final report

Project Code: V.EQT.1103

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Date published: January 2006

PUBLISHED BY
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Locked Bag 991
NORTH SYDNEY NSW 2059

Development of automated data analysis method, data evaluation and protocol development for use of SPME volatile collection in beef for linkage to consumer flavour evaluation

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

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1. Objectives

The purpose of this project is to develop flavour analysis methods which will be used for the further improvement of beef quality. The objectives for this work were to:

1. Develop an automated analysis method for GC/MS data files.
2. With Texas Tech personnel, analyse data supplied by Texas Tech University from V.EQT.1004.
3. Establish a standard objective flavour database.
4. Train Texas Tech personnel in use of the technique.
5. Publish protocols defining the analysis method and database structure.
6. Advise in regard to recommended further priority flavour research issues.

2. Progress against milestones

The progress against the milestones set in project V.EQT.1103 is summarised in Table 1 and explained further in the following sections.

Table 1. Progress against the milestones set in project V.EQT.1103

No.	Milestone and achievement criteria	Due date	Progress
1	Automated analysis method developed and tested.	21-July-2010	Done. Method works and is undergoing further refinement
2	Texas data received and personnel trained in analysis method.	21-July-2010	Done. Personnel trained. TTU data has undergone initial analysis. QA underway.
3	Interim report on procedures and analysis arising from joint TTU/AFBI work	31-July-2010	Report submitted July 2010
4	Publish protocols and database structure.	31-Oct-2010	Done. See Appendix I
5	Recommend further priority research actions.	31-Oct-2010	Done. See this report

2.1. Protocol for analysis and quantification of flavour volatiles

A protocol for the analysis of flavour volatiles from grilled beef cooked by MSA methods has been developed from the work conducted at TTU and at AFBI. The protocol is based on the collection of the volatile compounds by solid phase microfibre extraction (SPME) and analysis by GC-MS. The GC-MS used at both locations was an Agilent GC-MSD

with very similar specifications; that at AFBI is a 6890N GC connected to a 5975B MSD. While the details of method setup are specific to this make of instrument, the general protocol could be applied to other makes of GC-MS.

An automated data analysis method has been devised and tested. This involves setting up customised methods within the GC-MS software which integrates each compound in a data file for each run. A macro has been created by AFBI which collates the data from many such data files into a spreadsheet with one row per sample with reference to the EQSref code. This method means that the integration of peak areas from one GC-MS runs can be conducted in about one minute instead of one hour.

Having conducted the automated analyses it is then necessary to quality assure the numbers obtained. A system for quality assurance has been devised. The integration method records the retention time and a Q value, which is a measure of the fit of the mass spectrum. These values serve to highlight data values which require to be checked manually. Further information on these measures is available in Appendix I.

2.2. Training of TTU Personnel

Jerrad Legako, from TTU, spent two weeks in Belfast in early July to be trained in the techniques of GC-MS data analysis. In addition, the opportunity was taken to review the methods used to date and to devise an appropriate strategy for the standardisation of the methods using internal and/or external standards. A brief summary of the topics covered includes:

- Manual GC-MS data analysis
  - Training in methods, including ion extraction, subtraction, quantitation reports
  - Quality assurance of manual data analysis using retention times, ion areas
- Automatic GC-MS data analysis
  - Conduct of automatic method
  - Quality assurance using retention times, Q values, ion areas
- Analysis of TTU data
  - Manual method
  - Automatic method
- Mass spectral interpretation
  - Recommended reading
  - Tricks of the trade
- SIM method and its use
- Use of standards
  - Internal versus external
  - Authentic compounds – how to use

- Standard operating procedure for odour compounds
- Review of methods
- AFBI flavour research and beef research programmes

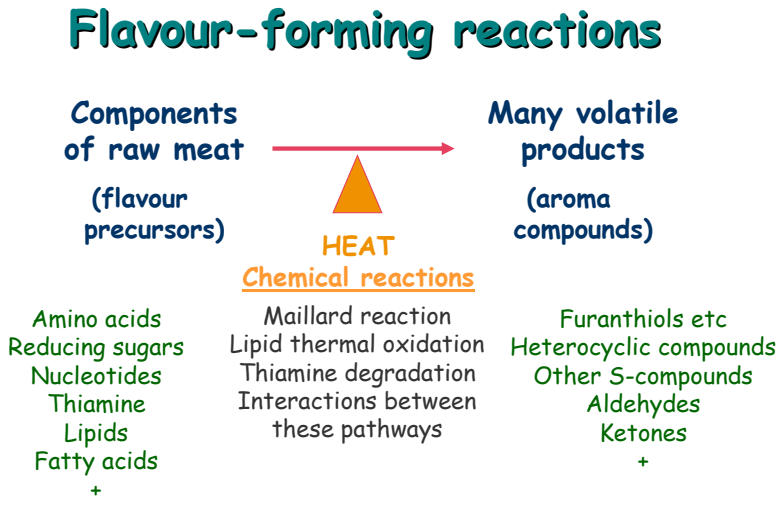
3. Flavour formation in cooked meat

The work conducted in the course of the MLA funded study with TTU, and other work conducted at AFBI, have generated important new information on how flavour differences contribute to the quality of beef. This section aims to summarise this knowledge and how it may be used to improve beef quality.

3.1. Overview

The volatile flavour compounds that characterise the odour and, in large part, the flavour of cooked beef are formed by a number of pathways and from a range of precursors (Figure 1). For example, the fatty acids, especially in the membrane lipids, react on heating to give lipid oxidation products such as aldehydes. The amino acids react with sugars to give a wide range of compounds by the "Maillard reaction". These pathways form a vast interacting network of reactions which between them give meat flavour (Farmer, 1992). Changes in the quantities of these precursors, the quantities of other meat components, the time or temperature of cooking or any additions to the meat can affect the balance of these compounds.

Figure 1. Formation of beef odour and flavour from precursors in raw meat



In addition to volatile compounds, flavour is affected by non-volatile taste compounds. These include salts, sugars, amino acids and nucleotides. These substances will confer sweet, sour, bitter, salty and "umami" taste on meat. Of these, the Japanese-defined

"umami" (deliciousness) is likely to have a special impact in cooked meat. It is known to be caused by natural glutamate and nucleotides, though the role of the quantities of these compounds is uncertain.

In addition, the quantity of fat present is likely to affect the release of flavour from the meat. It acts as a solvent, dissolving the flavour and slowing its release during chewing. Low quantities of fat in meat products causes excessively fast flavour release, giving a harsh flavour which disappears quickly on eating.

Thus, the final desirable flavour is a consequence of not only the quantity but also the balance of the flavour compounds formed and of the amount of fat present to dissolve them.

3.2. What have we learned?

A considerable body of data has been collected at TTU since December 2009 and the new automated method has been used to conduct an initial analysis. As an initial method of quality assurance, all data where Q<40 (low fit of mass spectrum) were removed before analysis. These preliminary analyses suggest that there is good evidence that treatments which affect flavour liking also affect the volatile compounds.

a) Intramuscular fat content has some effect on flavour liking and volatile compounds

MLA-funded studies at TTU and AFBI have suggested that the n-aldehydes (e.g. hexanal, heptanal, octanal, nonanal) are influenced by intra-muscular fat (IMF); high IMF samples do not give high levels of these compounds, but low levels can do so (Figure 2). These compounds are formed by the oxidation of fatty acids and will be higher from polyunsaturated fatty acids. They are believed to be formed primarily in the unsaturated membrane lipids rather than the more saturated marbling fat. At lower levels of IMF the aldehydes can be low or high.

Figure 2. Effect of IMF (%) on volatile compounds from grilled beef

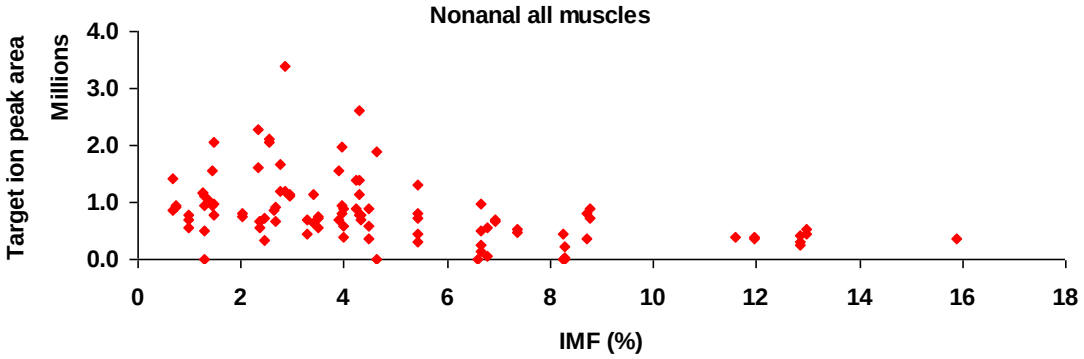
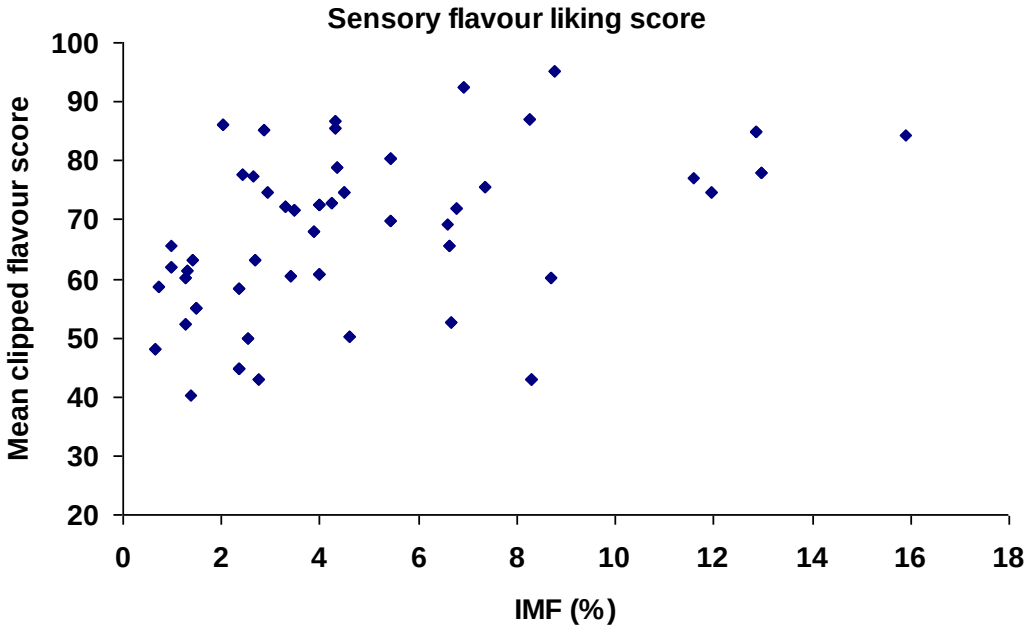


Figure 3. Effect of IMF (%) on the clipped MQ4 scores for grilled beef



The consumer scores for flavour liking (clipped mean scores) also appear to be influenced by IMF (Figure 3). It is difficult to separate the effect of IMF on tenderness and flavour. Thompson (Thompson, 2004) has reported that there is a curvilinear relationship between IMF and consumer scores for flavour liking after correction for shear force, with improvement in flavour scores plateauing at between 15% and 20%. This effect on flavour may be explained by the role of fat on flavour release, as observed previously in meat products (Chevance and Farmer, 1999). However, despite the apparent inverse relationship between the patterns in Figures 2 and 3 there is no direct correlation between consumer scores and volatile measurements (e.g. R^2 = 0.02 for nonanal), so the impact of IMF on flavour appears to be complex.

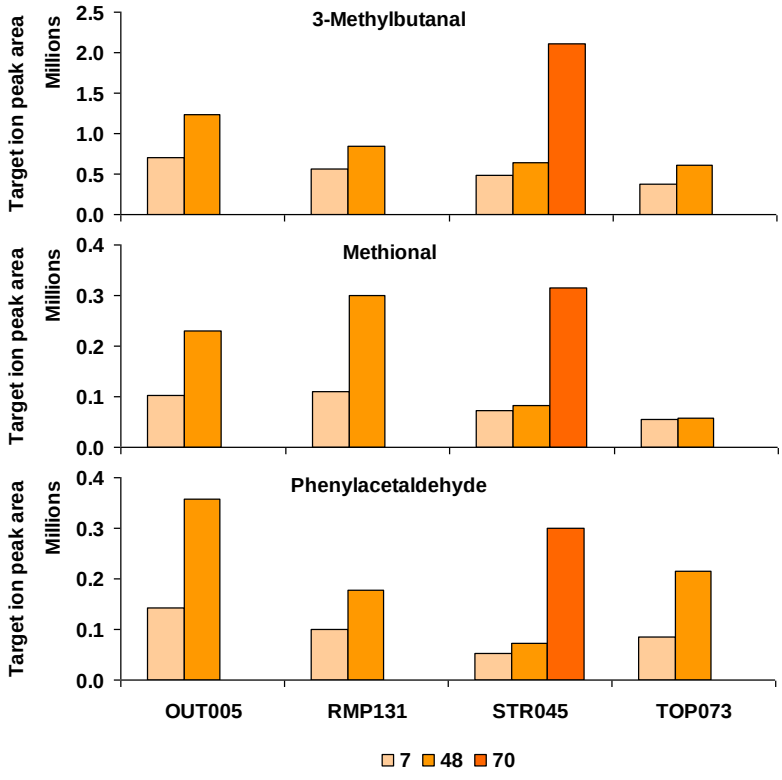
b) Ageing of beef increases the quantities of some flavour volatiles

There is much anecdotal evidence that increased ageing gives increased flavour in beef. Previous studies have reported conflicting findings for sensory measures of flavour; Yancey et al. (Yancey et al., 2005) studied the effect of ageing and found that neither "beef flavour identity" nor "brown roasted attribute" increased between 7, 14, 21 and 35d ageing. However, at 35d ageing, these attributes decreased and "metallic, "rancid" and "sour" flavours increased slightly. Bruce et al (Bruce et al., 2005) found that ageing up to 14 days increased fatty flavour.

During ageing, meat undergoes a wide range of enzymic breakdown pathways which convert large complex molecules to smaller ones. One example is proteolysis, which results in the breakdown of muscle proteins. However, other pathways will increase the concentrations of sugars, nucleotides, nucleosides and free fatty acids. These compounds then act as flavour precursors and react together during cooking to give flavour.

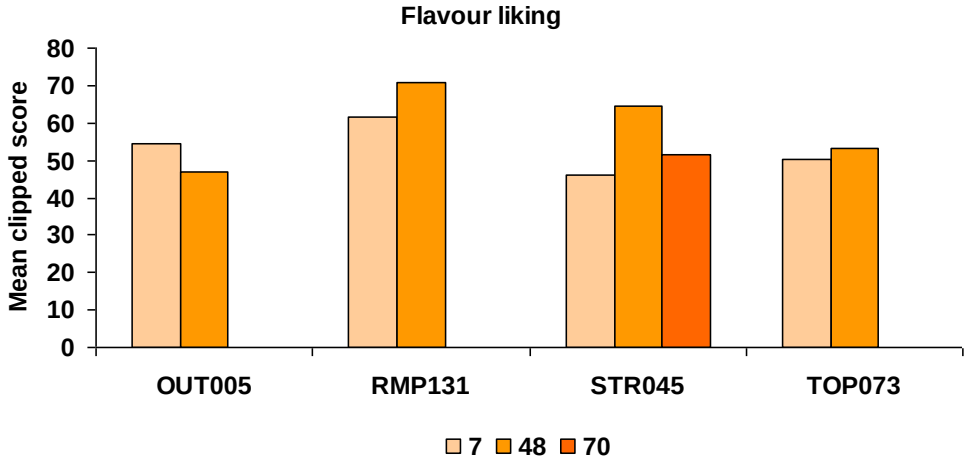
Analysis of the data from different muscles aged for different periods from the Australian meat in the MLA-funded studies at TTU and AFBI have suggest a trend for some flavour volatile compounds to increase with ageing (Figure 4). These compounds are known as "Strecker aldehydes" and are derived from the Maillard reaction between amino acids and sugars.

Figure 4. Effect of ageing on selected volatile compounds (Strecker aldehydes) from grilled beef (NB only STR045 was aged to 70d)



Flavour liking tends to increase from 7 to 48 days but thereafter decreases to 70 days (Figure 5). This could perhaps reflect the fact that some increase in flavour volatiles is liked but too much is not!

Figure 5. Effect of ageing on clipped flavour liking scores for grilled beef (NB only STR045 was aged to 70d)



It is known that the free amino acid and sugar concentrations in meat increases considerably during ageing (Farmer et al., unpublished data; (Koutsidis et al., 2008a; Koutsidis et al., 2008b)). It is highly likely that the increased availability of flavour precursors as ageing progresses will lead to increases in flavour volatiles and changes in the balance between them, which could lead to either improvements or detrimental effects.

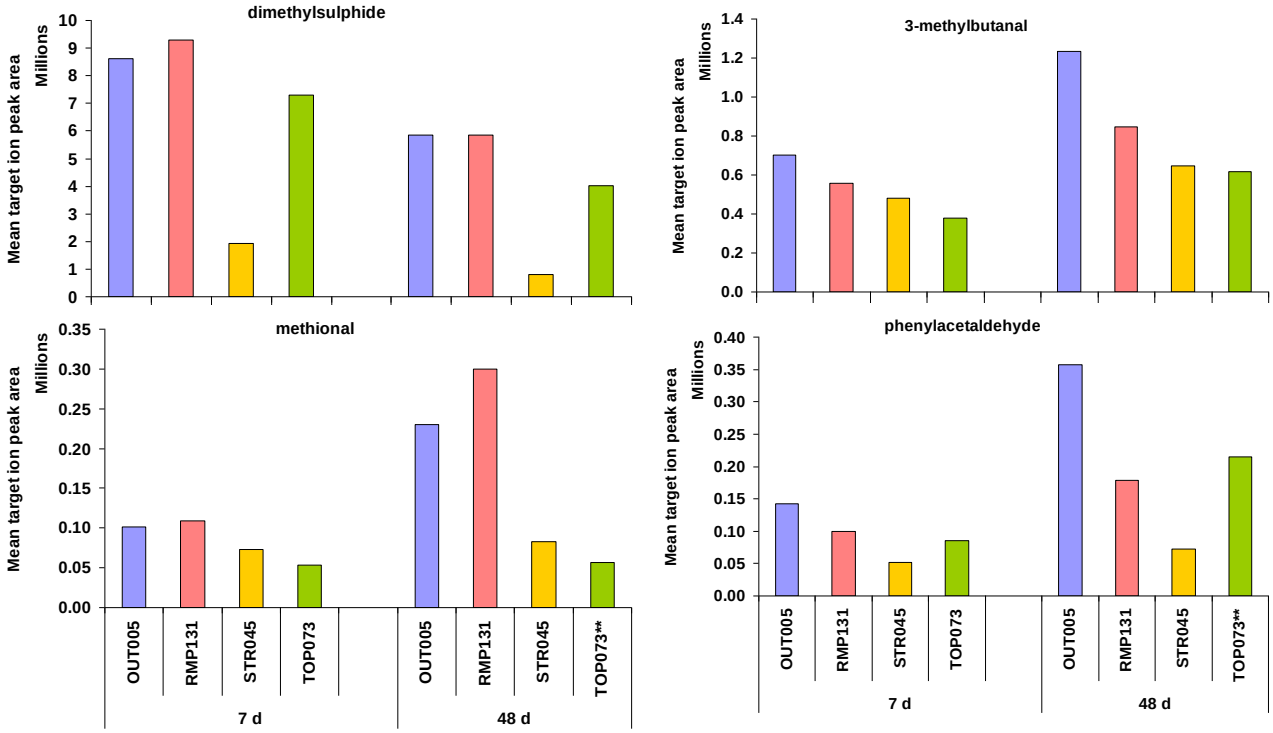
c) Flavour differences occur between muscles

Whilst the range of tenderness across muscles is well known, fewer authors have investigated differences in flavour between muscles and frequently they do not agree (Yancey et al., 2005). Although flavour scores differ between muscles, it is not clear to what extent differences in tenderness influence these flavour scores; this is known as the "halo effect". Methodologies need to be devised which separate the assessment of flavour and texture and instrumental analyses of texture and flavour help achieve this. Few authors have investigated the differences in flavour compounds. The data on possible differences in flavour volatiles between muscles obtained as part of MLA-funded studies at TTU and AFBI is therefore quite new.

The fact that similar trends occur between muscles at two ageing periods, when the data come from quite separate collections, suggests real effects. Certain compounds from different parts of the Maillard reaction show different effects of muscle (Figure 6). This suggests that small differences either in precursor

concentrations or other compounds between the muscles may be enough to cause measurable differences in flavour volatiles.

Figure 6. Effect of muscle on selected odour compounds in meat aged to 7 and 48 days



d) AFBI research on the relationship between consumer scores, flavour precursors and volatiles of beef sirloin

Recent studies not forming part of this research but reported elsewhere (Farmer et al., 2010) have shown that consumer scores for flavour liking of grilled beef are positively related to sweet flavour and negatively related to sour flavour. These studies also suggest that flavour liking is related to the content of certain precursors of flavour in the raw beef such as sugars and nucleotides. Fatty flavour is strongly related to total intramuscular fat but neither are related to consumer liking scores. This work has also been extended to include the flavour volatiles, with the aim of identifying key flavour volatiles which act as markers for sensory attributes and consumer liking (or disliking). This would supplement the work reported in this report in that it would help to focus analyses on those volatiles of specific relevance. These studies are ongoing.

e) Consumers from different countries have different flavour preferences

Research from around the world has shown that consumers from around the world have different likes and dislikes for flavour. It has been widely reported that American consumers prefer grain-fed beef to grass-fed beef whereas UK and Australian consumers like both. Studies comparing the response of Mediterranean and Northern European consumers to lamb (Sanudo et al., 2000; Sanudo et al., 2007) showed that consumers preferred what they were used to eating, namely Northern European consumers preferred grass-fed lamb while Mediterranean consumers preferred grain-fed lamb. In addition, data from MSA research have demonstrated that consumers from different countries may accord tenderness, juiciness and flavour liking a different relative weighting (Polkinghorne, personal communication). In addition, MSA consumer research has shown that the MSA model does not accurately predict eating quality for some consumers and it is suspected that this over-prediction is caused by flavour notes disliked by that consumer group.

Studies on beef identified different subgroups within countries that preferred different sources of beef (Oliver et al., 2006). Similar results have been found in research at AFBI on the characteristics of beef liked and disliked by different subgroups of the population (Farmer et al., unpublished data). Therefore, consumers from one country cannot be regarded as homogenous in their preferences.

It is because of these effects that it is important for the future development of MSA that the role of flavour in consumer liking is further understood.

3.3. What do we need to know?

There are a number of flavour issues that are causing some trouble to the MSA system because (a) their impact does not appear to be captured by MSA grading at present and (b) because consumers from different countries respond differently to these factors. These must, therefore, be the priorities for resolution.

- How does a grass diet affect flavour? What impact does quantity, type of grass (fresh or silage), growing conditions and length of feeding have?

- How does muscle impact on flavour? It is believed within the industry that different muscles have different flavours. Thus, while some muscles may have the same tenderness score, they may have different flavours. There is little scientific evidence on the nature for these differences, but that reported in Section 3.2 above suggests they exist.

- Ageing affects flavour and tenderness. While it is known that ageing affects tenderness more in some muscles than others, the role of such interactions for flavour formation is less well understood. Such information could help explain why the flavour of some muscles does not benefit from ageing and to predict those where different effects may be expected.

- Does hanging method have any effect on flavour?

- Different cooking methods deliver different flavours due to the differences in time, temperature and moisture of cooking. The degree of doneness will also have a strong impact. An understanding of the importance of these effects and their interaction with muscle and other factors would assist the use of muscles most appropriately for the target consumers. The impact of new cooking methods used in international cuisine can be tested.

- Enhancement of beef by injection with phosphates and other salts is very popular in USA. This would be expected to affect the quantities of volatile products of the Maillard reaction as this reaction is very sensitive to phosphate and pH.

- Marbling is known to affect eating quality and is expected to affect flavour release as explained earlier. However, the level of marbling needed to give optimum flavour release is unknown.

4. Options for improving flavour of MSA beef

The overall aim of this work must be to improve the delivery and consistency of good eating quality beef. This encompasses both the improved **prediction** of eating quality, so that an appropriate grade can be applied, and also the improved **management** of eating quality, so that the flavour produced is appropriate for the expected customer. The following options have been considered on how best to use the information on flavour and flavour volatiles to achieve this:

- Monitor volatile compounds on-line
- Modify MSA score based on grading predictors of beef flavour
- Qualify MSA score with flavour descriptors for beef with special flavour qualities

4.1. Monitor volatile compounds on-line

In this option, an instrument would be used to monitor the volatiles on-line. The beef would be sampled, heated on the instrument and volatiles collected, analysed and key compounds used to predict the flavour quality of the cooked beef. While this is

theoretically feasible, there are certain hurdles that would make this difficult to operate routinely:

- Using current GC-MS equipment, each analysis requires at least 20 minutes and usually more.
- The current instrumentation requires expert scientific operation and a clean environment and would be unsuited to meat plant use.
- The cost of sensitive GC-MS analyses would be too high for routine use.
- While some equipment, e.g. electronic noses, can measure volatiles more quickly, up until now it has lacked the sensitivity to determine the concentrations of most volatile compounds of importance for flavour.
- To develop a method for routine use would require the identification of marker compounds at sufficiently high concentrations to allow their monitoring by a rapid method such as electronic nose technology. This may be possible in the future and the development of this technology is being monitored.

The on-line measurement of meat volatiles, therefore, must await the development of new and more sensitive equipment, probably using electronic nose technologies.

4.2. Modify MSA score based on grading predictors of beef flavour

The current MSA model predicts eating quality by predicting the MQ4 score. This MQ4 score is based on individual scores for tenderness, juiciness, flavour liking and overall liking as follows:

$$MQ4 = 0.3\ TN + 0.1\ JU + 0.3\ FL + 0.3\ OL$$

MQ4 may be predicted from certain grading measures:

$$MQ4\ (pred) = constant + fn\ AGEING + fn\ pHu + fn\ HANG + fn\ HANG{*}AGE + \ldots\ etc\ etc$$

This prediction predicts tenderness very effectively but is less precise for the prediction of flavour liking. It would be possible to modify the model so that tenderness, juiciness and flavour liking are predicted separately and then combined:

$$TN\ (pred) = constant + fn^{t}\ AGEING + fn^{t}\ pHu + fn^{t}\ HANG + fn^{t}\ HANG{*}AGE + \ldots\ etc\ etc$$

$$JU\ (pred) = constant + fn^{f}\ AGEING + fn^{f}\ pHu + fn^{f}\ HANG + fn^{t}\ HANG{*}AGE + \ldots\ etc\ etc$$

$$FL\ (pred) = constant + fn^{f}\ AGEING + fn^{f}\ pHu + fn^{f}\ HANG + fn^{t}\ HANG{*}AGE + \ldots\ etc\ etc$$

$$MQ4\ (pred) = a * TE\ (pred) + b * JU\ (pred) + c * FL\ (pred)$$

Modification of the values of *a*, *b* and *c* would enable the predicted MQ4 to take into account the differences between populations in terms of the importance they place on flavour, but would not take into account the differences between populations in their liking for specific types of flavour. However, this last could be overcome by incorporating a term relating to population preferences in the Predicted FL equation:

$$FL\ (pred) = constant + fn^{f}\ AGEING + fn^{f}\ pHu + fn^{f}\ HANG + fn^{t}\ HANG*AGE + fn^{f}\ COUNTRY$$
$$\ldots\ etc\ etc$$

This would give an MSA score modified for the importance of flavour for specific populations, based on best animal/meat plant grading predictors of beef flavour and the relative importance of flavour forming pathways. The derivation of this equation would be achieved by a combination of two methods:

Prediction from MSA database. The MSA database contains many results for flavour liking for beef from many different treatments. However, the very high correlation between flavour liking and tenderness makes the prediction of flavour difficult. The results have to be either statistically adjusted for tenderness or data selected to have the same tenderness score. Nevertheless, analyses conducted on the MSA database show that, despite the correlation between consumer scores, the prediction equations for FL and TN differed in the weightings accorded to different factors (Polkinghorne, personal communication). Further analysis of this database may suggest which beef and consumer factors have most impact on flavour liking.

a) **Analysis of flavour compounds**. This will allow the underpinning science of flavour differences to be understood, which in turn will permit better management of the beef to achieve the desired quality. For example, if the flavour compounds important for the recognisable flavour of grass-fed beef are known and their formation understood, a knowledge of potential mitigating factors would assist when preparing beef for the US market. It also provides a basis for extrapolation to predict the impact of factors not tested.

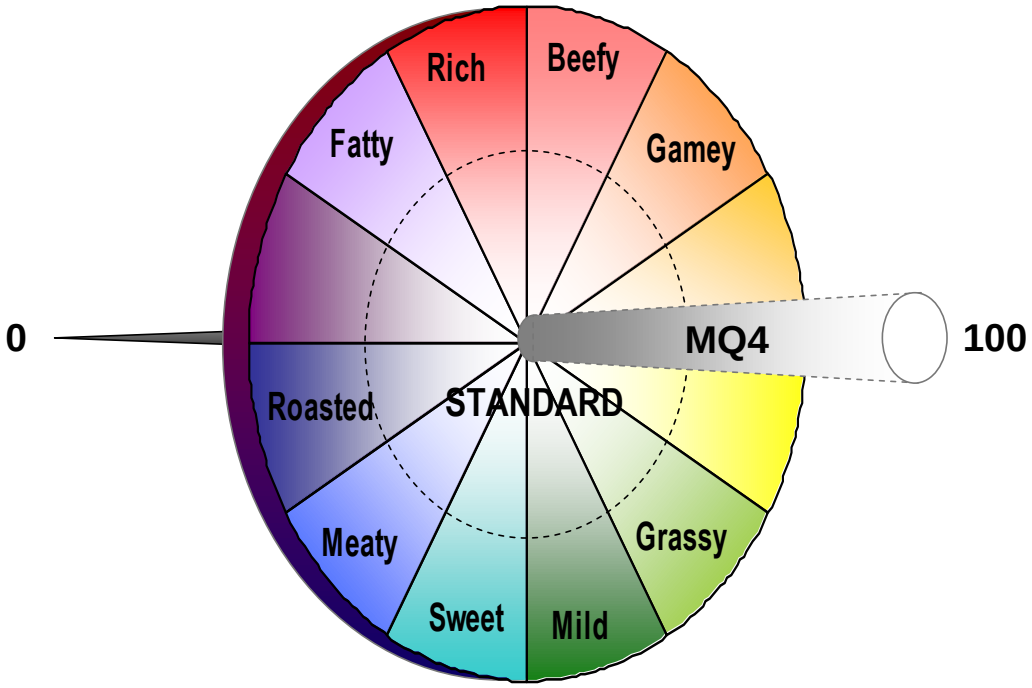
This method would fine tune the MSA model to better predict flavour as well as tenderness and to adjust the score for the majority of the population in a given country, where those preferences are known. It would not accommodate well the different tastes of individuals within a country.

4.3. Qualify MSA score with flavour descriptors for beef with special flavour qualities

An alternative approach is to capitalise on the diversity of flavours available from certain types of beef and to predict and highlight these so they can be marketed appropriately to consumers. Thus, instead of altering the MSA score as such, beef with

specific characteristic flavours would be classified according to its MSA grade and its specific flavour note. Figure 7 illustrates how this could work. Beef without an unusual flavour would be graded as MSA 4*, as previously (in the centre of the wheel in Figure 7) while other beef may be MSA 4* "rich and beefy" or MSA 4* "mild and sweet".

Figure 7. Example of MSA flavour grading system (descriptors are examples only)



A model in which the flavour score can be qualified by flavour descriptors would require an ability to predict these descriptors for that beef where a specific flavour characteristic is sufficiently pronounced to be worthy of identification. This would be achieved by two methods:

a) **Preference mapping**. MSA data from real consumers would be related to quantitative descriptive analysis (profiling) for the same meat using trained panellists. This enables the likes and dislikes of consumers to be expressed in terms of the descriptive language used by trained assessors. Some of these descriptors would provide valuable additional marketing information for subgroups of the population, while others would indicate the presence of some flavour which would be disliked by most people and thus should not be marketed as MSA beef.

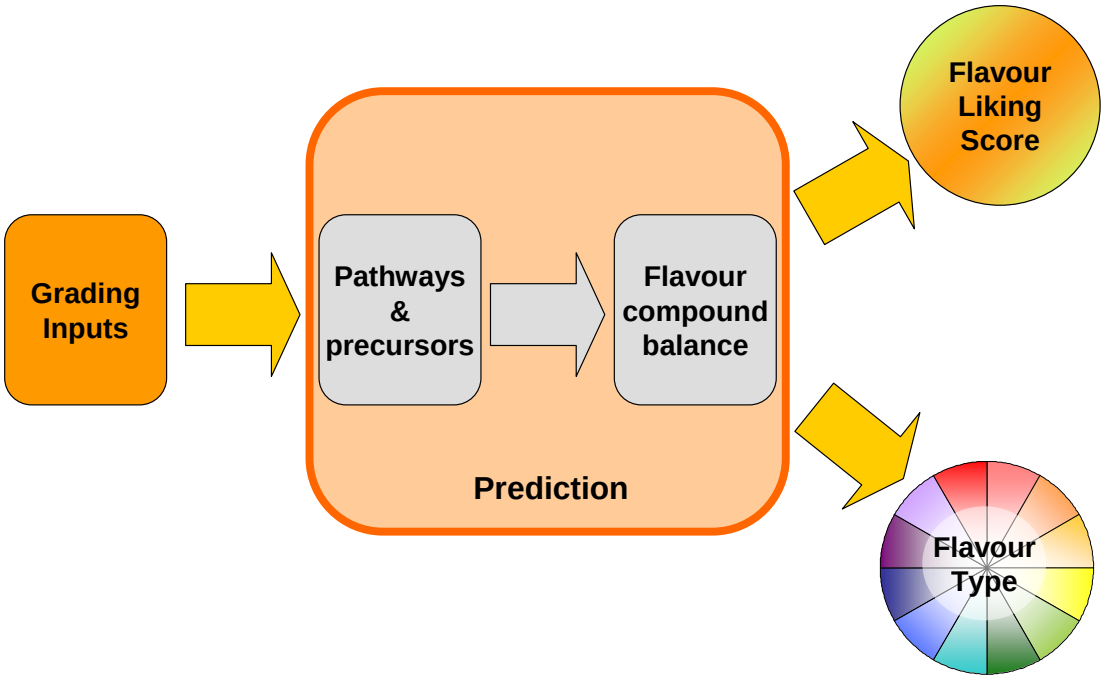
b) **Analysis of flavour compounds**. The relationship of the flavour compounds detected with the consumer data and profiling data would enable the causes of flavour differences to be better understood. This could enable the prediction of

the flavour impact of factors not yet tested. It would also enable practical advice to be provided on the impact of certain factors, such as the long-ageing of different muscles and different pack-types, and the reasons why this can be detrimental.

This approach would ensure that beef with marked differences in flavour, but similar MSA score, are not marketed as the same product thus causing confusion to consumers. Instead product predicted to have a specific characteristic flavour would be classified according to its MSA grade and also its specific flavour note.

The approaches outlined in Sections 4.2 and 4.3 could be used separately or together. Figure 8 illustrates how an understanding of the effect of animal and processing parameters on flavour volatiles could assist the prediction of both an overall flavour score and/or the flavour type.

Figure 8. Prediction of flavour score and/or flavour type from grading measurements using knowledge of flavour chemistry



5. Recommendations

a) A literature review should be commissioned on the likes and dislikes of different consumers from different countries or backgrounds for different flavour characteristics in beef.

b) Research should be commissioned to elucidate further the differences in volatile compounds between different muscles, ageing treatments, hanging

methods and other factors (see Section 3.3) and how these relate to consumer liking.

c) Research should be commissioned on the relationship of flavour descriptors and flavour liking (as perceived by consumers), according to clear protocols to ensure that appropriate methods are used. The consumer studies should be conducted in several countries to identify the different characteristics of importance to different cultures.

6. Conclusion

The method developed at TTU and AFBI for the analysis of volatile components of grilled beef works and delivers results that are sufficiently reproducible to highlight differences between treatments.

An automatic method for the analysis of the copious quantities of data obtained has been developed at AFBI and made available to TTU.
These methods demonstrate considerable capability to elucidate the differences ibn the main flavour-forming reactions between different muscles, ageing treatments, diets and other factors. This knowledge will help to understand the different likes and dislikes of consumers in different countries.

A further development of MSA is proposed which will enable the MSA predicted score to be either modified for the target consumers based on their perceptions of flavour, or to be qualified by descriptors that will enable consumers or purchasers to make their own decisions.

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Appendix I.

Protocol for the analysis and quantification of flavour volatiles

Protocol for the analysis and quantification of flavour volatiles

from beef prepared according to MSA cooking protocols

Linda Farmer and Terence Hagan, July 2010
Agri-Food and Biosciences Institute, Belfast, UK

conducted in collaboration with
Texas Technical University, Lubbock, USA

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1 Recommended method for objective flavour testing

The following is the method for the collection and analysis of volatile compounds developed following the studies at Lubbock and Belfast in December 2009 and 2010, as presented in the report on the project conducted at that time, with additions from work conducted since then.

1.1 Preconditioning of glassware and fibres

Vials (Supelco 15ml clear vial with screw top hole cap with PTFE silicone septa) are preheated at 95°C (preferably 120°C) in a clean laboratory oven for at least 60 minutes, to remove volatile compounds. The vials may be held in an oven at 60°C until required. Lids and septa are heated only to 60°C. SPME fibres are conditioned in a GC injector at 250°C for a minimum of 30 minutes and held in sealed glass tubes (preheated as for the vials above) for up to 12 hours. Before collecting volatiles the fibres were conditioned for a further 5 minutes.

1.2 Collection of volatile compounds on to SPME

Steaks are cooked to a "medium" cooking endpoint according to the MLA protocol for consumer panels. During the rest period, after the steaks are removed from the grill, three cores are removed from the centre of a steak using an Instron meat corer (half inch, 12.5mm). These cores are immediately cut in half to give six pieces, which are transferred to a preconditioned vial and the lid with septum screwed on.

The sealed vial is immediately placed in a water bath preheated to 65°C[1], fastened in a clamp, and left for 5 minutes to equilibrate. The SPME needle is then inserted through the cap and the fibre exposed for 10 minutes. After this time, the fibre is retracted into the needle, a septum placed over the end of the needle and the collected sample was stored in the glass tube at 4°C until analysis by GC-MS within 24 hours of collection time. The SPME fibre may be released from the holder at this stage to free holders for further use.

The method could be adapted for roast beef by taking one core of roast beef at the beginning of the panel and placing a specified number of pieces in a preconditioned vial as above. In contrast with the grilled samples, there is no outer surface present on the roast beef as presented to the consumers. This outer surface would be particularly rich in volatiles due to the higher temperatures attained. Tests would be needed to determine whether this roast sample would be sufficient to allow measurement of the volatiles or whether a larger vial would be required.

1.3 Use of internal and external standards

Two methods for monitoring the performance of the method and the instrumentation have been evaluated. An external standard at a known concentration, injected with a series of n-alkanes by manual liquid injection each day, is a good way of monitoring the performance of the instrument, both in terms of response and also chromatographic separation. This method assumed that the injection of a standard volume is accurate. Provided the user has been properly trained in the use of a manual GC injection syringe, this should be possible.

An internal standard added to each vial may be used to monitor the effectiveness of each SPME collection. This method assumes that the efficiency of collection of an internal standard from a liquid sample on the wall of the vial is related to the efficiency of collection of volatiles from the meat. This is as yet unproven.

Both methods are presented here for reference. It is recommended that an external standard and alkanes are used daily. Evidence suggests that the internal standard method lacks reproducibility, but may be used in addition to the external standard if desired.

[1] TTU are currently investigating whether a solid heater block can be used in place of the water bath. We have advised them that this could be helpful but that the consistency of temperature across the block and within the vial needs to be checked.

1.3.1 External standard and n-alkanes

A solution of n-alkanes (C6 – C22; 20 ng/μl) and bromobenzene (10 ng/μl) was prepared in 10ml pure pentane. This solution was held in the refrigerator (4°C) but raised to room temperature and agitated before use. Before a series of analyses and each morning, after the instrument has been calibrated, 1 μl of this solution is injected under the same conditions as used for SPME, except that the front end of the column is not cooled in liquid nitrogen or solid CO_2 before analysis and the split is set at 10:1.

The injection is made as follows:

a. A 5 μl gas chromatograph syringe is rinsed three times with pure pentane.
b. The syringe plunger is withdrawn by 0.5 μl to leave an air gap between the pentane and the sample.
c. The tip of the syringe needle is placed in the stock solution and the plunger withdrawn a further 1 μl.
d. The sample is withdrawn into the barrel, the needle wiped gently on clean tissue, and the volume of sample checked. If it is not 1 μl (± 0.05 μl), the sample is discarded and the process recommenced.
e. The needle is inserted through the injection port septum, the needle allowed to heat up for 5 seconds and the plunger depressed rapidly but steadily. The syringe is left in position for 5 seconds and withdrawn.

Examination of the chromatogram should show that the alkanes elute at regular intervals throughout the temperature programmed period of the run and that the peak areas for all the peaks are similar. There is usually some falling off of peak height as the later peaks broaden in shape. The bromobenzene should elute at its expected linear retention index (LRI 928). The peak shapes should be “normal” and should not show excessive tailing, though a little tailing is normal on a non-polar column. The operator should be content with the quality and reproducibility of this analysis before any true collections are commenced.

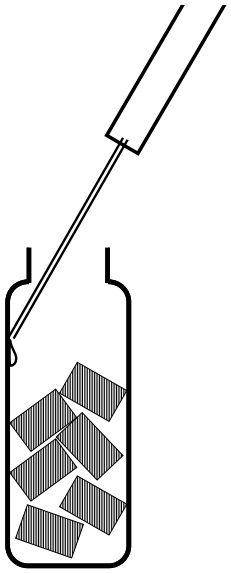
The split is set at 10:1 while the setting for SPME analysis is splitless. To obtain an accurate estimate of quantities the impact of this should be determined by injecting six external standard samples with and without the split. This will enable reproducibility to be determined as well as allowing a ratio to be calculated for split/splitless.

During a series of analyses the alkanes and external standard should be examined each day to ensure there is no deterioration in chromatography or response of the GC-MS. A quality control chart may be used to monitor the external standard.

1.3.2 Internal standard

An internal standard may be added to each vial as follows. The internal standard (4-octanol) is diluted 1/1000 by taking 2 μl 4-octanol, adding 48 μl ethanol and 1950 μl deionised water (818 μg/ml) and then by a further 1/1000 by diluting this solution 2 μl with 1998 μl (818ng/ml or 0.8 ng/μl). After the meat was added to the vial, 10 μl of this solution (8ng 4-octanol) was run down the side of the vial above the meat sample and its presence checked visually (Figure 1).

Figure 1. Addition of internal standard to vial containing meat sample.



1.4 Injection

The front of the GC column should be cooled either in liquid nitrogen or solid CO_2 for 5 minutes. The SPME needle is then inserted through the injector septum and the fibre exposed in an Agilent glass injector liner held at 250°C for 3 minutes with purge off (splitless) before the purge is activated. The SPME fibre held in the injector for 5 minutes to ensure volatile removal. The sample was collected on to the cooled portion of the column. After 5 minutes, the cooling agent was removed and the chromatographic program was started manually.

1.5 Chromatography

The sample is analysed on a 25m DB-5 equivalent capillary column using the following programme: 35°C for 5 minutes, ramped at 8°C/min until 220°C then 20°C/min to 290°C, giving a run time of 37 minutes. The solvent delay was 2.00 minutes. The GC-MS settings were: mass range 33-500, eV = fixed (2000eV used at Belfast, 2600 eV used at TTU). Best value yet to be ascertained).[2] The eluent from the column was monitored using simultaneous full scan and "single ion monitoring" (SIM) modes.[3] These methods were used to search for a list of compounds as discussed in Section 6. This list will evolve based on current and future work. GC-MS and data analysis are conducted using Agilent MSD Chemstation D.03.00.611 software.

[2] A setting of 3000eV was found to give maximum sensitivity but also too much noise. It is likely that a lower setting would be appropriate but it is recommended that this is fixed rather than variable. This best setting is currently under investigation.

[3] This list must be entered according to the retention times obtained on a given column, to ensure that each compound is within range. This can be done by reference to standard injections of n-alkanes C6 – C20.

1.6 Identification of compounds

All identifications should be by comparison of the mass spectra and linear retention indices with mass spectra of authentic compounds and should not depend on the library of mass spectra supplied with the instrument. For the work described herein, comparison was with data recorded previously at Belfast or, in some cases, from the literature.

1.7 Data analysis and transportability of data

The ion peak areas should be determined for the target ion for each compound. In general, SIM will be used for this. To calibrate these against the authentic compounds, it is proposed that an appropriate solution of authentic compounds be injected along with a standard (e.g. 4-octanol, bromobenzene). The standard should also be injected each morning with n-alkanes (C7 – C20) to check the condition of the column, calculate retention indices and quantification.[4] This will ensure that instruments from different laboratories which may have inherently different sensitivities will give comparable data.

It has been demonstrated that data runs from different Agilent instruments may be transferred from one to another and further analysis conducted of these at a remote location. This can be done by e-mail though the size of the files makes a physical data storage method more satisfactory. Transferring raw data between instruments from different manufacturers is unlikely to be possible.

1.8 Calibration standards

A solution of authentic compounds at known concentration with the internal standard 4-octanol should be prepared such that 1μl injected contains comparable quantities to those analysed by SPME. These should be injected and analysed at the beginning, middle and end of any series of analyses. Often an injection quantity of 1ng is a good starting point.

For more accurate calibration, a series of solutions with a range of concentrations of authentic compounds designed to bracket all the samples analysed, may be used.

The solvent used for the preparation of authentic compounds is usually a volatile solvent such as hexane. However, to aid sharing and transport of authentic standards, the use of an involatile solvent has been investigated. Tests with pentadecane indicate that this method could work, provided the MS source is disabled for the appropriate period of the GC-MS run using timed events in MS Instrument Parameters. Care needs to be taken as follows: (a) polar volatile compounds may show low solubility in pentadecane and this could make the concentrations inaccurate, (b) some volatile compounds may elute during the period the source is shut down and these would need to be injected separately, (c) a suitably pure source of pentadecane is needed.

2 Data analysis

Two data sets are available from each run: the full scan data and the SIM data. Both are held within the data folder for that run. The full scan data will be more complete but less sensitive, while the SIM data should be more sensitive but is very dependent on the correct

[4] It is proposed that an appropriate solution of authentic compounds be injected along with a standard compound. This should be the same compound as the external standard injected each morning with the n-alkanes. While the estimated quantitation of each compound obtained will not be as accurate as that determined with a calibration curve for each compound, it should be consistent and provide good relative estimates.

time settings being used and ensuring limited retention time variability between runs. The following method works for both sets of data. These methods have been tested on Chemstation version E.02.00.493.

The following procedures in Chemstation relate to the tabs and drop down menus at the top of the screen. Instructions to be clicked are in bold.

2.1 Set up of analysis method

A data run (full scan) is chosen for its good chromatography, representative retention times and good mass spectra. The compounds for quantitation have been chosen in turn and added to the quantitation method. This quantitation method has been prepared (**AFBIQUANT.M**) and may be transferred to other similar Agilent GC-MSD instruments and edited to correspond to the new elution times.

The following outlines how the file was created.

- Select data file
- Load default method as follows
- **Method**
- **Load**
- default.mth @msdchem/1/methods
- **Calibrate**
- **Set up quantitation**
- In Quantitation Database Globals
- Enter appropriate calibration name and integration file.
- Integrate
- **Calibrate**
- **Set up quantitation**
    - Select first peak of interest. Double R click
    - LR click on target ion
- LR click on qualifier ion
- Enter compound name in Quant Set Up Box
- Save
- Select next peak and repeat until final peak
- Save
- Select ions and compounds from the selected run (retention times and spectra: target ion and three qualifiers)

One method can cover a maximum of approximately 85 compounds. However, multiple methods and integration files can be created to cover as many compounds as required.

2.2 Editing analysis method and integration files

To edit details

- **Calibrate**
- **Edit compounds**
- Can edit the compound names, target and qualifier ions, retention times and windows

To add in new compounds

- **Calibrate**
- **Set up quantitation**
- Insert above
  - Quant set up
  - Select peak
  - Double R click
  - LR click on target ion
  - LR click on qualifier ion
  - Can edit the relative intensity and the permitted limits for this.
- Tab **Advanced**
- Set up individual integration parameter files (xxxx. E files)
  - For each compound either a default integration file can be used or an integration file can be set up for each compound. The method identifies and uses these to integrate these compounds. The default integration parameters used for many compounds is shown in Table 1.

Table 1. Default integration parameters

Parameter	Value
Initial Area Reject	1
Initial Peak Width	0.020
Shoulder detection	OFF
Initial threshold	10.00
Baseline all valleys on	3.00

2.3 Automatic data analysis for a run

- Load method, **AFBIQUANT.M**
- Select the signal (data file) to be integrated, which can be either full scan or SIM: e.g. DATA.MS or DATA.SIM
- **Quantitate**
- **Calculate**
  - This creates a text file, which can be useful for reference.
- **Quantitate**

- **Generate report**
- Style = detailed text only
- Destination – tick screen and file (select a filename, same for every run, xxx.txt)
- **Export results**
- **Quantitation results report to xls**
  - Creates MSRep.xls in every data folder.
- Repeat for each data file.

2.4 Use of AFBI macro to collate data from a number of runs

A macro has been created to collate the results from all the MSRep.xls files. It is called GC_MS.xls. Changing the name of this file causes it to fail.

- Save to your PC then open in Excel (enable macros).
- The macro prompts you to open the 1st results file then opens all higher numbered folders in same main folder e.g. if 1st file in folder *\BELFAST032.D then folders *\32, 33 etc are opened.
- Assumes Excel file names are all MSrep.xls and results are on sheet Qres.

The data is added into an excel spreadsheet of the format shown in Figure 2.

Figure 2. Format of volatile summary file

Code	Data File Name:	Acquired Date:	Method Name:	Sample Name:	1: 3-methylbutanal: RT (min)	1: 3-methylbutanal: area	1: 3-methylbutanal: Q	2: dimethyldisulphide: RT (min)	2: dimethyldisulphide: area	2: dimethyldisulphide: Q	Etc. up to perhaps 20-30 compounds
Leave blank	C:\Documents and Settings\Administrator\Desktop\BELFAST030.D	##########	C:\MSDCHEM\1\METHODS\polyethyl.M	Fibre 6 Syr3 Sample C92U	8.531	47813	67	10.152	15157	93	

It is envisaged that a further column will be added which could specific the general method, e.g. SPME, thermal desorption. This would allow analyses conducted using different methods to be incorporated into the database.

2.5 Quality assurance of data

While the above methods will fill a table with data, it is essential that the following checks are conducted before the data is used.

a. Three selected runs from start, end and middle of sequence should be checked manually for all compounds using same integration parameters

b. Low Q values – the identification should be checked.[5]

[5] Q is a measure of the comparison of the actual ratio of target and qualifier ions against the expected ratio as set in the quantitation file. Thus, a low Q value usually indicates a poor MS fit (correspondence with Agilent).

c. Unusual (outlier) ion areas should be checked, together with top and bottom of main range.

d. Runs with odd I.S. areas should be checked.

e. Statistical analyses will be conducted to compare effect of different fibres and other possible factors.

Where the volatile area spreadsheet is altered, a colour code will be used to show where an amendment has been made and an outline of the criteria used to obtain the replacement value will be noted.

2.6 Conversion of peak areas to quantities using internal / external standard

The conversion of peak areas to quantities collected will involve the use of an internal or external standard to provide the common reference point and coinjection of the same internal standard with authentic volatile compounds of interest at about the same concentration. This will permit "semi-quantitation" using authentic standards.

Possible uses of internal and external standards include:

a. Use internal standard (I.S.) in each run and calculate "quantity in sample" relative to the 8ng I.S. (4-octanal) added to the vial. This will not be accurate as rate of release from meat will be different to that from standard solution, but should be an underestimate. If occasional runs have "odd" I.S. standards take an average of the I.S area from the runs before and after.

b. Use internal standard average of good runs in a day and calculate "quantity in sample" relative to the 8ng I.S. (4-octanal) added to the vial. This will not be accurate as rate of release from meat will be different to that from standard solution, but should be an underestimate. This would be appropriate if the variation in I.S, area does not seem to reflect the variations between collections. This only adjusts for the calibration of the GC-MS that day, not any variability between runs.

c. Use external standard (E.S.) – Calculate "quantity in sample" relative to the quantity E.S. (e.g. bromobenzene, 10ng) injected with the alkanes. The resulting quantity should be a good estimate of the quantity collected from the sample and injected. An adjustment will be eneded to account for the difference between the splitless SPME injection and the split liquid injection of the external standard. This only adjusts for the performance of the GC-MS from day to day, not any variability between individual runs.

"Semi-quantitation" may then be achieved using authentic standards. A solution of all compounds of interest should be prepared at a known concentration.[6]

Calculate relative target ion (TI) response factor (R) for each compound:

For compound x versus standard s, if same quantity of each is injected:

$$\frac{\text{TI area x}}{\text{TI area s}} = R$$

[6] A full calibration curve could be used to give full quantitation but this would add little further information.

Calculate R from standard solution of all compounds with I.S. Quantities injected should be similar to those in real runs (i.e. peak sizes should be similar).

Then in a normal run, the quantity of compound x , unknown quantity (Q x) can be calculated from the TI area and that of the standard s, known quantity (Q s)

$$\frac{\text{TI area x}}{\text{TI area s}} = \frac{\text{R. Q x}}{\text{Q s}}$$

$$\text{Quantity injected, } Q\,x = \frac{\text{TI area x}}{\text{TI area s}} = \frac{\text{Q s}}{\text{R}}$$

For compounds for which standards are gaseous, e.g. methanethiol, specialised methods will be needed to inject a known concentration, standard solutions will need to be purchased or R estimated from a related compound?

Having determined R for each of the authentic compounds, these would be tabulated (as separate worksheet in the volatile summary file). The values of R would be looked up to calculate semi-quantitative values from R and area. These would be placed in extra columns in the volatile summary file.

3 The FLAVOURBLUE database

The outcome of the analyses of flavour compounds in beef by this method would be a FLABOURBLUE database. This will contain the quantities of each compound collected by SPME from three cores of beef by the method specified, as determined by the methods detailed above. This information will be available for each sample analysed, with reference to the EQS reference number also used for the sensory data.

It is intended that data acquired by different laboratories could be added to the database to build up a body of flavour data that will eventually provide more information than any one experiment alone.

Of crucial importance to the quality of this database will be the quality assurance of the data and the management of the database. It will be essential that some checks are conducted on each dataset before it is added to FLAVOURBLUE. Quality assurance will be a vital part of the data acquisition process and will be time consuming. This is best conducted by the laboratory acquiring the data but, where this is not possible or is not completed, this could be conducted by those running the database if the full data is available.

It is proposed that AFBI, with its experience in flavour chemistry, would be best placed to manage the database and perform any additional quality assurance needed.

Figure 2 summarises the process required for the collection of data, its collation, quality assurance and addition to FLAVOURBLUE.

Figure 2. Summary of process for the collection of flavour volatile data and its addition to FLAVOURBLUE, with an indication of which steps are most time consuming