THE MOLECULAR BASIS OF SAVOURY

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Not everything that can be counted counts and not everything that counts can be counted.

-William Bruce Cameron

Abstract

It is widely portrayed that health, sustainability and environmental concerns are driving consumers to choose a plant-based diet. However, they do form supplementary contributors to food choices, amongst other factors. Flavour ultimately decides whether a food will be acceptable at the point of consumption. Other than for the small percentage of neophobes, most of the world's growing middle class make themselves acquainted with the latest diet fads, as observed through the decades. If we are to stay on this path of promoting and advocating plant-based diets as healthy, sustainable, and environmentally friendly, it only seems pertinent that we deliver flavoursome products that are appreciated by most so that they remain trendy. After over a hundred years of being discovered, umami now forms the buzzword of the decade. However, it was observed that umami was only partially responsible for the appreciation of beef in high-temperature short-time (HTST) cooked foods such as grilled and fried burgers. Rather, the rapidly formed aroma volatiles from precursors are involved in simultaneous oxidation degradation and interaction with the Maillard reaction products (SODIM) process. This includes contributions from a myriad of seasonings and herbs added to meat during cooking, increasing its appreciability. Giving ‘mirepoix’ (pronounced ‘meer pu ah’) its fair dues, we ought to characterize the formation of aroma compounds in beef during HTST processing compared to plant-based products. Although comparisons between beef burgers and plant-based meats have been sought, the studies only compared commercial products. There is compelling evidence that aroma is a critical yet pervasive, if not an all-encompassing, attribute to decision-making in food choice.

Thus, this study was conceptualized to observe the formation and development of volatiles in plant-based ingredients and compare them to the aroma profile of beef mince. The key question to be addressed was, how can the aroma of a popular food be captured and analysed objectively? If aromas were based on volatiles. Could they be identified and measured on a molecular basis using chemometrics? More specifically, the study aimed to assess the use of head space Solid-phase Microextraction Gas Chromatography Mass Spectrometry (HS SPME GCMS) as a tool to study aroma profiles. Further, it proposed studying the different constituents of beef mince, namely the fat, sarcoplasm, and myofibrillar fractions, and their ability to promote aroma formation. Finally, it investigated the formation of volatiles in heat-treated plant-based lipids and proteins compared to beef mince in the formation of aromas.

The topic's significance is highlighted by the evidence noted in the literature of the challenges observed in the analysis of aroma volatiles. A comprehensive scoping review is presented on the current status of aroma analysis and its interpretation of volatile compounds found in plant-based meat and their likeness to animal meat. The key findings of the review suggest that the lack of standard definitions of common terms exacerbates differences in opinion. However, using advanced untargeted chemometric analysis may be able to observe, decipher and guide researchers and flavour chemists to be objective in creating appreciable plant-based foods.

Consequently, the importance of the methods of analysis using chemometrics highlighted the need for optimising analytical conditions. The application of HS-SPME-GCMS was chosen as a green technology with the ability to analyse wide-ranging volatiles from complex matrices. Due to the varied adsorption capability of the fibres, initially, both Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) and Carboxen/ Polydimethylsiloxane (CAR/PDMS) fibres were selected for the study. However, DVB/CAR/PDMS was incorporated in the study due to its ability to adsorb a wider range of volatiles from the headspace. The ability of the fibres to adsorb analytes was observed to be dynamic, depending on a range of factors, with time and temperature being the most effective. control A single time and temperature parameter for equilibration and concentration was observed to optimally extract analytes from the headspace. Volatiles observed from the method of cooking (pan-grilled vs heat treatment in vial) matched closely compared to the formats (Fresh whole vs lyophilised). Although not quantified, peak intensities for aldehydes in vail-cooked lyophilized samples suggest a lower rate of oxidative degradation. In relation to the formation of warmed-over flavours (WoF) and other degradative products during storage, the effect of chilling the sample was more pronounced compared to not chilling or the addition of water prior to analysis. The use of a single internal standard could not be justified due to the number of volatiles observed whilst conducting untargeted analysis. The adsorbability of the internal standard seemed to depend on the headspace environment and concentration of analytes, voiding its application.

The complexity of beef warranted its fractionation, as observed from the optimisation study. This enabled the observation of volatile formation in the different fractions. SDS PAGE highlighted two important aspects of fractionation: 1) the presence of many soluble proteins in addition to myoglobin and haemoglobin in the aqueous extract; and, 2) the efficacy of removing soluble proteins observed in the deblooded fraction. The separation enabled the observation of volatile formation from the different fractions. It resulted in five distinct essays with a

combination of either the sarcoplasmic, myofibrillar or lipid fractions. All five assays, including the whole meat samples, produced significantly different peaks, as observed from the chromatograms. The largest number of aldehydes were observed in the lipid fraction, followed by the deblooded and defatted myofibrillar fraction. Fresh and lyophilized whole beef mince also presented differences in volatile profiles. Fractionation demonstrated the limitations of analysing volatiles from whole foods.

The volatile profiles obtained from heat-treated coconut and canola oils and their admixtures were also significantly different to the volatiles observed from beef fat. Although beef fat is known to possess a high degree of saturated fats, the lipids extracted from beef mince produced volatiles similar to those observed from canola oil. Coconut oil produced the lowest number of volatiles compared to canola oil and beef fat. Volatiles from plant proteins, namely pea and soy, were also compared to those from the myofibrillar fraction of beef mince. Whilst volatiles from heat-treated pea and soy compared favourably with each other, they differed from beef. The largest number of volatiles was observed from pea protein. When proteins and lipids were combined, they followed a similar pattern of volatile formation.

The project established a method for objectively analysing aroma formation using chemometrics. It successfully observed the formation of volatile compounds from plant-based ingredients and compared them to volatiles from beef mince. However, the current research has limitations in identifying every molecule and understanding the dynamics that contribute to noticeable flavour differences. Further research could assist our understanding of whether the addition of flavour enhancers, such as yeast extracts and mushrooms, would produce volatiles similar to those found in beef and thereby invoke a similar perception.

Declaration

"I, Derrick D'Souza, declare that the PhD thesis entitled 'The Molecular basis of Savoury' is no more than 80,000 words in length, including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

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Thank you.

List of Abbreviations

3MCPD	3-monochloropropane-1,2-diol
ADH	Alcohol dehydrogenase
AED	Atomic emission detection
AEDA	Aroma extract dilution analysis
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
ARP	Amadori rearrangement products
ASE	Accelerated solvent extraction
AGE	Advanced Glycation End-products
CAMOLA	Carbon module labelling
CAR-	Carboxen
CaSR	Calcium-sensing receptor
-COOH	carboxylic acid functional group
DF	Degree of freedom
DVB-	Divinyl-benzene
EI	Electron ionization
ESI	Electrospray ionization
FAO	Food agricultural organization
FDBM	Freeze dried beef mince
FDBM-DB	Freeze dried beef mince- De blooded
FDBM-DF	Freeze dried beef mince- De fatted
FDBM-AE	Freeze dried beef mince- Aqueous extract
FDBM-DBDF	Freeze dried beef mince- De-blooded and defatted
FID	Flame ion detector (flame ionization detector
g	Grams

GCMS	Gas chromatography mass spectrometry
GMP	disodium guanylate (guanosine monophosphate)
GC-O	Gas chromatography olfactometry
HPL	hydroperoxide lyase
HTST	High temperature short time
HVP	Hydrolysed vegetable proteins
IFIC	International Food Information Council
IMF	Intramuscular fat
IMP	Disodium inosinate (Inosine monophosphate)
IMS	Ion Mobility Spectrometry
IS	Internal standards
LN_2	Liquid Nitrogen
LOP	Lipid oxidation products
LOXs	Lipoxygenases
LRI	Linear Retention Index
MAG	Mono ammonium glutamate
Mb	Myoglobin
MDGC	Multidimensional Gas Chromatography
mg	Milligrams
MRP	Maillard reaction Products
MSG	Monosodium glutamate
MLA	Meat & Livestock Australia
MW	Molecular weight
m/z	Mass to charge ratio
-N	Nitrogen substituted compounds
$-NH_2$	Amino group radical

NIST	National Institute of Standards and Technology
-O	Oxygen substituted compounds
-OH	Hydroxyl group
PA	Polyacrylate
PBMA	Plant based meat analogues
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
PG-Fr	Pan grilled -Fresh
PG-Ly	Pan grilled lyophilized
ppm	Parts per million
PTR-ToF-MS	Proton Transfer Reaction-Time of Flight-Mass Spectrometry
PUFA	Polyunsaturated fatty acids
RI	Retention Indices/Index
RT	Retention time
-S	Sulphur substituted compounds
SAFE	Solvent assisted flavour extraction
SCE	Super critical extraction
SCP	Single-cell protein
SD	Steam distillation
SDE	Simultaneous distillation -extraction
SDS- PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SEM	Standard error of mean
-SH	Sulfhydryl group (thiol)
SHS	Static head space
SIFT	Selected-Ion Flow-Tube

Sl.	slight
SODIM	simultaneous oxidation degradation and interaction with Maillard reaction products
SOP	Standard operating procedures
SPE	Solid phase extraction
SPME	Solid phase micro extraction
TIC	Total Ion Chromatogram
TVP	Texturized vegetable proteins
Vial- Fr	Vial -fresh
Vial- Ly	Vial lyophilized
WoF	Warmed over flavour
YEs	Yeast Extracts
µg	micrograms
µL	microliter
µm	micrometer

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Chapter 1

Introduction to the thesis

Background

Factors affecting food choices are varied (Mela, 1999; Köster, 2009). *Nouvelle* foods, the stability of food patterns, existing food choice beliefs and behaviour (except for the small percentage of the neophobes) are dependent on perspectives, initial experience and exposure (Martins and Pliner, 2005; Spence et al., 2016).

It is a popular view that health, sustainability and environmental concerns are driving consumers to choose plant-based diets. It is also said that flavour is the most important characteristic and ultimately decides the acceptability of a food. However, a current survey failed to show this trend (IFIC, 2024).

The presence of umami flavour- a taste, forms the buzzword of the decade to express Appreciability. Umamification is therefore thought to increase appreciation (Mourtisen, 2020). In this context, it could be implied that plant-based meat analogues are umamified by adding umami ingredients making them flavourful and hence are made to represent and taste like meat. The Ajinomoto group refers to umami (one of the five basic tastes) as a 'delicious savoury taste' and describes it as 'meaty, savoury deliciousness that deepens flavour'. Umami (Japanese) and savoury (Western) terms are also used interchangeably to signify wholesome flavour. The flavour is a combination of taste, aroma and trigeminal sensation (Gibson & Newsham, 2018). Savoury, on the other hand, is defined as salty or spicy but not sweet. Does savoury thus represent taste, smell, olfaction, trigeminal sensation or a combination has also been discussed extensively by Land (2008).

The sheer number of terms presented here can be confusing to decipher due to the lack of standard definitions (Spence et al., 2014). All too often, the terms aroma and flavour are also interchangeably used. When volatile/ aroma analysis is conducted, it is termed flavour analysis due to the involvement of olfaction with the use of sensory descriptors. Sensory descriptors of aroma volatiles have been observed to result from learnt behaviour and perceptions of past experiences. Prior negative experiences and beliefs had a more significant effect in choosing novel foods (Martins & Pliner, 2005). Although this effect is observed mainly in animal foods, could this mean that the attribution of legume proteins as being grassy, beany and soapy affects its credibility? It brings us to the conundrum of what comes first: perspective, perception, or impulse. Putting it into context, 'Do perspectives override impulses and vice versa? Similarly, can an impulse modify perceptual ability or the other way around'?

This is evident from sensory descriptors where sweaty, soapy, rubber and bleach are essentially non-food attributes given to aromas in beef by sensory panels. If a person were to never have tasted a food before, on what basis would the judgement be based? And on the other hand, once an attribution is made, does it affect decision-making? This was partly explained by Yeomans et al. (2008), where mislabelling led to increased dislike compared to labels with insufficient information. Also, perceptive values of hedonic identification of extraneous non-attributes increased significantly. Perception and expectation were strong indicators of negative responses, especially when expectation does not meet sensory evaluation.

In the case of plant-based meat, the justification for using the word meat has been debated widely (Gleckel, 2020; Milburn, 2023). Martins & Pliner (2005) discussed the effect of naming ingredients and foods. Marshall et al. (2022) found that the description of meat substitutes with meat-related labels increased the willingness to consume. Similarly, yeast extracts have been considered to possess meaty and brothy nuances. Likewise, mushrooms have also been known to possess meaty attributes. Considering the above, to what extent then does adding these ingredients evoke similar perceptions and perspectives across all modalities- chemical interactions amongst the compounds, physiological-olfaction and psychological bring about an accepted response? In this case, being the acceptance of plant-based meat.

Plant-based meat analogues (PBMAs) are marketed as meat alternatives and aim to please the palate with meat-like flavour and texture. Whilst meat flavour has been extensively studied in relation to its characteristic taste, the current trend of creating plant-based meat alternatives with meaty flavour has met with challenges (Thong et al., 2024). In this regard, we hypothesised that umami was only partially responsible for the appreciation of meat/beef. Rather, savoury aromas through olfaction contributed greatly to appreciating high-temperature short-time (HTST) cooked foods. This is evident because grilled beef was more appreciated than boiled beef. However, a myriad of factors alters Appreciability. Similarities and comparisons of volatiles in meat and its plant-based counterpart have been conducted (Kacmarska et al., 2021; He et al. 2021). However, the studies only compared commercially available PBMAs. The studies did not implicitly investigate the contributing factors leading to the formation of savoury compounds.

Hence, the overarching research questions were, ‘Does the addition of high umami value ingredients such as yeast extract, mushrooms and other flavour-enhancing ingredients increase savoury aroma compounds or mask off flavours, thus presenting an aroma profile akin to

meat?' In that sense, 'Does the mere presence of savoury aroma compounds relate to increased appreciability and, thus, the uptake of plant-based meats?'

This project was conceptualized to bridge the gap in existing knowledge of how our preconceived perceptions of beefy aroma compare to aromas from plant-based meats. Due to the exploratory scope, the design used for this study was mixed methodology but largely qualitative, with some semiquantitative measures using chemometrics. Several studies were conducted using similar methodology on beef and plant-based meats. Few side-by-side studies of beef burgers and plant-based meat burgers have also been undertaken. Thus, a correlational study using comprehensive untargeted chemometric analysis was ideal for establishing a framework for further research.

The project aimed to ascertain similarities and differences in assigning meat-like or beef-like labels to plant-based proteinaceous foods. It met the aim by attaining the following objectives:

1. Identifying pathways and development of volatile aromatic compounds in foods processed using High-Temperature Short time (HTST) format.
2. Determining the efficacy of the use of solid phase micro extraction (SPME) for volatile analysis in complex media
3. Ascertaining the contribution of fatty acids to the development of savoury aromas during thermal treatment.

Collectively, this research adds to the existing body of literature on how savoury aroma compounds are key to increasing Appreciability in foods. It also aims to demonstrate the link between umami taste compounds and how the formation of savoury aroma compounds may act synergistically in enhancing olfactory perception. The research findings highlight the implication of the need for further research into how perceptions play an important role in food choice. Regardless of the protein source, learnings from this study will help further research in plant-based meat aromas. It could also be applied to other meat alternative studies such as cellular, cultured, myco and insect proteins.

Thesis outline

The thesis is presented under the following six chapters

Chapter 1: Introduction to the Thesis

This chapter uses existing knowledge from literature and industry to provide background to research significance and the need for current and future research in the area. The aims and objectives of the research are discussed, and a thesis outline is presented.

Chapter 2: Perspectives on the formation of beef like aroma in plant-based meat analogues: A review

The chapter starts with a general overview of the broader literature surrounding the sense of smell and its association with other senses. The review discusses the importance of perspectives, likeness, appreciability and the general perception of meat flavour. It reviews existing consensus on definitions of terminology used in sensory science and its effect on assessment and analysis of beef and PBMAs. The chapter also includes a review of the factors affecting the formation of aromas during the High-Temperature Short Time (HTST) processing of beef and the efficacy of creating similar aromas in plant-based meat analogues as assessed by chemometrics. An extensive literature review is conducted on the use and application of headspace solid phase microextraction combined with gas chromatography-mass spectrometry in the analysis of volatiles.

Chapter 3: Optimizing Conditions for the Detection of Aroma Compounds in Beef Mince Using Head Space -Solid Phase Micro Extraction -Gas Chromatography /Mass Spectrometry

Several methods exist for detecting volatiles in food, as chromatography is the method of choice for such analysis; however, due to the complex matrix of whole foods, methods have to be optimized as observed in preliminary trials and presented in this chapter. The chapter presents similar techniques used in other studies, its advantages and limitations, sample preparation for extraction of compounds of interest from the matrix, selection of SPME fibers and the optimization of the equilibration, extraction and desorption conditions. It also includes method validation to enable its use in future studies.

Chapter 4: The origins of meaty aromas

Observation during the optimisation stage led to the need to establish the effectivity of SPME fibers in extracting compounds of interest from a complex media. This chapter forms one of the key chapters that address the limitations of the use of SPME and the role of sample preparation in conducting comprehensive untargeted volatile analysis for mapping purposes. Beef mince which can broadly be categorized into three main groups the sarcolemma, sarcoplasm and fat were fractionated and analysed using optimized conditions.

Chapter 5: Analysis of Volatiles from Plant-based Lipids and Proteins Compared to Beef Mince.

The composition of fatty acids in foods has been studied rigorously in terms of its deleterious implications on health such as from overconsumption of saturated fats and cholesterol acquired from the consumption of animal products, the formation of trans and its consumption from highly processed foods and the beneficial effects from the consumption of polyunsaturated fatty acids (PUFAs) such as Omega3 and Omega 6. However, the stability of PUFAs during HTST processing and its effect on the formation of aroma compounds compared to beef cooked at the same time and temperature have not been thoroughly studied. Results from this study could shed light on its interaction with other aroma compounds and the effect of fatty acids on the development of appreciable aroma.

Chapter 6: Conclusions and future directions

This chapter will present the overall conclusions of the research finding. It will also include opinions and future research directions critical to the success of the development of appreciable plant-based food formats in the future.

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Chapter 2

Literature review

Perspectives on the formation of beef like aroma in plant-based meat analogues: A review

Introduction

The annual global meat consumption has been recorded at 360 million tonnes, presenting a 58% increase in two decades to 2018. This has been mainly attributed to population growth and rising incomes (Whitnall & Pitts, 2019). Beef consumption rose by 1.1% in 2022 and is projected to increase by 0.75% annually to 2025. Australian per capita beef consumption was recorded thrice the global average, with Australian plant-based protein consumption at only 0.6% of fresh meat sales reported by Meat & Livestock Australia (MLA, 2022). Elsewhere, a 3.7% net revenue loss of alternative meat sales was observed by Beyond Meat for 2022 leading to a 4.9% decrease year-on-year to 2024 (Beyond Meat Reports, 2024). An explanation for the low uptake of overall plant-based foods and meat alternatives has been attributed to taste and ingredients used as the most critical (Barkho, 2023). A steady increase in the uptake of plant-based protein alternatives was reported by the Good Food Institute (Pierce et al., 2025) in global retail sales. Although, a nine percent drop in unit sales overall and twelve percent drop in plant-based meat was observed.

It is commonly noted in the literature that the flavour of the product is a crucial factor influencing food acceptance and purchase (Byrne, 2020; Genovese & Caporaso, 2022; Wang et al., 2022). However, a recent survey noted that organoleptic characteristics (21%) were less important compared to health concerns (49%) when choosing plant-based proteins (Passiou et al., 2023). Similarly, a survey by the International Food Information Council (IFIC) (2024) in the USA revealed that convenience, extended shelf life and taste were equally important factors in deciding to purchase processed and packaged foods. Saving money and time and being healthful weighted equally around 20%. The article also reveals that consumers were concerned and confused about how processed and packaged products are labelled and marketed. Almost 50 % believed plant-based meats were not processed foods. A likely change in view could affect the entire plant-based food movement. Does regular consumption of food make it appreciative, or is appreciable food chosen more often? Could creating better tasting and flavourful plant-based products that fit the healthy and nutritional needs, compared to something reminiscent of meat, increase the acceptance rate?

It was observed that regular consumption could increase acceptance but not necessarily because of its taste. Also, products didn't need to be claimed as meat for them to be chosen (Hoek et al., 2011). These observations are in agreement with the IFIC (2024) survey finding that only a third of the respondents valued taste to be an important characteristic. Studies have found the

word meat was less effective in choice decision-making (Hoek et al., 2011) compared to personal beliefs and values (Graham, 2015). Views have also been presented on the conflicting messaging, infringement of personal choice and traditional values by industry-driven advocacy on the reduction in red and processed meat consumption (Milford and Kildal, 2019; Sievert et al., 2022). An important point to consider is that constructs of meat demand analyses have been problematic (Alston and Chalfant, 1991). Maybe a holistic approach to food choice is needed to understand how the different sensory impulses impact decision making in product appreciation (Figure 2.1). Could food choice be entrenched in higher order decision making compared to just the impulses from sensory inputs (Mustapa et al., 2025)

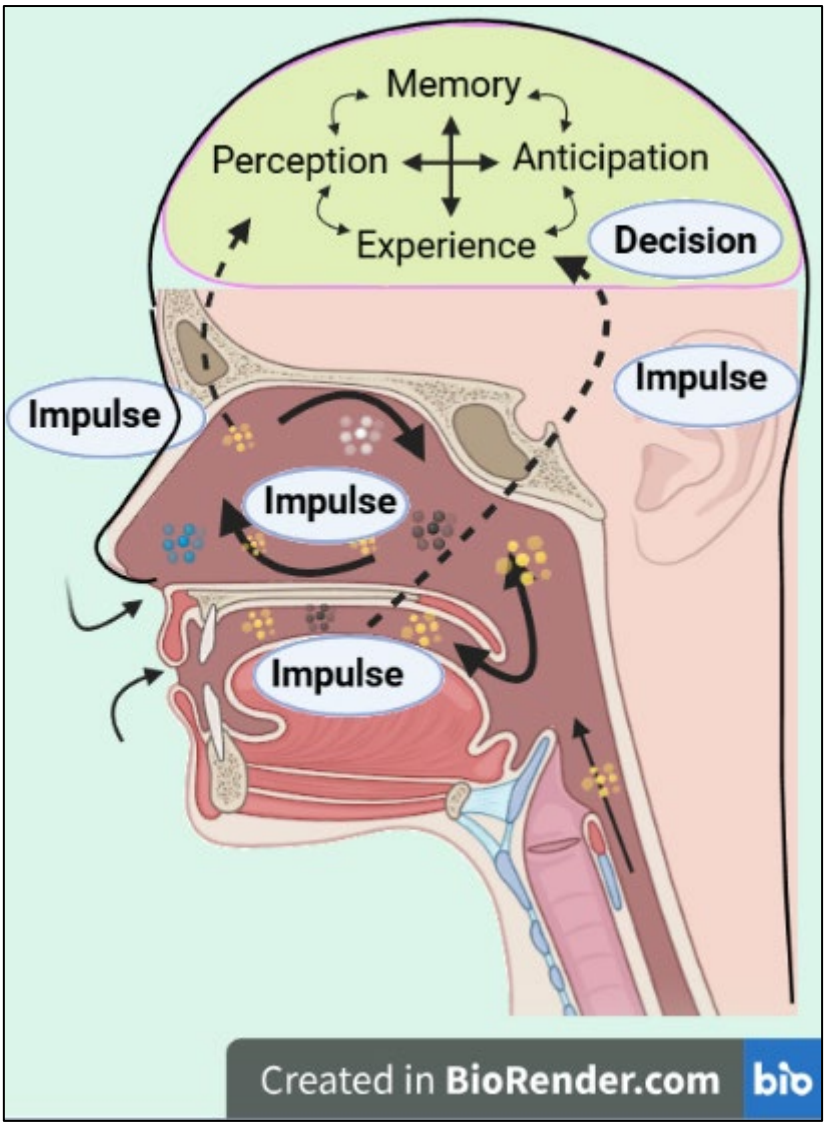

Figure 2.1. Effect of sensory inputs on food identification, perception and perspectives leading to food choice.

Although food products are created depending on the market demand, product development however is hierarchical, but there is no agreement on the approach undertaken (Rudder et al., 2001).

Table 2.1. Ingredients list of some commercially available plant-based meats.

Product Name	Ingredient list
Beyond meat Beyond Burger	Water, Yellow Pea Protein*, Avocado Oil, Natural Flavors, Brown Rice Protein, Red Lentil Protein, 2% or less of Methylcellulose, Potato Starch, Pea Starch, Potassium Lactate (to preserve freshness), Faba Bean Protein, Apple Extract, Pomegranate Concentrate, Potassium Salt, Spice, Vinegar, Vegetable Juice Color (with Beet).
Eaty Gourmet Burgers	Water, Textured vegetable protein (Wheat protein, wheat flour, Soy protein, Caramel colour), Seasoning (Maltodextrin, Pea protein, Coconut oil, Maize starch, Yeast, Yeast extract, Natural flavours, Flavouring (Fermented sugar), Dehydrated vegetables, Salt, Onion Extract, Fiber, Spices, Paprika Extract, Herb Extract, Natural Smoke flavour, Fermented rice, Mineral (Iron), Vitamin B12), Vegetable oil, Vegetable gum (Methylcellulose, Guar gum, Xanthan gum)
vEEF Burger patties	Water, Vegetable Protein (21%) (Soy, Pea), Vegetable Oil, Thickeners (Methylcellulose, Potato Starch), Yeast Extracts, Salt, Garlic, Herbs, Spices, Malt Powder (Barley), Mineral (Iron), Vitamins (B3, B6, B2, B1, B12).
Alternative Meat Co Alternative burger	Water, Vegetable Protein (27%) (Soy, Wheat, Pea, Hemp), Vegetable Oil, Natural Flavours, Caramelised Onion, Thickener (Methylcellulose), Pea Fiber, Natural Colours (Beetroot Red, Caramel I, Paprika Extract), Tomato Paste, Vegetable Extract, Parsley

Unreal Co Italian Beefy Burger	Water, Flavours (Onion, Salt, Garlic, Paprika, Thyme, Chives, Oregano, Cinnamon, Parsley, Pepper), Protein (**Soy**, Rice, Pea), Fat [Canola, Coconut], Starch (Corn, Tapioca, Potato, Nutritional Yeast, Chickpeas, Fermented Rice Powder, Maltodextrin, Fiber (Bamboo, Methylcellulose), Gums (Xanthum, Guar), Colour (Beet)
Naturli Burgers	Water, SOY PROTEIN (21%), rapeseed oil, onion, spices (including pap-rika), salt, cornstarch, garlic, tomato, apple extract, pea fiber, pea starch, natural flavouring, stabiliser (methyl-cellulose).
Impossible meats	Water, **Soy** Protein Concentrate, Sunflower Oil, Coconut Oil, Thickener (INS 461), Glutamic Acid, Natural Flavours, Cultured Dextrose, Modified Starch, Yeast Extract, **Soy** Leghemoglobin (genetically modified), Salt, Antioxidant (INS 307b), **Soy** Protein Isolate, Vitamins and Minerals (Zinc Gluconate, Niacin (Vitamin B3), Thiamine Hydrochloride (Vitamin B1), Pyridoxine Hydrochloride (Vitamin B6), Riboflavin (Vitamin B2), Vitamin B12).

Sources: Beyond meat beyond burger < https://rb.gy/m74bir>; Eaty Gourmet Burgers < https://eatynomeaty.com/>; vEEF < https://rb.gy/1rf72q>; Alternative meat co Alternative burger < https://rb.gy/s0xg56>; Unreal Co Italian beefy burger <https://rb.gy/8ds14a>; Naturli burgers: < https://rb.gy/4nh0sp>; Impossible meat< https://shorturl.at/Ava99>

The use of the voice of the consumer or models such as the quality function deployment method (Stewart-Knox & Mitchell, 2003) is seldom undertaken concurrently. Also, during recipe development, the key focus is on creating texture and taste (flavour), such as using ingredients possessing umami and kokumi effects, but not aroma. In relation to plant-based meats itself, several reviews have been published about the challenges of the creation and promotion of plant-based products albeit with opposing views (Pavagadhi & Swarup., 2020; Sogari et al., 2023; Wang et al., 2022). Starting with creating a recipe, the use of protein fractions of legumes, especially soy and pea, is widely proclaimed to possess beany, green and off flavours. Other legume-based protein fractions, such as from fava beans, chickpeas, lupin, etc, have had low commercial applications. Similarly, the use of vegetable oil, canola oil and coconut fat are

widely utilised (Table 2.1) to mimic the sensorial attributes of meat. Mushrooms, yeast extracts (YE) and other tastants are also added to the formulation to enrich the meaty flavour. Thereby attempting to match the aroma profile of a beef burger. On the other hand, the terms plant-based food and plant-based meat are loosely associated, leading to a wide generalization. Yet another systemic drawback is that terms such as flavour, aromas, odourants, and tastants are often used loosely and interchangeably due to a lack of standard definitions.

Beefy, brothy, brown, burnt, meaty, and roasted are regarded as appreciative sensory descriptors compared to cardboardy, green, hay-like, livery, musty, and warmed-over flavour to volatiles in beef (Miller et al., 2023a and Miller et al., 2023b). Interestingly, multiple compounds identified using chemometrics in varied ingredients cooked at elevated temperatures present similar sensory attributes e.g. 4-terpeneol, nonanal, 3-methyl-2-thiophenecarboxyaldehyde was observed as being beefy in simulated beef and yeast extracts, similarly octane, caryophyllene oxide, benzothiazole, 2-isobutylthiazole, 2-methyl-3-furanthiol, and dimethyl disulfide were observed as being meaty (Ames and Elmore, 1992; Lin et al., 2014, Mahadevan and Farmer, 2006, Mountford et al., 2014 and Soo-Yeun et al., 2006)

Meat, in general, is known to possess both appreciable and non-appreciable attributes. Some compounds, such as hexanal, are appreciated in smaller quantities but not in higher quantities (Hongsoongnern & Chambers IV, 2008; Chambers IV & Koppel, 2013). Whilst other compounds such as benzaldehyde with popcorn, caramel, burnt sugar and almond-like attributes or 3-pentyl furan with savoury, off, green, sweet, fruity, bitter, metallic, rancid, tea, fruity, green attributes (Table 2.2) were observed to be ubiquitous in cooked foods and not considered key savoury aroma compounds. Even if the identification of aromas from a single and binary mixture of compounds by Gas Chromatography-Olfactometry can be reasonably made, identification of aromas in a mixture of 3 or more compounds is often considered difficult (Dunkel et al., 2014; Rocha et al., 2022). According to Dunkel et al. (2014), individual component identification may not truly represent the whole food. Hence, what constitutes the applicability of meat, whether it be the flavour, taste, aroma or trigeminal senses has been difficult to pinpoint due to several reasons including a lack of definition, vast variability in terms of the methodology used to describe Appreciability in the absence of standard definitions; and chemometrically attributed to hundreds of aroma active compounds amongst over 800 volatiles identified in beef (Specht & Baltes, 1994).

Factors affecting the development and formation of aromas in beef have been studied and reviewed extensively (Chail, 2105; Gorraiz et al., 2002; Khan et al., 2015; Kerth, 2017, Watanabe et al., 2015). A few studies have also compared volatiles in beef burgers and plant-based meat alternatives (Van Vliet et al., 2021; Kaczmarska et al., 2021), but none have enumerated how composition, formulation and effect of cooking would affect the volatile profile. The use of aroma-active compounds and the associated odour descriptors extrapolated from meat studies for use in plant-based protein alternatives have been met with challenges (Thong et al., 2024). The vastness of flavour research, the complex matrix of meat, limits of detection and other technological and sensory challenges present challenges in systematic mapping of the development and formation of aroma compounds.

It can, therefore, be said that the challenges in creating a meaty experience in plant-based meat alternatives are multi-dimensional and non-hierarchical. Primarily, biological, chemical, and physical factors such as product composition (matrix and precursors), along with formulation and cooking techniques, are known to influence the development of aromas (Miller et al., 2023). Additionally, it is essential to consider consumers' perception of meatiness and their expectations when consuming the product (Miller et al., 2023a and Miller et al., 2023b). Discrepancy in any of these factors could potentially lead to an unfavourable reaction towards food acceptance or appreciation. In this context, the question is whether the addition of ingredients possessing meaty attributes when added to plant-based proteins expresses the same. Once a product is developed, how do we ascertain that it best replicates meatiness or beefiness? Reaching a consensus using a correlational theory could lead to a causality fallacy due to the multiple pathways of volatile formation and their interpretation using odour descriptors.

Therefore, this review aims to establish factors affecting the formation of savoury aroma compounds through the different stages of their creation through chemometrics. Especially composition, formulation, cooking and analysis. To do this, only studies of aroma compounds applicable to beef (mince/ground) or plant-based protein/meat alternatives, either roasted, grilled or fried, with substantial effects of Maillard and associated reaction products, are discussed. Thus, enabling an appreciable comparison of the aroma compounds formed during high-temperature processing. Studies possessing critical information but using other beef cuts or meats have been included with remarks. Health effects, nutritive values and quality are not discussed as is outside the scope of this review. Also, umami- a tastant and now Kokumi- a perception/ tastant associated with calcium-sensing receptor (CaSR) tripeptides (Forde & Stieger, 2024) and vitamin degradation compounds (non-aroma) are not discussed. With

respect to chemometrics, data relating to the use of Headspace Solid phase microextraction-gas chromatography-mass spectrometry (HS SPME -GCMS) is reviewed in detail and related methodology is in brief. Due to the rarity of studies that included both sensory and chemometric analysis of beef and plant-based meat, a direct correlation of data could not be undertaken. This review presents a comprehensive yet scoping review of comparable literature on aroma compounds akin to meaty, roasted and associated descriptors represented in beef and plant-based meat and ingredients.

Composition matrix and precursors

The composition of an ingredient, also known as the matrix, is made up of constituents ultimately responsible for the development of aromas in cooked foods. These dictate its value in food depending on the appreciability it creates (Ha et al., 2019). Beef comprises several constituents, including proteins, fats, sugars, salts, fiber, vitamins, minerals and several minor components (Williams et al., 2007b). For analytical and sample preparation purposes, the highly complex matrix of animal meat can be broadly categorised into insoluble sarcolemma-consisting of myofibrillar and stromal proteins (muscle and tendons) made up of actin, myosin and other structural proteins and tissues). The soluble- sarcoplasm consists of myoglobin, haemoglobin and several other components (Duarte, 1999; Tornberg, 2005; Jairath et al., 2024) and the hydrophobic fats and lipids (Cobos & Díaz, 2015). These are biologically linked and bound into a matrix, thereby providing the complexity observed in aroma formation upon cooking or heating (Li et al., 2023). The use of trimmings in comminuted meats adds to the variety of grades and quality, thereby further affecting the composition (Cobos & Díaz, 2015; Williams, 2007a). Nevertheless, another challenge is the use of the word meat rather than beef, signifying the possibility of the use of porcine meat in the preparation of a burger and the difference between ground and minced meat.

On the other hand, plant-based meats are created mainly using cereals, legumes or seeds containing 20-50% protein. Soy and pea (flour, concentrate, isolate and hydrolysates) are the most used plant-based proteins composed of up to 75% in concentrates and 80-95% in isolates (Klupšaitė & Juodeikienė, 2015). Wheat gluten and protein fractions from potatoes, rice and other legumes are used in smaller amounts (Bohrer, 2019). The use of protein fractions from Lupin (although containing a higher proportion of protein), lentils, fava beans, hemp and other legumes and seeds have not been observed in commercial plant-based meat alternatives and remain vastly underutilized. The protein types commonly found are globulins (35-72%),

albumins (15-25%), glutenins (10-20%) etc. However, a wide variation between breeds and cultivars is also observed (Sui et al., 2021). A deficiency in sulphur-containing amino acids (cysteine and methionine) compared to its animal counterpart is commonly observed and attributed to affecting aroma outcomes.

Extraction and process conditions of leguminous proteins can also affect specific functional properties and are optimised to preserve them (Sui et al., 2021). The extraction process could unstabilise the matrix, resulting in a higher rate of oxidative damage. However, it benefits by increasing the amino acid concentration, reducing tannins, phytates and trypsin inhibitor activity, thereby affecting the aroma output (Fernández-Quintela et al., 1997). Breeding, malting, fermentation and other methods have also been evaluated to reduce off notes (Ritter et al., 2024; Badjona et al., 2023; Mittermeier-Kleßinger et al., 2021; Roland et al., 2017). Damodaran et al., (2013) suggested the use of β-cyclodextrin in mitigating off flavours from protein-bound precursors in soy protein isolates.

Several studies have been published on the effect of processing conditions on structure-function modification, such as binding ability, emulsification, foaming, gelation, rheological properties, solubilization and mitigating off flavours (Rathnakumar et al., 2023; Wang et al., 2023; Akharume et al., 2021) but few addressed how modification affects their aroma profile compared to its meat counterpart. Regardless, large compositional differences exist between animal meat and plant-based meat. The study by Van Vliet et al. (2021), which used metabolomics, highlights the metabolite differences of over 90% between plant and animal meat. The differences relate not just to proteins, carbohydrates and fats but, more importantly, to smaller molecular weight compounds that may be crucial in the formation of aroma compounds in PBMA's, such as amino acids peptides, sugars, fatty acids, dicarboxylic acids, phenols, alcohols, vitamins, glycerides, minerals etc. flavonoids, phenols, tannins (Wang, 2022). What can be observed is that compounds that are completely absent in beef are present in plant-based meat and vice versa; compounds present in both plant and animal meat differ in varying degrees. Moreover, it should be noted that Van Vliet et al. (2021) compared ground meat (unseasoned, unformulated) to plant-based meat (seasoned, formulated). Also, comparing beef mince is difficult due to the varied quality and grades available, with different compositional values adding to the complexity. In beef, anserine had a greater brothy flavour than carnosine (Pereiralima & Ordonez, 2000). Creatinine, hydroxyproline, glucosamine and cysteamine are other metabolites found only in beef. On the other hand, phenols, tocopherols, and phytosterols can affect the aroma outcomes and can be found in meat or in large quantities

in plant-based meat. Although the differences in metabolites between meat and PBMA's have been established, their effect on aroma output is yet to be realized.

It is widely acknowledged that, of the total volatiles produced during heat treatment of an ingredient, only a few possess aroma activity and a few exhibit key impact or signature odours (Dunkel et al., 2014). In beef, over 1000 volatiles have been identified; however, only a handful are said to possess key aroma impact compounds (Mottram, 1998) that could give the beef its signature characteristic aroma, with little consensus. The challenge of finding which constituent/s contribute to specific aromas has been debated and given rise to discussions and experiments ascribing roles of specific constituents in the development of aromas called precursors (Hornstein & Crowe, 1960; Mahadevan & Farmer, 2006). Precursors are constituents of an ingredient or breakdown compound whose further oxidation, degradation, and interaction result in the development, promotion, or increase in the formation of the aroma active volatiles and key impact aroma compounds. It should be noted that the presence of precursors themselves does not directly relate to appreciable aroma outcomes unless subjected to a process. Two key reaction pathways (Enzymatic and non-enzymatic) are linked to the formation of aroma compounds (Jairath et al., 2024; Hemmler et al., 2018)

Enzymatic action is an inherent occurrence in all biological matter (mostly occurring prior to thermal processing). Both endogenous and exogenous enzymatic action have been extensively studied in relation to flavour formation. The action of endogenous enzyme activity such as glycolysis, lipolysis and proteolysis can positively or negatively affect aroma outcomes. In meat, post-mortem enzyme activity is affected by a range of factors, such as pre and post-slaughter conditioning (Koutsidis et al., 2008; Stetzer et al., 2008) and is responsible for turning 'muscle into meat' (Ferguson & Gerrard, 2014). In comminuted meats, due to the nature of processing and the area of exposure, the likelihood of enzymatic and oxidative reactions increases considerably (Jongberg et al., 2017). Similarly, in plants, pre- and post-harvest processing, handling, and storage affect several attributes that ultimately affect the final product (Roland et al., 2017).

Glycolysis is the breakdown of simple sugar glucose. In beef, it occurs briefly during postmortem and rigor mortis. Broadly speaking, glycogen, by the action of ATPases and other enzymes, induces glycolysis resulting in rephosphorylation of adenosine diphosphate to yield glyceraldehyde 3-phosphate and adenosine triphosphate, generating free sugars such as inosine 5′-monophosphate: a key source of the reducing sugar pentose accumulating in post-slaughter muscle by hydrolysis of Adenosine triphosphate (Madruga, 1997). The anaerobic metabolism

also leads to the build-up of lactate and hydrogen ions, thus reducing the pH of the postmortem muscle. The reduced pH promotes the proteolytic activity of endogenous proteases (calpains, cathepsins and calpastatin) (Wang et al., 2022), cleaving off peptides and increasing free amino acids in the muscle, which, along with the reducing sugars, are later implicated in the Maillard reaction during thermal processing. Use of Exogenous enzymes (proteases) such as papain, bromelain, trypsin, chymotrypsin etc have also been employed and achieve dual benefits of tenderizing the meat as well as promoting desirable volatile formation or mitigating unfavourable outcomes through the cleavage action. Zhao et al. (2020) studied the effect of Bromelain, Flavourzyme™, papain and proteinase-K on volatile development in beef with papain being the most effective in increasing desirable precursors up to 5 times compared to the control whilst also increasing the levels of undesirable amino acids such as valine, lysine, leucine, isoleucine and phenylalanine including creating bitter hydrophobic ends.

Research has examined the proteolytic effects of both endogenous and exogenous legume proteases (Dent et al., 2023; Chen et al., 2021). Su et al. (2023)has been shown to promote an increase in peptide chain lengths, which in turn enhances the level of compounds with umami potential while simultaneously reducing the number of bitterness-promoting peptides (Su et al. 2023). Additionally, aminopeptidases have been utilized to minimize bitterness in legume protein fractions (Lei et al., 2017).

Lipolysis is typically considered an undesirable consequence of lipid oxidation and degradation, particularly affecting the formation of volatile compounds. This issue is most significant in lipids that have a high degree of unsaturation (Tatiyaborworntham et al., 2022). In post-mortem muscle, the interruption of metabolic processes leads to the exposure of molecular oxygen, which, in combination with pro-oxidants and reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions, hydrogen peroxide, hydroperoxyl radicals, lipid peroxyl radicals, alkoxyl radicals, iron-oxygen complexes, and singlet oxygen (Min & Ahn, 2005), interacts with intramuscular lipids, particularly phospholipids. This interaction results in the production of undesirable volatile compounds (Domínguez et al., 2019; Min & Ahn, 2005; Tatiyaborworntham et al., 2022).

Lipoxygenase activity influences both plants and animals, including fungi (such as mushrooms). The enzymes involved include lipoxygenases (LOX1, LOX2, LOX3), hydroperoxide lyase (HPL), alcohol dehydrogenase (ADH), and phospholipases, which are non-heme metal oxidoreductive agents. These enzymes catalyse the oxidation of unsaturated fatty acids and esters using molecular oxygen, leading to the formation of double-bonded

conjugated hydroperoxy derivatives through various pathways. Plant-based proteins, particularly those derived from legume fractions, are significantly impacted by endogenous lipolytic activity. Soy, cowpea, and lentils exhibit a high degree of LOX activity, while Adzuki beans, fava beans, and kidney beans display medium levels of activity. Chickpeas, lima beans, and mung beans, on the other hand, show low LOX activity under specific conditions (Chang and McCurdy, 1985). Zhang et al., (2020) found that endogenous enzyme activity dominated the formation off-flavour precursors and compounds compared to lipid content in pea and soy.

Fermentation involves the enzymatic action of microorganisms during their metabolic processes, leading to various chemical changes. This process enhances the development of flavours and aromas by increasing the quantity of aroma precursors and by controlling antinutritional factors, such as phytates, tannins, and protease inhibitors (Senanayake et al., 2023). Traditionally fermented products, including soy sauce, tempeh, tofu, and Natto, have been utilized to improve the consumption of soybeans. Research indicates that differences in ingredient composition (Yamana et al., 2020), fermentation cultures (Schindler et al., 2012), and fermentation technologies (Terefe, 2022) can significantly influence aroma outcomes. In the creation of a soy sauce-like seasoning, it was found that both grain and legume seasonings had similar levels of sweetness; however, legumes exhibited a higher umami characteristic, attributed to the presence of glutamic acid, compared to the polysaccharides found in grains. With advancements in fermentation technology, the production of biomass from single-cell protein (SCP) sources — including yeast extracts, filamentous fungi, and microalgae — has been achieved (Anupama & Ravindra, 2000; Terefe, 2022). Currently, research is focused on precision fermentation to create targeted functional proteins (enzymes) and nutrients, such as Leghemoglobin™, aimed at producing components and precursors for flavour generation (Terefe, 2022).

Non-enzymatic precursors are characterised as non-volatile, water-soluble (Koutsidis et al., 2008; Mottram, 1998) and low molecular weight compounds (Resconi et al., 2013). These include sulphur-containing amino acids such as cysteine and methionine, along with reducing sugars (Mottram & Nobrega, 2002; Zhan, 2020), peptides and nucleotides (Spanier et al., 2004), unsaturated triglycerides, phospholipids, free fatty acids, and vitamins like thiamine (Mottram & Nobrega, 2002; Guentert et al., 1990), as well as metals (Skibsted et al., 1994). Notably, the nucleotide 5'IMP was found to be more effective than cysteine or thiamine in promoting volatiles with meaty nuances (Madruga, 1997); however, it was necessary for its concentration to be ten times higher than that typically found in beef for analysis purposes.

Additionally, downstream reactants, such as those from the Maillard reaction associated with Strecker degradation and Amadori rearrangement products (ARPs), are also regarded as precursors in the development of process flavours, particularly in relation to cooking effects (Cui et al., 2021).

The careful selection of ingredients can significantly enhance the final product by reducing, masking, or highlighting certain attributes. While intrinsic factors - such as composition, matrix, and precursors - play a crucial role in developing key aroma compounds and characteristics, extrinsic factors, like formulation and thermal processing (or cooking), are equally important in creating a final product that is more appealing. Studies analysing real food systems have acknowledged the role of ingredients beyond meat in improving, masking, or increasing the appeal of plant-based analogues (Devaere et al., 2022; Yuan et al., 2023)

In general, the macronutrients in beef - proteins, carbohydrates, and to a lesser extent, fats - have minimal impact on aroma. Instead, it is the presence of free amino acids, free fatty acids, and other low molecular weight compounds, such as nucleotides and ribonucleases, that are believed to contribute to the formation and development of savory aromas (Shahidi, 1986). During high-temperature processing, the rate of aroma formation peaks, primarily due to the stability of the matrix and its involvement in a process that could be known as Simultaneous Oxidation, Degradation, and Interaction with Maillard Reaction Products (SODIM). The SODIM effect, as coined here, reflects the simultaneous nature of aroma formation, which is typically discussed in a sequential manner in the literature for better understanding and mapping (Diez-Simon et al., 2019). This means that the development and formation of volatile compounds increase exponentially at temperatures above 120°C, as observed in model studies (Zhan et al., 2020). It is important to note that food is rarely consumed as isolated ingredients; rather, it is enjoyed as a carefully crafted mixture of selected ingredients that come together to create a recipe, which consists of a list of ingredients and a set of instructions aimed at achieving a desired outcome.

Effect of formulation

Although formulation is undertaken before processing, it's important to understand the effect of formulation on the outcomes of processing, i.e. when selecting ingredients to create a recipe. Several reviews have explored how formulation in meat burgers and patties interacts with factors like fat content and cooking methods (Berry & Leddy, 1984), nutrition and health (Small et al., 1991), and the nutritional quality of plant-based meats (Bohrer, 2019), including

their gastrointestinal fate (Ishaq et al., 2022), nutritional value, health safety, and legal regulations (Kołodziejczak et al, 2022), as well as texture (Kyriakopoulou et al., 2021). Although, justifications for the creation of meat alternatives are debated heavily, the primary goal in formulating plant-based meat is to create products that closely resemble the meat they aim to imitate by using effective functional ingredients. Compared to beef burgers, plant-based meat is a formulated product made from a blend of functional ingredients. These ingredients typically include protein fractions, simple and complex carbohydrates, fats, and oils, along with various additives such as acidifiers, humidifiers, emulsifiers, binders, colourants, flavour enhancers, and other novel components like leghemoglobin (Table 2.1). However, there is limited research available on how each ingredient contributes to the overall perception of meaty aroma.

Proteins

It is generally recognized that raw proteins have a neutral flavour. In the case of bulk and raw animal meat, the structural proteins produce little in terms of significant aromas, often described as bloody, serum-like, or metallic. Comminuted meat, made from quality cuts containing myofibrillar and stromal proteins, is formulated based on the percentage of fat used. However, controlling the sarcoplasmic proteins can be challenging due to the shear action that occurs during comminution, which can lead to drip loss or purging. This results in lower moisture levels in comminuted meat (around 64%) compared to whole meat (around 73%) (Cobos & Díaz, 2015; Jongberg et al., 2017; Warner, 2017; Williams, 2007a). Studies on the aromas produced when high-temperature processing is applied to the myofibrillar and stromal fractions of beef are limited. However, with the introduction of leghemoglobin™, several studies have explored the effect of myoglobin in food. For instance, Devaere et al. (2022) found that adding myoglobin to soy-based burgers enhanced the production of aroma volatiles similar to those found in meat. Additionally, research by Wu et al. (2021) indicated that myoglobin influenced lipid oxidation in washed muscle from cod and pork, which depended on the availability of phospholipids and the protein microstructure. Similarly, a study by Yancey et al. (2006) observed a low correlation between lipid oxidation and iron content (including free iron, myoglobin, and haemoglobin); however, a positive correlation was found between liver flavour and iron content. Overall, significant differences in the volatile profiles of beef mince have been noted when using various grades and quality trimmings.

In the context of Plant-Based Meat Alternatives (PBMA), Thong et al., (2024) found that meaty flavours did not correlate with plant proteins. Additionally, commonly used leguminous

proteins, including concentrates, isolates, or hydrolysates derived from raw and uncooked soy, peas, and other beans, have been associated with undesirable aromas such as beany, fatty, green, and grassy scents (Roland et al., 2017). However, various processing and extraction methods—such as enzymatic treatment (using exogenous flavour enzymes or endogenous sprouting), fermentation, and acid hydrolysis—are known to mitigate these issues (Saffarionpour et al., 2024). Protein structural modifications can influence functionality and the ability of volatile compounds to bind to the surface of the modified proteins (Zhang et al., 2023; Keppler et al., 2020). Several chemical interactions - ranging from ionic and covalent to hydrophobic and steric - are responsible for this effect (Saffarionpour et al., 2024; Wang et al., 2023). The impact on flavour retention and release has been studied, particularly concerning the binding of undesirable compounds such as saponins (Heng et al., 2004). Wang & Arntfield. (2016) observed that vicilin had a greater affinity for binding aldehydes compared to ketones due to the weaker interactions with ketones. However, this study was conducted at low temperatures (below 100°C), making the volatile outputs not directly comparable to those of other PBMA products. With the advancement in technology, off-flavours in leguminous proteins have been reduced; however, this is still a work in progress. Most of the research into protein-flavour binding is limited to extraction methodologies, storage, or low-temperature processing. It would be desirable to understand how modified protein fractions, on exposure to HTST processing, develop appreciable aroma.

Carbohydrates

Research on the impact of carbohydrates on aroma development primarily focuses on sugars, including reducing sugars, that are involved in the Maillard reaction, as well as the effects of caramelization. However, both simple and complex polysaccharides, such as native or modified starches from wheat, potato, maize, and other cereals, are utilized for various functions in plant-based and animal-based meat burgers (Kyriakopoulou et al., 2021; Rekola et al., 2023) their impact on the formation of aroma is less known. Furthermore, ingredients like methylcellulose, acacia gum, xanthan gum, and carrageenan are incorporated to enhance texture and binding. These ingredients can also be used for encapsulation, coatings, and creating scaffolding for novel components like oleogels and hydrogels (Bohrer, 2019; Kyriakopoulou et al., 2021; Huang et al., 2022). In comparison to traditional meat patties, plant-based meat analogues can contain 2-30% carbohydrates. Increasingly, novel applications of complex carbohydrates in PBMA are being explored to mitigate off-flavours and enhance texture, mouthfeel, and fat reduction (Jimenez-Colmenero et al., 2013). The dynamics of aroma

formation and development when intense heat is applied during grilling, particularly in the presence of a wide variety of ingredients, present a challenge that has yet to be fully understood.

Fats and oils

The oxidation of lipids contributes to high odour activity values, significantly influencing flavour (Shahidi et al., 1986; Frankel, 2005). Auto-oxidation initiates the formation of hydroperoxides, which decompose further to produce hydroxyl and peroxyl radicals. These radicals can cleave and interact with Maillard reaction products, resulting in the formation of various compounds, including aliphatic hydrocarbons, aldehydes, ketones, alcohols, carboxylic and fatty acids, esters, lactones, and alkyl furans. Some of these compounds can polymerise to form stable substances. The sources of lipids in comminuted meats include choice cuts and trimmings, composed of visceral, subcutaneous, and inter- or intramuscular fat (IMF). Adding trimmings increases the proportion of subcutaneous saturated fat, which is less prone to autoxidation and the formation of undesirable aroma compounds (Mottram, 1998). Depending on the chosen cuts, unsaturated triglycerides and phospholipids may be present in lean tissue. These compounds are more susceptible to rapid oxidation and degradation during high-temperature processing. Certain volatile compounds, such as 2-octenal, 2-nonenal, 2,4-nonadienal, 2,4-decadienal, and 1-octen-3-one, are known to impart intense flavours resulting from the breakdown of linoleic and arachidonic acids (Arshad, 2018). The choice cuts studied by Blackmon et al. (2015) exhibited unique volatile profiles. However, some disagreement was noted regarding the correlation of stearic, palmitoleic, oleic, linoleic, and arachidonic acids with beef and umami attributes in comparison to other studies. Fatty acid profiles have also been utilized for species-level identification; however, during sensory assessments, taste panellists were unable to distinguish between beef and pork fat (Mu et al., 2023). Barros et al. (2020, 2021) reported conflicting results, where substituting animal fat with algal and wheat germ oil emulsions was less accepted than substituting with tiger nut oil emulsions. Notably, volatile analysis was not conducted in either of these studies.

Formulating PBMA that mimic beef burgers' fat and oil composition has proven challenging. Successful texture development has been achieved by combining saturated fats (such as coconut oil, shea butter, cocoa butter, hydrogenated palm oil, and sal fat) with unsaturated lipids like sunflower and canola oil (Bohrer, 2019). However, the resulting aroma from these formulations has not yet been fully explored. While some research has been conducted using oleogels and hydrogels, commercial applications remain limited. Dreher et al. (2021) utilized a combination of canola oil and sal fat in varying proportions to produce a plant-based salami,

with a 1:1 ratio of canola oil to sal fat being the most favoured. However, this salami was not cooked, resulting in fewer Maillard reaction products, and thus it cannot be directly compared to cooked PBMA such as burgers. The effects of different fats and varying fat percentages on meat and PBMA have been extensively studied and reviewed (Arshad et al., 2018; Davis, 2019), but there is still little consensus on the best formulations.

pH

Fresh raw meat is considered unacceptable when its pH is below 5.3 or above 5.7 (MLA, nd.). Changes in the pH of meat can occur both intentionally and unintentionally due to various factors such as aging, marination (acidification), curing, and smoking. For instance, fermentation tends to lower pH values, while the use of certain phosphates can raise them (Gomez et al., 2020; Wu et al., 2014). Maintaining an appropriate pH range is crucial for ensuring the quality and acceptability of meat products. Alterations in pH can influence protein denaturation and affect the structure-function characteristics of the meat. This can lead to changes in surface properties, such as increased hydrophobicity (Saffarionpour, 2024) or irreversible covalent bonding (Anantharamkrishnan et al., 2020). The final pH, rehydration capability, and altered surface area of meat are all impacted by these processes (Jiang et al., 2010). Model systems have provided insights into the development of aroma compounds. In a mixture of amino acids and sugars, compounds such as furfurals (which have a bready aroma) and thiols and thiophenes (which smell meaty) are preferred at lower pH levels; their concentrations decrease as pH rises. In contrast, pyrazines (which have a nutty, roasted aroma) and disulphides (sulphurous and alliaceous scents) favour higher pH levels (Madruga & Mottram, 1995; Meynier & Mottram, 1995; Van Ba et al., 2013). A higher rate of Maillard reaction products is formed at alkaline pH levels (Ghorab et al., 2010). While the effects of pH on structural modifications and their associated changes have been studied, the specific impact of pH on aroma development and its subsequent effects on final products, such as PBMA, remains unclear.

Moisture

Moisture plays an indirect role in the formation and development of aroma in foods. In protein-rich foods, controlling moisture is primarily important for maintaining the product's water-holding capacity during cooking and enhancing the flavour-binding effect of the proteins. Although the moisture content in comminuted meat is typically consistent, variations can be introduced based on the ingredients, functionality, cooking methods, storage conditions, and

the sensory appeal of the final product (Akwetey & Knipe, 2012; Turhan & Biyik, 2022; Bunmee et al., 2022). The moisture content in PBMA can vary significantly, ranging from 50% to 80%. Despite this, these products have been criticized for being dry and not matching the sensory qualities of animal meat (Ahmad et al., 2022). Regarding protein structural changes, the covalent binding of macromolecular proteins through disulfide linkages has been found to reduce the amount of legumin in pea protein isolates due to the formation of aggregates. Additionally, conflicting findings have been reported on the impact of high-moisture extrusion on amino acid loss, which could affect the aroma profile of the final product (Osen et al., 2015).

Seasonings

Every consumable food is seasoned. Seasonings increase the palatability and appreciability of foods. Salt, sugar and vinegar are the commonly used seasonings. Although the volatile analysis was not conducted, Vázquez-Araújo et al. (2013) in a consumer survey conducted in three countries, observed that cultural and geographical differences play a distinct role in the type and strength of seasonings. Similarly, Hartley et al. (2022) found that people who consumed meat daily were less sensitive to salt and umami detection. Sodium, potassium and calcium chloride salts, along with salts of mono ammonium glutamate (MAG), monosodium glutamate (MSG), guanosine monophosphate (GMP), and inosine monophosphate (IMP), can be employed to facilitate enhancement and reduce salt simultaneously (Rocha et al., 2021). Salt reduction in processed foods is a challenging issue and is being addressed by industry and research (Lorén et al., 2023).

Flavour enhancers

The loss of food product flavours during processing is replaced and improved by the application of flavour enhancers. They play a crucial role in plant-based and meat alternative formulations by providing essential savoury aromas. Common flavour enhancers in plant-based meats include yeast extracts, mushrooms and their extracts, allium (such as onion and garlic), herbs and spices, and enzyme-hydrolysed vegetable proteins (HVP).

Yeast extracts (YEs) are, as the name suggests, derived from the cultivation of yeast (Khan et al., 2020). They contain high levels of precursors commonly found in meat, such as glutamic acid and other amino acids, along with 5'-ribonucleotides, peptides, and vitamins like thiamine. However, YEs are low in lipids (Alim et al., 2019). Despite their low lipid content, some aliphatic aldehydes and ketones have been identified in YEs. Their formation is associated with alternative pathways of glucose degradation. Compounds such as 2-methyl-3-

(methyldithio)furan, which has a very low odour threshold, and other furan derivatives like 2-methyl-3-furanthiol and 2-methyl-3-(methylthio)furan have also been detected in YEs (Ames, 1992). Although yeast extracts are low in cysteine, they contain amino acids like methional, which can produce sulphurous odours reminiscent of vegetables, onions, or cooked cabbage. Furthermore, because YEs are rich in thiamine, they may promote the formation of thiophenes and thiazoles, contributing roasted, cheesy, and meaty characteristics to their aroma (Ames, 1992).

High levels of a diverse range of pyrazines have also been observed in YEs, imparting nutty, roasted, and burnt notes. Excessive roasting may lead to strong, burnt, and nutty flavours, which can suggest a bitter perception due to olfactory responses. This aligns with findings by Alim et al. (2018) that indicate temperatures exceeding 130°C increase bitterness.

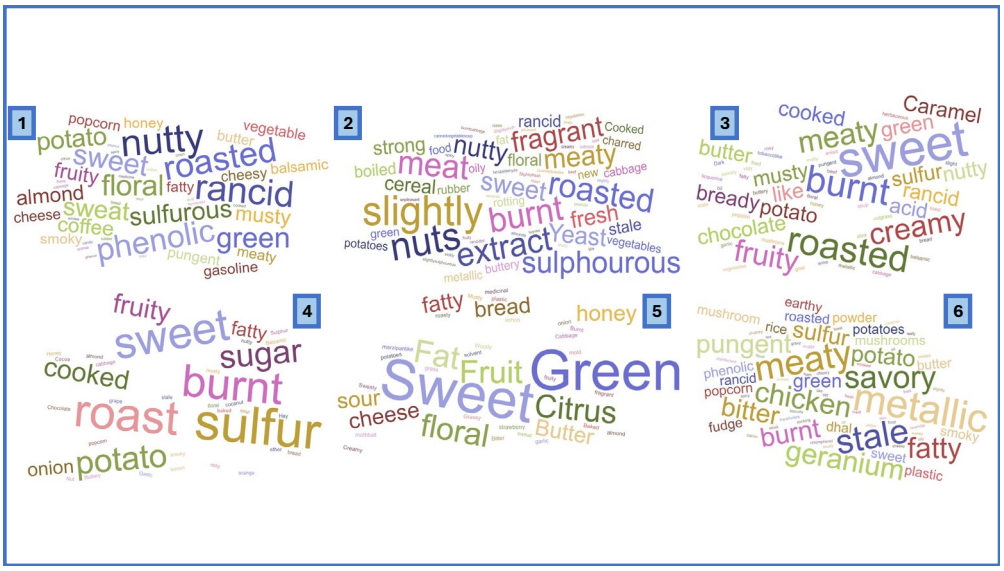

Figure 2.2 Word clouds of aroma descriptors for different yeast extracts found in the literature. Where the bigger and bolder words related to descriptors most observed. 1. Chen et al., 2023; 2. Wang et al., 2020; 3. Alim et al., 2018; 4. Lin et al., 2014; 5. Mahadevan and Farmer., 2006; 6. Ames and Elmore., 1992.

It is important to note that not all yeast extracts are the same; significant differences have been observed between different batches and processing conditions (Mahadevan & Farmer, 2006) (Figure 2.2). Recent biotechnological advances have led to the development of yeast extracts that contain higher protein levels with more specific peptide links or amino acids. These can elicit specific peptide expressions, resulting in intended savoury aromas through precision fermentation (Huling, 2023). Knowing that during processing and extraction of the yeast biomass a critical level of Maillard reaction products and aroma active products are already

present; One critical question remains unanswered is: to what extent does the addition of yeast extracts to plant-based formulas enhance desirable aromas? A thorough understanding of the ingredients and their interactions within the matrix is crucial for formulating a product since the outcome can only be realized after processing. Evidence suggests that although YEs possess savoury, meaty, and beefy nuances due to their composition. These have been observed to enhance savoriness in products particularly those processed at low temperatures, their use in products exposed to high temperatures has resulted in higher amounts of pyrazines and advanced Maillard reaction products, which may elicit bitter and unfavourable responses.

Mushrooms and other fungal products have traditionally been sought after for their unique flavours. In addition to using commercially grown mushrooms as food enhancers, the commercial fermentation and production of mycoproteins resembling animal meat have become popular. Various alternative meat products are now available on the market (Huling 2023). When it comes to their role as flavour enhancers, mushrooms contain limited amounts of sulphur-substituted amino acids, particularly cysteine. Dried mushrooms are low in free amino acids but are rich in aspartic acid, glutamic acid, and arginine, which contribute to the umami taste. They also provide a range of vitamins, including riboflavin, niacin, and folates. The co-fermentation of fungal and legume proteins has been shown to enhance antioxidant properties, thereby reducing or masking undesirable odours in legume proteins. Literature reviews highlight that mushrooms can offer meaty, beefy, and savoury aromas. Interestingly, the thermal processing of mushrooms has been found to decrease volatile compounds rather than increase them (Selli et al., 2021; Tian et al., 2016). Key aroma compounds, such as 1-octanol, 3-octanol, and 1-octen-3-ol, were reduced during cooking.

This raises questions about whether mushrooms might have a synergistic effect when paired with meats but potentially no effect or even negative effects when combined with legume proteins. What components of mushrooms act as flavour enhancers? Most studies and reviews have primarily focused on comparing the volatile compounds of raw and cooked mushrooms. The volatiles released when mushrooms are added to a formulation may yield significantly different outcomes.

Herbs and spices have long been utilized in meat preparation for their flavour-enhancing properties, as well as their roles as preservatives (through antibacterial and antioxidant effects) and colourants (Gottardi et al., 2016). These elements typically originate from various plant parts, including dried rootstock, tubers, bulbs, bark, leaves, flowers, buds, fruits, and seeds. Several spices are commonly used in comminuted meat preparations such as meat burgers,

processed meat-based alternatives, PBMA and extrudates. These include coriander, cumin, fennel, cinnamon, cloves, cardamom, chilli, paprika, pepper, nutmeg, mace, star anise, ginger, mint, thyme, oregano, rosemary, and alliums (onions, garlic, and chives). The choice of spices can vary based on geographical and cultural influences.

Beyond providing direct aromatic effects, these herbs and spices also offer antioxidant properties, which help reduce and mask rancid off-flavours, including hexanal, octanal, nonanal, 2-pentyl furan, and 1-octen-3-ol (Yuan et al., 2023; Dwivedi et al., 2006). While these flavourings do not contribute significantly to the characteristic aroma of beef, they often evoke memories of comfort foods, enhancing overall acceptability (Jiang et al., 2014).

Alliums contain s-substituted compounds such as bis(2-methyl-3-furyl) disulphide and 3-mercapto-2-methyl-1-pentanol, which provide a meaty sensory appeal in onions (Cerny, 2015; Stöppelmann et al., 2023). Studies have shown varying effectiveness in masking lipid oxidation off-odours in irradiated ground beef, with the order of effectiveness being onion < garlic < combined effects (Yang et al., 2011). Notably, there was a significant change in the concentration of alcohols, while the levels of hydrocarbons and aldehydes remained unchanged.

Hydrolysed vegetable proteins (HVP)

Hydrolysed vegetable proteins (HVPs) are produced through either acid or enzyme hydrolysis of proteins derived from sources such as corn, soybeans, peanuts, defatted rice bran, and others. A concern with acid hydrolysis is the development of the carcinogen 3-monochloropropane-1,2-diol (3MCPD), which is currently regulated under Australian Food Standards. During enzymatic hydrolysis, proteases facilitate the breakdown of proteins. Various proteases from animal, plant, and microbial sources have been studied (Tapal & Tiku, 2019). This digestion process results in the cleavage of specific amino acids and peptides, which are used in the production of targeted flavours. The most abundant amino acids found in flavorzyme™ hydrolysed defatted soybeans include leucine, phenylalanine, lysine, glutamine/glutamic acid, and alanine. Additionally, key aroma compounds identified include 2-methyl-3-furanthiol (associated with a meaty aroma), 3-mercapto-2-pentanone (catty, urine-like), 2-furan methanethiol (coffee-like and sulphurous), and 3-(methylation)propanal, along with methanethiol (Wu & Cadwallader, 2002).

Additives

The shelf life and storage capability of formulated products depend on their buffering capacity to prevent oxidation and degradation. Antioxidants and buffers used to help maintain product quality. Although they are not known to directly contribute to key savoury aroma attributes, they can indirectly influence these aromas by modifying oxidation and reduction reactions, thereby promoting or reducing oxidative, rancid, and stale aromas. This ability can enhance the perception of other volatile compounds.

Comminuted meat naturally contains a variety of prooxidants and antioxidants within its matrix. Free and bound iron, such as heme proteins found in myoglobin and haemoglobin (Wu et al., 2021), act as prooxidants in muscle meats, promoting the oxidation of unsaturated fats, free fatty acids, and phospholipids. On the other hand, phosphates, nitrates, and other metal chelators serve as antioxidants and buffers. In plant-based meat analogues, compounds such as phenols and polyphenols (flavonoids), as well as terpenoids (including terpenes and carotenoids) found in ingredients like curcumin and tomatoes, provide nutritional value and act as preservatives due to their antioxidant properties. These properties can indirectly affect oxidation rates, thus altering the volatile profile.

Extensive processing and the use of functional compounds and ingredients require careful consideration. Loss or creation of unwanted adducts can occur during further processing and cooking at high temperatures. Binders, emulsifiers, and texturisers offer multifunctional benefits and are widely applied in processed foods. They help provide structure and texture, maintain emulsions, increase water-holding capacity, reduce drip loss, minimize fat content, and ensure product stability from processing through to shelf life. Commonly used ingredients include polysaccharides like carboxymethyl cellulose, carrageenan, sodium alginate, and pectin (Moll et al., 2022), gums such as xanthan, guar, and locust bean (Han et al., 2023), as well as proteins like egg albumen, caseins, legume concentrates and hydrolysates, and gluten (Bohrer, 2019), along with various phosphates (Dykes et al., 2019).

In terms of sensory analysis of flavour, k-carrageenan has been found to be better appreciated compared to five other polysaccharide binders (locust bean gum, Arabic gum, gellan gum, guar gum, and xanthan gum) in constructing plant-based meat analogues (Han et al., 2023). A combination of pea protein and apple pectin has also shown promise (Moll et al., 2022), although its effect on aroma output has not been studied. The use of complex coacervation (protein-polysaccharide complex) and microencapsulation (hydrogels and oleogels), along

with fat reduction, could be beneficial in reducing oxidative degradation and managing flavour retention and release (Xiao et al., 2014). The presence of these compounds can have either a positive or negative effect on aroma output. For example, masking off-flavours from lipid oxidation can be viewed as beneficial, while reducing the effect of key aroma compounds due to binding could be seen as a drawback.

Research on steric hindrance (repulsive forces) effects in complex matrices and whole foods has been conducted using model compounds. Zhang et al. (2023) found that positional isomerism can negatively influence the binding of homologous ketones to soy protein isolates through weak hydrophobic or hydrogen bonding. If binding occurs, conformational changes can take place, as smaller aroma compounds tend to bind with hydrophobic proteins that have available hydrophobic pockets. Enzyme crosslinking can also be utilized to control the retention and release of flavours from complex matrices (Chen et al., 2023). However, very few studies have analysed the effect of binders and texturisers on the aroma output of formulations.

Effect of cooking

Cooking is seen as a method for making food more enjoyable by enhancing its flavour, digestibility, and safety. However, it can produce both positive and negative aromas. Research on the effects of heat on the development of meaty aromas highlights several key processes: 1. The Maillard reaction, 2. Strecker degradation, 3. Amadori rearrangement products, 4. Degradation of thiamine (which is pH-dependent), and 5. Degradation of fats and lipids (which leads to the formation of free fatty acids). Additionally, the interactions between these processes are also significant. Other factors that have been studied include the type of thermal input, cooking methods, flavour interactions, flavour binding and masking, warmed-over flavours, artifact production, and the formation of advanced glycation end products (AGE).

Fuel type

Flame grilling, oven grilling, pan frying, and electric conduction (ohmic or pulsed electric cooking) are the most common methods for preparing burger patties. Much of the research has focused on how different fuel types impact the formation of polycyclic aromatic hydrocarbons (PAHs), as well as the creation of furans and pyrazines that occur when exposed to high temperatures.

Method of cooking

The initial discussion highlighted that cooking comminuted meats with intense heat applied to small morsels affects several factors, ultimately increasing the rates of aroma formation. Research has shown that the method of cooking beef can significantly influence flavour development (Vázquez-Araújo et al., 2013). In contrast, He et al. (2021) found that the cooking method had a limited impact on flavour compared to the choice of ingredients, or formulation, in beef and plant-based burgers. Additionally, a study by Dreeling et al. (2000) revealed that deep frying was the least preferred method of cooking while griddling and grilling were the most acceptable (Cabral, 2019). Other cooking techniques, such as ohmic heating (Halleux et al., 2005), high hydrostatic pressure processing (Hayes et al., 2014), pulsed electric field (Kantono, 2019), and microwave cooking in combination with conventional heating (Drew & Rhee, 1979) have been tested with varying inferences.

Time and temperature

The effect of time and temperature on aroma are two of the most important factors in thermally processed foods. Although the Maillard reaction can occur at room temperatures, most comminuted meats are cooked at much higher temperatures, typically between 180-250°C, and for shorter periods of time, usually around 5-7 minutes, to achieve the internal temperature of (72-74ºC) required by regulations. The time needed to reach this temperature depends on the cooking temperature and the size of the portion.

High cooking temperatures can lead to an exponential increase in volatile compounds, which can produce unpleasant aromas (Specht & Baltes, 1994) due to increased oxidation and degradation rates (Wall et al., 2019). While the total protein concentration remains unchanged, the concentrations of peptides and free amino acids increase. This increase may be attributed to the folding, refolding, and aggregation of proteins, which release peptides and amino acids stored within the protein matrix. The relationship between surface hydrophobicity and aroma binding appears to be linear, initially increasing and then decreasing, possibly due to protein aggregation during heating. Additionally, factors such as sulfhydryl content, particle size, and protein secondary structures are affected by these changes (Wang et al., 2023).

Loss of moisture

The moisture content of lean raw beef cuts is between 70% and 75%, compared to 60% to 65% for comminuted meats. The observed loss of moisture is attributed to mechanical shearing during meat processing, a phenomenon referred to as drip loss or the purging effect (Warner,

2017). Thermal processing of comminuted meats at high temperatures further contributes to moisture loss (Wang et al., 2022). This loss can be explained by increased surface area, rapid restructuring, protein unfolding, and the formation of random coils and aggregates, which are common phenomena observed in thermally exposed proteins and the exposure of the meat to high temperature surfaces increasing moisture loss.

Moisture plays an indirect role in enhancing the aroma attributes of cooked meat. During intense heating, the purged exudates concentrate as they leave the meat coming into contact with the heated surroundings and being exposed to higher temperatures on the cooking surface. This may accelerate the reaction rates involved in the Maillard reaction, increasing the likelihood of reaching the end-stage products of this reaction. A study on low-fat burgers revealed that moisture loss during grilling and deep frying was significantly higher compared to roasting or griddling (Dreeling et al., 2000). This may be because, although moisture is lost during griddling, the expelled exudates remain, resulting in concentrated reactants on the griddle surface. In contrast, during deep frying or grilling, the expelled juices are lost along with the moisture as drippings. Furthermore, moisture loss in plant-based burgers was found to be lower than in beef counterparts (Vu et al., 2022), likely due to the inclusion of binders and the increased water-holding capacity of fractionated proteins.

In grilled beef, there is an increase in Maillard reaction products such as pyrazines and furans. Conversely, high-moisture foods, like boiled beef, contain compounds such as sulphur, ammonia, aldehydes, organic acids, and certain pyrazines, such as 2,5-dimethyl pyrazine and 3-mercapto-2-pentanone, which are not typically found in grilled or roasted beef. Plant-based proteins, on the other hand, are hydrated to appropriate levels for formulating meat analogues. However, during processing into concentrates, isolates, and hydrolysates, their hydrophobicity changes (Segura-Campos et al., 2012; Dent et al., 2023), resulting in increased hydrophilic amino acids and peptides, which enhance solubility.

Although this holds true, Dent et al. (2023) found that the method used to quantify hydrolysed soluble proteins can lead to inaccurate estimations. This suggests that aggregates of insoluble proteins may produce a higher quantity of aroma compounds associated with nutty, burnt, and bitter sensory qualities. Unfortunately, an analysis of the effect of moisture loss on aroma compounds resulting from different cooking methods for comminuted meats or formulated plant-based proteins could not be located.

Aromagenesis

Aromagenesis is likely influenced by all the factors discussed so far. Research has highlighted the similarities and differences in aroma compounds between PBMA and traditional animal meats (Kaczmarska et al., 2021) Proponents and opponents have debated about products derived from PBMA compared to beef. The important aspects of aroma generation in thermally processed PBMA include exposure to high temperatures, low free moisture content, and formulation similar to comminuted animal meat products. However, identifying the specific compounds responsible for producing savoury aroma notes has proven to be challenging. Several factors contribute to this complexity, including the multiplicity of reaction pathways (Cerny & Davidek, 2003), the speed of reactions at high temperatures, the volume of reactants produced at elevated temperatures, and varied responses at different pH levels (Parker, 2013).

The reaction between sugars and amino acids leading to Maillard reactions and associated pathways have been extensively studied in the context of food (Coultate, 2023; Parker 2013). In brief, the pathway for generating savoury aromas begins with a condensation reaction between the amino group of a free amino acid and the carbonyl group of a reducing sugar, known as the Maillard reaction. This reaction yields a compound called a Schiff's base (N-glycosylamine), which rearranges (via 1,2-enaminol) to form a deoxysone intermediate (1-amino-1-deoxy-2-ketose), categorized as an Amadori product. Depending on whether the reactant is an aldose or a ketose, a 2-amino-1-deoxyaldose (Heyns compound) may form as well. Further rearrangement, decomposition, and cyclization occur during the intermediate stage involving Amadori product rearrangement and Strecker degradation, resulting in compounds such as furfurals, furanones, and dicarboxylic acids (Macleod in Shahidi 1994; Coultate, 2023; Ruan et al., 2018). The Amadori rearrangement product transforms an α-hydroxy carbonyl to a 2-amino ketone through reductive amination. Additionally, free ammonia can interact with α-hydroxy carbonyls to produce intermediates, including compounds from Strecker degradation. Amadori rearrangement products have been studied (Heping Cui et al., 2021) for their role in creating and applying process flavours, showcasing significant commercial value,

Strecker degradation involves an α-dicarbonyl acting as an oxidizing agent that oxidatively decarboxylates amino acids. This process produces an imine, which can be hydrolysed to yield an α-keto amine and a (Strecker) aldehyde. The formation of Strecker aldehydes may occur through various pathways depending on the precursor and reaction conditions (Ruan et al 2018). These resulting products are considered important contributors to meaty aroma

compounds. Several classes of nitrogen (-N), sulphur (-S), and oxygen (-O) substituted heterocyclic compounds, including furans, furanones, alkanethiols, methylfuranthiols, pyrazines, pyridines, pyrroles, imidazoles, oxazoles, thiazoles, thiophenes, alkyl sulphides, and disulphides, have been identified.

In addition to the Maillard reactions and associated pathways, the presence of small molecular weight precursors - such as free fatty acids, phospholipids, nucleotides, organic salts, and sugars (Belitz et al., 2004) - can degrade, interact, and polymerize with compounds formed during the intermediate stages (Strecker degradation and Amadori rearrangement products) to create a complex matrix of interactions in actual foods. However, most studies on the Maillard reaction and its associated pathways regarding aroma generation have primarily focused on meaty aromas from meat. Their applicability to aroma generation in plant-based protein alternatives remains inconclusive, compounded by the sheer number and rate of reactions involved. Since these rates and interactions are nonlinear, traditional rate order kinetics modelling has proven inefficient (Zhan et al., 2020). The use of multi-response kinetic modelling (Martins, 2003) has been proposed as a pathway to unravel these complexities and effectively visualise flavour interaction outcomes within the proposed parameters.

Non Maillard Interactions

In addition to aroma production from Maillard reaction compounds and their interactions, both intentional and unintentional interactions within the endogenous and exogenous food matrix - comprising proteins, carbohydrates, lipids, minerals, salts, functional ingredients, novel substitutes, and other additives - can significantly modulate aroma output and perception. The four main types of interactions - crosslinking, adduct formation, binding/masking by adsorption, and retention and release—are critical to the outcome of food products (McGorrin & Leland, 1997).

Proteins are the most extensively studied macronutrients in relation to flavour interactions. Due to their complex structure and ability to unfold, form random coils, and create aggregates, proteins offer numerous binding sites compared to lipids and carbohydrates. Cooking food at high temperatures rapidly affects the denaturation of proteins. For instance, the core of a beef burger must reach a minimum temperature of 75°C, while the outer layers can reach temperatures of 180-200°C in grilled foods or even 600°C in the case of flame grilling. The characterisation and analysis of aroma or volatile compounds have not adequately considered this effect. Moreover, most studies in this field have focused on mitigating unintentional

interactions and examining the retention and release of flavour due to factors such as formulation, processing, storage, and eventual release during consumption in ready-to-eat foods like beverages (Ammari & Schroen, 2018) and plant-based foods (Wang & Arntfield, 2017), but not specifically on plant-based meats.

In addition, high-temperature processing of PBMA poses greater challenges, causing the reversibly bound compounds to be lost and the covalently bound compounds to persist, affecting the flavour. Several mechanisms such as covalent, non-covalent (Van der Waals forces, hydrophobic, hydrogen and ionic) bonding, electrostatic linking, Michael addition reactions, thiol and di-/trisulfide exchange reactions involving carbonyl or sulphur-containing compounds (Anantharamkrishnan et al., 2020; Wang & Arntfield, 2017) have all been indicated to play a role. Within the extensive literature available on flavour interactions, cross-referencing of flavour attributes is widely used between animal and plant-based products. However, large differences in the structure and conformation of proteins exist such as caseins and whey proteins in dairy, sarcoplasmic and myofibrillar proteins in meat and legumin, vicilin and glutenins in legume and seed proteins when subjected to varying temperature, pH and other modifications.

Although extensively used as a model compound in protein-flavour interaction studies, β-lactoglobulin's use may be cognisant in the study of protein interaction and storage stability of milk-based beverages but may not be as relevant in studying flavour interactions during the thermal processing of plant-based meats. Therefore, only a general hypothesis can be formed of the interactions involved during HTST processing of comminuted beef burgers and plant-based meat. In a study of commercial yellow pea flower, canola meal and wheat gluten- binding increased with increasing chain length of both homologous aldehydes and ketones with higher hydrophobic affinity to aldehydes compared to ketones. Reversible interaction occurs with -amino (NH_2), sulfhydryl (-SH), hydroxyl (-OH) and carboxylic (-COOH) functional groups and covalent bonding occur between -NH2, -SH functional groups (Wang & Arntfield, 2014). Legume proteins Vicilin (7S) and legumin (11S) were pH-sensitive in terms of binding ability with aldehydes and ketones. Unfolding of proteins during heating leads to aggregation and the formation of random coils. This phenomenon increases numerous binding sites. However, intramolecular binding, such as rearrangement, reduces the binding capability, thereby decreasing binding ability

Covalent bonding has been observed between aldehydes, hydrocarbons, functional groups, and S-substituted compounds (Wang et al., 2022). However, Anantharamkrishnan et al. (2020) did

not find evidence of hydrocarbons forming covalent bonds, attributing this to the lack of functional groups. Acids and alcohols do not form covalent bonds; instead, acids engage in hydrogen bonding and ionic interactions. Esters and lactones were found to be unreactive, as were pyrazines and pyridines. Diketones, such as diacetyl, were reactive, while mono-ketones were not. This reactivity is due to additional carbonyl crosslinking with either arginine or lysine residues, especially under acidic conditions, where they interact with primary amines through the Schiff base condensation reaction, forming adducts. Aldehydes like hexanal were found to interact more readily with proteins via the Schiff base formation than benzaldehyde or furfural. Compounds containing sulphur functional groups were less reactive compared to significant aroma compounds such as propanethiol, 2-furanmethanethiol , and thiophenol, which featured simple thiol groups forming covalent adducts with disulphide linkages. Dimethyl di- and trisulfides also formed covalent adducts. Flavour perception is influenced by strong binding interactions, in contrast to aromas that are not strongly bound and can reversibly bind and release through hydrophobic and hydrogen interactions in the mouth (Guichard, 2005).

Aroma analysis

In a broad sense, the analysis of odours, smells, and aromas involves identifying and detecting compounds through chemesthesis and sensory analysis (Labows and Cagan,1993). It occurs when chemical compounds present on the oral and nasal mucosal surfaces bind to specific receptors in the olfactory cavity, eliciting a sensory response that can be described using aroma descriptors. On the other hand, chemometrics employs instruments to analyse the compounds present in a sample (González-Domínguez et al., 2022). This approach offers a short analysis time and the possibility for automation. Detection and quantification of specific compounds can be achieved objectively, with accuracy and precision. While each compound can be isolated and identified for its respective odour activity, challenges arise due to the diversity of compounds, their concentrations, the specificity of the instrumentation, and the speed and order of elution. The diverse range of factors complicate the perception and identification of these compounds (Dunkel et al., 2014).

Both methods have their advantages and disadvantages, but when used in combination, they provide the most comprehensive information. This integrated approach is the most widely recognized for aroma analysis (Bueno et al., 2019). Therefore, complementary methodologies, along with chromatographic and spectroscopic tools, have been employed to analyse aromas in general, including those derived from animal and plant-based meats (Khan et al., 2015;

Bleicher et al., 2022). This discussion focusses on studies that measured aroma volatiles using chemometrics in the analysis of beef and PBMA cooked at temperatures above 100°C. It emphasizes the use and effectiveness of headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC/MS) as a tool for analysing volatiles including its limits. Although the review is centred on chemometric-based analysis, the significance of perceptual descriptive sensory analysis as reported in literature is acknowledged.

HS-SPME is a widely used technique for analysing volatile compounds in complex matrices. Its popularity stems from its ease of use, durability, and capability to analyze various compounds efficiently. A key advantage of HS-SPME is that it extracts and concentrates analytes in a single step. However, users should be aware of some limitations, which will be discussed further. Excellent resources are available for understanding the fundamental principles of the physical chemistry involved and its applications, including works by Eisert and Pawliszyn (1997) and Murtada and Pawliszyn (2024), as well as a sources focused on flavour analysis by Chiofalo & Presti, 2012. Merkle et al. (2015) have also provided a comprehensive review of the application of SPME in food science.

Since PBMA are formulated with functional ingredients to replicate meat, it is appropriate to apply similar methodologies for analysing their volatile profile. However, the matrix effect should be considered, since beef mince contains a variety of constituents within its intrinsic matrix and differs from PBMA. The behaviour of these compounds can vary significantly when subjected to thermal processing. Additionally, every step from sample preparation, equilibration, extraction and concentration, separation, detection, identification, and statistical analysis are critical in aroma analysis.

Sample preparation

While SPME is an effective technique that requires minimal sample preparation, there are several important criteria to consider when analysing complex media such as meat. These include the presence and concentration of lipids, the state of the samples (whether solid or liquid), expected volatility and degradative properties, and storage after heat treatment (which can contribute to warmed-over flavour development). Sample preparation varied considerably between studies (as shown in Table 2.3). For model studies involving yeast extracts, reagents such as amino acids and reducing sugars were dissolved in distilled water or a buffered solution and then thermally treated at temperatures exceeding 100°C to enhance the formation of

Maillard reaction products. For roast beef samples, cooking methods included preparing the meat whole, as burger patties, or as sliced steaks, all reaching an internal temperature of 70-74°C as recommended. These samples were then chopped or ground and sealed in glass vials for further analysis. Similarly, mushrooms were either roasted or freeze-dried, then ground and rehydrated to create a slurry. Additionally, PBMA were grilled on a pan or griddle and subsequently weighed into headspace vials for analysis.

Fiber selection

SPME fibers come with various coatings (Table 2.2), resulting in different affinities for volatile organic compounds. These coatings include Polydimethylsiloxane (PDMS), Carboxen (CAR), Divinylbenzene (DVB), and Polyacrylate (PA). and a combination of those. Among these, the DVB/CAR/PDMS fiber has been identified as the most used for analysing volatiles in beef, mushrooms, yeast extract, and PBMA. However, comparative studies revealed that CAR/PDMS fibers out-performed DVB/CAR/PDMS in terms of extraction yields, sensitivity, and the number of identified compounds (Mansur et al., 2018).

Table 2.2. Range and type of SPME fibers commonly used in the analysis of volatiles in beef and plant-based ingredients.

Fiber	**Thickness (µm)**	**Polarity**	**MW range**
PA	85	Polar	80-300
PDMS	7	Non-Polar	125-600
PDMS	100	Non-Polar	60-275
DVB/ PDMS	65	Bipolar	50- 300
CAR/ PDMS	75	Bipolar	30-225
CAR/ PDMS	85	Bipolar	30-225
DVB/CAR/PDMS	50/30	Bipolar	40-275

SPME for GC analysis (Merck nd). PA- polyacrylate, PDMS- polydimethyl siloxane, DVB-divinylbenzene, CAR- carboxen. MW-molecular weight.

In a study by Jung et al., (2019), CAR/PDMS was shown to have better adsorptive capabilities compared to PDMS and Polyacrylate when identifying volatiles in mushrooms. This discrepancy is likely due to the pore structure: DVB contains micropores measuring 2–20 Å, while CAR has micro (2–20 Å), meso- (20–500 Å), and macropores (>500 Å) (Chiofalo & Presti, 2012). Although we did not find studies using 7µm PDMS, Rochat et al. (2007) analysed sulphur compounds in roast beef using 100µm PDMS with unsatisfactory results. Additionally,

Alim et al. (2018), Chen et al. (2023), and Costa et al. (2013) employed DVB/PDMS fibers to analyse volatiles in yeast extracts and mushrooms. The temperature used by Chen et al. (2023) for analysing volatiles in yeast extracts was lower compared to that of Alim et al. (2018). Yeast extracts exposed to temperatures above 130°C produced strong nutty, roasted, burnt, and sour odours due to the formation of furans, pyrazines, and thiophenes, in contrast to unheated samples and those tested by Chen et al. (2023). Costa et al. (2019) successfully identified different mushroom strains using volatile profiles with the DVB/PDMS fiber. Similarly, Raza et al. (2019) tested four types of SPME fibers for their ability to adsorb pyrazines from yeast extracts and found that DVB/CAR/PDMS performed better. Overall, there are differing opinions regarding the selection of fibers and their effectiveness. Qualitatively, using multiple fibers provided the most comprehensive information about the volatiles present in the samples (Shirey 2012).

Equilibrium and extraction

The partitioning of volatiles from liquid/ solid samples into the headspace, the attainment of equilibrium conditions and the absorbability of analytes onto the polymeric (SPME) fibers are critical factors in the analysis of headspace volatiles by SPME. Volatilization depends on the microstructure of the sample, partition coefficient, phase ratio, vapour pressure, the polarizability of organic compounds and volatility of the analytes (Eisert & Pawliszyn, 1997; Seuvre et al., 2000; Machiels & Istasse, 2003; Chiofalo & Presti, 2012). Equilibrium refers to the time taken for the sample analytes to volatilize into the headspace of the vial before the SPME fiber is inserted into the vial for extraction and concentration. The application of heat reduces the time taken for the headspace analytes to reach equilibrium (Rocha 2022). However, increased temperatures can potentially present varied results due to the degradation of compounds or precursors (Spanier et al., 2004). CAR/PDMS required a longer extraction time compared to DVB/CAR/PDMS (Chiofalo & Presti, 2012). Also, the presence of lipids and other components alters the partition coefficient by reducing vapour pressure and promoting the formation of aroma compounds (Samavati et al., 2012). However, their lipophilic nature reduces volatility (Page and Lacroix, 1993). Hence, in complex matrices, the extraction or volatilization of aroma compounds remained challenging, meaning volatility was a crucial factor (Buttery et al., 1969; De Roos, 2006). A wide range of temperatures ranging from 40-100ºC was applied in the literature (Table 2.3).

Table 2.3: Protocols used in the analysis of volatiles in Beef and Plant based ingredients using HS- SPME- GCMS

Sample Type	Sample Preparation	SPME Fiber	Equilibrium and extraction condition	Desorption and separation (GC parameters)	Ref
Simulated and Roast beef	1. 2g simulated beef flavour + 5g distilled water. 2. 100 g raw beef steak, chopped 0.8cm³ roasted 190C-15 min, 5g analysed in vial.	DVB/CAR/PDMS	60 min at 60ºC water bath with ±2ºC. Stirring constantly @ 250rpm. 60ºC applied based on total area counts and number of peaks. Also veg hotdogs served at 60C	Injection- 250°C for 3min, splitless. GC Temperature ramp 40°C for 3min, then to 180°C @ 3 °C/min and then to 260 °C @ 10°C/min, Held at 260 °C for 2min. Carrier gas - Helium. Column: DB5/ FID	1
Model	Reagents buffered with sodium phosphate, then heated treated at 140 °C for 30 min.	DVB/CAR/PDMS	Equilibration at 50ºC for 20 min whilst stirring. IS (0.1 µL of 65 ng of 1,2-dichlorobenzene in 1 µL of methanol) added before desorption of the Fiber	Aroma extract dilution analysis/gas chromatography-olfactometry (AEDA/GC-O) Carrier gas- Helium. Column: DB-Wax	2
Grilled beef steaks	Aged beef steaks cooked to 71 °C, rested for 3 minutes. Only cores (1.27cm in diameter) were utilized. 15 mL glass vials	85 µm CAR/ PDMS	Equilibration: 5 min at 65 °C. Extraction: 10 min at 65ºC. water bath.	Sample injected onto a cryogenically cooled (-60ºC) column head. Initial hold at -60 °C for 3 min, @ 20 °C /min ramp to 20 °C, a 5 °C/ min to 100 °C, a 10 °C/ min to 125, and a 20 °C/ min to 260 °C with a 3-min hold. Ions within 33-500 m/z range. Column: VF-5ms	3

Grilled beef steaks	Aged beef (3 choice cuts) steaks 2.5 cms thick and grilled at 177ºC, 205ºC or 232°C. Steaks cooked to internal temp 71ºC.	75µm PDMS	CAR/	Equilibration: 60 min at 70 °C. Extraction: 2h at 70ºC. water bath.	Fiber was desorbed at 280°C for 3 min. multi-dimensional gas chromatograph. Columns: 1. BPX5-5% phenyl polysilphenylene-siloxane 2. BP20 -polyethylene glycol. Initial oven temp 40°C and increased at a rate of 7°C/min until reaching 260°C.	4
Hanwoo Beef	24 h postmortem beef. Longissimus muscle with Frozen samples (50g) were cut and powdered using liquid nitrogen and stored at -80ºC. 1gm of powdered weighed into 40ml headspace vial.	75µm PDMS	CAR/	Equilibration: 10 min at 60 °C. Extraction: 30, 45 and 60 min at 60ºC. water bath.	Fiber was desorbed at 250°C for 5, 10 min then retracted and left in injector port for 50 min. split ratio 10:1, flow rate 1ml/min. 3 GC programs 1. Oven 40ºC for 5 min-increased to 250ºC @8ºC/min and held for 5 min. 2. Oven 40ºC for 8min- increased to 200ºC @5ºC/min then increased to 250ºC@10ºC/min and held for 5 min. Carrier gas- Helium. Column: DB-5MS,	5

Roast beef	Beef sirloin roasted in custom oven, no salt no oil. Oven heated from cold to 250ºC in 2 min and maintained for 20 min.	100µm PDMS	1. Direct extraction from oven condenser (custom) 10 min. 2. Percolated condensate purified through Affi-gel-501 exposed to SPME fiber 5 min.	1. GC-Atomic emission detection (AED): Oven 50ºC for 5 min increased to 240ºC @5ºC/min. Carrier gas: Helium. Column: SPB1. 2. GCMS: Initial oven 50ºC for 5 min increased to 250ºC @5ºC/min. Carrier gas- Helium. Column: DB1-MS, 3. 2D-GC-ToF-MS: Injector 240ºC for 5 min splitless, Carrier gas- Helium. Column:DB1 and DB225	6
Yeast Extracts	6g YE +30ml water-high pressure reactor and heated to 100, 110, 120, 130 and 140ºC) for 1 h.	65µm DVB/PDMS	5ml heated YE sample + 1µL of IS (2-methyl-3heptanone) in 20ml vial. Equilibration: 20 min at 55 °C. Extraction: 40 min at 55ºC water bath	Oven 40ºC for 3 min increased to 230ºC @5ºC/min held for 5 min then increased to 260ºC @10ºC/min for 3 min. Carrier gas- Helium. Column: DB Wax and DB 5MS.	7
Yeast Extracts	Unheated YE1.5g+3 ml water in 20ml vial+	65µm DVB/PDMS	IS-10 µL of 2-methyl-3-heptanone (0.1 mg/mL in MeOH). Equilibration: 20 min at 45 °C. Extraction: 40 min at 45ºC water bath	Desorbed splitless @240ºC for 5 min. Initial oven 35ºC fo+E8:E9r 5 min increased to 250ºC @ 5ºC/min and held for 5 min. Carrier gas- Helium @ 1.04ml/min Column: ZB-wax	8

Mushrooms	Washed, dried and roasted @163ºC for 7-8 min then either roasted again for 9 min or steamed for 10 min and frozen. Defrosted samples were homogenized with a mortar and pestle. 1g sample added to 20ml vial. Quantification conducted with *A. bisporus* mushroom powder.	50/30 DVB/CAR/ PDMS	IS- 0.5 mg/ml of 1-octanol in MeOH Autosampler: agitation 250rpm Equilibration: 15 min at 40 °C. Extraction: 20 min at 40ºC water bath	Splitless injection GCMS Column: ZB-Wax	9
Mushrooms	6 types of fresh mushrooms were lyophilized and powdered. 200 mg was weighed in an amber vial (16ml) with 5 mL of 6% salt solution.	PA, PDMS and CAR/PDMS	Equilibration: 15 min at 100 °C. Extraction: 10 min at 100ºC using hot plate.	Splitless mode, desorbed at 280°C for 3 min. Oven 40°C for 5 min increased to 190°C @ 3°C/min and held for 15min Carrier gas- Helium @1.2ml/min. Column: Supelco-Wax10	10

Mushrooms	A. bisporus not heat treated divided in two groups. 1. mushroom, distilled water and peanut oil (2:1:1) was combined then homogenized; 2. Mushrooms coarsely chopped and then combined with water and oil in the same ratio as above. 0.1 g of sample added to 10ml crimped vial.	Tested: 50/30 DVB/CAR/ PDMS Used: 65µm DVB/PDMS	Autosampler: Agitated. Equilibration step omitted. Extraction: 20 min at 50 °C	GC-FID 330ºC @ 40ml/min H2 gas and 400ml/min air: Desorbed at 250ºC for 1 min in splitless mode during sampling then split 1:20. Oven 40ºC increased to 250ºC @3ºC/min held for 10 min. Carrier gas- Helium. Column: - SLB-5MS	11
Meat and PBMA	Assorted samples minced and formed into 20g patty, either uncooked, pan fried to internal temp 75°C or fried to golden. Cooked samples 1g+2g water prepared with IS	50/30 DVB/CAR/ PDMS	IS: 0.5µg/g 4-methyl-1-pentanol. Equilibration: step omitted. Extraction: 60 min at 40ºC	Desorbed splitless @240ºC for 5 min. Initial oven 35ºC for 5 min increased to 250ºC @ 5ºC/min and held for 5 min. Carrier gas- Helium @ 1.04ml/min Column: ZB-wax	12
Commercial PBMA	Assorted commercial meat and plant-based burger patties cooked as per instructions on electric gridle. 2g of cooked or uncooked sample weighed into 20ml vial	50/30 DVB/CAR/ PDMS	IS: 10µl of 1,2-dichlorobenzene (0.8µl/ml). Equilibration: 5min at 70ºC. Extraction: 50 min at 70ºC	desorbed splitless @250ºC for 2 min. Initial oven 40ºC for 2 min increased to 280ºC @ 5ºC/min and held for 5 min. Carrier gas- Helium @ 1.0ml/min Column: TG-5MS	13

PBMA +myoglobin	Plant based burgers prepared with texturized soy proteins- Burger's patty 30 g shaped manually and baked for 12 min at 250ºC in an oven. 3g cooked sample sealed in a 20ml vial.	50/30 DVB/CAR/ PDMS	Autosampler: agitated 250rpm. Equilibration: 20 min at 45ºC. Extraction: 40 min at 45ºC	desorbed splitless @ 250ºC for 5 min. Initial oven 35ºC for 5 min increased to 280ºC @ 5ºC/min and held for 5 min. Carrier gas- Helium @ 1.0ml/min Column: TG-5MS	14

1. Soo-Yeun et al. (2006); 2. Zhao et al. (2019); 3. Legako et al. (2016); 4. Wall and Kerth. (2019); 5. Van ba et al. (2010); 6. Rochat et al. (2007); 7. Alim et al. (2018); 8. Chen et al. (2023); 9. Davila et al. (2022); 10. Jung et al. (2019); 11. Costa et al. (2013); 12. Kaczmarska et al. (2021); 13. He et al 2021; 14. Devaere et al 2022.

In contrast, during sensory evaluation, the samples are presented at a much lower temperature. The flavour binding and interactions at these two temperature ranges could differ, leading to variations between sensory and chemometric data. Figure 2.3 below highlights the range of factors affecting the equilibrium and extraction of analytes using SPME especially when considering comprehensive untargeted analysis from complex media such as whole foods.

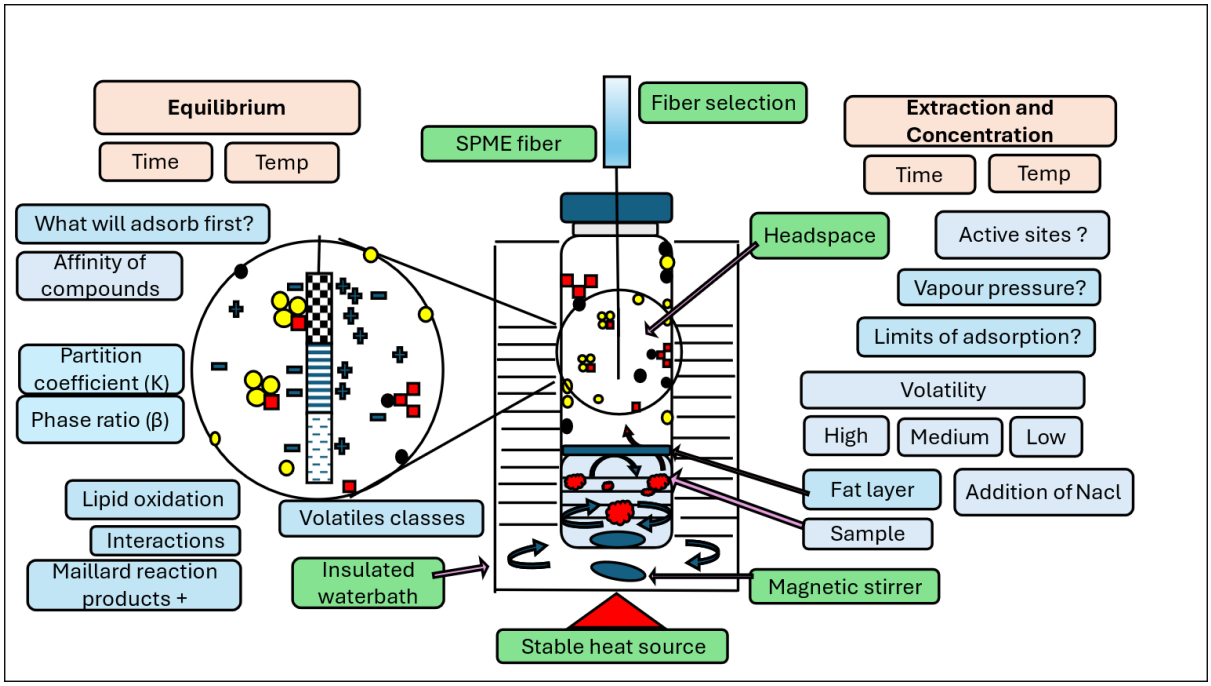

Figure 2.3. Factors affecting the analysis of volatiles using HS-SPME.

Separation, detection and identification

The three critical aspects - form the basis of chromatographic analyses. Separation is achieved by considering the polarity of analytes to eluate and complexity of the matrix by selecting a column of specific length, dimension and stationary phase. The most used columns identified were 5MS and WAX (Table 2.3). Although the use of hydrogen as a mobile phase is becoming increasingly popular, helium was predominantly used in these studies. The two most common detectors identified were Mass Spectrometers (MS) and Flame Ionization Detectors (FID). MS detectors are highly sensitive and can identify a wide range of compounds from complex media, especially when used in conjunction with a compound library. It was noted that electron ionization was consistently applied at 70 eV, though the mass-to-charge (m/z) values varied (35-600) depending on the volatiles being analysed. The temperature of the ion source and detector was also varied between studies. FIDs are particularly suitable for hydrocarbon analysis and are favoured for their stability and linearity in detection, particularly where quantitation was preferred, such as FAME analysis.

Isotope labelling studies (Zhao et al., 2019) have successfully detected and enumerated a greater number of compounds and formation pathways, possibly due to the reduced matrix effects. However, it remains uncertain whether these pathways and compounds can be identified within a whole sample. Other techniques, such as Electrospray Ionization (ESI), Atomic Emission Detection (AED), and Ion Mobility Spectrometry (IMS), have also been utilized. The use of novel methodologies and soft ionization tools, such as Selected-Ion Flow-Tube (SIFT), has enabled the analysis of sensitive organosulfur compounds in garlic (Ozcan and Barringer, 2020) as well as the investigation of aroma compound development during mastication through breath analyses (Sumonsiri & Barringer, 2013). Other tandem spectroscopic methods combined with chromatography, such as Proton Transfer Reaction-Time of Flight-Mass Spectrometry (PTR-ToF-MS) (Beauchamp et al., 2022), GC E-Nose (Ray 2011), GC-Olfactometry (GC-O) (Kanjana & Rouseff, 2011; Machiels et al., 2003; Rochat & Chainterau, 2005), have also been employed. Various separation methodologies are applied prior to GC-O screening to better enable odour analysis from complex matrix. Challenges in identifying compounds often arise from overlapping and combined effects when using Olfactometers and E-Noses. The significance of Multidimensional Gas Chromatography (MDGC) has also been reviewed, demonstrating its ability to resolve overlapping peaks, improve low-level detectability, enhance resolution, and reduce interferences (Amaral & Marriott, 2019). Identification of key aroma-active sulphur compounds, which are highly unstable and generally present in very low amounts, was made possible by Rochat et al. (2007), who utilized 2DGC-ToF-MS-Olfactometry and HS-SPME-GC-MS to analyse roast beef top notes. The application of multi-dimensional gas chromatography (MDGC) technology could increase consistency in compound identification thus providing a more accurate aroma perception helping improve separation and identification.

Compound libraries and GC retention databases, such as Wiley, NIST, Flavornet, Food db, etc. have assisted in the identification of compounds (Babushok 2015., Bizzo et al., 2023). Volatiles identified from the chromatogram were presented in classes for ease of analysis. The use of retention time along with reference material have been used as a tool to identify compounds of interest (Adams, 2007). However, the use of retention indices along with some considerations has been suggested to be more accurate compared to retention times (Bianchi et al 2007., Bizzo et al 2023).

Identification of volatiles

It is commonly presented in literature is that only a few volatile compounds are important amongst others, possess perceptual capability and serve as key aroma compounds (Rocha et al., 2022). However, compounds could become reactive due to changes in their environment (reaction/interaction) and yield significantly different outcomes during processing i.e. A key aroma compound present at a specific locus may not present significance at another. Hence the use of comprehensive untargeted analysis has gained popularity due to the information it delivers. Diez-Simon, Mumm and Hall (2019) reviewed studies using metabolomics of volatiles based on Mass spectrometry. Other more targeted use of omics such as "Foodomics"- was applied to analyse the effects of lipids in aroma generation in beef (Zhou et al, 2024). "Sensomics"- was used to study the loss of aroma during storage and identified key volatiles responsible for meaty notes (Zhang et al, 2023). "Flavouromics"- Integrated flavour and sensory analysis combined with chemometric studies using the metabolomic approach (Ötleş., and Özyurt., 2023). "Volatilomics"- A validated method was developed to discriminate between meat species namely beef and pork using multivariate data analysis (Pavlidis et al., 2019). Carbon module labelling (CAMOLA) was employed to observe beef-meaty aroma compounds in yeast extracts (Alim et al., 2019).

Table 2.4 Volatiles observed in various beef, mushroom and yeast extracts using HS-SPME-GCMS.

Aroma Compounds	Odour descriptor/ sensory	Sample type/ Ref
Aldehydes		
2-Methylpropanal	Brothy, **meaty,** Caramel, malty sweet, slight (sl) Cereal	Mushroom[2], grilled beef [13]
Benzene acetaldehyde	green, rosy	Yeast extract[15]
3-Methyl butanal	Chocolate, yeasty, malty, sour milk, potato, earthy, raw meat, caramel, dark chocolate, malty, fresh nuts, sweet, pleasant	Roast beef [1], mushroom[2] [8], grilled beef [5] [10] [11] [13], autoclaved beef [6], yeast extract [9] [15], cooked beef [14]
2-Methyl butanal	Cocoa, Yeasty, malty, sour milk, potato, earthy, raw meat, caramel, Chocolate, nutty	Roast beef [1], mushroom [2] [8], grilled beef [5] [10] [11] [13], autoclaved beef [6], yeast extract [9] [15], cooked beef [14]
Pentanal	almond, malt, pungent, acrid	Roast beef [1], mushroom [2], model [3], grilled b [5] [10] [11] [12], autoclaved beef [6], cooked beef[14]
Hexanal	fresh, grassy, green, barnyard, green, cut grass, floral, fragrant, slight (sl) fruity	Roast beef [1], mushroom [2] [8], model [3], grilled beef [5] [7] [10] [11] [13], autoclaved beef [6], cooked beef [14], yeast extract[15]
E-2-Hexenal	Livery	Autoclaved beef [6], mushroom [8], grilled beef[13]
Heptanal	Nutty, fatty, green, floral, fragrant, stale, rancid nuts	Roast beef [1], autoclaved beef [6], grilled beef [7] [10] [11] [12], mushroom [8], cooked beef [14]
2-Heptenal	Soapy, fatty, almond, fishy	Grilled beef [5] [12]
Octanal	Soapy, fatty, orange, soapy, lemon, green, sweet, citrus, hay/grainy	Simulated & roast beef [1], mushroom [2] [8], grilled beef [5] [7] [10] [11] [13], autoclaved beef [6], yeast extract [9], cooked beef [14]
Nonanal	Grassy, tea, vegetable, lemony, sour, **beefy,** hay, stale, plastic, metallic, hay/grainy, stale fat	Simulated, roast beef [1], mushroom [2], model [3], grilled beef [5] [7] [10] [11] [13], autoclaved beef [6], yeast extract [9] [15], cooked beef [14]
Nonenal	Tallow, fatty	Roast beef [1], grilled beef [5] [12],
2-Nonenal	Cardboard, paper, sl. fragrant, **sl. burnt meat**	model [3], grilled beef [5] [12]
(E)-2-Nonenal	Fatty, barnyard	Grilled beef [5] [7] [13], autoclaved beef [6], cooked beef[14]

(E,E)-2,4-Nonadienal	Savoury, potato, sweet, hay/grainy	autoclaved beef [6], grilled beef [7 13], Mushroom [8]
(E,E)-2,6-Nonadienal	Sweet, floral	Grilled beef [13]
Decanal	Rubber tubing, cooked veggie, sweet popcorn, smoky, soapy, fatty, sweet	Simulated & roast beef [1], model [3], grilled beef [5 7 10 11], autoclaved beef [6]
(E,E)-2,4-Decadienal	Musty, stale biscuits, stale, fatty, oily	Mushroom [2 8], autoclaved beef [6], grilled beef [13]
Benzaldehyde	Popcorn, caramel, Burnt sugar, almond, benzaldehyde	Simulated, roast and boiled beef [1], Mushroom [2 8], grilled beef [5 7 10 11], Autoclaved beef [6], yeast extract [9 15 19], cooked beef[14]
Benzeneacetaldehyde	Grainy, sweet	Mushroom [2], Grilled beef [12 13], cooked beef[14]
Phenylacetaldehyde	Herb, oil, burning, rosy, perfume, floral, honey, lavender, fragrant roses	Simulated & roast beef [1], grilled Beef [5 10 11], autoclaved beef [6], mushroom [8]
5-Methyl-2-phenyl-2-hexenal	Wax, **cured meat,** roasted	Simulated beef [1]
Alcohols		
3-Methylbutanol	Malty	Mushroom [2], yeast extract[15]
5-Methyl-2-furan methanol	Butter, creamy	Yeast extract[15]
Linalool	Lemon, Foral	Simulated beef [1], mushroom [2]
4-Terpeneol	Fried, barbecue, **beefy**	Simulated beef [1]
β-Fenchyl alcohol	Bleach, chlorine, wood, dandelion, **meat**	Simulated beef [1]
4-Methyl-5-thiazole ethanol	**Beef,** nutty	Yeast extract [15]
Phenylethyl alcohol	Honey, rosy	Mushroom [2], yeast extract [9]
2-Phenylethanol	Wine -like, floral, flowery	Yeast extract [15]
guaiacol	Grassy	Grilled beef [13]
Ethyl maltol	Caramel	Yeast extract[15]
Acids		
Acetic acid	Sour, acid, pungent	Roast beef [1], mushroom [2], grilled beef [5 11], autoclaved beef [6], yeast extract [9 15]
2-Hydroxy-propanoic acid	Fruity	Yeast Extract [15]

Butanoic acid	Putrid, vomit, musty, rancid, like parmesan cheese, sl. nutty	Mushroom [2], grilled beef [11] [12], yeast extract [15]
3-Methylbutanoic acid	Stinky, sweaty, cheese like, sl.cold meat fat, nutty	Mushroom [2], yeast extract [15]
Hexanoic acid	Rancid, goat-like	Roast beef [1], mushrooom [2], model [3], grilled beef [5] [11], yeast extract [15]
Propanoic acid	Acid, sour, cheese like	autoclaved beef [6], yeast extract [15]
2-Methylpropanoic acid	Fatty, cheese like	Mushroom [2], yeast extract [15]
Esters		
Acetic acid, methyl ester(methyl ethanoate)	Fruity	Yeast extract[15]
Methyl butanoate	Burnt, fruity, bubblegum	Grilled beef [13]
Ethyl butanoate	Bubblegum, sweet, vanilla	Grilled beef [13]
Ethyl 2-methylpropanoate	Fruity	Mushroom [8]
Ketones		
Acetophenone	Musty, almond	Autoclaved beef [6], yeast extract [9]
2,3-Butanedione	Buttery, caramel, sweet, hay/grainy, creamy, butanedione like	Grilled beef [5] [8] [10] [11] [13], Yeast extract [9] [15]
2,3-Pentanedione	Fudge, caramel, sweet**,** sickly, buttery, creamy	Cooked beef [14]
3-Hydroxy-2-butanone	Buttery, sweet, cabbage	Roast beef [1], grilled beef [5] [10] [11] [12], yeast extract[15]
4-Hydroxy-2-butanone	slight sweet	Yeast extract[15],
3-Mercapto-2-pentanone	Sulphury	Model [4]
2-Hexanone	Burnt, sl. HVP	Autoclaved beef [6]
2-Octanone	Fruity, musty	Model [3], autoclaved beef [6], grilled beef [7] [12]
2,3-Octanedione	Fragrant, **sl. Meaty**	Roast beef [1] mushroom [2] [8], grilled beef [5]
2-Nonanone	**Meaty**, green, burning wood	Mushroom [2] [8]
1-Octen-3-one	Mushroom, metallic, earthy	Mushroom [2] [8], autoclaved beef [6], grilled beef [13]
Dimethyl sulfone	Onion, cabbage	Yeast extract [9]

2-Methyl-3-thiolanone	Onion	Yeast extract [9]
Trans-β-damascenone	Honey, beer dregs, metallic, sweet, bitter, chutney (pickled), cooked apple	Yeast extract [15]
Furans		
2-Furanmethanol	Burnt, bready	Mushroom [2], autoclaved beef [6], yeast extract [9 15]
2-Furan methanethiol	Roasted, sulphur	Yeast extract[15]
2-Ethylfuran	Perfume, lemony, **beef**, chemical, beany, ethereal cocoa, bready, malty coffee nutty, cheesy, stale, mushroom, chicory	Simulated beef [1], cooked beef [14]
2-Acetylfuran	Sulphur, sweet, toffee, rancid, balsamic	Simulated beef [1], model [4], yeast extract [9 15]
2-Pentylfuran	Savory, off, green, sweet, fruity, bitter, metallic, rancid, tea,	Roast beef [1], mushroom [2 8], model [3], Grilled beef [5 7 11], autoclaved beef [6], cooked beef[14]
2-Furan carboxaldehyde	Bready, roasted, plastic, rubber, metallic, savoury, potatoes, dhal powder, **meat**, rice,	Grilled beef [5], autoclaved beef [6], yeast extract[15]
Furfural	Bread, sweet	Mushroom [2], Model [3 4], Yeast Extract [9]
5-Methylfurfural	Burnt sugar, caramel, sweet	Yeast extract [9 15]
2-Methyl-5-(methio)-furan	Roasted, **meaty**	Yeast extract[15]
Dihydro-2(3H)-furanone	Creamy	Yeast extract [15]
Dihydro-5-pentyl-2(3H)-furanone	Coconut,	Yeast extract [9]
Dihydro-5-methyl-3(2H)-furanone	Sweet, tobacco like	Yeast extract [15]
Hydrocarbons		
Aliphatic and aromatic hydrocarbons		
Tetradecane	Alkane	Roast beef [1], grilled beef [5 7], mushroom [8]
Pentadecane	Alkane	Simulated beef [1], grilled beef [7]
Calamenene	Herbal, savoury, spicy, yeasty, MSG, **beef**	Simulated beef [1]
Anethol	Liquorice, metallic	Yeast extract[15]

Trans-anethole	Sweet, sulphur	Simulated beef [1]
Styrene	Sl. cereal. Sweet	Grilled Beef [5], autoclaved beef [6]
Alicyclic hydrocarbons		
Terpenes		
α-Phellandrene	Fresh	Simulated beef [1]
2-Pinene	Pine	Simulated beef [1]
Sabinene	Woody	Simulated beef [1], mushroom [2]
δ-3-Carene	Floral, sugar, honey, smoky, **beef**, lemon	Simulated & roast beef [1], mushroom [2 8]
D-Limonene	Grassy, rancid, rubbery, lemon, cooked cereal, sweet	Mushroom [2], cooked beef[14]
ϒ-Terpinene	Grassy, plastic, smokey, cod liver oil, **beef**, lemon	Simulated beef [1]
α-Elemene	Barn yard, smelly socks, freshly cut wood, sulphur, burnt	Simulated beef [1]
β-Cubebene	Rotten, dusty, yeasty, rubbery	Simulated beef [1]
β-Elemene	Sewage, green tea, **meaty**, MSG	Simulated beef [1]
β-Caryophyllene	Soil, **meaty**	Simulated beef [1], Mushroom [8]
epi-Bicyclosesquiphellandrene	Ashy, yeast, sulphur	Simulated beef [1]
ϒ-Cadinene	Herb, burnt, sulphur, Woody	Simulated beef [1]
β-Selinene	Herbaceous	Simulated beef [1]
δ-Cadinene	Woody	Simulated beef [1]
1,2,3,4,4a,7-Hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	Medicine, caramelized, roasted	Simulated beef [1]
Oestragole	Herbaceous, anisic	Yeast extract[15]
Curcumene	Pungent	Yeast extract[15]
Octane	**Meaty**	Grilled beef [5 10 11 12], mushroom [8]
Nonane	Sour, burnt, cracker	Mushroom [8], grilled beef [10 11 12]
N containing compounds		
Pyrazines		
Acetylpyrrole	Unpleasant, plastic, antiseptic	Simulated & roast beef [1]

2-Acetyl-1-pyrroline	Popcorn, roasted, cooked rice, green, fatty, stale, mushroom, popcorn, old food, roasty,	Mushroom [8], grilled beef [13]
2-acetylpyrrole	Nutty, roast, toast	[1],Mushroom [2], grilled beef [5] [12], yeast extract [9] [15]
Pyrazines	Potatoes, dhal powder, green, leafy, roasted**,** sulfur	Cooked beef [6]
2-Ethyl-3-methyl-pyrazine	Strong roasted nuts	Grilled beef [5]
2-Ethyl-6-methylpyrazine	Fruity	Simulated beef [1], mushroom [2], grilled beef [5]
2-Ethyl-3,6-dimethyl pyrazine	**Meat**, smokey, sulphur, natural gas, soil, nutty, roasted, potato	Simulated & Roast beef [1], autoclaved beef [6]
Methyl pyrazine	Popcorn, burnt, roasted, nuts, sweet	Mushroom [2], grilled beef [5] [11], yeast extract [9] [15], cooked beef [14]
2,5-Dimethyl pyrazine	Sulphurous, cooked vegetables, **sl. meaty**	Grilled beef [5] [10] [11], autoclaved beef [6], cooked beef [14]
2,3,5-trimethyl-pyrazine	Burnt, bread, musty	Grilled beef [5], yeast extract[15]
Trimethyl pyrazine	Burnt, smokey, painty, solvent, nutty, roasted, potato, caramel	Simulated beef [1], grilled beef [5] [11] [12] [13], yeast extract [9]
2-Ethyl-5-methylpyrazine	Fruity, sweet	Yeast extract[9]
2-Ethyl-3-5/6-dimethyl pyrazine	Burnt nuts	Grilled beef [10]
2-Ethyl-3,5-dimethylpyrazine	Potatoes, dhal powder, **meat**, rice, potato**,** roasted, potato like, burnt nuts	Grilled beef [11] [13]
5-Ethyl,-2,3-dimethylpyrazine	Caramel	Grilled beef [13]
2,5-Dimethyl-3-(3-methylbutyl)-pyrazine	Fruity	Grilled beef [5], yeast extract [9]
2,6-Diethyl-3-methylpyrazine	Baked	Yeast extract [9]
2-Isoamyl-6-methylpyrazine	Rubbery, cabbage, sweet, mint	Simulated beef [1]
Trimethylpyrazine	Chocolate, earthy, caramel, mushrooms, fresh, metallic**,** hay**,** butter, nutty	Simulated beef [1], grilled beef [13]
2-Ethyl-3,6-dimethylpyrazine	Potato, roast, burnt potato skin, fatty, gravy, savory, metallic	Yeast extract [9]
3-Isopentyl-2,5-dimethylpyrazine	Yeasty, fermented, foral perfume	Simulated beef [1], mushroom [2]
Indole	Burnt	Yeast Extract [9]

S containing compounds		
Methional	Cooked Potato, roast potato, savoury, vegetable, dhal powder, meat, rice, potato, roasted, cooked potato, sulphurous, **boiled meat, meat extract**	Mushroom [2 8], yeast extract [9 15], grilled beef [11 13],
Methionol	Sweet soup, **meaty**	Mushroom [2], [15]
Dimethyl sulfide	Grassy, caramel. Blood	Grilled beef [5 7 10 11 13]
Dimethyl disulfide	**Meaty**, sulfur	Mushroom [2], autoclaved beef [6], grilled beef [10 11], Yeast extract[15]
Dimethyl trisulfide	Sulfur, garlic, cooked cabbage, stale, metallic, geranium, pungent, sl. sulphurous, canned vegetable soup	Mushroom [2], autoclaved beef [6], yeast extract [9 15]
Dimethyl tetrasulfide	**Meaty**, savory, burnt	Autoclaved beef [6]
Thiophene	Garlic	Model [4], yeast extract [9]
2-Methylthiophene	Floral, grass. **burnt beef**, sulfur, metallic, green, savory	Simulated beef [1], model [3 4], yeast extract [9]
2-Ethylthiophene	Rotting food	Model [4]
2-Acetylthiophene	Sulfur, chlorophenol, phenolic, disinfectant,	Yeast extract [9]
2-Furfurylthiol	Roast	Model [4], yeast extract [9]
3-Thiophenethiol	**Meaty,** roasted, chicken, metallic,	Model [4]
2,5-Dimethylthiophene	**Beef**, sweet, ham, rancid	Simulated beef [1], model [4]
2-Acetylthiazole	Popcorn, roasted, **meaty**, burnt, mealy, pungent, cooked rice	Model [4], autoclaved beef [6], grilled beef [13], cooked beef[14], yeast extract[15]
4,5-Dimethylthiazole	**Meaty**, sulphur, roast, smoky, **boiled meat**	Simulated beef [1]
5-Ethyl-2,4-dimethylthiazole	Burnt nuts	Model [4]
Benzothiazole	Chicken, metallic, geranium, phenolic, smoked ham, **meaty**, spicy, plastic, stock, rubber	Mushroom [2]
2-Thiophenecarboxaldehyde	Sulphur	Yeast extract[9]
3-Methyl-2-thiophenecarboxaldehyde	Ham, sweet, **beefy**, savoury	Simulated beef [1]

2-Methyltetrahydrothiophen-3-one	potato skins, pungent, savory, sulfur, roasted, metallic, oily, potato, earthy, rancid, roasted	Model [4]

1. Soo-Yeun et al. (2006); 2. Davila et al. (2022); 3. Zhang et al. (2021); 4. Zhao et al. (2019); 5. Wall et al. (2019); 6. Van Ba et al. (2010); 7. Wang et al. (2018); 8. Aisala et al. (2019); 9. Alim et al. (2018); 10. Legako et al. (2016); 11. Hunt et al. (2016); 12. Blackmon et al. (2015); 13. Mountford et al. (2014); 14. Ma et al. (2013); 15. Lin et al. (2014).

Herein, an extensive range of volatiles is compiled and presented in classes (Table 2.3). The studies form a comparative guide on the development and formation of savoury aroma compounds. The studies were selected based on the use of plant-based ingredients such as yeast extract and mushrooms frequently used in the formulation of meat alternatives to enhance meaty or beefy characteristics. The use of HS-SPME-GCMS and samples cooked at temperatures over 100ºC were considered. It is evident that even though ingredients such as yeast extract and mushrooms possess constituents found in meat, they may not promote a similar aroma profile as beef (Table 2.4). Wide variances within the methodologies were also observed among the assays and studies (Table 2.3), including the observed aroma compounds and their associated sensory descriptors. Meat/meaty or beef/beefy as a sensory descriptor was mentioned less than 10% of the times in over 150 volatiles presented. The S-substituted class of volatiles possessed the most meaty and beefy notes. 3-methylbutanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal and benzaldehyde were identified widely. Amongst them 2 and 3-methylbutanal and phenylacetaldehyde were observed to be Strecker aldehydes (Rainer and Eichner 2000). Whereas heptanal, octanal, nonanal and 2(E)-nonenal are formed from thermal degradation fatty acids (Legako et al 2016). Fatty acid when subjected to heat form intermediate hydroperoxides, these further decompose to form an aldehyde with a radical. Under certain conditions such as oxidation and dehydration it could decarboxylate to form another aldehyde (Specht & Baltes, 1994, Frankel, 2005). Lipid oxidation products (LOP) such as 4,5-dihydro-5-propyl2(3*H*)-furanone (γ-heptalactone) and 4,5-dihydro-5-butyl-2(3*H*)-furanone (γ-octalactone) are known to be potent odourants. These including γ-hepta-, γ-octa- and γ-nonalactone as well as δ-lactones were amongst the ketones observed in fats heated at 145ºC by Watanabe and Sato. (1971) in Specht & Baltes, (1994). C_5-C_{10} aldehydes and fatty aldehydes such as benzaldehyde, (E)-octenal and alcohols 1-pentanol,1-hexanol, 1-heptanol, 1-octanol,1-nonanol, 1-octen-3-ol and (*E*)-2-octen-1-ol can be formed from different pathways such as lipid oxidation and degradation of unsaturated fatty acids (linoleic acid and α-linolenic acid) but also from the degradation of carbohydrates and amino acids (Strecker degradation-

Maillard reactions). Individually, octanal emitted soapy, fatty, harsh, orange peel, lemon, green, honey, sweet, and fruity notes. However, a combination of octanal, 2,3,5-trimethylpyrazine, and 2-ethyl-5-methylpyrazine produced fruity, green, sweet, roasty, and pungent aromas.; 2,3,5-trimethylpyrazine revealed toasted and roasted coffee notes, while 2-ethyl-5-methylpyrazine invoked roasted nuances (Kerth, 2017; Van Ba et al., 2010; Specht & Baltes, 1994). It is noteworthy that Specht and Baltes, (1994) observed that the majority of the compounds possessing high aroma activity presented fatty, sweet or roasted nuances towards roasted meat character in shallow fried beef, but no beefy or meaty notes were observed. Also, they agreed with the findings of Cerny and Grosch (1992) that other than methional, s-substituted compounds did not produce meaty flavour notes rather a more roasty note similar to the one observed from 2-acetylthiazoline.

Aroma compounds from roasted mushrooms potentially possessed meaty aromatics from the presence of furans, pyrolles, pyridines and thiazoles (Davila et al 2022). One of the limitations of the studies reported in Table 2.4 and literature in general is that the volatiles may have been observed when cooked but not assessed for their effectivity in a formulation.

Conclusion

The challenge with comparing beef burgers to its plant-based counterparts is that there is no set standard for creating a beef burger patty let alone comparing aroma outcomes of beef burgers to plant-based meats. It is also important to note that beef burger patties are supplemented with several plant-based ingredients to achieve characteristic flavour. As such, some critical considerations need to be noted. 1. All or most of the plant-based ingredients are pre-processed unlike beef mince. They have been subjected to thermal processing and have undergone a significant amount of simultaneous oxidation, degradation and interaction with Maillard reaction products (SODIM) effect before being formulated to be further cooked. 2. Composition and the presence of precursors vary widely between the two. 3. Use of additives such as herbs & spices (allium, brassicas) and seasonings (salt, sugar, vinegar) Flavourings (Hydrolysed proteins, Yeast extracts, mushroom extracts, smoke flavour etc) remain the same. Meaning, a well-seasoned beef or plant-based patty would be more appreciated compared to an unseasoned patty. However, the addition of similar flavourants may not elicit similar outcomes due to the differences in their composition and formation of ternary complexes protein-carbohydrate-lipid interactions.

The findings of the review resonate with the findings of the numerous reviews and studies conducted to date. We are yet to come to a consensus on the appreciability of plant-based meat. Using a multifaceted approach by defining keywords such as Flavour, using a systematic methodology in sensory, chemometrics, and omics and identifying and mapping formation and related pathways of compounds in beef and relating them to plant-based protein alternatives at a molecular level could yield an objective comparison that can help formulators develop products with increased Appreciability without the need to ascribe meatiness to plant-based proteinaceous foods. Differences in aroma profile between plant-based meat and beef burgers have already been observed and their likelihood of acceptance rate is low. It seems counter intuitive to research and characterize meat, when we are trying to develop, promote and advocate the consumption of plant-based foods. It has also been pointed out as comparing apples to oranges. However, knowledge gaps prevent us from progressing to the next step due to the fragmented approach of research studies. Additionally, extensive studies have been conducted on protein and other interactions in meat, its direct correlation to the use of fractionated legume, cereal and seed proteins in creating plant-based meat is difficult due to the varied matrix. Increased use of fortification with novel substitutes and functional ingredients necessitates further research to be able to understand aroma- matrix interactions.

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Chapter 3

Optimizing Conditions for the Detection of Aroma Compounds in Beef Mince Using Head Space -Solid Phase Micro Extraction -Gas Chromatography /Mass Spectrometry

Introduction

Most plant-based meat analogues are designed to closely resemble their animal counterparts, particularly beef. Comminuted beef, such as minced or ground meat, is the key ingredient that is often replicated to create plant-based products like burgers, sausages, and meatballs. However, developing a comparable aroma profile has proven to be challenging for several reasons (Thong et al., 2024; Wang et al., 2022; Pavagadhi and Swarup, 2020). While raw meat does not have a significant aroma, the formation of important aroma compounds in cooked meat can vary based on factors such as composition, formulation, and processing, as discussed in Chapter 2 of the literature review.

Aroma analysis is commonly employed to evaluate the flavor of meat and meat products using established methodologies, such as hyphenated Gas Chromatography Mass Spectrometry (GC-MS). This technique generates accurate, repeatable, and reproducible data; however, the results can be highly specific, meaning they may vary between different equipment (Hübschmann, 2009). Coupled with the inherent variability of biological food samples within complex matrices, this makes the extraction and concentration of analytes for detection and analysis challenging (Mahattanatawee & Rouseff, 2011). Nevertheless, untargeted comprehensive analysis can reveal critical information.

Several extraction methods have been developed to isolate, extract, and concentrate analytes for analysis, with various comparisons made among them. These methods include Solvent-Assisted Flavor Extraction (SAFE), Accelerated Solvent Extraction (ASE), Supercritical Extraction (SCE), Steam Distillation (SD), Simultaneous Distillation-Extraction (SDE), Solid Phase Extraction (SPE), and Solid Phase Micro Extraction (SPME) (Cadwallader & Macleod, 1998; Bleicher et al., 2022). The volatiles in meat have been extensively studied using SPME, as discussed in Chapter 2. Among the array of methodologies available for determining volatiles, Headspace Solid Phase Micro Extraction (HS-SPME) is particularly advantageous, despite its limitations (Sun et al., 2021; Hartonen et al., 2019). Watkins et al. (2012) demonstrated that employing multiple methods can provide complementary results, rather than one method being unequivocally superior to another.

In analysing the study results, four important factors were identified as critical to the assessment.

1. **Sample Selection and Preparation**: It is essential to ensure that all total volatiles are captured, including evaluating the effects of storage on the samples.

2. **Equilibration and Extraction**: The method for equilibrating and extracting analytes must be tailored to the volatility of the compounds present in the complex matrix.

3. **Column Selection and Parameter Optimization**: Choosing the right column and optimizing the parameters are vital for achieving effective separation of the analytes.

4. **Detection and Data Analysis**: The final step involves the detection of the analytes and the thorough analysis of the obtained data.

Figure 3.1 below illustrates the challenges involved in establishing a starting point for developing optimized parameters for the analysis of volatiles using Headspace Solid-Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME-GC/MS).

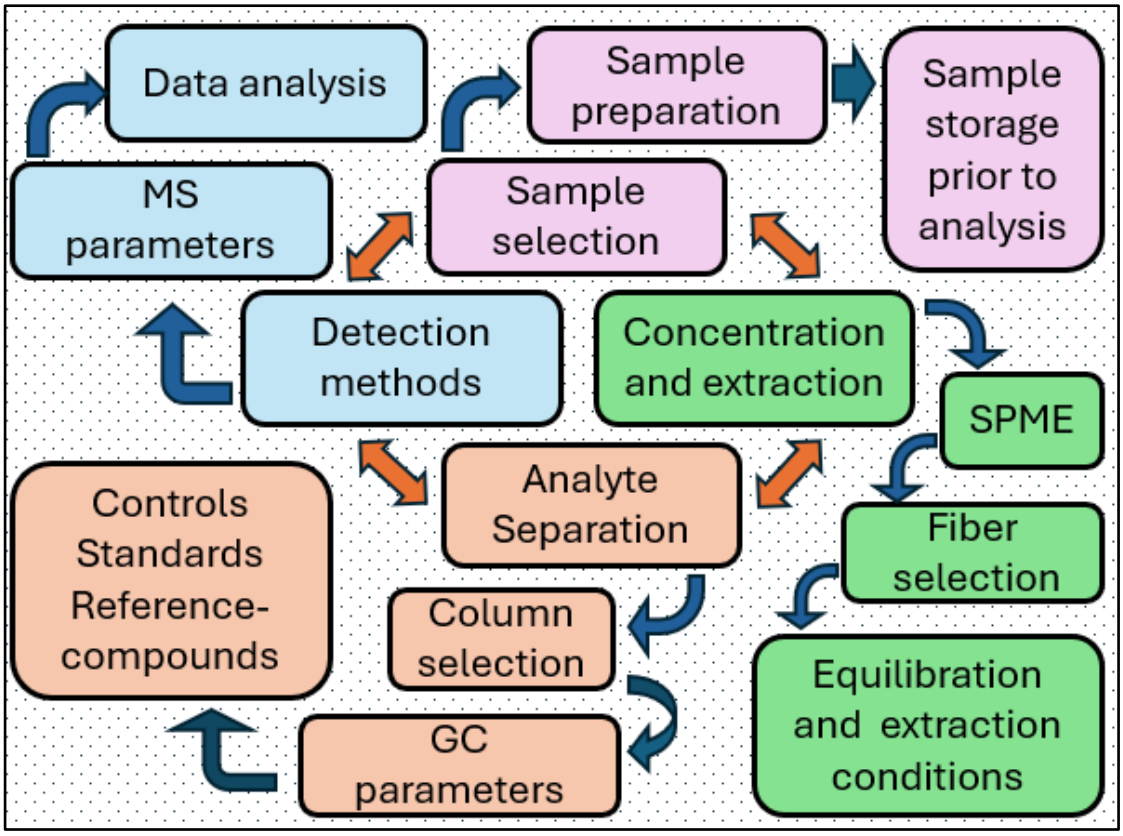

Figure 3.1 The challenges in ascertaining a starting point in developing optimized parameters for the analysis of volatiles using HS-SPME-GC/MS. Without prior knowledge, hierarchical workflow designs can be unproductive.

Sample selection and preparation

While some volatile compounds are commonly found in various foods, how samples are selected and prepared is crucial for the formation and development of key aroma-active compounds (Blackmon et al., 2015). The method of preparation and cooking can also result in

different aroma profiles, as extensively documented (Gardner & Legako, 2018; Cabral, 2019). Regarding the storage of cooked meat, the phenomenon known as warmed-over flavour can negatively impact eating quality (Younathan, 1985; Shahidi, 2020). This indicates that sample selection and preparation significantly influence the volatile compounds produced. Additionally, this process depends on various other factors, which will be discussed further.

Fiber selection

A variety of Solid Phase Microextraction (SPME) fibers have been studied for their effectiveness in adsorbing analytes of interest (see Chapter 2, Table 2.2). The adsorption capacity of SPME fibers is influenced by the range and polarity of the analytes (Kataoka et al., 2000). In food analysis, the most commonly used fibers are 50/30 µm DVB/CAR/PDMS (molecular weight 40-275) and 75 µm or 85 µm CAR/PDMS (molecular weight 30-225).

Wang et al. (2018) found that the 75 µm CAR-PDMS fiber performed better than the 50/30 µm DVB/CAR/PDMS fiber when equilibrated at room temperature and extracted for approximately one hour. Conversely, Machiels and Istasse (2003) reported that the 50/30 µm DVB/CAR/PDMS fiber outperformed the 75 µm CAR/PDMS fiber, demonstrating more efficient adsorption. Additionally, Elmore et al. (2001) utilized both fiber types simultaneously in order to achieve a more comprehensive aroma profile.

Equilibration and extraction

Solid phase microextraction (SPME) is a non-exhaustive analytical method that relies on the adsorption of volatile compounds reaching equilibrium during the extraction phase. While time and temperature are critical factors, several other variables also influence the rate of equilibrium, concentration, and, ultimately, extraction. These variables include vial size, sample size, sample type, analyte concentration, vapour pressure, partition coefficient, venting, and agitation or stirring (Hübschmann, 2009; Chiofalo & Presti, 2012).

A review of the literature reveals that samples have been equilibrated at a wide range of temperatures, including semi-thawed states (Flores et al., 2006), room temperature (Wang et al., 2018), 40°C-50°C (Chen et al., 2023; Davila et al., 2022; Kaczmarska et al., 2021), 51°C-60°C (Soo-Yeun et al., 2006; Zhao et al., 2019; Van Ba et al., 2010), 61°C-70°C (Legako et al., 2016; Wall & Kerth, 2019), and even 100°C (Jung et al., 2019) Choosing the appropriate temperature can be particularly challenging when dealing with complex matrices. Achieving consensus on the optimal equilibrium or extraction conditions has proven difficult, especially for whole beef samples, due to the various components present.

Relying on the area count of total ion chromatogram (TIC) as a measure can be inappropriate without understanding how volatility is affected by the presence of lipids and other constituents (Roberts et al., 2000). Most analyses emphasize the need to agitate or vibrate the sample (Nolvachai et al., 2023; Rocha et al., 2022). Consequently, samples often need to be ground, homogenized, and sufficiently liquefied for proper agitation. However, Januszkiewicz et al. (2008) noted that while agitation generally increases extraction, it may also reduce the extraction of certain compounds. Additionally, they found that the addition of 6M sodium chloride (NaCl) solution alone enhanced the extraction of analytes more effectively than agitation. The use of salt creates a "salting out" effect (Murtada & Pawliszyn, 2024), although it comes with some limitations. Moreover, various other factors, such as instrument capability, column selection, and detection methods, can also impact the analysis (Hübschmann, 2009).

Desorption and Separation

Desorption of the adsorbed analytes occurs in the injector port of the gas chromatograph. A suitable temperature in the injection port is essential for effective desorption of SPME (Solid Phase Microextraction) fibers. If the injection port temperature is too low, it may not effectively desorb the compounds, resulting in peak broadening or carryover. Conversely, if the temperature is too high, the desorbed compounds may undergo thermal degradation (Gaffke and Alborn, 2021).

The optimal temperature for the injector port depends on two key factors: the maximum temperature range of the column being used and the type of sample being analysed. Although the manufacturer recommends a temperature range of 250°C to 310°C for CAR/PDMS fibers and 230°C to 270°C for DVB/CAR/PDMS fibers, a generally adopted range is 240°C to 280°C (see Chapter 2, Table 2.3). However, an optimal temperature of 200°C was observed (Gaffke and Alborn, 2021).

Additionally, the length of time required for complete desorption of analytes from the fiber is crucial to minimize carryover and improve peak resolution (Gaffke and Alborn, 2021). Using an appropriate septum and liner designed for this purpose is also vital, as it can help reduce ghost peaks. A narrow bore straight liner can improve the resolution of early-eluting peaks without necessitating cryogenic cooling of the column head (Yang and Peppard, 1994).

As highlighted in the literature review, achieving separation involves using an appropriate stationary phase—either a non-polar (5-MS) or a slightly polar (Wax) column, typically ranging from 30 to 60 meters in length. In some cases, longer 100-meter columns have been

utilized for analyzing fatty acid methyl esters. Several parameters, including time and temperature ramps, flow rate, and on-column focusing, must be optimized to achieve relevant Detection

Several detection methods are employed to identify analytes separated by gas chromatography (GC). One of the most commonly used detectors in food flavour analysis is the mass spectrometer (MS). This tool provides information at a molecular level by breaking molecules into ions—either through electron or chemical ionization—and expressing them as a mass-to-charge ratio (m/z) (Soo-Yeun et al., 2004; Zhao et al., 2019)).

Detection is achieved by optimizing various parameters, such as ion source temperature, interface temperature, acquisition mode, scan speed, and mass-to-charge ratio range. While detecting targeted compounds may be simpler, optimizing parameters for untargeted analysis of complex media presents challenges. Molecules with diverse boiling points, molecular weights, and fragmented ions can complicate the process. Consequently, expanding the detection range may reduce sensitivity, risking the loss of the ability to identify compounds present in very low concentrations, which is particularly concerning for key aroma-active compounds. It is also crucial to ensure that the m/z range corresponds with the specifications of the solid-phase microextraction (SPME) fiber used.

This chapter focuses on optimizing the equilibrium and extraction conditions for applying SPME. Extensive method development was necessary for all aspects of gas chromatography-mass spectrometry (GC-MS) due to a lack of prior in-house studies and expertise in flavour analysis. The study offers insights into the steps required to develop a method for analysing volatiles from complex media. The primary aim of this chapter was to optimize the detection and analysis of volatiles in beef mince. Several tests were conducted to ensure the accuracy, repeatability, and reproducibility of the results. The outcomes and discussions for four specific tests are included in this chapter.

1. Fiber selection and optimizing equilibration and extraction conditions

2. Effect of cooking method on volatile output.

3. Effect of post-cooking storage

4. Quantification of volatiles using internal standards

with a brief description for the others

Material and Methods

Moisture content

The moisture content of native beef mince was determined using the AOAC method 950.46 (air oven drying). A total of 5.000 grams (±0.01) of minced meat was weighed in triplicate and placed in a pre-weighed aluminium dish (50 mm in diameter) with a cover. The samples were then placed uncovered in a preheated convection oven at 102°C for 16 hours. After the drying period, the samples were removed from the oven, covered, and placed in a desiccator to cool. Weights were taken until a constant weight was achieved.

Moisture content was calculated as a percentage by subtracting the weight of the empty dish from the weight of the dish plus the sample after drying. This difference was then divided by the weight of the sample and multiplied by 100.

Similarly, the moisture analysis of the freeze-dried (lyophilized) samples was performed by calculating the difference between the original sample weight and the weight of the lyophilized sample, and then obtaining a percentage based on this difference.

Sample selection and preparation

Regular fresh beef mince (3 × 500g) with 18% fat and 15.9% protein (nutrition panel) was purchased from different local retailers and thoroughly mixed. A sub-sample of 100 g was reserved at -20°C for additional tests. The remaining 400 g was wrapped in thin sheets of plastic cling wrap and placed in the freezer at -20°C for 24 hours. After freezing, the sample was transferred to pre-weighed freeze-dryer containers and lyophilized using a Christ Alpha 1-4 LSC plus (Martin Christ Gefriertrocknungsanlagen, GmbH, Osterode am Harz, Germany). The freeze-dried beef mince whole (FDBM-w) was weighed to determine moisture content, then ground with a coffee grinder (Breville, Australia), homogenized, and stored in the freezer at -20°C for further analysis.

Heat treatment

For comparison, two different cooking methods (pan frying vs. heating in a vial) and formats (native vs. lyophilized) were tested using a modified method based on Machiels and Istasse (2003).

Case1(Cooking in vial).

Five grams (±0.0020 g) of fresh native beef mince and 1.50 grams (±0.0020 g) of freeze dried beef mince- whole (FDBM-w) were weighed separately in triplicates into a flat-base 40 ml screw-cap headspace vial fitted with a single-use Teflon-faced silicone septum (23193-U SUPELCO, USA). The lyophilized samples were hydrated overnight by adding 3.5 ml of deionized water. The sealed vials were then heated in a paraffin oil bath at 160°C for 30 minutes (see Figure 3.2). At the end of the treatment, the vials were dipped into an ice bath to stop any further cooking and were immediately stored at -20°C until required for GC analysis.

Case 2 (Panfried and nitrogen cooled).

For comparison a portion of the whole beef mince (50g) and FDBM-w 18g was mixed with 36g deionized water and hydrated overnight. The meats were shaped into a patty 1.5cm thick and pan fried on a benchtop heater at gas mark 5, using a Teflon coated non-stick pan without any added oil. The patties were cooked (3 minutes on both sides) till the internal temperature monitored using a probe thermometer reached 74ºC (Figure 3.2). They were immediately dipped into Liquid Nitrogen (LN_2) and powdered using a Breville coffee grinder and stored at -20ºC for volatile analysis. Samples were analysed within 48hrs of being heat treated.

Fiber selection and optimization

A variety of manual SPME fibers were initially tested, including 100µm PDMS, 7µm PDMS, 75µm CAR/PDMS, and 50/30 DVB/CAR/PDMS (Supelco, USA), to extract and concentrate analytes onto the fibers (data not provided). However, only the 75µm CAR/PDMS and 50/30 DVB/CAR/PDMS fibers were selected for optimisation (Elmore et al., 2001). The frozen cooked samples were thawed for 2 hours in the refrigerator and then equilibrated using an insulated water bath (Figure 3.3C). A three-factorial design was employed, considering time intervals of 15, 30, and 45 minutes at temperatures of 45°C, 60°C, and 70°C. These parameters were chosen based on preliminary trials (data not shown) conducted on in-house instruments and a literature review (Ch 2, Table 2.3). Both CAR/PDMS and DVB/CAR/PDMS fibers can tolerate a pH range of 2-11; thus, the samples were not buffered or tested for pH, as they were expected to fall within the acceptable range of 5.6-6, given their exceptional retail quality.

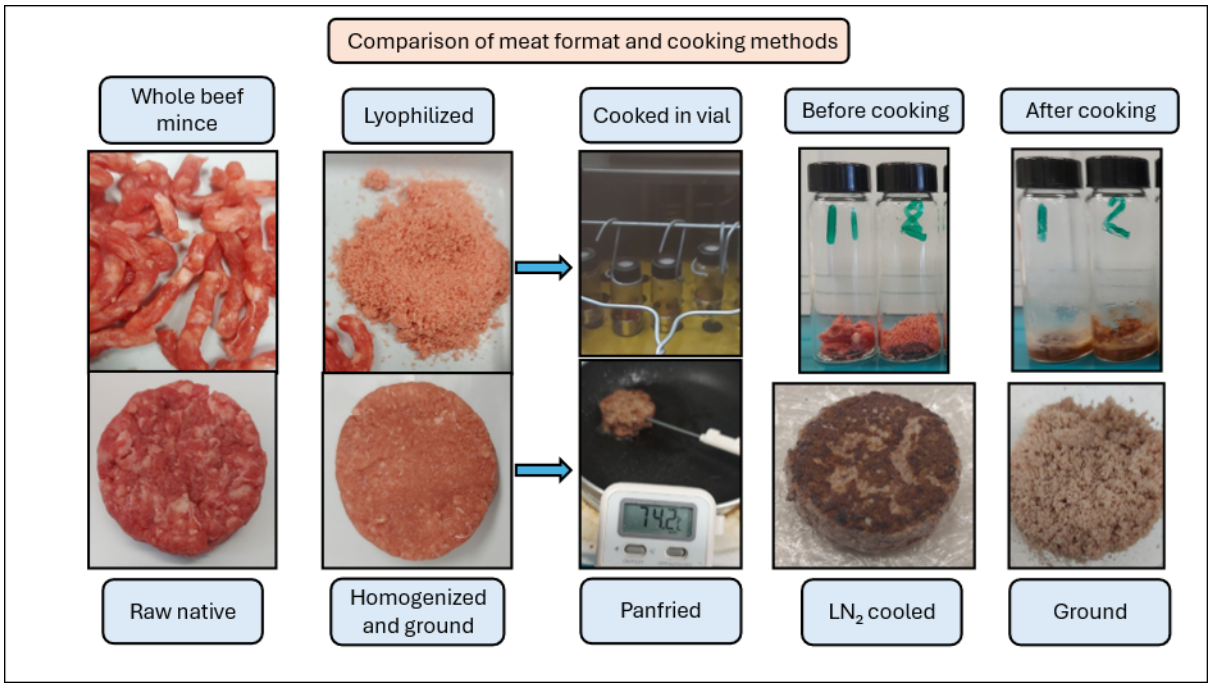

Figure 3.2 Sample preparation and heat treatment of beef mince of either native or lyophilized or pan fried or vial cooked for volatile analysis by HS-SPME-GC/MS

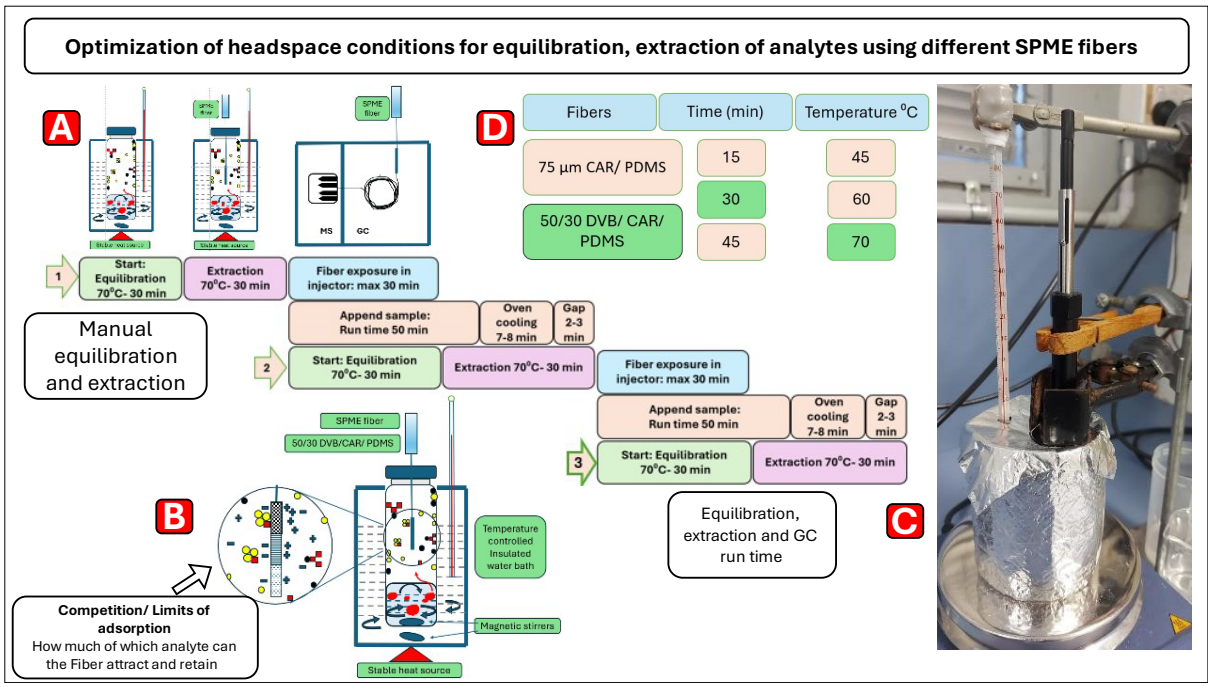

Figure 3.3 A. Schematic diagram showing the equilibration, extraction conditions for optimized GC run time management. B. Schematic diagram and C. Physical setup of water

bath to equilibrate and extract analytes from head space onto the SPME fibers D. Three factorial time and temperature conditions undertaken to optimize volatile extraction.

Establishing GCMS parameters for separation and detection of analytes

The fibers were manually exposed through the injection port, which was lined with a moulded Thermogreen® LB-2 septum (SUPELCO, USA) and a straight solid-phase microextraction (SPME) liner (0.75 mm × 5.0 × 95 mm, Restek Corporation, USA). A gas chromatograph (Shimadzu, QP2010, Japan) coupled with a mass spectrometer (Shimadzu, QP2010 plus, Japan) was utilized to separate and detect volatiles. Injection port temperatures of 180°C, 200°C, and 220°C were initially tested; however, data for these trials were not provided. The samples were optimized for an injection port temperature of 220°C to ensure maximum desorption of the analytes from the fiber onto the column (Gaffke & Alborn, 2020). Complete desorption of the analytes from the fibers occurred within a few minutes, although fibers were sometimes left in the injector port or a separate cleaning port for durations ranging from a few minutes up to 60 minutes (refer to Chapter 2, Table 2.3). In this study, the fiber was allowed to remain in the injector port for 30 minutes to optimize cleaning and maximize runtime efficiency while also coinciding with the gas chromatography (GC) runtime (see Figure 3.3A).

A SUPELCO-WAX 10 column (SUPELCO, USA), measuring 30 m × 0.25 mm ID × 0.25 µm, was employed for the separation of analytes. Although a 5-MS column was initially intended for use as part of a comprehensive untargeted approach, it was not utilized. Helium (99.99%) was used as the carrier gas at a constant flow rate, with a split ratio adjusted to 10:1. The initial column temperature was held at 40°C for 2 minutes before increasing to 240°C at a rate of 5°C per minute. The ion source temperature was set to 220°C, and the interface temperature was set to 260°C, with an electron energy of 70 eV. A solvent/acquisition delay of 0.50 minutes was implemented. Mass spectra were collected in SCAN mode over a range of m/z 30-500, at an acquisition rate of 0.20 spectra per second.

Establishing Retention Indices (RI)

In addition to the standard practices for comparing data from literature, databases, or other studies, the use of the Retention Index (RI) can be particularly beneficial, especially when instrument parameters require adjustment or when retention time drifts occur due to manual injection. Bianchi et al. (2007) demonstrated that by utilizing automated flow controllers, they could achieve a maximum change in RI (ΔRI) of 7 units. In this study, a maximum allowable ΔRI of 20 units was deemed acceptable, as noted in the literature. The RI values for the

observed volatiles were calculated using data from the NIST database, specifically the 'NIST Chemistry WebBook, SRD 69.' The calculation followed the formula established by Van Den Dool and Kratz (1963) and adapted by Babushok (2015):

$$RI\ (x) = 100\ \cdot z + 100\ \cdot \frac{RT(x) - RT(z)}{RT(z+1) - RT(z)}$$

where RI (x) is the retention index of the unknown compound x,

z the number of carbon atoms of the n-alkane eluted before the unknown compound x,

RT (z) n alkane eluted before the unknown compound x,

RT (z + 1) n-alkane eluted after the unknown compound x.

Peaks were tentatively identified by calculating the Retention Index (RI) against alkane standards C7-C40 (Sigma Aldrich, Australia) (Ahamed et al., 2024; Kaczmarska et al., 2018; Zhang et al., 2022). These were compared with mass spectral databases (Wiley, 2008; Linstrom & Mallard, n.d.). A minimum similarity match of 80% was deemed acceptable. Additionally, a threshold of 20,000 absorbance intensity units was established as the minimum acceptable limit. The raw total ion chromatograms (TIC) data were processed using Microsoft Excel and further analysed using the statistical software OriginPro 2024b student version (Northampton, MA, USA).

Miscellaneous tests

Ascertaining in-vial temperature during cooking (oil bath) and equilibrating (water bath)

Temperature and time are critical factors in the formation and development of aroma compounds. To measure the in-vial and headspace temperatures during heat treatment in the oil bath, a thermometer was inserted, and the time and temperature required to reach 160°C were recorded. The maximum temperature observed was 154°C after 16 minutes, leading to the conclusion that a cooking time of 30 minutes was sufficient, based on visual observations of browning and the progression of the Maillard reaction. Additionally, the headspace temperature reached 70°C within 15 minutes, confirming that 30 minutes was adequate for the sample to reach equilibrium. Although further optimization of the time could have been considered, the 30 minutes for equilibration and extraction was an effective approach to minimize errors related to manual handling during the GC run.

Vial cleaning

To reuse vials effectively, it is essential to validate the cleaning procedures for their efficiency, contamination carryover, and potential formation of active sites. The vials were soaked and washed with a standard laboratory detergent, then rinsed and dried in an oven at 200°C for 2 hours. After each wash cycle, three blank vials containing 10 ml of deionized water undergo the same heat treatment, equilibration, and extraction conditions as the samples to assess cleaning efficiency. Any peaks detected in these blank vials are subtracted from the area measured in the sample runs. Although silanisation of the vials could offer improved performance, it was deemed unnecessary due to the effectiveness of the cleaning procedure, the reduction in chemical use, and the cost savings involved.

Establishing blank runs

Several system peaks are known to appear in chromatograms due to various sources. Common contributors include siloxanes from column bleed, phthalates from septum bleed, and other extraneous ghost peaks. These peaks must be deducted from the final readings. To minimize their occurrence, the column temperature was limited to 20°C below the maximum allowable temperature. Septums were exposed to 250°C for 2 hours prior to use. Daily blank column and blank fiber checks were conducted before starting the sample runs. Isolated peaks identified in the blank runs were subtracted from the sample readings.

Setting standard operating procedures (SOP) for GCMS experiments

This note outlines the standard operating procedures established to ensure consistent and reproducible results. Each day, a blank column and fiber run were conducted before running any samples, as well as an end-of-day blank column run to confirm that all compounds had eluted. Before reusing washed vials, a blank vial run was performed on three randomly selected vials from the washed batch. Single-use PTFE-coated septa were utilized for each sample run. Additionally, a small magnetic stirrer was used to facilitate the stirring of the sample during equilibration. Each day, 6-7 samples were prepared to ensure that no samples were stored in the freezer for more than 48 hours.

Alkane standards (C_7-C_{40}) obtained from Sigma-Aldrich Australia were run in triplicate before each new experiment. For the alkanes, a direct injection method was employed, injecting 1 µL at a 100:1 split, using the same GCMS parameters as described earlier. The internal standard, 2-methyl-3-heptanone, was freshly prepared each week by diluting 100 µL of the standard in 10 mL of methanol to create a 10,000-ppm stock solution. A 10 µL aliquot of the 400 ppm (0.4

μg/mL) stock solution in methanol was added to each sample just prior to equilibration and extraction. A standard curve was constructed in the range of 0.2-0.8 μg/mL for semi-quantitative analysis.

Statistical analysis

Data were analysed using OriginPro 2024b student version (Northampton MA, USA). and presented as mean ± standard error (SEM) of peak area. Differences were determined using one-way ANOVA and Tukeys test. Significance was considered as ($P < 0.05$). Multivariate statistical analysis using Principal component analysis- two dimensional listwise biplot was employed to observe the relationship between the tests.

Assumptions, biases and limitations

Due to the nature of this study, any alternative measures, assumptions, biases, and limitations encountered have been presented here to the best of our knowledge. The data shown in the tables are raw peak intensity measurements from the Total Ion Chromatogram (TIC), used ad hoc for qualitative analysis due to the inability to procure certified reference materials for identifying and quantifying observed volatiles. The presence, increase, or decrease of specific volatiles serves only as markers of quality within this work.

It is widely accepted that not all volatiles possess aroma activity (Mottram, 1994). Furthermore, those that do exhibit aroma activity may do so depending on the matrix, their concentration, and the variability in perceptual ability within the sensory panel. Therefore, this work does not imply that similarities or differences in the volatile profiles of heated samples will translate to similar sensory nuances or appreciation in the final products. An increase or decrease in volatiles from individual ingredients may not have a linear effect on the sensory output of formulated products (Frankel, 2005). However, this study is a step toward building a database that enhances the understanding of the formation and development of volatiles and aromas in plant-based ingredients for creating meat analogues (Arshad et al., 2018; Münch et al., 2024). Comparisons of volatiles observed in this study with those found in existing literature were made solely to note their presence and should not be interpreted as having any relevance to the sensory attributes observed in different studies.

The cooking procedure in the vial aimed to capture the Maillard reaction products similar to those developed on the crust of a grilled burger. Though grilling typically requires a shorter cooking time, the cooking time in this study was extended due to the maximum heating capacity of the oil bath at 160°C. The prolonged cooking time resulted in pressure build-up

within the vial, leading to a visible loss of moisture (see Figure 3.4). The vials were not completely sealed to avoid the risk of explosion from this pressure. Variations in moisture levels may have caused differences in the peak intensities of the observed volatiles. Although no qualitative differences in volatiles were noted, vials with significant moisture loss were discarded and not included in this study.

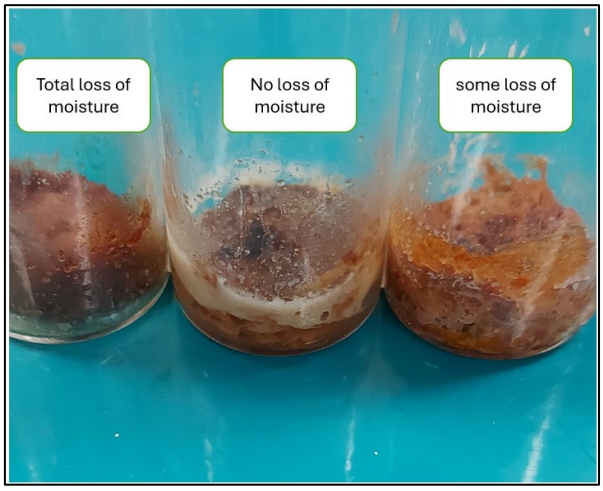

Figure 3.4 Varying degrees of moisture loss from the vial due to pressure build-up during heat treatment in an oil bath.

The effect of fat (lowering of volatility) forming a layer on top of the FDBM-w sample during equilibration and extraction was also of concern. Hence fractionation of the beef mince was sought as a measure to effectively analyse volatiles from the complex matrix.

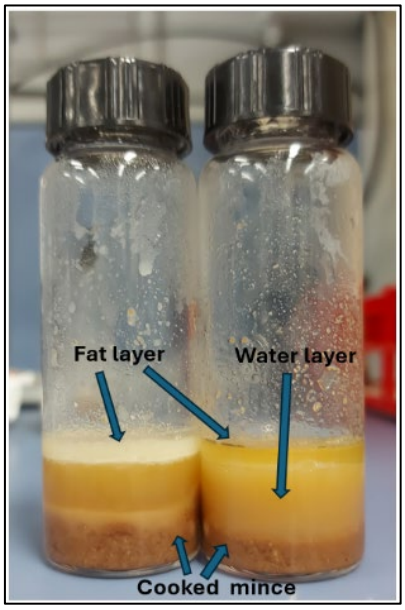

Figure 3.5. Post-analysis observation of FDBM-w sample vial containing the layer of fat, water and cooked mince.

Additionally, due to excessive fragmentation, electron ionization (EI) may not be suitable for analysing large molecular weight compounds. It has also been observed that performing quantitative analysis on the formation of hydroperoxides and secondary oxidative compounds using EI can be challenging without the use of stable derivatives.

The range of C_7-C_{40} linear alkanes used for calculating retention indices proved to be excessive, particularly when utilizing a WAX column. Compounds over C_{32} were difficult to elute due to their high boiling points and the temperature limitations of the WAX column. Several blank runs with extended run times were necessary after each alkane standard run to ensure that all alkanes and any fragments resulting from the long run time were eluted. For future studies, it may be beneficial to limit the alkane standard range to under C_{30}, especially when using a polar (WAX) column or a column with a low-temperature range.

Regarding statistical analysis, due to the nature of the study and the differences among samples, not all compounds were observed in every sample, and some volatiles were detected only in one sample. This variability affected the statistical analysis because of the presence of blanks and missing data. The introduction of a zero value instead of a blank helped mitigate this issue, although it affected the ratio of the coefficients. However, a qualitative assessment showed that the observable variance of the data across components did not change. Given the qualitative nature of the assessment, the application of principal component analysis was deemed acceptable.

Results and Discussion

This chapter presents the results and discussions on four key areas:

1. The rationale for selecting DVB/CAR/PDMS fiber, along with the equilibration and extraction of analytes at 70°C for 30 minutes.

2. The effectiveness of cooking reconstituted (rehydrated) FDBM-whole at 160°C in an oil bath as a method for analysing volatiles in cooked beef, which may also serve as a control.

3. The duration for which cooked samples can be stored under freezing conditions before analysis, without causing undesirable flavour changes.

4. The challenges associated with using internal standards for the quantification of volatiles from complex matrices.

Fiber selection and optimization of equilibration and extraction conditions

Initially, a comprehensive untargeted analysis using a variety of solid phase microextraction (SPME) fibers was planned for data collection. However, due to time and resource constraints, as well as challenges encountered during early method development, we decided to compare data collected with two specific fibers: 75 µm CAR/PDMS and 50/30 µm DVB/CAR/PDMS. These fibers were chosen because they exhibit dual polarity and possess a wide range of analyte adsorption capabilities (30 m/z to 275 m/z). Additionally, they have been consistently used in multiple studies (Chapter 2, Table 2.2) related to the analysis of key aroma compounds in beef, mushrooms, yeast extract, and meat analogues (Chapter 2, Table 2.3).
At the outset of fiber selection, the polarity of the column was not considered for two reasons:

1. Both the CAR/PDMS and DVB/CAR/PDMS fibers are bipolar and can adsorb a wide range of analytes (Chapter 2, Table 2.2).

2. While the initial analysis was expected to involve both polar and nonpolar columns, it was ultimately conducted only using a Wax column.

After selecting the fibers, we focused on equilibration and extraction conditions based on time and temperature. Both equilibration and extraction were performed at 70°C for 30 minutes. However, manual handling made it challenging to ensure consistent sample changeover. This included monitoring the equilibration time and temperature, adding the fiber for analyte extraction, injecting the sample, and controlling the desorption time.

Several different scenarios were modelled, but none were successful in producing reliable data (results not presented). Ultimately, a single time parameter (30 minutes) and temperature (70°C) provided the best qualitative data outcome (Figure 3.3A). Additionally, quantitative data may have been impacted due to our inability to control and retain moisture (Figure 3.4) in the sealed vials during thermal treatment at 160°C, which led to pressure buildup and subsequently resulted in anomalous data. While some compounds, such as aldehydes, appeared unaffected, other compound classes, including pyrazines and sulfur (S) and nitrogen (N) substituted compounds, were impacted.

Time and temperature are interdependent factors in the context of reaching equilibrium; however, they are not necessarily inversely related. This means that a higher temperature generally results in a shorter time required to reach equilibrium. Once equilibrium is achieved, extending the equilibration time does not increase the concentration of analytes in the

headspace. In our study, the 30 minutes allocated for equilibration and extraction coincidentally matched the GC run time of 50 minutes, along with additional time for oven cooling and manual changeover (see Figure 3.3A).

From Table 3.1, it is evident that increasing the equilibration and extraction time to 45 minutes did not significantly enhance the output, except for a few compounds. Literature indicates that equilibration and extraction temperatures between 60°C and 70°C yield the best results (refer to Chapter 2, Table 2.3). We chose 70°C as the optimal temperature based on the data presented in Table 3.1 and Figure 3.6.

The sample we used—regular beef mince with approximately 18% fat—may have affected the volatility of small molecular weight polar compounds. This is likely due to the high lipid content, which could form a layer on the surface of the sample (illustrated in Figure 3.5), thus reducing the adsorptive capability of the fibers. While increasing the temperature above 70°C could enhance volatility; it may also raise the vapour pressure within the vial, as well as the partition coefficient and oxidative rates. This could lead to the oxidation of fatty acids, resulting in the formation of aldehydes, alcohols, ketones, and other undesirable compounds associated with off-flavours.

It is important to note that although the chromatograms showed considerable consistency across repeats, minor dynamic drifts, combined with our interpretation of detected peaks using the mass spectrometer library, contributed to challenges in identifying compounds. Issues such as overlap and tailing of large peaks (e.g., aldehydes and extraneous peaks such as phthalates and siloxanes) complicated the identification process. Consequently, excluding certain data resulted in a significant number of peaks being classified as unknown, thereby creating gaps in the dataset.

SPME fibers have been found to be influenced by competition and affinity towards specific compounds in complex matrices (Flores et al., 2006; Thomas et al., 2016). Some aspects of competitive adsorption and its limitations were observed while establishing the calibration curve for the internal standard, which will be discussed later in this chapter. One question that remains unanswered is whether the adsorbed compounds detach themselves as time, temperature, and environmental conditions change within the vial. Understanding this could help optimize the equilibrium and extraction of significant volatiles. However, this phenomenon was not studied in detail, as it was beyond the scope of the research, and it

adversely affected our understanding of the dynamics between temperature, vapor pressure, volatility, and adsorption.

Additionally, the position of the fiber within the headspace is known to potentially impact the adsorption efficiency of the fiber (Brunton et al., 2001). To ensure consistent results, we manually marked the depth at which the fiber was inserted. However, the specific effect of fiber position on the volatile output was not tested.

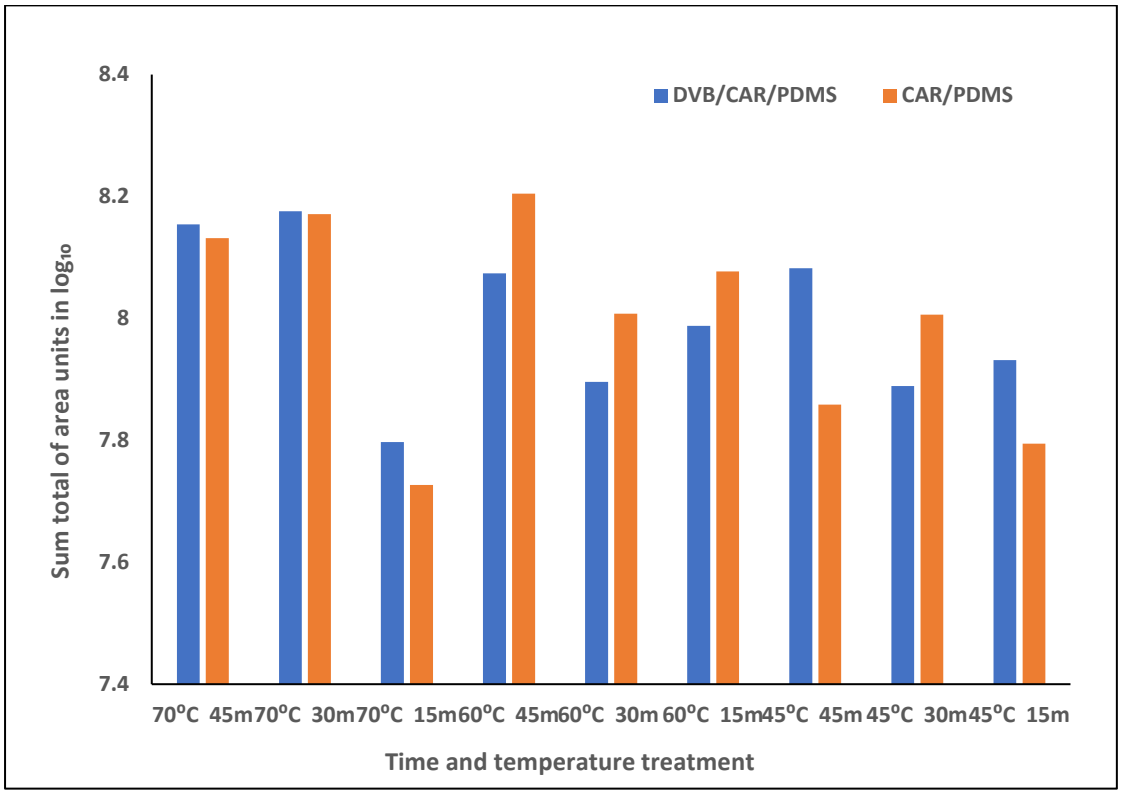

Figure 3.6: Comparison of CAR/PDMS and DVB/CAR/PDMS fibers for their ability to extract headspace volatiles.

The highest sum of the area of peaks tentatively identified was observed to be $\log_{10}$ 334.05 for DVB/CAR/PDMS fiber at a temperature of 60ºC for 45minutes. However, results observed for CAR/PDMS fiber from 70ºC at 30 min was observed to be $\log_{10}$ 321.70 (Figure 3.7). The overall one-way ANOVA test proved no significant differences existed between the population means at $P<0.05$ but significant differences were observed in the homogeneity of variance (Levene's test) possessing a prob>F = 0.01235. Further, the large number of missing values (1140) could indicate an unreliable ANOVA prediction. Hence a Kruskal-Wallis ANOVA was performed resulting in significant difference being observed between the population means with chi-square value of 61.67 and degree of freedom (DF)=17 thus rejecting the null

hypothesis. A Kurtosis value of 44.09 with a skewness of 6.38 was also observed using bootstrap sampling further demonstrating that a significant difference exists between the population means (Figure 3.8).

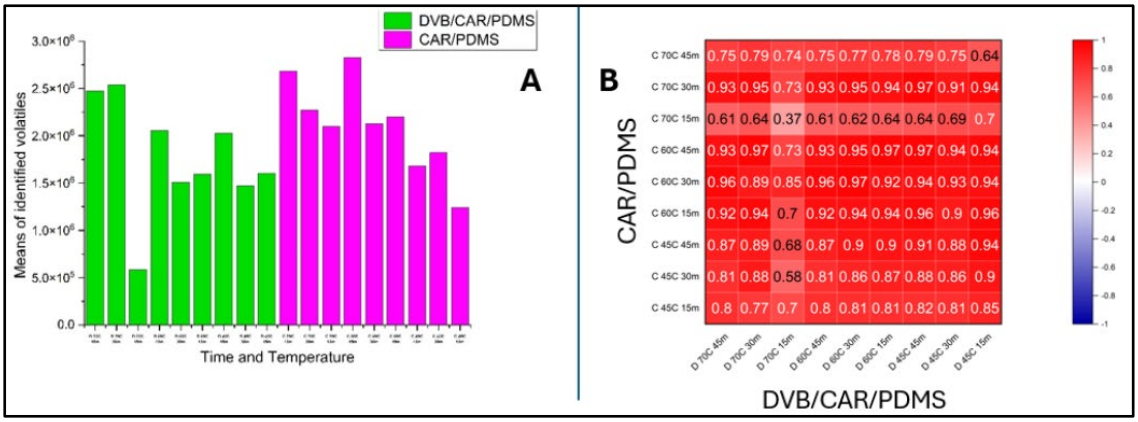

Figure 3.7 (A). Comparison of population means of tentatively identified volatiles between the various treatments observed for the two fibers. (B). Correlation plot of population means between DVB/CAR/PDMS and CAR/PDMS.

Data analysis revealed that the population means of the various treatments did not follow the expected linear progression; instead, they exhibited a random pattern with no clear trend or consistent relationship among the values presented. Brunton et al. (2001) noted that 75 µm CAR/PDMS performed better than DVB/PDMS (notably, not DVB/CAR/PDMS), which showed better performance at lower temperatures. However, in our study, DVB/PDMS performed poorly compared to its performance at higher extraction temperatures. Machiels & Istasse (2003) found that DVB/CAR/PDMS was more effective at various extraction times, especially for higher molecular weight compounds with lower volatility, though they used a non-polar (5MS) column. According to the total ion chromatogram and the alignment of the gas chromatography parameters for effective manual handling of samples, both 50/30 µm DVB/CAR/PDMS and 75 µm CAR/PDMS provided different equilibrations and extraction efficiencies for the analytes (see Figure 3.7 and Table 3.1).

Aldehydes such as pentanal, hexanal, heptanal, octanal, and nonanal derived from the oxidative and thermo-oxidative degradation of fatty acids were observed to be consistent with the literature (Kaczmarska et al., 2021). They were also the largest number of peaks as well as the area under peaks (Ahamed et al., 2023). Hexanal, octanal, benzaldehyde and tetradecanal were the most predominant aldehydes observed (Figure 3.8). SPME fibers adsorb analytes based on molecular weights. However, no differences in the adsorbability based on molecular weight

could be observed. E.g. 2-methylbutanal (MW. 86.132), benzaldehyde (106.121) and pentadecanal (MW. 226.398) were extracted equally. Hence, conclusions could not be drawn on the mechanism of extraction. A better understanding of SPME's ability and affinity to adsorb analytes could help in mapping flavour formation.

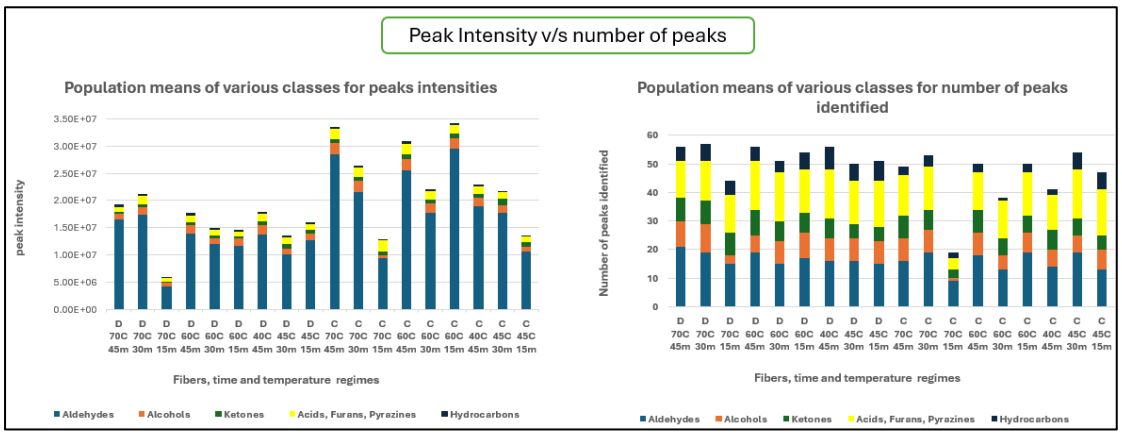

Figure 3.8 Comparison of population means of sum of peak intensities and the number of peaks for the various classes of compounds observed in two commercial SPME fibers, (C) indicates Carboxen/ Polydimethylsiloxane coating, (D) indicates divinylbenzene/ Carboxen/ Polydimethylsiloxane coating.

As expected, the number of acids, furans, thiophenes, and pyrazines combined was high compared to their peak area (Figure 3.8). This was attributed to the extended cooking at 160ºC for 30 minutes and the development of Maillard reaction products. Challenges in identifying hydrocarbons were also encountered due to our use of a Wax column, an MS library dedicated to the 5MS column, and low matches with NIST webbook data. Peaks that did not match NIST RI criteria were omitted or reported as unknown (Table 3.2).

Further investigation also shows some peaks have been elusive E.g. undecanal, which was observed in pre-trials to be a predominant compound, was not identified except with DVB/CAR/PDMS at 70 ºC and 45 min. Co-elution and or peak overlap due to large tailing peaks at similar retention time was identified as a possible cause of misidentification. Another shortcoming was compounds that that were not reproduced efficiently were redacted to minimize error. Approximately 80-90 compounds were separated and detected during the sample run. However, only an average of 50% of the volatiles were tentatively identified. This created larger uncertainty in the data due to the use of reduced population means.

Table 3.1 Fiber selection and optimizing equilibration and extraction parameters for the analysis of volatiles

Column: SUPELCO Wax10			DVB/CAR/PDMS									CAR/PDMS								
	LRI		70°C			60°C			45°C			70°C			60°C			45°C		
Compounds	(calc)	(Ref)	45 min	30 min	15 min	45 min	30 min	15 min	45 min	30 min	15 min	45 min	30 min	15 min	45 min	30 min	15 min	45 min	30 min	15 min
Aldehydes																				
Butanal, 2-methyl-	908	915	5.88	5.40	5.54	6.01	6.03	5.88	6.13	6.14	6.15	5.68	5.71	5.94	5.90	6.09	6.04	6.09	6.21	6.34
Butanal, 3-methyl-	912	920	6.39	5.89	5.98	6.51	6.53	6.37	6.58	6.61	6.59	6.12	6.18	6.36	6.36	6.56	6.41	6.45	6.63	6.78
Pentanal	975	983	5.80	5.92		5.91	5.93	6.03	6.14	6.09	6.21	6.30	6.35	6.47	6.47		6.44	6.28	6.64	6.40
2-Butenal	1041	1035																	5.54	
Hexanal	1078	1099	6.35	6.58	5.46	6.44	6.55	6.63	6.78	6.77	6.86	6.80	6.56	6.64	7.00	6.79	6.96	6.83	7.12	6.79
2-Butenal, 2-methyl-, (E)-	1096	1096											5.71		5.81		5.82			
Heptanal	1184	1188	6.48	6.68	5.41	6.55	6.66	6.85	7.00	6.83	6.99		6.96	6.67	7.09	6.79	7.04	6.79	7.03	6.67
4-Heptenal, (Z)-	1241	1234		5.31				5.14		5.26					5.59		5.35		5.24	
Octanal	1288	1299	6.63	6.90	5.58	6.78	6.79	6.99	7.13	6.85	6.95	7.04	6.98	6.69	7.15	6.79	7.03	6.71	6.91	6.53
2-Hexenal, 2-ethyl-	1336	1336											5.39							
Nonanal	1393	1411	7.01	7.24	6.05	7.08	7.05	7.18	7.24	6.84	6.99	7.07	7.23		7.28	6.93	7.12	6.71	6.85	6.47
2-Octenal, (E)-	1430	1425	6.02	5.89		5.95	5.90	6.08	6.05	5.55	5.81	6.34	6.18		6.16	5.82	5.95		5.52	
Decanal**	1498	1501	5.90	6.07	4.67	5.76	5.58	5.70	5.72	5.30	5.43	6.15	6.03		5.75	5.50	5.57		5.31	
Benzaldehyde	1525	1504	7.11	6.86	6.47	7.04	6.93	6.63	6.85	6.66	6.53	7.38	7.20	6.83	7.27	7.03	7.00	6.76	6.82	6.47
2-Nonenal, (E)-	1537	1524	6.41	6.48	5.17	6.03	6.07	6.36	6.23	5.76	5.96	6.58	6.45		6.43		6.09	5.39	5.69	
2-Furancarboxaldehyde, 5-methyl-	1576	1562	5.64		4.87	5.50	5.26	5.07	5.38		5.06	5.96	5.88			5.71	5.63	5.47	5.35	5.22
Undecanal	1604	1604	5.90																	
2-Decenal, (E)-	1645	1638	6.90	6.84		6.32	6.39	6.72	6.56	6.04	6.27	6.88							6.05	5.66
Benzeneacetaldehyde	1646	1652			5.83															
3-Thiophenecarboxaldehyde	1696	1687	5.97	5.68																
2-Thiophenecarboxaldehyde	1697	1702				5.49			5.24	5.03		6.16	5.94		5.99		5.69	5.52	5.30	5.13
Dodecanal	1710	1720	5.73	5.85	5.02	5.47							5.68		5.43					
3-Methyl-2-thiophenecarboxaldehyde	1714	1765	5.76	5.36		5.45	5.55	5.43	5.59	5.18	5.32	5.77	5.78		5.78		5.55	5.33	5.25	

Tridecanal	1818	1833														5.48				
Tetradecanal	1922	1940	6.46	6.34	6.09	6.19	5.93	5.76	5.61	5.35	5.40	6.42	6.16	5.84	6.04	5.95	5.68	5.24	5.40	5.35
Benzeneacetaldehyde, .alpha.-ethylidene-	1936	1939	5.22																	
Pentadecanal-	2015	1999	6.61	6.47	6.37	6.36		5.90				6.59	6.30	6.02	6.18	6.15	5.83	5.39	5.50	5.54
Heptadecanal	2068	2247	7.72	7.61	5.86	7.47														
Alcohols																				
1-Pentanol	1244	1251	5.30	5.81			5.43	5.56	5.71	5.79	5.87	5.74	5.96		5.99		5.94	5.82	5.98	5.71
1-Hexanol	1348	1353	5.54	5.89		5.61	5.67	5.88	6.03	5.82	5.96	5.81	5.86		5.95		5.98	5.74	5.87	5.56
1-Octen-3-ol	1446	1447	5.84	6.09	4.87	6.05	6.11	5.99	6.16	5.99	5.96	5.97	6.03	5.73	6.14	5.86	6.09	5.97	5.93	5.63
1-Heptanol	1450	1460	6.11	6.43		6.15	6.20	6.44	6.56	6.19	6.34	6.35	6.35		6.42		6.40	6.03	6.18	5.82
1-Octanol	1553	1546	6.31	6.59	4.84	6.34	6.32	6.50	6.52	6.06	6.21	6.53	6.46		6.50	6.18	6.41	5.95	6.06	5.71
Cyclooctyl alcohol	1610							5.34	5.38	5.06	5.10	5.59	5.48		5.45		5.29			
trans-2-Undecen-1-ol	1612		5.79			5.79	5.70	5.57	5.63	5.46	5.33	5.88			5.83	5.57				5.03
1-Nonanol	1655	1668	5.27	5.42				5.20												
2-Furanmethanol	1660	1678	6.45	6.42	6.31	6.52	6.45	6.40	6.57	6.49	6.46	6.87	6.84		6.85	6.75	6.69	6.71	6.56	6.42
1-Docosanol, methyl ether	1818												5.77							
2-Thiophenemethanol	1947	1937														5.18				
1-Dodecanol, 3,7,11-trimethyl-	2603		5.51	5.79																
Ketones																				
2-Butanone	900	919	5.80	5.72	5.37	5.83	5.83	5.74	5.95	5.96	5.94	6.17	6.16	6.07	6.32	6.29	6.35	6.43	6.50	6.46
2,3-Butanedione	973	989			5.26															
2,3-Pentanedione	1057	1071										5.80		5.85	6.05	5.95	6.01	5.81	6.19	5.95
3-Heptanone, 2-methyl-	1164	1164	5.64	5.68	5.98	5.93	6.00	5.84	6.03	6.19	6.09									
2-Heptanone	1180	1187	5.55	5.73	5.19	5.83	5.85	5.75	6.03	6.04	6.03		5.97	5.78	6.13	5.98	6.14	6.13	6.11	5.94
2-Octanone	1284	1285	5.24	5.43		5.49	5.50	5.51	5.75	5.49	5.57	5.58	5.86		5.76		5.71	5.53	5.54	5.25
5-Hepten-2-one, 6-methyl-	1337	1340					5.13		5.22	5.12									5.05	
2-Nonanone	1388	1394		5.88		5.99	5.87	5.76	5.98											
2-Decanone	1493	1515	5.66	5.98	4.88	5.69	5.39	5.16	5.29		4.93	5.84	5.68		5.45	5.31	5.30	5.29		

Ethanone, 1-(2-furanyl)-	1508	1511									5.80	5.50		5.66	5.29		5.15	5.13	
trans-3-Nonen-2-one	1512	1523	5.14	5.39	4.63														
2,3-Dimethylhydroquinone	1683					5.80													
1-(6-Methyl-2-pyrazinyl)-1-ethanone	1694	1679				5.46													
Ethanone, 1-(2-thienyl)-	1781	1782									5.97	5.78		5.70		5.29	5.28		5.09
2-Tridecanone	1810	1813									5.73	5.59		5.51					
Nona-3,5-dien-2-one	1891			5.53															
2-Pentadecanone	2010	2027	5.71		5.41	5.40		5.06			5.69				5.23				
2-Hexadecanone	2131	2130	5.36		5.20														
Esters, Thiophene, furans and pyrazines																			
n-Caproic acid vinyl ester	1637						5.25												
Thiophene	1020	1022							5.09		5.18				5.35		5.37		5.55	5.52
1-Pentanethiol	1037	1039																5.45	5.36
2-Thiopheneacetic acid, octyl ester	1090		5.29			5.37	5.43	5.54	5.85										
Thiophene, 2-methyl-	1092	1090								5.66	5.90	5.82	5.85		6.11	5.80	5.84	5.84	6.02	5.99
Dihydro-3-(2H)-thiophenone	1530	1547									5.20							5.21	5.16	5.05
2-Acetylthiazole	1648	1661	5.48	5.62		5.86	5.70		5.61	5.54	5.38	5.97						5.62	5.50	5.29
Furan, 2-ethyl-	945	945	5.88	5.67		6.09	6.13	6.07	6.29	6.32	6.35	6.41	6.45	6.47	6.68	6.38	6.78	6.73	6.92	6.73
Furan, 2-propyl-	1027	1033									5.11									
2-n-Butyl furan	1132	1122											5.43		5.61		5.59		5.54	5.29
Furan, 2-pentyl-	1229	1239	6.44	6.58	5.80	6.72	6.76	6.64	6.85	6.79	6.74									
trans-2-(2-Pentenyl)furan	1301	1282	5.73	5.97		6.06	6.06	5.98	6.17	6.01	6.00	6.29	6.27		6.38	6.00	6.28	6.06	6.09	5.78
2-n-Heptylfuran	1432	1429		5.94																
Furfural	1466	1467				5.53	5.50					5.91	5.86		5.97	5.72				
2-n-Octylfuran	1534	1530	5.83	6.02		5.68	5.45	5.55	5.39				5.95				5.59			
Pyrazine	1209	1209	5.48			5.48	5.47	5.41	5.64	5.58	5.56	5.99	6.00			5.93	5.98	6.00	5.92	5.83
Pyrazine, methyl-	1262	1267	6.09	5.94	5.86	6.16	6.12	6.00	6.21	6.15	6.06	6.59	6.78	6.18	6.57	6.49	6.45	6.41	6.30	6.22
Pyrazine, 2,5-dimethyl-	1318	1318	6.22	6.03	6.26	6.22	6.13	6.02	6.24	6.05	6.03	6.63	6.54	6.22	6.52	6.54	6.42	6.31	6.08	6.04
Pyrazine, 2,6-dimethyl-	1324	1325	6.12	6.04	6.19	6.28	6.27	6.13	6.30	6.19	6.06	6.60	6.55	6.24	6.55	6.54	6.40	6.27	6.16	5.99

Pyrazine, ethyl-	1331	1334			5.32															
Pyrazine, 2-ethyl-6-methyl-	1381	1402	5.78	5.60	6.12	5.84		5.72	6.02	5.77		6.00	6.10			6.21	6.04			
Pyrazine, 2-ethyl-5-methyl-	1388	1406			5.97							6.07				6.11				
Pyrazine, trimethyl-	1399	1401	6.00	5.95	5.91	6.06	5.98	5.95	6.07	5.84	5.79	6.31	6.27		6.22	6.16	6.10	5.88	5.80	5.62
Pyrazine, 3-ethyl-2,5-dimethyl-	1440	1439	6.06	6.93	5.74	6.05	5.97	5.76	5.93	5.68		6.27	6.14		6.17	5.96	6.12	5.87	5.82	5.59
Pyrazine, 2-ethyl-3,5-dimethyl-	1457	1443			5.43	5.45	5.25													
Pyrazine, 3,5-diethyl-2-methyl-	1488	1474		6.09																
2,3,5-Trimethyl-6-ethylpyrazine	1509	1502		5.88																
Pyrrole	1516	1525			5.01	5.39	5.27	5.29	5.57	5.55	5.58		5.63		5.74	5.76	5.57	5.60	5.84	5.87
4H-Thiopyran-4-one, tetrahydro-	1531																		5.64	
2-Acetyl-3-methylpyrazine	1694	1635			5.64															
Ethanone, 1-(1H-pyrrol-2-yl)-	1973	1960			5.02															
Disulfide, dimethyl	1070	1072				5.39	5.77	5.32	5.68	5.54	5.64	5.73	5.78		6.10		6.04		6.12	5.96
Hydrocarbons and miscellaneous																				
Benzene	935	936									4.84								5.24	5.19
Benzene, 1,3-dichloro-	1489	1477									5.66	5.63								
1,3-Octadiene	947	958			4.80						5.41									
Cyclopentane, butyl-	969	979						5.05	5.26	5.17										
Decane	991	1000		5.32	5.22			5.70	5.82			5.48	5.55	5.49	5.70		5.63	5.46	5.52	5.28
1-Ethyl-5-methylcyclopentene	1029																5.30		5.43	5.16
Toluene	1037	1035					5.21		4.95	5.41	5.09		5.43				5.39	5.49	5.43	5.40
1,3-Nonadiene, (E)-	1048	1047							5.07	5.06	4.97								5.00	4.87
2,3-Pentanedione	1055	1071	5.17	5.48	4.95	5.69	5.64	5.57	5.52	5.86	5.52									
Undecane	1096	1100							5.32											
Dodecane	1194	1200		5.67									5.64		5.89					
Tridecane	1295	1300		5.77		5.44				5.26										
1,3-Hexadiene, 3-ethyl-2-methyl-	1418							5.06	5.14											
Pentadecane	1496	1500	5.66																	
Cyclododecyne	1589		5.70																	

1,2,4-Trithiolane, 3,5-dimethyl-	1604	1610				6.12		5.94	6.05	6.03	5.83								5.72	5.52
2-Hexadecene	1866		5.83	5.72	5.52	5.54	5.21	5.18				5.83	5.55	5.38	5.44	5.34				
Indole	2097	2444	5.85	5.63	5.14	5.45	5.21													
Tentatively identifiable compounds			56.00	55.00	44.00	56.00	50.00	53.00	55.00	49.00	50.00	49.00	53.00	19.00	50.00	38.00	49.00	41.00	54.00	47.00
Sum of Peak area in Log_{10}			332.72	331.00	242.22	334.05	294.38	310.33	327.10	287.37	292.27	302.13	321.70	116.85	306.35	230.12	295.66	243.55	317.70	271.48

Effect of cooking method and format on volatile output of beef mince.

The effect of the cooking method (pan-grilled vs. heated in a vial) and format (native vs. lyophilized) on the volatile profile of beef mince was found to vary (see Figure 3.9 and Table 3.2). In the case of the pan-grilled patty, only the surface exposed to the pan reached 180 °C, while the interior maintained different relative temperatures, with the core monitored to reach 74 °C, indicating that cooking was complete. In contrast, the vial-heated sample achieved a maximum temperature of 160 °C, corresponding to the highest temperature of the oil bath. The sample was exposed to this temperature for 30 minutes to achieve sufficient browning. However, due to the small sample size and extensive heating, the entire sample reached higher temperatures, resulting in a different volatile profile.

Additionally, regarding the loss of aroma volatiles, the pan-grilled patty was exposed to the atmosphere during cooking and subsequent chilling using liquid nitrogen. It was further exposed during the grinding process, which likely led to a loss of aroma volatiles. On the other hand, the vial-heated samples remained sealed throughout the heating, cooling, and storage processes. They were only vented briefly just before equilibrium and extraction, during the insertion of the stirrer and addition of water, which likely helped retain most of the aroma compounds. The reduced exposure to the atmosphere may have limited oxidative degradation.

One area of concern was the buildup of pressure, which could cause the septum to rupture or deform. This was managed by not tightening the cap, thereby allowing for pressure release. However, this approach resulted in an uncontrolled loss of moisture from the vials. Although qualitative data were reproducible, inconsistencies in the quantitative data were observed. Differences in quantitative data could have arisen from several other manually controlled factors. Samples that completely lost moisture from the vial were not accounted for or reported.

Regular beef mince is a coarsely ground product made from trimmings and fats sourced from various parts of the carcass, and it has a limited shelf life. Studies have shown that oxidation can affect the aroma profile during freezing (Al-Dalali et al., 2022). It was hypothesized that lyophilizing beef mince would enhance its shelf life and homogeneity when ground, making it easier to weigh accurately. The altered surface structure and area of the lyophilized samples did not negatively impact the qualitative data, as evidenced by the correlation biplot of the tentatively identified compounds (Figure 3.9). The pan-grilled lyophilized (PG-Ly) sample closely matched the pan-grilled fresh (PG-Fr) sample, while the vial-cooked lyophilized (Vial-Ly) and fresh (Vial-Fr) samples showed similar results. This correlation may be attributed to

the inconsistencies mentioned earlier. Additionally, weighing the vials before and after heat treatment could have helped isolate the samples, thereby reducing inconsistencies.

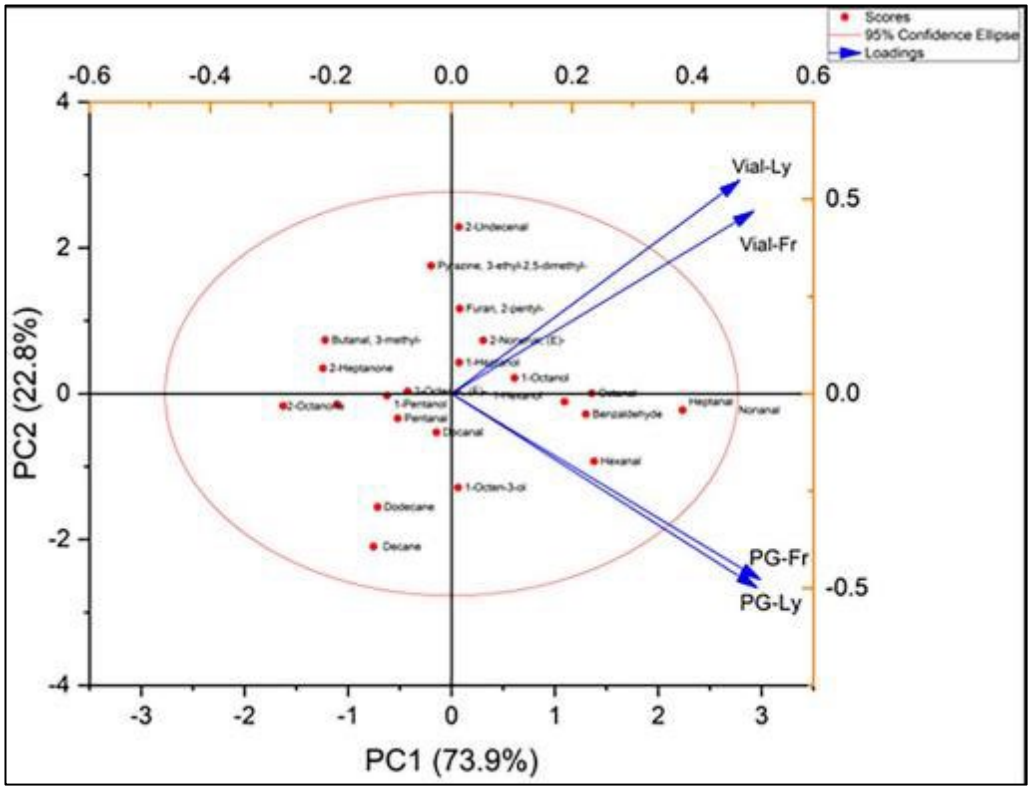

Figure 3.9 PCA biplot indicating the differences in volatile profile due to differences in cooking method and format.

It is important to note that the vectors shown in the PCA biplot only reflect complete data sets, as observations with missing data were excluded by the statistical software. Figure 3.10 illustrates the distribution of the sum of the area under peaks for different compound classes. Aldehydes were the most frequently observed compounds, both qualitatively and quantitatively. The correlation biplots showed a strong association with aldehydes such as hexanal, nonanal, heptanal, and 2-undecenal. Among the samples, nonanal was found in the highest concentration in vial-cooked beef mince, particularly in fresh/native products. This finding aligns well with the literature (Al Dalali et al., 2022) and is linked to fatty acid oxidation, which is typical for most aldehydes. The higher concentration observed in the vial-cooked format may be attributed to the preservation of more volatiles compared to the pan-cooked samples. Significant activity was also noted for hexanal, heptanal, octanal, 2-decanal, and 2-undecenal.

The decreased peak intensity of aldehydes in lyophilised samples may indicate reductions in oxidation rates or delays in the oxidation process. Similar patterns were observed for certain

alcohols, with 1-heptanol and 1-octanol exhibiting high peak intensities in vial-cooked samples. Furan and pyrazine compounds were exclusively found in vial-cooked samples, with the exception of 2-pentyl furan, which appeared in all sample types. Notably, the vial-cooked fresh sample exhibited the highest peak intensity for 3-ethyl-2,5-dimethyl pyrazine and was the only pyrazine detected across all four formats to varying degrees. Moreover, vial-cooked lyophilized samples contained a greater quantity of furans and pyrazines. A significant number of peaks remained unidentified and were categorized as unknowns. The sum of the area for the peaks under the pan-grilled fresh sample for the unidentified compounds was twice that of the other samples, indicating a critical loss of information (Table 3.2).

Table 3.2: Comparing effect of pan grilling vs cooking in the vial using native and lyophilized beef mince.

	Retention Indices (RI)		Pan Grilled LN2 cooled		Vial cooked Ice cooled	
Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Aldehydes						
Butanal, 3-methyl-	914	920	6.07E+04	5.67E+04	6.58E+05	1.83E+06
Butanal, 2-methyl-	920	915		1.44E+05	1.71E+06	6.33E+05
Pentanal	977	983	2.44E+05	2.36E+05	1.72E+06	8.18E+05
Hexanal	1081	1099	3.27E+06	2.73E+06	1.06E+07	3.07E+06
2-Butenal, 2-methyl-, (E)-	1093	1096			4.44E+05	
Heptanal	1186	1188	1.26E+06	1.36E+06	1.11E+07	4.28E+06
4-Heptenal, (Z)-	1241	1234				2.04E+05
Octanal	1290	1299	1.93E+06	1.45E+06	1.37E+07	6.71E+06
Nonanal	1395	1411	5.91E+06	4.70E+06	2.65E+07	1.57E+07
2-Octenal, (E)-	1432	1425	1.94E+05	2.37E+05	1.99E+06	1.33E+06
Decanal	1500	1501	6.37E+05	2.82E+05	1.92E+06	1.23E+06
Benzaldehyde	1527	1504	1.93E+06	1.90E+06	6.39E+06	9.31E+06
2-Nonenal, (E)-	1539	1536	3.11E+05	3.29E+05	7.57E+06	3.44E+06
2-Furancarboxaldehyde, 5-methyl-	1576	1591				5.30E+05
Undecanal	1606	1603	8.04E+04	4.44E+04		
2-Decenal, (E)-	1647	1644	3.83E+05		1.45E+07	1.03E+07
2-Thiophenecarboxaldehyde	1697	1695				5.30E+05
Dodecanal	1710	1709	8.17E+04		1.00E+06	6.97E+05

3-Methyl-2-thiophenecarboxaldehyde	1715	1714				2.88E+05
2-Undecenal	1755	1752	1.09E+05	8.69E+04	9.19E+06	1.08E+07
2,4-Dodecadienal, (E,E)-	1768	1812			3.88E+05	3.90E+05
Tridecanal	1818	1814	1.15E+05	9.82E+04		8.39E+05
2,4-Decadienal, (E,E)-	1815	1809	5.81E+04		2.92E+06	2.24E+06
Tetradecanal	1924	1921	1.95E+05			
Benzeneacetaldehyde, .alpha.-ethylidene-	1935	1939			4.94E+05	
13-Methyltetradecanal	1979	1977			3.10E+05	1.04E+06
Pentadecanal-	1994	1992			5.61E+05	1.53E+06
cis-9-Hexadecenal	2163	2147				8.18E+05
Heptadecanal	2189	2240				8.50E+05
Octadecanal	2349	2347	1.67E+05			4.24E+06
Sum			1.69E+07	1.37E+07	1.14E+08	8.36E+07
Count			18	14	20	26

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Alcohols						
1-Pentanol	1246	1243	1.10E+05	1.06E+05	1.27E+06	4.24E+05
1-Hexanol	1349	1347	1.68E+05	1.78E+05	2.57E+06	6.36E+05
1-Octanol, 2-methyl-	1434	1434				2.16E+05
1-Octen-3-ol	1448	1445	6.62E+05	1.04E+06	1.64E+06	9.25E+05
1-Heptanol	1452	1450	3.05E+05	2.86E+05	5.07E+06	2.24E+06
1-Hexanol, 2-ethyl-	1485	1479	8.28E+04			3.37E+05
1-Octanol	1555	1552	7.26E+05	5.38E+05	7.50E+06	3.48E+06
2-Octen-1-ol, (Z)-	1612	1616	1.32E+05	2.70E+05		
2-Octen-1-ol, (E)-	1613	1616	2.07E+05			6.58E+05
1-Nonanol	1657	1654	1.34E+05		6.90E+05	2.99E+05
2-Furanmethanol	1660	1658			1.60E+06	3.42E+06
1-Decanol	1758	1763			5.27E+05	
1-Dodecanol	1964	1969	5.88E+04			
Sum			2.59E+06	2.42E+06	2.09E+07	1.26E+07
Count			10	6	8	10

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Acids and Esters						
2-Thiopheneacetic acid, octyl ester	1090	1089				3.15E+05
Dodecanoic acid, methyl ester	1802	1799	1.84E+05	1.41E+05		
Isopropyl palmitate	2239	2237	1.22E+05	2.04E+05		
Sum			3.06E+05	3.45E+05	0.00E+00	3.15E+05
Count			2	2	0	1

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Furans						
Furan, 2-ethyl-	945	945			2.94E+06	6.93E+05
2-n-Butyl furan	1129	1122			3.48E+05	
Furan, 2-pentyl-	1231	1239	1.56E+05	2.24E+05	8.41E+06	3.27E+06
trans-2-(2-Pentenyl)furan	1301	1282			1.33E+06	8.60E+05
2-n-Heptylfuran	1432	1429			1.69E+06	8.63E+05
2-n-Octylfuran	1535	1530			2.01E+06	8.53E+05
Sum			1.56E+05	2.24E+05	1.67E+07	6.53E+06
Count			1	1	6	5

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Ketones						
2-Butanone	900	919	6.01E+04		5.41E+05	7.21E+05
2,3-Pentanedione	1054	1054			3.31E+05	
3-Heptanone, 2-methyl-	1164	1164	2.07E+05	3.30E+06		4.39E+05
2-Heptanone	1182	1187	6.21E+04	7.43E+04	1.61E+06	4.54E+05
2-Octanone	1286	1283	5.90E+04	6.24E+04	6.80E+05	2.69E+05
2,3-Octanedione	1326	1335	4.19E+05	8.23E+05		
5-Hepten-2-one, 6-methyl-	1338	1345	2.14E+05	2.27E+05		
2-Nonanone	1390	1387	5.03E+04		1.23E+06	7.59E+05
2-Decanone	1493	1492			1.60E+06	6.96E+05
Ethanone, 1-(2-furanyl)-	1506	1505				2.74E+05
trans-3-Nonen-2-one	1512	1512			3.14E+05	2.47E+05
3,5-Octadien-2-one	1571	1571			1.13E+06	
1-(6-Methyl-2-pyrazinyl)-1-ethanone	1694	1688				4.85E+05

Ethanone, 1-(4,5-dihydro-2-thiazolyl)-	1760	1740	6.37E+04			
Ethanone, 1-(2-thienyl)-	1779	1770				6.67E+05
2-Tridecanone	1809	1813			4.89E+05	5.65E+05
5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	1856	1835	6.01E+04	5.62E+04		
2-Pentadecanone	2020	2019			4.39E+05	4.46E+05
2(3H)-Furanone, dihydro-5-pentyl-	2035	2055			3.51E+05	
Sum			1.20E+06	4.54E+06	8.71E+06	6.02E+06
Count			9	6	11	12

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Thiophenes and S substituted						
2-Acetylthiazole	1648	1661				4.19E+05
Disulfide, dimethyl	1070	1069				3.60E+05
Sum			0.00E+00	0.00E+00	0.00E+00	7.80E+05
Count			0	0	0	2

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Pyrazines						
Pyrazine	1209	1209				4.52E+05
Pyrazine, methyl-	1262	1267			1.21E+06	1.44E+06
Pyrazine, 2,5-dimethyl-	1318	1318		6.40E+04	1.03E+06	1.84E+06
Pyrazine, 2,6-dimethyl-	1324	1325			1.51E+06	1.53E+06
Pyrazine, 2-ethyl-6-methyl-	1381	1402			3.46E+05	8.17E+05
Pyrazine, 2-ethyl-5-methyl-	1388	1406				8.64E+05
Pyrazine, trimethyl-	1400	1401		9.84E+04	8.11E+05	1.23E+06
Pyrazine, 3-ethyl-2,5-dimethyl-	1442	1439	6.88E+04	1.32E+05	7.90E+06	3.97E+06
Pyrazine, 2-ethyl-3,5-dimethyl-	1457	1443			1.15E+06	3.26E+05
Pyrazine, 3,5-diethyl-2-methyl-	1488	1480				6.43E+05
2,3,5-Trimethyl-6-ethylpyrazine	1509	1502				6.55E+05
Pyridine, 2-pentyl-	1570	1592				4.06E+05
Sum			6.88E+04	2.94E+05	1.40E+07	1.42E+07
Count			1	3	7	12

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Hydrocarbons						
Decane	993	990	4.14E+05	6.81E+05	5.39E+05	2.09E+05
Toluene	1038	1035	5.14E+04	3.64E+04		
Nonane, 2,5-dimethyl-	1042	1059	5.10E+04	5.73E+04		
Decane, 4-methyl-	1044	1054	7.43E+04			
Undecane	1092	1100	1.91E+05	1.00E+05	5.76E+05	
Benzene, 1,3-dimethyl-	1137	1142	8.31E+04			
Dodecane	1197	1194	4.41E+05	3.81E+05	5.81E+05	3.94E+05
1,2,4-Trithiolane, 3,5-dimethyl-	1625	1610			8.79E+05	7.49E+05
Indole	2454	2452				6.18E+05
Sum			1.31E+06	1.26E+06	2.58E+06	1.97E+06
Count			7	5	4	4

Compounds	(Calc)	(Ref)	PG-Fr	PG-Ly	Vial-Fr	Vial-Ly
Unidentifiable Peaks						
Unknown	1001		9.05E+04	1.59E+05		
Unknown	1004		1.13E+05	1.55E+05		
Unknown	1027		6.96E+04	1.20E+05		
Unknown	1071		5.58E+04			
Unknown	1077		5.48E+04	7.04E+04		
Unknown	1097		8.25E+04			
Unknown	1150		5.27E+04			
Unknown	1219		7.49E+04			
Unknown	1221		8.92E+04			
Unknown	1238		8.23E+04			
Unknown	1241		7.71E+04			2.04E+05
Unknown	1258				3.83E+05	
Unknown	1282		6.70E+04			
Unknown	1294		8.32E+04			
Unknown	1296		1.09E+05			
Unknown	1330		1.39E+05		2.72E+06	9.87E+05
Unknown	1383		9.41E+04			
Unknown	1429		3.01E+05	9.99E+04		

Unknown	1436	1.73E+05	1.59E+05		
Unknown	1490	1.04E+05	3.72E+05		
Unknown	1496	8.79E+04	3.16E+04	8.48E+05	5.06E+05
Unknown	1509			1.28E+06	6.55E+05
Unknown	1543			3.00E+05	
Unknown	1589				5.96E+05
Unknown	1608			6.26E+05	
Unknown	1609			7.49E+05	3.95E+05
Unknown	1639	1.40E+05	2.61E+05		
Unknown	1677				3.12E+05
Unknown	1695	8.84E+04			
Unknown	1696	7.16E+04			
Unknown	1740				1.04E+06
Unknown	1772	6.48E+04	8.62E+04		
Unknown	1789	1.81E+05		6.50E+05	4.57E+05
Unknown	1791	1.77E+05	1.97E+05		
Unknown	1795	8.12E+04	8.94E+04		
Unknown	1796	8.68E+04			
Unknown	1816	9.67E+04			
Unknown	1818	1.15E+05	9.82E+04		8.39E+05
Unknown	1868	3.01E+05	3.03E+05	5.03E+05	5.93E+05
Unknown	1874	5.88E+04		7.59E+05	2.46E+06
Unknown	1891				3.42E+05
Unknown	1922			1.94E+06	
Unknown	2019				3.60E+05
Unknown	2028	1.40E+05	2.40E+05	1.70E+06	2.97E+06
Unknown	2084			3.59E+05	1.18E+06
Unknown	2135	8.90E+05		1.88E+07	
Unknown	2136	1.06E+06			
Unknown	2190			4.05E+05	
Unknown	2206				1.45E+06
Unknown	2241	6.12E+04	4.82E+04	4.42E+05	
Unknown	2243	8.15E+04	1.01E+06		
Unknown	2347	1.51E+05	1.40E+05	2.08E+06	
Unknown	2372				1.56E+06

Unknown	2582	8.80E+04			
Unknown	2746	1.11E+05			
	Sum	6.04E+06	3.64E+06	3.45E+07	1.69E+07
	Count	40	18	17	18

[a] Volatile compounds tentatively identified using NIST library in Shimadzu LabSolutions.
[b] Retention Indices (calculated) using Van den Dool and Kratz (1963) and Babushok, 2015.
[c] Retention Indices (Reference) from NIST online (https://webbook.nist.gov/chemistry/)
[d] Beef mince -Fresh Pan grilled liquid nitrogen cooled **(PG-Fr)**
[e] Beef mince- Lyophilized- Pan grilled liquid nitrogen cooled **(PG-Ly)**
[f] Beef mince -Fresh- Vial cooked Ice cooled- **(Vial Fr)**
[g] Beef mince- Lyophilized- Vial cooked Ice cooled- **(Vial- Ly)**

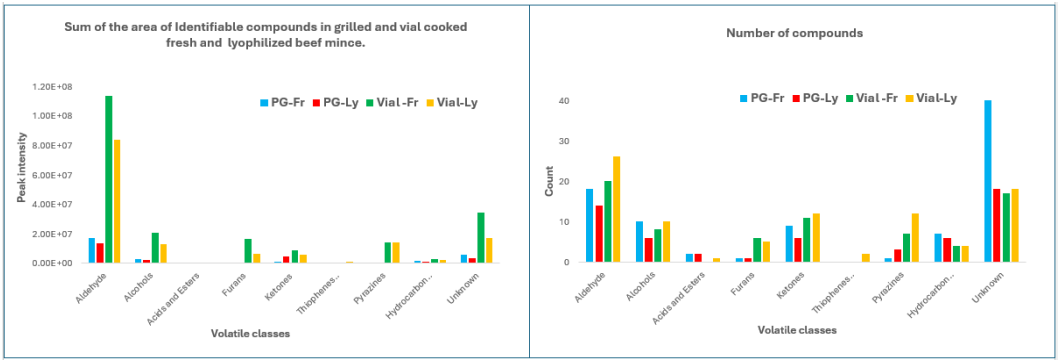

Figure 3.10 Sum of the area under the peak of tentatively identifiable compounds grouped in various compound classes in Fresh whole beef mince (Fr)- Pan grilled (PG) and loyphilized (Ly) compared to vial cooked (Vial) compared to the number of compounds observed for each class.

Effect of post cooking chilling, addition of water and storage on volatile output.

Microbial and autoxidative degradation occur simultaneously, making the deterioration of meat quality unavoidable, regardless of the storage method used. Most studies involving cooked meat samples were conducted with the samples stored in the refrigerator before HS-SPME-GC-MS analysis (Alim et al., 2018; Davila et al., 2022; Devaere et al., 2022; He et al., 2021; Van Ba et al., 2010; Wall and Kerth, 2019). Flores et al. (2006) found that freezing the samples enhanced the analysis of chiral compounds. In contrast, Al-Dalali et al. (2022) and Huang et al. (2013) reported that freezing can negatively impact various oxidative processes. Additionally, the formation of harmful volatile compounds related to warmed-over flavour (WoF) in stored, cooked meat is well documented. Key volatile classes associated with WoF in cooked meat include aldehydes, alcohols, and ketones (Chen et al., 2024).

Lyophilised beef mince (FDBM-w) was intended to serve as a control for comparing volatile formation and development in plant-based ingredients. To meet this objective, it needed to be stored over an extended period with minimal oxidative degradation before cooking. Additionally, when using Solid Phase Microextraction (SPME) as an extraction method, two important factors needed to be understood: 1. Would volatiles when condensed during chilling impact the aroma profile? 2. To what extent would the addition of water (to create a slurry and facilitate stirring the sample) influence volatilisation? To investigate these factors, rehydrated lyophilised beef mince was used to study the effects of not cooling the sample, cooling immediately after heat treatment, freezing, and adding water prior to equilibration and extraction.

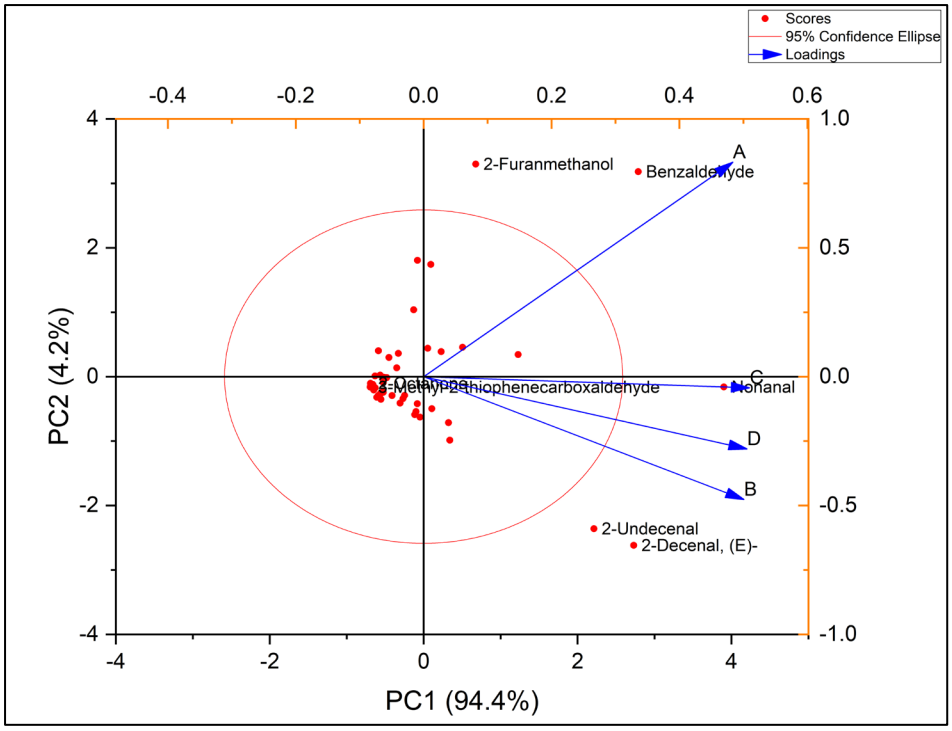

Figure 3.11 Correlation biplot of the effect of storage (no cooling/ cooling/ freezing) and the addition of water to rehydrated-lyophillized whole beef mince heat treated in an oil bath at 160ºC for 30 minutes. (A)- Not cooled, no water added, analysed same day. (B)- Chilled, no water added, analysed same day. (C)- Chilled, water added, analysed same day. (D)- Frozen, water added, analysed over multiple days.

Sample A appeared to be significantly different from Samples B, C, and D (Figure 3.11). Unlike the others, no water was added to create a slurry for Sample A, and it was not chilled. Instead, it was transferred directly from the oil bath at 160°C to a water bath to equilibrate and extract at 70°C for 30 minutes. In contrast, Sample B did not have water added either; however, it was immediately chilled in an ice bath after heat treatment. The results from Sample B aligned more closely with those of Samples C and D, where water was added to create a slurry and facilitate the agitation of the sample. This suggests that chilling or freezing the sample had a more significant impact than the addition of water. Unfortunately, adding water to Sample A was not feasible without allowing the sample to cool down sufficiently. Although it would have been possible to add boiling water to maintain a high temperature, the intent was to keep the temperature low enough to preserve and optimally extract the analytes

The sum of the area for peak intensities among the samples showed no significant differences. The compounds contributing to these differences included nonanol, benzaldehyde, 2-decenal

(E), 2-undecenal, furanmethanol, methylpyrazine, and 2,5-dimethyl pyrazine. The variations in volatile output could not be linked to the formation of WoF compounds due to a weak correlation. Additionally, the addition of water did not affect the volatile output as initially suspected. No variability was observed in samples stored in the freezer for over a week (data not shown). Therefore, it was decided to heat-treat the samples in batches of six, use an ice bath to chill them, freeze the samples immediately, and utilize them within 48 hours. The results of the study are summarized in Table 3.3 below.

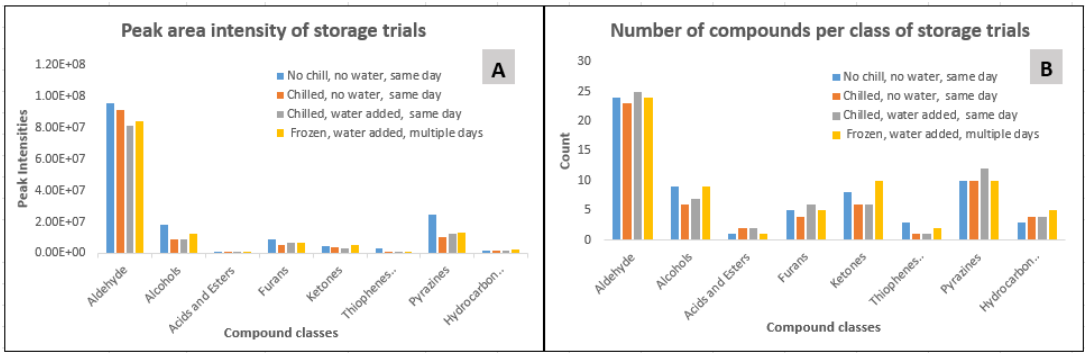

Figure 3.12 Effect of chilling and addition of water to volatile output of vial sealed lyophilized whole beef mince heat treated at 160ºC for 30 minutes in an oil bath. (A) Sum of area of the peaks for each compound class. (B) Total count of compounds under each class.

Aldehydes were the most prominent compounds observed both qualitatively and quantitatively. These compounds are generally known to result from lipid oxidation, particularly secondary oxidation of fatty acids (Skibsted et al., 1994; Huang et al., 2013; Shimizu & Iwamoto, 2022). Their significant presence may have influenced the analysis of compounds found in lower concentrations. The number of aldehydes appeared to remain consistent regardless of cooling, water addition, or storage conditions. However, their intensity decreased slightly over time, particularly in the frozen samples. Existing literature indicates that the concentration of aldehydes initially increases and then decreases during the storage of cooked meat (Skibsted et al., 1994). Our findings reveal that while the total area under the peaks for aldehydes increased slightly, the number of observed peaks decreased, suggesting a rise in peak areas. Specifically, pentanal, hexanal, heptanal, octanal, and nonanal all increased, while there was a slight decrease in the peak area for benzaldehyde. This decrease may be attributed to the beef mince from a supermarket shelf being predisposed to oxidative damage. Undecanal was not detected in the frozen samples, which may have been an analytical anomaly.

The presence of 2-methyl and 3-methyl butanal is associated with bacterial degradation in aerobically packaged meat and has also been identified as a confinement odour (Reis et al., 2016). Although Reis et al. (2016) observed this in vacuum-packaged lamb during storage, 2-methyl and 3-methyl butanal were consistently found in most samples, possibly indicating confinement due to the heat treatment of minced meat in sealed vials. Overall, the lipid content of the samples and the storage conditions were key factors in the development of compounds associated with off-flavours and undesirable odours (Chen et al., 2024; Al-Dalali et al., 2022).

Alcohols: The formation of alcohols can be quite complex, depending on the matrix. In cooked beef, alcohols may be produced through degradation processes such as oxidation or hydrolysis of fats, or from the further reduction of aldehydes and ketones. Additionally, their presence in high-temperature cooked foods could accelerate the formation of Maillard reaction products and advanced glycation end-products (AGEs) (Wu et al., 2020). The concentrations of hexanol, heptanol, and octanol remained unchanged. Nonanol was not detected in the no-water, no-chill, same-day sample, but it remained consistent in the other samples. A slight increase in 1-octen-3-ol was observed in the frozen sample. 2-octen-1-ol was only detected in the frozen sample, indicating its development as a confined volatile, which has also been noted in autoclaved beef (Van Ba et al., 2010). 2-Furanmethanol has been suggested to form through the Maillard reaction (Ames et al., 2001) and was consistently found in all samples except the non-chilled one. This suggests low oxygen levels in the frozen samples, leading to a slowdown in oxidative processes. Lastly, 5-methyl-2-furanmethanol was observed only in the non-chilled sample.

Acids and esters: Not many acids or esters were identified. Depending on the polarity analytes can be strongly or weakly bound to SPME fibers. Both CAR/PDMS and DVB/CAR/PDMS fibers moderately adsorb polar compounds (Kataoka et al., 2000). Also, the dynamism and reactivity between alcohols and acids and the formation of esters could affect identification in a complex matrix. Only two esters were identified, and no specific trend could be observed between the different treatments.

Ketones: Formed from the oxidative or lipid thermal degradation, ketones are believed to be reactive and interact with Maillard reaction products to form other products. This is evident from the decrease of ketones overall with storage, also observed by Zhang et al. (2022).

Furans: Identified as a key aroma class of compounds in beef flavour, their identification is difficult due to instability and low concentration (Maga & Katz, 1979). -Ethyl, -pentyl, -heptyl

and -octyl furans were identified along with furfural. Their concentration remained stable among the different treatments indicating there was no significant effect from the treatments**.**

Pyrazines. No pyrazines were lost by either chilling, addition of water or freezing. However, the concentration of pyrazines almost halved when the sample was chilled (Figure 3.12) indicating cooling had a negative effect. There were varied responses amongst the observed pyrazines. There was no consistency in the observation of pyrroles and pyridines. Methyl Pyrazine, 2,5-dimethyl pyrazine and 2,6-dimethyl pyrazine, trimethyl pyrazine decreased in chilled samples. 2-ethyl-6-methyl-pyrazine concentration was higher in chilled and water-added samples.

Hydrocarbons: Indole was the only hydrocarbon compound consistently observed in all studies. The mass spectrometry library was set to identify compounds separated on a 5MS column, which thereby limited the ability to identify hydrocarbons and other compounds using Lab solutions. Overall, the different treatments had no significant effect on chilling, water addition, or short-term storage.

Table 3.3: Effect of chilling, the addition of water and storage on the formation and identification of aroma compounds using DVB/CAR/PDMS fibers

	Retention Index		Water-No Chill-No	Water- Yes Chill-Yes	Water Yes Chill-Yes	Water Yes Frozen
Compounds by class	(Calc)	(Ref)	same day	same day	same day	1+ week
Aldehydes						
Butanal, 2-methyl-	908	915	6.61E+05	6.97E+05	1.01E+06	6.33E+05
Butanal, 3-methyl-	912	920	1.89E+06	2.13E+06	3.15E+06	1.83E+06
Pentanal	974	983	1.14E+06	5.22E+05	6.10E+05	8.18E+05
Hexanal	1078	1099	3.96E+06	2.14E+06	2.08E+06	3.07E+06
Heptanal	1183	1188	5.64E+06	3.85E+06	3.38E+06	4.28E+06
4-Heptenal, (Z)-	1241	1234				2.04E+05
Octanal	1288	1299	8.06E+06	6.23E+06	5.49E+06	6.71E+06

Nonanal	1393	1411	1.71E+07	1.55E+07	1.29E+07	1.57E+07
2-Octenal, (E)-	1430	1425	1.57E+06	2.13E+06	1.41E+06	1.33E+06
Decanal	1498	1501	1.30E+06	1.44E+06	1.08E+06	1.23E+06
Benzaldehyde	1524	1504	1.71E+07	9.81E+06	1.09E+07	9.31E+06
2-Nonenal, (E)-	1536	1524	3.70E+06	4.95E+06	2.66E+06	3.44E+06
Undecanal	1603	1604	6.42E+05	8.16E+05	7.92E+05	
2-Decenal, (E)-	1644	1638	9.96E+06	1.55E+07	9.99E+06	1.03E+07
3-Thiophenecarboxaldehyde	1695	1687	1.89E+06	1.01E+06	1.20E+06	5.30E+05
Dodecanal	1709	1720	6.02E+05	7.08E+05	6.75E+05	6.97E+05
3-Methyl-2-thiophenecarboxaldehyde	1714	1765	4.05E+05	2.85E+05	3.79E+05	2.88E+05
2-Undecenal	1752	1740	8.18E+06	1.17E+07	8.14E+06	1.08E+07
2,4-Decadienal, (E,E)-	1812	1827	2.36E+06	3.42E+06	1.84E+06	2.24E+06
Tridecanal	1814	1833			1.02E+06	8.39E+05
2-Dodecenal, (E)-	1859	1889			3.50E+05	
Tetradecanal	1921	1940	1.77E+06	2.14E+06	2.73E+06	2.17E+06
13-Methyltetradecanal	1977	1990	1.87E+06	1.06E+06	1.80E+06	1.04E+06
Pentadecanal-	1992	1999	1.45E+06	1.49E+06	2.20E+06	1.53E+06
cis-9-Hexadecenal	2161	2147	5.88E+05	7.53E+05	9.21E+05	8.18E+05
Heptadecanal	2240	2247	9.61E+05			
Octadecanal	2347	2343	2.51E+06	3.14E+06	4.36E+06	4.24E+06

			Sum	9.53E+07	9.15E+07	8.11E+07	8.40E+07
			Count	24	23	25	24
Alcohols							
1-Heptanol, 3-methyl-	1093			6.01E+05			
1-Pentanol	1243	1251		5.55E+05			4.24E+05
1-Hexanol	1347	1353		6.12E+05	4.10E+05	5.25E+05	6.36E+05
1-Octanol, 2-methyl-	1434						2.16E+05
1-Octen-3-ol	1445	1447		7.55E+05	7.27E+05	6.64E+05	9.25E+05
1-Heptanol	1450	1460		1.87E+06	1.64E+06	1.39E+06	2.24E+06
1-Octanol	1552	1546		2.88E+06	2.97E+06	2.41E+06	3.48E+06
2-Octen-1-ol, (E)-	1612	1616					6.58E+05
1-Nonanol	1654	1668			3.70E+05	3.75E+05	2.99E+05
2-Furanmethanol	1658	1678		1.03E+07	3.12E+06	3.36E+06	3.42E+06
2-Furanmethanol, 5-methyl-	1719	1711		3.58E+05			
2-Thiophenemethanol	1943	1937		6.33E+05		1.79E+05	
			Sum	1.85E+07	9.24E+06	8.90E+06	1.23E+07
			Count	9	6	7	9
Esters and Acids							
2-Thiopheneacetic acid, octyl ester	1089				3.64E+05	4.28E+05	3.15E+05
Dodecanoic acid, methyl ester	1799	1804		3.23E+05	3.94E+05	3.37E+05	
			Sum	3.23E+05	7.58E+05	7.64E+05	3.15E+05

Count			1	2	2	1
Ketones						
2,3-Pentanedione	1054	1071				3.02E+05
2-Heptanone	1180	1187	8.83E+05	4.06E+05	4.83E+05	4.54E+05
2-Octanone	1283	1285	5.10E+05	2.92E+05	3.60E+05	2.69E+05
2-Nonanone	1387	1394		8.35E+05		7.59E+05
2-Decanone	1492	1515	4.83E+05	6.04E+05	6.47E+05	6.96E+05
Ethanone, 1-(2-furanyl)-	1505	1511	6.45E+05		1.93E+05	2.74E+05
trans-3-Nonen-2-one	1512	1523	nd			2.47E+05
Dihydro-3-(2H)-thiophenone	1563	1547	3.25E+05			
3,5-Octadien-2-one	1571	1565				1.02E+06
Ethanone, 1-(3-thienyl)-	1770	1772	1.11E+06	8.98E+05	8.97E+05	6.67E+05
Ethanone, 1-(1H-pyrrol-2-yl)-	1971	1977	3.10E+05			
2-Pentadecanone	2019	2010	4.95E+05	5.05E+05	5.74E+05	3.60E+05
Sum			4.76E+06	3.54E+06	3.15E+06	5.05E+06
Count			8	6	6	10
Furans						
Furan, 2-ethyl-	945	945	1.15E+06	6.69E+05	1.13E+06	6.93E+05
Furan, 2-pentyl-	1229	1239	4.37E+06	2.72E+06	3.06E+06	3.27E+06
trans-2-(2-Pentenyl)furan	1300	1282	1.19E+06	8.01E+05	8.10E+05	8.60E+05
2-n-Heptylfuran	1430	1429			5.40E+05	8.63E+05

Furfural	1465	1467	1.04E+06		2.22E+05	
2-n-Octylfuran	1533	1530	9.24E+05	9.51E+05	8.39E+05	8.53E+05
Sum			8.67E+06	5.14E+06	6.60E+06	6.53E+06
Count			5	4	6	5
Thiophenes						
Thiophene, 2-methyl-	1090	1090	9.09E+05			
2-Acetylthiazole	1647	1661	1.40E+06			4.19E+05
Disulfide, dimethyl	1069	1072	5.43E+05	3.14E+05	5.70E+05	3.60E+05
Sum			2.85E+06	3.14E+05	5.70E+05	7.80E+05
Count			3	1	1	2
Pyrazines						
Pyrazine	1208	1209	1.63E+06	4.16E+05	4.30E+05	4.52E+05
Pyrazine, methyl-	1261	1267	5.42E+06	1.43E+06	1.62E+06	1.44E+06
Pyrazine, 2,5-dimethyl-	1317	1318	5.84E+06	1.93E+06	2.45E+06	1.84E+06
Pyrazine, 2,6-dimethyl-	1323	1325	4.14E+06	1.64E+06	1.74E+06	1.53E+06
Pyrazine, 2-ethyl-6-methyl-	1380	1402	1.34E+06	9.23E+05	9.30E+05	8.17E+05
Pyrazine, 2-ethyl-5-methyl-	1387	1406	1.34E+06	8.72E+05	1.10E+06	8.64E+05
Pyrazine, trimethyl-	1398	1401	2.42E+06	1.19E+06	1.38E+06	1.23E+06
Pyrazine, 3-ethyl-2,5-dimethyl-	1440	1439	1.87E+06	1.40E+06	1.48E+06	3.97E+06
Pyrazine, 2-ethyl-3,5-dimethyl-	1456	1443	4.67E+05	3.05E+05	3.34E+05	3.26E+05
Pyrazine, 3,5-diethyl-2-methyl-	1488	1480		3.18E+05	2.23E+05	6.43E+05

Pyrrole	1515	1525	4.34E+05		2.48E+05	
Pyridine, 2-pentyl-	1568	1592			4.92E+05	
Sum			2.49E+07	1.04E+07	1.24E+07	1.31E+07
Count			10	10	12	10

Hydrocarbons						
Decane	990	1000		3.13E+05	1.85E+05	2.09E+05
Dodecane	1194	1200	4.64E+05	3.10E+05		3.94E+05
Tridecane	1294	1300		3.78E+05	3.42E+05	4.39E+05
Nonane, 2-methyl-5-propyl-	1294		5.63E+05			
2,6,10-Trimethyltridecane	1433				1.77E+05	
1,2,4-Trithiolane, 3,5-dimethyl-	1625	1610				7.49E+05
Indole	2452	2444	7.91E+05	8.65E+05	1.04E+06	6.18E+05
Sum			1.82E+06	1.87E+06	1.75E+06	2.41E+06
Count			3	4	4	5

Note: Compounds presented are selected compounds tentatively identified from Total ion chromatogram in full scan mode. Retention Indices were calculated (Calc) using Bianchi et al (2007) and Reference (Ref) retention Indices were obtained from NIST web page, where possible and available; Ref RIs were matched with Van den dool and Kratz or Normal alkane, polar column (SUPELCO WAX 10 and those published by Bianchi et al (2007).

Establishing a calibration curve and retention indices.

Retention indices are calculated using the retention times of linear alkanes. They help in ascertaining and identifying volatiles based on retention time and elution order in conjunction with mass spectrum data. Key factors in selecting alkane standards depend on the range required for the study, the type of column used, GC run time and the maximum allowable oven temperature. In our case, the application of alkanes C_7- C_{40} was based on the intention of conducting untargeted analysis. However, it should be noted that SPME fibers possess only a

limited range for adsorbing analytes. Hence, the use of longer chain alkanes >C_{30} is a misapplication; however, it has been applied in literature (Ahamed et al., 2024; Zhang et al., 2023; Costa et al., 2019). Also, since alkane standards eluted depending on their boiling point, our use of the wax column could not elute the entire range of alkanes within the optimized GC run time due to the column's maximum temperature limit. Thereby requiring the run time to be increased significantly and the column to be held at the maximum temperature for an extended period to ensure the elution of the alkanes (Figure 3.13). Further, the mass spectrometer library contained spectrum references catered to the 5MS (nonpolar) column and as such, the retention indices did not match the elution times of alkanes from the Wax column for some higher molecular weight alkanes. Integration of the alkane standards into the spectrum process library was not possible further complicating identification. Retention indices of alkanes C_7-C_{33} (Figure 3.13) were achieved by comparing them to the NIST database. In essence alkane standards from C_7-C_{30} would be adequate to match the ability of DVB/CAR/PDMS fiber to adsorb analytes ranging from MW 40 -275.

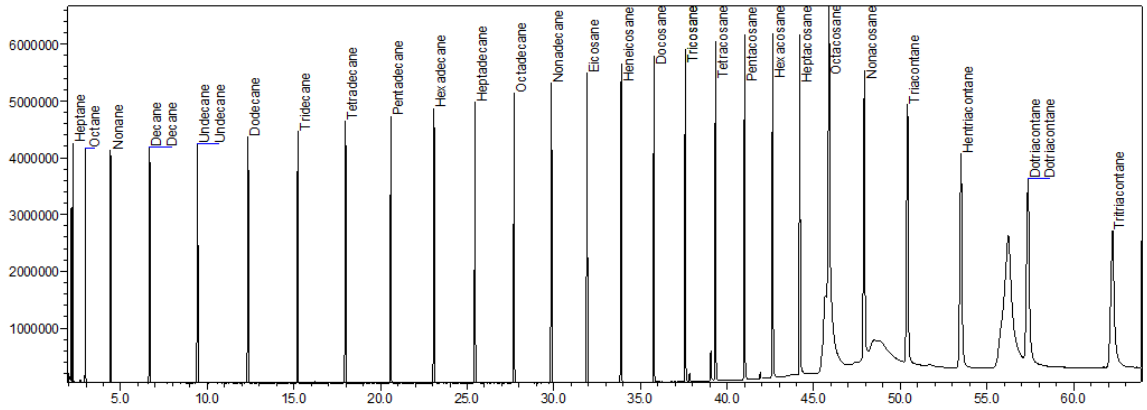

Figure 3.13 Inability to elute the complete range of C_7- C_{40} saturated alkanes on a SuplecoWax10 column even with extended GC run time at maximum allowable temperature.

Internal Standard (IS): 2-methyl-3-heptanone was trialled as an internal standard to calculate the calibration curve for semi-quantitative analysis as applied in literature (Liu et al., 2018; Sun et al., 2021; Tingle, 2023; Yuan et al., 2023; Zhang et al., 2022). The use and application of an internal standard by SPME have been reasoned (Eisert & Pawliszyn, 1997), debated (Nolvachai et al., 2023), and critically reviewed (Ouyang & Pawliszyn, 2008). Further questions led to the uncertainty of its use, especially in relation to quantification. 1. Since the IS was insoluble in water and would require a solvent as a dispersant, would the addition of the standard containing a solvent (methanol/ hexane) affect the volatility of the environment within the vial? Which analytes will the fibers adsorb first, and how much of it? 2. Does adding IS to the vial and

equilibrating at 70ºC affect fiber durability (swelling) or performance? 3. Can the IS calibration curve (peak area) be applied to the whole chromatogram- this is because absorbance intensities across the chromatogram vary considerably, especially in higher molecular weight compounds; hence, standards would need to be closer to the peak of interest or be the exact match.

It was also observed that recovery was dependent on the matrix. Recovery from water was much higher than when added to the lyophilised beef sample. Could this mean that recovery would be affected if the matrix changed? We did not further challenge recovery in different matrices. However, linearity was affected by the range of concentration in complex media which meant that we achieved excellent results when using 2-methyl-3-heptanone as a direct injection external standard with a concentration range from 0.01 ppm-1000 ppm compared to using it as an internal standard in water with a concentration range of 10 ppm-1000ppm and as an internal standard in heat treated lyophilised beef mince with a concentration range of 200-800 ppm. Also, due to the extensive GC run time (50 min) per sample and manual application of the procedure only a maximum of 6 samples per day could be analysed, making it difficult to conduct a calibration curve run each day prior to running the sample.

At this stage, several unknown mechanisms remain untested, and many questions remain unanswered, particularly regarding the use of an internal standard with the application of solid phase microextraction (SPME). Although our lyophilised samples performed well, we did not conduct rigorous performance or proficiency tests for their use. The repeatability and reproducibility of the qualitative analysis were excellent, as reflected by the consistent retention times. However, manually controlling the temperature and time during equilibration and extraction—combined with the manual addition of the sample—introduced variations in peak patterns and dynamic drifts in the chromatogram that were challenging to manage. This variability affected the detection and identification of volatiles. Additionally, the Shimadzu Lab Solutions library could only be utilized in conjunction with the 5MS column, further complicating identification efforts. In this context, internal standards were deemed inappropriate for quantifying volatiles due to their wide variations.

Conclusions

The objective of this study was to use beef mince as a control to compare aroma compounds—especially those formed during high-temperature cooking—with plant-based ingredients used in the production of meat analogues. Using untargeted analysis with Solid Phase Microextraction (SPME), we successfully extracted, separated, and tentatively identified

approximately 50% of the observed volatile compounds. The 50/30 µm DVB/CAR/PDMS fiber performed slightly better than the 75 µm CAR/PDMS fiber in extracting analytes, which contrasts with some findings reported in the literature.

The population means of various treatments did not follow a linear progression or demonstrate a consistent relationship as expected. Instead, the results exhibited a random pattern with no discernible trend. Through manual handling of samples, both the 50/30 µm DVB/CAR/PDMS and 75 µm CAR/PDMS fibers showed optimal equilibration and extraction of analytes when subjected to 70°C for 30 minutes. Heat-treating lyophilised beef mince in a vial using an oil bath yielded consistent qualitative results.

Preparing samples in small batches for analysis—ensuring they are not stored in the freezer for more than 48 hours—resulted in reliable outcomes. However, the use of an internal standard proved challenging and was more indicative of quality than of quantification. The conclusions drawn from this research are considered speculative, as there is a possibility of misinterpretation or loss of data from peaks that could not be identified. The following chapters aim to guide prospective researchers in developing methodologies for their research, highlighting the advantages of using SPME as an extraction tool while also noting its limitations.

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Chapter 4

The origins of meaty aromas

Introduction

A search within Google Scholar for open-access publications on 'plant-based meat' flavour from 2023-2025 returned 4,180 results. The number of publications on plant-based proteins and meat alternatives has increased considerably, and so has the interest (Karabulut et al., 2024; Mustapa et al., 2025). The consensus and conclusions are that there is a slow uptake of plant-based meats, and consumers are eager to try them. Overall, most research and reviews found that significant sensory and volatile profile differences exist between meat and plant-based meat, but more investment in research is needed to close the gap if we are to meet consumer preferences (Giezenaar et al., 2024; Hernandez et al., 2023; Sogari et al., 2023)). On the contrary, although willingness to change is evident, appreciation of beef has not diminished (Caputo et al., 2022; Cleland et al., 2025; Giezenaar et al., 2024) whilst what constitutes meaty still being debated (Carreño, 2022).

Beef Flavour Appreciation and Aroma Compounds

The appreciation of beef is a multifaceted process that engages preceptory as well as visual and olfactory senses (Fromson, 2015; Kerth & Miller, 2015). Olfaction plays a significant role in the sensory experience of eating (Dunkel et al., 2014). The flavour profile of beef is largely determined by a combination of the meat's biological constituents, such as specific precursors (Li et al., 2023; Spanier et al., 2004), recipe development and cooking conditions. Aroma compounds in beef exposed to high temperatures can be formed from various sources. This includes the ongoing enzymatic processes during post-mortem aging, the rapid oxidation and degradation of unsaturated lipids and proteins, the formation of Maillard reaction products (responsible for browning during cooking) and the concomitant interactions between the degradative products, including the post-heat treatment development of compounds observed as warmed-over flavours (WoF) (Kerler & Grosch, 1996; Resconi et al., 2013). These compounds are mainly small, volatile molecules that can be detected by the human olfactory system undeterred by relabelling (Cormiea & Fisher, 2023).

A significant portion of beef's characteristic aroma has been attributed to a few key volatile compounds, such as aldehydes (e.g., hexanal, nonanal), ketones (e.g., 2-heptanone), alcohols (e.g., 1-octen-3-ol), and sulphur-containing compounds (e.g., dimethyl disulphide), as well as carboxylic acids, esters, and hydrocarbons. These compounds contribute to beef's complex bouquet, ranging from grassy, fatty, and meaty notes to roasted and caramelized characteristics. Among these, aldehydes and ketones are often associated with the fatty and oxidative aspects

of beef aroma, while sulphur compounds are linked to more pungent and savory aspects. Alcohols, esters, acids, furans thiophenes and pyrazines are all intermediates of lipid oxidation and Maillard reaction products (Cabral, 2019; He et al., 2021; Wall & Kerth, 2019; Zhang et al., 2021). Given the intricate nature of beef flavour, detecting these volatiles requires sophisticated analytical techniques. Currently, no standard methods exist for isolating and identifying these compounds other than the widely used gas chromatography hyphenated with tools such as mass spectrometry for volatile analysis. Even so, the ability to concentrate and extract analytes of interest remains cumbersome and time-consuming (Ahamed et al., 2023; Beauchamp et al., 2022; Bizzo et al., 2023; Ismail et al., 2020; Kempińska-Kupczyk and Kot-Wasik, 2019).

Several methods exist for isolating analytes of interest, with the most used approaches being solvent-assisted flavour evaporation (SAFE), steam distillation extraction (SDE), and solid-phase microextraction (SPME). These methods offer varying levels of sensitivity, specificity, and convenience, with each having its own set of advantages and limitations. Understanding these methods is essential for selecting the most appropriate analytical technique for any given study of beef aroma volatiles (Ahamed et al., 2023, Liu et al., 2018; Sun et al., 2021; Watkins et al., 2012).

Analysis by SPME: Advantages, Disadvantages, and Challenges

Solid phase microextraction (SPME) is one of the most widely used methods for analysing volatile compounds. It has gained popularity with applications in varied foods and formats such as solid, liquid and gaseous (headspace) (Murtada & Pawliszyn, 2024). This method offers several key advantages; SPME's ability to extract analytes of interest based on a non-exhaustive, non-selective (limited by the range) from a small sample size, with minimal sample preparation and small chemical footprint makes it an attractive green technology (Merkle et al., 2015; Rocha et al., 2022). It can preserve the volatile profile of the sample without introducing external contaminants. Additionally, SPME can be easily integrated with gas chromatography-mass spectrometry (GC-MS), offering a high degree of versatility, sensitivity and specificity in identifying volatile compounds. These characteristics make SPME an attractive option for flavour analysis of whole foods like beef, where the complex volatile profile needs to be captured accurately (Kataoka et al., 2000; Roberts et al., 2000; Spietelun et al., 2013).

When comparing different extraction methods, Watkins et al. (2012) showed that SPME was able to extract more volatiles compared to steam distillation extraction (SDE) from beef and

lamb fat. Whilst Sun et al. (2021) compared SPME with Solvent assisted flavour extraction (SAFE) and found that volatile profiles between the two methods were different and may be complimentary rather than comparative. SDE and SAFE were however liquid based compared to headspace analysis by SPME. Madruga et al. (2009) compared SDE, entrainment on Tenax and SPME and observed that SPME (CAR/PDMS Fibers) produced comparably less favourable results. SPME was unable to detect aldehydes with high boiling points (>200ºC) with similar results for lipid-derived alcohols and ketones. Could this be due to the Fiber selection or affinity towards smaller molecular weight compounds, thereby getting saturated with no ability to extract larger molecules, which could be characteristic aroma compounds (Madruga et al., 2009)?

Hence, SPME has some notable limitations (Kaeppler & Mueller, 2013; Spietelun et al., 2013). One challenge is coelution, which occurs when two or more compounds elute simultaneously, making it difficult to distinguish them in the final analysis (Cserháti, 2010; Wieczorek et al., 2020). This can be particularly problematic when analysing complex matrices like beef, where many volatile compounds are present in similar concentrations. Coelution may result in peak overlap, leading to the loss of valuable information about individual aroma compounds. Large variations in peak intensities can affect sensitivity, leading to inaccurate analysis. Another issue is competition for binding sites for the extraction of fiber. SPME Fibers have a limited capacity for volatile compound adsorption, which means that volatile compounds in a sample can result in competitive interactions. This reduces the extraction efficiency of certain compounds, especially those in low concentrations (Shirey, 2012). Additionally, the fiber capacity can limit the ability to extract larger, less volatile compounds, which may be important in characterizing the full aroma profile of beef. Furthermore, the choice of fiber coating material can affect extraction efficiency. Different coatings have varying affinities for specific classes of compounds, and thus, optimizing the Fiber type is essential to ensure the extraction of the most relevant volatiles for beef flavour analysis. SPME is based on the equilibrium principle, and as such, extraction and concentration are dependent on attaining equilibrium in the headspace, thereby requiring a good understanding of the partitioning coefficient, effect of vapour pressure etc. Despite these challenges, SPME remains a powerful and versatile tool for volatile analysis, provided these limitations are carefully considered and addressed (Ai, 1997, Bleicher et al., 2022)

Effect of Beef's Complex Matrix on Aroma Analysis

The complexity of the beef matrix presents significant challenges for aroma analysis, particularly when using techniques like SPME. Beef is composed of a variety of components, including muscle tissue, fat, and sarcoplasmic proteins, each of which can influence the volatile profile (Cobos & Días, 2015; Mottram, 1998) The matrix's complexity arises from interactions between these components, as well as from the state and type of cooking (Dreeling et al., 2000). The muscle tissue contains various compounds that contribute to aroma, including amino acids, peptides, and lipids. When beef is cooked, these components undergo Maillard reactions, lipid oxidation, and protein degradation concomitantly, forming volatile compounds (Cobos & Díaz, 2015). Although volatile formation pathways have been mapped in model studies it is difficult to do so in whole foods due to the inability to isolate fractions and instrumentation limits (Nolvachai et al., 2023). Fat, which has a high capacity for retaining and releasing volatiles, is particularly important in beef flavour, as it stores many fat-soluble aroma compounds released during cooking. However, it also complicates the extraction process, as volatile compounds may be tightly bound in fat droplets or trapped within the muscle fibers, making them more difficult to extract and quantify accurately (Chevance et al., 2000).

Sarcoplasmic proteins, which include enzymes and other water-soluble proteins, also contribute to the volatile profile, particularly through enzymatic reactions that occur post-mortem. These proteins can catalyse the formation of specific volatiles, such as aldehydes and alcohols (Cobos & Días, 2015), which can significantly affect beef aroma. However, these proteins can also interact with other components in the matrix, altering the availability of volatiles for extraction (Reineccius, 2022, Wang et al., 2023). For example, the presence of fat can impede the release of water-soluble compounds, while muscle fibers may physically trap volatiles within the tissue (Domínguez et al., 2019)

The matrix effect is further complicated by the presence of varying fat content across different cuts of beef, as well as the animal's age, breed, and diet, all of which can influence the volatile profile. Additionally, aging and cooking temperature significantly impact the release and transformation of aroma compounds. As a result, achieving accurate and reproducible results when analysing beef volatiles requires careful consideration of these matrix effects. It often necessitates using optimized extraction protocols to ensure that all relevant compounds are adequately captured (Bleicher et al., 2022).

In conclusion, analysing the aroma volatiles in whole beef mince presents various technical and analytical challenges due to the complex nature of beef's chemical composition and the limitations of existing analytical methods. In lieu of such challenges, fractionation was sought into three distinct parts. Namely Fat, Sarcoplasmic (aqueous extract) and myofibrillar fractions. Although this has been effectively carried out before (Duarte et al., 1999), fractionation has not been attempted to analyse volatiles. Herein, fractionation was conducted to observe the formation of volatiles from lipid and protein oxidation, the Maillard reaction and their interaction.

Materials and Method

Lyophilisation

During pre-trials, it was observed that extraction and fractionation were easier when using freeze-dried Beef mince compared to native raw beef mince (Nowak & Jakubczyk. 2020). Hence, freeze-dried beef mince (FDBM) was prepared as described in Chapter 2 and in brief as follows: A local grocery store brought regular beef mince 18%fat (3×500g), which was combined and homogenized lightly. A sub-sample of 100g was stored at -80ºC. The remainder was flattened thinly between sheets of plastic film (Cling Wrap-Local store) and frozen at -20ºC overnight. The frozen samples were lyophilized using Christ Alpha 1-4 LSC plus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), following which it was ground using a Breville (Australia) coffee and spice control grinder and stored at -20ºC in small sample (50grams) containers.

Fractionation of beef mince

Fractionation was conducted using freeze-dried beef mince by mechanical and chemical separation. Five distinct fractions (Figure 4.1) were obtained by separating the muscle tissue, sarcoplasm and fat. A*). Aqueous extract (AE), b*). De-blooded muscle fraction (DB), c*). Fat extract (FAT), d*). De-fatted muscle fraction (DF), e). De-blooded and defatted muscle fraction (DBDF).

Lyophilised whole beef mince 100 grams was weighed (West lab balance series 200g, Victoria, Australia) into a beaker and 250ml of chilled deionized water (Milli-Q, Merck Millipore, Darmstadt, Germany) was added. The sample was held at 4 ºC and stirred occasionally for an hour. The sample was transferred to centrifuge bottles (250 mL, polypropylene bottle with Screw-On Cap)

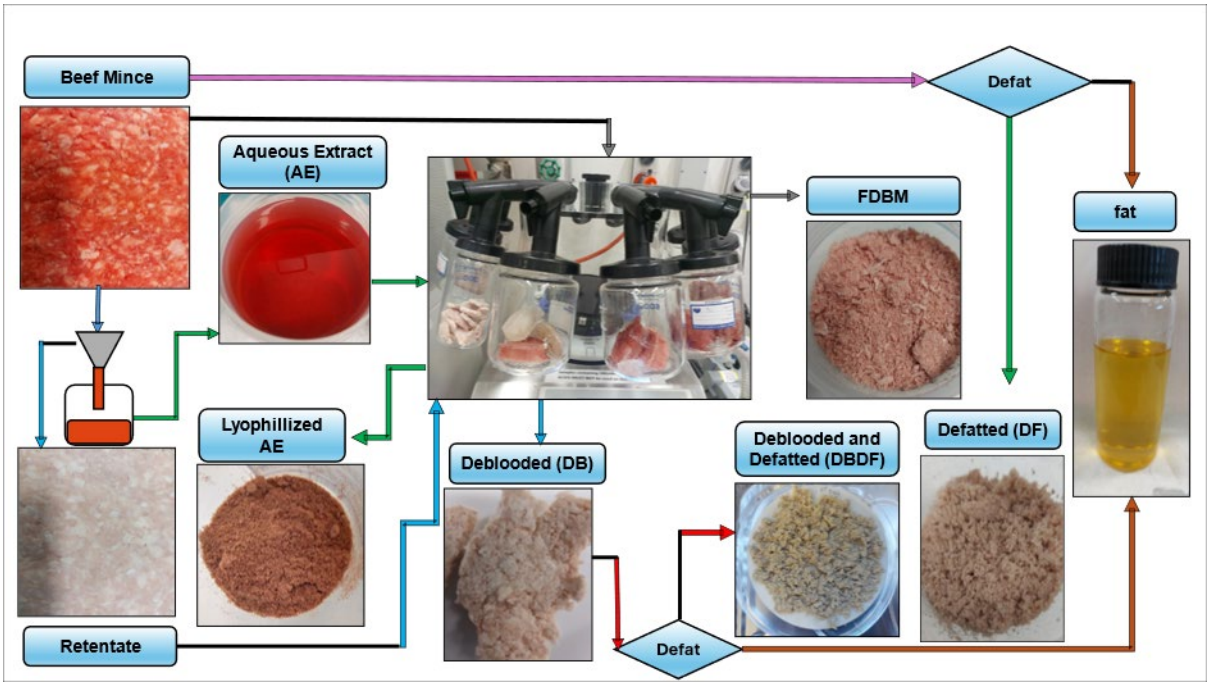

Figure 4.1 Process flow diagram of fractionation of beef mince by lyophilisation. Aqueous Extract (AE), Deblooded (DB), Deblooded and defatted (DBDF), Defatted (DF), Freeze dried beef mince (FDBM) and Fat

and centrifuged at 4000 rpm for 10 minutes at 4 ºC (Beckman Coulter, Avanti J-26S XPI, JA20, California, USA) and filtered through a Whatman glass microfiber filter (125mm diameter/ 1.6µm pore). The permeate was stored in the refrigerator at 4ºC whilst the retentate was redispersed with 250ml of chilled deionized water, and the process was repeated 4 times. At the end of the process, the permeate (a*) and retentate (b*) were re-lyophilised separately, weighed and stored at -20ºC till further analysis. This process created the a*, aqueous extract (AE) and the b*, de-blooded (DB) fractions.

Fat was extracted by adding 50 ml of diethyl ether (Merck Sigma-Aldrich, Melbourne, Australia) to 100 g of freeze-dried and ground sample in a stoppered Erlenmeyer flask and shaken for 2 min and allowed to rest for 15 min (repeated twice). 50 ml of petroleum ether (Merck Sigma-Aldrich, Melbourne, Australia) was subsequently added, followed by 2 minutes of shaking and 15 minutes of rest. The sample was centrifuged at 4000 rpm for 10 min and filtered over anhydrous sodium sulphate (Merck Sigma-Aldrich, Melbourne, Australia). The procedure was repeated thrice, and the extract was collected in a round bottom flask for rotary vacuum evaporation (Heidolph, Labrota 4000-efficient GMBH, Schwabach, Germany). Following evaporation, the fat was further dried under a nitrogen stream, which formed the c*,

fat extract. The retentate was carefully air-dried over filter paper in a darkened fume hood overnight at ambient temperature and formed the d*, de-fatted muscle fraction (DF). The e*. De-blooded and defatted muscle fraction (DBDF) was prepared by first de-blooding, followed by defatting 100 grams of lyophilised beef mince as described above.

Determination of residual fat

The de-blooded and defatted sample was subjected to Soxhlet extraction to gain an understanding of the effectiveness of the fat extraction process. AOAC (2000b) method was applied to determine crude fat in meat (MLA, 1998). Briefly, 5.0026g of ground DBDF sample was weighed into a thimble with cotton wool. Approximately 90 ml of petroleum spirit (Sigma Aldrich-Australia) was used as the extraction solvent. The sample was refluxed and extracted for 6 hours. The solvent was recovered using a rotary evaporator (Heidolph, Labrota 4000-efficient GMBH), following which the sample was dried in the oven at 100ºC till a constant weight was achieved.

Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was conducted to ascertain the effectivity of sarcoplasmic extraction from FDBM and if any residual soluble proteins were present in the DBDF fraction. Bis-acrylamide solution 30% (Bio-Reagent), tris-base, ammonium persulfate, TEMED, glycine, glycerol, bromophenol blue, Equine Myoglobin and Bovine Haemoglobin were all purchased from Sigma-Aldrich-Melbourne, Australia. FDBM fractions AE and DBDF were used for this study. Samples were only tested in the native non-reduced and unheated form. BioRad Mini-PROTEAN II vertical electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) with a Tris/glycine discontinuous PAGE system was used to carry out the PAGE analysis. Gels were cast, and electrophoresis was performed according to Bogahawaththa et al. (2017) & Patel (2007). Initially, 10 µl of 5mg/ml to 100 mg/ml of the AE in sample buffer were loaded onto the gel and tested for separation efficiency (Figure 4.3A). Finally, 5µl of 10mg/ml suspension of the samples in sample buffer and standards (equine myoglobin and bovine haemoglobin) in sample buffer were created. The filtered and optimized suspensions were loaded onto the pre-casted gel wells, and electrophoresis was conducted (Figure 4. 3B). Gel staining and destaining were performed as described in the literature cited above. Images of the gels were captured for qualitative assessment using the Chemidoc MP gel imaging system (Bio-Rad Laboratories, California, USA).

Sample preparation and thermal treatment

Due to the availability and the differences of the different fraction, 2 grams of fat, 1 gram each of Fresh (un-lyophilized) beef mince, FDBM, DB, DF fractions and 0.5 grams of AE and DBDF fractions were weighed individually (Figure 4.2) into 40ml head space vials (SUPELCO Bellefonte, PA, USA) fitted with black polypropylene screw type lid with hole and Teflon faced silicone septum (SUPELCO 23193-U). Samples were hydrated with 1 ml of milli-Q water overnight. The hydrated samples were heat treated at 160ºC for 30 minutes using a paraffin oil bath. Immediately after heat treatment, samples were plunged into an ice bath to cool and kept frozen for volatile analysis.

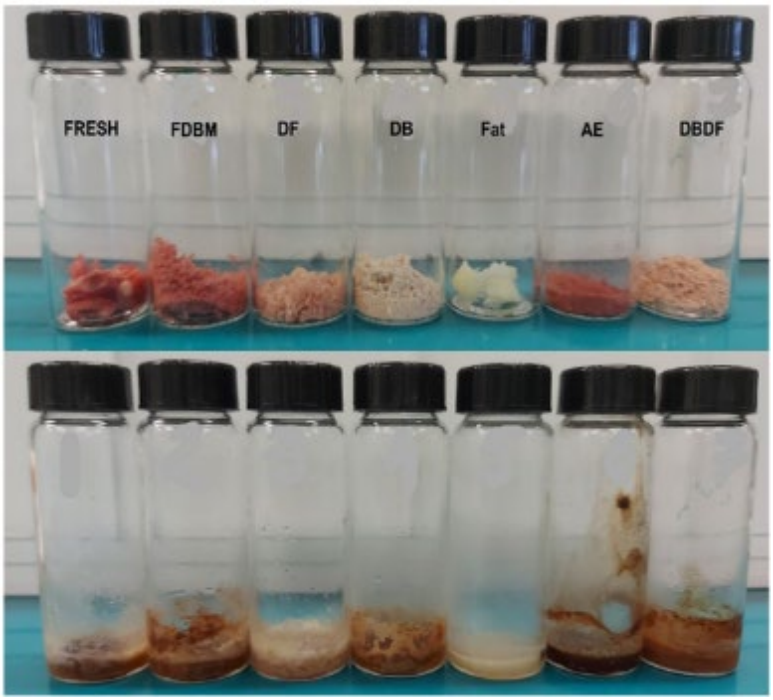

Figure 4.2 Before and after heat treatment (in vial 160ºC- 30 min) of rehydrated FDBM fractions prior to HS SPME-GCMS analysis.

Total Volatile Analysis

Overall, the volatile analysis followed a similar pattern and the same GCMS program optimised in the previous chapter was used throughout the project for conducting comparative studies. Except for the fat, 5 ml of deionized water and a magnetic stirrer were added to each vial and brought to room temperature. As and when required, the vials containing the samples were heated to 70ºC in a water bath for 30 minutes to equilibrate (Chapter 3. Figure 3.3). Analytes were adsorbed onto the fiber from the equilibrated sample headspace using a 30/50 µm

Divinylbenzene/carboxen/polydimethylsiloxane (24 Ga, 2 cm, manual holder, SUPELCO, Bellefonte, PA, USA) during the extraction process. The fiber was exposed for 30 minutes whilst the vial was held at 70ºC and stirred continuously using a magnetic stirrer. Analysis was conducted using a single quadrupole GC- 2010 (Shimadzu, Japan) with a mass spectrometer GCMS-QP 2010 Plus (Shimadzu, Japan). The injector temperature was held at 220ºC with a 1:5 split SPME fiber manually appended and allowed to desorb. The fiber was left in the injector for the next 30 min. Compounds were separated on a SUPELCOWAX 10 fused silica capillary column (length 30 m, ID 0.25 mm, and thickness 0.25 µm, Supelco, Sigma Aldrich, Bellafonte, PA, USA). Ultra-high purity helium at 1.04 mL/min was used as carrier gas. The initial column temperature was held at 40ºC for 2 minutes, followed by a temperature ramp of 5ºC/ min to 240ºC and held for 10 minutes with a total run time of 49 minutes. The interface and ion source temperatures were 300 and 220ºC respectively. MS analysis was carried out by electron ionization at 70eV. Spectra were scanned using TIC from 40 to 600 m/z. Peaks were identified by comparing them to the NIST library (2023) with ≥80% similarity index and compared to those found in the literature. Blank column and blank fiber checks were conducted at the start and the end of the day to ensure no carryover or contamination occurred. C_7-C_{40} Saturated alkane standards (Sigma- Aldrich Melbourne, Australia) were used to calculate retention indices (Ahamed et al., 2024; Kaczmarska et al., 2018; Zhang et al., 2022). The calculated retention indices were matched to reference retention indices from the NIST webbook (webbook.nist.gov) under Van Den Dool and Kratz RI, polar column, temperature ramp showing data for SUPELCOWAX10 30.m/0.25mm/0.25µm. The agreed maximum deviation between calculated and reference retention index was ≤20. Where retention index data for SUPELCOWAX10 column was not available DB-WAX or retention index for another suitable polar column was accepted. Hence the acceptance threshold or the maximum deviation of retention indices for non SUPELCOWAX10 columns was accepted at ≥30 (Bizzo et al., 2023).

Statistical analysis

OriginPro 2024b -student version (OriginLab Corporation, Northampton, USA) was utilized to conduct all statistical analyses. The same statistical analysis was conducted for all the studies for effective comparison. All assays for volatile analysis were carried out in triplicates, and peak intensity was reported as means of the area under the peak. Standard deviation was not reported due to large variations in the observed peak areas. If significant differences were observed during analysis between replicate samples, only duplicates were accepted, making it

difficult to report standard deviation. Hence, data was only qualitatively assessed. Also, due to the nature of the data and the number of blanks in the data, the non-parametric Kruskal-Wallis ANOVA was conducted to observe significant differences amongst the means. A 2-dimensional listwise correlation matrix was obtained using the multivariate principal component analysis (PCA). From the PCA, scree plot and biplot were used to observe correlations and differences amongst the fractions.

Results and discussion

Fractionation of beef mince

The moisture content of minced meat was compared effectively between the standard oven drying method, which showed 35.7%, and the lyophilised sample, which showed 35.9%. This ratio was used to rehydrate the various lyophilised fractions overnight before heat treatment. In the beef mince, there was 54.38% fat on a dry weight basis, which aligned well with the 18% fat content indicated on the nutrition panel label for fresh beef mince. Additionally, extraction using Soxhlet revealed trace amounts of fat (greater than 0.5%) in the defatted fraction. The aqueous extract resulted in a freeze-dried weight of 11.7%, corresponding to a recovery rate of 71.33% for the de-blooded (DB) fraction. It is important to note that some muscle and fat were lost during the extraction process. The myofibrillar (DBDF) fraction was approximately 41.23% after the removal of the aqueous extract and fat.

Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

During the pre-trials, SDS-PAGE was performed on fresh beef mince. However, the extraction efficiency was found to be better when using FDBM-AE (data not shown). Figure 4.3(A) displays different concentrations of the aqueous extract (AE) assay from L2 to L7, showing several proteins. Figure 4.3(B) illustrates the optimized concentrations (L1 and L2) and demonstrates the efficient separation of various proteins in the AE of FDBM, including myoglobin and haemoglobin. These proteins were confirmed by reference standards: L5 and L6 correspond to myoglobin, while L7 and L8 indicate haemoglobin. L3 and L4 contained the DBDF samples, and no proteins were detected, as expected, confirming that the extraction of sarcoplasm from the sample was efficient.

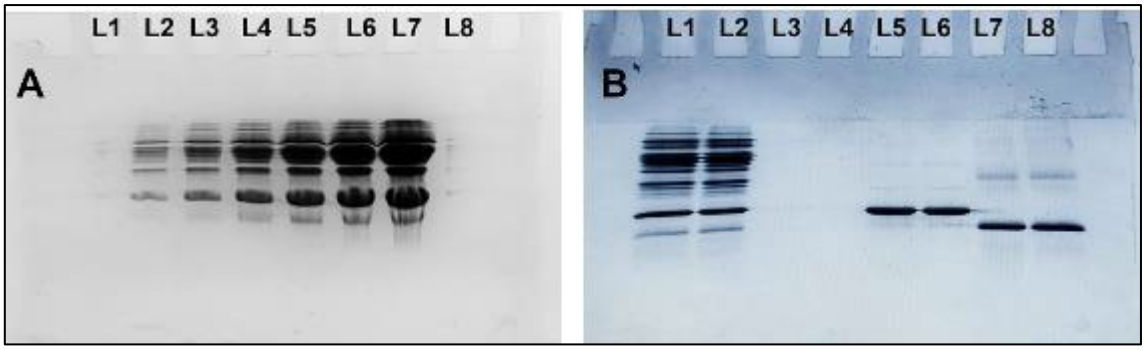

Figure 4.3 (A) SDS-PAGE of freeze-dried beef mince (sarcoplasm) aqueous extract (AE) in various concentrations; (B). Confirmation of extraction efficiency.

Volatile analysis

The TICs shown below in Figure 4.4 reveals that the seven different essays and fractions produced significantly varying qualitative and quantitative characteristics in their volatile compounds. Further, Figure 4.5 illustrates the count of volatiles tentatively identified in each class of compounds.

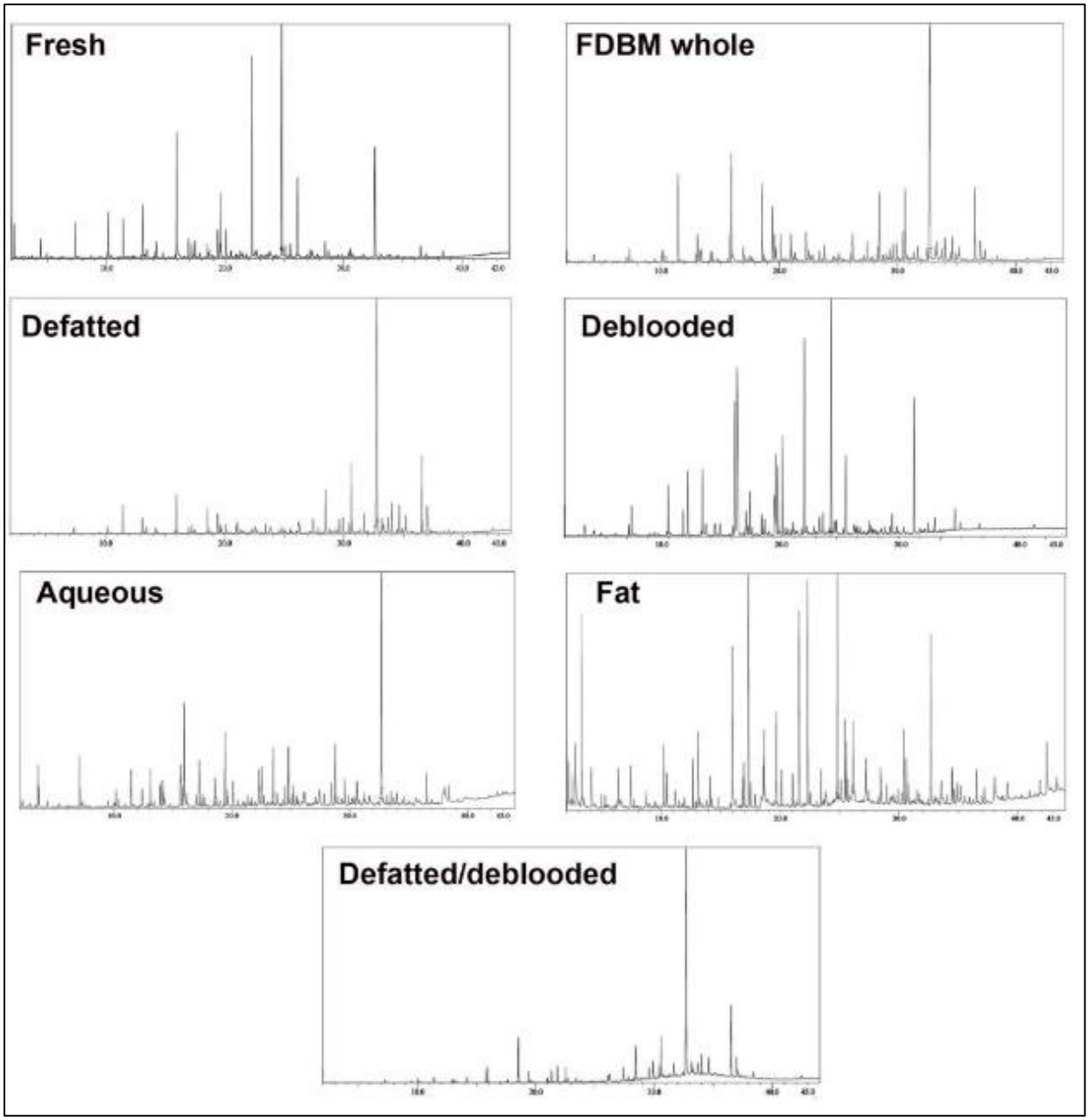

Figure 4.4 Total Ion Chromatogram of volatiles observed in an untargeted analysis using 50/30µm DVB/CAR/PDMS Fiber on a SUPELCO Wax10 column in the different fractions of Beef mince. Where, Fresh- Beef mince Native, FDBM whole- Freeze-dried Beef mince whole, De fatted- without fat, De blooded- without sarcoplasm, De blooded and defatted- without the sarcoplasm and fat, Fat- only lipid extract and Aqueous extract- Freeze dried sarcoplasm.

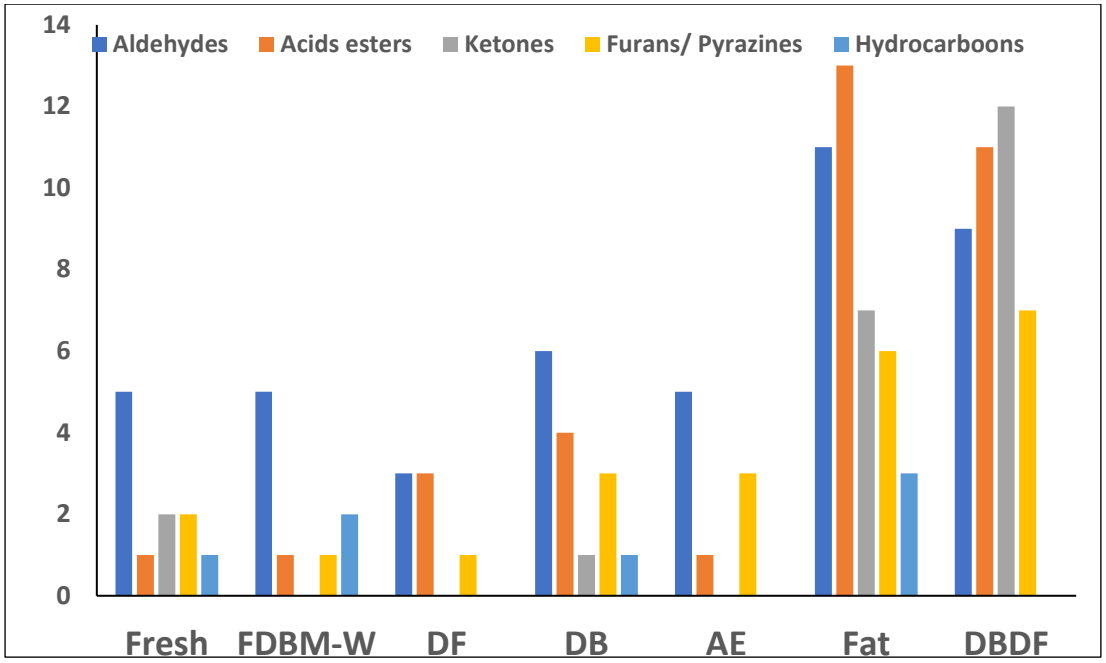

Figure 4.5 Total number of compounds observed in each class for the different fractions.

Notably, the fat and DBDF fractions yielded a considerably larger number of identifiable compounds compared to the other samples (Figure 4.5). The DBDF fraction exhibited the highest count of ketones, furans, thiophenes, and pyrazines, although no hydrocarbons were detected. DBDF fraction showed a higher number of ketones than aldehydes. In contrast, the AE and DF fractions, which lacked fat, produced the smallest total number of volatiles across all classes. No ketones were identified in the FDBM, DF, and AE fractions, and neither the DF nor the AE fractions contained any hydrocarbons. It was anticipated that FDBM would generate a larger variety of compounds, as lyophilization is known to alter the structure of meat, and grinding would further expose a larger surface area during heat treatment.

However as noted earlier, lyophilisation may have reduced the oxidative damage due to the decrease in water activity. Further, the effect of cooking in a sealed vial possibly reduced further oxidative degradation due to the equilibration being maintained. No documented evidence of the development of confinement odours of cooked meat in similar conditions was available in the literature except for that developed in packaged raw meat (Reis et al. 2016).

Figure 4.4 illustrates the total ion chromatogram (TIC) of various fractions of beef mince and highlights the significant differences between the samples. It also demonstrates that analysing whole samples might overlook analytes of interest, which could be crucial for reducing competition and coelution effects when using solid phase microextraction (SPME). Some

limitations of the study became apparent, particularly the finding that the volatile profile of frozen-dried beef mince (FDBM) differed from that of fresh whole beef, contradicting the original hypothesis. However, a study conducted by Chang (1973) found that no new volatiles were formed in cooked, freeze-dried or stored beef. Although differences were observed in the peak intensities of some of the volatiles. This discrepancy may have arisen from the challenge of accurately identifying the eluted peaks in this study. The use of a wax (WAX) column hindered the ability to match compounds using the NIST reference database. Additionally, the absence of reference compounds further contributed to the loss of data (as shown in Table 4.1).

A total of 123 peaks were resolved and tentatively assigned as aldehydes across the seven fractions; however, only 21 peaks could be definitively identified as aldehydes. Consequently, a significant number of peaks remained unidentified, leading to a lower-than-expected number of aldehydes reported for both fresh and FDBM fractions. A similar issue was observed with other classes of compounds listed in Table 4.1. Specifically, no pyrazines were objectively identified through cross-referencing. Out of 22 probable acids, only 2 were identified, along with 2 from hydrocarbons and 2 from miscellaneous compounds, with 95 and 29 peaks identified, respectively.

The slope of the scree plot (Figure 4.6) indicates the percentage of variance associated with each component and their corresponding eigenvalues. For further analysis, components 1 through 4 were selected. The most significant variance was observed between components 2 and 3 (Figure 4.7). Overall, the variance appears to be concentrated within three components. There was some correlation observed between the AE and DF components, which may relate to the fat fraction missing from the sample. Additionally, Fat and DBDF showed a correlation, but this could be attributed to the varying amounts of observed volatiles in those samples compared to others (see Table 4.1). On the other hand, FDBM-W did not exhibit a significant correlation with the fresh beef mince sample, possibly due to data loss resulting from the inability to confirm compound identity. Therefore, due to the substantial number of missing values across assays, the statistical observations presented here should be interpreted with caution. Table 4.1 below lists the tentatively identified volatile compounds in different fractions of beef mince. The fat fraction contained the highest number of identified volatiles (40), followed closely by DBDF with 39 identified volatiles. In contrast, the defatted (DF) sample had the fewest identified volatiles. The volatile profiles from fresh beef mince and FDBM-W did not match.

Table 4.1. Preliminary identification and cross-referencing of resolved peaks in the different fractions of lyophilized beef mince.

Compounds	Preliminary identification using MS	Cross-referenced using NIST
Aldehydes	123	21
Alcohols	50	17
Acids	22	2
Esters	3	1
Ketones	56	12
Furans	22	8
Thiophenes	7	2
Pyrazines	22	0
Hydrocarbons	95	2
Miscellaneous	29	2

Meatiness is known to be developed in cooked meat from precursors in the sarcoplasm undergoing the Maillard reaction and interacting with fat oxidation products. Myoglobin and haemoglobin have been constituents of interest in relation to providing the quintessential meatiness in meat and, more recently, leghaemoglobin in plant-based meat.

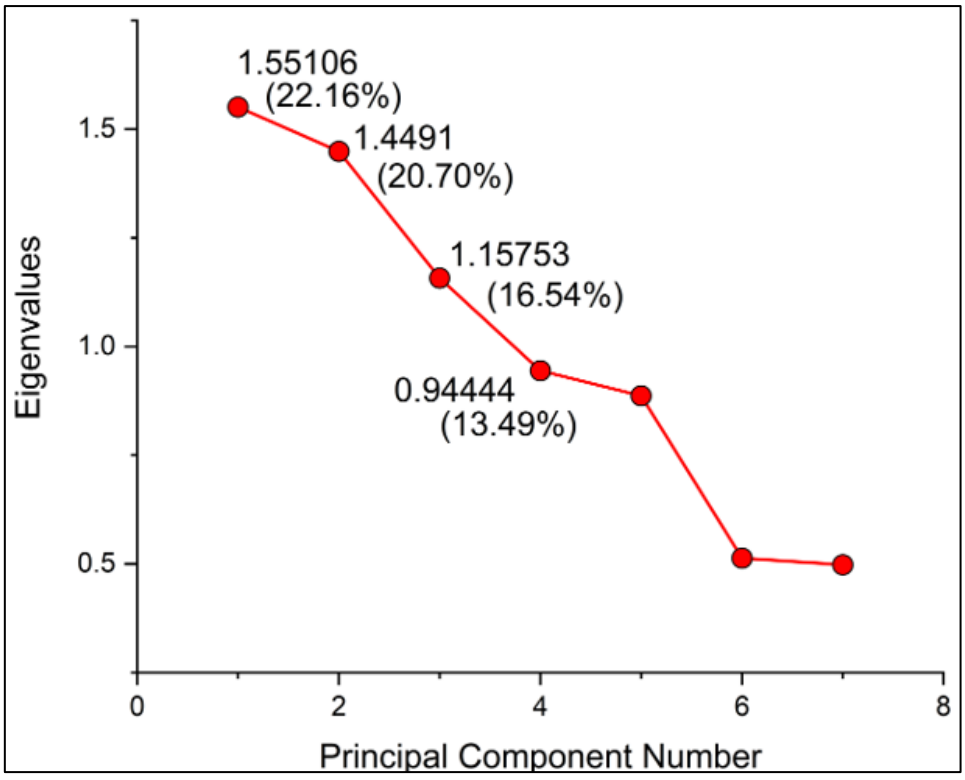

Figure 4.6 Scree plot showing principal components to consider for observing the correlation between assays for volatile analysis of different fractions of beef mince.

However, several compounds such as; but not limited to 2-methyl propanol, 3-methyl-2-furan carboxaldehyde, 2-ethyl-3,5-dimethyl pyrazine, dimethyl tetrasulfide, 2-methyl phenol, 2-(2-thiapropyl)but-2enal, 2,3-octanedione, 2-nonanone, 2-methyl-3-methylthiofuran, 2-methyl-3-methyldithiofuran, 2-methyl-3-methyltrithiofuran, 2-methyl-5-(methio)-furan, β-elemene, β-caryophyllene, 2-methyl-5-isopropylpyrazine, 2-thiophenemethanol, methionol, 3-methylthio-thiophene, 2-methyl-3-furanthiol, 4,5-dimethylthiazole, caryophyllene oxide, octane, etc. (Chapter 2. Table 2.2). have been noted to possess meaty attributes and are found in foods other than meat. As a first, fractionation, was an attempt at characterizing and resolving aroma active compounds using untargeted chemometric analysis in an aim to map the progression and formation of aroma compounds. In retrospect, was not attainable through a single study and led to more questions compared to the answers. However, the study does highlight the challenges faced and exercises caution when analysing whole foods. The TIC obtained from the different fractions (Figure 4.4) is evidence of the number of volatiles that presumably coelute, overlap or are masked (protein flavour interaction) and remain unresolved during whole food analysis using HS-SPME-GCMS.

Figure 4.5 shows the number (count) of volatiles tentatively identified in each class of compounds not the total area under the peak. Several limitations were observed in undertaking this study. Firstly, sample assay weights were different, this was intended due to the quantity of available fractions after lyophilizing. E.g. The aqueous (sarcoplasmic) extract collected was significantly low compared to fat. Also, the weight-to-volume ratio differed for other fractions. The DBDF fraction was lighter compared to samples that contained fat and had to be of a lower weight even though the volume was higher.

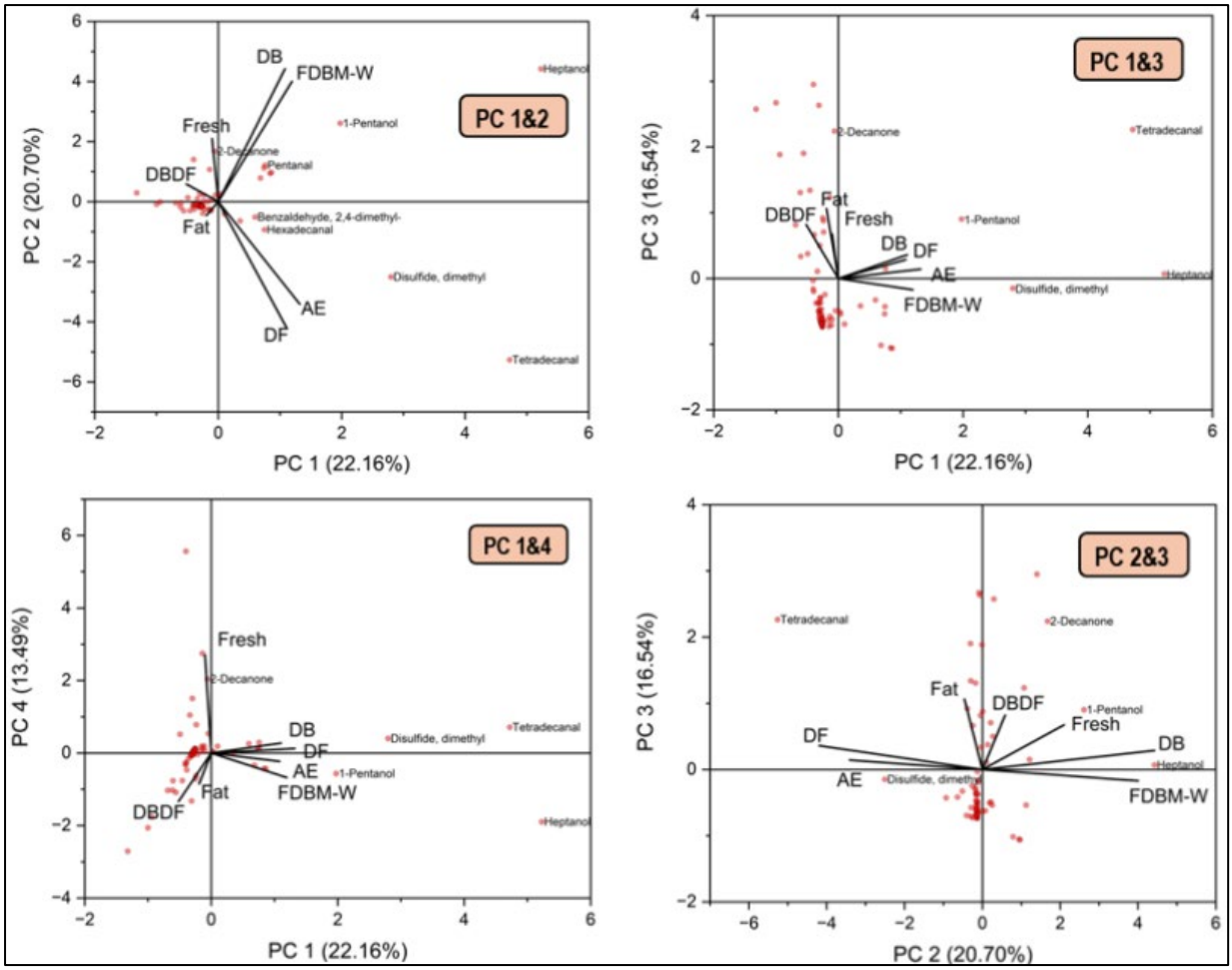

Figure 4.7 Two dimensional listwise correlational biplot between components 1- 4 and the observed variance between the assays is shown. Compounds of significance were labelled.

These factors likely influenced the partitioning of aroma compounds into the headspace during the equilibration and extraction processes. Although qualitative data should not have been affected, the inability to identify the peaks using the NIST database hindered identification efforts. When it comes to the extraction of various fractions, lyophilised samples performed better. This improvement may be attributed to a reduction in surface area and surface water in the lyophilised samples. In terms of protein stability and structural changes, studies have demonstrated that freeze-dried meat samples exhibit higher rehydration rates, lower shrinkage, and better maintenance of porosity and structure (Nowak & Jakubczyk, 2020; Lewis et al., 2010). This indicates that lyophilization is a viable method for fractionating and studying different components. Conversely, other studies have reported cell disruption when fractionating microalgae (Kasper et al., 2013; Grossmann et al., 2018). Additionally, protein structural changes due to nucleation were observed by Iyer et al. (2016) during the

lyophilization of myoglobin (Mb); however, the effects of these changes on flavour development have not yet been explored.

Table 4.2 Tentatively identified qualitative data of different fractions of beef mince using HS-SPME-GCMS.

Aldehydes									
Compound Name	Calc RI[a]	Ref RI[b]	Fresh	FDBM-W[c]	DF[d]	DB[e]	AE[f]	Fat	DBDF[g]
2-Propenal	810	840						1.62E+06	
Pentanal	971	983	4.08E+05	1.79E+05		4.85E+05		2.32E+06	3.44E+05
2-Butenal, 2-methyl-, (E)-	1029			2.07E+05					
2-Butenal	1034	1047						8.26E+06	
Hexanal	1078	1079					3.04E+05	4.85E+06	3.25E+06
2-Pentenal, (E)-	1128	1124						6.66E+05	
Heptanal	1185	1184						5.71E+06	4.96E+06
2-Hexenal, (E)-	1220	1224						4.78E+05	
Octanal	1291	1319						7.21E+06	1.28E+07
2-Heptenal, (E)-	1328	1323	6.67E+05			1.51E+05		2.06E+06	
2-Hexenal, 2-ethyl-	1334	1330				2.95E+05			7.64E+05
2-Octenal, (E)-	1435	1432	2.47E+06			4.40E+05		3.78E+06	
2,4-Heptadienal, (E, E)-	1424	1485	4.44E+05			3.78E+05		3.65E+06	
Benzaldehyde	1532	1529				1.57E+05		7.42E+05	2.50E+07
2-Nonenal, (E)-	1542	1543				5.31E+05		5.55E+06	1.12E+06
Undecanal	1609	1599						1.56E+06	2.79E+06
2-Decenal, (E)-	1651	1652				7.11E+05		1.28E+07	
2-Octenal, 2-butyl-	1670	1653							1.20E+06
Dodecanal	1715	1713						1.90E+06	
Benzaldehyde, 2,4-dimethyl-	1733	1742			5.31E+06	6.65E+05			
2-Undecenal	1756	1755						9.74E+06	1.44E+06
2,4-Decadienal, (E,E)-	1820	1798						5.63E+06	
Tetradecanal	1935				2.83E+07		2.22E+06	3.40E+06	
7-Tetradecenal, (Z)-	1963	1962			8.16E+05				
Pentadecanal-	1997	2000						8.94E+05	
Benzaldehyde, 4-pentyl-	2012	2003							3.93E+05
Hexadecanal	2105	2124					1.22E+06		1.23E+06
cis-9-Hexadecenal	2166	2147						1.06E+06	9.95E+05
Octadecanal	2352	2343						1.89E+06	8.87E+06

Alcohols, Acids and Esters									
Compound Name	Calc RI	Ref RI	Fresh	FDBM W	DF	DB	AE	Fat	DBDF

1-Butanol	1142	1152				7.45E+05		5.71E+05	
1-Penten-3-ol	1158	1175						4.04E+05	
1-Pentanol	1250	1251				6.16E+06		2.39E+06	
1-Hexanol	1351	1353	4.55E+05					1.33E+06	4.41E+05
Heptanol	1452	1449		5.24E+05	7.84E+05	5.44E+06	8.40E+05	9.63E+05	1.29E+06
2-Hexen-1-ol, 2-ethyl-	1533	1518			1.16E+06				
1-Pentadecanol	1542	2254				4.55E+05			6.32E+05
1-Octanol	1556	1546						3.84E+06	1.34E+07
1-Nonanol	1661	1668						8.06E+05	3.01E+06
2-Furanmethanol	1663	1678							1.00E+06
1-Decanol	1763	1763						3.49E+05	1.27E+06
Ethanol, 1-(2-butoxyethoxy)-	1798	1800						6.52E+05	
1-Undecanol	1863	1875							1.47E+06
1-Dodecanol					1.07E+06			5.28E+05	
1-Tetradecanol								3.71E+05	
1-Pentadecanol								7.03E+05	
1-Heptadecanol	2479	2496							3.39E+05
Pentadecanoic acid, methyl ester	2112	2138							5.09E+05
Hexadecanoic acid, methyl ester	2220	2217						6.45E+05	9.71E+05

Ketones

Compound Name	Calc RI	Ref RI	Fresh	FDBMW	DF	DB	AE	Fat	DBDF
2-Heptanone	1181	1187						3.80E+05	9.83E+05
2-Octanone	1286	1285						4.10E+05	1.45E+06
2-Nonanone	1392	1394	3.89E+05					6.82E+05	4.45E+06
2-Decanone	1494	1515	1.32E+06			2.01E+06			9.89E+06
2-Undecanone	1599	1602							9.17E+05
2-Tridecanone	1813	1814						6.50E+05	1.12E+06
5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	1857	1853							6.68E+05
2-Tetradecanone	1916	1907							5.06E+05
2-Pentadecanone	2025	2010						8.81E+05	3.16E+06
2(3H)-Furanone, dihydro-5-pentyl-	2043	2055						1.03E+06	1.24E+06
2-Hexadecanone	2126	2130							7.70E+05
2-Heptadecanone	2237	2243						4.21E+05	2.39E+06

Furans, Thiophenes and Pyrazine

Compound Name	Calc RI	Ref RI	Fresh	FDBM W	DF	DB	AE	Fat	DBDF
Furan, 2-methyl-	894	876					3.98E+05	8.98E+04	
2-Butanone	878	905						1.72E+05	4.52E+05
Furan, 2-ethyl-	940	945			6.68E+05			1.92E+06	
Furan, 2-ethyl-	950	945						1.88E+06	
Disulfide, dimethyl	1060	1077					3.56E+06		3.76E+05
Furan, 2-pentyl-	1232	1239						5.28E+05	7.83E+06
2-n-Heptylfuran	1433	1430					7.90E+05		1.87E+06
2-n-Octylfuran	1538	1530	1.27E+06			9.72E+05		3.17E+05	3.01E+06
Thiophene, 2-pentyl-	1458	1460				8.43E+05			7.66E+05
Thiophene, 2-hexyl-	1564	1564	2.75E+05	1.87E+05		2.39E+05			1.20E+06

Hydrocarbons and miscellaneous

Compound Name	Calc RI	Ref RI	Fresh	FDBM W	DF	DB	AE	Fat	DBDF
Undecane	1111	1100		2.12E+05					
Hexadecane	1612	1600	1.76E+05			5.73E+05			
Heptadecane	1709	1700		1.77E+05					
2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	1869	1884						6.66E+05	
Formamide, N,N-dibutyl-	1778	1773						3.45E+05	
Phenol, 2,4-bis(1,1-dimethylethyl)-	2317	2316						1.23E+06	

[a] Retention Indices (calculated) using Van den Dool and Kratz (1963). [b] Volatile compounds tentatively identified using NIST library in Shimadzu LabSolutions and from NIST online (https://webbook.nist.gov/chemistry/). [c] Freeze dried beef mince-Whole (FDBM-W), [d] Defatted (DF), [e] De-blooded (no sarcoplasm) (DB), [f] Aqueous extract – sarcoplasm (AE), [g] De-blooded and Defatted (DBDF).

Mottram and Edwards (1983) found no differences between freeze-dried, non-freeze-dried, and the sample defatted with a nonpolar solvent. Regardless, most studies have used freezing to store assays before and after cooking and prior to volatile analysis. Therefore, it may be necessary to study the effect of freezing and lyophilising on volatile formation in future studies.

Standard AOAC recommended methods for the extraction of fat from meat are acid hydrolysis, Folch or Soxhlet (AOAC, 2000b). The need to retain retentate (Defatted-DF fraction) for further analysis merited modification of extraction methods (FAO, 1986). The extraction efficiency of fat using diethyl ether and petroleum ether conducted using a non-standard method was confirmed using Soxhlet extraction of the retentate (DBDF). However, the

extraction of bound lipids, especially phospholipids found in meat, remained unvalidated due to the extraction with non-polar solvents only. This could be important because the oxidation of phospholipids is thought to promote the formation of aroma-active compounds. Mottram & Edwards (1983) studied the effect of lipids from fractionated and lyophilized beef cooked to 100ºC and found that triglycerides and phospholipids together impacted the volatile profile more than triglycerides alone and may be an important factor to consider when formulating plant-based meats. The results of the current study differed from those observed by Mottram & Edwards (1983), where the lyophilised, non-lyophilized and defatted samples varied significantly from each other.

For effective electrophoresis, solubilisation of proteins is a necessary step in preventing protein aggregation. The use of a solubilising agent or detergent is generally recommended to prevent ionic and hydrophobic interactions and disulphide bridges, as well as prevent other bonds (Baharuddin et al., 2021; Rabilloud, 2009). However, SDS-PAGE was successfully conducted as a qualitative confirmatory test to assess the validity of the presence of myoglobin in the aqueous extract and the effective removal of all sarcoplasmic material from the beef mince using native and non-reduced assay. Numerous higher molecular weight proteins were observed but remained uncharacterised. Further protein characterization and behaviour using 2D-Electrophoresis may help in validating and comparing the presence of soluble proteins in legumes or plant-based meat alternatives to sarcoplasmic proteins in meat extracts.

Several studies have now been conducted comparing animal and plant-based meat (Biazotto et al., 2025; Forster et al., 2024; Hernandez et al., 2023; Kaczmarska et al., 2021). A large variation in volatile profile was observed between traditional meat, substitutes and traditional plant-based products (Kaczmarska et al., 2021). Most studies used whole foods to compare volatile or sensory profiles. However, the contribution of the constituents of those foods towards key aroma compounds was not evaluated. Similarly, the efficacy of SPME fibers has been compared with whole foods to show differences in their ability to adsorb analytes of interest (Ahamed et al., 2023; Machiels & Istasse, 2003; Elmore et al., 2001). However, their limitations regarding coelution, competition, adsorption limits in relation to equilibrium constants (Bartelt & Zilkowski, 1999) etc have seldom been addressed. In essence, mapping the formation and development of meaty aroma remains challenging due to the matrix of the beef. Initial study and pre-trials showed that large aldehydic peaks dominated the TIC, including alcohols, ketones and acids. The reason could be because of the concentration of lipids in beef mince, leading to oxidation and breakdown of unsaturated lipids to free fatty

acids as well as the formation of other oxidative products, including products relating to WoF. Although not displayed in the results due to the inability to identify peaks, the differences between the fractions are observable when viewing the chromatograms (Figure 4.4). Observing the development of volatiles in different fractions could give a view of the source of aroma compounds. This knowledge could help greatly whilst developing new products.

The effect of lipids has long been viewed and analysed for its role in providing aroma-active compounds via oxidative products (Domínguez et al., 2019; Fu et al., 2022; Shahidi & Oh, 2020; Ueda et al., 2021; Zhang et al., 2021). Alternate views on lipid oxidation have been expressed on the peroxyl and alkoxyl pathways and the hydroperoxide and free radical formation via the hydrogen abstraction pathway. Nonetheless, no clear designation has yet been formalized (Schaich, 2013). Several aldehydes, alcohols, acids, esters and ketones were observed in the lipid fraction. However, a larger proportion of similar classes of volatiles were observed in the DBDF fraction, contrary to the expectation. Also, the number of compounds in the deblooded (DB) and FDBM-W fraction were low. A valid explanation for this phenomenon could not be expressed with any certainty and relegated to inaccurate identification. Although a significant number of compounds from the different classes were not identified, most of the identified volatiles eg. aldehydes represented ones that were related to off flavours and warmed-over flavours such as pentanal, hexanal, heptanal, octanal, 2-nonenal (E and 2-alkylfurans these are known to be secondary products of unsaturated fatty acid oxidation and free fatty acids (Shahidi, 1994, Zhang et al., 2021) except for a few such as (E,E)-2,4-decadienal (Stöppelmann et al., 2023). Several furans were observed in the lipid fraction as stated above in this study. Increased Maillard reaction activity was hypothesized in the aqueous extract and defatted fraction. However, although initially identified as pyrazines, none of the pyrazines could be correlated to the retention indices as observed and hence not reported. If viewed in the context of compounds noted earlier from literature (Ch 2, Table 2.2) for exhibiting meaty nuances, very few of the compounds relating to meaty were observed by fractionating. This could be due to the absence of precursor compounds in the fractions. Although the compounds were observed in several different studies and varied sampling using SPME-GCMS, the results explicitly portray the limits of analytical observations. Also, it is not known how many of the appreciable compounds and in what concentrations they should be to elicit meatiness, and no specific guidelines exist as such. Devaere et al. (2022) showed that the addition of myoglobin to plant-based (soy) burgers positively influenced the aroma profile towards conventional meat. Additionally, the influence of haemoglobin and free iron on the

aroma of cooked beef was studied by Li et al. (2024) and found that free iron increased oxidative compounds such as hexanal, 1-octen-3-ol, and 2-akylfurans whilst haemoglobin favoured Strecker and Maillard derived reaction products by binding aldehydes to proteins. It is important to note that although the study rationalized the spiking of haemoglobin and may well justify its approach for analytical purposes, the aqueous extract of beef contained more soluble proteins than just myoglobin and haemoglobin as observed in the SDS PAGE analysis. Hence the prelude that myoglobin and or haemoglobin provides the quintessential meaty flavour could be plausible. Overall, the fractionation of minced beef provided sufficient evidence that justifies further research to map the formation and development of aromas.

Conclusion

Fractionation of beef mince was more effective when using a lyophilised sample compared to a fresh native sample. SDS PAGE of aqueous extract showed the presence of several proteins in addition to myoglobin and haemoglobin. It also validated the effective separation of sarcoplasmic constituents from myofibrillar fraction. The volatile analysis of the various fractions did not correlate well with the hypothesis or the literature. Although the separation of the analytes on the gas chromatography was efficient, the identification of the Mass spectrometer data in conjunction with the NIST database in the absence of reference standards increased the likelihood of misidentification of compounds. Although the use of HS-SPME-GCMS has been undertaken in various studies, its limitations are less known and prove to be an obstacle in observing analytes of interest, especially when using untargeted analysis from whole foods. Several questions remained unanswered in relation to mapping the development and formation of meaty aroma in beef mince and warrant further study.

In clear terms, although the origins of meaty aromas remained elusive in this study, fractionating beef mince enabled the study of the constituents of beef mince that may contribute to the appreciable meaty aroma. Comprehensive untargeted analysis using a range of analytical methods, including but not limited to the analysis of volatiles, could further provide critical information.

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Chapter 5

Analysis of Volatiles from Plant-based Lipids and Proteins Compared to Beef Mince

Introduction

Meat is highly valued for its taste, aroma, and nutritional composition, providing excellent protein quality, including all essential amino acids, as well as other important components such as vitamins and minerals (Kaczmarska et al., 2021). However, its lipid composition, which includes high levels of saturated fats, cholesterol, and trans fats, has been linked to various chronic and preventable diseases (Bronzato & Durante, 2017). Minced or ground meat typically contains an average fat content of 8-18%. In contrast, plant-based meat alternatives offer a nutritious option that contains a balanced number of high-quality proteins (compared to traditional ingredients) and healthier fats. Nonetheless, some of these products have a fat composition similar to that of ground or minced meat (Romão et al., 2023). To mimic the texture and flavour of meat, formulations increasingly utilize polyunsaturated oils, oil emulsions (such as hydrogels, microencapsulated lipids, and coacervates), fat replacers, and saturated fats like coconut fat and cocoa butter. Achieving a flavour profile comparable to that of meat remains a challenging goal Kaczmarska et al., 2021; Thong et al., 2024; Wang et al., 2022). While some sources (Davis, 2021; Good Food Institute, 2025) argue that plant-based meats were not necessarily designed to mimic animal meat and that consumers should understand the differences in eating quality between plant-based alternatives and their meat counterparts, others, like 'LIKEMEAT,' suggest that evidence shows plant-based meats can look, feel, and taste similar to real meat (He et al., 2021). The variations in products and opinions may stem from differences in formulation and analysis methods used.

The two most important components in creating a meat mimetic are fats and proteins. Proteins contribute essential nutritional benefits, texture, and taste, while fats enhance mouthfeel, flavour, and satiety, also playing a role in nutrition (Kyriakopoulous et al., 2021). It is well established that cooking enhances the aromas and desirability of foods. However, the unavoidable, irreversible oxidative damage to proteins and fats can introduce off-flavours, which ultimately reduce product quality. In meat analogues, oxidative damage may be intensified due to processing conditions and high cooking temperatures. Consequently, the generation of aromas can be divided into three interrelated factors: thermal input (temperature and time), oxidation and degradation, and the interaction of developed volatiles.

Thermal generation of aromas

In relation to thermal aroma generation from fats and proteins in meat and meat alternatives, the presence of specific precursors, as discussed in the literature review, is of significance.

Unsaturated lipids (triglycerides and phosphoglycerides) with a high degree of unsaturation are prone to the production of lipid oxidation products (LOPs) such as α-dicarbonyl, α, β-unsaturated aldehydes, 2-butenal, pentanal, hexanal etc. Whereas the unfolding, formation of random coils and aggregation of proteins promote the dissociation of peptides and other small molecular weight compounds. The dissociated peptides and amino acids lead to the formation of Amadori and Heyns intermediates, Strecker's degradation compounds, followed by Maillard reaction compounds. This phenomenon has been credited to the generation of aromas from thermal input in meat (Bleicher et al., 2022; Parliament, 1989); however, the source of volatiles akin to providing likeness of meat are not so clear in plant-based meats (Thong et al., 2024).

Oxidation of lipids and proteins

The oxidation of lipids and proteins follows similar steps: initiation, propagation, and termination of free radical chain formation. Various mechanisms and pathways can influence this oxidation, including oxidation by triplet oxygen, thermal dissociation or decomposition of hydroperoxides, and photooxidation (Frankel, 2005). The rate of oxidation is affected by multiple factors. The composition of the food matrix, as well as the presence of antioxidants or pro-oxidants, can either promote or delay the oxidative process. In meat, pro-oxidants such as oxymyoglobin have been observed to initiate both lipid and protein oxidation. Reductions in cysteine levels and increases in free sulfhydryl groups may indicate protein oxidation, while the reduction of thiols is also seen as a sign of oxidation progression (Zhou et al., 2016).

Plant-based ingredients, particularly legumes like soy and peas, are vulnerable to the enzymatic action of lipoxygenase, which promotes the oxidation of unsaturated fats and leads to undesirable flavour changes (Tingle, 2023). Furthermore, thermal input—especially high-temperature cooking—can accelerate oxidative processes. Zhuang et al. (2022) found that while the content of saturated fatty acids increased, the levels of unsaturated fatty acids decreased. Notably, the α-dicarbonyl content of soy oil increased sevenfold compared to other oils when heated to 200°C. However, this study focused solely on the relationship between fatty acid composition and the resulting lipid oxidation products at various temperatures without examining its effects on protein oxidation.

Interaction and development of aroma-active compounds

In addition to thermally generated volatiles and the formation of oxidative products, research has focused on molecular interactions that lead to odour activity through processes such as binding, masking, retention, and release (Paravisini & Guichard, 2016; Wang and Arntfield,

2016; Wang and Arntfield, 2017). Studies investigating protein binding phenomena have primarily compared plant-based proteins with other functional ingredients, such as whey protein isolate (Barallat-Pérez et al., 2023), egg albumin, and bovine serum albumin (Weerawatanakorn et al., 2015), particularly under thermally induced conditions (above 100°C). Snel et al. (2023), while examining the effects of esters and ketones in unheated protein isolates, found that smaller molecular weight ketones and esters negatively impacted retention as protein concentration increased. In contrast, larger molecular weight ketones and esters were retained more effectively. This phenomenon was attributed to steric hindrance caused by large proteins and the size-exclusion effect of small hydrophilic molecules.

Barallat-Pérez et al. (2023) discovered that plant proteins displaced carbonyl compounds, and thermal treatment (above 100°C) caused conformational changes due to the binding of proteins to hexanal. Notably, their study did not include fat but rather examined the effects of residual fat from protein isolates. They also observed that the solubility and binding of legumin were influenced at low pH, whereas vicilin was not affected. Heat was found to decrease the binding of aldehydes and ketones. Additionally, increased molecular weight and concentration of volatile compounds enhanced protein binding (Heng et al., 2004). Covalent binding could mask off-flavours. It is widely studied in pharmacology to improve the acceptance of bitter medicines and in the formulation of beverages and other food products.

When creating plant-based meats, the focus may be more on controlling flavours rather than merely avoiding certain tastes. The Maillard reaction, which occurs during roasting and grilling, produces compounds like furans and pyrazines that can impart a bitter taste, influencing the overall flavour perception. Additionally, because the protein and lipid compositions differ between meat and its alternatives, the resulting molecular interactions that affect aroma perception must be taken into account (Paravisini & Guichard, 2016; Thong et al., 2024).

Most studies and research to date on lipids and proteins exposed to high temperatures, such as during roasting, grilling and frying, have been under scrutiny for the negative aspects of deterioration such as ill effects of nutrition, formation of off flavours during storage and food safety. However, research on lipid oxidation and concomitant protein oxidation and degradation in relation to the formation of odour compounds in plant-based ingredients has not been conducted and is being sought (Geng et al., 2023). However, due to the breadth of the subject and the interactions involved, it is extremely challenging to include every aspect of aroma formation and development in a single study. Considering such findings, this study

aimed to investigate the effect of heat treatment on the formation of volatiles in plant-based lipids (coconut fat and canola oil) and proteins (pea and soy) and their combinations regularly used in the formulation of plant-based meats (Thong et al., 2024). Further, the volatiles were compared to observations from Chapter 4, namely volatiles obtained from the fractionation of beef mince.

Limitations, biases and assumptions

Although using the GCMS program consistently throughout the study was expected to aid in comparing results, it may not have been effective for analysing lipid volatiles. The low volatility of lipids, due to their chemical composition, might have benefited from a more targeted approach. Comparing results using retention indices could have provided better insights. Additionally, analysing oxidative degradation through methods such as the Thiobarbituric Acid Reactive Species (TBARS) test, examining the fatty acid profile, or conducting a free fatty acid test could have complemented the findings from the GCMS study, particularly in understanding the formation of specific volatiles in plant-based ingredients. Adjusting the 1:1 mixture of coconut fat and canola oil used in the study could have lead to a better representation of volatiles similar to those found in beef fat. Moreover, incorporating pro-oxidants and antioxidants in the formulation could help promote a volatile profile more akin to that of meat.

Material and methods

Materials

Regular beef mince and canola oil were purchased from the local retail grocer. Yellow Pea protein (83%) was acquired from Australian Plant Protein Pty Ltd, Vic, Australia. Soy protein was obtained from Xin Soy, China. Methanol, dichloromethane, salt (NaCl) AR grade, C7-C40 Saturated Alkane standards, and coconut fat were all purchased from Sigma, Melbourne, Australia.

Lipid extraction from beef mince

Fat was extracted from beef mince using a modified method Folch et al. (1957). In brief, a mixture of Dichloromethane and methanol (8:2) (Segura &Lopez-Bote, 2014) was used to extract lipid fractions from the lyophilised mince. Samples were placed in Erlenmeyer flasks with the solvent for an hour. The mixture was agitated continuously and filtered through filter paper (Whatman 597, 110mm). The procedure was repeated three times to ensure all the lipids

were extracted from the samples. The lipid extract was added to a separatory funnel with 20 ml of 1M NaCl solution to create a biphasic separation, and the lipid fraction was decanted. This was repeated thrice. Further, the solvents were extracted using a rotary evaporator at 30ºC under vacuum. The extracted lipids were further subjected to nitrogen stream for 10 minutes at 30ºC and stored in the freezer for further analysis.

Sample preparation and thermal treatment

Two grams of protein powder (pea or soy) was weighed into 40 ml headspace vials (Supelco Bellafonte, PA, USA) fitted with black polypropylene screw type lid with a hole, and Teflon faced silicone septum (Supelco 23193-U) and hydrated to 75% with Milli-Q water overnight. 9.5% of either coconut or canola oil, or a combination of the two (1:1) was added to the hydrated proteins consistent with the total average fat percentage and values found in commercial plant-based mince. The hydrated samples were heat treated at 160ºC for 30 minutes using a paraffin oil bath. Immediately after heat treatment, samples were plunged into an ice bath to cool immediately and kept frozen for volatile analysis by SHS-SPME-GCMS.

Total volatile analysis

Exactly 5 ml of deionized water and a magnetic stirrer were added to the cooked and defrosted samples after bringing them to room temperature. The vials containing the samples were resealed and heated to 70ºC in a water bath for 30 minutes to equilibrate. Extraction of headspace analytes was conducted using a 30/50 µm Divinylbenzene/carboxen/polydimethylsiloxane (24 Ga, 2 cm, manual holder, SUPELCO, Bellefonte, PA, USA). The fiber was exposed to the equilibrated headspace for 30 minutes whilst the vial was held at 70ºC and stirred continuously using a magnetic stirrer at 250 rpm. Analysis was conducted using a GC-MS, Shimadzu QP-2010 Plus, (Tokyo, Japan). The fiber was manually appended and allowed to desorb into the injector held at 220ºC with a 1:10 split. Compounds were separated on a SUPELCOWAX 10 fused silica capillary column (length 30 m, ID 0.25 mm, and thickness 0.25 µm, Supelco, Sigma Aldrich, Bellafonte, PA, USA). Ultra-high purity helium at 1.04 mL/min was used as carrier gas. The initial column temperature was held at 40ºC for 2 minutes, followed by a temperature ramp of 5ºC/ min to 240ºC and held for 10 minutes with a total run time of 49 minutes. The interface and ion source temperatures were 300 and 220ºC, respectively. MS analysis was carried out by electron ionization at 70eV. Spectra was scanned using TIC from 40 to 600 m/z. Peaks were identified by comparing them to the NIST library (2023) with ≥80% similarity index and comparing them to those found in

the literature. The fiber was effectively cleaned by leaving the fiber in the injector port for 30 minutes. Blank column and blank fiber checks were conducted at the start of the day to ensure no carryover, or contamination occurred. C_7-C_{40} Saturated alkane standards (Sigma- Aldrich, Melbourne, Australia) were used to calculate retention indices. The calculated retention indices were matched to reference retention indices from the NIST webbook (webbook.nist.gov) under Van Den Dool and Kratz RI, polar column, temperature ramp showing data for SUPELCOWAX10 30.m/0.25mm/0.25µm. The agreed maximum deviation between the calculated and reference retention index was ≤20. Where retention index data for the SUPELCOWAX10 column was not available, DB-WAX or retention index for another suitable polar column was accepted. Hence, the acceptance threshold or the maximum deviation of retention index value was ≤30 for non-SUPELCOWAX10 columns (Bizzo et al., 2023).

Statistical analysis

Multivariate principal component analysis (PCA) was conducted to obtain a 2-dimensional listwise correlation matrix using OriginPro 2024b -student version (OriginLab Corporation, Northampton, USA). Correlations in the data were observed using the scree and bi-plot obtained from the PCA.

Results and discussion

A total minimum of 3 replications for the 12 combinations of lipid and protein samples were conducted. The formation of volatiles from oxidation, degradation and interaction reactions in heat-treated plant-based lipids and proteins and their combinations were studied. The results and discussion are presented in three parts. 1. Effect of composition on the formation of volatile profile in heat-treated lipids (Coconut fat and canola oil). 2. Proteins (pea and soy) and 3. The combination of lipids and proteins. These were compared to volatiles from beef fat (beef mince), deblooded and defatted beef mince (myofibrillar) and reconstituted whole freeze-dried beef mince. Data for beef fat, deblooded and defatted beef mince (myofibrillar) and reconstituted whole freeze-dried beef mince were used from Chapter 4- fractionation study.

Effect of lipid composition on the thermal generation of volatiles.

Listwise correlational biplot (Figure 5.1 A&B) created using principal component analysis was able to summarize 82.86% and 99.68% of the variation in the data within 2 and 3 principal components, respectively, observed from the scree plot. Significant differences in the volatiles formed from coconut fat and canola are evident from the biplot. A mixture of coconut fat and

canola oil, representing a balance between saturated and unsaturated fats, similar to beef fat, is used in commercial meat analogues to provide textural functionality. However, the volatiles formed from such a mix were significantly different to the volatiles generated from beef fat. Volatiles from canola oil correlated better with beef fat, presenting the fact that a higher degree of oxidative compounds were formed in canola oil, similar to Beef fat. A high degree of saturation, low oxidative damage and low volatility of compounds from coconut fat correlated negatively with beef fat and canola oil. It should be noted that any correlations reported from PCA analysis only relate to a one-dimensional view, i.e. numerically obtained data (area intensity) from the GCMS study. Any correlations and links made to key aroma compounds between meat and meat analogues are only arbitrary and made in reference to literature. The reason is that the increase or decrease or the presence or absence of a specific compound in a mixture does not necessarily indicate a positive outcome and, as such, must be viewed only in relation to the study discussion.

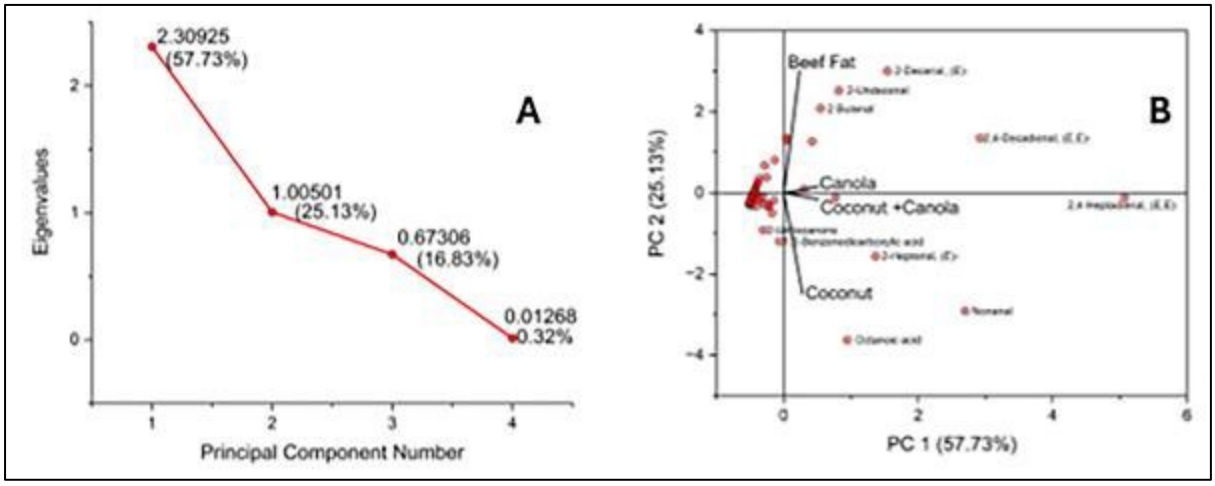

Figure 5.1 Scree plot (A) and Listwise correlational biplot (B) created using principal component analysis to observe similarities and differences in volatile formation and TIC output of heat-treated lipids

Table 5.1: Volatiles observed in plant-based fats and oils compared to beef fat from beef mince heated to 160C for 30 min, using 50/30 DVB/CAR/PDMS (SPME fiber) on a 30m. Supelco Wax10 column.

Names	Cal RI	Ref RI	Coconut	Canola	Coconut +Canola	Beef Fat
Aldehydes						
2-Propenal	810	840	1.61E+05	nd	nd	1.62E+06
2-Butenal	1034	1047	nd	6.38E+05	5.14E+05	8.26E+06
Pentanal	971	983	nd	nd	nd	2.32E+06
Hexanal	1078	1079	2.99E+05	6.26E+05	5.92E+05	4.85E+06
2-Pentenal, (E)-	1128	1124	nd	2.21E+05	1.94E+05	6.66E+05
Heptanal	1185	1184	nd	2.32E+05	1.45E+05	5.71E+06
2-Hexenal	1220	1224	nd	1.28E+05	1.12E+05	4.78E+05
Octanal	1291	1319	1.26E+05	3.76E+05	2.30E+05	7.21E+06
2-Heptenal, (E)-	1328	1323	4.51E+05	1.34E+06	1.24E+06	2.06E+06
Nonanal	1395	1411	6.36E+05	2.99E+06	2.47E+06	nd
2-Octenal, (E)-	1435	1432	1.68E+05	3.78E+05	3.60E+05	3.78E+06
2,4-Heptadienal, (E,E)-	1424	1485	2.04E+05	5.99E+06	5.89E+06	3.65E+06
Benzaldehyde	1532	1529	nd	nd	nd	7.42E+05
2-Nonenal, (E)-	1542	1543	nd	2.65E+05	1.75E+05	5.55E+06
Undecanal	1609	1599	nd	nd	nd	1.56E+06
2-Decenal, (E)-	1651	1652	1.02E+05	1.57E+06	8.61E+05	1.28E+07
Dodecanal	1715	1713	nd	nd	nd	1.90E+06
2-Undecenal	1756	1755	nd	1.00E+06	5.61E+05	9.74E+06
2,4-Decadienal, (E,E)-	1820	1798	nd	4.29E+06	2.96E+06	5.63E+06
Tetradecanal	1935		nd	nd	nd	3.40E+06
trans-4,5-Epoxy-(E)-2-decenal	2013	2006	nd	3.13E+05	4.06E+05	nd
cis-9-Hexadecenal	2166	2147	nd	nd	nd	1.06E+06
Octadecanal	2352	2343	nd	nd	nd	1.89E+06
Alcohols						
1-Butanol	1142	1152	nd	nd	nd	5.71E+05
1-Penten-3-ol	1158	1175	nd	4.85E+05	3.46E+05	4.04E+05
1-Pentanol	1250	1251	nd	1.25E+05	1.11E+05	2.39E+06
1-Octen-3-ol	1450	1447	nd	3.27E+05	2.67E+05	nd
1-Hexanol	1351	1353	nd	nd	nd	1.33E+06
1-Heptanol	1452	1449	nd	1.35E+05	nd	9.63E+05
1-Octanol	1556	1546	nd	1.89E+05	9.66E+04	3.84E+06
Propylene Glycol	1606	1611	nd	na	2.64E+05	nd
1-Nonanol	1661	1668	nd	nd	nd	8.06E+05
1-Decanol	1763	1763	nd	nd	nd	3.49E+05
Ethanol, 1-(2-butoxyethoxy)-	1798	1800	nd	nd	nd	6.52E+05

1-Dodecanol	1970	1969	nd	nd	nd	5.28E+05
1-Tetradecanol	2174	2173	nd	nd	nd	3.71E+05
1-Pentadecanol	2277	2270	nd	nd	nd	7.03E+05
Acids and esters						
Octanoic acid	2094	2104	8.09E+05	3.06E+05	3.81E+05	nd
Nonanoic acid	2192	2174	nd	4.61E+05	2.75E+05	nd
1,2-Benzenedicarboxylic acid	2552	2536	2.17E+05	1.44E+05	2.00E+05	nd
Ketones						
2-Heptanone	1181	1187	nd	nd	nd	3.80E+05
2-Octanone	1286	1285	nd	nd	nd	4.10E+05
2-Nonanone	1392	1394	nd	nd	nd	6.82E+05
2-Undecanone	1601	1602	1.50E+05	nd	nd	nd
2-Tridecanone	1813	1814	nd	nd	nd	6.50E+05
2-Pentadecanone	2025	2010	nd	nd	nd	8.81E+05
2(3H)-Furanone, dihydro-5-pentyl-	2043	2055	nd	nd	nd	1.03E+06
2-Heptadecanone	2237	2243	nd	nd	nd	4.21E+05
.gamma.-Dodecalactone	2389	2384	nd	nd	9.76E+04	nd
Furans						
Furan, 2-methyl-	894	876	nd	nd	nd	8.98E+04
2-Butanone	878	905	nd	nd	nd	1.72E+05
Furan, 2-ethyl-	940	945	nd	nd	nd	1.92E+06
Furan, 2-pentyl-	1232	1239	nd	1.74E+05	1.27E+05	5.28E+05
2-n-Octylfuran	1538	1530	nd	nd	nd	3.17E+05
Hydrocarbons amd miscellaneous compounds						
Formamide, N,N-dibutyl-	1778	1773	nd	nd	nd	3.45E+05
2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	1869	1884	nd	nd	nd	6.66E+05
Phenol, 2,4-bis(1,1-dimethylethyl)-	2317	2316	nd	nd	nd	1.23E+06

(Cal RI)- Calculated Retention Index using the Van den dool and Kratz formula,(Ref RI)- Reference Retention Index from NIST webbook for Polar column as indicated for Supelco wax10 column, in the absence of such reference data from DB-Wax or other appropriate polar column was used, nd- volatiles not detected, Data observed is area intensity from TIC analysis.

Many volatiles were isolated and separated from heat-treated lipids. However, only results for peaks that could be tentatively identified using reference indices have been reported herein and discussed further. A total of 57 compounds were tentatively identified and classified by class (Table 5.1). The 23 aldehydes accounted for the greatest number of compounds by class in the heated lipids followed by 14 alcohols, 10 ketones, 3 acids, 4 furans and 3 miscellaneous compounds. Of the 57 compounds, 29 compounds were exclusively detected in beef fat of which 17 compounds were highest in beef fat compared to other fats. Coconut fat produced (11 compounds) the least number of detectable volatiles and also had the lowest intensity of observed volatiles. Of the compounds identified only in beef fat, a significant number of them were ketones, except for 2-Undecanone detected only in coconut. Canola and canola mixed with coconut were devoid of ketonic compounds. Hexanal, octanal, 2-heptenal, (E)-, 2-octenal, (E)-, 2,4-heptadienal, (E, E)- and 2-decenal, (E)- were the only compounds (aldehydes) detected in all of the lipids. Nonanal was the only compound to be detected in all lipids except beef fat. In the canola and coconut mixed samples, canola had a greater impact on the overall volatile formation compared to coconut. Also, the area intensity of the volatiles formed in the mixed sample was slightly lower than the area intensity of the canola. 2-decenal, (E)- had the greatest impact on beef fat. 2-butenal, 2-pentenal, (E)-, heptanal, 2-hexenal, 2-nonenal, (E)-, 2-undecenal, 2,4-decadienal, (E,E)-, 1-penten-3-ol, 1-pentanol, 1-octanol and furan, 2-pentyl- were detected in all samples except coconut. Whereas, twenty-nine compounds made up of a large number of aldehydes, alcohols, all of the ketones except 2-undecanone and all of the furans namely, pentanal, benzaldehyde, undecanal, dodecanal, 2,4-decadienal, (E,E)-, cis-9-hexadecenal, octadecanal, 1-butanol, 1-hexanol, 1-nonanol, 1-decanol, ethanol, 1-(2-butoxyethoxy)-, 1-dodecanol,1-tetradecanol, 1-pentadecanol, 2-heptanone, 2-octanone, 2-nonanone, 2-tridecanone, 2-pentadecanone, 2(3H)-furanone, dihydro-5-pentyl-, 2-heptadecanone, furan, 2-methyl-, 2-butanone, furan, 2-ethyl-, 2-n octylfuran, formamide, N,N-dibutyl-, 2-hexadecene, phenol, 2,4-bis(1,1-dimethylethyl)- were found only in beef fat.

Volatile formation in plant proteins compared to meat.

With respect to comparing volatiles between plant-based and meat proteins, defatted – freeze-dried beef mince would have been a comparable approximation since it would comprise both the soluble and insoluble (myofibrillar) fraction. However, due to the lack of data points, it was not possible to conduct a PCA comparison. Instead, de-fatted and de-blooded (myofibrillar fraction) freeze-dried beef mince fraction was compared. Due to the low number of samples and available data points, the degree of correlation observed between the compounds identified

may lead to misrepresentation of outcomes. To avoid this, the results of the observed volatiles and DBDF beef mince have been discussed further.

Scree plot from PCA condensed the data to two significant components at 47.35% and 32.27%. A significant positive correlation was evident between components two and three (plant-based proteins) compared to component one- de-blooded and defatted beef mince (DBDF) (Figure 5.2). 1-octanal, octadecanal and 2-decanone positively corelated with DBDF.

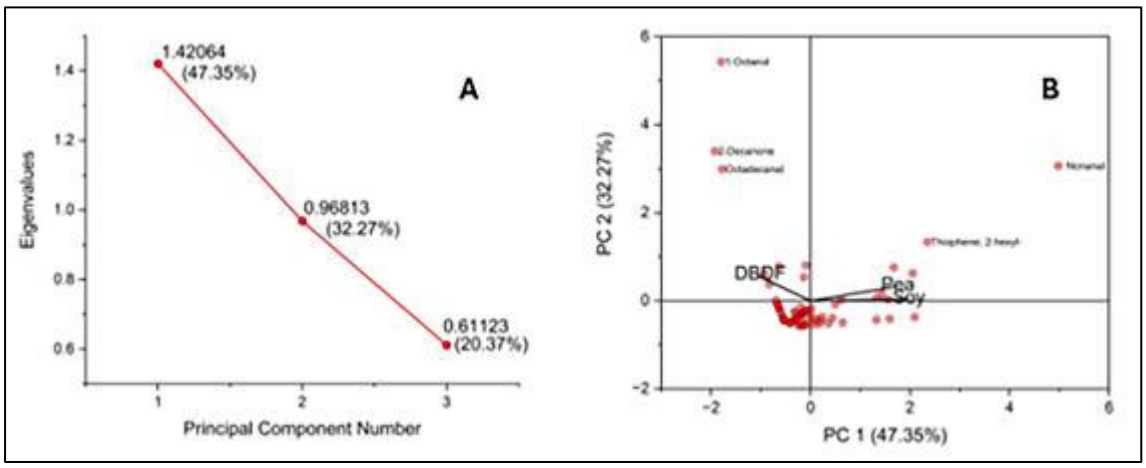

Figure 5.2 Scree plot (A) and Listwise correlational biplot (B) created using PCA of heat treated (160ºC- 30 min in vial) commercial pea and soy proteins and compared with de-blooded and de-fatted freeze-dried beef mince (myofibrillar fraction). Data for myofibrillar fraction was taken from the previous chapter.

Whereas nonanal, decanal, 2,4-decadienal, (E, E)-, pentadecanal-, 1-octen-3-ol and trans-3-nonen-2-one were negatively correlated. Pea and soy proteins were closely correlated compared to DBDF in relation to the observed volatiles. On the contrary, only three aldehydes, three alcohols and one furan was tentatively identified in defatted freeze-dried beef mince (Ch4. Tables 4.1-5), even though it contained both the myofibrillar and the sarcoplasmic fraction and could be a result of an analytical quirk or error and hence not considered for PCA comparison with plant-based meats.

Table 5.2. Tentatively identified volatiles in pea and soy proteins and compared to FDBM-DBDF.

Names	Calc RI	Ref RI	Pea	Soy	DBDF
Aldehydes					
Pentanal	958	983	8.61E+04		3.44E+05
Hexanal	1071	1079	9.93E+05	1.66E+06	3.25E+06
Heptanal	1181	1184	2.01E+05	2.90E+05	4.96E+06
Octanal	1288	1319	4.74E+05	3.45E+05	1.28E+07
2-Hexenal, 2-ethyl-	1334	1330	1.12E+05	4.32E+05	7.64E+05
Nonanal	1394	1411	1.50E+06	6.98E+05	
Decanal	1500	1501	2.72E+05	4.79E+05	
Benzaldehyde	1528	1529	1.58E+06	3.34E+06	2.50E+07
2-Nonenal, (E)-	1539	1543	8.91E+04	1.12E+05	1.12E+06
Undecanal	1606	1599	1.07E+05		2.79E+06
2-Decenal, (E)-	1648	1652	1.36E+05		
2-Octenal, 2-butyl-	1670	1653	2.97E+05	1.70E+06	1.20E+06
Dodecanal	1712	1713	1.47E+05		
Benzaldehyde, 4-ethyl-	1715	1742	1.24E+05		
3-Methyl-2-thiophenecarboxaldehyde	1736	1765	1.50E+05		
2-Undecenal	1756	1755	2.33E+05		1.44E+06
2,4-Decadienal, (E,E)-	1816	1798	4.70E+05	5.32E+05	
Tetradecanal	1924	1940	8.42E+04		
Benzaldehyde, 4-pentyl-	2012	2003			3.93E+05
Pentadecanal-	2030	2000	2.01E+05	1.89E+05	
Hexadecanal	2105	2124			1.23E+06
cis-9-Hexadecenal	2166	2147			9.95E+05
Octadecanal	2352	2343			8.87E+06
Acids and Esters					
Hexanoic acid, pentyl ester	1511	1525	7.35E+04		
Pentadecanoic acid, methyl ester	2112	2138			5.09E+05
Hexadecanoic acid, methyl ester	2220	2217			9.71E+05
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	2550	2536		1.33E+05	
Alcohols					
1-Pentanol	1247	1251	7.68E+04		
1-Hexanol	1351	1353			4.41E+05
1-Octen-3-ol	1449	1447	2.46E+05	4.61E+05	
Heptanol	1452	1449			1.29E+06

1-Octanol	1557	1546	2.66E+05		1.34E+07
1-Nonanol	1661	1668			3.01E+06
2-Furanmethanol	1665	1678		1.61E+05	1.00E+06
1-Decanol	1763	1763			1.27E+06
1-Undecanol	1863	1875			1.47E+06
Phenol, 2-methoxy-	1868	1849		4.26E+05	
Phenol, 2,6-dimethoxy-	2274	2269		6.84E+05	
trans-Isoeugenol	2359	2347		2.41E+05	
1-Heptadecanol	2479	2496			3.39E+05
Ketones					
2-Butanone	878	905			4.52E+05
2-Heptanone	1177	1187	2.72E+05	2.71E+06	9.83E+05
2-Octanone	1283	1285	6.22E+04	8.01E+05	1.45E+06
2-Nonanone	1389	1394	2.01E+05	6.04E+05	4.45E+06
3-Octen-2-one	1409	1415		1.93E+05	
2-Nonen-4-one	1483	1489		1.38E+05	
2-Decanone	1494	1515			9.89E+06
trans-3-Nonen-2-one	1514	1523	5.75E+04	1.77E+05	
2-Undecanone	1600	1602		2.37E+05	9.17E+05
1-Propanone, 1-phenyl-	1731	1737		9.11E+04	
Ethanone, 1-(3-thienyl)-	1783	1782	5.71E+04		
2-Tridecanone	1813	1814			1.12E+06
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	1826	1801	1.36E+05		
5,9-Undecadien-2-one, 6,10-dimethyl-	1857	1864	6.41E+04	1.39E+05	6.68E+05
2-Tetradecanone	1916	1907			5.06E+05
2-Pentadecanone	2022	2010	1.22E+05	1.13E+06	3.16E+06
2(3H)-Furanone, dihydro-5-pentyl-	2040	2055		4.88E+05	1.24E+06
2-Pentadecanone, 6,10,14-trimethyl-	2126	2110	7.60E+04	2.03E+05	
2-Hexadecanone	2126	2130			7.70E+05
1-Hexanone, 1-(2-thienyl)-	2134	2104	5.59E+04	1.47E+05	
2-Heptadecanone	2237	2243			2.39E+06
Furans					
Furan, 2-ethyl-	929	945	2.09E+05	5.83E+05	
2-n-Butyl furan	1125	1138	5.51E+04	2.98E+05	
Furan, 2-pentyl-	1228	1239	3.87E+06	1.59E+07	7.83E+06
2-n-Heptylfuran	1433	1430	2.76E+05		1.87E+06
2-n-Octylfuran	1536	1530	2.86E+05	1.21E+05	3.01E+06
2-Furanmethanol	1664	1666	1.18E+05		

Thiophenes					
Thiophene	1009	1022		3.21E+05	
Thiophene, 2-ethyl-	1168	1177		1.16E+05	
Thiophene, 2-methyl-	1080	1090	9.66E+04		
Thiophene, 2-methyl-5-propyl-	1321	1314	7.43E+04	9.44E+04	
Thiophene, 2-butyl-	1356	1351	3.33E+04	2.32E+05	
Thiophene, 2-pentyl-	1459	1460	2.06E+05	1.25E+06	7.66E+05
2-Acetylthiazole	1653	1661	5.79E+04		
Thiophene, 2-hexyl-	1677	1564	7.93E+05	3.49E+05	
Pyrazines					
Pyrazine, methyl-	1263	1267	8.27E+04		
Pyrazine, 2,5-dimethyl-	1319	1318	1.46E+05		
Pyrazine, 2-ethyl-6-methyl-	1383	1402	5.74E+04		
Pyrazine, trimethyl-	1402	1401	1.27E+05		
Pyrazine, 3-ethyl-2,5-dimethyl-	1444	1439	5.48E+05	3.17E+05	
Hydrocarbons and miscellaneous					
Toluene	1024	1057	5.15E+04		
Disulfide, dimethyl	1060	1070			3.76E+05
Dodecane	1192	1200	2.90E+04		
1-Pentadecene	1543	1526		1.28E+05	
Hexadecane	1595	1600		1.07E+05	
1,2,4-Trithiolane, 3,5-dimethyl-	1607	1610		7.85E+05	
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	1759	1751	1.61E+05		
cis-Calamenene	1837	1839	3.57E+04		
Indole	2459	2444		9.75E+05	

Pea proteins possessed the largest number of volatiles (54), followed by soy proteins (45) and FDBM-DBDF at 42. Aldehydes were the largest group of volatiles, with 19 compounds in peas, 11 in soy and 14 in DBDF fraction. Hexanal, heptanal, octanal, 2-hexenal, 2-ethyl-, benzaldehyde, 2-nonenal, (E) and 2-octenal, 2-butyl- found in all samples in varying concentrations, Peas had the lowest concentration whilst DBDF possessed the highest concentration overall. Smaller molecular, unlike larger molecular weight aldehydes, were evenly distributed. Peas contained aldehydes from C_{17}-C_{20} whereas DBDF contained aldehydes with molecular weight up to C_{23}. Also, aldehydes such as nonanal, decanal, 2,4-decadienal,

(E,E)- and pentadecanal were observed in both pea and soy protein samples only. DBDF had the highest concentration of benzaldehyde and octanal. Smaller molecular weight acids and esters were not observed in any samples. Pentadecanoic acid, methyl ester and hexadecanoic acid, methyl ester was observed in DBDF. Although peas contained greater number of aldehydes, it contained the least number of alcohols with DBDF possessing the most. Octanol was present in highest concentration and in DBDF. Alcohols were also widely dispersed; none were observed to be present in all three samples. Like aldehydes, ketones were present in higher concentration in DBDF sample compared to pea or soy. However, soy contained the same number of ketones as DBDF but not in similar configuration. Only, 2-heptanone, 2-octanone, 2-nonanone, 5,9-undecadien-2-one, 6,10-dimethyl- and 2-pentadecanone were observed in all three samples. 3-octen-2-one, 2-nonen-4-one and 1-propanone, 1-phenyl- were observed only in the soy sample. Peas contained more furans, thiophenes and pyrazines with DBDF possessing the least with 3 furans, one furan and no pyrazines. This was contrary to our hypothesis that myofibrillar fraction would possess higher Maillard reaction products. But agrees well with literature where pure proteins possessed lower Maillard reaction products. Dimethyl disulphide, a critical flavour active volatile, was observed only in DBDF. Since hydrocarbons and miscellaneous compounds possess low odour activity and are more stable, they were not discussed in detail.

Volatile formations in heated mixtures of plant-based proteins and lipids compared to meat.

PCA analysis was undertaken in three steps using heated mixtures of coconut fat and canola oil with pea and soy proteins. The results followed a similar trend as observed in previous studies of fats and proteins. The mixture of coconut and canola oil produced results similar to those of canola oil. This may be attributed to the susceptibility of the high unsaturated fatty acid content of canola oil to oxidation. Zhuang et al. (2022) found that oils high in polyunsaturated fatty acids generated higher levels of lipid oxidative products (LOP) when exposed to high temperatures. Similar LOP such as 2-butenal, 2-pentenal were present only in canola oil and canola oil mix but not in Coconut fat. Hexanal, and 2,4-heptadienal (E, E) were present in higher quantities in canola oil and canola oil mix compared to coconut fat alone.

Within the different classes of compounds, several volatiles were observed from various sources (Table 5.5). The formation of volatiles also followed a similar pattern in this study (Table 5.4). Aldehydes were commonly observed to have the highest peak concentration in most samples (Table 5.3). Similarly, the number of aldehydes in all the samples in this study except soy (high in ketones) was higher than in other classes of compounds (Table 5.4).

Coconut possessed the least number of aldehydes. Whereas the mixture of peas and canola had the most. The number of aldehydes and the total number of compounds seemed to decrease when the combination of both lipids (coconut and canola) was used along with proteins (pea or soy) compared to when they were cooked with a single lipid source. A higher number of aldehydes were observed when proteins were combined with canola oil compared to coconut fat. This could be related to the oxidative stability of saturated fat.

The total number of compounds in pea protein was higher compared to all other samples (Table 5.4). Although the number of volatiles was not significantly different between the groups, namely lipids, proteins, and admixtures, coconut fat had the least volatiles. Only one alcohol, 1-Octen-3-ol was observed to be present in all the mixtures except coconut fat. Except for a few volatiles, there was an overall decrease in the peak intensity of volatiles in the Coconut-Canola mix compared to canola oil by itself (Table 5.1). Pea protein and mixture with pea produced the most volatiles compared to soy and soy mixtures. It was expected that heated mixtures (lipid and protein combination) may produce a higher number of volatiles due to residual lipids. Münch et al. (2024)-explain that plant-based proteins are rich in non-protein constituents such as pro and antioxidants. This could affect the overall oxidative stability of the proteins. Although their study only related to oil in water emulsions- their findings could have a direct impact on how most chemometric analyses of heated plant-based proteins are conducted, such as by creating a slurry for increasing volatility and detection.

The number of ketones was considerably reduced in mixtures of proteins and lipids compared to proteins alone. Ketones were also negligible in both lipid samples. Although more hydrocarbons were expected in lipids, none could be identified. This could be due to the inability to find cross-references for the detected peaks whilst using the SOPELCOWAX10 column from the NIST webbook database. Except for 2-pentylfuran - no other furans, thiophenes or pyrazines were observed in the lipid samples. Peas and pea-lipid mixtures possessed a higher number of compounds compared to soy, although not significantly different. Low levels of indole were observed in lipid and fat mixtures with peas compared to none in peas. A mixture of lipids and soy had a higher concentration of indole; soy had the highest concentration.

As noted earlier, the oxidation of lipids is a sequential, unavoidable, non-stoppable and irreversible chain reaction by the action of molecular oxygen forming free radicals (Frankel, 2005). But also, one that co-exists or affects other oxidative (proteins) or reactive states (Maillard reaction products) (Bleicher et al., 2022). Whilst studying these phenomena,

qualitative similarities were observed in the volatiles formed in heated plant-based lipid and protein mixtures compared to beef mince in this study. Products of lipid oxidation, such as a large number of aldehydes, ketones and alcohols, are indicated in the formation of warmed-over flavour, thereby reducing the Appreciability of the product (Chen et al., 2024; Shahidi, 1994). These have generally been discussed extensively in meat and meat products (Chen et al., 2024; Dinh et al. 2021; Keller & Grosch, 1996). However, several aldehydes observed in this study such as hexanal, octanal, nonanal, 2-heptenal, (E)-, 2-octenal, (E)-, 2,4-heptadienal, (E,E)-, benzaldehyde, 2-nonenal, (E)-, undecanal and 2-decenal, (E)- including others were also observed in grilled beef (Wall et al., 2019), roast beef (Soo-Yeun et al., 2006), autoclaved

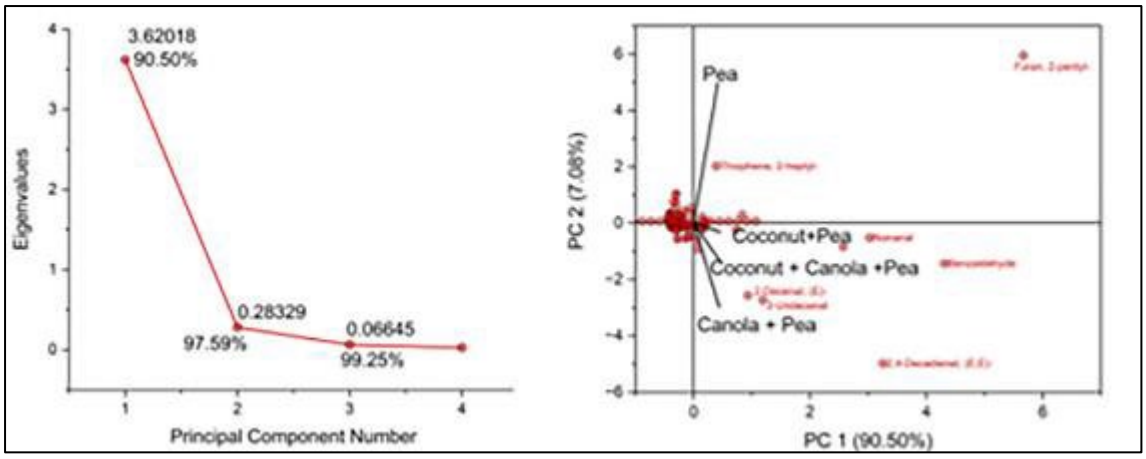

Figure 5.3: PCA of volatiles observed in a reaction of pea protein with plant based lipids at 160°C.

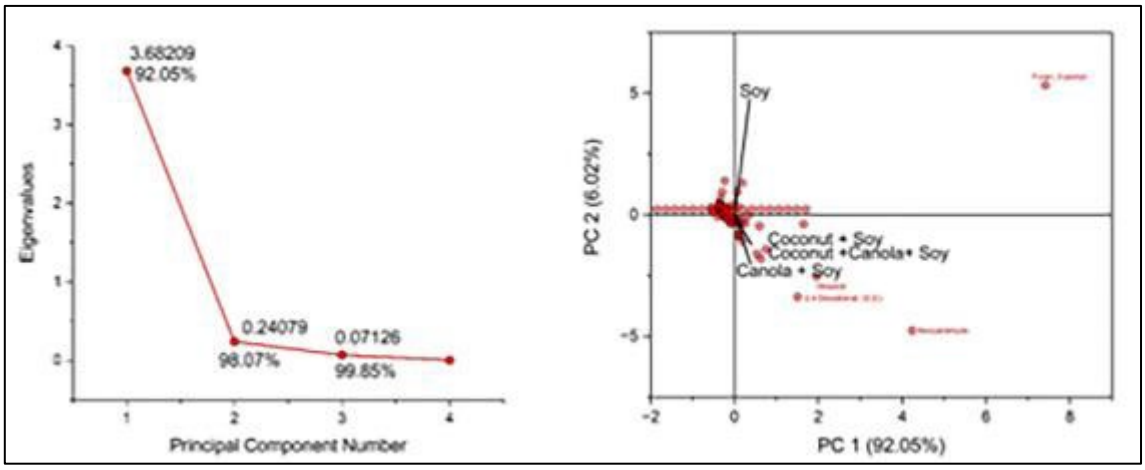

Figure 5.4: PCA of volatiles observed in a reaction of soy protein with plant based lipids at 160ºC.

beef (Van Ba et al., 2010), mushroom (Aisala et al., 2019; Davila et al., 2022), yeast extracts (Alim et al., 2019; Ames et al., 1992; Lin et al., 2014) and other studies (Table 5.5). Plant-based meat is regularly compared to ground or minced meat (Hernandez et al., 2023; Kaczmarska et al., 2021). However, a key point to note is that this comparison is rather arbitrary since plant-based meat is a formulated product that is seasoned and flavoured, compared to ground or minced meat. Although this can be debated, in the context that meat mince or ground meat possesses all necessary precursors intrinsically and plant-based formulations are created to mimic them; the studies in their conclusions noted that the volatile profile of plant-based foods contained increasingly diverse volatiles and were different to ground beef. In the case of Hernandez et al. (2023) the concentration of hexanal in plant-based products was thrice than of lean ground meat and close to twice that of regular ground meat. All other aldehydes were also observed to be at a higher concentration in plant-based meats. Kaczmarska et al. (2021) also observed hexanal to be three folds higher in substitutes compared to beef as well as total aldehydes. (E,E)-2,4-decadienal an off flavour and odour (Thong et al., 2024) and a well-recognized warmed over flavour compound was forty times higher in the meat substitutes compared to beef.

Some aldehydes may be more potent compared to others. As part of secondary lipid oxidation, aldehydes such as the potent trans-4,5-Epoxy-(E)-2-decenal (Table 5.1) were observed in heated canola and coconut and canola mix but not in coconut possessing low odour thresholds or high perceptual ability (Skibsted in Shahidi, 1994, Ch.10). They may also possess synergistic effect with other volatiles thereby either lowering odour threshold or masking aroma perception. Also, they may be implicated in promoting oxidative states, especially in food cooked at high temperatures, such as burgers. Furthermore, a study that compared beef burgers to plant-based burgers (Thong et al., 2024) observed that sensory evaluation did not necessarily match protein sources. This may well be related to the intrinsic composition of meat compared to the formulated plant-based meats (Kaczmarska et al., 2021).

The formation of compounds such as pentanal, 2-hexenal-2-ethyl, decanal, benzaldehyde, 3-thiophenecarboxaldehyde, dodecanal and benzaldehyde-4-ethyl were amongst the aldehydes observed in heat-treated pea and soy proteins and their combination with the lipids but not in either of the lipids. This could signify its formation from residual lipids and protein degradation (Barallat-pérez et al., 2023). Heat treatment of proteins in combination with lipids increases ongoing oxidative processes in both proteins and lipids and lends to possible interactions (binding) with their degradative products.

Table 5.3: Volatiles observed in mixtures of heat-treated plant-based lipids and proteins.

Names	Cal RI	Ref RI	CoPe	CaPe	CoCaPe	CoSo	CaSo	CoCaSo	Pea	Soy
Aldehydes										
Butanal, 3-methyl-	893	918		1.02E+05						
Pentanal	962	983	1.94E+05	2.76E+05	2.00E+05		1.30E+05	1.15E+05	8.61E+04	
Hexanal	1068	1079	1.79E+06	2.00E+06	1.91E+06	1.06E+06	2.09E+06	1.73E+06	9.93E+05	1.66E+06
Heptanal	1180	1184	3.12E+05	5.30E+05	3.54E+05	2.03E+05	3.49E+05	2.64E+05	2.01E+05	2.90E+05
Octanal	1298	1319	5.11E+05	1.07E+06	6.50E+05	1.39E+05	4.22E+05	2.76E+05	4.74E+05	3.45E+05
2-Heptenal, (E)-	1325	1323		5.18E+05			1.47E+05			
2-Hexenal, 2-ethyl-	1336	1334	1.14E+05	1.27E+05	1.42E+05	1.44E+05	1.50E+05	1.45E+05	1.12E+05	4.32E+05
Nonanal	1395	1411	1.43E+06	3.24E+06	1.80E+06	3.50E+05	1.40E+06	7.42E+05	1.50E+06	6.98E+05
2-Octenal, (E)-	1433	1432	3.10E+05	5.44E+05	3.78E+05	1.47E+05	2.57E+05	2.34E+05		
2,4-Heptadienal, (E,E)-	1499	1485		5.21E+05						
Decanal	1501	1501	3.51E+05	4.49E+05	4.12E+05	2.72E+05	4.66E+05	4.11E+05	2.72E+05	4.79E+05
Benzaldehyde	1530	1529	2.85E+06	3.36E+06	2.81E+06	2.69E+06	3.32E+06	3.31E+06	1.58E+06	3.34E+06
2-Nonenal, (E)-	1540	1542	2.36E+05	5.56E+05	3.13E+05	1.36E+05	2.84E+05	2.27E+05	8.91E+04	1.12E+05
Undecanal	1607	1604	1.73E+05	1.34E+05	1.08E+05				1.07E+05	
2-Decenal, (E)-	1649	1651	4.90E+05	1.93E+06	8.53E+05	1.62E+05	7.79E+05	4.42E+05	1.36E+05	
2-Octenal, 2-butyl-	1670	1659				2.12E+05	2.72E+05	2.64E+05	2.97E+05	1.70E+06
3-Thiophenecarboxaldehyde	1702	1687		1.64E+05		1.15E+05	9.08E+04	1.20E+05		
Dodecanal	1713	1720	1.17E+05	1.33E+05	1.13E+05				1.47E+05	
Benzaldehyde, 4-ethyl-	1717	1742	1.93E+05	3.95E+05	2.24E+05		1.08E+05		1.24E+05	
5-Methyl-2-thiophenecarboxaldehyde	1736	1767		1.89E+05		1.19E+05	9.23E+04	1.24E+05		

3-Methyl-2-thiophenecarboxaldehyde	1737	1765		2.01E+05					1.50E+05	
2-Undecenal	1757	1756	6.42E+05	2.31E+06	8.49E+05	1.47E+05	6.42E+05	3.62E+05	2.33E+05	
2,4-Decadienal, (E,E)-	1818	1820	1.91E+06	3.44E+06	2.77E+06	6.23E+05	2.42E+06	1.32E+06	4.70E+05	5.32E+05
2-Dodecenal, (E)-	1865	1889	1.03E+05	1.40E+05						
Tetradecanal	1924	1940							8.42E+04	
Benzeneacetaldehyde, .alpha.-ethylidene-	1939	1939					1.12E+05			
Pentadecanal-	2031	2041	9.11E+04						2.01E+05	1.89E+05
Alcohols, acids and Esters										
1-Pentanol	1247	1250	1.64E+05	2.27E+05	1.61E+05		8.94E+04		7.68E+04	
1-Octen-3-ol	1450	1447	3.24E+05	4.98E+05	3.43E+05	3.16E+05	4.08E+05	2.91E+05	2.46E+05	4.61E+05
1-Heptanol	1455	1460	1.87E+05	3.60E+05	1.93E+05		8.78E+04			
1-Octanol	1558	1556	2.55E+05	6.60E+05	2.95E+05		1.66E+05	1.11E+05	2.66E+05	
2-Furanmethanol	1664	1665				2.10E+05	1.81E+05	2.10E+05		1.61E+05
Phenol, 2-methoxy-	1868	1849								4.26E+05
Maltol	1984	1969				3.23E+05	3.67E+05	4.16E+05		
Phenol, 2,6-dimethoxy-	2274	2269								6.84E+05
trans-Isoeugenol	2359	2347								2.41E+05
Hexanoic acid, pentyl ester	1511	1525							7.35E+04	
Dodecanoic acid	2536	2516	4.15E+05			6.50E+05				
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	2552	2536	2.95E+05	2.36E+05	2.75E+05		1.23E+05			1.33E+05
Ketones										

2-Heptanone	1179	1187	3.42E+05	3.36E+05	3.60E+05	1.04E+06	1.46E+06	1.23E+06	2.72E+05	2.71E+06
2-Octanone	1282	1285				2.27E+05	2.89E+05	2.75E+05	6.22E+04	8.01E+05
2-Nonanone	1388	1394				1.56E+05	1.88E+05	1.75E+05	2.01E+05	6.04E+05
3-Octen-2-one	1410	1415	9.56E+04	1.08E+05	1.02E+05	1.42E+05	2.26E+05	1.91E+05		1.93E+05
2-Nonen-4-one	1482	1489					1.13E+05	9.83E+04		1.38E+05
trans-3-Nonen-2-one	1515	1523	1.04E+05	2.10E+05	1.15E+05	1.50E+05	1.98E+05	1.76E+05	5.75E+04	1.77E+05
3,5-Octadien-2-one	1524	1521	1.58E+05	1.21E+05	1.63E+05					
2-Undecanone	1601	1602	1.41E+05			1.09E+05				2.37E+05
1-Propanone, 1-phenyl-	1731	1737								9.11E+04
Ethanone, 1-(3-thienyl)-	1784	1782		1.95E+05					5.71E+04	
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	1825	1801	1.01E+05	1.57E+05	1.04E+05				1.36E+05	
5,9-Undecadien-2-one	1857	1864							6.41E+04	1.39E+05
2-Pentadecanone	2023	2010	9.93E+04						1.22E+05	1.13E+06
2(3H)-Furanone, dihydro-5-pentyl-	2039	2055				2.85E+05	2.96E+05	2.82E+05		4.88E+05
2-Pentadecanone, 6,10,14-trimethyl-	2126	2110							7.60E+04	2.03E+05
1-Hexanone, 1-(2-thienyl)-	2134	2104							5.59E+04	1.47E+05
.gamma.-Dodecalactone	2389	2384								
Furans, Thiophenes and Pyrazines										
Furan, 2-ethyl-	934	945	2.67E+05	5.36E+05	2.99E+05	2.86E+05	5.36E+05	4.71E+05	2.09E+05	5.83E+05
2-n-Butyl furan	1125	1138				1.04E+05	9.25E+04	1.03E+05	5.51E+04	2.98E+05
Furan, 2-pentyl-	1228	1239	3.03E+06	3.42E+06	2.93E+06	3.32E+06	4.70E+06	4.51E+06	3.87E+06	1.59E+07
2-n-Heptylfuran	1433	1430							2.76E+05	

Furfural	1471	1467	9.92E+04							
2-n-Octylfuran	1537	1530	1.47E+05	2.74E+05	1.60E+05				2.86E+05	1.21E+05
2-Furanmethanol	1665	1666	3.01E+05	2.87E+05	2.54E+05				1.18E+05	
2(3H)-Furanone, dihydro-5-pentyl-	2041	2055	1.08E+05	2.64E+05						
Thiophene	1006	1022				2.44E+05	3.08E+05	3.07E+05		3.21E+05
Thiophene, 2-methyl-	1082	1090		8.59E+04		1.40E+05		1.17E+05	9.66E+04	
Thiophene, 2-ethyl-	1168	1177								1.16E+05
Thiophene, 2-methyl-5-propyl-	1321	1314							7.43E+04	9.44E+04
Thiophene, 2-butyl-	1356	1351							3.33E+04	2.32E+05
Thiophene, 2-pentyl-	1460	1460	2.00E+05	1.87E+05	1.87E+05	1.83E+05	2.32E+05	2.21E+05	2.06E+05	1.25E+06
2-Acetylthiazole	1654	1661	2.14E+05	1.97E+05	1.74E+05				5.79E+04	
Thiophene, 2-heptyl-	1677	1652			9.20E+05	2.19E+05	5.04E+05	4.54E+05	7.93E+05	
Pyrazine	1210	1209	1.16E+05	1.30E+05	9.03E+04					
Pyrazine, methyl-	1264	1267	2.08E+05	2.17E+05	1.22E+05				8.27E+04	
Pyridine, 2-ethyl-	1277	1277					9.51E+04			
Pyrazine, 2,5-dimethyl-	1320	1318	3.10E+05	3.11E+05	2.36E+05	1.16E+05	9.23E+04	1.30E+05	1.46E+05	
Pyrazine, 2-ethyl-6-methyl-	1385	1402	1.25E+05	1.30E+05	8.59E+04				5.74E+04	
Pyrazine, trimethyl-	1403	1401	2.75E+05	2.87E+05	1.57E+05	1.10E+05	1.16E+05	1.31E+05	1.27E+05	
Pyrazine, 3-ethyl-2,5-dimethyl-	1445	1439	8.15E+05	7.59E+05	6.89E+05	2.76E+05	3.63E+05	3.77E+05	5.48E+05	3.17E+05
Pyrrole	1519	1525				1.18E+05	9.21E+04	1.52E+05		
Hydrocarbons and miscellaneous										

Disulfide, dimethyl	1059	1078					9.51E+04			
Dodecane	1192	1200							2.90E+04	
1-Pentadecene	1543	1526								1.28E+05
Hexadecane	1595	1600								1.07E+05
1,2,4-Trithiolane, 3,5-dimethyl-	1606	1610	1.20E+05			5.88E+05	2.03E+05	4.54E+05		7.85E+05
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	1760	1751	1.86E+05		1.65E+05				1.61E+05	
cis-Calamenene	1837	1839							3.57E+04	
Phenol, 2-methoxy-	1867	1849				7.70E+05	7.40E+05	7.35E+05		
Phenol	2014	2014				1.48E+05	1.47E+05	1.46E+05		
Phenol, 2,6-dimethoxy-	2273	2269				7.45E+05	6.44E+05	6.33E+05		
Indole	2460	2444	2.91E+05	2.89E+05	2.42E+05	5.96E+05	6.47E+05	6.40E+05		9.75E+05
Dodecanamide	2767	2784				2.10E+05				

Cal RI- Calculated Retention index, Ref RI- Reference retention Index, (CoPe – Coconut fat + Pea protein), similarly Ca- Canola oil and So- soy protein.

Table 5.4. Total number of volatiles by class from the various samples of plant-based lipids, proteins and their admixtures.

Compound class	Co	Ca	CoCa	Pea	Soy	CoPe	CaPe	CoCaPe	CoSo	CaSo	CoCaSo
Aldehydes	8	15	15	19	11	18	23	16	15	19	16
Alcohol acids and esters	3	8	9	4	6	6	5	5	4	7	4
Ketones	1	0	1	10	13	7	6	5	7	7	7
Furans, thiophenes and pyrazines	0	1	1	17	10	14	14	13	11	11	11
Hydrocarbons	0	0	0	3	4	3	1	2	6	6	5
	12	24	26	53	44	48	49	41	43	50	43

Co-Coconut, Ca-Canola, Pe-Pea and So-soy.

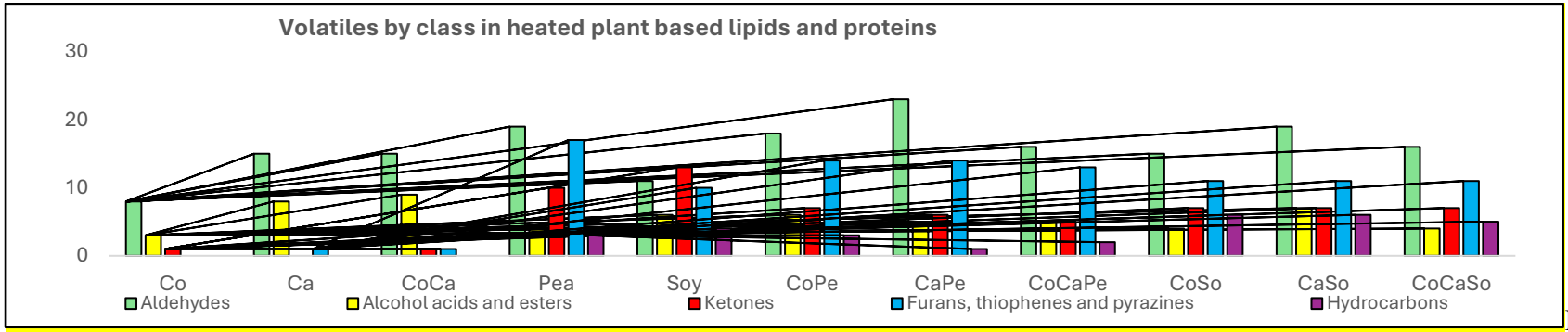

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Figure 5.5 Number of tentatively identified volatiles observed from each of the compound classes in plant-based lipids and proteins. Co-Coconut, Ca-Canola, Pe-Pea and So-soy.

Table 5.5. Volatiles found in this study compared to aroma active compounds observed from different sources.

Names	Sources of volatiles from literature*	References
Pentanal	Roast beef, Mushroom, Model, Grilled Beef, autoclaved beef, silicone bath	1,2,3,4,5,9,10,11,13
Hexanal	Roast beef, Mushroom, Model, Grilled Beef, autoclaved beef, silicone bath, Yeast Extract	1,2,3,4,5,6,7,9,10,12,13,14,16
2-Pentenal, (E)-	Yeast Extract	16
Heptanal	Roast beef, autoclaved beef, Grilled beef, Mushroom, silicone bath, Yeast Extract	1,5,6,7,9,10,11,13,16
2-Hexenal	autoclaved beef, Mushroom, Grilled beef	5,7,12
Octanal	Simulated & Roast beef, Mushroom, Grilled Beef, autoclaved beef, Yeast Extract, silicone bath	1,2,4,5,7,8,9,10,12,13
2-Heptenal, (E)-	Grilled Beef	4,11
Nonanal	Simulated, Roast beef, Mushroom, Model, Grilled Beef, autoclaved beef, Yeast Extract, silicone bath	1,2,3,4,5,6,8,9,10,12,13,14,16
2-Octenal, (E)-	Grilled Beef [5]	4
Benzaldehyde	Simulated, Roast and boiled beef, Mushroom, Grilled Beef, Autoclaved beef, Yeast Extract, silicone bath	1,2,4,5,6,7,8,9,10,13,14,16
2-Nonenal, (E)-	Grilled Beef, autoclaved beef, silicone bath	4,5,6,12,13
Undecanal	Roast beef, Model, Grilled Beef	1,3,4
2-Decenal, (E)-	Roast beef	1
Dodecanal	Roast beef, Grilled Beef	1,4
2-Undecenal	Grilled Beef	4
2,4-Decadienal, (E, E)-	Mushroom, autoclaved beef, Grilled beef, Yeast Extract	2,5,7,12,15
Tetradecanal	Grilled Beef, autoclaved beef	4,5,6
cis-9-Hexadecenal	Grilled beef	6
Octadecanal	Grilled beef	6
1-Butanol	Mushroom	2,7

1-Penten-3-ol	Simulated beef	1
1-Pentanol	Mushroom, Grilled Beef	2,4,7,12
1-Octen-3-ol	Roast beef, Mushroom, Grilled beef, silicone bath	1,2,4,6,7,10,12,13
1-Hexanol	Mushroom, Grilled Beef	2,7,10
1-Heptanol	Roast beef, Grilled beef, Mushroom, Yeast Extract	1,4,7,10,16
1-Octanol	Mushroom, Grilled Beef, autoclaved beef, silicone bath	2,4,5,6,7,13
Octanoic acid	Roast beef, Mushroom, Grilled beef	1,2,10
Nonanoic acid	Mushroom, Grilled Beef	2,6,10
2-Heptanone	Roast beef, Mushroom, Model, Grilled Beef, autoclaved beef, silicone bath, Yeast Extract	1,2,3,4,5,6,7,10,13,18
2-Octanone	Model, autoclaved beef, Grilled beef, Yeast Extract	3,5,6,11,16
2-Nonanone	Mushroom, Yeast Extract	2,7,15,16
2-Undecanone	Mushroom, Grilled beef	2,6,7
2-Tridecanone	Grilled beef	6
2(3H)-Furanone, dihydro-5-pentyl-	Yeast Extract	8
2-Butanone	Roast beef, Mushroom, Grilled Beef, Yeast Extract	1,2,4,9,10,16
Furan, 2-ethyl-	Simulated beef, silicone bath, Yeast extracts	1,13,15
Furan, 2-pentyl-	Roast beef, Mushroom, Model, Grilled Beef, autoclaved beef, silicone bath, Yeast extract	1,2,3,4,5,6,7,10,13,15,16
2-n-Octylfuran	autoclaved beef	5

*Compounds cross-referenced to sources from those observed in literature other than coconut fat, canola oil, pea and soy proteins using HS-SPME-GCMS.

References: 1. Soo-Yeun et al. (2006) 2. Davila et al. (2022), 3. Zhang et al. (2021), 4. Wall et al. (2019), 5. Van Ba et al. (2010), 6. Wang et al. (2018), 7. Aisala et al. (2019), 8. Alim et al. (2018), 9. Legako et al. (2016), 10. Hunt et al. (2016), 12. Blackmon et al. (2015), 12. Mountford et al. (2014), 13. Ma et al. (2013), 14. Lin et al. (2014), 15. Mahadevan & Farmer, (2006), 16. Ames & Elmore, (1992)

Binding is a significant phenomenon that involves masking, retention, and release. It can occur through various mechanisms, including the formation of Schiff bases, Michael additions, or disulphide linkages, which create strong and irreversible covalent bonds (Anantharamkrishnan et al., 2020). Alternatively, binding can involve weak, reversible non-covalent interactions, such as hydrogen and ionic bonding, hydrophobic interactions or Van der Waals forces. The interactions often occur through functional groups like amine (-NH2), carboxyl (-COOH), hydroxyl (-OH), or sulfhydryl (-SH) groups (Snel et al., 2023; Barallat-Pérez et al., 2023).

However, comparing the flavour profiles of plant-based meats to those of animal meat may be inappropriate due to the specificity of these interactions. Factors such as the type of protein, composition, and concentration can all influence binding pathways (Vatansever et al., 2024). This issue has been highlighted in several studies comparing commercial plant-based meats with animal meats (Biazotto et al., 2025; Karabulut et al., 2024; Thong et al., 2024). The composition of animal proteins, peptides, and amino acids, particularly in beef (Alekseeva & Kolchina, 2019; Li et al., 2023; Williams, 2007), varies significantly from legumes like soy and peas (Day et al., 2022). This variation can impact the overall ability of the product to bind volatiles and aroma compounds.

Legumin (11S) and vicilin (7S) found in pea proteins, as well as glycinin (11S) and β-conglycinin (7S) in soybeans, are recognized as active sites for protein binding (Heng et al., 2004). In terms of binding affinity, aldehydes, followed by alcohols and ketones in that order, are known to interact with proteins due to hydrophobic interactions (Guo et al., 2022). Additionally, esters may bind to the 11S globulins of protein isolates via the methyl functional group in the carbonyl compound (Guo et al., 2019; Reineccius, 2005). Moreover, multiple binding sites for flavour compounds on protein isolates can result from cooperative or competitive binding, influenced by the ratio and concentration of proteins to flavour compounds (Guo et al., 2022). The study also indicated that the 'salting out' effect might influence the release of flavour compounds. However, these interactions were primarily investigated in low-temperature systems, which likely resulted in minimal structural modifications to the proteins. The binding phenomena in heat-treated proteins, on the other hand, remain underexplored.

High thermal inputs, such as those employed in this study, can modify proteins by exposing hydrophobic regions, thereby enhancing flavour binding (Saffarionpour, 2024). In the context of protein oxidation, degradation, and the formation of Maillard reaction products in plant-based proteins, several furans, thiophenes, and pyrazines were detected in pea and soy samples,

as well as their mixtures with lipids, but not in lipids alone. Among the furans, 2-pentylfuran was the only one present in all samples except for coconut, while 2-ethylfuran was found in all samples except for lipids. No specific trend was noted for the other furans beyond their absence in lipid samples. The polarity and positioning of branched chains in furans may influence their interaction with amino acids. Cross-linking of oxidized furans with nitrogen-based compounds via a 1,4-dicarbonyl condensation can yield pyrroles (Saffarionpour, 2024). Low concentrations of pyrroles were detected only in soy-lipid mixtures, but not in pea or pea-lipid mixtures.

S-substituted compounds such as thiols, thiazoles, thiophenes, and sulphides are significant contributors to aroma (Mottram, 1998). Several thiophenes were observed in protein-lipid mixtures, but none were found in lipid samples when compared to beef. A clear trend was not evident. Notably, 2-pentyl thiophene was found in all samples, while 2-acetylthiazole was only present in pea samples. The formation of 2-alkylthiophenes can occur through the addition of H_2S, followed by a nucleophilic attack of sulphur on the carbonyl group to create an intermediate. The subsequent loss of water can lead to the formation of an alkylthiophene (Elmore & Mottram, 2000). These compounds also form adducts with proteins such as β-lactoglobulin (Anantharamkrishnan et al., 2020). While the formation and interaction of these compounds in meat and meat products have been discussed, their elucidation in plant-based ingredients remains limited.

Predominantly, non-covalent bonding through molecular docking was noted between hydrophobic interactions of methylpyrazines and hydrogen bonding among di-/trimethylpyrazines, depending on the alkyl structures of the pyrazine ring with pea protein isolate (Guo et al., 2024). As expected, no thiophenes or pyrazines were found in lipid samples. In pea samples, all pyrazines were present, except for pyrrole, which was observed in soy-lipid mixtures. Pyrazine, methylpyrazine, and 2-ethyl-6-methylpyrazine were exclusively observed in pea and lipid mixtures, while 2,5-dimethylpyrazine, trimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine were found in both pea and soy lipid mixtures. Due to the dynamic nature of quenching, higher molecular weight pyrazines exhibited a greater affinity for binding to pea proteins in hydrophobic regions (Guo et al., 2024).

Conclusions

In examining the effects of lipid composition on the formation of volatile compounds, canola oil showed a greater influence compared to other fats. Although the number of volatiles

produced from canola oil was similar to that of beef fat, the biplot obtained from the PCA analysis revealed significant differences among beef fat, coconut oil, and canola oil. Some similarities were observed between canola oil and a coconut-canola mixture. The volatiles generated from heated pea and soy proteins, as well as their mixtures, were significantly different from those of FDBM-DBDF (myofibrillar fraction). No significant similarities among any classes of compounds across the assays could be determined. Pea protein produced the highest number of volatile compounds compared to soy or FDBM-DBDF. Furthermore, the trends regarding the number of tentatively identified compounds were similar for protein and lipid mixtures. Generally, heat treatment of proteins combined with lipids increased the production of volatiles, with pea and pea-lipid mixtures yielding the largest number.

In this study, comparing volatiles between heat-treated plant-based lipids and proteins versus fractioned beef mince presented challenges and did not yield satisfactory results. This was primarily due to human error, analytical limitations, and low confidence in the data obtained from the fractions study. Manually interpreting mass spectrometry (MS) data was extremely time-consuming, resulting in unresolved data with numerous unidentified peaks, which increased the likelihood of inaccurate comparisons. Additionally, the lack of supplementary evidence regarding the progression of oxidation and degradation of lipids and proteins hindered the ability to make conclusive remarks. Future research needs to include scaled temperature studies, along with an assessment of lipid degradation through fatty acid profiles, to better understand the binding phenomena of various proteins in the presence of different lipid degradation products at varying temperatures.

As novel ingredients and processing techniques for protein extraction and the formulation of meat alternatives continue to evolve, further research is essential in characterizing and understanding the sensory output and consumer acceptance of these products if they are to become a staple in future diets. The study could also benefit from incorporating sensory analytical techniques in conjunction with chemometrics.

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Chapter 6

Conclusions and future directions

In view of the need for creating appreciable meat alternatives using plant-based ingredients amidst a lack of standard terminology and wide-ranging personal opinions on sensory descriptors, the current study sought to undertake a comparative study of aroma compounds in beef and plant-based ingredients associated with savoury perceptions. To achieve this, it investigated the formation of volatiles in the beef mince and plant-based ingredients using gas chromatography-mass spectrometry. Specifically, the project aimed to identify pathways and the development of volatile aromatic compounds in foods processed using the High-Temperature Short Time (HTST) format. Further, it studied the efficacy of using headspace solid-phase microextraction for volatile analysis in complex media. It ascertained the contribution of fatty acids to the development of savoury aromas during thermal treatment.

A comprehensive literature review was undertaken to build on current knowledge and understanding of volatile formation and analysis. It was found that the composition of meat is highly complex and varies greatly with plant-based ingredients that are essentially fractionated functional ingredients that need to be formulated. It was observed that although taste and texture may have been attained by formulation, the creation of a matching aroma profile was challenging. Fuel type, cooking method, the extent of cooking, moisture, etc, all played a significant role in the development of aroma compounds. The greatest challenge was observed to be an objective analysis of the volatiles. Although aroma compounds may be considered subjective due to large variances observed in sensory descriptors, using chemometrics to detect and identify volatiles using comprehensive untargeted analysis could be helpful. The review's findings resonate with the numerous studies on the need for further research on the holistic approach to advocating the inclusion of plant-based foods in the diet.

The extensive literature review revealed the wide use of qualitative and quantitative methodologies in analysing volatiles. Therefore, optimising conditions was necessary for the detection of aroma compounds in this study. The application of HS-SPME-GCMS as a green technology with the ability to analyse volatiles from whole foods was considered appropriate. However, the starting point in optimising conditions requires the careful selection of various conditions, such as sample selection, concentration and extraction, analyte separation, and ultimately detection.

Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) and CAR/PDMS fibers were selected to extract analytes from the headspace. The selectivity of the analytes of both SPME fibers was slightly different. The stationary phase further affects the ability to identify volatiles based on analyte retention, mass spectrometer library search, retention indices and

databases. Equilibration extraction time and temperature were optimised to 30 minutes at 70ºC. Several questions remained unanswered in relation to the use of SPME in undertaking comprehensive untargeted analysis from the complex matrix. Several conditions affected the effectiveness of SPME fibers, such as equilibration conditions, length of the fiber, vapour pressure, the complexity of the matrix, depth of fiber insertion, and durability of the fiber. Although using and applying an optimised equilibration and extraction condition for all essays was convenient, it could have affected the overall results. This was evident when samples with high-fat content produced low peak intensities in some compounds. Whether this phenomenon occurred due to the overall presence of a large number of aldehydes observed in samples with fat oxidation or due to competition was difficult to prove.

Further, two different cooking methods (pan-grilling and cooking in a vial using an oil bath) and formats (whole beef mince and lyophilisation) were undertaken to observe differences in the volatile profile. The cooking method was observed to have a greater effect than the format. This leads to the conclusion that lyophilisation did not significantly affect the volatile profile. In another study, it was found that chilling the sample post-heat treatment can affect the volatile profile. Adding water to the vial to stir the sample did not significantly impact the volatile profile. Freezing the sample for storage post-cooking for up to a week did not qualitatively impact the volatile profile. However, some compounds decreased significantly. It should be noted that the selection of an alkane standard should match the ability of the SPME fiber. Applying a large range of alkane standards proved erroneous when using a Wax column. Establishing a calibration curve using an internal standard also proved challenging due to the complexity of the sample matrix.

During sample characterization and analysis, it became evident that the high-fat content of approximately 18% by whole weight (54% by dry weight basis) would hinder the volatility of headspace analytes. Hence, fractionation of beef mice was proposed to enable the study of volatile formation in different fractions. Beef mince was successfully fractionated into sarcoplasmic, myofibrillar and fat fractions. SDS PAGE showed that the sarcoplasmic fraction (aqueous extract) contained several other soluble proteins besides haemoglobin and myoglobin. Effective separation of sarcoplasm (de-blooded and defatted fraction- DBDF) was also validated by SDS PAGE. Volatile analysis of the various fractions revealed that fat and DBDF provided most of the volatiles. The spread of volatiles is evident from the PCA biplots (1-4). Although Fat and DBDF had the highest number of volatiles. They differed qualitatively as well as quantitatively. DBDF fraction produced a greater number of ketones compared to all

other compounds. Interestingly, no pyrazines were observed in any samples. A view of the chromatograms of the various fractions reveals the extent of differences that could be lost when conducting an analysis of a whole beef mince sample using SPME. However, it should also be noted that a total ion chromatogram may consist of several volatiles that may not be relevant to the sample.

Since proteins are the key ingredient in producing meat alternatives, it seems pertinent that aroma generation from proteins be studied. However, it has been shown that pure proteins do not produce appreciable aromas; rather, the interaction of peptides and amino acids with reducing sugars produces the Maillard reaction and associated products. These then interact with lipid degradative products to yield aroma compounds that may be favourable or unfavourable.

This study compared the ability of plant-based lipids and proteins to yield volatiles similar to those found in beef mince. Coconut fat, canola oil, pea, and soy proteins were heat-treated in various combinations. It was observed that coconut fat produced the least volatiles. Canola oil influenced the volatile profile compared to coconut fat. Pea protein produced a higher number of volatiles compared to soy. Although numerous volatiles were observed to be common between plant-based ingredients and beef mince. It was difficult to decipher their importance in promoting a meaty or beefy aromatic experience.

It was successfully demonstrated to an extent that the use of SPME in the application of comprehensive untargeted analysis of volatiles could provide vital information. However, several shortcomings were observed in relation to the choice of analytics. Based on the results, further research could be undertaken by considering the shortcomings of this study. Overall, the loss of information relating to the ability to identify peaks hindered the ability to make conclusive remarks

Future directions

The future of the plant-based movement depends on several factors that are worth considering. While individual studies in this area may be limited in scope, the broader research highlights the role of flavour enhancers, such as yeast extracts, hydrolysed vegetable proteins, mushrooms and mushroom extracts, seaweed, and nucleotide salts, in enhancing the sensory experience, particularly taste. This process, known as 'umamification,' is primarily effective for foods cooked at temperatures below 100°C. However, when foods are grilled or roasted at higher temperatures, there are additional factors that contribute to taste and aroma that need to be

taken into account. This is important because, at the moment of consumption, aromas (volatiles) are perceived before taste and provide a much stronger stimulus. Although studies on consumer perspectives and perceptions exist, they tend to focus on psychological aspects and rarely incorporate technological factors.

Interdisciplinary and cross-functional research is essential to examine the various factors influencing food choices. Factors such as convenience and price are already recognised as critical in determining choices and repeat purchases. Therefore, advocates for plant-based foods should adopt a more inclusive approach, moving beyond the narrow focus on creating products that mimic meat, such as burgers, sausages, and mince. This broader strategy could involve clearly defining key concepts that are commonly debated, such as the terms "meat," "savoury," and "umami." These terms often reflect subjective opinions and personal tastes, rather than universally agreed-upon markers of quality.

Based on the results of studies conducted, several suggestions have been made to enhance further research and the application of HS-SPME-GCMS for flavour analysis.

Effective use of HS-SPME-GCMS for volatile analysis in complex matrices

The findings from the study, along with the broader literature review referenced, highlight the need for careful consideration when using and applying Solid Phase Microextraction (SPME) for comprehensive untargeted analysis. SPME fibers have a limited capacity for adsorbing analytes, and several factors can influence their effectiveness. Therefore, future studies should focus on reviewing and optimising conditions irrespective of previous research. Although this project utilised a single SPME fiber, employing a variety of fibers may provide a more comprehensive understanding of the analytes present, particularly for untargeted analysis. Existing literature supports this perspective. Additionally, it is crucial to assess the performance of SPME fibers, as their adsorptive qualities can deteriorate due to various factors.

In the context of GC-MS (Gas Chromatography-Mass Spectrometry), careful consideration should be given to column selection, particularly its length, when conducting untargeted analyses. Using a column that is longer than the standard length of 30 meters can help elute analytes more effectively and reduce the risk of overlapping peaks. Additionally, employing Multi-Dimensional Gas Chromatography (MDGC) or a high plate-number column can aid in resolving eluates and peaks, minimising the need to repeat experiments with different columns. The polarity of the columns must also be taken into account, depending on the available

certified reference materials and the reference mass spectrometer library. A non-polar column is preferable if the NIST webbook database is being used as a reference for retention indices.

Incorporating sensory analytical techniques, such as olfactometry or electronic noses (E-noses), alongside gas chromatography would yield valuable information in addition to chemometric analysis. Relying on a single GC-MS program for all studies could lead to erroneous results; therefore, it is essential to use different GC-MS programs tailored to the specific matrix being analysed, as this may provide critical insights.

Further research is necessary, including the reapplication of the current study with ingredients such as yeast extracts, mushrooms, and other commonly used flavour enhancers. This could offer deeper insights into the pathways involved in aroma formation. The use of omics technologies might also enhance our understanding of volatile compound formation. With sufficiently large datasets, we may be able to decipher the simultaneous effects of oxidation, degradation, and interactions with Maillard reaction products (referred to as the SODIM effect).

Comprehensive untargeted volatile analysis of complex media

Fractionating beef mince allowed for the observation of different volatile compounds that were not detected in whole beef mince. However, weighing and cooking the fractions in the vial presented challenges, particularly due to moisture control issues resulting from pressure buildup and the subsequent loss of volatiles. For future studies, using equipment that can capture volatiles online to minimize their loss would be beneficial. Additionally, complementary studies employing HPLC and FTIR techniques to examine the effects of cooking on non-volatile components could provide valuable insights.

Simultaneous oxidation, degradation and interactions with Maillard reaction products (SODIM)

Lipid oxidation products are strong odorants and are often associated with undesirable flavours. Some of these products interact with Maillard reaction products, leading to the formation of aromatic compounds. Although these reactions occur in a rate-limiting and stepwise manner, it is important to note that the process is dynamic and happens simultaneously. For instance, while Maillard reaction products can act as antioxidants and limit lipid peroxidation, lipid oxidation is an ongoing process and can vary in concentration depending on the sample composition and environment. This process is accelerated during high-temperature heat treatment.

Future studies should systematically utilise data from untargeted analyses to create neural mappings that can enhance our understanding of the formation and pathways of volatile compounds. Furthermore, studies could be expanded to include flavour enhancers such as yeast extracts. The results would provide valuable information on the aroma profile comparability between beef and plant-based meats.