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PhD THESIS

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QUANTIFYING MEAT SPOILAGE WITH AN ARRAY OF BIOCHEMICAL INDICATORS

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ABSTRACT

Freshness and safety of muscle foods are generally considered as the most important parameters for the food industry. It is crucial to validate and establish new rapid methods for the accurate detection of microbial spoilage of meats. In the current thesis, the microbial association of meat was monitored in parallel with the chemical changes, pH measurements and sensory analysis. Several chemical analytical techniques were applied to explore their dynamics on quantifying spoilage indicators and evaluate the shelf life of meat products. The applied analytical methods used were Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, image analysis, high performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC/MS).

The first component of the study was designed to evaluate the potential of FTIR spectroscopy as a rapid, reagent-less and non-destructive analytical technique in estimating the freshness and shelf life of beef. For this reason, minced beef samples (survey from the Greek market), beef fillet samples stored aerobically (0, 5, 10, 15 and 20ºC) and minced beef samples stored aerobically, under modified atmosphere packaging (MAP) and active packaging (0, 5, 10, and 15ºC), were analysed with FTIR. The statistical analysis from the survey revealed that the impact of the market type, the packaging type, the day and the season of purchase had a significant effect on the microbial association of mince. Furthermore, the Principal Components Analysis (PCA) and Factorial Discriminant Analysis (FDA), applied to the FTIR spectral data, showed discrimination of the samples based on freshness, packaging type, the day and season of purchase. The validated overall classification accuracies (VCA) were 61.7% for the freshness, 79.2% for the packaging 80.5% for the season and 61.7% for the day of purchase. The shelf life of beef fillets and minced beef was evaluated and correlated with FTIR spectral data. This analysis revealed discrimination of the samples regarding their freshness (VCA 81.6% for the fillets, 76.34% for the mince), their storage temperature (VCA 55.3% and 88.1% for the fillets and mince, respectively) and the packaging type (VCA 92.5% for the mince). Moreover, estimations of the different microbial populations using Partial Least Squares Regression (PLS-R) were demonstrated (e.g. Total viable counts-TVC: RMSE 1.34 for the beef fillets and 0.72 for the mince).

Subsequently, Raman spectroscopy was examined as a rapid, non-destructive analytical technique and compared with FTIR spectroscopy. The spectroscopic, microbiological and sensory analysis data obtained from minced beef samples during storage in aerobic and MAP conditions at 5 °C were correlated using different machine learning methods. These methods included PLS regression and support vector machines regression (SVR) using linear and non-linear functions. Both the FTIR and Raman models were capable of estimating the microbial counts and the sensory scores of the tested minced beef samples, whereas the FTIR models performed slightly better in comparison with the Raman models. For FTIR models, PLS-R, SVR_L (linear function) and SVR_P (polynomial function) gave better estimations (TVC RMSE 0.55, 0.50 and 0.51, respectively). For the Raman models, SVR_R (radial basis function) and SVR_P gave the better estimations (TVC RMSE 0.56 and 0.57, respectively). For the classification models, slightly better performance was observed for the SVR_R (VCA 87.50%) and SVR_P (VCA 87.50%) models regarding the FTIR data and for the Raman data, the SVR_P (VCA 84.62%) models performed slightly better.

VideometerLab (image analysis), a rapid and non-destructive technique, was also used to analyse samples obtained from minced beef during storage in aerobic and MAP conditions at 5 °C. The data derived were correlated with microbiological and sensory analysis data. Image analysis gave useful information about the changes on the surface of the meat, whereas the PLS-R models built using the spectra collected, provided sufficient estimations about the microbial load (TVC RMSE 0.48) and the sensorial status of the meat sample (VCA 86.5%).

Finally, two further studies were conducted using rapid analytical methods such as HPLC analysis of organic acids and GC/MS to monitor the changes in organic acids and volatile compounds, respectively, that are present in the meat substrate during storage. Minced beef samples stored under aerobic, MAP and active packaging at 0, 5, 10, and 15ºC were monitored with both analytical methods, and several compounds found to be possible chemical indicators. Interesting information about the evolution of spoilage under different temperature and packaging conditions was demonstrated. Moreover, the applied PLS-R models gave estimations of the different microbial populations (e.g. TVC: RMSE 0.88 for the HPLC and 0.81 for the GC/MS models) and the FDA facilitated the discrimination of the samples regarding their freshness (VCA 88.8% for HPLC and 79.2% for GC/MS).

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List of Abbreviations

A_f	accuracy factor
ANOVA	analysis of variance
ATR	attenuated total reflectance
B	butcher shops
B_f	bias factor
ESO	ephemeral spoilage organisms
GC/MS	gas chromatography/mass spectroscopy
AHC	hierarchical agglomerative clustering
HPLC	high performance liquid chromatography
HS/SPME	headspace/solid phase micro-extraction
IS	internal standard
F	fresh
FDA	factorial discriminant analysis
FTIR	Fourier transform infrared spectroscopy
LAB	lactic acid bacteria
lag	lag phase
μ_{max}	maximum specific growth rate
MAP	modified atmosphere packaging
MAP/OEO	MAP volatile compounds of Oregano Essential oil/ active packaging
PLS-R	partial least squares regression
PC	principal component
PCA	principal components analysis
$\mathbf{R^2}$	square of the correlation coefficient
% RE	percent relative error
RMSE	root mean square error
RT	retention time
S	spoiled
SD	standard Deviations
SF	semi-fresh
SM	supermarkets
SPME	solid phase micro-extraction
SVR	support vector machines regression
$\mathbf{SVR_L}$	SVR using linear function
$\mathbf{SVR_P}$	SVR using polynomial function
$\mathbf{SVR_R}$	SVR using radial basis function
$\mathbf{SVR_S}$	SVR using sigmoid function
TVC	total viable counts
VCA	validated overall classification accuracies
VP	vacuum packaging

Chapter 1

Literature Review and Objectives

Introduction

Spoilage of meat can be considered as an ecological phenomenon that encompasses changes in the available components (e.g., low molecular weight compounds) during proliferation of bacteria present in the microbial association of stored meat. A general feature of microbial spoilage is its relatively sudden onset, it does not appear to develop gradually, but more often as an unexpected and unpleasant revelation. This is a reflection of the exponential nature of microbial growth and resulting in microbial metabolism also proceeding at an exponentially increasing rate. If a microbial product associated with spoilage, e.g., an off-odour, has a certain detection threshold, the level will be well below this threshold for most of the product's acceptable shelf-life.

1.1. The ecology of the spoilage microbiota

1.1.1. Factors determining microbial spoilage of foods

It is well established that in any food ecosystem there are five key ecological components which determine the impact on quality (e.g., intrinsic, processing, extrinsic, implicit, and the emergent effects) (Nychas and Skandamis, 2005). Therefore the overall effect of a combination of parameters is generally much higher than the perceived effect of each individual one.

The most important intrinsic factors are water activity, acidity, redox potential, available nutrients and natural antimicrobial substances. Extrinsic parameters are factors in the environment in which a food is stored, notably temperature, humidity and atmosphere composition. Physical or chemical treatments often result in changes

in the characteristics of a food product, determining the microbiota associated with the product. Implicit parameters are mutual influences, synergistic or antagonistic, among the primary selection of organisms resulting from the influence of the above mentioned parameters. Thus, implicit parameters are the result of the development of a microorganism which may have a synergistic or antagonistic effect on the microbial activity of other microbial communities present in the food product (Huis in't Veld, 1996). Often, these individual factors interact to produce an additive or synergistic effect on final meat quality (Nychas and Skandamis, 2005).

1.1.2. Microbial association under different storage conditions

Understanding how different properties of a food, its environment and its history can influence the microbiota that develops in storage is an important first step towards being able to make estimations concerning shelf-life, spoilage and safety. The food industry is continually creating new microbial habitats, either by developing new products and reformulating traditional ones, or by chance, as a result of the composition of raw materials or in production. The microbiological quality of meat depends on the physiological status of the animal at slaughter, the spread of contamination during slaughter and processing, the temperature and other conditions of storage and distribution (Nychas *et al*, 2008). These ecological determinants influence the establishment of a particular microbial association and determine the rate of attainment of a maximum populations known as the 'Ephemeral (specific) spoilage micro-organisms- E(S)SO (i.e., those which fill the niche by adopting ecological strategies) (Nychas and Skandamis, 2005). *Pseudomonas* sp., *Brochothrix thermosphacta*, lactic acid bacteria and *Shewanella putrefaciens* are considered to be the main spoilage bacteria of low and high pH raw meat, stored in chill temperature

aerobically or vacuum/map conditions (Garcia-Lopez *et al.*, 1998; Stanbridge and Davis, 1998). It needs to be noted that the final composition of the microbiota eventually characterizes the type of spoilage (Nychas *et al.*, 1998).

Chill storage

Temperature seems to be the most important factor that influences spoilage as well as the safety of meat (Mossel, 1971; McMeekin, 1982; Lambert *et al*., 1991; McDonald and Sun, 1999). Chill storage can change both the nature of spoilage and the rate at which it occurs. There may be qualitative changes in spoilage characteristics as low temperatures exert a selective effect, preventing the growth of mesophiles and leading to the microbiota being dominated by psychrotrophs. Although psychrotrophs can grow in chilled foods they do so relatively slowly so that the onset of spoilage is delayed. In this respect temperature changes within the chill temperature range can have pronounced effects.

There seems to be no taxonomic restriction of psychrotrophic organisms, resulting in yeasts, moulds, Gram-negative and Gram-positive bacteria being found. One feature they all share is that in addition to their ability to grow at low temperatures, they are inactivated at moderate temperatures. Although mesophiles cannot grow at chill temperatures, they are not necessarily killed. Chilling will produce a phenomenon but its effects are not predictable in the same way as heat processing. The extent of cold shock depends on a number of factors such as the type of organism (Gram positives appear more susceptible than Gram negatives), its phase of growth (exponential-phase cells are more susceptible than stationary phase cells), the temperature differential and the rate of cooling (in both cases the larger it is, the greater the damage), and the

growth medium (cells grown in complex media are more resistant) (Adams and Moss, 1995). Yeasts do not outgrow bacteria on muscle products unless a bacteriostatic agent is included in specific products (e.g., British fresh sausages) or the product is stored for extended periods in cold and dry environments (Nychas *et al.*, 2007).

Storage under aerobic conditions

A consortium of bacteria, commonly dominated by *Pseudomonas* spp, are usually responsible for spoilage (-1 – +25ºC) providing the atmosphere is moist. Odour and slime production causes spoilage in 10d at 0 and 5d at 5 ºC (Hood and Mead, 1993). The type of muscle spoilage under aerobic conditions is characterized by putrefaction and is related to proteolytic activity and off odour production by gram-negative bacteria that dominate (Nychas *et al.*, 1998). The microbial associations developing on muscle tissues stored aerobically at cold temperatures are characterized by an oxidative metabolism. The gram-negative bacteria that spoil meat are either aerobes or facultative anaerobes. Pseudomonads tend to dominate the microbial consortium in aerobically stored meats, especially *Ps. fragi*, *Ps. fluorescence*, *Ps. putida*, and *Ps. ludensis*. *Brochothrix thermosphacta* and cold-tolerant *Enterobacteriaceae* (e.g. *Hafnia alvei*, *Serratia liquefaciens* and *Enterobacter agglomerans*), which may also occur on chilled muscle foods stored aerobically (Borch *et al.*, 1996; Nychas *et al.*, 1998). However, in terms of population numbers, they do not contribute to the microbial associations (Nychas *et al.*, 2008). Lactic acid bacteria (LAB), although they have been detected in aerobically spoiled chilled meat, are not considered to be important in spoilage except possibly for lamb (Drosinos, 1994; Holzapfel, 1998).

Storage under Vacuum or MAP

A combination of gases, commonly a mixture of oxygen, carbon dioxide and nitrogen, is normally used in modified atmosphere packaged (MAP) meats. Shelf life in MAP is determined by the choice of atmosphere, storage temperature and the meat type. Vacuum (VP) and modified atmosphere packaging of meat changes the meat microbiota and sequentially the time course and character of spoilage. As the numbers of bacteria (particularly pseudomonads) are restricted by the high relative concentration of carbon dioxide, the spoilage of meat stored under MAP occurs later than that stored aerobically. Gram positives, particularly homo- and heterofermentative LAB (e.g., *Lactobacillus, Leuconostoc, Lactococcus* and *Carnobacterium* spp.) typically develop on meat under enriched carbon dioxide atmospheres. Both lactic acid bacteria and *Br. thermosphacta* are the main, if not the most important, causes of spoilage characterized by muscle souring (Adams and Moss, 1995; Davies and Board, 1998).

1.2. Meat composition and chemical changes in the meat substrate

The approximate chemical composition of typical adult mammalian muscle after rigor mortis is presented on Table 1.1. Its high water activity and abundant nutrients make meat an excellent medium to support microbial growth, since changes that occur during spoilage take place in the aqueous phase of the meat. These substrates are catabolized by almost all bacteria in muscle food microbiota (Drosinos and Board, 1995; Gill, 1976; Nychas *et al.*, 2007).

Although many of the microorganisms that grow on meat are proteolytic, they grow initially at the expense of the most readily utilized substrates-the water soluble pool of carbohydrates and non-protein nitrogen (Adams and Moss, 1995). The order in which these compounds are catabolized by the major meat spoilage organisms is summarized in Table 1.2.

Table 1.1. Chemical composition of typical mammalian muscle after rigor mortis [a]

Component	**% weight**
Water	75.0
Protein	19.0
Myofibrillar	11.5
Sarcoplasmic	5.5
Connective tissue and organelle	2.0
Lipid	2.5
Carbohydrate and lactic acid	1.2
Lactic acid	0.90
Glucose-6-phosphate	0.15
Glycogen	0.10
Glucose and glycolytic intermediates	0.05
Miscellaneous soluble nonprotein substances	2.3
Nitrogenous	1.65
Creatinine	0.55
Inosine	0.30
Monophosphate ATP, AMP	0.10
Amino acids	0.35
Carnosine, anserine	0.35
Inorganic	0.65
Total soluble phosphorus	0.20
Potassium	0.35
Sodium	0.05
Magnesium	0.02
Other metals	0.23
Vitamins	Traces

[a] Modified from Adams and Moss (1995) and Nychas *et al.* (2007)

Table 1.2 Order of substrate utilization during growth of major muscle spoilage bacteria [a]

	Aerobic					Anaerobic [b]				
Substrate	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
Glucose	1	1	1	1	1	1	1	1	1	1
Glucose-6-phosphate	2			2					2	
Lactate	3	2		3						
Pyruvate	4	3				2				
Gluconate	5	4				2				
Gluconate-6-	6									
Propionate		5								
Formate							1			
Ethanol		6								
Acetate		7				2				
Amino acids	7	8	2	4		2	1		3	2
Serine and Cysteine							1			
Creatinine	8									
Citrate	9									
Aspartate	10									
Glutamate	11									
Ribose	12		3			2				
Glycerol			4							
Lipids										

[a] Adapted from Nychas et al. (2007). In the order, 1 is first, 12 is last. A, *Pseudomonas* spp.; B, *Shewanella putrefaciens*; C, *Br. thermosphacta*; D, *Enterobacter* spp.; E, lactic acid bacteria.
[b] Under oxygen limitation and/or CO_2 inhibition.

The concentration of glucose, lactic acid, and certain amino acids followed by nucleotides, urea and water-soluble proteins can affect the type (e.g., saccharolytic, proteolytic), the rate of spoilage and, moreover, seems to be the principal precursor(s) of those microbial metabolite(s) that we perceive as spoilage (Nychas *et al.*, 1998; Nychas *et al*, 2008). Extensive proteolysis only occurs in the later stages of decomposition when the meat is usually already well spoiled from a sensory point of view. (Adams and Moss, 1995).

Glucose is the preferred energy substrate and the first to be used by various microorganisms growing on muscle foods. Its limitation can cause a switch from a

saccharolytic to an amino acid-degrading metabolism in at least some bacterial species (Borch *et al.*, 1991). Lactate is almost exclusively the second energy source utilized by the microbial association of muscle under aerobic and anaerobic conditions. The general conclusion is that the lactate concentration decreased following glucose utilization in muscle samples stored aerobically, in vacuum packages, or under other MAP conditions. Amino acids in meat are the third main energy pool for bacteria in muscle foods. Storage of beef, pork, poultry skin and fillets has revealed that the sum of the free amino acid and water-soluble protein content increases during storage, which is associated in colony counts (Nychas *et al*, 2008).

1.2.1. Substrate conversion to spoilage compounds

It is known that endogenous enzymatic activity within muscle tissue post-mortem can contribute to changes during storage (Koohmaraie, 1994; Jackson *et al.*, 1997; Alomirah *et al*, 1998). The post-mortem glycolysis, caused by indigenous enzymes, ceases after the death of the animal. Glycogen is a polymer of glucose held in the liver and muscles as an energy store of the body. During life, oxygen is supplied in muscle cells in the animal by the circulatory pathways to yield carbon dioxide and water. After death the supply of oxygen to the muscles is cut off, the redox potential falls and respiration ceases, but the glycolytic breakdown of glycogen continue until the glycolytic enzymes are inactivated by the low pH developed. In a typical mammalian muscle the pH will drop from an initial value of 7 to 5.4-5.5 with the accumulation of about 1% lactic acid (Adams and Moss, 1995).

Subsequently, the contribution of meat indigenous enzymes in its spoilage is negligible compared to the microbial action of the microbiota (Nychas and Tassou, 1997; Tsigarida and Nychas, 2001). However, it should be clarified that, it is the microbial activity (growth) per se, rather than the activity of microbial enzymes and as a consequence, it is the accumulation of metabolic by-products that characterizes food spoilage (Nychas *et al.,* 2008).

The production of metabolic compounds that can eventually spoil muscle tissues is the outcome of substrate interactions with the developed microbial association and with the indigenous muscle enzymes as well as of nonenzymatic chemical reactions and physical changes.

Chemical changes under aerobic conditions

Under aerobic conditions none of the bacteria are known to cease growth because of substrate exhaustion at the muscle surface; oxygen availability, however, has been suggested to be a limiting factor. The pseudomonads predominate because of their higher growth rates and greater affinity for oxygen, over the other muscle spoilage bacteria (Gill and Newton, 1977). The sequential catabolism of D-glucose and L- and D-lactic with D-glucose used preferentially to lactate, while the oxidation of glucose and glucose 6-phosphate via the extracellular pathway caused a transient accumulation of D-gluconate and an increase in the concentration of 6-phosphogluconate (Nychas *et al,* 2008).

Odours of by-products such as sulfides and methyl esters are usually the first manifestation of spoilage of chilled meat stored under aerobic conditions (Dainty *et*

al., 1985a, 1989a; Dainty *et al,* 1989b); the compounds involved are mostly products of amino acids. *Pseudomonas,* particularly *Ps. fragi*, are the major and possibly the sole producer of ethyl esters in aerobically stored meat (Dainty *et al.,* 1985a; Edwards *et al.,* 1987, McMeekin, 1975). Concentrations of four of the volatile compounds, acetone, methyl ethyl ketone, dimethyl sulfide, increase continuously during aerobic storage of ground beef at 5, 10, or 20 ºC (Stutz *et al,* 1991). Hydrogen sulfide, another potential indicator of spoilage combines with the muscle pigment to give a green discoloration. Puterscine, cadaverine, histamine, tyramine, spermine and spermidine were found to be present in ground pork, beef, poultry, and fish stored either aerobically or in vacuum packages (Nychas *et al.,* 2007).

Br. thermosphacta has much greater spoilage potential than lactobacilli and can be important in both aerobic and anaerobic spoilage of muscle foods. This bacterium utilizes glucose and glutamate but no other amino acid during aerobic incubation (Gill and Newton, 1977), and produces a mixture of end products. The assimilation of glucose and production of formic and acetic acids in a model system (gel cassette) are affected by the presence of other spoilage bacteria (e.g., pseudomonads and *Shewanelle* spp.) (Tsigarida and Nychas, 2001)

In general, Gram positive bacteria, especially the LAB are not important contaminants of muscle foods stored under aerobic conditions. *Br. thermosphacta* may have some importance in the spoilage of pork, lamp and fish, particularly on fatty surfaces (Nychas *et al.,* 2007).

Chemical Changes under oxygen limitation or anoxic conditions

Under vacuum and in MAP, the putrid odours associated with storage in air are replaced by relatively inoffensive sour/acid odours. Such odours have been assumed to arise from the acidic end products of glucose fermentation, which is the primary source of energy for microbial growth (Gill, 1983; Gill and Newton, 1977). However, the production of such off odours is difficult to explain in terms of accumulation of acetic, isobutanoic, L-isopentanol, and D-lactic acids because the amounts are relatively small compared to the amount of the endogenous L-lactic acid of normal pH muscle. The dairy/cheesy odours found in beef stored in gas mixtures with CO_2 were produced by *Br. thermosphacta* and lactic acid bacteria, both of which can produce diacetyl or acetoin and alcohols (Nychas *et al.,* 2007).

Members of the family *Enterobacteriaceae* produce ammonia and volatile sulfides, including hydrogen sulfide and malodorous amines, from amino acid metabolism. Indeed, it was reported that under oxygen-limiting conditions, although the order of substrate utilization remains the same with the exception of glucose and lactate, this group can use alternative carbon sources (Gill and Newton, 1979; Newton and Gill, 1980; Tsigarida and Nychas, 2001). The production of tyramine, putrescine and cadaverine has also been attributed to lactic acid bacteria in meat stored under vacuum or MAP conditions (Dainty *et al.,* 1985).

Since Gram negative bacteria such as pseudomonads are inhibited in reduced-oxygen environments, the spoilage of muscle foods stored under oxygen environments, spoilage under oxygen limitation and carbon dioxide-enriched atmospheres is due to undefined actions of lactic acid bacteria and/or *Br. thermosphacta*, (Nychas *et al.*,

1998; Tsigarida and Nychas, 2001). However, the presence of sulfur compounds, such as propyl esters and 3-methylbutanol compounds, as well as the production of formic and acetic acids lead again to the crucial question as to whether this inhibition is due to carbon dioxide enrichment or oxygen limitation.

Homofermentative or heterofermentative types of metabolism and their ecological determinants are of importance. Different LAB produce L or D forms of lactate depending on the presence of D-nLDH and/or L-nLDH (specific NAD+- dependent lactate dehydrogenases). The increase of acetate in beef, pork and fish stored under different vacuum or MAP conditions could be also attributed to a shift from homo- to heterofermentative metabolism of lactic acid bacteria. Finally, alcohols, particularly ethanol and propanol, appear to be the most promising compounds as indicators of spoilage in meat and meat products stored under vacuum or MAP conditions (Nychas *et al.*, 2007).

Tables 1.3 to 1.6 present the end-products with their factors and precursors, produced under different storage conditions by common spoilage bacteria of meat.

Table 1.3 End-products of Gram-negative bacteria (e.g., *Pseudomonas* spp., *Shewanella putrefaciens*, *Moraxella spp*, *Serratia spp*) inoculated in broth, a sterile model system, and naturally spoiled muscle and factors and precursors affecting their production [a].

End-product	Factors	Precursors
Sulfur compounds		
Sulfides	Temperature	Cysteine, cystine, methionine
Dimethylsulfide	and substrate	Methanethiol, methionine
Dimethyldisulfite	(glucose)	Methionine
Methyl mercaptan	limitation	nad
Methanethiol		Methionine
Dimethyltrisulfide	nad [b]	Methionine, methanothiol
Carbon disulfide	nad	nad
1-Propanthiol	nad	nad
2-Pentyl thiophene	nad	nad
Methylethylsulfide	nad	nad
2-Methyl undecanthiol		
Esters		
Methyl esters (acetate)	Glucose (l)[c]	nad
Ethyl esters (acetate)	Glucose (l)	nad
Ethyl hexanoate	nad	nad
Ethyl octanoate	nad	nad
Ethyl nonanoate	nad	nad
Ethyl decanoate	nad	nad
2-Hexen-1-ol propanoate	nad	nad
Isoamyl acetate	nad	nad
2-Ethylhexyl-2-ethyl	nad	nad
hexanoate	nad	nad
Hexyl formiate	nad	nad
Ketones		
Acetone	nad	nad
2-butanone	nad	nad
Acetoin/diacetyl [c]	nad	nad
3-Octanone	nad	nad
5-Methyl-4-hepten-3-one	nad	nad
2-Octen-2-one	nad	nad
2,5-Octandione	nad	nad
2-Methyl-3-decen-5-one	nad	nad
3-Decen-2-one	nad	nad
2-Methyl-3-decen-5-one	nad	nad

Table 1.3 *(continued)*

End-product	Factors	Precursors
Hydrocarbons		
Hexane	nad	nad
2,4 dimethylhexane	nad	nad
and methylheptone	nad	nad
9-Methyl-2-decene	nad	nad
4-Dodecene	nad	nad
5-Butyl-4-nonene	nad	nad
2-Methyl-2-decene	nad	nad
2-Methyl-2-dodecene	nad	nad
5-Methyl undecene	nad	nad
3-Tetradecene	nad	nad
Undecene	nad	nad
Dodecene	nad	nad
1-Butene	nad	nad
2,4,4-Trimethyl-1-pentene	nad	nad
2-Decene	nad	nad
Aromatic Hydrocarbons		
Diethylbenzene	nad	nad
Trimethylbenzene	nad	nad
p-Dimethylbenzene	nad	nad
o-Dimethylbenzene	nad	nad
1-Methyl-2,1-	nad	nad
methylethylbenzene	nad	nad
Toluene	nad	nad
Aldehydes		
2-methylbutanal	nad	iso-leucine
Hexanal	nad	nad
4-Idroxy-3-methylbutanal	nad	nad
Nonanal	nad	nad
Decanal	nad	nad
2-Butyl octenal	nad	nad
10-Undecenal	nad	nad
Tridecanal	nad	nad
Alcohols		
Methanol	nad	nad
Ethanol	nad	nad
2-methylpropanol	nad	Valine
2-methylbutanol	nad	iso-leucine
3-Methyl-1-butanol	nad	Leucine
2-Hexen-1-ol	nad	nad
1-Octen-3-ol	nad	nad
2-Octen-1-ol	nad	nad
2-Nonen-1-ol	nad	nad
2-Hexyl-1-decanol	nad	nad
2-Ethyldodecanol	nad	nad
2-Butyl-1-octanol	nad	nad
1-Dodecanol	nad	nad
1,9-Nonandiol	nad	nad
6-Dodecenol	nad	nad
Hexadecandiol	nad	nad
2-Pentadecanol	nad	nad
2-Methyl-1-decanol	nad	nad
2-Methyl-3-buten-1-ol	nad	nad
1-Nonanol	nad	nad

Table 1.3 *(continued)*

End-product	Factors	Precursors
1-Undecanol	nad	nad
1,2-Dodecandiol	nad	nad
5-Octen-2-ol	nad	nad
2-Ethyl-1,3-hexandiol	nad	nad
2-Ethyl-1-decanol	nad	nad
Terpene compounds		
Caryophyllene	nad	nad
Citronellyl acetate	nad	nad
Limonene	nad	nad
Linalool	nad	nad
Menthol	nad	nad
α-Terpineol	nad	nad
Phenol compounds		
4-Methylguaiacol	nad	nad
Butylhydroxytoluene	nad	nad
4-Methoxybenzhydrol	nad	nad
4(1,1,3,3)-Tetramethylbutyl)phenol	nad	nad
Other compounds		
Ammonia	Glucose (l)	Amino acids
Trimethylamine	nad	Trimethylamine oxide
Dibutylphtalate	nad	nad

[a] Modified from Nychas *et al.,* 2007, Ercolini *et al.,* 2009

[b] nad = no available data, [c] (l) low concentration of glucose

Table 1.4 End-products of lactic acid bacteria (*Lactobacillus* sp., *Leuconostoc* sp., *Carnobacterium* sp.) inoculated in broth, a sterile model system, and naturally spoiled muscle and factors and precursors affecting their production [a].

End-product	Factors	Precursors
Organic acids		
L-lactic acid	nad[b]	Glucose
D-lactic acid	nad	Glucose
Acetic acid	Glucose (l), O_2 (h), E	Glucose, lactate, pyruvate
Tetradecenoic acid	nad	nad
Formic acid	nad	Glucose, acetic acid
Sulfur compounds		
Carbon disulfide	nad	nad
1-Propanthiol	nad	nad
2-Pentyl thiophene	nad	nad
Methylethylsulfide	nad	nad
4-Methylthiophenol	nad	nad
Undecanthiol	nad	nad
Methoxybenzenthiol	nad	nad

Table 1.4 *(continued)*

End-product	Factors	Precursors
Esters		
Ethyl hexanoate	nad	nad
Ethyl octanoate	nad	nad
2-Tetradecylmethoxyacetate	nad	nad
9,12-Tetradecadien-1-ol acetate	nad	nad
Terbutyl cyclohexyl acetate	nad	nad
Ethenyl decanoate	nad	nad
Ketones		
2-Nonanone	nad	nad
Acetoin	pH (l), glucose (h)	Pyruvate
Diacetyl	pH (l), glucose (h)	Pyruvate
Hydrocarbons		
9-Methyl-2-decene	nad	nad
5-Butyl-4-nonene	nad	nad
2-Methyl-2-decene	nad	nad
2-Methyl-2-dodecene	nad	nad
Decane	nad	nad
2-Dodecene	nad	nad
1-Tetradecene	nad	nad
3-Tridecene	nad	nad
5-Undecene	nad	nad
8-Methyl-1-undecene	nad	nad
5-Octadecene	nad	nad
9-Octadecene	nad	nad
Aromatic Hydrocarbons		
Toluene	nad	nad
p-Dimethylbenzene	nad	nad
o-Dimethylbenzene	nad	nad
1-Methyl-2,1-methylethylbenzene	nad	nad
Aldehydes		
Hexanal	nad	iso-leucine
4-Idroxy-3-methylbutanal	nad	nad
Nonanal	nad	nad
Decanal	nad	nad
2-Ethyl hexanal	nad	nad
Tetradecanal	nad	nad
2-Ethenylbutenal	nad	nad
7-Hexadecenal	nad	nad
Alchohols		
Ethanol	nad	Glucose (aerobic storage)
1-Octen-3-ol	nad	nad
2-Octen-1-ol	nad	nad
2-Hexyl-1-octanol	nad	nad
2-Methyl-1-dodecanol	nad	nad
2-Hexyl-1-decanol	nad	nad
2-Ethyldodecanol	nad	nad
2-Butyl-1-octanol	nad	nad
1-Dodecanol	nad	nad
4-Methyl-1-dodecen-3-ol	nad	nad
2-Methyl-1-undecanol	nad	nad
ter-Butylcyclohexanol	nad	nad

Table 1.4 *(continued)*

End-product	Factors	Precursors
Isotridecanol	nad	nad
2-Buten-1-ol	nad	nad
2-Ethyl-1-hexanol	nad	nad
5-Methyl-1,5-hexadien-3-ol	nad	nad
1-Undecanol	nad	nad
Hexadecanol	nad	nad
Terpene compounds		
Caryophyllene	nad	nad
Citronellyl acetate	nad	nad
Limonene	nad	nad
Linalool	nad	nad
Linalyl propanoate	nad	nad
Menthol	nad	nad
Borneol	nad	nad
α-Terpineol	nad	nad
Isobornyl acetate	nad	nad
Limonene oxide	nad	nad
Phenol compounds		
4-Methylguaiacol	nad	nad
Butylhydroxytoluene	nad	nad
4-Methoxybenzhydrol	nad	nad
Other compounds		
δ-Nonalactone	nad	nad
Dibutylphtalate	nad	nad
Hydrogen peroxide	nad	nad

[a] Modified from Nychas *et al.,* 2007, Ercolini *et al.,* 2009

[b] nad = no available data; (h), high oxygen; (l), low concentration of glucose; E, appropriate enzymes - (iLDH, NADH peroxidase, lactate, or pyruvate oxidase

Table 1.5 End products formed by *Brochothrix thermosphacta* in naturally spoiled meat or in model muscle systems (e.g., broth or gel cassette). Factors and precursors of these end products are also presented [a]

End-product	M/P	Factors	Precursors
Aerobically			
Acetoin	+	Glucose (h), pH (h/l), T (h/l)	Glucose (mj), alanine (mn), diacetyl
Acetic acid	+	Glucose (h), pH (h/l), T(h/l)	Glucose (mj), alanine (mn)
L-Lactic acid [np]	(np)	T (h), pH (h), O_2 (l)	Glucose
Formic acid	+	T(h), pH(h),	Glucose
Ethanol	+	T(h), glucose	nad
CO_2	+	nad	Glucose
iso-Butyric acid	+	Glucose (l), T (l), pH (h)	Valine, leucine
iso-Yaleric acid	+	Glucose (l), T (l), pH (h)	Valine, leucine
2- Methylbutyric	+	Glucose (l), pH (h)	iso-leucine
3- Methylbutanol	+	Glucose (h), pH (l)	nad
2,3- Butanediol	+	Glucose (h), T (h/l)	Diacetyl
Diacetyl	+	nad	nad
2-Methylpropanol	+	Glucose (h)	Valine
In different gaseous atmospheres			
L-Lactic acid	+	Glucose (h), pH (h),T(ns)	Glucose
Acetic acid	+	O_2 (h), glucose (l)	Glucose
Ethanol	+	T (h), pH (h)	nad
Formic	+	T (h), pH(h)	nad

[a] Modified from Nychas *et al.*, 2007

(h), high pH, concentration of glucose, or storage temperature; (l), low pH, concentration of glucose or storage temperature; (h/l), contradictory results; (ns), not significant factor; (mj), major contribution; (mn), minor contribution; (np), no production under strictly aerobic conditions; nd, not-determined; nad, no available data; T, temperature.

Table 1.6 Production of biogenic amines by muscle microbiota in muscle foods and broths [a]

		Storage condition		Factors
Biogenic amine	Bacteria	T(°C)	Medium/ pack	
Putrescine	*Hafnia alvei, Serratia liqúefaciens, Shewanella putrefaciens*	1 1	VP[b]	pH, ornithine (arginine) utilization
Cadaverine	*H. alvei, S. liquefaciens, S. putrefaciens*	1 1	VP	pH, lysine utilization
Histamine	*Proteus morganii, Kebsiella. pneumoniae, H. alvei, Aeromonas hydrophila, S. putrefaciens*			Temperature, pH, histidine utilization
Spermine				pH, spermidine
Spermidine				pH, agmatine, arginine
Tyramine	*Lactobacillus* sp., *L. carnis, L. divergens, Enterococcus feacalis*	1 20	VP Air [c]	pH
Tryptamine				pH

[a] Modified from Nychas *et al.*, 2007

[b] Vacuum pack, [c] Aerobic storage.

1.2.2. Detection of spoilage compounds

The correlation between microbial growth and chemical changes during spoilage has been continuously recognised as a means of revealing indicators that may be useful for quantifying muscle tissue quality as well as the degree of spoilage (Nychas *et al.*, 2007). However, the different hurdles imposed (e.g. packaging, temperature, preservatives), as well as intrinsic factors (e.g. pH, glucose concentration) can influence the succession of the components of the microbial association, particularly of the ESO (Nychas *et al.*, 2008, Ercolini *et al.*, 2009). The unpredictable dominance of one or other bacterial species, due to both the above mentioned extrinsic as well as intrinsic factors influence both the quantitative and qualitative production of potential indicators (Nychas and Tassou, 1997; Nychas *et al.*, 2008). This case demonstrates the need for a more holistic approach, where more than one indicator could be used at the same time, in order to minimise the inaccurate spoilage information provided.

Recently some interesting analytical approaches have been put forward for the rapid and quantitative monitoring of meat spoilage. These include biosensors (enzymatic reactor systems), electronic noses (array of sensors), high performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS) and Fourier transform infrared (FT-IR) spectroscopy (Nychas *et al.,* 2007). Some potential indicators that have been investigated with different analytical techniques are summarised in Table 1.7. The development of an "expert system" that can automatically classify the sensorial input into a "diagnosis" based on extracted pre-processing features is necessary before widespread adoption is possible. The application of advanced statistical methods (discriminant function analysis, clustering algorithms, chemometrics) and intelligent methodologies (neural – artificial or not- networks, fuzzy logic, evolutionary algorithms, and genetic programming) may be used for qualitative and quantitative indices in parallel with unsupervised or supervised learning algorithms (Nychas *et al.,* 2007).

Table 1.7. Compounds potentially useful for the assessment of shelf life of raw meat and fish under different packaging conditions [a]

Compound	Test	Packaging conditions [b]
Glucose	Enzymatic kit	Air, VP, MAP
Acetate	Enzymatic kit, HPLC	VP, MAP
Gluconate	Enzymatic kit	Air, VP, MAP
Total lactate	HPLC	VP, MAP
D-Lactate	Enzymatic kit	VP, MAP
Lactic acid	HPLC	Air, MAP, MAP/OEO
Citric acid	HPLC	Air, MAP, MAP/OEO
Formic acid	HPLC	Air, MAP, MAP/OEO
Acetic acid	HPLC	Air, MAP, MAP/OEO
Propionic acid	HPLC	Air, MAP, MAP/OEO
Ethanol	Enzymatic kit, GLC	VP, MAP
Free amino acids	Chromatometric	Air
Ammonia	Enzymatic, colorimetric	Air
Acetone, methyl ethyl ketone, dimethyl sulfide, dimethyldisulfide, hydrogen sulfide	GLC, GC/MS, Sulfur selective detector	VP, MAP
Diacetyl, acetoin	Colorimetric	VP, MAP
Biogenic amines	HPLC, sensors, enzymic test, GLC, Enzyme electrodes, test strips	Air, VP, MAP
Microbial activity	Impedance - capacitance	Air

[a] Modified from Nychas *et al.*, 2007, Skandamis and Nychas 2001,2002, unpublished data from Lab of Food Microbiology and Technology , AUA

[b] VP : vacuum packaged; MAP: modified atmosphere packaged; MAP/OEO :MAP with the presence of the volatile compounds of oregano essential oil.

In this thesis, several quick/rapid analytical techniques were investigated for their potential in evaluating the spoilage of meat such as FTIR and Raman spectroscopy, image analysis (videometer), HPLC and GC/MS analysis.

Fourier transform infrared (FT-IR) spectroscopy has attracted considerable interest since it is a rapid and non-destructive method and has been identified as having considerable potential for applications in food and related industries, with several reports on muscle food analysis (van Kempen, 2001). FT-IR spectroscopy involves

the observation of vibrations of molecules that are excited by an infrared beam, and an infrared absorbance spectrum represents a "fingerprint" which is characteristic of any chemical or biochemical substance. Al-Jowder *et al.* (1997, 1999) reported that FTIR with attenuated total reflectance (FTIR-ATR) has the potential to be established as a technique for meat authentication. Yang and Irudayaraj (2001) demonstrated the dynamics of FTIR-ATR and FTIR-PAS (photoacoustic spectroscopy) techniques for beef and pork quality control through a multi-layer analysis. In addition, according to Karoui *et al.* (2007), FTIR-ATR can be adapted for on-line detection of freeze–thawed fish from fresh fish samples. Finally, the first studies correlating the microbial spoilage of meat with biochemical changes within the meat substrate have been conducted on chicken (Ellis *et al.,* 2002) and beef tissues (Ellis *et al.,* 2004) that were ground to a paste and stored aerobically in ambient temperature. More recent studies of this type correlate the spoilage status of beef stored under different conditions (i.e. temperature and packaging) with the biochemical fingerprints observed with FTIR (Ammor *et al.,* 2009, Argyri *et al.,* 2010).

Raman spectroscopy is a vibrational spectroscopy method that is complementary to absorbance. In general, symmetric vibrations can be thought of as Raman active, whilst asymmetric vibrations are active for absorbance. Typically, an inelastic scattering event (Stokes Raman scattering) is measured, as an incoming photon from a monochromatic light source exchanges energy with a molecule, the resultant scattered photon has a lower energy than that of the incident light, and this energy difference is reflected as a peak on a spectrograph. Raman spectroscopy has gained increasing interest as a versatile and rapid tool for analysis of biological samples. Potential applications have been found in areas ranging from medical diagnostics and tissue or

organism characterization, to analysis of food and agricultural products. Examples of this technique for muscle food analysis, include studies upon the authenticity of poultry species (Ellis *et al.,* 2005), the sensory quality of beef (Beattie, 2004), the structural changes in fish proteins (Herrero *et al,* 2004), the quality screening of fish (Marquardt and Wold, 2004), the changes in pork proteins (Bocker *et al.,* 2007) and the texture of pork muscle (Herrero *et al.,* 2008). Though, no reported studies using Raman spectroscopy seem to have been conducted on the spoilage of muscle foods.

Videometer is a colour and texture measurement vision system that constitutes an image analysis technique. The system is based on a high-intensity integrating sphere illumination featuring light emitting diodes (LED) together with a high-resolution B/W camera. The well-defined and diffuse illumination of the optically closed scene enhances the true colour as well as colour variation of samples. Image processing techniques have been applied increasingly for food quality evaluation in recent years (Du and Sun, 2004). It has been mainly applied for evaluating the quality of muscle tissues, such as separating fat and lean tissues on beef cut surface (Chen *et al.,* 1995), revealing indicators for beef tenderness (Li *et al.,* 2001; *Li et al.,* 1999). Additionally, it has been applied for evaluating pork quality (Lu *et al.,* 1997) and predicting consumer responses to fresh pork colour (Lu *et al.,* 2000) or assessing quality of fried minced beef and diced turkey (Daugaard *et al.,* 2010). However, no studies seem to be reported in the literature regarding the use of image analysis in evaluating the spoilage of muscle foods.

Different microbial metabolites, volatile and non-volatile, have been detected in naturally contaminated samples of meat with GC, GC-MS or HPLC (see section 1.2.1).

Nychas and Tassou (1995) have investigated the profile of water-soluble proteins as detected by HPLC during storage of chicken fillets in air, VP and MAP. Skandamis and Nychas (2001, 2002) have studied the HPLC profile of organic acids of minced beef stored in air and MAP treated or not with oregano essential oil. Tsigarida and Nychas (2001) have investigated in parallel the HPLC profile of organic acids and the profile of the volatile compounds as attributed from GC analysis of sterile beef fillets inoculated with meat spoilage bacteria and stored in air and in MAP. The volatile compounds using GC or GC/MS analysis have also been studied for inoculated and/ or naturally contaminated beef stored in air (Dainty *et al.,* 1985a; Dainty *et al.,* 1989b) and in MAP and/ or VP (Stutz *et al.,* 1991; Jackson *et al.,* 1992; Insauti *et al.,* 2002), for chicken stored in MAP (Eilamo *et al.,* 1998) and for cooked ham stored in MAP (Leroy *et al.,* 2009). Finally, the GC-GC/MS volatiles profile of different types of fresh or processed meat (chicken, beef or pork) has been studied, to assess their quality characteristics, without subsequent storage of the samples (Wettasinghe *et al.,* 2001; Marco *et al.,* 2004; Xie *et al.,* 2008; Rivas-Cañedo *et al.,* 2009). However, knowledge gaps related to HPLC or GC, GC/MS analysis need to be addressed since the above studies do not include combinations regarding numerous compounds, several storage conditions (i.e. temperature and packaging), microbiological and sensory evaluation.

1.3. Objectives of the project

The overall objective in this project was the evaluation of meat spoilage using different analytical techniques. The microbial association of meat was monitored in parallel with its metabolic profile. Several chemical rapid analytical techniques were applied to explore their dynamics on quantifying spoilage indicators and evaluate the

shelf life of the meat products. The applied analytical methods were Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, image analysis, high performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC/MS).

To achieve these aims the following studies have been carried out:

(a) The first study was designed to evaluate the potential of FTIR spectroscopy as a rapid, reagent-less and non-destructive analytical technique in estimating the freshness and the shelf life of beef. For this reason, minced beef samples (survey from the Greek market), beef fillet samples stored aerobically (0, 5, 10, 15 and 20ºC) and minced beef samples stored aerobically, under modified atmosphere packaging (MAP) and active packaging (0, 5, 10, and 15ºC), were analysed with FTIR. The microbiological and sensory analysis data were correlated to the FTIR spectral data using different mathematical approaches such as Principal Components Analysis (PCA), Factorial Discriminant Analysis (FDA) and Partial Least Squares Regression (PLS-R). The aim of these correlations was to achieve estimations of the microbiological and sensory analysis data only by using the FTIR spectral data.

(b) Subsequently, Raman spectroscopy was examined as a rapid, non destructive analytical technique and compared with FTIR spectroscopy. In this study, time series spectroscopic, microbiological and sensory analysis data were obtained from minced beef samples during storage in aerobic and MAP conditions at 5 °C, and analysed using different machine learning methods. These methods included PLS-R and support vector machines regression (SVR) using linear and non-linear functions. These analyses were performed to evaluate Raman

spectroscopy in comparison with FTIR spectroscopy applying different types of calibration models for estimating the microbial counts and sensory scores.

(c) VideometerLab (image analysis), a rapid and non- destructive technique, was also used to analyse samples obtained from minced beef samples during storage in aerobic and MAP conditions at 5 °C. The data derived were correlated with microbiological and sensory analysis data, applying PCA, FDA and PLS-R.

(d) Finally, two further studies were conducted using rapid analytical methods such as HPLC analysis of organic acids and GC/MS to monitor the changes of organic acids and the volatile compounds, respectively, that are present in the meat substrate during storage. Minced beef samples stored under aerobic, MAP and active packaging at 0, 5, 10, and 15ºC were monitored with both of the analytical methods in an attempt to; i) explore the evolution of spoilage under different temperature and packaging conditions through the detection of spoilage indicators, ii) associate the identified detected compounds with the different microbial groups, iii) correlate the HPLC and GC/MS data with the microbial counts and the sensory scores, using Hierarchical Agglomerative Clustering (AHC), PCA, FDA and PLS-R.

Chapter 2

Materials and Methods

2.1. Microbiological analysis

2.1.1. Samples preparation

(a) Survey of Minced Beef from the Greek Market

A total number of 56 samples of minced beef were obtained from supermarkets (31) and butcher shops (25) in Athens (Greece) and transported under refrigeration to the laboratory within 30 min. All samples were cut and weighted at the time of purchase and then put in a plastic bag or rapped in a permeable film.

Samples (25 g) from minced beef were weighed aseptically, added to sterile quarter strength Ringer's solution (225 ml) (LAB M), and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature. The enumeration (see 2.12.) that included counts of Total Viable Counts (TVC), *Pseudomonas* spp., *Brochothrix thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria (LAB) (pH 5.2, 5.7), H_2S-producing bacteria was performed in parallel with pH measurements (see 2.2) and sensory analysis (see 2.3).

The above study was a part of an extensive experiment (in the frames of the EU projects ProSafeBeef and SYMBIOSIS). This experiment included the analysis of 150 samples of minced beef, of which 50 were bought from traditional Butcher shops, cut and weighted at the time of purchase and 100 from supermarkets that included three forms of packaging. From the 100 samples bought from supermarkets 13 were packaged in polystyrene trays wrapped in flexible transparent film (aerobic storage), 24 were packaged in plastic containers with modified atmosphere (MAP) and 63 were cut and weighed at the time of purchase. These 150 samples were further analysed

with FTIR (see Section 2.5) to exploit the dynamics of the method in estimating the quality of samples coming from different origin. Further analysis of these samples was conducted using Raman (see Section 2.6) and VideometerLab (see Section 2.7).

(b) Shelf life Beef fillets stored in air at 0, 5, 10, 15 and 20˚C

Fresh deboned pieces of beef bought local butcher's shop transported under refrigeration to the laboratory within 30 min, where it was held at 1 ˚C for 1–2 h. The samples were prepared by cutting the meat pieces into portions (4.0 x 5.0 cm, thickness 1.0 cm). The portions placed onto Petri dishes and stored at 0, 5, 10 15 and 20˚C for 350, 240, 148, 114 and 72 h respectively.

For microbiological analysis a portion (4.0 x 5.0 cm, thickness 1.0 cm) was added to 150 ml of Ringer solution (LAB M, code LAB100Z, Athens Greece) and homogenized in a stomacher (Lab Blender, Seward, London, UK) for 60 s at room temperature. The enumeration (see 2.1.2.) that included counts of total viable microbiota (TVC), *Pseudomonas* sp., *Brochothrix thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria (LAB), and yeasts/moulds was performed in parallel with pH measurements (see 2.2) and sensory analysis (see 2.3). Samples from this study were also used for FTIR analysis (see 2.5) to exploit the dynamics of the method in evaluating the quality of beef samples during storage. Further analysis of these samples was conducted using Raman (see 2.6) and VideometerLab (see 2.7).

(c) Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

This study was conducted in the frames of the EU projects ProSafeBeef and SYMBIOSIS. The microbiological and sensory analysis was not a part of this thesis, though the experimental design and indicative results are reported briefly to support the rest types of analysis.

Fresh minced beef was obtained from the central meat market in Athens (Greece) and transported under refrigeration to the laboratory within 30 min, where it was held at 1°C for 1–2 h. Two portions of 75 g each were placed onto styrofoam trays. The trays with the meat samples were subsequently packaged under three packaging conditions, namely air, modified atmospheres (40% CO_2/ 30% O_2/ 30% N_2) (MAP), and modified atmospheres with the presence of volatile compounds of oregano essential oil (MAP/OEO) –active packaging- and stored at 0, 5, 10 and 15˚C for 650, 482, 386 and 220 h respectively. The microbiological analysis included counts of total microbiota (TVC), *Pseudomonas* spp., *Brochothrix thermosphacta*, lactic acid bacteria and *Enterobacteriaceae.* Minced beef samples from this study were used for FTIR analysis (see 2.5), HPLC analysis (see 2.8) and GC/MS (see 2.9) analysis to exploit the biochemical fingerprints of beef during storage and evaluate the beef shelf life in conventional and non-conventional (active packaging conditions) and in different temperatures (0, 5, 10, 15 °C) that represent good and bad storage practices in a distribution chain for meat products.

(d) Shelf life of minced beef stored in air and MAP at 5˚C

Fresh minced beef was obtained from a retail market in Athens (Greece) and transported under refrigeration to the laboratory within 30 min, where it was held at 1 ˚C for 1–2 h. Two portions of 75 g were placed onto styrofoam trays. These samples were packaged under two packaging conditions, i.e. air and MAP (40% CO_2/ 30% O_2/ 30% N_2), and stored at 5 ˚C. For the aerobic storage, the samples were placed into permeable polyethylene bags for domestic use (Fino, Sarantis S.A., Greece). For the MAP the samples were packed into plastic pouches (200 mm wide -240 mm long - 90 μm thickness), of gas permeability at 20 ˚C and 50 % relative humidity. ca. 25,90, and 6 cm^3 /m^2 per day/ 10^5 Pa for CO_2, O_2 and N_2 (data provided by the manufacturer - Flexo-Pack S.A., Greece), respectively, using a HenkoVac 1900 Machine(Howden Food Equipment B.V., The Netherlands).

The minced beef samples were stored for 144 h in both packaging conditions, until spoilage was pronounced, whilst a total of 13 sampling points were collected for each condition with a sampling frequency of 12h. The enumeration (see 2.1.2) that included counts of total viable microbiota (TVC), *Pseudomonas* sp., *Brochothrix thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria (LAB), and yeasts/moulds was performed in parallel with pH measurements (see 2.2) and sensory analysis (see 2.3). Samples from this study were used for FTIR (see 2.5) and Raman (see 2.6) analysis to compare the two spectroscopic techniques in evaluating the beef shelf life. VideometerLab was also tested using these samples for evaluating the beef shelf life.

2.1.2. Enumeration

Serial decimal dilutions in Ringers solution were prepared and 1 or 0.1 ml samples of appropriate dilutions were poured or spread on non-selective and selective agar plates. (i) Total viable counts were determined on Tryptic Glucose Yeast Agar (402145, Biolife, Milan, Italy), incubated at 30˚C for 48 h; (ii) *Pseudomonas* spp. on Pseudomonas Agar Base (CM559 supplemented with selective supplement SR103, Oxoid, Basingstoke, UK), incubated at 25 ˚C for 48 h, incubated at 25˚C for 48 h; (iii) *Brochothrix thermosphacta* on STA Agar Base (402079 supplemented with selective supplement 4240052, Biolife, Milan, Italy), incubated at 25 ˚C for 48 h; (iv) lactic acid bacteria in MRS agar (401728, Biolife, Milan, Italy) (pH = 5.7 and/or pH = 5.2) overlayed with the same medium and incubated at 30˚C for 72 h; (v) *Enterobacteriaceae* on Violet Red Bile Glucose Agar (402188, Biolife, Milan, Italy) overlayed with the same medium and incubated at 37 ˚C for 24 h, (vi) yeasts and moulds on Rose Bengal Chloramphenicol Agar Base (LAB 36 supplemented with selective supplement X009, LAB M, UK), incubated at 25 ˚C for 72 h and (vii) Iron agar (made from basic ingredients), for the enumeration of hydrogen sulfide-producing bacteria, overlaid with the same medium and incubated at 25 ˚C for 72 h.

2.1.3. Growth Kinetics

The growth data from plate counts of the different spoilage bacteria of meat were transformed to $\log_{10}$ values (log cfu g^{-1} or log cfu cm^{-2}) and were modelled as a function of time using the primary model of Baranyi and Roberts (1994), and the kinetic parameters of maximum specific growth rate (μ_{max}) and lag phase (lag) were estimated. For curve fitting the in-house Institute of Food Research program DMFit,

was used, which was kindly provided by Dr. J. Baranyi (Institute of Food Research, Norwich, United Kingdom).

2.2. pH measurement

The pH value was recorded by a pH meter (Metrohm 691 pH meter), the glass electrode being immersed in the homogenate of minced meat after the end of microbiological analysis.

2.3. Sensory evaluation

Sensory evaluation of meat samples was performed during storage according to Gill and Jeremiah (1991) by a sensory panel composed of five trained staff members from the laboratory. The same individuals participated in each evaluation, which was conducted blind. The sensory evaluation was carried out in artificial light and the temperature of packaged product was similar to ambient temperature. The colour and odour were described before and after cooking (20 min at 180 ˚C in preheated oven), whereas taste was described after cooking. Odour characteristics of minced beef, as determined by special samples kept frozen and thawed prior to each sensory evaluation, were considered as fresh. Putrid, sweet, sour, or cheesy odours were regarded as indicative of microbial spoilage and such samples were classified as spoiled. Bright colours typical of fresh oxygenated meat were considered indicative of fresh meat, whereas a persistent dull or unusual colour rendered the sample spoiled. Each attribute was scored on a three-point hedonic scale corresponding to: 1=fresh; 2=marginal; and 3=unacceptable. To characterise intermediate sensory qualities two additional discrete points of 1.5 and 2.5 were employed. A score of 1.5 was

characterized as semi-fresh and was the first indication of change from that of typical fresh meat (i.e., less vivid red colour, odour and flavour slightly changed, but still acceptable by the consumer). Scores >2 rendered the product spoiled and indicated the end of its shelf life.

2.4. Statistical analysis

The data obtained from the **Survey of Minced Beef from Greek market** were subjected to descriptive statistical analysis, F-test and one way analysis of variance (ANOVA) dividing the samples in different groups regarding the origin of the samples' purchase: Supermarkets, Butcher Shops, Season (Spring, Summer, Autumn, Winter) and Day (Monday, Tuesday, Wednesday, Thursday and Friday) of purchase.

2.5. FTIR analysis

2.5.1. Samples preparation

All the beef samples for the FTIR measurements were obtained at the same sampling times that microbiological analyses took place and were stored at -20°C until the analysis.

(a) Survey of Minced Beef from Greek Market

150 samples of minced beef (with their replicates) were collected for FTIR analysis from an extensive experiment which formed a part of the **Survey of Minced Beef from Greek Market (see Section 2.1.1).** The number of the spectra collected was 300. The dimensions that samples were cut were approximately 8 x 1 x 0.5 cm.

(b) Shelf life Beef fillets stored in air at 0, 5, 10, 15 and 20˚C

For the beef fillets FTIR measurements, a thin slice of the aerobic upper surface of the fillet was cut so as to measure 8 x 1 x 0.5 cm and 72 samples (with replicate samples being used at some time points for confirmation reasons) were collected in total.

(c) Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

186 samples (with replicate samples being used at some time points for confirmation reasons) were collected for FTIR analysis from minced beef stored aerobically and under modified atmosphere with and without the presence of the volatile compounds of oregano essential oil (2% v/w) - active packaging- and stored at 0, 5, 10 and 15˚C until the spoilage was very pronounced. The dimensions of the samples were as detailed above.

Except from the raw mince, 20g of minced beef from each sampling time were freeze-dried (freeze drying at -40°C for 30h) were collected and stored at -20°C until the analysis. The analysis of these results is in progress and thus indicative results are given in the Appendix A1 section.

(d) Shelf life of minced beef stored in air and MAP at 5˚C

104 samples were collected for FTIR analysis from minced beef stored aerobically and under modified atmosphere at 5˚C. These samples included 2 replicates from different mince samples (biological replicates) and 2 replicates from each sample. The resulting number of the spectra was 104. The samples of mince were collected at the same time that the microbiological and sensory analyses took place. The samples were cut to the same dimensions as described previously.

2.5.2. FT-IR/ATR spectroscopy

FT-IR analysis was carried out using a ZnSe 45° ATR (attenuated total reflectance) crystal on a Nicolet 6700 FT-IR Spectrometer equipped with a DLaTGS Detector with KBr Window. The samples were placed on the ZnSe ATR crystal so that the aerobic upper surface of the meat was placed in intimate contact with the crystal. The sample then was pressed with a gripper so as to have better possible contact with the crystal. The spectrometer was programmed with Omnic Software-version 7.3 to collect spectra over the wave number range 4,000 to 400 cm-1, whilst the scans per measurement were 100 with a resolution of 4 cm^{-1}, resulting in a total integration time of 2 min. The ZnSe ATR crystal was capable of 12 external reflections, with the evanescent field affecting a depth of 1.01 μm. For the experiments of the **Survey of Minced Beef** and **Shelf life of minced beef stored in air and MAP at 5˚C**, a Smart ARK Base was used and according to the default of the new automated accessory, the spectrometer was programmed to collect spectra over the wavenumber range 4,000 to 650 cm-1 to reduce the noise. Reference spectra (background spectra) were acquired by collecting a spectrum from the cleaned blank crystal prior to the presentation of

each sample replicate. At the end of each sampling, the crystal surface was cleaned with detergent, washed with distilled water, dried with lint-free tissue, cleaned with ethanol and finally dried with lint-free tissue at the end of each sampling interval.

2.5.3 Mathematical treatment of the data

(a) Survey of Minced Beef from Greek Market, Shelf life of Beef fillets stored at 0, 5, 10, 15 and 20˚C, Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

Prior to data analysis, the FT-IR spectral data were mean-centered and standardized. Then the corrected spectral data were subjected to a principal component analysis (PCA). PCA is a bilinear modeling method that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components (PCs). The number of principal components is less than or equal to the number of original variables. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on. Thus, in this study, PCA was applied to investigate differences between samples and thus reduce the size of the data set. The variables, for which the communality values of each of the first three principal components (PCs) were higher or equal to 0.6 were considered as significantly explaining the variance of the standardized data set. A second PCA with the selected variables (wavenumbers) revealed the PCs that were further used for analysis.

For qualitative analysis, the later PCs were subjected to factorial discriminant analysis (FDA) in attempt to estimate the quality of a sample that was pre-characterized as Fresh (F), Semifresh (SF) or Spoiled (S) from the sensory analysis. Moreover, the

same data were subjected to a FDA based on the defined packaging type (air, MAP, and active packaging) constituting the dependent variable and to an FDA based on the storage temperature. For the **Survey of the minced beef,** FDA analysis included also the discrimination of the Season and the Day of purchase on which each sample was bought. FDA is a supervised discrimination or classification technique that is used in situations where the groups are known a priori. The aim of discriminant analysis is to classify an observation, or several observations, into these predifined groups. The discrimination rules are based on linear combinations of the observed variables, called discriminant factors. The FDA requires the knowledge of a well classified set of data (matrix X), divided in g groups. Groups are stored in a vector Y with values from 1 to g and data classified in group i is $X_{(Y=i)}$. FDA seeks for factors where the projections of data are, as well as possible, well classified according to the g a priori known groups. These factors divide the multivariable space of data using hyperplanes. The selection of the factors (or orthogonal hyperplanes) is made in order to minimize the probability of misclassification.

For quantitative analysis, PCs significantly contributing to the variance of the data set were regressed using a partial least squares regression (PLS-R) onto viable counts. PLS is a fixed linear regressor of the type Y = AX + B, which works on the basis of extracting a smaller number of orthogonal latent components (A) that are linear combinations of the original (X). As a supervised technique i.e. one which uses *a priori* knowledge of the solution to a problem in order to optimize calibration, it is important to include a set of validation data against which to select the optimal number of latent variables. Model training is then based on the minimum root mean

squared error (RMSE) for validation predictions. In this experiments, leave-one-out cross validation technique was applied to evaluate the performance of the model.

Performance criteria: The criteria for evaluating and comparing the models were the root mean square error (RMSE), the square of the correlation coefficient R^2 for the known values versus validation estimates and the percent relative error (% RE) between predictions and observations (Ross 1996; Singh et al. 2009). The performance of the model was also graphically illustrated by the percent relative error (% RE) where % RE <0 are fail-safe predictions and % RE > 0 are fail dangerous predictions.

$$\%RE = \left(\frac{y_{observed} - y_{predicted}}{y_{observed}} \right) \cdot 100 \quad (1)$$

The PCA, FDA, and PLS-R calculations were performed using Statistica® v6.0 (Statsoft, OK, USA), XLSTAT® v2006.06 (Addinsoft, Paris, France), and Unscrambler® v9.6 (Camo, Oslo, Norway), respectively.

(b) Shelf life of minced beef stored in air and MAP at 5˚C

Prior to data analysis, the FTIR spectra were mean-centered and standardized. For quantitative estimation of the counts of all the microbial groups and of the sensory scores from the FT-IR spectra the data were divided in two parts regarding the different biological replicates. One of these data sets was used for the calibration and the other was used for the validation of the models. Then the data were subjected to the following linear and non-linear regression models:

Partial least squares regression (PLS-R): The basic principals of PLS is described above in the previous experiments. The number of latent variables used in each model was the point at which the lowest RMSE in the validation data was seen.

Support Vector Machines Regression (SVR): support vector machines are a relatively new tool, originally designed for classification problems involving large multidimensional data sets. The basic idea is to map the data X into a high-dimensional feature space via a nonlinear mapping function (kernel function) and perform a linear regression in this feature space. The dimensionality of the feature space is determined by the choice of kernel function and its parameters while the complexity of the model is determined by an extra penalty parameter. Such method can also be applied to the case of regression, maintaining all the main features that the SVM algorithm has; a non-linear function is learned by a linear learning machine in a kernel-induced feature space while the capacity of the system (i.e. the complexity of the model) is controlled by a parameter that does not depend on the dimensionality of the space. The model produced by SVR depends only on a subset of the training data, because the cost function for building the model ignores any training data close to the model prediction (within a threshold ε). One of the most important ideas in SVR is that presenting the solution by means of small subset of training points gives enormous computational advantages. Using the epsilon intensive loss function we ensure existence of the global minimum and at the same time optimization of reliable generalization bound. A good review of SVR can be found in Smola and Scholkopf (2004).

In this study, different kernel functions including linear (SVR_L), polynomial (SVR_P), radial basis (RBF) (SVR_R) and sigmoid (SVR_S) functions, where tested for their capability on fitting Raman and FTIR data. The different SVM parameters needed for each model were optimized with leave-one-out cross-validation for the training set in terms of RMSE. During this process, a broad range of parameter settings was investigated with large steps and after identifying a promising region, this region was searched in more detail. To test the validity of the supervised models, the data were partitioned into model training and calibration sets selection of subsets of biological replicates as described at the beginning of this section. All the above calculations were performed using MATLAB® v. 7.0 (Mathworks, Inc., Massachusetts, USA).

Performance criteria: The criteria for evaluating and comparing the models were the root mean square error (RMSE) and the square of the correlation coefficient R^2 for the known values versus validation predictions for both the viable counts and the sensory scores. In addition, the percentage of relative error (% RE) was used for the viable counts whilst the confusion matrix was used to evaluate the correct classification of the predicted sensory scores.

The confusion matrix for the sensory scores was derived using a 0.25 cut-off value for estimated values with respect to the closest sensory value. For example, a sample with an estimated score of 1.35 was classified as 1.5. Samples with a score of 1 were categorised as fresh, samples scoring 1.5 were categorised as semi-fresh and scores above 2 that pointed the end of the products' shelf life were categorised as spoiled. Finally, the accuracy of the model was visualized with the % recognition rate.

2.6. Raman analysis

2.6.1. Samples preparation

Shelf life of minced beef stored in air and MAP at 5˚C

52 samples were collected for Raman analysis from minced beef stored aerobically and under modified atmosphere at 5˚C. These samples included 2 replicates from different mince samples (biological replicates), whilst up to 5 replicate Raman measurements were taken from the surface of each sample. Thus, the number of the spectra collected was 260. The samples of mince were collected at the same time as the microbiological and sensory analyses. Prior to analysis, the samples were placed into 9 cm Petri dishes.

Survey of Minced Beef from Greek Market and Shelf life of Beef fillets stored at 0, 5, 10, 15 and 20˚C

In order to enhance the data collected from Raman analysis samples that were kept at -80°C from the experiments **Survey of Minced Beef** (150 samples) and **Shelf life of Beef fillets** (72 samples) were collected placed into Petri diches. Up to 3 replicate Raman measurements were taken from the surface of each sample. Thus, the number of the spectra collected was 450 for the survey and 216 for the beef fillets. The statistical analysis of these results is in progress and indicative results are given in the Appendix A2 section.

2.6.2. Raman spectroscopy

A 633 nm DeltaNu Advantage probe with a right-angled sampling attachment was used for data collection, with the aperture postioned 16 mm above the surface of the meat sample delivering ~ 6 mW laser power. Five replicate spectra were collected from each biological replicate sample, from randomly selected positions on the sample surface. Spectra were acquired over a Stokes Raman shift range of 200 to 3400 cm^{-1} at medium resolution (6 cm^{-1}). Each spectrum was integrated for 60 s and the spectra exported from the DeltaNu control software as ASCII-XY files for subsequent numerical analysis.

2.6.3 Mathematical treatment of the data

The treatment of the Raman data was performed in the same way described above for the FTIR data in the section **2.5.3., Shelf life of minced beef stored in air and MAP at 5˚C.**

2.7. Videometerlab analysis

2.7.1. Samples preparation

(a) Shelf life of minced beef stored in air and MAP at 5˚C

52 samples were collected for VideometerLab analysis from minced beef stored aerobically and under modified atmosphere at 5˚C. These samples included 2 replicates from different mince samples (biological replicates). Thus, the number of the spectra collected was 104. The samples of the mince were collected at the same

time that the microbiological and sensory analyses took place. Prior to analysis, the samples were placed into Petri dishes.

(b) Survey of Minced Beef from Greek Market and Shelf life of Beef fillets stored at 0, 5, 10, 15 and 20˚C

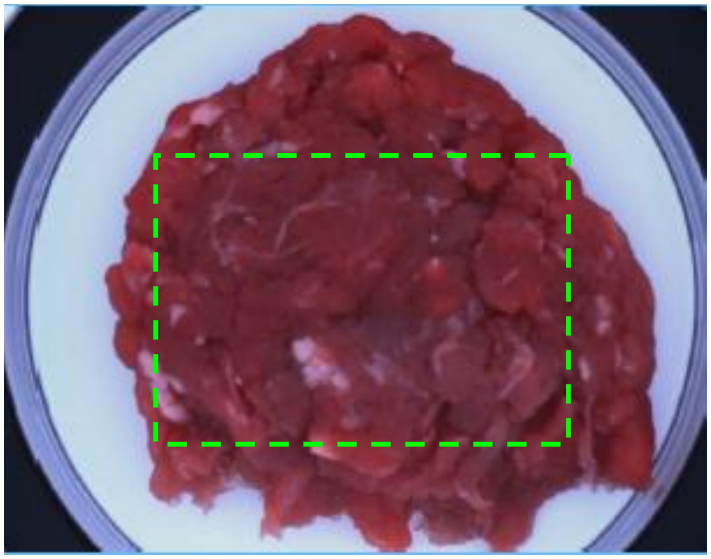
In order to enhance the data collected from Videometer analysis samples that were kept at -80°C from the experiments **Survey of Minced Beef** (150 samples) and **Shelf life of Beef fillets** (72 samples) were collected placed into Petri dishes. Thus, the number of the spectra collected was 150 for the survey and 72 for the beef fillets. The statistical analysis of these results is in progress and indicative results are given in the Appendix A3 section.

2.7.2. Visible and near-infrared sample acquisition

The multi-spectral images were acquired using the VideometerLab camera, a multi-spectral camera for laboratory analysis which can take mono-chromatic photos at 18 wavelengths spanning from Ultra Blue (UB) to Near-Infrared (NIR) in a 960 x 1280 pixel resolution. The wavelengths used are given in Table 2.7.1. After the acquisition of the multi-spectral images, an average of each the sample's picture was acquired using the instruments supplementary software (Picture 2.7.1). This resulted in a set of 18 variables for each sample. In total, 104 spectra were collected (including 2 replicates from different samples).

Table 2.7.1. VideometerLab wavelengths.

No	Wavelength (nm)	Colour
1	430	UB
2	450	Blue
3	470	Blue
4	505	Green
5	565	Green
6	590	Amber
7	630	Red
8	645	Red
9	660	Red
10	700	Red
11	850	NIR
12	870	NIR
13	890	NIR
14	910	NIR
15	920	NIR
16	940	NIR
17	950	NIR
18	970	NIR



Picture 2.7.1. Example of a multi-spectral image acquired using the VideometerLab camera. Green line shows the selection of a representative area for which the average spectrum of each wavenumber was calculated

2.7.3. Mathematical treatment of the data

Prior to data analysis, the Videometer spectral data were mean-centered and standardized. Then the corrected spectral data were subjected to a principal component analysis (PCA) to investigate differences between samples and thus reduce the size of the data set. The variables, for which the communality values of the first five principal components (PCs) were higher or equal to 0.6 were considered as significantly explaining the variance of the standardized data set. Since all the tested variables were found to be significant, all of them were used for further analysis.

For qualitative analysis, the above PCs were subjected to factorial discriminant analysis (FDA) in attempt to estimate the quality of a sample that was pre-characterized as Fresh (F), Semi-fresh (SF) or Spoiled (S) from the sensory analysis. Moreover, the same data were subjected to a FDA based on the defined packaging type (air, MAP, and active packaging) constituting the dependent variable and to an FDA based on the storage temperature. For quantitative analysis, the standardised spectral data were regressed using a partial least squares regression (PLS-R) onto viable counts. The leave-one-out cross

validation technique was applied to evaluate the performance of the model.

Performance criteria: The criteria for evaluating and comparing the models were the root mean square error (RMSE) and the square of the correlation coefficient R^2 for the known values versus validation predictions for both the viable counts and the sensory scores. In addition, the percentage of relative error (% RE) was used for the viable counts whilst the confusion matrix was used to evaluate the correct classification of the predicted sensory scores.

The PCA, FDA, and PLS-R calculations were performed using Statistica® v6.0 (Statsoft, OK, USA), XLSTAT® v2006.06 (Addinsoft, Paris, France), and Unscrambler® v9.6 (Camo, Oslo, Norway), respectively.

2.8. HPLC analysis for organic acids

2.8.1. Samples preparation

Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

For sample preparation, 2 g of mince was homogenized manually with a glass rod in 4 mL of dH_2O for 2 min and centrifuged (5 min at 9000 rpm at 5°C). Two (2) mL of the supernatant were transferred to an Eppendorf tube and then 20 μl 1% solution of sodium azide (as a preservative) and 20 μl of trifluoroacetic acid (TFA) (for protein precipitation) were added. Stirring and centrifugation (5 min at 9000 rpm at 5^oC) of the above was followed by filtration of the final supernatant through a 0.22 μm filter (Millipore). In total 77 samples were analysed in duplicate (2 samples from time 0h from which the mean values were used, 6 samples for

each packaging condition at 0, 5, 10 ^{o}C and 7 samples for each packaging condition at 15 ^{o}C). The coefficient of variation (CV%) of the results was always lower than 5%.

Shelf life of Beef fillets stored at 0, 5, 10, 15 and 20˚C

The same procedure described above was followed for the beef fillets as well**.** In total 52 samples were analysed in duplicate (2 samples from time 0h from which the mean values were used and 11, 9, 10, 11 and samples from 0, 5, 10, 15 and 20 ^{o}C respectively). These results are similar to those obtained with minced beef and the mathematical analysis of the data is in progress. The results are given in brief in the Appendix A4 section.

2.8.2. HPLC analysis

The analysis was performed as described by Skandamis and Nychas (2001) using a Jasco (Japan) HPLC equipped with a Model PU-980 Intelligent pump, a Model LG-980-02 ternary gradient unit pump and a MD-910 multiwavelength detector. The injection valve was connected with a 20 μl loop, whilst 50 μl of the sample were injected each time. The sample was eluted isocratically with a solution of 0.009N H_2SO_4 (using HPLC grade solvent and ultra pure water) through an Amminex HPX-87H column (300 x 7.8 mm, Bio-Rad Laboratories, Richmond, CA) at a rate of 0.7 mL/min and oven temperature set at 65 °C. The software used for the collection and the processing of the spectra was the Jasco Chrompass Chromatography Data system v1.7.403.1. Spectral data were collected from 200 to 600 nm, however chromatogram integration was performed at 210 nm and the purity of the peaks was examined through the software using all spectral ranges. Solutions of oxalic, citric, malic, lactic, acetic, formic, tartaric, succinic and propionic acids (HPLC grade) were used as reference substances, analysed using the same programme and their spectra were compared with the samples for the identification of the peaks.

2.8.3 Mathematical treatment of the data

Raw spectral data from HPLC analysis (areas under peaks) were initially mean-centered and standardized to facilitate comparison of the peaks with different magnitude. The standardized data were subjected to principal component analysis (PCA) to investigate whether the peak areas had significantly changed during storage. The variables (peaks), for which the communality values of the first three principal components (PCs) were higher or equal to 0.5 were considered as significantly explaining the variance of the standardized data set. A second PCA using the selected variables (peaks) revealed the PCs that were further used for analysis. In this case, the total variance (100%) of the data set could be explained by 13 PCs from which the first five were extracted and used for further analysis, accounting for 84.0% of cumulative variance observed in the experiment. Initially, Hierarchical Agglomerative Clustering (AHC) was employed using the Euclidian distance and Ward's linkage measure in an attempt to explore the unsupervised discrimination of the samples. Subsequently, the selected PCs were submitted to Factorial Discriminant Analysis (FDA) in order to qualitatively estimate the spoilage status of a sample belonging to a previously-defined sensory group. The groups of fresh, semi-fresh and spoiled samples constituted the three classification clusters. Moreover, in an effort to estimate the counts of the different microbial groups, the above PCs were regressed using a partial least squares regression (PLS-R) model onto total viable counts (TVC), *Pseudomonas* spp, *Br. thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, yeasts and moulds. The leave-one-out cross validation technique was applied to evaluate the performance of the models.The PCA, AHC, FDA, and PLS-R calculations were performed using Statistica® v6.0 (Statsoft, OK, USA), XLSTAT® v2006.06 (Addinsoft, Paris, France), and Unscrambler® v9.6 (Camo, Oslo, Norway), respectively.

Performance criteria: The performance of the PLS-R model was evaluated using four different criteria, namely, the RMSE, the bias factor (B_f), the accuracy factor (A_f) and the percent relative error (% RE) between predictions and observations (Ross 1996; Singh et al. 2009). The bias factor (B_f) indicates how much the observed values lie above or below the line of equity ($y = x$) and provide an indication of the structural deviation of the model. A bias factor = 1 indicates no structural deviation between observations and predictions, i.e., on average the model is exact, while a bias factor < 1 indicates under-prediction of the model ("fail safe" predictions).

$$B_f = 10^{\left(\frac{\sum_{i=1}^{N}\left(y_{predicted}/y_{observed}\right)}{N}\right)} \quad (2)$$

The accuracy factor (A_f) indicates the average deviation between predictions and observations, i.e. how close predictions are to observations. The larger the value the less accurate is the average estimate.

$$A_f = 10^{\left(\frac{\sum_{i=1}^{N}\left|y_{predicted}/y_{observed}\right|}{N}\right)} \quad (3)$$

The performance of the models was also graphically illustrated by the percent relative error (% RE) where % RE <0 are fail-safe predictions and % RE > 0 are fail dangerous predictions.

2.9. GC/MS analysis

2.9.1. Headspace SPME

Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

The volatile compounds of meat were isolated by the headspace (HS) solid phase micro-extraction (SPME) method (HS/SPME). The fibre used for the absorption of volatiles was a

DVB/CAR/PDMS - 50/30μm (needle length 1 cm, needle size 24 ga) (Sigma Aldrich, Greece).

The conditions of HS/SPME sampling used were as follows: 5 g of minced beef, 10 mL of 25% NaCl solution and 10 μL of internal standard (4-Methyl-1-pentanol, final concentration 1000 μg/L) were homogenized with a glass rod for 2 min into a 20 mL glass vial. The vial was closed hermetically using a mininert valve (Sigma Aldrich, Greece) and the contents were magnetically stirred for 15 min at 39 °C. Then, the fiber was exposed to the headspace for another 30 min, under the same conditions. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the GC/MS for 1 min. Before each analysis, the fiber was exposed to the injection port for 10 min to remove any volatile contaminants.

2.9.2. Gas chromatography/Mass spectrometry

GC/MS analyses were performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5973C mass spectrometer. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The injection port was equipped with a SPME liner (0.75 mm x 6.35 mm x 78.5 mm) suitable for SPME analysis. It was operated in splitless mode for 1 min at 250 ^{o}C. Separation of compounds was performed on a HP-5MS column (30 m, 0.25 mm i.d., 0.25μm film thickness, Agilent). Oven temperature was maintained at 40 ^{o}C for 5 min, programmed at 4 °C/min to 150 °C and then it was raised to 250 °C with a rate of 30 °C/min and held for 5 min. The interface temperature was set at 280°C. The mass spectrometer was operated in electron impact mode with the electron energy set at 70 eV and a scan range of 29–350 m/z (scan rate: 4.37 scans/sec, gain factor: 1, resulting EM voltage: 1188V). The temperature of MS source and quadrupole was set at 230 and 150 °C, respectively.

Identification of the compounds was effected by comparing: (i) the linear retention indices (LRI) based on an homologous series of even numbered n-alkanes (C8–C24, Niles, Illinois, USA) with those of standard compounds and by comparison with literature data, and (ii) MS data with those of reference compounds and by MS data obtained from NIST library (NIST/EPA/NIH Mass Spectral Library with Search Program, data version NIST 05, software version 2.0d). Amdis software (version 2.62, http://chemdata.nist.gov/mass-spc/amdis/) was used for the deconvolution of mass spectra and identification of target components.

The volatile compounds were semi-quantified by dividing the peak areas of the compounds of interest by the peak area of internal standard (IS) and multiplying this ratio by the initial concentration of the IS (expressed as μg/L). The peak areas were measured by selecting single ions.

2.9.3 Mathematical treatment of the data

The data from GC/MS analysis (concentrations) were initially mean-centered and standardized to facilitate comparison of the peaks with different magnitude. To simplify the analysis and create global models that are not biased from the presence/absence of a given metabolite due to different storage conditions, some compounds were kept out of the chemometrics analysis. These compounds were detected in small quantities and where either in traces or not detected at all at some storage conditions (e.g. present only in small quantities under aerobic storage and not detected at all under MAP, or present only at some temperatures in MAP). These compounds, along with compounds detected in traces were used only for qualitative analysis. Moreover, samples that were stored under MAP/OEO were characterized by high amounts of essential oil components leading to significant overlapping

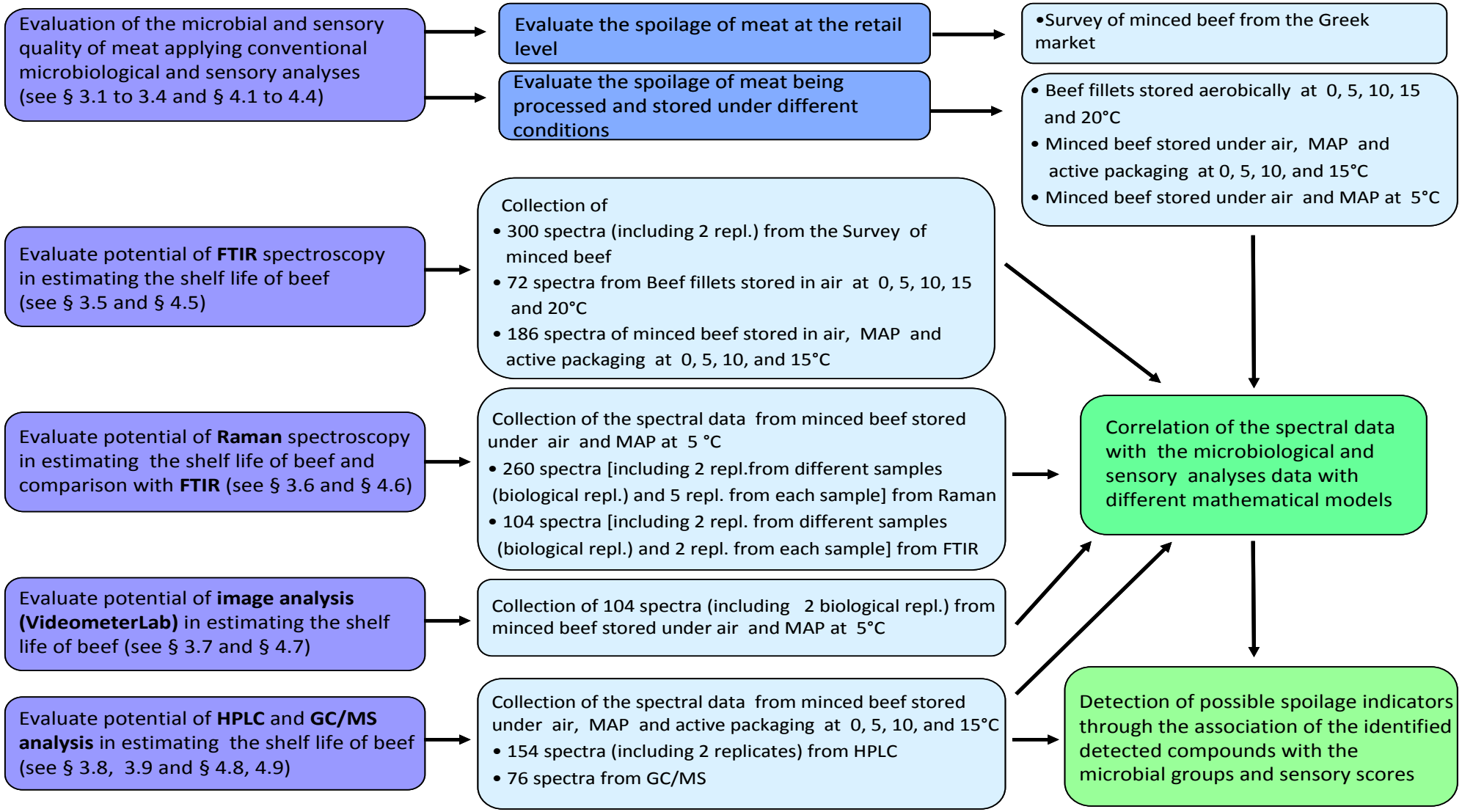
in chromatograms. These samples were as well kept out of analysis. In total 74 samples were analysed (2 samples from time 0h from which the mean values were used, 38 samples from aerobic storage and 34 samples from MAP storage).

The standardized data of the chosen compounds were subjected to principal component analysis (PCA) to investigate which compounds had significantly changed during storage. The variables (peaks), for which the communality values of the first five principal components (PCs) were higher or equal to 0.5 were considered as significantly explaining the variance of the standardized data set. All the tested variables were found to be significant and thus all of them were used for further analysis. Initially, Hierarchical Agglomerative Clustering (AHC) was employed using the Euclidian distance and Ward's linkage measure in an attempt to explore the unsupervised discrimination of the samples. Subsequently, the selected PCs were submitted to Factorial Discriminant Analysis (FDA) in order to qualitatively estimate the spoilage status of a sample belonging to a previously-defined sensory group. The groups of fresh, semi-fresh and spoiled samples constituted the three classification clusters. Moreover, in an effort to estimate the counts of the different microbial groups, the standardised variables were regressed using a partial least squares regression (PLS-R) model onto total viable counts (TVC), *Pseudomonas* spp., *Br. thermosphacta*, LAB, *Enterobacteriaceae*, yeasts and moulds. The leave-one-out cross validation technique was applied to evaluate the performance of the model.The PCA, AHC, FDA, and PLS-R calculations were performed using Statistica® v6.0 (Statsoft, OK, USA), XLSTAT® v2006.06 (Addinsoft, Paris, France), and Unscrambler® v9.6 (Camo, Oslo, Norway), respectively.

Performance criteria: The performance of the PLS-R model was evaluated using four different criteria, namely, the RMSE, the bias factor (B_f), the accuracy factor (A_f) and the

percent relative error (% RE) between predictions and observations (Ross 1996; Singh et al. 2009). The bias factor (B_f) indicates how much the observed values lie above or below the line of equity ($y = x$) and provide an indication of the structural deviation of the model. A bias factor = 1 indicates no structural deviation between observations and predictions, i.e., on average the model is exact, while a bias factor < 1 indicates under-prediction of the model ("fail safe" predictions). The accuracy factor (A_f) indicates the average deviation between predictions and observations, i.e. how close predictions are to observations. The larger the value the less accurate is the average estimate. The performance of the model was also graphically illustrated by the percent relative error (% RE) where % RE <0 are fail-safe predictions and % RE > 0 are fail dangerous predictions.

Flow diagram of the experimental procedure



Chapter 3

Results

3.1. Survey of minced beef from the Greek Market

The descriptive statistical analysis of the microbial load on all the samples (56) and those purchased from supermarkets (31) or Butcher shops (25) are presented in Tables 3.1.1 and 3.2.1, respectively. In general, the microbiological analysis of the 56 samples of minced beef showed a TVC range between 4.71-7.99 log cfu g^{-1}. The samples obtained from supermarkets showed slightly larger mean microbial populations than the Butcher shops, while the Butcher shops had a wider microbial population range. Moreover, the F-test (Table 3.1.3) revealed significant differences between the TVC, *Pseudomonas* spp, and *Br. thermosphacta*.

Table 3.1.1 Descriptive statistics of microbial load, pH and Sensory scores on the total of the minced beef samples

Microorganisms	Minimum	Maximum	Range	Mean	Standard deviation
TVC	4.708	7.987	3.279	6.238	0.787
Pseudomonas spp.	3.000	7.792	4.792	5.673	1.016
Br. thermosphacta	3.079	6.748	3.669	5.161	0.937
H_2S-producing bacteria	1.000	8.301	7.301	3.614	1.357
LAB pH 5.2	2.778	6.908	4.130	4.889	0.846
LAB pH 5.7	2.968	7.204	4.236	4.884	0.819
Enterobacteriaceae	1.301	6.748	5.447	3.721	1.094
pH	5.360	6.290	0.930	5.607	0.158
Sensory	1.000	3.000	2.000	1.795	0.562

Table 3.1.2 Descriptive statistics of microbial load, pH and Sensory scores of the minced beef samples purchased from supermarkets (SM) and Butcher shops (B).

Microorganisms	Minimum	Maximum	Range	Mean	Standard deviation
TVC **SM**	5.415	7.929	2.514	6.432	0.615
TVC **B**	4.708	7.987	3.279	5.998	0.915
Pseudomonas spp. **SM**	4.286	7.310	3.024	5.898	0.797
Pseudomonas spp. **B**	3.000	7.792	4.792	5.393	1.193
Br. thermosphacta **SM**	4.173	6.462	2.289	5.396	0.688
Br. Thermosphacta **B**	3.079	6.748	3.669	4.869	1.123
H_2S-producing bacteria **SM**	1.301	8.301	7.000	4.021	1.363
H_2S-producing bacteria **B**	1.000	6.826	5.826	3.110	1.192
LAB pH 5.2 **SM**	3.477	6.908	3.431	5.093	0.766
LAB pH 5.2 **B**	2.778	6.342	3.564	4.636	0.887
LAB pH 5.7 **SM**	3.531	7.204	3.673	5.123	0.765
LAB pH 5.7 **B**	2.968	5.973	3.005	4.586	0.800
Enterobacteriaceae **SM**	2.491	6.748	4.257	4.021	1.134
Enterobacteriaceae **B**	1.301	5.435	4.134	3.349	0.935
pH **SM**	5.360	6.290	0.930	5.598	0.167
pH **B**	5.375	5.980	0.605	5.619	0.148
Sensory **SM**	1.000	3.000	2.000	1.871	0.547
Sensory **B**	1.000	2.500	1.500	1.700	0.577

Table 3.1.3 F-Test to compare the Standard Deviations (supermarket-Butcher shops).

Microorganisms	**Ratio of Variances**	**F**	**P-value**
TVC	**0.169708, 0.800699***	0.37488	**0.011719**
Pseudomonas spp.	**0.20233, 0.954613**	0.446942	**0.0377308**
Br. thermosphacta	**0.169708, 0.800699**	0.37488	**0.011719**
H_2S-producing bacteria	0.591297, 2.7898	1.30616	0.50637
LAB pH 5.2	0.337048, 1.59023	0.744531	0.440219
LAB pH 5.7	0.413783, 1.95227	0.914036	0.80669
Enterobacteriaceae	0.406699, 1.91885	0.898387	0.772576
pH	0.576703, 2.72094	1.27392	0.547566
Sensory	0.54681, 1.45328	0.909784	0.680894

**Bold cells indicate significant effect of the market type*

The ANOVA conducted to define the effect of the Day of purchase on the samples, showed no significant differences (95% probability level) between the means of the samples (Figure 3.1.1.). In contrary, the Season was found to exhibit a significant effect on the microbial association (Table 3.1.4; Figure 3.1.2.), especially for *Pseudomonas* spp, H_2S-producing bacteria, LAB grown at pH 5.2 and *Enterobacteriaceae* pH was also affected by the Season.

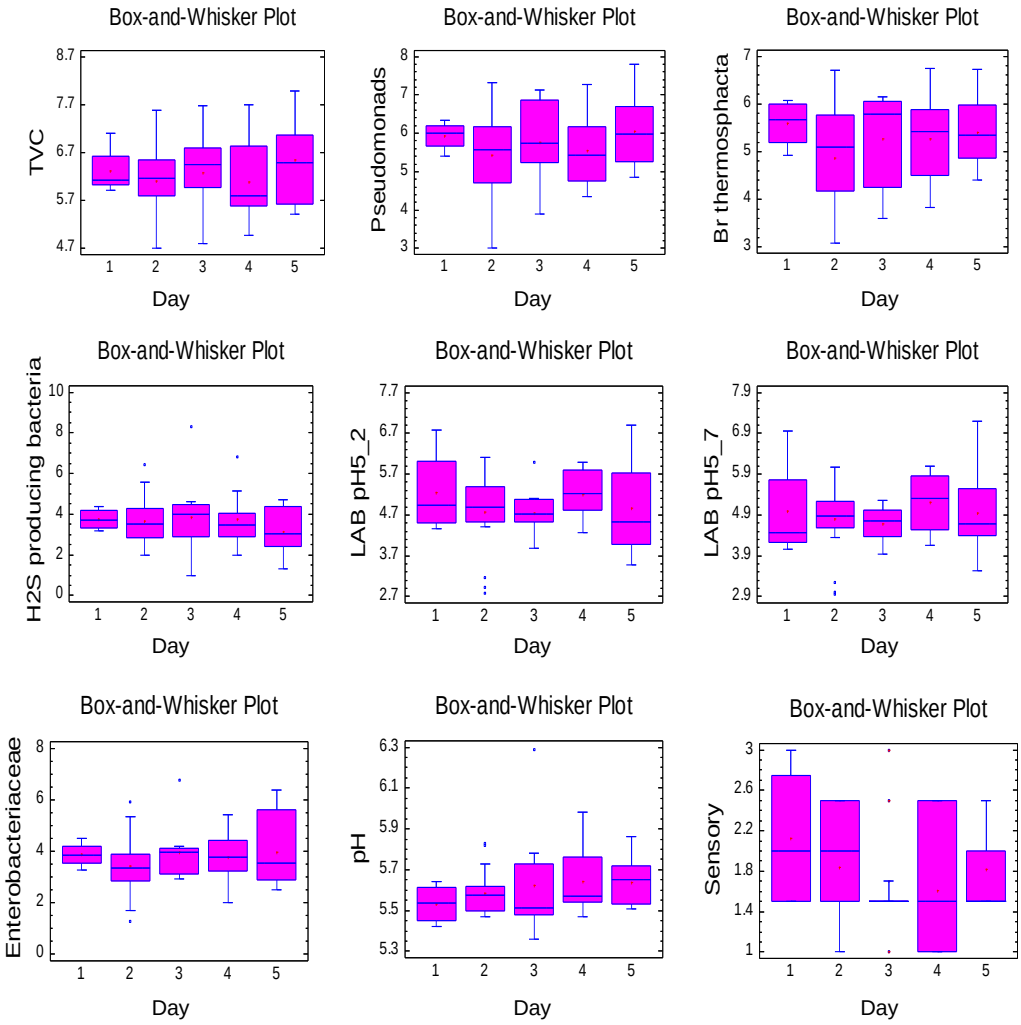


Figure 3.1.1. Distribution of the means concerning the Day of purchase; Monday (1), Tuesday (2), Wednesday (3), Thursday (4) and Friday (5). The ends of the box are the upper and lower quartiles, the median is marked by a horizontal line inside the box. The whiskers are the two lines outside the box that extend to the highest and lowest observations

Table 3.1.4 The effect of the Season on the different microorganisms, pH and sensory scores according to the ANOVA analysis

Microorganisms	**Season**			
	Spring	Summer	Autumn	Winter
TVC	x*	x	x	x
Pseudomonas sp.	x	x, y	x, y	y
Br. thermosphacta	x	x	x	x
H2S-producing	x	x, y	y	y
bacteria	a	a, b	a, b	b
LAB pH 5.2	x	y	y	x, y
LAB pH 5.7	x	x	x	x
Enterobacteriaceae	x	y	x, y	y
	x	y	y	y
pH	a	a, b	b	b
Sensory	x	x	x	x

**Different letters indicate significant differences at probability levels of 95% (x,y,z) or 99% (a,b,c)*

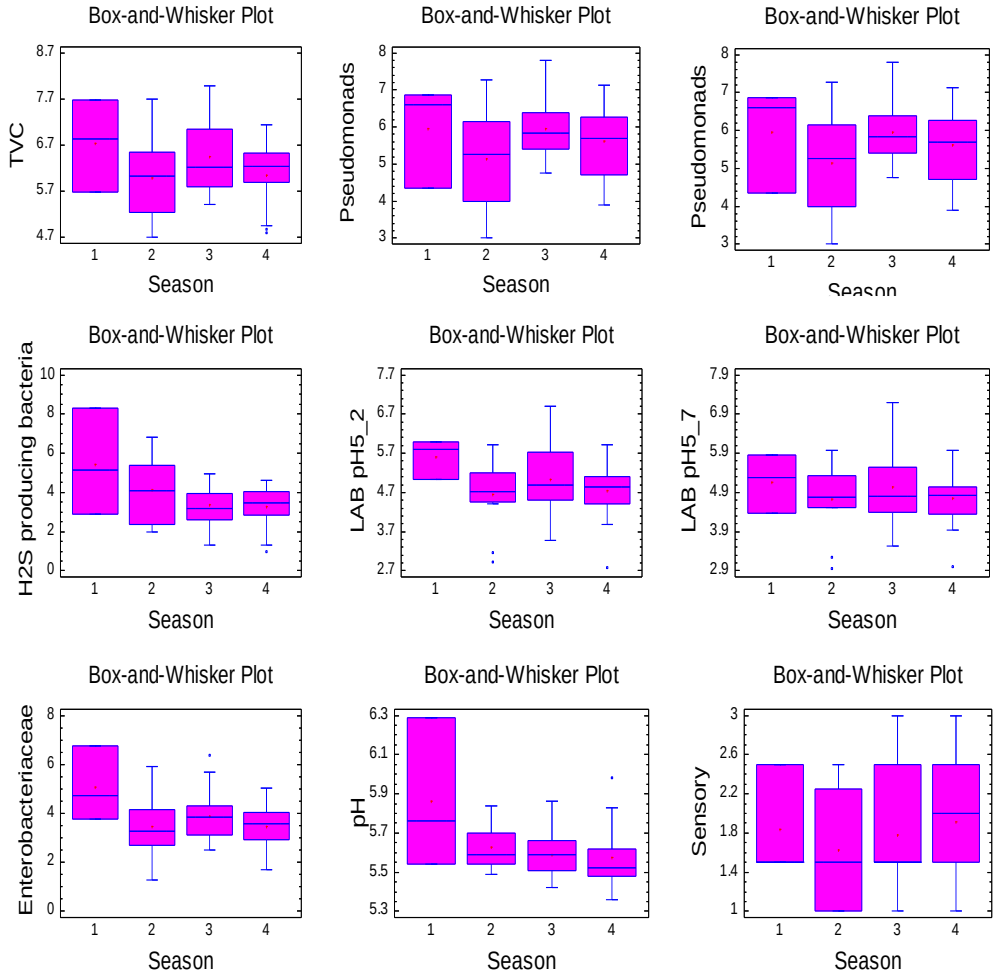


Figure 3.1.2. Distribution of the means concerning the Season of purchase. 1: Spring, 2: Summer, 3: Autumn and 4: Winter

The above statistical analysis was also applied to all 150 samples that were subsequently used for FTIR, Raman and Videometer analysis to explore the variations between the samples. The TVC varied from 4.18 to 8.87 for the SM samples and between 4.60 to 8.40 for the B samples, whilst the F-test revealed that the type of Market (SM vs. B), had a significant effect on the populations of *Pseudomonas* sp., *Br. thermosphacta*, LAB (pH 5.7) and on the pH. The ANOVA conducted to define the effect of season on the samples, showed significant differences between the means of all the microorganisms tested, whilst no effect was observed of the pH or sensory evaluation. The Day of purchase was found to have a significant effect on all the microbial groups except for the H_2S-producing bacteria and the pH values. Finally, the 'packaging type' was found to affect the microbial association, the pH and sensory values.

3.2. Shelf life of beef fillets stored in air at 0, 5, 10, 15 and 20˚C

3.2.1. Microbial association and growth kinetics

Figure 3.2.1. presents the growth curves of TVC, *Pseudomonas* spp., *Br. thermosphacta*, lactic acid bacteria and *Enterobacteriaceae*. Table 3.2.1 summarizes kinetic parameters of the natural microbiota groups, following data fitting using the Baranyi and Roberts' model (1994). The initial microbiota of the beef fillets comprised, in decreasing order of magnitude, of *Pseudomonas* spp., *Br. thermosphacta*, lactic acid bacteria (LAB) and *Enterobacteriaceae*. The contribution of these groups to the natural microbiota depended strongly on the temperature (Table 3.2.1).

During the aerobic storage of the fillets, the maximum specific growth rate (μ_{max}) of pseudomonads was higher than of that of the rest microorganisms, with *Br. thermosphacta* following very closely. The μ_{max} of *Enterobacteriaceae* becomes similar to that of pseudomonads and *Br. thermosphacta* as the storage temperature increases, decreasing its lag phase. Finally, the LAB growth rate was always lower than the others, but the difference decreased with storage temperature.

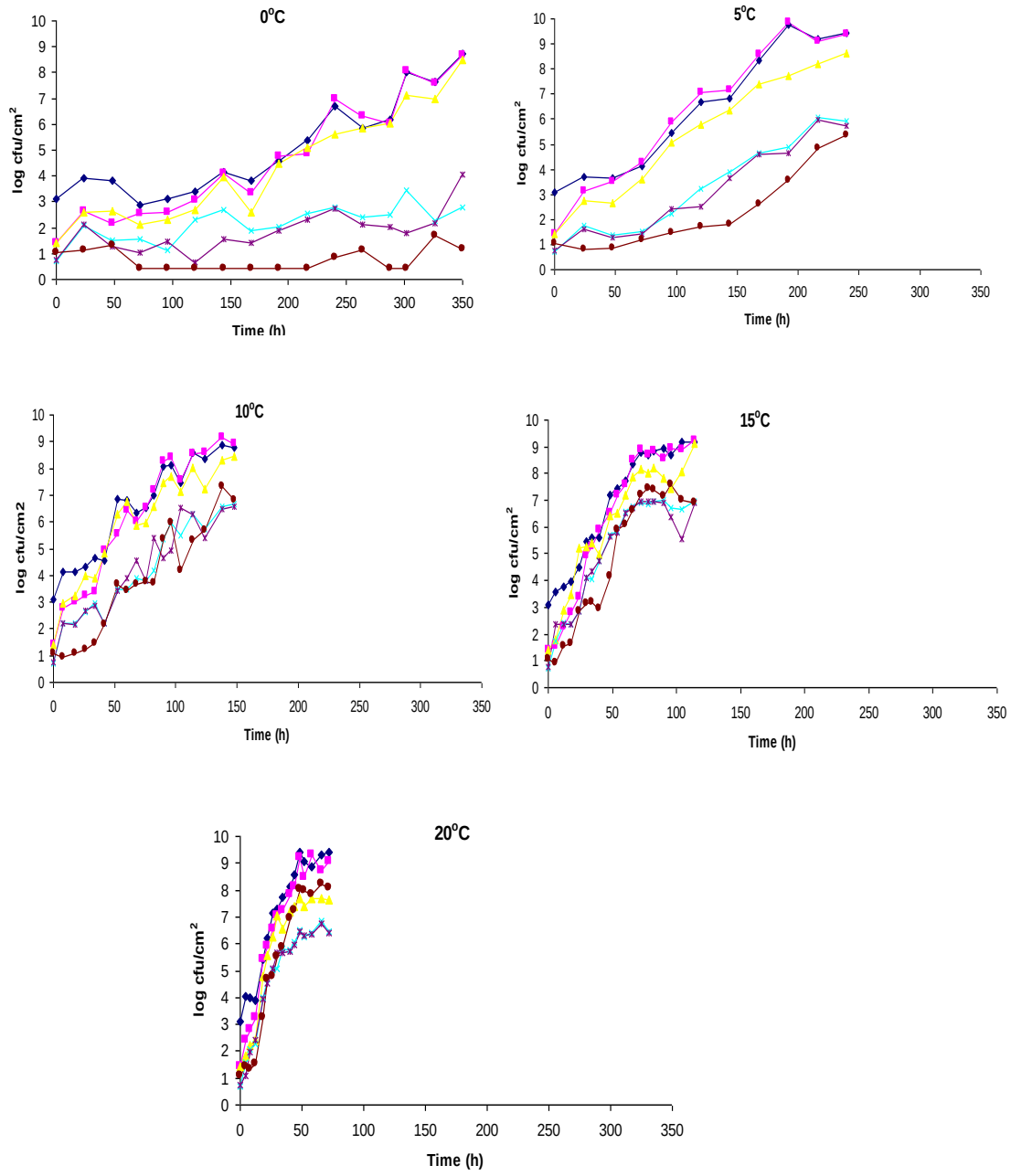


Figure 3.2.1. Growth curves of TVC (◆); pseudomonads (■); *Br. thermosphacta* (▲); LAB pH 5.2 (✱); LAB pH 5.7(×); and *Enterobacteriaceae* (●) at 0, 5, 10, 15 and 20ºC.

Table 3.2.1 Kinetic parameters of the natural microbiota following data fitting (Baranyi's model)

Temperature (°C)	Microorganisms	Initial Population (log cfu cm^{-2})	Final Population (log cfu cm^{-2})	μ_{max} [c]	lag	R^2
0	TVC	2.89	8.71 [a] (∞)[b]	0.0575	125.20	0.95
	Pseudomonads	1.41	8.69 (∞)	0.0562	75.68	0.95
	Br.thermosphacta	1.41	8.48 (∞)	0.0617	119.50	0.94
	Enterobacteriaceae	1.01	1.19 (∞)	0.0365	298.70	0.15
	LAB 5.2	1.11	2.80 (∞)	0.0119		0.40
	LAB 5.7	1.01	2.79 (2.83)	0.0117		0.74
5	TVC	2.89	9.44 (9.47)	0.0908	42.54	0.97
	Pseudomonads	1.41	9.37 (9.46)	0.1010		0.98
	Br.thermosphacta	1.41	8.62 (8.66)	0.0803		0.98
	Enterobacteriaceae	1.01	5.38 (∞)	0.0933	130.90	0.97
	LAB 5.2	1.11	5.72 (∞)	0.0552	45.30	0.95
	LAB 5.7	1.01	5.92 (∞)	0.0595		0.95
10	TVC	2.89	8.78 (8.78)	0.1110		0.92
	Pseudomonads	1.41	8.93 (8.85)	0.1561		0.95
	Br.thermosphacta	1.41	8.44 (8.32)	0.1308		0.95
	Enterobacteriaceae	1.01	6.78 (∞)	0.1079	16.63	0.95
	LAB 5.2	1.11	6.57 (6.55)	0.1026		0.93
	LAB 5.7	1.01	6.67 (∞)	0.0855		0.94
15	TVC	2.89	9.15 (9.07)	0.1936		0.98
	Pseudomonads	1.41	9.22 (8.93)	0.2685		0.98
	Br.thermosphacta	1.41	9.14 (8.04)	0.2272		0.95
	Enterobacteriaceae	1.01	6.91 (7.43)	0.2623	19.48	0.97
	LAB 5.2	1.11	6.93 (6.89)	0.2068		0.97
	LAB 5.7	1.01	6.96 (6.90)	0.2193		0.98
20	TVC	2.89	9.43 (9.18)	0.3115		0.98
	Pseudomonads	1.41	9.08 (9.10)	0.4069		0.97
	Br.thermosphacta	1.41	7.64 (7.50)	0.4527		0.98
	Enterobacteriaceae	1.01	8.10 (8.08)	0.4110	4.74	0.98
	LAB 5.2	1.11	6.42 (6.29)	0.3898		0.97
	LAB 5.7	1.01	6.47 (6.42)	0.3518		0.97

[a] Determined experimentally , [b] Estimated by the Baranyi model, ∞ Fitted curve was completed without upper asymptote (semisegmoidal), [c] μ_{max} : maximum specific growth rate

3.2.2 Sensorial evaluation of beef fillets spoilage in comparison with TVC and pH

The shelf life of beef fillets stored aerobically was found to be 10d, 4d, 82h, 60h, and 40h at 0, 5, 10, 15 am 20 °C based on sensory evaluation. The sensory evaluation of the spoilage was not always correlated with the same microbial load but depended on the temperature. The products microbial load at the time of the first sensorial detection of spoilage (meat characterized as semi-fresh) increased with temperature (from 4.01 to 7.17), and so did the pH values with minor differences. Also, the end shelf life of the product (meat characterized as Spoiled) corresponded to different microbial loads (from 6.68 to 8.15 cfu cm^{-2}) that also increased with temperature.

Table 3.2.2 Sensorial evaluation of beef fillets spoilage in comparison with TVC and pH

	Initial Point	**0˚C**		**5˚C**		**10˚C**		**15˚C**		**20˚C**	
		SF	**S**	**SF**	**S**	**SF**	**S**	**SF**	**S**	**SF**	**S**
TVC (log cfu cm^{-2})	3.10	4.01	6.68	5.44	6.80	4.89	7.02	5.62	7.74	7.17	8.15
pH	5.59	5.55	5.60	5.54	5.66	5.53	5.92	5.56	5.62	5.72	6.00
Time (h)	0	168 [a]	240 [b]	96	144	42	82	40	60	26	40

[a] The time (h) that the panellists characterised the product as semi-fresh (SF)

[b] The time (h) that the panellists characterised the product as spoiled (S)

3.3. Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

3.3.1. Microbial association and shelf life

The microbiological analysis revealed that the initial microbiota of minced beef consisted of *Pseudomonas* spp., *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria, yeasts and moulds. The succession of these groups and their contribution to the final microbiota was greatly influenced by temperature and type of storage. When minced beef was stored in air, all microbial groups had higher viable counts compared with the packaging under MAP and with the presence of the volatile compounds of oregano essential oil (MAP/OEO-active packaging). Aerobic storage accelerated spoilage due to the fast growing *Pseudomonas* spp., which was found to be the dominant microorganism at all temperatures tested. Packaging under modified atmospheres (MAP) delayed the growth rates of all members of the microbial association, as well as the maximum population attained by each microbial group compared with aerobic storage. Moreover, modified atmosphere packaging favoured the emergence of facultative anaerobic populations including lactic acid bacteria and *Br. thermosphacta*. The most profound changes were evident in samples treated with volatile compounds of oregano essential oil (OEO). This significantly affected the development of the microbial communities of minced beef stored under modified atmospheres. In this case, the final counts of all members of the microbial association, with the exception of lactic acid bacteria, were even lower when compared with the samples stored under MAP. It should be noted that under MAP, and even more so under MAP/OEO, the inhibition of the *Enterobacteriaceae* group was observed in samples stored at low temperatures in comparison with samples stored at abuse temperatures.

According to the sensory analysis, the end of shelf life was strongly dependent on packaging and temperature conditions. The shelf life of the minced beef decreased with increasing storage temperature. Samples stored under aerobic conditions were scored as semi-fresh and spoiled more rapidly than samples stored under MAP conditions regardless of storage temperature. In addition, the combination of MAP and oregano essential oil allowed an increased shelf life of minced beef compared to aerobic or MAP packaging and positively affected the odour and colour of minced beef (pleasant aroma and vivid red colour). In comparison with aerobic packaging, the extension of shelf life in meat samples stored with oregano essential oil was 12, 21, 106 and 176 h at 15, 10, 5, and 0°C, respectively. Similarly, but to a lesser extent, MAP, when compared with aerobic packaging, enabled the extension of mince shelf life for 6, 9, 82 and 95h at 15, 10, 5, and 0°C, respectively. The type of muscle spoilage under aerobic conditions was characterized by putrefaction, whereas in the case of MAP and MAP/OEO, spoilage was characterized by muscle souring.

3.4. Shelf life of minced beef stored in air and MAP at 5˚C

3.4.1. Microbial association and shelf life

The microbiological analysis revealed that the initial microbiota of the minced beef consisted of *Pseudomonas* spp., *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria (LAB), yeasts and moulds. During the aerobic storage of minced beef, *Pseudomonas* spp. were the dominant microorganisms, followed by *Br. thermosphacta*, yeasts and moulds, LAB and *Enterobacteriaceae* (Figure 3.4.1). Packaging under MAP delayed the growth of the pseudomonads, yeasts and moulds, and *Enterobacteriaceae* and suppressed the maximum level of the aerobic counts compared with the aerobic storage, whilst affected positively the growth of *Br. thermosphacta* and LAB (Figure 3.4.1.; Table 3.4.1). Table 3.4.1 summarizes the estimates by the primary model of Baranyi and Roberts (1994) for the final population, lag phase and maximum specific growth rate for the total viable counts (TVC), pseudomonads, *Br. thermosphacta*, *Enterobacteriaceae*, LAB, yeasts and moulds for each of the packaging condition tested.

The sensory evaluation of the mince defined the end of shelf life at 60 h for aerobic storage and 72 h for storage under MAP at 5 °C (Figure 3.4.1.). The type of muscle spoilage under aerobic conditions was characterized by putrefaction, whilst in the case of MAP the spoilage was characterized by muscle souring.

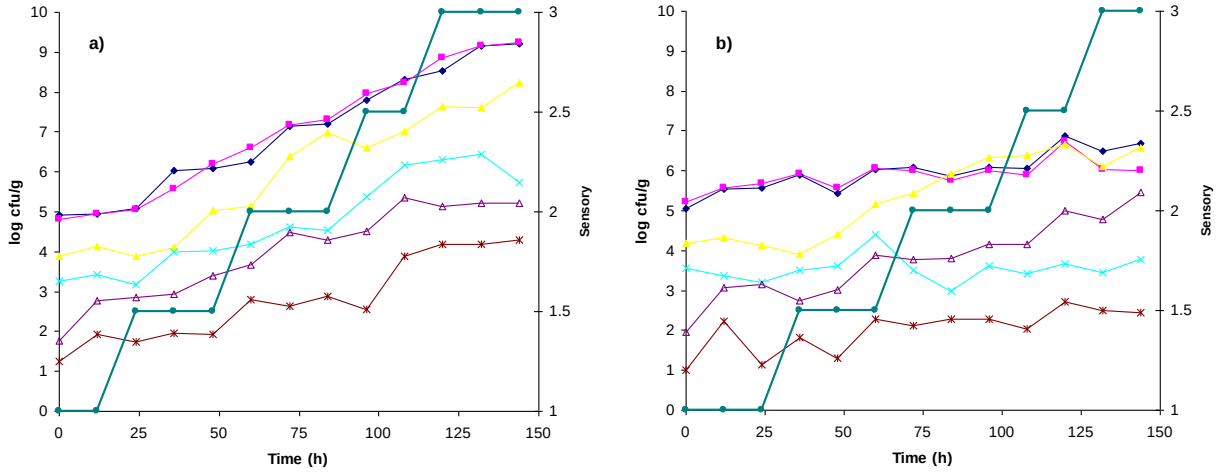


Figure 3.4.1. Growth curves of all the tested microbial groups (primary axis) and sensory scores (secondary axis) for the minced beef stored aerobically and under MAP at 5ºC; (♦) Total Viable Counts, (■) pseudomonads, (▲) *Br. thermosphacta*, (x) yeasts and moulds, (△) LAB, (*) *Enterobacteriaceae*, (●) Sensory scores.

Table 3.4.1 Kinetic parameters of the microbial association of the meat estimated by the primary model of Baranyi and Roberts (1994).

Temperature (ºC)	Microorganisms	Initial Population (log cfu cm^{-2})	Final Population (log cfu cm^{-2})	μ_{max} [c]	lag	R^2
Air	TVC	4.91	9.22 [a](∞)[b]	0.0789	8.99	0.98
	Pseudomonads	4.80	9.23 (∞)	0.0823	12.66	0.96
	Br.thermosphacta	3.89	8.24 (∞)	0.0829	21.55	0.94
	Yeasts-Moulds	3.25	5.74 (6.19)	0.0906	43.69	0.92
	LAB	1.78	5.22 (6.28)	0.0679	1.39	0.94
	Enterobacteriaceae	1.24	4.29 (∞)	0.0586	30.12	0.85
MAP	TVC	5.05	6.68 (∞)	0.0264	43.56	0.67
	Pseudomonads	5.21	6.00 (∞)	0.0159	30.90	0.52
	Br.thermosphacta	4.18	6.57 (6.44)	0.1064	42.60	0.92
	Yeasts-Moulds	3.57	3.77 (∞)	0.0081	23.28	0.42
	LAB	1.95	5.47 (∞)	0.0509	-	0.94
	Enterobacteriaceae	1.00	2.43 (∞)	0.0295	13.68	0.80

[a] Determined experimentally , [b] Estimated by the Baranyi model, ∞ Fitted curve was completed without upper asymptote (semisegmoidal), [c] μ_{max} : maximum specific growth rate

The pH values at the beginning of storage were within the normal range for fresh beef (Borch *et al.,* 1996), with the initial mean value of 5.75. There was a decrease in pH for all samples stored under MAP in relation to storage time, whilst an increase was observed in pH values of all samples stored aerobically. The final mean pH values for aerobic storage and storage under MAP were 6.11 and 5.60 respectively.

3.5. FTIR analysis

The raw meat samples from each experiment which included 150 minced beef (survey of minced beef), 72 from beef fillets (stored aerobically) and 186 minced beef (stored under air, MAP and MAP/OEO) samples were analysed using FTIR. Figure 3.5.1 presents spectra collected from samples of beef fillets stored aerobically at 5ºC. A major peak at 1640 cm^{-1} due to the presence of moisture (O-H stretch) with an underlying contribution from amide I in the meat sample was apparent, whereas a second peak at 1550 cm^{-1} appeared due to the absorbance of amide II (N-H bend, C–N stretch). A second amide vibration was shown at 1400 cm^{-1} (C–N stretch), followed by amide III peaks at 1315 and at 1240 (C-N stretch, N-H bend, C-O stretch, O=C-N bend). A small feature seen at about 1745 cm^{-1} is due to fat (C=O ester) and the peaks at 1460, 1240 and 1175 cm^{-1} can also be attributed to fat. Finally, the peaks arising from 1025 to 1140 could be absorbance due to amines (C-N stretch) (Ellis *et al.,* 2002, 2004; Rajamäki *et al.,* 2006; Socrates 2001; Chen *et al.,* 1998). More details about the possible assignments of each peak observed in the FTIR spectra are given in Table 3.5.1.

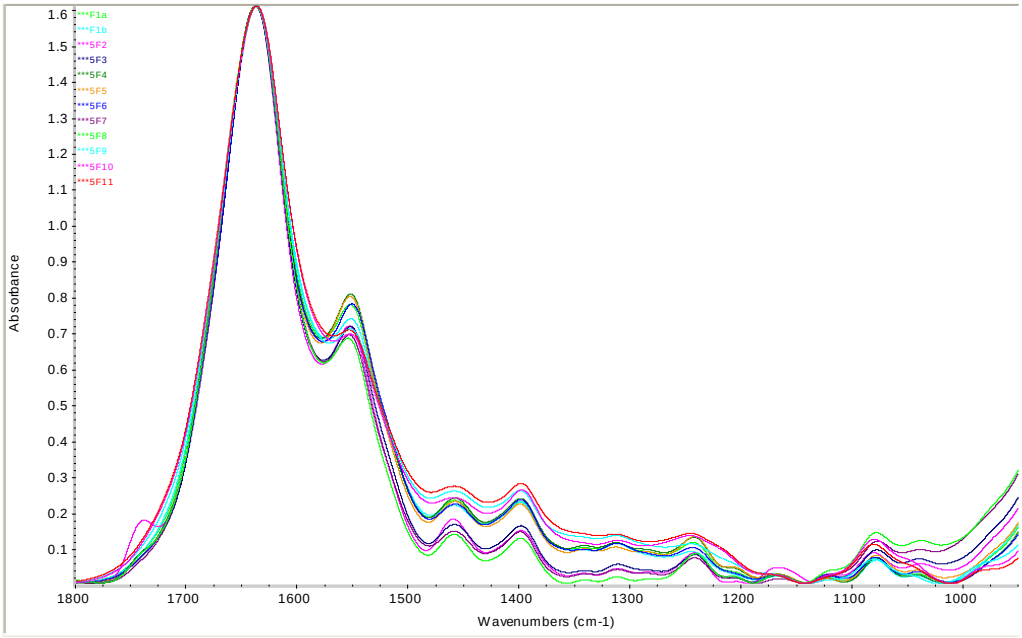


Figure 3.5.1. Typical FTIR spectra in the range of 1800 to 900 cm^{-1} collected from beef fillets stored at 5°C for 10 days. Different colours represent different sampling days

Table 3.5.1 Observed FTIR frequencies and possible assignments of the vibration modes

Frequency (cm^{-1})	Assignment	Reference
1720-1580 (1640)	water (H-O-H def vib) and amide I band (80% C=O stretch, 10% C-N stretch, 10% N-H bend)	Socrates 2001, Wu *et al.* 2006
1580-1520 (1550)	amide II band (40%CN str, 60%NH bend vib)	Bocker *et al.* 2007
1520-1480 (1520 sh)	amide II (combination of C–N stretch and N–H bend)	Ellis *et al.* 2004, Kaiden *et al.* 1987
1480-1461 (1468 sh, 1461 sh)[a]	Lipids (CH_3 asym def, CH_3 asym bend, C-H def of CH_2,CH_2 scissoring vib, C-H bend) or amines (asym CH3 def vib)	Socrates 2001, Chen *et al.* 1998, Bocker *et al.* 2007
1460-1432 (1456, 1440sh)	CH2 bending	Bocker *et al.* 2007
1430-1413 (1413 sh)	amide (C–N stretch) or lipids (RCOO- sym str COO- , -CH_2-COOR def vib of CH_2)	Ellis *et al.* 2004, Socrates 2001
1413-1371 (1396)	amino acid side chains, lipids (CH bend or C-O stretch in carboxylates) or nitro group (NO_2 sym stretch)	Pappas *et al.* 2008, Pistorius 1995, Ellis *et al.* 2004
1370-1365 (1371sh)	free amines (C–N stretch)	Ellis *et al.* 2004
1365-1358 (1365 sh)	primary and secondary aliphatic amines (X-sensitive band)	Socrates 2001
1355-1344 (1344 sh)	amines -CH_2NH_2 (CH_2 twisting vib)	Socrates 2001
1344-1331 (1338)	CH_2 side-chain vibrations, free amino acids/long chain aliphatic carboxylic acids (CH stretch /CH_2 def vib)	Bocker *et al.* 2007, Socrates 2001
1330-1294 (1310)	amide III a-helical structures (30% C-N stretch, 30% N-H bend,10% C=O-N bend, 20% other)	Bocker *et al.* 2007
1293-1275 (1284)	amide III β-turn (30% C-N stretch, 30% N-H bend,10% C=O-N bend, 20% other)	Bocker *et al.* 2007
1273-1263 (1263 sh)	amide III random coil (30% C-N stretch, 30% N-H bend,10% C=O-N bend, 20% other)	Socrates 2001
1262-1212 (1240)	lipids, nucleic acids (asym PO_2- stretch), amide III P=O stretch (30% C-N stretch, 30% N-H bend,10% C=O-N bend, 20% other), amines from free amino acids (C-N stretch)	Socrates 2001, Pappas *et al.* 2008, Pistorius 1995, Chen *et al.* 1998, Ellis *et al.* 2002
1211-1199 (1205 sh)	amide III (30% C-N stretch, 30% N-H bend,10% C=O-N bend, 20% other), amines (CN stretch)	Bocker *et al.* 2007, Socrates 2001
1200-1190 (1196 sh)	amines -$N(CH_3)_2$ (CH_3 rock and asym CCN stretch)	Socrates 2001
1185-1143 (1165 double)	fat related (C-O stretch), esters (C-O-C), carbohydrates (C-O stretch), –NH_2 def	Chen *et al.* 1998, Bocker *et al.* 2007, Pappas *et al.* 2008
1140-1110 (1124)	riboses (C-O stretch), amines (NH_2 rock/twist)	Bocker *et al.* 2007, Socrates 2001
1108-1065 (1078)	nucleic acids and phospholipids (PO_2 sym stretch) / C-O stretch	Bocker *et al.* 2007
1052-1036 (1043)	lipids, polysaccharides (C-O , C-O-P stretch)	Bocker *et al.* 2007
1036-1020 (1032, -[b])	polysaccharides (C-O stretch), amines (CN stretch)	Socrates 2001, Bocker *et al.* 2007
1020-1008	Polyglycines	Socrates 2001
983-965	a, b, pyranose compounds (ring vib), aromatic carboxylic acids	Socrates 2001

[a] two peaks observed at this region, with the same possible assignment. [b] second peak with no well schemed edge. Abbreviations: def: deformation, vib: vibration, stretch: stretching, bend: bending, sym: symmetric, asym: asymmetric, rock: rocking, twist: twisting, sh: shoulder.

3.5.1. Survey of minced beef from the Greek Market

The wavenumbers that were selected from the first PCA to be significant in this data set ranged from 1718 to 1207 cm^{-1} and were selected for further analyses. The FDA provided classifications of the samples based on the Freshness (Figure 3.5.2.), the type of Packaging (Figure 3.5.3), the Season (Figure 3.5.4) for each sample bought and the Day of purchase . The performance of each model (correct classification the built model and validations) is presented in Table 3.5.2. The performance of the PLS-R models was inadequate to estimate the microbial counts (low R^2 and relative high RMSE values) (Table 3.5.3) and thus the results are further analysed.

Table 3.5.2 Quality of fit of the FDA models for minced beef samples of the Survey and validations

Type of Analysis	Correct Classification (Train) (%)	Correct Classification (Validation) (%)
Freshness	91.28	61.74
Packaging	92.62	79.19
Season	93.96	80.54
Day	84.56	61.74

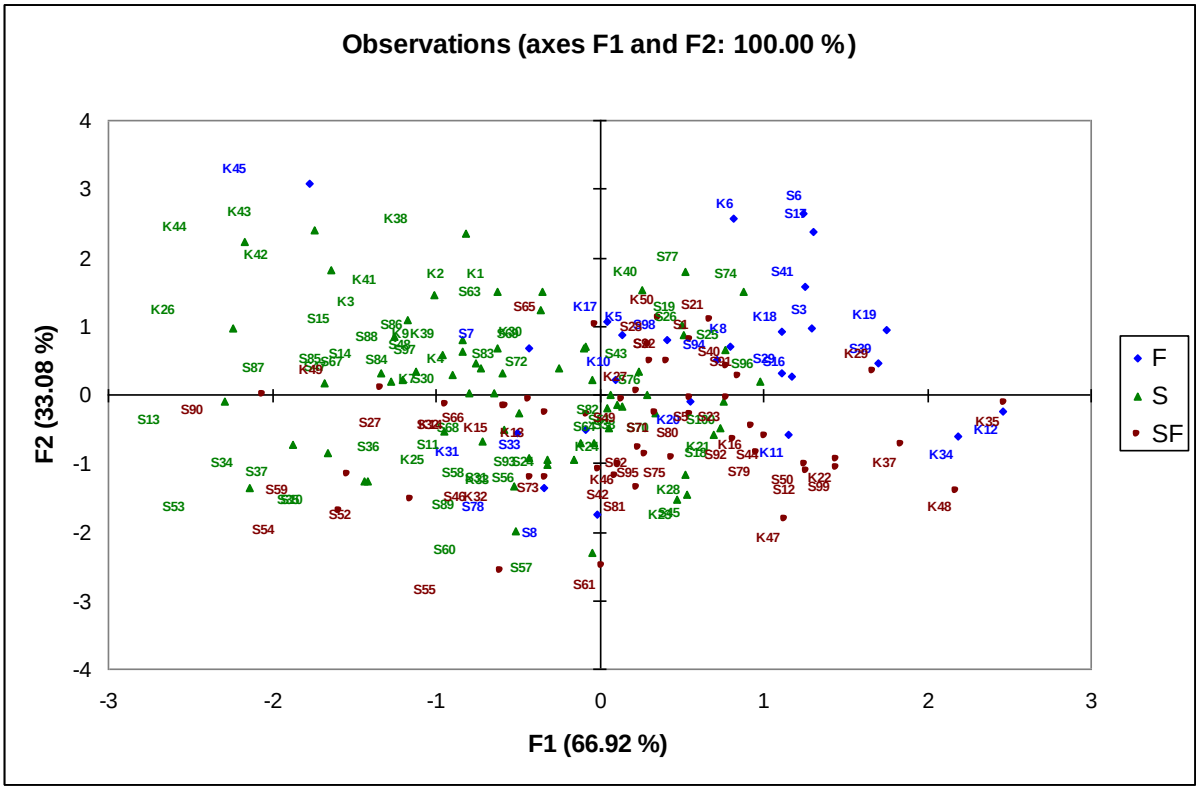


Figure 3.5.2. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 different minced beef (Survey samples) freshness groups : Fresh (F), Semi-fresh (SF), and Spoiled (S).

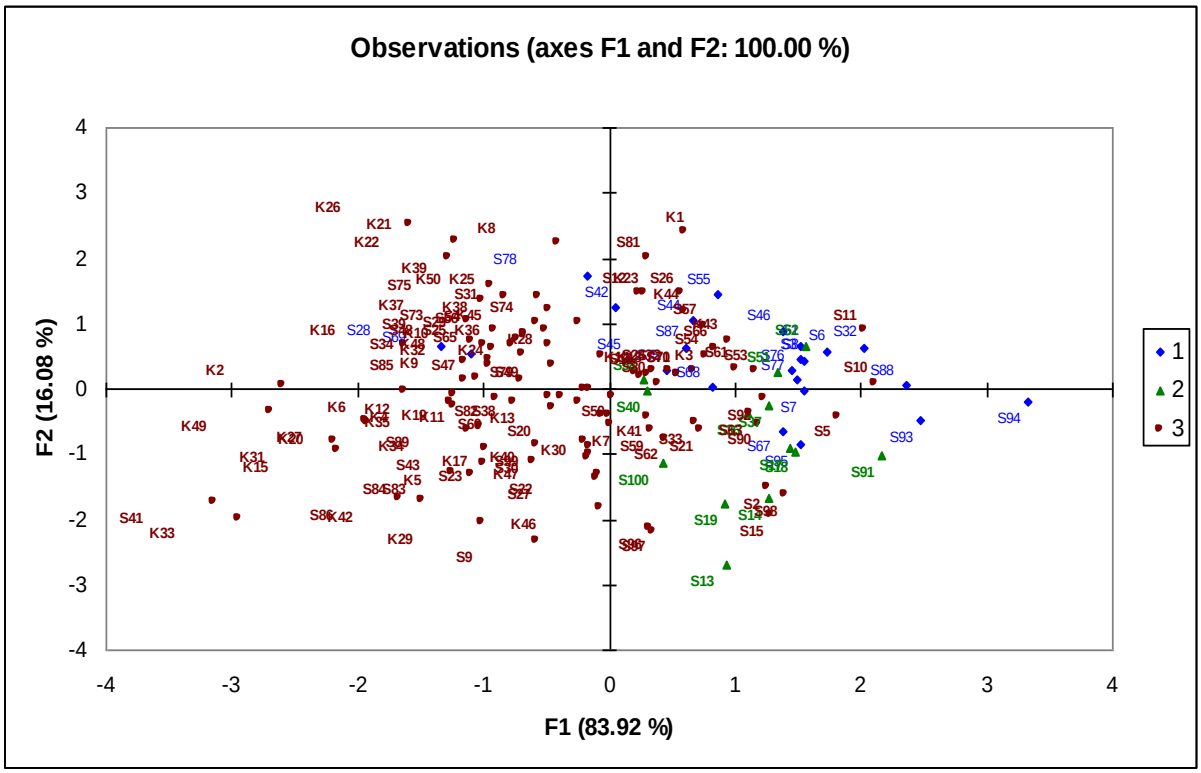


Figure 3.5.3. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 packaging groups of the minced beef Survey: Modified Atmosphere Packaging (1), Wrapped with flexible transparent film (2), and freshly cut at the time of purchase (3).

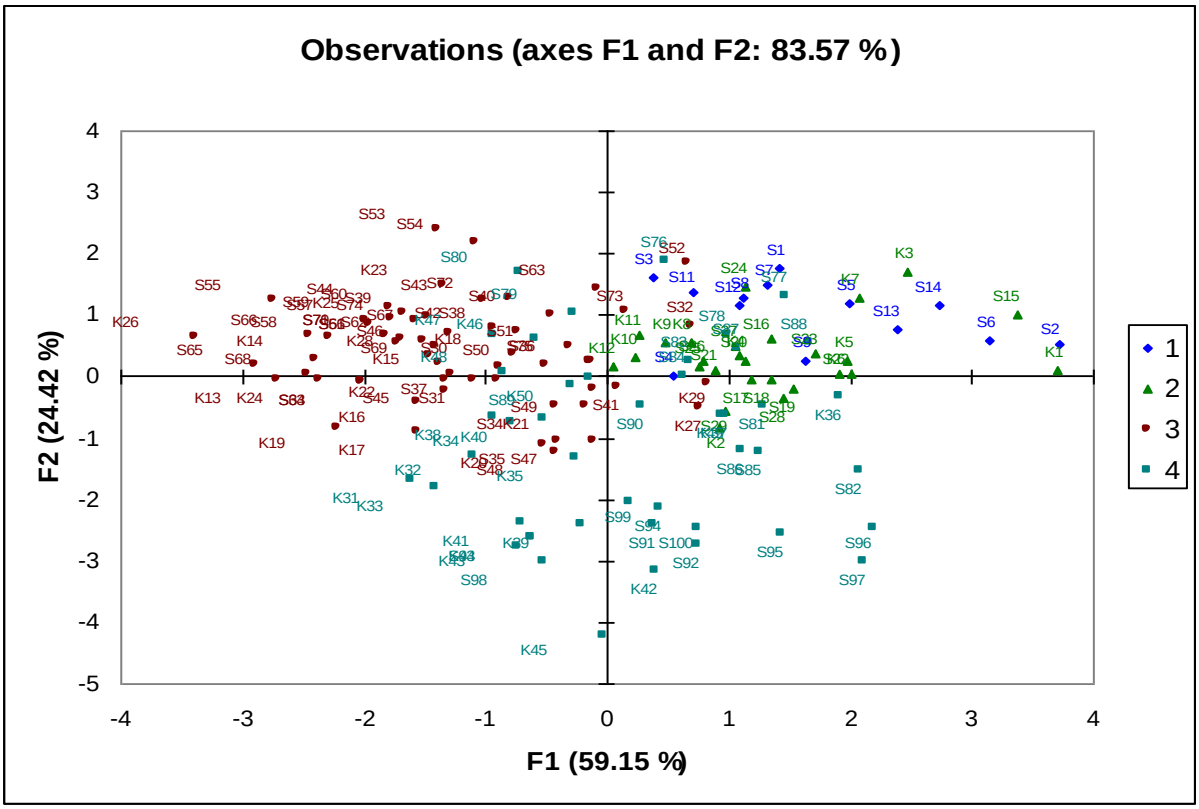


Figure 3.5.4. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 4 different Seasons that the minced beef Survey samples where purchased: Spring (1), Summer (2), Autumn (3) and Winter (4).

Table 3.5.3 Root mean square errors and R^2 for the calibration and validation estimates of each PLS-R model built from the Survey minced beef FTIR measurements

Microbial group	Train		Validation	
	R^2	RMSE	R^2	RMSE
TVC	0.42	0.67	0.07	0.86
Pseudomonads	0.46	0.70	0.08	0.92
Br. thermosphacta	0.61	0.60	0.60	0.61
H_2S-producing bacteria	0.59	0.85	0.28	1.14
LAB (pH 5.7)	0.46	0.75	0.13	0.96
LAB (pH 5.2)	0.48	0.75	0.17	0.95
Enterobacteriaceae	0.54	0.70	0.29	0.90

3.5.2 Shelf life of beef fillets stored in air at 0, 5, 10, 15 and 20˚C

The wavenumbers that were selected from the first PCA to be significant in this data set ranged from 1718 to 1203 cm^{-1} and from 1020 to 1001 cm^{-1} were selected for further analyses. The FDA exhibited a correct classification of 98.68% (training), whilst the validated FDA provided 81.58% correct classification, showing a good correlation between the sensory detection of the spoilage status and that of chemical metabolites during spoilage as detected from the FTIR. The classification accuracies of the FDA regarding the sensory groups are presented in detail in Table 3.5.4. Figure 3.5.5. depicts the easily distinguished group of Spoiled samples from Fresh and Semi-fresh ones. The results for the FDA regarding the discrimination due to the temperature were 93.42% for the training and 55.26% for the validation (Figure 3.5.6.).

Table 3.5.5 presents the RMSE, the R^2 and the % RE values for the models built for the FTIR beef fillets measurements. In general, the performance was found to be similar between the different models, whilst it was observed that better estimations were obtained for LAB, followed by the the TVC. Indicatively, Figure 3.5.7 shows the percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota of minced beef samples according to the PLS-R models. The distribution of percent relative error (% RE) values above or below 0 showed if there was an under- or over-prediction. Regarding the estimations given by all models, the % RE values were distributed above and below 0, with a general over-prediction of the models at lower population densities and a trend of under-prediction especially at higher population densities; TVC estimates showed these trend for counts less than 6 log cfu g^{-1} and more than 6 log cfu g^{-1}, whilst LAB, pseudomonads, *Br. thermosphacta* and *Enterobacteriaceae* estimates showed this trends for counts less than 5 log cfu g^{-1} and more than 5 log cfu g^{-1}.

Table 3.5.4 Confusion matrix according to the FDA for the validation of the beef fillets sensory estimates

True class	Estimated class			Correct Classification (Sensitivity %)
	Fresh	Semi-fresh	Spoiled	
Fresh (n = 26)	18	0	8	69.23
Semi-fresh (n=16)	1	13	2	81.25
Spoiled (n =34)	3	0	31	91.18
Total (n =76)	22	13	41	81.58
Specificity (%)	81.81	100.00	75.61	

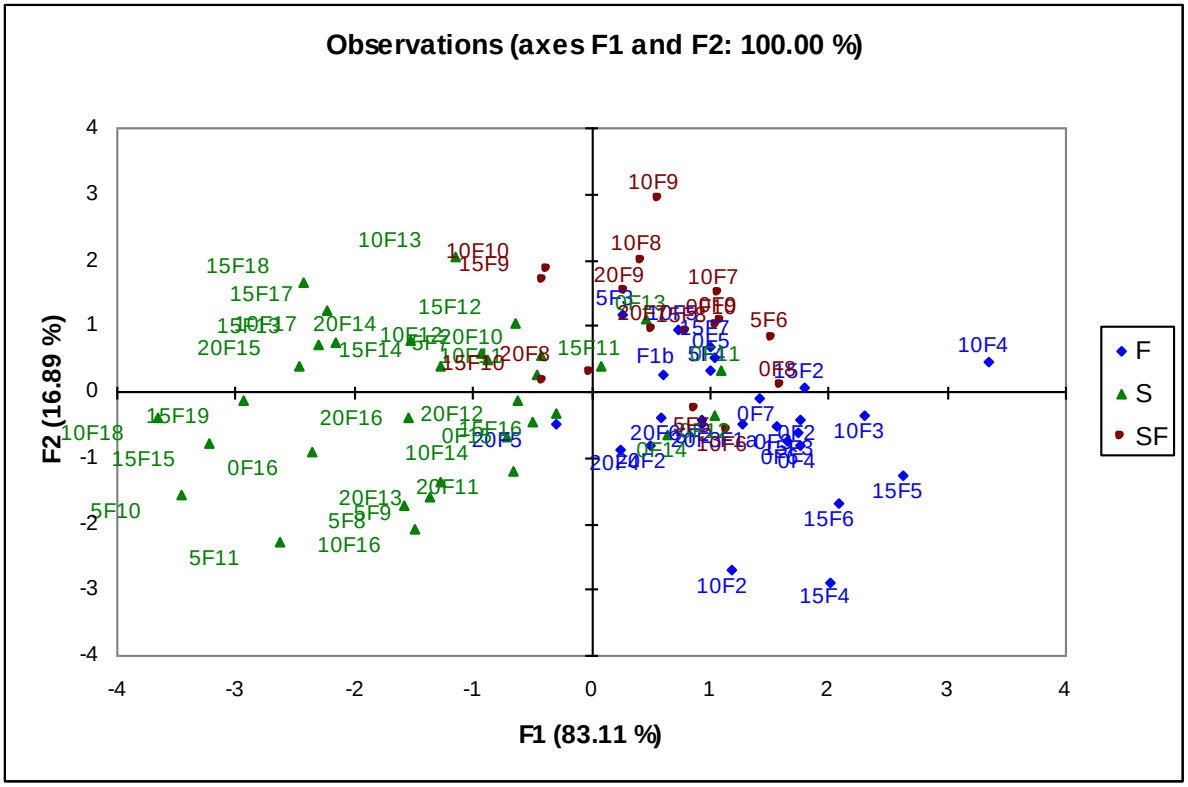


Figure 3.5.5. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 different beef fillets freshness groups: Fresh (F), Semi-fresh (SF), and Spoiled (S).

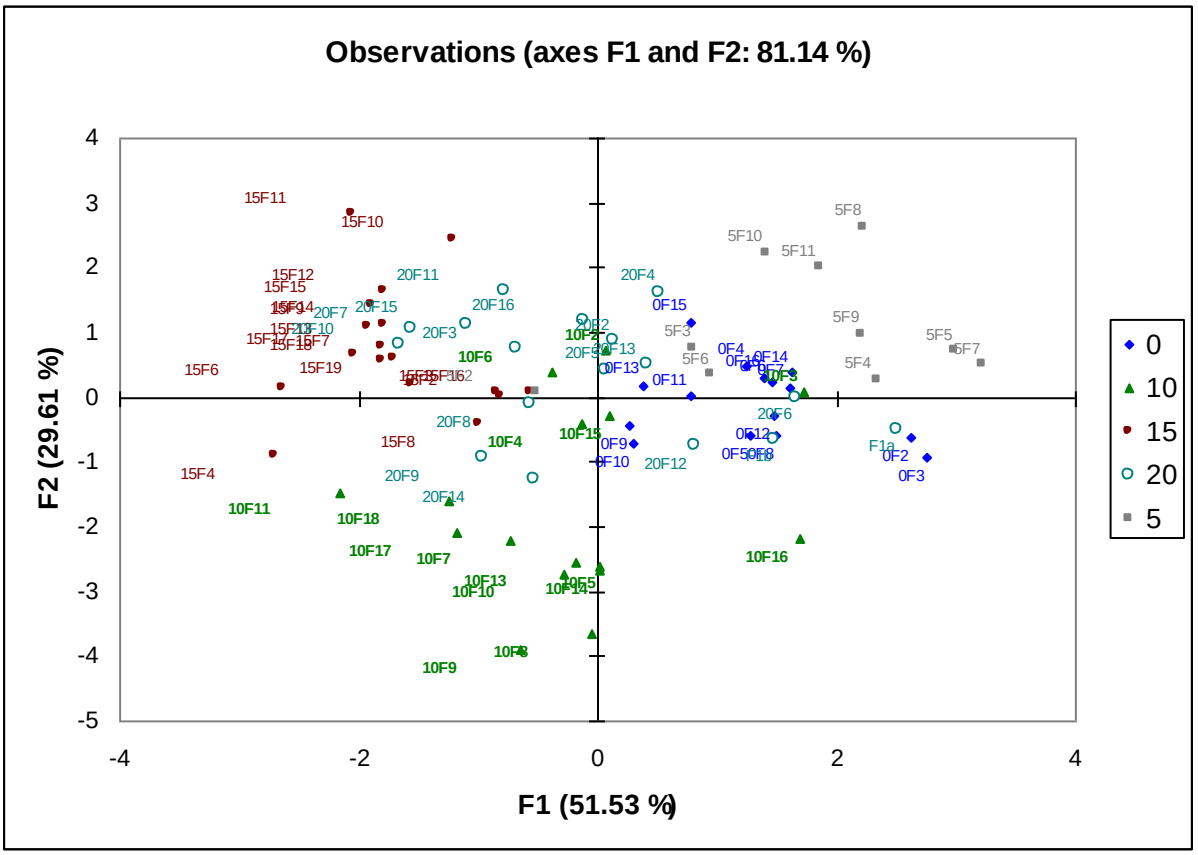


Figure 3.5.6. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 5 different storage temperatures of beef fillets: 0, 5, 10, 15 and 20°C. The samples stored at abuse temperature conditions (10, 15, 20°C) are distributed to the left side of the map, whereas the samples stored at chill temperatures (0, 5°C) are distributed to the right.

Table 3.5.5 Comparison of calculated performance indices for the estimation of the microbial population in beef fillet samples using the validation estimates from the PLS-R /FTIR models

Microbial group	No of latents	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	1	61.04	37.66	0.60	1.34
***Pseudomonas* spp.**	1	59.74	37.66	0.59	1.59
Br. thermosphacta	1	62.34	27.27	0.55	1.47
LAB pH 5.7	1	61.04	37.66	0.65	1.17
LAB pH 5.2	1	61.04	37.66	0.68	1.15
Enterobacteriaceae	1	62.34	27.27	0.64	1.57

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

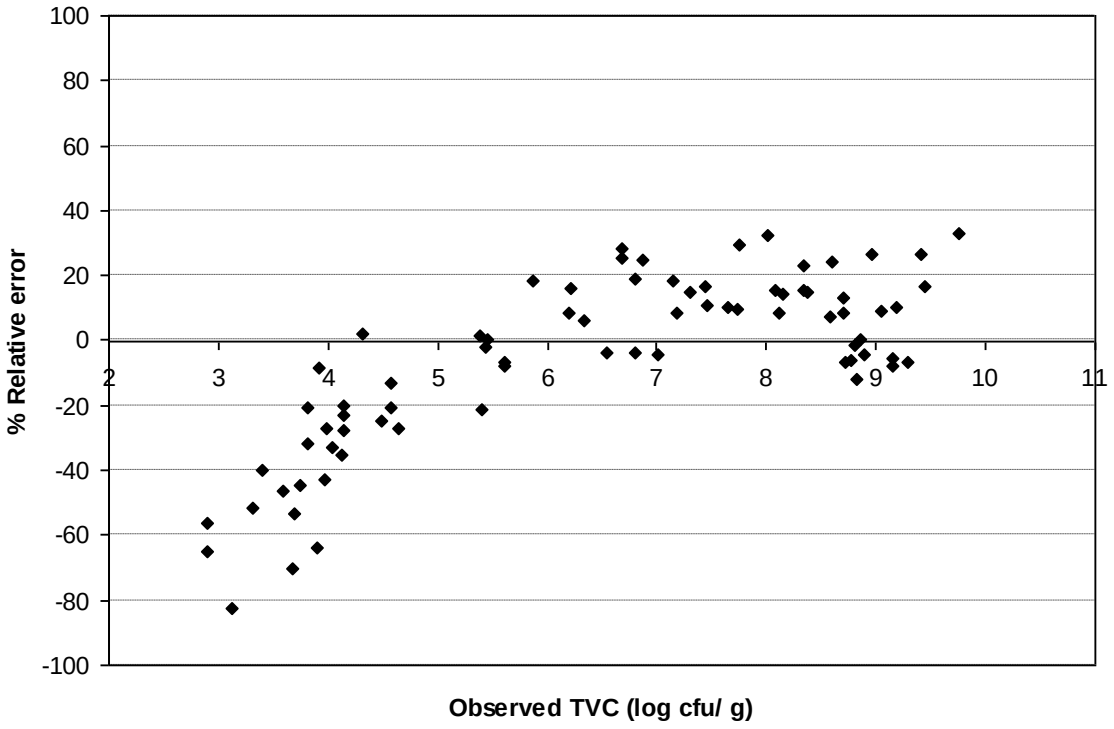


Figure 3.5.7. Percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota (TVC) of beef fillets samples according to the PLS-R /FTIR model

3.5.3. Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

The wavenumbers that were selected from the first PCA to be significant in this data set ranged from 1714 to 1710 cm^{-1}, 1614 to 1211 cm^{-1} and from 1031 to 1000 cm^{-1} were selected for further analyses. The FDA provided classifications of the samples regarding the Freshness (Figure 3.5.8), the type of Packaging (Figure 3.5.9), the storage Temperature (Figure 3.5.12) and the combination of the storage temperature and packaging. The percentages of the correct classification (the built model) and of the validations of each model are presented in Table 3.5.6, whilst Table 3.5.7 presents the performance of the FDA according to the correct classification of the sensory scores for each class.

Table 3.5.8 present the RMSE, the R^2 and the % RE values for the models built for the FTIR minced beef measurements. In general, it was observed that better estimations were obtained for LAB, followed by the *Enterobacteriaceae* and the TVC. Indicatively, Figure 3.5.10 shows the percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota of minced beef samples according to the PLS-R models. The distribution of percent relative error (% RE) values above or below 0 showed if there was an under- or over-prediction. Regarding the estimations given by all models, the % RE values were distributed above and below 0, with a general over-prediction of the models at lower population densities and a trend of under-prediction especially at higher population densities; TVC, LAB and yeasts/moulds estimates showed these trends for counts less than 7 log cfu g^{-1} and more than 7 log cfu g^{-1}, whilst pseudomonads, *Br. thermosphacta* and *Enterobacteriaceae* estimates showed this trends for counts less than 5 log cfu g^{-1} and more than 5 log cfu g^{-1}.

Table 3.5.6 Quality of fit of the built FDA /FTIR models for minced beef samples and validations

Type of Analysis	Correct Classification (Train) (%)	Correct Classification (Validation) (%)
Freshness	100.00	76.34
Packaging	100.00	92.47
Temperature	100.00	88.11
Temperature and Packaging	98.38	71.89

Table 3.5.7 Confusion matrix according to the FDA /FTIR models for the validation of the sensory estimates

True class	Estimated class			Correct Classification (Sensitivity %)
	Fresh	**Semi-fresh**	**Spoiled**	
Fresh (n = 65)	45	20	0	69.23
Semi-fresh (n=66)	7	53	6	80.30
Spoiled (n =55)	0	11	44	80.00
Total (n =186)	52	84	50	76.34
Specificity (%)	86.53	63.10	88.00	

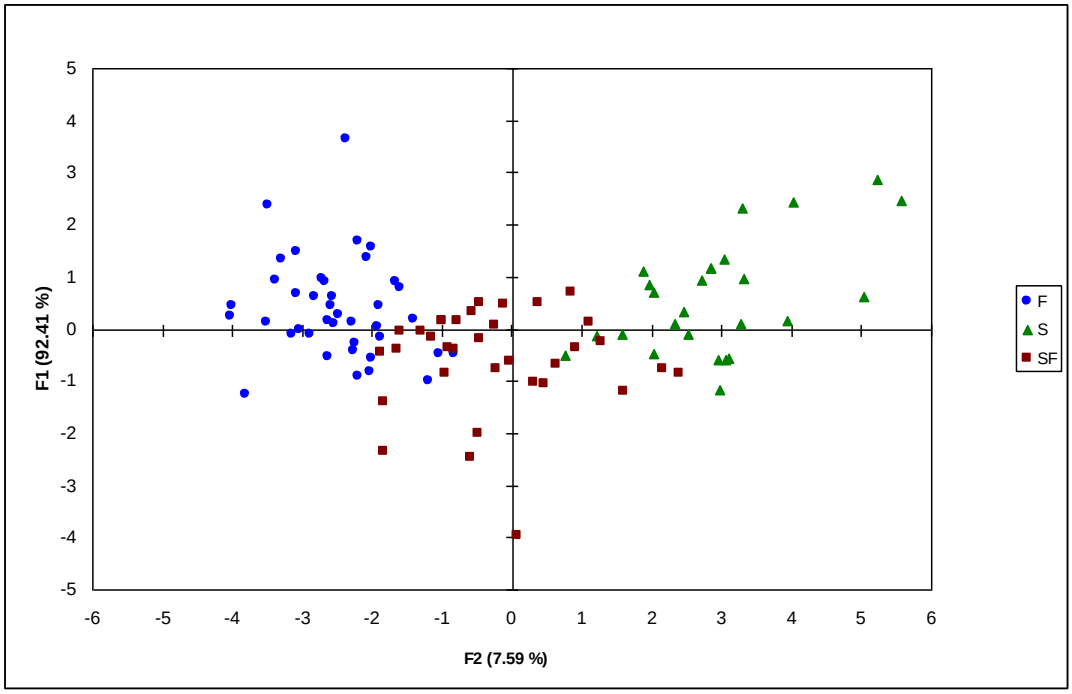


Figure 3.5.8. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 different minced beef freshness groups: Fresh (F), Semi-fresh (SF), and Spoiled (S).

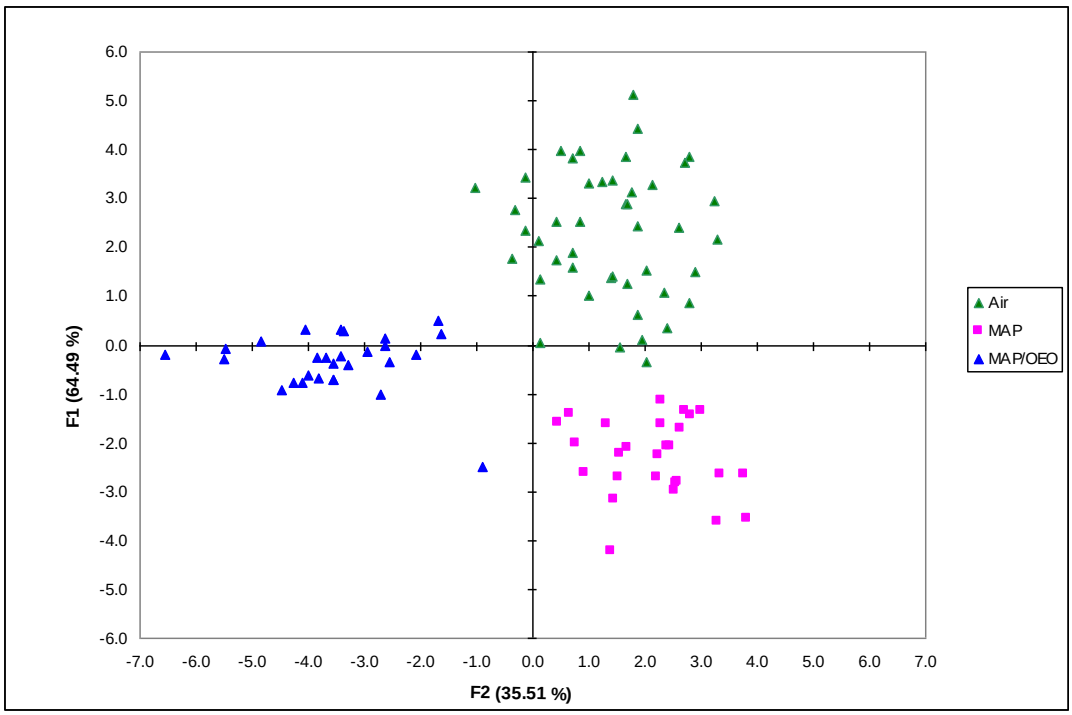


Figure 3.5.9. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 different minced beef packaging groups: aerobic packaging (Air), modified atmosphere packaging (MAP), active packaging (MAP/OEO)

Table 3.5.8 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the PLS-R /FTIR models

Microbial group	No of latents	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	2	95.16	73.66	0.72	0.72
Pseudomonas spp.	2	87.10	64.52	0.81	0.88
Br. thermosphacta	1	75.27	46.24	0.67	0.63
LAB	2	94.09	75.27	0.50	0.96
Enterobacteriaceae	2	88.17	66.67	0.84	0.69
Yeasts & Moulds	2	86.02	60.22	0.74	0.84

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

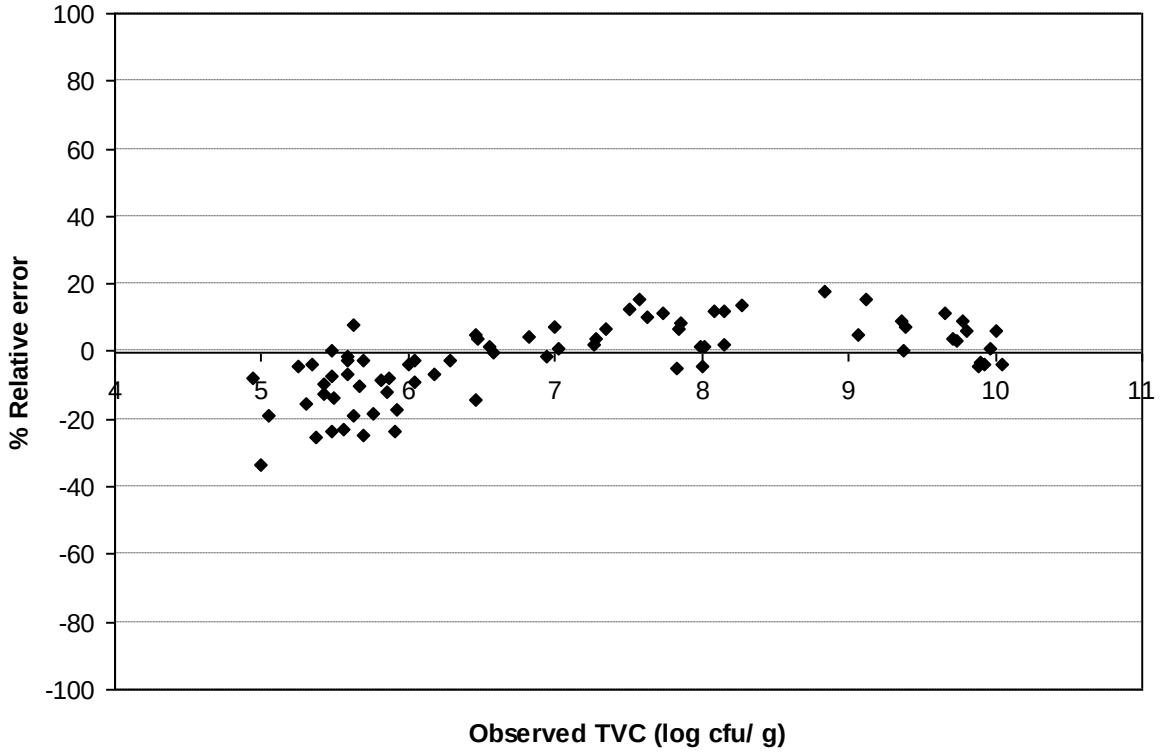


Figure 3.5.10. Percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota (TVC) of minced beef samples according to the PLS-R /FTIR model

3.6. Raman analysis and comparison with FTIR

3.6.1. Raman analysis

Typical spectral data obtained from the Raman in the range of 3400 to 200 cm^{-1} collected from minced beef stored aerobically and under MAP at 5 °C are shown in Figure 3.6.1. Tentative assignments for the observed peaks in the Raman spectra are shown in Table 3.6.1. and have been referenced in the literature. It has to be noted that for this experiment and for both Raman and FTIR data all the range of the wavenumbers were used for the calibrationmodels.

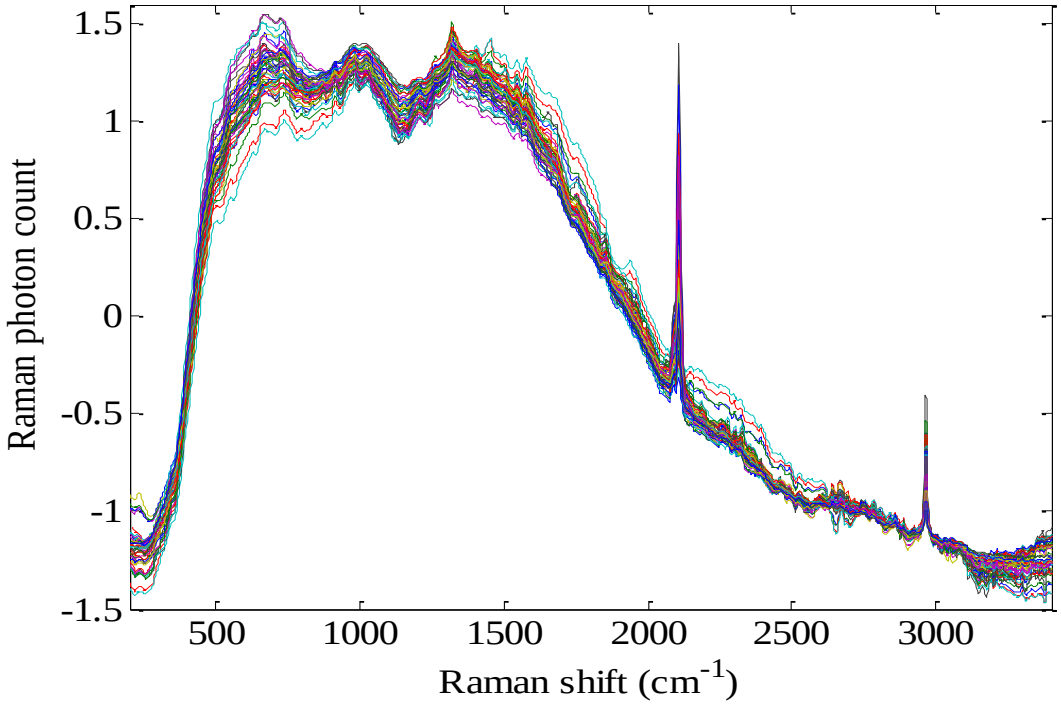


Figure 3.6.1. Raman spectra collected from minced beef samples stored aerobically and under MAP at 5ºC

Table 3.6.1 Observed Raman frequencies and possible assignments of the vibration modes

Frequency (cm^{-1})	Assignment	Reference
3397-3309 (num)[a]	amido acids, amino acids, carbohydrates, polypeptides,amines (NH stretch)/ water, carbohydrates, lipids (OH stretch)	Socrates 2001, Yang *et al.* 1993
3309-3291 (3300)	amino acids/proteins/amines (NH stretch)	Socrates 2001
3284-3250 (3275, 3261)[b]	amines (N-H stretch), peracids (O-H stretch)	Socrates 2001
3250-3219 (3247, -[c])	water, lipids (OH stretch)	Socrates 2001
3228-3100 (num)	free amino acids, lipids (NH_3^+ stretch)	Socrates 2001
3100-3088	aliphatic amino acids, peptides, and proteins(C-H stretch vib)	Herrero *et al.* 2004
3088-3075 (3081)	polyglycines (NH_2 stretch)	Socrates 2001
3072-3041 (3066, 3050)	ketones (CH stretch)	Socrates 2001
3041-3022 (3028)	lipids (NCH_3, CH_3 stretch)	Socrates 2001
3019-3009	amines (CH_3 stretch), ketones (CH stretch), lipids (=C-H stretch)	Sarkadei & Howel 2007, Socrates 2001
3006-2991 (2994)	free amino acids (CH str)	Wong *et al.* 2007, Socrates 2001
2988-2947 (2966)	aliphatic amino acids,amines, proteins, lipids (CH_3 stretch)	Socrates 2001, Herrero 2008b
2947-2800 (num)	polyglycines, proteins, amines, kerones, aldehydes, lipids (CH_3, CH_2, CH stretch)	Sarkardei & Howell, 2007, Socrates 2001, Herrero *et al.* 2004, Greve *et al.* 2008
2797-2769 (2784, 2778)	aryl aldehydes (overtone CH in-plane def vib)	Socrates 2001
2769-2703 (num)	free amino acids (sym -NH_3^+ stretch), aldehydes (CH stretch, overtone CH in-plane def vib)	Socrates 2001
2700-2684 (2691)	Lipids RCOOH (O-H stretch, hydrogen bonded)	Socrates 2001
2684-2628 (2672, 2640)	free amino acids	Socrates 2001
2628-2597 (-, 2625,2602)	amido acids	Socrates 2001
2594-2547 (2591,1572,2556)	cysteinyl residues (stretch vib of the S-H group)	Herrero 2008b
2547-2263 (num)	nd	
2263-2225 (2254, -)	nitrile group (–C---N)	Thygesen *et al.* 2003
2244-2141 (num)	nd	
2141-1997 (num)	free amino acids (NH_3^+ stretch)	Socrates 2001
1997-1950	nd	
1950-1884 (1941,1910,-)	amido acids	Socrates 2001
1881-1866	cyclopropenones (C=O)	Socrates 2001
1866-1838	aliphatic aminoacids (C-H stretch, CH_2 groups)	Socrates 2001
1834-1803	cyclopropenones, cyclobutenediones (C=O stretch)	Socrates 2001
1791-1766	cyclobutanone derivatives	Socrates 2001
1766-1747 (1753)	ketones, free amino acids, peroxy acids, carbohydrates, nucleic acids (C=O stretch)	Socrates 2001
1747-1734	cyclopentanones, ketones, amido acids/lipids ester (C=O stretch)	Socrates 2001
1731-1708 (1708sh)	a-diketones-CO-CO	Socrates 2001

Table 3.6.1 *(Continued)*

Frequency (cm^{-1})	Assignment	Reference
1708-1691 (1691sh)	a,b- unsat carboxylic acids (as dimer)	Socrates 2001
1691-1672 (1681)	unsuturated ketones, aldehydes, lipids (C=C stretch)	Socrates 2001, Herrero 2008a
1672-1644 (1659)	proteins with high a-helical content, amide I band (80% CO stretch, 10%CN stretch, 10%NH bend vib)	Socrates 2001, Herrero 2008a
1634-1603 (1622sh)	amino acids (ring stretch)	Herrero 2008b
1603-1588 (num)	amide II (mixtures of C–N stretch and N–H bend coordinates)	Balakrishnan *et al.* 2008
1494-1447 (1482, 1456)	amines (CH_3, CH_2 asym def vib), lipids (CH_2 scissoring vib, CH3 asym bend), bending vibrational modes of amino acid functional groups	Wong *et al.* 2007, Socrates 2001
1450-1434 (1450 sh)	aliphatic aminoacids, proteins (CH_3, CH_2, CH bend)	Herrero 2008b, Wong *et al.* 2007
1425-1394 (1406)	amino acids, lipids (RCOO- sym str COO- , -CH2-COOR def vib of CH_2)	Socrates 2001, Herrero 2008b
1391-1381	amide S (Ca–H bending), CH_2 (CH_2 sym bend)	Balakrishnan *et al.* 2008, socrates
1372-1359	amino acids (stretch and bend), aliphatic amines	Wong 2007, Herrero 2008b, Socrates 2001
1359-1347	amines	Socrates 2001
1347-1297 (1322)	amino acids, proteins, carboxylic acids (CH_3, CH_2, CH vib),	Herrero 2008b, Socrates 2001, Greve *et al.* 2008
1341-1313	free amino acids (CH stretch)	Socrates 2001
1313-1288	aryl aldehydes/ ketones	Socrates 2001
1288-1275	amide III (α-helix structure which overlaps with the region assigned for β-turns)	Herrero 2008b
1275-1250 (1266)	amide III (random coil, β-sheets), fat (=C–H symmetric rock (cis))	Herrero 2008b, Marquardt & Wold 2007
1250-1234 (1236)	amide III (b-sheets, undefined or random coil structures)	Herrero 2008b, Wong *et al.* 2007
1234-1219	amide III (β-sheet structure -10% CO stretch, 30% CN stretch, 30% NH bend, 10% O=C-N bend, rest other vib), free amino acids - dicarboxylic (C-O stretch)	Socrates 2001
1219-1197 (1210)	amines, lipids (CN stretch), amino acids	Bocker *et al.* 2007, Socrates 2001
1194-1178	amines (CN str), carbox acids (C-O str)	Socrates 2001
1166-1138 (double)	C-C stretch, COH def, saccharide components (ring stretch)	Bocker *et al.* 2007, Socrates 2001
1138-1122	aliphatic amines (CN stretch)	Socrates 2001
1122-1106 (1109)	aromatic amines (NH_2 rocking/twisting), saturated primary amines , Saccharide components (ring stretch vib), PO_2- (sym PO_2- stretch)	Socrates 2001
1106-1091 (1097)	nucleic acids (sym stretch of phosphate group), lipids (PO_2-, CO stretch)	Socrates 2001
1091-1066	amines (CN stretch, CH_3 rock)	Socrates 2001
1066-1047 (1056, 1047sh)	amines (CN stretch, CH_3 rock) , lipids/proteins (CC, C-O, C-O-P stretch)	Marquardt & Wold 2004, Bocker *et al.* 2007, Herrero 2008b, Socrates 2001

Table 3.6.1 *(Continued)*

Frequency (cm^{-1})	Assignment	Reference
1047-1013 (1031)	Saccharide components (ring stretch vib), C-C skeletal stretch (in aggregated b-structures)	Socrates 2001, Bocker *et al.* 2007
1013-1005	amino acids (ring stretch)	Herrero 2008a, Wong *et al.* 2007, Marquardt & Wold 2004
1003-975 (984)	aromatic carbox acids	Socrates 2001
975-953 (968)	a,b-pyranose compounds (ring vib), amino acids pyranose ring (term CH_2 def vib), lipids (CN stretch)	Socrates 2001
953-934	a-helical structure (C-C stretch)	Herrero 2008b
934-903 (915)	amino acids pyranose ring (asym ring)	Herrero 2008b, Socrates 2001
903-887 (893)	amino acids pyranose ring (anomeric C-H def), C-C stretch in a-helix	Socrates 2001, Bocker *et al.* 2007
887-862 (875)	CH bend, CH_2 rock, amino acids pyranose ring (equatorial C-H def other than anomeric C-H def /ring vib)	Wong *et al.* 2007, Bocker *et al.* 2007, Herrero 2008b
862-850 (856)	C–C stretch	Yang *et al.* 2003
850-828 (818)	sulfolipids (C-O-S stretch), lipids (P-O asym stretch)	Socrates 2001
812-784 (800)	primary aliphatic amines	Socrates 2001
784-765	amino acids pyranose ring (sym ring breathing)	Socrates 2001
765-750 (756)	amino acids side chain and C–H bend vib	Herrero *et al.* 2004, Wong *et al.* 2007
750-734 (740)	secondary amines	Socrates 2001
734-712 (734sh)	proteins amide V (N-H bend)	Socrates 2001
712-681 (696, -)	amino acid residues (C-S stretch), polyglycines (CH2 rock vib, NH def vib), aromatic carboxylic acids (CO2 in-plane def vib)	Herrero 2008b, Bocker *et al.* 2007, Socrates 2001
680-643br (648)	amino acid residues (C-S stretch), aliphatic aldehydes/ketones (C-C-CO in-plane def vib)	Herrero 2008b, Bocker *et al.* 2007, Socrates 2001
640-618 (631)	amino acid residues (C-S str), proteins- amide IV (40% O=C-N bend, rest other vib)	Herrero 2008b, Bocker *et al.* 2007, Socrates 2001
600-565	aldehydes/ketones (C-CO in-plane def vib), amino acids and C–H bending vibrations	Wong et al, 2007, Socrates 2001
565-518 (556)	carbox acids (CO2 out-of-plane rock def vib), free amino acids (CO2- or C-C-N def vib), aldehydes (C-CO in-plane def vib)	Socrates 2001
498-256 (num)	amines/carboxylic acids	Socrates 2001
253-221 (240)	secondary aliphatic amines (Sat.)NHCH3(CH_3 torsional vib)	Socrates 2001

[a] a number of peaks (more than 3) observed at this region, with the same possible assignment. [b] two peaks observed at this region, with the same possible assignment. [c] two peaks observed at this region, second peak with no well schemed edge. Abbreviations: num: a number of peaks, def: deformation, vib: vibration, stretch: stretching, bend: bending, sym: symmetric, asym: asymmetric, rock: rocking, twist: twisting, sh: shoulder.

3.6.2. Estimation of the microbial populations and sensory scores using the Raman data

Tables 3.6.2 to 3.6.5 present the RMSE, the R^2 and the % RE values for the models built for the Raman measurements. It was observed that better estimations were obtained for TVC, LAB and *Enterobacteriaceae*. The SVR_S, performed worse than the other SVR models (exhibited high RMSE) and the results were not further analysed for this case. A trend of underprediction of all the counts was observed especially in larger microbial loads in some cases, which was more intense for the estimations of yeasts and moulds. Indicatively, Figure **3.6.2.** shows the percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota of minced beef samples according to the SVR_R. For all the Raman models more than 70% of the estimated microbial counts were included within the ± 20% RE zone, whilst the SVR_R and SVR_P models showed better performances (Tables 3.6.4 and 3.6.5).

Moreover, the distribution of percent relative error (% RE) values above or below 0 showed if there was an under- or over-prediction. Regarding the estimations given by all models, for TVC the % RE values were distributed above and below 0, with a trend of under-prediction was evident especially at higher population densities (counts more than 7 log cfu g^{-1}). As far as the models of the remaining microbial groups are concerned (data not shown), the % RE values were distributed above and below 0, with a general over-prediction of the models at lower population densities (counts less than 7 log cfu g^{-1}) and a trend of under-prediction especially at higher population densities (counts more than 7 log cfu g^{-1}).

The classification accuracies of the sensory estimates regarding the Raman models for each class and in total is shown in Table 3.6.6. A slightly better performance was observed for the SVR_P models (overall performance of 84.62% correct classification for the validation estimates).

Table 3.6.2 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the PLS-R /Raman models

Microbial group	No of latents	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	6	95.16	73.66	0.73	0.63
Pseudomonas spp.	3	87.10	64.52	0.52	0.81
Br. thermosphacta	6	75.27	46.24	0.74	0.55
LAB	6	94.09	75.27	0.71	0.73
Enterobacteriaceae	9	88.17	66.67	0.72	0.52
Yeasts & Moulds	5	86.02	60.22	0.62	0.68

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

Table 3.6.3 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the SVR_L /Raman models

Microbial group	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	93.85	78.46	0.72	0.68
Pseudomonas spp.	85.38	60.77	0.59	0.85
Br. thermosphacta	82.31	59.23	0.72	0.53
LAB	85.38	56.92	0.69	0.83
Enterobacteriaceae	72.31	41.54	0.61	0.65
Yeasts & Moulds	82.31	56.15	0.75	0.34

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

Table 3.6.4 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the SVR_R /Raman models

Microbial group	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	96.15	85.38	0.80	0.56
Pseudomonas spp.	89.23	75.38	0.70	0.71
Br. thermosphacta	86.92	57.69	0.79	0.46
LAB	90.77	66.92	0.73	0.71
Enterobacteriaceae	72.31	43.85	0.72	0.50
Yeasts & Moulds	82.31	56.92	0.63	0.63

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

Table 3.6.5 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the SVR_P /Raman models

Microbial group	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	96.15	82.31	0.79	0.57
Pseudomonas spp.	89.23	71.54	0.68	0.73
Br. thermosphacta	84.62	58.46	0.76	0.51
LAB	87.69	60.00	0.73	0.72
Enterobacteriaceae	70.00	42.31	0.77	0.43
Yeasts & Moulds	82.31	56.15	0.72	0.55

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

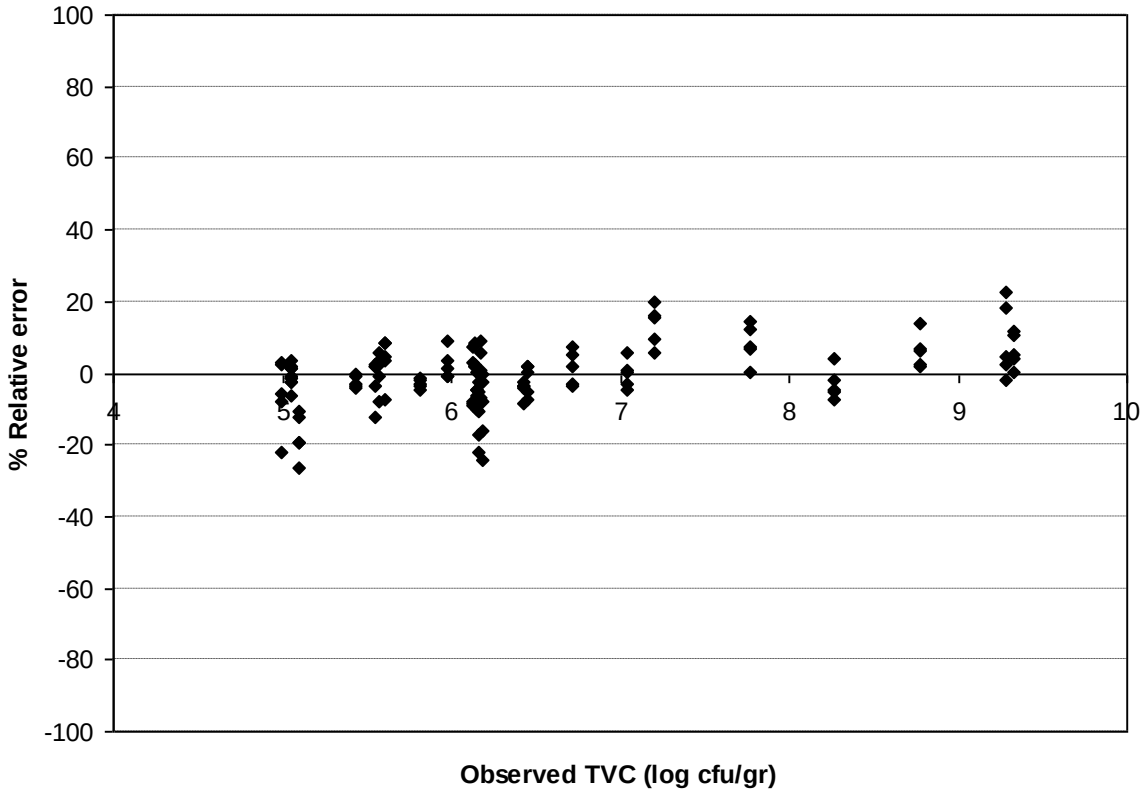


Figure 3.6.2. Percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota (TVC) of minced beef samples according to the SVR_R /Raman model

Table 3.6.6 Percentage of the correct classification of the validation sensory estimates for the Raman models

Class	**Correct Classification (%)**			
	PLS	**SVR_L** [a]	**SVR_R**	**SVR_P**
Fresh (*n* = 26)	80.77	73.08	69.23	73.08
Semi-fresh (*n*=30)	56.67	70.00	66.67	80.00
Spoiled (*n* =74)	90.54	90.54	87.84	90.54
Total (*n* =130)	80.77	82.31	79.23	84.62

3.6.3. Comparison of FTIR and Raman spectroscopy

Typical spectral data obtained from the FTIR in the range of 1800 to 900 cm^{-1} collected from minced beef stored aerobically and under MAP at 5 °C are shown in Figure 3.6.3. Tentative possible assignments for the observed peaks in the FTIR spectra are analysed in paragraph 3.5. and shown in details in Table 3.5.1. For comparison reasons, Tables 3.6.7 to 3.6.9 present the RMSE, the R^2 and the % RE values for the models built for both FTIR and Raman measurements. In general, it was observed that for both FTIR and Raman calibration models, better estimations were obtained for TVC, LAB and *Enterobacteriaceae*, whilst the FTIR models performed in general slightly better in estimating the microbial counts compared to the Raman models. The SVR_S, performed worse than the other SVR models (exhibited high RMSE for both FTIR and Raman data) and the results were not further analysed for this case. Regarding the FTIR models, no particular trend of over- or under-prediction was observed, except from estimates of the yeasts and moulds that were always under-predicted irrespective of the model and the *Br. thermosphacta* that showed a general trend of over-prediction in the case of PLS. For the Raman models, a trend of under-prediction of all the counts was observed especially in larger microbial loads in some cases, which was more intense for the estimations of yeasts and moulds. Table 3.6.9 that depicts the % RE values of the models, indicates that also for all the FTIR models, more than 70% of the estimated microbial counts were included within the ± 20% RE zone, except from the counts of yeasts and moulds that were underestimated and were totally outside of the acceptable range, no matter the model used. For the FTIR, the models used showed similar performances, whilst for the Raman the SVR_R and SVR_P models showed the better performances (Tables 3.6.7 to 3.6.9).

The classification accuracies of the sensory estimates regarding the FTIR and Raman models for each class and in total is shown in Table 3.6.10. A slightly better performance was

observed for the SVR_R and SVR_P models regarding the FTIR data (both showed overall performance of 87.5% correct classification for the validation estimates) and for the Raman data, the SVR_P models performed slightly better (overall performance of 84.62% correct classification for the validation estimates).

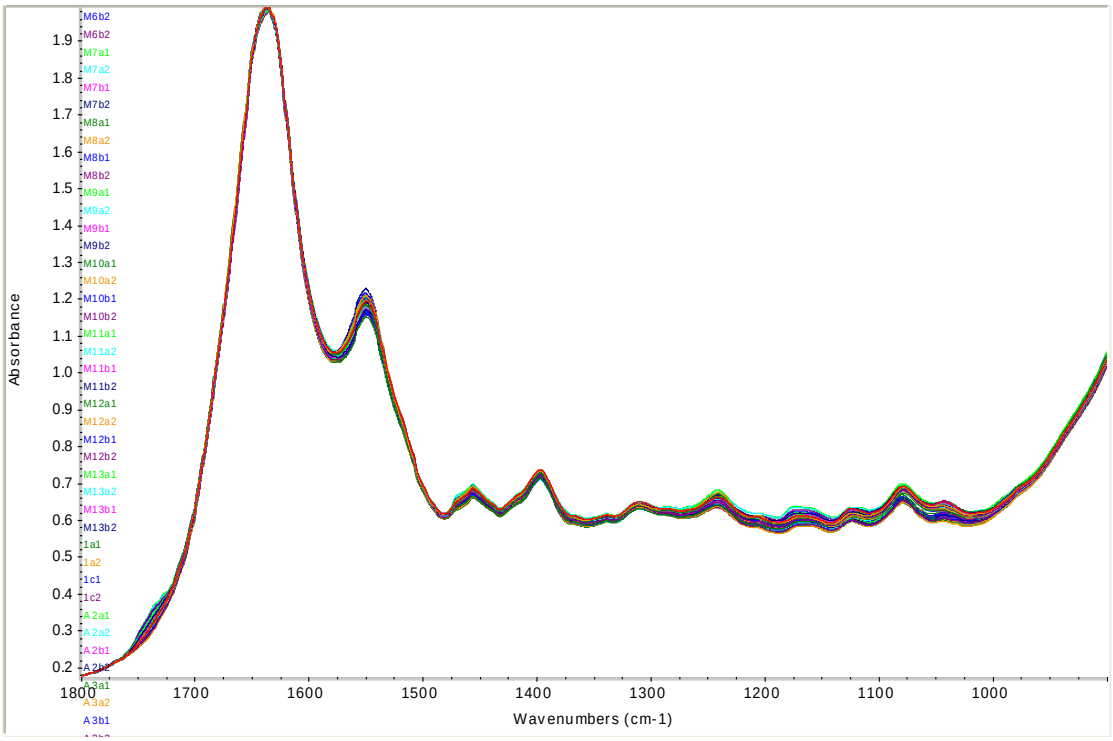


Figure 3.6.3. FTIR spectra collected from minced beef samples stored aerobically and under MAP at 5ºC

Table 3.6.7 Root mean square errors for the validation estimates for each FTIR and Raman model

	Model [a]	TVC	Pseudomonads	LAB	*Br. thermosphacta*	*Enterobacteriaceae*	Yeasts & Moulds	Sensory
FTIR	PLS	0.5472 (9[b])	0.6007 (9)	0.4368 (9)	0.6886 (9)	0.4442(4)	0.5478(7)	0.3937(9)
	SVR_L	0.5040	0.5662	0.4162	0.7846	0.4345	0.5516	0.3932
	SVR_R	0.5109	0.5793	0.4111	0.6849	0.4382	0.5154	0.3941
	SVR_P	0.5098	0.5648	0.4153	0.6842	0.4394	0.5475	0.3908
Raman	PLS	0.6301 (6)	0.8122 (3)	0.5513 (6)	0.7280 (6)	0.5245 (9)	0.6789 (5)	0.3228 (6)
	SVR_L	0.6777	0.8494	0.5328	0.8269	0.6502	0.3445	0.3932
	SVR_R	0.5629	0.7060	0.4626	0.7054	0.4961	0.6291	0.3277
	SVR_P	0.5713	0.7252	0.5107	0.7245	0.4345	0.5516	0.3932

[a] Number of latent variables used to calculate the PLS model. [b] SVR_L = linear. SVR_R = radial basis function. SVR_P = polynomial.

Table 3.6.8 R^2 for the validation estimates for each FTIR and Raman model

	Model [a]	TVC	Pseudomonads	LAB	*Br. thermosphacta*	*Enterobacteriaceae*	Yeasts & Moulds	Sensory
FTIR	PLS-R	0.8066	0.7885	0.8392	0.7269	0.7562	0.7172	0.6453
	SVR_L	0.8368	0.8129	0.8163	0.6693	0.7740	0.7240	0.6660
	SVR_R	0.8316	0.8036	0.8178	0.7329	0.7600	0.7629	0.6580
	SVR_P	0.8329	0.8147	0.8167	0.7347	0.7682	0.7201	0.6659
Raman	PLS-R	0.7259	0.5183	0.7449	0.7142	0.7169	0.6169	0.7834
	SVR_L	0.7205	0.5940	0.7220	0.6856	0.6068	0.7531	0.6660
	SVR_R	0.7951	0.7003	0.7874	0.7317	0.7232	0.6254	0.7781
	SVR_P	0.7893	0.6835	0.7649	0.7333	0.7740	0.7240	0.6660

[a] SVR_L = linear. SVR_R = radial basis function. SVR_P = polynomial.

Table 3.6.9 Percent of the samples in ± 20% relative error zone (20 % RE) for the validation estimates for each FTIR and Raman model

	Model [a]	TVC	Pseudomonads	LAB	*Br. thermosphacta*	*Enterobacteriaceae*	Yeasts & Moulds
FTIR	PLS-R	95.83	91.67	68.75	89.58	70.83	0.00
	SVR_L	95.83	91.67	89.58	89.58	72.92	0.00
	SVR_R	93.75	91.67	91.67	87.50	70.83	0.00
	SVR_P	95.83	91.67	87.50	81.25	75.00	0.00
Raman	PLS-R	95.16	87.10	75.27	94.09	88.17	86.02
	SVR_L	93.85	85.38	82.31	85.38	72.31	82.31
	SVR_R	96.15	89.23	86.92	90.77	72.31	82.31
	SVR_P	96.15	89.23	84.62	87.69	70.00	82.31

[a] SVR_L = linear. SVR_R = radial basis function. SVR_P = polynomial.

Table 3.6.10 Percentage of the correct classification of the validation sensory estimates for the FTIR and Raman models

	Class	Correct Classification (%)			
		PLS	SVR_L [a]	SVR_R	SVR_P
FTIR	**Fresh** (*n* =6)	33.33	16.67	33.33	33.33
	Semi-fresh (*n*=12)	83.33	91.67	100.00	100.00
	Spoiled (*n* =30)	90.00	93.33	93.33	93.33
	Total (*n* =48)	81.25	83.33	87.50	87.50
Raman	**Fresh** (*n* = 26)	80.77	73.08	69.23	73.08
	Semi-fresh (*n*=30)	56.67	70.00	66.67	80.00
	Spoiled (*n* =74)	90.54	90.54	87.84	90.54
	Total (*n* =130)	80.77	82.31	79.23	84.62

[a] SVR_L = linear. SVR_R = radial basis function. SVR_P = polynomial.

3.7. Image analysis

3.7.1. VideometerLab analysis

The spectral data obtained from the VideometerLab for the 18 wave numbers collected from minced beef stored aerobically and under MAP at 5 °C are shown in Figure 3.7.1a and Figure 3.7.1b respectively. Regarding the values of the spectra obtained for each storage condition some noticeable differences were visually observed. It should however be noted that for both cases, an increase was observed within the first 12h for the values of tested bands. The values of several bands exhibited a decrease during aerobic storage of meat, whereas an increase was observed in the case of MAP. More specifically, in the case of aerobic stored samples, the values of the bands 470 (blue), 645, 660, 700 (red), and 850, 870, 890 (NIR) showed a decrease, and so did the bands 505, 565 (green), 590 (amber) but to a lesser degree. The rest of the bands remained stable or showed a very low decrease during storage. In the case of MAP stored samples, the values of the bands 450, 470 (blue), and 870 (near infrared) showed an increase, and so did the bands 430 (ultra blue), 505, 565 (green) and 630 (red), but in a less degree. The rest of the bands remained stable or showed a very low increase during storage. The above changes were observed more intensely at aerobic stored samples.

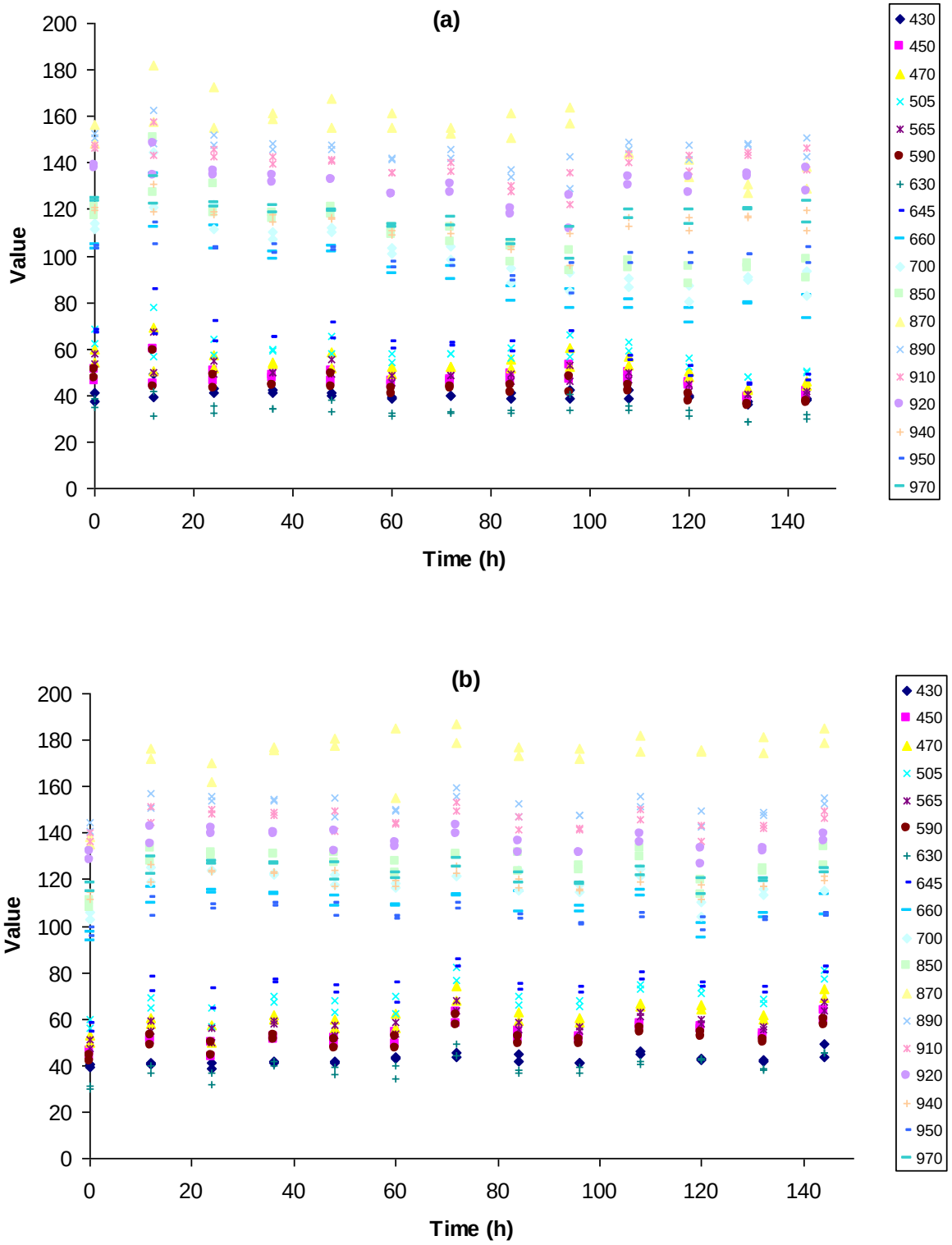


Figure 3.7.1. VideometerLab spectra (in replicates) collected from minced beef samples stored aerobically (a) and under MAP (b) at 5ºC. The legend on the right depicts each wavelength (nm) that was applied to the measurements.

3.7.2. Estimation of the microbial populations and sensory scores

The first PCA showed that all the bands were found to be significant and thus all of them were used for further analyses. The FDA provided classifications of the samples with an overall correct classification for the validation sensory scores of 71.15%, whilst the provided estimations from the PLS-R model were better regarding the overall classification (86.54%) and each sensory group separately (Tables 3.7.1; 3.7.2).

Table 3.7.3 presents the RMSE, the R^2 values and % RE values for the models built for the Videometer measurements. In general, it was observed that better estimations were obtained for TVC, pseudomonads, LAB and yeasts and moulds. Indicatively, Figure 3.7.2 illustrates the distribution of the percent relative error (% RE) of the estimated (validated) values for total viable counts compared to the observed ones. Moreover, the distribution of percent relative error (% RE) values above or below 0 showed if there was an under- or over-prediction. Regarding the estimations for TVC the % RE values were randomly distributed above and below 0, with 100% of estimated microbial counts included within the ± 20% RE zone. As far as the models of the remaining microbial groups are concerned (data not shown), the % RE values were also distributed above and below 0.

Table 3.7.1 Confusion matrix according to the FDA /Videometer model for the validation of the sensory estimates

True class	Estimated class			Correct Classification (Sensitivity %)
	Fresh	**Semi-fresh**	**Spoiled**	
Fresh (n = 10)	5	2	3	50.00
Semi-fresh (n=12)	1	4	7	33.33
Spoiled (n =30)	2	0	28	93.33
Total (n =52)	8	5	38	71.15
Specificity (%)	62.50	80.00	73.68	

Table 3.7.2 Confusion matrix according to the PLS-R /Videometer model for the validation of the sensory estimates

True class	Estimated class			Correct Classification (Sensitivity %)
	Fresh	**Semi-fresh**	**Spoiled**	
Fresh (n = 10)	6	4	0	60.00
Semi-fresh (n=12)	2	9	1	75.00
Spoiled (n =30)	0	0	30	100.00
Total (n =52)	8	13	31	86.54
Specificity (%)	75.00	69.23	96.77	

Table 3.7.3 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the PLS-R /Videometer models

Microbial group	No of latents	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	14	100.00	82.69	0.85	0.48
Pseudomonas spp.	12	96.15	82.69	0.83	0.54
Br. thermosphacta	14	90.38	76.92	0.80	0.48
LAB	12	88.46	61.54	0.81	0.59
Enterobacteriaceae	11	61.54	50.00	0.71	0.50
Yeasts & Moulds	6	90.38	65.38	0.79	0.47

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

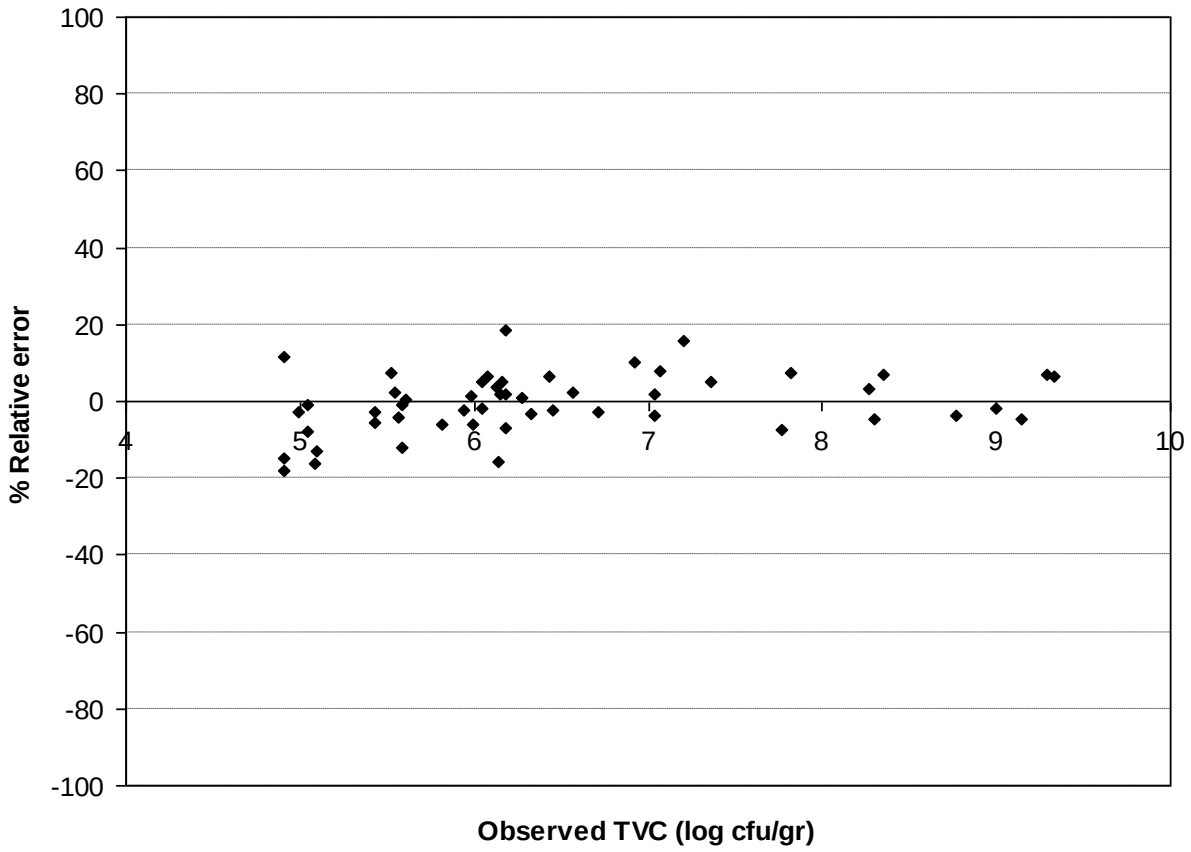


Figure 3.7.2. Percent relative errors (% RE) between observed and estimated (validated) counts of the total microbiota (TVC) of minced beef samples according to the PLS-R /Videometer model

3.8. HPLC analysis of organic acids

3.8.1. HPLC analysis

The analysis of chromatograms from HPLC resulted in the selection of 17 pure peaks (with purity >99%). The selected peaks had the following retention times (RT) (min): 6.2, 6.9 (citric acid), 7.0, 7.9, 8.3, 9.7, 10.9 (lactic acid), 11.9 (formic acid), 12.9 (acetic acid), 14.9, 15.1 (propionic acid), 16.1, 17.8, 18.6, 20.5, 24.6 and 28.1. An indicative metabolic profile from HPLC analysis is shown in Figure 3.8.1. In particular, Figure 3.8.1a illustrates the metabolic profile of a fresh minced beef sample at the onset of storage, while Figures 3.7.1b - 1d depict the profile of spoiled beef stored under the three different packaging conditions at 15°C.

The chromatographic profile of the same peaks differs according to storage conditions. When samples were stored aerobically, an increase was observed in the peaks with RT of 7.0, 7.9 and 17.8 min. The peak with RT of 20.5 min was present only when spoilage was pronounced and exclusively at aerobic storage (data not shown). The values of lactic acid area (Figure 3.8.2.) as well as the values of the peak with RT of 9.7 min declined in all samples stored aerobically. The propionic acid chromatographic areas remained stable until the end of the shelf life and increased afterwards at chill temperatures (0 and 5 °C). In contrast, at 10°C a decrease was observed until the end of shelf life, followed by a subsequent increase, whereas at 15ºC an increase was exhibited throughout storage.

In comparison with aerobic storage, the peaks with RT 7.0, 7.9 and 17.8 min, showed no particular trend under MAP or MAP/OEO at all temperature conditions, with the exception of 15ºC where an increase was observed in most cases. It has to be noted though, that the peaks with RTs of 7.0 and 7.9 min showed a similar trend between the different storage conditions.

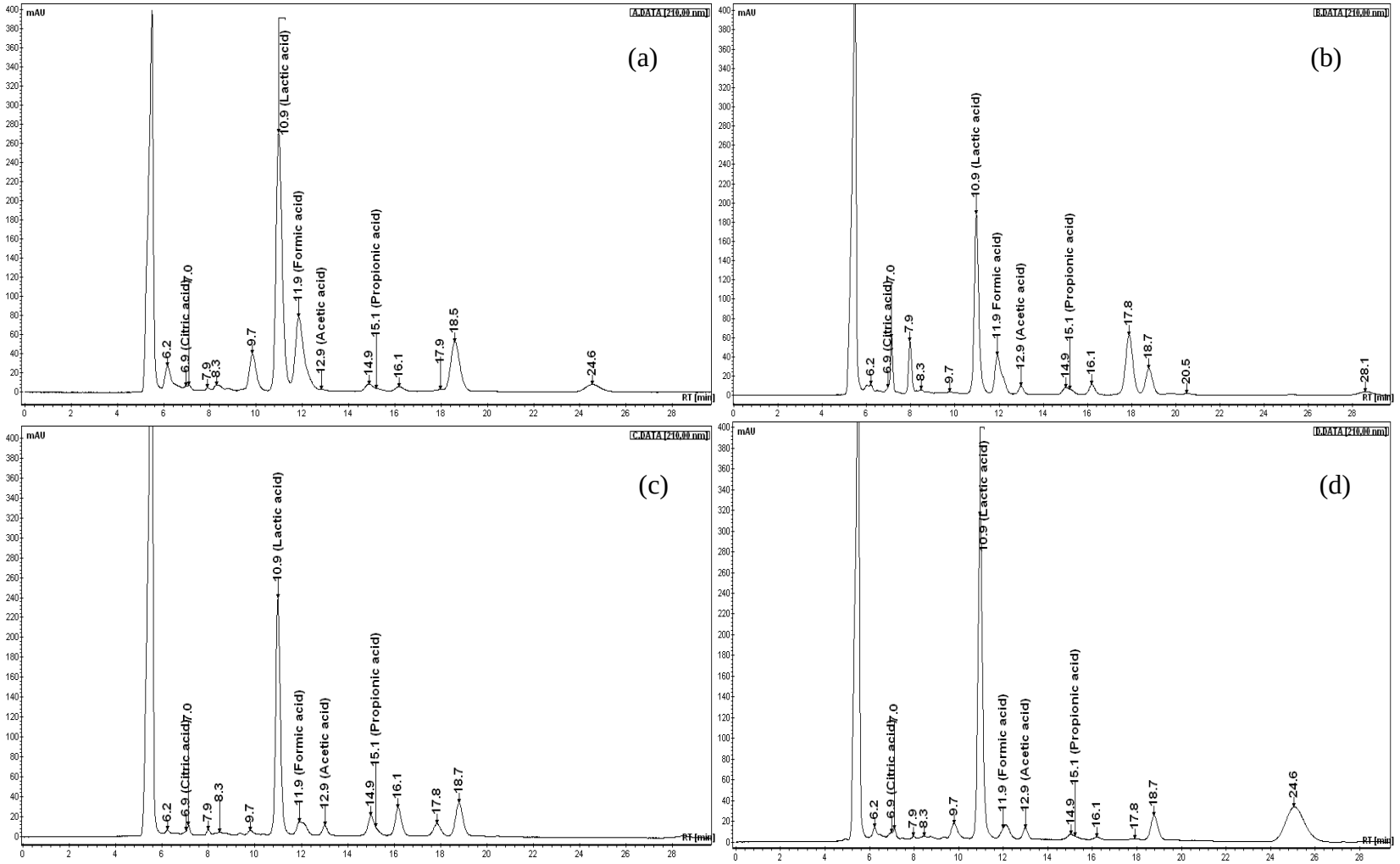


Figure 3.8.1. Typical HPLC metabolic profiles of minced beef at the onset of storage (a) and after 78h at 15ºC stored aerobically (b), under MAP without (c) and with (d) the presence of volatile compounds of oregano essential oil

The values of lactic acid area remained mostly unchanged, with a slight increase towards the end of the storage period for MAP samples and a noticeable increase for MAP/OEO samples over time (Figure 3.8.2.). The peak with RT of 9.7 min increased in samples stored at chill temperatures (0, 5°C) for both MAP and MAP/OEO, and subsequently decreased after the end of shelf life. In contrast, at abuse temperatures (10, 15°C) a decrease was observed in the case of MAP, with a concurrent increase for MAP/OEO. The total amounts of propionic acid showed an increasing trend over storage time when mince was stored under MAP at all temperatures with the exception of 0°C where it remained constant. In contrast, for MAP/OEO samples, there was a decline of propionic acid at all temperatures with the exception of 15°C where it remained stable until the end of shelf life and increased afterwards.

The total amounts of acetic acid exhibited an increase during storage at all temperatures and packaging conditions assayed (Figure 3.8.3.). This pattern was more evident at aerobic storage compared to MAP and MAP/OEO when samples were stored at 0°C, although higher values were observed at 5, 10 and 15°C in samples stored under MAP and MAP/OEO. On the other hand, the chromatographic areas of peaks with RT of 6.2 and 11.9 min (formic acid) showed a decreasing trend, with formic acid exhibiting an increase only after the end of shelf life in some storage conditions. The peaks with RT of 6.9 (citric acid), 8.3, 16.1, 18.6 and 24.6 min showed no particular trend over time between the different storage conditions. Finally, the peak with RT of 28.1 min was evident only when spoilage was pronounced (data not shown). The changes in the values of chromatographic areas from the above-described peaks at the onset of storage and the end of shelf life of the mince under all temperatures and packaging conditions are summarized in Table 3.8.1.

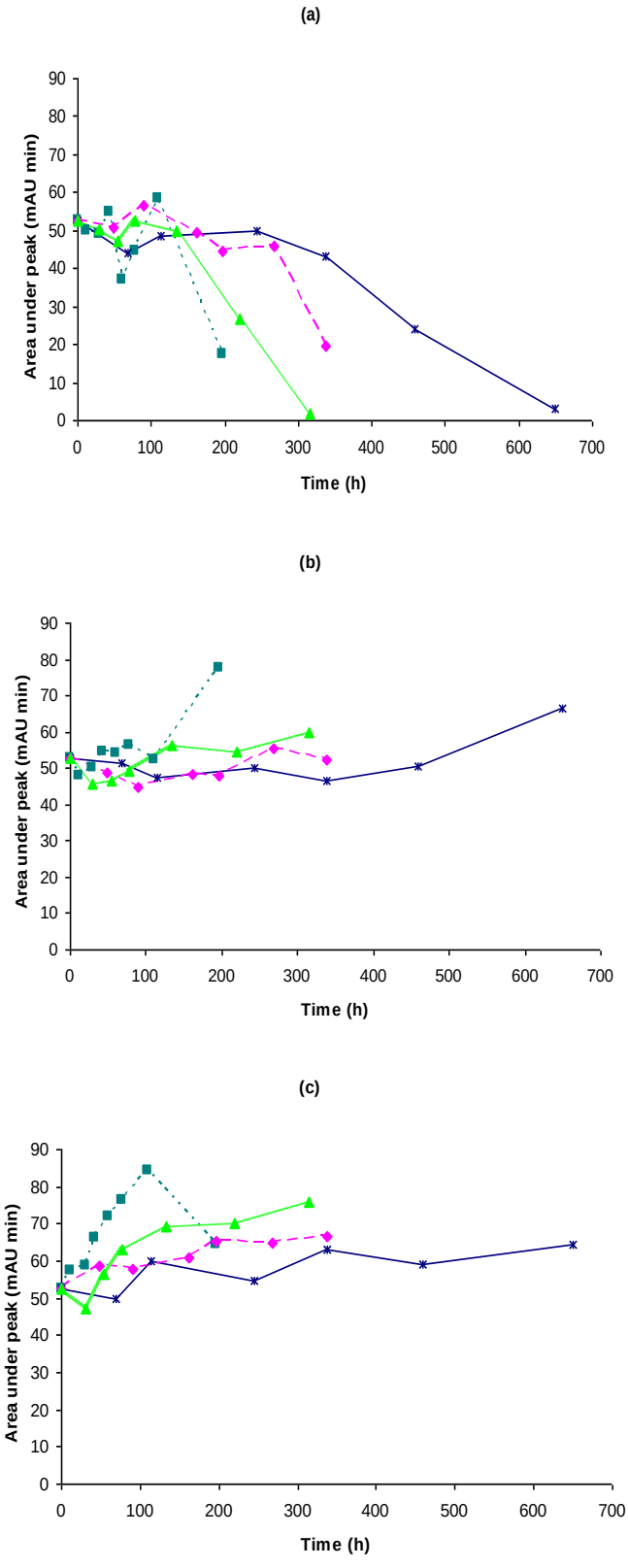


Figure 3.8.2. Changes of chromatographic area under peak of lactic acid in minced beef stored aerobically (a), under MAP without (b) and with (c) the presence of volatile compounds of oregano essential oil at 0 °C (*,—)for 650h, 5 °C (◆,– –) for 482h, 10 °C (▲,〰) for 386h and 15 °C (■,- -) for 220h.

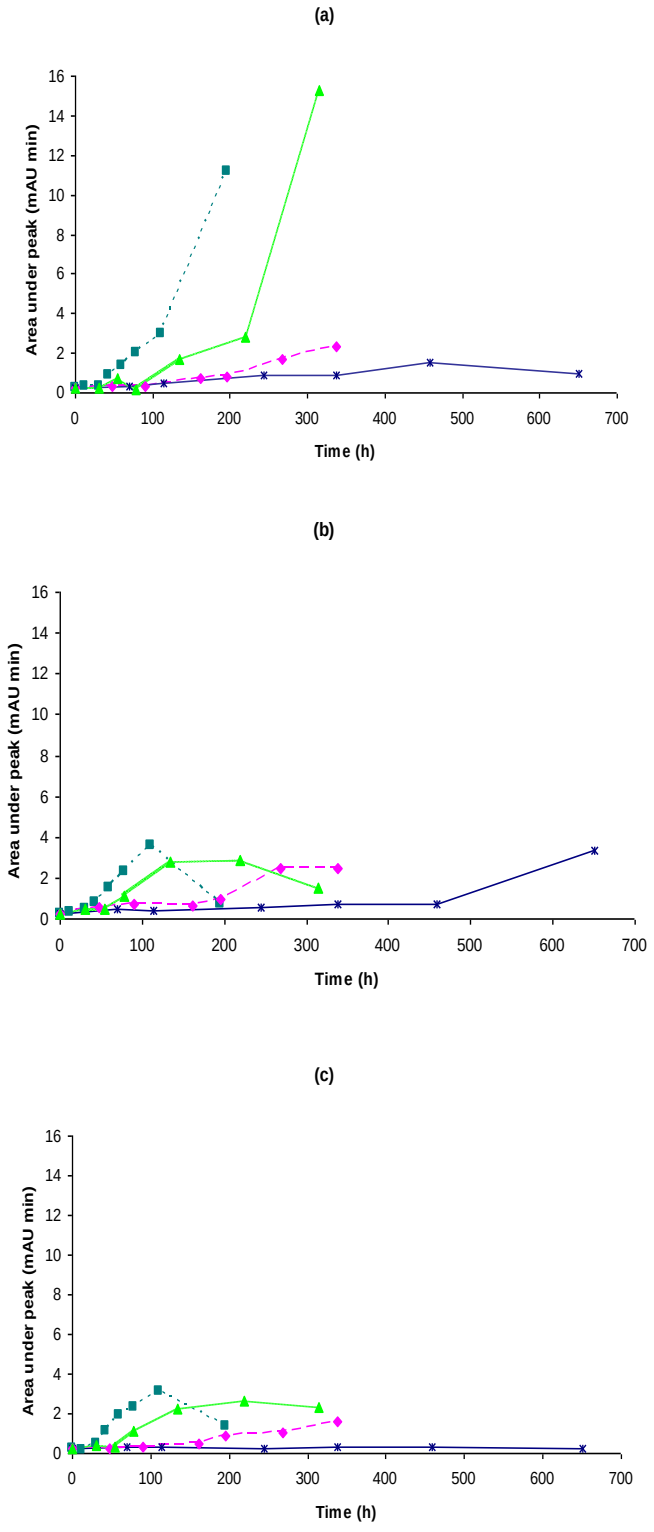


Figure 3.8.3. Changes of chromatographic area under peak of acetic acid in minced beef stored aerobically (a), under MAP without (b) and with (c) the presence of volatile compounds of oregano essential oil at 0 °C (*,—)for 650h, 5 °C (◆,– –) for 482h, 10 °C (▲,〰) for 386h and 15 °C (■,- -) for 220h.

Table 3.8.1 Changes in the chromatographic areas under peaks at the beginning of the storage (time 0h) and at the end of shelf life of each storage condition.

RT (Peak ID) (min)	Chromatographic Area under peak (mAU min)												
	Fresh (0h)	End of shelf life											
		0 °C			5°C			10°C			15°C		
		Air	MAP	MAP / OEO	Air	MAP	MAP / OEO	Air	MAP	MAP / OEO	Air	MAP	MAP / OEO
6.2 (unknown)	7.90	2.65	2.20	2.60	2.25	2.75	3.15	3.16	2.55	3.65	3.15	1.35	2.6
6.9 (Citric)	1.05	2.50	1.85	1.35	0.85	1.65	0.75	1.60	0.45	0.90	1.00	0.55	0.9
7.0 (unknown)	1.25	24.50	0.50	0.20	9.00	0.60	0.85	3.05	0.50	1.05	2.80	1.25	1.4
7.9 (unknown)	0.55	23.95	nd [a]	0.55	0.70	0.50	1.15	1.15	0.15	0.50	2.00	0.75	0.5
8.3 (unknown)	0.60	0.90	0.45	0.75	0.80	0.80	1.25	0.85	0.40	0.70	0.45	0.45	0.4
9.7 (unknown)	7.40	2.75	7.65	8.90	2.20	6.65	11.3	4.50	2.40	8.15	0.40	1.90	5.9
10.9 (Lactic)	52.60	43.25	50.7	58.95	44.70	55.25	64.85	49.95	49.20	69.15	36.80	53.95	71.95
11.9 (Formic)	21.55	2.35	4.20	4.75	1.10	2.75	6.15	7.75	7.15	7.05	15.70	4.45	4.05
12.9 (Acetic)	0.25	0.85	0.75	0.35	0.80	1.70	1.05	0.70	1.15	2.20	1.35	1.55	1.95
14.9 (unknown)	2.50	2.30	3.70	2.30	2.70	4.80	1.80	3.00	3.40	1.45	2.50	4.20	1.85
15.1 (Propionic)	0.55	0.85	0.45	0.20	0.60	1.00	0.35	0.15	1.05	0.20	0.70	1.00	0.50
16.1 (unknown)	1.40	0.60	1.10	0.60	1.95	2.65	0.45	0.40	3.70	0.45	3.70	5.10	0.30
17.8 (unknown)	0.30	6.7	nd	nd	16.95	1.20	0.15	12.30	1.50	nd	110.8	5.15	0.55
18.6 (unknown)	14.80	10.85	13.45	10.70	13.95	24.25	11.35	12.55	12.40	10.35	13.30	5.65	9.10
24.6 (unknown)	4.25	0.90	nd	nd	nd	nd	3.05	7.80	5.00	13.65	0.65	0.20	9.60

[a] nd: not detected

3.8.2. Estimations of the microbial populations and the sensory scores

An analysis was undertaken to correlate the shelf life of the mince regardless of the storage conditions (i.e. temperature, packaging) with the HPLC profile of organic acids. The above 17 pure peaks were initially subjected to PCA and the peaks with RTs of 6.2, 6.9 (citric acid), 7.0, 7.9, 8.3, 9.7, 10.9 (lactic acid), 12.9 (acetic acid), 15.1 (propionic acid), 16.1, 17.8, 20.5 and 24.6 min were selected as significant, and subjected to a second PCA. Figures 3.8.4a-c illustrate the loading plots of the selected variables (peak areas) and their contribution to each one of the first 3 PCs, whereas Figures 3.8.5a-c depict the scores plot for the first 3 PCs labelled with the sensory scores of the samples. Figure 3.8.4 reveals the relationships of the selected 13 peaks depending on the pair of PCs being plotted each time. Variables contributing similar information are grouped together meaning that they are correlated. When a numerical value of one variable increases or decreases the number of the other one has a tendency to change in the same way. For example, peaks with RTs of 7.0 and 7.9 that are grouped together in all graphs of Figure 3.8.4 have similar behaviour throughout spoilage. When variables are negatively correlated they are positioned on opposite sides of the plot origin, in diagonally opposed quadrants. The further away from the plot origin a variable lays, the stronger impact that variable has on the model. Figures 3.8.5 a-c showed that the first PC (explaining 36.4% of total variance) correlated positively with acceptable samples [fresh (sensory score 1) and semi-fresh (sensory score 1.5)] and negatively with spoiled ones. The second PC (explaining 16.8% of total variance) is negatively correlated with acceptable samples and positively with spoiled ones, and finally the third PC (explaining 15.4% of total variance) is positively correlated with acceptable samples and positively or negatively with spoiled ones. Taking into account both Figures 3.8.4 and 3.8.5, useful information about the impact of each variable (peak area) on the spoilage status of a given sample can be drawn.

Observing Figures 3.8.4a and 3.8.5a, it can be inferred that peaks with RTs of 7.0, 7.9 and 17.8 min are more associated with spoiled samples, lactic and citric acids with semi-fresh samples and peaks with RTs of 24.6, 6.2, 9.7 min are associated with fresh samples. Figure 3.8.6 depicts the discrimination of the samples according to AHC. Three major groups can be visualised, from which the first two groups correspond to acceptable mince samples from a sensory point of view (fresh and semi-fresh) and the third one corresponds to spoiled samples, with a 10.5% of the samples being misclassified.

The FDA provided classifications of the samples regarding their spoilage status, providing a correct classification rate of 93.3% (fit of the model) and a validation of 88.0%. Specifically, the classification of the samples after the validation of the model was 88.46% correct for the fresh samples, 81.82% for the semi-fresh and 89.47% for the spoiled ones. Figure 3.8.7 demonstrates the discrimination map of the samples regarding their spoilage status (fresh, semi-fresh and spoiled). The map reveals the transition of meat samples from fresh status to semi-fresh and finally to spoiled. These results revealed a good correlation of the sensory estimates of the spoilage with the dynamic changes of the chromatographic areas of organic acids that were present at different times through storage.

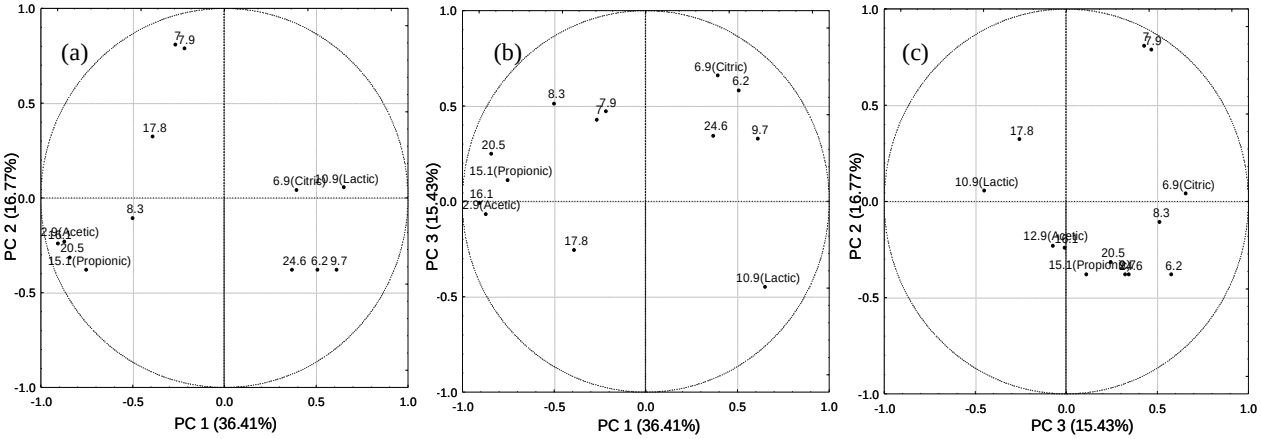


Figure 3.8.4. Loadings plot with the significant variables to each extracted principal component (PC) according to principal components analysis (PCA): (a) PC 1 vs. PC 2, (b) PC 1 vs. PC 3, (c) PC 3 vs. PC 2

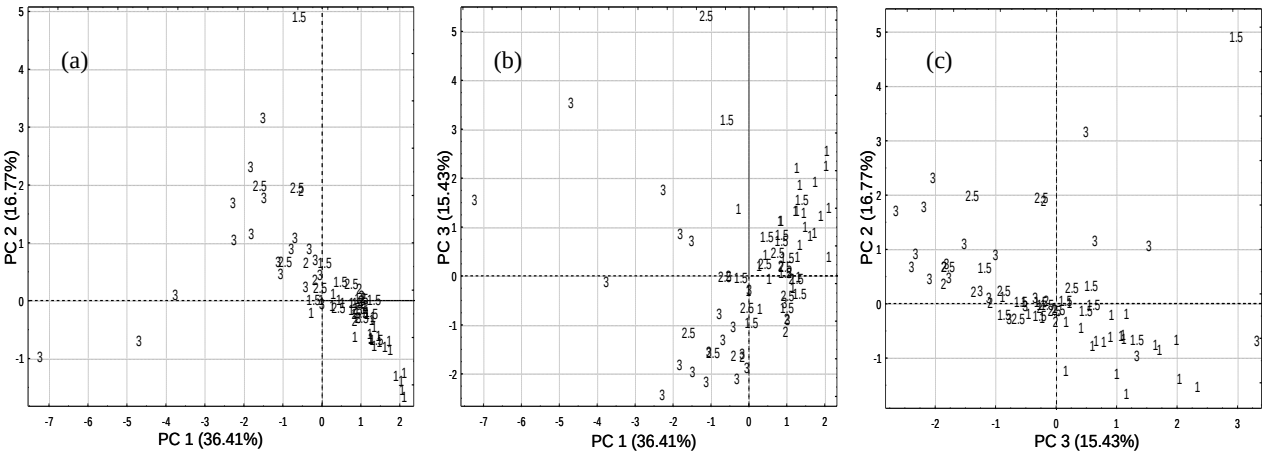


Figure 3.8.5. Scores plot for the first 3 PCs labelled with the sensory scores of the samples (a) PC 1 vs. PC 2, (b) PC 1 vs. PC 3, (c) PC 3 vs. PC 2. Sensory scores scale: 1=fresh; 2=marginal; and 3=unacceptable. Score of 1.5 was characterized as semi-fresh and was the first indication of microbial proliferation. Scores above 2 rendered the product spoiled and pointed the end of the products' shelf life.

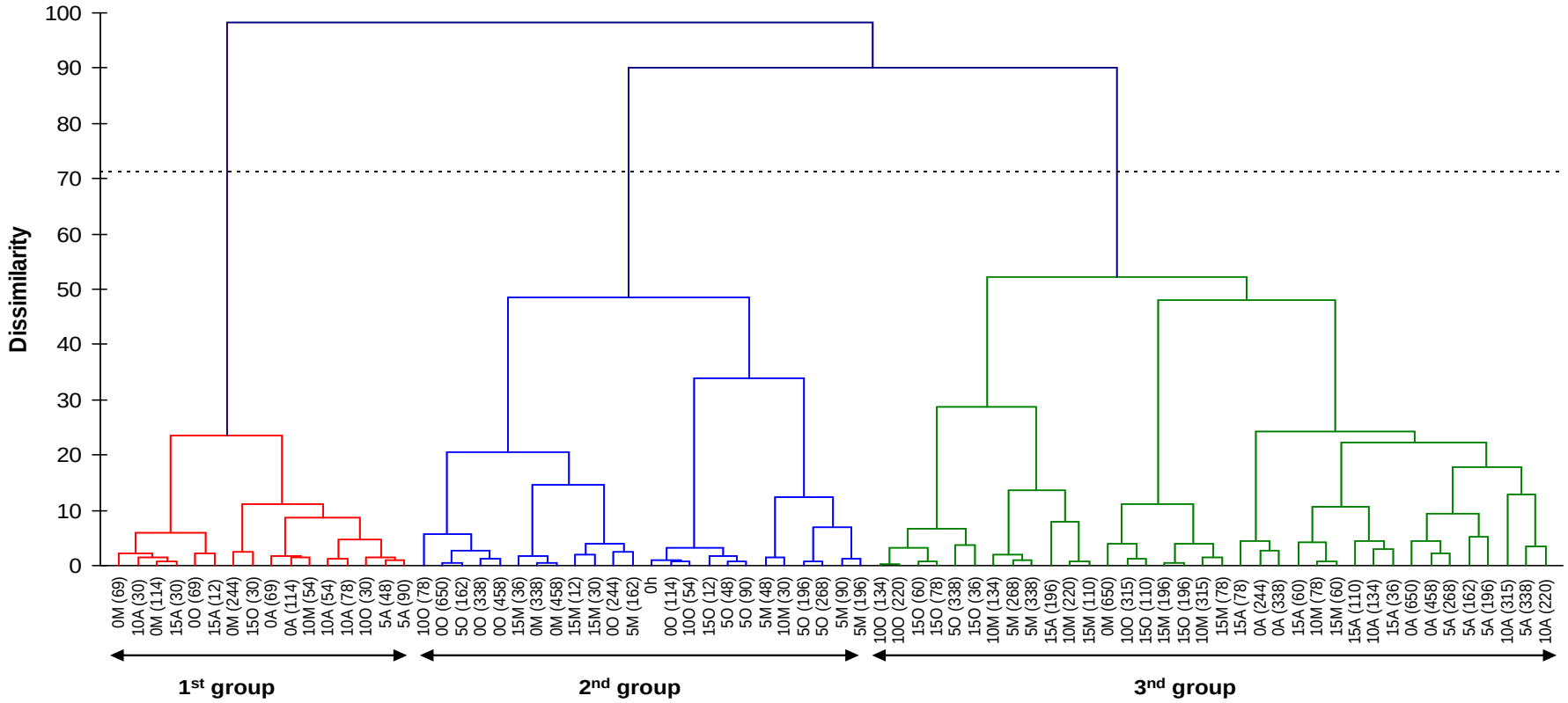


Figure 3.8.6. Cluster analysis dendrogram discriminating the acceptable (1st and 2nd group) from the unacceptable (3nd group) minced beef samples. Sample coding corresponds to storage temperature (first digit), packaging condition (A for air, M for MAP and O for MAP/OEO) and storage time in hours (last digit).

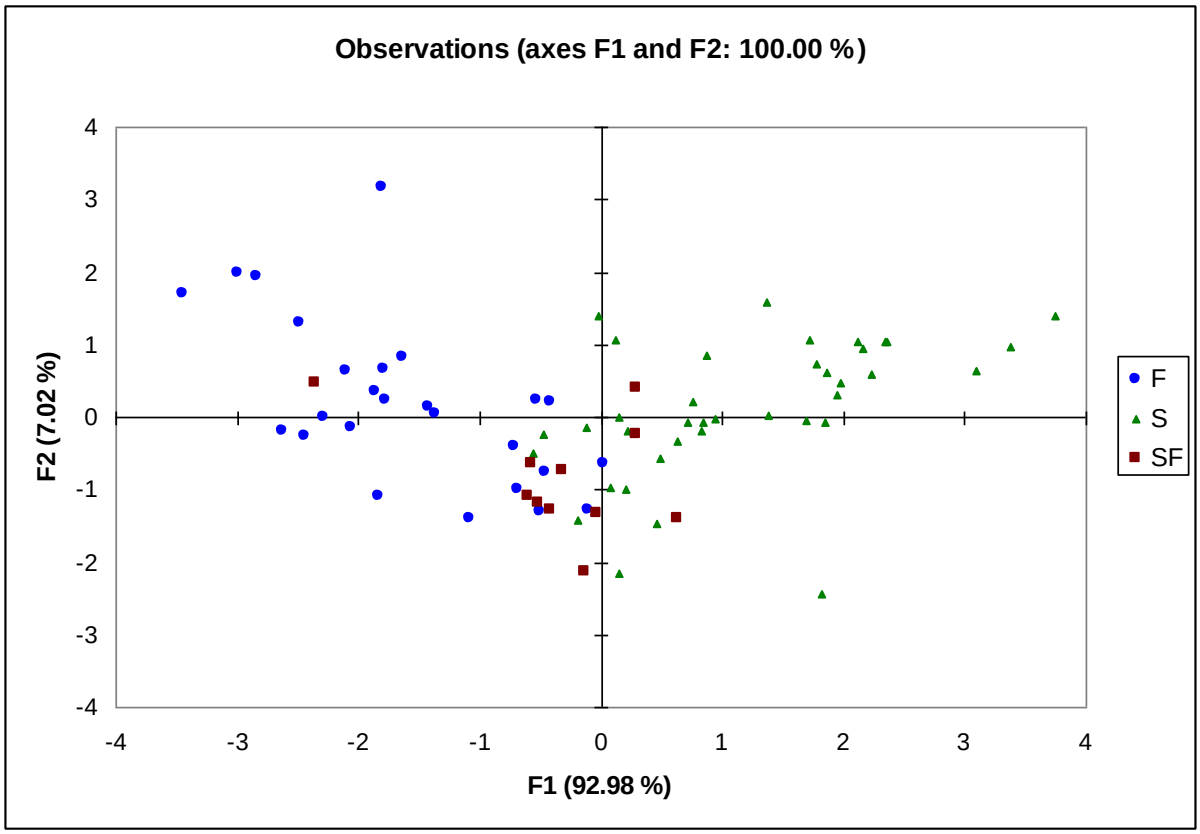


Figure 3.8.7. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 different minced beef freshness groups: Fresh (F), Semi-fresh (SF), and Spoiled (S).

The potential of PLS-R analysis to estimate the population of selected microbial groups of the indigenous microbiota of meat samples such as total viable counts (TVC), *Pseudomonas* spp, *Br. thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, and yeasts/moulds was also demonstrated. The calculated values of bias and accuracy factors as well as the root mean square error index that demonstrated the performance of the model built for every group of microorganisms are shown in Table 3.8.2. Figure 3.8.8 illustrates the distribution of the percent relative error (% RE) of the validated values for total viable counts compared to the observed ones.

The values of B_f were generally close to unity, indicating good agreement between observations and estimations (Table 3.8.2), with almost no structural correlation between them, whilst the fact that in certain cases it is slightly below 1 indicates a 'fail-safe' model (Ross, 1996). In addition, the values of the accuracy factor indicated that the average deviation between estimations and observations of the various microbial groups enumerated ranged from 9.0% (either above or below the line of equity) for lactic acid bacteria to 17.9% for *Br. thermosphacta* (Table 3.8.2). Moreover, the distribution of percent relative error (% RE) values above or below 0 showed if there was an under- or over-prediction. Regarding the estimations for TVC the % RE values were randomly distributed above and below 0, with 90.8% of estimated microbial counts included within the ± 20% RE zone. However, according to the % RE values a trend of over-prediction was evident especially at lower population densities (counts less than 7 log cfu g^{-1}) and a trend of under-prediction was observed especially at higher population densities (counts more than 7 log cfu g^{-1}). As far as the models of the remaining microbial groups are concerned (data not shown), the % RE values were distributed mostly above 0, presenting thus a general under-prediction of the models.

Table 3.8.2 Comparison of calculated performance indices for the estimation of the microbial population in beef samples using the validation estimates from the PLS-R /HPLC models*

Microbial group	B_f [a]	A_f [b]	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE [d]
TVC	0.993	1.100	90.78	64.47	0.58	0.88
Pseudomonas spp	0.996	1.175	69.73	43.42	0.61	1.30
Br. thermosphacta	0.999	1.179	68.42	38.15	0.41	1.09
LAB	1.001	1.090	88.16	71.05	0.50	0.75
Enterobacteriaceae	0.993	1.157	75.00	47.36	0.57	1.18
Yeasts and moulds	0.994	1.147	75.00	50.00	0.60	1.01

* In all cases one latent variable was used in the PLS-R models

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

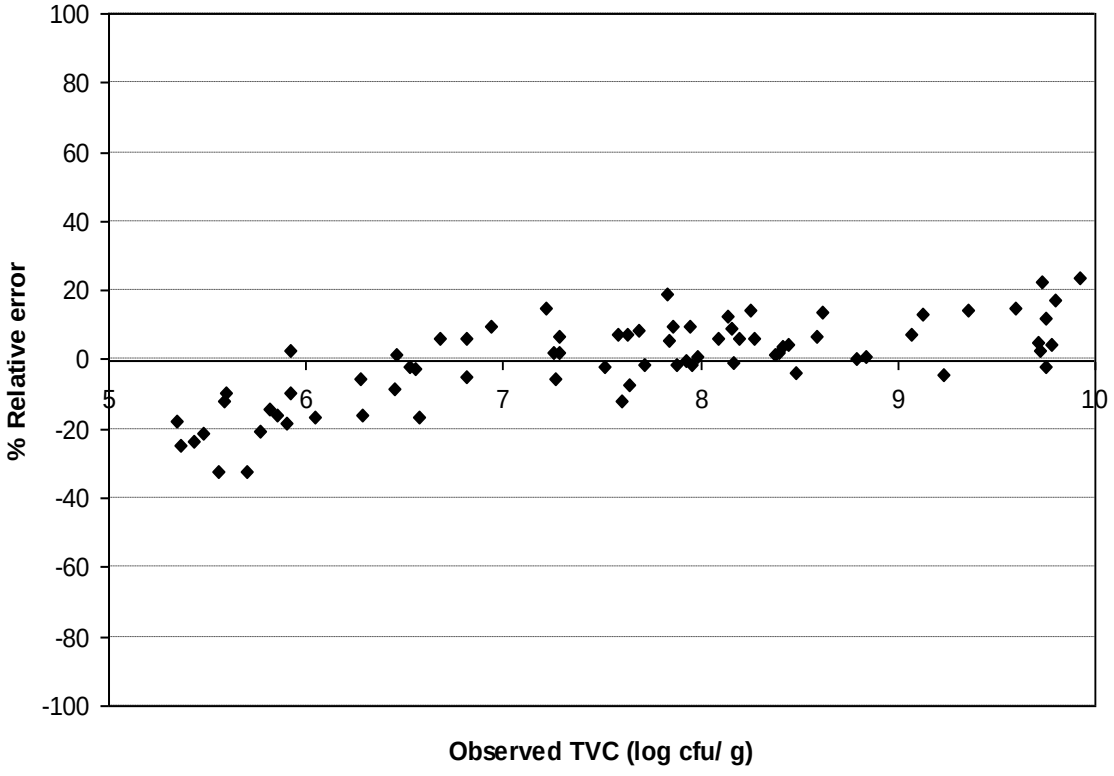


Figure 3.8.8. Percent relative errors (% RE) between observed and estimated counts of the total microbiota (TVC) estimated from the validated values of the PLS-R /HPLC model

3.9. GC/MS analysis

3.9.1. GC/MS analysis

The volatile compounds of beef that were isolated using the HS/SPME method and those detected by GC/MS are listed in Table 3.9.1, whilst Figure 3.9.1 shows indicatively the volatile metabolic profile of a fresh minced beef sample at the onset of storage (a) and of spoiled minced beef samples stored under Air and MAP at 5 °C. Table 3.9.1 demonstrates the presence/absence of each compound detected during minced beef storage under different temperature and packaging conditions and which compounds were further used for the estimations of the sensory scores and microbial counts.

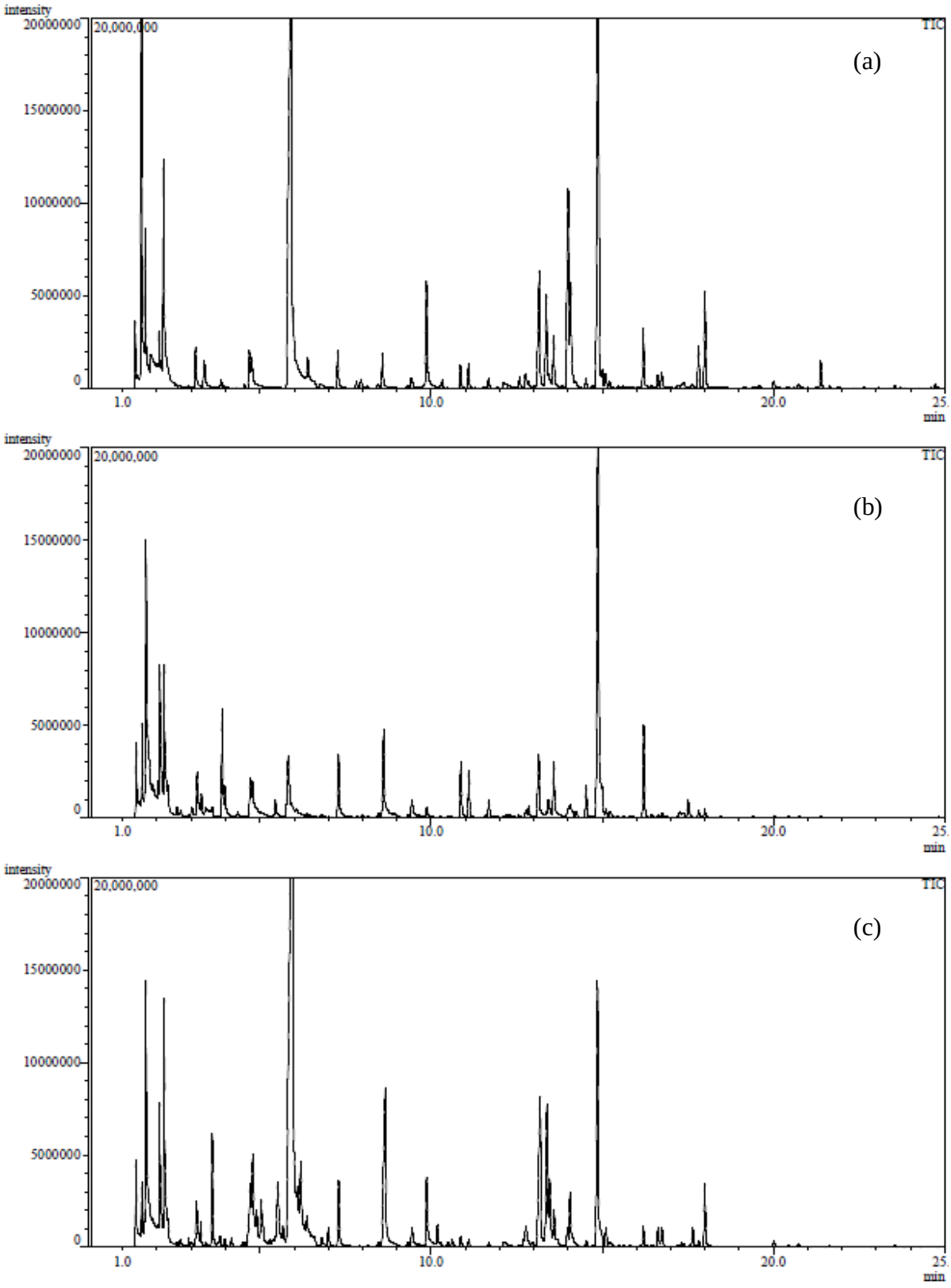


Figure 3.9.1. Typical GC/MS volatile metabolic profiles of minced beef at the onset of storage (a) and after 268h at 5ºC stored aerobically (b) and under MAP (c).

Table 3.9.1 Volatile compounds detected by GC/MS in minced beef stored under aerobic and modified atmosphere packaging conditions at 0, 5, 10 and 15 ºC.

Compound	LRI [a]	RT (min)	Identification [b]	Target ion [c] (m/z)	AIR				MAP			
					0	5	10	15	0	5	10	15
Alcohols												
Ethanol *	513.3	1.596	A	31	+	+	+	+	+	+	+	+
Propanol	566	1.930	A		+	+	+	+	+	+	+	+
2-Butanol	602.7	2.170	A	59	+	+	+	+	+	+	+	+
1-Butanol	656	2.770	A		+	+	+	+	+	+	+	+
1-Penten-3-ol *	677.2	3.011	A	57	+	+	+	+	+	+	+	+
Isoamyl alcohol (3-Methyl-1-butanol) *	727.4	4.024	A	55	+	+	+	+	+	+	+	+
2-Methyl-1-butanol *	730	4.110	A	56	+	+	+	+	+	+	+	+
Amyl alcohol (pentanol) *	760.4	4.930	A	42	+	+	+	+	+	+	+	+
2,3-Butanediol (1)	783	5.542	B	45	+	+	+	+	+	+	+	+
2,3-Butanediol (2)	795.4	5.895	B	45	+	+	+	+	+	+	+	+
1-Hexanol *	869.4	8.897	A	56	+	+	+	+	+	+	+	+
Heptanol *	971.8	13.260	A	70	+	+	+	+	+	+	+	+
1-octen-3-ol *	980.5	13.640	B	57	+	+	+	+	+	+	+	+
3-Octanol	996.3	14.315	B	59	+	+	+	+	+	+	+	+
2-Ethyl-1-hexanol	1030	15.690	B	57	+	+	+	+	+	+	+	+
2-Octen-1-ol *	1068.8	17.270	B	57	+	+	+	+	+	+	+	+
1-Octanol *	1071.9	17.391	A	56	+	+	+	+	+	+	+	+
Nonanol	1172.5	21.259	B	56	+	+	+	+	+	+	+	+
4-Carvomenthenol (Terpinen-4-ol)	1177	21.431	B		+	+	+	+	+	+	+	+
Aldehydes												
Acetaldehyde	500	1.515	A		+	+	+	+	+	+	+	+
3-Methylbutanal (Isovaleraldehyde)	646.4	2.664	B	44	+	+	+	+	+	+	+	+
2-Methylbutanal	657.6	2.790	B	57	+	+	+	+	+	+	+	+
Pentanal *	698.2	3.248	A	44	+	+	+	+	+	+	+	+
Hexanal *	802	6.110	A	82	+	+	+	+	+	+	+	+
trans-2-Hexenal	852.9	8.192	B		+	+	+	+	+	+	+	+
trans-2-Heptenal*	956.6	12.610	B	83	+	+	+	+	+	+	+	+
cis-4-Heptenal	900.5	10.190	B		+	+	+	+	+	+	+	+
Heptanal *	901.6	10.235	B	70	+	+	+	+	+	+	+	+
Benzaldehyde	958.1	12.666	B	106	+	+	+	+	+	+	+	+

Table 3.9.1 (*Continued*)

Compound	LRI [a]	RT (min)	Identification [b]	Target ion [c] (m/z)	AIR				MAP			
					0	5	10	15	0	5	10	15
Octanal *	1003.1	14.600	A	43	+	+	+	+	+	+	+	+
trans-2-octenal *	1058.4	16.850	B	70	+	+	+	+	+	+	+	+
Nonanal *	1104.3	18.700	B	57	+	+	+	+	+	+	+	+
trans-2-Nonenal *	1160.5	20.799	B	55	+	+	+	+	+	+	+	+
3-Phenylpropionaldehyde	1162	20.874	B		+	+	+	+	+	+	+	+
cis-4-Decenal	1194.1	22.080	B		+	+	+	+	+	+	+	+
n-decanal *	1206	22.510	B	57	+	+	+	+	+	+	+	+
trans,trans-2,4-Nonadienal	1213.8	22.790	B	81	+	+	+	+	+	+	+	+
trans-2-Decenal	1262.1	24.480	B	55	+	+	+	+	+	+	+	+
trans,trans-2,4-Decadienal (1)	1293.5	25.585	B	81	+	+	+	+	+	+	+	+
trans,trans-2,4-Decadienal (2)	1316.3	26.350	B	81	+	+	+	+	+	+	+	+
Ketones												
Diacetyl (3-hydroxy-2-butanone) *	591.5	2.084	A	86	+	+	+	+	+	+	+	+
Methyl ethyl ketone (2-Butanone) *	600.7	2.150	B	72	+	+	+	+	+	+	+	+
2-Pentanone *	685.6	3.107	B	43	+	+	+	+	+	+	+	+
Acetyl propionyl (2,3-Pentanedione) *	634.297	3.220	B	100	+	+	+	+	+	+	+	+
Acetoin (3-Hydroxy-2-butanone) *	725	3.956	B	45	+	+	+	+	+	+	+	+
3-Methyl-2-pentanone	745.3	4.516	B		+	+	+	+	-	-	-	-
2-Heptanone *	890.9	9.790	B	43	+	+	+	+	+	+	+	+
6-Methyl-2-heptanone	956	12.616	C	58	+	+	+	+	+	+	+	+
2,3-Octanedione or 2,5- *	986.1	13.880	C	43	+	+	+	+	+	+	+	+
3-Octanone *	987.5	13.935	B	72	+	+	+	+	+	+	+	+
2-Octanone *	992	14.130	B	58	+	+	+	+	+	+	+	+
3-Octen-2-one	1040	16.095	B	55	+	+	+	+	+	+	+	+
Acetophenone	1065	17.093	A	105	+	+	+	+	+	+	+	+
2-Nonanone *	1093.1	18.251	B	58	+	+	+	+	+	+	+	+
trans,trans -3,5-Octadien-2-one	1093	18.249	B		+	+	+	+	+	+	+	+

Table 3.9.1 (*Continued*)

Compound	LRI [a]	RT (min)	Identification [b]	Target ion [c] (m/z)	AIR				MAP			
					0	5	10	15	0	5	10	15
Hydrocarbons												
Hexane	600	2.143	A		+	+	+	+	+	+	+	+
Benzene	654.4	2.754	B	78	+	+	+	+	+	+	+	+
Heptane	700	3.270	A	71	+	+	+	+	+	+	+	+
Cyclohexane, methyl	717.3	3.744	B	83	+	+	+	+	+	+	+	+
Cyclopentane, ethyl	726.3	3.992	B	69	-	-	-	-	+	+	+	+
Alkane 1	732	4.148	C	70	-	-	-	-	+	+	+	+
Alkane 2	739	4.344	C	56	-	-	-	-	+	+	+	+
Alkane 3	752	4.686	C	56	-	-	-	-	+	+	+	+
Toluene	758.6	4.882	B	91	+	+	+	+	+	+	+	+
Alkane 4	767	5.116	C	85	+	+	+	+	+	+	+	+
Alkane 5	773	5.252	C	97	+	+	+	+	+	+	+	+
Isomer of Alkane 5	774	5.300	C	97	+	+	+	+	+	+	+	+
Alkane 6	782	5.516	C	97	+	+	+	+	+	+	+	+
1-Octene	791	5.756	B	56	+	+	+	+	+	+	+	+
Alkane 7	796	5.919	C	97	+	+	+	+	+	+	+	+
Alkane 8	800	6.058	C	112	+	+	+	+	+	+	+	+
n-Octane	800	6.020	A	114	+	+	+	+	+	+	+	+
Trans-4-Octene	806.5	6.289	B	112	+	+	+	+	+	+	+	+
Alkane 10	810	6.450	C	112	+	+	+	+	+	+	+	+
cis-2-Octene	813.7	6.587	B	112	+	+	+	+	+	+	+	+
Cyclohexane, ethyl	830.3	7.275	B	83	+	+	+	+	+	+	+	+
Ethyl benzene	858.4	8.442	B	91	+	+	+	+	+	+	+	+
Xylene 1	866.5	8.777	C	91	+	+	+	+	+	+	+	+
Styrene	888.3	9.680	B	104	+	+	+	+	+	+	+	+
Xylene 2	890.2	9.761	C		+	+	+	+	+	+	+	+
n-Nonane	900	10.170	A	57	+	+	+	+	+	+	+	+
Alkane 11	988	14.022	C	56	+	+	+	+	+	+	+	+
n-Decane	1000	14.470	A	57	+	+	+	+	+	+	+	+
Benzene, 1,2,3-trimethyl	1020.1	15.307	B	105	+	+	+	+	+	+	+	+
Indane	1033	15.805	B	117	+	+	+	+	+	+	+	+
3a,4,5,6,7,7a-Hexahydro-4,7-methanoindene	1064	17.074	B	66	+	+	+	+	+	+	+	+
1-Undecene	1092.6	18.233	B	55	+	+	+	+	-	-	-	-
Naphthalene	1180.7	21.570	A	128	+	+	+	+	+	+	+	+

Table 3.9.1 (*Continued*)

Compound	LRI [a]	RT (min)	Identification [b]	Target ion [c] (m/z)	AIR				MAP			
					0	5	10	15	0	5	10	15
n-Dodecane	1200	22.295	A	57	+	+	+	+	+	+	+	+
Tridecane	1300	25.810	A	57	+	+	+	+	+	+	+	+
n-Tetradecane	1400	29.100	A	57	+	+	+	+	+	+	+	+
Terpenes												
a -Thujene	925.8	11.277	A	93	+	+	+	+	+	+	+	+
a -Pinene	931.6	11.530	A	93	+	+	+	+	+	+	+	+
Camphene	945.6	12.1280	A	93	+	+	+	+	+	+	+	+
Sabinene	973.9	13.350	B	93	+	+	+	+	+	+	+	+
Myrcene	991.4	14.102	A	93	+	+	+	+	+	+	+	+
a -Phellandrene	1003	14.789	B	93	+	+	+	+	+	+	+	+
delta-3-carene	1007.9	14.794	B	93	+	+	+	+	+	+	+	+
a -Terpinene	1015	15.082	B	121	+	+	+	+	+	+	+	+
p-Cymene	1023.3	15.420	A	134	+	+	+	+	+	+	+	+
Limonene	1027.3	15.580	A	93	+	+	+	+	+	+	+	+
Eucalyptol	1029.7	15.680	A	81	+	+	+	+	+	+	+	+
Ocimene (cis)	1038.9	16.052	B	93	+	+	+	+	+	+	+	+
γ-Terpinene	1058.4	16.850	A	93	+	+	+	+	+	+	+	+
Linaloloxide (cis, isomer B)	1072	17.431	B		+	+	+	+	+	+	+	+
Terpinolene	1088	18.033	B		+	+	+	+	+	+	+	+
4,7-Methano-1H-indene, octahydro-	1089	18.080	C	95	+	+	+	+	+	+	+	+
Linalol	1099.5	18.513	A	71	+	+	+	+	+	+	+	+
Esters												
Methyl acetate	546.3	1.804	B		+	+	+	+	+	+	+	+
Ethyl acetate *	800.5202	2.280	A	61	+	+	+	+	+	+	+	+
Ethyl propionate *	709.7	3.536	A	57	+	+	+	+	+	+	+	+
n-Propyl acetate	712.7	3.620	A	61	-	-	-	-	+	+	+	+
Methyl butyrate	719.1	3.796	A		+	+	+	+	+	+	+	+
Ethyl isobutyrate	753.1	4.730	A	116	+	+	+	+	+	+	+	+
Ethyl butyrate (butanoate) *	804.5	6.209	A	88	+	+	+	+	+	+	+	+
Ethyl lactate *	815.7	6.680	A	45	+	+	+	+	+	+	+	+
Ethyl 2-methylbutyrate	851.4	8.150	B	57	+	+	+	+	+	+	+	+
Ethyl 3-methylbutyrate	855	8.300	B	88	+	+	+	+	+	+	+	+

Table 3.9.1 (*Continued*)

Compound	LRI [a]	RT (min)	Identification [b]	Target ion [c] (m/z)	AIR				MAP			
					0	5	10	15	0	5	10	15
Isoamyl acetate	878.8	9.284	A	70	+	+	+	+	+	+	+	+
Ethyl pentanoate	903.3	10.308	A	88	+	+	+	+	+	+	+	+
Pentyl acetate	917.5	10.919	A	70	+	+	+	+	+	+	+	+
Methyl caproate (methyl hexanoate)	926.8	11.321	A	74	+	+	+	+	+	+	+	+
Ethyl hexanoate *	1001.1	14.520	A	88	+	+	+	+	+	+	+	+
Hexyl acetate	1015.8	15.113	A	43	+	+	+	+	+	+	+	+
Ethyl heptanoate	1099.7	18.520	A	88	+	+	+	+	+	+	+	+
Ethyl octanoate	1198.3	22.230	A	88	+	+	+	+	+	+	+	+
Ethyl nonanoate	1297	25.723	A	88	+	+	+	+	+	+	+	+
Miscellaneous												
Dimethyl sulfide	541.6	1.775	B	62	+	+	+	+	+	+	+	+
Furan, 2-methyl	605.7	2.203	B	82	+	+	+	+	+	+	+	+
Furan, 3-methyl	610	2.270	C		+	+	+	+	+	+	+	+
Furan 2-ethyl	701.9	3.321	B	81	+	+	+	+	+	+	+	+
2-n-Butyl furan	891.5	9.814	B	81	+	+	+	+	+	+	+	+
Furan, 2,5-diethyltetrahydro	897	10.045	C	81	+	+	+	+	+	+	+	+
Unknown 1	910		C		-	-	-	-	+	+	+	+
Unknown 2	920	11.041	C		-	-	-	-	+	+	+	+
Furan 2-pentyl	991.8	14.119	B	81	+	+	+	+	+	+	+	+
2,3,5,6-Tetramethylpyrazine	1085.2	17.933	B	136	+	+	+	+	-	-	+	+
Unknown 3	1096		C	45	+	+	+	+	+	+	+	+

* These compounds were used for the estimation of the sensory scores and the microbial counts

[a] LRI: Linear retention indexes

[b] A: Identification based on LRI and MS data of reference compounds, B: Identification based on LRI and MS data of NIST/WILEY libraries and literature data, C: Tentative identification on based on LRI and MS data of NIST/WILEY libraries

[c] Ions used for quantification

To describe the behaviour of the volatiles that were detected and quantified, each group of compounds was divided into sub-groups according to the similarity in the 'kinetics' between the compounds during storage:

Alcohols

1) 1-Penten-3-ol, 1-pentanol (Figure 3.9.2.), heptanol, 1-octen-3-ol, 2-octenol and 1-octanol exhibited a similar profile. In MAP higher amounts were observed, with an increasing trend. In Air at 0, 5 °C an increase was observed only at the beginning of storage followed by a decrease, whereas at 10, 15 °C a mixed profile was observed.

2) Hexanol showed an increasing trend at all storage conditions, a phenomenon that was more intense at abuse temperatures. Moreover, higher amounts were observed in MAP than in Air (Figure 3.9.3.).

3) 1-Nonanol showed an increasing trend at MAP, a phenomenon that was more intense as the temperature increased. An increase was observed at 15 °C in Air as well, but at 0, 5, 10 °C it was detected in traces.

4) 2, 3-butanediols (2 different enantiomers) presented an increasing trend in Air where higher amounts than MAP were detected. In MAP an increase was observed towards the end of storage. Due to bad chromatographic behavior, these compounds were not further used for quantitative analysis.

5) 3-Methyl-butanol and 2-methyl-butanol showed an increase in Air, where higher amounts than MAP where observed. 2-Methyl-butanol was detected in traces in MAP at all temperatures, whilst 3-Methyl-butanol was observed in traces in MAP at 0, 5 °C and at 10, 15 °C its amounts showed an increase during storage.

6) 3-Octanol exhibited a mixed trend, mostly increased during storage at all conditions.

7) 2-Ethyl-1-hexanol and ethanol, 2-butanol showed a mixed profile.

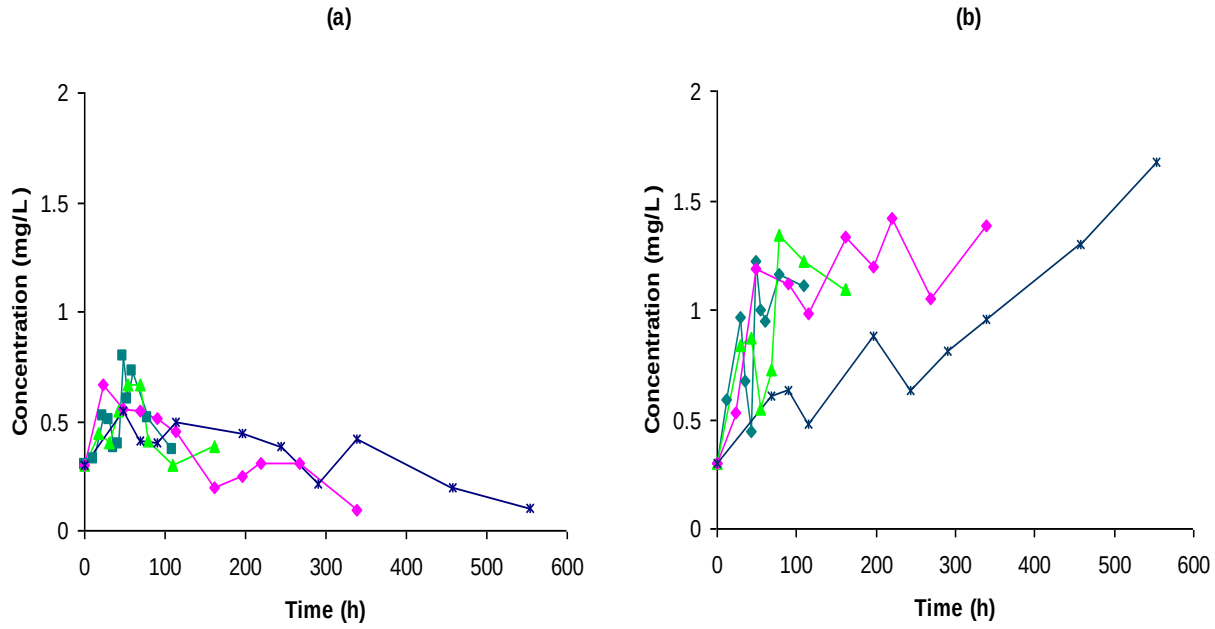


Figure 3.9.2. Changes of the concentration of 1-pentanol in minced beef stored aerobically (a) and under MAP (b) at 0 °C (*) for 554h, 5 °C (◆) for 338h, 10 °C (▲) for 162h and 15 °C (■) for 110h.

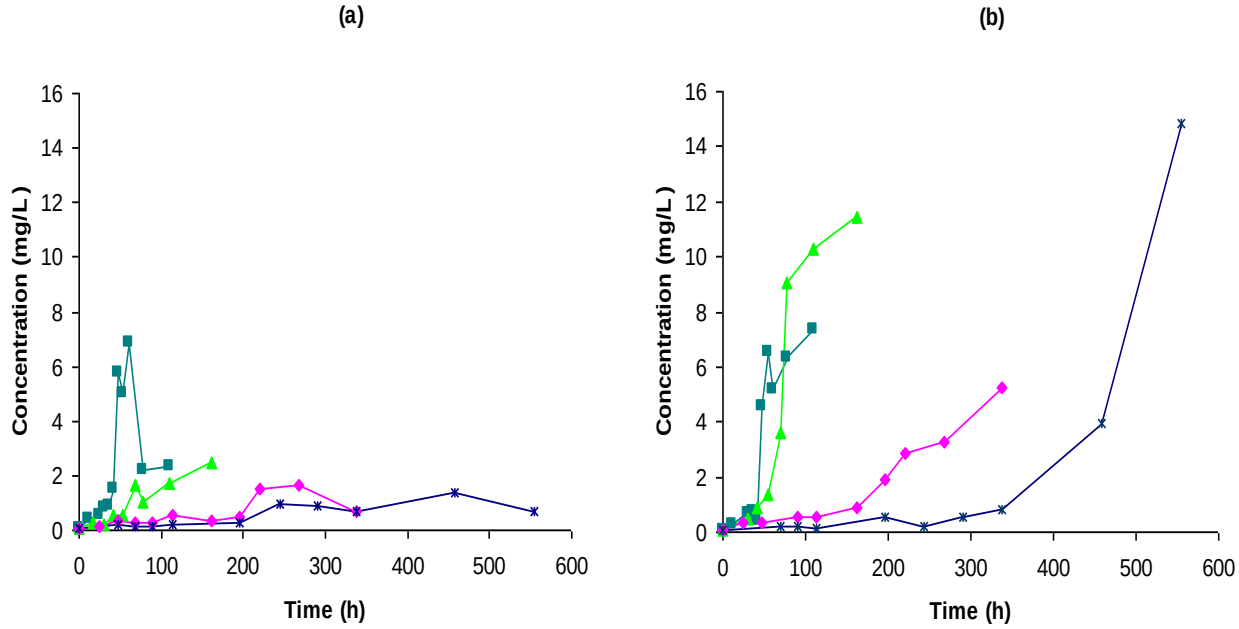


Figure 3.9.3. Changes of the concentration of hexanol in minced beef stored aerobically (a) and under MAP (b) at 0 °C (*) for 554h, 5 °C (◆) for 338h, 10 °C (▲) for 162h and 15 °C (■) for 110h.

Carbonyls

Aldehydes

1) Pentanal (Figure 3.9.4.), hexanal, heptanal, octanal, nonanal, decanal, trans, trans-2, 4-nonadienal (1) and trans, trans-2, 4-decadienal (2) showed a similar mixed trend. In MAP higher amounts were observed than in Air, except from 15 °C in Air that showed the higher values of all the conditions tested. In Air an increase was observed at the beginning of storage followed by a decrease until the compounds were found in traces or not detected at 0, 5, and 10 °C.

2) Benzaldehyde, trans-2-heptenal, trans-2-octenal, trans-2-nonenal and trans-2-decenal showed a similar mixed trend that was analogous to the previously described sub-group 1.

3) 3-methyl-butanal and 2-methyl-butanal showed a similar trend. An increase was observed in Air, and a mixed trend was observed in MAP.

Ketones

1) Acetoin (3-hydroxy-2-butanone) and diacetyl (2, 3-butanedione) showed a similar trend, analogous to aldehydes (sub-group1).

2) 3-Octen-2-one, 2, 5-octanedione and 2, 3-pentanedione showed a similar trend, analogous to aldehydes (sub-group 1).

3) 3-Methyl-2-pentanone was observed only in Air in small quantities, and increased towards the end of shelf life.

4) 2-butanone exhibited a mixed trend but mostly decreased.

5) 2-Pentanone, 2-heptanone, 3-octanone (Figure 3.9.5.) and 2-nonanone showed a similar profile with an increasing trend. 2-Pentanone exhibited higher amounts in Air, whereas 2-nonanone showed higher amounts in MAP.

6) 2-Octanone, 6-methyl-2-heptanone showed a similar profile with group (5) regarding all temperatures in MAP and 15°C in Air, but at 0, 5, 10 °C in Air showed an increase at the beginning of storage and afterwards remained constant.

7) Acetophenone demonstrated a mixed profile

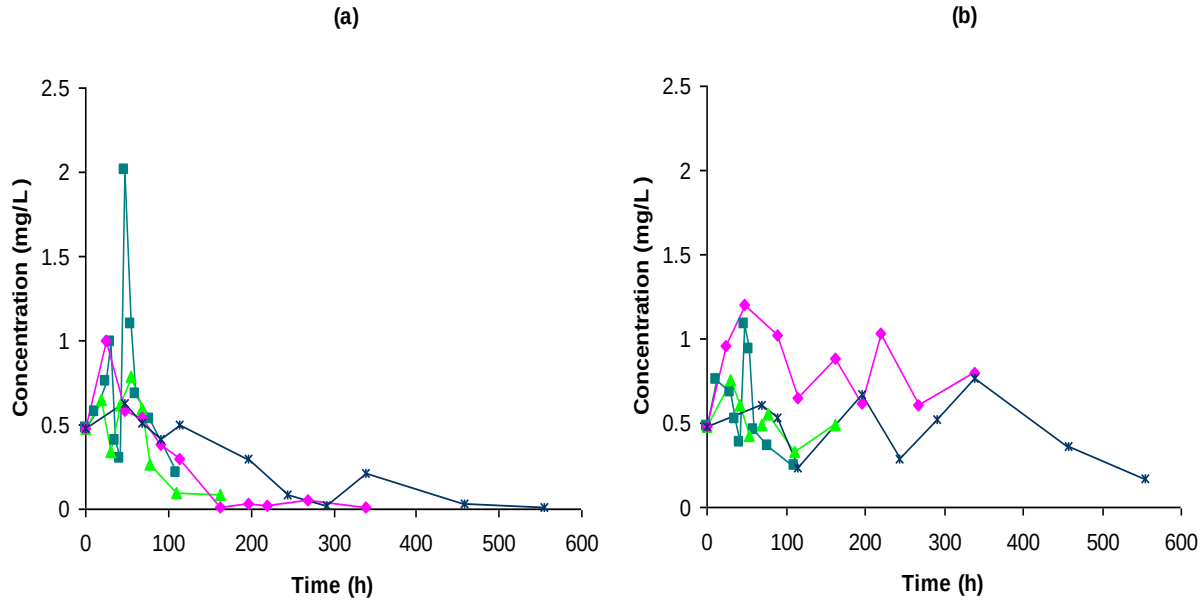


Figure 3.9.4. Changes of the concentration of pentanal in minced beef stored aerobically (a) and under MAP (b) at 0 °C (*) for 554h, 5 °C (◆) for 338h, 10 °C (▲) for 162h and 15 °C (■) for 110h.

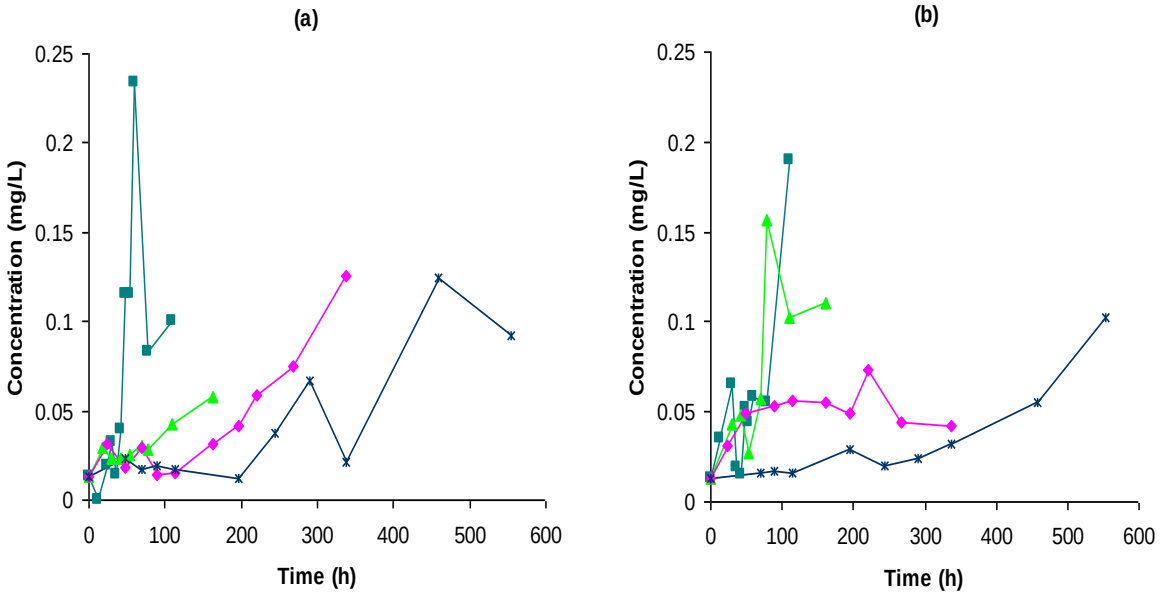


Figure 3.9.5. Changes of the concentration of 3-octanone in minced beef stored aerobically (a) and under MAP (b) at 0 °C (*) for 554h, 5 °C (◆) for 338h, 10 °C (▲) for 162h and 15 °C (■) for 110h.

Hydrocarbons

In general terms, hydrocarbons exhibit a similar mixed trend, with some being present only under MAP and in traces/or not detected at all in Air.

1) Heptane, octane, cyclohexane, methyl (MAP>Air), trans-4-octene, alkane 11, benzene 1, 2, 3-trimethyl, styrene, napthalene, benzene (Figure 9.3.6), ethyl benzene, toluene (MAP>Air) and indane 3, 4, 5, 6, 7, 7a-hexahydro-4, 7-methanoindene were detected at both packaging conditions.

2) The following compounds showed a similar trend to the above in MAP samples; in Air cyclopentane, ethyl, alkanes 1, 2 and 3 were not detected at all, whereas alkanes 4, 5, 6, 7, 8, and 10, cis-2-octene, cyclohexane, ethyl and 1-octene were detected or detected in traces.

3) 1-Undecene was detected only in Air samples, exhibiting an increase after some time of storage depending on the temperature

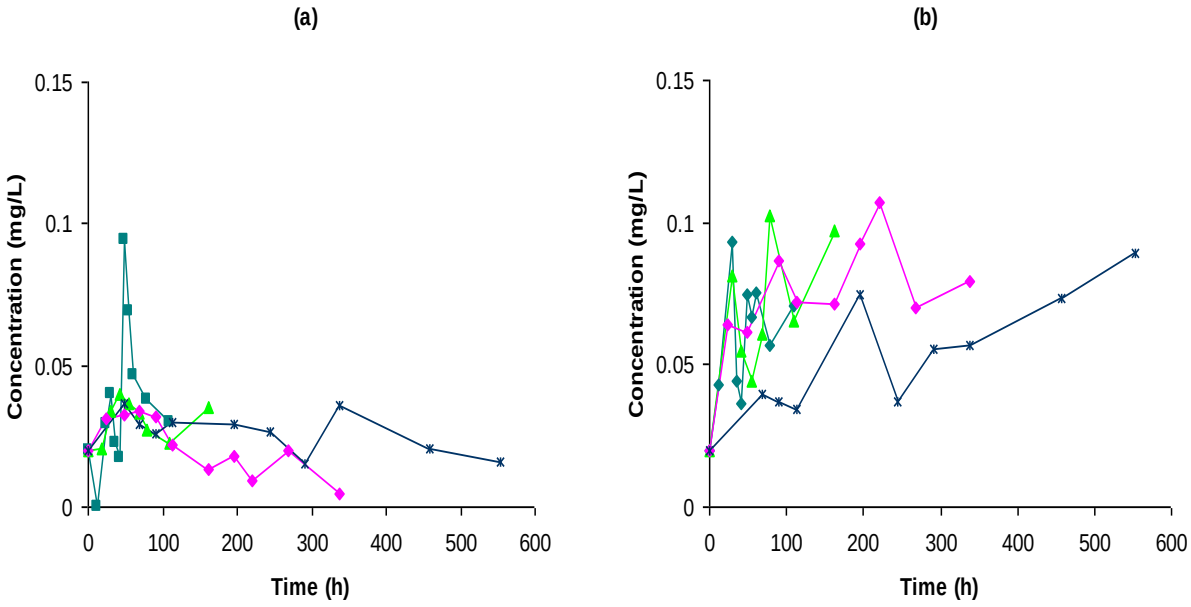


Figure 3.9.6. Changes of the concentration of benzene in minced beef stored aerobically (a) and under MAP (b) at 0 °C (*) for 554h, 5 °C (◆) for 338h, 10 °C (▲) for 162h and 15 °C (■) for 110h.

Terpenes

All the detected terpenes, namely *a*-thujene, *a*-pinene, camphene, sabinene, myrcene, a-phellandrene, 3-carene, *a* -terpinene, limonene, *a* -terpinene, linalool, p-cymene, gave a mixed profile, similar to the above described hydrocarbons. Moreover, the compound cis -ocimene was observed in traces or small quantities at all storage conditions, whereas the Terpene was detected in small amounts/traces at 0, 5 °C in MAP and eucalyptol was detected 0, 5 °C in Air and MAP in small amounts/traces and in higher amounts at the rest storage conditions tested.

Esters

1) Ethyl lactate exhibited a mixed trend with higher quantities being observed in Air at 15 °C.

2) Methyl hexanoate showed also a mixed profile.

3) Ethyl acetate demonstrated an increasing trend at MAP, which was more intense as the temperature increased. Similar profile was demonstrated in Air, except from low temperatures

that a decrease was observed towards the end of storage. In both Air and MAP higher amounts were observed at 15 °C.

4) Ethyl propanoate, ethyl butanoate and ethyl hexanoate showed a mixed trend, with higher amounts being observed with the temperature increase. The highest amounts were detected at 15 °C in Air.

5) Ethyl pentanoate and ethyl heptanoate showed a mixed trend, and detected in traces at 0, 5 °C in Air and MAP, with higher amounts observed at 15 °C in Air. Ethyl octanoate showed a similar mixed trend, and detected in traces at 0 °C in Air and at 0, 5 °C in MAP, with higher amounts observed at 15°C in Air. Ethyl isobutyrate was detected in traces at all storage conditions except from 15°C in Air.

6) Pentyl acetate (Figure 3.9.7.) and hexyl acetate exhibited an increasing trend in MAP, detected in traces at 0, 5, 10 °C in Air, and increased followed by a decrease at 15°C in Air. Isoamyl acetate was detected in traces in MAP and increased only at the end of the storage. In Air an increase was observed, followed by a decrease at 0, 5 °C. In general it was observed in small quantities. Propyl acetate was detected in traces in MAP and increased only at the end of storage, whereas it was not detected in Air.

7) Ethyl 2-methyl butyrate was detected in traces at 0, 5 °C in both Air and MAP and exhibited a mixed trend but mostly increase at 10, 15 in both Air and MAP Ethyl 3-methyl butyrate showed a mixed trend in both Air and MAP at all temperatures, but mostly increased at 10, 15 in both Air and MAP

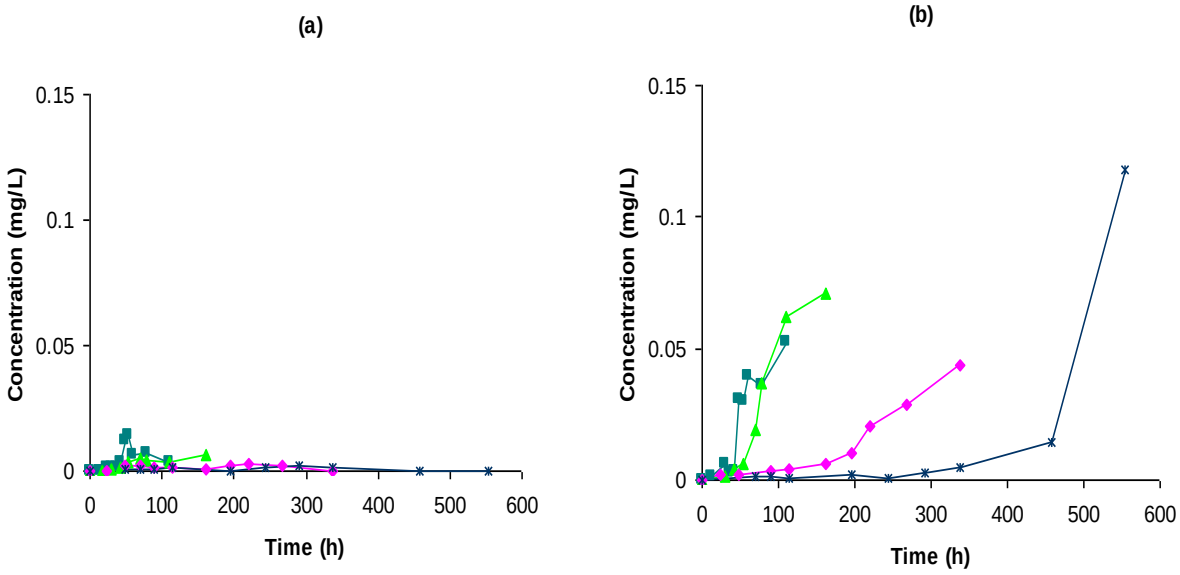


Figure 3.9.7. Changes of the concentration of pentyl acetate in minced beef stored aerobically (a) and under MAP (b) at 0 °C (*) for 554h, 5 °C (◆) for 338h, 10 °C (▲) for 162h and 15 °C (■) for 110h.

Miscellaneous

1) Dimethyl sulphide showed a mixed trend

2) Unknown 1, furan-2-pentyl, furan-2-ethyl, 2n-butyl furan showed an increasing trend in MAP and a mixed trend in Air

3) Furan, 2, 5-diethyltetrahydro and furan, 2-methyl showed a mixed trend, at both storage conditions, with similar aerobic profile to the above described group (1), whereas furan, 2-methyl was observed in very small amounts or in traces in MAP.

4) Unknown 2 and unknown 3 demonstrated an increase in MAP whereas they were not detected in Air.

5) Pyrazine 2, 3, 5, 6-tetramethyl was not detected at 0, 5 °C in MAP, and at 10, 15 °C showed an increase at the end of storage. In Air an increase was observed near the end of shelf life.

3.9.2. Evaluation of the microbial populations and the sensory scores

An analysis was undertaken to correlate the shelf life of the meat regardless of the storage conditions (i.e. temperature, packaging) with the volatile metabolic profile obtained using the GC/MS analysis. All the above peaks that were selected for quantitative analysis are shown depicted in Table 3.9.1. They were all found to be significant based on the PCA and thus all were used for the predictive models.

Figures 3.9.8a-c illustrates the loading plots of the selected variables (peak concentrations) and their contribution to each of the first 3 PCs, whereas Figures 3.9.9a-c depicts the scores plot for the first 3 PCs labelled with the sensory scores of the samples. These figures show the correlations of the different compounds with the sensory scores, as described in the previous section for HPLC analysis (section 3.8.2). From Figures 3.9.8a and 3.9.9a, it can be inferred that 2-butanone is correlated with acceptable samples (fresh and semi-fresh), a peak that showed a decrease during storage. 2-Pentanone, 2-nonanone, 2-methyl-1-butanol, 3-methyl-1-butanol are associated with spoiled samples which increased during storage at the most storage conditions. Similar correlations with the later are observed for the ethyl hexanoate, ethyl propanoate, ethyl lactate, ethyl acetate, ethanol, 2-heptanone, 3-octanone. Likewise, similar conclusions are defined from Figures 3.9.8b and 3.9.9b. Finally, Figures 3.9.8c and 3.9.9c correlate the most of the acceptable samples with 2-butanone, 2,3-pentanedione, 2,5-octanedione, pentanal, hexanal, trans-2-heptanal, trans-2-octenal that all showed a mixed, mostly decreasing trend during storage. On the other hand the compound that are allocated at the two bottom quandrants are positively associated with spoiled samples.

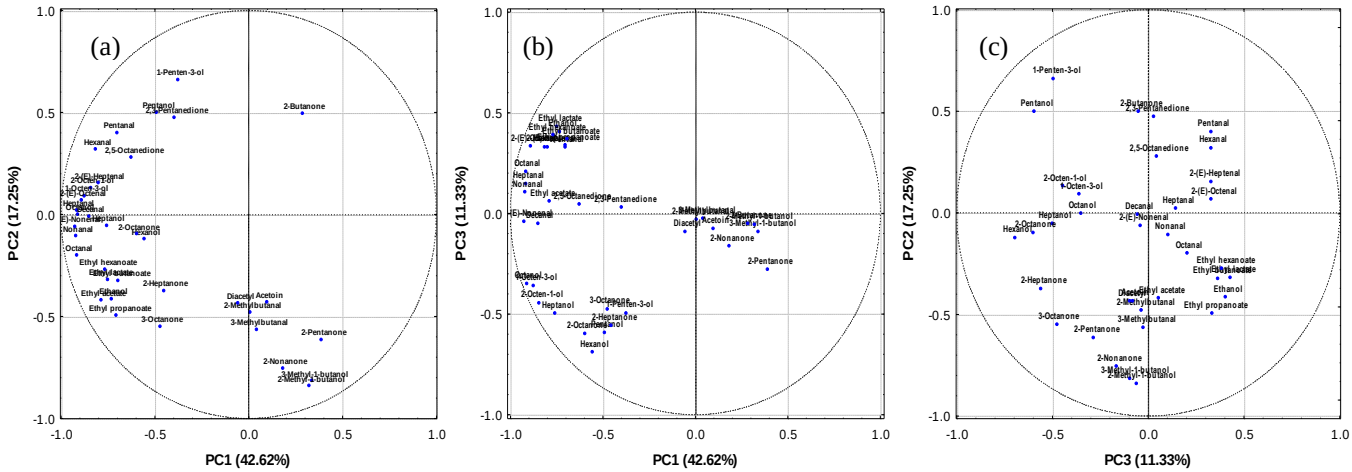


Figure 3.9.8. Loadings plot with the significant variables to each extracted principal component (PC) according to principal components analysis (PCA): (a) PC 1 vs. PC 2, (b) PC 1 vs. PC 3, (c) PC 3 vs. PC 2

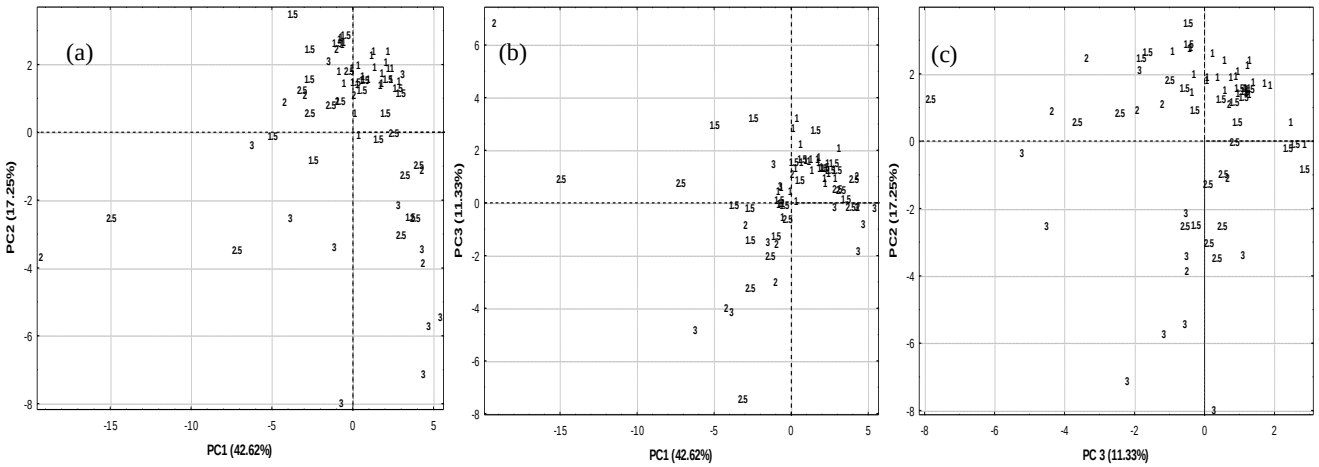


Figure 3.9.9. Scores plot for the first 3 PCs labelled with the sensory scores of the samples (a) PC 1 vs. PC 2, (b) PC 1 vs. PC 3, (c) PC 3 vs. PC 2. Sensory scores scale: 1=fresh; 2=marginal; and 3=unacceptable. Score of 1.5 was characterized as semi-fresh and was the first indication of microbial proliferation. Scores above 2 rendered the product spoiled and pointed the end of the products' shelf life.

According to AHC (data not shown), three major groups could be visualised, one groups corresponds to acceptable mince samples from a sensory point of view (fresh and semi-fresh), one that corresponds to spoiled samples and the third one included 3 sub-groups where 2 of

them corresponded to acceptable samples and another that contains spoiled samples. Due to these defined groups, a 10.8% of the samples were misclassified.

The FDA provided classifications of the samples with an overall correct classification for the validation sensory scores of 79.17% (Table 3.9.2). Figure 3.9.10 demonstrates the discrimination map of the samples regarding their spoilage status (fresh, semi-fresh and spoiled). The map reveals the transition of meat samples from fresh status to semi-fresh and finally to spoiled.

Table 3.9.2 Confusion matrix according to the FDA / GC/MS model for the validation of the sensory estimates

True class	**Estimated class**			**Correct Classification (Sensitivity %)**
	Fresh	**Semi-fresh**	**Spoiled**	
Fresh (n = 27)	21	0	6	77.78
Semi-fresh (n=29)	1	10	5	62.50
Spoiled (n =16)	1	2	26	89.66
Total (n =72)	23	12	37	79.17
Specificity (%)	91.30	83.33	70.27	

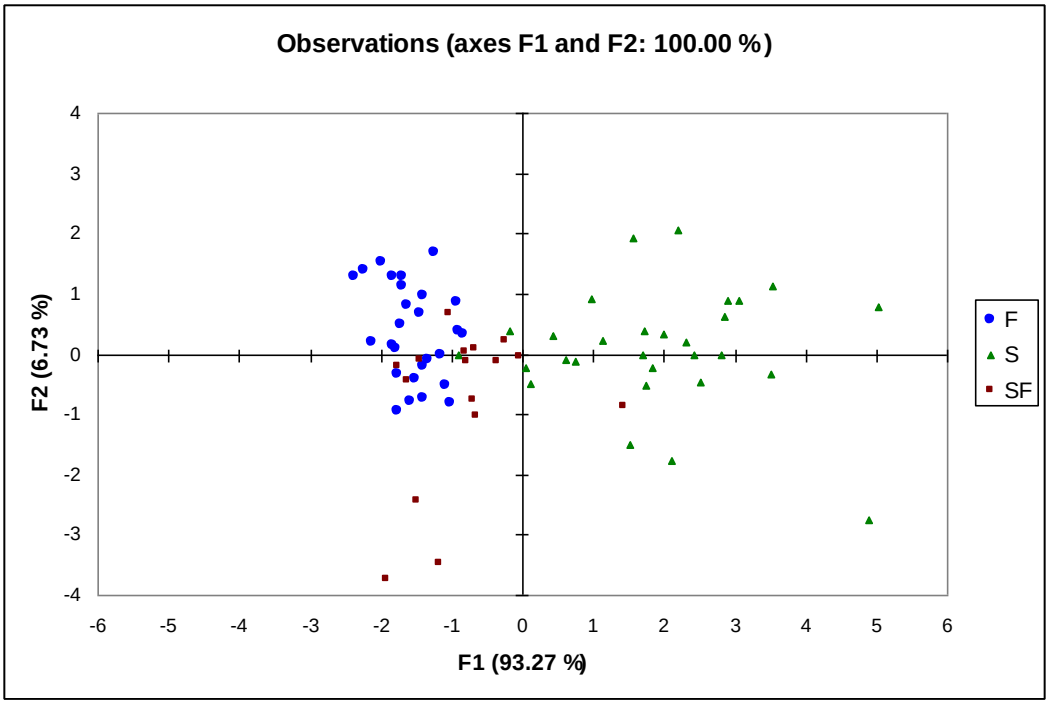


Figure 3.9.10. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the 3 different minced beef freshness groups: Fresh (F), Semi-fresh (SF), and Spoiled (S).

The potential of PLS-R analysis to estimate the population of selected microbial groups of the indigenous microbiota of meat samples such as total viable counts (TVC), *Pseudomonas* spp, *Br. thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, and yeasts/moulds is demonstrated in Table 3.9.3 through the calculated values of bias and accuracy factors as well as the root mean square error index. Figure 3.9.11 illustrates the distribution of the percent relative error (% RE) of the validated values for total viable counts compared to the observed ones.

The values of B_f were generally close to unity, indicating good agreement between observations and estimations (Table 3.9.3), with almost no structural correlation between

estimations and observations. The fact that in certain cases it is slightly above 1 indicates a slight 'fail-dangerous' model (Ross, 1996). In addition, the values of the accuracy factor indicated that the average deviation between estimations and observations of the various microbial groups enumerated ranged from 9.3 % (either above or below the line of equity) for TVC to 14.0% for *Br. thermosphacta* (Table 3.9.3). Moreover, the distribution of percent relative error (% RE) values above or below 0 showed whether there was an under- or over-prediction. Regarding the estimations for TVC the % RE values were distributed above and below 0, with 91.78 % of estimated microbial counts included within the ± 20% RE zone. However, according to the % RE values a trend of over-prediction was evident especially at lower population densities (counts less than 7 log cfu g^{-1}) and some times an under-prediction was observed at higher populations. As far as the models of the remaining microbial groups are concerned (data not shown), the % RE values for the *Pseudomonas* spp., Br. *thermosphacta* and yeasts/moulds were distributed mostly above 0, presenting a general under-prediction of the models, whilst the % RE values for the LAB showed a trend of over-prediction at the lower populations densities and an equal distribution of the rest values above and below the line of equity.

Table 3.9.3 Comparison of calculated performance indices for the estimation of the microbial population in minced beef samples using the validation estimates from the PLS-R / GC/MS models

Microbial group	No of latents	B_f [a]	A_f [b]	% of the samples in ± 20% RE [c] zone	% of the samples in ±10% RE zone	R^2	RMSE[d]
TVC	2	1.001	1.093	91.78	76.71	0.65	0.81
Pseudomonas spp	3	1.012	1.125	83.56	60.27	0.78	0.97
Br. thermosphacta	2	1.010	1.140	75.34	58.90	0.54	0.94
LAB	3	1.008	1.099	90.41	65.75	0.47	0.81
Enterobacteriaceae	2	1.008	1.112	80.82	65.75	0.71	0.84
Yeasts and moulds	3	1.009	1.111	84.93	78.08	0.74	0.78

[a] bias factor, [b] accuracy factor, [c] relative error, [d] root mean square error

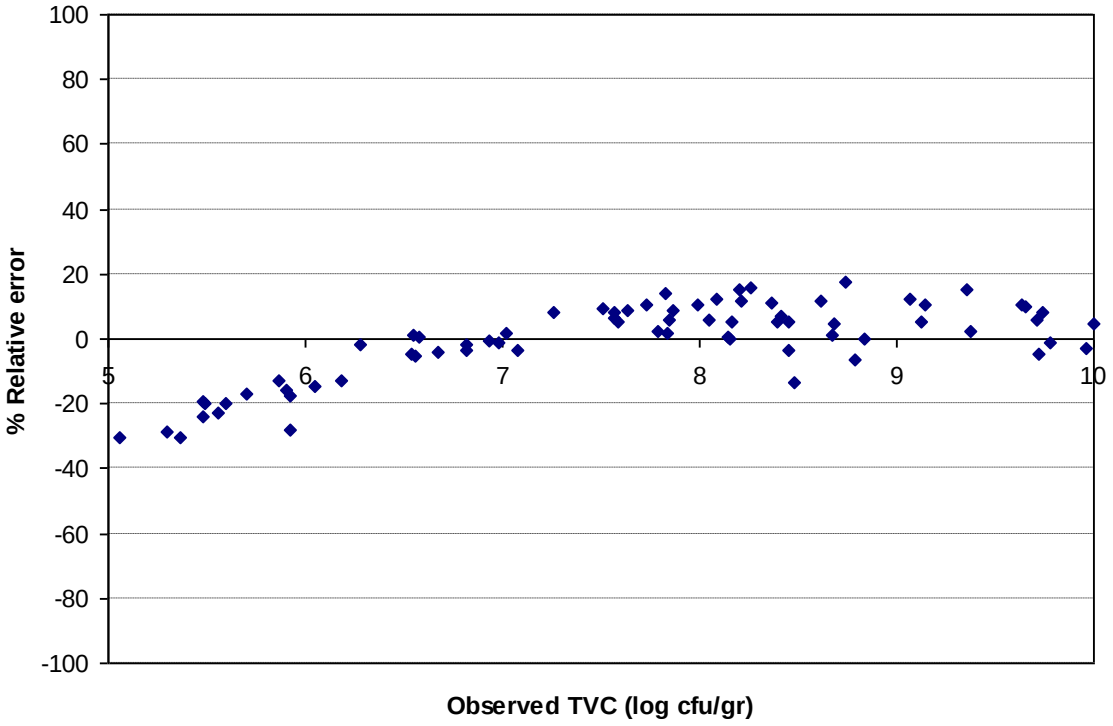


Figure 3.9.11. Percent relative errors (% RE) between observed and estimated counts of the total microbiota (TVC) estimated from the validated values of the PLS-R / GC/MS model

Chapter 4

Discussion

4.1. Survey of minced beef from the Greek Market

The microbiological analysis of the 56 samples of minced beef showed a TVC range of between 4.71-7.99 log cfu g^{-1} The samples obtained from a supermarket, ranged between 5.42 - 7.93 log cfu g^{-1} (mean 6.43 log cfu g^{-1}), had a slightly larger mean microbial populations than from the Butcher shops, that ranged between 4.71-7.99 log cfu g^{-1} (mean 6.00 log cfu g^{-1}). These population levels are lower when compared to other studies (Nychas, 1984; Tsigarida, 2000). *Pseudomonas* spp. exhibited the larger populations, followed very closely by *Br. thermosphacta* especially in the supermarkets' samples. The same trend was observed for LAB that were very close to the ones of *Br. thermosphacta*. This trend was also observed elsewhere (Tsigarida, 2000) and may be attributed to the fact that today supermarkets obtain whole parts of meat pre-packaged in MAP. This may allow a change in the microbial association and induce better competitive growth of *Br. thermosphacta* and LAB. In contrast, this was not observed in the past, as this pre-packaging approach was not used (Nychas, 1984). The F-test also showed a significant effect of the market type on the TVC, *Pseudomonas* spp. and *Br. thermosphacta*. Finally, the mean values of the Butcher shops samples' sensory scores were slightly lower than those of the supermarkets, but their difference was not found to be statistically significant.

When applying ANOVA to the data of the 56 samples in relation to Day of purchase this showed that this factor did not have a significant effect on the microbial association, pH or sensory characteristics of the product. In contrary, regarding the total 150 samples (Section 3.3.1) ANOVA showed that the Day of purchase had a significant effect on the product. This confirms previous studies by Nychas, Robinson and Board (1991). Moreover, for the 56 samples, there was a significant effect of

Season on *Pseudomonas* spp., H_2S-producing bacteria, LAB, *Enterobacteriaceae* and pH. It has to be noted that the samples obtained in the Spring were the most spoiled. ANOVA conducted to define the effect of the season on all 150 samples, showed significant differences between the means of all the microorganisms tested, whilst no effect was observed in relation to pH or sensory scores. This may be due to the increased number of the samples resulting in an increase in variance of the examined variables and the 'sample population' becoming more representative.

4.2. Shelf life of beef fillets stored in air at 0, 5, 10, 15 and 20˚C

The initial microbiota of the beef fillets comprised, in decreasing order of magnitude, of *Pseudomonas* spp., *Br. thermosphacta*, lactic acid bacteria and *Enterobacteriaceae*, whilst the contribution of these groups to the natural microbiota depended strongly on temperature. During aerobic storage of the fillets, it was demonstrated that pseudomonads μ_{max} was higher than of that of the other microorganisms, with *Br. thermosphacta* following very closely. Pseudomonads maintained a growth rate advantage over competing psychrotrophs and mesophiles at temperatures approaching 20°C and are usually the predominant spoilage micro-organisms found in chilled meat stored under aerobic conditions (Lambert *et al*, 1991). Furthermore, the capability of *Br. thermosphacta* to grow on meat during both aerobiosis and anaerobiosis makes it a significant meat colonizer and an important member of the spoilage-related biota (Ercolini *et al*, 2006).

It was also shown that the μ_{max} of *Enterobacteriaceae* becomes similar to that of pseudomonads and *Br. thermosphacta* as the storage temperature increases, and its lag phase decreased. Additionally, the LAB growth rate was always below that of the rest

of the microbial groups, but as the storage temperature increased, showed less deviation from the growth rate values of the other bacteria. This can be of importance since it has been stated that many members of the *Enterobacteriaceae* and LAB can play a role in meat spoilage under certain circumstances (Ercolini et al, 2006; Drosinos, 1994; Holzapfel, 1998). Regarding the sensory evaluation of spoilage, it was demonstrated that it was not always correlated with the same microbial load but depended on the temperature. Dainty *et al.* (1985) reported that with storage, the odour of meat undergoes normal changes gradually from a fresh 'meaty' smell ($\leq 10^7$ bacteria/g) to an inoffensive but definitely non-fresh one, to a dairy/buttery/fatty/cheesy (10^8), and finally to a putrid ($>10^9$) odour. This common aspect was not reproduced in this study, and sensory analysis demonstrated that the products' microbial load at the time of the first sensorial detection of spoilage (meat characterized as Semi-fresh) increased with the temperature (from 4.01 to 7.17). Also, at the end of shelf life of the product (meat characterized as Spoiled) corresponded to microbial loads that ranged between 6.68 (0 ºC) to 8.15 log cfu cm^{-2} (20 ºC) and increased with the temperature. This finding is consistent with an indication that the population threshold that depicts the shift of a sample from fresh to semi-fresh and then from semi-fresh to spoiled is temperature dependant. Consequently, one should take into consideration that introducing a global population threshold may not be accurate enough to define the end of the shelf life of a meat product. In this case, the sensory evaluation can provide complementary information to give more accurate and thus safer conclusions upon the evolution of meat spoilage.

4.3. Shelf life of minced beef stored in air, MAP, and in active packaging at 0, 5, 10 and 15˚C

The microbiological analysis revealed that the initial microbiota of minced beef consisted of *Pseudomonas* spp., *Br. thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria, yeasts and moulds. When minced beef was stored in air, all microbial groups had higher viable counts compared with the packaging under MAP and MAP/OEO. Aerobic storage accelerated spoilage due to the fast growing *Pseudomonas* spp., which were found to be the dominant microorganism at all temperatures tested. Similar results for meat have been reported previously (Tsigarida et al., 2000; Skandamis and Nychas, 2001; Sakala *et al*., 2002; Ercolini *et al*., 2006, 2009). Packaging under modified atmospheres (MAP) delayed the growth rates of all members of the microbial association, as well as the maximum population attained by each microbial group compared with aerobic storage. Moreover, modified atmosphere packaging favoured the emergence of facultative anaerobic populations including lactic acid bacteria and *Br. thermosphacta*. The microbial profiles described above are similar to those found in pork, beef and fish (Koutsoumanis *et al*., 2000; Koutsoumanis *et al*., 2006; Nychas *et al*., 2008; Ercolini *et al*., 2009). In the case of minced beef stored in MAP/OEO the development of the microbial association was highly affected, with the final counts of all the microbial groups (except from the LAB) being ever lower compared to the samples stored in MAP. Similar studies with fish and beef showed that MAP acts synergistically with the essential oil (irrespective of the application method, either in vapour or in direct liquid contact), since only a selected proportion of microbiota, when compared to aerobic storage, is allowed to develop (Skandamis and Nychas, 2001).

According to the sensory analysis, the shelf life of the minced beef decreased with increasing storage temperature and samples stored under aerobic conditions were scored as semi-fresh and spoiled earlier than samples stored under MAP conditions and even earlier than samples stored under MAP/OEO, regardless of storage temperature. The type of muscle spoilage under aerobic conditions was characterized by putrefaction, which is related to proteolytic activity and off odour production by Gram-negative bacteria that dominate. In the case of MAP and MAP/OEO, spoilage was characterized by muscle souring, mostly due to the activity of both lactic acid bacteria and *Br. thermosphacta* (Nychas *et al.*, 1998). These undesirable sensory changes are related to the members of the microbial association, their succession, the type and availability of energy substrates in meat. The initial concentration of glucose, lactic acid, and certain amino acids followed by nucleotides, urea and water-soluble proteins can affect the character (e.g., saccharolytic, proteolytic) and the rate of spoilage and, moreover, seems to be the principal precursor(s) of the microbial metabolite(s) perceived as spoilage (Tassou and Nychas, 1997). Additionally, due to succession of the ephemeral spoilage organisms, there is a change in the metabolic activity of one or more members of the microbial association of meat. This may influence the type (e.g. acidic or alkaline metabolites) as well as the rate of microbial activity (e.g. nutrient consumption) (Skandamis and Nychas, 2001).

4.4. Shelf life of minced beef stored in air and MAP at 5˚C

During the aerobic storage of minced beef at 5 °C, *Pseudomonas* spp. were the dominant microorganisms, followed by *Br. thermosphacta*, yeasts and moulds, LAB and *Enterobacteriaceae*. Packaging under MAP delayed the growth of the pseudomonads, yeasts and moulds, and *Enterobacteriaceae*, suppressed the maximum

level of the aerobic counts compared with the aerobic storage and affected positively the growth of *Br. thermosphacta* and LAB. This particular shift in the microbial association and growth rates between the two packaging conditions has been reported previously (Tsigarida *et al.,* 2000, Skandamis and Nychas 2001, Skandamis and Nychas, 2002, Ercolini *et al.,* 2006). The pH values at the beginning of storage were within the normal range for fresh beef (Borch *et al*., 1996), with the initial mean value of 5.75. There was a decrease in pH for all samples stored under MAP in relation to storage time, whilst an increase was observed in pH values of all samples stored aerobically. These changes in the pH may be attributed to the depletion of glucose and lactate and the formation of organic acids and a-amino groups (Skandamis and Nychas, 2001).

As in the previous studies, the type of muscle spoilage under aerobic conditions was characterized by putrefaction, which is related to proteolytic activity and off odour production by the dominant gram-negative bacteria (Nychas et al., 1998). In the case of MAP the spoilage was characterized by muscle souring, of which main cause, if not the most important are both LAB and *Br. thermosphacta* (Adams and Moss, 1995; Davies and Board, 1998).

4.3. FTIR analysis

In this study FTIR spectroscopy was used to exploit the metabolic fingerprints of different minced beef samples obtained from the Greek Market, beef fillets stored aerobically at five different temperatures, and minced beef stored under aerobic, MAP and MAP /OEO at four different temperatures.

The minced beef samples obtained from the Greek Market were discriminated based on their freshness, the packaging type, the Season and the Day of purchase from the spectral data collected from the FTIR. Also, the ANOVA conducted on the microbiological data of the samples, showed a significant effect of packaging type, the Season and the Day of purchase on the microbial association of the mince. These findings that the ephemeral spoilage organisms (ESO) that were selected in each sample induced the production of different spoilage compounds within the meat substrate. These chemical changes can be reflected by the metabolic fingerprint of a meat sample as pictured by an FTIR spectrum.

The results from the analysis of the FTIR spectra collected from the beef fillets and minced beef spoilage experiments were complementary to each other. In both experiments, there was a well discrimination between the fresh samples from the semifresh ones and the spoiled, regardless the microbial load of a sample. The classification accuracies varied between the different classes for both of the studies, probably due to the different number of the examined samples within each class. This variability is attributed to the fact that the spoilage rate was different between the different temperature and packaging conditions. Thus, no matter how frequently the sampling times were, the sigmoid growth curve that the ESO follow, 'forces' the samples from the one class to the other suddenly, through the sequential accumulation of the spoilage metabolites.

In contrast, the total microbial load was not correlated with the same sensory evaluation of the spoilage but depended on the storage temperature and the packaging. Thus, it was confirmed that the microorganisms may be the main cause of spoilage of

meat, but it is the accumulation of metabolic by-products that characterizes food spoilage (Nychas *et al.*, 2008). For both the spoilage experiments, the performance of the PLS-R models that were built to estimate the microbial counts, was found to be similar between the different microbial groups with the LAB-model and the TVC performing slightly better. Moreover, the performance of the minced beef models was better than for the beef fillets models. This may be attributed to the higher number of the samples tested in the former case (186 vs. 72 samples). Additionally, it can be attributed to the mincing process which results in a more homogenised meat product and thus a more homogeneous evolution of the spoilage process. These results indicate that FTIR spectroscopy is a promising technique, able to develop in a rapid, non-invasive and low cost microbiological and sensory analysis, thus replacing the expensive and time-consuming microbiological analysis and the sometimes subjective sensory analysis. Finally, the discrimination of the samples being stored in different temperatures or in different packaging systems indicates the FTIR as a help-tool to be used in every step of the chill chain, as rapid, quality control point, showing the 'past' of a sample.

Ellis and coworkers (Ellis *et al.*, 2002, 2004) have been the pioneers suggesting that FTIR spectroscopy can be used directly on the surface of food to produce biochemical interpretable ''fingerprints" (metabolic snapshot) enabling early detection of microbial spoilage of chicken breast and beef rump steaks held from freshness to spoilage during 24 h aerobically and at ambient temperature. However, in both studies freshness was neither assessed for non- aerobically packaged products, nor at chill temperatures, i.e. the standard conditions for beef storage. The focus was more on the rapid detection of bacterial spoilage, in terms of microbiological analyses (only TVC),

whereas no attempt was made to correlate spectral data with quality classes defined by sensory assessment of the samples. In addition, spoilage was monitored in only one storage temperature (room temperature), whereas in the current studies different packaging (i.e. aerobic, MAP, MAP/OEO) and temperature (i.e. chill and abuse) conditions were assayed representing the different treatments that a sample may undergo during the chill chain. Also, authors have used 7 log cfu g^{-1} as threshold above which the sample is considered to be spoiled, which was not always the case in the current studies.

The quantitative estimation of the microbial counts was successful in the case of the spoilage experiments, but not in the case of the Survey. This can be attributed to the fact that the samples from spoilage experiments originated from a same 'batch' of beef that had undergone the same post-mortem chemical changes, as the glycogen breakdown (Adams and Moss, 1995). On the other side, the samples of the Survey originated from different parts of beef, which had undergone different types of treatment and been subjected to different levels and types of spoilage. These different types of spoilage probably originated from different post mortem chemical changes and the growth of different ESO. Finally, the different fat content of the samples affects the spectrum of each sample, since fat absorbs in several areas of the studied spectral range (see Table 3.5.1) and may overlap some regions of interest. Consequently, there is a need to study in more depth the whole range of the spectra (1800-900 cm^{-1}) and investigate the areas that are spoilage associated and are not affected by the fat content of the sample.

4.6. Raman analysis and comparison with FTIR

In this study, time series spectroscopic, microbiological and sensory analysis data were obtained from minced beef samples during storage in aerobic and MAP conditions at 5 °C, and analysed using different machine learning methods. These methods included PLS regression and support vector machines regression (SVR) using linear and non-linear functions. These analyses were performed to evaluate Raman spectroscopy in comparison with FTIR spectroscopy applying different types of calibration models for estimating the microbial counts and sensory scores.

In general, it was observed that for both FTIR and Raman calibration models, better estimations were obtained for TVC, LAB and *Enterobacteriaceae*, whilst the FTIR models performed in general slightly better in estimating the microbial counts compared to the Raman models. Regarding the FTIR models, no particular trend of over- or under-prediction was observed, except from estimates of the yeasts and moulds that were always under-predicted irrespective of the model and the *Br. thermosphacta* that showed a trend of over-prediction in the case of PLS. For the Raman models, a trend of under-prediction of all the counts was observed especially at bigger microbial loads in some cases, which was more intense for the estimations of yeasts and moulds. For FTIR models, PLS, SVR_L (linear function) and SVR_P (polynomial function) gave for all the counts sufficient estimates, except from the counts of yeasts and moulds that were underestimated and were totally outside of the acceptable range, no matter the model used. For the Raman models, SVR_R (radial basis function) and SVR_P gave for all the counts sufficient estimates. For the classification models, slightly better performance was observed for the SVR_R and

SVR_P models regarding the FTIR data and for the Raman data, the SVR_P models performed slightly better.

These results indicate that in the case of the FTIR data, two linear models (PLS-R and SVR_L) and one non-linear (SVR_P) were more accurate for the estimation of the microbial counts, whereas two of the SVR non-linear models (SVR_R and SVR_P) gave more accurate estimates for the sensory scores. In the case of the Raman, two non-linear models (SVR_R and SVR_P) estimated more accurately the microbial counts, whereas the non-linear SVR_P gave better estimations for the sensory scores, indicating SVR models as more capable of fitting the Raman data. The possible explanation for the above conclusion lies between the next lines.

In many industrial (spectroscopic) applications, PLS is used to make regression models because of its simplicity to use, speed, relative good performance and easy accessibility. However, non-linear relationships can only be modelled in a limited way (i.e., weak nonlinearities) by taking into account more latent variables (Thissen *et al.*, 2004). Support Vector Machines (SVMs), originally proposed by Vapnik *et al.*, in 1995, might also be useful for spectral regression purposes (support vector regression: SVR). A possible large advantage of SVR is its ability to model nonlinear relations and it is gaining popularity due to its attractive features and promising empirical performance (Thissen *et al.*, 2004; Shinoda *et al.*, 2008). SVM is a new type of machine learning theory based on statistical learning theory, which emphasizes statistical learning in the case of fewer samples. The structural risk minimization principle derived from statistical learning theory takes this as the foundation, as compared with artificial neural networks (ANNs), giving the SVMs prominent

advantages. Firstly, the strong theoretical basis provides a high generalization capability and avoids overfitting. Secondly, the global model is capable of dealing efficiently with high-dimensional input vectors. Third, the solution is sparse and only a subset of training samples contributes to this solution, thereby reducing the workload (Zou *et al*,. 2006).

Taking into account the above observations, Raman spectroscopy can be used as a rapid, non-invasive technique for estimating the spoilage of meat, giving similar and complementary information with FTIR spectroscopy. Since this is the first time that this technique (Raman) was investigated for its capability in estimating the meat spoilage, additional studies are needed to enhance the number of the input data with samples stored under several packaging and storage conditions, and to explore the type of the best applied machine learning method.

4.7. Image analysis

Regarding the values of the spectra obtained for each storage condition some noticeable differences were visually observed. The values of several bands exhibited a decrease during aerobic storage of meat, whereas an increase was observed in the case of MAP. These changes were observed more intensely in aerobic stored samples. More specifically, the most profound changes in the case of aerobic stored samples, were shown in the values of the bands 470 (blue), 645, 660, 700 (red), and 850, 870, 890 (NIR) that showed a decrease, and so did the bands 505, 565 (green), 590 (amber) but in a less degree. In the case of MAP stored samples, the values of the bands 450, 470 (blue), and 870 (near infrared) showed an increase, and so did the bands 430 (ultra blue), 505, 565 (green) and 630 (red), but in a less degree. These differences in

the progress of the colours' changes between the different packaging conditions are indicating the different discolouration processes between the different packaging techniques, probably due to the emergence of the ESO at each case.

Many studies upon meat spoilage refer to green discolouration, as a typical undesirable change on the meat surface, caused by different types of microorganisms. This phenomenon has been attributed to the presence of H_2S, which may interact with myoglobin to form sulfmyoglobin, which deteriorates the meat colour by 'green discolouration' (Gill and Newton, 1979). Microorganisms that are referred to produce H_2S and thus cause 'greening' are *Sh. putrefaciens*, members of the *Enterobacteriaceae* (*Serratia liquefaciens, Hafnia alvei* and *Enterobacter* (Pantoea) *agglomerans*) and some strains of *Saccharomyces cerevisiae* and *S. bayanus* (Dainty et al., 1985, 1989a; Jay, 2000; Fleet, 2006). Moreover, certain obligatory or facultatively heterofermentative LAB, such as *Leuconostoc*/ *Weissella* and *Lactobacillus sakei/curvatus* respectively, were found to be responsible for meat pigment discoloration (greening) at package opening due to H_2O_2 formation (Schillinger and Lucke, 1987a,b; Borch *et al.*, 1996). Additionally, when fresh meat is stored anaerobically, certain LAB such as *Lact. sakei* may cause or accelerate spoilage by formation of H_2S in package, which results in black spots on meat due to reaction of H_2S with the iron present in myoglobin (Shay and Egan, 1981; Egan *et al.*, 1989).

Finally, yeasts (*Candida lipolytica, Candida zeylanoides* or *Yarrowia lipolytica*) may cause also pigment discolouration through the pigmented spots from their surface colonization. Moulds commonly associated with surface colonization of aerobically

stored meats include *Thamnidium*, *Mucor*, *Penicillium* and *Rhizopus*, as well as *Cladosporidium herbarum* and *Sporotorichum carnis*, which cause 'black spot' and 'white spot' spoilage, respectively (Gill *et al.*, 1981; Mossel *et al.*, 1995).

Regarding the evaluation of the sensory estimates, it was found that the PLS-R model provided better estimations from the FDA model (correct classification for the validation sensory scores of 86.54% vs 71.15%). Moreover, the former was able to not to miss-classify fresh samples as spoiled and spoiled samples as fresh or semi-fresh, whilst many fresh samples were miss-classified as semi-fresh. It has to be stressed out though, that the number of the fresh and semi-fresh samples tested were less than the spoiled ones, due to the reason explained above in the section 4.5. On the other hand, concerning the estimation of the microbial counts, it was observed that better estimations were obtained for TVC, LAB and yeasts and moulds. All the above results, consist an indication that the image analysis may be proved as an accurate, rapid and non- destructive method that gives useful information about the changes on the surface of the meat and delivers a direct answer about the microbial load and the sensorial status of a given unknown meat sample.

4.8. HPLC analysis of organic acids

The results derived from HPLC analysis demonstrated changes of the chromatographic areas under the peaks of the eluted acids, that were associated or not with the storage conditions (e.g. temperature, packaging). For example, acetic acid exhibited an increase in all cases, that was more intense in samples stored under MAP and MAP/OEO at 5, 10, and 15 °C. This increase may be attributed to the fact that acetic acid is produced by several members of the microbial association, namely

Pseudomonas spp., *Br. thermosphacta*, *Enterobacteriaceae*, LAB and it was noticeable in meat stored under aerobic or modified atmosphere conditions (Lampropoulou *et al*., 1996; Garcia-Lopez *et al*., 1998; Zaunmüller *et al*., 2006). Moreover, the increased yields observed under MAP and MAP/OEO, could be associated with several lactic acid bacterial strains that oxidize indigenous (meat) and microbial L(+)- and D(-)-lactic acid, to acetic acid under atmospheres enriched with CO_2 once the glucose sources are depleted (Drosinos and Board, 1995).

Regarding the total amounts of lactic acid, a decline was evident at all temperatures when samples were stored aerobically. However, in MAP stored meat samples, the total amounts of lactic acid remained unchanged with an increase towards the end of shelf life, whereas a noticeable increase was observed in samples stored under MAP/OEO. This fact may be associated with changes in the metabolic pathways of some members of the microbial association, such as those in the homofermentative or heterofermentative pathway of glucose metabolism by lactic acid bacteria (Nychas *et al*., 1998). Therefore, if heterofermentative strains of lactic acid bacteria are selected under MAP, the oxidative pentose–phosphate pathway is used and lactic acid is produced with other intermediates such as ethanol and acetaldehyde (Zaunmüller *et al*., 2006). On the other hand, if more homofermentative strains are selected when OEO is present, then more lactic acid is produced as main end product from Embden – Meyerhof – Parnas pathway (Holzapfel, 1998).

Another bacterium, *B. thermosphacta*, produces anaerobically L-lactate and ethanol as its main end-products from glucose, but aerobically, it produces L-lactate as well as a mixture of acetic and butyric acids, acetoin and alcohols which significantly

contribute to off-odour development (Samelis, 2006). It also utilizes glutamate aerobically, while it may produce small amounts of formic acid irrespective of gaseous atmosphere. Thus, *Br. thermosphacta* has the ability to possess and activate various metabolic pathways for sugar breakdown; It generally behaves as a homofermentative bacterium under low oxygen and glucose availability, while it shifts to heterofermentative under high oxygen and/or glucose-limiting conditions (Samelis, 2006).

Similarly, the profile of the rest organic acids of the product is changing, as the microbiota utilize or produce glycolitic compounds through the glycolytic pathway and organic acids are perceived produced as intermediates of citric acid cycle. From this point of view, when a metabolic pathway changes and different intermediate products or by-products occur during the different dynamic storage conditions, the characterization of the spoilage on its onset and at the end of sensory shelf life is a complicated issue.

Subsequently, an analysis was undertaken to correlate the shelf life of the mince regardless the storage conditions (i.e. temperature, packaging) with the HPLC profile of organic acids. According to the PCA, the derived loading plots and scores plot gave useful information about the possible correlations of the compounds used for the analysis in correlation with the sensory scores. In line with the identified peaks, it was shown that lactic and citric acids were correlated with semi-fresh samples, whereas acetic and propionic acids were correlated with the spoiled samples.

The AHC, which is an unsupervised method, could provide a discrimination between acceptable and spoiled samples, whereas the FDA analysis –a supervised method- could provide discriminations between the three different sensory classes; fresh, semi-fresh and spoiled samples. The correct classification rate for the validation of the FDA model was 88.46% correct for the fresh samples, 81.82% for the semi-fresh and 89.47% for the spoiled ones, with an overall performance of 88% accuracy. These results revealed a good correlation of the sensory estimates of the spoilage with the dynamic changes of the chromatographic areas of organic acids that were present at different times through storage.

The potential of PLS-R analysis to estimate the population of selected microbial groups of the indigenous microbiota of meat samples such as TVC, *Pseudomonas* spp, *Br. thermosphacta*, LAB, *Enterobacteriaceae*, and yeasts/moulds was also demonstrated in this work. The values of B_f were generally close to unity, indicating good agreement between observations and estimations, with almost no structural correlation between estimations and observations, whilst the fact that in certain cases it is slightly below 1 indicates a 'fail-safe' model (Ross, 1996). In addition, the values of the accuracy factor indicated that the average deviation between estimations and observations of the various microbial groups enumerated ranged from 9.0% (either above or below the line of equity) for lactic acid bacteria to 17.9% for *Br. thermosphacta*. Regarding the estimations for the TVC the % RE values were randomly distributed above and below 0, with 90.8% of estimated microbial counts included within the ± 20% RE zone, with a trend of over-prediction being evident especially at lower population densities (counts less than 7 log cfu g^{-1}). Moreover, for

the remaining microbial groups a general under-prediction of the models was observed.

These findings indicate that HPLC analysis of organic acids can be utilised as a potential technique for meat analysis to estimate the spoilage status and the microbial load of a meat sample regardless of storage conditions.

4.9. GC/MS analysis

HS/SPME-GC/MS analysis facilitated the investigation of the volatile compounds that are present during the storage of beef under different temperature and packaging conditions. The detected compounds were found most of the times to be temperature and/ or packaging dependant. Some of the identified compounds have been referred in the past to be present during storage of meat or meat products under several temperature and packaging conditions and their behaviour through time is most of the times in accordance with the current results (Dainty *at al.*, 1985, 1989; Stutz *et al.*, 1991; Edwards *et al.*, 1987; Jackson *et al.*, 1992; Tsigarida and Nyghas, 2001; Insausti *et al.*, 2002; Leroy *et al.*, 2009; Ercolini *et al.*, 2009). In each study, a plethora of different compounds were identified that depending on the analytical method used, the type of the meat and on the storage conditions, making the correlations difficult. The main groups of volatile compounds that have been found during spoilage of meat are alcohols, aldehydes, ketones, hydrocarbons, esters and sulphur compounds (Nychas *et al.*, 2007; Ercolini *et al.*, 2009; Leroy *et al.*, 2009).

According to the profiles of alcohols, different trends and amounts were observed between the same compounds at different packaging conditions. Hexanol, 2, 3-

butanediols (2 different enantiomers) and 3-octanol showed generally an increase at all storage conditions. 1-Penten-3-ol, 1-pentanol, heptanol, 1-octen-3-ol, 2-octenol, 1-octanol and 1-nonanol exhibited an increase mainly in MAP samples, whereas 3-methyl-butanol and 2-methyl-butanol showed an increase in Air. 2-Ethyl-1-hexanol and ethanol, 2-butanol showed a mixed profile.

All alcohols may be ascribed to glucose and amino acid metabolism of the microorganisms present or/and to the oxidation of fatty acids. More specifically, gram negative bacteria (e.g. *Pseudomonas, Shewanela*, *Moraxella*) have been found to produce ethanol, methanol, 2-methyl propanol (precursor valine) and 2-methylbutanol (precursor isoleucine) (Nychas *et al*., 2007). *Br. thermosphacta*, produces different types of alcohols according to the storage conditions due to the change of its metabolism. Anaerobically, it produces mainly ethanol (precursor glucose), whereas aerobically, it produces ethanol, 3-methylbutanol (precursor leucine), 2-methylbutanol (precursor isoleucine), 2,3-butanediol (precursor diacetyl) and 2-methylpropanol (precursor valine) (Nychas *et al*., 2007; Samelis, 2006). Homofermentative and heterofermentative LAB were as well reported to produce ethanol which probably originated from the glucose catabolisms and/or the microbial conversion of threonine (Nychas *et al*., 2007, Tsigarida and Nychas 2001, Leroy *et al*., 2009). Ethanol has been releted to 'bread dough-yeast like' sweet odour (Marco *et al.*, 2007, Insausti et *al.*, 2002). 3-methyl butanol and 2-methyl propanol are possible bacterial conversion products of leucine and valine, respectively, following the Strecker degradation of amino acids during the proteolysis (Smit *et al*., 2005; Luna *et al*., 2006). 3-Methyl-1-butanol can be associated with a whiskey-like/green odour (Luna *et al*., 2006; Lattouture-Thiveyrat *et al*., 2003).

Some of the detected alcohols have been also reported as possible products of lipid oxidation. These are 1-propanol, 1-butanol, 1-heptanol, 1-octanol, 1-octen-3-ol, and 2-ethyl hexanol (O'Sullivan *et al.*, 2005; Leroy *et al.*, 2009). 1-octanol and 1-octen-3-ol may attribute mushroom odours (Marco *et al*., 2007; Luna *et al*., 2006; Insausti et *al.*, 2002), 1-octanol may give mushroom/sharp/ fatty odours (Marco *et al*., 2007; Luna et al., 2006) and 2-ethyl hexanol can be responsible for ''earthy'' off-odours (Leroy *et al*., 2009). Finally, out of the rest detected alcohols, 1-butanol, 1-pentanol, 1-hexanol and 1-penten-3-ol may attribute to meat fruit/medicinal, roasted meat/balsamic, green grass and butter odours, respectively (Marco *et al*., 2007; Insausti et *al.,* 2002).

Aldehydes, similarly to alcohols, may be produced by glucose and amino acid metabolism and lipid oxidation. Aldehydes are reported as particularly important components in imparting fatty flavours; the short-chain aldehydes tend to be rather sharp or acid with the fattiness increasing with chain length and degree of non-saturation (Leroy *et al*., 2009). Xie *et al.* (2008) have reported that the linear saturated and unsaturated aldehydes with more than five carbon atoms can be produced from fat oxidative degradation, whilst the methyl-branched aldehydes (3-methylbutanal) and phenyl aldehydes (phenyl acetaldehyde) are probably related to amino acid degradation. The origin of branched aldehydes and their corresponding alcohols is also attributed in Strecker degradation reactions of amino acids (Ventanas *et al*., 1992). In the current study, pentanal, hexanal, heptanal, octanal, nonanal, decanal, trans, trans-2, 4-nonadienal (1), trans, trans-2, 4-decadienal (2), benzaldehyde, trans-2-heptenal, trans-2-octenal, trans-2-nonenal and trans-2-decenal showed a similar

mixed trend with higher amounts being observed in MAP and a decrease being observed in Air.

Hexanal has been described as a product deriving from the unsaturated omega-6 fatty acids oxidation (Brewer and Vega, 1995). Montel *et al*. (1998) have also reported that hexanal can derive from hydrolysis of triglycerides or from amino acid degradation. Hexanal and 2, 4-decadienal may contribute positively to beef flavour, but may produce undesirable flavours at higher concentrations (Melton, 1983). High quantities of hexanal can produce rancid, fresh cut grass off-flavour (Luna *et al*., 2006; Marco *et al*., 2007).

Nonanal was reported as the decomposition product of the 10-hydroperoxide produced from the oxidation of oleic acid, which is the main fatty acid in beef fat (Insausti *et al.* 2002), as well as a product from amino acid degradation (Mondel et al., 1998). Dainty *et al.* (1984) and Edwards *et al.* (1987) have identified nonanal, to be among the volatile compounds isolated from samples of sterile beef which were inoculated with various strains of *Pseudomonas.* It has been described to give pelargonium, soapy, rancid odour and was related to unpleasant sensory answer (Ercolini *et al.* 2009; Insausti et *al.*, 2002). Benzaldehyde has been ascribed as possible bacterial conversion product of phenylalanine (Smit *et al*., 2005), whilst it contributes with almond odours in meat (Luna *et al.* 2006).

3-methyl-butanal and 2-methyl-butanal have showed an increase in aerobically stored samples and a mixed trend under MAP. 3-methylbutanal has been reported to be produced under aerobic conditions by *B. thermosphacta* aerobically (Samelis, 2006),

whereas 2-methylbutanal (precursor isoleucine) can be produced by several Gram negative bacteria (eg *Pseudomonas, Shewanela, Moraxella*) (Nychas *et al*., 2007). 3-methylbutanal and 2-methylbutanal are related to fruity, cheese, toasted and malty odour (Insausti et *al.,* 2002; Hinrichsen and Pedersen, 1995; Marco *et al*., 2007).

The rest aldehydes may contribute to meat odours with rancid, green (pentanal); fatty, green, roasted, rancid (heptanal); salty (trans-2-hexenal); rancid, dirty (trans-2-heptenal); geranium, herbal, floral, soapy (octanal and decanal); roasted, cured (trans-2-octenal); and green, fatty (trans-2-nonenal) notes (Luna *et al.* 2006; Marco *et al*., 2007; Xie *et al*., 2008).

Ketones probably originate from several fatty acid oxidation reactions chemical autooxidation, enzymatic α- or β-oxidation (Leroy *et al*., 2009; Ercolini *et al*., 2009). Some methyl ketones such as 2-nonanone can derive from a lypolytic process but also from several other possible pathways, such as alkane degradation by *Pseudomonas* through a unique alpha-oxidation, with no change in the carbon skeleton (Ercolini *et al*., 2009). Methylketones are also formed by bacterial dehydrogenation of secondary alcohols, a reaction that appears to be part of the alkane oxidation sequence (Ercolini *et al*., 2009).

In the present work, acetoin (3-hydroxy-2-butanone), diacetyl (2, 3-butanedione), 3-Octen-2-one, 2, 5-octanedione and 2, 3-pentanedione showed a similar trend, analogous to aldehydes. *Br. thermosphacta* has been reported to produce acetoin (presurcors: glucose, alanine, diacetyl) and diacetyl under aerobic conditions Inoculation experiments with *Enterobacteriaceae* and *B. thermosphacta* showed an

initial increase in the levels of acetoin and diacetyl. Moreover, several Gram negative bacteria (e.g. *Pseudomonas, Shewanela, Moraxella*) and homofermentative LAB (*Lactobacillus, Leuconostoc* and *Carnobacterium*) have been found able to produce acetoin and diacetyl (precursor pyruvate) (Nychas *et al*., 2007). Leroy *et al.*, (2009) reported that acetoin can originate from the microbial degradation of aspartate and/or from the glucose catabolism of *Br. thermosphacta, Carnobacterium* spp., and *Lactobacillus* spp. The accumulation of acetoin and diacetyl was related by Dainty *et al.*, (1989) to the detection of creamy, dairy, and cheesy odours. The later led to the characterisation of meat as not fresh but were not sufficiently intense or unpleasant to characterise it as spoiled.

2-butanone exhibited a mixed trend but mostly decreased, whilst 2-Pentanone, 2-heptanone, 3-octanone, 2-nonanone, 2-Octanone, and 6-methyl-2-heptanone showed mainly an increasing trend at both storage conditions of the current study. These ketones probably originate from several fatty acid oxidation reactions (Leroy *et al*., 2009; Yu *et al*., 2008). 2-butanone was reported to be produced by Gram negative bacteria (eg *Pseudomonas, Shewanela, Moraxella*) (Nychas *et al*., 2007) and may contribute to the sensory perception ‘‘ethereal’’ (Luna *et al*., 2006). 2-pentanone may give roasted, sweet odours (Marco *et al*., 2007), 2-heptanone gives spicy, fruity, cinnamon flavour, (Luna *et al*., 2006; Insausti et *al.*, 2002; Marco *et al*., 2007) and 2-octanone has been characterised by the sensory attribute ‘‘green herbaceous’’ (Luna *et al*., 2006). 2-nonanone, can be formed through the beta-oxidation of decanoic acid derived from lipolysis and in a second step through beta-ketoacid decarboxylation (Ercolini *et al*., 2009) and is characterised by a roasted/burnt odour (Marco *et al*., 2007)

In general, hydrocarbons exhibited a similar mixed trend, while some of them were present mainly under MAP. Heptane, octane, cyclohexane, methyl, trans-4-octene, alkane 11, benzene 1, 2, 3-trimethyl, styrene, napthalene, benzene, ethyl benzene, toluene and indane 3, 4, 5, 6, 7, 7a-hexahydro-4, 7-methanoindene were detected at both packaging conditions. Cyclopentane, ethyl, alkanes 1, 2, 3, 4, 5, 6, 7, 8, 10, cis-2-octene, cyclohexane, ethyl and 1-octene were mainly detected in MAP stored samples. In contrary, 1-Undecene was detected only in Air samples, exhibiting an increase after some time of storage depending on the temperature. Aromatic compounds such as benzene and toluene, may play a role in the overall flavour of beef although no individual compound of this group has a meat-like odour itself (Insausti *et al*., 2002). Benzene contributes to butter flavour, and toluene is related to painty odour and to sour and bloodlike flavour descriptors (Insausti et al., 2002). Toluene can be produced by various strains of *Pseudomonas (*Dainty *et al.*, 1984; Edwards *et al.*, 1987), and by several other Gram negative bacteria such as *Shewanela* or *Moraxella*) (Nychas *et al*., 2007).

All the detected terpenes, gave a mixed profile, similar to the above described hydrocarbons. They have been found to contribute with citrus, spicy, floral, green, wood odour to meat (Xie *et al*., 2008; Marco *et al*., 2007; Luna *et a*l., 2006).

Esters may be produced by esterification of the various alcohols and carboxylic acids found in beef (Insausti *et al*., 2002). Ethyl esters were reported to be formed by microbial esterase activity, through chemical esterification of alcohols and carboxylic acids following (Ercolini *et al*., 2009). Methyl esters and ethyl esters can also be

produced by microorganisms such as *Pseudomonas, Shewanela, Moraxella* (Nychas *et al*., 2007).

In the present study, ethyl lactate, ethyl propanoate, ethyl butanoate, methyl hexanoate ethyl hexanoate, ethyl pentanoate, ethyl heptanoate, and ethyl octanoate exhibited a mixed trend with higher quantities being observed at most cases in Air at 15 °C. Ethyl acetate demonstrated an increasing trend at both conditions, except from chill temperatures in air that a decrease was observed at the end of storage, with higher amounts being observed at 15 °C. Pentyl acetate and hexyl acetate exhibited an increasing trend in MAP and increased followed by a decrease at 15°C in Air. Ethyl 2-methyl butyrate was detected in traces at chill temperatures and increased at abuse temperatures in both packaging conditions. Ethyl 3-methyl butyrate showed a mixed trend in both Air and MAP at all temperatures, but mostly increased at abuse temperatures in both Air and MAP. According to the above, esters were found to exhibit a diverse behaviour and be highly depended to temperature and packaging conditions. They were detected with higher amounts in one packaging condition, whereas when they were detected in traces in one packaging condition was usually for the lower storage temperatures. These compounds may contribute with fruity, sweet, sour, and citrus to meat odour/flavour (Marco *et al*., 2007; Takeoka *et al*., 1996)

Sulfur compounds usually arise from sulforated amino acids catabolism (Ercolini *et al*., 2009). In this study, dimethyl suplphide was a sulphur compound that exhibited a mixed trend. It most likely originated from sulfur containing amino acids such as cysteine and methionine developed by psycrotrophic bacteria, which can produce proteolytic enzymes in beef (Insausti *et al*., 2002). Dimethyl disulfide may be

produced by LAB through the degradation of cysteine or by Gram negative bacteria (except from *Enterobacteriaceae*) through the degradation of methenethiol and/or methionine (Montel *et al*., 1998; Ardö, 2006; Nychas *et al*., 2007). It has been related to cabbage and rotting odour and to sour, bloodlike, and cooked beef fat flavour descriptors (Larick and Turner, 1990; Leroy *et al*., 2009).

Furan-2-pentyl, furan-2-ethyl, 2n-butyl furan, and Unknown 1, showed an increasing trend in MAP and a mixed trend in Air. Also, furan, 2, 5-diethyltetrahydro and furan, 2-methyl showed a mixed trend, at both storage conditions, with similar aerobic profile to the previously described group, whereas furan, 2-methyl was observed in very small amounts or in traces in MAP. Furan-2-ethyl was detected in cooked meat and was identified as contributor to beef flavour with some other heterocycles (St. Angelo *et al*., 1987).

Two unknown compounds, Unknown 2 and unknown 3 demonstrated an interesting increase in MAP whereas they were not detected at all in Air. The only compounds that were also not detected under aerobic conditions were cyclopentane-ethyl and alkanes (1), (2), (3).

Pyrazine 2, 3, 5, 6-tetramethyl was not detected at 0, 5 °C in MAP, but at 10, 15 °C showed an increase at the end of storage. In Air a sharp increase was observed near the end of shelf life, that may introduce this compound as a spoilage indicator for aerobic storage of beef.

According to the PCA, the derived loading plots and scores plot gave useful information about the possible correlations of the compounds used for the analysis in correlation with the sensory scores. 2-butanone was correlated with acceptable samples (fresh and semi-fresh) and showed a decrease during storage. Moreover, the most of the acceptable samples were correlated with 2-butanone, 2, 3-pentanedione, 2, 5-octanedione, pentanal, hexanal, trans-2-heptanal, trans-2-octenal that all showed a mixed, mostly decreasing trend during storage. 2-Pentanone, 2-nonanone, 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl hexanoate, ethyl propanoate, ethyl lactate, ethyl acetate, ethanol, 2-heptanone, 3-octanone, diacetyl, acetoin were associated with spoiled samples which increased during storage at the most storage conditions.

According to AHC the samples were categorised into two groups; acceptable mince samples (fresh and semi-fresh), and spoiled samples whereas FDA provided classifications of the samples within the three different sensory classes; fresh, semi-fresh and spoiled. The correct classification rate for the validation of the FDA model was 77.78% correct for the fresh samples, 89.66% for the semi-fresh and 62.50% for the spoiled ones, with an overall performance of 79.17% accuracy. These results revealed a good correlation of the sensory estimates of the spoilage with the dynamic changes of the amounts of the volatiles compounds.

The potential of PLS-R analysis to estimate the population of selected microbial groups of the indigenous microbiota of meat samples such as total viable counts (TVC), *Pseudomonas* spp, *Br. thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, and yeasts/moulds was also demonstrated in this study. The values of B_f were generally close to unity, indicating good agreement between

observations and estimations. The fact that in certain cases it is slightly above 1 indicates a slightly 'fail-dangerous' model (Ross, 1996). In addition, the values of the A_f indicated that the average deviation between estimations and observations of the various microbial groups ranged from 9.3 % (either above or below the line of equity) for TVC to 14.0% for *Br. thermosphacta* (Table 3.9.3). Regarding the estimations for TVC, the % RE values were distributed above and below 0, with 91.78 % of predicted microbial counts included within the ± 20% RE zone. However, a trend of over-prediction was evident especially at lower population densities (counts less than 7 log cfu g^{-1}) and some times an under-prediction was observed at higher populations. As far as the models of the remaining microbial groups are concerned, the *Pseudomonas* spp., *Br. thermosphacta* and yeasts/moulds were generally under-estimated, whilst the LAB were slightly over-estimated at the lower populations densities. These results indicate, that the HS/SPME- GC/MS analysis can provide useful information about the dynamic changes of the volatile metabolic compounds being present in the meat substrate during storage and provide estimations about the microbial populations and the sensory scores.

Chapter 5

Conclusions and Future Work

5.1. Conclusions

In the present thesis, a holistic approach was followed in order to evaluate the spoilage of beef. The microbial association was monitored in parallel with the metabolic profile of meat. For this reason, a number of quick/ rapid analytical techniques were explored for their potential on evaluating the freshness/ spoilage of meat. Through a series of experiments preformed, the following remarks were summarized.

- The type of market (super-markets/ butcher shops), the type of packaging (aerobic, MAP), the day and the season of randomly purchased samples (from Greek market) may affect significantly the microbial association of meat and the pH values.
- The initial microbiota of the beef fillets (stored in air at 0, 5, 10, 15 and 20˚C) comprised, in decreasing order of magnitude, of *Pseudomonas* spp., *Br. thermosphacta*, LAB and *Enterobacteriaceae*, whilst the contribution of these groups to the natural microbiota depended strongly on the temperature. The dominant microorganism was found to be pseudomonads, followed by *Br. thermosphacta*. Moreover, the sensory evaluation of the spoilage was not always correlated with the same microbial load but depended on the storage temperature.
- FTIR spectroscopy was used as rapid, non-destructive, economic tool to evaluate the freshness of minced beef exploiting the metabolic fingerprints of i) minced samples beef samples obtained from Greek Market, ii) beef fillets stored aerobically at five different temperatures, and minced beef stored under aerobic, MAP and MAP /OEO at four different temperatures. The minced beef samples obtained from Greek Market were well discriminated regarding their freshness, the packaging type, the season and the day of purchase using the spectral data

collected from the FTIR, whilst it was not feasible to estimate the microbial counts. The results from the analysis of the FTIR spectra collected from the beef fillets and minced beef spoilage experiments were complementary to each other. In both experiments, a well discrimination between the sensory classes of the beef samples was observed, while the microbial counts were sufficiently estimated.

- Raman spectroscopy was also evaluated as rapid, non-destructive, analytical technique and compared with FTIR spectroscopy. The spectroscopic, microbiological and sensory analysis data obtained from minced beef samples during storage in aerobic and MAP conditions at 5 °C, were sufficiently correlated using different machine learning methods. The FTIR models performed in general slightly better in estimating the microbial counts compared to the Raman models. Though, both of the methods were able to estimate the microbial counts and the sensory scores of the tested minced beef samples.
- VideometerLab (image analysis), a rapid and non- destructive technique, was also used to analyse samples obtained from minced beef samples during storage in aerobic and MAP conditions at 5 °C. Image analysis gave useful information about the changes on the surface of the meat, whereas the models built using the spectra collected, estimated sufficiently the microbial load and the sensorial status of the meat sample.
- HPLC analysis of organic acids was found to be a potential technique for meat analysis on estimating the spoilage status and the microbial load of a meat sample regardless of storage conditions (minced beef stored under aerobic, MAP and MAP /OEO at four different temperatures). Additionally, it provides interesting information about the evolution of spoilage under different temperature and packaging conditions.

- The HS/SPME- GC/MS analysis provided useful information about the dynamic changes of the volatile metabolic compounds during meat storage (under aerobic and MAP at four different temperatures). Many of the identified and semi-quantified compounds were correlated with the sensory scores, depicting possible spoilage indicators. Finally, the microbial counts and the sensory scores were estimated, through the use of the GC/MS data.
- The results obtained in this study, demonstrated that the afformentioned analytical techniques could be utilised to evaluate the spoilage of meat as rapid method in comparison with the existing laborious, time consuming and provided retrospective results e.g. conventional microbiological analysis and the highly demanding trained personnel methodologies. The efficacy of these methods though, was evident within single batches and it is crucial to evaluate the built models using independent data sets, from samples that originate from different batches. Moreover, further studies are required to validate and expand this approach with meat with differend initial characteristics (e.g. different origin, pH, colour, fat, contaminated with different microbial loads).

5.2. Future work

In the current thesis, several chemical analytical techniques were applied to explore their dynamics on quantifying spoilage indicators and evaluate the shelf life of the meat products. The applied analytical methods were Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, image analysis, high performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC/MS). All the methods were found to be able to estimate more or less the meat spoilage. Nevertheless, further studies are needed to enhance the input data and explore in more

depth the spoilage succession. To achieve this, the following studies should be carried out:

✓ Explore the metabolic fringerprint of meat during storage analysing freeze-dried samples beef samples on FTIR.

The presence of moisture has a significant effect on the FTIR spectum of a meat sample and interesting information is hidden due to the water overlapping the rest compounds. A study is already conducted (see Appendix A) and series of freeze dried samples (stored under different temperature and packaging conditions) were analysed on FTIR. Even though this method can not be seen as rapid (due to the freeze-drying procedure), the analysis of the collected spectra will give more details about the spoilage evolution.

✓ Explore Raman spectroscopy and image analysis using meat samples stored under several packaging and temperature conditions.

The aforementioned analytical methods were applied for the first time for the evaluation of meat spoilage. Both were found to be promising to be applied as rapid analytical methods for evaluating meat spoilage. However, additional studies would be useful to establish the accuracies of the methods, using meat samples stored under several storage conditions and originate from different batches of meat. A part of these analyses have already be conducted (see Appendix A), whilst the mathematical treatment of the data is in progress.

✓ Investigate the key metabolic compounds produced by selected species of bacteria that dominate during storage of meat.

This can be achieved by inoculating sterile meat with single cultures or cocktail cultures of different strains of *Pseudomonas*, *Brochothrix thermosphacta*, lactic acid bacteria and enterobacteria. Subsequently, these meat samples will be stored under different conditions and the microbiological analyses, will be conducted in parallel with chemical analyses. The use of HPLC and GC/MS will provide chemical indicators that characterise the metabolic activity of the aforementioned microorganisms under different storage conditions and in the absence/presence of other spoilage microorganisms.

✓ Assess the impact of the variability in initial characteristics of the meat in the efficacy of the methods

The efficacy of the applied methods should be assessed with meat samples that have different initial characteristics (e.g. different origin, pH, colour, fat, microbial loads) and stored under different temperature and packaging conditions. Subsequently, the spoilage succession will be evaluated with the same methods and the models built will be intergraded and validated with more data sets.

✓ Intergrate the obtained results from the different instruments into combined models

It was demonstrated in this study that the different analytical methods were found to give complementary information about the evolution of meat spoilage and were able to estimate the microbial counts and/ or the sensory scores with different performances. An interesting approach to follow could be an intergrated analysis of the obtained results into one or more combined models. Such an application that would combine the inputs of several chemical indices of different origin (e.g. non-volatile and volatile compounds) could reveal more accurate shelf life indicators and minimise the error of the built models.

Chapter 6

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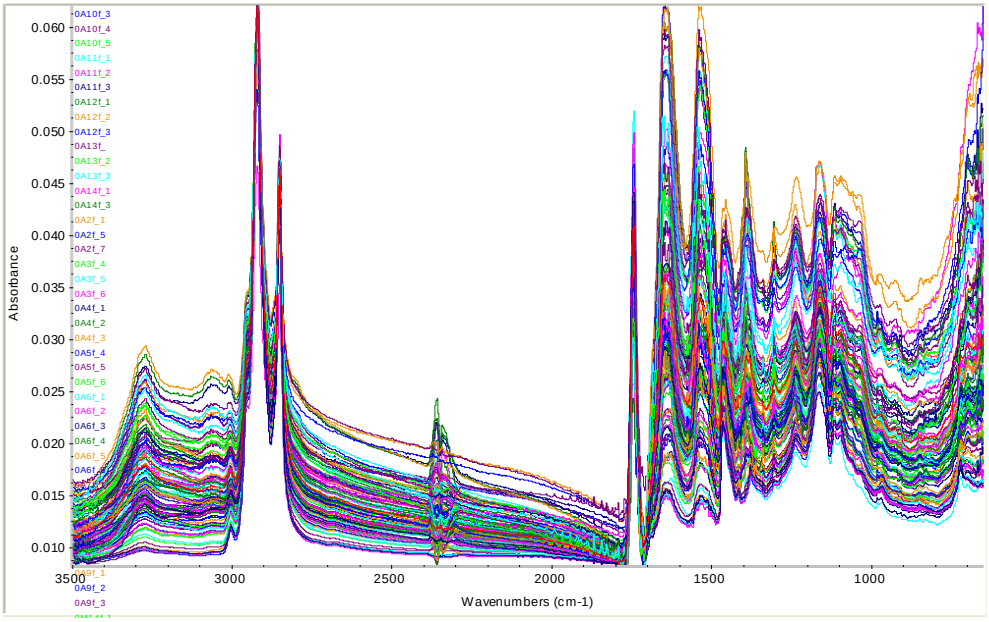
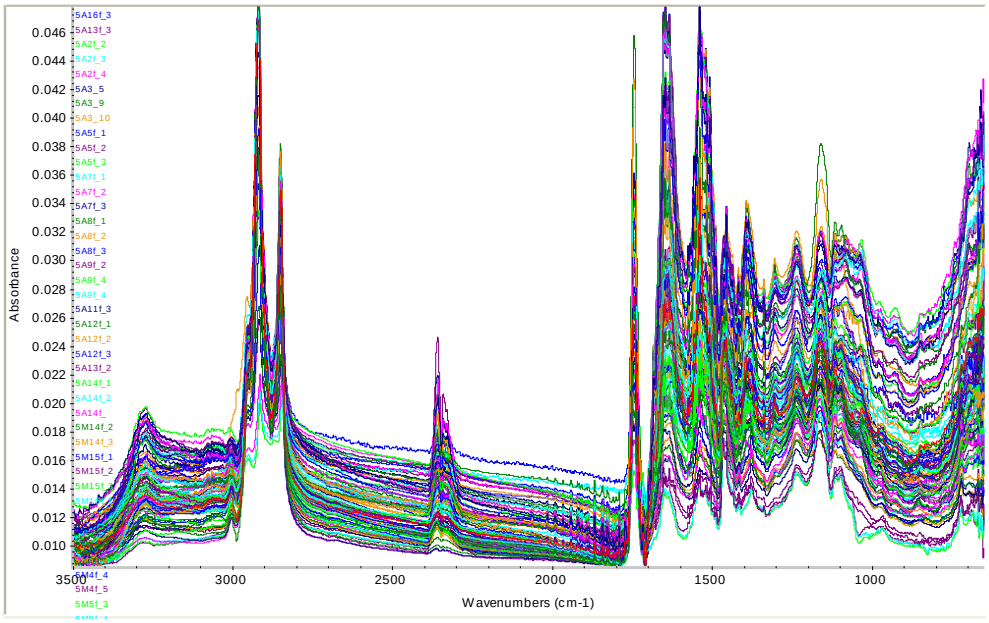
*A*PPENDIX A

Raw data

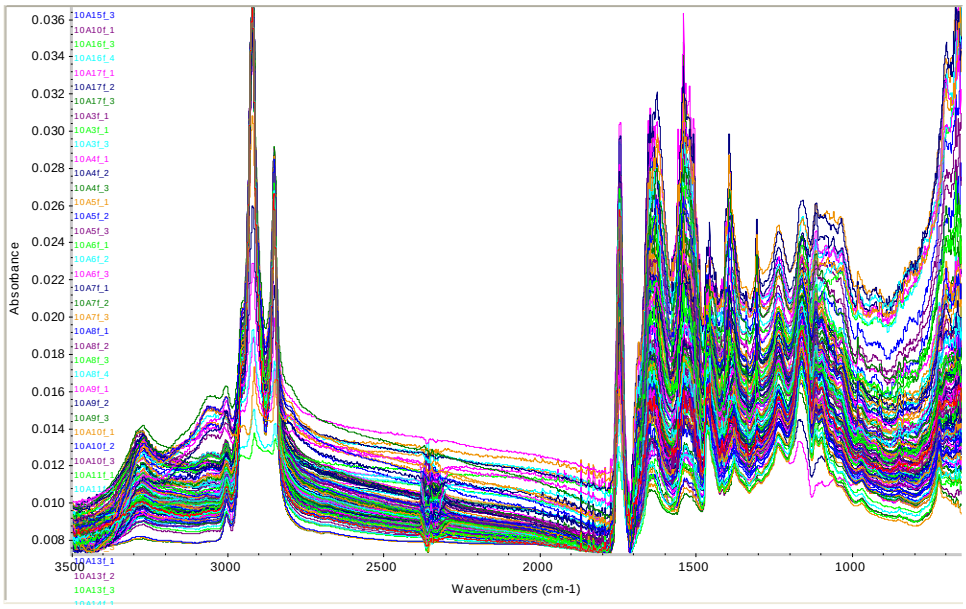
Appendix A1: FTIR analysis



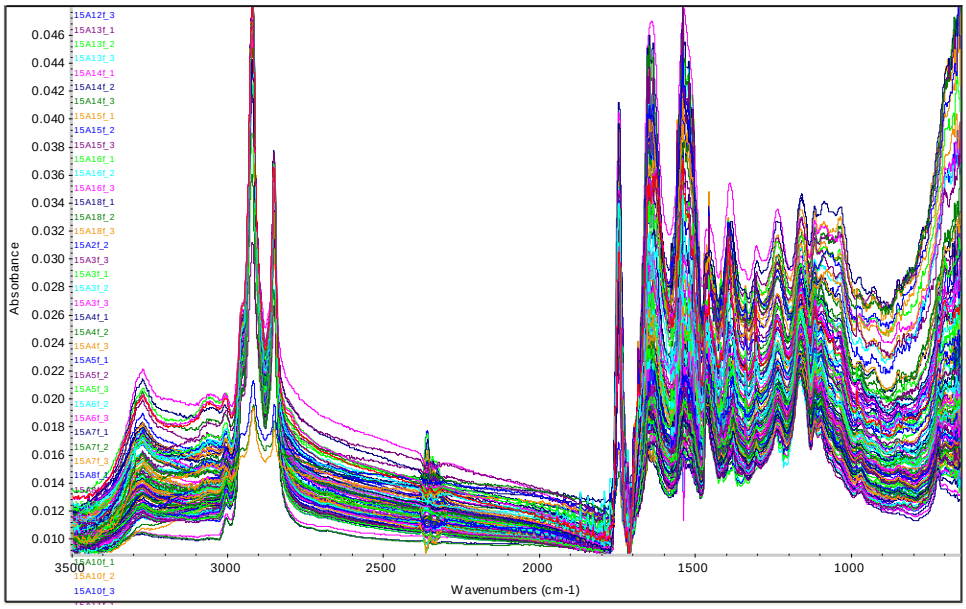
Appendix A1 - Figure 1. Raw FTIR spectra in the range of 3500 to 650 cm^{-1} collected from freeze-dried minced beef samples (in triplicates) stored in air, MAP, and MAP/OEO at 0˚C



Appendix A1 - Figure 2. Raw FTIR spectra in the range of 3500 to 650 cm^{-1} collected from freeze-dried minced beef samples (in triplicates) stored in air, MAP, and MAP/OEO at 5˚C

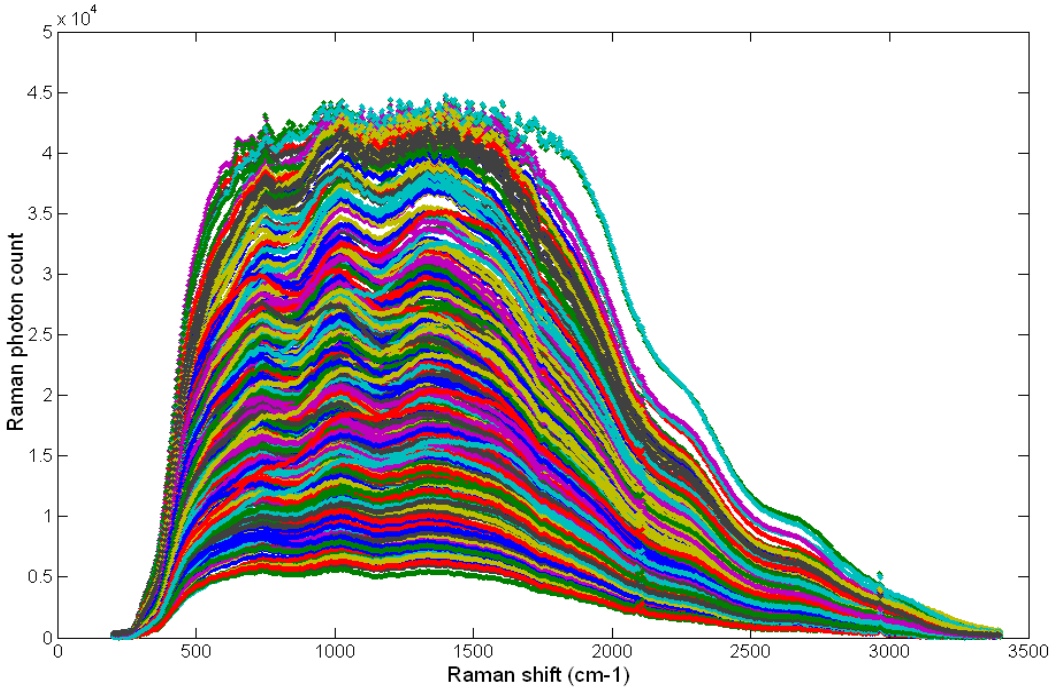


Appendix A1 - Figure 3. Raw FTIR spectra in the range of 3500 to 650 cm^{-1} collected from freeze-dried minced beef samples (in triplicates) stored in air, MAP, and MAP/OEO at 10˚C

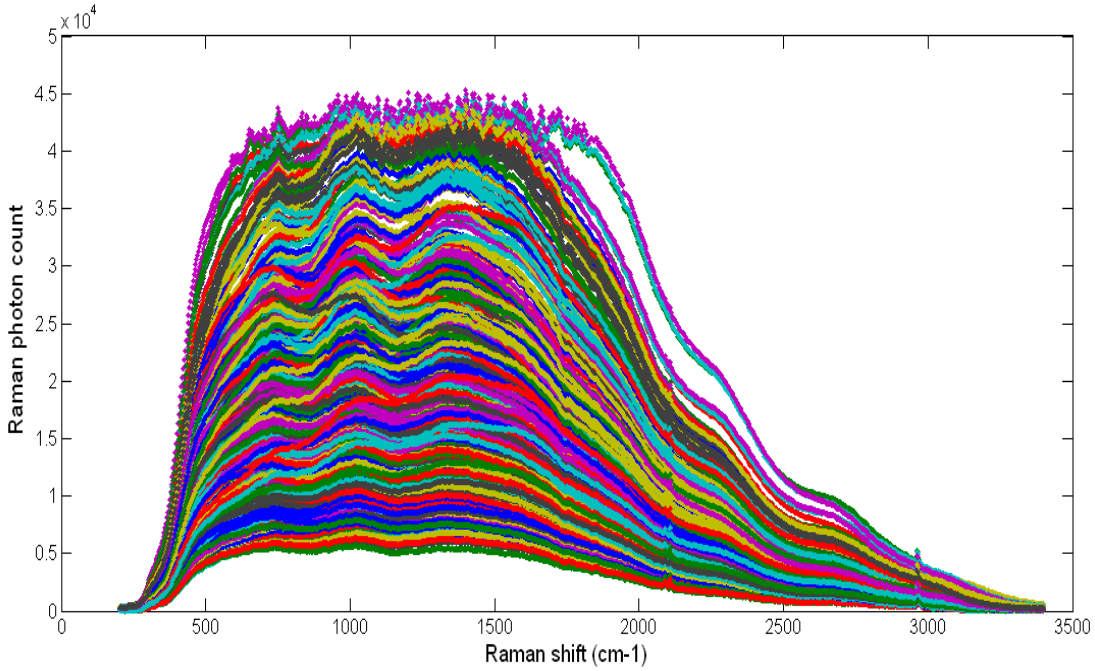


Appendix A1 - Figure 4. Raw FTIR spectra in the range of 3500 to 650 cm^{-1} collected from freeze-dried minced beef stored samples (in triplicates) in air, MAP, and MAP/OEO at 15˚C

Appendix A2: Raman analysis



Appendix A2 - Figure 1: Raw Raman spectra collected from minced beef samples (150x3 replicates) collected from the Survey of Minced Beef from the Greek Market



Appendix A2 - Figure 2: Raw Raman spectra collected from beef fillets samples (72x3 replicates) stored aerobically at 0, 5, 10, 15 and 20˚C

Appendix A3: VideometerLab analysis

Appendix A3 – Table 1. Mean values spectra of minced beef samples collected from a survey from Greek market using VideometerLab (S: Supermarkets, B: Butcher shops)

Sample	Wavenumber values																	
	405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
S1	12.8	19.7	23.4	27.5	28.9	28.8	29.3	39.4	45.7	51.2	56.7	93.5	86.0	82.8	78.0	71.3	70.0	62.6
S2	13.5	18.2	21.2	24.9	26.1	25.7	24.7	32.7	38.6	44.4	49.6	79.9	79.8	76.9	72.3	65.3	63.6	55.4
S3	11.1	14.9	18.0	22.2	24.6	23.8	20.6	34.6	47.4	53.6	59.4	94.3	83.9	80.6	75.9	69.2	67.7	59.8
S4	9.8	12.2	14.3	17.1	18.3	17.8	16.7	25.3	32.5	37.3	42.2	76.8	66.9	63.5	58.9	53.2	51.8	45.5
S5	11.7	14.3	16.9	21.0	23.8	22.8	18.9	30.7	46.0	53.4	58.1	81.2	85.5	83.1	78.8	71.0	69.0	59.4
S6	11.1	15.5	18.4	22.0	23.5	23.1	22.1	32.9	40.4	45.9	51.5	89.3	78.5	75.2	70.4	64.1	62.7	55.6
S7	12.8	18.9	22.4	26.5	27.6	27.2	26.6	37.2	43.9	49.4	55.2	93.8	81.6	78.1	72.9	66.0	64.3	56.5
S8	11.3	14.5	17.2	20.8	22.2	21.5	19.2	30.3	39.0	44.4	49.8	86.2	72.7	69.2	64.2	57.6	56.1	48.4
S9	10.5	15.7	18.2	20.8	21.6	22.2	27.4	31.5	33.2	37.9	42.8	77.3	74.9	72.0	67.7	61.5	59.9	52.2
S10	13.1	18.9	22.2	26.0	26.9	26.6	26.3	35.7	42.1	48.4	54.6	91.8	82.0	78.7	73.9	67.1	65.8	58.6
S11	11.1	14.8	17.4	20.7	21.8	21.4	20.1	30.4	38.2	44.1	50.0	87.7	76.5	73.1	68.3	62.0	60.6	53.7
S12	13.1	17.2	20.3	24.1	25.2	24.3	21.4	33.4	41.8	47.7	53.5	88.4	78.2	74.7	69.6	62.4	60.7	52.2
S13	12.1	20.1	23.5	26.9	27.6	28.2	33.2	37.8	40.4	46.8	52.6	87.8	86.1	83.3	78.9	72.2	70.7	62.7
S14	12.4	20.8	24.3	27.5	28.3	28.9	34.0	38.0	40.2	46.4	52.1	86.8	84.6	81.8	77.3	70.8	69.1	61.2
S15	12.8	20.4	24.5	28.4	29.7	29.8	32.4	41.6	46.1	51.9	58.0	94.1	89.0	85.8	81.0	73.8	72.4	64.6
S16	12.4	17.9	21.7	25.7	27.0	26.6	26.3	36.2	42.5	47.9	53.6	89.0	79.8	76.2	70.9	63.1	61.1	52.0
S17	12.8	19.4	23.5	27.6	28.7	28.3	28.9	39.8	45.9	51.8	58.3	98.0	86.3	82.7	77.5	69.8	68.1	59.7
S18	12.8	19.0	23.3	28.1	30.1	29.3	27.4	40.2	50.1	57.2	63.8	98.9	89.8	86.6	81.6	73.9	72.2	63.8
S19	11.7	17.7	21.8	26.5	28.6	28.0	26.3	39.5	49.7	56.8	63.5	100.0	90.4	87.1	82.0	74.2	72.6	64.0
S20	11.4	16.1	19.2	22.1	22.6	22.4	23.5	31.1	35.2	40.3	46.0	83.1	75.1	71.5	66.4	59.3	57.6	49.7
S21	12.5	17.8	21.5	25.3	26.6	26.1	25.5	36.1	43.3	49.0	54.9	90.3	82.9	79.5	74.4	66.7	65.0	56.5
S22	10.5	13.8	16.4	19.2	20.2	19.8	19.5	27.6	33.7	38.6	43.9	78.8	70.3	66.9	62.0	55.5	53.9	46.6
S23	11.5	15.8	18.8	21.8	22.3	22.0	22.4	30.2	34.6	39.6	45.2	83.7	70.9	67.2	62.0	55.4	53.8	46.6
S24	10.9	14.7	17.0	19.1	18.9	18.9	21.1	26.8	28.8	33.6	39.2	79.6	67.3	63.5	58.4	52.1	50.6	43.8
S25	10.8	14.1	16.6	19.3	19.9	19.6	19.7	27.4	32.5	37.5	42.9	78.2	68.7	65.1	59.9	52.9	51.0	42.9
S26	12.1	17.4	20.7	23.9	24.4	24.2	25.4	33.3	37.4	42.5	48.3	87.4	73.9	70.2	65.1	58.2	56.5	48.8
S27	11.8	17.9	21.4	24.7	25.3	25.4	27.6	35.2	39.0	44.2	50.1	89.6	78.3	74.6	69.4	62.2	60.5	52.3
S28	11.1	14.5	17.1	19.9	20.5	20.1	19.9	28.3	33.2	37.9	43.4	83.3	67.0	63.1	58.0	51.5	49.9	42.9
S29	11.7	15.0	17.6	20.4	21.0	20.5	19.9	27.8	34.0	39.1	44.3	74.5	71.3	68.0	63.0	55.8	54.0	45.9

Appendix A3 – Table 2. (*Continued*)

Sample	Wavenumber values																	
	405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
S30	10.6	13.6	16.2	19.9	22.1	21.4	18.9	31.3	42.8	49.0	54.9	90.1	79.1	75.9	71.4	65.2	63.6	56.4
S31	10.4	13.0	15.3	18.5	20.2	19.6	17.5	27.8	37.2	42.7	48.1	82.7	70.8	67.5	63.0	57.3	55.9	49.5
S32	12.2	17.0	20.8	26.3	30.0	28.8	23.7	42.6	62.7	70.1	76.3	106.7	97.1	94.3	90.0	83.0	81.3	72.9
S33	11.4	15.8	19.2	23.8	26.4	25.7	23.1	36.2	48.2	54.0	59.3	90.0	84.2	81.3	77.1	70.9	69.2	61.5
S34	9.4	10.8	12.4	15.0	16.3	15.7	13.5	23.0	32.9	38.9	44.4	79.0	65.2	61.9	57.5	52.0	50.5	44.2
S35	11.3	15.2	18.2	22.2	24.0	23.3	20.7	33.3	44.0	49.8	55.6	92.4	79.0	75.5	70.7	64.0	62.3	54.5
S36	11.9	16.0	19.3	23.9	26.3	25.5	22.7	37.5	50.2	56.9	63.2	99.8	88.8	85.5	80.9	74.1	72.5	64.8
S37	12.4	16.8	20.1	24.5	26.6	25.7	22.6	36.3	48.1	54.5	60.7	97.6	85.8	82.4	77.5	70.3	68.6	60.6
S38	10.6	12.6	14.8	18.2	20.1	19.3	16.7	28.1	38.9	44.8	50.2	82.2	75.5	72.2	67.4	60.6	58.8	50.7
S39	11.0	13.8	16.1	18.6	19.3	18.9	18.0	25.2	31.1	35.6	40.3	69.4	65.8	62.7	58.0	51.8	50.3	43.4
S40	11.7	17.3	20.9	24.6	25.9	25.5	25.1	35.3	41.8	46.9	52.4	88.5	77.2	73.8	68.9	62.5	61.1	54.0
S41	9.6	10.9	12.6	14.8	15.9	15.4	14.0	21.9	28.9	33.1	37.6	68.3	58.0	54.8	50.4	45.5	44.4	39.2
S42	11.7	14.4	17.1	20.3	21.6	20.7	17.7	28.6	37.4	42.4	47.8	85.0	67.5	63.7	58.3	51.8	50.2	43.2
S43	12.6	18.0	21.5	25.2	26.1	25.6	25.0	33.8	40.9	47.1	53.1	86.9	78.6	75.3	70.3	63.1	61.5	53.4
S44	12.2	17.0	20.9	25.9	29.1	27.8	23.5	40.3	56.4	63.1	69.3	102.6	90.1	86.8	81.9	74.6	73.0	64.6
S45	11.7	14.5	17.1	20.2	21.2	20.5	18.3	28.3	35.5	40.4	45.9	82.1	68.3	64.4	59.0	52.2	50.6	43.2
S46	12.9	17.9	22.1	27.3	30.4	29.1	24.4	41.5	57.8	64.4	70.6	104.0	91.0	87.7	82.7	75.1	73.5	65.0
S47	11.1	13.8	16.2	19.2	20.6	19.8	17.5	27.4	37.2	42.9	48.2	79.2	72.4	69.0	64.0	56.9	55.1	47.1
S48	10.9	14.1	16.3	18.4	18.3	18.1	19.0	24.6	27.6	31.8	36.8	73.3	61.1	57.5	52.6	46.8	45.4	39.3
S49	12.8	18.7	22.9	28.0	30.6	29.6	26.7	40.9	53.0	59.2	65.3	100.1	88.0	84.8	80.0	72.9	71.2	62.8
S50	11.5	16.0	19.5	23.7	25.2	24.5	23.3	35.0	43.2	49.0	55.2	93.3	80.5	76.7	71.3	63.2	61.2	51.6
S51	11.3	16.3	20.2	24.9	27.1	26.2	23.7	38.0	49.9	56.2	62.5	98.7	89.1	85.8	80.7	73.1	71.5	62.7
S52	12.5	18.6	22.6	26.9	28.1	27.3	25.1	37.9	46.3	52.7	59.2	97.2	84.0	80.4	75.0	67.8	66.4	58.7
S53	12.6	17.7	21.6	25.6	26.6	26.0	25.4	35.7	43.5	49.9	56.0	90.4	85.9	82.3	76.9	68.4	66.6	58.0
S54	13.0	17.2	21.0	25.3	27.2	26.1	23.0	36.1	47.2	53.5	59.4	91.9	84.3	80.6	75.3	67.2	65.3	57.1
S55	13.5	19.1	23.4	28.0	30.0	29.0	26.9	41.1	50.9	57.0	63.3	99.5	87.3	83.3	78.1	70.2	68.3	60.2
S56	13.2	19.6	23.8	28.0	28.7	28.0	28.0	37.5	44.7	50.9	56.8	89.1	87.7	84.1	78.8	69.9	67.8	58.5
S57	13.7	19.9	24.5	29.3	30.8	29.8	27.9	40.3	51.2	57.9	64.0	93.9	94.0	90.6	85.4	76.1	73.8	63.8
S58	12.8	18.7	23.0	27.3	28.8	28.0	27.2	39.3	47.6	53.7	60.1	96.5	87.9	84.0	78.7	70.6	68.7	60.4
S59	12.3	15.7	18.7	22.1	23.1	22.2	20.1	28.7	39.6	45.8	50.7	76.4	81.1	77.9	73.0	64.6	62.4	53.2
S60	11.8	15.4	18.2	21.0	21.2	20.6	20.1	26.8	34.7	40.8	45.8	73.2	76.0	72.8	67.9	60.0	58.0	49.6

Appendix A3 – Table 2. (*Continued*)

Sample	Wavenumber values																	
	405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
S61	11.8	15.1	18.3	22.6	23.2	22.0	19.7	27.6	41.0	47.8	52.3	74.7	84.0	81.2	76.6	68.2	66.0	56.8
S62	12.3	16.2	19.8	24.7	25.4	24.1	21.9	30.5	44.0	51.2	56.2	81.1	87.7	84.7	79.8	70.7	68.3	57.9
S63	12.1	16.9	20.2	23.2	23.4	23.1	23.7	31.2	37.1	42.9	48.4	79.6	79.5	76.1	71.0	63.1	61.2	52.9
S64	11.3	14.2	16.9	19.9	20.9	20.2	19.2	28.4	34.7	39.8	45.4	82.0	69.3	65.3	60.1	53.4	51.7	45.1
S65	11.4	15.3	18.3	21.2	21.8	21.4	21.7	30.0	34.9	40.0	45.6	83.4	72.0	68.1	63.0	56.5	55.1	49.0
S66	11.0	14.5	17.2	19.7	19.8	19.5	20.2	26.8	31.9	37.2	42.5	74.5	73.1	69.6	64.6	57.5	55.8	48.6
S67	12.8	19.0	23.4	28.0	29.7	28.8	27.5	40.5	48.9	54.7	60.9	97.9	86.8	82.9	77.7	70.2	68.7	61.9
S68	12.4	19.2	23.4	27.3	28.0	27.6	28.5	39.0	44.1	49.9	56.2	96.2	84.4	80.4	75.0	67.5	66.0	58.9
S69	12.8	18.4	22.5	26.9	28.4	27.5	25.6	38.9	48.7	54.8	61.2	100.0	85.0	80.9	75.5	67.6	65.8	57.6
S70	13.1	19.6	23.5	27.3	28.0	27.7	28.7	38.4	43.9	49.6	55.8	93.3	84.9	81.2	75.9	68.1	66.5	58.0
S71	11.2	13.8	16.3	19.2	20.3	19.6	17.6	27.1	34.9	40.2	45.7	79.5	68.3	64.6	59.5	52.8	51.1	43.6
S72	12.7	18.1	21.9	26.1	27.4	26.7	25.1	36.4	44.4	50.4	56.7	94.7	82.9	79.2	74.0	66.8	65.3	57.8
S73	12.5	16.2	19.2	22.6	23.5	22.8	21.0	29.2	35.8	42.9	49.0	80.5	74.2	70.9	65.9	59.0	57.3	49.3
S74	11.7	14.0	16.1	18.6	19.2	18.6	17.0	24.2	31.1	36.0	40.7	68.7	65.3	62.1	57.4	51.4	50.0	43.6
S75	12.2	16.7	19.9	23.5	24.3	23.8	23.1	33.1	39.5	45.1	51.1	86.4	77.1	73.3	67.9	59.9	57.9	48.4
S76	13.7	19.8	23.9	28.4	30.5	29.7	27.5	41.1	50.5	56.3	62.2	98.3	87.2	83.8	78.8	71.8	70.4	62.8
S77	13.1	19.3	23.4	28.0	30.0	29.3	27.8	40.8	49.3	54.8	60.7	96.9	86.8	83.5	78.5	71.4	70.0	62.6
S78	13.0	16.3	19.4	23.3	25.0	23.9	20.2	33.3	43.6	49.0	54.7	90.7	75.2	71.5	66.2	59.3	57.9	50.5
S79	12.5	16.9	20.1	23.5	24.4	23.8	22.9	32.3	38.8	44.0	49.7	85.2	73.9	70.2	64.9	57.6	55.8	47.4
S80	13.5	18.4	22.2	26.7	28.8	27.9	25.5	37.5	49.9	56.4	61.7	87.8	88.5	85.7	80.8	71.8	69.3	57.7
S81	12.7	15.6	18.4	21.9	23.4	22.3	19.0	30.2	42.9	49.4	54.3	77.9	77.2	74.1	68.9	60.4	58.0	47.2
S82	11.5	14.0	16.4	19.4	20.6	19.8	17.4	26.7	37.1	42.6	47.6	74.4	69.9	66.6	61.5	53.8	51.7	42.3
S83	12.6	17.2	20.8	25.0	26.8	25.9	23.4	35.1	43.9	49.2	54.7	90.0	78.4	74.8	69.6	62.5	60.9	53.0
S84	12.8	17.5	21.0	24.9	26.4	25.6	23.3	34.2	42.3	47.6	53.2	87.6	77.9	74.3	69.1	61.7	60.0	51.7
S85	12.1	15.1	17.7	20.9	21.8	21.1	18.9	28.3	36.2	41.7	47.1	78.8	73.4	69.8	64.4	56.7	54.8	46.0
S86	11.7	16.3	19.8	23.9	25.4	24.7	22.7	33.9	43.0	49.0	54.8	87.4	79.5	76.1	70.7	62.7	60.7	51.0
S87	12.9	17.8	21.6	26.1	28.2	27.2	23.6	38.9	52.3	58.9	65.3	101.3	85.9	82.5	77.1	69.6	68.0	59.6
S88	12.2	16.5	20.4	25.5	29.0	27.7	22.4	41.3	61.5	69.1	75.6	106.7	93.7	90.5	85.5	77.8	76.3	67.8
S89	11.6	15.5	18.9	23.1	25.3	24.3	21.3	34.9	46.1	52.1	58.0	93.7	79.7	76.1	70.7	63.1	61.3	52.4
S90	11.7	15.9	19.2	23.3	25.2	24.4	21.5	34.4	45.2	51.2	57.2	93.9	79.0	75.5	70.3	63.5	62.0	54.4
S91	13.3	19.5	23.8	28.9	31.5	30.6	27.3	42.3	54.8	62.1	68.7	103.5	91.6	88.5	83.5	76.2	74.9	67.1

Appendix A3 – Table 2. (*Continued*)

Sample	Wavenumber values																	
	405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
S92	12.4	16.1	19.0	22.4	23.4	22.7	20.7	30.7	38.5	43.8	49.4	84.0	71.7	68.0	62.7	55.8	54.1	46.3
S93	13.0	16.9	20.5	25.3	28.3	26.9	21.6	39.2	58.0	65.0	71.2	101.8	87.4	84.1	78.9	71.3	69.8	61.3
S94	12.3	16.2	19.7	24.4	27.4	26.0	21.0	38.9	58.3	65.9	72.6	105.0	90.4	87.1	82.0	74.4	72.8	64.4
S95	10.4	12.7	15.1	18.4	20.4	19.4	15.8	29.2	44.0	50.5	56.5	90.5	73.6	70.1	65.0	58.3	56.6	48.9
S96	11.7	15.8	18.7	22.0	23.0	22.4	20.8	30.3	37.3	43.0	48.8	85.7	70.6	67.1	62.1	56.0	54.7	48.3
S97	10.4	12.4	14.4	16.8	17.5	16.9	15.3	23.4	30.0	34.8	40.0	76.0	60.6	57.0	52.1	46.5	45.2	39.2
S98	13.8	19.2	23.1	27.7	30.0	29.1	26.4	40.4	50.3	56.6	62.6	96.3	86.5	83.1	77.9	70.1	68.3	59.1
S99	14.0	18.1	21.5	25.8	27.8	26.8	23.5	36.6	46.4	52.5	58.3	91.3	80.4	76.9	71.6	63.7	61.7	52.2
S100	10.9	13.5	16.2	19.7	22.0	21.1	18.1	31.3	43.9	49.9	55.6	89.0	75.6	72.4	67.7	61.6	60.2	53.2
K1	12.3	17.5	21.1	25.9	28.6	27.8	25.3	38.9	50.1	56.0	61.4	92.7	87.0	84.1	79.8	72.8	71.1	63.1
K2	12.1	16.3	19.0	22.0	22.4	22.2	23.3	30.1	34.3	39.1	44.3	78.6	71.6	68.1	63.4	56.4	54.5	46.1
K3	12.7	17.6	20.7	24.3	24.8	24.4	24.4	33.0	37.6	42.8	48.4	85.0	75.5	71.7	66.5	58.9	57.0	48.2
K4	12.7	18.9	22.9	27.9	30.1	29.6	29.2	40.5	47.7	53.0	58.5	92.1	84.8	81.5	76.9	69.1	67.0	56.9
K5	11.3	14.5	17.1	20.6	22.2	21.7	20.3	29.4	36.9	41.7	46.4	76.5	71.4	68.3	63.9	57.5	55.9	48.6
K6	11.4	14.5	17.1	20.7	22.3	21.8	20.3	29.5	37.0	41.8	46.5	76.6	71.4	68.2	63.8	57.5	55.9	48.6
K7	13.1	19.0	22.5	26.4	27.5	27.2	27.4	37.0	43.3	48.3	53.7	89.5	81.1	77.7	73.0	66.3	64.7	57.1
K8	11.9	15.8	19.0	23.4	25.6	24.9	22.6	35.1	45.2	50.8	56.3	89.8	80.3	77.0	72.3	65.3	63.5	55.0
K9	12.1	16.1	18.7	21.7	22.0	21.8	22.5	29.3	35.5	40.9	46.0	75.2	76.4	73.4	68.9	61.4	59.3	50.1
K10	12.1	16.1	18.7	21.7	22.0	21.7	22.5	29.2	35.4	40.8	45.9	75.1	76.3	73.3	68.7	61.2	59.1	49.9
K11	12.1	14.6	17.1	20.7	22.1	21.2	18.8	28.4	40.5	46.6	51.4	77.2	79.2	76.2	71.6	63.4	61.0	50.8
K12	11.8	14.4	17.0	20.9	22.9	21.9	18.5	29.6	43.8	50.0	54.6	78.5	79.0	76.1	71.5	63.1	60.5	49.6
K13	11.4	16.2	19.5	23.8	25.6	25.1	24.3	35.6	44.5	50.5	56.2	89.0	84.9	81.7	76.9	68.9	66.8	56.8
K14	11.5	15.8	19.1	23.6	26.0	25.3	23.1	36.6	47.3	53.7	59.6	93.2	85.4	82.3	77.8	70.7	69.0	60.2
K15	11.5	16.8	20.3	24.6	26.4	26.0	25.9	36.9	44.5	50.4	56.4	92.1	84.2	80.9	76.2	68.5	66.5	56.8
K16	10.8	15.4	18.9	23.5	26.1	25.4	23.3	35.6	46.1	52.0	57.5	89.0	82.1	79.0	74.5	66.9	64.7	54.7
K17	11.2	15.6	18.9	23.2	25.4	24.7	22.7	35.7	46.1	52.2	57.9	91.4	84.0	80.8	76.1	68.6	66.7	57.6
K18	11.2	15.6	18.9	23.3	25.5	24.8	22.8	35.9	46.3	52.4	58.1	91.5	84.2	81.0	76.3	68.8	66.9	57.8
K19	11.0	13.9	16.4	19.9	21.2	20.5	18.8	28.8	37.0	42.3	47.6	81.1	73.3	69.8	64.9	57.7	55.8	47.2
K20	9.9	14.2	16.8	19.9	20.6	20.6	22.4	29.8	33.8	38.7	44.0	81.4	72.8	69.5	64.8	58.3	56.6	48.8
K21	11.8	14.6	17.2	20.9	22.6	21.7	18.8	30.7	41.6	47.5	52.9	83.6	75.8	72.4	67.4	59.6	57.4	47.7
K22	11.5	14.2	16.8	20.7	22.9	21.9	18.1	31.4	44.7	50.8	56.2	86.3	77.4	74.1	69.3	62.0	60.1	51.1

Appendix A3 – Table 2. (*Continued*)

Sample	Wavenumber values																	
	405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
K23	11.7	14.6	17.2	21.1	23.3	22.3	19.0	32.1	45.4	52.0	57.7	88.8	78.6	75.4	70.7	63.3	61.2	51.8
K24	11.7	14.5	17.2	21.0	23.2	22.2	18.9	32.0	45.2	51.8	57.5	88.7	78.5	75.3	70.6	63.1	61.0	51.5
K25	11.3	14.5	17.2	20.8	22.1	21.4	19.5	28.7	37.9	43.7	48.6	76.5	75.2	71.9	67.1	59.1	56.8	47.0
K26	11.7	15.6	18.7	22.5	23.6	23.0	21.6	32.0	40.4	45.9	51.7	87.4	79.5	75.8	70.7	63.2	61.3	52.7
K27	12.3	16.1	19.3	23.5	25.2	24.3	21.7	33.0	44.5	50.7	56.0	83.4	81.9	78.8	74.0	65.6	63.2	52.8
K28	13.5	19.1	22.6	26.7	27.7	27.3	27.3	37.0	43.7	49.3	55.1	90.0	84.7	81.3	76.3	68.4	66.6	57.8
K29	12.4	16.9	19.9	23.4	24.0	23.7	23.9	32.5	38.3	43.8	49.4	84.8	79.9	76.3	71.2	63.4	61.5	52.7
K30	12.7	17.0	20.1	23.9	24.9	24.4	23.9	32.6	41.6	47.6	52.8	81.3	84.6	81.8	77.4	69.5	67.4	57.9
K31	11.9	15.2	17.9	21.2	22.1	21.6	20.8	29.1	38.2	44.1	49.1	76.6	78.5	75.6	71.0	62.7	60.4	50.0
K32	11.9	14.8	17.3	20.7	21.8	21.0	18.7	27.7	40.1	46.3	50.8	75.0	80.6	77.9	73.3	64.9	62.5	52.1
K33	12.3	16.3	19.6	24.1	25.9	25.1	23.3	34.6	43.5	48.9	54.4	86.9	82.0	78.6	73.5	65.2	63.0	52.8
K34	12.4	17.5	20.9	24.9	25.7	25.4	26.0	35.2	41.4	47.2	53.0	86.1	84.2	80.8	76.0	67.7	65.4	55.2
K35	12.3	17.2	20.5	24.5	25.4	25.0	25.4	34.5	40.6	46.3	51.9	85.7	82.7	79.3	74.4	66.2	64.0	54.0
K36	15.9	23.5	28.1	33.6	35.6	34.8	32.5	46.0	54.5	60.7	66.6	99.5	93.0	89.8	85.1	77.5	75.8	66.9
K37	14.9	21.9	26.2	31.4	33.3	32.5	30.6	44.0	52.9	59.3	65.3	98.0	92.2	89.1	84.3	76.4	74.5	65.1
K38	13.4	19.7	23.6	28.4	30.2	29.7	29.1	41.2	48.8	54.8	60.9	95.9	88.1	84.8	79.9	71.8	69.7	59.7
K39	11.9	14.5	16.8	19.9	20.9	20.2	18.4	27.5	38.2	44.4	49.2	74.9	77.6	74.6	69.9	61.9	59.6	49.5
K40	13.6	19.5	23.9	29.7	32.6	31.6	28.2	43.0	54.9	60.7	66.2	99.8	89.7	86.4	81.6	74.0	72.0	62.4
K41	13.5	19.4	23.6	28.8	31.3	30.5	28.1	41.7	51.4	57.1	62.8	98.5	89.0	85.6	80.7	73.3	71.6	62.9
K42	12.7	18.1	22.0	26.9	29.0	28.4	27.2	38.6	46.7	52.2	57.8	94.8	83.3	79.8	74.9	67.5	65.6	56.5
K43	12.2	17.1	20.4	24.7	26.5	25.9	24.4	37.1	46.1	52.1	58.2	95.1	85.2	81.8	77.0	70.1	68.6	61.0
K44	12.5	18.2	21.7	25.9	26.5	26.0	26.1	35.2	41.9	47.8	53.7	88.7	84.7	81.3	76.1	68.2	66.3	57.1
K45	11.3	14.6	17.3	21.2	23.0	22.2	19.8	31.3	40.6	46.0	51.4	87.6	73.1	69.4	64.3	57.3	55.6	47.5
K46	13.2	17.3	20.7	25.2	27.2	26.2	22.9	34.9	45.9	52.0	57.2	85.3	82.1	79.0	74.0	65.4	63.0	52.0
K47	11.7	14.7	17.4	21.3	23.1	22.2	19.0	29.3	42.3	48.3	52.7	76.3	78.6	75.9	71.3	63.4	61.1	51.0

Appendix A3 – Table 2. Mean values spectra of beef fillets stored aerobically at 0, 5, 10, 15 and 20˚C the wavenumbers tested using VideometerLab

Tempera-ture	Time (h)	Wavenumber values																	
		405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
	0	10.7	13.2	15.6	18.5	19.3	18.8	18.2	26.0	35.4	41.3	46.7	76.1	74.4	71.4	66.7	59.6	57.5	47.9
0°C	24	11.1	12.8	14.9	17.6	18.6	18.1	17.0	25.0	33.2	38.6	43.8	75.8	72.9	69.7	64.6	58.1	56.5	48.4
	48	11.4	14.5	17.4	20.9	22.6	22.1	21.5	29.8	37.4	42.4	47.2	75.1	74.5	71.8	67.2	61.1	59.6	51.8
	72	11.6	14.0	16.3	19.0	19.9	19.6	19.4	26.6	32.6	37.4	42.2	74.1	68.3	65.2	60.6	55.0	53.6	46.5
	96	11.2	14.2	16.9	19.9	20.8	20.5	20.7	29.2	35.7	40.8	46.2	80.8	71.6	68.4	63.7	58.4	57.2	50.6
	120	11.3	14.3	16.5	18.5	18.5	18.4	20.1	25.1	28.3	32.5	37.4	72.5	63.9	60.5	55.5	50.1	49.0	42.9
	144	12.2	15.2	17.6	20.1	19.9	19.6	19.9	26.4	32.5	37.6	42.8	74.3	70.8	67.6	62.4	55.3	53.5	44.4
	168	11.2	13.6	15.5	17.1	16.5	16.5	18.6	22.6	25.6	30.5	35.9	72.9	66.2	62.8	57.6	51.6	50.3	43.4
	192	12.4	14.6	17.1	19.9	20.6	20.0	18.1	26.6	36.0	41.4	46.3	76.4	73.2	70.0	64.8	57.9	56.4	48.3
	216	11.8	14.8	17.7	21.2	22.8	22.3	21.0	31.0	38.9	44.0	49.2	81.8	74.7	71.5	66.7	60.6	59.3	51.7
	240	12.0	17.0	20.0	22.4	21.6	21.7	25.1	29.9	32.2	37.1	42.8	81.1	71.5	67.7	62.1	55.1	53.5	45.2
	264	11.9	14.8	17.1	19.2	18.7	18.5	19.1	24.8	30.0	35.1	40.2	71.5	67.8	64.6	59.5	52.8	51.2	42.9
	288	11.3	16.4	20.3	24.7	26.9	26.5	26.5	36.8	44.0	49.0	54.1	87.3	79.0	76.0	71.6	66.2	65.1	58.3
	302	11.0	13.7	16.0	18.3	19.0	19.3	23.0	28.8	32.0	36.8	42.0	78.4	69.9	67.2	63.1	59.1	58.5	53.8
	326	11.2	15.2	17.6	19.5	19.0	19.2	22.9	26.5	27.9	32.0	36.9	75.8	62.5	59.0	54.0	49.1	48.0	42.4
5°C	24	12.7	17.6	20.6	23.3	23.2	23.3	26.5	31.3	33.9	38.9	44.5	81.4	74.4	70.9	65.7	58.8	57.3	48.9
	48	12.6	18.8	22.8	26.6	27.7	27.5	28.8	35.8	40.4	44.6	49.5	84.6	73.8	70.5	65.7	60.0	58.8	52.0
	72	12.4	16.2	19.4	22.8	23.8	23.2	21.8	30.7	40.2	45.9	51.2	79.8	74.9	71.8	66.9	59.9	58.0	48.6
	96	11.2	13.0	14.9	17.0	17.3	16.9	16.9	23.4	28.7	33.6	39.0	73.5	63.7	60.3	55.4	49.3	47.8	40.2
	120	12.9	14.8	17.0	19.3	19.1	18.6	18.0	25.0	32.5	38.5	44.1	75.7	71.2	67.8	62.4	54.9	53.0	43.8
	144	13.9	16.0	18.4	21.4	21.9	21.2	19.5	28.4	37.3	43.1	49.3	83.0	71.3	67.6	62.4	55.0	53.0	43.4
	168	11.1	13.8	15.9	17.8	17.4	17.4	19.6	23.5	26.0	30.3	35.2	70.1	62.6	59.1	53.9	47.8	46.3	39.3
	192	12.3	12.9	14.9	17.9	19.9	19.2	17.6	27.3	39.8	46.0	51.6	83.5	76.2	73.0	68.1	61.1	59.2	49.9
	216	11.4	12.7	15.3	19.0	20.3	19.3	17.1	23.5	38.8	45.5	49.6	71.3	81.9	79.1	74.1	65.6	63.3	51.9
	240	11.9	12.8	13.4	17.2	19.9	18.6	15.8	22.3	39.4	44.9	47.0	56.8	86.0	84.8	81.0	73.0	70.8	59.6

Appendix A3 – Table 2. (*Continued*)

Tempera-ture	Time (h)	Wavenumber values																	
		405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
	0	10.7	13.2	15.6	18.5	19.3	18.8	18.2	26.0	35.4	41.3	46.7	76.1	74.4	71.4	66.7	59.6	57.5	47.9
10 °C	8	12.5	16.9	20.9	26.1	29.7	29.0	26.4	40.5	54.0	60.5	66.0	94.9	92.4	89.7	85.4	79.0	77.7	69.2
	18	12.3	15.0	17.2	19.3	19.2	18.9	18.7	25.2	32.5	38.1	43.2	71.7	70.7	67.8	62.5	55.7	54.2	45.5
	26	11.7	13.9	16.3	19.0	19.9	19.3	17.9	26.2	36.0	41.8	46.6	73.5	73.8	71.1	66.1	59.3	57.9	49.3
	34	11.0	12.4	14.2	16.2	16.6	16.2	15.6	22.2	29.7	35.1	40.2	70.0	66.1	63.2	58.2	52.4	51.0	43.6
	42	10.4	12.1	14.0	16.3	16.9	16.6	15.7	22.9	30.8	36.1	41.0	69.7	67.0	64.1	59.4	53.7	52.5	45.5
	52	10.7	12.3	14.5	17.4	19.0	18.4	16.5	24.8	35.7	41.1	45.5	69.4	68.5	66.0	61.6	56.3	55.1	48.1
	60	11.0	13.6	15.9	18.5	19.3	19.0	18.5	25.6	31.9	36.7	41.5	72.7	66.1	63.1	58.2	52.6	51.3	44.7
	68	11.0	14.8	18.2	22.4	24.8	24.2	22.8	33.7	43.2	48.3	53.4	87.0	75.4	72.4	67.8	62.8	61.9	55.6
	76	11.0	15.6	19.0	23.0	25.3	25.1	25.0	35.8	43.8	49.5	54.9	85.7	80.8	78.1	73.7	68.0	66.8	59.3
	82	11.3	14.3	16.8	19.4	19.9	19.7	20.2	27.2	32.5	37.6	43.0	78.0	69.4	66.2	61.1	55.0	53.6	46.0
	90	11.4	14.2	16.6	19.3	20.0	19.8	20.1	27.6	33.9	39.6	45.1	76.0	68.9	65.7	60.5	53.9	52.3	43.5
	96	11.3	13.7	15.8	17.9	18.0	17.7	17.5	24.2	31.5	37.2	42.8	76.1	70.5	67.3	62.1	55.9	54.5	47.0
	104	11.2	15.1	18.1	21.4	22.8	22.6	22.8	31.7	38.0	43.3	48.7	83.1	75.1	72.1	67.3	61.4	60.1	52.5
	114	11.6	12.7	14.5	17.0	18.0	17.3	15.0	22.2	34.8	41.1	45.6	70.6	73.1	70.6	65.7	59.1	57.7	49.4
	124	11.3	15.7	19.0	22.7	24.3	24.3	25.9	35.0	42.0	47.5	53.1	87.5	80.3	77.7	73.4	68.7	68.0	62.2
	138	13.3	15.9	18.8	22.4	23.4	22.5	20.4	29.0	43.2	50.7	55.8	81.0	83.6	80.8	75.3	66.5	64.4	52.6
	148	11.1	12.2	14.7	18.5	22.3	21.4	18.7	28.9	49.0	56.0	59.6	75.5	84.7	83.1	79.7	74.6	73.5	66.2

Appendix A3 – Table 2. (*Continued*)

Tempera-ture	Time (h)	Wavenumber values																	
		405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
	0	10.7	13.2	15.6	18.5	19.3	18.8	18.2	26.0	35.4	41.3	46.7	76.1	74.4	71.4	66.7	59.6	57.5	47.9
15°C	6	11.2	14.3	17.1	20.3	21.4	21.0	21.1	28.1	35.2	40.7	45.5	72.6	74.8	72.2	67.8	61.3	59.6	51.0
	12	11.6	14.2	16.5	18.9	19.1	18.8	19.1	25.7	32.9	38.7	44.0	73.3	73.7	70.8	65.8	58.8	57.2	48.4
	18	11.3	13.3	15.2	17.2	17.3	17.1	17.4	22.7	28.8	33.9	38.7	67.1	67.8	65.0	60.3	54.6	53.3	46.5
	24	11.9	15.6	18.4	21.1	21.2	21.1	23.0	28.5	33.6	39.1	44.5	77.4	76.9	73.8	68.7	61.7	60.1	51.4
	30	12.7	16.7	19.6	22.5	22.6	22.3	23.4	29.8	36.4	42.0	47.2	77.2	77.4	74.4	69.4	62.3	60.6	51.5
	34	13.0	17.4	20.5	23.5	23.4	23.2	24.6	30.9	38.3	44.2	49.6	79.3	82.9	80.2	75.2	68.1	66.6	57.6
	40	12.2	14.6	16.9	19.4	20.2	20.1	21.2	27.3	32.7	37.7	42.4	69.2	68.5	66.1	62.1	57.2	55.9	49.6
	48	11.3	12.6	14.4	16.4	16.3	16.0	16.1	21.6	29.1	35.0	40.0	68.6	75.5	72.8	67.9	61.3	59.9	51.7
	54	13.3	17.9	21.1	24.4	25.0	24.4	22.6	30.2	38.5	43.3	47.5	71.1	74.5	72.1	67.9	62.8	61.9	56.0
	60	13.0	14.9	16.7	18.6	18.4	18.1	18.1	23.3	30.5	35.8	40.3	67.0	68.1	65.4	60.6	54.5	52.9	45.2
	66	11.6	13.8	15.9	18.1	17.9	17.6	17.9	23.4	29.5	35.1	40.1	68.7	69.3	66.4	61.4	54.6	53.0	44.6
	72	10.3	10.2	11.4	13.1	14.0	13.6	12.5	16.9	25.7	30.9	34.8	57.1	62.3	59.9	55.7	50.2	48.8	41.6
	78	11.3	13.2	15.1	16.9	16.7	16.5	17.2	21.7	27.5	32.6	37.3	65.4	66.4	63.6	58.9	53.1	51.7	44.8
	82	11.6	14.9	17.5	20.0	20.1	19.9	20.6	26.9	33.0	38.6	43.8	73.1	72.6	69.5	64.3	57.1	55.3	45.9
	90	11.8	16.3	19.0	21.1	21.0	21.3	25.4	28.9	30.3	34.4	39.1	76.2	64.1	60.8	56.1	51.5	50.6	45.4
	96	13.3	17.0	20.2	24.1	24.2	23.3	22.0	27.1	37.3	43.6	48.0	70.3	78.9	76.5	72.0	64.5	62.4	52.5
	104	11.8	15.2	18.0	21.3	21.6	21.3	22.0	26.9	33.9	39.4	44.2	71.6	74.1	71.3	66.5	60.0	58.4	50.1
	114	12.1	16.3	19.8	24.0	26.3	26.0	25.9	35.8	45.2	51.0	56.2	86.0	81.3	78.8	74.7	69.4	68.3	61.3

Appendix A3 – Table 2. (*Continued*)

Tempera-ture	Time (h)	Wavenumber values																	
		405	**435**	**450**	**470**	**505**	**525**	**570**	**590**	**630**	**645**	**660**	**700**	**850**	**870**	**890**	**910**	**940**	**970**
	0	10.7	13.2	15.6	18.5	19.3	18.8	18.2	26.0	35.4	41.3	46.7	76.1	74.4	71.4	66.7	59.6	57.5	47.9
20°C	4	12.5	15.7	18.3	21.1	21.1	20.5	20.0	26.7	35.9	42.0	46.9	73.0	77.1	74.3	69.3	61.3	59.2	48.7
	8	12.8	14.1	16.0	18.2	18.4	17.7	15.7	22.0	34.0	39.7	43.7	65.5	71.5	68.9	64.3	57.7	56.0	47.5
	12	11.3	12.6	14.3	16.5	16.9	16.5	15.7	21.9	30.1	35.2	39.7	65.0	66.9	64.2	59.6	53.3	51.6	43.6
	18	11.6	12.4	14.1	16.2	16.5	15.8	14.4	20.4	32.3	38.5	42.8	66.6	73.2	70.5	65.7	58.6	56.9	47.9
	22	13.1	15.7	18.3	21.3	21.7	21.0	19.6	27.6	39.2	45.3	49.8	73.1	75.6	72.9	68.1	60.5	58.4	48.1
	26	12.5	15.7	18.3	21.0	21.1	20.9	21.4	27.8	34.2	39.8	45.0	73.7	73.8	70.9	66.1	58.8	56.9	47.3
	30	12.4	15.5	18.1	20.8	21.5	21.4	22.3	28.7	33.7	38.5	43.5	73.2	72.0	69.2	64.8	59.0	57.5	49.8
	34	14.1	21.8	27.2	33.2	36.6	36.1	34.8	46.8	57.4	62.8	66.8	89.2	89.3	87.5	84.5	80.7	80.0	75.3
	40	13.3	18.6	21.6	24.3	24.3	24.4	27.3	31.6	34.0	38.7	43.8	76.6	73.2	70.0	64.8	57.8	56.0	47.1
	44	13.2	17.5	20.6	23.5	23.6	23.4	24.7	30.9	37.5	43.2	48.4	77.6	78.9	76.0	71.1	63.6	61.7	52.1
	48	11.6	13.6	15.8	18.6	18.9	18.2	16.6	21.7	32.1	38.3	42.5	64.4	71.1	68.7	64.3	57.6	55.8	46.9
	52	10.9	12.0	14.1	17.2	19.8	19.2	17.8	26.8	40.0	45.9	50.2	72.1	73.9	72.3	69.3	65.7	64.8	59.3
	58	14.0	18.8	22.1	25.7	26.8	26.5	26.8	33.8	41.4	47.4	52.6	81.1	79.6	76.7	72.0	64.9	63.1	53.6
	66	11.5	12.9	14.9	17.3	17.8	17.2	15.7	20.2	29.9	36.7	41.0	62.5	71.4	69.1	64.6	58.0	56.4	48.0
	72	15.3	22.7	26.7	30.5	32.1	32.4	35.4	41.4	46.3	51.8	56.9	86.0	82.7	80.0	75.6	69.4	67.9	59.5

Appendix A4: HPLC analysis

Appendix A4 – Table 1. Changes in the chromatographic areas under peaks during storage of beef fillets stored aerobically at 0, 5, 10, 15 and 20˚C

Tempera-ture	Time (h)	Chromatographic Area under peak (mAU min)																	
		6.2	6.9 (Citric)	7.0	7.5	7.9	8.1	8.4	9.2	9.7	10.9 (Lactic)	11.7 (Formic)	12.9 (Acetic)	14.9	15.1 (Propionic)	16.0	17.7	18.7	20.0	25.9
	0	23.1	1.1	1.3	0.0	0.4	1.0	1.1	0.2	5.8	50.3	22.2	0.4	2.4	0.5	1.1	0.4	15.9	0.0	158.2
0°C	24	10.0	1.1	1.1	0.0	1.1	0.6	0.6	0.4	6.8	35.6	23.0	0.3	2.9	0.9	1.7	0.3	11.6	0.0	154.0
	72	15.6	0.9	1.3	0.0	0.4	0.5	0.6	0.2	7.9	52.5	39.9	0.3	2.9	0.3	0.2	0.3	12.1	0.0	158.1
	72	9.2	1.3	1.0	0.0	0.4	0.5	0.9	0.3	7.5	37.3	16.6	0.4	2.8	0.6	0.3	0.3	10.7	0.0	14.4
	120	6.6	1.2	0.9	0.0	0.7	0.6	0.8	0.4	8.5	41.6	7.3	0.3	3.9	0.7	0.4	0.4	10.2	0.0	4.1
	144	9.0	1.4	1.0	0.0	0.4	0.3	0.6	0.3	7.6	43.8	13.2	0.3	2.3	0.7	0.4	0.2	10.8	0.0	49.0
	168	21.2	2.2	1.6	0.4	1.8	0.3	0.9	0.6	3.0	39.3	4.3	0.5	4.0	1.2	3.3	0.8	11.4	0.0	1.3
	192	8.6	1.9	1.4	0.1	0.7	0.4	0.8	0.4	8.9	48.8	5.2	0.5	2.1	0.5	0.5	0.3	13.1	0.0	25.3
	216	10.0	1.6	1.3	0.1	0.6	0.1	0.6	0.3	8.3	49.9	4.3	0.3	3.9	0.6	0.4	0.4	14.6	0.0	12.3
	240	3.1	1.0	1.2	0.3	1.9	0.0	0.7	0.4	7.6	41.4	3.4	0.3	3.6	0.5	0.4	0.4	15.6	0.0	0.9
	288	2.0	1.3	2.6	0.5	17.5	0.0	0.7	0.9	7.2	41.8	1.2	0.2	1.7	0.8	0.8	0.4	15.4	0.0	0.6
	350	4.0	3.0	15.0	0.9	21.6	0.0	1.5	1.2	4.3	28.9	0.9	0.3	1.7	1.6	1.3	1.1	14.8	0.1	0.2
5°C	24	6.5	0.9	1.0	0.0	0.4	0.8	0.7	0.3	6.2	48.6	28.6	0.3	2.7	0.2	0.1	0.1	12.8	0.0	150.0
	48	8.7	0.7	1.1	0.0	0.6	0.6	0.8	0.4	6.1	42.7	16.3	0.3	2.4	0.4	0.7	0.2	14.5	0.0	10.1
	72	6.7	1.2	1.2	0.0	0.6	0.6	0.7	0.3	4.7	37.0	13.8	0.3	2.9	1.0	1.5	0.6	11.3	0.0	8.0
	96	17.6	1.5	1.6	0.0	0.7	1.2	1.6	0.5	4.7	45.5	19.7	0.5	4.4	1.1	1.9	0.5	12.6	0.0	10.0
	120	9.4	2.0	3.0	0.6	3.0	0.0	1.7	0.6	1.4	34.4	6.2	0.6	3.0	1.9	3.9	1.4	12.7	0.0	0.6
	144	4.3	2.1	1.9	0.6	4.4	0.0	1.2	0.5	4.9	38.0	5.2	0.3	3.4	1.0	1.8	1.1	14.2	0.0	0.4
	168	3.3	1.7	11.0	1.0	23.7	0.0	1.2	0.8	6.4	39.0	1.7	0.3	2.8	0.7	1.0	0.8	13.6	0.0	1.2
	192	6.4	0.7	4.6	0.7	2.7	1.5	1.3	0.9	1.3	17.3	13.9	1.2	1.3	2.3	3.0	1.1	12.0	0.2	1.7
	240	4.8	1.2	2.8	1.4	2.1	1.6	1.0	1.3	1.2	7.3	13.7	1.8	0.7	4.7	4.4	1.3	15.3	0.2	1.7

Appendix A4 – Table 1. ***(Continued)***

Tempera-ture	Time (h)	Chromatographic Area under peak (mAU min)																	
		6.2	6.9 (Citric)	7.0	7.5	7.9	8.1	8.4	9.2	9.7	10.9 (Lactic)	11.7 (Formic)	12.9 (Acetic)	14.9	15.1 (Propionic)	16.0	17.7	18.7	20.0	25.9
	0	23.1	1.1	1.3	0.0	0.4	1.0	1.1	0.2	5.8	50.3	22.2	0.4	2.4	0.5	1.1	0.4	15.9	0.0	158.2
10°C	8	19.3	1.9	1.2	0.0	0.7	0.5	0.9	0.3	9.3	52.9	16.6	0.7	2.5	0.6	0.4	0.3	15.4	0.0	127.8
	26	6.7	0.9	1.0	0.0	0.5	0.7	0.7	0.2	7.5	43.0	20.4	0.2	2.8	0.9	0.7	0.3	12.0	0.0	66.8
	34	9.1	1.1	1.3	0.0	0.4	1.0	0.9	0.3	6.3	38.1	20.7	0.4	3.9	0.8	1.2	0.3	11.7	0.0	22.0
	42	5.4	0.9	1.1	0.0	0.5	0.9	0.7	0.3	4.5	37.6	19.1	0.3	5.1	1.5	3.5	0.5	11.2	0.0	2.0
	52	4.7	1.2	1.0	0.0	0.6	0.4	0.8	0.4	8.7	55.5	15.0	0.3	2.1	0.4	1.3	0.4	13.4	0.0	94.6
	68	9.4	1.3	1.1	0.0	0.6	1.6	1.0	0.4	3.3	40.0	37.1	0.4	5.0	1.5	5.2	0.6	16.5	0.0	0.8
	90	5.3	0.8	1.7	0.3	1.5	1.3	0.9	0.5	1.4	27.9	26.6	0.5	3.3	1.9	5.3	1.1	15.8	0.0	0.3
	104	6.2	2.1	4.3	1.0	34.2	0.2	1.3	0.4	8.4	43.3	13.6	0.4	0.7	0.9	1.2	0.7	13.9	0.0	33.1
	124	6.2	2.3	7.3	0.9	13.7	0.0	1.5	2.7	3.9	19.3	6.3	0.5	0.7	1.6	1.2	20.8	17.0	0.2	4.8
	148	6.8	1.7	0.1	0.7	3.1	0.0	0.8	2.1	0.8	1.7	4.0	1.2	0.5	5.6	24.2	1.9	12.1	3.8	3.6
15°C	18	5.7	1.0	1.4	0.0	0.6	1.0	1.0	0.4	8.1	41.2	17.2	0.3	2.3	0.7	1.5	0.3	14.2	0.0	13.4
	24	5.6	1.7	1.4	0.0	0.7	0.6	0.9	0.4	7.8	46.8	6.4	0.4	3.7	0.5	1.0	0.4	18.4	0.0	2.8
	30	5.0	1.0	1.1	0.0	0.6	0.6	0.7	0.5	8.4	53.4	10.0	0.4	1.9	0.4	0.7	0.3	15.8	0.0	92.7
	34	6.8	1.0	1.2	0.0	0.3	0.6	0.7	0.3	6.9	40.6	26.0	0.4	1.9	0.6	0.5	0.4	14.4	0.0	41.7
	40	8.1	1.8	1.2	0.0	0.6	0.5	0.9	0.3	7.8	46.6	27.0	0.5	2.1	0.5	0.4	0.4	12.3	0.0	46.0
	48	7.6	0.8	1.4	0.0	1.2	0.4	0.6	0.3	6.4	49.8	38.2	0.6	3.0	0.7	0.8	0.3	12.5	0.0	57.3
	54	3.7	1.1	1.2	0.0	1.4	0.8	0.6	0.4	5.5	40.1	13.5	0.4	3.6	0.9	3.6	1.1	11.2	0.0	0.2
	60	2.8	2.3	11.4	0.7	17.3	0.3	0.8	0.5	7.4	41.0	2.8	0.4	3.3	0.9	1.8	2.0	16.3	0.0	0.6
	72	5.7	2.3	5.6	0.7	6.5	0.0	0.7	0.7	4.9	45.6	15.4	0.5	1.8	1.6	0.9	1.2	14.5	0.0	4.9
	90	2.3	1.3	6.0	0.7	5.2	0.0	0.5	0.7	1.1	24.1	6.0	0.7	1.8	1.5	4.9	16.0	13.9	0.3	0.0
	114	3.9	1.3	4.9	0.7	3.7	0.0	0.9	0.5	1.6	12.5	6.6	1.1	1.7	5.4	5.5	1.2	12.1	1.0	1.1

Appendix A4 – Table 1. ***(Continued)***

Tempera-ture	Time (h)	Chromatographic Area under peak (mAU min)																	
		6.2	6.9 (Citric)	7.0	7.5	7.9	8.1	8.4	9.2	9.7	10.9 (Lactic)	11.7 (Formic)	12.9 (Acetic)	14.9	15.1 (Propionic)	16.0	17.7	18.7	20.0	25.9
	0	23.1	1.1	1.3	0.0	0.4	1.0	1.1	0.2	5.8	50.3	22.2	0.4	2.4	0.5	1.1	0.4	15.9	0.0	158.2
20°C	8	10.2	0.8	1.0	0.0	0.4	0.3	0.8	0.2	6.8	50.6	48.2	0.3	2.2	0.3	0.2	0.3	14.1	0.0	155.6
	12	4.9	0.9	0.9	0.0	0.4	1.2	0.6	0.3	5.9	43.0	27.9	0.4	3.0	0.4	1.9	0.4	15.7	0.0	95.0
	18	15.4	1.2	1.5	0.0	0.4	1.1	1.0	0.2	6.9	45.1	14.9	0.3	3.4	0.8	1.2	0.5	14.8	0.0	77.1
	26	5.5	1.8	1.2	0.0	0.9	0.8	1.1	0.6	8.0	55.8	16.5	0.5	2.0	0.7	0.5	0.5	15.0	0.0	84.4
	30	3.0	0.4	1.4	0.0	1.6	0.7	0.7	0.3	2.6	23.8	12.7	0.3	2.7	1.2	4.9	0.9	18.2	0.0	0.0
	34	5.2	0.5	1.3	0.0	1.2	0.9	0.8	0.4	2.3	28.6	22.9	0.4	3.5	1.6	5.8	0.9	16.6	0.0	0.0
	40	3.3	0.8	2.6	0.4	4.5	0.8	0.9	0.6	2.4	32.9	12.5	0.6	3.4	1.6	4.2	3.2	13.9	0.3	0.1
	52	4.1	1.2	5.2	0.5	3.0	0.6	0.8	0.6	1.5	16.7	10.7	1.5	2.3	2.8	7.0	1.5	13.4	0.9	0.6
	72	1.7	1.1	2.3	0.6	2.7	0.7	0.6	0.9	0.5	23.1	10.6	2.6	0.5	2.3	3.1	18.1	16.6	0.7	0.0

APPENDIX B

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Rapid monitoring of the spoilage of minced beef stored under conventionally and active packaging conditions using Fourier transform infrared spectroscopy in tandem with chemometrics

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ABSTRACT

Fourier transform infrared (FTIR) spectroscopy was exploited to measure biochemical changes within fresh minced beef in an attempt to rapidly monitor beef spoilage. Minced beef packaged either aerobically, under modified atmosphere and using an active packaging were held from freshness to spoilage at 0, 5, 10, and 15 °C. Frequent FTIR measurements were collected directly from the sample surface using attenuated total reflectance, in parallel the total viable counts of bacteria, the sensory quality and the pH were also determined.

Principal components analysis allowed illuminating the wavenumbers potentially correlated with the spoilage process. Qualitative interpretation of spectral data was carried out using discriminant factorial analysis and used to corroborate sensory data and to accurately determine samples freshness and packaging. Partial least-squares regressions permitted estimates of bacterial loads and pH values from the spectral data with a fit of $R^2 = 0.80$ for total viable counts and fit of $R^2 = 0.92$ for the pH.

Obtained results demonstrated that a FTIR spectrum may be considered as a metabolic fingerprint and that the method in tandem with chemometrics represents a powerful, rapid, economical and non-invasive method for monitoring minced beef freshness regardless the storage conditions (e.g. packaging and temperature).

1. Introduction

The spoilage of meat may arise from several intrinsic and extrinsic factors including physical damage (e.g. freezing) or chemical changes (e.g. slime production due to proteolysis). It is well established however that microbial activity is by far the most significant cause of meat deterioration (Nychas, Douglas, & Sofos, 2007). Microbial spoilage leads to the development of off-odors and off-flavors and often slime formation, which make the product undesirable for human consumption (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). These changes are the expression of the biochemical activity of the developed microbial association, which will dominate at the end of the storage as a consequence of the main man-imposed extrinsic conditions (e.g. temperature and atmosphere). Although in general, meat (beef) spoilage is not always evident, consumers would agree that gross discoloration, strong off-odors, and the development of slime would constitute their main qualitative criteria for meat rejection (Nychas et al., 2007). In general, spoilage is a subjective judgment by the consumer, which may be influenced by cultural and economic considerations and background as well as by the sensory acuity of the individual and the intensity of the change.

More than 50 methods have been used for the detection of microbiologically spoiled or contaminated meat (e.g. organoleptic, microbiological, and physico-chemical) and which are well documented (for a review see Ellis and Goodacre (2001)). Due to their limitations (e.g. time-consuming, labor-intensive, retrospective information, and require highly trained panelists), they are unattractive for routine analysis (Dainty, 1996; Ellis, Broadhurst, & Goodacre, 2004; Ellis, Broadhurst, Kell, Rowland, & Goodacre, 2002; Nychas, Drosinos, & Board, 1998). Additional tools/methodologies using mathematic equations have been applied to describe the kinetics of ephemeral/specific spoilage organisms (E(S)SO) with the purpose to predict spoilage of various foods (McMeekin et al., 2006; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008; Nychas et al., 2007).

The idea of using microbial metabolites as potential indicators of spoilage was introduced in the 1970s and expanded in the last

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decades (Gill, 1986; Jay, 1986; McMeekin, 1982; Nychas et al., 1998). Metabolomics as well as analytical methods (e.g. Fourier transform infrared spectroscopy and fluorescence spectroscopy) boosted this concept and this approach was re-addressed (Ellis, Broadhurst, Clarke, & Goodacre, 2005; Ellis et al., 2004).

Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectroscopy represents a cheaper (reagent less), easy-to-use, faster and non-destructive way of obtaining compositional information on food samples, and indeed, the usefulness of this analytical technique in tandem with chemometrics has also been demonstrated for several foods, namely virgin olive oils (Manaf, Man, Hamid, Ismail, & Abidin, 2007; Tay, Singh, Krishnan, & Gore, 2002), fruit products (He, Rodriguez-Saona, & Giusti, 2007; Kelly & Downey, 2005), corn starch (Dupuy, Wojciechowski, Ta, Huvenne, & Legrand, 1997), coffee (Briandet, Kemsley, & Wilson, 1996; Downey, Briandet, Wilson, & Kemsley, 1997), honey (Kelly, Petisco, & Downey, 2006), wine (Edelmann, Diewok, Schuster, & Lendl, 2001), and beef (Al-Jowder, Defernez, Kemsley, & Wilson, 1999; Al-Jowder, Kemsley, & Wilson, 2002), to tackle authentication and adulteration problems. Recently, this technique has been also used to early detect bacterial spoilage of chicken breast and beef rump steaks (Ellis et al., 2004, 2005). Although both studies were pioneering in the early detection of meat spoilage, under aerobic conditions, other storage conditions (e.g. packaging systems and temperature) were not investigated. This is of great importance since the changes and development in technologies for food processing and preservation [e.g., vacuum packaging (VP), modified atmosphere packaging (MAP), and active packaging] make it evident that the meat industry needs rapid analytical methods or tools for quantification of spoilage regardless the packaging system used.

Therefore the purpose of this study was to explore the potential of FTIR-ATR as a rapid and accurate method for monitoring the spoilage of minced beef samples stored under different storage conditions (i.e. packaging and temperature).

2. Material and methods

2.1. Essential oil

Samples of *Origanum vulgare* ssp. *hirtum* were collected and their essential oil was obtained by steam distillation in Ecofarm Hellas. The latter kindly provided the oregano essential oil (EO).

2.2. Minced beef preparation

Fresh minced beef of normal pH (pH 5.5) was purchased from the retail central market in Athens (Greece) and transported under refrigeration to the laboratory within 30 min, where it was held at 1 °C for 1–2 h. The minced beef was divided into portions of 75 g and packaged individually either aerobically and under modified atmosphere with and without an oregano essential oil (2% v/w) slow releasing system. Samples stored aerobically were enclosed into permeable polyethylene bags, while samples packed under modified atmospheres packaging (MAP) were incorporated into plastic pouches (200 mm wide × 240 mm long × 90 μm thickness), with oxygen permeability of 6 $cm^3\ m^{-2}\ 24\ h^{-1}$ at 20 °C and 50% RH, and flushed with 40% CO_2, 30% O_2, and 30% N_2 using a Henco-Vac Machine (Howden Food Equipment BV, The Netherlands). The application of volatile compounds of EO inside the packaging was a modification of the procedure applied by Skandamis and Nychas (2002). Briefly, the essential oil was distributed on a Whatman paper No. 6 in a final concentration of 2% v/w and then the paper was placed within the container but not in contact with the minced meat.

In both groups of MAP samples, the pouches were evacuated and flushed three times before being filled (3 l). After filling, the pouches were double heat-sealed. A limited number of samples were freeze-stored to serve as controls during sensory evaluation of color and odor.

2.3. Experimental design

A factorial experiment (3 × 4) was designed and performed. Minced beef was stored under three packaging conditions (aerobic, MAP without EO and MAP with EO (active packaging). Samples were divided into groups and stored at 0, 5, 10, and 15 °C until the spoilage was very pronounced. Sampling was performed as detailed in Table 1.

2.4. Microbiological analysis and pH measurement

Samples (25 g) from meat were weighed aseptically, added to sterile quarter strength Ringer's solution (225 ml), and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature. Decimal dilutions in quarter strength Ringer's solution were plated on plate count agar (PCA; Biolife) and incubated at 30 °C for 48 h for enumerating total viable counts (TVC). Duplicate packages from each storage temperature as well as from each type of packaging were taken at appropriate time intervals (Table 1) to allow for efficient kinetic analysis of total viable counts and sensory characteristics. Enumeration of TVC and pH analyses were performed on these duplicate samples and results are displayed as the mean of both measurements. Each sample was analyzed in duplicate (coefficient of variation of samples from the same experiment, <0.35%).

Resulting data (growth counts) were transformed to $\log_{10}$ values. TVC data were fitted using the Baranyi and Roberts' model (Baranyi & Roberts, 1994). For curve fitting, the in-house program

Table 1
Sampling frequencies along the spoilage process depending on the storage temperature.

Time (h)	Storage temperature (°C)			
	0	5	10	15
0	+	+	+	+
6			+	+
12				+
18			+	+
24	+	+		+
30			+	+
36				+
42			+	+
48	+	+		+
54			+	+
60				+
69	+	+	+	+
78			+	+
90	+	+	+	+
110			+	+
114	+	+		
134			+	+
162		+	+	
196	+	+	+	+
220		+	+	+
244	+	+	+	
268		+	+	
291	+	+		
315		+	+	
338	+	+		
386	+	+	+	
482		+		
458	+			
554	+			

DMFit (Institute of Food Research, Reading, UK) was used, which was kindly provided by Dr. J. Baranyi, available also in the internet (http://www.ifr.ac.uk/safety/DMFit/).

The pH value was recorded by a pH meter (Metrohm 691 pH meter), the glass electrode being immersed in the homogenate of minced beef after the end of microbiological analysis.

2.5. Sensory analysis

Sensory evaluation of meat samples was performed during storage according to Gill and Jeremiah (1991) by a sensory panel composed of four members (staff from the laboratory). The same trained persons were used in each evaluation, and all were blinded to the sample tested. The sensory evaluation was carried out in artificial light and the temperature of packaged product was similar to ambient temperature. Special attention was given to the color and the presence of exudate in the pack prior to opening and the assessment of abnormal odors during the opening of the pack (Skandamis & Nychas, 2002). Each attribute was scored on a three-point hedonic scale where: 1 = acceptable (fresh); 2 = marginal (semi-fresh); and 3 = unacceptable (spoiled). Assessment was designed to identify spoilage conditions exclusively. Odor characteristic of minced beef, as exemplified by special samples from frozen storage that were thawed prior to each sensory evaluation, was regarded as acceptable. Distinct putrid, sweet, sour or cheesy odors were regarded as indicative of spoilage and therefore unacceptable. Bright colors typical of fresh oxygenated meat were considered acceptable. A persistent dull appearance, or unusual color or appearance was considered unacceptable. The time in days before the panel considered the quality to be at the limit of acceptability (score = 2–1) was defined as the sensory shelf life of samples. The shelf life limit was defined as the point when 50% of the panelists rejected the sample.

2.6. FTIR-ATR measurement

Samples of approximately 8 cm × 1 cm × 0.5 cm were excised in parallel to the microbiological analysis (Table 1). FTIR spectra were collected using a ZnSe 45° ATR crystal on a Nicolet 6700 FTIR spectrometer equipped with a DLaTGS Detector with KBr beamspliter and controlled by Omnic Software – v7.3. The ZnSe ATR crystal was capable of 12 external reflections, with the evanescent field effecting a depth of 1.01 μm. FTIR-ATR measurements were collected directly from the sample surface at 20 °C in the spectral range of 4000–400 cm^{-1}, by accumulating 100 scans with a resolution of 4 cm^{-1}. The collection time for each sample spectrum was 2 min. Each sample was measured in duplicate and results are displayed as the mean of both measurements. Reference spectra were acquired by collecting a spectrum from the cleaned blank crystal prior to the presentation of each sample replicate. At the end of each sampling the crystal surface was cleaned with detergent, washed with distilled water, dried with lint-free tissue, cleaned with ethanol and finally dried with lint-free tissue following collection of each spectrum.

2.7. Mathematical treatment of the data

Mean-centered and standardized (1/SD) spectral data collected between 1800 and 1000 cm^{-1} were subjected to a principal component analysis (PCA) to investigate differences between samples. The PCA transforms the large number of potentially correlated factors into a smaller number of uncorrelated factors (i.e. principal components), and thus reduces the size of the data set. For qualitative analysis, principal components (PCs) significantly contributing to the variance of the data set were subjected to factorial discriminant analysis (FDA) in an attempt to predict the likelihood of a sample belonging to a previously-defined qualitative group. Since the raw spectral data could not be used because of the strong correlation between the variables (the wavenumbers), the uncorrelated PCs resulting from PCA were employed. For quantitative analysis, PCs significantly contributing to the variance of the data set were regressed using a partial list squares regression (PLS-R) onto TVC or pH data. PCA, FDA, and PLS-R calculations were performed using the Statistica® v6.0 package (Statsoft, OK, USA), the XLSTAT® v2006.06 package (Addinsoft, Paris, France), and The Unscrambler® v9.6 package (Camo, Oslo, Norway), respectively.

3. Results

Table 2 summarizes kinetic parameters of TVC following data fitting using the Baranyi and Roberts' model (Baranyi & Roberts, 1994). Lag phase was observed only at 0 °C, while there was an increase in the maximum specific growth rate (μ_{max}) of TVC correlated to the increase of the storage temperature regardless the packaging type. The influence of the packaging type was also evident. In fact at the four different storage temperature, TVC μ_{max} followed the increase order active packaging < MAP < aerobic.

Table 3 recapitulates, for each storage temperature and packaging type, time cut-off, TVC, and pH corresponding to each hedonic

Table 2
Kinetic parameters (following data fitting) of total viable counts (initial counts 5.48 ± 0.2 log cfu) as function of the storage temperature and the packaging type.

Curve	μ_{max}[b]	Lag phase (h)	y_0[c] (log cfu/g)	y_{END}[c]/observed counts[d] (log cfu/g)	Standard error (SE) of fitting	Adjusted R^2 statistics of the fitting
0 °C_Air*	0.05	100	5.58	9.78/10.04	0.27	0.98
0 °C_MAP**	0.02	207	5.47	8.29/8.27	0.18	0.97
0 °C_AP***	0.01	220	5.47	N.C.[a]/7.34	0.20	0.92
5 °C_Air	0.05	NO	5.34	9.86/9.96	0.15	0.99
5 °C_MAP	0.03	NO	5.37	8.00/8.15	0.16	0.97
5 °C_AP	0.02	NO	5.41	7.73/7.85	0.14	0.98
10 °C_Air	0.09	NO	5.72	9.61/9.89	0.25	0.97
10 °C_MAP	0.10	NO	5.37	8.48/8.66	0.18	0.98
10 °C_AP	0.07	NO	5.57	7.71/7.95	0.18	0.95
15 °C_Air	0.16	NO	5.27	9.51/9.72	0.30	0.96
15 °C_MAP	0.16	NO	5.54	8.32/8.48	0.15	0.98
15 °C_AP	0.12	NO	5.63	7.75/7.94	0.17	0.94

*, **, and *** are aerobic, modified atmosphere (MAP), active packaging (AP) coupling MAP and the slow releasing oregano essential oil, packaging, respectively.
NO, not observed.
[a] Non-computable – fitted curve was completed without asymptote (semisegmoidal).
[b] Maximum specific growth rate h^{-1}.
[c] Estimated by the Baranyi model.
[d] Determined experimentally.

Table 3
Time needed (days) for the sensory panel to marginally consider (cut-off) the product acceptable (hedonic mark 2) or spoiled (hedonic mark 3). TVC (log cfu/g) and pH at the same time points are also shown.

Sample	Hedonic mark 2 = marginal spoiled			Hedonic mark 3 = spoiled		
	Time (d) cut-off[a]	TVC	pH	Time (d) cut-off	TVC	pH
0 °C_Air*	12.1	9.4	6.0	16.1	9.4	6.0
0 °C_MAP**	16.1	7.0	5.5	27.1	8.3	5.1
0 °C_AP***	19.1	6.5	5.5	27.1	7.3	5.5
5 °C_Air	6.8	9.1	6.0	10.2	9.7	6.1
5 °C_MAP	10.2	7.9	5.5	12.1	8.0	5.3
5 °C_AP	11.2	7.7	5.3	16.1	7.6	5.2
10 °C_Air	2.9	8.7	5.6	5.6	9.2	6.2
10 °C_MAP	3.3	8.5	5.4	6.8	8.4	5.4
10 °C_AP	3.8	7.8	5.3	9.2	7.7	5.0
15 °C_Air	2.0	8.7	5.3	3.3	9.7	6.1
15 °C_MAP	2.3	7.8	5.1	3.8	8.4	5.3
15 °C_AP	2.5	7.9	5.0	3.8	7.6	4.9

* Air, aerobic packaging.
** MAP, modified atmosphere packaging.
*** AP, active packaging coupling modified atmosphere and the slow releasing oregano essential oil.
[a] This time is considered as the sensory shelf life above which the product is rejected.

mark scored by the panel. In comparison with the aerobic packaging, it was observed that the active packaging enabled extension of the sensory shelf life of minced beef by 9 h, 3.5 days, 6 days, and 9 days when stored at 15, 10, 5, and 0 °C, respectively. No significant differences in the sensory shelf life, between samples stored under active packaging and under MAP, were observed at 0 and 15 °C, i.e. extreme temperatures for the growth of meat flora. However, at 5 and 10 °C, the active packaging extended the sensory shelf life of minced beef by 3 and 2.5 days in comparison to the MAP, respectively. Moreover, no correlation was observed between TVC levels and the spoilage detection time. Indeed, depending on the packaging type and the storage temperature some samples were rejected at 7, 8, or 9 $\log_{10}$ cfu/g.

Furthermore, it was observed that for each storage temperature, pH values recorded for the three different packaging were, in general, similar and started to increase for aerobically packaged samples, while it dropped for MAP and active-packaged samples. Interestingly, for the aerobically packaged samples, pH values increased at 42, 78, 114, and 196 h of storage, respectively, when stored at 15, 10, 5, and 0 °C. Except for samples stored at 10 °C, these matched exactly with the cut-off time separating fresh from semi-fresh aerobically packaged samples (Table 3).

3.1. FTIR spectra and multidimensional data analysis

Fig. 1 shows spectra collected from 1800 to 1000 cm^{-1} for fresh (24 h of storage time) and spoiled (23 days of storage time) beef MAP samples stored at 0 °C. These spectra correspond, respectively, to 5.4 and 8.1 $\log_{10}$ cfu/g of TVC, and to 5.4 and 5.2 pH values. A major peak at 1640 cm^{-1} due to the presence of moisture (O–H) in the meat sample is apparent in the ATR spectra. Two others peaks corresponding to the absorption of amide II (C–N stretch) and fat (C=O ester) were observed at 1550 cm^{-1} and at 1745 cm^{-1}, respectively.

3.2. Spoilage monitoring using FTIR spectral data

Mean-centered and standardized FTIR spectral data collected between 1800 and 1000 cm^{-1} of the 188 mean spectra (62 from air, 62 from MAP, 62 from active packaging, and two from 0 h; ex-

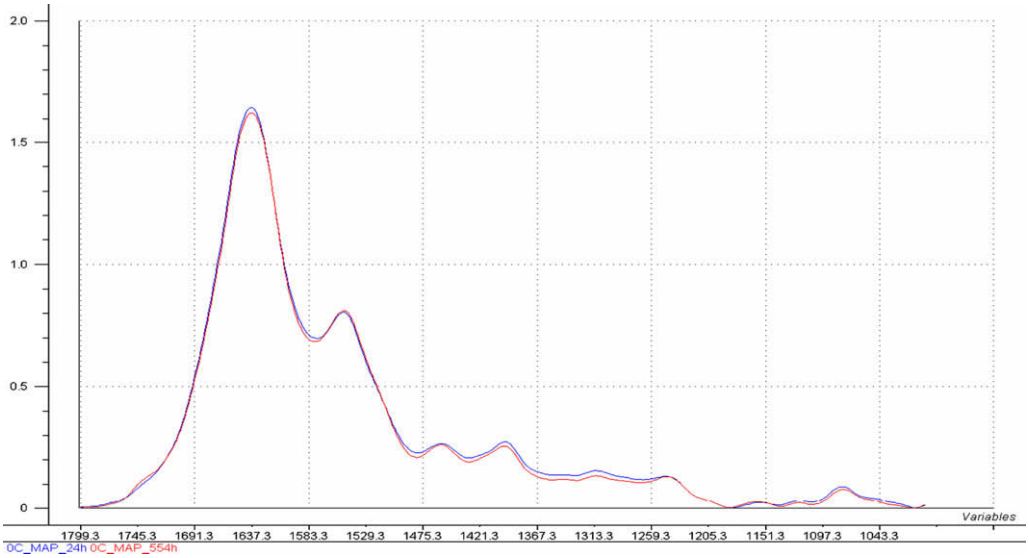

Fig. 1. FTIR-ATR spectra collected for fresh (24 h) and spoiled (554 h) minced beef packaged under modified atmosphere and stored at 0 °C. The fresh sample corresponded to 5.4 $\log_{10}$ cfu/g total viable counts bacteria (TVC) and pH 5.4, while the spoiled sample corresponded to 8.1 $\log_{10}$ cfu of TVC and to pH 5.2.

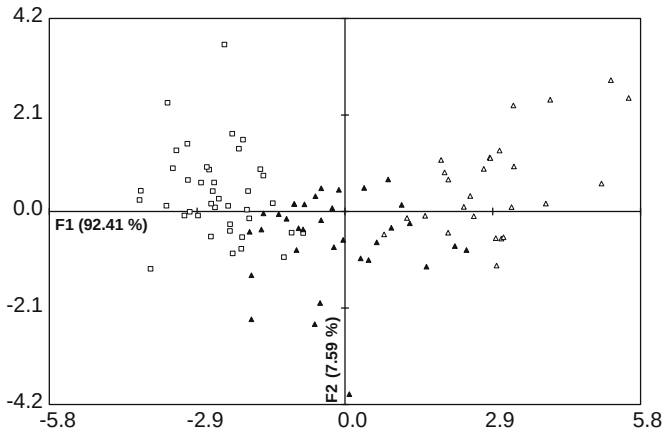

Fig. 2. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the three different minced beef freshness groups: □, fresh; ▲, semi-fresh; and △, spoiled.

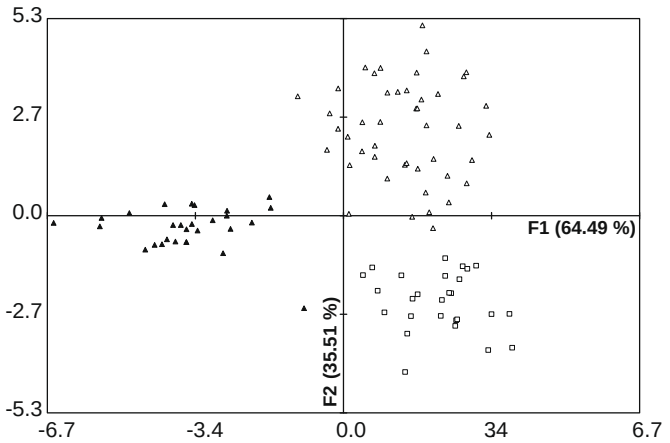

Fig. 3. Discriminant analysis similarity map determined by discriminant factors 1 (F1) and 2 (F2) for FTIR-ATR spectral data of the three different minced beef packaging groups: □, modified atmosphere packaging; ▲, active packaging; and △, aerobic packaging.

cept two samples 5 °C/MAP/69 h and 10 °C/air/386 h) representing combinations of the packaging type, the storage temperature, and the storage time were subjected to PCA. It was shown that the total variance of the data set could be explained by 65 PCs among which the five first PCs explain 90.9% of the total variance. In this regard, variables (wavenumbers) for which the communality value of each PC out of the five was higher or equal to 0.6, (i.e. the absolute value of each of the five PCs coordinates was higher than 0.78) were considered as significantly explaining the variance of the spectral data set and hence were considered as potential wavenumbers associated with the biochemical changes happening during the spoilage process. These wavenumbers ranged from 1714 to 1710 cm^{-1}, 1614 to 1211 cm^{-1}, and 1031 to 1000 cm^{-1} and were selected for further analyses. These wavenumbers corresponded mainly to the absorption of amide II (C–N stretch; peak at 1550 cm^{-1}), fat (CH_2 scissoring; peak at 1458 cm^{-1}), amides (C–N stretch; peak at 1398 cm^{-1}), and amines (C–N stretch; peaks at 1311 cm^{-1} and at 1246 cm^{-1}) (Al-Jowder et al., 1999; Ellis et al., 2002, 2004; Socrates, 2001). It is noteworthy that the most significant (each of the five PCs communality value was higher or equal to 0.95) wavenumbers explaining the variance of the data set ranged from 1402 to 1371 cm^{-1} and 1321 to 1303 cm^{-1} and corresponded to amides and amines as aforementioned.

Spectral data corresponding to the selected wavenumbers (230 variables) were subjected to a new PCA, which showed that the total variance of the data set could be explained by 39 PCs. These factors data were then subjected to a FDA based on the defined sensory groups (fresh, semi-fresh, and spoiled) constituting the dependent variable. Fig. 2 shows the factorial map defined by the two discriminant factors (DF), DF1 and DF2, which explained 100% of the total variance. The classification table (Table 4A) resulting from the FDA provided 100% correct classification and 76.3% correct classification when cross-validated. Even though 23.7% of samples could not be cross-validated, no fresh sample was reclassified as spoiled or *vice versa*.

The same approach was followed taken type of packaging as a factor. PCs resulting from the PCA were subjected to a FDA based on the defined packaging type (air, MAP, and active packaging) constituting the dependent variable. Fig. 3 shows the factorial map defined by DF1 and DF2, which explained 100% of the total variance. The classification table (Table 4B) resulting from the FDA provided 100% correct classification and 92.5% correct classification when cross-validated.

3.3. Supervised PLS-R for the prediction of TVC and pH based on FTIR spectral data

PLS-R was performed on PCs data resulting from PCA in an attempt to predict either TVC or pH based on FTIR spectral data. Interestingly, only two regression factors stood in either the TVC or pH prediction models. The slope R^2 (R is the correlation coefficient) and the root mean squared error (RMSE) indicating the quality of fit were, respectively, about 0.80 and 0.58 for the TVC fit and

Table 4
(A) Classification table for the learning sample and the cross-validation results as regards the sensory group (rows: observed classifications; columns: predicted classifications) and (B) classification table for the learning sample and the cross-validation results as regards the packaging type (rows: observed classifications; columns: predicted classifications).

From/to	Learning sample					Cross-validation				
	Fresh	Semi-fresh	Spoiled	Total	% Correct (%)	Fresh	Semi-fresh	Spoiled	Total	% Correct (%)
Panel A										
Fresh	65	0	0	65	100	45	20	0	65	69.2
Semi-fresh	0	66	0	66	100	7	53	6	66	80.3
Spoiled	0	0	55	55	100	0	11	44	55	80.0
Total	65	66	55	186	100	52	84	50	186	76.3
	Air	MAP	Active packing			Air	MAP	Active packing		
Panel B										
Air	63	0	0	63	100	60	2	1	63	95.2
MAP	0	61	0	61	100	4	55	2	61	90.2
Active pack	0	0	62	62	100	3	2	57	62	91.9
Total	63	61	62	186	100	67	59	60	186	92.5

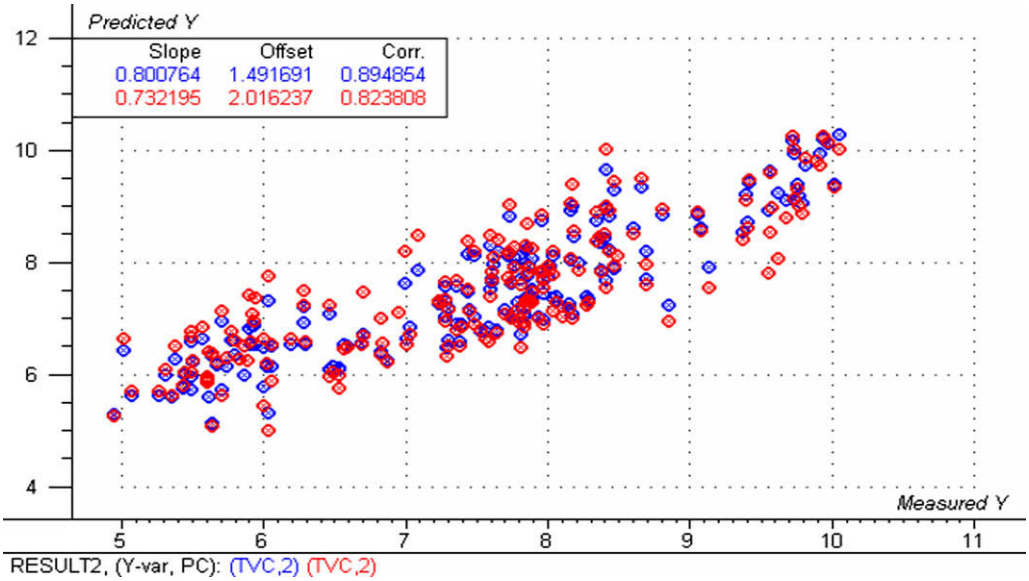

Fig. 4. The estimates from partial least-squares regression vs. observed total viable counts (TVC) levels. In blue are estimates from the learning sample and in red are cross-validated estimates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

0.93 and 0.12 for the pH regression. Cross-validations (Figs. 4 and 5) decreased slightly the quality of TVC prediction with $R^2 = 0.73$ and RMSE = 0.71, and the quality of the pH fit with R^2 and RMSE of about 0.85 and 0.20, respectively.

4. Discussion

The evaluation of meat freshness, spoilage or safety is based on, sensory and microbiological analyses (Nychas et al., 2008). The disadvantages of sensory analysis, which is probably the most acceptable and appropriate method, is its reliance on highly trained panellists, a procedure which makes it costly and unattractive for routine analysis. On the other hand, unfortunately, microbiological analyses are either lengthy (traditional and conventional microbiology), or costly and high-tech (molecular tools), as well as destructive to products tested, while not able to give the 'immediate answer required' (McMeekin et al., 2007). This is also applied to databases such as ComBase (www.combase.cc) and Sym'previus (www.symprevius.net) that are designed to describe the kinetics of microorganisms in order to determine the shelf life of various foods. This is due to the fact that the mathematical models (i.e. quantification of microbial behavior) on which these databases are structured are also based on invasive, time-consuming, tedious and costly microbiological analyses (i.e. traditional–conventional microbiology and to a less extent molecular tools).

Despite the progress made by the predictive microbiology, spoilage models provide only specific information and so far underestimated factors, e.g. meat matrix and microbial population dynamics (i.e. changes of this association in space and time, changes in microbial metabolites occurring due to the interaction on meat surfaces). Thus, efforts have been made to replace microbiological analyses with (bio)chemical changes occurring in muscle

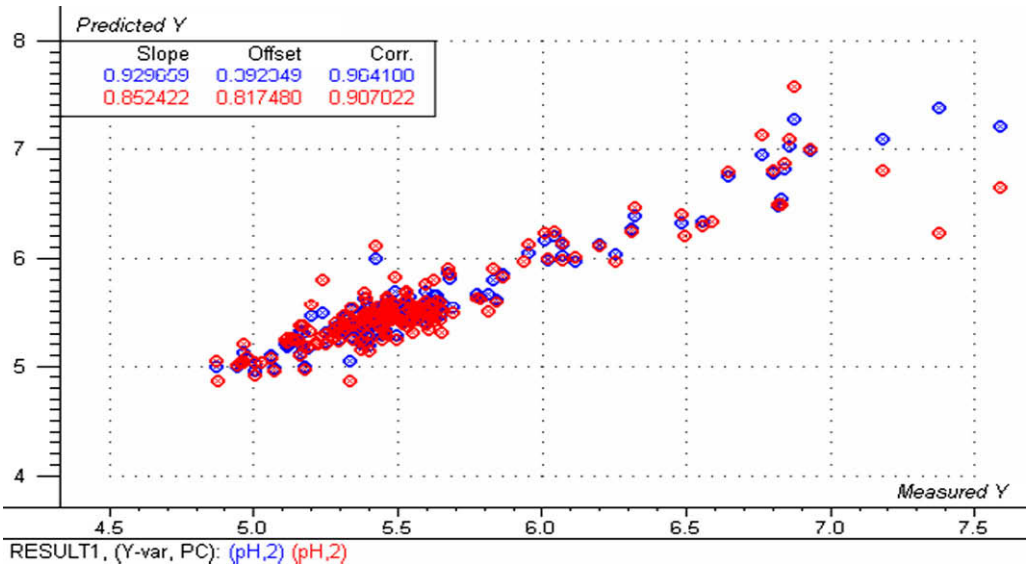

Fig. 5. The estimates from partial least-squares regression vs. observed pH values. In blue are estimates from the learning sample and in red are cross-validated estimates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(e.g. various microbial metabolic products, termed as chemical spoilage indices – CSI), as potential tools to assess meat spoilage. The attempts that have been made over the last two decades to associate given metabolites with the microbial spoilage of meat have not been very much appreciated, due to low understanding of the phenomena (Nychas et al., 2007). The basic concept for these methods, that has been reviewed recently (Ellis & Goodacre, 2001; Nychas et al., 2007) is that as the bacteria grow on meat, they utilize nutrients and produce by-products.

The metabolomic concept in food microbiology was introduced by Goodacre and coworkers (Goodacre, Vaidyanathan, Dunn, Harrigan, & Kell, 2004). Chemometric methods (e.g. PCA and hierarchical cluster analysis), in parallel with mathematical models (e.g. artificial neural networks (ANNs) and fuzzy logic), have started being applied in the evaluation of meat quality (Ellis et al., 2002). These approaches could rapidly provide information related to the contribution of the ephemeral spoilage organisms (ESO) in meat spoilage or to the categorization of meat with regard to (i) type of meat and (ii) spoilage (Ellis et al., 2005; Mataragas, Skandamis, Nychas, & Drosinos, 2007). Ellis and coworkers (Ellis et al., 2002, 2004) have been the pioneers stipulating that FTIR spectroscopy can be used directly on the surface of food to produce biochemical interpretable "fingerprints" (metabolic snapshot) enabling an early detection of microbial spoilage of chicken breast and beef rump steaks held from freshness to spoilage during 24 h aerobically and at ambient temperature (Ellis et al., 2002, 2004). However, in both studies freshness was neither assessed for non-aerobically packaged products, nor at chill temperatures, i.e. the standard conditions for beef storage. Also, authors have used 7 $\log_{10}$ cfu/g as threshold above which the sample is considered to be spoiled. This might be true for aerobically stored samples but not for modified atmosphere or active-packaged samples stored at chilling temperatures.

In this study, FTIR spectroscopy was used to obtain metabolic snapshots (Ellis et al., 2002) of minced beef during storage under different storage conditions (e.g. temperature packaging system) in attempt to monitor spoilage. In fact, it was shown that the comparison of FTIR spectra could enlighten certain biochemical changes occurring during meat spoilage. However the visual analysis of the huge FTIR spectral data set (415 variables, i.e. wavenumbers for each spectrum) obtained along the spoilage process for the different storage conditions, especially at close storage periods, was not at all feasible. Therefore chemometric methods, namely PCA, FDA, and partial least square regression (PLS-R) were used to process and extract the biological information encrypted by the FTIR spectrum, with the purpose to correlate growth of total viable microbial association (e.g. countable in a very generic medium, such as plate count agar), metabolic compounds and sensory analysis with spoilage in order to accurately predict the spoilage level of an unknown product. In particular, the application of PCA showed that few wavenumbers were highly (negatively or positively) related to the spoilage and depended on storage time and conditions applied.

These wavenumbers corresponded mainly to the absorption of amide II (N–H), fat (CH_2), amides (C–N), and amines (C–N) (Al-Jowder et al., 1999; Ellis et al., 2002, 2004; Socrates, 2001). Freshness-dependent FDA performed on the data set resulted in accurate discrimination between fresh and spoiled products; hence demonstrating the effectiveness of the method to monitor minced beef spoilage. The good discrimination of the model is thought to be linked mainly to the onset of proteolysis (Nychas & Tassou, 1997) resulting in changes in the levels of amides and amines as it has already been reported by Ellis and coworkers (Ellis & Goodacre, 2001; Ellis et al., 2002), but also to the rate of endogenous glucose consumption, and the changes in organic acids (Dainty, 1996; Nychas et al., 1998).

The need for an objective evaluation of meat spoilage should be able to face the advanced technologies that could be or already have been applied for meat preservation (e.g. vacuum pack, modified atmospheres, and release of antimicrobial compounds) (Ercolini, Storia, Villani, & Mauriello, 2006; Stanbridge & Davies, 1998). This study revealed the ability of FTIR spectroscopy in tandem with chemometrics to accurately predict the packaging type. This issue although at the moment is not among the priorities of the authorities/meat industries can be reserved as additional/alternative information to monitor meat shelf life. In particular this study showed that the developed model could discriminate accurately minced beef samples according to their type of packaging. This very good discrimination could be structured on two levels. A major level separated the active packaging from the MAP and the aerobic packaging, and a second level separated MAP-based packaging from the aerobic packaging. These findings in the authors' opinion are of great importance since they show that there are more than one metabolic fingerprint describing meat spoilage. It is reported that this was due to ephemeral spoilage microbial ecology which is developed and influenced the type (e.g. acidic or alkaline metabolites) as well as the rate of microbial activity (e.g. nutrient consumption) (Skandamis & Nychas, 2001, 2002, 2005; Tsigarida & Nychas, 2001)

In this study, FTIR has been shown to represent an economical, rapid and non-invasive tool for the monitoring of minced beef spoilage *via* the measurement of biochemical changes occurring in the meat substrate rather than enumerating bacteria *per se*. Each FTIR-ATR spectrum may be considered as a metabolic fingerprint (snapshot; Ellis et al., 2002) of which can be easily transformed to useful information related to degree of spoilage. Additional work has to be performed in order to enlarge the spectral data for more accurate results.

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Rapid qualitative and quantitative detection of beef fillets spoilage based on Fourier transform infrared spectroscopy data and artificial neural networks

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ABSTRACT

A machine learning strategy in the form of a multilayer perceptron (MLP) neural network was employed to correlate Fourier transform infrared (FTIR) spectral data with beef spoilage during aerobic storage at chill and abuse temperatures. Fresh beef fillets were packaged under aerobic conditions and left to spoil at 0, 5, 10, 15, and 20 °C for up to 350 h. FTIR spectra were collected directly from the surface of meat samples, whereas total viable counts of bacteria were obtained with standard plating methods. Sensory evaluation was performed during storage and samples were attributed into three quality classes namely fresh, semi-fresh, and spoiled. A neural network was designed to classify beef samples to one of the three quality classes based on the biochemical profile provided by the FTIR spectra, and in parallel to predict the microbial load (as total viable counts) on meat surface. The results obtained demonstrated that the developed neural network was able to classify with high accuracy the beef samples in the corresponding quality class using their FTIR spectra. The network was able to classify correctly 22 out of 24 fresh samples (91.7%), 32 out of 34 spoiled samples (94.1%), and 13 out of 16 semi-fresh samples (81.2%). No fresh sample was misclassified as spoiled and vice versa. The performance of the network in the prediction of microbial counts was based on graphical plots and statistical indices (bias and accuracy factors, standard error of prediction, mean relative and mean absolute percentage residuals). Results demonstrated good correlation of microbial load on beef surface with spectral data. The results of this work indicated that the biochemical fingerprints during beef spoilage obtained by FTIR spectroscopy in combination with the appropriate machine learning strategy have significant potential for rapid assessment of meat spoilage.

1. Introduction

In most developed countries meat consumption is very high mainly due to its high nutritional value in the human diet. The great variability in raw meat in terms of chemical composition, technological and chemical attributes results in highly variable end products which are marketed without a desired and controlled level of quality [1]. In order to maintain quality standards, control procedures must be carried out on meat comprising chemical analyses, instrumental methods, organoleptic evaluation, and molecular screening methods. More than fifty methods have been used for the detection of microbiologically spoiled or contaminated meat (e.g. organoleptic, microbiological, and physico-chemical), all of which are well documented [2]. However, these techniques are invasive, time consuming, labour intensive, demand highly trained personnel, and thus they are unsuitable for online application and routine analysis [3–7]. The lack of general agreement on the early signs of incipient spoilage for meat makes more difficult the task to evaluate it objectively, mainly due to changes in the technology of meat preservation (e.g. vacuum, modified atmospheres, etc.). The use of microbial metabolites as a consequence of microbial activity in meat has been continuously recognized as a potential means for assessing meat quality [4,6,8]. The attempts that have been made over the last two decades to associate given metabolites with microbial spoilage of meat have not been very much appreciated, due to low understanding of the underlying phenomena [6].

Recently, some interesting analytical approaches using mathematical equations have been applied to describe the kinetics of ephemeral/specific spoilage organisms (E(S)SO) with the purpose to predict spoilage of various foods [7,9]. Other approaches are based on the use of biosensors (enzymatic reactor systems), electronic noses (array of sensors), and vibrational spectroscopy methods (e.g. FTIR, Raman spectroscopy) [10–12] for the same purpose. In contrast to conventional methods, Fourier transform infrared (FTIR) spectroscopy is rapid, non-invasive, requires no specific consumable or reagent permitting users to collect full spectra

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in a few seconds allowing simultaneous assessment of numerous meat properties [4,5,13]. The basic concept underlying this method stipulates that as bacteria grow on meat, they utilize nutrients and produce by-products that cause spoilage. The quantification of these metabolites represents a fingerprint characteristic of any biochemical substance, providing thus information about the type and the rate of spoilage [2,13]. The integration of the FTIR Attenuated Total Reflectance biosensors or other biosensors in tandem with an information platform would result in the development of an "expert system" that would be able to qualitatively and/or quantitatively discriminate between meat samples based on extracted pre-processing features.

The application of advanced statistical methods (e.g. discriminant function analysis, clustering analysis, partial least square regression, chemometrics) and intelligent methodologies (neural networks, fuzzy logic, evolutionary algorithms, genetic programming) can be used as qualitative indices rather quantitative since their primary target is to distinguish objects or groups or populations [14,15]. Nowadays, machine learning strategies are based on supervised learning algorithms [16]. The last mentioned approach together with the development of artificial neural networks (ANN) could be used effectively in the evaluation of meat spoilage. Interest in using artificial neural networks in food science is increasing in the last years, as they have shown promising results in several applications such as sensory analysis, pattern recognition, classification, microbial predictions, and food process optimization [17–21]. Essentially, ANNs are computing algorithms that attempt to imitate the computational capabilities of large, highly connected networks of relatively simple elements such as neurons in the human brain [22]. They contain a series of mathematical equations that are used to simulate biological processes such as learning and memory. Their development first involves a learning process that adaptively responds to the input variables according to a learning rule. The network has the ability to learn from its environment and adapt to it similar to its biological counterparts [23]. An ANN normally has no restriction on the type of relationship between the growth parameters (input patterns) and the desired output. In contrast to conventional models in which a mathematical equation must be stated beforehand, ANNs directly explore the knowledge contained in the input–output patterns by adjusting the highly nonlinear topology, as the input–output patterns are repeatedly presented to the network [24].

The aim of the present study was to build upon previous experience undertaken in our laboratory on beef spoilage using FTIR spectroscopy and develop an artificial neural network that could (i) discriminate between different quality classes of beef fillets during aerobic storage at chill (0, 5 °C) and abuse (10, 15, and 20 °C) temperatures, and (ii) predict the microbial load on the surface of meat samples directly from FTIR spectral data.

2. Materials and methods

2.1. Sample preparation

Fresh deboned pieces of beef were purchased from a local meat retail outlet and transported under refrigeration to the laboratory within 30 min. On arrival, the samples were prepared by cutting the meat pieces into portions (40 mm wide × 50 mm long × 10 mm thick) and maintained at 4 °C for 1 h until use. The portions were subsequently placed into 90 mm Petri dishes and stored at 0, 5, 10, 15, and 20 °C in high-precision (±0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan) for an overall period of 350 h, depending on storage temperature, until spoilage was pronounced. Meat samples were not subjected to any prior pre-treatment such as fat and connective tissue removal, or inoculation with selected species of bacteria. For the FTIR measurements, a thin slice of the aerobic upper surface of the fillet was excised and used for further spectral analysis.

2.2. Microbiological analyses

For microbiological analysis a portion (40 mm wide × 50 mm long × 10 mm thick) was added to 150 ml sterile quarter strength Ringer's solution, and homogenized in a stomacher (Lab Blender 400, Seward Medical, London, UK) for 60 s at room temperature (*ca.* 20 °C). Further decimal dilutions were prepared with the same diluent, and duplicate 0.1 ml samples of three appropriate dilutions were spread in triplicate on plate count agar (PCA 4021452; Biolife, Italy) for counts of total viable bacteria (TVC), which was incubated at 30 °C for 48 h. Duplicate samples from each storage temperature were analyzed at appropriate time intervals to allow for efficient kinetic analysis of total viable counts. Specifically, meat samples stored at 0 and 5 °C were analyzed every 24 h, whereas samples stored at 10, 15, and 20 °C were analyzed every 8, 6, and 4 h, respectively. Growth data from plate counts were log transformed and fitted to the primary model of Baranyi and Roberts [25] using the in-house program DMFit (Institute of Food Research, Norwich, UK) to determine the kinetic parameters of microbial growth (maximum specific growth rate and lag phase duration).

2.3. Sensory analysis

Sensory evaluation of meat samples was performed during storage according to Gill and Jeremiah [26] by a sensory panel composed of five members (staff from the laboratory) at the same time intervals as for microbiological analyses. The same trained persons were used in each evaluation, and all were blinded to the sample tested. The sensory evaluation was carried out in artificial light and the temperature of all samples was close to ambient. The descriptors selected were based on the perception of colour, smell, and taste. The first two descriptors were assessed before and after cooking for 20 min at 180 °C in a preheated oven, while the last descriptor was evaluated only after cooking. Each sensory attribute was scored on a three-point hedonic scale corresponding to: 1 = fresh; 2 = marginal; and 3 = spoiled. Score of 1.5 was characterized as semi-fresh and it was the first indication of meat spoilage. Odour characteristics of beef fillets, as determined by special samples kept frozen and thawed prior to each sensory evaluation, were considered as fresh. Putrid, sweet, sour, or cheesy odours were regarded as indicative of microbial spoilage and classified the samples as spoiled. Bright colours typical of fresh oxygenated meat were considered fresh, whereas a persistent dull or unusual colour rendered the sample spoiled. Overall, 74 meat samples were assessed by the sensory panel and classified into the selected three groups as fresh (n = 24), semi-fresh (n = 16), and spoiled (n = 34).

2.4. FTIR/ATR spectroscopy

Meat samples were analyzed in parallel to the microbiological and sensory analyses. FTIR spectra were collected using a ZnSe 45° ATR (Attenuated Total Reflectance) crystal on a Nicolet 6700 FTIR Spectrometer equipped with a DLaTGS (deuterated L-alanine doped triglycene sulphate) Detector with KBr beamspliter. The samples were placed on the ZnSe ATR crystal so that the aerobic upper surface of the meat was in intimate contact with the crystal, and then pressed with the machine's gripper in order to obtain the best possible contact with the crystal. The ZnSe ATR crystal was capable of 12 external reflections, with the evanescent field effecting a depth of 1.01 μm. The spectrometer was controlled by Omnic Software-version 7.3 to collect spectra over the wavenumber range of 4000–400 cm^{-1}, by accumulating 100 scans with a

resolution of 4 cm^{-1}. The collection time for each sample spectrum was 2 min. Each sample was analyzed in duplicate and results are displayed as mean value of both measurements. Reference spectra were acquired by collecting a spectrum from the cleaned blank crystal prior to the presentation of each sample replicate. At the end of each sampling, the crystal surface was cleaned with detergent, washed with distilled water, dried with lint-free tissue, cleaned with ethanol and finally dried with lint-free tissue at the end of each sampling interval.

2.5. Pre-treatment of the data and neural network development

The FTIR spectra collected between 1800 and 1000 cm^{-1} were initially submitted to smoothing based on the Savitzky-Golay algorithm. Subsequently, mean-centred and standardized spectral data were subjected to principal components analysis (PCA). The PCA is an unsupervised method that transforms a large number of potentially correlated factors into a small number of orthogonal (uncorrelated) factors (i.e. principal components), reducing thus the size of the initial dataset and optimizing the feature vector [27]. Since the raw spectral data could not be used because of the strong correlation among the variables (wavenumbers), the uncorrelated principal components from PCA analysis were employed for this purpose. The variables (wavenumbers), for which the communality values of the first three PCs were higher or equal to 0.6 were considered as significantly explaining the variance of the spectral data, and hence they were considered as potential wavenumbers associated with the biochemical changes during meat spoilage. The wavenumbers that were selected from the first PCA to be significant in this data set ranged from 1718 to 1203 cm^{-1} and 1020 to 1001 cm^{-1} and were selected for further analyses. A second PCA with the selected variables (wavenumbers) revealed the Principal Components (PCs) that significantly contributed to the variance of the data set. In our case, the total variance (100%) of the data set could be explained by 37 principal components (PCs) from which the first five were extracted and used as input to the developed neural network, accounting for 98.08% of cumulative variance observed in the experiment (data not shown).

A multilayer perceptron (MLP) network based on backpropagation was developed to determine the applicability of neural networks as a meat quality classifier. The network consisted of an input layer with seven input nodes for storage temperature, sampling time, and the five principal components (Fig. 1). The output layer consisted of two nodes, one for the quality class (fresh, semi-fresh, spoiled), and another for the predicted total viable counts of the meat sample. The class membership of a single sample pattern was coded in a numerical format by assigning 1 for "fresh" samples, 2 for "semi-fresh", and 3 for "spoiled" samples with a cut-off value of 0.5. In order to keep the neural network as simple as possible one hidden layer was selected with a varying number of neurons. The network configuration was approached empirically by testing different possibilities (i.e. neurons in the hidden layer, learning rate, and momentum) and selecting the one that provided the best classification accuracy. In a fully interconnected network, all neurons in the hidden layer are connected to all neurons in the input and output layers, but no connections are allowed between neurons in one layer or from one neuron to itself, or directly between input and output layer neurons. The hidden neurons are in fact the elements in a neural network that provide high degree of nonlinearity (24). In these networks each node receives signals through connections with other nodes or the outside world in the case of the input layer. The net input to node j has the form:

$$I_j = \sum_{i=1}^{n} w_{ij} \cdot x_i + \theta_j \quad (1)$$

where x_i are the inputs, w_{ij} are connection weights associated with each input/node and θ_j is the bias associated with node j. The output from each node is used as an input in a nonlinear transfer function:

$$O_j = f(I_j) \quad (2)$$

The most commonly used transfer functions are sigmoidal, hyperbolic tangent and linear function. In our work the sigmoidal and hyperbolic tangent were selected as transfer functions in both hidden and output layers. All inputs were normalized in the range from 0.1 to 0.9 and −0.9 to +0.9 for sigmoidal and hyperbolic tangent functions, respectively, to avoid saturation problems in their performance due to different value ranges of the inputs.

The standard backpropagation algorithm for network training is based on the steepest-descent gradient approach applied to the minimization of the error function defined as:

$$E = \frac{1}{2}\sum_{s=1}^{3}(d_{qs} - y_{out,s})^2 \quad (3)$$

where d_q represents the desired network output for the q^{th} input pattern in the s network layer and y_{out} is the network output. The generalized delta rule was applied for adjusting the weights of the feedforward networks in order to minimize Eq. (3). The rule for adjusting weights was given by the following equation:

$$w_{ij}^s(t+1) = w_{ij}^s(t) + \eta\delta_j^s y_j^s + \alpha\Delta w_{ij}^s(t) \quad (4)$$

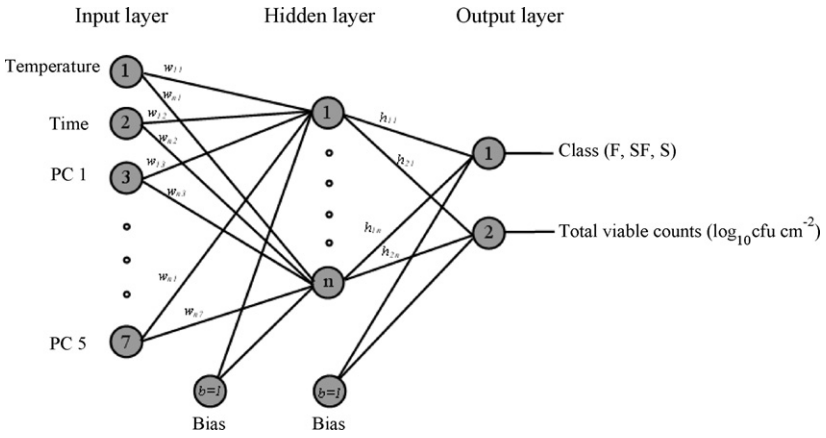

Fig. 1. Schematic structure of the developed neural network. The input layer contains the incoming signals of the network corresponding to storage temperature, time, and the values of the five principal components. The output layer contains two nodes, one for the predicted quality class (fresh, semi-fresh, spoiled) of meat samples and one for total viable counts. w_{ij}: synaptic weights with i being the index of the input signal neuron and j being the output signal neuron; b: bias term.

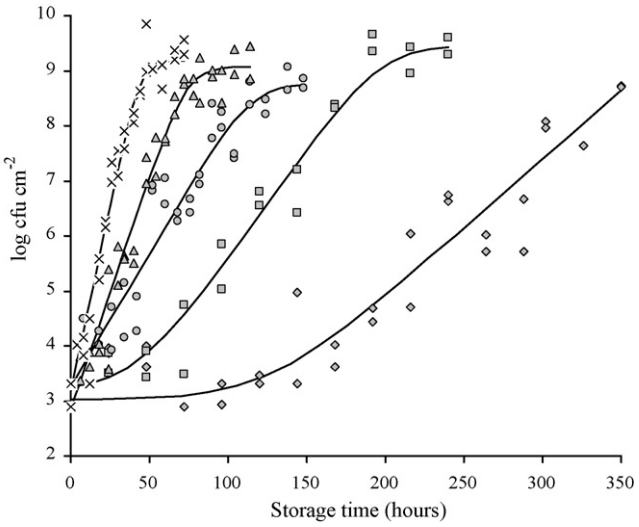

Fig. 2. Changes of total viable counts (TVC) obtained from beef fillets stored under aerobic conditions at 0 °C (♦), 5 °C (■), 10 °C (●), 15 °C (▲), and 20 °C (×). Data points are values from duplicate meat samples after incubation at 30 °C for 48 h. Lines represent growth curves fitted with the Baranyi primary model.

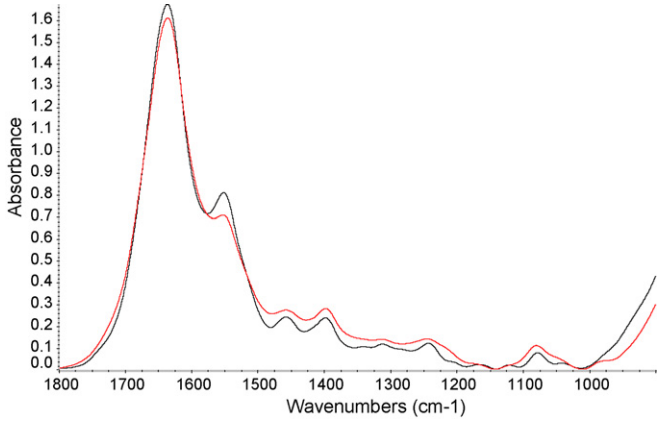

Fig. 3. Typical FTIR spectra in the range of 1800–1000 cm^{-1} collected from fresh (black line) and spoiled (red line) beef fillets stored at 5 °C for 10 days.

where η is the learning rate parameter, α the momentum term, and δ the negative derivative of the total square error with respect to the neuron's output.

The entire database consisted of 74 meat spectral patterns corresponding to different storage temperatures and sampling times. As the number of observations was small, separation of the dataset into training and testing subsets (hold-out method) would further reduce the number of data and would result in insufficient training of the network. Therefore, in order to improve the robustness of classification, the leave-1-out cross validation technique was employed to evaluate the performance of the developed network. The classification accuracy of the MLP network was determined by the number of correctly classified samples in each sensory class divided by the total number of samples in the class. The performance of the neural network in the prediction of total viable counts for each meat sample analyzed was determined by the bias (B_f) and accuracy (A_f) factors [28], the mean relative percentage residual (MRPE) and the mean absolute percentage residual (MAPR) [29], and finally by the root mean squared error (RMSE) and the standard error of prediction (SEP) [30]. The MLP network was developed in MATLAB version 7.0 code (Mathworks, Inc., Massachusetts, USA).

3. Results

The population dynamics of total viable counts (TVC) during beef fillet storage at different temperatures is presented in Fig. 2, whereas the estimated kinetic parameters after fitting with the primary model of Baranyi and Roberts are shown in Table 1. Lag phase was observed only at 0 and 5 °C, while a progressive increase of maximum specific growth rate (μ_{max}) values with storage temperature was evident. The aerobic plate counts of meat samples indicated that the total microflora ranged from 2.9 to 3.3 $\log_{10}$ cfu cm^{-2} at the onset of storage (fresh samples) to 8.7–9.4 $\log_{10}$ cfu cm^{-2} for samples characterised as spoiled.

Typical FTIR spectral data in the range of 1800–1000 cm^{-1} collected from fresh and spoiled beef fillet samples stored at 5 °C for 10 days are shown in Fig. 3. These spectra can be employed to obtain meabolic snapshots (fingerprints) of beef fillets during storage at different temperatures in an attempt to monitor meat spoilage. The temperature of 5 °C was chosen as a typical chill storage temperature for meat. The comparison of FTIR spectra could provide information on certain biochemical changes occurring during meat spoilage. Hence, based in Fig. 2, a major peak at 1640 cm^{-1} due to the presence of moisture (O–H stretch) with an underlying contribution from amide I in the meat sample was apparent, whereas a second peak at 1550 cm^{-1} appeared due to the absorbance of amide II (N–H bend, C–N stretch). A second amide vibration was shown at 1400 cm^{-1} (C–N stretch), followed by amide III peaks at 1315 and at 1240 (C–N stretch, N–H bend, C–O stretch, O=C–N bend). The peaks at 1460, 1240 and 1175 cm^{-1} can be attributed to fat (C=O ester). Finally, the peaks arising from 1025 to 1140 could be absorbance due to amines (C–N stretch) [4,5,13,31,32].

An MLP neural network based on back propagation was used to classify beef fillet samples into three sensorial categories (fresh, semi-fresh, spoiled) from the metabolic fingerprints of FTIR spectral data after dimensionality reduction with principal components analysis. The classification performance of the MLP network with variable number of neurons in the hidden layer and different transfer functions (logistic sigmoid and hyperbolic tangent) is presented

Table 1
Estimated kinetic parameters of total viable counts (TVC) by the Baranyi model as a function of storage temperature (initial counts 3.10 ± 0.30 $\log_{10}$ cfu cm^{-2}).

Temperature (°C)	μ_{max} (h^{-1})[a]	Lag phase (h)	y_0 ($\log_{10}$ cfu cm^{-2})[b]	y_{end} ($\log_{10}$ cfu cm^{-2})[b]		Standard error of fit	R^2
				Observed	Predicted		
0	0.057	125.2	3.03	8.71[c]	–[d]	0.445	0.953
5	0.091	42.5	3.26	9.44	9.47	0.384	0.974
10	0.111	–[e]	3.26	8.77	8.78	0.522	0.924
15	0.194	–	2.87	9.15	9.07	0.338	0.975
20	0.312	–	3.17	9.42	9.18	0.278	0.982

[a] Maximum specific growth rate.
[b] Initial and final total viable counts estimated by the Baranyi model.
[c] Mean value from two independent experiments.
[d] Not computed as fitted curve presented no upper asymptote.
[e] Not observed.

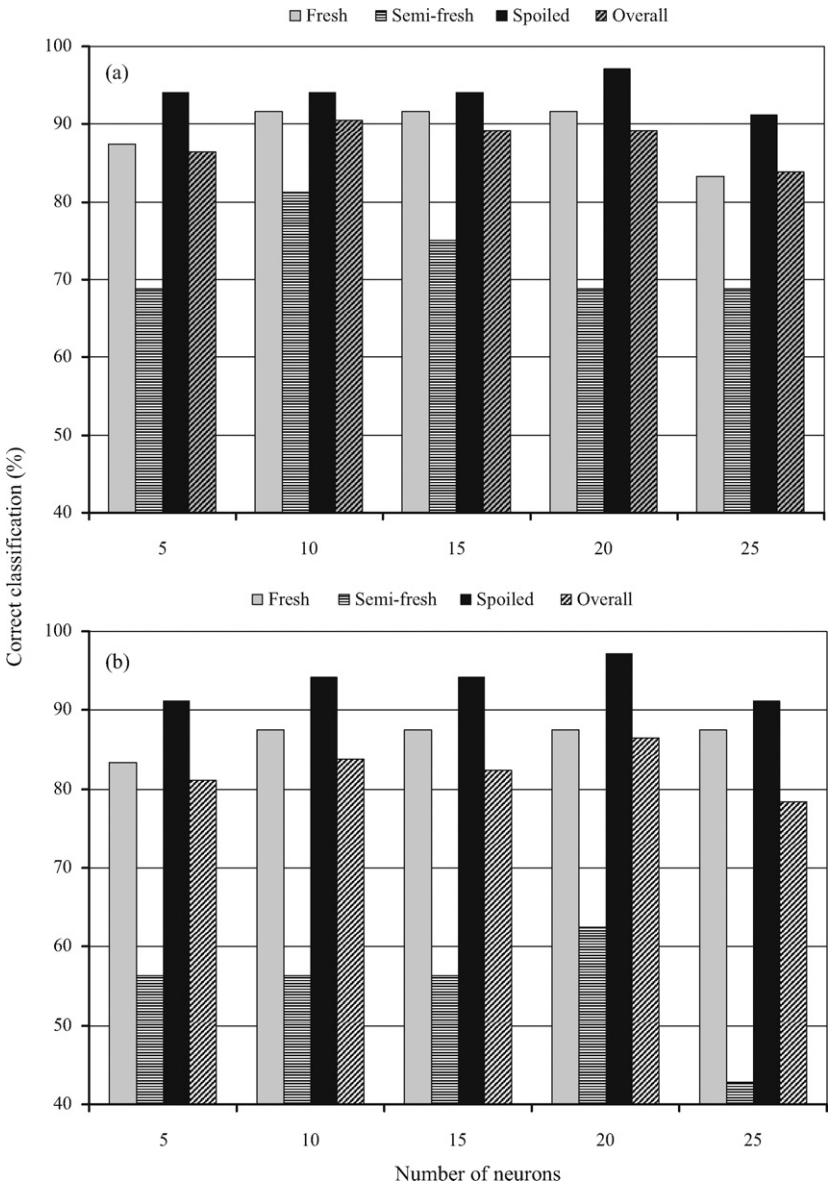

Fig. 4. Classification performance of neural networks with variable number of neurons in the hidden layer according to logistic sigmoid (a) and hyperbolic tangent activation transfer functions.

in Fig. 4. The learning rate ($n = 0.10$) and momentum ($\alpha = 0.20$) parameters were selected to ensure that the convergence of the learning process was achieved. Generally, the classification performance of the network obtained for the meat samples stored at different temperatures and cross-validated with leave-1-out method was lower when the selected transfer function was hyperbolic tangent despite the fact that the algorithm converged faster. The highest overall correct classification with hyperbolic tangent transfer function (86.5%) was obtained with 20 neurons in the hidden layer (Fig. 4b), however within the individual classes performance was low, especially for semi-fresh meat samples (62.5%). The best performance of the classifier was obtained with 10 neurons in the hidden layer and a logistic sigmoid transfer function (Fig. 4a) providing a 90.5% overall correct classification, which within the selected classes corresponded to 91.7%, 94.1%, and 81.3% for fresh, spoiled, and semi-fresh meat samples, respectively. The classifica-

Table 2
Confusion matrix of the 7-10-2 MLP classifier performing the task of discrimination of meat samples based on the leave-1-out cross validation method.

True class	Predicted class			Row total (n_i)	Sensitivity (%)
	Fresh	Semi-fresh	Spoiled		
Fresh ($n = 24$)	22	2	0	24	91.7
Semi-fresh ($n = 16$)	1	13	2	16	81.2
Spoiled ($n = 34$)	0	2	32	34	94.1
Column total (n_j)	23	17	34	74	
Specificity (%)	95.6	76.5	94.1		

Overall correct classification (accuracy): 90.5%.

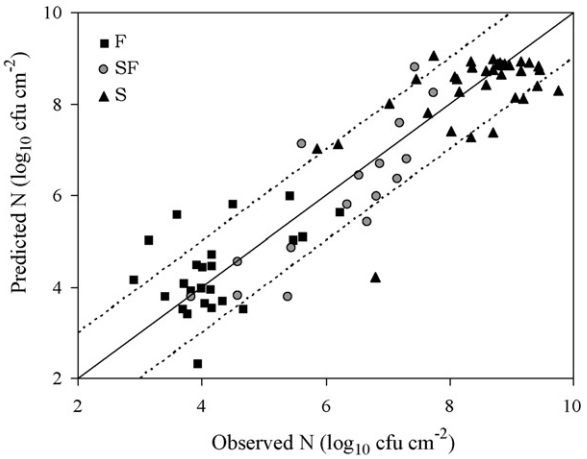

Fig. 5. Comparison of total viable counts (TVC) of beef fillets generated by the ANN model against experimentally observed values during storage at aerobic conditions (F: fresh; SF: semi-fresh; S: spoiled meat samples).

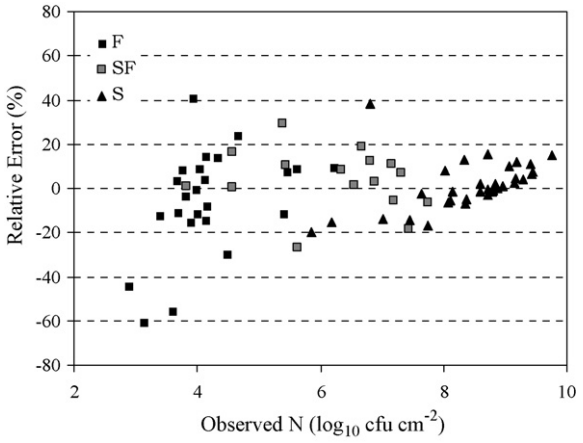

Fig. 6. Percent relative errors between observed and predicted by the neural network total viable counts (TVC) during storage of beef fillets at aerobic conditions (F: fresh; SF: semi-fresh; S: spoiled meat samples).

tion accuracies obtained from this network, designated as 7-10-2, are presented in the form of a confusion matrix in Table 2. The sensitivities (i.e. how good the network is at correctly identifying the positive samples) for fresh and spoiled meat samples were 91.7% and 94.1%, respectively, representing 2 misclassifications out of 24 fresh meat samples, and also 2 misclassifications out of 34 spoiled samples. In the case of semi-fresh samples the respective figure was somehow lower (81.2%). In this case 3 samples out of 16 were misclassified, 1 as fresh and 2 as spoiled. The specificity index (i.e. how good the network is at correctly identifying the negative samples) was also high especially in fresh and spoiled samples, indicating satisfactory discrimination between these two classes (Table 2).

The plot of predicted versus observed total viable counts (Fig. 5) showed reasonably good distribution around the line of equity ($y = x$), with the majority of data (*ca.* 78%) included within the ±1 log unit area, although some over-prediction was evident in the case of fresh meat samples, especially with low observed initial counts. The performance of the MLP network is also presented in Fig. 6 where the % relative error of prediction is depicted against the observed microbial population. Based on this plot, data were almost equally distributed above and below 0, with approximately 88% of predicted microbial counts included within the ±20% RE zone. It needs to be emphasized though that the network over-estimated the bacterial population for certain fresh samples, especially at lower observed microbial counts, corresponding to low temperature (0 °C) and short storage time. The performance of the MLP network to predict total viable counts in meat samples in terms of statistical indices is presented in Table 3. Based on the calculated values of the bias factor (B_f) it can be inferred that the network under-estimated total viable counts in semi-fresh and spoiled samples ($B_f < 1$), whereas for fresh samples over-estimation of microbial population was evident ($B_f > 1$). In addition, the values of the accuracy factor (A_f) indicated that the predicted total viable counts were 18.1%, 12.2%, and 8.4% different (either above or below) from the observed values for fresh, semi-fresh, and spoiled meat samples, respectively. The mean relative percentage residual index (MRPR) also confirmed the under-prediction for semi-fresh and spoiled samples (MRPR > 0) and over-prediction for fresh samples (MRPR < 0), whereas the values of mean absolute percentage residual (MAPR), representing the average deviation between observed and predicted counts, verified the information provided by the accuracy factor. The standard error of prediction (SEP) index is a relative typical deviation of the mean prediction values and expresses the expected average error associated with future predictions. The lower the value of this index is, the better the capability of the network to predict microbial counts in new meat samples. The value of the index was less than 10% in spoiled samples indicating good performance of the network for microbial count predictions in this class (Table 3). Comparable results were observed for semi-fresh samples (SEP 13.6%), but for fresh samples the index gave higher values as the network over-estimated microbial counts for some fresh samples, particularly those stored at 0 °C and for short storage time (Fig. 5).

4. Discussion

A major challenge facing the meat industry today is to obtain reliable information on meat quality throughout the production, distribution, and storage chains, and turn this information into decision support systems which would ultimately provide a guaranteed quality of meat products for consumers [1].

Table 3
Performance of the 7-10-2 MLP classifier for the prediction of total viable counts in meat samples (fresh, semi-fresh, spoiled, overall) analyzed by FTIR.

Statistical index	Mathematical expression	Fresh	Semi-fresh	Spoiled	Overall
Bias factor (B_f)	$10^{\sum \log(P/O)/n}$	1.031	0.951	0.982	0.991
Accuracy factor (A_f)	$10^{\sum \lvert\log(P/O)\rvert/n}$	1.181	1.122	1.084	1.123
Mean relative percentage residual (MRPR%)	$\frac{1}{n}\cdot\sum\frac{100\cdot(O-P)}{O}$	−5.572	3.971	1.082	−0.451
Mean absolute percentage residual (MAPR%)	$\frac{1}{n}\cdot\sum\frac{100\cdot\lvert(O-P)\rvert}{O}$	17.564	11.078	7.869	11.708
Root mean squared error (RMSE)	$\sqrt{\frac{\sum(O-P)^2}{n}}$	0.872	0.846	0.835	0.850
Standard error of prediction (SEP%)	$\frac{100}{\bar{O}}\cdot\sqrt{\frac{\sum(O-P)^2}{n}}$	20.861	13.622	9.917	12.937

The metabolomic concept in food microbiology which has been introduced recently [14] improved the concept of using single biochemical indicators as proposed in late 80s and 90s [3,33–36]. Chemometrics (e.g. principal components analysis – PCA, hierarchical cluster analysis – HCA, discriminant function analysis – DFA, partial least square regression – PLSR), in parallel with machine learning approaches based on soft computing (e.g. artificial neural networks – ANN, genetic algorithms, support vector machines – SVM) have been applied as data mining techniques in bioprocess data [16,37]. These approaches could rapidly provide information related to the contribution of the ephemeral spoilage organisms (ESO) in meat or to the categorization of meat with regard to (i) type of meat and (ii) spoilage [3,15,38,39]. Ellis et al. [4,5] have been the pioneers stipulating that FTIR spectroscopy can be used directly on the surface of food to produce biochemical interpretable "fingerprints" (metabolic snapshots), enabling thus early detection of microbial spoilage of chicken breast and beef rump steaks.

In this work, FTIR spectroscopy was employed to obtain metabolic fingerprints of beef fillets during storage in aerobic conditions at five different storage temperatures (0, 5, 10, 15, and 20 °C). A machine learning approach was then followed to develop a pattern recogniser based on a simple multilayer perceptron (MLP) neural network, in an attempt to classify meat samples in three quality classes (fresh, semi-fresh, spoiled) as judged previously by a taste panel. The classification performance of the MLP network was very good for fresh and spoiled samples with correct classification rates exceeding 91% (Table 2) after leave-1-out cross validation of the dataset. It is characteristic that no fresh samples was misclassified as spoiled and vice versa, indicating that the biochemical fingerprints provided by FTIR spectral data could discriminate these two classes quite accurately. Lower percentages were obtained for semi-fresh samples (*ca.* 81%) with erroneous classifications in the other two classes. It must be emphasized however that the number of examined samples within each class was not equally distributed, due to the different spoilage rate of beef samples at the different temperatures assayed (Table 2). This may have affected the learning process of the neural network, which is basically a data driven approach [40], and thus could account for the lower classification accuracies observed for this class. It is also worth noting that the logistic sigmoid transfer function employed in the neurons of the hidden layer gave higher classification accuracies compared with the hyperbolic tangent transfer function (Fig. 4), despite the fact that the latter results in faster convergence of the training algorithm [24]. It is worth noting that initially two independent neural networks were developed for the prediction of either quality class or TVC counts, with lower prediction accuracies each (data not shown). Moreover, as both output parameters are not independent, in the sense that quality class is related to microbiological counts and vice versa, a network that would combine both outputs would be more efficient. The relatively lower accuracies obtained in the semi-fresh class could also be attributed to the performance of the sensory evaluation process, as the difference between "fresh" and "semi-fresh" class is sometimes not very clear. So further improvement on prediction could be based on better training of sensory evaluation panels in combination with the development of an improved/standardised protocol for meat assessment.

The application of machine learning to correlate FTIR spectral data with meat spoilage is not new and it has been tackled in the past [2,4,5]. However, in these works, the focus was given on the rapid detection of bacterial spoilage, in terms of microbiological analyses, whereas no attempt was made to correlate spectral data with quality classes defined by sensory assessment of the samples. In addition, spoilage was monitored in only one storage temperature (room temperature), whereas in our work five different storage temperatures have been assayed (0, 5, 10, 15 and 20 °C). In this way spoilage has been monitored not only at abuse temperatures but also at chill temperatures. Concerning TVC counts indicating beef spoilage it was found by sensory evaluation that the respective values ranged from 7.0 to 8.2 $\log_{10}$ cfu cm^{-2}, depending on storage temperature. In a previous work undertaken in our lab [13], FTIR snapshots were taken into account for the characterization of minced beef samples into the same quality classes using linear discriminant function analysis (DFA) analysis. Results showed that the classification accuracies of the MLP classifier were better compared with DFA in the characterization of meat samples, indicating the advantage of ANN approach in tackling complex, nonlinear problems as meat spoilage.

Another challenge from the microbiological perspective would be the implementation of machine learning approaches to correlate FTIR spectral data to bacterial counts on meat samples. As reported in previous works [5], spectra collected from the surface of beef contained biochemical information that could be correlated with the spoilage status of the samples. In this way, expensive and time-consuming microbiological analysis could be replaced in the long term by an on-line system based on spectroscopic data, providing rapid, non-invasive, and low cost microbiological analyses [4,7]. To investigate this issue, the MLP classifier was designed with two nodes in the output layer, one corresponding to the sensorial class of beef fillets, and another one for the prediction of microbial counts for each sampling time and storage temperature, based on TVC measurements. The comparison of observed and predicted bacterial counts, based on calculated statistical induces and plots (Figs. 5 and 6, Table 3), presented reasonably good agreement, showing that the developed neural network approach could be used effectively to assess the spoilage condition of beef fillets. The plots of the estimates versus observed bacterial counts were within *ca.* 1 $\log_{10}$ cfu cm^{-2} from the line of equity, which is comparable with a value of *ca.* 0.5 $\log_{10}$ cfu cm^{-2} for beef steaks reported previously [5]. These results were also confirmed by the percent relative error index (%RE) between observed and predicted values (Fig. 6) with the exception of three samples corresponding to fresh beef fillets with low initial counts. The calculated validation indices showed acceptable performance of the developed neural network in predicting total viable counts of beef samples directly from FTIR spectral data. The values of the bias factor (B_f) were close to unity indicating good agreement between predictions and observations when the three quality classes were taken together (Table 3). However, within classes, underestimation was evident for spoiled and semi-fresh samples, while TVC for fresh samples were over-estimated. However, the calculated values B_f are within the range of 0.9–1.0 or 1.0–1.05 which are considered adequate [41], whereas other authors have accepted B_f values of between 0.75 and 1.25 as being acceptable for spoilage microorganisms [42]. Generally, the highest prediction accuracy of the neural network was observed in the case of spoiled samples as this class presented the lowest values of indices compared to the other two classes. Concerning the values of the accuracy factor (A_f) it has been reported [43] that an increase of 0.15 (15%) would be acceptable for each independent variable included in model development. Therefore, in our study, with only one independent variable (temperature) we would expect A_f up to 1.15, which is in good agreement with the calculated values for the three classes and the overall model as well (Table 3). The mean relative percentage residual and the mean absolute percentage residual are statistics similar to the bias and accuracy factors [44] which provided similar information as the other two indices about the performance of the neural network.

5. Conclusion

In conclusion, these data demonstrate the utility of the analytical approach based on FTIR spectroscopy which in combination with an appropriate machine learning strategy (artificial neural

networks) could become an effective tool for monitoring beef fillets spoilage during aerobic storage at chill and abuse temperatures. The collected spectra could be considered as biochemical fingerprints containing valuable information for the discrimination of meat samples in quality classes corresponding to different spoilage levels, and also could be used to predict satisfactorily the microbial load directly from the sample surface.

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A comparison of artificial neural networks and partial least squares modelling for the rapid detection of the microbial spoilage of beef fillets based on Fourier transform infrared spectral fingerprints

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ABSTRACT

A series of partial least squares (PLS) models were employed to correlate spectral data from FTIR analysis with beef fillet spoilage during aerobic storage at different temperatures (0, 5, 10, 15, and 20 °C) using the dataset presented by Argyri et al. (2010). The performance of the PLS models was compared with a three-layer feed-forward artificial neural network (ANN) developed using the same dataset. FTIR spectra were collected from the surface of meat samples in parallel with microbiological analyses to enumerate total viable counts. Sensory evaluation was based on a three-point hedonic scale classifying meat samples as fresh, semi-fresh, and spoiled. The purpose of the modelling approach employed in this work was to classify beef samples in the respective quality class as well as to predict their total viable counts directly from FTIR spectra. The results obtained demonstrated that both approaches showed good performance in discriminating meat samples in one of the three predefined sensory classes. The PLS classification models showed performances ranging from 72.0 to 98.2% using the training dataset, and from 63.1 to 94.7% using independent testing dataset. The ANN classification model performed equally well in discriminating meat samples, with correct classification rates from 98.2 to 100% and 63.1 to 73.7% in the train and test sessions, respectively. PLS and ANN approaches were also applied to create models for the prediction of microbial counts. The performance of these was based on graphical plots and statistical indices (bias factor, accuracy factor, root mean square error). Furthermore, results demonstrated reasonably good correlation of total viable counts on meat surface with FTIR spectral data with PLS models presenting better performance indices compared to ANN.

1. Introduction

One of the most commonly consumed food commodities on a global basis is meat, due to its high nutritional value in the human diet. In the USA alone the retail market of beef industry amounted to $76 billion in 2008 with an overall consumption of approximately 27.3 billion pounds in that year (USDA, 2008). During meat production/processing quality assurance is difficult due to the heterogeneous nature of the raw material, since the chemical composition, technological and sensory attributes are highly influenced by pre-slaughter (e.g., breed, age, environment) intrinsic (e.g., pH, available nutrients) and extrinsic (e.g., storage method, period and temperature of storage) factors (Damez and Clerjon, 2008; Nychas et al., 2008; Prieto et al., 2009). Consequently, in order to keep the quality standards as close as possible to the preference of the consumer, control procedures must be undertaken including sensory, microbiological and physico-chemical analysis. Today, more than 50 such methods have been employed for the characterization of microbiologically spoiled or contaminated meat (Ellis and Goodacre, 2001; Nychas et al., 2008). However, these methods suffer certain disadvantages as they are time-consuming, destructive, require highly trained personnel, provide retrospective information, and hence they are unsuitable for online monitoring (Dainty, 1996; Nychas et al., 1998, 2008; Ellis et al., 2002, 2004; Liu et al., 2004).

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Nowadays, various rapid, non-invasive methods based on analytical instrumental techniques, such as Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, near infrared spectroscopy, and electronic nose technology are being researched for their potential as reliable meat quality sensors (Ellis et al., 2005; Rajamäki et al., 2006; Damez and Clerjon, 2008; Ammor et al., 2009; Argyri et al., 2010; Balasubramanian et al., 2009; Prieto et al., 2009). The principle underlying this approach is based on the assumption that the metabolic activity of microorganisms on meat results in biochemical changes with the concurrent formation of metabolic by-products which may indicate or may contribute to spoilage. The quantification of these metabolites constitutes a characteristic fingerprint providing information about the type and rate of spoilage (Ellis and Goodacre, 2001; Nychas et al., 2008).

The introduction of converging technologies in the food industry is among the priorities of the 7th Framework Programme and they are anticipated to predominate in the future and result in substantial changes in the manner in which researchers design their research (Hair et al., 1998; NBIC report USA, 2002). This can be achieved thorough the integration of modern analytical and high throughput platforms with computational and chemometric techniques. Multivariate statistical analyses (e.g., partial least square regression, discriminant function analysis, cluster analysis) and intelligent methodologies (e.g., artificial neural networks), can result in the development of a decision support system for timely determination of safety/quality of meat products, and also prevent unnecessary economic losses (Mataragas et al., 2007; Nychas et al., 2008; Guillén et al., 2010). Furthermore, the development of computational research platforms and online experimental databases such as Combase (Baranyi and Tamplin, 2004) and Sym'Previus (Leporq et al., 2005), provide research scientists with fast and efficient means of storing and exchanging knowledge despite their geographic distribution.

Partial least squares discriminant analysis (PLS-DA) and artificial neural networks (ANNs) are widely employed modelling approaches due to their ability to relate the input and output variables without having any prior knowledge on the system under study, provided that an accurate and adequate amount of data on the system variables is available (Singh et al., 2009). Compared to other areas, the application of ANNs in the field of food science is still in the early development stage. Nevertheless, interest in using ANNs as secondary models in food microbiology is increasing as they have shown promising results in several applications such as growth parameter estimation of microorganisms (Geeraerd et al., 1998; Hervás et al., 2001; García-Gimeno et al., 2005), bacterial heat resistance (Lou and Nakai, 2001; Esnoz et al., 2006), production of metabolites (Poirazi et al., 2007), and simulation of survival curves (Palanichamy et al., 2008; Panagou, 2008). The multi-layer perceptron (MLP) is the most frequently used type of neural network in practical applications (Siripatrawan et al., 2006). The basic structure is comprised of three distinctive layers, the input layer where the data are introduced to the model and computation of the weighted sum of the input is performed, the hidden layer or layers where data processing takes place, and the output layer where the results of the neural network are produced (Bishop, 2004; Huang et al., 2007).

The purpose of the present study was to compare the performance of a multi-layer perceptron (MLP) neural network and partial least squares (PLS) regression models in order to (i) classify beef fillets stored aerobically at different temperatures (0, 5, 10, 15, and 20 °C) in terms of quality classes (i.e., fresh, semi-fresh, spoiled), and (ii) predict the total viable counts on the surface of meat samples directly from FTIR data.

2. Materials and methods

2.1. Experimental design

A detailed description of the methodology employed in this work is presented elsewhere (Argyri et al., 2010). In brief, fresh deboned pieces of beef were purchased from a local butcher shop and transported under refrigeration to the laboratory within 30 min. The samples were prepared by cutting meat pieces into portions (40 mm wide × 50 mm long × 10 mm thick) that were subsequently placed into 90 mm Petri dishes and stored at 0, 5, 10, 15, and 20 °C in high-precision (±0.5 °C) incubation chambers until spoilage was evident.

For the FTIR measurements, a thin slice (0.5 cm thickness) of the aerobic upper surface of the fillet was excised and used for further analysis. Spectra were collected using a ZnSe 45° ATR (Attenuated Total Reflectance) crystal on a Nicolet 6700 FTIR Spectrometer, collecting spectra over the wavenumber range of 4000 to 400 cm^{-1}, by accumulating 100 scans with a resolution of 4 cm^{-1}. The collection time for each sample spectrum was 2 min. Spectra collected between 1800 and 1000 cm^{-1} were initially subjected to smoothing according to the Savitzky–Golay algorithm prior to further analysis.

For microbiological analysis a portion (40 mm wide × 50 mm long × 10 mm thick) was added to 150 ml sterile quarter strength Ringer's solution, and homogenized in a stomacher for 60 s at room temperature. Further decimal dilutions were prepared with the same diluent, and duplicate 0.1 ml samples of three appropriate dilutions were spread in triplicate on plate count agar for counts of total viable bacteria, incubated at 30 °C for 48 h.

Sensory evaluation of meat samples was performed during storage, based on the perception of colour and smell before and after cooking (20 min at 180 °C in preheated oven) (Gill and Jeremiah, 1991). Each sensory attribute was scored on a three-point hedonic scale corresponding to: 1 = Fresh; 2 = Marginal; and 3 = Spoiled. Score of 1.5 was characterized as Semi-fresh and it was considered as the early detection of meat spoilage. Overall, 76 meat samples were evaluated by the sensory panel and classified into the selected groups as fresh ($n = 26$), semi-fresh ($n = 16$), and spoiled ($n = 34$).

2.2. Partial least squares (PLS) modelling

The partial least squares regression (PLS-R) derives its usefulness from its ability to analyze data with strongly collinear, noisy and numerous variables in the predictor matrix X (i.e., independent variables) and responses Y (i.e., dependent variables) (Eriksson et al., 2001). The PLS-R method projects the initial input-output data down into a latent space, extracting a number of principal factors (also known as latent variables) with an orthogonal structure, while capturing most of the variance in the original data. In brief, it can be expressed as a bilinear decomposition of both X and Y as:

$$X = \boldsymbol{TW}^T + E_X \quad (1)$$

$$Y = \boldsymbol{UQ}^T + E_Y \quad (2)$$

Therefore, the scores in the X-matrix and the scores of the yet unexplained part of Y have maximum covariance. In equations (1) and (2), $\boldsymbol{T}$ and $\boldsymbol{W}$, $\boldsymbol{U}$ and $\boldsymbol{Q}$ are the vectors of X and Y PLS scores and loadings, respectively, and E_X, E_Y are the X and Y residuals (Singh et al., 2009). The aim of PLS method is to find a linear (or polynomial) relationship between X and Y matrices, so that:

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$$Y = bX + E \quad (3)$$

where b is the regression coefficient. The PLS models are developed in two stages; the initial dataset is divided into training and testing subsets. The former dataset is used to build the models and compute a set of regression coefficients ($\boldsymbol{b}_{PLS}$), which are subsequently used to make a prediction of the dependent variable in the test subset. The initial dataset consisted of 74 beef fillet spectral patterns corresponding to different storage temperatures (0, 5, 10, 15, and 20 °C) and storage times (up to 350 h depending on storage temperature). The database was randomly partitioned into a training and testing subset representing 75% ($n = 57$) and 25% ($n = 19$) of the data, respectively. Test data were not employed in any step of training the PLS model but they were used exclusively to determine its performance. A series of PLS models were created using a number of latent variables ranging from 1 to 25, hence 25 models were developed in total. The performance of each generated model was calculated using leave-one-out cross validation. The optimum numbers of components were used to build the final model. The resulting model was then tested with the independent dataset.

This procedure was repeated two times for predicting the predefined sensory class: *i*) based on storage time and temperature as two input variables in addition to the FTIR dataset, and *ii*) based entirely on the FTIR data where no storage condition data was included to build the models. Similarly, two sets of models were developed to predict the total viable counts (TVC), firstly based on including the storage conditions as additional input variables, and secondly based entirely on the FTIR data. Therefore four sets of models were developed in total.

2.3. Artificial neural networks modelling

Mean-centered and standardized spectral data were initially subjected to principal components analysis (PCA) for dimensionality reduction, and the variables (wavenumbers) for which communality values were less than 0.6 were excluded from further analysis, as they were considered to contain not enough information to explain the variance of spectral data. The remaining wavenumbers (from 1718 to 1203 cm^{-1} and 1020 to 1001 cm^{-1}) were subjected to a second PCA, where the total variance (100%) of the dataset was cumulatively explained by 37 principal components (PCs). The scores of the first five PCs were extracted and used in further analysis as they explained a cumulative variance of 98.08% of the dataset.

The selected network was a multi-layer perceptron (MLP) based on back propagation. The basic element in an MLP is the "neuron" that receives a set of input signals (x_i) with weight (w_i), calculates their impact using the summation function ($I = \sum x_i \cdot w_i$), and finally produces an output using some activation function ($y = f(I)$). The determination of the weights is achieved through training of the system. Normally, supervised training is performed in such a way as to minimize the difference between the network output and the measured value:

$$\mathrm{MSE} = \frac{1}{n}\sum_{i=1}^{n}\left(y_{predicted,i} - y_{observed,i}\right)^2 \quad (4)$$

where, $y_{predicted,i}$ and $y_{observed,i}$ represent the predicted and observed values of the variable, respectively, and n is the number of observations. Back propagation (BP) is the most commonly used training algorithm in neural networks, also employed in this work. It works on the principle that after the information has gone through the network in a forward direction and an output has been produced, the error associated with this output is redistributed backwards through the model and weights are adjusted accordingly. Minimization of the error occurs through several iterations (training cycles) (Ham and Kostanic, 2001).

Two separate networks were developed in this work comprising of an input layer with seven nodes, one for temperature and storage time, respectively, and the remaining five for each one of the five PCs. The output layer contained one node for the prediction of either meat quality class (i.e., F, SF, S) or total viable counts on the surface of meat samples ($\log_{10}$ cfu cm^{-2}). In addition two other similar neural networks were developed in which storage time and temperature were excluded from the input layer as dependent variables, in an attempt to investigate the performance of the network to discriminate meat samples based only on FTIR data. Therefore four neural networks were developed in total. Based on previous work (Argyri et al., 2010) the best performance of the network was obtained with 10 neurons in the hidden layer. To facilitate comparison between the two models, the database was also randomly divided into a training subset with 75% of the data, and a test subset with the remaining 25%. These data were not employed at all in the training session of the network but they were used to assess its capability to foresee for unknown cases. The MLP network was developed using NeuralTools version 1.0 (Palisade Corp., Ithaca, NY, USA).

2.4. Evaluation of model performance

The classification accuracy of the neural network and PLS model was determined by the number of correctly classified meat samples in each sensory class divided by the total number of samples in the class. The overall correct classification (accuracy, %) of the model was determined as the number of correct classifications in all classes divided by the total number of samples analyzed (Panigrahi et al., 2006). For the prediction of total viable counts (TVC) in each meat sample three performance indices were calculated, namely the bias (B_f) and accuracy (A_f) factors (Ross, 1996) and the root mean squared error (*RMSE*).

The bias factor (B_f) indicates whether, on average, the observed TVC counts are above or below the line of equity ($y = x$), and if so, by how much. The index is defined as:

$$B_f = 10^{\left(\frac{\sum \log\left(\frac{\log N(t)_{predicted}}{\log N(t)_{observed}}\right)}{n}\right)} \quad (5)$$

where n is the number of observations. A bias factor = 1 indicates a perfect model where the predictions are in full agreement with observations. Values < 1 indicate that the observed total viable counts are larger than predicted ones.

The accuracy factor is a measure of the average deviation between predictions and observations, i.e., how close predictions are to observations.

$$A_f = 10^{\left(\frac{\sum \log\left(\left|\frac{\log N(t)_{predicted}}{\log N(t)_{observed}}\right|\right)}{n}\right)} \quad (6)$$

The values of this index are ≥ 1. The larger the value the less accurate is the average estimate.

The goodness of fit of the modelling approach was also evaluated by the root mean square error (RMSE), which measures the average deviation between observed and predicted values (Ratkowsky, 2004). The smaller the value of this index the better the fit of the model to the experimental data:

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$$\mathrm{RMSE} = \sqrt{\frac{\sum\left(\log N(t)_{predicted} - \log N(t)_{observed}\right)^2}{n}} \quad (7)$$

where n is the number of observations.

3. Results

Typical FTIR spectral data from 1000 to 1800 cm^{-1} collected from beef fillets stored at 0 °C for different storage times are presented in Fig. 1. The selected spectra correspond to each one of the three quality classes (i.e., fresh, semi-fresh, spoiled) employed in this work. Based on Fig. 1, a major peak at 1640 cm^{-1} was apparent in the meat sample due to the presence of moisture (O–H stretch) with an underlying contribution from amide I, whereas a second peak at 1550 cm^{-1} appeared due to the absorbance of amide II (N–H bend, C–N stretch). A second amide vibration was observed at 1400 cm^{-1} (C–N stretch), followed by amide III peaks at 1315 and at 1240 (C–N stretch, N–H bend, C–O stretch, O=C–N bend). The peaks at 1460, 1240 and 1175 cm^{-1} can be attributed also to fat. Finally, the peaks arising from 1025 to 1140 could be absorbance due to amines (C–N stretch) (Chen et al., 1998; Ellis et al., 2002, 2004; Ammor et al., 2009; Argyri et al., 2010).

A PLS model performance evaluation was performed using leave-one-out cross validation for the prediction of sensory class of beef samples. The number of latent variables (LVs) was selected on the basis of the highest number of correctly classified samples of the testing subset. For this reason, different models were developed with the LVs ranging from 1 to 25. For each model, the number of correctly classified samples in both the training and test dataset was calculated. When the PLS models were built based entirely on the FTIR data (i.e., no storage time and temperature was included), a number of 21 LVs was finally selected presenting the highest correct classification (%) in the training (98.2%) and test (68.4%) subsets (Fig. 2, Table 1). For the training subset, the PLS approach provided 100% correct classification for fresh and semi-fresh meat samples, whereas for spoiled samples the respective number was 96.1%, representing 1 misclassification out of 26 spoiled samples (Table 1). However, for the testing subset the relative percentages were lower, which is not unusual as these data were not involved at all in model development but provided as unknown cases for prediction. Specifically, the highest correct classification was observed in spoiled (71.4%) and fresh (75%) samples, with 2 samples misclassified as semi-fresh out of 7 and 8 samples, respectively. The lowest performance was obtained in semi-fresh samples with 2 misclassifications out of 4 samples. However, the performance was slightly improved when storage time and temperature were associated with the training data prior to building the model. The best performance in this case was monitored when 20 LVs (Fig. 2), showing a performance of 94.7% on the training and 70.0% on the independent testing dataset. For the training dataset, the PLS approach provided 18 out of 20 correct classification for fresh meat samples (Table 2), whereas for semi-fresh and spoiled samples, the respective numbers were 5 and 6 misclassifications out of 15 semi-fresh and 22 spoiled samples, respectively.

Similar performance was obtained for the ANN model developed entirely on the FTIR dataset (i.e., storage time and temperature were excluded from model development as dependent variables). The obtained correct classifications were 98.2% and 63.1% for the training and test datasets, respectively (Table 1). Within each sensory class in the training dataset, the ANN model provided 100% correct discrimination for fresh and semi-fresh samples, whereas for spoiled samples there was 1 misclassification out of 27 meat samples (96.3%). However, for the test dataset the performance of the ANN was lower but still comparable with the PLS model. Specifically, the highest correct classification was obtained for the fresh and spoiled sensory class where 2 samples were misclassified as spoiled and fresh, respectively (Table 1). Less consistent results were obtained for the semi-fresh class with 3 misclassifications out of 5 samples which is quite reasonable taking into account that sensorial discrimination of this class is rather difficult and requires highly trained taste panels. The performance of the ANN model was slightly improved when storage time and temperature were included as additional inputs in model development (Table 2). The obtained results indicated that correct classification increased by approximately 2% and 10% for the training

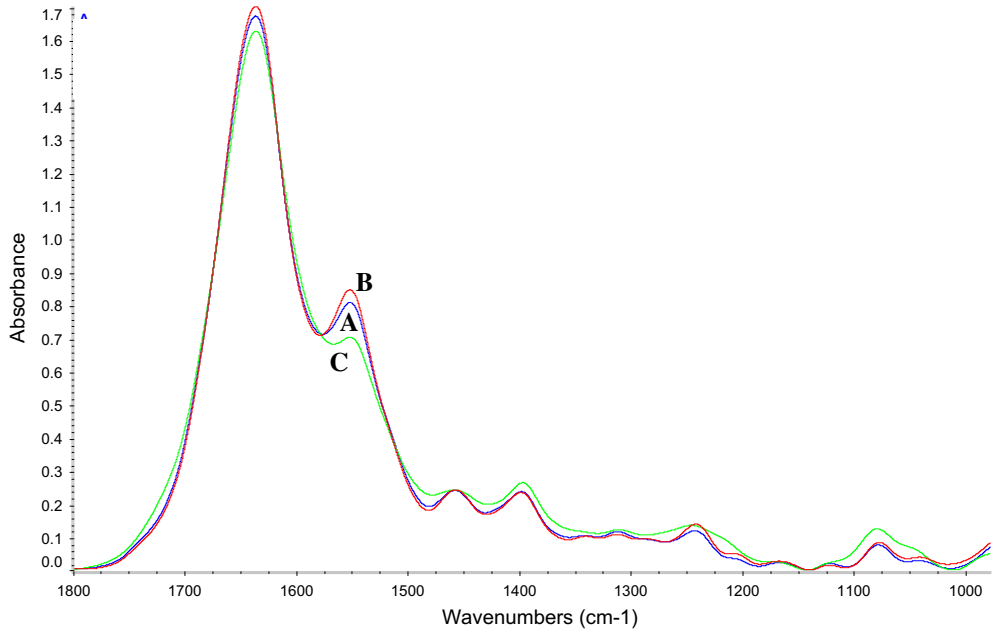

Fig. 1. Typical FTIR spectra in the range of 1800–1000 cm^{-1} collected from beef fillets stored at 0 °C at the beginning of storage (A; Fresh), after 96 h (B; Semi-fresh), and 216 h (C; Spoiled).

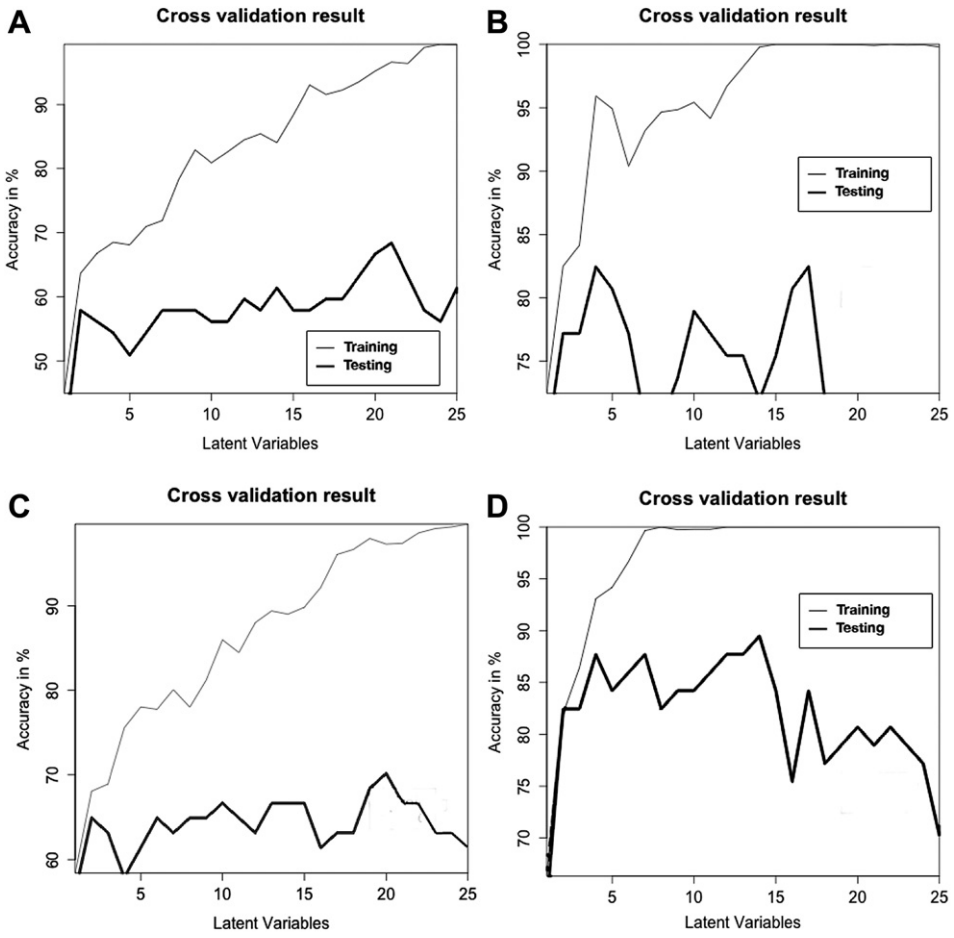

Fig. 2. Optimization of the PLS-DA classification models using latent variables ranging from 1 to 25 for the training (grey line) and test (black line) subsets after leave-one-out cross validation. (A) sensory class prediction based on FTIR data; (B) total viable counts prediction based on FTIR data; (C) sensory class prediction based on FTIR data plus storage time and temperature as additional inputs; (D) total viable counts prediction based on FTIR data plus storage time and temperature as additional inputs.

and test datasets, respectively. In this case, the ANN provided 100% correct classification for all sensory classes in the training dataset. With regard to the test dataset, classification performance was improved by approximately 14% for the spoiled meat samples, compared with the ANN model developed on FTIR data only, with 1 misclassification out of 7 samples. For fresh and semi-fresh meat samples, the calculated correct classifications were 71.4% and 60.0%, representing 2 misclassifications out of 5 semi-fresh and 7 spoiled meat samples, respectively (Table 2).

Table 1
Confusion matrix of the ANN classifier and the PLS model regarding sensory quality discrimination of beef fillets based on FTIR spectral data.

From/to	ANN training ($n = 57$)				
	Fresh	Semi-fresh	Spoiled	Total	Correct (%)
Fresh	19	0	0	19	100
Semi-fresh	0	11	0	11	100
Spoiled	1	0	26	27	96.3
	ANN testing ($n = 19$)				
Fresh	5	0	2	7	71.4
Semi-fresh	2	2	1	5	40.0
Spoiled	2	0	5	7	71.4
	PLS training ($n = 57$)				
Fresh	18	0	0	18	100
Semi-fresh	0	13	0	13	100
Spoiled	0	1	25	26	96.1
	PLS testing ($n = 19$)				
Fresh	6	2	0	8	75.0
Semi-fresh	2	2	0	4	50.0
Spoiled	0	2	5	7	71.4

Overall correct classification (accuracy) for ANN train and test datasets: 98.2% and 63.1%, respectively. Overall correct classification (accuracy) for PLS train and test datasets: 98.2% and 68.4%, respectively.

Table 2
Confusion matrix of the ANN classifier and the PLS model regarding sensory quality discrimination of beef fillets based on FTIR spectral data together with storage time and temperature as additional inputs to the models.

From/to	ANN training ($n = 57$)				
	Fresh	Semi-fresh	Spoiled	Total	Correct (%)
Fresh	19	0	0	19	100
Semi-fresh	0	11	0	11	100
Spoiled	0	0	27	27	100
	ANN testing ($n = 19$)				
Fresh	5	0	2	7	71.4
Semi-fresh	2	3	0	5	60.0
Spoiled	1	0	6	7	85.7
	PLS training ($n = 57$)				
Fresh	18	2	0	20	90.0
Semi-fresh	2	10	3	15	66.7
Spoiled	1	5	16	22	72.7
	PLS testing ($n = 19$)				
Fresh	5	1	0	6	83.4
Semi-fresh	0	1	1	2	50.0
Spoiled	2	1	8	11	72.7

Overall correct classification (accuracy) for ANN train and test datasets: 100.0% and 73.7%, respectively. Overall correct classification (accuracy) for PLS train and test datasets: 77.2% and 73.6%, respectively.

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The PLS approach was also used to associate spectral data with total viable counts (TVC) on the surface of meat samples. The model was developed on the assumption that when the difference between individual predictions and observations was higher than a threshold value of 1 log unit, then the prediction was false. When PLS was applied using only the FTIR data (i.e., no storage time and temperature was included within the input matrix), the model correctly predicted 87.7% of the training data, and 60% of the independent testing data. In the case of including the storage time and temperature within the input dataset, the model showed an increase in performance, reaching 100% and 84.2% for the training and testing, respectively.

For models developed on FTIR data only, the calculated value of the bias factor for the ANN training dataset was close to 1 indicating no systematic bias (under or overprediction) (Table 3), whereas for PLS model a slight underestimation was evident (B_f 0.967). The values of bias factor were improved when storage time and temperature were included as inputs in model development, especially for the PLS approach (Table 4). For the test datasets, underprediction ($B_f < 1$) was observed for the PLS models whereas overprediction ($B_f > 1$) was evident in ANN models, regardless of the approach employed in model development (i.e., inclusion or not of storage time and temperature as inputs). These calculations were also graphically verified by the comparison of the observed vs. predicted total viable counts (TVC) plots (Figs. 3 and 4).

Moreover, based on the calculated indices for the test datasets between ANN and PLS models that were developed on FTIR data only, it can be concluded that the PLS model presented a comparatively better performance as it yielded lower values for accuracy factor (1.321) and root mean square error (1.993) (Table 3). However, when storage time and temperature were included as input parameters to the models, then the best performance was obtained for ANN based on the comparison of the same indices (Table 4).

4. Discussion

So far the assessment of meat quality and safety is based on sensory and retrospective microbiological analyses (Nychas et al., 2008). Sensory analysis is an important and common method to evaluate quality of food commodities since the consumer is the ultimate judge of quality of a product (Lee and O'Mahony, 2005). However, the method has certain disadvantages as it relies on highly trained taste panels, a procedure which makes it costly and unattractive for daily analysis. In addition, a limited number of samples can be analysed daily due to the fatigue of the senses of the panellists. Finally, sensory evaluation has a subjective connotation, although this effect could be reduced by applying scientific protocols under carefully controlled conditions. On the other hand, microbiological analyses are laborious, time-consuming, costly and highly technical (molecular tools), as well as destructive to products analysed, requiring in most cases a complex process of sample preparation, while not able to give the 'immediate answer required' (McMeekin et al., 2007).

Table 3
Comparison of validation indices between the PLS and ANN models for total viable counts (TVC) predictions in meat samples based on FTIR spectral data.

Parameter	ANN		PLS model	
	Train	Test	Train	Test
Bias factor (B_f)	1.002	1.034	0.967	0.854
Accuracy factor (A_f)	1.291	1.390	1.090	1.321
RMSE	1.821	1.978	1.073	1.993

Table 4
Comparison of validation indices between the PLS and ANN models for total viable counts (TVC) prediction in meat samples based on FTIR spectral data together with storage time and temperature as additional inputs to the model.

Parameter	ANN		PLS model	
	Train	Test	Train	Test
Bias factor (B_f)	1.008	1.038	0.996	0.833
Accuracy factor (A_f)	1.118	1.166	1.003	1.409
RMSE	0.852	0.921	0.092	2.501

A major challenge of the meat industry in the 21st century is to obtain reliable information on meat quality and safety throughout the production, processing, and distribution chain, and finally turn this information into practical management support systems to ensure high quality final products for the consumer (Damez and Clerjon, 2008; Sofos, 2008). These systems must be readily available to the industry, and easy-to-use without requiring special expertise form the end-users. Certain databases are available today, such as the Combase (www.combase.cc) and Sym'Previus (www.symprevius.net) providing information on growth/death kinetics of microorganisms in order to define the shelf-life of various foods incorporating mathematical models (Baranyi and Tamplin, 2004;

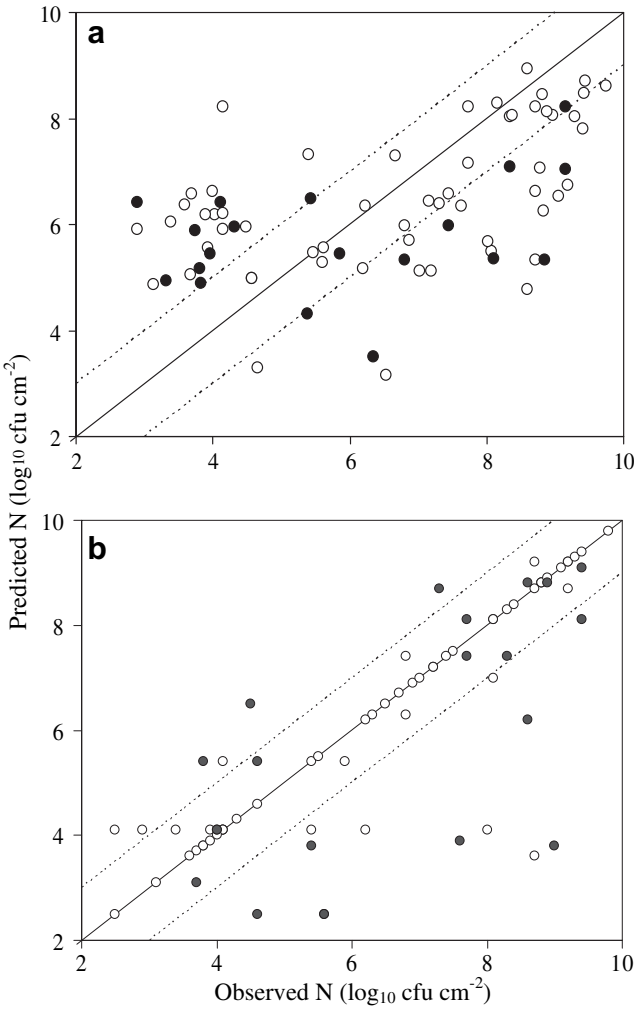

Fig. 3. Comparison between observed and predicted total viable counts (TVC) of beef fillets by the ANN (a) and the PLS-DA (b) model based on FTIR spectral data (open symbols: training data; solid symbols: test data; dotted lines are ± 1 log units area).

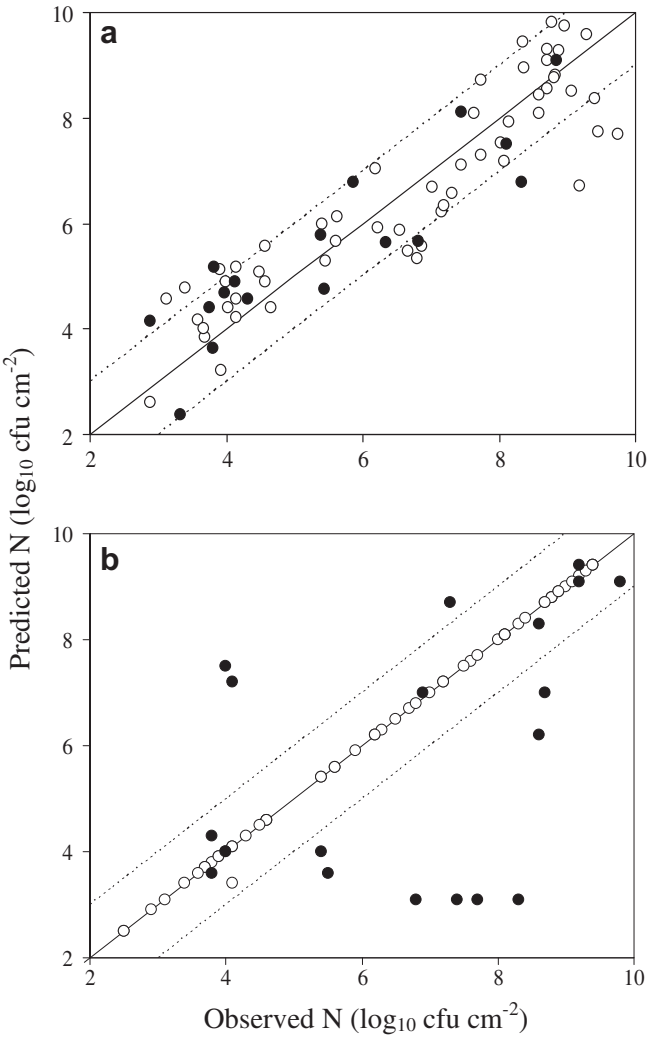

Fig. 4. Comparison between observed and predicted total viable counts (TVC) of beef fillets by the ANN (a) and the PLS-DA (b) model based on FTIR spectral data with storage time and temperature as additional inputs to the models (open symbols: training data; solid symbols: test data; dotted lines are ± 1 log units area).

Leporq et al., 2005). It must be stressed however, that the existing predictive microbiology spoilage models tend to underestimate important factors such as microbial interaction among the members of the microbial association as well as with the food matrix (Wilson et al., 2002; Koutsoumanis et al., 2004). In the latter case the changes in the concentration of microbial metabolites on meat surface due to microbial activity can be used to monitor quality deterioration. There is thus a need to replace, or at least limit, the number and extent of microbiological analyses, with (bio) chemical analyses in an attempt to define metabolic indices as potential indicators of spoilage. The concept is not new and it was proposed as a promising alternative to monitor meat spoilage in the late 80s and 90s (McMeekin, 1982; Gill, 1986; Nychas et al., 1988; Kakouri and Nychas, 1994; Dainty, 1996). However, the idea of a single biochemical substance as spoilage indicator put forward at that time, has been replaced today by the metabolomic concept which is based on a holistic approach of spoilage profile (Goodacre et al., 2004; Nychas et al., 2008).

Recent developments in sensor technologies and data analysis procedures have stimulated interest in developing rapid and non-invasive techniques to monitor changes in meat quality. Among these, spectroscopic methods are widely used for muscle food quality assessment and control, in both laboratory and meat industry installations (Hildrum et al., 2006). In contrast to conventional methods for the determination of meat quality parameters, Fourier transform infrared spectroscopy (FTIR) is a sensitive, rapid and non-destructive analytical technique, with simplicity in sample preparation, allowing simultaneous assessment of numerous meat properties. This technique has found numerous applications in foods such as olive oil (Maggio et al., 2010), honey (Kelly et al., 2006), wine (Versari et al., 2010), coffee (Briandet et al., 1996). Ellis et al. (2002, 2004) have been the pioneers to report that FTIR spectral data collected directly from the surface of meat could be used as biochemical interpretable "fingerprints" to provide information on early detection of microbial spoilage of chicken breast and rump steaks. However, the amount of information provided by spectral data require special data mining techniques based on multivariate statistical analysis (e.g., cluster analysis, principal components analysis, discriminant function analysis, partial least squares regression) and/or soft computing methodologies (e.g., artificial neural networks, genetic algorithms, support vector machines) to provide information related to (a) the responses of specific spoilage microorganisms in meat and (b) the discrimination of meat samples in quality classes (Goodacre, 2000; Mataragas et al., 2007; Verouden et al., 2009).

In the present work, FTIR spectral data from beef fillets stored under aerobic conditions at five different storage temperatures were analyzed by partial least squares regression in an effort to classify meat samples in three sensorial categories (fresh, semi-fresh, spoiled) as defined by a taste panel. The performance of the PLS approach was compared with a multi-layer perceptron (MLP) neural network. Two different approaches were followed in model development. Firstly, storage time and temperature were treated as input variables and associated with FTIR spectral data during model development. However, in practice, the history of a meat sample in terms of storage temperature and time is not always known, and hence meat quality must be assessed by spectral data only. To cope with this issue separate models were developed based on the FTIR data only and the two approaches were compared.

Results showed relatively better performance when storage time and temperature were included as inputs in model development, as a more precise dataset was used for the training of models. Good classification accuracies were obtained for fresh and spoiled meat samples, demonstrating the effectiveness of the method to discriminate samples between these two classes (Tables 1 and 2). The high classification rate of both models (i.e., PLS and ANN) could be associated to the beginning of proteolysis in meat (Nychas and Tassou, 1997) resulting in changes in the concentration of amides and amines (Ellis and Goodacre, 2001), as well as to glucose consumption and the resulting changes in the levels of organic acids (Dainty, 1996; Nychas et al., 1998). It must be emphasized however that the number of examined samples within each class was not equal due to the different spoilage rate of beef samples at different storage temperatures resulting in variable number of samples in each class. This may have affected the training process which is basically a data driven approach (Basheer and Hajmeer, 2000), and could thus account for the lower classification accuracies observed in certain classes (e.g., fresh and semi-fresh) (Tables 1 and 2). Finally, the lower accuracies observed in the semi-fresh class could also be attributed to the performance of the taste panel, as the difference between "fresh/semi-fresh" and "semi-fresh/spoiled" is sometimes subjective and affects the overall classification, as the developed models are based on supervised training for parameter optimization.

Another interesting perspective from a microbiological point of view would be the correlation of FTIR spectra to bacterial

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population counts on the surface of meat samples. In this way laborious and time-consuming microbiological analyses could be replaced in the long term by spectral data in order to provide rapid, low cost and non-invasive microbiological analyses (Nychas et al., 2008). The graphical plots between observed and predicted total viable counts as well as the calculated performance indices showed that for models developed on FTIR spectral data alone better performance was obtained by the PLS model (Table 3; Fig. 3) although the model had a tendency to underestimate total viable counts. However, when storage time and temperature were included in model development together with FTIR data the best performance was obtained by ANN (Table 4; Fig. 4). Generally, ANN models tended to overestimate microbial counts ($B_f > 1$) in contrast to PLS models where underestimation of total viable counts was evident ($B_f < 1$). An interesting alternative approach to evaluate the effectiveness of FTIR spectral data in the determination of sensory rating and total viable counts prediction in meat samples, would be the implementation of experimental studies in which meat samples would have been artificially contaminated with spoilage bacteria at different initial populations. Further research is needed in this direction as results from such studies would be valuable in the evaluation of the robustness of the FTIR approach.

In conclusion, the correlation between microbial growth and chemical changes during storage has been recognized as a way to identify indicators that could be employed to quantify quality as well as the degree of spoilage. Spectral data collected from FTIR analysis combined with an appropriate machine learning strategy (partial least squares regression, artificial neural networks) could become an interesting tool to monitor beef fillets spoilage through the measurement of biochemical changes occurring in meat substrate. Future work should also focus on the association of specific microbial groups (e.g., lactic acid bacteria, pseudomonads, enterobacteria) with FTIR spectral data in an attempt to increase the prediction performance of the models.

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Potential of a simple HPLC-based approach to quantify spoilage of minced beef stored in different temperatures and packaging systems

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ABSTRACT

The shelf life of minced beef stored (i) aerobically, (ii) under modified atmosphere packaging (MAP), and (iii) under MAP with oregano essential oil (MAP/OEO) at 0, 5, 10, and 15 ºC was investigated. The microbial association of meat and the temporal biochemical changes were monitored. Microbiological analyses, including total viable counts (TVC), *Pseudomonas* spp., *Brochothrix thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, and yeasts/moulds, were undertaken, in parallel with sensory assessment, pH measurement and HPLC analysis of the organic acid profiles. Spectral data collected by HPLC were subjected to statistical analysis, including Principal Components Analysis (PCA) and Factorial Discriminant Analysis (FDA). This revealed qualitative discrimination of the samples based on their spoilage status. Partial Least Square Regression (PLS-R) was used to evaluate quantitative predictions of TVC, *Pseudomonas* spp., *Br. thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, and yeasts/moulds. Overall, the HPLC profile of organic acids, was found to be a potential method to evaluate the spoilage and microbial status of a meat sample regardless of storage conditions. This could be a very useful tool for monitoring the quality of meat batches during transportation and storage in the meat food chain.

Proceedings

Oral presentations

- **Argyri, A.A**., Mallouchos, A., Panagou, E.Z., and Nychas, G-J. E. (2010). The dynamics of the HS/SPME-GC/MS as a tool to detect and monitor the spoilage of minced beef stored under different packaging and temperature conditions. 2nd Hellenic Congress on meat and meat products thereof «from stable to table», 24-26 September, Athens, Greece.
- **Argyri, A.A**., Panagou, E.Z., Jarvis, R., Goodacre, R. and Nychas, G-J.E. (2010). The dynamics of Fourier Transform Infrared spectroscopy (FT-IR) and Raman spectroscopy on the rapid prediction of meat shelf life 3rd National Conference of Interdisciplinary Society of Food Hygiene Assurance, 4-6 June 2010, Thessaloniki, Greece
- **Argyri, A.A**., Doulgeraki, A.I., Blana, V. A, Panagou, E.Z.,, and Nychas, G-J. E. (2010). The potential of HPLC analysis of organic acids on predicting the shelf life of minced beef stored under conventional and active packaging conditions 3rd National Conference of Interdisciplinary Society of Food Hygiene Assurance, 4-6 June 2010, Thessaloniki, Greece
- Bigwood T., **Argyri, A. A**., Doulgeraki, A. I., Blana, V. A, Papadopoulou O., Tassou C., Panagou, E. Z., & Nychas, G-J. E. (2010). The fate of Escherichia coli on beef fillets with the presence of the E. coli T4 bacteriophage and of the volatile compounds of oregano essential oil. 3rd Pan – Hellenic of Interdisciplinary Society of Food Hygiene assurance, 4- 6 of June, 2010, Thessaloniki, Greece.
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