
I_{max} Post	12.4	12.2	13.4	**15.4**	13.8	**16.2**	1.52	<0.001	0.77	0.01
T_{max} Pre	**18.16**	13.95	**16.7**	13.3	**21.3**	16.65	4.3	0.004	-0.40	ns
T_{max} Post	37.6	32.9	29.3	27.9	38.4	32.9	6.7	0.012	-0.37	ns

m/z 73	AGL n=30	AGH n=30	AGRNL n=30	AGRNH n=30	WGL n=30	WGH n=30	LSD	P sample	*r* n=6	P IMF n=210
MSA-MB score	**360**	**670**	**430**	**950**	**530**	**1170**				
AUC Pre	50	60	61	68	72	89	6.1	<0.001	0.82	<0.001
AUC Post	129	151	133	186	220	242	22	<0.001	0.72	<0.001
AUC Total	179	**211**	195	**254**	292	**331**	23.3	<0.001	0.75	<0.001
I_{max} Pre	5.11	7.71	7.41	12.7	11.93	17.05	2.4	<0.001	0.83	<0.001
I_{max} Post	2.12	3.4	3.25	5.29	4.87	6.76	1.4	<0.001	0.85	<0.001
T_{max} Pre	27	20	24	21	28	23	9.2	0.005	-0.61	0.015
T_{max} Post	47	42	42	34	45	35	7.6	<0.001	-0.88	<0.001

m/z 87	AGL n=30	AGH n=30	AGRNL n=30	AGRNH n=30	WGL n=30	WGH n=30	LSD	P sample	*r* n=6	P IMF n=210
MSA-MB score	**360**	**670**	**430**	**950**	**530**	**1170**				
AUC Pre	48	**63**	55	**72**	88	**99**	9	<0.001	0.72	<0.001
AUC Post	17	**22**	22	**27**	27	**36**	2.8	<0.001	0.86	<0.001
AUC Total	66	**85**	76	**100**	116	**136**	9.4	<0.001	0.76	<0.001
I_{max} Pre	2.3	**3.9**	3.2	**5.3**	5.3	**7.2**	0.92	<0.001	0.81	<0.001
I_{max} Post	0.9	1.5	1.5	**2.4**	2.1	**3.5**	0.68	<0.001	0.86	<0.001
T_{max} Pre	**35**	27	28	25	**30**	24	4.30	<0.001	-0.83	<0.001
T_{max} Post	16	13	14.5	13.3	17.3	15.6	4.35	ns	-0.16	ns

LSD = least significant difference for comparing means. P $_{sample}$ = p value for comparison between sample types. r = Pearson's correlation coefficient for relationship between IMF (MSA-MB) and release parameter (mean data only). P $_{IMF}$ = p value for correlation between IMF (MSA-MB) and release parameter (all data, n=210). LSD = least significant difference for comparing between samples. AGH (AngusGrass High Fat), AGL (AngusGrass Low Fat), AGRNH (AngusGrain High Fat), AGRNL (AngusGrain Low Fat), WGH (WagyuGrass High Fat) & WGL (WagyuGrass Low Fat).

9 Free Amino Acids & other Non-Volatile Flavour Compounds in Raw and Grilled Beef

9.1.1 *Contribution of free amino acids to meat flavour*

The sensory panel data strongly indicated that the overall "*Flavour Impact"* increased with increasing marbling. As this attribute was assessed in-mouth, this attribute is likely to be a composite sensation of olfactory and taste stimuli. *Dairy Fat* flavour and *Sweet* taste also increased strongly with MSA-MB, whereas *Acidity* and *Astringency* decreased (**Figure 5**). Unlike the aroma related flavour attributes, these flavour modalities are expected to be perceived primarily through taste receptors on the tongue and in the oral cavity, rather than through olfactory receptors. It is well known that the free amino acids and small peptides contribute considerably to the flavour and orosensory properties of meat (Dunkel & Hofmann 2009, Chen & Zhang 2007, Pereira-Lima *et al.* 2000, Schlichtherle & Grosch 1998). Most free amino acids are flavour-active in their own right, depending on their concentration in the food matrix. Free amino acids are also important precursors to volatiles formed during the Maillard reaction, such as alkylpyrazines and Strecker aldehydes. The volatile aldehyde 3-methylbutanal is formed directly from the Strecker degradation of the amino acid leucine, 2-methylpropanal from valine and methional from methionine. Hence, any differences in the concentrations of free amino acids and other non-volatile sapid compounds in the raw and cooked meat may contribute to the flavour potential either through providing a greater supply of substrates for the formation aroma volatiles and in their own right as sapid non-volatile components.

9.1.2 *Derivatisation of free amino acids and analysis by GC-MS*

Semi-quantitative measurement of free amino acids and other non-volatiles of potential interest was achieved by adapting the method described by Smart *et al.* 2010 and Leggio *et al.* 2012. Non-volatile primary amines and compounds containing a free carboxyl group are readily converted to volatile derivatives using methyl chloroformate in alkaline aqueous medium, making these compounds sufficiently volatile for analysis by GC-MS. Most free amino acids (with the exception of arginine) and some small peptides (e.g. carnosine) and flavour-active organic acids such as lactic and succinic acid can be derivatised under these conditions. Some free fatty acids can also be derivatised.

Three high-IMF and three low-IMF samples from different animals from each of the sample types, WagyuGrass, AngusGrass and Angus Grain were selected. For each sample four adjacent steaks taken. For the raw sample analysis, approximately 20 mm slices were excised from the middle two separate steaks and further reduced into small pieces and prepared for analysis. A total of 3 x animals x 2 replicates x 2 marbling levels = 12 raw samples were prepared. In the case of grilled meat samples, the remaining adjacent steaks were weighed and grilled until a final internal temperature of 57 ^{o}C. After resting, ~20 mm sections were taken from the middle of each steak and prepared for amino acid analysis (total of 12 grilled samples). Raw or grilled meat was immediately suspended in a 70 % ice-cold methanol solution and macerated to fine slurry. The samples were centrifuged and a volume of the supernatant was filtered through a 0.4 µm filter. A 500 µL volume of the meat suspension was transferred to a reaction vial and a 50 µL volume of norvaline internal standard solution was added. A semi-quantitative estimation of the concentration of free amino acids (mg/kg) was

achieved against the norvaline standard. The concentration of lactic acid was quantified by adding a volume of lactic acid stable isotope internal standard (sodium L-lactate-3-^{13}C, Sigma Aldrich). The meat extract was derivatised in a reaction described in and adapted according to Smart *et al*. 2010. After derivatisation, the volatile derivatives were extracted into 1000 µL of chloroform and dried with anhydrous sodium sulphate for analysis by gas chromatography-mass spectrometry (QP 2010 Plus GC-MS, Shimadzu). An aliquot (1 µL) of sample was injected at 250 ^{o}C in splitless mode. Derivatives were separated on a Sol-Gel Wax column (SGE, Australia, 30 m, 0.25 id, 0.25 µm film) using temperature programming; initial temperature 45 ^{o}C (held 2 minutes) and then heated at 9 °C/min to 180 °C (held 5min), 40°C/min to 220°C (held 5min). The mass spectrometer was programmed to scan the mass range *m/z* 40-300. Reference compounds were used to establish retention times of most compounds and quantified using a characteristic ion fragment. Integration of peaks was achieved using the Shimadzu proprietary software "LabSolutions" (Version 2.53). The identity of most analytes was established by reference compounds. In some cases published mass spectral data was used (Smart *et al.* 2010, Leggio *et al.* 2010). A total of 72 separate data for each sample type (WagyuGrass, AngusGrass & AngusGrain), from different fat levels (low and high) and state (grilled or raw) were used in the statistical analysis. The main effects of sample type, fat level and state and their interactions were analysed by MANOVA.

9.1.3 *Results -Non-volatiles in raw and grilled beef*

The estimated means for free amino acids, carnosine and other non-volatile analytes of interest in raw and grilled samples are summarised in **Table 16**. Based on the semi-quantitative data, the most abundant free amino acids in raw and cooked beef samples were: alanine, valine, isoleucine, proline, tryptophan and methionine. The dipeptide, carnosine (*beta*-alanyl-L-histidine) was also present at relatively high concentration in the extracts. The relative concentrations were similar to those reported by Leggio *et al*. (2012). Significant differences were measured between raw and cooked sample types for a number of amino acids and organic acids. The concentration of nearly all analytes increased significantly after grilling. As has been noted earlier, there was significant moisture loss during grilling, which alone contributed to the relative concentration of free amino acids. The data presented in **Table 16** were not adjusted for this loss, although a separate analysis on adjusted data indicated that most free-amino acids increased significantly regardless (data not shown).

In general, the effect of fat on amino acid concentration alone was not significant; however, the sample type x fat interaction was often highly significant. The interactions are further explored graphically in boxplots (**Figure 33**) & (**Figure 34**). Raw and cooked, high and low fat samples are plotted separately for each sample type. Interpreting the data from MANOVA analysis and the plots allows a better understanding of the complex effects of marbling and grilling on the free amino acids and other analytes in the samples. It is clear that the average concentration of free amino acids increased after grilling regardless of sample type or marbling level. The initial concentrations of some free amino acids in the raw meat were comparable (e.g. serine, glutamic acid, aspartic acid, glycine) and varied between sample types for others.

Table 16: Semi-quantitative data for free amino acids, lactic acid and other non-volatile compounds of interest in raw (R) and grilled beef (G).

Non-volatile Compound	AngusGrain R n=12	AngusGrain G n=12	AngusGrass R n=12	AngusGrass G n=12	WagyuGrass R n=12	WagyuGrass G n=12	P raw	P Grilled	Raw n=36	Grilled n=36	P State	High Fat n=36	Low Fat n=36	P Fat	P Type * State	P Type * Fat
	mg.kg															
Glycine (sweet)	0.026	0.03[b]	0.026	0.037[b]	0.028	0.05[a]	—	0.003	0.026	0.041	<0.001	0.034	0.034	—	0.009	0.05
Alanine (sweet)	40	48[b]	35	44[b]	41	55[a]	—	0.001	38.5	48.9	<0.001	43.1	44.2	—	—	0.008
Valine (bitter)	15.7	19.3	15.8	19.1	12.4	21.2	0.03	—	14.46	19.87	<0.001	17.7	16.7	—	0.007	0.004
Leucine (bitter)	7.6	9.9	7.9	10.1	6.1	10.9	0.02	—	7.2	10.3	<0.001	9.1	8.5	—	0.008	<0.001
Serine (sweet)	0.24	0.45	0.22	0.42	0.19	0.4	0.05	—	0.23	0.42	<0.001	0.35	0.31	—	—	0.009
Isoleucine (bitter)	9.7	14.5	9.5	15.3	6.9	15.2	0.004	—	9.1	15	<0.001	12.3	11.7	—	—	0.001
Threonine (sweet)	0.86	1.3	0.66	1.1	0.74	1.3	—	—	0.84	1.2	<0.001	1.08	0.98	—	0.02	—
Proline (sweet)	7.2	8.3[a]	6.7	6.9[b]	5.4	8.3[a]	—	0.008	6.3	7.8	<0.001	6.7	7.4	0.05	0.02	0.04
Asparagine (umami)	0.07	0.1[a]	0.04	0.08[b]	0.037	0.12[a]	<0.001	0.005	0.05	0.1	<0.001	0.07	0.08	—	—	0.01
Aspartic acid (umami)	0.37	0.95[b]	0.28	0.81[b]	0.5	1.1[a]	0.003	0.008	0.37	0.96	NS	0.68	0.66	—	—	0.04
Glutamic Acid (umami)	0.43	1.6	0.37	1.4	0.27	1.1	—	—	0.402	1.368	<0.001	0.97	0.79	—	—	—
Carnosine (sweet)	8.7	11.6[b]	6.2	10.5[b]	5.9	18.3[a]	0.04	0.006	6.3	13.49	<0.001	9.7	10.1	—	0.001	0.02
Methionine (meaty)	4.5	6.8	4.7	6.8	3.7	7.6	0.02	—	4.6	7.1	<0.001	6.05	5.6	—	0.05	0.005
Phenylalanine (bitter)	8.8	11[b]	7.1	9.7[b]	8.4	12.6[a]	0.09	0.007	8.3	11.1	<0.001	10.1	9.27	—	—	<0.001
Lysine (enhanced salt)	0.6	0.85	0.3	0.82	0.4	0.82	<0.001	—	0.47	0.83	<0.001	0.61	0.69	—	—	—
Tryptophan (bitter)	15.2	8.8[b]	16.8	18.2[a]	11.1	14.7[b]	0.08	0.03	12.4	13.9	—	12.4	13.9	—	0.02	—
Total Umami/Sweet AAs	63	80[b]	55	72[b]	59	94[a]	—	<0.001	58	82	<0.001	69	71	—	0.02	<0.001
Total Bitter AAs	73	84	72	91	59	97	0.008	NS	67	91	<0.001	80	77	—	<0.001	<0.001
Total AAs	135	164[b]	127	164[b]	118	191[a]	—	0.002	125	173	<0.001	150	148	—	0.002	<0.001
Lactic acid (sour)	1786	2063[b]	1877	2101[b]	1566	1826[a]	0.03	0.003	1689	1996	<0.001	1843	1843	—	—	—
α-ketoglutarate	0.63	1.3[a]	0.44	0.4[b]	0.61	1.1[a]	—	0.002	0.53	0.93	<0.001	0.77	0.69	—	0.03	—
Fumarate	0.2	0.2[b]	0.1	0.1[b]	0.23	0.64[a]	<0.001	<0.001	0.16	0.3	0.003	0.27	0.19	—	<0.001	—
Palmitate	0.76	0.98[a]	0.32	0.47[b]	0.52	1.2[a]	<0.001	0.006	0.53	1.2	0.05	0.78	0.92	—	—	—
Succinate (sweet)	0.93	3.3[a]	0.45	1.4[b]	0.74	2.3[a]	0.02	0.004	0.67	2.33	<0.001	1.61	1.39	—	0.03	—
Stearate	0.98	2[a]	0.4	0.75[b]	0.72	1.7[a]	<0.001	0.007	0.701	1.5	<0.001	1.0	1.1	—	—	0.04
Niacinamide	0.3	0.28	0.18	0.25	0.26	0.32	—	—	0.24	0.28	—	0.28	0.25	—	—	—

Superscripts denote differences between grilled samples.

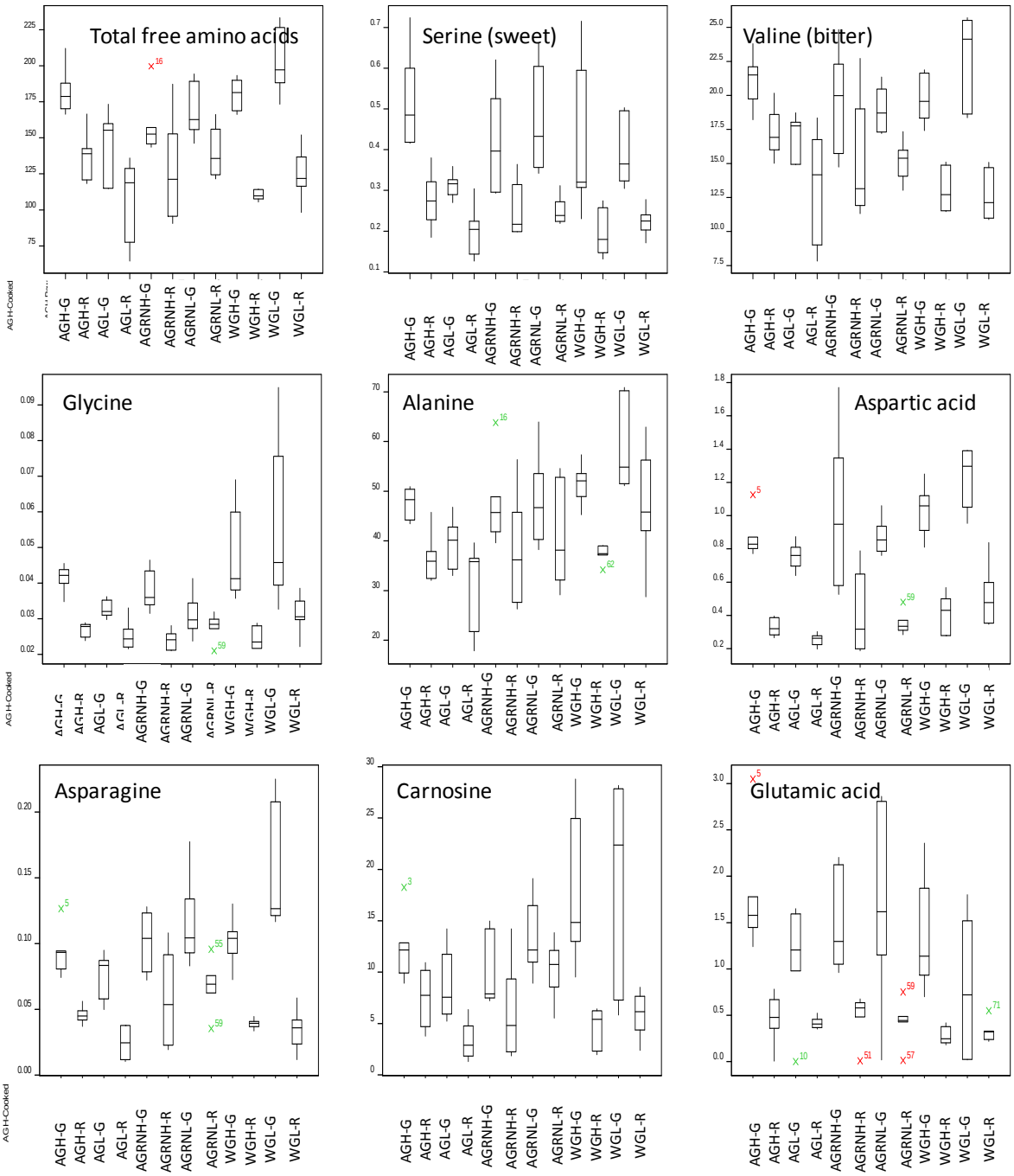

Figure 33: Boxplot showing the semi-quantitative concentration of free amino acids and carnosine in raw (R) and grilled (G) high (H) and low (L) fat beef samples. AGH = AngusGrass high fat (MSA-MB 727), AGL = AngusGrass low fat (MSA-MB 323), AGRNH = AngusGrain high fat (MSA-MB 828), AGRNL = AngusGrain low fat (MSA-MB 502), WGH = WagyuGrass high fat (MSA-MB 1106) and WGH = WagyuGrass low fat (MSA-MB 620).

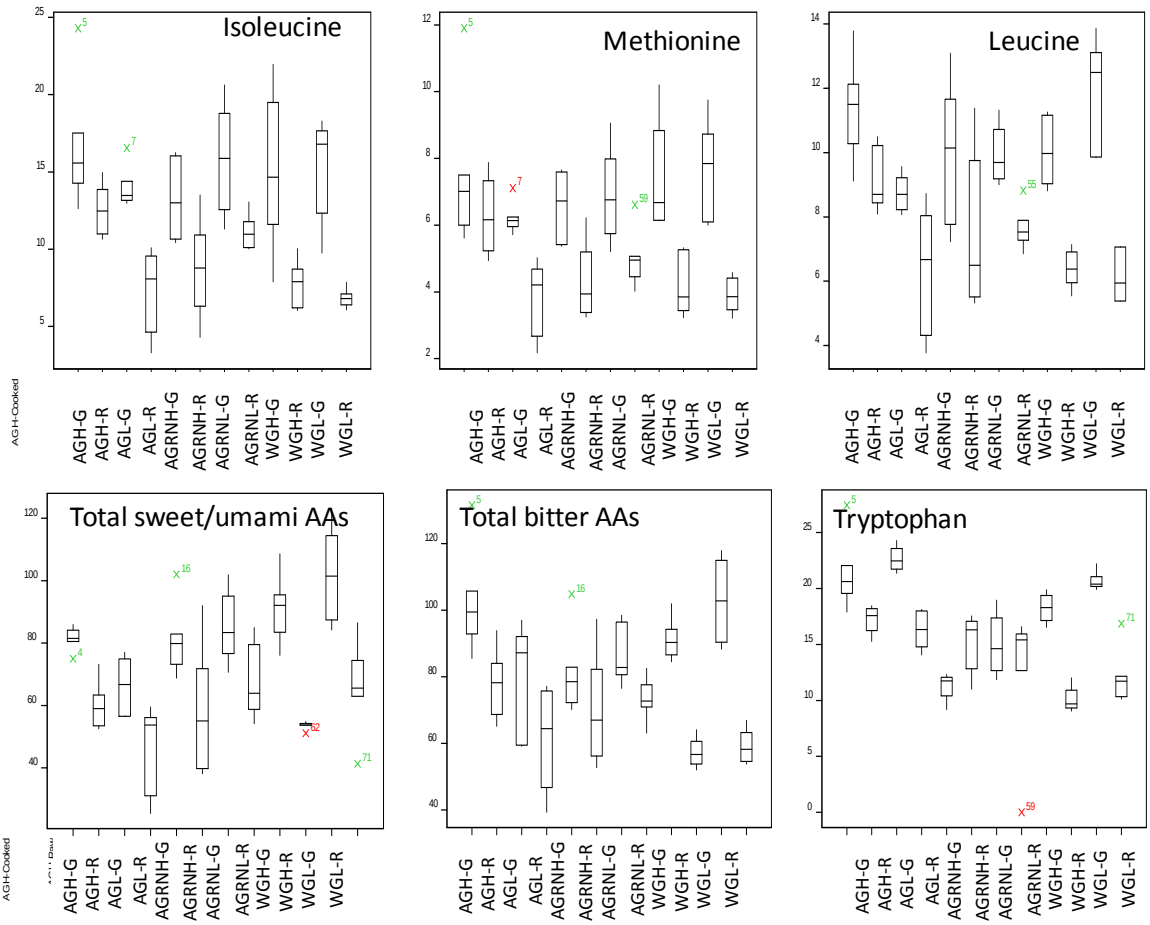

Figure 34: Boxplot showing the semi-quantitative concentration of free amino acids in raw (R) and grilled (G) high (H) and low (L) fat beef samples. AGH = AngusGrass high fat (MSA-MB 727), AGL = AngusGrass low fat (MSA-MB 323), AGRNH = AngusGrain high fat (MSA-MB 828), AGRNL = AngusGrain low fat (MSA-MB 502), WGH = WagyuGrass high fat (MSA-MB 1106) and WGH = WagyuGrass low fat (MSA-MB 620).

After grilling, the increases in free amino acids was greater in the higher marbled samples relative to the low marbled samples mainly for the WagyuGrass and AngusGrain samples, but not always for the AngusGrass samples. In most cases, the increases in amino acids from raw to cooked was lowest in the lowest-IMF AngusGrass low fat samples (MSA-MB score ~). It can be seen in **Table 16**, that on average, the free amino acids in the AngusGrass samples were lower than the other two sample types. It can be seen that the level of tryptophan was higher on average in the AngusGrass samples. For the higher IMF beef samples, the proportion of fat is higher in the meat, meaning that there is less protein present as a percentage of mass. Assuming that free amino acids are produced both through heat induced proteolysis and meat shrinkage, it would be reasonable to assume that the lowest fat (i.e. highest protein) samples (e.g. AGL, AngusGrass low fat) would produce the greatest amount of free amino acids. In general, it appeared that the opposite was the case, with higher amounts produced in the higher IMF samples.

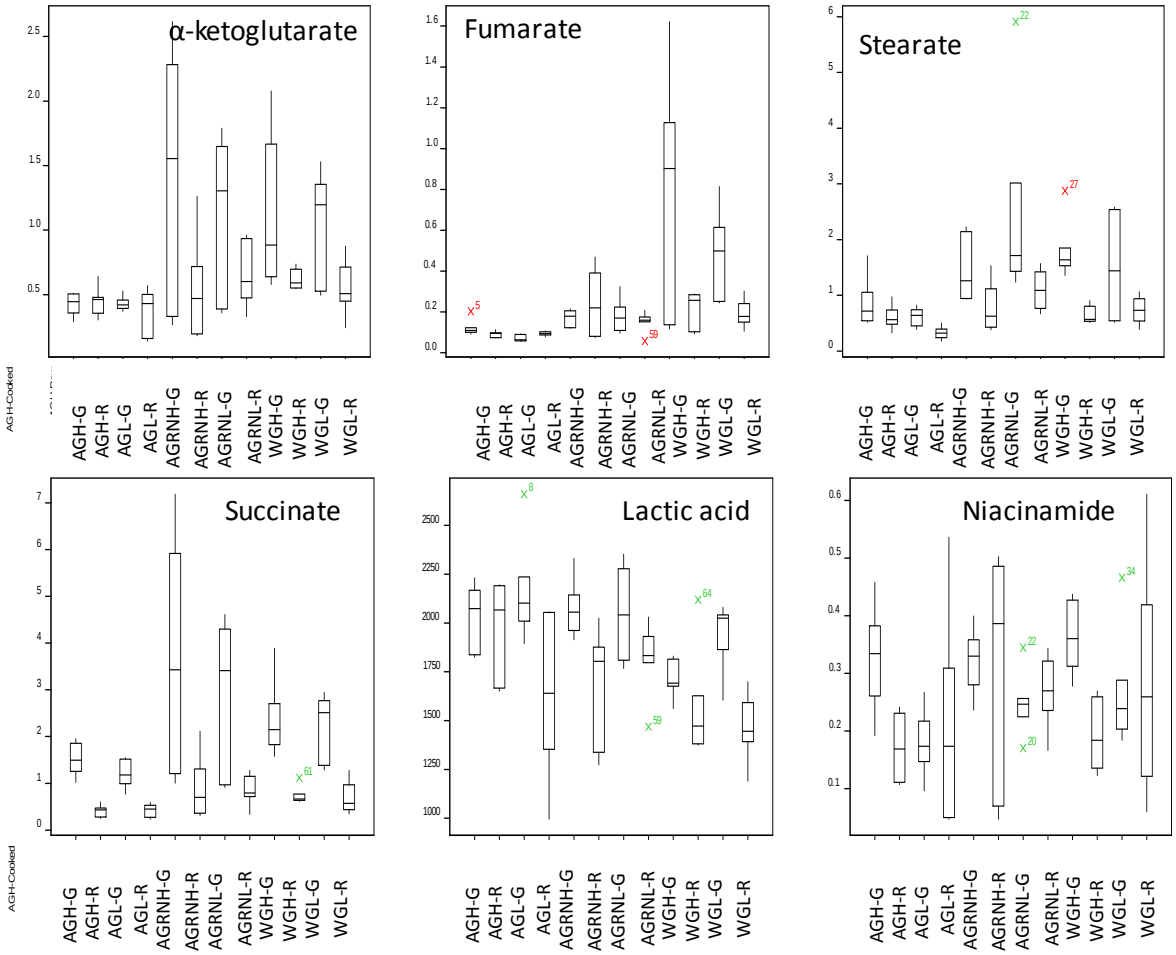

Figure 35: Boxplot showing the semi-quantitative concentration of organic acids and other non-volatile analytes in raw (R) and grilled (G) high (H) and low (L) fat beef samples. AGH = AngusGrass high fat (MSA-MB 727), AGL = AngusGrass low fat (MSA-MB 323), AGRNH = AngusGrain high fat (MSA-MB 828), AGRNL = AngusGrain low fat (MSA-MB 502), WGH = WagyuGrass high fat (MSA-MB 1106) and WGH = WagyuGrass low fat (MSA-MB 620).

Since the liquid loss during resting was lower for the high fat samples, this may partially explain the observed effect of greater retention of non-volatile compounds. It is also possible that the proteolysis reactions are catalysed by reactive lipid radical intermediates formed during grilling, leading to greater increases in the pool of free amino acids in high IMF samples, although this is speculation. The TBARS values for the WagyuGrass samples were significantly higher, suggesting that the WaguGrass fat was more susceptible to oxidation.

It is possible to assign a general flavour note to specific free amino acids (Watkins *et al.* 2013, Pereira-Lima *et al.* 2000). For example glycine, alanine, serine, threonine, proline and carnosine are perceived as sweet. Glutamic acid, asparagine, aspartate, methionine and lysine are associated with umami and meaty flavours. Sweet and umami amino acids may be considered as desirable flavour characters in grilled beef. Valine, isoleucine, leucine, phenylalanine and tryptophan have bitter and astringent tastes. If these bitter compounds are

completely absent from beef broth, it leads to abnormal flavours, however when present at elevated concentration, or 'out-of-balance' they can impart negative or undesirable flavour attributes (Pereira-Lima *et al.* 2000). The total umami and sweet free amino acids and the total bitter free amino acids in the meat samples were calculated. Although the average bitter free amino acids were similar in the sample types, the AngusGrass samples were significantly lower in sweet free amino acids compared to the WagyuGrass and AngusGrain samples (**Figure 34**). Previous research has suggested that changing the overall balance of free amino acids in beef broths lead to either undesirable or more desirable beef flavour. Higher levels of carnosine, glutamic acid, lysine, methionine and aspartic acid were positively associated with richer beef flavour (Pereira-Lima *et al.* 2000).

A number of organic acids relevant to beef flavour were also measured by the derivatisation method (**Figure 35**). Primarily, lactic acid was present at significantly lower concentration in WagyuGrass samples and highest in the AngusGrass. Lactic acid increased with grilling in all samples; however the final concentration was lowest in the high fat WagyuGrass. Note that the WagyuGrass samples were rated as having the lowest *Acidity* and lowest *Acidic aftertaste* by the sensory panel. The *Acidity* in the AngusGrain and AngusGrass were similar. It can be clearly seen that α-ketoglutarate increased with grilling only in the AngusGrain and WagyuGrass samples. Although the role of this compound in beef flavour is uncertain, Tjener *et al.* (2004) demonstrated that addition of this compound to fermented meat increased volatile flavour. Fumarate also increased significantly on grilling, especially in the WagyuGrass; apart from being sour, fumaric acid is known to enhance flavour perception. Although succinic acid increased in all samples with grilling, the final amounts in the cooked meat were highest in the WagyuGrass and AngusGrain samples. The flavour of succinic acid is described as both sweet and umami depending on the studies (Watkins et al. 2013, Schlichtherle & Grosch 1998). Although succinate was present in the raw beef samples, the concentration increased markedly with grilling and was highest in the AngusGrain and WagyuGrass samples. Of interest was that these samples were rated as significantly *Sweeter* than the AngusGrass samples.

The unsaturated stearic fatty acid (C16:0) was tentatively identified as a major peak in the beef extracts based on mass spectral data reported by Leggio *et al.* (2012). Stearate increased with grilling in mainly the AngusGrain and WagyuGrass samples. Finally, niacinamide (Vitamin B3) was tentatively identified in extracts, based on mass spectral data. Most literature reports the taste of this compound as bitter; it is unclear whether it is present above its taste threshold in beef. This water soluble compound did not differ between sample type and the effect of cooking and fat were not overall significant.

A very comprehensive study of non-volatile flavour-active compounds in stewed beef juice was published by Schlichtherle & Grosch (1998). They calculated taste activity values for individual compounds and used model systems and "omission tests", where single flavour compounds were selectively removed from artificial reconstructed broths to assess their flavour impact. Taste activity values (TAVs) are the ratio of the actual concentration of an analyte in the sample (stewed beef juice) divided by its corresponding taste threshold in solution. Compounds with a TAV > 1 are likely to have a perceivable impact on flavour. From their analysis, they estimated that lactic acid (TAV 10), phosphate (TAV 11.3), carnosine (TAV 8.6) and succinic acid (TAV 5.4) had by far the highest flavour impact. They also concluded

that glutamic acid, aspartic acid and alanine were very important. Other non-volatile compounds not measured in this study, such as 5'-inosine monophosphate, creatinine, creatine and various inorganic ions such as sodium, potassium, magnesium and chloride also played a role in stewed beef flavour. Based on the conclusion of Schlichtherle & Grosch and the sensory and non-volatile data found in this study, the following statements seem likely to apply. The higher *Acidity* and *Acid aftertaste* in the AngusGrass and AngusGrain were probably due to higher concentrations of lactic acid in these samples. The high *Sweetness* measured in the AngusGrain and WagyuGrass are likely due to higher concentrations of succinic acid and possibly the combined effect of numerous sweet tasting free amino acids. The overall lower *Flavour intensity* in the AngusGrass samples, especially apparent in the low-fat samples, appears to relate to a lower overall concentration of multiple non-volatile components. The very high *Lingering aftertaste* measured in the low fat AngusGrass (AGL) may be driven by a higher ratio of bitter amino acids to sweet/umami compounds.

9.2 Free Amino Acids in beef – Summary

- *Small differences in the concentration of free amino acids or other non-volatiles were measured in the raw and cooked meat using the derivatisation method*
- *Free amino acids and other non-volatile flavour compounds generally increased with grilling*
- *After grilling total free amino acids, organic acids and carnosine were highest in WagyuGrass*
- *After grilling, the concentration of sweet amino acids was higher the WagyuGrass and AngusGrass compared to the AngusGrass*
- *Lactic acid was lowest in the grilled WagyuGrass (low acidity), compared to the AngusGrain and AngusGrass (high acidity)*
- *Succinic acid was higher in the WagyuGrass and AngusGrain compared to the AngusGrass*
- *Smaller losses, or greater retention of free amino acids and other non-volatiles in higher fat meat may partially explain the higher flavour impact of highly marbled beef.*

10 *In vitro* measurement of generation of beef volatiles

10.1.1 *In vitro PTR-MS method development*

GC-MS volatile data indicated that some key fat-derived volatiles, such as hexanal and nonanal, did not appear to increase with the level of marbling (MSA-MB). This finding was somewhat at odds with expectations and in the light of some previous literature findings. The *in vivo* PTR-MS method was only sufficiently sensitive to pick up only the most abundant volatiles (i.e. *m/z* 73 and *m/z* 87). Further investigation of the effect of increased meat fat content on the temporal generation of volatiles was investigated using a novel *in vitro* real-time measurement approach with PTR-MS monitoring.

Pilot experiments indicated that at least 10 ions were present at sufficiently high concentration the headspace of grilled beef samples to enable real-time monitoring (**Table 17**). Identities of the most likely volatile compounds were made based on data from GC/MS profiles and literature.

Table 17: List of the ten ions (*m/z*) resolved in the headspace of grilled beef and volatile compounds most likely responsible for the signal.

Ion *m/z*	Most Likely Compounds	Chemical precursor if known
69	Intermediate fragment, furan	lipid
71	*(E)*-2-butenal	
73	2-methylpropanal, 2-butanone	valine, lipid
83	hexanal (fragment)	
87	2-methylbutanal, 3-methylbutanal, 2,3-butanedione	Isoleucine, leucine, lipid
89	2-hydroxy-3-butanone	oxidation of 1,3-butanediol
95	2-methylpyrazine, dimethyl disulphide	various amino acids, methionine
115	heptanal, 2-heptanone	lipid
129	octanal, 2-octanone	lipid
143	nonanal, 2-nonanone	lipid

A purpose built stainless steel inlet was engineered in-house at CSIRO to sit within the Silex grill. The inlet was engineered to connect with a Schott bottle water filter in series with the PTR-MS inlet. A 13 mm diameter filter was placed in-line to prevent liquid or fat molecules from entering the PTR-MS drift tube. The experimental setup is illustrated in **Figure 36**.

Figure 36: Photograph of the experimental set-up for real time measurement of volatiles during beef grilling. A stainless steel inlet with an opening of 1 mm was attached via a water filter to the PTR-MS inlet.

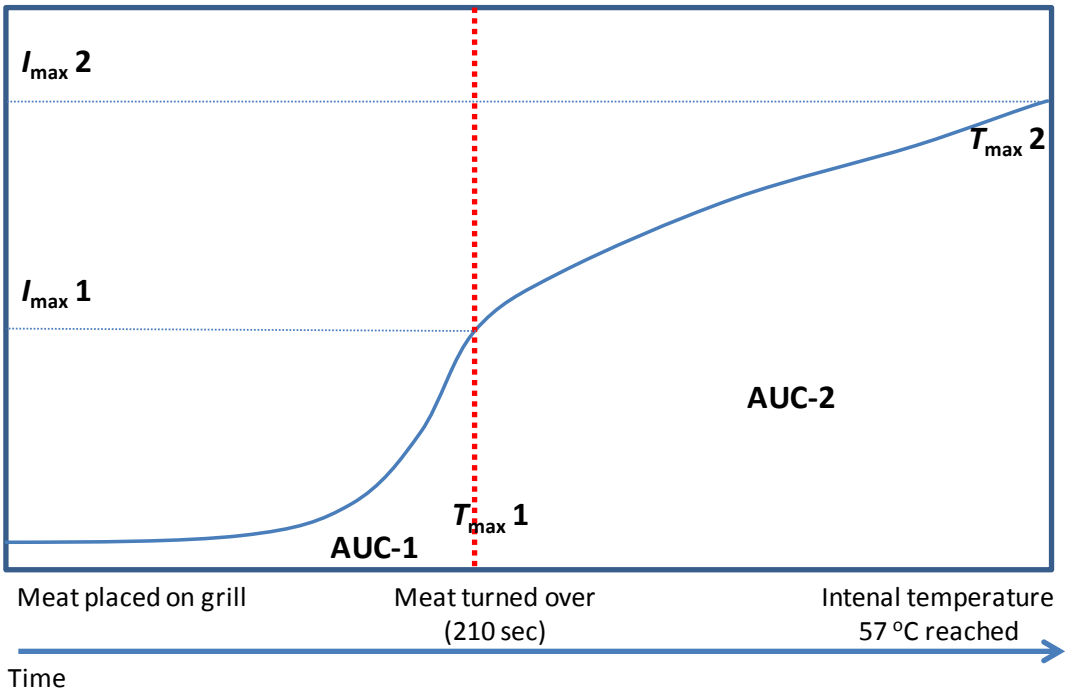

Figure 37: Diagram of idealised volatile generation curves. After grilling for 210 seconds, steaks were flipped (dashed red line) and cooked until an internal temperature of 57 °C was reached.

Standard i*n vitro* PTR-MS parameters were used for the measurements. (Frank *et al.* 2012). During initial piloting, with the grill plates closed, the amount of volatiles generated saturated the PTR-MS detector andit was quickly established that measuring volatile generation with the lid closed was not possible (data not shown). After the grill surface had reached 220 ^{o}C, olive oil spray was applied to the surface. After 800 seconds a single steak was placed on the grill surface at a distance of ~5-10 mm from the inlet plate. Samples were cooked for 210 seconds, flipped over and then cooked until the internal final temperature had reached 57 ^{o}C . Meat samples were then taken off the grill and recording of volatiles was stopped. At least 5 replicates of each of high and low fat WagyuGrass, AngusGrass and AngusGrain samples were measured. For each of the samples, 10 volatile ions were monitored during grilling. The volatile release curves were divided into pre- and post- flip periods as described in (**Figure 37**). As discussed previously, the critical curve parameters, I_{max}, T_{max} and AUC were calculated for each separate curve and used in statistical analyses. The replicate curve parameters were analysed by MANOVA using a 'sample type × ion' factor design. Breed and feed effects were evaluated using reduced data sets and MSA-MB scores coded as a covariate.

10.1.2 *In vitro PTR-MS results*

Typical volatile generation curves obtained for beef samples during grilling are shown in **Figure 38**. All of the ions listed in **Table 17** could be resolved in the grilled samples. Very clear differences were observed between low and high fat samples. The grilling times required to reach an internal temperature of 57 ^{o}C increased significantly with IMF (r = 0.39, p = 0.02) (**Figure 39)**. These data confirm the earlier finding that cooking times were longer for high fat samples (using the sensory grilling method **Figure 22**). Longer cooking times for higher fat samples was reported previously (Luchak *et al.* 1998).

Replicate curve parameters for sample types and also breed and feed comparisons were subjected to MANOVA analysis (**Table 18**). The data table summarises average differences between samples for all ions. The largest and most consistent differences were measured in the post-flip period (AUC-2, I_{max}-2 and T_{max}-2); highly significant differences were found between samples for all parameters during this time. All post-flip parameters were strongly correlated with IMF ($p < 0.001$).

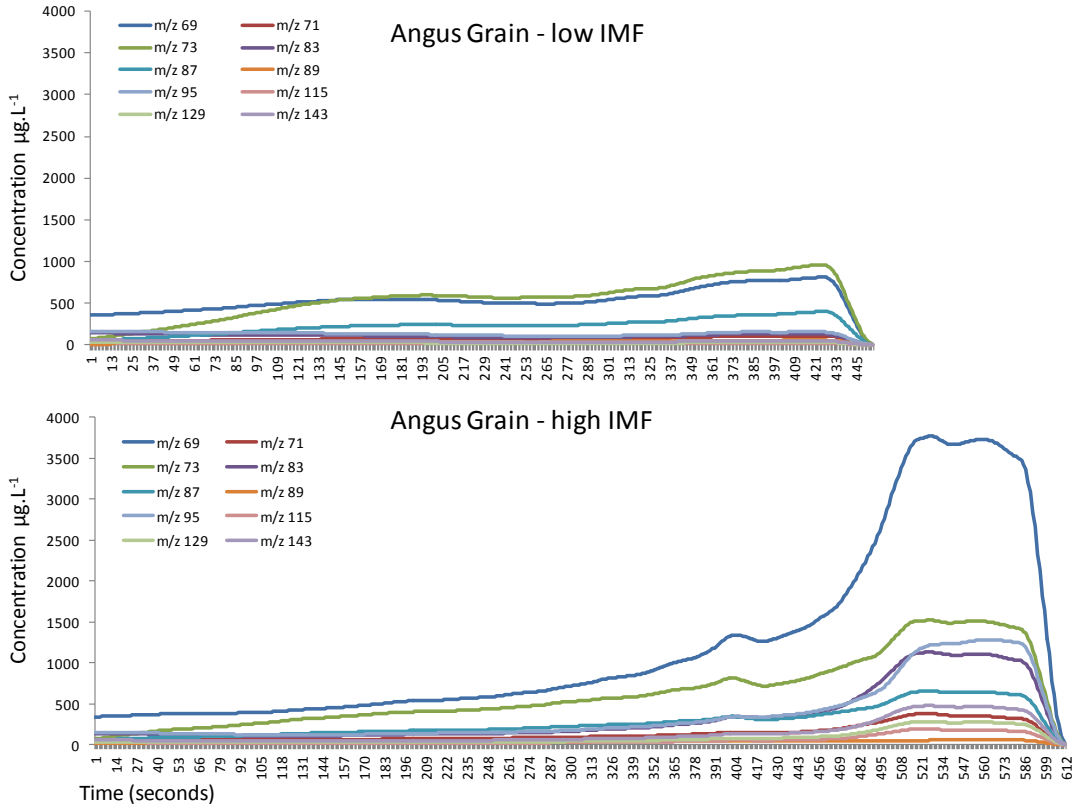

Figure 38: Typical volatile generation curves for low-IMF (top) and high-IMF (bottom) samples (AngusGrain). The low fat samples required shorter heating to reach an internal temperature of 57 ºC.

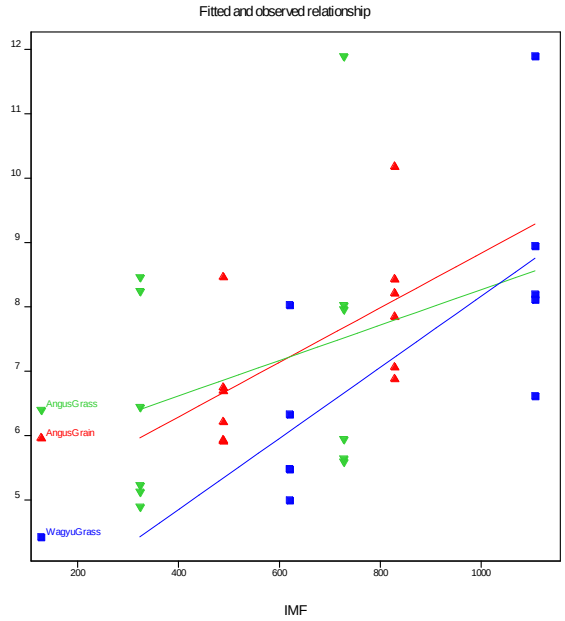

Figure 39: Scatterplot and fitted regression curves for the relationship between MSA-MB and cooking time (minutes) to reach internal temperature of 57 ºC.

Table 18: Summary of mean values for AUC, I_{max} and T_{max} for the post- flip period.

	Angus Grass		Angus Grain		Wagyu Grass				
	Low n=6	High n=6	Low n=6	High n=6	Low n=5	High n=5	LSD	P Sample	P IMF
AUC-2	21,504	38,558	29,057	**82,651**	19,851	**78,139**	18,066	<0.001	<0.001
I_{max}-2	173	**293**	225	**593**	193	**527**	118	<0.001	<0.001
T_{max}-2	264	**414**	307	**463**	261	**439**	35	<0.001	<0.001

After correction for the effect of marbling level (MSA-MB covariate), MANOVA indicated differences between sample types in volatile generation parameters. Overall the Angus Grain produced most volatiles (AUC-2) followed by WagyuGrass and then AngusGrass (average AUC-2 519750 = 47410 > 31618 p <0.001, LSD 15616). Similarly, the I_{max}-2 was highest for the AngusGrain compared to WagyuGrass and AngusGrass (I_{max}-2 average, 385 = 342 > 253, p = 0.02, LSD 104). Finally the estimated mean for T_{max}-2 suggested that the rate of increase in volatile generation with increasing IMF was different according to sample type; AngusGrain, WagyuGrass, AngusGrass, (T_{max}-2 370, 407, 238, p <0.001, LSD 26.6)

The differences between low and high fat samples applied mainly to WagyuGrass and AngusGrain. Differences in volatile generation between low and high fat samples were much smaller for the AngusGrass samples. The greater T_{max}-2 values for the higher fat samples corresponded with the average longer grilling times to reach the internal temperature of 57 ^{o}C. For example, the maximum volatile concentration was reached later (longer T_{max}) with the AngusGrain compared to the WagyuGrass.

The relationship between volatile generation and IMF was further explored for each of the 10 volatile compounds monitored in the *in vitro* experiments (**Figure 40 & Figure 41**). Similar patterns for AUC-2, I_{max}-2 and T_{max}-2 were observed for each individual compound; with increasing IMF, volatile production increased. In general, the fitted regression lines indicated the rate of volatile production was similar in the grass-fed samples and perhaps higher in the AngusGrain samples (red regression line) with increases in IMF. Each replicate experiment for a given nominal fat level was performed using steaks from the sample striploin. Careful observation of the data indicated that the variance in volatile production appeared to be greater for the highest fat samples; i.e. the WagyuGrass high fat and the AngusGrain high fat. The variability for some volatiles also appeared to be greater than others, e.g. *m/z* 87 compared to *m/z* 71. Volatile data from the GC-MS analysis of headspace volatiles (SPME & Tenax) also suggested differences between sample type in the rate of production (i.e. slope of fitted regression line) with increasing IMF. For example, the fitted regression lines for the WagyuGrass samples were often lower than those for the AngusGrain (**Figure 26** & **Figure 27**). The GC-MS volatile data also indicated a similar ranking in volatile production (AngusGrain, AngusGrass > WagyuGrass). The GC-MS volatile data also indicated that variability in volatile concentration became greater at higher IMF levels, supporting similar observations in the *in vitro* PTR-MS data.

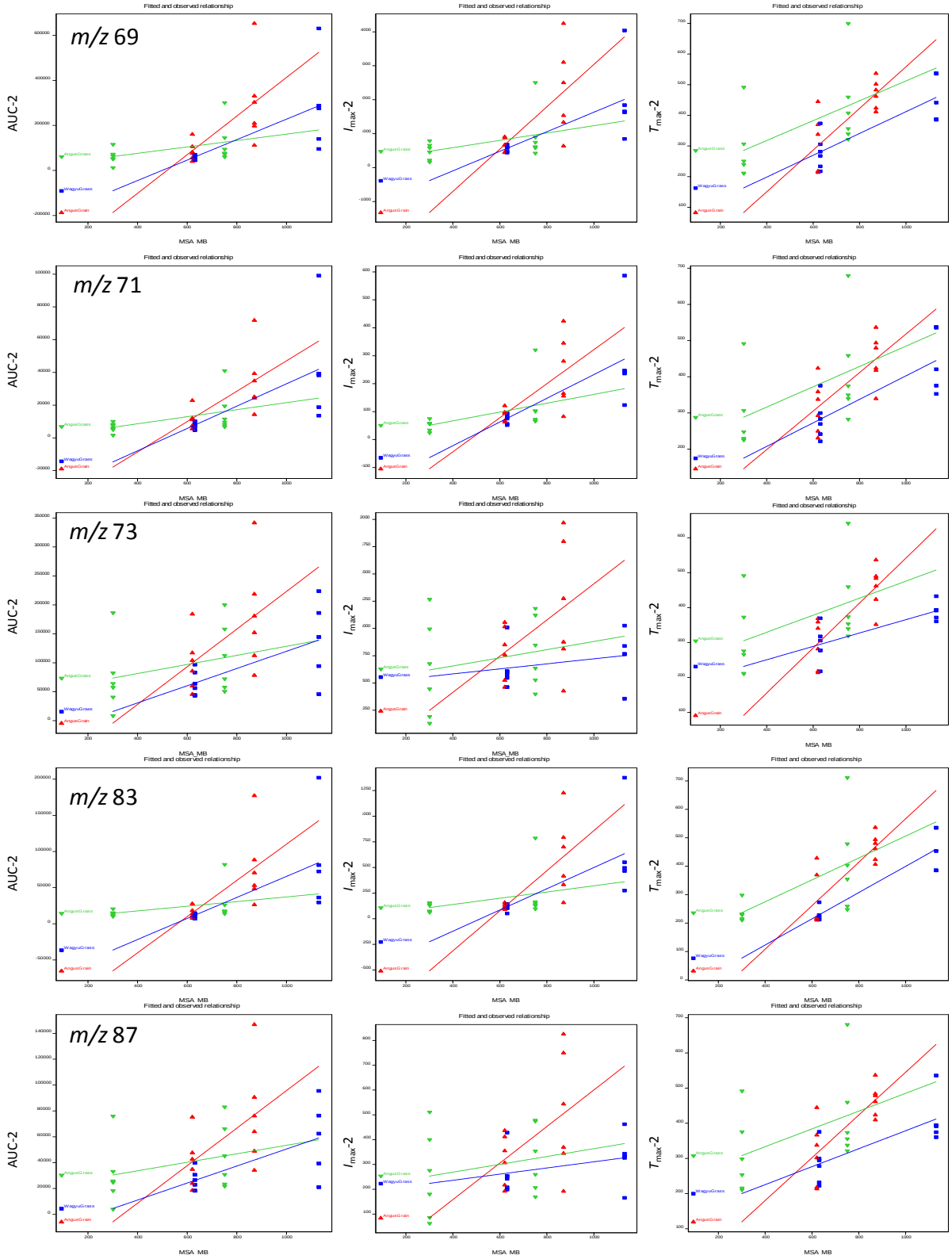

Figure 40: Scatter plots and regression models showing the relationship between volatile generation curve parameters and IMF (MSA-MB) for ions *m/z* 69 to m/z 87.

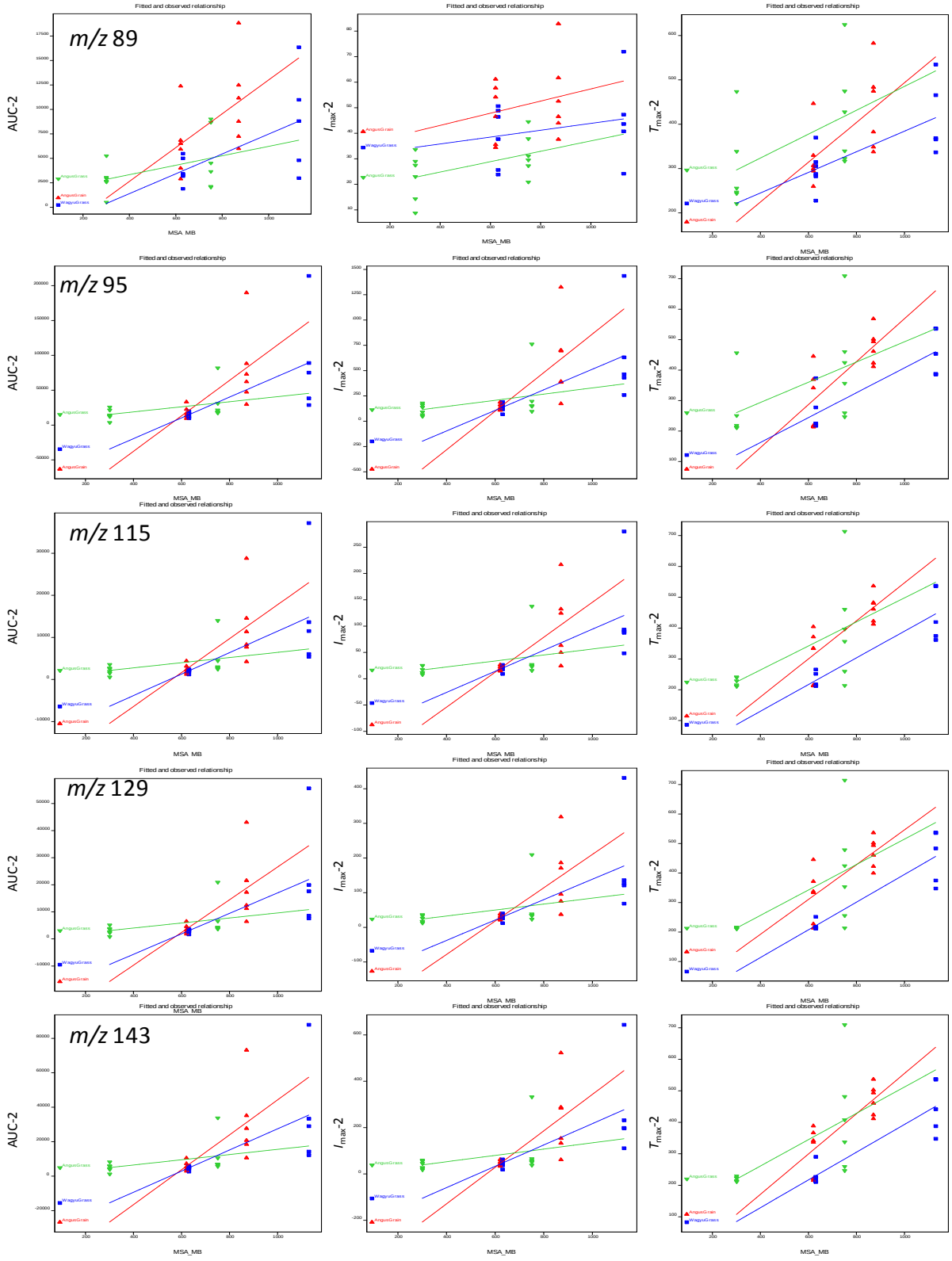

Figure 41: Scatter plots and regression models for the relationship between volatile generation curve parameters for ions *m/z* 89 to *m/z* 143.

10.1.3 ***Summary of PTR-MS volatile generation experimental results***

- *The PTR-MS method demonstrated the application of a new technology to quantitatively measure volatile production in grilling meat in real-time*

- *Since volatiles were highly correlated to MSA-MB, using a similar rapid approach on meat samples could provide the basis of an objective measure of IMF and perhaps beef quality*

- *In general, the amount of grilling time required to reach an internal temperature of 57 ^{o}C increased with marbling (MSA-MB) using the open grilling method — probably more relevant to barbequing at home or pan-frying/chargrilling in restaurants than the Silex grilling method*

- *The production of volatile compounds also increased with increasing IMF; this was most dramatic for samples with the highest marbling levels (MSA-MB > ~500)*

- *The rate of increase in volatile production with increasing marbling appeared to be slightly different, depending on sample type, suggesting subtle breed and feed effects*

- *The kinetics of volatile production was similar within each meat sample type across a suite of 10 volatiles*

- *The volatiles produced on the surface of the steaks by the heat of the grill are likely to dissipate rapidly away from the cooking meat. The amount of volatiles lost may not necessarily reflect the amount retained in the samples after resting — this may explain why the headspace concentration of octanal and nonanal did not increase with marbling when measured by DHS-Tenax*

11 Meat oral breakdown & non-volatile flavour release

Small differences in release rates of non-volatile components from chew-to-chew during eating may be lead to perceivable sensory differences. The trained panel sensory data clearly showed that *Sweet* taste increased, whereas *Acid* and *Astringent* taste decreased with increasing IMF. The analysis of free amino acids and non-volatiles indicated significant differences between sample types that supported the sensory findings for *Acid, Sour/acidic, Astringent aftertaste* and *Sweet*. Other attributes, such as *Dairy-fat* and *Oily mouthcoating* are also all likely to be related to perception of non-volatile components by taste receptors on the tongue and rather than by olfactory receptors. Furthermore, the texture related sensory data indicated that after correction for the effect of IMF, the AngusGrass samples were less *Tender* after 3 and 10 chews, required a greater *Number of Chews* and had more *Connective Tissue*. The temporal release of the volatiles identified in the previous section were further explored in this section.

11.1.1 *Oral Breakdown Methodology*

Meat was grilled and allowed to rest according to the sensory protocol. A ~12 g piece was cut from the centre of the steak and immediately weighed. One human subject (Caucasian male, 46 years old, normal dentition) was used for all experiments. The subject was required to thoroughly brush their teeth with standard toothpaste and rinse with water 40 minutes prior to experiments. A control blank of baseline saliva was obtained before each chewing experiment by transferring saliva to a pre-weighed plastic cup (baseline saliva). The whole hot piece of grilled steak was then placed in the subject's oral cavity and chewed once (1 chew). All of the combined juice and saliva from the first chew was transferred to a cup and weighed. After the second chew (2 chews) the meat liquid and saliva was once again transferred to a cup and weighed. The same process was continued after 4, 6, 10 and 20 chews. A 500 μL of the saliva/meat juice liquid was transferred into plastic Eppendorf tubes and 200 μL of HPLC grade methanol was added. The tube was vortexed and centrifuged (speed?) for 10 minutes and stored at -20 ^{o}C for 1 hour until a band of fat formed.

The fat layer was carefully removed and weighed. The mass of the pellet of remaining insoluble particle was determined as well as the total liquid (sum of saliva and meat juice) per sample. The total amount of saliva produced for each sample was also calculated. The relative percentage of solids, fat and liquid for each chew was determined. After passing the supernatant through a 0.4 μm Teflon filter, a 500 μL aliquot of the liquid was transferred to a glass test tube for methyl chloroformate derivatisation and analysis by GC/MS as previously described . Typical examples of the non-volatile derivatives present in saliva are shown in **Figure 42**. Clear differences between the blank saliva and after 1 and 10 chews can be seen. Integrated areas for non-volatile compounds were determined and used in statistical analyses.

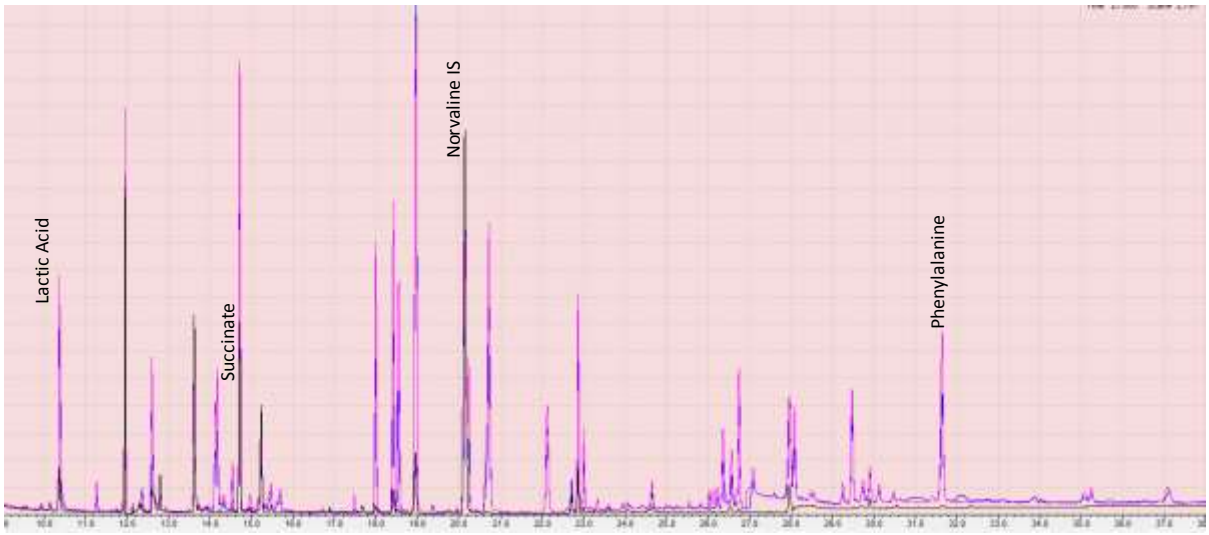

Figure 42: Typical total ion chromatogram profile of saliva and meat methyl chloroformate derivatives. Black trace = saliva blank, pink trace = chew 1, blue trace = chew 10.

Four replicates at baseline (blank saliva) and at each of the chew times (1, 2, 4, 6, 10 & 20) were analysed by GC-MS for three types of beef samples (i.e. 4 replicates x 7 time points x 3 sample types = 84 samples). Meat with the lowest MSA-MB score, i.e. AngusGrass low fat (AGL) and the sample with the highest MSA-MB score – i.e. WagyuGrass high fat (WGH) were analysed, to compare the effects of fat on non-volatile release. The highest fat grain-fed sample – AngusGrain high-fat (AGRNH) was also analysed to enable comparisons between high fat grain and grass fed samples and to also confirm high fat effects and test for any unique "Wagyu effects".

Replicate chew data for each sample were subjected to analysis by MANOVA, using sample type x chew number as fixed factors. LSDs were calculated to compare between sample types at each chew and between each chew.

11.1.2 *Oral Breakdown and non-volatile release Results*

The proportion of liquid (%) within the collected saliva and meat juice for each chew was highest for the low-fat AngusGrass samples compared to the high-fat AngusGrain and WagyuGrass samples. Small differences between the two high fat samples were evident within the first two chews, with more liquid released in the WagyuGrass sample. The amount of fat released (%) was highest in the AngusGrain and more fat was released earlier, than for the WagyuGrass sample. The amount of small insoluble particles was higher in the two high fat samples compared to the low fat AngusGrass, indicating more rapid breakdown of muscle tissue in higher marbled meat. The effect of sample type on amount of total saliva produced during eating approached significance ($p = 0.08$); the amount of saliva produced for the WagyuGrass high-fat sample was highest (3.99 g), compared to the AngusGrass low fat (2.97 g) and AngusGrain (2.13 g). The cumulative plot of the mass of meat juices and saliva together (**Figure 43**), shows the additive amount of total liquid produced per chew. The amount of meat juice/saliva produced by the grass-fed samples was higher compared to the grain-fed Angus,

mainly due to the production of more saliva. The sensory data showed that the *Oily mouthcoating* attribute was highest in the AngusGrain samples. The fat (%) released from the AngusGrain sample was higher in the initial chews compared to the other samples. Grain-fed beef is known to have a higher ratio of saturated fat than grass-fed beef (Scollan, Hocquette *et al.* 2006). The cold fat removed from the samples was also noticeably harder than for the grass fed samples, consistent with a greater proportion of saturated fat. The high *Oily-mouthcoating* scores for the AngusGrain sample may have been due to a higher proportion of saturated fat and more rapid release of fat in the first two chews. The slight differences between the high fat AngusGrain and WagyuGrass samples may have also been due to differences in the distribution of the IMF within the muscle structure.

Almost 50 non-volatile compounds were resolved in the saliva using the derivatisation method; however most remain unidentified and are not discussed here. Release curves for selected identified free amino acids and other non-volatiles of interest are summarised in **Figure 44**. Average non-volatile release profiles for WagyuGrass-high fat (WGH, blue line), AngusGrain high fat (AGRNH, red line) and AngusGrass low fat (AGL, green line) are shown in **Figure 44**. For some compounds significant differences were measured in release between sample types, designated by asterisks on the graphs (LSD, upper error bar). The effect of chew was highly significant for most graphs (lower error bar). The release profiles differed according to the non-volatile analyte and sample type.

For most compounds, the saliva concentration reached a maximum at chew 1 or 2 and then decreased. The maximum peak concentration/ intensity occurred earlier (on chew 1) for the high fat samples (AngusGrain and WagyuGrass) compared to the low fat AngusGrass (on chew 2) in many cases (e.g. glutamic acid, isoleucine, methionine, phenylalanine and valine). The I_{max} was higher for many analytes in the high-fat samples compared to the low-fat sample (e.g. glutamate, methionine, phenylalanine, lysine). Lactic acid is the compound most likely to be associated with *Acidic/sour* taste and *Acidic aftertaste.* The data clearly shows that the amount of lactic acid released from the low-fat AngusGrass meat was higher, compared to both the high fat samples. It was of interest that the WagyuGrass sample was rated lowest in lactic acid release and was also rated as the lowest in *Sour/Acidic* and *Acidic aftertaste (***Table 5**). Similarly, much greater release of serine (sweet), succinic acid (umami) and aspartic acid (umami) was measured in the WagyuGrass, compared to the AngusGrain and AngusGrass samples – this may also have contributed to the lower perceived *Sour/acidic* and *Acid Aftertaste* in the WagyuGrass samples. Greater concentrations of tryptophan were measured in the saliva from AngusGrass and AngusGrain compared to the WagyuGrass, perhaps explaining the slightly higher *Astringent aftertaste* measured in these samples. Two unsaturated long chain fatty acids, tentatively identified as palmitic (C16:0) and stearic (C18:0) were measured in the saliva extracts. These compounds appeared to follow a different release pattern; generally increasing in the saliva throughout the chews, but this was most obvious for the AngusGrain samples. It is tempting to relate these differences to the significantly higher *Oily mouthcoating* perceived in the AngusGrain samples. In general there was broad agreement between the semi-quantitative non-volatile data and the present in mouth data. The former sample preparation was more aggressive, i.e. involved maceration of the whole samples before extraction and may explain some of the differences observed.

Overall, the non-volatile release data provided further evidence for temporal and quantitative differences in oral breakdown and non-volatile release between the lowest fat samples (AngusGrass) and highest fat samples (AngusGrain and WagyuGrass). Some differences in

non-volatile release between the two high fat samples WagyuGrass and AngusGrain were also measured.

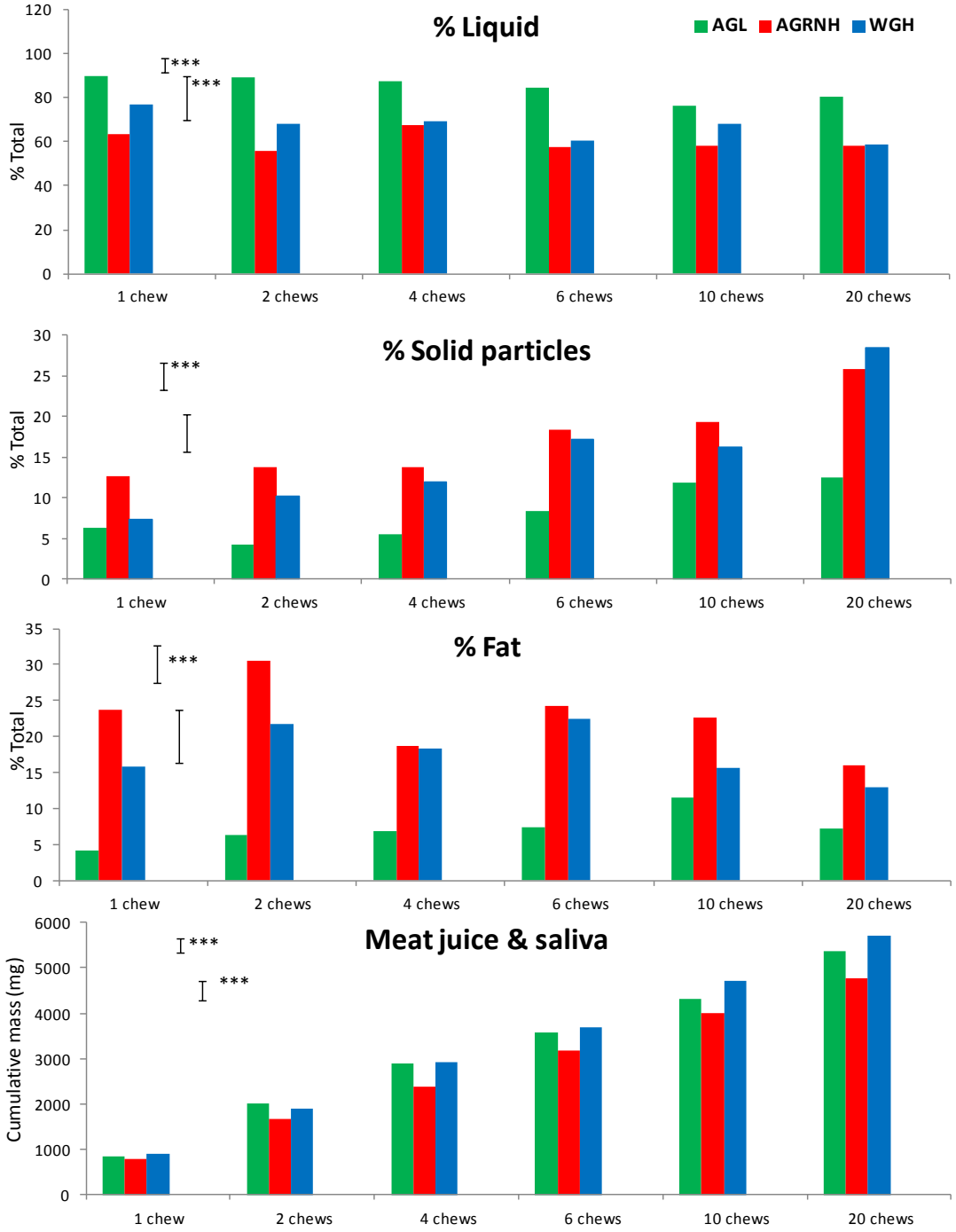

Figure 43: Average proportion of liquid, fat and solids (% mass) in saliva after 1, 2, 4, 6, 10 and 20 chews for AngusGrass low fat (AGL), AngusGrain high fat (AGRNH) and WagyuGrass high fat (WGH) grilled beef. Each data point is the average of four independent replicates. The least significant difference (LSD) is denoted by the bars for comparing sample type (low *vs* high fat) and between chews. Note use of different scales.

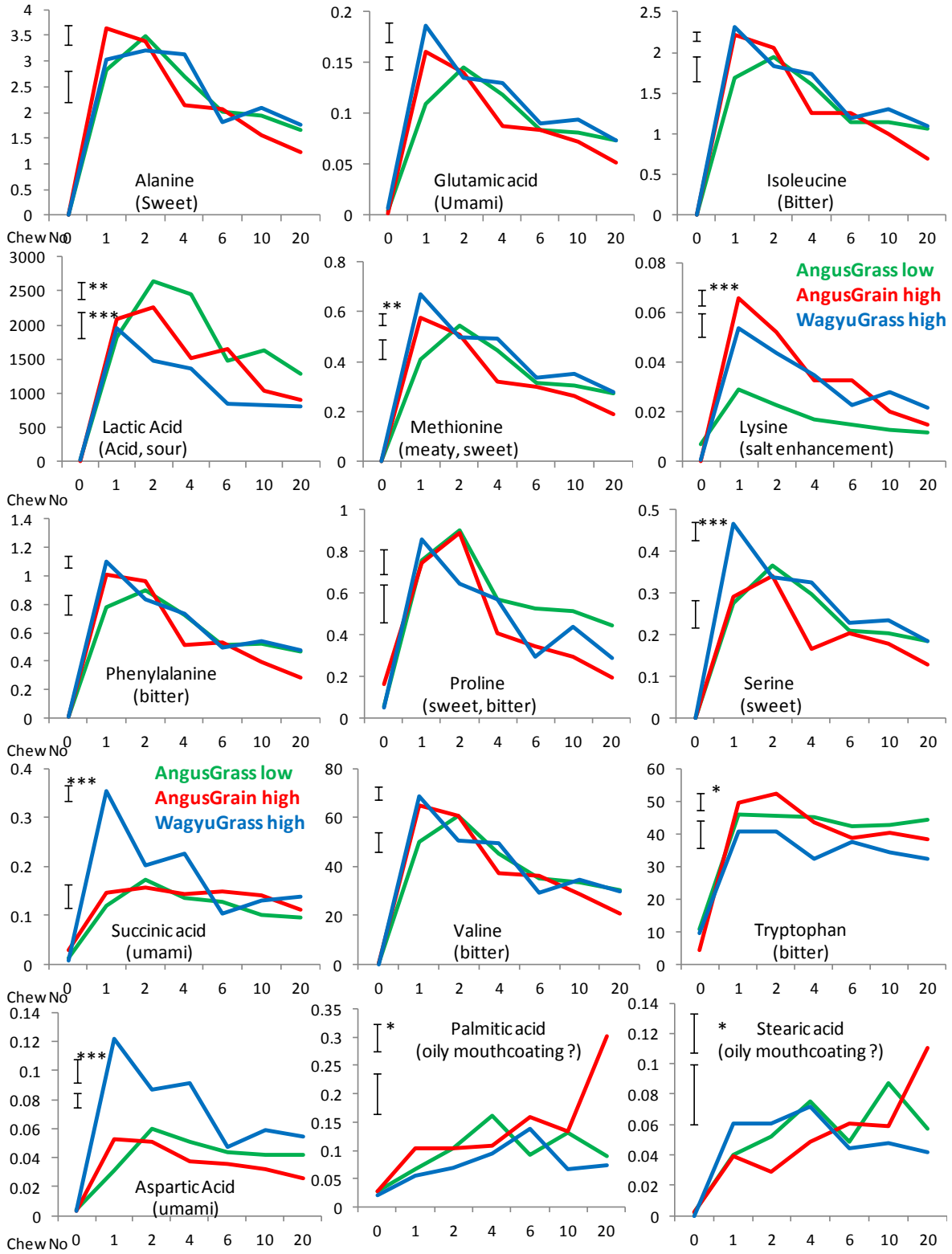

Figure 44: Release profiles of selected free amino acids and other non-volatile flavour compounds in saliva at baseline and at various chew numbers. AngusGrass low fat (AGL), AngusGrain high fat (AGRNH) and WagyuGrass high fat (WGH). Each data point is the average of four independent measures. The upper bar represents the least significant difference (LSD) for comparing samples types and the lower bar is the LSD for comparing chews. $P < 0.05$ = *, $p < 0.01$**, $p < 0.001$ = ***.

11.2 Summary of Non-Volatile Release

- *Differences were measured between the samples in terms of the amount of fat released per chew and the rate of formation of fine particles. More fat was released earlier from the high-fat AngusGrain compared to the high-fat WagyuGrass. The AngusGrain broke down slightly faster than the WagyuGrass*

- *More saliva was produced for the grass-fed samples compared to the grain-fed samples.*
- *Some amino acids were released faster in the higher fat samples compared to the low fat sample. The maximum in mouth concentration was reached at chew 1 in high fat samples compared to chew 2, in low fat samples. This small change in timing of release alone may result in perceptible flavour differences*

- *Lactic acid was significantly higher in the AngusGrass and AngusGrain samples, and lowest in the WagyuGrass, consistent with the sensory data for Acidity attributes*

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13 Appendix 1

Table 19: Average carcass characteristics for AngusGrass, AngusGrain and WagyuGrass samples within low, medium and high IMF categories.

Sample type	AngusGrass			AngusGrain			WagyuGrass			
Fat level	Low	Med	High	Low	Med	High	Low	Med	High	
CODE	AGL	AGM	AGH	AGRNL	AGRNM	AGRNH	WGL	WGM	WGH	P value
Sex	**M**	**M**	**M**	**M**	**M**	**M**	**F**	**F**	**F**	
AUS-MB	1	3	4.7	2.8	5	5.8	3.8	6.2	8.7	<0.001
MSA-MB	323	536	727	502.5	747.5	828	620	874	1106.7	<0.001
% Fat	5.2	7.8	9.9	10.2	13.7	14.9	7.8	10.9	17.5	<0.001
EMA	58.7	61.4	67.5	67.8	78	76	63	67.8	77.3	<0.001
CWT	361.6	318.6	328.3	417.4	405.4	410.6	287.1	287.9	258.3	<0.001
Dentition	3.7	2.8	2.7	1	1.5	0.8	5	4.8	4.7	<0.001
Hump	45	51	46.7	43.8	52.5	50	41.7	42	42.5	0.065
Oss	156.7	152	165	142.5	147.5	138	163.3	178	130.8	0.148
pH	5.58	5.58	5.56	5.54	5.5	5.51	5.52	5.56	5.55	0.398
L*	33.8	36.1	35.8	39.4	44.6	43.3	32.8	38.4	38.1	<0.001
a*	30.2	30.7	31.5	34	33.2	32.7	28.9	30.3	31.2	0.148
b*	22.5	23.4	24.2	26.9	25.7	24.9	22	23.1	23.9	0.185

Table 20: Summary of breed and feed comparisons using reduced data sets. The differences are essentially the same as those found using the full dataset.

ODOUR	Grain	Grass	P Feed	Angus	Wagyu	P Breed
Overall Impact	58.49	58.88	—	58.95	59.31	—
Grilled Beef	49.33	49.24	—	**10.81**	9.09	0.03
Livery	13.29	13.16	—	**12.36**	10.33	0.02
Bloody	**17.36**	15.43	0.02	15.83	15.73	—
Fishy	4.16	5.26	—	5.18	4.27	—
Hay Grain	17	16.07	—	15.78	**17.3**	0.05
Barnyard	12.28	12.67	—	12.37	10.97	—
Caramel	9.57	8.05	0.024	8.32	**10.21**	0.01
Metallic	10.42	10.73		**10.81**	9.09	0.03

FLAVOUR	Grain	Grass		Angus	Wagyu	
Overall Impact	58.27	56.61	0.043	57.09	**60.1**	<0.001
Grilled Beef	49.63	48.04	—	48.45	**50.92**	0.02
Livery	12.44	12.46	—	12.99	13.54	—
Bloody	**20.12**	18.31	0.05	18.73	19.41	—
Fishy	3.86	3.25	—	3.13	4.07	—
Hay Grain	15.6	15.8	—	15.63	15.03	—
Dairy Fat	18.67	15.18	<0.001	15.92	**18.5**	0.008
Grassy	14.95	12.36	0.007	12.76	14.38	—
Metallic	14.23	13.32	—	13.05	11.99	—

TASTE/AFTERTASTE	Grain	Grass		Angus	Wagyu	
Salty	14.9	14.75	—	14.66	14.97	—
Sour/Acidic	15.21	13.93	—	13.61	12.08	—
Sweet	14.91	13.26	0.002	13.43	15.38	<0.001
Acidic AT	14.64	13.41	—	13.23	12.05	—
Astringent AT	17.93	17.02	—	16.48	16.21	—
Lingering AT	31.07	30.3	—	30.11	29.64	—
Metallic AT	13.04	13	—	13.37	12.42	—
Oily Mouthcoating	15.86	13.41	<0.001	13.79	13.72	—

TEXTURE	Grain	Grass		Angus	Wagyu	
Juiciness 3 chews	40.12	36.99	0.011	38.37	41.19	0.04
Juiciness 10 chews	34.74	32.22	0.028	33.27	36.47	0.01
Tenderness 3 chews	50.8	47.61	0.007	48.88	52.38	0.005
Tenderness 10 chews	47.67	43.97	0.003	45.26	49.7	<0.001
Number of chews	26.1	26.9	—	26.29	24.5	<0.001
Connective Tissue	27.92	30.94	0.004	29.94	26.53	0.003

Table 21 Semi-quantitative data for volatiles measured in the headspace of grilled beef samples by either Tenax-DHS or SPME. Relative concentration units only.

	Alcohols	AngusGrain	AngusGrass	WagyuGrass	LSD	P Sample	P Breed	P Feed	$R_{I\ MF}$	P IMF
SPME	**1-hexanol**	29.91	6.19	**45.84**	24.74	0.01	<0.001			
SPME	**1-octanol**	2.24	1.89	**3.89**	1.07	0.002	<0.001			
SPME	**1-octen-3-ol**	25.91	**15.51**	29.16	10.94	0.039	<0.001			
SPME	**1-pentanol**	10.54	8.15	**15.21**	4.38	0.011	0.002			
SPME	**1-heptanol**	4.70	2.94	**9.56**	2.96	<0.001				
SPME	**2-penten-1-ol**	0.87	1.02	**1.98**	0.00	<0.001				
Tenax	**1-hexanol**	0.88	1.73	1.21	0.93				*-0.28*	
Tenax	**1-octen-3-ol**	3.71	7.91	3.97	4.60				*-0.46*	0.002
SPME	**4-methylphenol**	1.85	2.18	2.48	0.39				*0.33*	0.03
Tenax	**guaiacol**	0.09	0.08	0.08	0.05					
	Aldehydes	**AngusGrain**	**AngusGrass**	**WagyuGrass**	**LSD**	**P sample**	**P Breed**	**P Feed**	$R_{I\ MF}$	P IMF
SPME	**2-methylpropanal**	**4.57**	5.93	6.72	1.25	0.003		0.004	*0.5*	<0.001
SPME	**2-methylbutanal**	6.94	8.55	6.04	3.10				*0.57*	<0.001
SPME	**3-methylbutanal**	4.43	5.59	4.29	0.00				*0.56*	<0.001
SPME	**z-4-heptenal**	0.07	**0.55**	**1.27**	0.43	<0.001				
SPME	**hexanal**	74.29	55.26	70.26	35.98		ns			
SPME	**heptanal**	10.27	11.72	**23.73**	7.46	0.002	0.003			<0.001
SPME	**octanal**	4.34	4.79	**7.28**	2.36	0.05	0.025		*-0.418*	<0.001
SPME	**nonanal**	12.83	15.67	**22.77**	6.12	0.008	0.04		*-0.53*	<0.001
Tenax	**2-methylpropanal**	13.02	8.81	9.20	3.44	0.032	0.02		*0.5*	<0.001
Tenax	**2,3-methylbutanal**	**265.00**	**224.00**	183.00	63.20	0.037			*0.46*	0.002
Tenax	**(E)-2-octenal**	0.53	**1.00**	0.56	0.38	0.03	0.04		*-0.45*	0.003
Tenax	**(E)-2-nonenal**	1.06	1.58	0.88	0.67				*-0.42*	0.006
Tenax	**heptanal**	5.21	6.74	5.08	2.00					
Tenax	**hexanal**	19.00	29.90	21.10	17.62				*-0.31*	0.04
Tenax	**octanal**	1.76	**2.54**	1.37	0.98	0.05	0.055		*-0.26*	
Tenax	**nonanal**	0.64	**1.06**	0.55	0.42	0.037	0.04		*-0.45*	0.003
Tenax	**decanal**	0.05	0.04	0.04	0.04				*0.29*	
Tenax	**benzaldehyde**	8.13	**10.54**	5.69	3.18	0.011	0.007		*-0.25*	0.09
Tenax	**benzeneacetaldehyde**	**5.67**	**5.37**	2.70	1.54	<0.001	0.001		*0.566*	<0.001

Table 22: Semi-quantitative data for volatiles measured in the headspace of grilled beef samples by either Tenax-DHS or SPME. Relative concentration units only.

	Dienals	AngusGrain	AngusGrass	WagyuGrass	LSD	P sample	P Breed	P Feed	$R_{I\ MF}$	P $_{IMF}$
SPME	**2,4-hexadienal**	1.07	**2.18**	**3.25**	0.92	<0.001	0.015	0.004	*-0.25*	<0.001
Tenax	**(E,E)-2,4-nonadienal**	1.41	2.55	1.57	1.45				*-0.36*	0.017
Tenax	**(E,E)-2,4-decadienal**	0.59	0.72	0.55	0.32				*0.25*	
	Ester	**AngusGrain**	**AngusGrass**	**WagyuGrass**	**LSD**	**P sample**	**P Breed**	**P Feed**	$R_{I\ MF}$	P $_{IMF}$
SPME	**methyl acetate**	4.03	7.48	1.15	5.78					
SPME	**methyl butanoate**	0.58	0.63	0.29	0.20	0.005	0.017	ns	*0.35*	<0.001
SPME	**ethyl butanoate**	0	0.83	0.81	0.32	<0.001		<0.001	*-0.41*	<0.001
	Ketone	**AngusGrain**	**AngusGrass**	**WagyuGrass**	**LSD**	**P sample**	**P Breed**	**P Feed**	$R_{I\ MF}$	P $_{IMF}$
SPME	**acetone**	4.83	**8.60**	7.01	2.46	0.006		0.001	-	ns
Tenax	**2,3-butanedione**	6.23	8.54	7.10	2.28			0.04		
SPME	**2,3-pentanedione**	1.16	1.41	2.26	0.58	0.002	0.015		*0.34*	0.007
SPME	**3-hydroxy-2-butanone**	**47.12**	14.23	24.01	22.4	0.007		0.01		
SPME	**butyrolactone**	1.46	1.69	**2.54**	0.00	<0.001	<0.001		*0.28*	0.03
	Pyrazines	**AngusGrain**	**AngusGrass**	**WagyuGrass**	**LSD**	**P sample**	**P Breed**	**P Feed**	$R_{I\ MF}$	P $_{IMF}$
Tenax	**2,3-dimethylpyrazine**	8.71	**9.91**	5.63	3.74	0.06	0.02		*0.49*	<0.001
Tenax	**2,6-dimethylpyrazine**	4.37	3.19	2.78	2.74				*0.37*	0.02
Tenax	**2,3-diethyl-5-methylpyrazine**	0.25	0.31	0.31	0.24				*0.54*	<0.001
Tenax	**2-ethyl-5-methylpyrazine**	2.39	**2.47**	1.39	1.03	0.06	0.04		*0.51*	<0.001
Tenax	**3-ethyl-2,5-dimethylpyrazine**	**2.60**	2.36	1.63	0.73	0.02			*0.57*	<0.001
Tenax	**2-methylpyrazine**	**3.16**	2.84	1.73	1.25	0.05			*0.56*	<0.001
Tenax	**trimethylpyrazine**	1.27	**1.60**	0.91	0.60		0.004		*0.55*	<0.001
Tenax	**2-methylpyrrole**	0.09	0.17	0.24	0.17				*0.57*	<0.001
Tenax	**3-methylpyrrole**	0.16	0.21	0.15	0.11				*0.59*	<0.001
Tenax	**2-acetyl-1-pyrroline**	0.18	0.23	0.13	0.14				*0.1*	
Tenax	**2-acetyl-2-thiazoline**	0.08	0.05	0.03	0.04	0.05			*0.25*	
	Sulphur	**AngusGrain**	**AngusGrass**	**WagyuGrass**	**LSD**	**P sample**	**P Breed**	**P Feed**	$R_{I\ MF}$	P $_{IMF}$
Tenax	**dimethylsulphide**	1.14	0.63	**1.72**	0.80	0.025	0.025	0.015	*0.51*	<0.001
Tenax	**dimethyldisulphide**	1.61	1.05	0.61	0.80	0.045			*0.52*	<0.001
Tenax	**dimethyltrisulphide**	0.11	0.05	0.04	0.12				*0.257*	
Tenax	**methional**	**0.15**	**0.16**	0.10	0.04	0.027	0.022		*0.45*	0.004