FLAVOUR PRODUCTION OF STILTON BLUE CHEESE MICROFLORA

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The Flavour Production of Stilton Blue Cheese Microflora

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Abstract

In the blue cheese Stilton the starter mould *Penicillium roqueforti* grows and sporulates during the ripening period and is considered to be responsible for the unique blue cheese aroma. However, the sporulation of the mould, which results in the formation of blue veins, takes place in a fraction of the Stilton matrix which overall is very heterogeneous. Most blue cheeses develop a secondary microflora of yeasts which may affect their aroma. The aim of this study was to investigate the yeast flora of Stilton, the aroma profile of the cheese and the role of the yeasts in the aroma production.

The approach in this work was to study individually the different sections of Stilton (the blue veins, the white core and the outer crust) as previous studies have demonstrated each section has a differing bacterial flora. In addition to the classical microbiology, a series of molecular techniques (Denaturing Gradient Gel Electrophoresis, Restriction Fragment Length Polymorphism and Terminal RFLP) were compared and applied for the screening of the local fungal communities in the cheese. The results showed that the two approaches were complementary. It was concluded that the structure of the fungal community was different for each section of the cheese.

The aroma profiles of the three different sections of Stilton were studied using solvent extraction Gas Chromatography-Mass Spectrometry (GC-MS), a headspace GC-MS technique (SPME GC-MS) and direct headspace analysis (Atmospheric Pressure Chemical Ionisation [APCI]-MS). The different sections of Stilton presented different aroma profiles. Overall, the blue and the outer crust had similar profiles. These two sections contained higher amount of ketones while the white contained higher amounts of alcohols and aldehydes.

Yeast isolates and the starter *Penicillium roqueforti* were cultivated alone and in combination in a cheese model and the aroma production was studied with SPME GC-MS analysis. The co-culture of the starter *Penicillium roqueforti* and individual yeast isolates resulted in aroma profiles different from those that were produced by the mould or the yeasts individually.

The model of *Penicillium roqueforti* with *Yarrowia lipolytica* resulted in an aroma more similar to blue cheese than produced by the mould alone. Sensory analysis (Flash profile technique) was used in order to compare the aroma of this model with the aroma of blue cheeses and the perception of the combined culture was found to be similar to Stilton cheese, whereas that of the mould alone was not.

Yeasts are a significant part of the microflora of Stilton and they are able to affect the aroma production. Selected isolates of *Yarrowia lipolytica* could be used in combination with *Penicillium roqueforti* for the production of blue cheese aroma e.g. as a starter culture.

1 GENERAL INTRODUCTION

1.1 Description of Stilton cheese

Stilton is a 'Protected Designation of Origin' product of the Midlands and therefore very important for the local economy. Stilton is a semi-soft blue cheese, of an internally mould-ripened variety. A good Stilton will have a creamy texture with adequate and evenly distributed internal growth of the mould *Penicillium roqueforti* leading to a blue veined mass. The flavour is typical of blue cheese with creamy notes. The texture and the flavour of the cheese can vary among the producers even amongst the batches of the same producer. This is because of the complex production procedure of blue cheeses in general and Stilton in particular.

1.2 History and current status of Stilton

Stilton was first made in the early 18th Century in the area around Melton Mowbray in the Midlands of England. Stilton takes its name from the village Stilton which is located about 80 miles north of London. Stilton village was a popular stopping point for the coaches travelling from London to northern cities and at the same time it was conveniently located close to the Melton Mowbray area. Thus it became the central market place for the cheese and Stilton became known as Stilton cheese without ever having been produced there (Hallas, 1997).

Originally, Stilton was produced from unpasteurised milk. The production process changed between November 1988 and January 1989 when 36 outbreaks were

reported, with a total of 155 people suffered gastrointestinal symptoms associated with eating Stilton cheese (Maguire *et al*., 1991). It was then decided to use pasteurized milk in the production of the cheese.

Blue Stilton was granted the status of a product of protected designation origin (PDO) by the European Commission in 1996. Only cheese produced in the three counties of Nottinghamshire, Leicestershire and Derbyshire and made according to a standard code may be sold as Stilton. Nowadays, there are six dairies licensed to make Stilton and they are represented by the Stilton Cheese Makers Association (SCMA) that was formed in 1936. They are subject to audit by an independent inspection agency accredited to European Standard EN 45011 (SCMA, 2006).

1.3 Production procedure

Stilton is produced with pasteurized cows' milk. Acidification of the cheese is carried out by the addition of lactic acid bacteria (mainly *Lactococcus lactis*) as a starter culture, while the ripening is promoted by the development of the mould *Penicillium roqueforti*.

Describing briefly the production procedure, fresh pasteurized milk is transferred into an open vat to which the acid-forming *Lactococcus lactis*, rennet (milk clotting agent) and *Penicillium roqueforti* spores are added. Once the curds have formed, the whey is removed and the curds are allowed to drain overnight. The next day, the curd is cut into blocks to allow further drainage before being milled and salted. Each cheese is made with about 11 kg of salted curd that is fed into cylindrical moulds. The moulds are then placed on boards and turned daily to allow natural

drainage for 5 or 6 days. This ensures an even distribution of the moisture throughout the cheese. As the cheeses are not pressed, they create a flaky open texture which is required for the important stage of sporulation of the *P. roqueforti*. After 5-6 days the cylindrical moulds are removed and the coat of each cheese is sealed by smoothing and wrapping. In this way the air is prevented from entering the core of the cheese. The cheeses are then stored in controlled temperature and humidity (approximately 10^{o}C / 85% humidity) and they are turned regularly during this ripening period. At about 6 weeks, the cheeses form a hard brown crust which is characteristic of the variety. At this stage the cheeses are pierced with stainless steel needles. This allows air to enter the core of the cheese and thus allows the *P. roqueforti* to sporulate which creates the blue veins (Whitley, 2002; SCMA, 2006).

At about 9 weeks of age the product is ready to be sold unless further maturation is required. At this age the weight of each cheese is reduced to about 8kg and the pH should be within the range 5.5-6.1 depending on the region of the cheese (the higher values are in the blue veins). Cheeses that do not meet the quality standards of the Stilton variety (appearance, blue veining, aroma etc.) are sold as blue cheese (Whitley, 2002; SCMA, 2006).

1.4 The bacterial flora of Stilton

The number of studies on Stilton microbiology is very limited. Whitley (2002) focused on the correlation between the microflora and the quality of Stilton cheese. By using culture dependent techniques, the microflora of low quality batches of cheese with defective mould sporulation and poor blue vein development were

compared to batches of good quality. It was observed that, despite the lactic acid bacteria starter cultures which consisted mainly of mixed *Lactococcus* species (*Lactococccus lactis* subsp. *lactis, Lactococccus lactis* subsp. *lactis* var. *diacetylactis, Lactococccus lactis* subsp. *cremoris* and *Leuconostoc mesenteroides* subsp. *cremoris*), the final cheese microflora tended to be dominated by *Lactobacillus* species: *Lb. plantarum*, *Lb. brevis* and *Lb. curvatus*.

While *Lactobacillus* species were always dominating the cheese matrices, the balance between the different *Lactobacillus* species was related to the cheese quality as well as differentiating between the profiles associated with individual dairies. In most of the cases *Lb. plantarum* was present and in high quantity. *Lb. plantarum* coincided with the good quality cheeses where it was present alone or it was the dominant *Lactobacillus* species. In contrast, the heterofermentative *Lb. brevis* was connected with cheeses of poor quality. Whenever *Lb. brevis* was co-present with *Lb. plantarum* in good quality cheeses, they were accompanied by other homofermentative lactobacilli (*Lb. curvatus*, *Lb. casei*). In the poor quality cheeses, there was *Lb. brevis* alone or co-present with *Lb. plantarum* without being accompanied by other homofermentative species. However the *Lb. brevis* strains isolated in poor quality cheeses did not present the same assimilation patterns to those of *Lb. brevis* in good quality cheeses. *Lb. plantarum* isolates generally presented a wide range of different assimilation patterns no matter where they where coming from. Rennet has been found to be the main source of origin of *Lactobacillus* in the Spanish blue veined cheese, Cabrales (Florez *et al.*, 2006).

In a subsequent study, Ercolini *et al.* (2003) employed molecular analysis techniques for the analysis of the bacterial profile of Stilton. Denaturing Gradient Gel Electrophoresis (DGGE) of the V3 and V4-V5 regions of the 16S rDNA was applied

for the analysis of DNA extracted directly from the cheese and from bulk cells from culture media. The DGGE analysis revealed that the bacterial flora of Stilton was very complex, consisting of close relatives of *Lactococcus lactis*, *Enterococcus faecalis*, *Lactobacillus curvatus*, *Staphylococcus equorum*, *Staphylococcus* sp., *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. Again, *Lactococcus lactis* was not detected in the profiles retrieved from the culture media but only with the direct analysis of the cheese. However, analysis of bulk cells from plates of lower dilutions (outside the countable range) revealed the presence of this species. This suggests that *Lactococcus lactis* might not have been detected in the previous study (Whitley, 2002) because of its presence in the final product in low counts and demonstrates the importance of the combination of the two approaches (direct population analysis of cheese and after cultivation) for overcoming possible biases. The combination of more than one approach helps with presenting a more detailed picture of the microflora of samples with complex communities. If only culture media were used no *Lactococcus lactis* would have been isolated and it would be mistakenly concluded that the starter *Lactococcus lactis* does not survive in the final product.

Ercolini *et al.* (2003) further studied the matrix of Stilton regarding the bacterial differentiation within the different locations of the cheese by using fluorescence *in situ* hybridization (FISH). The distribution of the bacteria was not found to be the same in all the parts of the cheese. Lactococci were found in the internal part of the veins as mixed colonies and as single species colonies within the core. *Lactobacillus plantarum* was detected only underneath the crust surface, while *Leuconostoc* microcolonies were homogeneously distributed in all parts observed. The core had a simple composition in microbial species. *Lactococcus lactis* and *Leuconostoc* colonies represented 70% and 15% of the detected colonies respectively,

while the rest were coccus-shaped unidentified microorganisms. The veins and the external crust were also characterized by the presence of large colonies of unidentified cocci. Large colonies of rods detected in the veins were suggested to be colonies of *Lactobacillus curvatus*. A few *Lactococcus lactis* microcolonies were also found mixed with other unidentified coccus-shaped microorganisms; their detection by this approach further supports the idea that these organisms are viable within the matrix as the site of probe binding was ribosomal RNA, found only in viable cells. The surface presented an external layer of large yeast-like cells as well as cocci similar to those in the veins (Ercolini *et al*., 2003). The use of FISH revealed that the different sections of Stilton can develop different local bacterial communities. Despite the fact that a lot of the colonies remained unidentified this study clearly demonstrated the high level of Stilton's polymorphism.

1.5 Fungi as part of the microflora in cheeses

In addition to the bacterial flora, yeasts and moulds are present in cheeses affecting the ripening, maturation and the final properties of the product. They can be an important part of the starter flora and they can be encountered and used as culture adjuncts. Fungi are used as adjuncts in Brie, Camembert, Pont l'Eveque, Maroilles and Reblochon, Herve and Limburger and Tallegio (Fox *et al*., 2004). They are particularly important in mould and bacterial surface-ripened cheeses because they promote the growth of other microorganisms. For the Tilsit cheese, yeasts can be responsible for colour and flavour formation during ripening (Rademaker *et al*., 2005). The mould *Penicillium camemberti* is one of the most important parts of the

microflora of Camembert and Brie and the growth of its white mycelia on the surface gives to these cheeses their characteristic appearance, typical aroma and taste (Fox *et al*., 2004).

Fungi, and yeasts in particular, can be an important part of the secondary flora of cheeses and they spontaneously occur in almost all the cheese varieties. Yeasts can colonise any part of the cheese, especially the surface, and they can grow during the early stages of cheese making (Fox *et al*., 2000). In a study of fourteen different Hungarian commercial cheese products coming from eight different cheese varieties, all samples were found to contain several yeast species which were not part of the starter flora. Twenty-six different species were identified with *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Geotrichum candidum* and *Candida catenulata* being predominant (Vasdinyei *et al*., 2003). By reviewing other studies on yeasts in cheeses (Fleet, 1990; Fox *et al*., 2000; Viljoen *et al*., 2003; Fox *et al*., 2004; Fleet, 2007) it is noticeable that for each variety there are species that constantly appear in their microbial communities. The species *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Geotrichum candidum* and *Candida catenulata*, *Candida zeylanoides*, *Saccharomyces cerevisiae* are among the most frequently occurring. This is mainly attributed to a particular set of parameters and conditions in the production procedure of cheeses that favour the growth of yeasts. Generally, the low pH, the high salt content and the storage temperatures allow for competitive yeast growth. Therefore there are yeasts which, despite the fact that they are not part of the starter culture flora, constantly affect the production of the cheeses and their characteristics. Yeasts show particularly abundance in blue cheeses (Fleet, 1990). So far there has been emphasis on the study of *D. hansenii* because it is

one of the most dominant species in the yeast communities of the blue cheeses (Fleet, 1990). However, the role of less dominant species can be equally important.

In the past yeasts were associated with defects in cheese (e.g. undesirable gas production and off-flavours). However, in recent years, especially for blue cheeses, they have been increasingly considered to participate in enhancing the ripening, the maturation and the flavour formation because of the high proteolytic and lipolytic activity that some of the species present and the metabolism of lactose and its substitutes (Van den Tempel *et al*., 1998; Van den Tempel *et al*., 2000a; Van den Tempel *et al*., 2000b). The lipolytic activity, and the lipolysis particularly, is important in blue cheeses during ripening for the release of fatty acids. The fatty acids in addition to being aroma compounds by themselves also act as precursors for a series of desirable groups of compounds including methyl ketones, alcohols, lactones and esters (Molimard & Spinnler, 1996). *D. hansenii* and *Y. lipolytica* isolates from blue cheeses have been demonstrated to hydrolyse tributyrin and showed strong assimilation of lactose, galactose, lactate and citric acid under environmental conditions similar to those in Danablu maturation (Van den Tempel *et al*., 2000a). *Candida catenulata* presented a similar activity with the only exception that it did not assimilate lactose (Van den Tempel *et al*., 1998). The breakdown of tributyrin results in butanoic acid which has a cheese-like odour and is an important part of the flavour of many types of cheese including Cheddar and mould ripened cheeses such as Camembert (Curioni & Bosset, 2002). The products of citrate metabolism and the metabolism of lactose to lactate are also important in the formation of the aroma precursors. Some microorganisms can also metabolise lactose to alcohol and acetate which are part of the aroma profiles in many cheese varieties (McSweeney *et al.*, 2000). The assimilation of galactose by yeasts can be very important as some starter

bacteria (e.g. *Streptococcus thermophilus*) are unable to metabolise it, and galactose can accumulate in the curd (McSweeney *et al.*, 2000) changing the organoleptic properties of the cheese. *Y. lipolytica* degrades all components of casein (Van den Tempel *et al*., 2000a). The metabolism of casein and production of amino acids is important in blue cheeses as *Penicillium* species further metabolise them with parallel production of NH_3 which deacidifies the curd as a result. As a result of the awareness of the important properties of the yeasts occurring in blue cheese a lot of research has been conducted on the yeasts present in the different blue cheese varieties (Roostita *et al*., 1996; Van den Tempel *et al*., 1998; Addis *et al*., 2001; Wojtatowicz *et al*., 2001; Viljoen *et al*., 2003; Florez *et al*., 2006). However, to date there has not been any detailed study of the yeasts in Stilton.

The impact of yeasts on aroma formation is of great interest (Marilley *et al*., 2004). In the past, the quality evaluation of cheeses was mainly based on the absence of faults or the presence of potentially harmful or spoilage microorganisms. However, in recent years the production is very controlled and products less often fail to meet a basic level of quality. The focus is now on producing cheeses of high quality and aroma enhancement has a main role in this. The connection between the microflora and aroma formation in cheeses is well established (McSweeney *et al*., 2000), however, the impact of the secondary yeast flora on the aroma formation is not well studied. Until recently, research was mainly focused on studying the structure of the microbial populations in dairy products (e.g. developing of molecular profiling tools) (Fleet, 1999) but there was not much focus on assessing their capability for aroma production. However, the role of the secondary flora on the aroma production is likely to be important (Addis *et al*., 2001).

In addition to their individual properties and contribution to the aroma, the yeasts could indirectly influence the properties of the cheese by affecting the growth of other species, especially the starter mould, *P. roqueforti*. It is established that, during the ripening of blue cheeses, interactions between the lactic starter culture and *P. roqueforti* determine the maturation time, aroma, texture and appearance of the final cheese (Hansen *et al.*, 1997). Interaction between *P. roqueforti*, *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Lactococcus* ssp resulted into positive interactions including increase of the growth rate of *P. roqueforti* and, more interestingly, the increase of sporulation and blue colour intensity of the spores (Hansen *et al.*, 1997). As the yeasts in blue cheeses are predominant (Van den Tempel *et al.*, 1998) their interactions with the starter flora and the associated biochemical activities are also expected to influence the cheese properties including the aroma and consequently the sensory characteristics and final quality (Fox *et al.*, 2000; 2004).

1.6 Stilton cheese and other blue cheese varieties

All the blue cheese varieties present a series of common characteristics including the high number of yeast counts. However, there are some differences between the blue cheese varieties which could differentiate their secondary yeast flora composition and the way that this influences the properties of the cheese.

All the blue cheeses have a high salt content (2-5%) but the salting procedure (or brining) can be different. In the case of Stilton the salt is mixed with the curd before this is transferred into the cylindrical moulds and therefore an equal distribution of the salt is ensured in the whole mass of the cheese from the very

beginning of the ripening. The Stilton cheese producers know by experience that the even mixing of the salt in the curd is crucial for the production of good quality Stiltons (SCMA, 2006). In other blue cheese varieties the salting may be applied on the cheese surface by dry salting or by immersing the cheeses in brine (Fox *et al*., 2000; 2004). In these cases a gradient of salt is created from the surface to the core of the cheeses. In addition, the salt diffusion into the cheese core can be faster in the pierced parts creating an even more uneven salt distribution. The gradient concentrations in the different sections equilibrate slowly during ripening (Fox *et al*., 2000). In Danablue for example the difference in the salt concentration between the core and the surface layer was found to be as much as 2% at some stages of ripening (Hansen, 2001). The salting procedure is very important in the manufacture of blue cheeses. On one hand, the high salt content enhances the presence of yeasts which are generally salt tolerant. On the other hand the salting is one of the main sources of contamination of blue cheeses with yeasts (Viljoen *et al*., 2003). Only low populations of yeasts were found in Danablu cheeses before brine-salting compared with those after the addition of salt (van den Tempel, 2000). Therefore the stage and the way that the salt is applied (homogenous mixing or gradual distribution) could affect the stage at which and the way that the yeast flora develops in the cheese.

Another important difference between the blue cheese varieties is the way that the starter mould *P. roqueforti* is added. Stilton belongs to the category of the blue cheeses where the spores of *P. roqueforti* are added from the beginning of the production, in the milk, and therefore they are distributed throughout the cheese. In other varieties, such as Danish Blue cheese, rods are used to pierce the formed curds and distribute the mould in the cheese through the holes (Fox *et al*., 2000; Zerfiridis, 2001, Fox *et al.*, 2004). In the case of Roquefort cheese the mould may be added to

the curd or introduced as an aerosol through holes in the rind (Fox *et al*., 2000; Zerfiridis, 2001). In the case of the spores added in the milk the interactions of the mould and the yeasts could start in the very first stage of the production. In addition, the mould would grow throughout the core (without sporulating because of lack of air) and therefore the mycelium of *P. roqueforti* may have a more significant role than in the cases of where the mould spores are added later by piercing. This is important as it was found that the enzymatic activity of the ordinary mycelium and the sporulating mycelium of *P. roqueforti* is different (Lawrence, 1966; Lawrence *et al.*, 1968).

Finally, the outer section of Stilton is different from most of the other blue cheese varieties. A characteristic hard brown crust is formed on the 6th week of the production procedure. This makes the matrix of the Stilton cheese even more heterogeneous than the other blue cheese varieties. The outer sections of other blue cheese varieties were found to contain higher yeast populations than the inner core (Van den Tempel *et al*., 1998; Viljoen *et al*., 2003). It would be interesting to see if similar observations exist in Stilton and if these are affected by the crust.

1.7 Summary

The blue cheeses present a series of common characteristics which allow the significant development of yeast secondary flora. The production procedure of Stilton and the final product present a combination of characteristics (outer crust, addition of *Penicillium roqueforti* spores in the milk, homogenous salting etc.) which differentiates this variety from the rest. As the properties of the cheeses are closely related with its microflora it could be suggested that similar differentiation in the microbiology, including the secondary yeast flora, may exist between Stilton and other blue cheese varieties.

In contrast to other blue cheese varieties, the yeasts in Stilton have not been studied in detail yet. Previous studies on Stilton (Whitley, 2002; Ercolini *et al*., 2003) demonstrated that the bacterial flora of the final cheese can be very extremely complex despite the simple starter culture that is used. The balance between some species (e.g. lactobacilli) was found to differ between cheeses of different quality (Whitley, 2002). More interestingly, the bacterial distribution was shown to be different in different sections of the cheese (Ercolini *et al*., 2003). The way that the yeast flora is distributed in the cheese could be of the same complexity.

One of the most important parameters for the quality of the cheese is the aroma production. The microflora of the cheese, including the secondary yeast flora, is known to be mainly responsible for the flavour of the cheese including the aroma production. Taking into account the strong differentiation in the Stilton matrix (the blue veins, the white core and the outer crust), it could be suggested that in order to understand the role of yeasts in depth, it would not be enough to study them in the whole cheese overall, but it is important to understand how they are located in the

different sections of the cheese matrix. If it is shown that the different sections of the Stilton cheese contain different fungal communities, then it is possible these would result in different aroma profiles. The combination of knowledge on the microbial and aroma differentiation in the different sections of the cheese would allow for an in-depth understanding of how the aroma develops in Stilton and how it is affected by the secondary yeast flora.

The variation between Stilton from different producers is much greater than for the other well-known blue cheese varieties (e.g. Danish blue, Roquefort) which tend to be more standardised. Stilton dairies produce cheeses with individual characteristics. Part of the variation comes because of the complex production procedure of Stilton and the traditional techniques and equipment that are used that make the production less easily controlled. In addition, Stilton with different characteristics may be produced from the same dairy in order to meet the needs of different customers. The variation of cheeses from different producers is known to be affected by the different secondary flora. Therefore knowledge of the secondary flora of Stilton would be essential for the producers in order to understand and control the individual characteristics of their cheeses. Methodology for the effective characterisation of the fungi in Stilton and its aroma formation would be very helpful.

This study focuses on the fungal community in Stilton and its local differentiation in the three different parts of the cheese matrix: blue veins, white core and outer crust. It investigated the hypothesis that spatial differentiation of the yeast secondary flora exists and is accompanied by aroma differentiation between these sections. The possible impact of the yeast secondary flora on the starter mould *P. roqueforti* and the aroma production is also investigated. Several techniques were tested and employed

for this study providing an evaluation of their capability to analyse and compare the different section of the blue cheese.

2 STILTON FUNGAL FLORA

2.1 INTRODUCTION

2.1.1 General

In previous microbiological analyses of blue cheeses, the whole cheese matrix was sampled as one (Viljoen *et al.*, 2004) or the outer part and the inner core were studied separately (Roostita *et al*., 1996; Van den Tempel *et al*., 1998; Addis *et al*., 2001; Wojtatowicz *et al*., 2001; Viljoen *et al*., 2003; Florez *et al*., 2006). The approach used here was to study the three different parts of Stilton, the blue veins, white core and outer crust separately. In this way it was possible to focus on the differentiation between the white core, a part with limited presence of air, and the blue veins, where the starter *P. roqueforti* sporulates in the presence of air (Fox *et al*., 2004).

As discussed in the introduction, the information available on Stilton microbiology is very limited compared to other blue cheese varieties which have been extensively studied. There has been no detailed study of the fungal flora distribution in Stilton, or other blue cheeses, taking into account the differentiation between the blue veins, the white and the outer crust. Overall there have been few studies of Stilton's fungal flora apart from a few early studies (Percival *et al*., 1912; Brindley, 1954). However, Stilton cheeses in those days were produced from unpasteurised milk without using starter cultures and therefore they were much different from today's product. Nevertheless, these studies agree that yeasts constantly occur in Stilton cheeses of all ages and qualities and they have a major contribution in the microbial

profile of good quality cheeses (Percival *et al*., 1912) as well as being present in cheeses with defects (Brindley, 1954).

Whitley (2002) recently examined the microflora present in Stilton with limited blue vein development. Yeasts were isolated from different batches of Stilton and identified using culture dependent techniques. The most commonly occurring species were *Candida famata* (anamorph of *Debaryomyces hansenii*) and *Candida sphaerica* (anamorph of *Kluyveromyces lactis*) while *Candida catenulata*, *Candida lipolytica* (anamorph of *Yarrowia lipolytica*), *Candida dubliniensis*, *Candida boidinii* occurred less frequently. *C. famata* was the most frequently isolated species of yeasts including in cheeses with poor development of blue veins. For this reason it was suspected to inhibit the growth of *P. roqueforti*. Tests were conducted on culture media in order to investigate possible inhibition of *P. roqueforti* by *C. famata*. However, inhibition was observed under anaerobic conditions only and just for one strain of the total that were tested.

The application of molecular biology techniques to blue cheese fungal studies is generally limited. In the present study culture dependent techniques and molecular analysis (RFLP, PCR-DGGE, TRFLP) were employed and compared for studying the fungal flora in the different parts of Stilton. This was particularly important if the limited selection of culture media for yeast species is considered. It has become apparent that microbial communities can be enormously complex and present a high diversity of species and physiological characteristics with only a fraction of the total flora being culturable (Cocolin *et al.*, 2007). This is because of a lack of knowledge of the conditions under which most microorganisms are growing in the natural environment and therefore the difficulty in developing cultivation media which simulate these conditions. Even for the part of the microflora that can be cultivated,

there is a need for differentiating the species but their physiological similarities makes this task very challenging. In contrast to bacterial media, there are few media selective for specific yeasts species. Generally, the classical plating methodologies only allow the measurement of microbial groups or genera and rarely are sensitive enough to differentiate down to the species level (Giraffa, 2004).

In addition, further identification and classification of fungi, without using molecular techniques, requires the study of morphological, physiological and biochemical characteristics. This process can be extremely complex, laborious and time consuming. It could require as many as 60-100 tests and several weeks of analysis (Esteve-Zarzoso *et al*., 1999). The results are not always reliable and many studies report differences between the classification using classical microbiology and DNA analysis (Guarro *et al*., 1999). Various kits have been developed for the rapid identification of yeasts based on their biochemical properties e.g. the API strips (Biomerieux). However, these kits were designed for clinical diagnosis and identification of species of medical interest without including many of the species that occur in food (Tornadijo *et al*., 1997; Ramani *et al*., 1998; Esteve-Zarzoso *et al*., 1999). Furthermore, isolates with similar biochemical features may be differentiated into different species on morphological features e.g. pseudomycelium production, which these kits do not take into account and therefore a morphological study may be required.

All the above have made the use of molecular techniques for studying yeasts even more necessary. As a result the approaches for studying microorganisms have changed dramatically during the last few decades. Molecular methods have become a valid support to traditional techniques with a growing trend towards molecular approaches and the replacement of classical microbiological methods. The advances

in molecular biology have provided reliable and effective methods for detection, identification and typing of food microorganisms (Ercolini 2004; Cocolin *et al*., 2007) many of which are based on rRNA or rDNA sequences and PCR techniques (Florez *et al*., 2006). Their use for studying the diversity of microorganisms in dairy products is of major interest (Ercolini 2004; Rademaker, *et al*., 2005; Florez *et al*., 2006; Rademaker, *et al*., 2006; Cocolin *et al*., 2007). This is because of the need to investigate the correlation between the microorganisms and key food properties and it is a matter of discussion in this study.

2.1.2 PCR – Denaturing Gradient Gel Electrophoresis

PCR-DGGE was one of the molecular techniques that was employed here for the study of the fungi in Stilton. PCR-DGGE is one of the most common molecular techniques used for food analysis, mostly for screening and comparing microbial population profiles at different fermentation stages or from different sources (batch, geographical origin, variety etc.) (Ercolini, 2004). It was previously successfully used for the study of bacteria in Stilton (Ercolini *et al*., 2003).

Denaturing Gradient Gel Electrophoresis **(**DGGE) is an electrophoretic method capable of detecting differences between PCR amplimers of the same size but with differences in their sequences. The PCR amplimer can come from single or mixed DNA templates. The most commonly employed target for PCR amplification prior to DGGE is the ribosomal DNA. This is because it is a conserved region of the genome that also includes variable regions. Therefore, universal primers can be designed for hybridizing to conserved regions but spanning variable regions in order

to obtain PCR amplicons with species-specific differences in base pair composition that can be separated by DGGE. The three precursors of the eukaryotic ribosome molecules, 26S, 18S and 5.8S subunits, have undergone a relatively slow evolution, allowing for their use in comparison of organisms. So far work has focused primarily on regions of the 26S rDNA and the 18S rDNA (Ercolini, 2004).

The separation of PCR amplimers is based on the differential melting temperatures (Tm) of such DNA fragments. The melting temperature, and therefore the electrophoretic mobility in polyacrylamide DGGE gels, is sequence-dependent. Consequently, decreased electrophoretic mobility occurs in linear gradient polyacrylamide gels with denaturing agents. Specifically, the amplimer becomes partially melted once it reaches the Tm of the lowest melting domain. One of the primers for the DGGE is required to contain an additional GC clamp at its 5' end as this prevents the complete detachment of the double DNA strands (Shieffield *et al*., 1989). As a result of the partial melting, the mobility of the amplimer is reduced and separation can be achieved for fragments of the same size but different base pair sequence. The denaturing conditions are created with urea and formamide (100% chemical denaturant contains 7 M urea and 40% formamide). Then low and high denaturing solutions are prepared according to the desired gel denaturing range. Electrophoresis takes place at a constant temperature within the 55-65^{o}C range (Ercolini, 2004). Each sample results in a fingerprint consisting of DNA bands and the profiles can be compared directly on the gel. Individual bands can be excised and directly sequenced or sequenced after cloning.

2.1.3 Terminal Restriction Fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (TRFLP) is another technique that can be used for direct DNA analysis of food samples. TRFLP analysis is a combination of three technologies: PCR amplification of DNA, restriction enzyme fragmentation of the PCR amplicon and electrophoresis of the fragments. TRFLP can be used for the characterization of single strains, complex microbial communities and comparative community analysis (Rademaker *et al*., 2006).

As with the DGGE analysis, selective PCR amplification of target genes, usually rDNA, takes place, but in this case using primer pairs where one primer is fluorescently labelled (Figure 2.1). This is followed by digestion of the amplicons with one (or more than one) appropriately selected restriction endonucleases. For each amplicon one fluorescently labelled terminal restriction fragment (TRF) is generated. The length of the TRF depends on the DNA sequence of each microorganism and the enzyme used to cut the sequence. The digests are then separated with high-resolution sequencing gel electrophoresis and the length of each digest fragment is measured from the time of the fluorescence signal on automated DNA sequencers. The laser scanning system of the DNA sequencer detects the fluorescently labelled probes attached to the fragment and from their signal intensity the sequencer can record their relative abundances. The results are presented in electropherograms where the profile of each microbial community is as a series of peaks of varying heights/areas. The position of each peak corresponds to the length of the fragment and its height/area to its quantity. Potentially all the TRFs that are generated from the digestion of the PCR amplicon could be compared with the terminal fragments available in databases and are supposed to be species-specific.

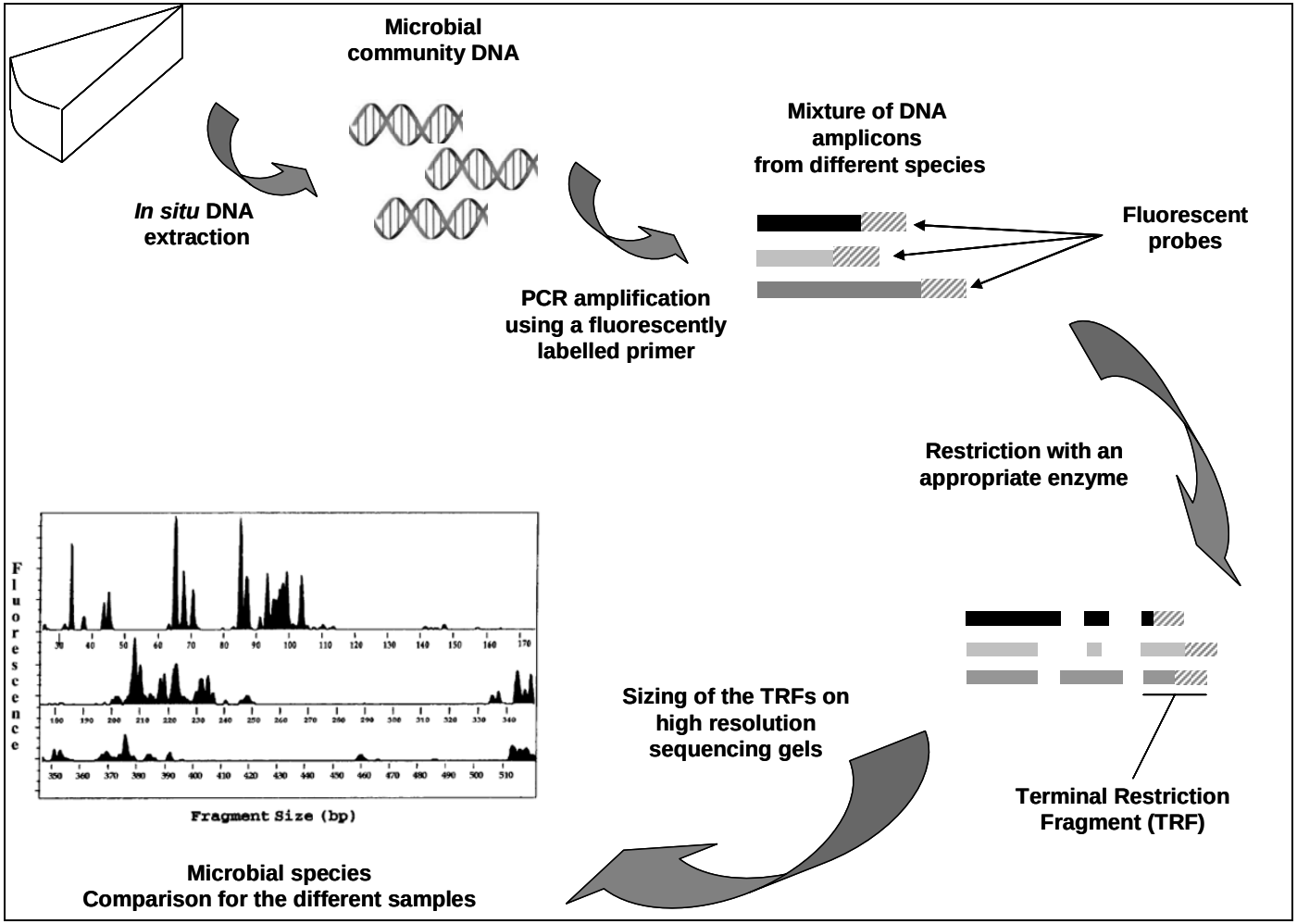


Figure 2.1 Flow diagram of the application of TRFLP analysis to Stilton cheese samples.

Semi-quantification of each species in the mixed community can be obtained since the fluorescence intensity, and consequently the size of each peak, is proportional to the amount of each TRF present (Blackwood *et al*., 2003; Cocolin *et al*., 2007). The data can be easily statistically analysed and/or rapidly compared by visual interpretation. The use of one fluorescently labelled primer restricts the analysis to the terminal restriction fragments (TRFs) only and provides simplicity in the analysis. Size markers, bearing different fluorophores than those for the samples, can be included in every lane making the measurement of the terminal fragments very accurate (±1 base).

TRFLP can be applied in two different ways. One application is the analysis of peak profiles without the identification of species. This allows the screening of differences in the microbial communities of the same type of ecosystem or for the comparison of the profiles of different ecosystems (Marsh, 1999; Osborn *et al*., 2000; Blackwood *et al*., 2003; Tiquia, 2005). Alternatively, the type of species can be detected when analysis focuses on species identification (Marsh, 1999; Rademaker *et al*., 2005; Rademaker *et al*., 2006). While the principal of the two applications is similar, the outputs are different and they are subject to different types of errors.

Because of its simplicity, TRFLP is among the molecular methods that have been widely applied for the study of bacteria in environmental and plant ecosystems (Tiquia, 2005; Dickie *et al*., 2007; Pandey *et al*., 2007). However, the applications of the technique for studying fungal communities are fewer than those on bacteria. Recently, TRFLP has been employed for profiling microbial populations of foods, mainly dairy samples. Currently, only a few food studies have been reported and they are exclusive to bacterial communities. Rademaker *et al*. (2005) has used TRLP to study and compare Tilsit cheese surface microflora composition between different

stages of ripening. In a subsequent study (Rademaker *et al*., 2006) TRFLP was used for assessing the microbial population dynamics during yoghurt and hard cheese fermentation and ripening. Sanchez *et al*. (2006) combined reverse transcriptase PCR with TRFLP and performed semi-quantitative analysis of metabolically active bacteria which are used in dairy fermentations.

The aim of the current study was to use TRFLP both for identifying the fungi in Stilton cheese as well as screening the differences between the fungal profiles of the different parts of the cheese. The results can be compared with those from the culture-dependent analysis and DGGE analysis. At the time that this study took place it was the first attempt to apply TRFLP analysis on yeasts in dairy foods.

2.1.4 Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) refers to variation in restriction fragment lengths and is the technique on which the TRFLP technique was based and developed. In many RFLP applications rDNA is extracted followed by the amplification of coding and conserved genes and neighbouring non-coding and variable internal transcribed spacers (ITS) regions (Deak *et al*., 2000; Vasdinyei *et al*., 2003; de Llanos Frutos *et al*., 2004; Trost *et al*., 2004). The PCR products can then be digested using restriction enzymes. The length of the fragments depends on the DNA sequence of each microorganism and the enzyme selection. The size of the fragments is calculated with gel electrophoresis in parallel with a DNA ladder and compared with databases.

Ribosomal regions show low intraspecific polymorphism and high interspecific differences (Li, 1997) and they have been effective for screening genealogical relationships in several fungus species (Esteve-Zarzoso *et al*., 1999). The size of the PCR product is usually enough for species differentiation within a narrow microbial community. However, the following restriction patterns with endonucleases yield unique profiles resulting in further differentiation and identification of species. The number and the size of the fragments in each pattern as well as the size of the initial PCR product compose a characteristic profile for each microbial species.

Esteve-Zarzoso *et al*. (1999) tested the application of the RFLP on the rDNA region spanning the 5.8 rRNA gene and the two ITS (ITS1, ITS2) for identifying 132 species of yeasts from 25 different genera. These authors provided a database with a wide range of teleomorphic and anamorphic ascomycetous and basidiomycetous yeast entries including those of interest for dairy science. Generally, the size of the PCR products and the restriction patterns obtained by using the enzymes *Cfo*I, *Hae*III and *Hinf*I provided unique profiles for each species. Accordingly, the use of this molecular approach was proposed as a new rapid and easy method for routine yeast identification.

The capability of the ITS1-5.8S-ITS2 approach for effective differentiation and identification of yeasts is confirmed by the results of several studies (Deak *et al*., 2000; de Llanos Frutos *et al*., 2004; Trost *et al*., 2004). As an alternative to the choice of the 5.8S gene region, RFLP analysis of the 18S rDNA-ITS1 region was employed for the characterization of yeast isolates from various dairy products (Vasdinyei *et al.*, 2003). In all cases, the main bias is that many species present similar RFLP profiles which the technique is not sensitive enough to differentiate. To recapitulate, the convenience and rapidity of the RFLF is clearly demonstrated in various applications.

However, optimum screening of the microbial communities can be obtained only after careful consideration of the primer and enzyme combination.

2.1.5 Conclusions

Stilton has received very little attention compared to other blue cheese varieties of similar economical significance. Even more the structure and the role of the fungal secondary flora has not been investigated in depth. The development of molecular biology provides analytical tools that could allow a better understanding of the fungal flora of Stilton.

So far the blue cheese studies have focused on the microbial profiles of the whole final product. It seems that the differentiation that may exist within the cheese matrix, including between the sections of the core, is underestimated. Such differentiation has already been demonstrated for the bacterial community of Stilton.

This chapter focuses on the fungal community in Stilton and its local differentiation in the three different parts of the cheese matrix: blue veins, white core and outer crust. This work investigated the first part of the hypothesis that spatial differentiation of the secondary microflora exists and is accompanied by aroma differentiation between these sections and that the secondary microflora is related to the aroma production and differentiation.

2.2 MATERIALS AND METHODS

2.2.1 Cheese sample origin and preparation

A whole 45 days old Stilton cheese (7.56 kg) was purchased from a retail outlet. The cheese was divided under aseptic conditions into different regions, outer crust (outer), pure white core (white) and blue veined core (blue). Initially the outer part of the cheese was separated from the core. Then a layer of 1 cm width was removed in order to ensure the differentiation between the inner parts (blue veined and white core) and the outer part. Finally, the inner parts were separated into white and blue parts. The samples were then stored in sterile plastic containers at -80^{o}C until analysis. On the day of analysis they were defrosted at 4^{o}C.

2.2.2 First set of microbial counts

Rose Bengal Chloramphenicol Agar (RBCA; Oxoid), Dichloran Rose Bengal Chloramphenicol agar (DRBC; Oxoid), Dichloran-Glycerol (DG18; Oxoid) and Malt Extract agar (Oxoid) were used for fungal analysis. All media were prepared according to the manufacturer's instructions. Aliquots of 15 to 20 ml sterile media were dispensed into Petri dishes (90 mm diameter) and allowed to dry at room temperature. RBCA and DRBC media were kept in the dark to prevent photodegradation of Rose Bengal and breakdown to products that are toxic or inhibitory to fungi (King *et al*., 1979).

For each analysis, samples (10 g) of each part of the cheese, blue, white and outer, were defrosted and homogenised with 9 ml of Maximum Recovery Diluent (MRD; Oxoid) in sterile filter Stomacher bags (Stomacher 400, Seward) using a stomacher (Stomacher 400 Circulator, Seward) at 230 rpm for 5 min. Additional 10-fold serial dilutions were prepared in MRD and aliquots of 0.1 ml spread plated in triplicate onto each of the selected media using a bent glass rod. All the spread plated media, together with blank media for control, were incubated for colony development at 25^{o}C for 5 days in the dark without being disturbed. Results were calculated as the means of three determinations.

2.2.3 Microbial counts of precisely sampled parts of cheese

A series of micro-samples (130-190 mg) were precisely taken from each part of the cheese by scratching with a sterile scalpel. The samples were weighted directly in sterile microcentrifuge vials with o-ring seals (Biospec Products). Nine parts of MRD and 4 glass beads (diameter: 2 mm; acid washed) were added. Two samples were prepared for each section. The samples were homogenised using a Mini-Beadbeater-1 (Biospec Products) at 2500 rpm for 2 x 40 s, cooling on ice between each treatment. The samples of the same section were then combined and volumes of 1 ml used for further 10-fold serial dilutions. These were then cultivated both on RBCA (Oxoid) and Malt Extract Agar (Oxoid) as described in §2.2.2.

2.2.4 Isolation of strains

After viable counts on RBCA plates from both series of counts, those bearing between 30 and 300 colony forming units (cfu) were used for isolate collection. A total of 88 randomly selected yeasts and two of *P. roqueforti* were isolated from all parts of the cheese. The yeast colonies were purified by repetitive streaking on RBCA plates and incubation at 25^{o}C. One colony of each of the final pure cultures was used to inoculate 10 ml of Yeast Peptone Dextrose broth (YPD; BD Biosciences) and incubated at 25^{o}C for 48 hours. A final volume of 1 ml (containing 20% glycerol) was stored in a sterile microcentrifuge vial with o-ring seal at -80^{o}C for further study.

2.2.5 Media for *Debaryomyces hansenii*

Media for testing *Debaryomyces hansenii* species were Yeast Peptone Dextrose agar (YPD) (Yeast extract (Oxoid) 1%, neutralized bacteriological peptone (Oxoid) 2%, glucose (Fisher Scientific) 2%, agar No 3 (Oxoid) 1.5%) for general growth and Acetate agar (KAc agar) (Yeast extract (Oxoid) 0.1%, potassium acetate (Oxoid) 1%, glucose (Fisher Scientific) 0.05%, agar No 3 (Oxoid) 1.5%) for enhancement of sporulation.

D. hansenii isolates were grown on YPD agar for 5 days at 25^{o}C (general growth conditions) and 38^{o}C (selective temperature for *D. hansenii* variety *fabryii* under which *D. hansenii* variety *hansenii* is not able to grow) and on Acetate agar for 8 days at 21^{o}C.

2.2.6 API Strips

API 20C AUX (bioMerieux, Inc.) was used for the identification of yeast isolates according to the manufacturer's instructions. The data were analysed using the *apiweb* software available online at the company's website (https://apiweb.biomerieux.com).

2.2.7 DNA extraction from pure yeasts and *P. roqueforti* cultures

From the yeast isolate pure cultures, grown on RBCA at 25°C for 48 h, 2-3 colonies were suspended in sterile PCR tubes containing 50 µl of extraction buffer (0.02M NaOH, 0.01% N-Lauroylsarcosine) using sterile pipette tips. The cell suspension was heated at 99^{o}C for 10 minutes in a PCR thermal cycler (TC-312; Techne) and then centrifuged at 16000 x g for 5 minutes (Biofuge Pico; Heraeus) in order to remove the non-soluble cellular material. The supernatant (35 µl) was transferred to a sterile Eppendorf tube. The extracts were used directly as a template for PCR or stored at -20^{o}C for future use (unpublished method; Gibson, B. Personal communication).

2.2.8 DNA extraction from cheese

DNA was extracted from Stilton cheese using the method of Rose *et al*. (1990) with minor modifications and after cheese samples were subjected to a preliminary

treatment with sodium citrate solution suitable for products with high lipid and protein content like blue cheeses (Florez *et al*., 2006).

The different parts of the cheese were precisely and aseptically sampled using sterile scalpels. The samples were weighed directly into 2.0 ml o-ring seal microcentrifuge vials suitable for use with a Mini-Beadbeater-1. Suspensions (10% w/v) were prepared by adding sodium citrate solution (2% [w/v] trisodium citrate dehydrate; Sigma). Four glass beads (2mm diameter; acid washed) were also added and the cheese suspensions incubated for 5 min at 45^{o}C. The samples were homogenised at 2500 rpm for 40 s using a Mini-Beadbeater-1 and further incubated for another 5 min at 45^{o}C. After vortexing for 10 s, the cheese solids were allowed to set for one minute, then 1 ml of cell suspension was transferred to a new microcentrifuge vial. Cells were collected by centrifugation at 16000 x g for 10 min (Biofuge Pico – Heraeus) and the supernatant was discarded. The cell pellets were washed with 1 ml of PBS by centrifugation at 16000 x g for 10 min and the liquid layer was discarded again. To the cell pellet in the microcentrifuge vial were added: 0.3 g of glass beads (0.5 mm diameter; Biospec), 0.2 ml of lysis buffer (10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100) and 0.2 ml of 1:1 mix of phenol-chloroform. Lysis was achieved by shaking the tubes twice in the Beadbeater at 4600 rpm for 1 min and by cooling the tubes on ice in between. TE buffer (0.2 ml; 10 mM Tris, 1 mM EDTA, pH 8.0) was added and the samples vortexed for a few seconds. The tubes were centrifuged for 5 min at 16000 x g. The aqueous phase was transferred to a sterile Eppendorf tube where 2 volumes of 100% ethanol were added. The samples were mixed thoroughly and centrifuged for 5 min at 21000 x g. The supernatant was discarded and the DNA pellet was rinsed with 0.5 ml

of cold 70% ethanol. The supernatant was discarded and DNA pellets in the tubes left to dry. The DNA pellets were left overnight to dissolve in 100 µl TE buffer at 4^{o}C.

2.2.9 PCR amplification for DGGE analysis

18S rDNA-PCR

The set of eukaryal primers that was used for amplifying variable regions of 18S rDNA was the forward Ef 1427 – 1453 (5′ TCTGTGATGCCCTTAGATG 3′) and the reverse Er 1616 – 1637 (5′ GCGGTGTGTACAAAGGGCAGGG 3′) (Van Hannen *et al*., 1998). A GC clamp was added to the forward primer (Muyzer *et al*., 1993).

Each 50 µl of mixture contained 20 ng of template DNA, each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 5 µl of Buffer IV (Abgene), and 3 units of *Taq* polymerase (Abgene).

Amplifications were performed in a TC-312 Techne thermal cycler. Template DNA was initially denatured for 5 min at 94°C followed by 10 cycles of touchdown PCR at 94°C for 1 min, 66°C for 1 min decreasing 1°C every cycle and 72°C for 3 min. Finally, 20 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 3 min were performed and completed with a final extension step of 72°C for 10 min (Ercolini *et al*., 2003).

26S rDNA-PCR

The NL1 (5' GCCATATCAATAAGCGGAGGAAAAG 3') and the reverse LS2 (5' ATTCCCAAACAACTCGACTC 3') set of primers was used for amplifying variable regions of 26S rDNA (Cocolin *et al*., 2002). A GC clamp was added to the forward primer (Muyzer *et al*., 1993; Cocolin *et al*., 2002).

Each 50 µl of mixture contained 20 ng of template DNA, each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.5 mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl and 1.5 U of *Taq* polymerase (Abgene).

Amplifications were performed in a TC-312 Techne thermal cycler. Template DNA was initially denatured for 5 min at 95°C followed by 30 cycles of 95°C for 1 min, 52°C for 45 s and 72°C for 1 min, completed with a final extension step of 72°C for 7 min.

Electrophoresis

Aliquots (5 µl) of PCR products mixed with 1 µl 6X loading dye (Promega, UK) were separated by electrophoresis on 1.5% agarose gel with 1 x TAE at 70 mA for 1 h. Ethidium bromide (40 µg per 100ml of gel) was used to stain DNA bands. A 100 bp molecular weight marker (Promega, UK) was included to determine the size of the amplified DNA fragments, which were viewed under UV light using an ImageMaster® VDS system (Pharmacia Biotech).

2.2.10 DGGE analysis

DGGE analysis of PCR products was performed using a Dcode apparatus (Bio-Rad, UK). Samples were applied to 8% (wt vol^{-1}) polyacrylamide gels in 1 x TAE buffer. Parallel electrophoresis was performed at 60°C by using gels containing a range of concentrations of urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% [wt vol^{-1}] formamide); 20-60% and 30-45% for 18S rDNA DGGE analysis of Stilton isolates, 5-60% for 26S rDNA DGGE analysis of different parts of Stilton cheese. The electrophoretic conditions were 50 V for 10 min followed by 130 V for 360 min. The gels were stained with 100 ml ethidium bromide solution (0.1 mg / 100 ml) for 5 min, rinsed in distilled water for 20 min and viewed under UV light using an ImageMaster® VDS system (Pharmacia Biotech).

2.2.11 Sequencing of DGGE amplimers

DNA amplimers to be sequenced were excised from 26S PCR DGGE gels using sterile scalpels and transferred into sterile Eppendorf tubes containing 20 µl of sterile ultra pure deionised water. Each DNA sample was eluted into the aqueous phase overnight at 4^{o}C. Depending on the DNA concentration (estimated based on the brightness of the band), 1-2 µl of the aqueous phase was used as DNA template for a new PCR reaction in order to obtain a sufficient amount of template DNA for sequencing. The PCR protocol followed was the same but the respective primers were without the GC clamp. Following re-amplification, the PCR product was loaded on 1% agarose (Biogene) gel in 1 X TAE buffer supplied with 70mA for 30 min. The

DNA bands were cut after minimum possible exposure to UV and purified with QIAquick Gel Extraction Kit (Qiagen) or Wizard PCR Preps DNA Purification system (Promega) according to the manufacturer's instructions. The DNA was collected in 50 µl of water.

In order to concentrate the DNA, the purified DNA solutions were mixed with 0.1 volumes of 3 M sodium acetate (pH 5) and 2 volumes of 100% ethanol and then stored at -20^{o}C for 1 h. Samples were incubated at room temperature for 5 min and centrifuged at 13000 x g at 4^{o}C for 45 min. The pellet was washed twice with 70% ethanol and allowed to dry at room temperature for 20 min.

The samples were sequenced at MWG (Germany) laboratory and the sequences were compared with those in the GeneBank database (National Center for Biotechnology Information) using the Basic Local Alignment Search Tool (Altschul *et al*., 1990; NCBI, 2007) in order to determine their closest known relatives.

2.2.12 RFLP analysis

RFLP analysis of DNA from yeast isolates was conducted using primers for the 5.8S rRNA gene. These were the ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Esteve-Zarzoso *et al*., 1999). PCR amplifications were performed in a TC-312 Techne thermal cycler. Each 50 µl of mixture contained 20 ng of template DNA, each primer at a concentration of 0.5 µM, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 2.5 mM $MgCl_2$, 5 µl of Buffer IV (Abgene), and 2 U of *Taq* polymerase (Abgene). Template DNA was initially denatured for 5 min at 95°C followed by 35 cycles of 94°C for 1 min, 55°C

for 2 min and 72°C for 2 min, completed with a final extension step of 72°C for 10 min. Aliquots of PCR products (10 µl) mixed with 2 µl 6X loading dye (Promega, UK) were separated by electrophoresis on 1.5% agarose gel with 1 x TAE at 85 mA for 1.5 h.

The restriction digest reactions consisted of 20 µl PCR product, 10 units of one of the restriction enzymes; *Hae*III (5'…GG▼CC…3'), *Cfo*I, (5'…GCG▼C…3'), *Hinf*I (5'…G▼ANTC…3') in the appropriate buffer. All the above reactions were incubated at 37°C for 3 h. From each digest 10 µl aliquots were mixed with 2 µl 6X loading dye and were run on a 3 % gel with 1 x TAE for 2 h at 85 mA. For agarose gel electrophoresis ethidium bromide (40 µg per 100 ml of gel) was used to stain DNA bands. A 100 bp molecular weight marker (Promega, UK) was included to determine the size of the amplified DNA fragments which were viewed under UV light using an ImageMaster® VDS system (Pharmacia Biotech). The sizes of the bands were calculated using the Gel Analysis Kit software (Wang, 2008).

2.2.13 TRFLP

T-RFLP analysis of fungal communities was performed using the 5.8S forward (5' TCGATGAAGAACGCAGG 3') (Dickinson, 2007) and FITS reverse 1 (5' ATATGCTTAAGTTCAGCGGGT 3') (Ranjard *et al*., 2001; referred to as 3126T) set of primers targeting the 5.8S rRNA gene (see Figure 20.9). The FITS reverse 1 was labelled with green D3 dye (Sigma Proligo). PCR amplifications were performed in a TC-312 Techne thermal cycler. Each mixture (final volume 30 µl) contained 20 ng of template DNA, each primer at a concentration of 0.33 µM, each

deoxynucleoside triphosphate at a concentration of 0.25 mM, 2 mM $MgCl_2$, 3 µl of Buffer IV (Abgene), and 0.3 U of *Taq* polymerase (Abgene). Template DNA was initially denatured for 3 min at 94°C followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, completed with a final extension step of 72°C for 15 min.

Aliquots of PCR products (5 µl) were mixed with 1 µl 6X loading dye (Promega, UK) and separated by electrophoresis on 2 % agarose gel with 1 x TAE at 85 mA for 1h. Ethidium bromide (40µg per 100ml of gel) was used to stain DNA bands. A 100 bp molecular weight marker (Promega, UK) was included to determine the size of the amplified DNA fragments, which were viewed under UV light using an ImageMaster® VDS system.

The restriction digest reactions consisted of 5 µl PCR product, 5 units *Hae*III (5'…GG▼CC…3'), 1 µl of 10X NE Buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9 at 25°C) and 3.5 µl of dH_2O (all the material purchased from New England, Biolabs). All the above reactions were incubated at 37^{o}C for 3 h. From each digest 5 µl were then run on a 2 % gel with 1X TAE to determine their intensities. These were then diluted appropriately and loaded into a 96-well plate with each well containing 40 µl of Genome Lab sample loading solution and 0.5 µl of Genome Lab DNA size standard-600 bp (Beckman-Coulter, UK). The samples were then overlaid with one drop of mineral oil and sent for fragment analysis on CEQ 8000 fragment analysis system (Beckman Coulter, UK) in the Plant Sciences Division, School of Biosciences, University of Nottingham. Sample data consisted of the size (length in base pairs) and the peak high for each TRF, analysed using the CEQ 8000 operation software.

2.2.14 *Candida catenulata* reference strains

Candida catenulata reference strains were provided from the National Collection of Yeast Cultures for TRFLP analysis. These were the strains NCYC 39 and NCYC 1369. The cultures, which were freeze dried, were rehydrated with 0.5ml YPD broth, sub-cultured in 10ml YPD broth thrice and twice on plates at 25^{o}C. Individual colonies were then used for DNA extraction and TRFLP analysis.

2.3 RESULTS AND DISCUSSION

2.3.1 Enumeration of fungi in Stilton cheese

The fungal populations of the three different sections of Stilton cheese, blue, white and outer, were compared using several selective media. Ideally, the selective medium for enumerating yeasts and moulds should support the growth of all species uniformly and regardless of individual pH, nutrient, a_w and growth temperature requirements. The growth of bacteria should be suppressed for unbiased fungal counts.

However, ideal media do not exist and a choice, based on its selectivity performance in recovering counts and the highest diversity of recovered species, was needed for the analysis of Stilton. Four media were assessed for their efficiency in analysing the fungal communities. These were Rose Bengal Chloramphenicol Agar, Dichloran Rose Bengal Chloramphenicol agar, Dichloran Glycerol agar and Malt Extract agar. The selection was based on a previous study on the recovery of fungal populations from blue veined cheeses (Viljoena *et al*., 2004).

The colony forming units (cfu) for the three parts of Stilton are presented in Table 2.1. Yeasts and mould were distinguished by colony morphology and counted in separate. These are given as means based on three replicates expressed as $\log_{10}$ cfu g^{-1} accompanied by standard deviations (SD). Fungi in all parts of the Stilton cheese exhibited populations greater than 10^6 cfu g^{-1}. Yeasts were within the range 10^6-10^8 cfu g^{-1}. The number of yeasts in the outer crust was about 10-fold higher than those in the blue veins and about 50-fold more than those in the white core. This higher count in the outer part is in agreement with other studies of blue cheeses produced in

Table 2.1 Viable counts of fungi from three Stilton regions.

		RBCA*		DRBC*		DG18*			
Targeted group	**Sample**	**Log cfu g^{-1}**	**SD**	**Log cfu g^{-1}**	**SD**	**Log cfu g^{-1}**	**SD**	**F value**	**Pr > F**
Yeasts	Outer	7.86 (a)	0.05	7.88 (a)	0.05	7.85 (a)	0.05	0.393	0.691
Yeasts	Blue	7.03 (a)	0.12	7.03 (a)	0.08	6.9 (a)	0.07	1.791	0.246
Yeasts	White	6.57 (a)	0.02	6.51 (b)	0.02	6.48 (b)	0.03	11.853	0.008
Moulds	Outer	-	-	-	-	-	-		
Moulds	Blue	7.42 (a)	0.05	7.32 (ab)	0.06	7.29 (b)	0.19	3.152	0.116
Moulds	White	6.95 (a)	0.04	6.91 (a)	0.05	6.92 (a)	0.03	0.623	0.573

* RBCA ~ Rose Bengal Chloramphenicol Agar; DRBC ~ Dichloran Rose Bengal Chloramphenicol Agar; DG18 ~ Dichloran-Glycerol Agar. All media were incubated at 25^{o}C. The data are the means based on three replicates.

SD: Standard deviation

Pr>F: Probability that there is a difference between means. Means in the same row labelled with different letters (a, b) are significantly different ($P < 0.05$) as shown by ANOVA.

different countries. It is well established in the literature that yeast counts differ significantly between the exterior and interior for most of the blue cheese varieties (Italian Gorgonzola, Danish blue, Australian blue cheeses, Polish Rokpol, blue cheeses produced in South Africa), with the exterior exhibiting higher yeast populations (a minimum of 10-fold) (Roostita *et al*., 1996; Gobbetti *et al*., 1997; Van den Tempel *et al*., 1998; Wojtatowicz *et al*., 2001; Viljoen *et al*., 2003). The higher number of yeasts in the outer part of the blue cheeses is often explained by the presence of oxygen, the exposure to the environment and the inoculation from the brine. However, there is not a lot of information on the difference between the populations in the veins and the white core of blue cheeses. The blue part in Stilton had 5-fold higher yeast counts than the white core. This could also be because of the presence of oxygen, introduced when the cheese is pierced, which favours their growth.

The counts of moulds were broadly at the same levels with yeasts. Moulds were higher in the blue part than in the white part. Surprisingly, there was no mould recovery in the outer part samples on any type of medium. Based on morphological examination *Penicillium roqueforti* was the only mould species that was observed. This is supported by the later molecular analysis of the cheese where no other mould species were detected either.

Regarding the comparison of the tested media (Table 2.1), RBCA tended to result in higher counts, however, the differences where not statistically significant in all the cases. The RBCA yeast count for the white part was significantly higher than with the other two media. The RBCA mould count for the blue part was higher than the DG18 count but not higher than the DRBC. Bacterial colonies were detected on ME (mainly Gram positive rods) and therefore its use was rejected. The selectivity of

media supplemented with antibiotics, such as RBCA, DRBC, is reported to be superior to media based on acidification, such as ME. The latter is reported to yield lower yeast counts (Beuchat, 1993). ME is an acidified medium and therefore it should be less efficient for suppressing lactic acid bacteria.

In addition, Dichloran and Rose Bengal suppress the spreading of moulds, and thus would facilitate the counting of yeasts in the co-presence of the starter *P. roqueforti*. DG18 agar has been shown to perform well for enumerating moderately xerotolerant yeasts (Beuchat, 1993).

Stilton contains significant concentrations of NaCl. It was considered that its microflora might be adapted to salt and therefore salt-supplemented media could be required for its enumeration. However, Addis *et al*. (2001) examined this possibility using media supplemented with NaCl and found no significant differences between blue cheese populations obtained on supplemented and non-supplemented media.

Isolates of yeasts and mould from both RBCA and DRBC were observed macroscopically and microscopically. There was no obvious difference in the types of isolates that were recovered. RBCA was therefore selected for use in subsequent experiments. RBCA plates from several dilutions from the three parts of the cheese were used for the collection of yeast isolates. A collection of 48 (10 from blue, 19 from white, 19 from outer) yeast isolates was randomly selected and sub-cultured for further study.

2.3.2 Micro-sampling of different sections of Stilton for fungal counts

A series of counts was obtained after micro sampling of the different parts of the cheese and ensuring no contamination from different sections. The aim of this

experiment was to confirm the trends that were found with the previous standard counting method as well as to isolate more yeast species which could be precisely attributed to the specific region.

The results (Table 2.2) confirmed that the outer part contained higher yeast counts and the blue part contained higher counts than those in the white core. The differences between the parts were greater. The range of the counts was larger (10^5-10^8) but still within the values reported in the literature (Roostita *et al*., 1996; Gobbetti *et al*., 1997; Van den Tempel *et al*., 1998; Viljoen *et al*., 2003). With this sampling method the blue part appeared to have higher counts than before (7.6 log cfu g^{-1} instead of 7.03 log cfu g^{-1}) and the white significantly lower (5.07 log cfu g^{-1} instead of 6.57 log cfu g^{-1}). The higher counts for the white part in the previous counting could be because of poorer separation of the sections. Moulds were higher in the blue part than in the white but once again absent from the outer samples. It should be remembered that the sample quantity is small and higher variation could be expected. Nevertheless, the results of the analysis demonstrated clearly the higher fungal populations in the blue part than those in the white.

RBCA plates from several dilutions from the three parts of the cheese were used for the isolation of yeasts. A collection of 40 yeast isolates (12 from blue, 15 from white, 13 from outer) was randomly selected and sub-cultured for further study. Two isolates of *P. roqueforti* (one from the blue veins and one from the white core) were also collected.

Table 2.2 Viable counts of fungi in different Stilton parts after micro-sampling.

Targeted group	**Sample**	**Log CFU g^{-1*}**	**SD**
Yeasts	Outer	8.32	0.02
Yeasts	Blue	7.60	0.02
Yeasts	White	5.07	0.02
Moulds	Outer	-	-
Moulds	Blue	7.87	0.05
Moulds	White	4.93	0.09

*RBCA, 25^{o}C. The data are the means based on three replicates.

2.3.3 Grouping of yeast isolates macroscopically and microscopically

The collection of 88 Stilton yeast isolates were classified into five groups (Group 01 – 05) according to their examination macroscopically and microscopically. All the groups presented vegetative reproduction by budding except Group 05 which presented both splitting and budding. Groups 01-04 also produced ascospores and therefore these were ascomycetous yeasts.

The first group (Group 01; 8 isolates) consisted of big (0.5 mm) circular-oval shaped colonies, with undulate edges and butyrus consistency (Figure 2.2, a). Their colour was white to cream. The shape of the cells was oval and pseudohyphae and

septate hyphae were developed occasionally. There were asci containing one to four ascospores.

The second group (Group 02; 24 isolates) consisted of small (0.2 mm) pink circular colonies, with entire edges and butyrus consistency (Figure 2.2, b). The shape of the cells was oval.

The third group (Group 03; 18 isolates) had medium size (0.3 mm), white, conical shaped colonies (Figure 2.2, c). The surface of the colony was fluted vertically. The cells were circular and the asci were containing one or two ascospores. The cells tended to form clusters. The result of this could be observed in the colony consistency which was butyrus but with clots.

The fourth group (Group 04; 36 isolates) consisted of medium size (0.3 mm), circular, convex colonies with entire edges (Figure 2.2, d). Their colour was white to cream when grown on Sabouraud Dextrose and white with a slightly pink centre when grown on RBCA. The consistency was butyrus. Despite the strong differences in the colony morphology, the cell morphology of this group was identical to *Group 03* but without the formation of clusters.

The last group (Group 05; 2 isolates) consisted of medium (0.3 mm) circular, white, umbrinate colonies (Figure 2.2, e). The colonies were dry. The shape of the cells was oval and there was true hyphae and rectangular arthroconidia.

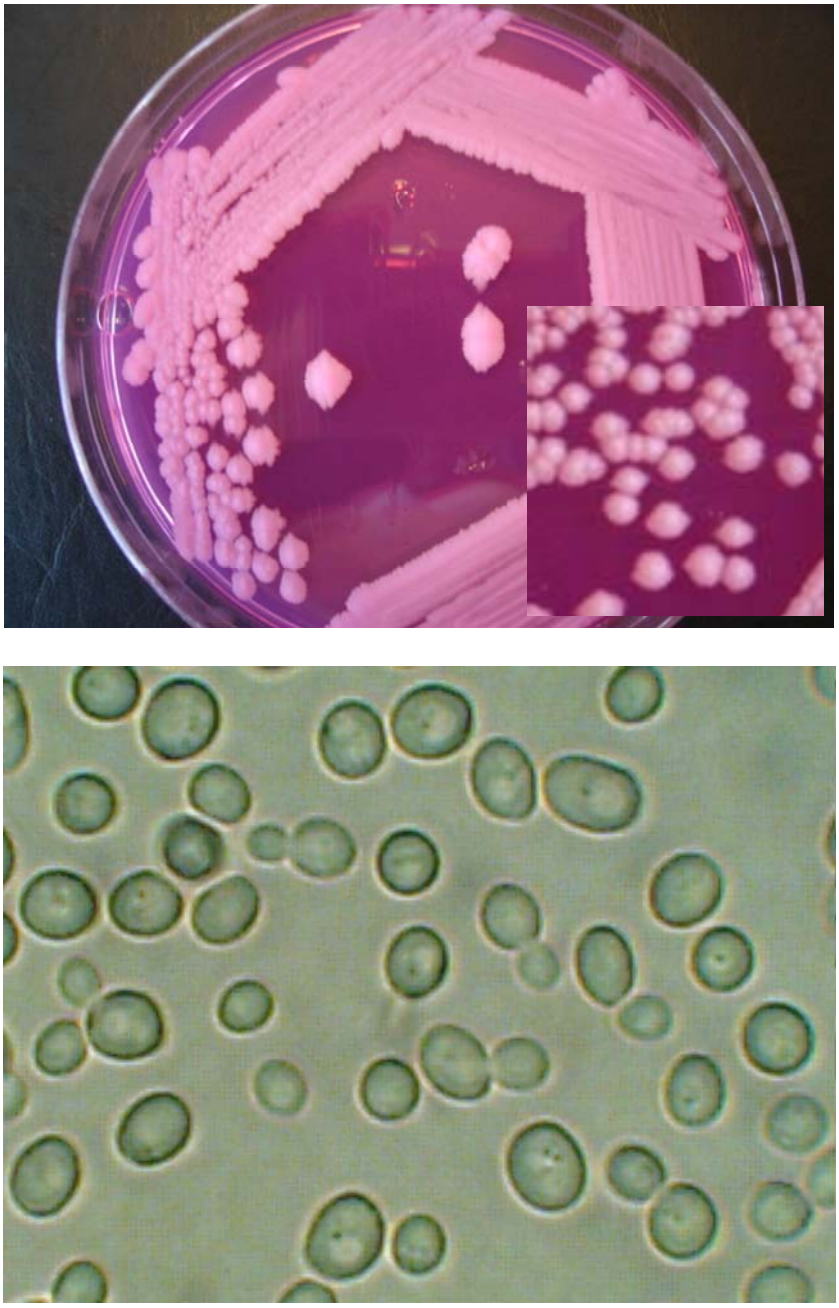


Fig. 2.2a. Colony and cell morphology of *Group 01* Stilton isolate on RBCA

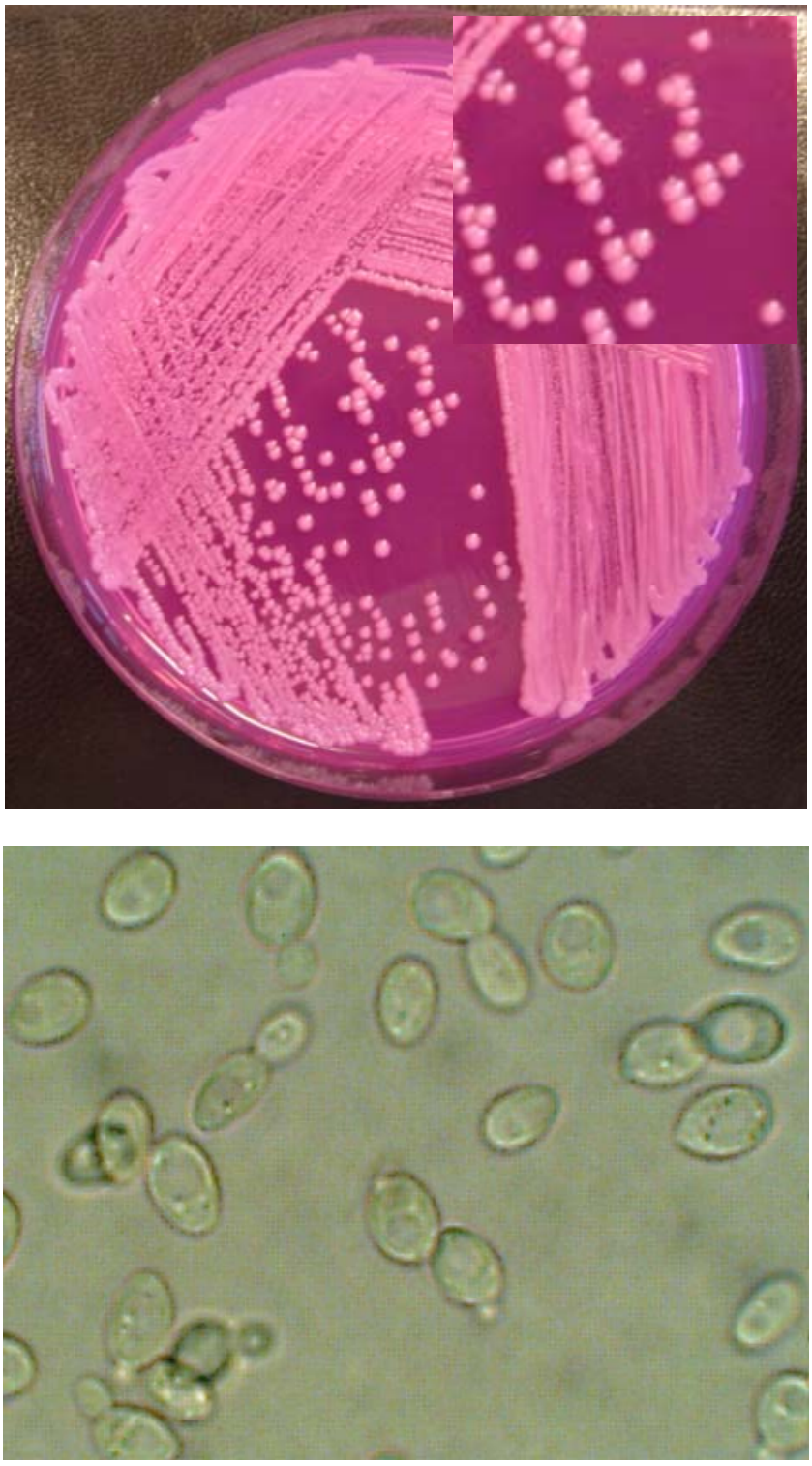


Fig. 2.2b. Colony and cell morphology of *Group 02* Stilton isolate on RBCA

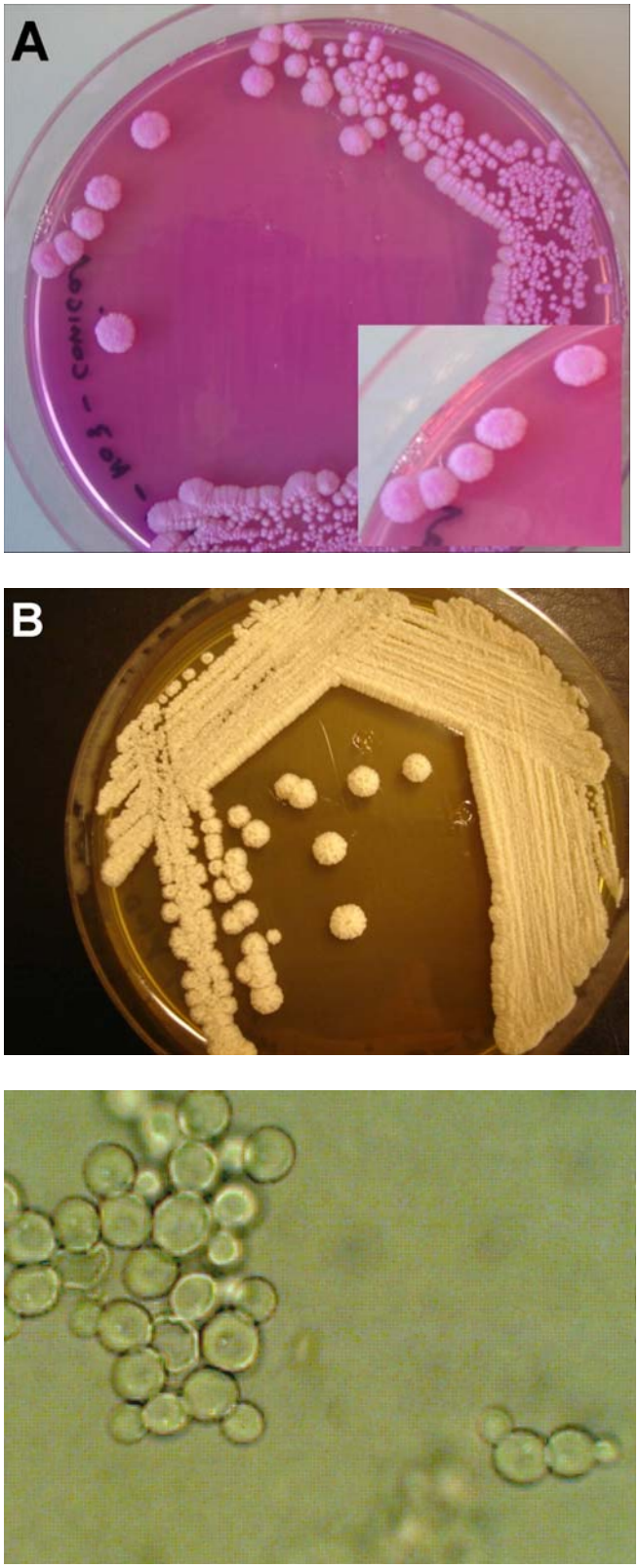


Fig. 2.2c. Colony and cell morphology of *Group 03* Stilton isolate on (A) RBCA and (B) Sabouraud Dextrose media.

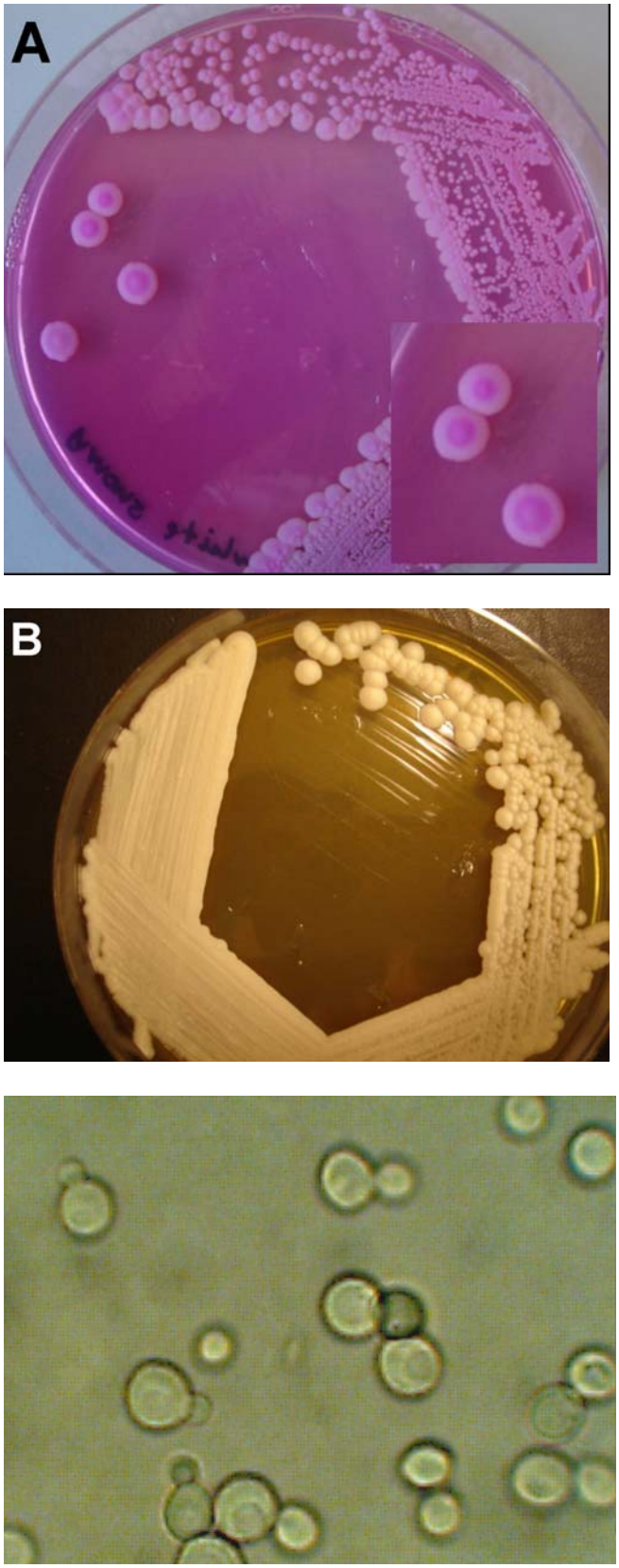


Fig. 2.2d. Colony and cell morphology of *Group 04* Stilton isolate on (A) RBCA and (B) Sabouraud Dextrose media.

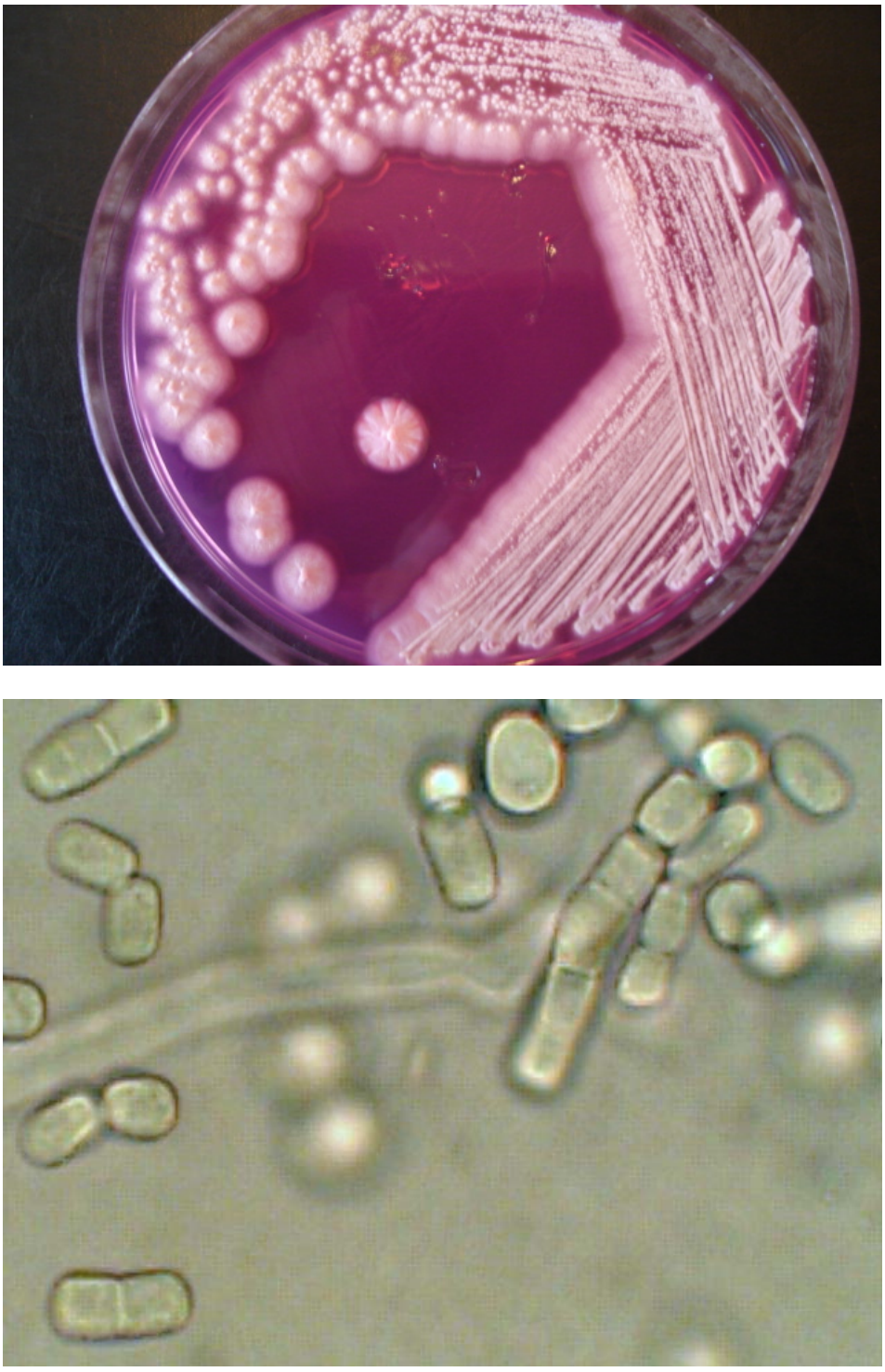
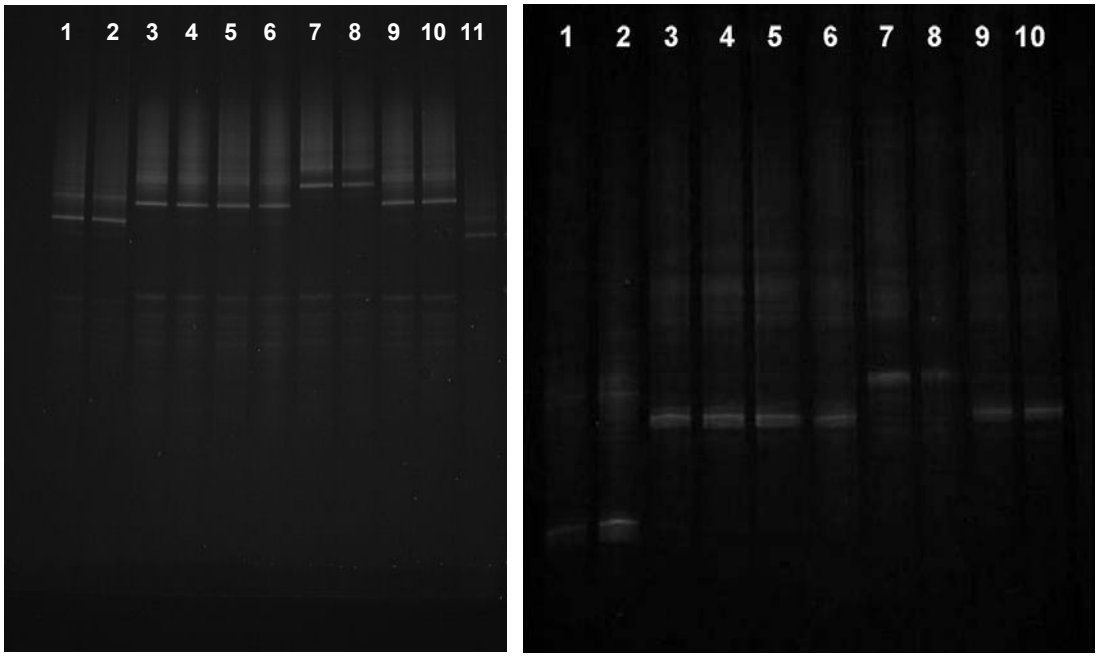


Fig. 2.2e. Colony and cell morphology of *Group 05* Stilton isolate on RBCA media.

2.3.4 18S PCR DGGE on yeast isolates from Stilton

Following the grouping of the isolates according to their macroscopic and microscopic characteristics, 18S PCR DGGE was applied for molecular analysis. The aim of this analysis was to group the isolates according to their molecular similarity and compare it with the results that were obtained macroscopically and microscopically. In addition, it was intended to optimise the technique for future analysis of mixed communities directly from the different sections of the cheese. Using the 18S primers the discrimination between the five groups was poor (Figure 2.3a). Three of the five groups of isolates, *Group 02* with *Group 03* and *Group 04*, showed bands which were co-migrating. Overall the range of the separation on the DGGE gel (range 20%-60%) was narrow with most of the yeasts and *P. roqueforti* banding close together. There was no significant improvement in separation when a narrower denaturing range (30%-45%) was applied (Figure 2.3b).

The results are in agreement with previous studies reporting the limited analytical efficiency of DGGE when combined with primers targeting the 18S gene. The DGGE analysis of the eukaryotic community of fermented maize (ben Omar *et al*., 2000) using 18S primers resulted in a limited number of intense bands, and undesirable bands, corresponding to nuclear DNA from maize, dominated the profiles. Similar problems were observed in 18S DGGE analysis of fungi in cassava. All the distinguishable bands in the DGGE profiles were corresponding to non-microbial DNA (Ampe *et al*., 2001). DGGE analysis of 18S rDNA PCR products also failed for direct analysis of vanilla beans (Röling *et al*., 2001). Therefore, it was concluded that the 18S DGGE would not be appropriate for the study of the pure Stilton isolates and later the mixed microbial samples extracted from the different Stilton sections.



(a) (b)

Figure 2.3 18S rDNA DGGE analysis of Stilton isolates. (a) Gel range 20-60%. (b) Gel range 30-45%. Running conditions 130 V for 360 min. Lanes 1-2: *Group 01*; 3-4: *Group 03*; 5-6: *Group 04*; 7-8: *Group 05*; 9-10: *Group 02*; 11: *Penicillium roqueforti*.

2.3.5 RFLP on isolates from Stilton

ITS-PCR RFLP was applied to all 88 Stilton yeast isolates in order to group them according to their molecular similarity. In addition, the restriction patterns of the ITS-PCR amplicons would potentially allow identification of species by comparing them with results available in the literature. The ITS-PCR amplicons and their subsequent restriction patterns were generated from the region spanning the ITS1 and ITS2 and the 5.8S rRNA gene (also referred to as 5.8S±ITS region).

Each yeast isolate produced a single amplified ITS-5.8S rDNA PCR product (Figure 2.4) and the molecular sizes of the PCR products were identical for isolates of the same morphological groups. The PCR amplicons showed satisfactory size variation, with molecular sizes ranging from 350 bp to 720 bp (Table 2.3). In contrast to the DGGE results, *Group 02* isolates were differentiated from the rest of the morphological groups; however, *Group 03* and *Group 04* isolates resulted in PCR products of the same molecular size. Therefore, the size of the PCR amplicon was capable of separating the isolates to a certain degree but it was not able to separate these two groups which had different morphological appearance.

Restriction patterns of the PCR products from each group of isolates were obtained (except for the *Group 05* isolates for which only ITS-PCR was performed as only a few isolates were recovered) with the *Cfo*I, *Hae*III, and *Hinf*I enzymes (Figure 2.5) in order to identify the isolates further and examine any possible differentiation within each group or between *Group 03* and *Group 04*. The summary of the ITS-PCR fragments and the restriction fragments with each endonuclease can be seen in Table 2.3. Fragments smaller than 100bp were not considered as they could not be measured accurately. No differences were found between patterns from isolates of the same

morphological group which had been isolated from different sections of the cheese. The digestion of the PCR products of *Group 03* and *Group 04* isolates with 3 restriction endonucleases resulted again in identical profiles suggesting that they could be two different morphological groups but of the same species. Their digestion patterns with *Hinf*I (Figure 2.5; lanes 9 and 10) resulted in only one visible fragment of 315bp, while the initial ITS-PCR product was 630bp long. Therefore it was concluded that the band after digestion was composed of double fragments. The occurrence of double fragments is usual in restriction patterns of RFLP analysis (Esteve-Zarzoso *et al*., 1999).

The level of discrimination with RFLP was greater than with DGGE. RFLP was an affordable and fast way for comparing all the isolates according to their molecular similarity. However, the RFLP results could not be assigned with confidence with any of the patterns that were previously reported for RFLP analysis of yeasts using the same primers and restriction enzymes (Esteve-Zarzoso *et al*., 1999) and further methods were required to provide an identification.

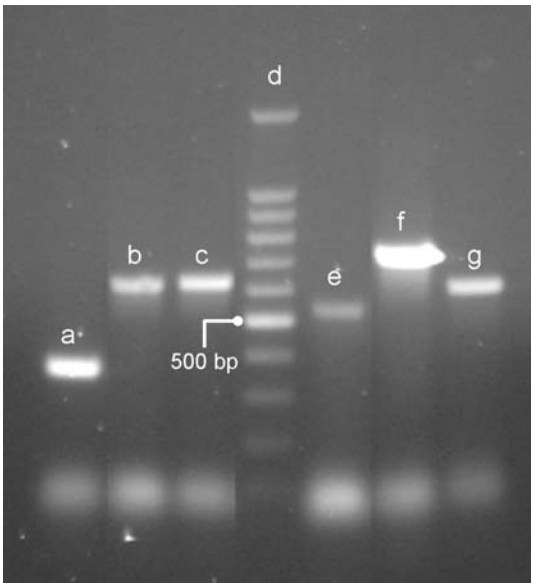


Figure 2.4 ITS-PCR amplicons before RFLP analysis (1.5% agarose gel). (a) *Group 01*, (b) *Group 03*, (c) *Group 04*, (d) 100bp ladder, (e) *Group 05*, (f) *Group 02*, (g) *Penicillium roqueforti*.

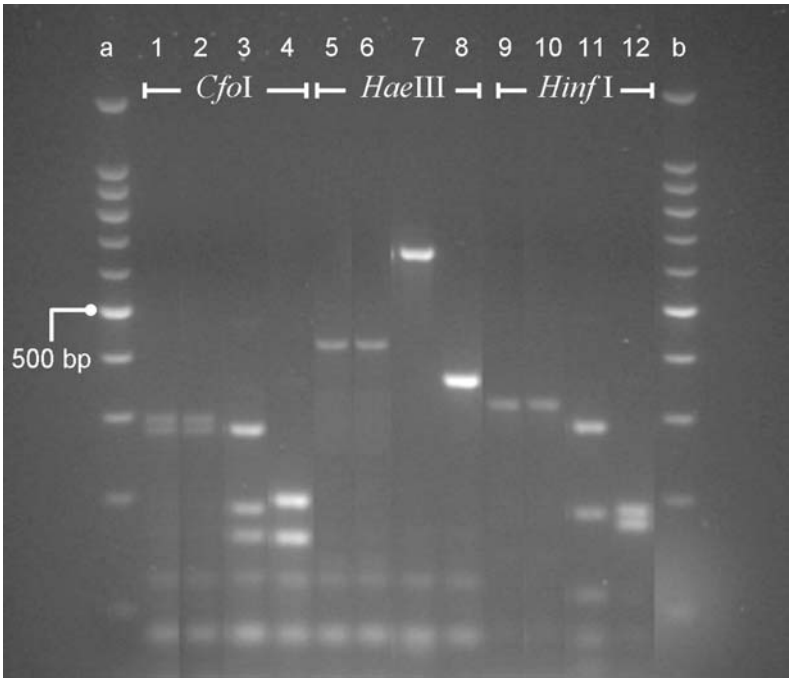


Figure 2.5 RFLP analysis of Stilton isolates using *Cfo*I, *Hae*III and *Hinf*I. (3% agarose gel). *Group 03* lanes 1, 5, and 9; *Group 04* lanes 2, 6 and 10; *Group 02* lanes 3, 7 and 11; *Group 01* lanes 4, 8 and 12. Lanes a and b are 100bp ladders.

Table 2.3 Size (bp) of PCR amplicons and RFLP fragments of the main fungi found in Stilton.

Species	**PCR amplicon**	***Cfo*I**	***Hae*III**	***Hinf*I**
Group 01	350	190, 150	350	180, 160
Group 02	720	280, 180, 150	610, 110	280, 180, 110
Group 03	630	300, 300	415, 130	315, 315
Group 04	630	300, 300	415, 130	315, 315
Group 05	540	-	-	-
Penicillium roqueforti	600	-	-	-

2.3.6 26S PCR-DGGE analysis of yeast isolates from Stilton

Preliminary PCR-DGGE analysis of Stilton yeast isolates using 18S primers (Section 2.3.4) resulted in poor discrimination of bands and co-migration of *Group 02*, *Group 03* and *Group 04*. Subsequent RFLP analysis showed higher diversity between the groups. Equally good discrimination needed to be achieved with DGGE before this could be applied for the direct analysis of the yeast communities in the cheese. For this reason 26S PCR DGGE was examined using the yeast isolates and the starter *P. roqueforti* using the NL1GC/LS2 set of primers. In addition, bands corresponding to representative isolates from each group were excised from the gel and sequenced to obtain an identification (Table 2.4). For isolates from *Group 01*, *Group 02* and *Group 05* the sequences were performed on the D1 region of the 26S subunits using the forward primer only. For *Group 03* and *Group 04,* longer sequences were obtained by sequencing using both forward and reverse primers and then the two sequences were combined using the BioEdit biological sequence alignment editor. From these results the isolates were found to be closest relatives of *Yarrowia lipolytica* for *Group 01*, of *Kluyveromyces lactis* for *Group 02*, *of Trichosporon ovoides* for *Group 05*, and of *Debaryomyces hansenii* for both *Group 03* and *Group 04*. These were designated *Debaryomyces hansenii* group A and *Debaryomyces hansenii* group B respectively. Comparison of the *Debaryomyces hansenii* groups' sequences did not demonstrate any genetic differentiation within the analysed regions (248 bp). *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Debaryomyces hansenii* are ascomycetous yeasts and match the microscopic observation of ascospores in these groups of isolates.

Table 2.4 Identities of bands obtained from 26S PCR DGGE analysis of the D1 region of DNA extracted from Stilton isolates recovered after cultivation of different parts of the cheese on RBCA.

Group	Closest match	Origin	Homology (%)	GenBank Accession no.*
01	*Yarrowia lipolytica*	RBCA – outer part of the cheese	100	EF362750.1
02	*Kluyveromyces lactis*	RBCA – blue part of the cheese	99	AF374614.1
03	*Debaryomyces hansenii*	RBCA – outer part of the cheese	100	DQ409146.1
04	*Debaryomyces hansenii*	RBCA – outer part of the cheese	100	DQ409146.1
05	*Trichosporon ovoides*	RBCA – blue part of the cheese	99	EF537891.1

The use of 26S primers (Figure 2.6; lanes 1-4) overcame the co-migration problem and satisfactory differentiation was obtained between *Debaryomyces hansenii* and *Kluyveromyces lactis*. However the bands from *D. hansenii* strains from groups A and B co-migrated in all cases. Occasionally DGGE profiles of pure *D. hansenii* and *K. lactis* cultures included a second band (Figure 2.6, lane 4; Table 2.5, No 1-2). Multiple bands from a single species have been observed in many DGGE studies of fungi. Masoud *et al*. (2004) reported the presence of three *Pichia kluyveri* bands that migrated very close to each other in the profile of coffee beans. The sequences of the bands were differing in one to two base pairs and it was suggested that they represent different strains of the same species. However, in the present study double bands were observed even for DGGE analysis of pure cultures of *D. hansenii* and *K. lactis* (Figure 2.7; lane 4) and therefore they could not be the product of different strains. The presence of multiple bands for pure cultures of the same strain has been reported previously and was suggested to be the result of amplification of DNA molecules with slight differences in migration behaviour due to incomplete extension of the same template caused by the GC clamp (Nubel *et al*., 1996; Rosado *et al*., 1998). Another study reported four bands for *Geotrichum candidum* (Florez *et al*., 2006). In contrast to previous reports, in the present study the secondary bands were not close to the main bands. Like all Eukaryotes, yeasts can be polyploid with rDNA operons which would not necessary be exactly identical on each chromosome and this could result in multiple bands. Therefore, for DGGE results from mixed communities careful interpretation is needed in order not to overestimate the community diversity.

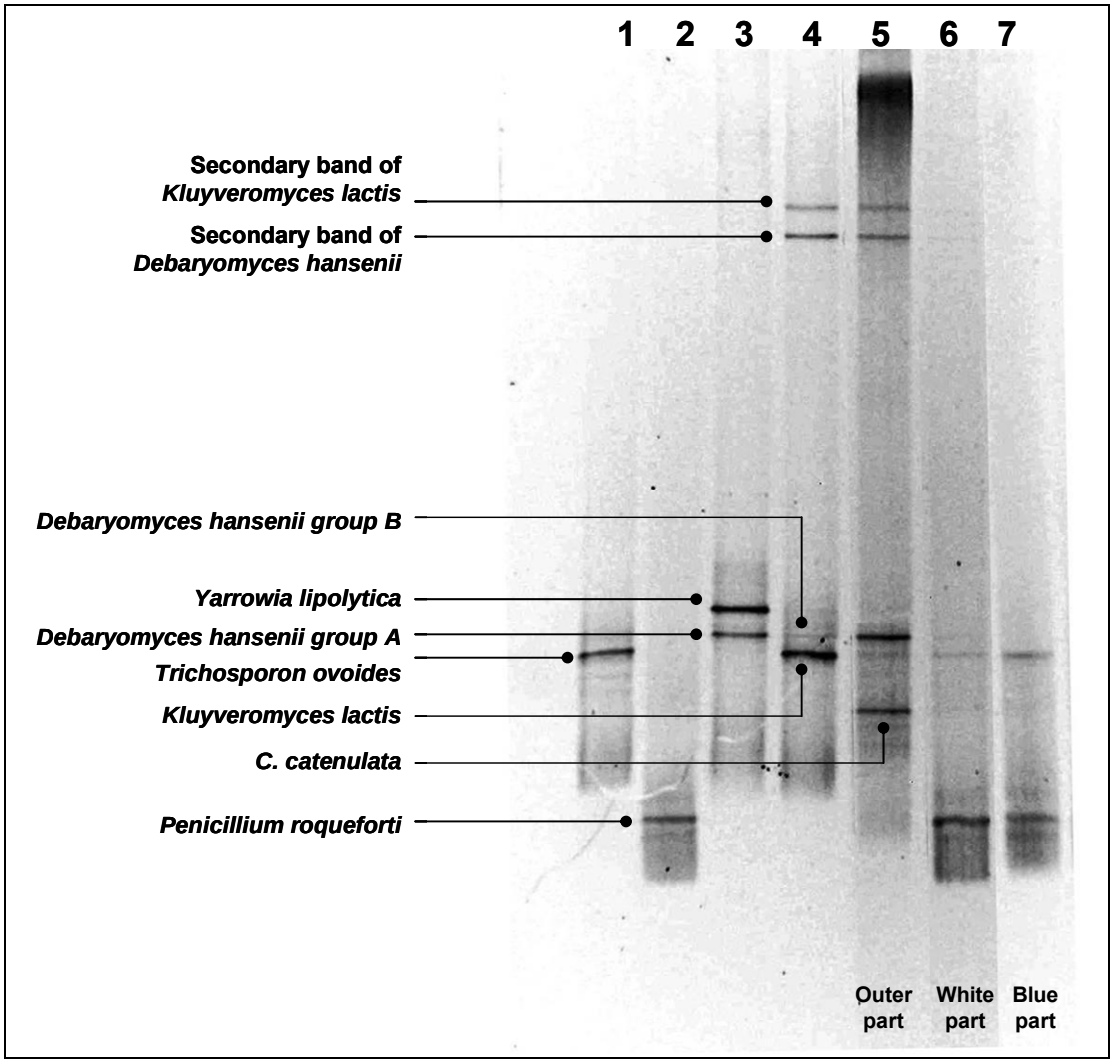


Figure 2.6. Gel (5-60% range) of 26S rDNA DGGE analysis of pure yeast isolates and different parts of Stilton cheese. Running conditions 130 V for 360 min. Lanes 1-4 marker strains. Lanes 5-7 Stilton patterns.

Table 2.5 Sequencing results of DNA bands from the D1 region of 26S rDNA subunit of yeasts present in Stilton

No.	Closest match	Homology (%)	GenBank Accession no.*	Part of cheese used
1	*Kluyveromyces lactis*	98	EU186075.1	Outer, blue
2	*Debaryomyces hansenii*	99	FJ473450.1	Outer
3	*Candida catenulata*	99	FJ473449.1	Outer

2.3.7 API

Selected isolates were further characterised by using the API 20C AUX assimilation kit. The API tests were not able to identify successfully all the selected isolates but there was still a good indication of the degree of variation in the assimilation profiles for isolates from the same groups. The assimilation profiles of 12 isolates are summarised in Table 2.6.

For *Group 01* isolates identified by sequencing as *Yarrowia lipolytica* the identification was of limited value. The isolates were identified as *Candida krusei/inconspicua* with the possibility of *Candida lipolytica* (anamorph of *Yarrowia lipolytica*). No correct identification was possible without the further required tests (glucose, erythritol, thiamine, and actidion test). There were no differences between the profiles from the *Yarrowia lipolytica* group.

Table 2.6 Assimilation profiles of yeast isolates from the 5 groups isolated from Stilton cheese.

	Groups of isolates											
SEQUENCIING ID	***Kluyveromyces lactis***		***Yarrowia lipolytica***				***Debaryomyses hansenii* group A**		***Debaryomyses hansenii* group B**		***Trichosporon ovoides***	
API ID	***Candida sphaerica***		***Candida krusei / inconspicua***				***Candida famata***		***Candida famata***		***Cryptococcus terreus***	
%ID - COMPOUND	**77.4**	**99.2**	**98.9**	**98.9**	**98.9**	**98.9**	**-**	**99.9**	**99.9**	**99.9**	**99.9**	**99.9**
None	-	-	-	-	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	+	+	+	+	-	+	+	+	-	-
Calcium 2-Keto-Gluconate	-	-	-	-	-	-	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	+	+
Adonitol	-	-	-	-	-	-	-	+	+	+	-	-

Xylitol	-	-	-	-	-	-	-	-	-	+	-	-
D-Galactose	+	+	-	-	-	-	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	+	+	-	-	-	-	+	+	+	+	-	-
Methyl-αD-Glucopyranoside	-	-	-	-	-	-	+	+	+	+	-	-
N-Acetyl-Glucosamine	-	-	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	-	+	-	-	-	-	+	+	+	+	+	+
D-Lactose	+	+	-	-	-	-	+	+	+	+	+	+
D-Maltose	+	+	-	-	-	-	+	+	+	+	-	-
D-Saccharose	+	+	-	-	-	-	+	+	+	+	-	-
D-Trehalose	-	-	-	-	-	-	-	+	+	+	+	+
D-Melezitose	-	-	-	-	-	-	+	+	+	+	-	-
D-Raffinose	+	+	-	-	-	-	-	-	-	-	-	-
Hyphae / pseudohyphae	-	-	-	-	-	-	-	-	-	-	+	+

The *Group 02* isolates of *Kluyveromyces lactis* were identified as *Candida sphaerica* (anamorph of *Kluyveromyces lactis*). The test performed well for one of the isolates (%ID 99.2) but was poorer for the other (%ID 77.4). This was because of its inability to ferment D-cellobiose. These results would suggest that isolates of *Group 02* are not identical which the molecular methods did not indicate.

Identification of the *Group 05* isolates by API gave an identification of *Cryptococcus terreus* which did not correspond with the sequencing identification of *Trichosporon ovoides*. Both isolates had the same profile suggesting a single population. Finally, three of the four *D. hansenii* isolates were identified as *Candida famata* (anamorph of *D. hansenii*) (%ID 99.9). All four isolates of *D. hansenii* groups A and B gave different profiles suggesting a mixed population. The diversity was caused because of differences in the ability to grow in glycerol, L-arabinose and xylitol. There was no obvious differentiation between the profiles of *D. hansenii* isolates coming from groups A and B. However, the number of isolates studied was low.

The API 20C AUX was designed for use with clinical strains and does not perform well with all food isolates (Ramani *et al*., 1998). For the Stilton isolates the API tests were able to provide information on the metabolic properties of the isolates and satisfactory identification of three groups of isolates after taking in account their sexual stage that was observed microscopically.

2.3.8 Distribution of yeasts in different parts of Stilton

The collection of 88 Stilton isolates (22 from blue, 34 from white, 32 from outer) was studied regarding their distribution in the different parts of the cheese after

division into the 5 groups based on their macroscopic and microscopic observation (see §2.3.3): *Kluyveromyces lactis, Yarrowia lipolytica*, *Trichosporon ovoides*, and two *Debaryomyces hansenii* groups. The two *D. hansenii* groups (A and B) have been studied separately because of their obvious morphological differences. The abundance of each yeast group in the different parts of the cheese was calculated as a percentage of the total number of isolates (Figure 2.7).

The results from the two different batches of viable count plates were broadly the same. The yeast communities in each part of the cheese consisted of different species and/or had common species but in different quantities. There were species dominating the outer part which were present in the white core but absent from the blue; vice versa, yeast species dominant in the blue veins were absent from the outer part.

The blue part was dominated by *K. lactis* (68%) which was also present in the white part (26%). However, *K. lactis* was absent from the outer part. Overall, it was the most abundant species in the interior of the cheese. A few isolates of *Trichosporon ovoides* were occasionally isolated from the blue part.

D. hansenii group B was the second largest group in the blue veins and together with *K. lactis* this group dominated the population. It was present in the white core at the same level (~25%) and was also the dominant species in the outer part. Considering that the yeast counts for the outer part were at least 10-fold higher than in the other parts, it could be concluded that *D. hansenii* group B was one of the most abundant yeast species in the cheese matrix.

Both *D. hansenii* group A and *Y. lipolytica* were isolated from the outer and white parts but not from the blue veins. *D. hansenii* group A consisted of 32% of the

white part population and 22% of the outer while *Y. lipolytica* was present in lower levels (16% and 9% respectively).

D hansenii isolates overall dominated the Stilton cheese. They consisted of half of the flora of the white part and the majority of the outer part. The uneven distribution of *D. hansenii* group A and *D. hansenii* group B in the different parts of the cheese suggested that their strong morphological differentiation is accompanied by differentiation in their physiological and/or biochemical properties. Indeed, the four isolates of *D. hansenii* that were examined with API (Table 2.6) presented different biochemical profiles.

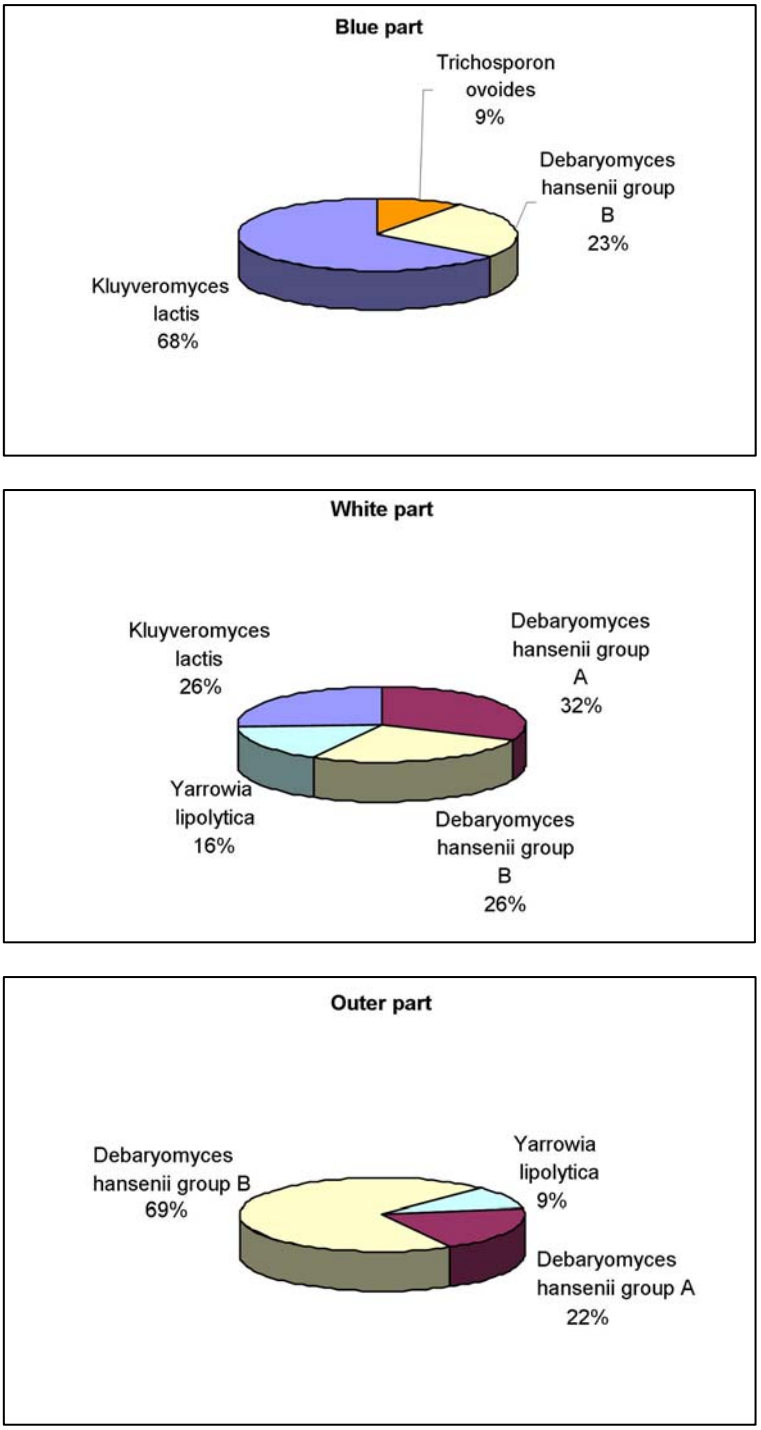


Figure 2.7 Relative concentrations of groups of Stilton isolates in each part of Stilton expressed as percentages of the total number of isolates.

2.3.9 *D. hansenii* group A and B

Isolates of *D. hansenii* group A and B did not present any genetic differentiation with RFLP analysis despite their strong morphological differences. RFLP analysis would not allow differentiation between isolates from different subspecies or between different strains of the same species (Petersen *et al*., 2001). For this reason some further physiological tests were performed in order to investigate the possibility of the isolates in groups A and B belonging to two known subspecies of *D. hansenii*: *D. hansenii* variety *fabryii* or *D. hansenii* variety *hansenii*.

Three isolates randomly selected from each *D. hansenii* group were grown on YPD medium at 25^{o}C (unbiased growth conditions for all the varieties) and 38^{o}C (selective growth temperature of *D. hansenii* variety *fabryii*; Barnett *et al*., 1990) in order to check whether they belonged to different subspecies. None of the isolates was able to grow at 38^{o}C.

2.3.10 PCR-DGGE analysis of cheese

Having optimized the 26S PCR DGGE conditions using isolates from the five groups, this DGGE approach was then used for direct analysis of the Stilton fungal flora, using fungal DNA directly extracted from the three parts of Stilton cheese. Three independent DNA extractions from each Stilton part were performed and two replicates run on DGGE gels. The DGGE profiles of the different cheese parts are presented in Figure 2.6 (lanes 5-7).

The microbial diversity obtained by PCR-DGGE was similar to that obtained with culture dependent techniques. *K. lactis*, *Y. lipolytica* and *D. hansenii* species recovered with the culture media were also present in the direct extraction DGGE profiles. However, no *T. ovoides* was detected with direct extraction DGGE analysis. A *Candida catenulata* band (identified by sequencing), a species not included in the collection of isolates, was present in the outer part profiles (Table 2.5, No 3).

From the three parts of the cheese, the blue part was the one with the least complex community. It comprised only the yeast *K. lactis* and the starter mould *P. roqueforti*. This is in agreement with the culture-based results where *K. lactis* dominated the blue parts (68%); however the other major species detected by culturing, *D. hansenii* (23%), was not detected with DGGE.

The DGGE profiles of the white core samples consisted of a strong *P. roqueforti* band and two other weak bands of *D. hansenii* and *K. lactis*. Both species were detected by culturing from these samples. In contrast to the culture results, no *Y. lipolytica* was detected in the DGGE profiles of the white part.

The outer part profile was the most complex. It included a strong *D. hansenii* band and a very weak band of *Y. lipolytica*. This is in agreement with the culture-based results where these species were 91% and 9% of the profile respectively. In addition, *K. lactis* was only present in the DGGE profiles from this area. This was a very weak band which suggests that this species is present in this part of the cheese but not in high amounts and therefore can not be recovered on culture media. Two secondary bands, one for *D. hansenii* and one for *K. lactis*, were also seen in the outer part profiles but no *P. roqueforti* bands were present. This is in agreement with the culture-dependent analysis where *P. roqueforti* was not cultured from the outer samples. Interestingly, a new strong band belonging to *C. catenulata* yeast species

was present in the outer part DGGE profiles. This was a species that was not detected in any of the Stilton parts using culture-dependent techniques.

DGGE provided data on the yeast communities in the different parts of Stilton additional to those obtained with culture-dependent techniques. In particular it detected a species not found by the culture based methods. There are numerous studies which use DGGE for fungal analysis of food samples including coffee beans (Masoud *et al*., 2004), wine (Cocolin *et al*., 2000; Di Maro *et al*., 2007) and vanilla beans (Röling *et al*., 2001). In addition, the DGGE technique was recently used for studying the fungi of microbial communities in dairy foods including blue cheeses (Florez *et al*., 2006) and raw milk (Cocolin *et al*., 2002). However, by reviewing the results available in the literature it can be concluded that in many cases the discrimination power of 18S PCR-DGGE was poor and non-microbial DNA bands were recovered. Such problems were not observed with 26S PCR-DGGE. In addition the D1/D2 domain of the 26S rRNA is acknowledged to give best phylogenetic discrimination. It is now accepted as the main tool for yeast taxonomy and databases include all yeast species described to date (De Llanos Frutos *et al*., 2004).

2.3.11 TRFLP analysis

A TRFLP technique was developed and modified using yeast isolates obtained from the culture-dependent technique. The aim of this study was to semi-quantify and compare the amount of each species in the different parts of the cheese. Representatives from each of the 5 groups of Stilton yeasts and the starter *P. roqueforti* were used. The technique was then used to characterize the members of the

mixed microbial communities present in the different parts of Stilton as well as comparing the quantities of each species within each profile and comparing between profiles.

2.3.11.1 TRFLP analysis on pure cultures

PCR templates (Figure 2.8) were generated using a set of primers targeting the 5.8S rRNA gene. In general, analysis of the 5.8S region has been shown to result in improved resolution between the taxonomic groups compared to restriction analysis of the 18S rDNA region (Lord *et al*., 2002). The forward primer was newly developed (Dickinson, 2007) and applied for food analysis for the first time. It amplifies from the 5.8S rRNA gene towards the ITS2 without including any of the ITS1 region (Figure 2.9). Therefore the pair of primers targets the 5.8S rRNA-ITS2 region.

The main TRFs produced by using the restriction enzyme *Hae*III ranged from 68 to 418 bp (Table 2.7). All individual strains of the same species yielded the same TRFLP profile using the *Hae*III restriction enzyme. The different *D.hansenii* groups A and B also yielded identical TRFP profiles and could not be distinguished. Therefore, four distinct TRFLP profiles were produced (Figure 2.10). *D. hansenii* and *P. roqueforti* species resulted in more than one TRF. The electropherograms of *D. hansenii* comprised a main TRF (68bp) but occasionally a secondary fragment was produced (367bp). The same fragment was produced in many independent replicate runs and therefore it was considered as a real TRF and not a pseudo-TRF or PCR artefact. The same pair of fragments was also detected later in the TRFLP profiles of

the cheese sections. The TRF_{68}/TRF_{367} ratio ranged from 20 to 30. *P. roqueforti* had a more

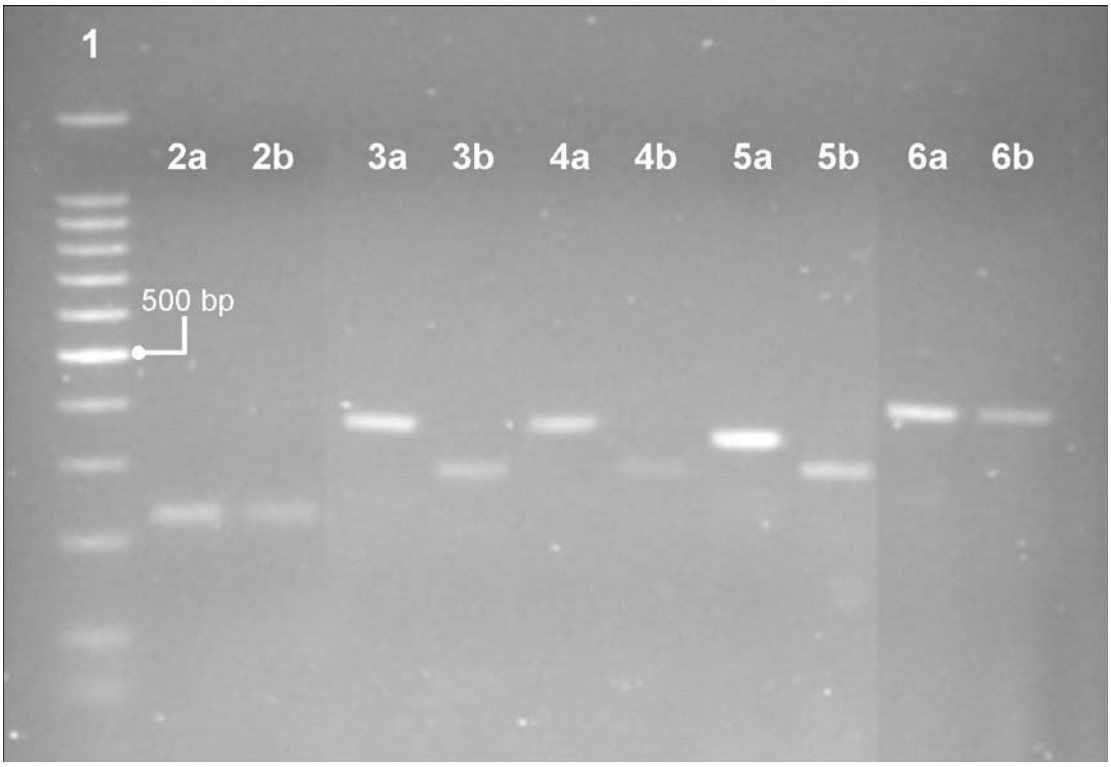


Figure 2.8 Gel of PCR products and their digests of Stilton yeast isolates for T-RFLP analysis (2 % agarose gel). Products before and after digestion with *Hae*III (a and b respectively). From left to right: lane 1, 100bp ladder; lanes 2, *Yarrowia lipolytica*; lanes 3, *Debaryomyces hansenii* group A; lanes 4, *Debaryomyces hansenii* group B; lames 5, *Trichosporon ovoides*; lanes 6, *Kluyveromyces lactis.*

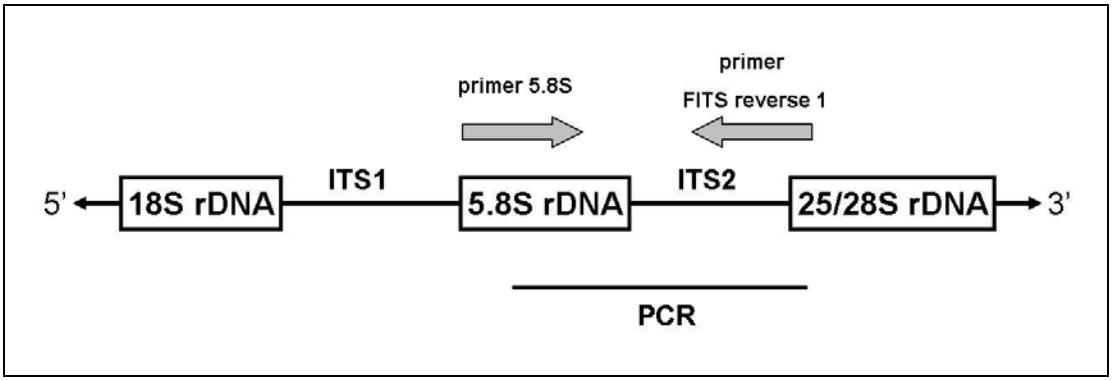


Figure 2.9 Target area within the ribosomal genes for the TRFLP pair of primers that was used for the analysis of Stilton fungal isolates (Dickinson, 2007).

complex TRFLP profile composed of 4 TRFs (Figure 2.10). The TRF_{77}/TRF_{84} ratio ranged from 10 to 20. As in the case of multiple DGGE bands, this could be explained from the fact that polyploid eukaryotes have a greater number of chromosomal pairs (diploid, triploids, etc) and this could result in multiple bands.

Replicate TRFs from isolates of the same species differed by less than 1 bp. The same was true for TRFLP analyses of the same samples carried out on different days indicating that the size of the fragments is highly reproducible. Therefore one characteristic TRF profile was obtained for each group of isolates.

Table 2.7 Fragments (bp) of TRFLP analysis of fungi species found in Stilton.

Species	**Main fragments**	**Secondary fragments**
Yarrowia lipolytica	225	
***Debaryomyces hansenii* A**	68	367
***Debaryomyces hansenii* B**	68	367
Trichosporon ovoides	343	
Kluyveromyces lactis	418	
Penicillium roqueforti	77, 78.5	84, 85

Several non reproducible weak but detectable peaks were present besides the main TRFs of the pure cultures. These smaller peaks may correspond to TRFs which are a result of sequence variations in different operons or PCR artefacts such as non-specific primer annealing. This phenomenon is well documented in bacterial TRFLP studies (Rademaker *et al*., 2005; Rademaker *et al*., 2006).

The PCR amplification of the DNA templates consisted of 30 cycles. The number of PCR cycles can be critical in TRFLP. A high number of cycles can result in re-annealing of gene products, formation of chimeric artifacts and pseudo-TRFs. Previous studies suggest the use of a low number of cycles in order to minimize these effects (Sanchez *et al*., 2006). However, there are cases where reducing the number of PCR cycles resulted in similar results (Osborn *et al*., 2000). The number of secondary fragments in the present study was limited and their size negligible compared to the size of the main TRFLP peaks.

The enzyme *Hae*III was used for the restriction of the PCR products. The choice of the restriction enzyme may affect the efficiency of TRFLP analysis. An optimal restriction enzyme would produce a wide range of fragment sizes, without variation within species, and should have recognition sites in most of the analysed fungi. *Hae*III was found to have the highest number of usable fragments within 26 tetrameric RFLP enzymes (Dickie *et al*., 2007). This is in agreement with a computer based study where restriction digests of published sequences where simulated for six restriction enzymes and *Hae*III generated the highest number of different TRFLP profiles (Edwards *et al*., 2005). Finally, the use of *Hae*III was successfully applied for TRFLP analysis of fungi in salads use (Dodd, C. E. R. Personal communication).

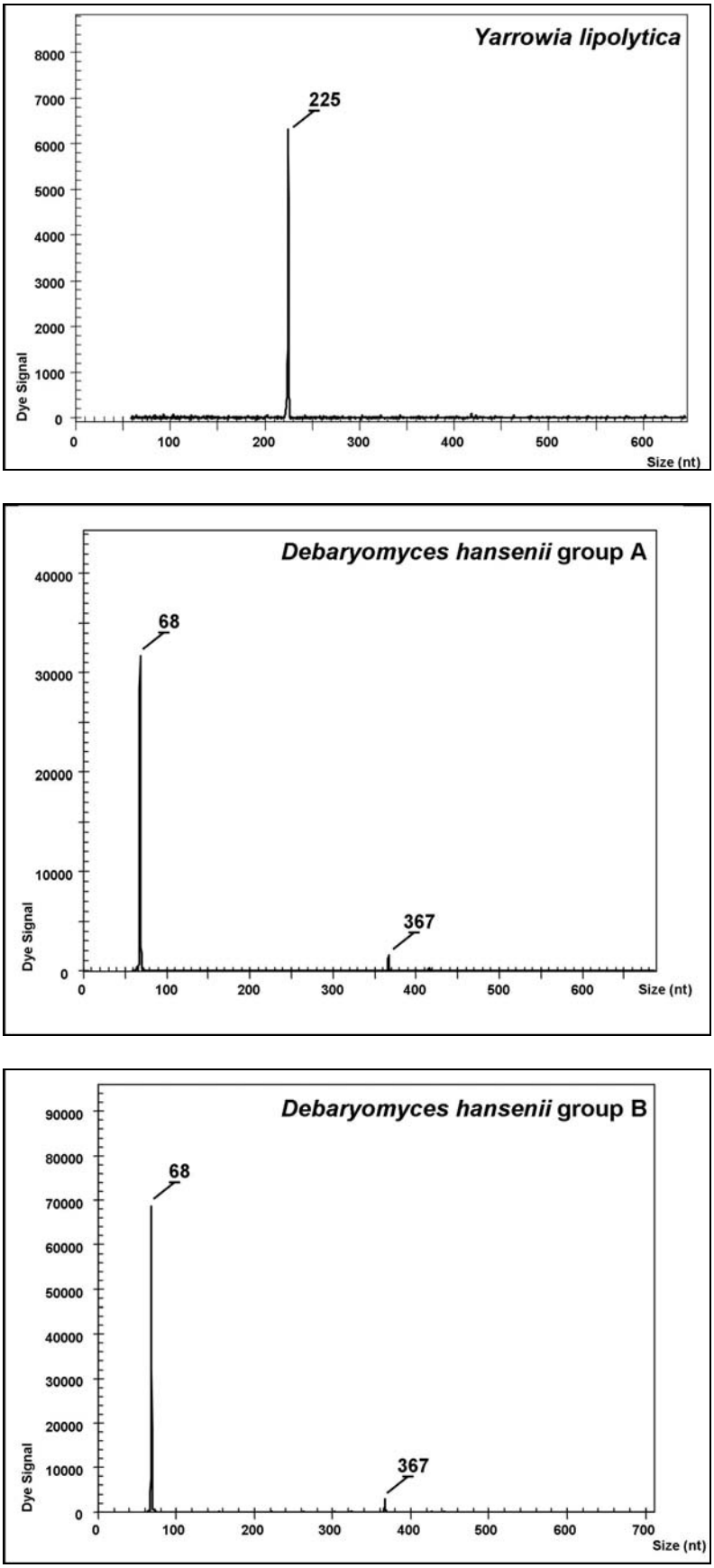
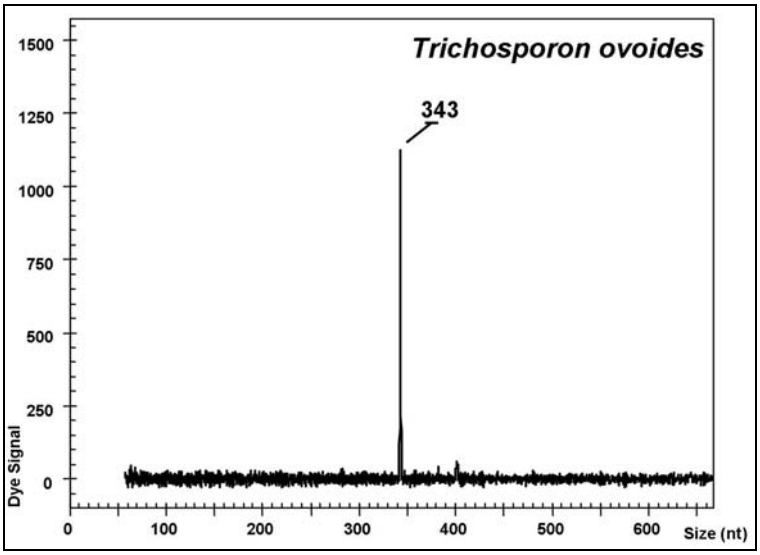
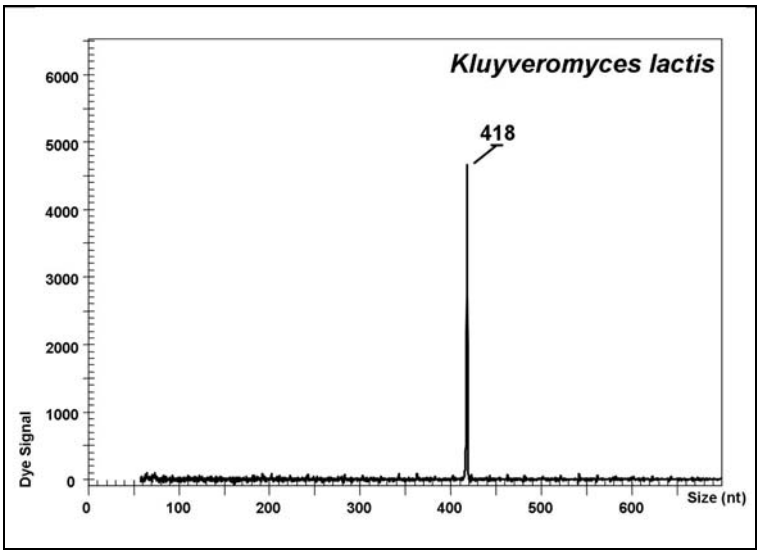


Figure 2.10 Electropherograms of TRFLP analysis of pure isolates.





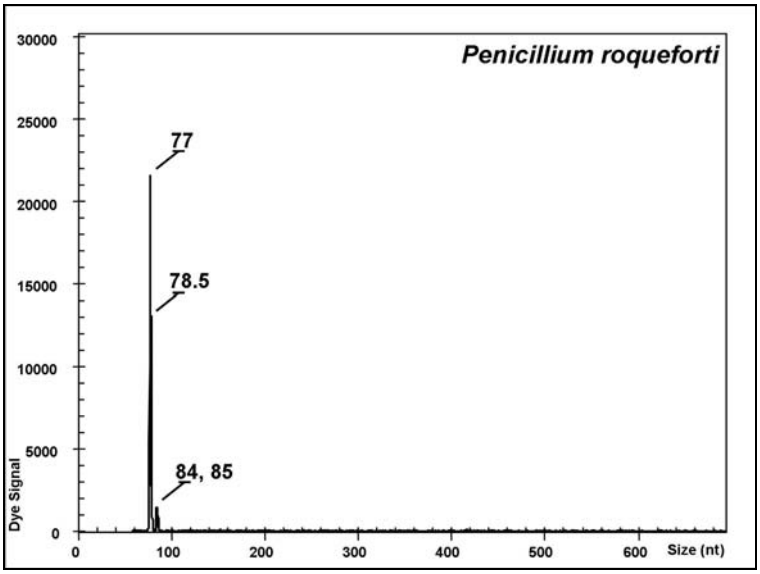


Figure 2.10 continue. Electropherograms of TRFLP analysis of pure isolates.

2.3.11.2 TRFLP analysis of cheese

TRFLP analysis of the fungal communities in the different parts of Stilton resulted in three profiles with distinct differences. Figure 2.11 displays the electropherograms from TRFLP analysis of the blue, white and outer parts of Stilton. The differences were both qualitative and quantitative. The results of the TRFLP analysis are summarised in Table 2.8.

The majority of the TRFs correlated with peaks in the profiles of the cultured Stilton isolates indicating the presence of *K. lactis*, *D. hansenii*, *Y. lipolytica* and the starter mould *P. roqueforti*. As with the DGGE analysis, *T. ovoides* was not present in the profiles.

A new TRF (257bp), not corresponding to any of the yeast isolates, was also present in the profiles. It was hypothesized that this TRF could correspond to the *C. catenulata* species that was detected by DGGE analysis. Since *C. catenulata* had not been isolated on culture media in the current study, strains from the National Collection of Yeast Cultures (NCYC 39 and NCYC 1369) were used as TRFLP references. Indeed, the TRF for *C. catenulata* was 257, the same as the new fragment present in the cheese profiles (Figure 2.12).

The signal intensities of the peaks were used to evaluate the relative concentrations of each species in the different parts of the cheese (Table 2.8). Overall the total signal intensities for the outer part were significantly higher than for the blue and white (10-fold higher) which would correlate with the higher microbial counts seen in the outer part. The starter *P. roqueforti* was the dominant species in both the blue and white parts. Its signal intensity in the blue part was approximately 3 times

higher than the white part (Table 2.8). Once again it was not detected in the outer part.

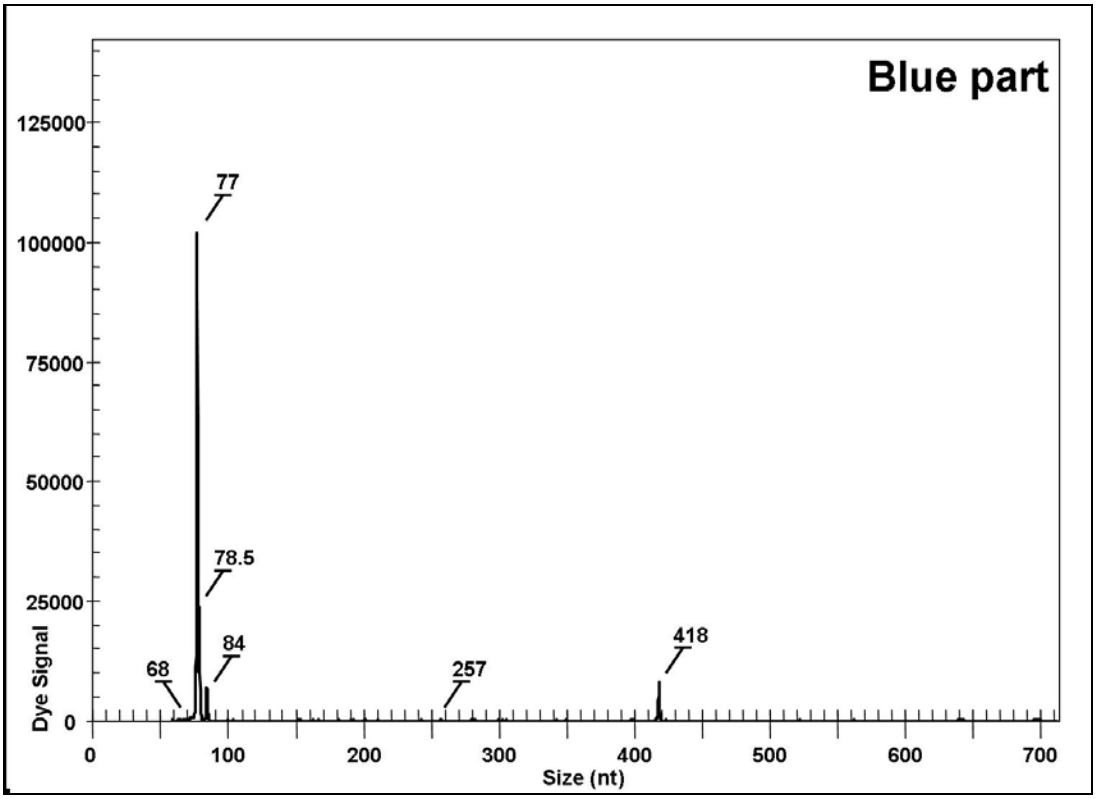
The charts in Figure 2.13 present the relative abundance of the yeast species without including the mould *P. roqueforti* and therefore they are directly comparable with those from the culture dependent analysis (Figure 2.7). The blue region profile consisted of *K. lactis*, *D. hansenii* and *C. catenulata*, with *K. lactis* dominating. Its TRF signal intensity was 10 times higher than the total signal intensity of the other two species. In contrast, DGGE was able to detect only the dominant species in the blue part (*P. roqueforti* and *K. lactis*) but not *D. hansenii* and *C. catenulata*. The white part had a more complex community including the species *K. lactis*, *D. hansenii*, *Y. lipolytica* and *C. catenulata,* with *K. lactis* and *C. catenulata* dominant. *Y. lipolytica* and *C. catenulata* were not detected with DGGE but *Y. lipolytica* was detected with the culture dependent techniques; however both in TRFLP and by culture dependent techniques this species was present in low amounts. However, *C. catenulata* was one of the dominant species in the TRFLP profiles of the white part and it is surprising that it could not be recovered with DGGE in the white part.

The outer part consisted of the same species as the white part but in different relative levels. *C. catenulata* had the higher intensity followed by *D. hansenii*, of which the intensity was three times lower. *K. lactis* and *Y. lipolytica* signals were respectively 10 fold and 100 fold lower than the signal of *D. hansenii*. Once again *P. roqueforti* was not detected in the outer region. This is in agreement with the DGGE results and the culture media findings.

Table 2.8 Summary of results of TRFLP analysis of yeasts from different parts of Stilton cheese.

	White part		Blue		Outer part	
Microorganism	**Peak height (rfu)**	**% Abundance**	**Peak height (rfu)**	**% Abundance**	**Peak height (rfu)**	**% Abundance**
P. roqueforti	40989		111570		-	
D. hansenii	863	21	437	9	12692	30
C. catenulata	1705	42	110	2	28306	67
K. lactis	1147	28	4133	89	899	2
Y. lipolytica	356	9	-	-	134	1
Total	**4071**	**100**	**4679**	**100**	**42031**	**100**

* rfu: relative fluorescent units



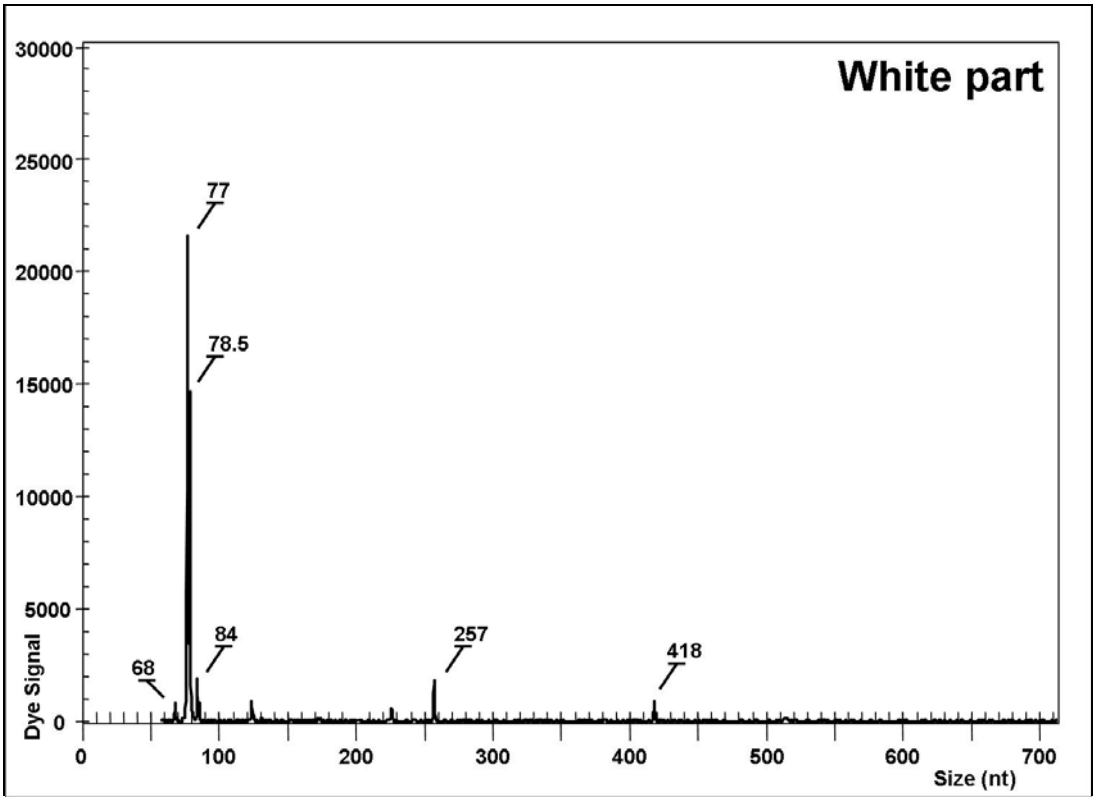


Figure 2.11 Electropherograms of TRFLP profiles in different parts of Stilton cheese.

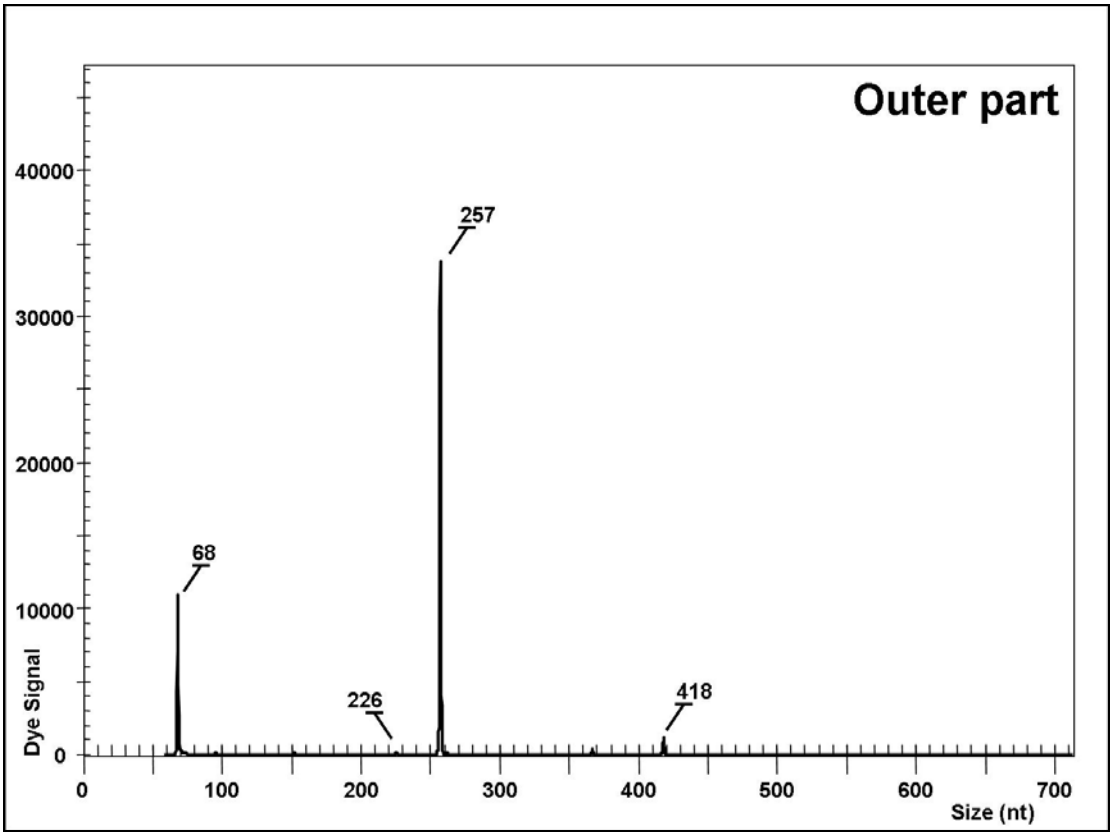


Figure 2.11 continue. Electropherograms of TRFLP profiles in different parts of Stilton cheese.

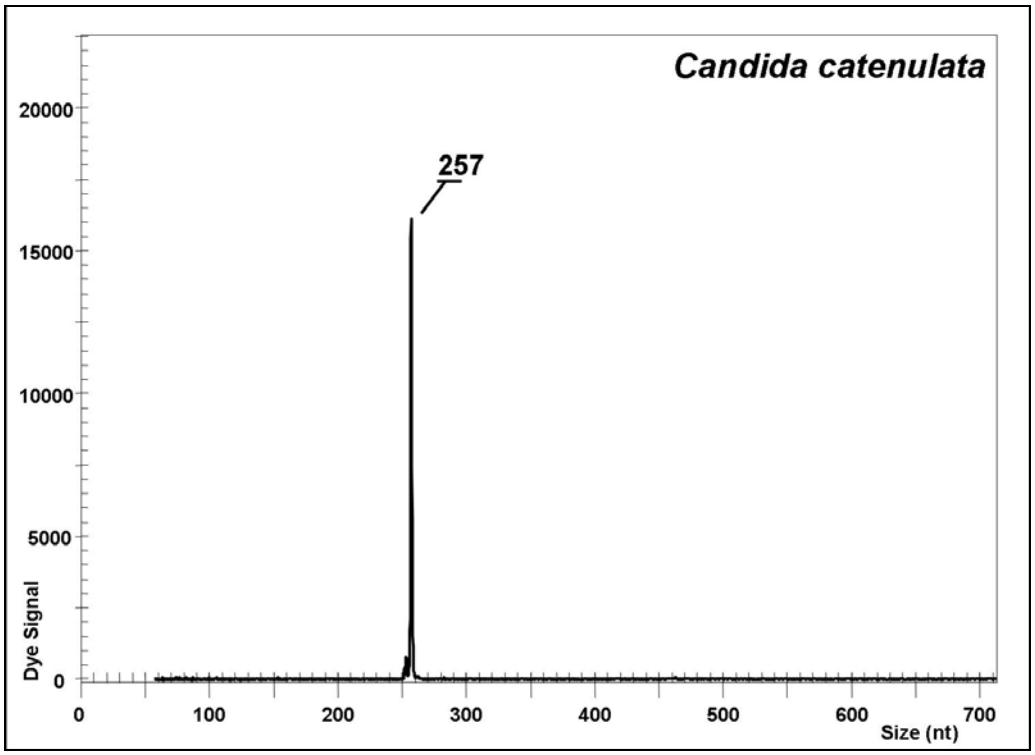


Figure 2.12 Electropherograms of TRFLP analysis of *Candida catenulata* isolate.

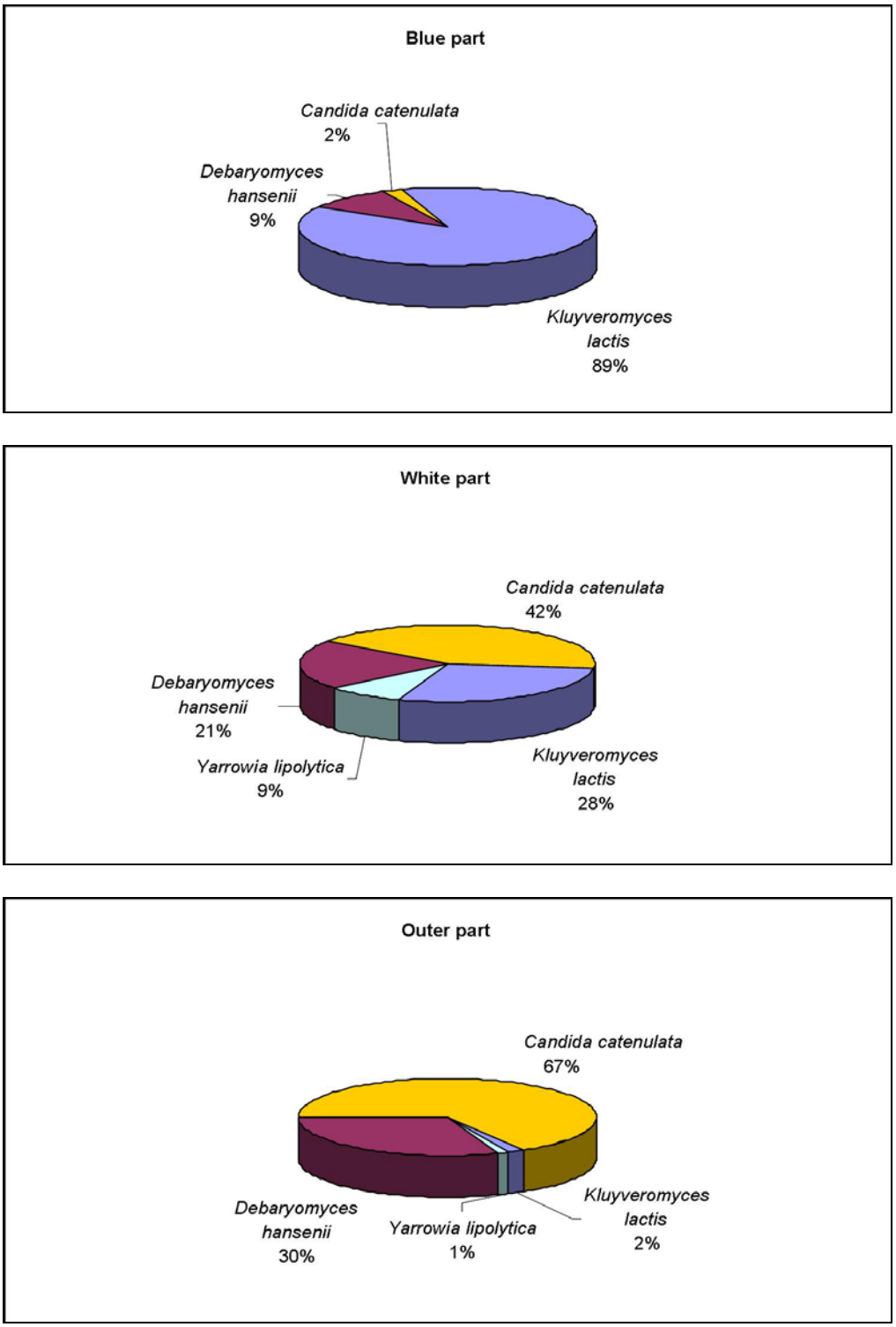


Figure 2.13 Relative abundance of Stilton species in TRFLP analysis of each part of Stilton. Results are expressed in percentages of total isolated strains.

The correlation of the peak heights (or areas) to the relative concentrations of each species in a mixed population has been applied in many studies, assuming two things: that the ratios of the amplicons in the final PCR product are proportional to the ratios of the templates in the mixed DNA and that the template ratios are proportional to the population in the food sample. In this way TRFLP is suggested for the semi-quantification of food-related microbial communities (Rademaker *et al*., 2006; Sanchez *et al*., 2006). Therefore, differences in the amount of a member of the microbial community relative to another can be observed by following the differences in height or surface area of their peaks. Comparisons can also take place between profiles of different samples.

TRFs differing by less than 1 bp were considered to be the same. The profiles of each cheese region were reproducible as regards the presence of the TRFs and their relative abundances. Reproducibility was achieved for both replicates of the TRFLP analysis of the same DNA extract and analysis of different DNA extracts from the same cheese samples. As in the case of the TFLP analysis of pure cultures, a few random non-reproducible TRFs were present among the replicates. These were restricted to small peaks.

2.3.11.3 TRFLP analysis of P. roqueforti spores

The starter *P. roqueforti* was not detected in the outer part with any of the applied techniques (culturing on media, PCR-DGGE, TRFLP). This was surprising as *P. roqueforti* spores are added to the milk during the production of Stilton. It was necessary to understand whether the mould is totally absent from the outer part or its

spores are present but do not develop. One possibility is that the efficiency of DNA extraction from mould spores with mechanical disrupting might be poor. For this reason TRFLP analysis was performed on *P. roqueforti* spores. Spores were carefully harvested from cultures grown on RBCA medium and examined microscopically in order to ensure the absence of hyphae. DNA was successfully extracted from the spores using the method used for DNA extraction from cheese and the resultant TRFs were the same as those in the previous analysis demonstrating that the analysis used on Stilton would be able to detect the presence of *P. roqueforti* spores and therefore their absence from the outer part profiles was not a result of any bias in the techniques.

2.3.12 Evaluation of the boiling based method for DNA extraction from yeast isolates

In this study, a boiling-based method was used for the DNA extraction from pure cultures of yeast isolates. The method was capable of extracting DNA from all the yeast isolates and the mould *P. roqueforti*. This DNA was successfully amplified with PCR protocols for use in DGGE, ITS-RFLP and later TRFLP analysis.

DNA extracted by boiling yeast cell suspensions has been successfully used for PCR in the last decade (Maiwald *et al*., 1994; Okhravi *et al*., 1998; Masoud *et al*., 2004). Many studies reported that boiled extracts of yeast cells can be used reliably for PCR amplification, particularly for ITS-PCR techniques. It was suggested that the density of the cell suspension is crucial for the success of the amplification (Deak *et al*., 2000), but no detailed studies on the influence and optimisation of the cell density

are available. However, there are reports that the reproducibility of boiling techniques is inefficient when combined with RAPD (Howell *et al*., 1996). In contrast to the techniques that were applied in this study (DGGE, ITS-RFLP and TRFLP), RAPD can differentiate microorganisms further than species level. It might be possible then that the quality of the DNA with this kind of extraction is not suitable for analysis at subspecies level.

In this study the amplified extracts from Stilton yeasts and *P. roqueforti* were successfully used in ITS-RFLP DGGE and in TRFLP experiments. It was noted that some PCR reactions failed when the DNA that was used had been extracted from old cultures (>3 days at 25^{o}C). However, this was not investigated in any trial. This failure could be because of the well developed cell walls seen in old cells or that lysis enzymes could be released destroying the polymerase or release of DNAses.

The application of such DNA extractions methods is of great significance in commercial laboratories where saving time is essential. Standard DNA extraction and purification protocols may require 24 h to complete while extraction by boiling and the following centrifugation is complete in less than half an hour. The time required for identification can be even more important when there is a need to confirm the presence of pathogens or identify the source of spoilage.

2.3.13 Comparison of molecular techniques

RFLP was applied for the screening and grouping of isolates according to their molecular similarities. It was an affordable and easy to perform technique on large number of isolates. It provided better differentiation than 18S DGGE by separating *K.*

lactis from *D. hansenii*. Previous studies have demonstrated the efficiency of the 5.8S±ITS region in measuring close fungus genealogical relationships since they exhibit greater interspecific differences than the conserved sequences from the 18SrRNA and 25S rRNA genes (Kurtzman, 1992; Kurtzman, 1993; Cai *et al*., 1996; James *et al*., 1996; Esteve-Zarzoso *et al*., 1999). Thus, 5.8S±ITS has been proven successful for the classification of a wide range of yeast species and databases including a great range of fungal species are nowadays available (Esteve-Zarzoso *et al*., 1999; de Llanos Frutos *et al*., 2004). However, this technique is not suitable for the analysis of mixed microbial communities and therefore its application could not be expanded to the direct analysis of the cheese.

Digestion of the PCR products with 3 restriction endonucleases resulted in identical profiles for *D. hansenii* isolates from groups A and B; therefore strain differentiation between these two groups was not possible. RFLP previously performed on the ITS1-5.8S rDNA-ITS2 region of *D. hansenii* has demonstrated no genetic diversity between strains (Petersen *et al*., 2001).

The main bias of the RFLP technique was its limited accuracy in measuring the fragment sizes. The results depend on the gel performance and in some cases varied as much as ±20bp for the same isolates analysed on different days. However, profiles of species available in the databases consist of fragments which can differ as little as 10bp with fragments from other species. Therefore sole application of RFLP analysis would not be efficient for the identification of species with confidence without previously sequencing the DNA of representative isolates.

For *Y. lipolytica* the ITS-PCR amplicon was 350bp and *Hinf*I digestion pattern was 180+160bp and differed with those reported by Esteve-Zarzoso *et al*. (1999) which were the 380bp and 190bp-190bp respectively. In contrast, the data are in

agreement with a later study (Deak *et al*., 2000) on nine *Y. lipolytica* isolates while the former study referred only to one strain. This difference could be because of the latter study being on an exceptional strain. In the current RFLP study of various Stilton isolates of *Y. lipolytica,* the size of fragments was consistent amongst all strains.

For *K. lactis,* the ITS-PCR amplicon (720bp) and digestion patterns with *Cfo*I, *Hae*III and *Hinf*I (fragment sizes of 280, 180, 150; 610, 110; 280, 180, 110 bp respectively) varied from those reported from Esteve-Zarzoso *et al*. (1999) (fragment sizes of 740; 285-190-165-90; 655-80; 290-180-120-80-65 bp respectively). Consequently, RFLP analysis results (fragment sizes of PCR amplicons and digestion patterns) of the same species may differ slightly between studies. Petersen *et al*. (2001) improved the performance of the RFLP by using fluorescently labelled primers and accurately measuring the PCR fragments based on the fluorescence signal intensity. Overall, for the present study the RFLP technique was a very useful and affordable way for the rapid screening of the genetic relatedness of the isolates.

Two culture-independent techniques, DGGE and TRFLP, were performed and compared for the analysis of Stilton fungal communities in this study. DGGE separates PCR products based on sequence-dependent double strand denaturation which is followed by changes in the electrophoretic mobility. TRFLP is a more recent technique following the latest trend for coupling restriction fragment analysis with automated sequencing separation. Both techniques have advantages, disadvantages and biases.

The fungal diversity of Stilton cheese was studied with DGGE and TRFLP analysis of the 26S and 5.8S-ITS2 fingerprints respectively. Significant differences were found between the microbial profiles of the different parts of Stilton with both techniques. Although the two methods amplified different regions of the DNA, they

led to comparable results. The outer part DGGE and TRFLP profiles consisted of the same species. For the blue part, although both techniques indicated the domination of *K. lactis,* it was only TRFLP that recovered other less dominant species (*D. hansenii* and *C. catenulata*). The same was true for the white part, where *Y. lipolytica* was present in small amounts in the TRFLP profile but it was not detected with the DGGE analysis. Overall, it could be concluded that qualitative results were better with TRFLP than with DGGE. This could be because of better resolution and sensitivity.

Sensitivity is a main concern in any molecular application on mixed microbial communities for the detection of species present in low abundance. As a result of the greater TRFLP sensitivity, a wide range of TRF signal intensities could be detected. The lowest TRF intensity was 300 times smaller than the highest. In another study the detection sensitivity of the TRFLP method was tested by performing standard PCR reactions with seeded population comprising 0.1-1% of the total community and these were successfully detected (Dunbar *et al*., 2000). In the current study TRFLP was able to detect a species present in the cheese in low amounts as well as identifying differences in quantities between the three parts of the cheese.

Another advantage of TRFLP is that the resolution in electrophoresis is superior to DGGE. The nucleic acid sequencing technology has considerably greater resolution than the electrophoretic systems of DGGE. With TRFLP the lengths of the TRFs could be measured with high accuracy (±1bp). In DGGE the identification of bands is based on the comparison of the migration of bands unless sequencing is conducted. Bands of different species can co-migrate with reference strains of other species or a band can be a combination of multiple bands of different co-migrating species (Sekiguchi *et al*., 2001) without this being visible on the gel. In our case the DGGE bands of *K. lactis* co-migrated with *D. hansenii* when using 18S primers. In

both 18S and 26S analysis all the bands were restricted to a relatively narrow range of the gel.

Random fragments appeared in TRFLP profiles even for pure cultures. These could be due to formation of chimeric molecules and pseudo-TRFs derived from partly single-stranded 5.8S rRNA amplicons and were observed in all species, although in minor amounts. This was confirmed by repeating the analysis of samples and observing that these fragments were not consistently present in the profiles. In general, pseudo-TRFs frequently appear in the TRFL profiles. The presence of pseudo-TRFs may result in overestimation of the sample biodiversity. These have also been detected in previous studies in TRFLP profiles of pure cultures (Egert *et al*., 2003; Rademaker *et al*., 2005; Sanchez *et al*., 2006). These peaks are called pseudo-TRFs since they are detected as terminal fluorescently labelled fragments but sequence data analysis has indicated that they do not represent the primary terminal restriction site. Egert *et al*. (2003) showed that pseudo-TRFs can be completely eliminated after digestion of the PCR amplicons with the single-strand-specific mung bean nuclease prior to TRFLP analysis. This treatment was not tested in the current study. Alternatively, TRFLP profiles of the same samples at different times of analysis were compared and pseudo-fragments (fragments that were not consistently present in the profiles) were not taken into account.

A significant advantage of TRFLP is that it can provide semi-quantitative data (Rademaker *et al*., 2006). The gel analysis is direct and digital. The results on the gel are transformed to data in an electropherogram form where the position of each peak corresponds to the TRF length and its height (or area) to the intensity of the fragment. With DGGE only reference to the species presence/absence in the analysed community can be done although many researchers consider the intensity (brightness)

of the bands as an indication of the DNA concentration of each species in the total mixture. Even so the intensities of the bands can not be transformed into quantitative data and one band can be the result of more than one species (Sekiguchi *et al*., 2001).

The quantification capability of TRFLP can be expanded if combined with other molecular tools. Interestingly, Sanchez *et al*. (2006) coupled TRFLP with RT-PCR for characterization of metabolically active LAB used in cheese manufacture and the semi-quantitative evaluation of their metabolic activity**.** However, in the same study PCR biases affected the quantification of the composition of the microbial community.

TRFLP has some general advantages compared to DGGE. Firstly, direct reference to previously published sequences available in databases can be done. This allows the use of software tools for estimating TRF sizes of species that would result from each primer-restriction enzyme combination (Maidak *et al*., 2000; Cocolin *et al*., 2007). These can be used prior to the analysis for evaluating their power to discriminate the microorganisms of interest. However, not all the TRFs for all the species can be predicted with the information available in the databases, especially for yeasts for which the information available is much more limited compared to bacteria. In this study the sizes of TRFs were generated in the laboratory by performing preliminary TRFLP analysis on Stilton isolates identified by sequencing.

However, for *C. catenulata,* a species detected in the outer part of Stilton with DGGE which was not isolated on culture, preliminary TRFLP could not be conducted as no cultured isolates were obtained and reference strains were needed to confirm the peak identity. This can be challenging when unknown species are found in the microbial profiles. In contrast, reference strains can be loaded on DGGE gels together with the analysed samples. DGGE also has the great advantage that unknown bands

can be extracted and sequenced and in this way the detection of *C. catenulata* was established. In DGGE excision of selected bands, re-amplification and sequencing made the phylogenetic identification fast and easy. TRFLP is based on capillary electrophoresis and its main drawback is that bands cannot be directly extracted from the gel for DNA sequencing. However, they can still be cloned and subsequently sequenced (Mengoni *et al*., 2002) but this approach is more time consuming than sequencing DGGE bands.

TRFLP seems to be more appropriate for analysis of complex communities consisting of known species especially when including species in low amounts. In addition, it lacks the difficulties arising from variation between gels by taking advantage of the high sensitivity of the sequencer in combination with the presence of internal markers in each sample.

2.3.14 Comparison of culture-dependent techniques and culture-independent approaches

Culture-dependent and culture-independent approaches were employed for the evaluation of Stilton fungal flora. Results from direct cheese analyses by TRLP and DGGE were compared with culturing on media followed by RFLP screening of isolates.

All the species isolated with culture-dependent techniques were also detected with DGGE and TRFLP analysis with the sole exception being *C. catenulata*. Despite the fact this species was detected with the molecular techniques it was not recovered by cultivation of cheese samples. However, this organism presented adequate growth

when pure cultures were grown on the same media and under the same incubation conditions. *C. catenulata* is a yeast species which has been extensively reported to be present in food including the Dana blue and Australian blue cheeses as part of their secondary flora (Fox *et al*., 2004). Therefore it is important it is detected and this illustrates an advantage of the molecular methods over the plating approach.. In contrast, *Trichosporon ovoides* was only detected by culture. However, only a few isolates of this species were occasionally isolated on media from the blue part samples.

Both count media and TRFLP analysis indicated the significantly higher fungal populations in the outer part of the cheese, with yeast counts 10-100 times higher than in the other two areas. With TRFLP the total signal intensities of the yeast species in the outer part was 10-fold higher but there were not differences between the blue and the outer parts. Such comparisons were not possible with DGGE. In addition, TRFLP and media cultivation allowed the comparison of abundances of individual species in the three different parts of the cheese. Both techniques suggested that *K. lactis* was dominating the blue part of the cheese while *D. hansenii* species were also present but in much lower amounts (68% and 23% for count media - 90% and 10% for TRLP, *K. lactis* and *D. hansenii* respectively). Cultivation of strains revealed that only *D. hansenii* group B was present in the blue veins; this fine distinction could not be observed with the molecular techniques as they do not differentiate beyond the species level.

According to culture media analysis the community from the outer part was dominated by *D. hansenii* (groups A and B) with *Y. lipolytica* being co-present but at a much lesser level. TRFLP was also able to detect a small amount of *K. lactis* but the main difference was that it detected *C. catenulata*, also present in DGGE profiles, that

was not recovered on culture media. According to the TRFLP results this was the dominant species in the outer part and therefore it could not be suggested that its absence in the culture dependent technique was because of low counts. However, it may be possible that the population of this species developed in the early stages of the cheese ripening but it did not remain viable until the final stage and maturation. In this case the DNA of the dead cells could still be present and detectable with molecular analysis. The results from both the molecular techniques and culture media suggest that the white core had the most variable community.

Although it is frequently stated that the molecular techniques are overcoming the biases of the culture dependent techniques it should be carefully considered that important biases may well apply with the molecular techniques. Each of the techniques has its own biases but the most common, and maybe the most important, are related to the DNA extraction and PCR amplification (von Wintzingerode *et al*., 1997; Becker *et al*., 2000). The importance of combining both culture-dependent and culture-independent approaches is acknowledged in this study.

Since both DGGE and TRFLP are based on PCR amplification, they are limited by the biases of this technique. PCR is more controlled when analysing DNA from pure cultures but several problems can arise when it is applied to mixed communities. The most common can be the selective amplification of specific templates and the formation of PCR artefacts (von Wintzingerode *et al*., 1997). The selection of primers can be one of the parameters that affects the results and particularly the TRFLP quantification. This is because of the differences in the efficiency with which templates compete in mixed DNA samples when different primers are used (Hodgetts *et al*., 2007). One reason is that different primers can present different hybridization efficiencies and specificities to each of the components

of the mixed templates. Therefore amplification would be favoured for templates with optimum binding with the primer (von Wintzingerode *et al*., 1997)

The applied DNA extraction may also affect the recovered microbial community. In this study mechanical lysis of cells using glass beads was applied. Insufficient or preferential disruption of cells can take place and bias the realistic reflection of the microbial diversity and relative abundances. An alternative could be lysis using enzymes. Leff *et al*. (1995) compared the DNA extraction method based on mechanical lysis with the lysis using enzymes on the basis of yield, purity and quality of the recovered DNA. These authors found that the bead beater resulted in the release of a significant amount of DNA although badly sheared while the samples treated with enzymes revealed DNA of lower purity.

Culture-dependent techniques overcome all these biases as they directly analyse the microbial community and not the DNA extracted from it. The culture dependent technique allowed the examination of isolates macroscopically and microscopically. As a result, *D. hansenii* isolates could be divided into two main morphological groups (group A and B). This was very important because isolates from group A were not recovered from the blue part samples, suggesting that they might be unable to populate the blue veins. This group of isolates was recovered in association with *P. roqueforti* or *K. lactis* species in the outer and white parts and therefore their lack of cullture from the blue parts could not be due to inhibition of growth by these organisms. As already discussed, this differentiation into groups was not possible with any of the molecular approaches. This is in agreement with previous RFLP studies where the restriction analysis of the ITS1-5.8S rDNA-ITS2 region of *D. hansenii* strains was not capable of typing at strain level no matter the restriction enzyme choice. Only one out of the nine enzymes tested resulted in diverse restriction

patterns but even in this case the strains grouped randomly without presenting the same biochemical properties within each group (Petersen *et al*., 2001).

2.3.15 Fungi present in Stilton

Different fungal communities exist in the three different sections of Stilton. The yeast communities in Stilton consisted of *K. lactis, Y. lipolytica*, *C. catenulata*, *T. ovoides* and two types of *D. hansenii* named groups A and B. Teleomorphs and anamorphs of these species (Table 2.9) have been reported to be present in many types of blue cheeses including Danish blue (Denmark), Gorgonzola (Italy), Roquefort (France), Rokpol (Poland) and blue cheeses produced in Australia and South Africa (Roostita *et al*., 1996; Gobbetti *et al*., 1997; Van den Tempel *et al*., 1998; Addis *et al*., 2001; Wojtatowicz *et al*., 2001; Vasdinyei *et al*., 2003). Similar yeast flora, although less diverse, have been detected in blue cheeses made from raw milk (Florez *et al*., 2006). One species frequently present in other blue cheeses that was not detected in this study is *Candida zeylanoides*.

Several studies have addressed the origin of yeast secondary flora in blue cheeses and it was suggested that there can be more than one source. An extensive study on the development of yeast during production of blue veined cheeses (Viljoen *et al*., 2003) showed that brine was a major source of yeast contamination. This is in agreement with other studies on cheese brines where the yeast populations were considerable (10^4 to 10^6 cfu/ml) and species with enhanced salt tolerance, like *D. hansenii*, were abundant (Seiler *et al*., 1990, Van den Tempel *et al*., 1998; Addis *et al*., 2001). In Stilton production, dry salting is used instead of brining and therefore

other ways of contamination may be the primary sources of contamination with yeasts. High numbers of yeasts can be introduced from post-production contamination, like contact with surfaces and equipment (Viljoen *et al*., 2003). Viljoen *et al*. (2003) showed that the yeast counts present in milk were secondary as a source of contamination and air contamination was almost non-existent. Finally, yeasts occur at low populations (10^2-10^4 cfu/ml) in both raw and pasteurized milk (Fleet, 1990; Van den Tempel *et al*., 1998; Cocolin *et al*., 2002). The present study of the distribution of the yeasts in the different parts of the cheese could provide some deeper insight into the source of the yeasts. When yeasts occur in the white region they are not likely to come from surface contamination but from raw materials, while in the outer part it could be surface contamination; in the blue it could be in the raw material or introduced during piercing.

Although variation exists in yeasts communities within dairies it seems that the main flora of yeasts present in blue cheeses is fairly consistent. The consistent and selective growth of particular yeast species in blue-veined cheeses is because of their physical and chemical properties. These are high concentrations of fat and protein, high concentrations of lactic acid, the presence of citric and acetic acid which the yeasts can utilise, residual unfermented lactose, high concentrations of salt, pH and storage conditions (Roostita *et al*., 1996).

The yeast species that was dominating the outer part of Stilton was *D. hansenii*. This is the most abundant and more frequently detected species in most of the blue cheeses (van de Tempel *et al*., 1998; Addis *et al*., 2001; Wojtatowicz *et al*., 2001; Vasdinyei *et al*., 2003). This could be because of its ability to grow at low temperatures, the ability to utilise lactic and citric acids and its high salt tolerance (Fleet *et al*., 1990). The drying of the surface of Stilton and the formation of the crust

Table 2.9 Teleomorphs, anamorphs and synonyms of main yeast species of interest in blue cheeses

Teleomorph name	**Anamorph name**	**Synonyms**
Debaryomyces hansenii	*Candida famata*	*Torulaspora hansenii*
Yarrowia lipolytica	*Candida lipolytica*	*Saccharomycopsis lipolytica*
Kluyveromyces lactis	*Candida sphaerica*	*Kluyveromyces marxianus, Candida kefyr*
-	*Candida catenulata*	*Candida / Mycotorula / Blastodendrion brumpti*
-	*Candida boidinii*	*Candida olivarium / quereta / methanolica*
Zygosaccharomyces rouxii	*Candida mogii*	*Saccharomyces rouxii, Zygosaccharomyces barkeri*
Trichosporon beigelii	-	*Trichosporon ovoides*

based on Barnett *et al*., 1990; Deak *et al*., 1996.

would lead to low aw and higher salt concentration. This should be an advantage for *D. hansenii* and could explain its higher presence in the outer part.

The role of *D. hansenii* has been investigated in several studies all reporting absence of extra-cellular proteolytic activity (Roostita *et al*., 1996; Addis *et al*., 2001) but intra-cellular proteolytic activity was reported (Roostita *et al*., 1996; Wojtatowicz *et al*., 2001). This activity could contribute to the proteolysis of the cheese after cell lysis. Lipolytic activity, both intra-cellular and extra-cellular, seems to vary and is probably strain dependent (Roostita *et al*., 1996; Addis *et al*., 2001; Wojtatowicz *et al*., 2001).

Y. lipolytica was present in Stilton in the outer and white parts only. It is a species also detected in almost all studies of blue cheeses although in much lower amounts than *D. hansenii*. This is in agreement with the present study where this species was only a fraction of the total flora. In other studies on blue cheese it is reported to be higher in the outer part than the inner core (Van den Tempel *et al*., 1998; Addis *et al*., 2001). However, the study of two inner sections separately allowed the observation that this species was not present at all in the blue veins. This is very important as this yeast is reported to inhibit the sporulation of *P. roqueforti* in *in vitro* studies (Juszczyk *et al*., 2005). It is reported to have a strong extra-cellular proteolytic and lipolytic activity as its name indicates (Roostita *et al*., 1996; Addis *et al*., 2001). Normally the population of this organism in the flora increases later in the maturation process (Roostita *et al*., 1996) and this might be the reason for its lower counts. Its strong extra-cellular enzymic activity suggests that it has an important role in maturation. This is supported by its ability to present strong growth at low temperatures (5-10°C) (Roostita *et al*., 1996) typical for the conditions that exist during maturation.

K. lactis dominated the blue part and was also present in the white and had some limited presence in the outer part. The presence of *K. lactis* can be explained by its strong assimilation and fermentation of lactose. It is also able to utilise lactic acid, citric acid, proteins and fats but to a lesser extent (Roostita *et al*., 1996). In the future it would be interesting to investigate the reason for the favourable growth of this species in the blue veins section and any possible interactions with the growth and sporulation of the starter mould. Interactions of *K. lactis* and *P. roqueforti* will be discussed in a later chapter (Chapter 4).

C. catenulata was detected only with molecular analysis. One explanation is that its population in Stilton reaches its peak during ripening and maturation but no significant number of viable cells is present in the final product. In this case it could be suggested that changes in the cheese matrix during ripening (e.g. pH, salt concentration) could favour the growth of other competitive species. This species frequently occurs in cheeses and has strong extra-cellular lipolytic and proteolytic activity. It can also utilise lactic and citric acids (Roostita *et al*., 1996).

T. ovoides was detected only with culture. This species has previously been reported to be present on the surface of Gorgonzola and its origin was found to be the surfaces of the plant and the equipment (Viljoen *et al.*, 2003). It was also found in Gouda cheese as a contaminant coming from the surfaces (Welthagen and Viljoen, 1998). However, in Stilton it was present in the blue part of the cheese and not in the outer crust, and it may be introduced into the interior by piercing. In Kopanisti cheese it was found to be the dominant yeast species (Kaminarides and Anifantakis, 1989). Its presence on the surfaces of dairies (and from there its transfer to the cheeses) could be attributed to the wide occurrence of this species in nature, especially humans, soil, and animals (Kurtzman and Fell, 1998).

The starter *P. roqueforti* was the only mould detected. Usually the blue cheese mould flora exclusively consists of the starter *P. roqueforti* (Gobbetti *et al*., 1997; Addis *et al*., 2001). Surprisingly there was mould detected in the blue and white part but not in the outer with any of the techniques used. Studies report the presence of *P. roqueforti* on the surface of other blue cheeses (Gobbetti *et al*., 1997).

No matter the source and the type, yeasts present their maximum development as secondary flora after the bacterial growth during ripening, maturation and storage. Several studies report the dynamics of yeasts during processing. These are getting to typical levels (10^7 – 10^8 cfu/ml) after 1 month of maturation (Gobbetti *et al*., 1997; Van den Tempel *et al*., 1998; Florez *et al*., 2006). In addition it was found that some yeasts in blue cheeses present significant growth during storage (Roostita *et al*., 1996).

2.4. CONCLUSIONS

Both molecular and culture-dependent techniques were used and compared for the analysis of the fungal profiles in the different parts of Stilton. Overall, the molecular techniques gave a more complete picture of the fungal community. Molecular methods were able to detect *C. catenulata*, which was a major part of the flora and for which cultivation was not possible either because of competition from other species on the medium or because the cells were not alive.

Since both culture-dependent and molecular methods have their drawbacks, and are subject to different biases, it should be concluded that none of the methods alone demonstrated the complete community. Both culture-dependent techniques and molecular analysis were required in order to obtain the most accurate possible conclusions. There is a growing trend for the molecular approaches to be suggested as those that reliably reflect the microbial community diversity. However, the results showed that the role of both approaches is complementary and their combination is required.

As in the case of the Stilton bacterial community (Ercolini *et al*., 2003), the structure of the fungal community was different for each section of the cheese. The outer crust was the part with the higher number of yeasts but there was no starter mould. This observation is particularly important from a technological point of view as the outer crust starts to form much earlier than the piercing and blue veining. Significant differences were found between the microbial profiles of the different parts of Stilton using both DGGE and TRFLP and although the two methods amplified different regions of the DNA, they led to comparable results. A significant advantage of TRFLP was that, because of its better resolution and sensitivity, it was

able to detect species that were present in low amounts and provided semi-quantitative data.

Both DGGE and TRFLP were able to investigate the microbial communities in the different sections of the cheese at species level. It remains unknown the degree of strain differentiation within the species detected. The same applies for the five groups of isolates that were recovered by culture which may be composed by one or more strains. Further study of the strain differentiation would be needed for a deeper appreciation of the fungal community diversity in the different parts of the cheese. In order this to be achieved the methodology should be expanded in order to include techniques suitable for the study of strains. Appropriate techniques to be used for this purpose could be the Pulse Field Gel Electrophoresis (PFGE) and Random Amplification of Polymorphic DNA (RAPD). These were found to be effective for the study of the yeast species that were identified in Stilton including *Y. lipolytica* (Deak *et al*., 2000) and *D. hansenii* isolated from surface-ripened cheeses (Petersen *et al*., 2000; Mounier *et al*., 2006).

The purpose of this part of the study was to determine whether different sections of Stilton cheese present different fungal communities in order later to understand the effect of this local differentiation on flavour production. The maximum development of the yeast flora follows the bacterial growth during ripening and maturation. In addition yeasts in blue cheeses present significant growth during storage. Therefore their contribution to the blue cheese properties should be important.

3. STILTON AROMA

3.1. INTRODUCTION

3.1.1 The aroma of blue cheese

The aroma of blue cheeses is mainly a result of the action of the starter microorganisms and the secondary flora. Both of them determine the cheese variety (Fox, 2000; Fox 2004). Other parameters such as enzymes from the rennet, lipases and secondary enzymes from the milk may have an impact on the final cheese aroma, however, these are considerably reduced by pasteurization (Urbach, 1997).

The aroma of Stilton cheese has not been extensively studied, despite being a protected designation origin product. Madkor, Fox, Shalabi and Metwalli (1987) studied part of the flavour in Stilton but focused on the analysis of FFA/ketone conversion during ripening. At that time Stilton was made using unpasteurised milk.

Acidification in Stilton, like in all blue cheeses, is carried out by the addition of a *Lactococcus lactis* starter culture, while the ripening is promoted by the development of the mould *Penicillium roqueforti*. The enzyme complex of *Penicillium roqueforti* is responsible for a major part of the proteolytic and lipolytic activity and the typical blue cheese flavour formation. Both conidia and mycelia are capable of producing aroma compounds (Fan, Hwang & Kinsella, 1976). The mould grows and sporulates during the ripening period forming blue veins in the core of the cheese. The procedure includes the catabolism and metabolism of a series of amino acids and fatty acids and flavour compounds are generated (Kinsella & Hwang, 1976a).

The sporulation of the mould takes place in a fraction of the total mass and the blue cheese matrix is highly heterogeneous. In addition, Stilton cheeses form a brown outer crust during ripening increasing the spatial heterogeneity. In Chapter 2 it was demonstrated that the presence of the starter mould *P. roqueforti* in the outer part is limited. In addition, high heterogeneity in the spatial distribution of the fungal flora was observed in all the different sections of Stilton. The relation between the fungal flora and the flavour formation in fermentation processes (McSweeney & Sousa, 2000) introduces the question of how homogeneous is the volatile composition of Stilton between the outer crust and core and between the predominantly blue/white sectors of the cheese.

The approach was to study the aroma profile of the three different sections of the cheese: blue veins, outer crust and white core so as to overcome possible misleading conclusions of studies on whole cheese samples. Cheeses from different dairies were also analysed allowing the question of how similar are different batches of cheese from different dairies.

3.1.2 Flavour analysis

Most of the techniques for analysing the aroma of cheese are based on mass spectrometry (MS) which is usually combined with gas chromatography (GC) (Careri, Bianchi & Corradini, 2002). GC can resolve complex mixtures, in addition, it has high resolution and trace components can be detected. By coupling GC with MS many of the components can be identified. In addition, direct MS techniques can be used for the analysis of food (Taylor *et al*., 2000; Careri *et al*., 2002). Direct MS

techniques overcome the time consuming analysis procedures associated with chromatography and they produce spectral profiles of ions formed by the volatiles, but at the expense of identification.

The aroma profiles of the three different sections of Stilton cheese, blue veins outer crust and white core, were studied using solvent extraction GC–MS, a headspace GC–MS technique (Solid Phase Microextraction (SPME) GC–MS) and direct headspace technique (Atmospheric Pressure Chemical Ionisation (APCI) MS). Their effectiveness in analysing the cheese aroma and evaluating the possible differentiation of the aroma within the different sections of the cheese were compared.

3.1.2.1 Solvent Extraction GC-MS

Solvent extraction is one of the simplest approaches for aroma isolation. One of the advantages of the solvent extraction techniques is that concentration steps can be applied without affecting the relative proportions of the aroma compounds. In addition, the recovery of the aromas and their concentrations do not depend on their volatility, a factor which affects the headspace analysis.

The biases imposed are related to the relative solubility of the various aroma compounds in the organic/aqueous phases that are involved in the extraction (Taylor, 2002). Furthermore, there are limitations for food matrices with high lipid content such as the blue cheeses. Lipid would also be extracted along with the aroma compounds and this must be separated from the extract prior to injection in the GC. Distillation can be used in order to separate the aroma compounds from the fat-containing solvent extract. This step may result in the loss of highly volatile

compounds and/or thermal degradation of compounds sensitive to heat treatment. In some cases the use of organic solvents in analysis can be problematic as they may be toxic and/or lead to chromatographic contamination, and/or masking of peaks in the solvent peak.

3.1.2.2 Solid Phase Microextraction (SPME) GC-MS

Headspace sampling is one of the most common methods that has been applied to the extraction of volatiles prior to GC-MS analysis (Mariaca & Bosset, 1997; Careri *et al*., 2002; Fox *et al*., 2004). SPME is a simple and sensitive headspace technique for the analysis of volatile compounds. It was originally developed and described by Pawliszyn's group for environmental analysis (Pawliszyn, 1997). Its main advantage is that it does not require the use of solvents which minimises contamination. In addition, the lack of solvent extraction simplifies the method and the extraction can be readily automated.

Briefly, an inert fibre which is coated with an adsorbent is used. This coated fibre is housed in the needle of a modified syringe. The fibre can be retracted into the needle or exposed for volatile sampling. The retractable feature protects the fibre against physical damage and contamination while it is penetrating the vial that contains the sample for analysis. The fibre is exposed in the headspace of the sample and allowed to adsorb volatiles.

The sample must be equilibrated. Two types of equilibration take place. The sample-headspace and the headspace-fibre equilibration. These processes are highly dependent on the chemical properties of the volatile compounds and the adsorbent

coat. Compounds with higher fibre affinity may demonstrate larger adsorption capacity compared to compounds with lower affinities. There are several different commercial fibre types available with different affinities and the selection of the fibre should be made according to the aim of the study.

Alternatively, the fibre can be directly immersed in the sample. This is not appropriate for solid matrices but can be used for aqueous samples which must also be free of fat, proteins and carbohydrates. Therefore the headspace mode is more appropriate for SPME on cheese samples. Use of headspace SPME analysis for the analysis of volatiles from Camembert cheese showed only a slight difference in sensitivity compared to direct SPME analysis (Jaillais, Bertrand & Auger, 1999). In both direct and headspace SPME the volatiles are then thermally desorbed into a GC column.

3.1.2.3 Atmospheric Pressure Chemical Ionisation – Mass Spectrometry (APCI-MS)

No matter the method of extraction of volatiles they all introduce biases (Taylor, 2002). This is mainly because of competition of the aromas and/or their partial recovery during extraction. An alternative in order to overcome these biases is direct MS analysis of the whole mixture of volatile compounds without prior separation. APCI-MS is such a technique, characterised by rapidity of analysis, but the lack of chromatographic separation and mode of ionisation results in limited structural information and identification of compounds.

A detailed description of the APCI-MS is available from Linforth *et al*. (1998). The APCI-MS consists of a simple system for headspace sampling in which the headspace is sucked into the source of the mass spectrometer and ionised without any chromatographic separation. An ion profile can be obtained in a few seconds allowing fast sample profiling. Figure 3.1 presents a brief description of the ionisation procedure and ion detection. The ionisation is controlled through the operating conditions within the interface and is low energy, minimising fragmentation. An initial reagent ion (H_3O^+) is formed as the water molecules in the gas phase pass through a point-to-plane corona discharge (4kV) at atmospheric pressure. Then the reagent ion transfers its charge to the volatiles with higher proton affinity, typically resulting in the protonated molecular ion (MH^+), although some compounds such as alcohols can dehydrate (Taylor *et al*., 2000). The soft ionisation is essential since fragmentation would make the spectra extremely complex and it would not be possible to assign ions to compounds unequivocally and therefore quantification would not be possible. Ideally one characteristic ion is produced from each component in the volatile mixture and the signal intensities of the ions are proportional to the quantity of the assigned compounds (Taylor *et al*., 2000). This results in minimal information on compound identification (the ion masses) but a potentially simpler system for sample profiling. Because of its rapidity it could be very useful for the routine analysis of cheese where specific information is required but for a large number of samples. However, this potential has not been studied so far.

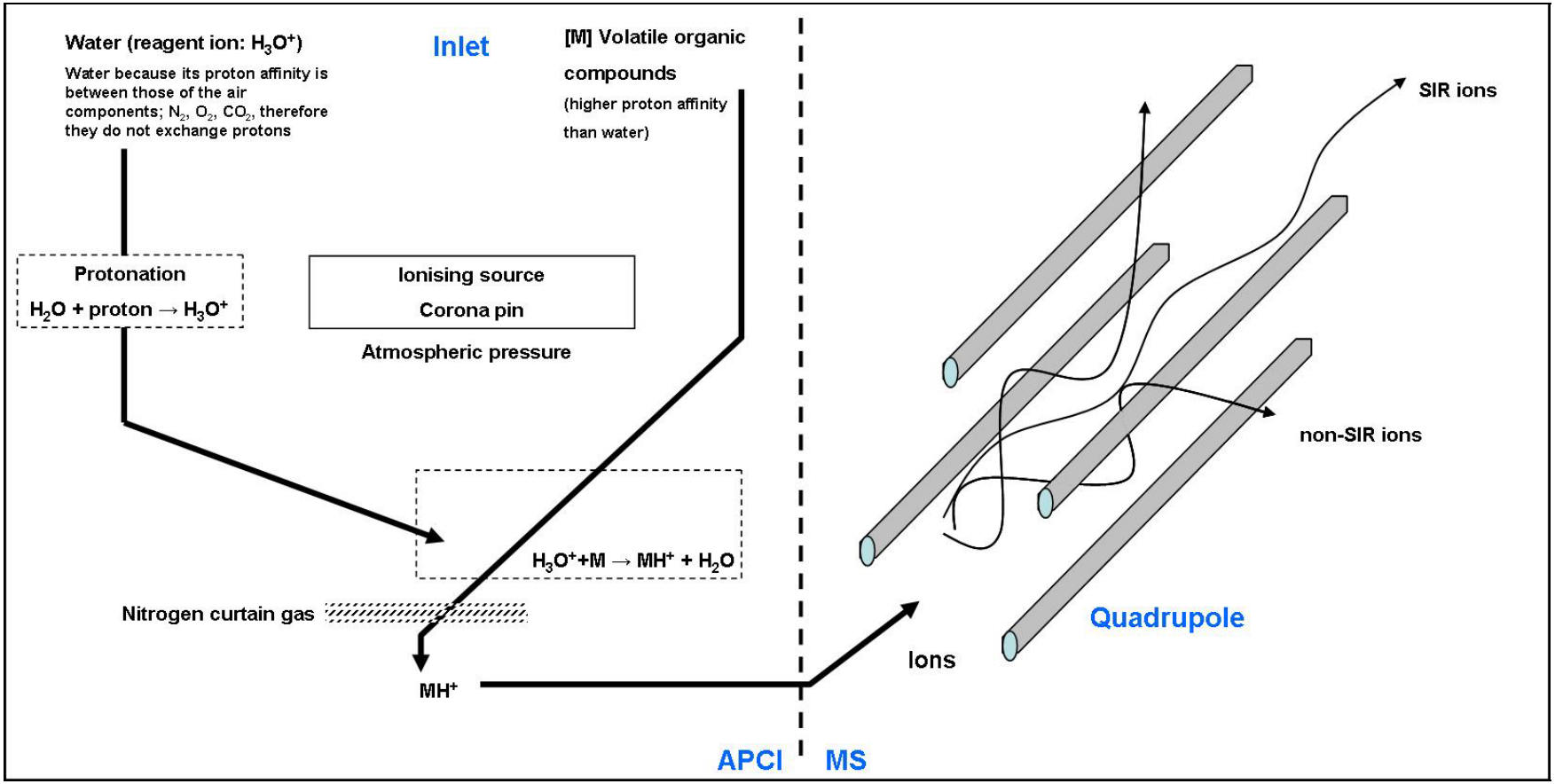


Figure 3.1 Ionisation and selected ion detection with APCI-MS.

3.2 MATERIALS AND METHODS

3.2.1 Cheese samples origin and preparation

Seven Stilton blue cheese samples were purchased from several retail outlets. The samples were produced at three different cheese factories (A, B and C) including both mature and standard cheeses. The cheeses were divided into different parts: outer crust (outer), pure white core (white) and blue veined core (blue). Initially the outer part of the cheese was separated from the core. Then a layer of 1 cm width was removed in order to ensure the differentiation between the inner parts (blue veined and white core) and the outer part. Finally, the inner parts were separated into white and blue parts. The samples were then stored at -80^{o}C. On the day of analysis they were defrosted at 4^{o}C and cut into pieces measuring approximately 3 mm across. One of the seven cheese samples was used for the preliminary aroma analysis and the comparison of the techniques, this was the same sample that was used for the microbial analysis in Chapter 2.

3.2.2 Chemicals

All the chemicals that were used as standards were GC grade (≥99.5%) purchased from Sigma-Aldrich (Gillingham, UK). The dichloromethane (DCM) used for solvent extraction was 99.9+ % capillary GC grade (Sigma-Aldrich, Gillingham, UK).

3.2.3 Atmospheric Pressure Chemical Ionisation - Mass Spectrometry (APCI-MS)

Four replicate samples (6g) of each cheese part were placed in 100 ml flasks (Schott, Stafford, UK), sealed and allowed to equilibrate at 22°C for 30 min. The lid of each flask had a 4 mm hole through which the transfer-line of the APCI-MS could be introduced for headspace sampling. Headspace was drawn from the flasks at 5 ml/min into the source of the Platform II mass spectrometer (VG, Manchester, UK) through a heated (120°C) deactivated fused silica transferline 1m x 0.53mm ID. The source was heated to 75°C and operated in positive ion mode (4kV) with a cone voltage of 12V.

Data were collected in full scan mode or in selected ion recording mode with a dwell time of 0.05 s for each ion (ions listed in Table 3.3). Masslynx 3.2 (Micromass, Manchester, UK) was used to process the data. The signal intensity for each ion was expressed relative to that observed when the headspace above a 5μg l^{-1} 2-nonanone solution was sampled.

3.2.4 Solvent extraction GC-MS

Solvent extraction was performed using DCM: cheese, water and DCM were mixed in a ratio of 2g/4ml/1ml respectively. A 5-nonanone internal standard was added at a concentration corresponding to 10 μg g^{-1} of cheese. Firstly, the water and cheese were mixed using an Ultra-Turrax homogenizer (T 25; IKA-WERKE, Staufen,

Germany). Then the DCM was added and the sample agitated on a flask shaker (SF1, Stuart Scientific, Stone, UK) for 20 min at 800 OSC/min. Thereafter, the samples were centrifuged at 1500g for 30 min and were separated into three layers: an aqueous top layer; a middle layer consisting of the cheese solids; and a DCM layer at the bottom. The lower DCM layer was collected and distillation under vacuum was used to separate the volatile compounds from lipids within the samples. A receiving flask (cooled in ice) was connected to the sample flask (heated on a water bath 98^{o}C) and the apparatus evacuated to 80 kPa using a Laboport pump (KNF Neuberger, Witney, UK) prior to sample introduction. After the apparatus was isolated from the pump, the DCM extract was injected via a septum into the sample flask. After 8 min the DCM and volatiles were collected from the receiving flask and transferred into GC vials. For all the samples, four replicates were prepared. The vials were stored at -80^{o}C prior to injection.

GC-MS settings: 1µl of sample was injected in splitless mode with a Fisons AS800 autosampler. The temperature of the injection port was 250^{o}C. Chromatography was carried out with a GC 8000 series gas chromatograph (Fisons) using a BP5 capillary column (30m x 0.25mm I.D.; film thickness: 1µm) from SGE (Milton Keynes, UK). Helium was employed as the carrier gas, at a constant pressure of 17 psi. The oven temperature programme was as follows: an initial temperature of 40^{o}C was maintained for 2 min, increasing at a rate of 8^{o}C/min to a final temperature of 220^{o}C. The transfer line from the gas chromatograph to the mass spectrometer was held at 250^{o}C. Mass spectrometry (MS) was performed with a Fisons MD 800 mass spectrometer. The mass spectrometer was operating under positive ionisation electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2 scans/s) scanning from m/z 20 to 250. Source temperature was 200^{o}C.

Compounds were identified by comparing their retention times and mass spectra with those of standards or their retention indexes (RI) with those published in databases and their mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library. The amounts of each compound present were estimated by comparison of the Total Ion Current (TIC) chromatograph peak area for each compound with those for the internal standard. All data were processed with MassLynx 3.2 (Micromass, Manchester, UK) software.

3.2.5 SPME GC-MS

Samples (1.5g) were placed in 20 ml Headspace vials (22.5 mm x 75.5 mm, Grace Alltech, UK). The vials were immediately sealed with a magnetic cap (20 mm diameter, 5 mm centre, PTFE / Silicone Liner; Grace Alltech) and they were allowed to equilibrate at 22^{o}C for 30 min before analysis. For all the samples, four replicates were prepared.

A 1-cm Stableflex fibre coated with 50/30 μm divinylbenzene-carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA, USA) was used for the extraction of the volatiles. Prior to use, the fibre was conditioned for 90 min in the injection port (300°C). Optimization of the extraction time (range tested 5-30 min) and extraction temperature conditions (22°C or 45°C) was performed. The extraction for 20 min at room temperature was finally selected. For all analyses, desorption time was set to 10 min at 230^{o}C.

GC-MS was carried out using a Trace GC Ultra gas chromatograph (Thermo Electron Corporation) and a DSQ mass spectrometer (Thermo Electron Corporation). The GC-MS settings were the same as those used for the solvent extracts.

Compounds were identified as in §3.2.4 and the TIC signal intensity for each compound was expressed relative to that observed when the headspace above a 5 μg l^{-1} 2-nonanone solution was sampled. Masslynx 3.2 (Micromass, Manchester, UK) was used to process the data.

3.2.6 Statistical analyses

Analysis of variance (ANOVA) was performed with XLSTAT (Addinsoft, USA) in order to examine the significance of the differences between the quantities of the aroma compounds in the different parts of the cheese.

The relationship between the different sections of the Stilton cheeses and their volatiles (variables) detected with SPME GC-MS analysis was evaluated by Principal Component Analysis (PCA) using Unscrambler v.9.0 (Camo Process AS., Norway). All data were standardised (1/standard deviation) prior to analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 Analysis of Stilton by solvent extraction, GC-MS

Eleven main peaks were observed in the GC-MS profiles of the solvent extracts (Table 3.1). Nonanone dominated the volatile profile followed by heptanone, pentanone and 3-methyl butanol. Overall, the ketones were the major aroma compounds found for all the cheese sections with alcohols and aldehydes comprising the rest of the profile. All samples contained a higher percentage of ketones than other compound groups. They represented 72%, 75% and 55% of the total volatile profile of the blue, outer and white parts respectively. Gallois and Langlois (1990) studied the volatile compounds of French blue cheeses and reported ketones to be 50-75% of the total profile. Moio, Piombino and Addeo (2000) reported ketones to comprise 55% and 47% of the aroma profile of the natural and soft Gorgonzola cheese. 2-nonanone and 2-heptanone were the most abundant ketones in both types of Gorgonzola (soft and natural) and Roquefort. The same is reported for Danish Blue cheeses although in some of them the 2-undecanone was equally high (Alewijn, Sliwinski, & Wouters, 2002; Trihaas, Vognsen, & Nielsen, 2005).

The proportion of alcohols in the white core of Stilton was three times higher (30%) than in the volatile profile of the outer crust (10%) or the blue veined core (10%). Alcohols were >30% of the total profile in Gorgonzola and 15-20% in Roquefort, although there is no data to determine whether this was from the white or the blue parts of these cheeses.

The blue part was found to contain the highest amount of volatiles. This was mostly because of the high amounts of 2-heptanone and 2-nonanone. The

Table 3.1 GC-MS analysis of solvent extracts from the three regions of Stilton Cheese.

Compound	LRI [a]	Blue		Outer		White	
		Mean	SD	mean	SD	mean	SD
3-Methyl-butanal	659 [2]	1.2	0.8	1.3	0.8	1.3	0.5
2-Pentanone	689 [1]	2.7	0.7	2.2	0.2	2.3	0.4
3-Hydroxy 2-butanone	711 [2]	1.1	0.5	1.3	0.4	1.4	0.1
3-Methyl-1-butanol	735 [2]	2.6	1.5	1.9	0.9	4.3	1.0
2-Heptanone	892 [1]	8.0	4.1	4.4	1.9	3.1	0.7
2-Heptanol	904 [1]	1.6	0.8	0.66	0.26	0.96	0.18
unidentified 1	1006	3.0	1.4	0.70	0.23	0.40	0.18
unidentified 2	1013	1.6	0.6	0.41	0.25	0.33	0.16
4-methylanisole	1034 [2]	2.0	1.2	1.0	0.3	0.94	0.17
8-Nonen-2-one	1086 [2]	2.3	1.2	1.4	0.3	0.72	0.18
2-Nonanone	1094 [1]	17.6	8.3	9.0	2.3	2.9	0.4

Average amounts (μg g^{-1}, and standard deviations, SD) for the compounds detected in solvent extracts of the outer crust, blue veins/mixed core (blue) and white core of Stilton cheese. Each value is based on four replicates.

[a] LRI: Linear Retention Indexes of the compounds relative to an alkane series. Superscripts show identification using either authentic standards [1] or retention indices from the literature and their mass spectra with the NIST mass spectral library [2]

concentration of 2-nonanone in the different sections of the cheese sample was around 3 to 18 μg g^{-1} which is comparable with the concentrations found in the French blue cheeses (2 to 10μg g^{-1}), Gorgonzola varieties (1 to 3 μg g^{-1}) and Danish blue (10 to 40 μg g^{-1}). Nonanone was present at twice the concentration of heptanone in the outer and the blue part. The same ratio was found in Danish blue cheese, in creamy Gorgonzola and in the Roquefort cheese when made with *P. roqueforti* strains with both high proteolytic and lipolytic strains (Gallois *et al*., 1990; Moio *et al*., 2000; Alewijn *et al*., 2002; Trihaas *et al*., 2005). Similar types of *P. roqueforti* starter are used in Stilton production (Whitley, 2002).

There was clear differentiation between the different zones of the cheese. Surprisingly, the volatile profile of the blue and outer were more similar containing high amounts of ketones while the white was different having a higher proportion of alcohols. This was unexpected since the blue veins are distributed within the white core and the outer crust has a very different appearance and microflora.

3.3.2 Analysis of Stilton by SPME GC-MS

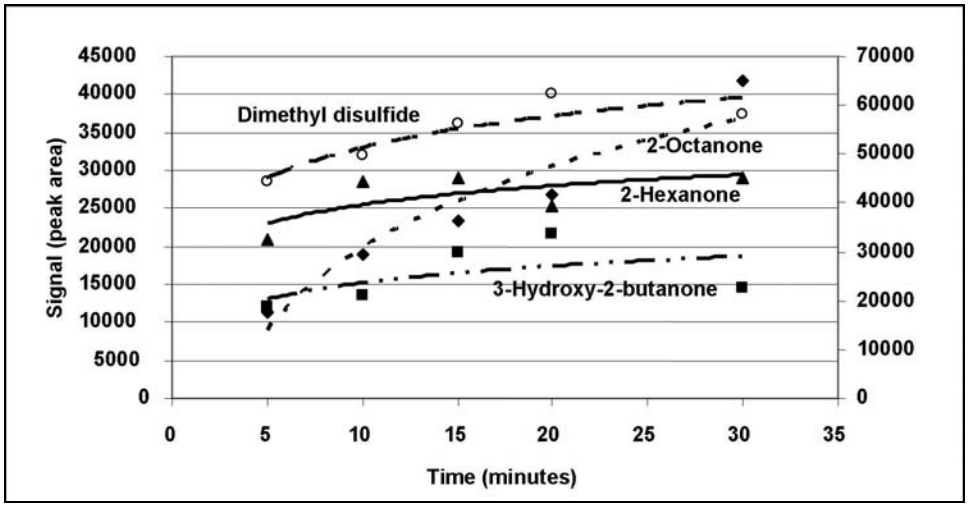
3.3.2.1 Preliminary SPME GC-MS

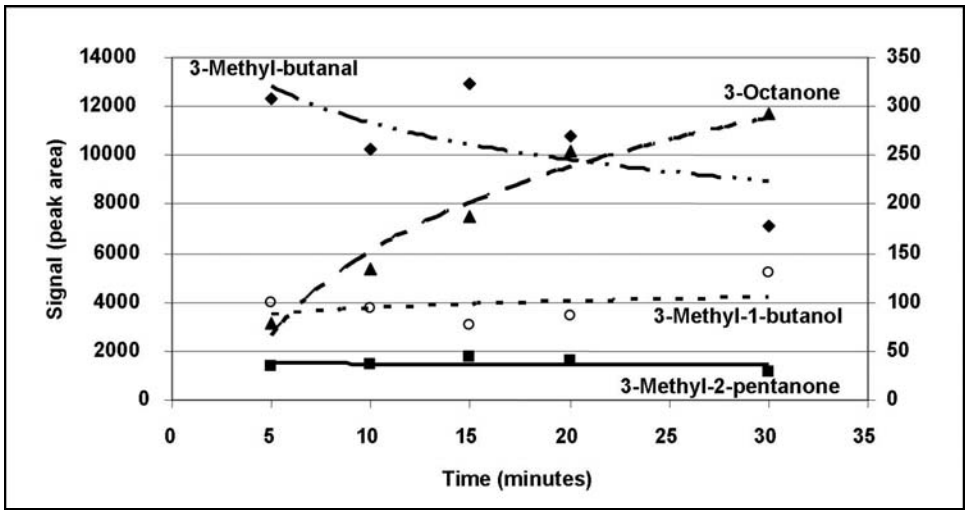
SPME as a method is simple. However, it is important to understand the effect of the physical chemistry of the food-headspace-fibre complex. It is important to assess the extraction time and temperature parameters in order to apply the method more effectively (Roberts, Pollien & Milo, 2000b; Frank, Owen & Patterson, 2004). The extraction time was considered in order to determine the best conditions for the

SPME analysis of volatile compounds from Stilton's headspace. For the trials the extraction temperature was held constant at 22^{o}C and the time of extraction was varied between 5-30 min. The results (Figure 3.2) demonstrated that when the time was increased the amount of volatiles extracted changed but the trend was not the same for all the compounds. For some of the compounds the quantities were increased but the rate of the increase was different. Compounds such as 2-Heptanone, 2-Nonanone, 3-Octanone and 2-Octanone seemed to follow a logarithmic-type trend while other compounds (3-Methyl-1-butanol, Dimethyl disulfide, 2-Hexanone, 3-Hydroxy-2-butanone and Acetone) presented lower rates of increase.

Other compounds (e.g. 3-Methyl-butanal, 3-Methyl-2-pentanone, 2-Pentanone) demonstrated decreasing trends. This can be explained as a displacement phenomenon (sometimes referred to as competitive adsorption). The occurrence of this phenomenon is well reported in several SPME studies (Roberts *et al*., 2000b). In the complex volatile composition of Stilton cheese, numerous compounds can be in competition for the adsorption sites of the fibre and displacement phenomena can take place when the SPME fibre reaches its maximum capacity. The porous structure of the fibre can easily become saturated and once this occurs, compounds with a higher affinity for the fibre will eventually displace those compounds with lower affinity.

Saturation is more likely to occur when using long extraction times (Lestremau *et al*., 2003) and therefore displacement phenomena can be minimized when shorter extraction times are used (Roberts *et al*., 2000b; Lestremau *et al*., 2003). On the other hand, shorter extraction times might have a cost in the sensitivity which is usually decreased since concentrations of analytes will not have approached their maximum levels within the fibre.





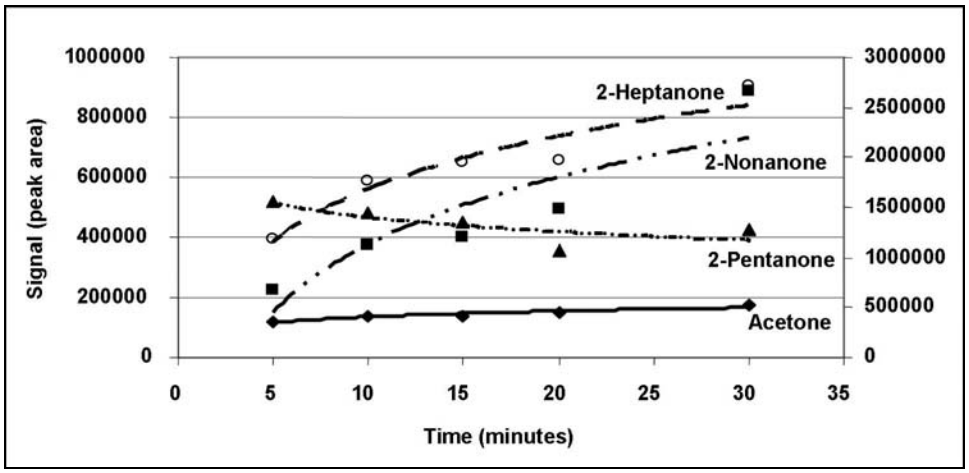


Figure 3.2 Effect of extraction time on absorption of Stilton volatile compounds on to the SPME fibre. Left axes: Acetone, 2-Pentanone, 2-Nonanone, 3-methyl-2-pentanone, 3-methyl-1-butanol, 3-Octanone, 3-Hydroxy-2-butanone, 2-Hexanone, 2-Octanone; right axes: 2-Heptanone, 3-methyl-butanal, Dimethyl disulfide.

In all cases, adsorption rates progressively changed with no immediate evidence of reaching equilibrium within the tested time range. No evidence of reaching equilibrium was observed in SPME study on cheddar cheese even when prolonged extraction times (up to 2 h) were applied (Burbank & Qian, 2005) or even after 16 hours for some compounds in blue mould, parmesan and pecorino style cheeses (Frank *et al*., 2004). In this study, for the compounds which were increasing with increase of extraction time, significant improvement in extraction efficiency was observed when the fibre exposure time was increased from 5 to 20 min. For many of them the detector response was found to be sufficient with extraction times as low as 5 min. In order to avoid extended displacement phenomena and for practicality reasons, 20 min was chosen as a good compromise between overall sensitivity and runtime efficiency for the instrument. A similar approach and extraction time (30 min) was previously applied successfully for cheddar cheese (Burbank *et al*., 2005).

The alteration of extraction temperature was also taken into consideration in order to enhance the flavour release of less volatile compounds. However, altered temperatures resulted in changes in the samples (separation of fats, melting etc.). For this reason 22^{o}C was preferred as a temperature which does not introduce major physical changes in the samples and allows the aroma release to take place in a temperature close to those at which the product is consumed.

3.3.2.2 Main SPME GC-MS

The SPME GC-MS chromatograms consisted of 29 main peaks including 12 ketones, 5 alcohols, 2 aldehydes and 2 sulfur-containing compounds (Table 3.2). Most

Table 3.2 Analysis of the three regions of Stilton cheese by SPME GC-MS.

Compounds	LRI [a]	Blue		Outer		White			Presence in blue cheese varieties
		mean	SD	mean	SD	mean	SD	Pr > F	
Methanethiol	<500 [1]	0.35 b	0.18	4.1 a	1.13	0.16 b	0.04	< 0.0001	1
Ethanol	<500 [1]	0.11 b	0.09	0.14 b	0.06	1.6 a	0.49	< 0.0001	-
Acetone	<500 [1]	2.5 b	0.97	19 a	5	1.7 b	0.52	< 0.0001	2
undentified 3	604	0.41 b	0.17	0.35 b	0.06	2 a	0.26	< 0.0001	-
2-Butanone	610 [2]	0.67 b	0.35	17 a	10	3.2 b	0.49	0.0075	1, 3
2-Methyl-1-propanol	634 [2]	0.86 b	0.25	ND c	-	1.5 a	0.23	< 0.0001	3, 4
3-Methyl-butanal	659 [2]	1.3 b	1.19	1.6 b	1.06	6.5 a	2.21	0.0019	2, 3, 4, 5
2-Methyl-butanal	668 [2]	0.18 b	0.17	0.19 b	0.11	0.81 a	0.42	0.0143	5
2-Pentanone	688 [1]	5.2 b	3.67	56 a	42	2.1 b	0.77	0.0194	1, 2, 3, 4, 5, 6
unidentified 4	690	49 a	16	39 a	22	28 a	3	0.2371	-
2-Pentanol	698 [2]	0.2 a	0.04	0.28 a	0.16	0.23 a	0.06	0.5489	2, 3, 4, 5
3-Hydroxy-2-butanone	709 [2]	3.5 a	1.19	3.8 a	0.39	6.4 a	3.63	0.1833	3, 4, 5
3-Methyl-1-butanol	733 [2]	10 b	2.35	1.3 c	0.63	18 a	2	< 0.0001	2, 3, 4, 5
2-Methyl-1-butanol	737 [2]	2.8 b	0.59	0.41 c	0.1	4.7 a	0.74	< 0.0001	2, 3, 5
Dimethyl disulfide	750 [2]	1.8 b	0.82	4.9 a	1.19	1.8 b	0.2	0.0006	3, 4
3-Methyl-2-pentanone	754 [2]	ND b	-	1.6 a	0.78	0.17 b	0.09	0.0011	3, 5
2-Hexanone	789 [2]	0.38 ab	0.33	2.3 a	1.85	0.07 b	0.02	0.0331	2, 3, 4, 5, 6
Octane	800 [1]	0.51 a	0.38	0.78 a	0.46	0.22 a	0.09	0.1300	-
2,4-Dimethyl-heptane	822 [2]	0.95 a	0.59	1.2 a	0.81	0.25 a	0.09	0.1123	-
2-Heptanone	890 [1]	23 ab	19	116 a	83	4.3 b	1.1	0.0228	1, 2, 3, 4, 5, 6, 7
α-pinene	944 [2]	0.05 b	0.03	1.2 a	0.35	0.04 b	0.01	< 0.0001	-
3-Octanone	988 [2]	ND b	-	1.2 a	0.27	ND b	-	< 0.0001	3, 4

Table 3.2 continue. Analysis of the three regions of Stilton cheese by SPME GC-MS.

Compounds	LRI [a]	Blue		Outer		White			Presence in blue cheese
		mean	SD	mean	SD	mean	SD	Pr > F	varieties
2-Octanone	992 [2]	0.35 ab	0.37	1.2 a	0.68	0.05 b	0.02	0.0169	1, 2, 3 ,4, 5
4-methylanisole	1030 [2]	0.83 a	0.46	0.53 ab	0.12	0.14 b	0.01	0.0182	3, 5
unidentified 5	1034	0.35 a	0.11	0.26 a	0.1	0.27 a	0.07	0.3326	-
unidentified 6	1039	0.13 b	0.04	0.27 a	0.08	0.1 b	0.05	0.0075	-
8-Nonen-2-one	1085 [2]	ND b	-	1.7 a	1.17	ND b	-	0.0089	3, 5
2-Nonanone	1093 [1]	8.6 ab	7.49	27 a	16	1.7 b	0.63	0.0142	1, 2, 3, 4, 5, 6, 7
2-Undecanone	1298 [2]	ND b	-	0.58 a	0.27	ND b	-	0.0007	2, 3, 4, 5, 6, 7

Average SPME GC-MS signal intensities (and standard deviations, SD) for the compounds detected when headspace samples of outer crust, blue veins/mixed core (blue) and white core of Stilton cheese were analysed. The values are relative to the peak area observed when the headspace above a 5μg l^{-1}, 2-nonanone solution was analysed. Each value is based on four replicates. Means in the same row labelled with different letters (a, b, c) are significantly different ($P < 0.05$) as shown by ANOVA.

[a] LRI: Linear Retention Indexes of the compounds relative to an alkane series. Superscripts show identification using either authentic standards [1] or retention indices from the literature and their mass spectra with the NIST mass spectral library [2]

ND: not detected.

[1] Frank, Owen, & Patterson, 2004.
[2] Day, & Anderson, 1965.
[3] Gallois *et al*., 1990.
[4] Trihaas *et al*., 2005.
[5] Moio *et al*., 2000.
[6] Gonzales De Llano *et al*., 1990.
[7] De Frutos, Sanz & Martinez-Castro, 1991.

of these have previously been reported as being present in blue cheese varieties (Table 3.2). Methanethiol, acetone, ethanol and α-pinene are compounds detected in Stilton (Table 3.2) but they are not frequently referred to as being present in other blue cheeses. However, these compounds (except the α-pinene) elute very early and not all the blue cheese studies have been set-up to detect compounds of very low linear retention indices (LRIs). Methanethiol is a particularly important compound to detect because of its odour and sensory properties (see §3.3.8).

The SPME GC-MS confirmed the differentiation within the zones of the cheese. ANOVA demonstrated that 23 of the compounds were found to be at significantly different levels ($P < 0.05$) in at least one of the three parts (Table 3.2). The ketones were dominant in the outer crust and the blue part of the cheese (percentage of headspace total signal intensity: outer 80%; blue 40%; white 20%) while the alcohols were mostly present in the white core (percentage of headspace total signal intensity: outer 1%; blue 10%; white 30%). The increased amount of ketones in the headspace of the outer and the blue parts and of alcohols in the white part was confirmed with later SPME analysis in a series of Stilton samples of different origin (see §3.3.6.1).

3.3.3 Atmospheric Pressure Chemical Ionisation - Mass Spectrometry (APCI-MS)

3.3.3.1 Preliminary APCI-MS

A series of parameters were considered in order to set an APCI-MS method appropriate for analysing Stilton. Initially, the spectral profiles at different cone voltages (CV: 12 V, 18 V and 30 V; constant flow: 10ml/min) for the same sample

were compared. At 12 V the spectra had a higher number of detected ions and higher signal intensities (Figure 3.3). It was observed that at the high CV the spectra were dominated by low mass ions, particularly by the m/z 18 that would correspond with protonated ammonia. The application of 12 V presented higher variation in ion intensities than the 18 V and 30 V and was selected for the rest of the analysis.

The sensitivity of APCI-MS can be increased by increasing the sample's headspace flow rate into the source (Taylor & Linforth, 2003). For this reason the selected cone voltage (12 V) was tested under different flow rates (Figure 3.4). However, in the present study the increase of the flow rate selectively enhanced the signal intensities of the even mass ions only. For this reason a low flow rate was preferred.

3.3.3.2 Main APCI-MS

Initially, headspace samples were introduced into the APCI-MS recording data in full scan mode (m/z 20 to 220). From these spectra, a series of ions were selected representing the most abundant ions in the spectra of the three parts of the cheese; the average intensity of these ions are shown in Table 3.3.

The profile for the blue and white parts of the cheese were broadly similar in the higher masses showing major ions at m/z 115 and 143. These masses would correspond to compounds with molecular weights of 114 and 142. This is consistent with the presence of 2-heptanone and 2-nonanone which would be expected in the headspace of a blue cheese (Madkor *et al*., 1987). There appeared to be greater differences between the blue and white parts of the cheese for the mid mass range

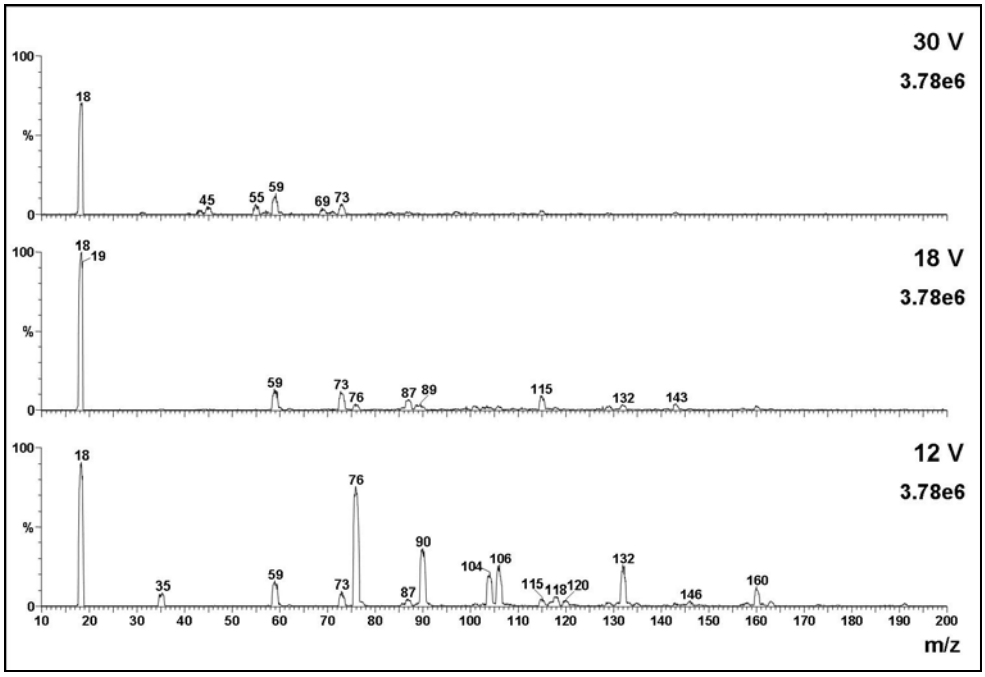


Figure 3.3 Effect of different cone voltages (12V, 18V, 30V) on ion intensities in spectra of the outer part of Stilton.

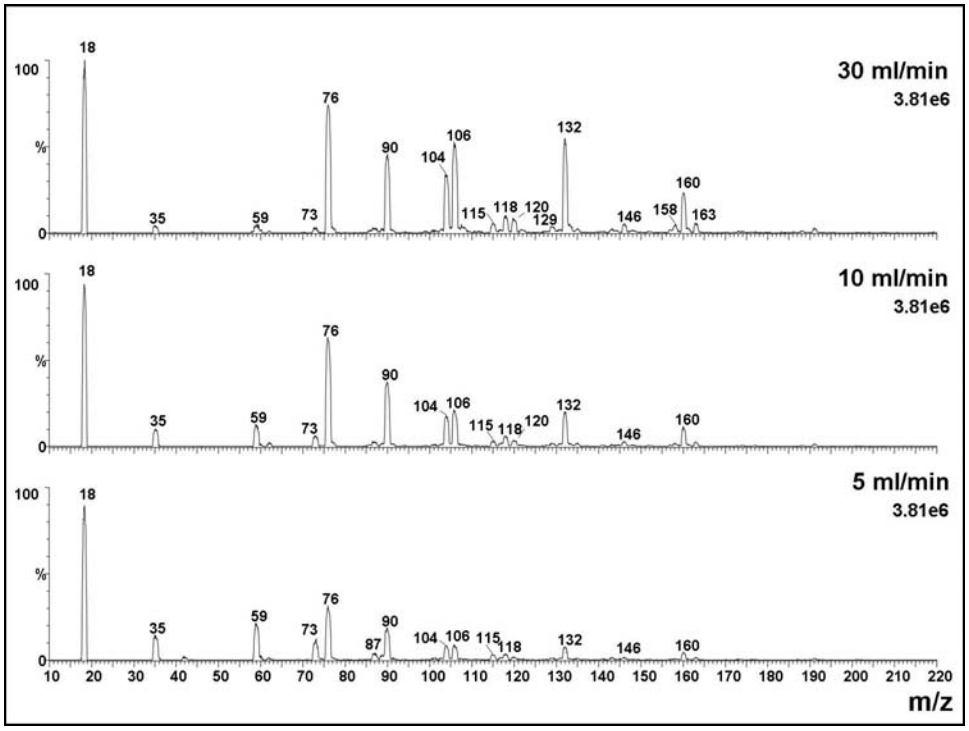


Figure 3.4. Effect of different flow rates (5, 10 and 30 ml/min) on ion intensities in spectra of the outer part of Stilton (CV: 12V).

Table 3.3 Average APCI-MS signal intensities (and standard deviations, SD) for the ions monitored in headspace samples of the outer crust, blue veins and white core of Stilton cheese.

m/z	Blue		Outer		White	
	mean	SD	Mean	SD	mean	SD
33	141	10	0.60	0.76	116	28
35	2.4	0.53	14	1	1.3	0.47
51	38	2.6	0.27	0.17	31	6.8
59	16	2.3	18	4.5	52	4.4
65	11	0.4	ND	-	7.4	2.47
69	1.2	0.36	0.10	0.09	3.3	0.93
71	6.4	1.70	ND	-	12	4
73	1.8	0.46	11	6	39	1.9
76	0.64	0.13	16	3.4	1.8	0.48
87	12	2	3.9	0.96	30	6.8
89	13	2.9	1.2	0.91	27	3.9
90	0.38	0.16	8.4	3.09	2.3	0.47
101	0.51	0.28	0.48	0.17	1.9	0.33
103	0.96	0.19	0.39	0.06	4.2	1.69
104	0.56	0.04	4.1	0.46	1.3	0.45
106	1.6	0.29	4.5	0.55	2.5	0.67
115	12	2.4	2.4	0.64	12	2.7
129	0.65	0.16	1.4	0.57	0.64	0.04
131	0.13	0.14	0.63	0.21	0.87	0.03
132	1.2	0.15	5.5	0.34	1.0	0.34

Table 3.3 continue. Average APCI-MS signal intensities (and standard deviations, SD) for the ions monitored in headspace samples of the outer crust, blue veins and white core of Stilton cheese.

m/z	Blue		Outer		White	
	mean	SD	Mean	SD	mean	SD
143	3.4	0.57	0.95	0.27	2.5	0.69
145	ND	-	ND	-	0.60	0.10
159	ND	-	0.05	0.04	0.39	0.11
160	0.49	0.11	4.1	0.82	0.32	0.21
173	0.10	0.04	0.34	0.03	0.38	0.16
187	0.03	0.04	0.23	0.07	0.24	0.07

The values are relative to the signal intensity observed when the headspace above a 5μg l^{-1}, 2-nonanone solution was sampled into the APCI-MS. Each value is based on four replicates.

ND: not detected

compounds. m/z 87 and 73 were higher for the white than the blue cheese parts; these would correspond to 5 and 4 carbon carbonyl compounds such as aldehydes or ketones. Both of these might be expected in the headspace above a cheese, however, APCI-MS cannot differentiate between isobaric compounds since they give the same mass ions.

There were substantial differences in the ion profiles of the blue and white parts compared with the outer (Table 3.3). The outer appeared to produce a series of ions of even mass. These were not expected since this is indicative of the presence of nitrogen containing compounds. Compounds containing only carbon, oxygen and hydrogen have even masses and protonate to produce odd mass ions. A single nitrogen in a molecule results in an odd molecular weight and hence an even mass ion.

Overall, the APCI-MS system appeared to produce a profile that could discriminate between samples of different parts of the cheese. The three cheese parts were different, but using this technique it was uncertain as to the exact form of the chemical substances responsible. Gas chromatography combined with mass spectrometry (GC-MS) was needed in conjunction with these studies in order to reveal their identities and enable the use of APCI-MS for rapid sample profiling.

3.3.4 Comparison of SPME and solvent extraction

Many more aroma compounds were detected with SPME than were detected with solvent extraction (21 extra compounds) potentially increasing the information in each analytical run. Some of these were compounds that elute early from the GC

column which may have been masked by the solvent such as ethanol. However, many additional compounds with LRI>600 were also detected.

From the 11 main compounds detected with the solvent GC-MS, six of them had a similar blue-outer-white pattern with both SPME and solvent extraction (for the 2-heptanone, 2-nonanone and 8-nonen-2-one the high standard deviation in SPME analysis should be taken in account). Solvent extraction showed that nonanone was present in higher quantities than heptanone whereas it was the other way round in both SPME and APCI-MS analyses. This may be due to differences in volatility and hydrophobicity affecting headspace partitioning above a cheese matrix rich in lipid (typically 35%).

Nonanone, heptanone and several other compounds were present at their highest levels in the SPME analysis of the outer, relative to the white and blue parts (Table 3.2). Such differences were not observed in the solvent extraction (Table 3.1). This is most likely caused by differences in headspace partitioning of aromas from the different parts. Care should be taken in the interpretation of data from the analysis of samples with different matrices. However, the headspace SPME quantification may reflect better the way the aromas are released into the gas phase and therefore delivered to the olfactory system. It takes into account the effect of the cheese matrix components (e.g. fats, proteins) on the volatility of the aroma compounds (Roberts & Taylor, 2000a).

Nevertheless, both solvent extraction GC-MS and SPME GC-MS demonstrated similarities between the outer and the blue parts of the cheese and their domination by ketones. In contrast, the white part was more responsible for the alcohols present in the Stilton aroma profile. The sporulation of the starter mould could be an explanation of the aroma differences between the blue veins and the white

core. However, it could not explain the high quantity of ketones in the outer crust. For this it might be useful to consider the high yeast counts that were detected in the outer part including species with strong proteolytic and lipolytic activity (see Chapter 2).

The average coefficient of variation was 45% and 35% for SPME and solvent extraction respectively. SPME was simpler to perform and despite the fact that the SPME data were semi-quantitative, it was better regarding the number of compounds detected and the overall amount of extractable data. The primary aim of this study was to screen the differences between the different sections of the Stilton matrix. For absolute quantification of volatiles by SPME the conduction of isotope dilution assays could be applied, as this approach is not subject to biases caused by excess compound concentrations or complex matrices (Teranishi, Wick & Hornstein, 1999; Roberts *et al*., 2000b).

3.3.5 SPME-APCI comparison

SPME GC-MS and APCI-MS are both headspace techniques and they should be expected to give similar results. The number of ions in the APCI-MS spectrum (26) was similar to the number of peaks detected with SPME (29). However, in APCI analysis analyte-analyte interactions can occur during ionisation if they are present at high concentrations in the source. In addition, one ion entry can include more than one isobaric compound. Therefore it would not be possible to differentiate stereo and positional isomers e.g. 2- and 3-methyl-butanal. These could only be measured as total methylbutanals. A similar situation exists for compounds which are not isomers

but of the same molecular weight e.g. 2-pentanol, 3-hydroxy-2-butanone, 3-methyl-1-butanol and 2-methyl-1-butanol should result in the same m/z value.

Overall most of the expected masses associated with the major ketones, alcohols and aldehydes in Stilton SPME headspace analysis were observed by APCI-MS. Alcohols and aldehydes often dehydrate resulting in more than one ion (MH^+ and $M\text{-}H_2O+H^+$) (Taylor *et al*., 2000). Indeed the pattern of m/z 69 and 87 (protonated 3-methyl-butanal and 2-methyl-butanal with and without dehydration) for the three different parts of Stilton was the same. The same was found for m/z 89 and m/z 71 (protonated 2-pentanol, 3-methyl-1-butanol and 2-methyl-1-butanol masses with and without dehydration). These profiles are in agreement with the SPME data and confirm the presence of higher amounts of alcohols and aldehydes in the headspace of the white section of the cheese.

However, the nitrogen containing compounds (even masses) seen by APCI-MS are not evident in either SPME (Table 3.2) or solvent extraction (Table 3.1). In addition, few volatile nitrogen-containing compounds have been reported for blue cheeses in the literature. The ions in Table 3.3 that correspond to the ketones were present in much lower amounts in the outer than in the other sections (m/z: 59, 73, 87, 115, 143; protonated acetone, 2-butanone, 2-pentanone, 2-heptanone and 2-nonanone respectively) relative to what was expected from Table 3.2 and 3.1. The main even mass ions in Table 3.3 appear to correspond to the masses expected for the ketones plus 18 mass units (58-76; 72-90; 86-104; 114-132; 142-160). Mass 18 could correspond to the addition of NH_4^+ to the molecule during ionisation (water is also mass 18 but as an uncharged species it would not ionise the compounds). Blake, Wyche, Ellis and Monks (2006) have used protonated ammonia as a reagent gas in chemical ionisation of volatile organic compounds. The spectra of the compounds had

the typical $(M+NH_4)^+$ and associated $([M+NH_4]+NH_3)^+$ forms. Therefore the APCI profiles could be the result of ionisation of the ketones with ammonium (NH_4^+; the protonated form of ammonia).

The even ion masses corresponding to ketones aducted with ammonia were abundant in the outer part of the cheese. This suggestion is supported by the presence of m/z 35 which is typical of the ammonia dimer ($NH4^+$.[NH3]) formed by high levels of ammonia in the source. Furthermore in Figure 3.4 it is demonstrated that the increase in the flow rate reduced the signal of m/z 35 and increased the signals for the aducted compounds. Ammonia is a compound present in cheeses, especially in the mould-ripened cheeses, coming from the deamination of amino acids and/or oxidative deamination of amines (Fox *et al*., 2000; 2004).

There are not any previous reports of the presence of ammonia in blue cheeses and the APCI-MS scan range (m/z 20-220) did not include mass 18. Therefore m/z 18 was not included in the selective ion list for the analysis of this cheese sample. Later APCI-MS analysis of Stilton showed the presence of m/z 18 (see Appendix 4). Ammonia could not be measured by either GC-MS method used and APCI-MS may be used as a method to follow ammonia development in cheese and consequently protein degradation.

Overall, sample analysis by GC based techniques is potentially better under these circumstances. However, by taking into account the GC-MS data the assignment of possible IDs to the different m/z values could be attempted (Table 3.4). The combined direct MS and GC-MS approach that was applied in this study demonstrates the role of these techniques can be complimentary.

Table 3.4 Possible IDs for m/z values detected with APCI-MS

m/z	Possible ID
33	Methanol
35	$NH4^+$.[NH3]
51	-
59	Acetone
65	Dimer of methanol
69	-
71	-
73	2-Butanone
76	Acetone+$[NH4]^+$
87	3-Methyl-butanal; 2-Methyl-butanal; 2-Pentanone
89	2-Pentanol; 3-Hydroxy-2-butanone; 3-Methyl-1-butanol; 2-Methyl-1-butanol
90	2-Butanone+$[NH4]^+$
101	3-Methyl-2-pentanone; 2-Hexanone
103	
104	Mass 87+$[NH4]^+$ (mainly 2-Pentanone)
106	Mass 89+$[NH4]^+$ (mainly 3-Hydroxy-2-butanone)
115	2-Heptanone
129	2,4-Dimethyl-heptane; 3-Octanone; 2-Octanone
131	-
132	2-Heptanone+$[NH4]^+$
143	2-Nonanone
145	-
159	-
160	2-Nonanone+$[NH4]^+$
173	-
187	-

3.3.6 Stilton cheeses from different producers

By comparing Tables 3.1, 3.2, and 3.3 it was concluded that SPME GC-MS was superior to the other two techniques for analysing the aroma profile as an overall method for discriminating differences within the different sections of the cheese. For this reason it was chosen for the analysis of a series of Stilton blue cheese samples from different producers. APCI-MS was also performed. Six more Stilton blue cheeses were selected to represent a range of different Stilton types from different producers (A, B and C). The range included unpackaged cheeses (A2), mature cheeses (A4, B1), two standard cheeses (A3, B2) and a handmade cheese (C1). The cheese that was initially used was from producer A and coded as A1.

3.3.6.1 SPME GC-MS on Stilton cheeses from different producers

The SPME GC-MS chromatograms for the new Stilton samples (Table 3.5) consisted of the same peaks present in the initial cheese (Table 3.2) although there were differences in the quantities of the compounds between samples. There were no new major peaks detected in the new samples. It seems that although variation exists between different cheeses the general aroma profile of the variety was consistent. The only exceptions were 8-nonen-2-one and the unidentified-6 which were absent in cheeses A4 and B2 respectively. 8-nonen-2-one was present in low amounts in all the cheeses from producer A (compared to the cheeses from producers B and C).

The SPME analysis of the additional Stilton cheeses confirmed the strong differentiation within the zones of Stilton that was initially observed after the analysis

Table 3.5. Average SPME GC-MS signal intensities (and standard deviations, SD) for the compounds detected when headspace samples of outer crust, blue veins/mixed core (blue) and white core of all Stilton cheeses that were analysed.

	Cheese B1			Cheese C1			Cheese A4			Cheese A3			Cheese A2			Cheese B2		
Compounds	**Blue**	**Out**	**White**	**Blue**	**Out**	**White**	**Blue**	**Out**	**White**	**Blue**	**Out**	**White**	**Blue**	**Out**	**White**	**Blue**	**Out**	**White**
	Mean	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
Methanethiol	0.16 b	8.75 a	0.03 b	0.13 b	2.75 a	0.02 b	1.27 a	0.37 b	0.37 b	0.16 b	0.49 a	0.03 b	0.41 a	0.97 a	0.56 a	0.05 b	0.84 a	0.05 b
Ethanol	0.00 a	3.02 a	0.00 a	0.00 b	5.38 a	0.17 b	0.00 b	16.6 a	0.00 b	0.00 b	12.9 a	0.39 b	0.00 b	4.14 a	0.29 b	0.00 b	0.00 b	0.08 a
Acetone	12.2 ab	19.6 a	7.99 b	16.5 a	1.73 b	12.0 a	2.19 a	2.09 a	4.22 a	6.11 a	1.90 a	2.10 a	17.2 a	4.81 a	3.80 a	12.4 b	17.2 a	4.65 c
undentified 3	0.25 b	0.14 b	1.39 a	0.20 b	0.11 b	0.66 a	0.09 b	0.00 b	1.06 a	0.24 b	0.09 b	2.89 a	0.11 a	0.11 a	0.54 a	0.08 b	0.17 b	1.07 a
2-Butanone	0.63 b	14.5 a	0.47 b	0.88 b	1.86 a	1.49 ab	0.00 a	2.40 a	4.63 a	2.46 c	12.1 b	66.2 a	0.56 b	11.9 ab	22.9 a	0.75 b	12.7 a	2.17 b
2-Methyl-1-propanol	0.00 b	0.00 b	0.25 a	0.00 b	0.21 a	0.00 b	3.65 a	1.47 b	3.62 a	0.64 b	0.85 ab	1.91 a	0.72 b	0.32 b	2.89 a	0.00 b	0.00 b	0.15 a
3-Methyl-butanal	0.96 b	0.00 b	23.4 a	2.48 ab	0.00 b	9.14 a	1.24 a	0.75 a	1.03 a	1.26 a	0.70 a	1.31 a	1.09 a	1.03 a	1.94 a	0.00 b	0.00 b	11.1 a
2-Methyl-butanal	0.12 b	0.00 b	4.47 a	0.28 b	0.00 b	1.44 a	0.11 a	0.15 a	0.12 a	0.15 a	0.12 a	0.16 a	0.13 a	0.17 a	0.23 a	0.00 b	0.00 b	1.51 a
2-Pentanone	135.0 a	74.8 ab	10.7 b	195.9 a	130.4 b	175.1 ab	0.18 c	7.20 a	3.17 b	17.6 a	14.8 a	3.43 a	33.6 a	12.9 a	2.98 b	224.7 a	109.7 b	14.2 c
unidentified 4	0.13 ab	0.19 a	0.06 b	0.68 a	0.38 a	0.38 a	0.00	0.00	0.00	0.05 a	0.04 ab	0.00 b	0.09 a	0.07 a	0.00 a	0.97 a	0.26 b	0.08 b
2-Pentanol	1.39 a	2.69 a	0.00 a	1.41 b	6.23 a	8.89 a	0.00 b	0.86 a	0.00b	0.40 b	2.06 a	0.00 b	1.65 a	1.36 a	0.25 a	5.32 a	1.90 b	0.00 c
3-Hydroxy-2-butanone	2.61 b	1.06 b	17.9 a	1.48 ab	0.49 b	2.74 a	0.46 b	1.13 b	6.90 a	3.56 b	1.75 b	13.1 a	1.61 a	1.73 a	4.08 a	0.91 b	1.89 b	8.39 a
3-Methyl-1-butanol	3.26 b	0.00 b	36.2 a	2.30 ab	0.60 b	4.56 a	46.0 ab	35.5 b	55.3 a	7.92 b	10.2 b	29.7 a	14.1 b	7.22 c	60.9 a	3.24 b	0.87 b	15.5 a
2-Methyl-1-butanol	0.75 b	0.00 b	6.22 a	0.00 b	0.00 b	1.16 a	5.45 a	6.50 a	5.82 a	1.82 b	2.21 b	3.75 a	3.04 b	1.77 b	7.34 a	0.49 b	0.00 c	1.76 a

Dimethyl disulfide	0.54 b	6.17 a	0.31 b	0.39 b	1.83 a	0.17 b	4.62 a	0.47 b	3.77 a	1.38 a	0.50 b	1.01 ab	3.30 a	2.44 a	3.70 a	0.22 b	0.88 a	0.46 b
3-Methyl-2-pentanone	0.21 b	2.82 a	0.23 b	0.38 b	1.53 a	0.35 b	0.00 c	3.33 a	0.94 b	0.00 b	2.11 a	0.60 b	0.44 b	2.05 a	0.47 b	0.00 b	0.91 a	0.00 b
2-Hexanone	5.82 a	3.47 ab	0.50 b	5.32 a	2.46 b	3.05 b	0.00 c	0.24 a	0.12 b	0.86 a	0.46 a	0.00 a	2.45 a	0.27 a	0.11 a	8.49 a	5.66 b	0.58 c
Octane	0.35 c	0.64 b	0.99 a	0.30 b	1.12 a	0.80 ab	0.45 b	1.41 a	0.51 b	0.31 b	0.61 a	0.34 b	0.34 b	0.94 a	0.39 b	0.28 b	0.42 b	1.10 a
2,4-Dimethyl-heptane	0.47 b	0.84 b	2.04 a	0.35 b	0.20 b	1.32 a	0.48 a	0.62 a	0.68 a	0.38 a	0.33 a	0.44 a	0.51 a	0.75 a	0.75 a	0.40 b	0.48 b	2.16 a
2-Heptanone	240.4 a	160.1 a	27.4 b	139.6 b	56.5 a	64.7 a	0.74 b	8.03 a	7.09 a	44.1 a	20.4 a	4.08 b	90.6 a	8.02 b	5.99 b	349.2 a	314.8 a	28.6 b
α-pinene	0.15 c	0.34 a	0.24 b	0.47 b	2.87 a	0.25 b	0.19 b	2.38 a	0.24 b	0.62 b	2.55 a	0.46 b	0.91 b	3.35 a	0.52 b	0.12 b	0.22 a	0.06 b
3-Octanone	1.26 b	2.58 a	0.23 c	0.39 b	0.93 a	0.10 b	0.00 b	0.13 a	0.00 b	0.25 a	0.10 b	0.00 c	0.40 a	0.00 b	0.00 b	1.50 b	2.47 a	0.19 c
2-Octanone	4.24 a	2.63 ab	0.40 b	1.28 a	0.89 a	0.58 a	0.00 b	0.06 a	0.00 b	0.58 a	0.19 a	0.00 a	1.36 a	0.10 b	0.08 b	5.98 a	4.02 b	0.35 c
4-methylanisole	0.53 a	0.17 b	0.09 c	0.82 a	0.10 b	0.13 b	0.00 b	0.19 a	0.00 b	0.51 a	0.17 b	0.00 c	0.64 a	0.14 b	0.00 b	0.61 a	0.18 b	0.12 b
unidentified 5	0.80 a	0.72 a	0.72 a	0.23 b	0.70 a	0.00 b	1.24 a	1.23 a	1.54 a	1.80 a	1.08 b	1.63 ab	2.55 a	1.48 b	3.46 a	0.10 a	0.00 b	0.00 b
unidentified 6	0.93 a	0.88 a	1.09 a	0.23 b	1.99 a	0.22 b	1.23 a	1.45 a	1.51 a	3.39 a	2.07 b	3.03 ab	2.95 b	2.40 b	4.34 a	0.00	0.00	0.00
8-Nonen-2-one	13.11 a	7.14 ab	0.00 b	2.01 a	2.31 a	1.34 a	0.00	0.00	0.00	1.33 a	0.00 a	0.00 a	3.34 a	0.00 b	0.00 b	19.0 a	9.99 b	0.00 c
2-Nonanone	128.0 a	70.2 ab	13.0 b	20.3 a	22.5 a	12.0 b	1.08 a	2.22 a	1.30 a	20.2 a	6.40 ab	1.07 b	45.5 a	2.85 b	1.98 b	195.0 a	107.2 b	11.1 c
2-Undecanone	3.75 a	2.65 a	0.21 b	0.27 b	0.99 a	0.23 b	0.00 b	0.08 a	0.00 b	0.33 a	0.21 a	0.00 a	1.76 a	0.14 b	0.00 b	5.94 a	3.12 ab	0.24 b

The values are relative to the peak area observed when the headspace above a 5µg l^{-1}, 2-nonanone solution was analysed. Each value is based on four replicates.

Means in the same row labelled with different letters are significantly different ($P < 0.05$) as shown by ANOVA.

of the first cheese sample, but not all the cheeses presented the same patterns. Heptanone and nonanone tended to be higher in the blue and/or outer parts than in the white. The amounts of heptanone in the blue and outer parts of cheeses from producer B were similar and significantly higher than in the white. However, there were cheeses from other producers (A2, A3 and C1) where heptanone was much higher in the blue than in the outer part. One interesting exception was cheese A4 which had lower amounts of ketones in the blue part than in the white. No matter the variation between samples, all the methyl-ketones detected (2-pentanone, 2-hexanone, 2-heptanone, 3/2-octanone, 2-nonanone, 2-undecanone) tended to be higher in the outer and/or blue part of the cheese than in the white. However, 3-hydroxy-2-butanone (acetoin) was higher in the white part.

Branched alcohols (3-methyl-butanol, 2-methyl-butanol, 2-methyl-1-propanol) tended to be higher in the white part irrespective of the origin of production. However, 2-pentanol did not follow the same trend. 3-methyl-butanal and 2-methyl-butanal were also higher in the white part in the cheeses from producer B and C but only in one cheese (A1) from producer A.

There were compounds that were related to one part of the cheese only. α-pinene was a compound that was constantly higher in the outer part. 4-methylanislole was higher in the blue part. Methanethiol tended to be higher in the outer part no matter the origin of production. Dimethyl disulfide, another sulfur-containing compound, was higher in the outer crust for cheeses from producer B and C but for the samples from producer A it was broadly higher in the core of the cheese (blue and white).

3.3.6.2 PCA analysis of SPME GC-MS of seven Stilton cheeses

PCA was applied to SPME GC-MS data for all seven Stilton samples (Figure 3.5) in order to investigate the differentiation of the aroma compounds between the different parts of the cheeses. The scores were coded with a letter corresponding to the dairy (A, B, C) accompanied by the replicate number and b, o or w to indicate the blue, outer or white part respectively.

All the samples were grouped according to the three sections (blue, white and outer) indicating that this was the main trend in the aroma differentiation irrespective of the origin of production. At the top right quarter of the PCA plot (Figure 3.5, within discontinuous line) are located most of the samples coming from the white core while those from the outer are mainly in the two lower quarters (Figure 3.5, within continuous line). The samples from the blue part are located in an area between these two groups. Aroma compounds were clustered within these three areas. A group of non-branched ketone compounds (acetone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 8-nonen-2-one, 2-nonanone, 2-undecanone) was clustered together which may occur as a result of a similar metabolic origin for these compounds. In general, the ketones were related with the outer and blue veined parts. There was a second group of compounds (methanethiol, dimethyl-disulfide, ethanol, α-pinene and 3-methyl-2-pentanone) which appeared to be more related with the outer crust especially with the samples from producer A. A third group (3-methyl-butanal, 2-methyl-butanal, 3-hydroxy-2-butanone, 3-methyl-butanol and 2-methyl-butanol) was positioned in the white part area again showing this region's higher alcohol and aldehyde content. Dairy B displayed the sharpest differentiation with the white parts being clearly different from the blue and outer which had similar profiles.

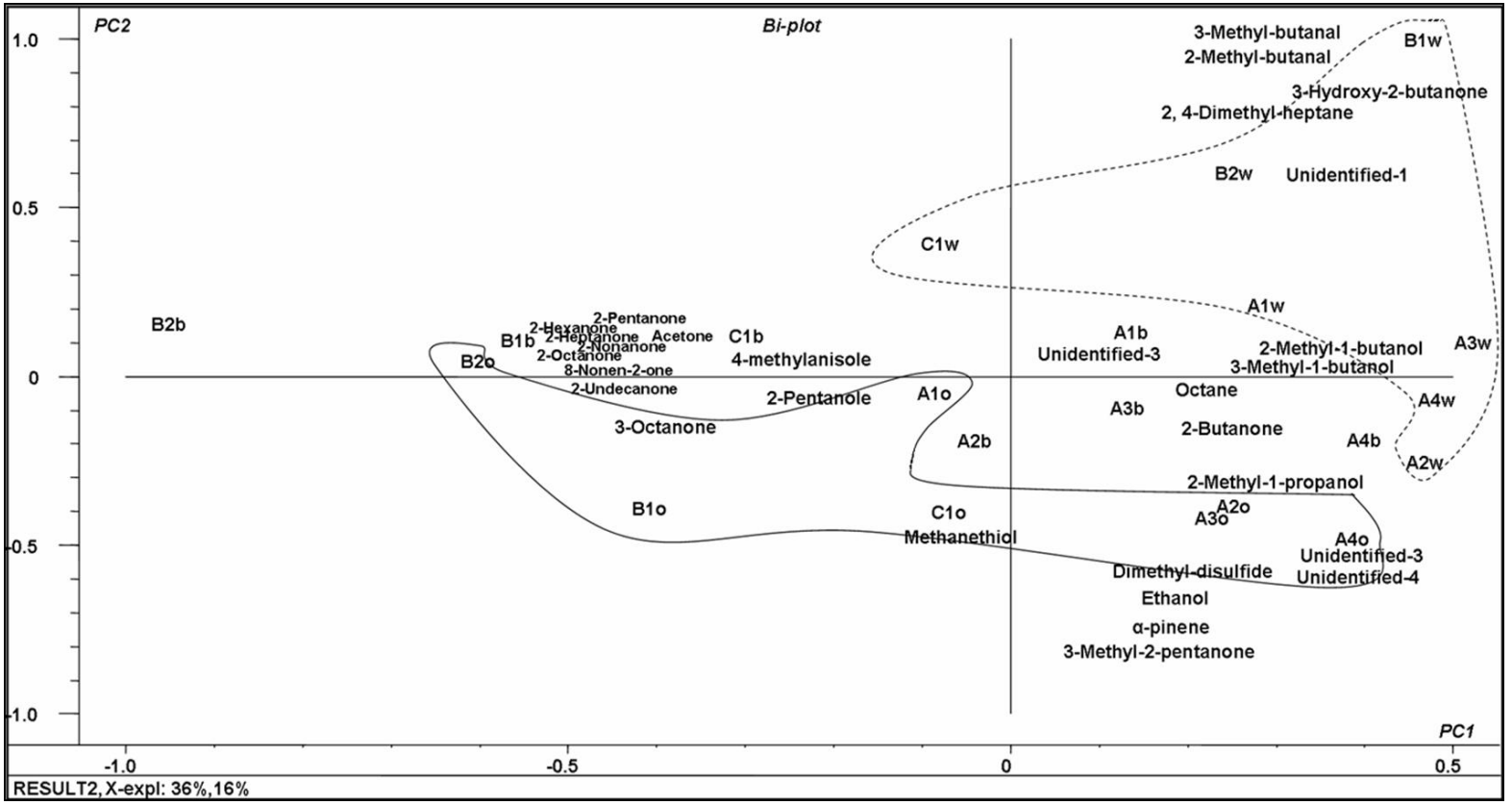


Figure 3.5. PCA plot of the compounds and the SPME GC-MS peak area data from the headspace analysis of the outer, blue and white parts of seven Stilton cheeses; A1, A2, A3, A4, B1, B2 and C1. Letters b, o and w refer to blue, out and white part respectively. Scores and loadings are based on the average of four replicate samples for each part of the cheeses.

3.3.6.3 PCA of SPME GC-MS data for individual parts of different cheese samples

PCA analysis of the data of each section of the cheese was individually investigated for similarities within the seven samples respective to the origin of production. The plot for the blue part, which is the most characteristic part for the blue cheese variety, is presented in Figure 3.6. The samples for the three different dairies were clearly differentiated from each other. Dairy A samples are to the right of the plot, B to the left and C in the middle. The differentiation (PC1) was caused by differences in the ketone-alcohol/aldehyde balance. Dairy B was associated with a higher level of ketones whereas dairy A had more alcohols and aldehydes. PC2 did not substantially resolve the samples from dairy B, but dairy A showed major differences with the mature cheese A4 correlating strongly with branched alcohols and sulfur compounds. This was not however the case with B1 which was also a mature Stilton. The blue parts are those where the starter mould *P. roqueforti* has high activity. The lipolytic activity of commercial strains of *P. roqueforti* can differ significantly. As a result different amounts of free fatty acids, precursors for aroma compound formation, are produced (Lopez-Diaz *et al*., 1996) and this could explain the differences in the aroma profiles of the blue parts of the cheeses.

The PCA plot for the outer part (Figure 3.6) presented similar trends with the plot for the blue. This was expected as it was previously observed that the blue and outer parts presented similarities in their aroma profiles. Again, in this plot the mature cheeses from producers A and B did not demonstrate any similarities. The PCA plot for the white part (Figure 3.6) demonstrated a strong differentiation between the

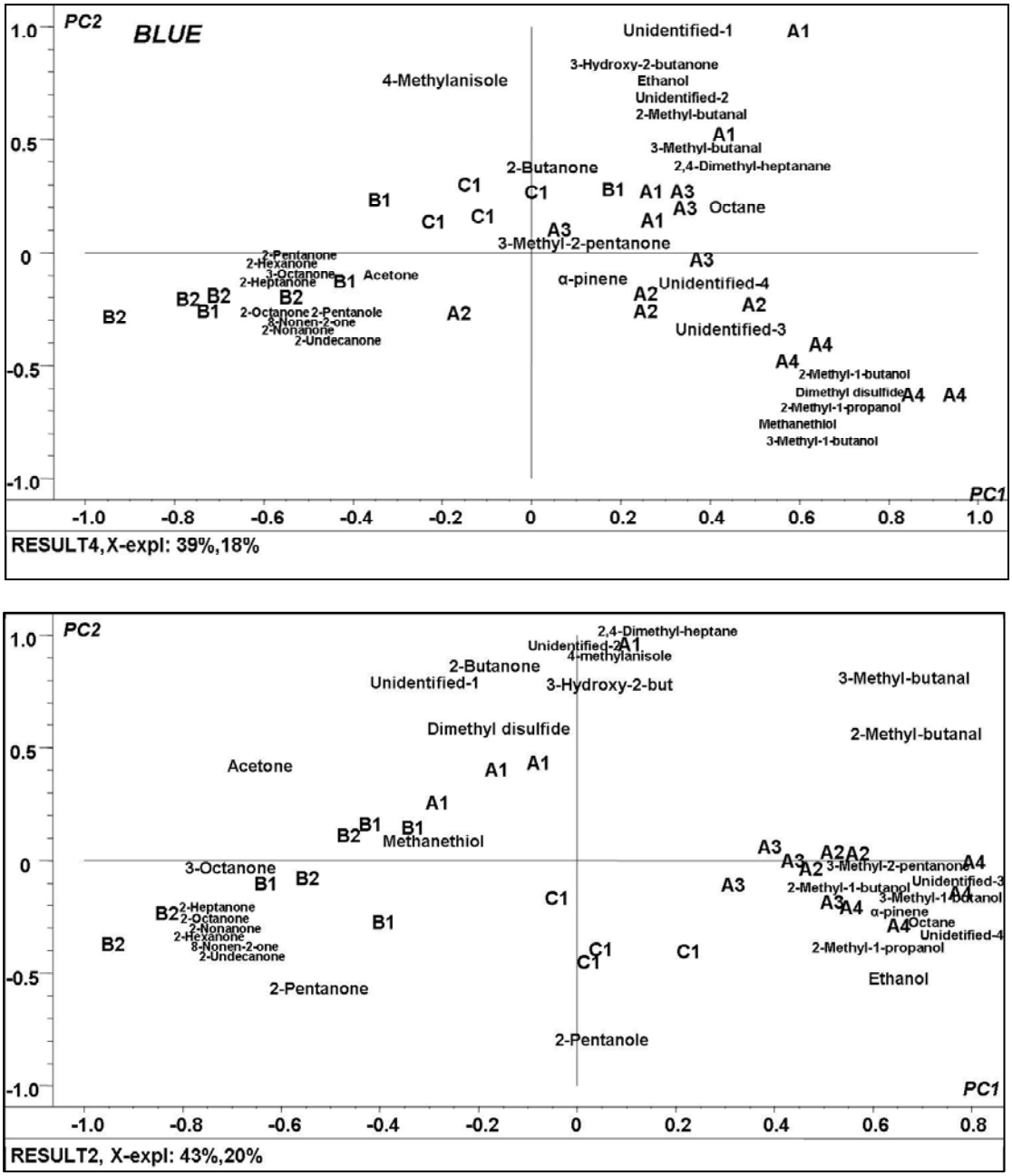


Figure 3.6 PCA plot of the compounds and the signal intensities detected when headspace samples of blue, out and white parts of seven Stilton cheeses (A1, A2, A3, A4, B1, B2, C1) were analysed with SPME GC-MS. Scores and loadings of four replicates of each cheese are shown.

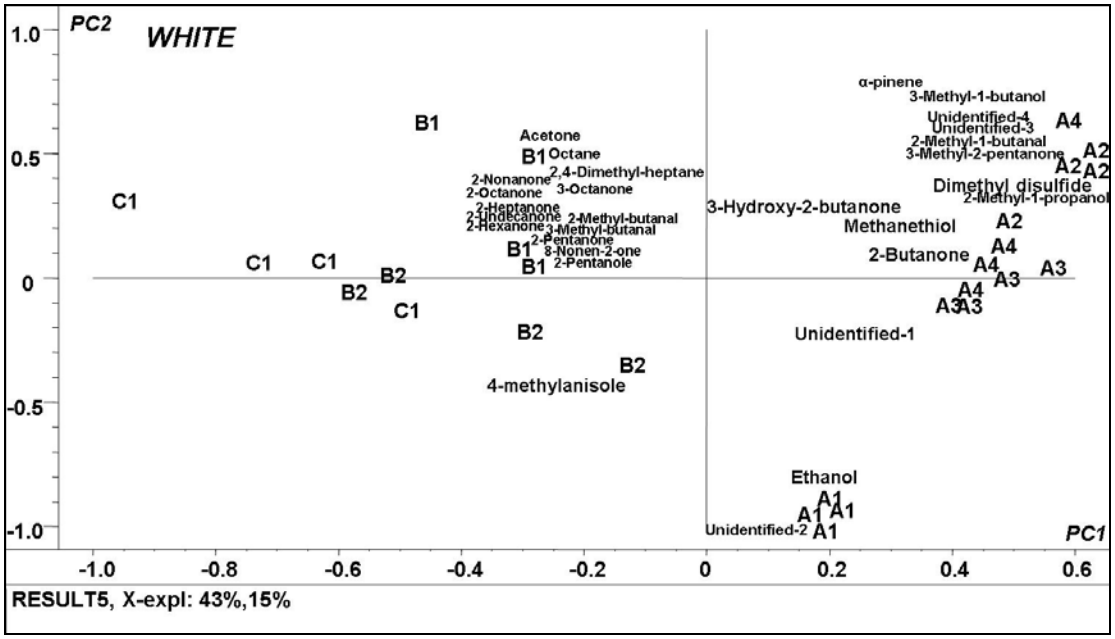


Figure 3.6 continue. PCA plot of the compounds and the signal intensities detected when headspace samples of blue, out and white parts of seven Stilton cheeses (A1, A2, A3, A4, B1, B2, C1) were analysed with SPME GC-MS. Scores and loadings of four replicates of each cheese are shown.

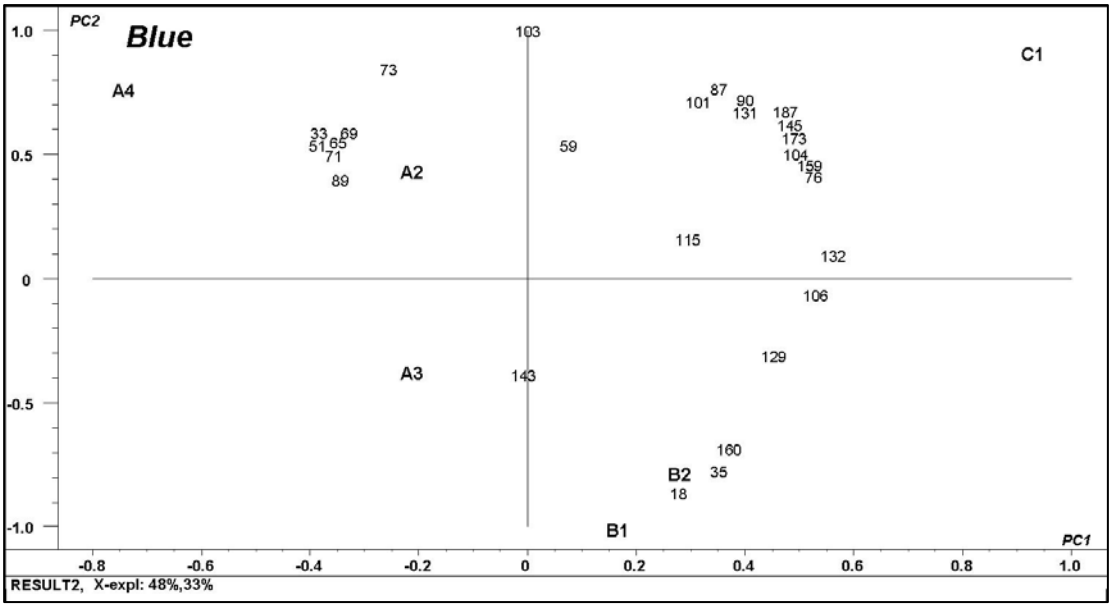
cheese from dairy B and C with those from dairy A. The cheeses from dairies B and C were more related with the methyl ketones as seen in the other two plots.

3.3.6.4 APCI-MS of 7 Stilton cheeses

APCI-MS analysis was applied to Stilton cheeses from different producers. The results (Appendix 4) confirmed the high amounts of m/z 18 (related with ammonia) in the headspace of Stilton cheeses. Mass 18 was not scanned in the analysis of the first cheese but was suspected because of the ammonium effect observed (see 3.3.3.2).

PCA analysis of the data of each of the three sections of the cheeses was conducted (Figure 3.7). In the PCA plots for the blue and white parts of the cheese (inner core) dairy B was strongly related with mass 18 (ammonia) and the secondary ammonia ion 35 ($NH4^+$.[NH3]).

The most important observation in the PCA plots was that the APCI-MS managed to differentiate the samples according to the origin of production. This observation was common in all the plots no matter the part of the cheese. Differentiation according to the origin of production was initially observed with the SPME GC-MS (see 3.3.6.3). Because APCI-MS is a direct MS technique it lacks the accuracy and amount of information of GC-MS. It was questionable whether the same level of discrimination could be observed with both techniques. The results demonstrate that APCI-MS can be very effective for the rapid differentiation of cheeses according to the producers. Its analytical ability was not affected by the high presence of ammonia.



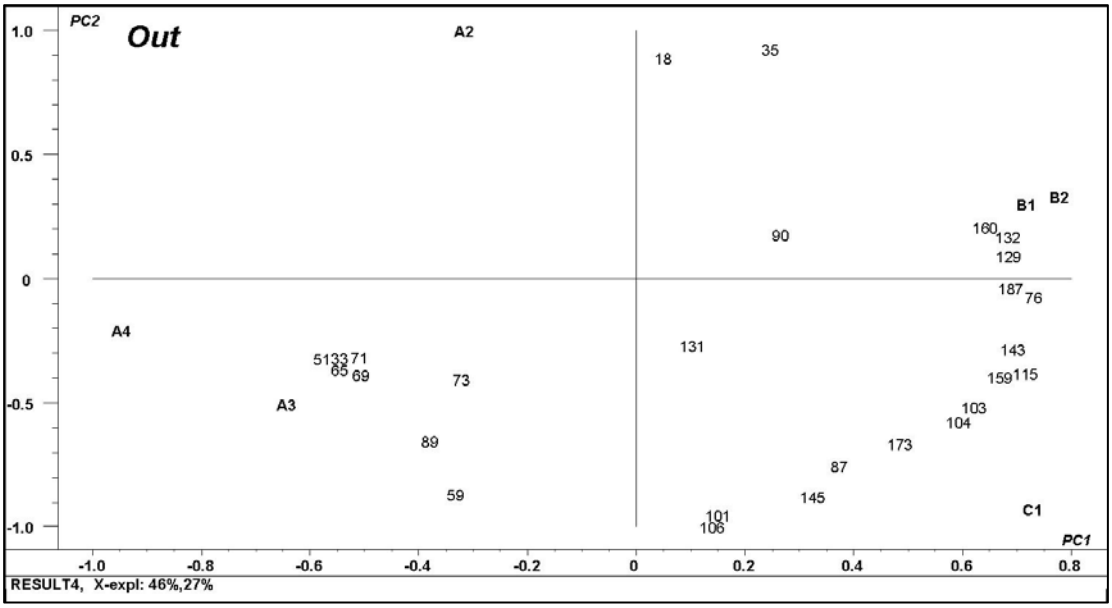


Figure 3.7 PCA plot of the main ions and the signal intensities detected when headspace samples of blue, out and white parts of Stilton cheeses (A2, A3, A4, B1, B2, C1) were analysed with APCI-MS.

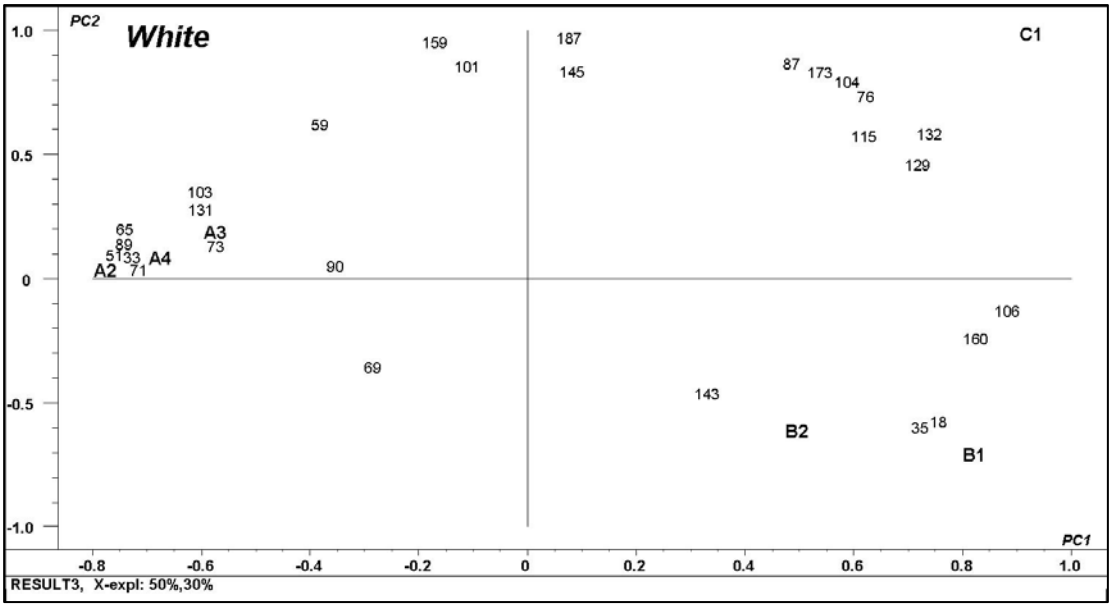


Figure 3.7 continue. PCA plot of the main ions and the signal intensities detected when headspace samples of blue, out and white parts of Stilton cheeses (A2, A3, A4, B1, B2, C1) were analysed with APCI-MS.

APCI-MS could be very useful for Stilton cheese production as the retailers request Stilton cheeses with specific characteristics. Furthermore, in a previous study it was suggested that direct injection of the volatile fraction of a product into a mass spectrometer produces a characteristic spectrum (sometimes referred to as the 'signature' or 'spectral fingerprint') which could be used for classification and estimation of the products' sensory and technological properties (Peres *et al*., 2003). Therefore the potential for the APCI-MS as a tool for classification of cheeses may not be limited to differentiation according to the producers but also according to the individual properties of cheeses from the same producer. Miniature MS systems are being developed at the moment (Taylor, Srigengan & Syms, 2001) which will allow portability of these systems. Then the applications could increase and it might be possible for these instruments to become routine analysis tool for quality control.

3.3.7 Origin of compounds in Stilton aroma

The blue and outer part aroma profiles were largely dominated by the ketones. In contrast to the domination of this group by ketones, there was a second group of compounds more related to the white part, mainly consisting of alcohols and aldehydes (3-methyl-butanal, 2-methyl-butanal, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 2-methyl-1-butanol).

The predominance of ketones and alcohols in Stilton's aroma profile should be expected as numerous studies report the intense lipid metabolism taking place in cheeses, in which the fatty acids are precursors of methyl ketones and alcohols. In blue cheeses, where the ketones are characteristic of the aroma profile, their main

pathway of aroma formation is attributed to the metabolism of *P. roqueforti*. High concentrations of free fatty acids (FFA) have been shown to inhibit the growth of *P. roqueforti* and their conversion to methyl ketones has been proposed to be a detoxifying mechanism (Kinsella and Hwang, 1976b). The presence of enzymes involved in *P. roqueforti*'s sporulation (blue part) and mycelium (white part) able to convert FFAs into methyl ketones was reported by Lawrence (1966) and Lawrence and Hawke (1968) respectively.

Figure 3.8 shows the main pathway of blue cheese aroma compound formation. Briefly, lipases hydrolyse the ester linkages of triacylglycerols in milk fat and fatty acids are formed (Chalier & Crouzet, 1998). These then enter a β-oxidation pathway which seems to favour the partial oxidation and formation of β-keto-acids instead of the complete oxidation to acetyl-CoA and acyl-CoA forms. β-keto-acids are decarboxylated to 2-alkanones after further reduction and these, in turn, are partially reduced to 2-alkanols (Kinsella *et al*., 1976a). The conversion of 2-akanones to the corresponding secondary alcohols (2-alkanols) is a step which is reversible under aerobic conditions (McSweeney *et al*., 2000).

Hence, the majority of the metabolites contain an odd number of carbon atoms. Furthermore, selective conversion of the C8:0 and C10:0 fatty acids is taking place during ripening (Anderson & Day, 1966) and therefore excess amounts of 2-heptanone and 2-nonanone are formed. However, in several studies the levels of the precursor FFAs (C_n) could not explain the excess production of the corresponding ketones (C_{n-1}), especially 2-heptanone and 2-nonanone, and it was suggested that long-chain FFAs (C_{n+}) were also catabolised (Madkor *et al*., 1987).

Aldehydes originating from amino acids can also degrade to alcohols by transamination and oxidative deamination followed by decarboxylation (Moio *et al*.,

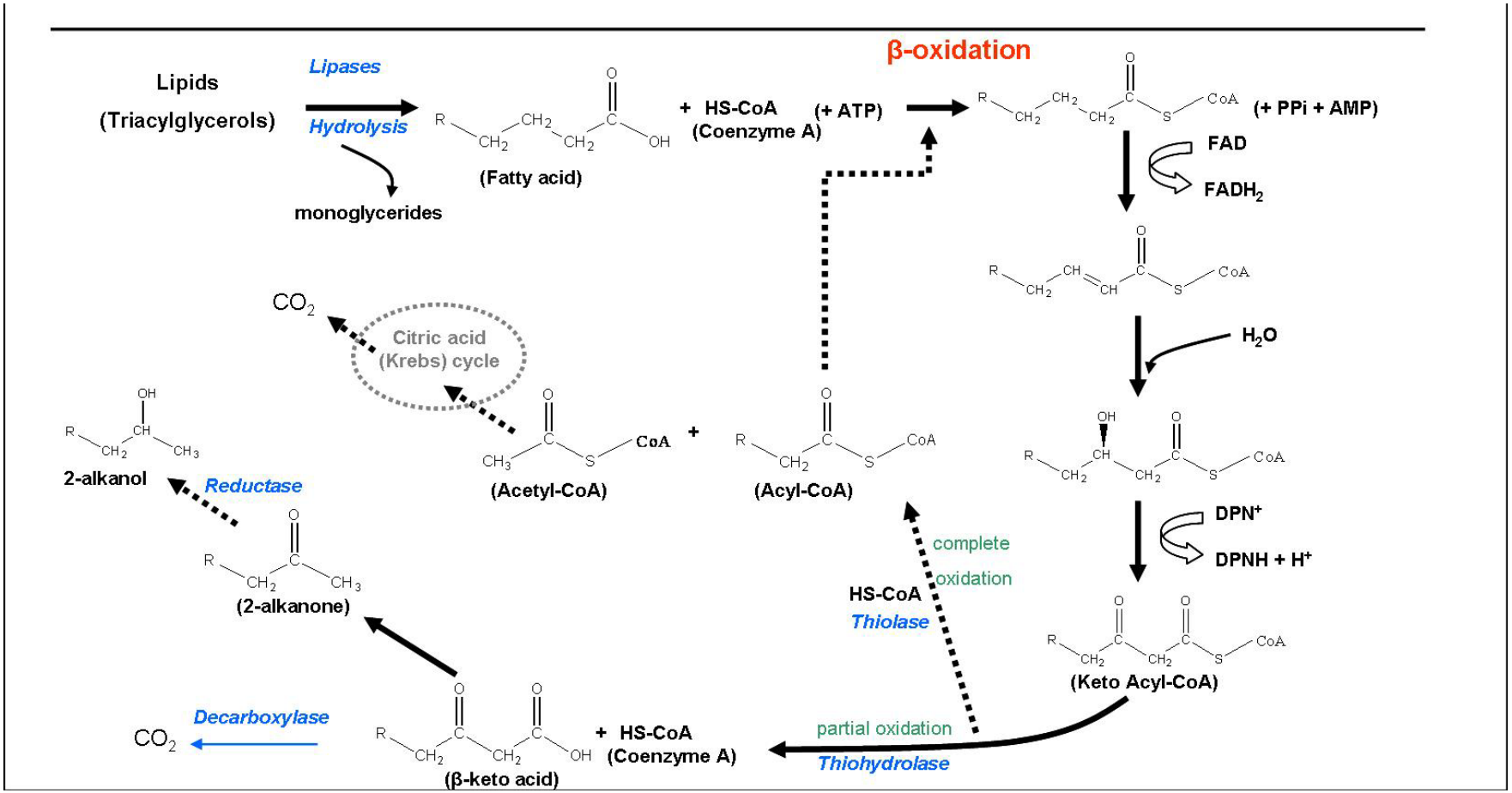


Figure 3.8 Main metabolic pathway of blue cheese flavour formation.

2000). Other major compounds of the aroma profile are the 3-methyl-butanal, 2-methyl-butanal, 3-methyl-1-butanol and 2-methyl-1-butanol and they are likely to be the result of protein degradation. Branched-chain aldehydes are believed to originate from enzymatic amino acid degradation (Moio *et al*., 1993). The 2-methylbutanal and 3-methylbutanal are formed from isoleucine and leucine respectively (Curioni *et al.*, 2002). Branched-chain aldehydes can also be products of Strecker degradation (nonenzymatic reaction) (Griffith & Hammond, 1989). The presence of branched-chain primary alcohols, such as 3 or 2-methyl-1-butanol, indicates the reduction of the aldehyde produced from isoleucine and leucine (Curioni *et al*., 2002).

Alcohols have been reported to be 15 to 30% of the total volatile compounds in blue cheese (Gallois *et al*., 1990; Moio *et al*., 2000). However, these studies did not refer to individual parts of the cheese and it not possible to demonstrate that the production of the alcohols and aldehydes is mostly related with the white core. The significant presence of alcohols in the white part of the blue cheese could be explained by the fact that methyl ketones can be reduced to secondary alcohols under anaerobic conditions (Fox *et al*., 2004). The white part is the part of the blue cheese where anaerobic conditions are favoured throughout the production and maturation in contrast to the blue veins where oxygen is present. For the same reason *P. roqueforti* is exclusively present in mycelium form in the white part while the blue veins allow the mould to sporulate. The mycelia of *P. roqueforti* are more active than the sporulating regions in reducing methyl ketones to secondary alcohols (Anderson *et al*., 1966). Finally, yeasts related with blue cheeses are also capable of reducing methyl ketones to secondary alcohols (Anderson *et al*., 1966). It was demonstrated that the yeast flora in the white part is different than the other two parts. The yeast

species present in the white part might be another reason for the higher amount of alcohols in the white core.

Methanethiol is a sulfur containing compound that is not frequently reported in blue cheeses. Methanethiol is derived from methionine and has important odour properties (see §3.3.8). There are several micro-organisms that are able to produce methanethiol from methionine degradation which starts with the enzymatic cleavage of the bond between carbon and sulfur (Molimard *et al*., 1996; Marilley *et al*., 2004). Methanethiol may result in further sulfur compounds e.g. dimethyldisulphide and dimethyl trisulfide via oxidative reactions. Indeed, dimethyl disulfide was present in Stilton and in most of the samples the patterns of these two compounds for the different parts of the cheeses were similar (Table 3.5).

3.3.8 Odour properties of compounds present in Stilton aroma

Most of the compounds detected in this study have previously been reported as present in blue cheese varieties (Table 3.2). Therefore they could be important for the blue cheese aroma. In general, a great number of volatile compounds coming from a broad range of chemical groups have been identified in cheeses. However, not all of the compounds detected in cheeses are odour active and responsible for the cheese flavour or at least not in the quantities present in the cheese (Curioni *et al*., 2002). Abundant volatiles may have little or no odour impact on the aroma of product. This leads to confusion in the literature between odour-active compounds and those which are insignificant. For this reason it is important to discuss some of the odour properties of the compounds that were found in the aroma profile of Stilton cheese.

The odour characters of the group of compounds that dominated the blue and outer parts consisted of blue cheese, green, and fruity attributes while odours of the group of compounds related to the white part were mostly characterised by fruity, alcoholic and malty attributes (Table 3.6).

Incorrectly, in early cheese aroma studies, it was thought that the aroma resulted from a single or a few compounds of the same class (McSweeney *et al*., 2000). This is partially true for blue cheeses where ketones are dominant. Among the dominant ketone compounds in the blue and outer parts, 2-heptanone and 2-nonanone are of the greatest interest. In sensory tests 2-nonanone has been found to be a key character of blue cheese flavour and 2-heptanone had an important contributory effect (Rothe, Kornelson & Schrodter, 1994). However, it is now well established that the total aroma results from the combination of a number of compounds and, most importantly, their ratios and concentrations are essential to the final aroma. (principle of the 'component balance theory'; Mulder, 1952). In another study supporting this, the aroma profiles of six different cheese varieties were found to consist of the same compounds but in different concentrations and the flavour did not seem to depend on the concentration of individual compounds but on the balance between the components (Bosset & Gauch, 1993).

Therefore the choice of the extraction method of the cheese aromas is very important. It must be appropriate in order for the extracted aroma profiles to be representative of the actual aroma profile of Stilton including the ratios and the balances between compounds. Thus in this study three different analytical approaches (solvent extraction, headspace extraction and direct injection) were used and compared. Furthermore it is demonstrated that the investigation of the balances of the

Table 3.6 Main odours characteristics for the aroma compounds detected in Stilton.

Compound	**Odour characteristics**	**Threshold** [b]
2-butanone	sweet, apricot [b]	n/a
2-heptanone	blue cheese, fruity, musty [a]	1-1.33 ppm
2-hexanone	fruity [a]	41-81 ppm
2-methyl-1-butanol	alcoholic, banana, iodoform, vinous, fusel oil, sweet [a] cooked, roasted, alcoholic/fruity undernotes [b]	0.14 mg/m^3; recognition at 0.83 to 1.7 mg/m^3
2-methyl-butanal	almond, burnt, choking, cocoa, estery apple, fermented, fruity [a] chocking, peculiar cocoa/coffee like when diluted, [b]	n/a
2-nonanone	blue cheese, fatty, fruity, green, ketone, musty	5-200 ppm
2-octanone	floral, bitter green, fruity [b]	41-62 ppm
2-pentanone	acetone like, wine, characteristic [b], sweet fruity ketone [a]	70 ppm
2-undecanone	dusty, floral, fruity, green, musty, rose	7-82 ppm
3-hydroxy-2-butanone	butter, fresh, fruity, green, mould, slightly rancid, woody [a] bland, woody, yogurt, fatty creamy 'tub' butter taste [b]	5-10 ppm
3-methyl-1-butanol	alcoholic, banana, green, malt, sweet, vinous [a] fusel oil, whisky characteristic pungent odour [b]	4.1-250 ppm

Table 3.6 continue. Main odours characteristics for the aroma compounds detected in Stilton.

Compound	Odour characteristics	Threshold
3-methyl-butanal	almond, chocolate, malt [a] choking, acrid, pungent apple-like [b]	n/a
3-octanone	strong, penetrating, fruity odour [b], mushroom, earthy [a]	21-50 ppm
4-methylanisole	pungent odour suggestive of ylang-ylang (plant) [b]	200 ppm
8-nonen-2-one	blue veined cheese like [c]	n/a
Acetone	flavour enhancer [b]	40-476 ppm
a-pinene	pine [b]	2.5-62 ppm
Ethanol	characteristic strong alcoholic [b]	8-900 ppm
Methanethiol	boiled-cabbage, sulfurous [a]	0.02-4 ppm

[a]: http://www.odour.org.uk

[b]: Burdock (2005)

[c]: Urbach, G., (1997)

different groups of compounds between the different sections of the cheese can help to understand how the overall aroma of the blue cheese develops.

A combination of compounds can result in an overall aroma very different from that of the compounds individually. The organoleptic perception of a given compound can be modified by other cheese aroma components (Curioni *et al*., 2002). An example of such compounds is the sulfur-containing methanethiol which was detected in all the seven Stilton samples (Table 3.5). It may be possible that this compound modifies the overall flavour of the cheese. As an individual compound its characteristic boiled-cabbage like odour is usually related with decomposition and it is unpleasant (Table 3.6). However, in combination with other compound(s) it loses its individual odour characteristics and it was found to be part of the typical aromas of cheddar cheese (Wijesundera & Urbach, 1993). Similar properties were observed for non-cheese aromas. The odour of beef stew juice created by a mixture of volatiles was lost completely when methanethiol was removed from the mixture (Guth & Grosch, 1994). It was observed that the presence of methanethiol in the outer part of Stilton was higher for most of the samples. However, previous studies suggest that the exact proportion does not seem to affect its properties and therefore its interaction with the other compound(s) is probably physiological. The concentration of methanethiol in full fat and low fat cheddar cheeses was correlated with the flavour grade, with the low fat (and poor in aroma) cheddars containing less methanethiol (Dimes, Urbach & Miller, 1996). Both sulfur containing compounds detected in Stilton, methanethiol and dimethyl disulfide, are considered to be important odour contributors (Curioni *et al*., 2002; Frank *et al*., 2004). The different levels of methanethiol and dimethyl disulfide was a characteristic that distinguished French blue cheese varieties (Gallois *et al*., 1990). Considering the above information on the role of the sulfur compounds

in the cheese aroma it could be concluded that further sensory research on their role in Stilton would be very interesting.

The role of the cheese matrix should also be considered. The perception threshold of a compound is strongly dependent on the matrix properties. The thresholds reported for specific aroma compounds in water and oil matrices vary significantly. For example, 2-heptanone has detection threshold of 140 ppb in water (Buttery, Turnbaugh & Lingl, 1988) and 1500 ppb in oil (Preininger & Grosch, 1994). The aroma differences detected between the different parts of the cheese and the overall flavour reflect differences in the compounds, their concentrations and their interactions with the matrix.

3.4 CONCLUSIONS

One aspect that was discussed in this chapter is the comparison of different analytical approaches for the analysis of the blue cheese flavour. From the different techniques that were compared for the Stilton aroma analysis APCI-MS was good for rapid discrimination of the different cheese profiles. The analysis was affected by the presence of ammonia in the headspace which was altering the reagent ion profile and affected the ionisation and ion profile. However, a low sample flow rate can minimise this effect and APCI-MS can be a rapid authentication method. SPME was simpler to perform than solvent extraction and contained a large amount of information in the profile. SPME GC-MS resulted in satisfactory qualitative results and was able to screen for differences between the sections of the cheeses and dairies. The combined direct MS and GC-MS approach and the different observations with each of them demonstrates the complimentary role of the techniques. Their combination could allow the rapid screening of the profiles with direct MS e.g. screen for maturation indicator compounds during ripening, taken that identities could be assigned to ions with prior GC-MS analysis.

So far in the literature emphasis has been focused on the role of the ketones in the blue cheese flavour, the role and contribution of compounds from other chemical groups (e.g. alcohols and aldehydes) is not well discussed. There is also a general trend for the production of the ketones to be attributed to metabolic pathways related with the *P. roqueforti,* mainly to the β-oxidation of fatty acids. Despite the fact that the blue cheese is a highly heterogeneous matrix this has not been taken into account. The aroma profile of the whole cheese was studied instead.

In this study it was demonstrated that the aroma profiles of the different sections of Stilton differ significantly. Overall, the blue and the outer crust had similar profiles. These two sections were more related with the ketones. The white part contained high amounts of alcohols and aldehydes. This characteristic of the white part has not attracted as much attention and much work has focused on the ketones, despite the fact that the white part is the biggest part of the cheese.

4. STUDY OF THE INTERACTIONS BETWEEN STILTON ISOLATES REGARDING THE AROMA PRODUCTION

4.1 INTRODUCTION

4.1.1 General

In the previous chapters it was demonstrated that the fungal communities between the three different sections of Stilton are very different (Chapter 2) and that these sections also have very different aroma profiles (Chapter 3). Ketones, which are important compounds for the blue cheese aroma and traditionally assigned to the activity of the starter *P. roqueforti*, were detected in high amounts in the outer section of the cheese where the presence of the *P. roqueforti* was limited. Considerable and diverse counts of yeast secondary flora were detected in the outer section of Stilton. In contrast, the formation of alcohols was favoured in the white core where the mould exists in mycelium form and does not sporulate because of the lack of air.

The fungal flora is known to have a major role in the aroma formation in fermented products including cheese (McSweeney *et al*., 2000). Therefore it could be hypothesised that the aroma differentiation between the sections of Stilton is related to the differences in the fungal communities. The question of what and how significant is the impact of the yeast flora on the aroma formation is introduced.

The impact of the secondary flora, especially the yeasts, on the flavour has been investigated in the past. However, much of this work focused on the way that the yeasts could contribute to the cheese ripening with regard to lipolysis, proteolysis and glycolysis and interactions in growth such as the suppression or the enhancement of

the microbial counts, the suppression of *P. roqueforti*'s hypha development etc. (Van den Tempel *et al*., 2000a; Van den Tempel *et al*., 2000b; Addis *et al*., 2001). It was suggested that the role of some yeast species may be beneficial by contributing to the ripening and aroma formation because of their considerable proteolytic and lipolytic activity. Consequently, there might also be a positive impact on the aroma production.

However, in contrast to the other aspects relatively little work has been done on the interactions of microorganisms regarding the formation of aroma compounds. Indeed, several yeast species have been found to produce interesting aroma compounds when they were studied outside the cheese matrix. Many yeast species were able to produce significant amounts of a range of compounds including esters, free fatty acids, alcohols and ketones which are particularly important for the blue cheese flavour (Spinnler *et al*., 2001; Buzzini *et al*., 2005). Most of the studies concern the aroma production from yeasts species individually. However, the aroma formation is a result of a complex of metabolic pathways which can follow different routes when different species are co-present. Synergistic effects expressed through mutual use and production of nutrients can take place. Metabolic products (intermediate or final) from one species could trigger the beginning or enhance another metabolic pathway from another species.

Therefore in order to understand any possible link between the aroma profiles and the yeast species it would be useful to focus not only on the study of the aroma production of individual species but also on how this is affected by the co-presence of the different species as part of the complex communities of Stilton. The most interesting would be the interactions between the starter *P. roqueforti* and the secondary yeast flora. These are known to determine many aspects of the blue cheese during the production and ripening e.g. the maturation time, the texture (Fox *et al*.,

2004). Thus it would be interesting to study how the aroma production of the starter *P. roqueforti* may be affected by the presence of the secondary yeast flora detected in Stilton.

In contrast to the benefits that may occur, the secondary flora could also have a negative impact by suppressing the growth of the starter cultures. Antagonism and negative interactions expressed though the production of antimicrobial/mycostatic metabolites or competition for nutrients can take place. Indeed, the blue cheese producers often have to deal with batches of cheese with poor blue veining as a result of the lack of *P. roqueforti*'s sporulation. This takes place at a late stage of the production (piercing after the 6^{th} week of production) and therefore after the yeast flora has reached considerable population counts.

4.1.2 The choice of a model for studying the aroma formation from fungi

The properties and interactions regarding the growth and the aroma production between microorganisms in cheeses are very complex. It would not be possible to study them without being affected by other parameters involved in the cheese production (e.g. enzymatic aroma production because of rennet present during ripening). Studies directly on cheeses are expensive. In addition, blue cheese production is time consuming and there is variation between batches. This would affect the comparability and the reproducibility of the results. Therefore in order to study the aroma production of the blue cheese fungal community it is preferable to grow the microorganisms in simpler matrices.

UHT milk is considered to be an appropriate medium for studying interactions of microorganisms of dairy origin. It has a composition similar to that of raw milk (Rosenthal, 1991) which is used for the production of cheese. As milk is the main ingredient that is used in cheese production its use would ensure that similar precursors of aroma compounds are provided in the model study. UHT milk has been used previously for the study of yeasts originating from mould-ripening cheeses and it was successfully applied for the evaluation of their lipolytic and proteolytic activity and the metabolism of important compounds e.g. free fatty acids, free amino acids, and ethanol (Roostita *et al.*, 1996a). UHT milk was also used for studying lactic acid bacteria in combination with yeasts and their potential interactions in a fermented milk product (Gataga *et al*., 2001). Among the species studied were yeasts and bacteria that are also present in Stilton. By growing the microorganisms individually and in combinations the authors were able to study the production of volatile organic compounds using headspace gas chromatography (HS-GC).

Alternative models that have been used are cheese media and semi-synthetic cheese media. These media are created by mixing real cheese or cheese curd with a synthetic medium (Hansen & Nielsen, 1997a; Leclercq-Perlat, Corrieu & Spinnler, 2004; Juszczyk *et al*., 2005). They present the advantage that their composition, water activity (aw) and pH can be easily and accurately adjusted by adding NaCl and HCl or NaOH. However, their composition is set to meet the particular growth requirements of each study and this makes them very specific. Their composition can be significantly different than the real milk or cheese. They were preferred for studying particular properties of microorganisms such as the growth of fungi in the presence of special nutrients, the comparison of the production of specific groups of volatile compounds and the potential of yeast as starter cultures (Hansen *et al*., 1997a; Van

den Tempel *et al.*, 2000a; Van den Tempel *et al*., 2000b; Leclercq-Perlat *et al*., 2004; Juszczyk *et al*., 2005). All the cheese media had different compositions from each other.

Other alternatives that have been used for studying microorganisms are butterfat emulsions for studying the effect of the environmental conditions on the lipolytic activity of *Penicillium roqueforti* (Larsen & Jensen, 1999) and whey for the characterisation of lactic acid bacteria strains on the basis of their neutral volatile compound production (Mauriello *et al.*, 2001). Finally, laboratory media have been used for studying interactions between *Penicillium roqueforti* and lactic acid bacteria, however, the interactions were highly affected by the choice of medium (Hansen *et al*., 1997b).

In another approach, the measurement of enzymes involved in the metabolic pathways can be carried out as an indication of the ability to produce flavours. This method was good for differentiation between strains (Marilley *et al*., 2004). However, this approach is limited because it is very time consuming (the preparation of cell-free extracts is required). In addition, many enzymes are involved in pathways that produce non-volatile taste compounds. Therefore this approach would be more appropriate for studies that focus on the flavour and not the aroma production.

4.1.3 Outline of study

The aim of this study was to investigate the properties of the Stilton fungal flora in terms of aroma production and particularly any interactions between the yeasts and the starter mould *P. roqueforti*. Representative isolates from the five

groups detected in Chapter 2 were grown individually and together with *P. roqueforti*. One *Lactococcus lactis* reference strain was also included in the study as this species together with the *P. roqueforti* are the main starter microorganisms of Stilton.

The use of UHT milk was preferred as a simple model which could provide a matrix capable for growing both the fungi and the starter *Lactococcus lactis* individually and in combinations without being particular selective for any of the microorganisms and having a composition close to the raw material used in cheese production. Because of the low aroma profile of milk its contribution to the final chromatographs would be relatively low making their analysis easier.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of inoculants

Representative yeast isolates of the groups that were recovered from Stilton (*D. hansenii* group A, *D. hansenii* group B, *Y. lipolytica*, *K. lactis* and *T. ovoides*) were grown on YPD agar at 25^{o}C for 5 days. *P. roqueforti* isolates from the blue veins of Stilton were grown on RBCA agar at 25^{o}C for 7 days. The *Lactococcus lactis* subsp. *lactis* NCIMB 4918 reference strain was grown on BHI at 30^{o}C for 48 hours.

4.2.2 Preparation of models

Yeast and bacterial bulk cells and conidia of *P. roqueforti* were harvested using sterile swabs and suspended in Phosphate Buffered Saline (PBS, Oxoid). The concentration of cells/conidia in the PBS suspension was calculated using a counting chamber (Weber Scientific International). The suspensions were then further diluted with PBS to a concentration of 1 x 10^{7} conidia ml^{-1} or cells ml^{-1}.

Models were prepared using UHT-treated milk purchased from a local retailer (3.6% fat, pH 6.65 at 21^{o}C). Milk (100 ml) was aseptically dispensed into sterilised Duran bottles (Schott;250 ml volume) containing a magnetic follower. The milk was inoculated with cells/conidia suspensions in PBS to a concentration of 1x10^{5} cells ml^{-1} and/or 1x10^{5} conidia ml^{-1}. The magnetic followers in the flask allowed the models to be stirred and remain homogenous. Four replicates were prepared for each model and

they were incubated at 25°C for 10 days. The bottles were observed daily for mould sporulation. The pH was measured on day 10.

Samples from the models (10 ml) were transferred in 20 ml Headspace vials (22.5 mm x 75.5 mm) for SPME analyses. The vials were immediately sealed with a magnetic cap (20mm diameter, 5mm centre, PTFE / Silicone Liner) and they were stored at -80^{o}C until analysis.

4.2.3 Cheese samples

A set of cheese samples was purchased from local retailers in order for their aroma to be analysed together with the models. These were 5 different blue cheese varieties (Stilton, Roquefort, Danish blue, Blacksticks blue, Shropshire blue), one blue cream cheese (spread cheese containing 50% blue cheese) and two typical non-blue cheeses (standard and extra mature cheddar cheese).

4.2.4 SPME GC-MS analysis

The SPME vials with the models were defrosted at 4^{o}C overnight before analysis. Cheese samples (1.5 g) were also prepared. All the samples were allowed to equilibrate at 22°C and SPME GC-MS analysis was conducted as described in § 3.2.5.

4.2.5 Statistical analyses

The relationship between the models and their volatiles (variables) detected with SPME GC-MS analysis was evaluated by Principal Component Analysis (PCA) using Unscrambler v.9.0 (Camo Process AS., Norway). All data were standardised (1/standard deviation) prior to analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 Model combinations and growth conditions

Representative isolates from each of the five yeast groups that were detected in Stilton (Chapter 2; *D. hansenii* group A, *D. hansenii* group B, *Y. lipolytica*, *K. lactis* and *T. ovoides*) were grown individually and in combination with *Penicillium roqueforti* in order to observe how their interactions affect the aroma production in comparison with when they grow individually. *Penicillium roqueforti* and *L. lactis* were also grown individually and in combination to establish the baseline that the established starter cultures would produce. Samples without any inoculant were also included as control samples.

Six blue cheeses were also included in the analyses in order to compare the similarities of the aroma profiles of the model systems with those from the real blue cheeses. Two typical non-blue cheeses (Cheddar standard and mature) were also included in the study. This was in order to check whether any similarity between the aromas of the models and the blue cheeses would be because of characteristics regarding the blue cheese variety or the cheese aroma in general.

The growth of the microorganisms was selected to take place in UHT milk at 25^{o}C and each trial lasted 10 days and without the addition of salt. Since blue-veined cheeses contain significant concentrations of salt it could be suggested that their microflora is salt-adapted and models supplied with salt should be used. However, Roostita *et al*. (1996a) tested the impact of a range of added sodium chloride (0-15%, w/v) on the growth of yeasts in milk. The rates of growth and the maximum cell densities decreased as the concentration of NaCl in the milk was increased from 0 to

15%. When salt was added the changes in milk composition were similar with those without salt but the strength of these changes was decreased making their observation more difficult.

In another study (Roostita *et al*., 1996b) a number of dairy yeasts originating from mould-ripening cheeses were examined regarding the influence of salt concentration and temperature on their growth. Several of the species that were studied were common with those detected in Stilton (*Kluyveromyces lactis*, *Candida lipolytica*, *Debaryomyces hansenii*). All species gave decreased growth rates and cell yields in milk at 10°C containing added NaCl compared with growth at 25°C. At 25°C they reached maximum populations of 10^7-10^8 cfu/ml in 2-3 days and retained high levels for 10 days. Thus 10 days incubation at 25°C was selected as an appropriate time length for the trails in the present study.

In fact yeasts that can grow at low a_w do not have an absolute requirement for reduced a_w and can grow most rapidly at high a_w (Beuchat, 1993). Mistakenly, they are referred as osmophilic and not xerotolerant. Similarly, no changes were found between the populations that were obtained from another study on blue cheeses on salt supplemented and non-salt supplemented media (Addis *et al*., 2001). Therefore the use of a medium without salt would not be expected to inhibit the growth of these yeast species.

4.3.2 SPME GC-MS analysis

The aroma analysis of the model systems included the compounds that were previously detected in Stilton cheese (Chapter 3) as well as new compounds that were

detected in significant amounts in the headspace of the models (Appendix 5). The model of the starter mould *P. roqueforti* resulted in an aroma profile which was characterised by the presence of high levels of ketones (2-heptanone, 2-nonanone, 2-pentanone, 8-nonen-2-one, 2-octanone, 2-decanone, 2-undecanone). This was expected as *P. roqueforti* is considered responsible for the main core of the blue cheese flavour, especially for the ketones which are formed through the β-oxidation of fatty acids (Kinsella *et al*., 1976a). The amounts of 2-heptanone and 2-nonanone in the *P. roqueforti* model were particularly high and at quantities similar to those in the real blue cheeses. The production of the full set of ketones that were detected in Stilton, in the *P. roqueforti*'s model was an indication that the growth of Stilton isolates in UHT milk could result in aroma profiles relevant to those that are produced in the real cheese production. A new compound, 1-pentanol, was produced uniquely as it was not detected in considerable amounts in any of the other model systems nor in the headspaces of the real cheeses.

L. lactis individually produced considerable amounts of 3-hydroxy-2-butanone while the production from *P. roqueforti* was minimal (Appendix 5). However, the production of this compound was four times higher when *L. lactis* and *P. roqueforti* grew together in the same model (50.8 compared to 12.6 for the *L. lactis* individually). In contrast, the presence of *L. lactis* suppressed *P. roqueforti*'s production of 2-heptanone (68.7 and 28.2 for individual *P. roqueforti* and with *L. lactis* respectively) and 2-nonanone (70.9 and 9.1 for individual *P. roqueforti* and with *L. lactis* respectively). These ketones are important for the blue cheese flavour. However, in the blue cheese production *L. lactis* would grow faster than the fungal flora (typically in the first 24 hours after the milk is inoculated with the starters) and would dominate the cheese curd during the first days of production. It has a major role

in the formation of appropriate conditions and compounds (e.g. production of lactic acid and the decrease of the pH in the curd) in order for the rest of the microflora to develop (McSweeney *et al*., 2000). The co-presence of *L. lactis* with *P. roqueforti* in a model resulted in significantly higher production of ethanol than in the models of *L. lactis* or *P. roqueforti* individually.

The models with *Y. lipolytica* presented the most interesting properties from all the yeast species that were detected in Stilton. The co-presence of *Y. lipolytica* with *P. roqueforti* dramatically increased the amount of 2-heptanone and 2-nonanone in the headspace of the models. The production of 2-heptanone and 2-nonanone by *P. roqueforti* individually was much higher than that for *Y. lipolytica* (68.7 compared to 5.7 and 70.9 compared to 3.3 respectively). But the greater production of 2-heptanone and 2-nonanone in the *P. roqueforti+Y. lipolytica* model (364.8 and 141.6 respectively) could not be explained as the result of the addition of the species' individual contributions. Similar trends were observed for a range of compounds that were detected in blue cheese aroma, especially the ketones that were previously detected in Stilton, while the same effect was not evident when *P. roqueforti* was grown in co-presence with other yeast species (Figure 4.1). In particular 2-pentanone and 8-nonen-2-one were two ketone compounds whose production was considerably higher in the co-presence of *P. roqueforti* with *Y. lipolytica*. A similar observation was previously reported between *P. roqueforti* and another yeast species. Prior lipolysis of oil by an enzyme of yeast origin (from the species *Candida cylindracea*) enhanced the subsequent production of methyl-ketones by *P. roqueforti* (Chalier *et al*., 1998).

As the results for the *P. roqueforti+Y. lipolytica* model were of particular interest, further studies were set up to look at a more complex flora system. *L. lactis*,

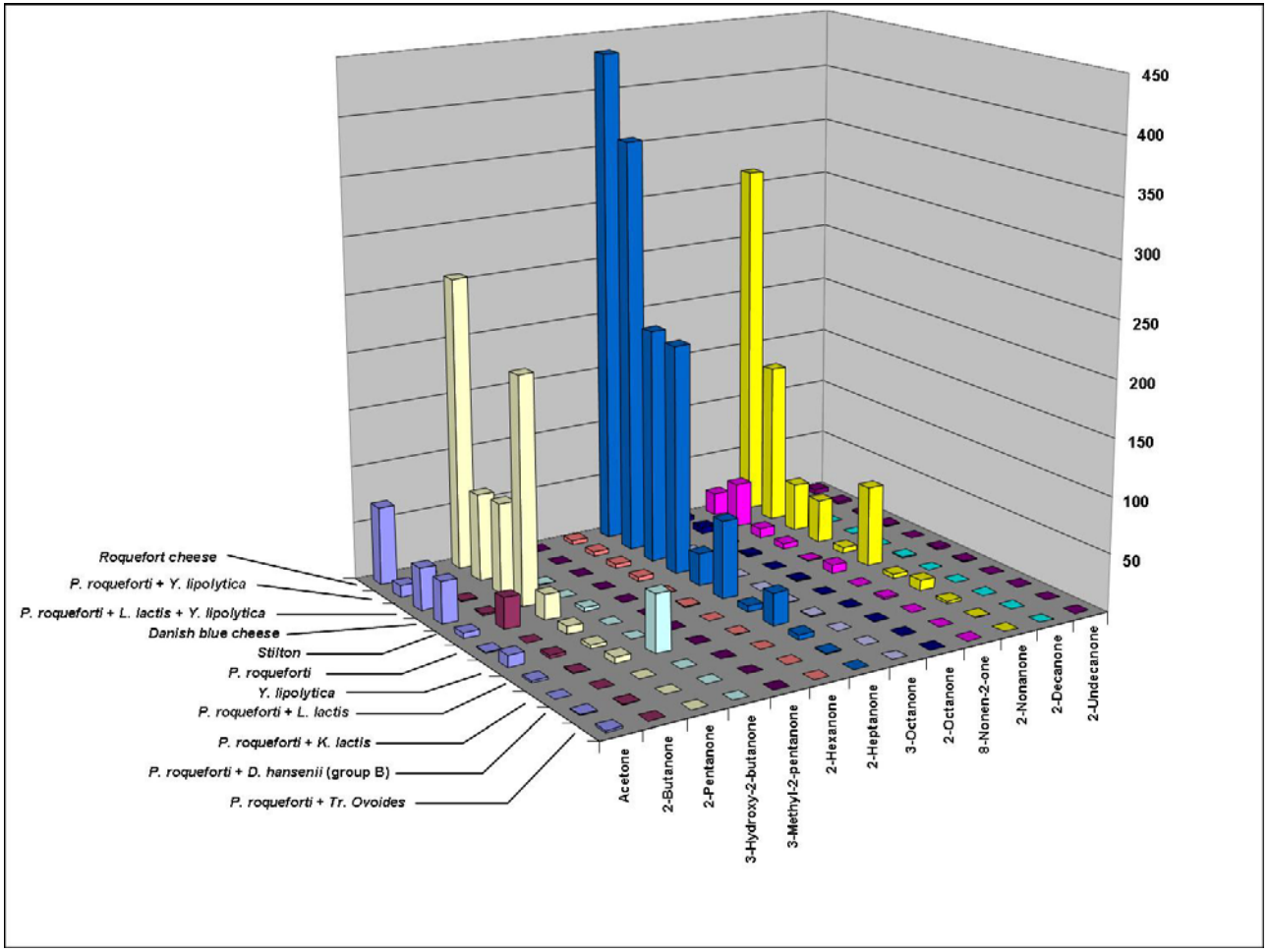


Figure 4.1 Comparison of quantities of the ketones detected in the blue cheeses (Stilton and two strong blue cheeses; Danish blue and Roquefort) and model systems. *Y. lipolytica* in co-presence with the *P. roqueforti* increased the quantities of ketones in the models. The values are relative to the peak area observed when the headspace above a 5µg/l, 2-nonanone solution was analysed.

was included in the model with *P. roqueforti* and *Y. lipolytica.* The presence of *L. lactis* with *P. roqueforti* and *Y. lipolytica* decreased the amount produced for some of the ketones (2-heptanone, 2-octanone, 8-nonen-2-one, 2-nonanone, 2-decanone, 2-undecanone; Appendix 5). This was in agreement with the previous observation in the *L. lactis*+*P. roqueforti* model where *L. lactis* had the same impact. However, even with the *L. lactis* co-present, 2-heptanone and 2-nonanone remained at high levels when *P. roqueforti* and *Y. lipolytica* grew in the same model. The production of 8-nonen-2-one was also affected by the presence of *L. lactis.* The presence of *L. lactis* resulted in similar amounts to those in the *P. roqueforti* model but the production of 2-pentanone was unaffected. Similarly, a decrease was observed in the amounts of another important ketone compound, 2-undecanone, however, the high standard deviation values did not allow conclusions to be made convincingly.

The individual growth of *Y. lipolytica* resulted in high quantities of several acids and esters (butanoic acid, 3-methyl-1-butanol acetate, 3-methyl-butanoic acid, 2-methyl-butanoic acid, hexanoic acid butyl ester, octanoic acid, octanoic acid ethyl ester, 3-methybutyl hexanoate, acetic acid 2-phenylethyl ester; Appendix 5). This could be because *Y. lipolytica* presents high lipolytic activity and could break the milk fat into fatty acids. The quantities of these acids were decreased in the *P. roqueforti*-*Y. lipolytica* and *P. roqueforti*-*Y. lipolytica*- *L. lactis* models. An explanation could be that fatty acids act as precursors for the formation of other aroma compounds by being metabolised by the starters *P. roqueforti* and *L. lactis* through numerous pathways (Gehrig & Knight, 1963; Urbach, 1995). This also explains their limited presence in the real cheeses' headspace.

Acetone and 2-heptanol production was enhanced in the *P. roqueforti*-*Y. lipolytica*- *L. lactis* model. These two compounds were also high in the blue cheese varieties with strong aroma (Roquefort and Danish blue; Appendix 5).

K. lactis was characterised by the production of high levels of ethanol. In addition, synergy was observed between *K. lactis* and *P. roqueforti* as this model system produced significantly higher amounts of a group of compounds while the production by *K. lactis* individually was significantly lower and by *P. roqueforti* insignificant. The majority of these compounds were acids, acetates and esters: acetaldehyde, ethylacetate, ethyl ester propanoic acid, n-propyl acetate, isobutyl acetate, 2-phenylethyl ester acetic acid, pentanoic acid, hexanoic acid ethyl ester, butanoic acid 3-methylbutyl ester, octanoic acid ethyl ester, propanoic acid 2-methyl-ethyl ester, 3-methyl-butanol, 2-methyl-1-butanol. This synergistic phenomenon is particularly interesting as in Chapter 2 it was demonstrated that *K. lactis* dominated the yeast community of the blue part where *P. roqueforti* has its higher presence and sporulates. It was present in lower amounts in the white core where *P. roqueforti* was also present in lower amounts and had limited presence in the outer part were *P. roqueforti* was not detected. Thus this co-existence of the two species is typical of what is seen in a final Stilton and the altered end products may be a potential feature of the Stilton blue-vein aroma. Indeed, compounds such as 3-methyl-butanol and 2-methyl-1-butanol were present in high amounts in the white part and the blue veins of Stilton but not in the outer part.

One possible explanation for the increased production of some of these compounds may be given through the metabolism of lactose and its related pathways. *K. lactis* produce strong assimilation and fermentation of lactose (Roostita *et al.*, 1996b). Lactose in cheese is metabolised to lactate. Depending on the cheese variety

(and consequently the starter microorganisms and/or secondary flora that is present in the cheese) lactate may be the start of several different pathways and result in compounds which contribute to the cheese flavour. It would be interesting to cite one of the major pathways regarding how lactate can be oxidised to acetate by LAB described by McSweeney *et al*. (2000) (Figure 4.2). It could be possible that similar pathways are taking place in the presence of *K. lactis*. The left branch of the pathway could explain the high presence of ethanol and acetaldehyde while the right branch is related to the high number of esters of acetic acid and the other esters of carboxylic acids.

It is suggested that the occurrence of this pathway (Figure 4.2) in cheese could be low because of the low redox potential and the limited presence of oxygen in the inner core of the cheese matrix (Fox *et al*., 1995). However, after piercing the veins in blue cheeses allow the presence of oxygen and the pathway would not be suppressed. This conclusion together with the synergy observed between the *K. lactis* and *P. roqueforti* may be the explanation of the dominance of *K. lactis* in the blue veins of Stilton and it lower presence in the white core where the presence of oxygen and the mould are lower.

On the other hand by comparing the *P. roqueforti* + *K. lactis* model with the *P. roqueforti* model it was observed that the presence of *K. lactis* suppressed the ability of *P. roqueforti* to produce all the major ketones (acetone 2-pentanone 2-hexanone 2-heptanone 2-octanone, 8-nonen-2-one, 2-nonanone, 2-decanone, 2-undecanone) which are an important part of the blue cheese aroma.

The models systems with *D. hansenii* isolates from groups A and B resulted in aroma profiles with similar patterns (Appendix 5). They produced some acetaldehyde and ethanol, but not as much as *K. lactis*. As in the case of *K. lactis*, *P. roqueforti*

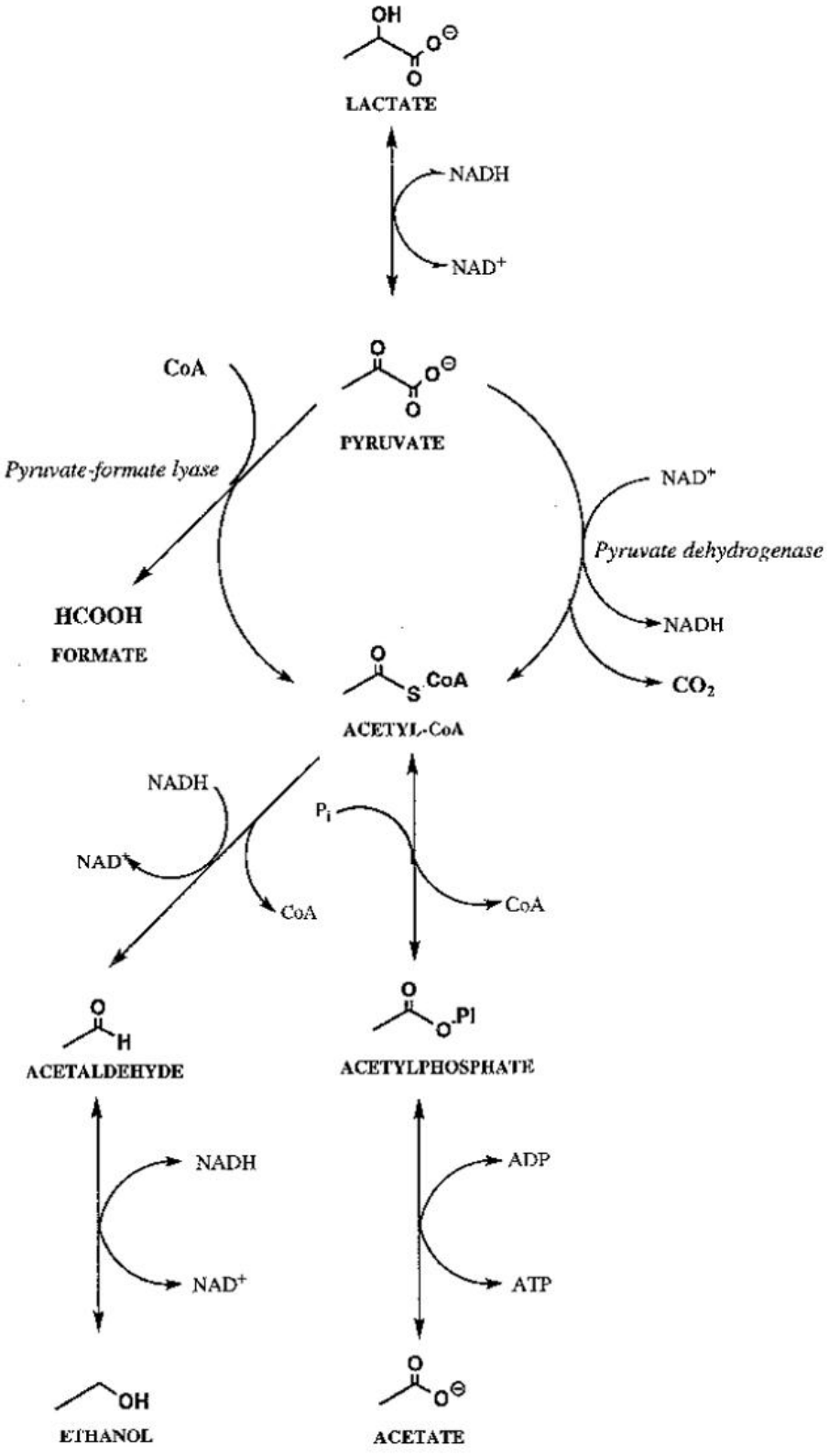


Figure 4.2 Metabolism of lactate (from McSweeney *et al*., 2000)

produced lesser amounts of ketones, especially heptanone and nonanone, in the presence of *D. hansenii*.

The model systems with *Tr. ovoides* resulted in very low quantities of aroma compounds that were previously detected in Stilton, when analysed with SPME GC-MS. In addition, the samples at the end of day 10 had a very strong off-milk aroma.

4.3.3 Principal Component Analysis

Principal Component Analysis (PCA) was applied in order to visualise the relationship between the aroma profiles of the model systems and the aroma profile of the real blue cheeses, particularly for the compounds that were previously detected in Stilton cheese (Figure 4.3).

PC1 (33% of the data) separated the aroma compounds in to two groups. On the right side of PC1 there was a cluster of aroma compounds the majority of which was methyl ketones (2-heptanone, 2-nonanone, acetone, 2, pentanone, 2-hexanone, 2-octanone, 2-undecanone, 8-none-2-one, 2-pentanol, 2-heptanol). On the left side of PC1 was a group of compounds including the main alcohols and aldehydes (3-methy-butanol, 2-methy-butanol, 3-methy-butanal, 2-methy-butanal) and the sulfur containing compounds (dimethyl-disulfide and methanethiol) that the were detected in Stilton. PC2 separated a group of alcohols (3-methy-butanol, 2-methy-butanol, ethanol and 2-methyl-propanol) from the rest of the compounds and associated them with *K. lactis*; however, PC2 explained only 13% of the data.

In agreement with the observations in § 4.3.2, there was strong differentiation between the aroma production by models containing *P. roqueforti* in the presence of

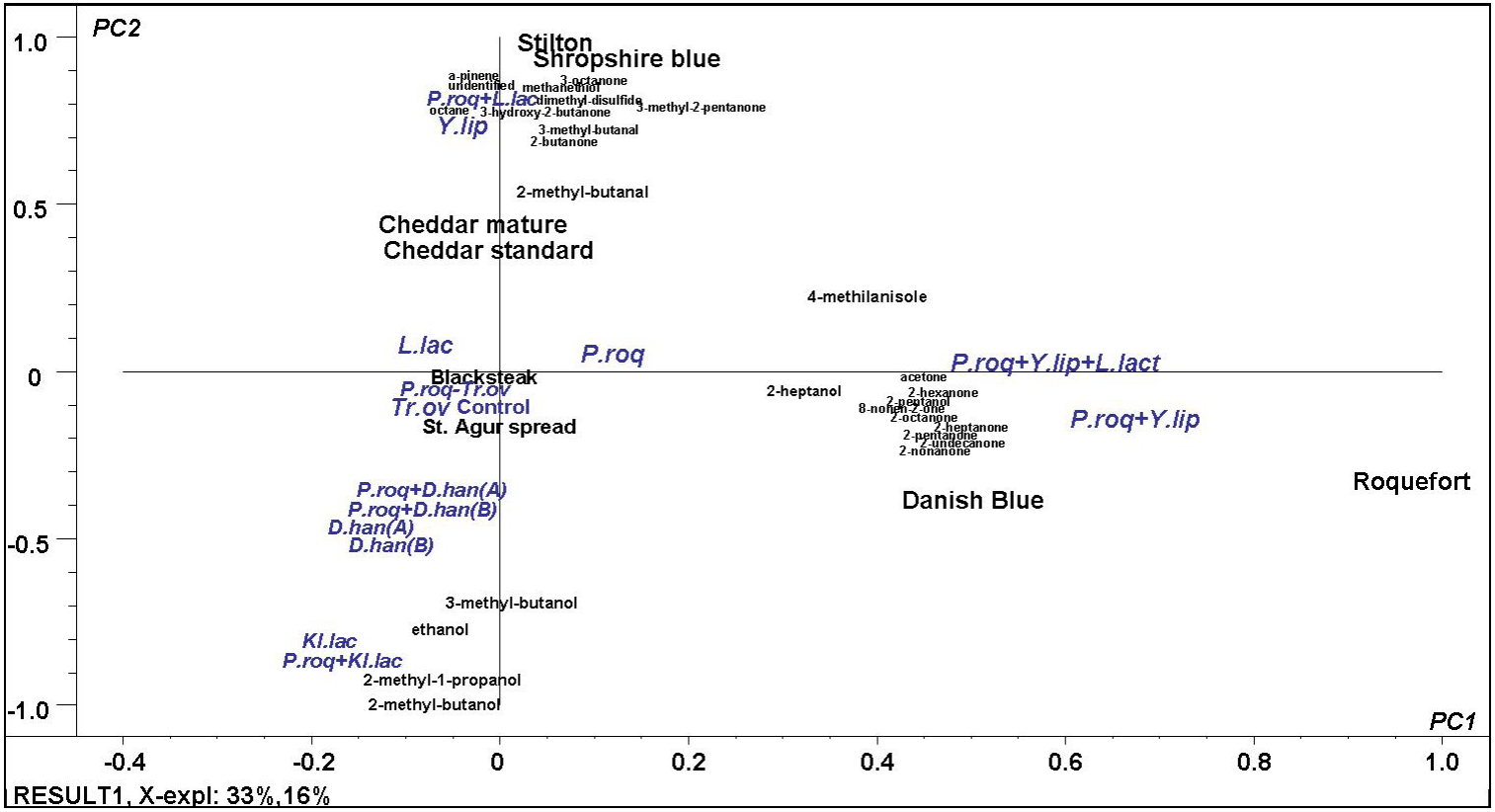


Figure 4.3 PCA plot of the SPME GC-MS peak area data from the headspace analysis of the cheeses and model systems for the aroma compounds that were previously detected in the headspace of Stilton. P. roq: *Penicillium roqueforti*; Y. lip: *Yarrowia lipolytica*; D. han: *Debaryomyses hansenii*; Tr. ovo: *Trichosporon ovoides*; L. lac: *Lactococcus lactis*.

Y. lipolytica (models *P. roqueforti*+*Y. lipolytica*+*L. lactis* and *P. roqueforti*+*Y. lipolytica*) and those containing *P. roqueforti* or *Y. lipolytica* individually. Models *P. roqueforti*+*Y. lipolytica*+*L. lactis* and *P. roqueforti*+*Y. lipolytica* were clustered in the right side of PC1 together with the ketone compounds. As discussed in Chapter 3, most of these compounds are characteristic of blue cheese varieties. The PCA of the aroma compounds detected in the three sections of Stilton (§ 3.3.6.2) demonstrated that these compounds were associated with the blue veins and the outer part. Their production was mainly associated with the metabolic pathways involving *P. roqueforti* (conversion of fatty acids through β-oxidation). However, in Figure 4.3 it is demonstrated that it is the model system with *Y. lipolytica* co-present with *P. roqueforti* that enhances the production of these compounds. *P. roqueforti* alone resulted in lesser amounts of these compounds compared with when growing together with *Y. lipolytica* or *Y. lipolytica* and *L. lactis*.

The models *P. roqueforti*+*Y. lipolytica* and *P. roqueforti*+*Y. lipolytica*+*L. lactis*, were not only clustered close to the ketones group but also between the Danish blue and Roquefort cheese varieties. This is interesting as later sensory analysis of the cheeses (see Chapter 5) ranked these two varieties as those with the strongest 'blue cheese' notes. Indeed, casual assessment of the aroma of the models showed that the model system of *P. roqueforti*+*Y. lipolytica* had a typical blue cheese smell while the rest of the models did not; the aroma of the *P. roqueforti*+*Y. lipolytica*+*L. lactis* model was not reminiscent of a blue cheese aroma despite its aroma profile and the aroma profile of *P. roqueforti*+*Y. lipolytica* model had broad similarities. This was surprising the *P. roqueforti*+*Y. lipolytica* and *P. roqueforti*+*Y. lipolytica*+*L. lactis* models were close in the PCA plot. However, it should be taken into account that PC1 explains only 33% of the data. The SPME analysis (Appendix 5) revealed differences

between the aroma profiles of these two models. *P. roqueforti*+*Y. lipolytica*+*L. lactis* produced more hexanoic acid-ethyl ester, octanoic acid-ethyl ester and alcohols, ethanol, 2-pentanol and 2-heptanol which were two times higher than in the model *P. roqueforti*+*Y. lipolytica* (Appendix 5). The *P. roqueforti*+*Y. lipolytica* model produce more ketones than *P. roqueforti*+*Y. lipolytica*+*L. lactis* model; especially 2-heptanone (365 and 207 respectively), 8-none-2-one (39 and 8 respectively), 2-nonanone (142 and 42 respectively). However, the PCA data were normalised and differences between the amounts of dominant compounds would affect the plot in the same degree with differences between less dominant compounds in the headspace. During the formation of blue cheese aroma (Figure 3.8) methyl-alcohols are the products of reduction of methyl-ketones and their increased formation in *P. roqueforti*+*Y. lipolytica*+*L. lactis* model could explain the lower amount of the ketones.

In general, the instrumental analysis of headspace does not necessarily reflect the sensory perception of the aroma of food (Linforth *et al.*, 2002; Cook *et al.*, 2005) and the concentrations of aroma compounds absorbed by the SPME fibre may differ from those that are transported to the olfactory system by sniffing.

The models of the other three yeast species detected in Stilton (*D. hansenii*, *K. lactis* and *T. ovoides*) did not present strong differentiation when *P. roqueforti* was co-present and they clustered together. There was no differentiation between the aroma profiles of the two models with *D. hansenii* group A and B. The isolates of these two groups did not present any differentiation with molecular analysis either (Chapter 2). The models containing *K. lactis* were clustered in the bottom left part of the PCA plot together with the alcohols; ethanol, 3-methyl-butanol, 2-methyl-butanol and 2-methyl-propanol.

4.3.4 Sporulation of the *P. roqueforti* in the model systems and pH

The model systems were observed daily for mould sporulation and their pH was measured at the end of the incubation (Table 4.1). The models with *P. roqueforti* co-present with other species resulted in pH values lower than those for the individual species or *P. roqueforti* only. This observation confirms that the metabolism of the medium that takes place as a result of the interactions between two species present in the model is different from when the species grew individually. The pH values of *D. hansenii* models were affected less by the presence of *P. roqueforti*. The pH of the models with *D. hansenii* group A and B did not differ significantly.

When *P. roqueforti* grew individually sporulation was observed on day 5. This was not affected by the presence of *L. lactis* and *K. lactis*. However, *D. hansenii* delayed the mould's sporulation (day 7) while the presence of *Y. lipolytica* and *T. ovoides* did not allow the mould to sporulate at all. In the model with *P. roqueforti*, *Y. lipolytica* and *L. lactis* there was limited sporulation on day 10. *D. hansenii* was previously reported to stimulate the growth and sporulation of *P. roqueforti* isolates from blue cheese (Van den Tempel, 2000a; Whitley, 2002). The phenomenon was strain specific. In addition, *D. hansenii* was the dominant yeast species of both good and poor blue veined cheeses.

On the other hand *Y. lipolytica* completely inhibited the sporulation of *P. roqueforti*. This yeast species was found in lower amounts in Stilton. Interestingly, *Y. lipolytica* was not detected in the blue veins of the cheese where the mould sporulates but it was present in the white core where the *P. roqueforti* is also present but does not sporulate because of the lack of air. *Y. lipolytica* has been reported to exhibit similar negative

Table 4.1 *P. roqueforti*'s sporulation during incubation and models systems' pH. The pH of the models on day 0 was 6.65 at 21°C.

Model system	pH (SD)	Sporulation
P. roqueforti	6.43 (0.03)	day5
Y. lipolytica	5.26 (0.09)	-
***D. hansenii* (group A)**	6.10 (0.05)	-
***D. hansenii* (group B)**	6.23 (0.14)	-
K. lactis	5.90 (0.02)	-
Tr. ovoides	5.78 (0.04)	-
L. lactis	5.84 (0.08)	-
P. roqueforti* + *Y. lipolytica	5.42 (0.06)	NO
P. roqueforti* + *L. lactis* + *Y. lipolytica	4.98 (0.09)	day10
P. roqueforti* + *L. lactis	4.62 (0.21)	day5
***P. roqueforti* + *D. hansenii* (group A)**	6.05 (0.32)	day7
***P. roqueforti* + *D. hansenii* (group B)**	6.12 (0.11)	day7
P. roqueforti* + *K. lactis	5.78 (0.03)	day5
P. roqueforti* + *Tr. Ovoides	5.54 (0.39)	NO

influences on the growth and sporulation of *P. roqueforti* in previous studies, however, it was reported that this effect was reduced in the presence of 5% salt and some strains of *P. roqueforti* were resistant (Van den Tempel, 2000; Van den Tempel *et al*., 2000a; Juszczyk *et al*., 2005).

4.4 CONCLUSIONS

The aroma profile of the model system with the starter mould *P. roqueforti* contained high amounts of ketones that are formed through the β-oxidation of fatty acids. This was an indication that the growth of isolates in UHT milk allowed them to execute metabolic pathways similar to those in the real blue cheese environment.

The models with *Y. lipolytica* presented the most interesting properties from all the yeast species that were detected in Stilton. The presence of *Y. lipolytica* with *P. roqueforti* dramatically increased the production of a series of compounds that are characteristic for the blue cheese variety including 2-heptanone and 2-nonanone. It is possible that *Y. lipolytica* plays a major role in the formation of adequate blue cheese flavour and it might be related with the production of cheeses with strong blue cheese flavour. In contrast, the presence of *K. lactis*, *D. hansenii* and the starter *L. lactis* inhibited the production of ketones.

Strong synergy was observed between *K. lactis* and *P. roqueforti* in the production of acetate related compounds and esters of carboxylic acids. This synergistic observation is in addition to the similar distribution that the populations of these two species present in the different sections of Stilton.

The model systems with *D. hansenii* isolates from groups A and B resulted in aroma profiles with similar patterns despite their strong morphological differentiation and the different distributions of their populations in the Stilton parts that was observed in Chapter 2.

The yeasts *K. lactis*, *D. hansenii* and the starter bacterium *L. lactis* suppressed the production of ketones by the mould *P. roqueforti*. *D. hansenii* and *L. lactis* also suppressed the sporulation of the mould. On the other hand *Y. lipolytica* completely

inhibited the sporulation of *P. roqueforti* but enhanced the aroma production. The PCA analysis revealed that the interactions that took place in the models systems that contained the starter mould *P. roqueforti* and *Y. lipolytica* resulted in an aroma profile similar to those of the cheese varieties with strong blue cheese aromas.

Y. lipolytica was not detected at all in the blue veins where the mould sporulates but it was present in the white core where the mould exists in the mycelial form. Any possible inhibition of *P. roqueforti*'s sporulation in the real cheese environment would be of great interest for blue cheese production. It would be very interesting in future work to investigate further the relationship between these two species and if the inhibition phenomenon is strain specific, if there is a critical level of the *Y. lipolytica* population and/or concentration of salt above which the inhibition takes place.

It could be that when the *P. roqueforti* grows together with *Y. lipolytica* in the white part where there is a lack of air, and the mould can not sporulate and take advantage of the enzymes produced in the sporulating regions, the latter enhances the production of important aroma compounds for the blue cheese flavour. Ironically, this yeast species might be the reason that cheese batches fail to form blue veins and result in high economic losses for the producers. It would be interesting to study in future more strains as this study is based on a few isolates of each species. If these phenomena are strain specific (either *Y. lipolytica* or *P. roqueforti*) then *Y. lipolytica* and *P. roqueforti* strains that are able to grow together delivering enhanced aroma production and no sporulation inhibition would have excellent potential for starter cultures. On the one hand the aroma production would be enhanced and on the other hand the cheese would be dominated by *Y. lipolytica* strains synergistic to the starter

mould which could compete against external *Y. lipolytica* contamination that may present the potential for inhibition.

5. BLUE CHEESE FLAVOURING SENSORY ANALYSIS

5.1 INTRODUCTION

5.1.1 The use of the cheese model with *Y. lipolytica-P. roqueforti* for blue cheese aroma production

From the model systems that were analysed in Chapter 4 those with *Yarrowia lipolytica* in co-presence with *Penicillium roqueforti* showed increased amounts of aroma compounds that were detected in the real blue cheeses including ketones that are important for the blue cheese flavour. Subsequent PCA analysis of the data revealed that the models *P. roqueforti+Y. lipolytica* and *P. roqueforti+Y. lipolytica+L. lactis* clustered not only with the ketone compounds but also with two blue cheese varieties with strong aroma (Danish blue and Roquefort). In particular the SPME data pattern of the *P. roqueforti+Y. lipolytica* model presented similarities with those from the real blue cheeses and casual assessment by sniffing indicated that its aroma was reminiscent of a blue cheese aroma. For this reason it was decided to be studied further in order to asses the degree of the similarity of the aroma of the model and the blue cheese aroma.

It is known that aroma similarities that are observed with instrumental analysis are not always evident in the perception of the aroma with the human senses. In addition, the aroma perception of individual people can vary. For this reason sensory analysis would be required in order to assess the degree of similarity of the aroma of the model system with those from the real cheese. In the case of a strong aroma similarity being seen with real cheeses, systems based on the *P. roqueforti+Y.*

lipolytica model could be used for the production of blue cheese flavour. Previous studies in this field have been conducted on the basis that *P. roqueforti* by itself is sufficient for the production of adequate blue cheese flavour. In these studies, the medium had to be pre-treated with enzyme in the case where copra oil was used (Chalier *et al*., 1998) or a high concentration of fat content had to be added in another case (Dwivedi & Kinsella, 1974). In general, such kinds of systems have great commercial interest and patents for the production of blue cheese flavour (Kosikowski & Jolly, 1979) or the production of blue cheese (Kosikowski & Jolly, 1976) were based on systems like those described above.

5.1.2 Sensory methods

5.1.2.1 Qualitative sensory evaluation

A preliminary sensory analysis was needed in order to confirm that the aroma profile of the model *P. roqueforti+Y. lipolytica* was reminiscent of blue cheese aroma, by a great number of people and under the controlled conditions of a sensory trial. A rapid qualitative sensory evaluation was designed based on the comparison of descriptive terms for the aroma of model and blue cheese.

The aim of the trial was to compare the responses (description with terms) of the panel for the aroma of real blue cheese and the aroma of the model without the evaluation or quantification of any differences/similarities. The appearance of the model differ significantly from blue cheese. Therefore the panellists should smell the samples without being able to see them in order that the descriptions be based entirely

on the aromas and not the appearance. The principle of this trial was that if a panel would smell two different samples with similar aroma their description would be based on similar words no matter how relevant the words would be to the blue cheese aroma characteristics.

5.1.2.2 Flash profile technique

A sensory method would be required to assess the degree of similarity of the aroma of the *P. roqueforti+Y. lipolytica* model with the aroma of the real blue cheese. Various methods are available for the characterisation and quantification of the sensory similarities and differences between products. Conventional descriptive profile methodologies are the most established and they involve comprehensive sensory descriptions of products by trained panellists and rating of sensory characteristics based on a consensual vocabulary (Stone *et al*., 1974). All of them require extensive training of the panellists in order to develop a common language for the description of the samples, learn how to evaluate the generated attributes and of course the conduction of the evaluation. These methods are very time consuming and of high cost. Alternative techniques, simpler and less time consuming, which do not require the use of trained panels have been described (Perrin *et al*., 2008, Tuorila & Monteleone, 2009) and one of these, the Flash profile, was employed in the current study.

The Flash profile is a technique that can be applied in order to investigate the degree of similarity between samples. The technique provides a relative sensory positioning (mapping) of samples. The method was developed by Sieffermann (2000)

and it is based on the Free Choice Profiling in combination with comparative evaluation. It involves ranking of a simultaneously presented sample set but no rating. The simultaneous presentation of the samples allows discrimination by direct comparison. Therefore the technique does not require trained panellists.

Each panellist is allowed to generate their own list of attributes for the evaluation of the product set through the Free Choice Profiling. All the attributes are combined in a total list available for the whole panel. The panellists individually select and use a list of attributes from the main list, and rank a set of simultaneously presented samples for each of the attributes. This lack of a common language between the panellists and the large number of terms without definitions does make the interpretation of sensory characteristics difficult.

The panel for a Flash profile analysis can be composed from both sensory evaluation experts and product experts (e.g. manufacturers/producers of the studied samples). This characteristic in combination with the rapidity of the technique makes it ideal for use in industry. The method is relatively new but it has been applied for the analysis of a great variety of products: jam, jellies, wine, chewing gums and bread products (Dairou & Sieffermann, 2002; Delarue & Loescher, 2004a; Blanche *et al*., 2007; Fan, Tsai & Qian, 2007; Poinot *et al*., 2007). So far it has been compared with conventional descriptive sensory analysis (Quantitative Descriptive Analysis) and the sensory maps of the two techniques were found to be similar or with small differences (Dairou *et al*., 2002; Delarue and Sieffermann, 2004b).

The Flash profile technique was not developed in order to replace the conventional techniques. It is often suggested that the Flash profile can act as language development and a mapping tool in preliminary studies prior to thorough

sensory evaluation and generally whenever a relative sensory positioning of samples is needed in a short time (Delarue *et al*., 2004b).

5.1.3 Outline of the study

The main aim of this study was to investigate the level of similarity between the aroma of the *P. roqueforti+Y. lipolytica* model and the aroma of real blue cheeses. First, a simple qualitative sensory comparison was conducted in order to investigate similarities in the aroma profiles. Subsequently, Flash profile analysis was employed in order to evaluate the degree of similarity. Real blue cheeses were included in the study. The resultant relative sensory positioning of real blue cheeses and the model would demonstrate the degree of similarity between the aroma profiles.

5.2 MATERIALS AND METHODS

5.2.1 Qualitative sensory analysis

Qualitative sensory testing was used in order to compare the odour of the model *P. roqueforti+Y. lipolytica* to a Stilton blue cheese as a standard (the A1 Stilton sample that was used in aroma analysis, Chapter 4). Two different panels were used for the qualitative sensory analysis. The first panel consisted of 17 subjects aged 20-40 years old and with a food sciences background. All of them were familiar with sensory procedures (they have participated in sensory panels in the past and some have conducted their own sensory trials). All the subjects had included blue cheese and cheeses in general as part of their diet. The second panel consisted of 16 subjects of the same range of ages but people from both a food sciences and a non-food related background were included. Subjects whose background did not include the consumption of blue cheeses were also included. All the sessions were conducted in standard individual sensory booths at the Sensory Science Centre, part of the Division of Food Sciences, University of Nottingham.

Different amounts of cheese and model systems were tested in order to achieve similar odour intensity for both types of sample. The samples were prepared by transferring 4 g of blue cheese cut into pieces measuring approximately 3 mm across and 50 ml of model system respectively in 100 ml sealed flasks. The flasks were covered with aluminium foil in order to conceal their contents. Each flask was marked with a 3-digit random code and kept at 4^{o}C until used. The samples were allowed to reach room temperature and equilibrate before the sensory sessions.

The panellists were provided with two samples, one model and one real cheese sample. They were asked to sniff them in the order that the samples were provided and describe them without using hedonic terms. The order of the samples (real cheese / model) was randomised. The subjects were allowed to sniff the samples more than once. The subjects were not allowed to taste the samples or look inside the container. The panellists were informed that the samples were 'food-related'.

The vocabulary that was used from the subjects in order to describe the samples was grouped in three categories. The first (A) contained words very relevant to blue cheese odour (e.g. blue cheese, Stilton cheese, Roquefort cheese etc.). The second group (B) contained characterisations related to general cheese odour but not to blue cheese varieties (e.g. cheese, cheesy, parmesan etc.). The third group (C) contained general dairy characterisations (e.g. dairy, milk etc) or other less relevant terms.

For each subject the description for the model was compared with the one for the real cheese. Whenever a subject described the model using terms that belonged to a category of the same or higher relevance than those that were used for the real cheese the response was considered as positive (equally relevant or more relevant description to the blue cheese odour for the model than for the real cheese). This means that in order to consider a description as a positive the response for the model should be either category B and B/C for the real cheese or category A for the model and A/B/C for the real cheese. In the cases that the descriptions for the model were related to the category C they were considered as negative responses even if the description for the real cheese was also related to category C.

5.2.2 Flash profile

5.2.2.1 Samples

Models of *P. roqueforti+Y. lipolytica* and *P. roqueforti* were prepared as described in Chapter 4 and the same set of cheese samples was used. Appropriate amounts of samples were chosen in order to achieve similar odour intensity for all type of samples (50 ml model systems, 7 g cheddar cheeses, 6 g blue cheese spread, 4 g blue cheeses cut into pieces measuring approximately 3 mm across). They were all transferred into 100 ml sealed flasks and the same procedure followed as in §5.2.1,

5.2.2.2 Panel

A panel comprised of 9 subjects (judges), 7 women and 2 men, from 22 to 55 years old was employed for the Flash Profile analysis. Their selection was made based on their experience in evaluating flavour and/or descriptive sensory analysis. Eight of them had already participated in sensory profiling and other types of sensory analysis. All of them were familiar with the blue cheese aroma including three who were familiar with a great variety of cheese types. However, they were not specifically trained in the evaluation of blue cheeses and they had not participated in the evaluation of cheese products at least during the last 6 months before the study.

5.2.2.3 Sensory sessions

The procedure performed was based on Delarue *et al*. (2004b). The flash profile analysis consisted of three sessions. To introduce the Flash Profile technique, the judges were given a brief outline of the procedures prior to each session. All the sessions were conducted in standard individual sensory booths at the Sensory Science Centre. During all sessions the panel was informed that the samples were related to cheese aroma but there was no information on the individual characteristics of each sample (e.g. real cheese or model, blue cheese or non-blue cheese aroma etc.).

During the first session each subject had to smell the samples and individually generate their own provisional list of attributes that best described the differences between the aromas of the samples. All the 10 samples were presented simultaneously. There was no limitation regarding the number of sensory attributes that they could generate but they were instructed to avoid hedonic terms. The subjects were asked to focus on attributes that discriminate/characterise the overall set of the samples and not on those which concerned individual or a few samples. This session lasted about 30 min.

Between the first and the second session the attributes generated by the whole panel were pooled and presented in a single list. At the beginning of the second session, the subjects had to choose their definitive list of attributes from the pooled list. They were asked to read the panel's list and, if desired, to review and update their personal list by adding, excluding or replacing attributes.

Then the subjects proceeded to the evaluation of the samples on a ranking mode, using their own definitive list of terms. All the ten samples were presented simultaneously in a randomised order. A 170 mm scale was used for each attribute,

marked at the left end with '-' for the attribute not perceived and at the right end '+' for the attribute strongly perceived. Ties were allowed. The judges could smell the samples as many times as they liked. Pauses were allowed during the evaluation and the subjects could take as much time as they needed to evaluate them. The third session was a repeat of the second session. The time length of the evaluation sessions (2 and 3) varied for each subject. The subjects did not necessarily participate in the sensory sessions at the same time but they all had to wait for the whole panel to complete the first session before proceeding to the following sessions in order to ensure that attributes from the whole panel were communicated to each member.

5.2.2.4 Data treatment

The discrimination efficiency of the attributes for each judge was tested by a one-way analysis of variance (ANOVA) on the rank data. Attributes that were found not to discriminate the samples were excluded from the concerned judge's list. Judges' repeatability between the two sessions was tested by Spearman correlation test. Only the attributes with reproducible ranking between the sessions were considered. Judges with poor overall performance were excluded from the data set.

Generalised Procrustes Analysis (GPA) was applied for the consensus configuration between judges' sensory maps. The GPA calculates a consensus from data matrices of a sensory profiling experiment. In the case of Flash profile a data matrix corresponds to each judge. The GPA plot demonstrates how similar or different the samples were to each other according to their schematic interpretation.

The data were collected on Microsoft Excel spreadsheets. ANOVA, Spearman correlation test, and GPA were performed with XLSTAT add-in for Microsoft Excel (Addinsoft).

5.3 RESULTS AND DISCUSSION

5.3.1 Qualitative sensory analysis

A preliminary sensory analysis was needed in order to investigate if the perception of the aroma of the model *P. roqueforti*+*Y. lipolytica* was similar with that of a real blue cheese. For this reason a rapid qualitative sensory evaluation was employed. Two different panels were employed for the sensory trials. The trial was based on the comparison of their responses for the real blue cheese and the model's aroma. The subjects were not provided with detailed information about the samples. Therefore the terms that were used and their relevance to the blue cheese aroma description did not matter as long as the descriptions were the same for both samples. The results are summarised in Table 5.1. The response from each panellist was taken as positive when the description for the model's aroma was equally or more relevant to the blue cheese than the description for the real blue cheese sample. The number of the positive responses was 12 out of a total of 17 (71%) for the first panel and 14 out of 16 (88%) for the second panel. Therefore there was good indication that the aroma of the model was comparable to the aroma of the real blue cheese.

For the first panel the number of the descriptions of the samples of model that were allocated in categories A and B was balanced (7 and 7 respectively) while for the second panel the responses in category B were much higher (3 and 12 respectively). The same was true for the descriptions of the real cheese samples that were allocated in groups A and B in the first panel (8 and 6 respectively) compared to the second panel (2 and 12 respectively). This could be explained by the fact that the first panel consisted of experienced subjects, familiar with the blue cheese aroma, and therefore

Table 5.1 Qualitative sensory comparison of the perception of the aroma of Stilton and the model *P. roqueforti+Y. lipolytica*.

Categories:	**A**	**B**	**C**
Description of categories:	blue cheese / blue cheese related	non-blue cheese varieties / mould related cheeses / mature cheese / cheesy	not related to cheese at all
Examples of key-words for each category:	blue cheese odours, *Stilton*, other blue cheese varieties	mature' odours, *parmesan cheese*, *Brie*, *cheesy*, *mouldy*, *Camembert*, *mould cheese*	dairy, other random characterisations
1st panel			
Non-cheese samples	7	7	3
Real cheese samples	A: 6, B: 1	A: 2, B: 5	B: 2 , C: 1
Positive responses:	7 / 7	5 / 7	0 / 3
Total positive responses:	**12 / 17**		
2nd panel			
Non-cheese samples	3	12	1
Real cheese samples	A: 2, B: 1	B: 11, C: 1	C: 1
Positive responses:	3 / 3	11 / 12	0 / 1
Total positive responses:	**14 / 16**		

able to identify it without visualisation or taste. The second panel consisted of less experienced panellists and may have been less familiar with cheeses. However, the ratios of the positive responses to the total responses were similar in both cases. This confirms that the fact that the method is based on comparisons and not individual assessments makes it independent of the subjects' experience and minimum familiarisation with the sample is required.

In the present study the technique was used in order to indicate the similarities between the aromas of the two samples before proceeding with further and more sophisticated quantitative sensory analysis. In this context it proved very effective and similar approaches could be used for the rapid assessment of similarities between other kinds of samples.

5.3.2 Flash profile

5.3.2.1 Samples, panel and diversity of generated attributes

The descriptive evaluation of the samples was performed using the Flash profile technique. As the aim of this trial was to investigate the degree of the aroma similarity between the *P. roqueforti+Y. lipolytica* model and the real blue cheeses, in addition to the mixed model the sample set included the models of the individual species as a control. The *P. roqueforti* model was included in order to discriminate between the aroma of the mixed model and that associated with ketone production of the starter mould. The set of the cheeses used for comparison (Table 5.2) included blue cheeses that varied in the country of origin, the raw material and their colour

Table 5.2 Cheese samples and their characteristics that were used for Flash profile

Type of sample	Stilton	Danish blue	Blacksticks	Shropshire blue	Roquefort
Milk type	Cow milk	Cow milk	Cow milk	Cow milk	Sheep milk
Crust	Yes	No	No	Yes	No
Core colour	white/creamy	white/creamy	yellow	yellow	white/creamy
Origin	UK	Denmark	UK	UK	France

(white and yellow core blue veined cheese) and two cheddar cheeses which would provide a comparison with general cheese aromas.

The combination of both sensory and product expertise within the panel was important in order to ensure the generation of original attributes during the first session and the efficient discrimination of the samples in the subsequent sessions without the need for training in the particular set of samples. In the present study, in the first session, each of the subjects generated 9–17 attributes resulting in a total of 69 attributes for the whole panel (Appendix 6). These included a diversity of characteristics covering the cheese aroma, dairy/milk related notes, and mould related notes but also other general terms that could not be directly correlated to any of the samples. As in Free-Choice profiling, the semantic interpretation of the attributes is impossible because of the large number of terms and secondly because the meaning of the term for each subject is unknown. The attributes that were selected by more than one subject are summarised in Table 5.3. The most common were related to the blue cheese aroma which is something that would be expected for this set of samples.

5.3.2.2 Assessment of each judge: discrimination ability and repeatability between sessions

The discrimination ability of attributes per judge was evaluated using one-way analysis of variance (ANOVA) (Table 5.4). The aim was to check discrimination ability for each of the attributes according to the panellists' ranking. The attributes that did not contribute to differentiation among products were excluded (significant level $p<0.05$). The application of ANOVA for this purpose is in contrast to the

Table 5.3 List of the attributes that were used from more than one subject from the Flash profile panel

Attribute	**Number of judges using the attribute**
Blue cheese / Blue cheese aroma / Blue cheesy	8
Cheese / Cheesy	5
Musty-fusty	5
Milk - Milky	5
Butter / Buttery	4
Cheddar / Cheddary / Cheddar cheese	3
Stilton	3
Rancid	3
Mould / Mouldy	3
Creamy	3
Mature	2
Fruity	2
Sweet / Sweety	2
Nutty	2
Acid / Acidic	2
Old socks / Smelly socks	2
Sharp – smooth	2
Paint	2

Table 5.4 F value from ANOVA and Spearman correlation coefficients (SCC) on sensory attributes of each judge of Flash profile.

Judge 1	**F**	**SCC**
Blue cheese	4.80*	0.62*
Fresh	7.22**	0.73*
Lingering	7.05**	0.73*
Mould(y)	9.61***	0.79**
Musty-fusty	6.33**	0.70*
Sour	1.32	

Judge 2	**F**	**SCC**
Blue cheese	24.10***	0.91***
Fermented milk	4.32*	
Hot/spicy odour	19.52***	0.89**
Rancid	0.40	

Judge 3	**F**	**SCC**
Blue cheese	7.62**	0.75*
Butter(y)	6.90**	0.74*
Cheddar(y)	5.40**	0.70*
Milk(y)	4.00*	

Judge 4	**F**	**SCC**
Alcohol–fermented smell	7.92**	0.75*
Blue cheese	4.71*	0.62
Cheddar(y)	3.77*	0.54
Cream cheese	5.05**	0.64*
Damp-mouldy	7.59**	0.74*
Malty	4.00*	0.57
Milk(y)	6.15**	0.69*
Stilton	10.38***	0.81**
Yoghurty/creamy aroma	5.12**	0.64*

Judge 5	**F**	**SCC**
Acid(ic)	6.14**	0.69*
Blue cheese	4.93*	0.63*
Butter(y)	3.12*	
Cheese rind-parmesan	2.85*	0.44
Cream cheese	10.43***	0.81**
Salty	4.29*	0.59

Judge 6	**F**	**SCC**
Blue cheese	44.44***	0.95***
Butter(y)	16.72***	0.88**
Cheddar(y)	88.89***	0.98***
Cream cheese	2.01	
Creamy	6.22**	0.70*
Feet like	4.27*	0.59
Mature	23.95***	0.91***
Mould(y)	15.79***	0.87**
Smelly	33.70***	0.94***
Sour and acidic	7.07**	0.73*
Stilton	21.53***	0.9***

Judge 7	**F**	**SCC**
Acid(ic)	2.35	
Blue cheese	102.06***	0.98***
Cheesy	14.44***	0.86**
Fat	35.56***	0.94***
Mould(y)	21.67***	0.90***
Rancid	16.40***	0.87**
Salty	2.22	
Sour	18.65***	0.89**

F values: *p<0.05, **p<0.01 and ***p<0.001

SCC: *p<0.06, **p<0.01 and ***p<0.001

common practice of using Friedman's non-parametric test for rank sensory data analysis. However, this test can not be applied in Flash profile because it cannot handle replications (Dairou *et al*., 2002).

Spearman correlation test (Table 5.4) was applied in order to test the repeatability of the attributes ranking between the two sessions. The rankings at the first session should be correlated at a significant level. The significance level was set to $p<0.06$ because the 'blue aroma' related attributes for most of the judges were $0.05<p<0.06$ while the remaining non-blue note related attributes presented p values much higher than 0.06. The task of discriminating the aroma of 6 different blue cheese varieties is considered a difficult task and therefore higher p values could be tolerated.

The F values of ANOVA showed that judges varied in their ability to discriminate between samples. There were no attributes which presented the same level of discrimination for all the judges. However, it was observed that the attributes with high F values tended to be accompanied by high SCC (and p values). In this context judges 6 and 7 presented the best performance despite the fact that they dealt with a higher number of attributes than other panellists who performed less well. Two poorly performing judges (both in terms of discrimination and reproducibility) were excluded from the data set in order to ensure the smooth interpretation of the averaged data in further analyses (GPA analysis) (Delarue *et al*., 2004b). Finally, seven judges from the initial total of nine were retained, and 24 attributes were used to determine the sensory position of the samples.

5.3.2.1 GPA analysis and relative positioning of the samples

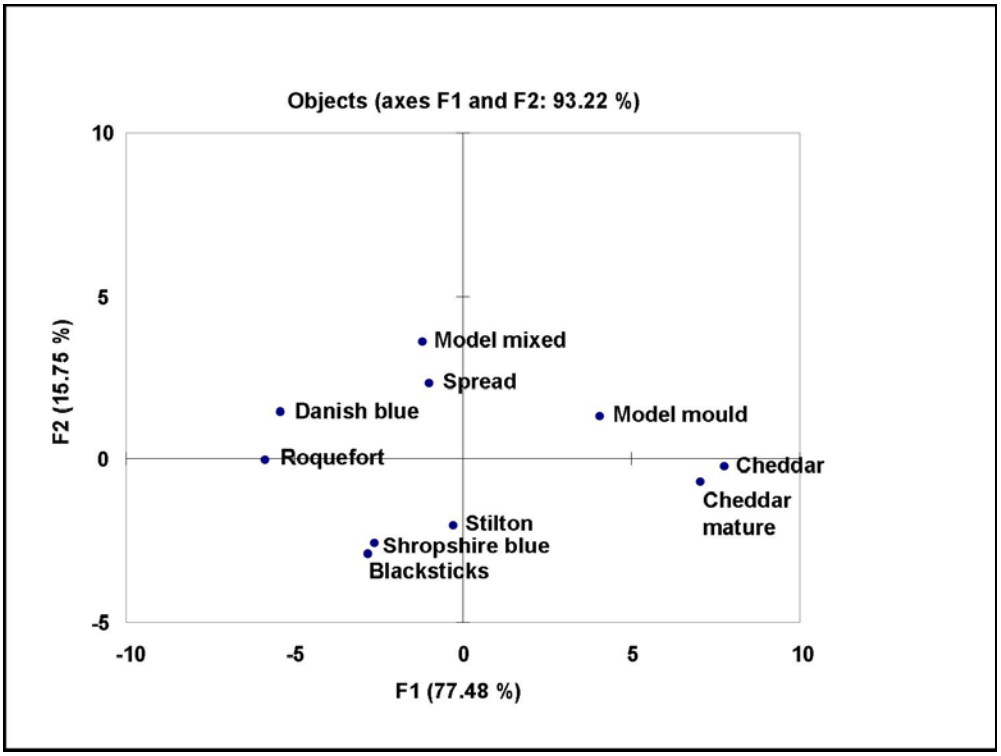
The GPA analysis of the Flash profile data provided the relative positioning of the samples. The plots defined by the first two factors of the GPA analysis (Figure 5.1) explained a high percentage of the total variance (93.22%; 77.48% and 15.75% for F1 and F2 respectively).

The first axis (Figure 5.1a) discriminated the real blue cheeses and the *P. roqueforti+Y. lipolytica* model ('Model mixed' on the plot) on one side and the cheddar cheeses with the individual *P. roqueforti* model ('Model mould' on the plot) on the other side. The *P. roqueforti+Y. lipolytica* model was not only grouped with the real blue cheese samples but was also ranked higher than the Stilton and the blue cheese spread. On the top of the scale were Roquefort and Danish blue, two varieties with strong blue aroma, followed by the blue cheeses with a yellow core, Blacksticks and Shropshire blue. The model of individual *P. roqueforti* was ranked outside the blue cheese range suggesting that the similarity of the model's aroma with the real blue cheeses could not be achieved just with the *P. roqueforti*.

The first axis of the variables plot (Figure 5.1b) was mainly characterised by blue cheese related attributes on the negative side of the variables' plot and presented strong opposition to the non-blue cheese and general dairy-related attributes on the positive side of the plot. Therefore the main trend in the differentiation between the samples in Figure 5.1a was related to the blue cheese aroma.

Regarding the relative positioning of the samples on the two dimensions of the plot (Figure 5.1a) the mixed model system was clustered with the blue cheese spread, while Stilton was grouped with the yellow core cheese and Roquefort with the Danish blue. By comparing the F2 axes of the two plots a differentiation of the samples int

two groups could be observed with the model and the blue spread on one side and all the yellow blue cheeses – Stilton, Shropshire and Blacksticks - on the other. This separation was driven by attributes related with dairy fermentation (on the top side) and with fat on the bottom side.



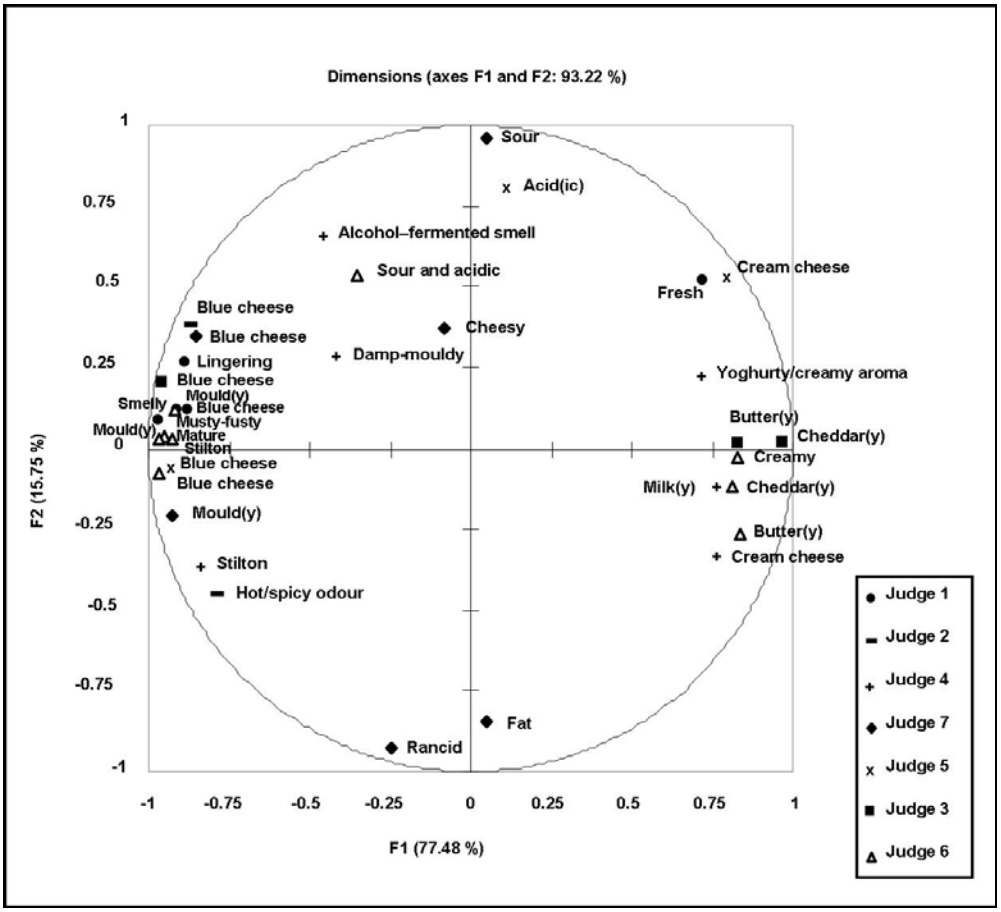


Figure 5.1 Plots of the GPA (first two principal axes) performed on the Flash profile data: (a) Mean configuration of samples; (b) Variables plot.

5.4 CONCLUSIONS

The flash profile analysis demonstrated the significant similarity of the aroma of the *P. roqueforti+Y. lipolytica* model with the aromas of the real blue cheeses. Notably the model's 'blue' notes were ranked higher than those of the blue cheese spread and Stilton cheese. It can be concluded then that the similarities that were observed with the instrumental analysis in Chapter 4 can be detected by human perception. As the same observation was not in evidence for the *P. roqueforti* model it must be the combination of *Y. lipolytica* with the starter mould that is responsible for the production of the strong blue cheese aroma and not the *P. roqueforti* alone.

The aroma similarity between the model and the real Stilton cheese is particularly interesting if the simplicity of the production of the model system is contrasted to the complexity of the real Stilton production which includes long ripening and maturation periods. However, the relative position of the model on the GPA plot was closer to the blue cheese spread than to the blue cheeses. This shows that despite the fact that the model presented stronger blue cheese notes than Stilton, its overall sensory perception was closer to a blue cheese product than to a pure blue cheese.

This work suggests that products based on the model could be used as an alternative to blue cheese flavouring in the food industry. Most of the existing flavourings are based entirely on the use of *P. roqueforti*'s spores and present the disadvantage that their production is based on media containing a high fat content.

In terms of methodology Flash profile was found adequate for the assessment of the similarity of the aroma profiles between a blue cheese aroma model and real blue cheeses. Using this technique the subjects managed to rank the samples

according to their blue cheese aroma intensity without these being separated into blue cheeses and non-blue cheese samples (models and cheddars) or real cheeses (blue cheeses and cheddars) and non-cheese samples (models). This is interesting as it was the first time that the technique was used for samples that are not of the same nature.

In previous studies Flash profile was suggested as complementary to the more adapted and accurate conventional profiling (Dairou *et al*., 2002). In the present study it is demonstrated that the time consuming conventional profile can be substituted by the Flash profile technique in cases where the objective is the investigation of the degree of similarity between samples, without the need for specifying which are the sensory characteristics that drive this similarity. Even though some basic information is still provided e.g. the main trend that drove the ranking in the present study were the blue cheese related attributes.

6. GENERAL DISCUSSION

The present study has demonstrated that yeasts are an important part of the secondary flora of Stilton. The different sections of Stilton (the blue veins, the white core and the outer crust) were studied separately. The fungal flora was complex; it consisted of *Kluyveromyces lactis, Yarrowia lipolytica*, *Candida catenulata Trichosporon ovoides* and *Debaryomyces hansenii*. As in the case of the Stilton bacterial community (Ercolini *et al*., 2003), the structure of the fungal community was different for each section of the cheese. Aroma analysis of these sections demonstrated that similar differentiation exists in the aroma profiles of each section, which overall contained high amounts of ketones, alcohols and aldehydes. Model systems of the starter mould *Penicillium roqueforti* and yeast isolates from Stilton showed that the aroma production was significantly different when the organisms were cultivated individually and in different combinations. This suggests that these yeasts do affect the aroma production in Stilton and the differentiation of the structure of the fungal communities and the aroma profiles in the different sections is related.

Both molecular and culture-dependent techniques were used and compared for the analysis of the fungal communities in the different parts of the cheese. Overall, the molecular techniques gave a more complete picture of the fungal community; however, none of the methods alone demonstrated the complete community and both culture-dependent techniques and molecular analysis were required in order to obtain the most accurate possible conclusions. TRFLP had the advantage of providing semi-quantitative data which the other molecular community analysis did not. However, for the species *Candida catenulata*, which was not isolated on culture and for which there were no isolates for reference, DGGE had the great advantage that the unknown band

could be extracted and sequenced allowing the identification of *C. catenulata* to be established. TRFLP also had the advantage of being very sensitive. It was able to detect a wide range of signal intensities of species; the lowest signal intensity was 300 times smaller than the highest. It seems that this technique is more appropriate for the analysis of complex communities consisting of known species, especially when the relative amounts of each species in the mixed communities needs to be calculated. It could be applied in future studies on Stilton in order to screen and compare the fungal communities and the quantities of each species during the different stages of production.

One explanation for *Candida catenulata* not being detected by culture is that its population might develop during the first stages of production but changes in the cheese matrix during ripening (e.g. pH, salt concentration) favour the growth of other competitive species in the final product. During ripening, aroma compounds which are characteristic of each cheese variety and precursors of aroma compounds that develop during the maturation are produced. Therefore, yeast species like *C. catenulata*, that may be present during ripening but do not survive in the final stage can be equally important as those species that are present in the final product. Indeed, *C. catenulata* has strong extra-cellular lipolytic and proteolytic activity and has been reported to be an important part of the microflora of other cheeses (Roostita *et al*., 1996). In milk it has demonstrated a strong ability to increase the concentrations of free fatty acids (Roostita *et al*., 1996). The breakdown of the milk fat and the release of fatty acids is an essential part of lipolysis and the creation of precursors of aroma compounds. The present study focused on the final aroma of Stilton. It would be interesting in future to study the development of the fungal flora of Stilton from the raw material to the final product in order to investigate the life cycle of the *C.*

catenulata population through the different stages of production and its role in the aroma development.

The approach that was followed in this work, to study separately the different sections of Stilton, allowed the observation of significant differences in the distribution of the yeasts in the different sections of the cheese. There were some species present in more than one section; however, their quantities were different. *Debaryomyses hansenii* was a species detected in significant amounts in all the parts of the cheese with both molecular and culture techniques. In previous studies on blue cheese varieties, *D. hansenii* was the most frequent and abundant yeast detected in the whole of the cheese (van de Tempel *et al*., 1998; Addis *et al*., 2001; Wojtatowicz *et al*., 2001; Vasdinyei *et al*., 2003). In the present study, *D. hansenii* was found to be dominant in the outer crust of Stilton where the number of yeasts was about 10-fold higher than in the blue veins and about 50-fold more than those in the white core. However, *D. hansenii* was not dominant in the blue part where *Kluyveromyces lactis* was the dominant species. If all areas of the cheese were studied as a single sample, the samples would be dominated by *D. hansenii* and the abundance of *K. lactis* in the blue part of the cheese would have been underestimated. The exterior of other blue cheeses also exhibit higher yeast populations (Roostita *et al*., 1996; Gobbetti *et al*., 1997; van den Tempel *et al*., 1998; Wojtatowicz *et al*., 2001; Viljoen *et al*., 2003) and similar misleading conclusions could take place if only the whole cheeses are studied. The abundance of *K. lactis* may have particular significance for *P. roqueforti*. *K. lactis* presents extracellular proteolytic activity (Roostita *et al.*, 1996) while *D. hansenii* does not (Roostita *et al.*, 1996; Van den Tempel *et al.*, 2000a; Addis *et al.*, 2001); studies on the effect of starter lactic acid bacteria on *P. roqueforti* in cheese based model systems, indicated that the production of the amino acids arginine and

leucine (as a result of bacterial proteolytic activity), was related with the stimulation of *P. roqueforti*'s growth and sporulation (Hansen *et al.*, 1997). The proteolytic activity of *K. lactis* may release amino acids with similar impact. *K. lactis* was not only dominant in the blue regions but its distribution followed the same pattern as *Penicillium roqueforti*. It was present at lower levels in the white part where the mould is present but does not sporulate. The observation of *K. lactis* dominance in the blue regions may be important in the manufacture of the cheese, particularly where cheeses fail to develop adequate blue veining as a result of the lack of sporulation by *P. roqueforti*. It would be interesting in future to study the role of *K. lactis* on the blue veining and particularly the effect of its proteolytic activity on *P. roqueforti*. In addition, the yeast communities in the blue regions of good quality cheeses could be compared with those in cheeses with defects in order to investigate any possible connection of the abundance of *K. lactis* with the blue veining.

Penicillium roqueforti was the only mould that was detected in Stilton. The mould was detected in the blue and white parts but not in the outer crust; this demonstrates the advantage of studying the different parts of the cheese separately as otherwise this observation would not be possible. The same result was observed both with molecular and culture-dependent techniques. The result is in some ways surprising as in Stilton production the spores of the mould are added in the milk and therefore it would be expected to be uniformly distributed in the whole cheese matrix and its oxygen requirement would suggest it is more adapted to grow on the surface. Indeed in studies of other blue cheese varieties where the mould flora has been found to consist exclusively of the starter *P. roqueforti* (Gobbetti *et al*., 1997; Addis *et al*., 2001) this was detected on the surface of the cheeses (Gobbetti *et al*., 1997) as well as in the interior. There is not any identified explanation for the absence of *P. roqueforti*

from the surface of Stilton, however one possible explanation is that the spores on the surface of the cheese produce mycelia during the early stages of the production, but their further growth and sporulation on the final product is inhibited by a parameter that takes place later. This could be another species present on the surface of the cheese such as *Yarrowia lipolytica*. This species was detected in the outer part, and was less present in the white part, where the mould is present but does not sporulate, and was not detected at all in the blue veins were the mould is present and sporulates. *Y. lipolytica* has previously been reported to inhibit the sporulation of *P. roqueforti* when tested *in vitro* (van den Tempel, 2000; van den Tempel *et al*., 2000a; Juszczyk *et al*., 2005) and in the present study this yeast inhibited the sporulation of the mould in the model systems, with no sporulation observed in the *P. roqueforti* + *Y.lipolytica* model after ten days of incubation. However, to date there is no study which demonstrates this inhibition directly in the cheese. A future study on the development of the fungal flora from ripening to maturation would reveal if the mould is present in the outer part at any stage of the production and if its decline coincides with the development of *Y. lipolytica*. Additionally, the distribution of *Y. lipolytica* in good quality cheeses could be compared with low quality cheeses (that fail to develop sufficient blue veining) to determine if inhibition of the sporulation of the mould is because of changes in the distribution of *Y. lipolytica* (or any other species typical of the outer part), as the outer crust starts to develop earlier than the piercing and blue veining.

Debaryomyces hansenii was the species with the highest counts in the outer part and was detected in all parts of the cheese. There were two different groups of this species (here named A and B) differentiated by their colony morphology. The two groups were not distinguishable by their biochemical and molecular analysis profiles;

the latter techniques were focused on identifying the species and were thus not methods optimized to differentiate strains. Interestingly, the distribution of the two groups in the different sections was different, with both groups present in the crust and the white part of the cheese, but group A not present in the blue part. However, the differing colony morphology and the different distribution of these two groups does suggest that strain differences may exist within this species which adapt them to growth in the different conditions of the cheese sections. These could be differences in the physiological conditions due to the growth of the mould and the production of ammonia which raises the pH and may be stronger in the sporulating regions.

D. hansenii is one of the most frequently reported yeasts in blue cheeses (van de Tempel *et al*., 1998; Addis *et al*., 2001; Wojtatowicz *et al*., 2001; Vasdinyei *et al*., 2003). For this reason and because of its good proteolytic activity (Roostita *et al*., 1996; Addis *et al*., 2001) it has been suggested that it should be used as part of the starter culture. The results in this study suggest that careful consideration should be taken when selecting isolates from this species for starters, as strains of group A, for example, might not be able to colonise the blue veins. It would also be important to determine which properties are important for the selection of starter cultures and the functions the starter is designed to promote, as these may differ between strains of the species. Future work could investigate if either of the two groups presents higher proteolytic and/or lipolytic activity and the desirability of these for optimizing product characteristics. Nevertheless, depending on whether the presence of *D. hansenii*, as a starter culture, is desirable in the blue veins, strains from the groups A and B could be used accordingly. In a previous study it was found that strains of *D. hansenii* exhibited weak inhibition of *P. roqueforti* in the presence of oxygen (as would be present in the blue veins), while positive interactions were observed at 25% carbon

dioxide and 0.3% oxygen (van den Tempel et al., 2000). In the present study, *D. hansenii* from groups A and B also exhibited weak inhibition of *P. roqueforti* sporulation in model systems (requiring seven days compared to five days when the mould was cultivated alone) and resulted in the same aroma profiles. If *D. hansenii* strains were required as a starter culture in order to take advantage of its high proteolytic activity, but without the cost of the inhibition of sporulation of the mould, then the selection of strains that do not populate the blue veins (such as group A) would be more appropriate.

Different analytical approaches for the analysis of the blue cheese aroma were compared. The combined direct MS and GC-MS approach and the different observations with each of them demonstrate the complementary role of the techniques. APCI-MS was good for rapid discrimination of the different cheese profiles; however, the analysis was affected by the presence of ammonia in the headspace. From the GC-MS techniques SPME gave satisfactory qualitative results and was able to screen for differences between the sections of the cheeses.

The individual study of the aroma of the different sections of the cheese allowed the observation of similarities between the outer and the blue parts of the cheese. These parts were dominated by ketones while the white part had increased amounts of alcohols. The production of ketones in the outer part could not be assigned to the starter *P. roqueforti* as this organism was not detected in this section. In contrast, a great variety and high counts of yeasts were present in the outer part. *Yarrowia lipolytica*, a species present in the outer part, produced significant amounts of ketones in model systems when it was grown individually. Therefore, members of the yeast community in the outer part may be responsible for the production of aroma compounds similar to those that *P. roqueforti* produces. Furthermore, the production

of aroma compounds was even higher when *Y. lipolytica* was cultured together with *P. roqueforti*. It seems that despite the fact that *Y. lipolytica* inhibited the sporulation of *P. roqueforti*, there is a kind of synergy regarding the aroma production. In contrast the presence of *K. lactis* and *D. hansenii* inhibited the production of ketones. It is possible that *Y. lipolytica* has a major role in the formation of the blue cheese aroma in Stilton. The enhanced aroma production by the combination of *Y. lipolytica* and *P. roqueforti* could be utilised in starter cultures. If the synergistic effect were confirmed in the cheese production then *Y. lipolytica* would be ideal for becoming part of the starter culture (or adjunct culture). Careful consideration should be taken in selecting the appropriate strains as they can inhibit the sporulation of the mould. However, the level of inhibition has been found to vary between strains (Juszczyk et al., 2005). Isolation of strains of *Y. lipolytica* with limited inhibition effect and/or resistant strains of *P. roqueforti* would be valuable for the blue cheese production. *Y. lipolytica* could also be applied as a smear ripening culture.

Flash profile analysis of the model system of *P. roqueforti* +*Y. lipolytica* and of blue cheeses demonstrated that the similarities in the aroma profiles that were observed with instrumental methods were reflected in sensory perception. Therefore products based on this model could be used as an alternative to blue cheese flavouring in the food industry. The present work focused on the aroma of the models, however, the sensory perception of the taste should be investigated too. Another key factor would be the aroma retention in the model. Preliminary trials of spray-drying of the *P. roqueforti* +*Y. lipolytica* model resulted in poor aroma retention and further work on the encapsulation of the aroma would be required to produce a commercially viable product. Carbohydrates, such as starches, are used extensively in spray-dried encapsulations of food ingredients in order to retain and protect volatile compounds.

Starch and starch-based ingredients can act as carriers for aroma encapsulation and also stabilise emulsions (Madene *et al*., 2006) and their addition may improve the model.

The biochemical pathways that are involved in the synergy between *Y. lipolytica* and *P. roqueforti* should be investigated. It could be possible that *Y. lipolytica*, which is highly lipolytic (Roosita *et al*., 1995; Van den Tempel *et al*., 2000a), releases significant amounts of free fatty acids in the cheese and these are β-decarboxylated by *P. roqueforti* to methyl ketones. The enzymes that are responsible for the increased production of aroma could be characterised and used for the improvement of the enzyme modified cheeses, which require mixing of cheese curd and high amounts of enzymes.

In order to study the role of the yeasts in the Stilton aroma production representative isolates were used from each of the different group of isolates. Isolates that belong to the same species would be expected to present similar properties; however, quite often the properties and/or the degree of expression of the properties that could affect the cheese (proteolytic/lipolytic activity, flavour production, mould inhibition etc.) are strain dependent (Van den Tempel *et al.*, 2000a). The assessment of the intraspecific variability of the different yeast groups that were detected in Stilton in combination with the comparison of flavour interactions in models composed of different strains from each species would allow to appreciate at what degree the findings reflect the properties of species or strains. Stain typing techniques such as PFGE and RAPD could be employed for the assessment of the strain differentiation within each group of isolates. Differences in the properties of technological interest, such as the lipolytic activity (and consequently the flavour

development) between strains of the same species could be observed although these would not necessarily correspond to the genetic diversity (Suzzi et al., 2001).

This study has given information on the fungi in Stilton and particularly the yeast secondary flora. So far it is common practice for the secondary flora of the blue cheeses not to be controlled. From the current study it can be concluded that the yeasts are a major and important part of the microflora of Stilton. Many issues have been raised which need to be investigated further. Finally, many observations in this study would not have been possible if the different parts of the cheese were not studied separately. Stilton has a very complex matrix and therefore similar approaches might prove equally useful in studies of other properties of Stilton.

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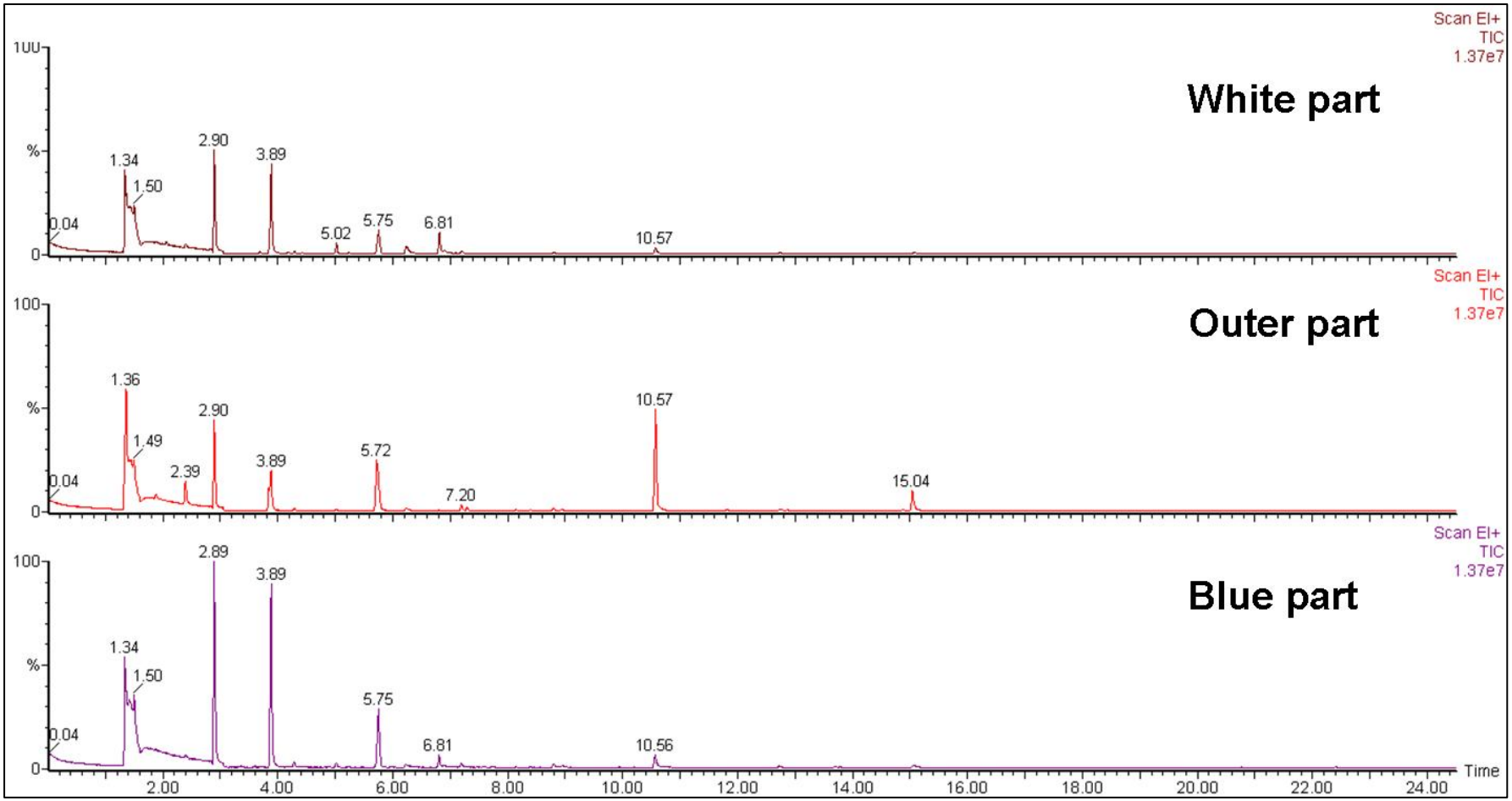
APPENDICES

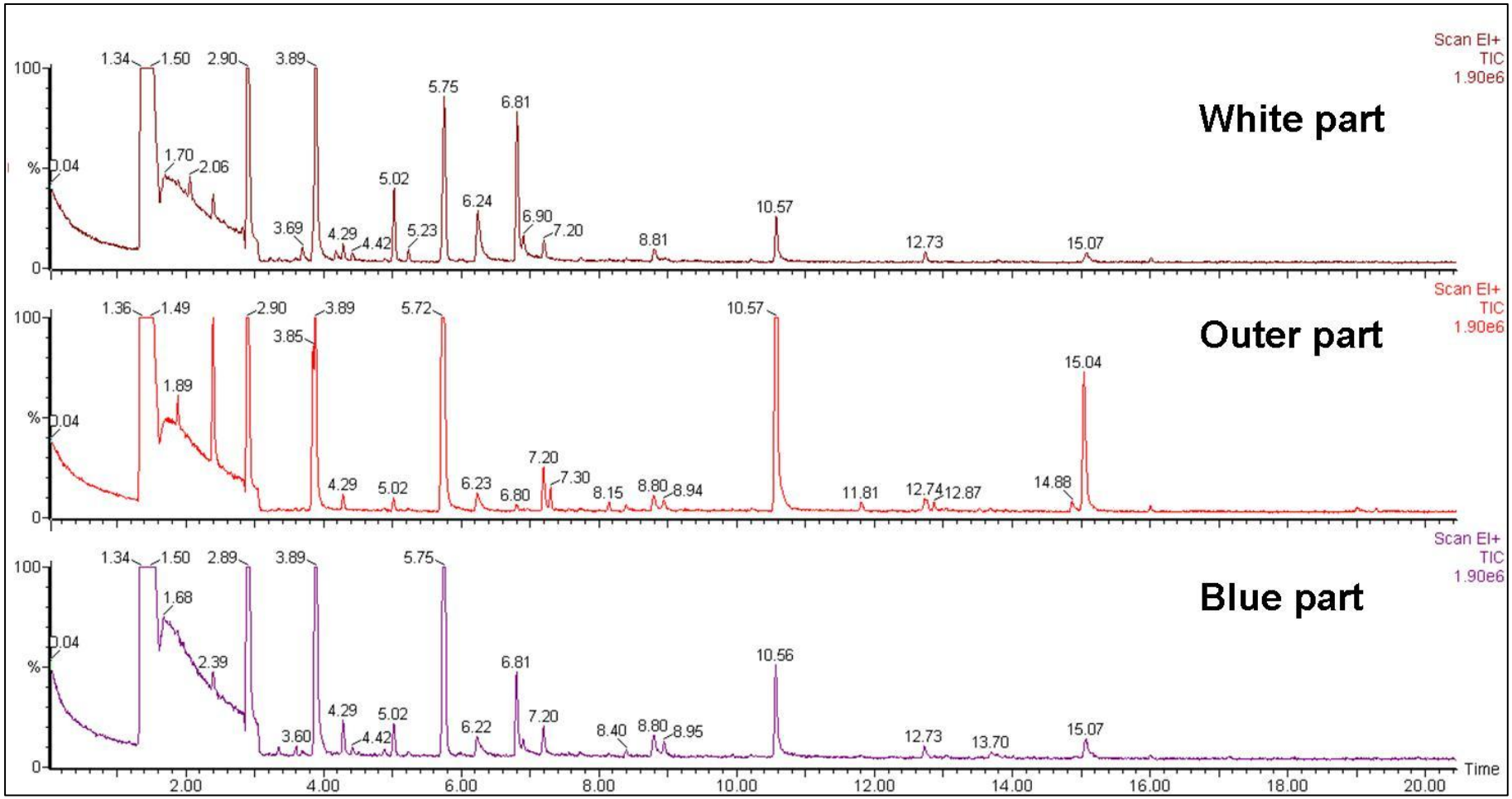
Appendix 1. Information on API reports.

Sequencing ID	***Profile number***	***% ID***	***T Index***	***First significant taxa***
Kluyveromyces lactis	2042432	77.4	0.75	*Candida sphaerica 2*
	2042632	99.2	0.7	*Candida sphaerica 2*
Yarrowia lipolytica	6000100	98.9	0.91	*Candida krusei / inconspicua*
	6000100	98.9	0.91	*Candida krusei / inconspicua*
	6000100	98.9	0.91	*Candida krusei / inconspicua*
	6000100	98.9	0.91	*Candida krusei / inconspicua*
Debaryomyses hansenii (group A)	2146731	-	-	*Candida famata*
	6356771	99.9	0.81	*Candida famata*
Debaryomyses hansenii (group B)	6356771	99.9	0.81	*Candida famata*
	6376771	99.9	0.89	*Candida famata*
Trichosporon ovoides	-	99.9	0.54	*Cryptococcus terreus*
	-	99.9	0.54	*Cryptococcus terreus*

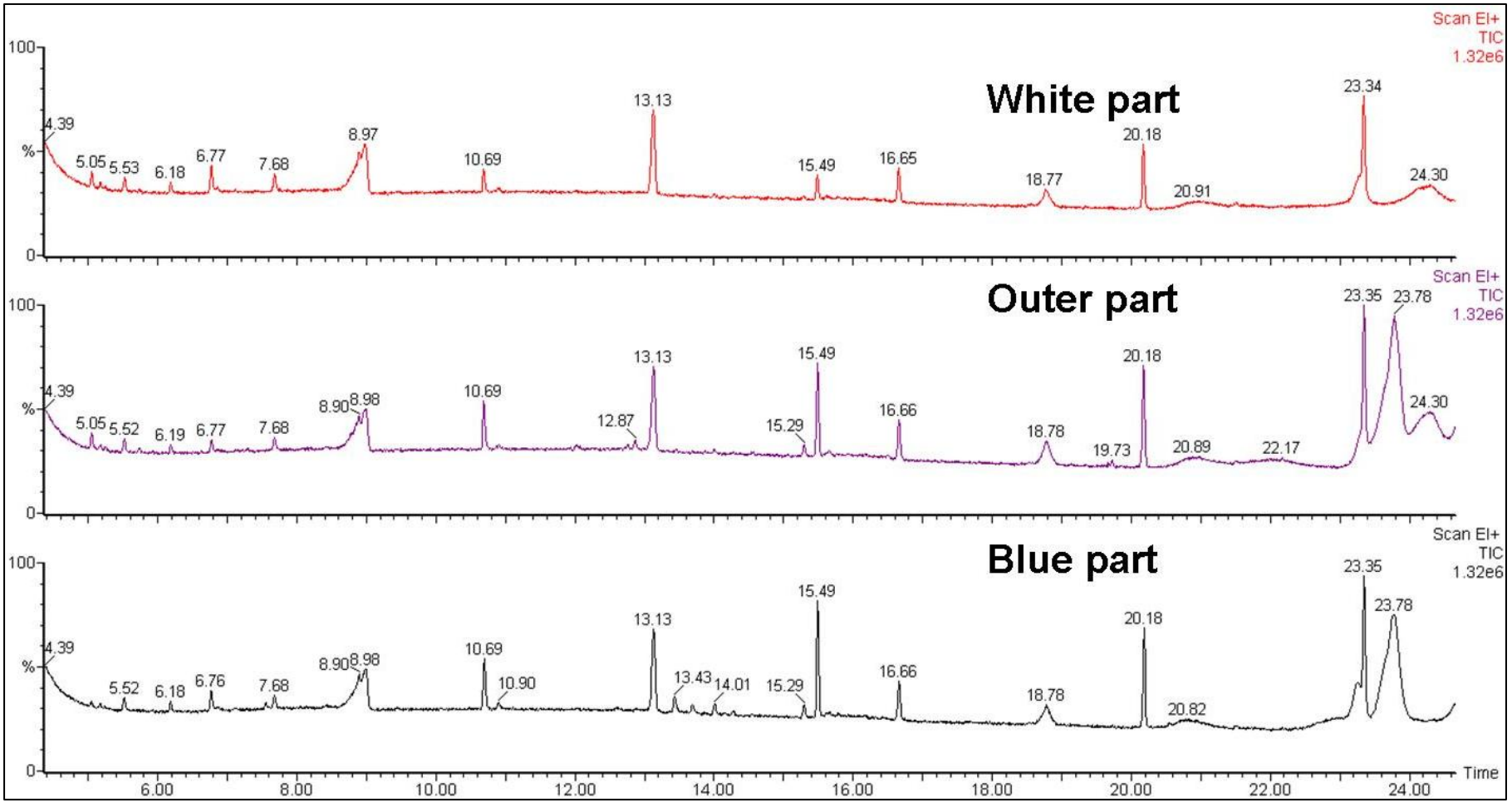
Appendix 2. Typical TIC chromatograms from the SPME GC-MS data set of white, outer and blue parts.



Appendix 2 continue. Magnification of TIC chromatograms from the SPME GC-MS data set of white, outer and blue parts.



Appendix 3. Typical TIC chromatograms from the solvent extraction GC-MS data set of white, outer and blue parts.



Appendix 4. Average APCI-MS signal intensities for the ions monitored in headspace samples of the outer crust, blue veins and white core of additional Stilton cheeses from different producers. The values are relative to the signal intensity observed when the headspace above a 5µg/l, 2-nonanone solution was sampled into the APCI-MS.

m/z	B1			A2			C1			B2			A4			A3		
	Blue	Out	White	Blue	Out	White	Blue	Out	White	Blue	Out	White	Blue	Out	White	Blue	Out	White
18	110.1	78.7	87.1	41.6	112.7	1.14	46.3	53.4	34.6	68.4	56.5	61.2	3.05	55.5	0.62	52.9	46.7	0.85
33	0.25	0.00	1.68	30.3	0.14	33.5	0.00	0.00	0.00	0.66	0.00	2.58	50.9	5.51	30.6	13.3	2.79	17.5
35	3.50	3.15	2.10	0.52	5.61	0.07	1.83	2.06	0.98	3.13	3.44	2.11	0.12	1.45	0.07	0.94	0.95	0.06
51	0.10	0.00	0.62	9.77	0.08	10.3	0.00	0.00	0.00	0.34	0.00	0.99	16.3	1.86	9.88	4.45	0.98	5.90
59	2.11	0.60	5.05	10.9	0.40	18.0	5.31	4.06	15.0	2.90	0.00	2.63	3.33	3.98	21.2	0.22	6.25	8.36
65	0.00	0.00	0.00	1.62	0.00	2.40	0.00	0.01	0.11	0.03	0.00	0.05	6.18	0.25	2.51	0.70	0.13	1.51
69	0.03	0.03	0.68	0.29	0.02	0.53	0.05	0.03	0.17	0.07	0.03	0.20	0.71	0.16	0.82	0.04	0.09	0.28
71	0.12	0.00	1.00	2.13	0.03	6.42	0.09	0.09	0.45	0.42	0.02	1.22	6.36	1.87	7.25	0.87	0.63	3.43
73	0.06	0.83	0.63	0.93	1.43	62.8	0.51	1.32	1.97	0.08	1.83	4.62	1.21	3.56	14.8	0.48	21.4	84.7
76	1.59	7.93	0.80	0.46	1.76	0.39	11.6	7.82	7.13	2.88	11.5	1.30	0.08	1.44	0.23	0.19	1.84	0.28
87	1.08	2.47	4.82	14.9	0.23	5.84	22.2	22.0	54.6	2.03	1.53	1.17	3.73	1.39	9.36	1.24	2.22	4.82
89	0.39	0.06	2.86	4.76	0.21	12.6	0.34	1.20	1.36	0.47	0.06	1.76	3.48	1.60	12.6	3.48	4.08	15.3
90	0.10	3.09	0.16	0.30	2.17	0.96	0.74	1.23	0.62	0.16	10.6	1.38	0.21	1.10	0.76	0.28	6.22	1.90
101	0.11	0.43	0.25	0.74	0.12	0.63	0.80	0.94	1.33	0.14	0.27	0.12	0.16	0.63	1.39	0.08	0.43	0.73
103	0.02	0.29	0.11	0.27	0.00	0.87	0.36	0.33	0.39	0.06	0.16	0.01	0.43	0.09	1.54	0.05	0.07	0.74
104	1.08	12.2	1.26	1.20	0.74	0.16	44.4	38.1	36.3	2.19	13.2	0.87	0.12	0.62	0.24	0.23	0.87	0.22

106	1.00	1.03	1.43	0.78	0.75	0.38	1.56	3.23	1.40	1.05	1.29	1.25	0.46	1.49	0.45	1.18	2.34	0.66
115	2.44	3.70	4.04	7.87	0.09	1.73	5.26	4.37	7.07	5.28	3.43	2.19	0.54	0.76	3.93	1.23	0.78	1.48
129	0.49	1.44	0.47	0.42	0.01	0.22	0.59	0.72	0.62	0.76	1.50	0.20	0.06	0.15	0.24	0.10	0.09	0.22
131	0.06	0.56	0.14	0.08	0.07	1.90	0.25	0.29	0.44	0.11	0.96	0.20	0.13	0.21	0.76	0.04	1.31	2.15
132	5.68	27.6	2.76	1.46	0.65	0.21	16.0	12.4	8.42	9.21	32.1	1.63	0.02	0.73	0.16	0.60	0.76	0.23
143	1.64	1.60	2.11	2.44	0.08	0.76	0.60	1.32	0.78	2.19	1.07	0.55	0.93	0.30	0.98	0.65	0.39	0.83
145	0.06	0.81	0.19	0.46	0.04	3.61	6.02	4.25	10.5	0.22	1.01	0.10	0.10	0.15	0.81	0.04	2.39	10.32
159	0.14	0.71	0.04	0.10	0.07	0.68	0.54	0.91	1.01	0.21	1.03	0.05	0.11	0.14	0.53	0.07	0.52	0.92
160	6.20	18.9	2.66	0.89	0.73	0.07	3.55	6.48	1.75	6.66	13.9	1.16	0.07	0.63	0.14	0.60	0.63	0.15
173	0.23	2.12	0.48	0.86	0.07	0.24	12.3	11.0	26.1	0.57	1.66	0.05	0.14	0.12	0.61	0.00	0.17	0.25
187	0.05	0.86	0.00	0.14	0.02	0.33	0.57	0.64	0.76	0.07	1.20	0.12	0.04	0.05	0.26	0.02	0.25	0.50

Appendix 5. Average SPME GC-MS signal intensities (and standard deviations, SD) for the compounds detected when headspace samples of model systems and real cheeses were analysed. The values are relative to the peak area observed when the headspace above a 5µg/l, 2-nonanone solution was analysed.

Suggested compound ID		Acetaldehyde	Methanethiol	Ethanol	Acetone	Unidentified	2-Butanone	Ethylacetate	2-Methyl-1-propanol	3-Methyl-butanal	2-Methyl-butanal	2-Pentanone	2-Pentanol	3-Hydroxy-2-butanone	Ethyl ester propanoic acid	n-Propyl acetate	3-Methyl-butanol	2-Methyl-1-butanol	Dimethyl-disulfide
LRI		0	0	0	0	586	596	614	624	652	663	686	698	710	711	713	733	737	748
Stilton	Mean	0.03	0.14	0.14	4.94	0.28	27.15	0.01	0.17	0.88	0.10	22.15	0.74	2.84	0.00	0.00	6.37	0.36	0.21
	SD	0.01	0.01	0.05	0.45	0.06	5.59	0.00	0.01	0.34	0.05	8.92	0.30	0.80	0.00	0.00	1.03	0.07	0.02
Danish blue cheese	Mean	0.14	0.03	3.05	37.18	0.05	1.11	0.16	0.96	0.38	0.04	200.18	2.99	0.07	0.01	0.04	26.09	1.13	0.07
	SD	0.07	0.04	3.70	34.42	0.03	0.80	0.27	0.37	0.50	0.04	47.31	1.77	0.04	0.01	0.01	12.64	0.53	0.03
Roquefort cheese	Mean	0.10	0.01	0.04	68.48	0.04	1.19	0.00	0.07	0.01	0.00	257.40	2.93	0.23	0.00	0.00	1.49	0.07	0.03
	SD	0.05	0.00	0.01	3.77	0.01	0.10	0.00	0.02	0.02	0.00	20.11	0.43	0.03	0.00	0.00	0.25	0.02	0.01
Shropshire cheese	Mean	0.21	0.72	0.85	16.01	1.00	0.29	0.01	0.66	5.17	0.88	38.57	0.43	15.33	0.01	0.02	18.39	0.99	0.68
	SD	0.04	0.20	0.33	2.79	0.15	0.02	0.00	0.13	0.77	0.26	10.67	0.10	3.84	0.00	0.01	6.74	0.23	0.10
St. Agur cheese spread	Mean	0.13	0.01	6.98	2.24	0.13	0.73	0.02	0.82	3.31	0.55	2.05	0.62	0.16	0.00	0.00	22.55	1.29	0.02
	SD	0.02	0.00	1.27	0.24	0.02	0.06	0.01	0.12	0.45	0.07	0.32	0.11	0.01	0.00	0.00	3.74	0.17	0.01
Blacksteak cheese	Mean	0.22	0.00	0.11	0.46	0.02	0.01	0.00	0.05	0.13	0.02	0.72	0.03	0.02	0.00	0.00	0.18	0.03	0.13
	SD	0.22	0.00	0.09	0.08	0.02	0.00	0.00	0.03	0.12	0.02	0.46	0.00	0.02	0.00	0.00	0.07	0.02	0.09
Cheddar cheese (mature)	Mean	0.09	0.08	3.04	4.36	0.18	1.47	0.18	0.02	1.65	0.63	0.72	0.05	1.78	0.00	0.96	0.10	0.01	0.04
	SD	0.04	0.01	0.46	0.61	0.03	0.12	0.02	0.01	0.10	0.04	0.10	0.01	0.26	0.00	0.11	0.01	0.00	0.01
Cheddar cheese (standard)	Mean	0.03	0.03	2.83	3.62	0.67	1.36	0.09	0.02	0.68	0.14	0.38	0.02	11.87	0.00	0.24	0.02	0.00	0.07
	SD	0.02	0.01	0.37	0.40	0.14	0.17	0.01	0.01	0.20	0.04	0.08	0.01	0.84	0.00	0.04	0.01	0.00	0.01
P. roqueforti	Mean	0.07	0.00	0.95	0.96	0.02	0.13	0.01	0.14	3.15	0.16	7.01	0.16	0.01	0.00	0.01	3.19	0.24	0.00
	SD	0.07	0.00	0.38	0.19	0.03	0.04	0.00	0.05	2.21	0.08	4.39	0.04	0.01	0.00	0.01	2.29	0.09	0.00

Y. lipolytica	**Mean**	0.38	0.02	2.48	10.43	0.01	2.19	0.05	0.60	14.27	3.91	3.11	0.03	0.03	0.00	0.00	14.73	1.11	0.02
	SD	0.16	0.02	1.58	5.99	0.01	0.38	0.02	0.43	8.62	2.40	0.71	0.01	0.02	0.00	0.00	11.68	0.82	0.01
D. hansenii **(group A)**	**Mean**	0.78	0.00	16.10	0.93	0.02	1.24	0.10	7.62	0.05	0.10	0.26	0.01	0.42	0.00	0.00	11.41	3.97	0.00
	SD	0.22	0.00	1.95	0.17	0.02	0.23	0.03	1.60	0.05	0.09	0.04	0.00	0.09	0.00	0.00	2.20	0.57	0.00
D. hansenii **(group B)**	**Mean**	0.42	0.00	9.43	0.90	0.01	1.05	0.03	5.76	0.27	0.11	0.19	0.00	0.19	0.00	0.00	13.95	4.03	0.00
	SD	0.11	0.00	0.47	0.06	0.00	0.21	0.01	0.52	0.44	0.04	0.01	0.00	0.13	0.00	0.00	1.00	0.46	0.00
P. roqueforti* + *L. lactis	**Mean**	0.69	0.01	27.87	1.82	3.30	0.15	0.16	0.16	0.24	0.01	5.27	0.02	50.82	0.02	0.01	3.14	0.19	0.03
	SD	0.14	0.00	0.92	0.13	0.63	0.02	0.06	0.02	0.16	0.00	1.19	0.01	9.03	0.00	0.00	0.07	0.01	0.01
Tr. ovoides	**Mean**	0.16	0.00	3.34	0.96	0.00	0.16	0.04	0.25	0.06	0.01	0.45	0.02	0.11	0.00	0.00	0.54	0.11	0.00
	SD	0.11	0.00	0.76	0.09	0.00	0.03	0.01	0.05	0.03	0.00	0.05	0.00	0.10	0.00	0.00	0.14	0.03	0.00
K. lactis	**Mean**	0.68	0.01	260.91	0.04	0.00	0.04	54.76	3.73	0.03	0.03	0.01	0.00	0.09	1.01	0.27	26.20	6.11	0.01
	SD	0.26	0.01	5.99	0.01	0.00	0.01	3.99	0.39	0.02	0.02	0.00	0.00	0.01	0.07	0.03	2.53	0.54	0.01
L. lactis	**Mean**	0.23	0.00	4.44	2.83	0.82	1.18	0.02	0.07	0.01	0.01	0.16	0.00	12.60	0.00	0.00	0.06	0.01	0.01
	SD	0.13	0.00	0.72	0.66	0.20	0.19	0.01	0.04	0.01	0.01	0.04	0.00	6.14	0.00	0.00	0.05	0.01	0.00
P. roqueforti* + *Y. lipolytica	**Mean**	0.12	0.00	1.93	10.24	0.01	0.51	0.03	0.29	2.58	0.77	76.64	0.79	0.01	0.00	0.03	8.95	0.59	0.00
	SD	0.07	0.00	0.69	5.47	0.00	0.14	0.01	0.07	1.35	0.48	15.71	0.68	0.01	0.00	0.01	1.74	0.15	0.00
P. roqueforti* + *L. lactis* + *Y. lipolytica	**Mean**	0.56	0.00	5.27	37.50	0.09	1.12	0.04	0.20	2.92	0.55	79.25	2.03	0.32	0.00	0.01	2.26	0.29	0.00
	SD	0.19	0.00	1.28	4.29	0.01	0.14	0.00	0.04	0.46	0.13	12.13	0.31	0.06	0.00	0.01	0.32	0.06	0.00
P. roqueforti* + *K. lactis	**Mean**	5.18	0.01	232.86	0.13	0.01	0.09	120.67	4.62	0.64	0.68	0.15	0.02	0.37	2.69	0.54	45.53	8.66	0.01
	SD	4.90	0.01	4.20	0.02	0.00	0.01	20.59	0.59	0.72	0.77	0.01	0.01	0.07	0.43	0.14	4.36	0.24	0.01
P. roqueforti* + *D. hansenii **(group A)**	**Mean**	0.46	0.00	4.68	0.98	0.02	0.30	0.01	4.48	0.69	0.19	0.44	0.02	0.66	0.00	0.00	18.09	3.27	0.00
	SD	0.25	0.00	3.26	0.42	0.02	0.18	0.01	1.23	0.62	0.12	0.40	0.02	0.78	0.00	0.00	10.25	1.32	0.00
P. roqueforti* + *D. hansenii **(group B)**	**Mean**	0.34	0.00	8.38	0.85	0.01	0.43	0.04	4.94	0.14	0.06	0.27	0.01	0.14	0.00	0.00	14.80	3.75	0.00
	SD	0.07	0.00	2.68	0.21	0.00	0.27	0.02	0.57	0.11	0.03	0.08	0.00	0.09	0.00	0.00	5.09	0.56	0.00
P. roqueforti* + *Tr. Ovoides	**Mean**	0.03	0.00	0.93	1.48	0.00	0.10	0.03	0.20	0.01	0.00	0.54	0.01	0.02	0.00	0.00	0.87	0.14	0.00
	SD	0.01	0.00	1.30	0.43	0.00	0.05	0.02	0.08	0.02	0.00	0.22	0.00	0.02	0.00	0.00	0.33	0.09	0.00
Control	**Mean**	0.03	0.00	0.32	1.83	0.00	1.43	0.34	0.00	0.03	0.00	0.17	0.00	0.01	0.00	0.00	0.02	0.00	0.01
	SD	0.01	0.00	0.16	0.24	0.00	0.23	0.08	0.00	0.04	0.00	0.03	0.00	0.00	0.00	0.00	0.03	0.00	0.01

Suggested compound ID		3-Methyl-2-pentanone	2-Methyl-ethyl ester propanoic acid	1-Pentanol	Isobutyl acetate	2-Hexanone	Octane	Butanoic acid	3-Methyl-butanoic acid	2-Methyl-butanoic acid	Pentanoic acid	3-Methyl-1-butanol, acetate)	2-Heptanone	Styrene	2-Heptanol	a-Pinene	Unidentified	Unidentified	3-Octanone
LRI		**753**	**758**	**765**	**772**	**788**	**799**	**799**	**838**	**846**	**875**	**875**	**891**	**898**	**899**	**945**	**952**	**955**	**988**
Stilton	**Mean**	0.19	0.06	0.00	0.00	0.50	0.08	0.00	0.00	0.00	0.02	0.00	28.16	0.06	0.50	0.03	0.02	0.00	0.12
	SD	0.03	0.00	0.00	0.00	0.27	0.01	0.00	0.00	0.00	0.00	0.00	13.83	0.01	0.19	0.01	0.01	0.00	0.03
Danish blue cheese	**Mean**	0.00	0.06	0.00	0.06	3.79	0.05	41.84	2.46	0.20	0.79	0.16	202.37	0.01	6.94	0.00	0.02	1.49	0.03
	SD	0.00	0.03	0.00	0.05	2.31	0.03	12.97	0.55	0.06	0.28	0.08	36.99	0.00	0.64	0.00	0.00	0.16	0.01
Roquefort cheese	**Mean**	0.02	0.00	0.00	0.01	3.46	0.02	0.00	0.08	0.03	0.01	0.00	435.16	0.62	4.86	0.00	0.00	0.03	0.07
	SD	0.01	0.00	0.00	0.00	0.45	0.01	0.00	0.05	0.02	0.00	0.00	35.58	0.04	0.53	0.00	0.00	0.01	0.03
Shropshire cheese	**Mean**	0.05	0.00	0.02	0.02	0.74	0.08	0.00	0.00	0.00	0.03	0.00	40.33	0.04	0.52	0.01	0.04	0.01	0.15
	SD	0.01	0.00	0.00	0.01	0.12	0.00	0.00	0.00	0.00	0.02	0.00	4.80	0.01	0.07	0.00	0.01	0.00	0.02
St. Agur cheese spread	**Mean**	0.01	0.00	0.00	0.00	0.06	0.01	0.04	0.00	0.00	0.04	0.00	3.44	0.02	0.36	0.01	0.00	0.04	0.01
	SD	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.00	0.00	0.01	0.00	0.54	0.00	0.08	0.00	0.00	0.01	0.01
Blacksteak cheese	**Mean**	0.00	0.00	0.01	0.00	0.04	0.01	0.00	0.00	0.00	0.00	0.00	3.35	0.01	0.10	0.01	0.00	0.00	0.01
	SD	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	2.26	0.01	0.04	0.01	0.00	0.00	0.00
Cheddar cheese (mature)	**Mean**	0.00	0.00	0.05	0.00	0.01	0.18	0.00	0.00	0.00	0.01	0.00	0.75	0.00	0.00	0.03	0.00	0.00	0.00
	SD	0.00	0.00	0.01	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.01	0.00	0.00	0.00
Cheddar cheese (standard)	**Mean**	0.00	0.00	0.05	0.00	0.00	0.15	0.01	0.00	0.00	0.00	0.00	0.58	0.01	0.00	0.02	0.00	0.00	0.00
	SD	0.00	0.00	0.01	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.01	0.00	0.00	0.00
P. roqueforti	**Mean**	0.01	0.00	0.34	0.02	0.88	0.08	0.00	0.00	0.00	0.00	0.00	68.71	0.01	0.37	0.01	0.01	0.00	0.13
	SD	0.00	0.00	0.09	0.00	0.34	0.01	0.00	0.00	0.00	0.00	0.00	4.07	0.00	0.08	0.00	0.00	0.00	0.04
Y. lipolytica	**Mean**	0.09	0.02	0.03	0.02	0.13	0.23	37.24	0.83	0.28	0.04	0.44	5.68	0.01	0.92	0.01	0.18	0.23	0.21
	SD	0.04	0.01	0.01	0.01	0.08	0.06	4.85	0.78	0.30	0.04	0.22	0.62	0.01	0.26	0.00	0.17	0.13	0.12

***D. hansenii* (group A)**	**Mean**	0.00	0.02	0.01	0.02	0.01	0.09	0.01	0.00	0.00	0.00	0.00	0.56	2.03	0.04	0.01	0.00	0.01	0.00
	SD	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.06	0.07	0.01	0.00	0.00	0.00	0.00
***D. hansenii* (group B)**	**Mean**	0.00	0.01	0.00	0.01	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.40	1.52	0.04	0.00	0.00	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.17	0.01	0.00	0.00	0.00	0.00
P. roqueforti* + *L. lactis	**Mean**	0.00	0.00	0.03	0.00	0.37	0.06	0.06	0.00	0.00	0.00	0.00	28.18	0.01	0.28	0.00	0.12	0.01	0.75
	SD	0.00	0.00	0.01	0.00	0.09	0.06	0.01	0.00	0.00	0.00	0.00	6.12	0.00	0.03	0.00	0.01	0.00	0.08
Tr. ovoides	**Mean**	0.00	0.01	0.00	0.00	0.00	0.04	0.05	0.00	0.00	0.00	0.00	0.14	0.01	0.01	0.01	0.00	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
K. lactis	**Mean**	0.00	0.46	0.00	0.34	0.00	0.03	0.14	0.00	0.00	2.19	0.01	0.23	0.00	0.02	0.00	0.00	0.01	0.00
	SD	0.00	0.01	0.00	0.02	0.00	0.01	0.02	0.00	0.00	0.06	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00
L. lactis	**Mean**	0.00	0.00	0.02	0.01	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.71	0.21	0.00	0.00	0.00	0.00	0.00
	SD	0.00	0.00	0.01	0.01	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.06	0.09	0.00	0.00	0.00	0.00	0.00
P. roqueforti* + *Y. lipolytica	**Mean**	0.06	0.00	0.04	0.05	4.35	0.09	14.69	1.17	0.37	0.08	0.13	364.76	0.02	9.21	0.01	0.14	0.13	0.09
	SD	0.05	0.00	0.02	0.01	1.68	0.08	7.41	1.25	0.33	0.07	0.07	24.50	0.01	1.04	0.00	0.04	0.02	0.11
P. roqueforti* + *L. lactis* + *Y. lipolytica	**Mean**	0.12	0.00	0.03	0.29	3.89	0.06	15.54	0.53	0.04	0.01	0.13	206.55	0.01	31.16	0.00	0.23	0.13	0.10
	SD	0.04	0.00	0.02	0.03	0.42	0.03	2.06	0.12	0.02	0.01	0.04	14.14	0.00	2.07	0.00	0.07	0.02	0.08
P. roqueforti* + *K. lactis	**Mean**	0.00	0.51	0.00	1.07	0.02	0.05	0.59	0.00	0.00	5.17	0.02	4.60	0.02	0.82	0.01	0.00	0.48	0.00
	SD	0.00	0.07	0.00	0.19	0.00	0.01	0.21	0.00	0.00	0.31	0.01	0.29	0.01	0.09	0.00	0.01	0.35	0.00
***P. roqueforti* + *D. hansenii* (group A)**	**Mean**	0.00	0.01	0.00	0.01	0.02	0.05	0.00	0.03	0.04	0.00	0.00	3.37	1.43	0.27	0.01	0.01	0.01	0.05
	SD	0.00	0.00	0.00	0.00	0.03	0.01	0.00	0.06	0.08	0.00	0.00	2.90	0.40	0.10	0.01	0.02	0.01	0.03
***P. roqueforti* + *D. hansenii* (group B)**	**Mean**	0.00	0.01	0.00	0.01	0.01	0.06	0.00	0.00	0.00	0.00	0.00	1.07	1.91	0.08	0.01	0.00	0.00	0.02
	SD	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.41	0.08	0.01	0.00	0.00	0.00	0.03
P. roqueforti* + *Tr. Ovoides	**Mean**	0.00	0.01	0.00	0.00	0.00	0.05	0.01	0.08	0.01	0.00	0.00	0.31	2.48	0.00	0.01	0.00	0.00	0.00
	SD	0.00	0.01	0.00	0.00	0.00	0.01	0.02	0.08	0.01	0.00	0.00	0.23	4.06	0.00	0.00	0.00	0.00	0.00
Control	**Mean**	0.00	0.01	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.57	0.01	0.00	0.00	0.00	0.00	0.00
	SD	0.00	0.01	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00

Suggested compound ID		Hexanoic acid	2-Octanone	Hexanoic acid, ethyl ester	4-Methylanisole	Butanoic acid, 3-methylbutyl ester	8-Nonen-2-one	2-Nonanone	Benzeneethanol	Hexanoic acid, butyl ester	Octanoic acid	2-Decanone	Octanoic acid, ethyl ester	3-Methybutyl hexanoate	Acetic acid, 2-phenylethyl ester	2-Undecanone	Unidentified
LRI		**990**	**992**	**997**	**1029**	**1056**	**1085**	**1094**	**1127**	**1150**	**1166**	**1194**	**1195**	**1250**	**1266**	**1296**	**1394**
Stilton	**Mean**	0.00	0.21	0.01	0.18	0.02	0.70	5.13	0.02	0.00	0.00	0.01	0.00	0.00	0.01	0.05	0.00
	SD	0.00	0.08	0.00	0.02	0.00	0.32	2.21	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Danish blue cheese	**Mean**	0.00	1.41	2.89	0.11	14.34	5.45	38.01	0.33	0.25	0.00	0.16	0.40	0.89	0.02	1.35	0.35
	SD	0.00	0.98	0.22	0.02	0.76	4.33	4.67	0.10	0.02	0.00	0.08	0.03	0.12	0.01	0.46	0.04
Roquefort cheese	**Mean**	0.05	3.91	0.29	1.64	0.34	19.99	314.91	0.01	0.02	0.00	0.63	0.23	0.06	0.01	3.98	0.22
	SD	0.02	0.47	0.11	0.20	0.10	2.73	44.98	0.00	0.00	0.00	0.21	0.04	0.02	0.00	1.61	0.05
Shropshire cheese	**Mean**	0.00	0.33	0.01	0.64	0.11	1.42	9.76	0.01	0.00	0.00	0.02	0.01	0.00	0.00	0.21	0.01
	SD	0.00	0.02	0.01	0.10	0.06	0.20	0.97	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
St. Agur cheese spread	**Mean**	0.00	0.02	0.31	0.20	0.60	0.09	1.08	0.20	0.01	0.00	0.00	0.04	0.03	0.01	0.03	0.03
	SD	0.00	0.00	0.07	0.06	0.09	0.02	0.20	0.08	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.01
Blacksteak cheese	**Mean**	0.00	0.02	0.01	0.33	0.00	0.11	1.25	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.01	0.01
	SD	0.00	0.00	0.01	0.13	0.00	0.04	0.50	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.01	0.01
Cheddar cheese (mature)	**Mean**	0.00	0.00	0.02	0.01	0.00	0.03	0.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
	SD	0.00	0.00	0.01	0.01	0.01	0.02	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cheddar cheese (standard)	**Mean**	0.00	0.00	0.01	0.02	0.00	0.04	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
	SD	0.00	0.00	0.00	0.01	0.00	0.03	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
P. roqueforti	**Mean**	0.00	1.46	0.01	0.07	0.04	7.57	70.88	0.07	0.00	0.00	0.38	0.01	0.00	0.00	1.53	0.01
	SD	0.00	0.81	0.00	0.03	0.01	4.91	5.73	0.04	0.00	0.00	0.31	0.01	0.00	0.00	1.08	0.01
Y. lipolytica	**Mean**	42.43	0.05	1.37	0.00	0.78	0.23	3.28	2.91	0.20	5.88	0.04	1.10	0.25	0.00	0.51	0.45
	SD	9.50	0.04	0.65	0.00	0.49	0.27	1.56	1.96	0.13	2.02	0.02	0.60	0.18	0.00	0.29	0.30

***D. hansenii* (group A)**	**Mean**	0.00	0.00	0.03	0.00	0.01	0.00	0.11	0.05	0.00	0.00	0.00	0.01	0.00	0.08	0.01	0.01
	SD	0.00	0.00	0.01	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.01	0.00	0.09	0.00	0.01
***D. hansenii* (group B)**	**Mean**	0.00	0.00	0.01	0.00	0.00	0.00	0.05	0.05	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
P. roqueforti* + *L. lactis	**Mean**	0.00	0.26	0.16	0.48	0.04	1.26	9.05	0.00	0.00	0.00	0.01	0.05	0.00	0.02	0.07	0.02
	SD	0.00	0.07	0.02	0.08	0.01	0.30	1.75	0.00	0.00	0.00	0.01	0.03	0.00	0.02	0.02	0.01
Tr. ovoides	**Mean**	0.00	0.00	0.03	0.00	0.01	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
	SD	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
K. lactis	**Mean**	0.00	0.00	1.00	0.00	0.01	0.00	0.04	0.00	0.00	0.00	0.00	0.70	0.00	1.84	0.00	0.22
	SD	0.00	0.00	0.11	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.34	0.00	0.07
L. lactis	**Mean**	0.00	0.00	0.00	0.00	0.00	0.01	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.01	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. roqueforti* + *Y. lipolytica	**Mean**	13.90	5.53	0.75	0.04	0.72	39.21	141.55	3.87	0.16	3.22	0.49	0.81	0.24	0.01	2.22	0.36
	SD	13.83	5.08	0.32	0.03	0.16	39.76	30.33	1.12	0.04	2.51	0.39	0.10	0.08	0.00	1.24	0.10
P. roqueforti* + *L. lactis* + *Y. lipolytica	**Mean**	20.70	1.50	5.67	0.15	0.18	8.19	42.27	0.53	0.09	3.05	0.21	4.18	0.04	0.17	1.39	1.40
	SD	3.59	0.98	0.14	0.04	0.03	5.71	2.81	0.12	0.04	0.65	0.06	0.66	0.01	0.08	0.30	0.28
P. roqueforti* + *K. lactis	**Mean**	0.00	0.05	2.94	0.00	1.38	0.27	2.45	0.72	0.02	0.00	0.02	1.50	0.01	9.43	0.06	0.85
	SD	0.00	0.01	0.66	0.00	1.10	0.05	0.43	0.24	0.02	0.00	0.01	0.24	0.01	1.46	0.03	0.14
***P. roqueforti* + *D. hansenii* (group A)**	**Mean**	0.00	0.04	0.01	0.03	0.01	0.18	1.87	1.62	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.00
	SD	0.00	0.04	0.01	0.02	0.01	0.16	1.06	1.31	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.00
***P. roqueforti* + *D. hansenii* (group B)**	**Mean**	0.00	0.01	0.01	0.00	0.00	0.01	0.42	0.28	0.00	0.00	0.00	0.01	0.00	0.09	0.01	0.00
	SD	0.00	0.00	0.01	0.01	0.00	0.01	0.21	0.31	0.00	0.00	0.00	0.01	0.00	0.09	0.00	0.01
P. roqueforti* + *Tr. Ovoides	**Mean**	0.00	0.00	0.01	0.00	0.01	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00
	SD	0.00	0.00	0.01	0.00	0.00	0.01	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
Control	**Mean**	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
	SD	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00

Appendix 6. Complete list of attributes generated from Flash profile panel

1. Blue cheese / Blue cheese aroma / Blue cheesy
2. Cheesy / Cheesy
3. Cheddar / Cheddary / Cheddar cheese
4. Stilton
5. Musty-fusty
6. Butter / Buttery
7. Rancid
8. Mould / Mouldy
9. Mature
10. Milk - Milky
11. Creamy
12. Fruity
13. Sweet / Sweety
14. Nutty
15. Mushroom
16. Cream cheese
17. Sweet – sour
18. Yoghurty / creamy aroma
19. Fermented yogurt drink
20. Fermented milk
21. Alcohol – fermented smell
22. Sour cream
23. Sour
24. Sour and acidic
25. Acid / Acidic
26. Mild apricot
27. Feet like
28. Smelly
29. Damp / mouldy
30. Damp shoes
31. Smelly feet
32. Damp woodland
33. Wet damp washing
34. Old socks / Smelly socks
35. Farm
36. Piggy / farmyard
37. Tree bark
38. Cooked milk
39. Off milk
40. ‘Gone off’ food
41. Compost
42. Flowers
43. Burnt wood-bonfires
44. Chestnuts
45. Cabbage/green vegetable/grass
46. Malty
47. Caramel – tropical fruit
48. Crisp
49. Herbs
50. Hot – spicy odour
51. Fat
52. Old
53. Salty
54. Cooked - not cooked
55. Sharp – smooth
56. Ketone
57. Paint
58. Bread
59. Cheese rind – parmesan
60. Stale
61. Lingering
62. Ripe
63. Fresh
64. Metallic
65. Lactic
66. Mellow
67. Ripe
68. Earthy
69. Rubber like