Biographical sketch

Teresa Rapisarda was born in Catania, Italy, on November 04, 1976. In 1996, she received her high school education at the Scientific Liceo in S. G. La Punta, Catania. In 2001, Teresa Rapisarda graduated in Food Science and Technology.

Since June 2001 to nowadays, Ms. Rapisarda worked in Consorzio Ricerca Filiera Lattiero-Casearia (Ragusa, Italy) on flavours analyses with the supervision of Dr. Stefania Carpino, my guidance and support during my work experience.

Since January 2008 she started her Ph.D, working at CoRFiLac and collaborating with scientific partners of great international relevance. Ms. Rapisarda was visiting researcher at Agroscope Liebefeld Posieux (ALP), in Bern, for the acquisition of a new extraction technique (Purge and Trap System) from March to June 2005. She got a stage at the Food Science and Technology Department by Cornell University, Geneva (NY, USA) from July to August 2006 and from June to July 2009. There, she had the great opportunity to work on dairy products flavour profile with the supervision of Professor Terry E. Acree and the researcher E. Lavin.

In March 2008, she got a stage at SMart Nose laboratory in Neuchâtel (Switzerland) for the acquisition of new knowledge on SMart Nose system.

Since the beginning of her Ph.D. program, she specialized on studying flavour profile of cheeses by Gas Chromatography Olfactometry. In 2008, in collaboration with Prof. A. Cannas, Sassari University (Italy), she also made experience on studying “natural” volatile compounds contained in animal feeds for small ruminants.

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December 09/12/2010

Teresa

A Gianni
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List of Abbreviations

AD: Dehydrated Alfalfa

ADF: Acid Detergent Fiber

ADL: Acid Detergent Lignin

AED: Atomic Emission Detector

AEDA: Aroma Extract Dilution Analysis

BP: Beet Pulps

BW: Body Weight

CG: Corn Grains

Charm Analysis: Combined Hedonic Aroma Response Measurement

CM: Corn Middlings

CN: Canola Meal

CoRFiLaC: Consorzio Ricerca Filiera Lattiero Casearia

CP: Crude Protein

DFA: Discriminant Function Analysis

DM: Dry Matter

DMDS: Dimethyl Disulphide

DMI: Dry Matter Intake

DMS: Dimethyl Sulphide

DMTS: Dimethyl Trisulphide

EE: Ether Extract

FAA: Free Amino Acid

FD: Flavour Dilution Factor

FFA: Free Fatty Acid

FID: Flame Ionisation detector

FPD: Flame Photometric Detector

GC-MS: Gas Chromatography Mass Spectrometry

GC-O: Gas Chromatography Olfactometry

GL: Corn Gluten Meal

HP: Soybean Meal 49

Ile: Isoleucine

LAB: Lactic Acid Bacteria

Leu: Leucine

Met: Methionine

MSD: Mass Selective Detector

NDF: Neutral Detergent Fiber

NFC: Non-Fiber Carbohydrate

NPD: Nitrogen Phosphorus Detector

OAV: Odour Activity Value

OG: Oat Grains

OSV: Odour Spectrum Value

P&T: Purge and Trap

PAB: Propionic Acid Bacteria

PCA: Principal Components Analysis

PG: Pea Grains

Phe: Phenilalanine

RAS: Retronasal Aroma Simulator

SD: Steam Distillation

SDE: Simultaneous Distillation Extraction

SH: Soybean Hulls

SM: Sunflower Meal

SN: Soybean Meal 44

SPME: Solid Phase Microextraction

Tyr: Tyrosine

Val: Valine

VOC: Volatile Organic Compound

WB: Wheat Brans

WG: Wheat Grains

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Summary

The acquisition of knowledge of the chemicals responsible for the characteristic flavours of foods and other products has long been the aim of researchers. In the last fifty years, numerous studies have been performed in attempts to find correlations between sensory qualities and objective instrumental measurements. Although modern instrumental techniques have considerably accelerated the steps of flavour research, there still remains much to be discovered in the field of flavour biochemistry. The valuation of a flavour is an usual human experience due to the response to a complex mixture of stimuli, originating from chemical substances, the volatile compounds of food and beverages before and during consumption, on the sensitive nerves in the mouth (taste) and nose (smell). Aroma intensity and flavour are the most important properties for the consumers to define good quality foods.

Various procedures have been applied to the isolation of volatiles from complex matrices, such as, steam distillation and high-vacuum distillation techniques, direct extraction techniques, supercritical fluid extraction, simultaneous distillation extraction methods and headspace techniques. However, no ideal universal method exists for the simultaneous analysis of volatile (flavour) compounds, because each extraction technique presents advantages and at the same time disadvantages. Several instrumental analyses have been applied to isolate and identify the volatile compounds, and the most commonly used are gas chromatography and mass spectrometry. In order to study the odour active compounds, gas chromatography olfactometry has been used, where the final detector is represented by trained human nose. The other instrumental approach recently proposed is the so-called 'electronic' or 'artificial' nose (SMart Nose), which allows to investigate the fingerprint of the volatile compounds of a food matrix.

CoRFiLaC has invested part of its economic resource, equipping an “aroma” laboratory, provided by a Gas Chromatography/Mass Spectrometry/Olfactometry and a SMart Nose system to study, respectively, the aroma active compounds and the volatile fingerprint and of milk and Historical Sicilian Cheeses.

The present PhD dissertation explains and summarizes the aroma research conducted in CoRFiLaC lab, in collaboration with the Food Science and Technology Department, at Cornell University, Geneva (NY, USA) for the odours’ recognition training and analyses by Gas Chromatography/Mass Spectrometry/Olfactometry;

Zootechnical Science Department of Sassari University (Sassari, Italy) for the experimental work about the relationship between palatability tests and Volatile Organic Compounds content. Hereafter, a concise outline about the dissertation organization will follow.

The first chapter will provide an introductive overview on *flavour*, discussing on their pathways' origin, and the mechanism of human perception.

The second chapter will give a detailed description of physical principles, advantages and drawbacks of the most used extraction methods for the isolation of volatile compounds, especially used in dairy field.

In the third chapter, advanced tools for the analysis and identification of volatile compounds will be introduced.

Chapters four and five are related to several experiments I dealt with, along these three past years. Representative examples of studies have been used to demonstrate the applications of Gas Chromatography Olfactometry and SMart Nose System to determine the odour active compounds and the fingerprint of volatile composition in dairy products and animal feeds, respectively. Chapter four is focused on an experimental study of the volatile profile of Historical Sicilian Piacentinu Ennese cheese. Chapter five is the study of flavour impact on animal feeds for small ruminants and the relationship between the different volatile compounds and the palatability of these feeds.

The last subject has been recently studied, therefore, the results reported in this dissertation cannot be supported by bibliographical data.

PART I: FLAVOUR MEASUREMENTS

Chapter 1
Flavour volatile compounds

1.1 What is the flavour?

"it cannot be disputed that it is chemistry which will reveal the cause of basic elements of flavour" (Brillat-Savarin, 1825).

Flavour is a response to a complex mixture of stimuli primarily on the sense of smell (odour) and taste (Moncrieff, 1967). The odour of natural materials is due to the presence of volatile compounds that are detected in the nose, whereas the taste (i.e. sweetness, bitterness, sour, etc.) is detected in the mouth, due to non volatile compounds. The word "aroma" is best reserved for the smell of food before it is put in the mouth and odour for the retronasal smell of food.

The chemicals that cause flavour in raw matrix are still largely unknown, and there are indications that only a small fraction of the large number of volatile compounds occurring in food actually contributes to the odour and aroma (Guth and Grosch, 1999). Therefore, it is necessary to make distinction between odour active compounds and the whole range of volatiles present in a raw matrix. However, the number of chemicals isolated and identified in raw matrix that contribute to flavour increases rapidly (Van Straten and Maarse, 1983) and the correlation between chemical composition and sensory attributes is coming better understood. Concerning the nature of the flavour constituents, it is not completely clear if all flavours are composed of the same chemical compounds present in different proportions or if the aroma profile depends on specific chemical entities (Rohan, 1970). Causing the heterogeneous flavour characteristics for each food, it is probably that both the two concepts are correct.

It is generally possible to distinguish the "nature" of flavours, them on their biochemical origin. It is, also, possible to classify them based on their biogenetic pathways from known precursors or by processing imposed on the initial materials. The generation of flavour compounds might be realized by normal plant metabolism during its growth, microbiological fermentations and their linked enzymatic

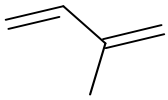
reactions, non-enzymatic reactions, such as heat treatments, and oxidative reactions that lead to flavour degradation.

Due to the wide field of flavour's origin and considering the general aim of this dissertation, it necessary to focus the attention on the flavour's processes occurring in plants and dairy products.

1.1.1 Origin of flavour in plants

The aromatic profiles of plants are due to a complex mixture of organic compounds that take place during the normal growth cycle. The synthesis of the aromatic compounds is influenced by the environment conditions, such as the territory, the season and the soil conditions in which the plants growth, and their stage of maturity. The essential oils are the most important flavour constituents which give the plant its distinctive and typical odour profile. The principal odorants of essential oils are represented by terpenes, derived from secondary plant metabolites, produced to attract specific insects for pollination or otherwise to expel certain animals employing these plants as food.

The term terpenes originates from turpentine (lat. *balsamum terebinthinae*); they are hydrocarbons derived from several isoprene (C5H8) units (up to 8) through condensation, with or without subsequent cyclization steps (Wallach, 1887).



isoprene

An important subclass of terpenes is represented by the terpenoids, derived from terpenes from hydroxylations, oxidations, dehydrogenations reactions. Depending on the number of isoprene (C5H8) units, it is possible to distinguish the terpenes as monoterpenes/-oids (C10, 2 isoprene units), sesquiterpenes/-oids (C15, 3 isoprene units), diterpenes/-oids (C20, 4 isoprene units), triterpenes/-oids (C30, 6 isoprene units), tetraterpenes/-oids (C40, 8 isoprene units).

Several studies performed on spontaneous plants present in the mountain area demonstrated that the pasture is rich in terpenes, in particular in monoterpenes (β-myrcene, β-limonene, β-terpinene, α and β-pinene) (Mariaca et al., 2001). The terpenes have an important chemical property represented by chirality and therefore different enantiomers of the same molecule may differ in taste, aroma or bioactivity.

Several studies performed on the effect of the spontaneous plants on the flavour profile of dairy products confirmed that the terpenes present in pastures influence the chemical composition of cheeses (Dumont et al., 1981; Bosset et al., 1999). These chemical compounds, especially mono and sesquiterpenes, are transferred from pasture plants, grazed by the animals, to milk and cheese, as reported by several authors (Bugaud et al., 2001a, 2001b, 2001c; deFrutos et al., 1991; Mariaca et al., 1997).

Carpino et al. (2004) gave a contribute to the characterization of Hyblean native species, demonstrating the presence of 40 odour compounds belonging to the aldehyde, ester, alkyl pyrazine, hydrocarbon, ketone and lactone, besides the terpenoid chemical classes.

When fresh pasture plants are consumed by cows, the damaged plants quickly activate the lipoxygenase system (Galliard and Chan, 1980; Belitz and Grosch, 1986; Wu and Robinson, 1999) that begins to breakdown carotenoids and lipids into a range of important volatile aroma compounds (Wache` et al., 2002) and non-volatile compounds that can have important sensory impacts in food systems. The carotenoids, lipids, and related degradation products of these compounds are consumed by the cow, and then they are taken up at the intestinal mucosa, incorporated in chylomicra, which are transferred through the lymphatic system and then to the bloodstream (Noble, 1981; Parodi, 1996). A second possible mechanism of uptake (Dougherty et al., 1962) is from volatile plant odours that are inhaled by the cow during the consumption of the forage. These compounds could pass very quickly through the bloodstream into the milk. The terpene composition in dairy products depends on the type of pasture. In detail, pastures rich in dicotyledons, in particular Apiaceae, contain a greater quantity and a wider variety of terpenes compared to pastures rich in Gramineae (Bugaud et al., 2001a).

Other studies showed the relations between the odour-active compounds present in fresh pasture plants with their presence in cheese, demonstrating a difference in odour-active compounds in cheese produced from cows eating only TMR versus those consuming fresh pasture as part of their diet (Carpino et al., 2004). The terpene composition in dairy products depends on the clime, the type of pasture and especially, on the territory. For this reason, these compounds can be used as valuable biomarkers for dairy products with Protected Designation of Origin (Buchin et al., 2002).

1.1.2 Origin of flavour by microbiological fermentation and enzymatic reactions in dairy products

There has been extensive research in the recent decades on dairy products flavour and agents responsible for the production of sapid compounds confirming that the flavour of most dairy products results from the combination of a large number of flavourful compounds present in the correct ratios and concentrations (Kosikowski et al., 1957; Bosset and Gauch, 1993;). The chemistry of the flavours in dairy products originates in milk, a complex system where reactions take place, modifying the flavour profile of dairy products (Kinsella, 1975).

Kosikowski (1957) suggested that natural milk enzymes, rennin, bacteria and microbial enzymes are involved in the formation of the characteristic flavour profiles of dairy products.

The principal biochemical ways for the formation of flavour compounds in dairy products, expecially in cheese, are the glycolisis, the lipolysis and the proteolysis processes. In detail, lipolysis and proteolysis during cheese ripening are the primary drivers.

These biochemical reactions might contribute in different ways to the formation of flavours or off-flavours in cheeses, depending on the type of milk, microflora (starter or no starter) and ripening conditions.

1.1.2.1 Glycolisis

Several studies have been conducted on the glycolysis in different types of cheeses. Steffen et al. (1987) and Turned et al. (1983) described the fermentation of lactose and lactate, in Swiss-type cheeses, carried out by Propionibacterium sp., assuming that propionate, and acetate as primary fermentation products are responsible of the typical odour and taste of these cheeses.

In Camembert and Brie (Karahadian et al., 1987; Lenoir, 1984; Noomen, 1983), the metabolism of lactate by the moulds lead to deacidification of the media that allows the growth of coryneform bacteria which are responsible for the characteristic flavour of these mould-ripened cheeses.

However, the metabolism of lactate or glucose could produce some off-flavours: Clostridium sp. produce two bad odour molecules, butyric acid and H_2. Another small source in the milk for glycolysis process is citrate.

The principal flavour compounds produced by citrate (Cogan, 1985, 1993; Fox et al., 1990; Hugenholtz, 1993), metabolized by mesophilic lactobacilli, are acetate, diacetyl, acetoin and 2,3-butanediol that contribute to good flavour profile in some cheeses varieties (Dimos et al., 1996).

1.1.2.2 Lipolysis

The lipolysis is the breakdown of lipids through the hydrolysis of triglycerides into free fatty acids and glycerol, mono or diglycerides. This process is essential to flavour development in many cheese varieties. The lipolysis is carried out by indigenous lipases that might derive from the milk, rennet, starter and non-starter bacteria. Therefore, milk fat is the starting point for the development of flavours in milk and cheeses. Foda et al. (1974), Ohren et al. (1967), Wijesundera et al. (1998) demonstrated that Cheddar and other cheeses from whole milk present a richer flavour profile than cheeses from skim milk. Milk fat contains high concentrations of short- and intermediate-chain fatty acids which, when released by lipolysis, contribute directly to cheese flavour. Free fatty acids (FFA) play also an important role as precursor of other flavour compounds. An example is given by the flavour profile of blue mould cheeses that is mainly represented by 2-methyl ketones. These odour compounds are due to the metabolism of the FFA by *P. roqueforti* (Gripon, 1993; McSweeney, et al., 1997; Molimard and Spinnler, 1996). Others chemical classes derived from the lipolysis are represented by lactones and esters. The first ones are formed by intramolecular esterification of hydroxyl fatty acids. The principal lactones that might be found in cheeses are γ- and δ-lactones. In rich-fat cheeses, the δ-decalactone increased to a maximum at about 14 weeks of production and then decreased, whereas in low-fat cheeses remained constant during ripening (Urbach, 1993). The esters are the consequences of the reaction between FFA and alcohols, giving to the cheeses a high flavour impact. Fourteen different esters were found in Emmental cheese (Bosset et al., 1995, 1997; Imhof and Bosset, 1994); esters were also found in Parmigiano-Reggiano as important contributors to its flavour profile (Meinhart and Schreier, 1986).

1.1.2.3 Proteolysis

Proteolysis is a more complex biochemical process than lipolysis. It is considered as an index of cheese maturity and quality and gives a direct contribution to flavours and, sometimes, off-flavours generation.

Similarly to lipolysis, the proteolysis is also catalyzed by several enzymes from the coagulant (i.e. chymosina, pepsina etc), the milk (i.e. plasmin, cathepsin D, etc), starter and non-starter microflora, and exogenous proteinases.

The beginning of the proteolysis is due to the coagulant enzymes that hydrolyze the caseins to generate large and intermediate peptides, after being degraded by the microflora enzymes. The final products of the proteolysis reaction are free amino acids (FAA), indices of ripening (Aston and Douglas, 1983; McSweeney and Fox, 1997) and several amino acids, both representing the precursors of flavour compounds (Urbach, 1995).

The catabolism of amino acids might also produce amines pyrazines, ammonia, keto acids, aldehydes and sulphur compounds.

Decarboxylation process of amino acids (i.e. tyramine from Tyrosine (Tyr) amino acid) explains the formation of most amines in cheese, like acetamine, propionamide, butyramide, isobutyramide and isovaleramide found in Cheddar, Emmental and Manchego cheeses (Ney, 1981). Several pyrazines were found in Swiss Gruyère (Liardon et al. 1982), Emmental (Sloot and Hofman, 1975) and Parmesan cheeses (Meinhart and Schreier, 1986). They are likely produced by the microorganisms.

Deamination of amino acids leads to the production of ammonia and α-keto-acids. α-keto-3-methyl butanoic and α-keto-3-methyl pentanoic acids have an intense cheese-like odour notes, but the concentration of these compounds might change widely among the different types of cheeses (Muller et al., 1971).

Transamination of amino acids and decarboxylation of the intermediate compound (imide) by Strecker reaction (Barbieri et al., 1994), leads to aldehydes compounds origin. Phenylacetaldehyde, isobutanal, 3-methylbutanal and methional might originate from Phenilalanine (Phe), Leucine (Leu)/Isoleucine (Ile), Valine (Val) and Methionine (Met), respectively (Adda et al., 1982). Benzaldehyde might be produced by the α-oxidation of phenylacetaldehyde or from the β-oxidation of cinnamic acid (Casey and Dobb, 1992). The aldehydes can not accumulate during cheese ripening because they are fast converted in the corresponding acids (Dunn and Lindsay, 1985; Lemieux and Simard, 1992).

The catabolism of the sulphur amino acids produces sulphur compounds, like dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS), that give a great contribution to the flavour profile of several cheeses.

Dimethyl sulphide is produced by propion acid bacteria from Metionine. DMS was found as a component of Swiss cheese flavour (Adda, 1982) and in Cheddar cheese (Aston and Douglas, 1983).

Dimethyl disulphide and DMTS have been identified in Parmesan (Barbieri et al., 1994) Cheddar (Barlow et al., 1989) cheeses, likely due to the action of starter and non-starter bacteria and their enzymes.

1.1.3 Origin of flavours by non-enzymatic reaction: heat treatments

It is well known that, in general, the flavour of many raw food changes during several processes, such as maturation, ripening and heat treatments (Rohan, 1970) and that these changes derive from precursors, many of which are odourless and tasteless. The heat treatment of a general food leads to chemical transformations with production of new flavour profile and brown color. These processes are known as non-enzymatic browing reactions and may be either due to caramelization of sugars or to the Maillard reaction between reducing sugars, amino acids, amines, peptides and proteins which lead to the production of colored melanoidins as well as a complex mixture of flavourful compounds. The caramelization reaction produces several compounds such as ketones, furanes, acetals and also change the colour of the products that become brown (Ohloff, 1972). Anyway, caramel flavours are formed when the sugars are heated to 150°C.

Milk is a complex entity and contains many components that may be precursors of chemicals off-flavour (proteins, fat, lactose, etc.). Reactions occurring between milk components during the heat treatments or storage of milk products result in off-flavours belonging to several chemical class, like ketone, lactone, aldehyde, furan, alcohol, acid and sulfur compounds.

1.1.3.1 Ketones

Among the ketone class, diketones, cyclic ketones, and methyl ketones are formed in heat processed milk and they are the major contributors to their cooked and stale flavours (Adda, 1986; Van Straten and Maarse, 1983).

2,3-butanedione (diacetyl) and 2,3-pentanedione diketones contributed significantly to the heated, burnt, and fermented notes in heated milk (Shibamoto, 1980). Diacetyl could be formed by α-dicarbonyl, an intermediate compound formed during nonenzymatic browning reactions. Bennett et al. (1965) and Scanlan et al. (1968) identified a small amount of diacetyl (3-5 ppb) in raw milk, but at a concentration below its flavour threshold in milk (10-19 ppb).

Cyclopentanone and 2-methyl-tetrahydrofuran-3-one, both cyclic ketones, also contribute to the heated, burnt, and fermented notes in heated milk (Shibamoto, 1980). However, the mechanism producing these compounds are formed in heated milk is not well documented.

Methyl ketones were not found in raw milk, but they developed during storage of heat-processed milk. Among methyl ketones, 2-heptanone, 2-nonanone, and 2-undecanone, were found in UHT milk, representing the most powerful odorants in this treated milk (Badings et al., 1981; Moio et al., 1994). Methyl ketones are formed by the thermal decarboxylation of β-keto acids (Badings et al., 1981; Schwartz et al., 1966), that are biosynthesized in the bovine mammary gland from acetate (Lawrence and Hawke 1966).

1.1.3.2 Lactones

Lactones are generally formed in dairy products from the thermal breakdown of γ- and δ-hydroxyacids and they are not identified in raw milk (Dimick et al., 1969). In fact, latones start to generate when the milk undergoes heat treatments.

δ-decalactone, γ-dodecalactone, 5-methyl-2-(5H)-furanone, 2-butenoic acid-γ-lactone, and α-methyl-γ-butyro-lactones were identified in heated milk by Shibamoto, (1980); Dimick et al. (1969); Keeney and Patton (1956). Lactones contribute to the formation of milky, buttery, coconut-like flavour notes in milk. The presence of lactones may contribute to the stale flavour of heated milk, but to a lesser extent than ketones.

1.1.3.3 Aldehydes

During the heat treatments, the reaction between reducing sugars, amino acids, amines, peptides and proteins leads to the well known Maillard reaction. The first steps of this browning non-enzimatic reaction are known as Strecker degradation that leads to the formation of several odorant aldehydes by amino acid-specific catabolism: phenylacetaldehyde is originated by Phe, isobutanal by Leu/Ile, 3-methylbutanal and methional by Val and Met, respectively (Adda et al., 1982). Benzaldehyde is also formed during the caramelization of sugars (Hodge, 1967).

1.1.3.4 Furans

Similarly to lactone compounds, furans are not present in raw milk and they appear in milk heated above 90°C (Shibamoto, 1980). Burton (1988) demonstrated that furfural and hydroxymethylfurfural are major volatile constituents of heated milk. The mechanism of generation of the furan derivatives was explained by Ferretti and Flanagan (1971). They could be formed in heated milk by the lactose-casein browning system, that occurs from the casein catalyzed degradation of at high temperatures (>80°C).

1.1.3.5 Alcohols

The flavour of heated milk is partly due to the formation of acetol, with its characteristic notes of yougurt. This alcohol is presents in low concentration in raw milk and increases at temperatures above 90°C (Shibamoto, 1980), as the product of carbohydrate fragmentation or degradation during non-enzymatic browning reactions (Shibamoto, 1980; Hodge, 1967; Heyns et al., 1966; Ferretti and Flanagan, 1971; Heyns and Klier, 1968; Shaw et al., 1968). The taste of acetol is characterized by different descriptors depending upon the medium used in testing. In aqueous solutions, acetol is described as sweet and roasted. In contrast, acetol is described as yogurt-like notes in emulsions. Other sugar alcohol compounds, like maltol and iso-maltol, were found in heated milk, but these compounds are usually present at subthreshold concentrations and do not contribute significantly to heated milk flavour (Badings et al., 1981; Shibamoto, 1980).

1.1.3.6 Acids

When milk is exposed to temperatures above 100°C the concentrations of acetic, butyric, hexanoic, octanoic, and decanoic acids increase (Shibamoto, 1980). Butyric and hexanoic acids are characterized by dirty sock and sweaty-like odours and contribute to the rancid flavour of heated milk.

1.1.3.7 Sulfur Containing Compounds

Studies performed on the oxidative stability of milk (Shipe, 1980) showed that sulfhydryl groups activated by heating may contribute to the off-flavours and to oxidative stability of milk. Hydrogen sulfide is formed indirectly by the release of free sulfhydryl groups arising from the denaturation of β-lactoglobulin (Hutton and Patton, 1952). The concentration of hydrogen sulfide in milk increases linearly with the intensity of heating (Badings et al., 1981). Dimethyl sulfide is another important constituent of raw milk and is also found at lower concentrations in heated milk. Despite that, dimethylsulfone is found in heated milk at higher concentrations. Shibamoto (1980) suggests that dimethyl sulfide is oxidized to dimethylsulfone via dimethyl sulfoxide as the intermediate.

Besides, sulphur-amino acids bring to the formation of highly odour thioaldehydes: methional, characterized by cooked potato notes, is produced by Methionine (Self, 1967).

1.1.4 Origin of flavours by non-enzymatic reaction: lipids oxidations, in milk and dairy products.

Autoxidation products are formed from unsaturated fatty acids by non-enzymatic autocatalytic oxidation reaction resulting in the formation of hydroperoxides (Badings, 1991). The hydroperoxides are very unstable flavourless compounds and induce to the production of secondary oxidation products such as aldehydes and ketones. Flavours and odours developed from the autoxidation of lipids make a food, especially milk and dairy products, unpalatable and described as rancid or oxidized.

The chemistry of lipid oxidation is complex and has been the subject of several researches (Shultz et al., 1962). The main reaction responsible is between polyenoic fatty acids of lipids and the atmospheric oxygen. The lipid oxidation involve free-

radical (RO•, ROO•) chain reactions until forming hydroperoxides and finally the formation of highly odours reaction products, like the aldehydes.

Aliphatic aldehydes are the most important breakdown products of hydroperoxides because they are major contributors of unpleasant odours and flavours in food products. Due to their prevalence in milk products (Forss, 1981), the most important precursors of the aldehydes are represented by the polyunsaturated acids: oleic, linoleic, linolenic, and arachidonic.

Aldehydes resulting from autoxidation may undergo further reaction and contribute to the flavour of dairy products: for example, nonanal might be oxidized to the acid. Other aldehydes may arise through a secondary reaction such as isomerization or from other isomeric polyunsaturated fatty acids present in trace amounts. Aldehydes derived from amino acids or lipids may react with themselves or with other carbonyl compounds by aldol condensation to a wide range of compounds (Forss, 1981).

Hydroperoxides are also formed by photo-induced oxidation of fatty acids and are the principle source of off-flavours developed by lipid oxidation.

When the milk is exposed to sunlight, the burnt or cooked cabbage flavours develope. These flavoura are largely attributed to the concentration of methional present in the matrix (Adda, 1986; Patton, 1954; Allen and Parks 1975; Samuelsson, 1962). The methional, with dimethyl disulfide, and many other aldehyde and sulfur compounds, is a product of Strecker degradation in the presence of singlet-oxygen, starting by amino acid Methionine (Forss, 1979; White and White, 1995; Parks, 1965).

1.2 How do chemical flavours work?

Flavour is important for human health and stimulates the appetite, preparing the digestive human process. It is also one of factors, along with others such as health, goodness, likeable, texture, color, that determines food quality and permits to find the presence of anti-nutrients preventing food alterations.

Flavour is defined as the sensation arising from integration or interplay of signals produced as a consequence of sensing chemical substances by smell (figure 1.1), taste and irritation stimuli from food and beverage (Laing and Jinks, 1996).

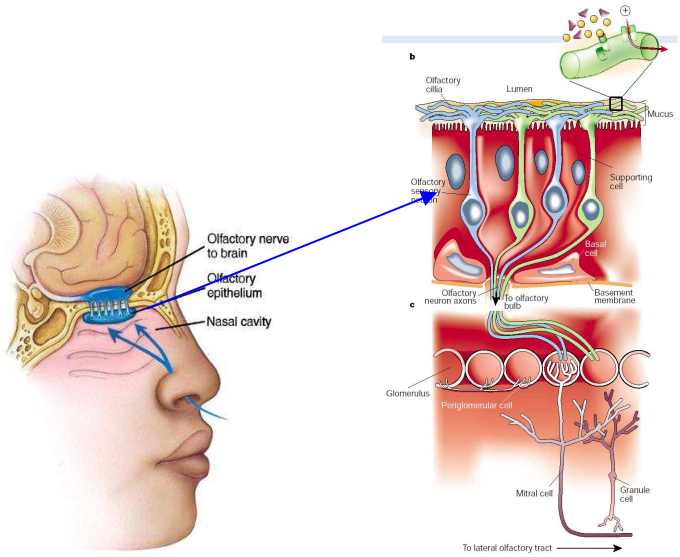


Figure 1.1 – Location of the sense of smell.

The flavour perception mechanism is not completely known, but for simplicity the flavour reaction can be resumed as follow:

$$\text{Flavour-active chemical + human} \rightarrow \text{flavour response}$$

To produce a flavour response, a chemical compound must have vapor pressure to enable it to vaporize and be carried in the air stream, a limited molecular weight (< 300), soluble in water or in lipids and must have odour-active structure containing functional or unsaturated carbon-carbon bonds (Moncrieff, 1967).

The sensory mechanisms by means of which the flavours are perceived and recognized is not completely known. It is a very specialist field of physiology-psychology, (awareness, emotion, memory, and cognition) and structural chemistry. Several theories on perception mechanisms have been developed, but, as part of the total process, the odour perception mechanisms are very complex and cannot easily be explained in detail. However, it is possible to affirm that flavour reactions (Acree, 1993):

- come in different qualities or modalities that show structural specificity toward stimulus;
- responses to mixtures of stimuli are characterized by inhibition and suppression and not synergy;
- show a sigmoid dose-response behavior.

Human chemosensory responses are studied with bioassays, defined by Finney (1978) as follows: “a biological assay (bioassay) is an experiment for estimating the nature, constitution, or potency of a material (or of a process) by means of the reaction that follows its application to living matter”.

The figure 1.2 shows a typical curve of dose-response behavior (Marin et al., 1991), which is used to quantitatively describe the potency of a flavour chemical.

The abscissa axis is a plot of amount of chemical compound used in a test for flavour activity. The ordinate axis is given by flavour response assigned by human behavior undergone to a proper psychological test.

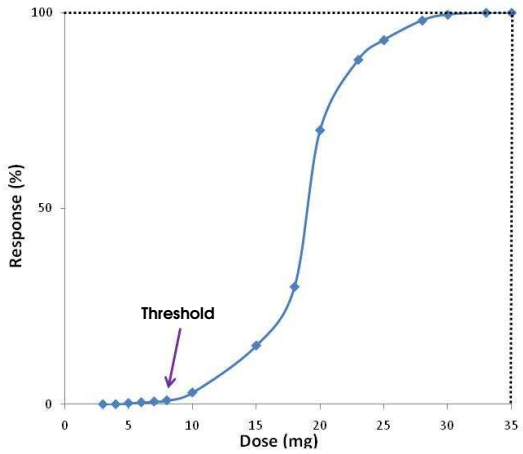


Figure 1.2 – Typical dose-response curve.

What does flavour activity mean? The flavour activity is measured as follows:

$$\text{flavour activity} = [1/(\text{threshold})] \times [\text{concentration}]$$

where the threshold is the lowest concentration of a compound in a matrix which can be perceived and below which a matrix has no more flavour; the concentration factor is the amount of the compound detected in a matrix.

In this way, a compound at low concentration has a good perception if its inverse threshold factor is high.

Flavour compounds are often present in trace quantity making isolation and identification very difficult. The development of several extraction methods and efficient analytical tools, such as gas chromatography (GC) combined with mass spectrometer (MS) and olfactometry detectors, has greatly contributed to progress in this area, gaving the “name” of the identified chemical compound, and information about its sensorial property. This object will be discussed in more detail in the following chapter.

1.3 Conclusions

Flavour is a response to a complex mixture of stimuli primarily on the sense of smell and taste due to the presence of volatile and non-volatile compounds that are detected in the nose, and in the mouth, respectively.

Flavours are generated by enzymatic and non-enzymatic reactions detectable through not yet known mechanisms. Sometimes these reactions might lead to produce off-flavours making unpalatable the product subjected to flavour degradations. Several theories on perception mechanisms have been developed, but none of them explain in details what happens. The flavour response depends on the balance between the characteristics of the organic compounds and their combined effect upon the receptors, both in mouth and nose.

Chapter 2
Techniques in flavour research

2.1 Extraction methods in flavour research

Aroma intensity and flavour are among the most important properties for consumers to define good quality foods. The flavour evaluationis an usual human experience due to the response to a complex mixture of stimuli, originating from chemical substances, the volatile compounds, of food and beverages, before and during consumption, on the sensitive nerves in the mouth (taste) and nose (smell).

The acquisition of knowledge of the chemical compounds responsible for the characteristic flavours of foods and other materials has been from early 60s the aim of further to forty researchers (Day, 1967; Schwartz et al, 1968; Forss, 1969, 1971, 1972; Ney, 1973; Lacrampe et al, 1975; Jennings and Filsoof, 1977; Bemelmans, 1979; Benkler and Reineccius, 1980; Lamparsky and Klimes, 1981 Cronin, 1982; Manning and Priee, 1983; Reineccius and Anandaraman, 1984; Schreier and Idstein, 1985; Grosch, 1990; Xanthopoulos et al, 1994; Urbach, 1996). Although modern instrumental techniques have considerably accelerated the steps of flavour research, there still remains much to be investigated in the field of flavour biochemistry. In the last fifty years, numerous studies have been carried out in order to find correlations between sensory qualities and objective instrumental measurements. Flavour measurements have enabled the discovery and quantification of many of the key taste and aroma compounds known today in food.

In general, volatile compounds are lipophilic and dissolved in the original fatty phase, so that the aim of the extraction is to isolate them from the complex matrix. The detection of flavour compounds greatly depends on their concentration and vapour pressure, as well as on the temperature and matrix of the food product. Various procedures have been applied to the isolation of volatiles from complex matrices. In matrices rich in fats, it is particularly difficult to separate smell-active chemicals from the multiple phases (i.e. whole milk and cheese), especially if the aim is to obtain an extract, fraction or aliquot that is both enriched and representative. Due to the sensitivity of some compounds to heat and/or oxygen, precaution have to be taken during the preparation of the sample and the isolation of

the volatiles to ensure that they remain unchanged and to minimise their losses (Mariaca and Bosset, 1997).

2.2 Extraction techniques of volatile compounds in dairy and animal feeding products.

The majority of techniques applied in flavour research are basically analytical methods able to isolate and study the volatile and non-volatile compounds for a subsequent identification. Although the remarkable progress and development in flavour field, there is no ideal universal method for the simultaneous analysis of volatile (flavour) compounds. It is well known that very small amounts of some material can have deeply affect taste and smell sensations. Therefore, it is obvious that only small concentrations of effective compounds are present in foods. To study these compounds, a big of amount of matrix must be extracted, and the extract need to be concentrated. Several methods have been used for the isolation of the volatile compounds of foods. The most commonly used methods, techniques and equipments for instrumental analysis of volatile (flavour) compounds in dairy products and animal feeding products are mainly consisting in steam distillation and high-vacuum distillation techniques, direct extraction techniques, supercritical fluid extraction, simultaneous (steam) distillation extraction methods (SDE) and headspace techniques.

Steam distillation at atmospheric pressure is the most common and favorite method of isolation and recovery of volatiles compounds, whereas the high vacuum distillation is used to allow the extraction of high weight molecules, producing small volumes of concentrated aqueous extracts. Both distillation methods require precautions to limit thermal degradation or generation of artifacts. Steam distillation technique has been applied to cheese varieties such as Swiss Gruyere cheese (Rychlik and Bosset, 2001). High vacuum distillation has been largely applied to cheese varieties such as Grana Padano cheese (Moio and Addeo, 1998), Gorgonzola cheese (Moio, et al. 2000), Cheddar cheese (O`Riordan et al., 2001), and smear-ripened cheese (Lecanu et al., 2002).

The direct extraction technique is a rapid and efficient method obtained by liquid/liquid or liquid/solid partitioning. However, this thechnique is not recommendable for samples with a very low fat content. Besides, the concentration step might cause the loss of volatile components, when the solvent has a relatively high boiling point. This technique has been, also, applied by Moio (1998, 2000) to study the Grana Padano cheese aroma and the Odour-impact compounds of Gorgonzola cheese.

Supercritical fluid extraction method is a rapid and efficient technique that permits to analyse trace components. Using carbon dioxide as solvent, this method avoids the problems of concentrating the extracts. However, similary to direct extraction technique, this thechnique is not efficient for samples with a very low fat content.

Simultaneous (steam) distillation extraction methods (SDE) uses only very low-boiling solvents such as pentane for the concentration of the aroma volatiles. Low temperature and low pressure prevent thermal generation of artifacts. This technique has been applied to cheese varieties such as Manchego cheese (Gomez-Ruiz et al., 2002).

Headspace techniques have been distinguished in static and dynamic form. The static solid phase microextraction (SPME) (Shooter et al., 1999), is the most known extraction technique useful for extraction and concentration of analyses either by submersion in a liquid phase or by exposure to a gaseous phase. Dynamic headspace methods have been developed to obtain more concentrated extracts. The volatile components of the gas phase are continuously removed and concentrated in a cold trap or desorbed onto an inert support, and finally recovered by thermal desorption (Mariaca and Bosset., 1997). Among the dynamic headspace techniques, the purge and trap system (Povolo and Contarini, 2003), is one of the most efficient concentration system of aroma compounds, that can be used for long investigations, giving reproducible results. Purge and trap has been largely applied to cheese varieties such as Cheddar cheese (O'Riordan, P.J. et al., 2001) and Manchego cheese (Gomez-Ruiz, J.A. et al. 2002). The Retronasal Aroma Simulator (RAS) implemented by flavour chemists of Cornell University, is a dynamic headspace extraction used to isolate the retronasal aroma developed during eating. This system has been used for the extraction of the retronasal volatile compounds from white and red wine (Genovese et al., 2009) and from Piacentinu Ennese cheese (Horne et al., 2005).

Steam distillation (SD), SPME, P&T and RAS techniques, the most used techniques in my experimental work, will be discussed in detail in the next paragraphs.

2.2.1 Steam Distillation technique (SD)

Steam distillation extraction technique, at atmospheric pressure, is the most common method for isolating volatiles. Many organic compounds tend to decompose at high temperatures, so the separation by normal distillation is not a good solution, and, for this reason, water or stream is introduced into the distillation apparatus (figure 2.1). By adding water or steam, the boiling points of the compounds are depressed, allowing them to evaporate at lower temperatures than their appreciable deterioration temperature. In most cases the volatiles are collected in a dilute solution and must therefore be subject to further concentration steps such as solvent extraction or adsorption.

In 1997, Carpino implemented a steam distillation apparatus (figure 2.1) for the isolation of aroma compounds in native Sicilian pastures. Steam distillation technique enables to isolate volatile substances like acetaldehyde, ethanol, diacetyl and acetoin in dairy products (Xanthopoulos et al., 1994), but presents several problems: highly volatile compounds may have low recoveries and/or may be masked by the chromatographic peak of the solvent. The solvent may give artifacts formation and it may, also, cause the thermal decomposition of compounds. Besides, big amount of sample is needed. This technique has been largely applied to several cheese varieties: Emmental (Vâmos-Vigyazô and Kiss-Kutz, 1974), Limburger (Parliment et al., 1982), Fontina (Ney and Wirotama, 1978), Cheddar (Vandeweghe and Reineccius, 1990), Swiss Gruyere (Rychlik and Bosset, 2001), and Ragusano (Carpino et al, 2004) as well as to milk (Jeon et al., 1978; Alm, 1982) and other milk products: butter (Stark et al., 1978), ghee (Jain and Singhal, 1969), butter oil (Urbach et al., 1972) and milk powder (Ferretti and Flanagan, 1972). Steam distillation apparatus has been used for the isolation and characterization of aroma profile of spontaneous essences of Hyblean pasture (Carpino et al., 2004).

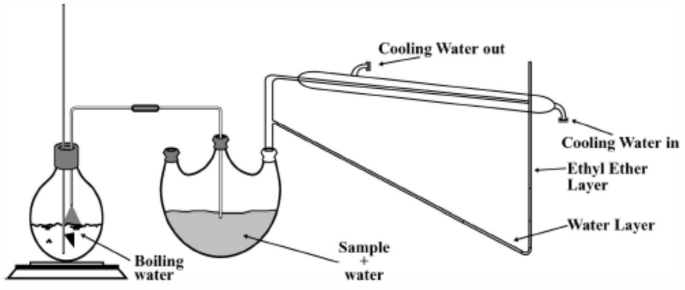


Figure 2.1 - Steam Distillation apparatus.

2.2.2 Headspace Techniques

Headspace techniques are based on adsorption and absorption principles for the extraction of volatiles released from the condensed phase. The adsorption is a physical adhesion of vapour or dissolved matter to the surface of a solid gas or liquid solute accumulates on the surface of a solid or a liquid (adsorbent), forming a film of molecules or atoms (the adsorbate). The absorption is a physical incorporation of one substance into or through another of a different state (e.g., liquids in solids, gases in liquids).

Several sampling methods have been developed for sorption-based analyte extraction following two basic principles: the static and the dynamic extraction modes. In the static technique (i.e. solid phase micro-extraction), the entire adsorbent volume is exposed to the sample in a single step and the equilibrium between the adsorbent and the sample is established after a certain time and no more analyte will then be extracted by the adsorbent. The amount of extracted material is dependent on its original concentration in the sample and the principle can therefore be used for quantitative analysis. In their classical static form, this technique is restricted to the most volatile components.

To obtain more concentrated extracts, dynamic headspace methods (i.e. purge and trap) have been developed. By dynamic extraction mode, the entire adsorbent volume is not immediately brought into contact with the entire sample, and the volatile components of the gas phase are continuously removed and concentrated in a cold trap or desorbed onto an inert support, and finally recovered either by thermal desorption (Reineccius and Anandaraman, 1984; Mariaca and Bosset, 1997).

The detection of flavour compounds greatly depends on their concentration and vapor pressure, as well as on the temperature and matrix of the food product. In general, the amount of volatile compound extracted, n_a, from a sample volume V_s with an initial analyte concentration C_0 into a sorbent volume V_f might be calculated according to the following equation (Zhang and Pawliszyn, 1995):

$$n_a = \frac{K_{fs}\, V_f \quad C_0\, V_s}{K_{fs}\; V_f \; + \; V_s}$$

K_{fs} is the partition coefficient of the volatile compounds between the sorbent (fiber) and the sample ($[analyte]_f/[analyte]_s$). When $K_{fs}\ V_f >> V_s$ all the analytes are transferred from the sample into the sorbent phase and the extraction is exhaustive, whereby $n_a = C_0V_s$.

2.2.2.1 Solid Phase Micro-Extraction (SPME)

Solid Phase Micro-Extraction (SPME) was developed in the early 1990s at the University of Waterloo by Dr. Pawliszyn's group. Solid Phase Micro-Extraction is a sample preparation technique based on absorption, which is useful for extraction and concentration of analyses either by submersion in a liquid phase or by exposure to a gaseous phase. It is a fast and simple technique of extraction that eliminates the solvents, reduces analysis costs and the time required for sample preparation. Solid Phase Micro-Extraction (Woolley and Mani, 1994) has been commercially available since 1993 and now is available with various adsorbent materials and various coating liquid (polymer) or solid (sorbent) thicknesses.

Aroma molecules distribute themselves so that their chemical potential is in equilibrium in all phases, depending on the affinity of molecules for each phase. The partitioning of aroma between two phases is described as the ratio of its concentrations in the phases. The first equilibrium of the volatile compounds is established between the sample and the headspace, and is represented by the K_{gp} partition coefficient

$$K_{gp} = \frac{C_g}{C_p}$$

where C_g and C_p are the concentration in the headspace and sample phases, respectively. This ratio is important because aroma perception occurs for those molecules that are in the headspace, whereas those entrapped in the sample phases will not. The equation describes the thermodynamics of flavour binding: a food product with high affinity for the volatile molecules will have a low K_{gp} and consequently few aroma molecules will be smelled (Ghosh and Coupland, 2007). K_{gp} can be affected by temperature as described in Meynier et al. (2003).

The second equilibrium is based on the diffusion and partitioning of the analytes (Zhang and Pawliszyn, 1995), volatile and non-volatile compounds, from the liquid or gas phase of sample to the fiber (figure 2.2). After the SPME extraction, the analytes are transferred to the injection port of separating instruments, such as a Gas Chromatograph, to allow the analysis.

In conclusion, the SPME technique presents many advantages (Zhang and Pawliszyn, 1994; Deibler et al.,1999), such as, the simplicity of use; cost-saving for solvents and matrix to analyze; the application on various matrices such as air,

water, soils and very complex matrices, like foods. Moreover, the using of fiber chemically inert and very stable at high temperature; the direct transferring of adsorbed analytes into a gas chromatograph injector. Solid Phase Micro-Extraction is also a solventless technique, non artifact forming method and requires a minimal manipulation of the sample (Fernandez-Garcia, 1996). The Solid Phase Micro-Extraction technique, on the other side, does not give reproducible results over a long period of time, due to the short age of the fibers. Another critical point for the SPME is the small volume of the sorbent, usually less than 1 μl, that allows an exhaustive extraction only for analytes with a very high partition coefficient (e.g. 100% extraction of analytes from a 10 ml sample using a 1 μl sorbent coating requires a partition coefficient of 105).

This method has been used for the extraction of volatile compounds from Camembert cheese (Jaillais et al., 1999), Cheddar cheese (Milo and Reineccius, 1997), smear-ripened cheese (Lecanu et al., 2002), milk proteins (Fabre et al., 2002). Carpino et al. (2010) applied SPME technique to study the aroma compounds of the Trachanas, a traditional fermented food product of Cyprus. A rapid and sensitive solid phase method was developed by Coulibaly and Jeon (1992) for the extraction of lactones at ppb levels. Solid Phase Micro-Extraction has been also used for the isolation of odour active compounds of several animal feeds for small ruminants (Mereu, 2009).

Figure 2.2 - Schematic illustration of SPME sampling (CoRFiLaC Laboratory).

2.2.2.2 Purge and Trap (P&T)

Purge and Trap (P&T) Thermal Desorption is also a very popular technique and is routinely used for the analysis of volatiles in samples of several nature as well as food samples (figure 2.3). Through the proper selection of adsorbent resins such as Tenax, water can be eliminated from being introduced into the GC. This is important for the analysis of high water content samples such as dairy products. The P&T technique is more sensitive by at least a factor of 1000 over headspace techniques: its sensitivity is measured in the ppb range. However, it is not useful for the detection of low levels of highly volatile organics due to breakthrough on the adsorbent resin beds. By purging samples at higher temperatures, higher molecular weight compounds can be detected. However, the P&T technique requires more time for sample preparation and can not normally be automated. In addition, very light volatiles and gases will not be trapped on the adsorbent resins and therefore will be missed in the analysis. The P&T technique has proven to be the optimal method for the analysis of flavours in food due to its high sensitivity and the ability to eliminate the water from the sample for analysis. Volatiles ranging from pentane through the terpenes can readily be analyzed with this technique. The advantages (Mariaca and Bosset 1997) of this technique are represented by the reproducibility of results and useful for long investigation (i.e. cheese ripening). However, it is not possible to use autosamplers with dairy products and technical modifications of the injection-port of GC are necessary.

The dynamic headspace methods have been used for the extraction of volatile compounds from milk, fermented milks (Imhof and Bosset, 1994), and cheese varieties such as Cheddar cheese (O'Riordan and Delahunty, 2001) and Manchego cheese (Gomez-Ruiz et al., 2002). Purge and Trap also has been used to determine the aroma profile of milk (Rapisarda, 2010 submitted).

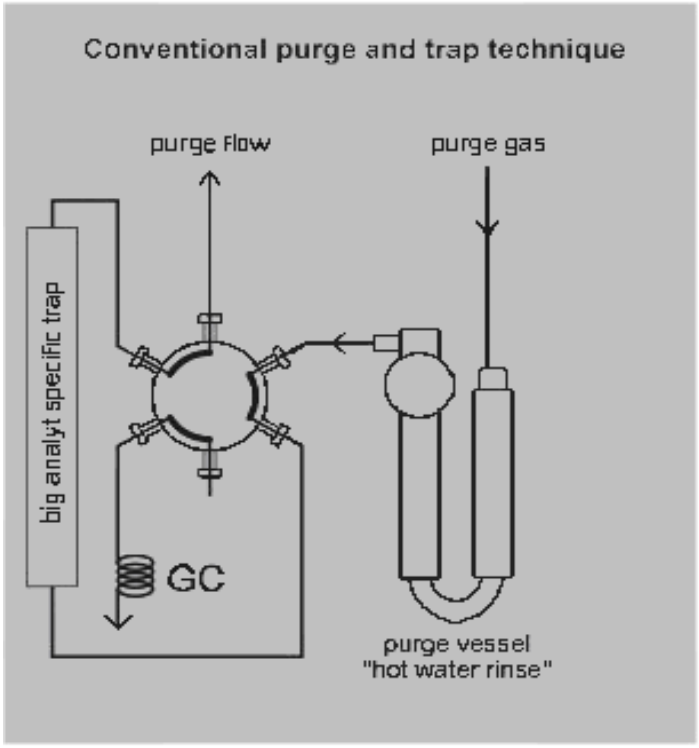


Figure 2.3 – Conventional Purge and Trap system.

2.2.2.3 Retronasal Aroma Simulator technique (RAS)

Retronasal aroma is an indirect perception due to the less volatile compounds vapors that are perceived as retro-olfaction, coming back to the esophagus after deglutition. Retronasal aroma is the odour impression during food consumption. The retronasal aroma is affected by several factors, such as pH of saliva, chewing, and temperature of the mouth, in contrast to the orthonasal aroma that derives directly from odorants of food, interacting with the specific receptors of the nasal cavity. The temperature of the mouth, the high polarity and neutral pH of saliva can change the volatility of some flavours, especially in foods with high fat or low pH. For example, the ice cream, if sniffed in its frozen state, does not reveal all the aromas to hint at its taste sensations. But warmed in the mouth, ice cream is a whole different experience.

Previous research and common experience showed that often a food has a different flavour during eating than when sniffed (Roberts and Acree, 1995). Only a few analytical methods of flavour release have incorporated the crushing, mixing, dilution, and temperature required to adequately simulate retronasal volatile release. There is a need for a device that produces headspaces similar to those occurring in the mouth during eating. A large-scale retronasal aroma simulator (RAS) was thus designed to simulate the mouth conditions of mastication, salivation, and temperature change and to provide sensitivity to detect odorants active at 10-9g/g in foods.

Flavour chemists at Cornell University's Agricultural Experiment Station in Geneva, NY, have developed an artificial mouth, called the Retronasal Aroma headspace Simulator (RAS). The RAS apparatus (figure 2.4) consisted of a water-jacketed stainless steel blender operating at body temperature (~ 37°C). It presents a screw-top lid having a regulated gas source attachment to provide airflow (laboratory purified air) and another stoppable attachment with a self-sealing silicone septum to hold the SPME fibre in place. For the extraction, an odourless artificial saliva (Roberts and Acree, 1995) is mixed with the sample being tested. This mixture is placed into the blender and agitated for 10 min at 60% power with the lid and stopcock closed (water temperature 37 1C; airflow20 mLmin–1). These conditions simulate the mastication and breathing that take place at the natural temperature of the mouth when eating. A syringe holding the SPME fibre is then fitted into place, the stopcock was opened and 1 cm of the fibre is exposed to the dynamic headspace of the sample for an additional 10 min. The syringe is removed from the septum and the volatiles are analysed. The dynamic headspace of the sample is maintained during

this time by closing the stopcock and leaving the blender and airflowrunning. Due to dynamic headspace mode, and using the same fiber for the SPME extraction, the RAS presents the similar advantages of the P&T system and the same disadvantages of SPME technique.

The RAS apparatus has been lately used to sudy the retronasal volatile compounds from Piacentinu Ennese cheese (Carpino et al., 2010).

Figure 2.4 - Retronasal Aroma Simulator apparatus
(CoRFiLaC Laboratory)

2.3 Conclusions

Flavour chemistry research studies the correlations between sensory qualities and objective instrumental measurements, assuming that all volatiles compounds occurring in food concur to its aroma.

Many volatile compounds are dissolved in the original fatty phase, and various procedures have been applied to the isolation of volatiles from complex matrices such as milk and dairy products.

Several extraction methods have been studied and used for the isolation of the volatile compounds of foods: steam distillation and high-vacuum distillation techniques, headspace extraction, in static and dynamic mode. The dynamic headspace technique have been developed to obtain more concentrated extracts. Each method presents advantages and disavantages, thereby, no method is considered ideal for the wide diversity of physical and chemical properties of these compounds and their interaction with the complex matrix such as milk and dairy products.

In general, a satisfying extraction and a good detection of volatile compounds depend on their property (concentration and vapour pressure), as well as on the characteristics of the substance (temperature and complexity of the matrix) from which they derive.

Chapter 3

Instrumental analysis for volatile compounds

3.1 Gas Chromatography

Gas chromatography (GC) is a chromatographic technique that can be used to separate volatile organic compounds and was invented by A. J. P. Martin (Martin and Synge, 1941).

The most important parts of a gas chromatograph are represented by a flowing mobile phase (Nitrogen, Helium, Argon, and Carbon dioxide) that also contains a molecular sieve to remove water and other impurities, an injection port that can be used in split or splitless mode, a separation capillary column containing the stationary phase, and a detector. The separation of the organic compounds is due to the interactions between the stationary phase and the analytes, due to their characteristics (i.e. polarity, molecular weight). After the components of a mixture are separated using gas chromatography, they need to be detected.

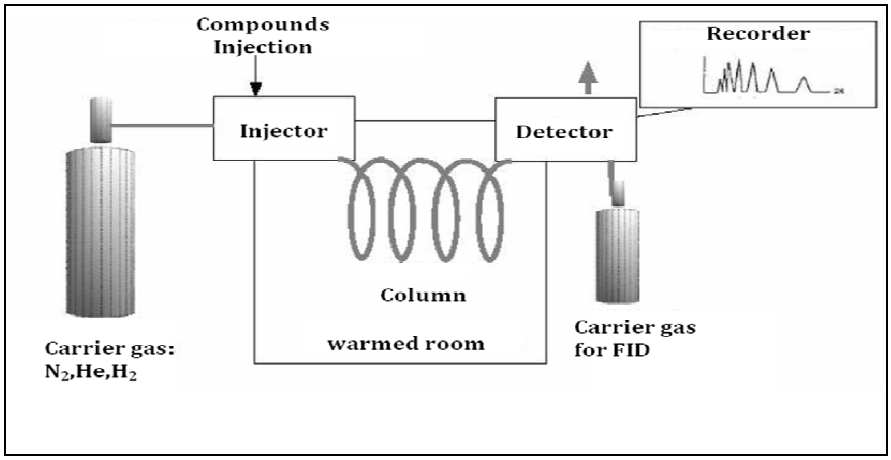


Figure 3.1 - Conventional Gas Chromatographic system

3.2 Detection Systems

Chromatography detector is a device that locates in the dimensions of space and time, the positions of the components of a mixture that has been subject to a chromatographic process and, thus, permits the senses to appreciate the nature of the separation. There are several methods to classify the detector system, one of them distinguishes in non-selective detectors that respond to all compounds, except the carrier gas, and selective detectors that respond to a range of compounds with a common physical or chemical property. There are also specific detectors, responding to a single chemical compound or non-specific. In general (though not always), non-specific detectors have lower sensitivities than the specific detectors. The ideal GC detector should have a sensitivity of about 10-12 - 10-11 g/ml.

Following is a serias of detectors that have been most frequently quoted in the literature for the qualitative and quantitative analysis of volatile (flavour) constituents of raw matrix.

3.2.1 Flame Ion Detector

The Flame Ion Detector (FID) is the most popular system used for GC analysis, due to its universal applicability, long-term stability and low cost. It has also high sensitivity, a large linear response range, and low noise. However, it is a non-specific and not selective detector (Figure 3.2). Besides, FID is a destructive technique causing the total burning of the eluate by a mixture of hydrogen and air. GC-FID systems have been applied to dairy products (Barbieri et al., 1994; Careri et al., 1994; Preininger et al., 1994), especially to investigate the volatile compounds in fermented milk by static headspace techniques (Monnet et al., 1994), for assay of flavour compounds in ewe's milk and yoghurt (Georgala et al.,1995), to detect flavour defects caused by lipid oxidation (Shipe et al., 1978; Ulberth and Roubicek, 1995) and to study the volatile organic compounds (VOCs) in Gorgonzola cheese during ripening and to study the odor profile of typical Sicilian cheeses (Carpino 2002).

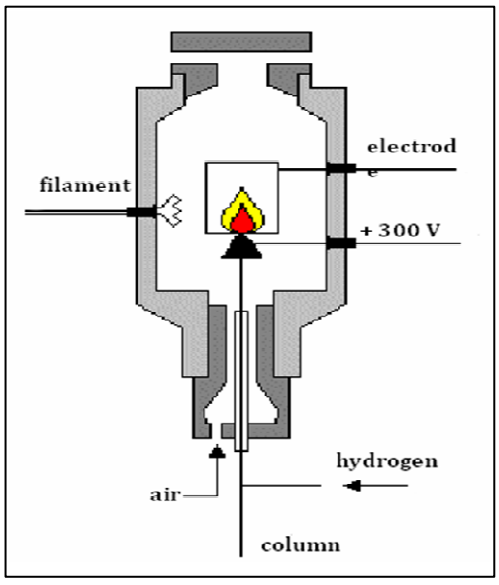


Figure 3.2 - Flame Ion Detector

3.2.2 Mass Selective Detector

The mass selective detector (MSD) is used for qualitative and quantitative determinations (Figure 3.3). GC-MSD technique is the most popular technique used to identify volatile compounds in dairy products. Moio et al. (1993) detected neutral volatile compounds in fresh bovine, ovine, caprine and water buffalo raw milks. Shiratsuchi et al. (1994) studied off-flavour compounds in spray-dried skim milk powder. Dynamic headspace analysis with MS detection was also used by Laye et al. (1995) to identify 33 volatile compounds from commercial whey protein concentrate.

Figure 3.3 - Mass Selective Detector (CoRFiLaC Laboratory)

3.2.3 Atomic Emission Detector

The atomic emission detector (AED) determines the atomic emission of many elements in analytes that, eluted from a GC capillary column, are sent into a microwave powered plasma where the compounds are destroyed and their atoms are excited by the energy of the plasma. The AED (Figure 3.4) allows to make quantitative and trace-level analysis, analyzes higher-boiling compounds and is 5-fold more sensitive than GC-FID for carbon. However, the AED show a great limit of the linear dynamic range that is 6 to 8-fold greater than for the FPD.

The evaluation of the various detection systems for the determination of volatile sulphur compounds in foods was reviewed by Mistry et al. (1994).

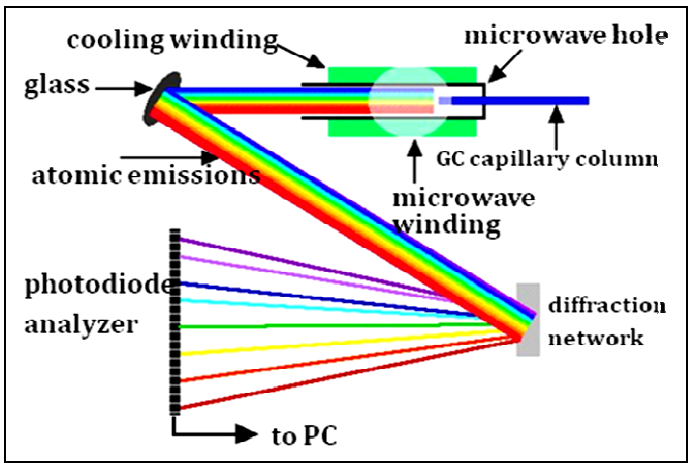


Figure 3.4 - Atomic Emission Detector

3.2.4 Nitrogen Phosphorus Detector

The nitrogen phosphorus detector (NPD) is similar in design and in operating conditions to the FID, but it's highly selective for nitrogen-containing compounds, like amines, pyridine, pyrazines, etc (Figure 3.5).

Nitrogen-containing volatiles of Swiss Gruyère cheese have been investigated by Liardon et al. (1982). Flavour defects in milk and dairy products due to pyrazines have been reported by Morgan (1976). The determination of 2-methoxy-3-alkyl pyrazines have been detected by Lund (1994).

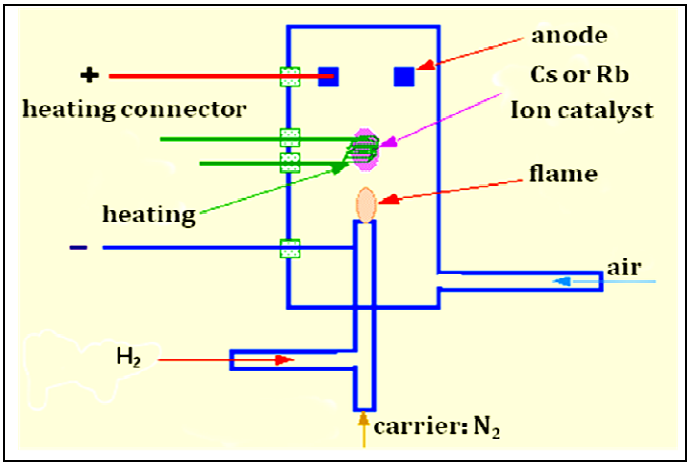


Figure 3.5 - Nitrogen Phosphorus Detector

3.2.5 Flame Photometric Detector

The flame photometric detector (FPD) is highly selective for sulphur-containing components. However, it suffers from defects, like the non linear response of output signal versus the concentration of sulphur species (Burdge and Farwell, 1994). This detector (Figure 3.6) was used by Manning and Moore (1979) to determine hydrogen sulphide and methanethiol by headspace analysis of hard cheese, and by Aston and Douglas (1981) to describe the detection of carbonyl sulphide in Cheddar cheese also using a headspace technique.

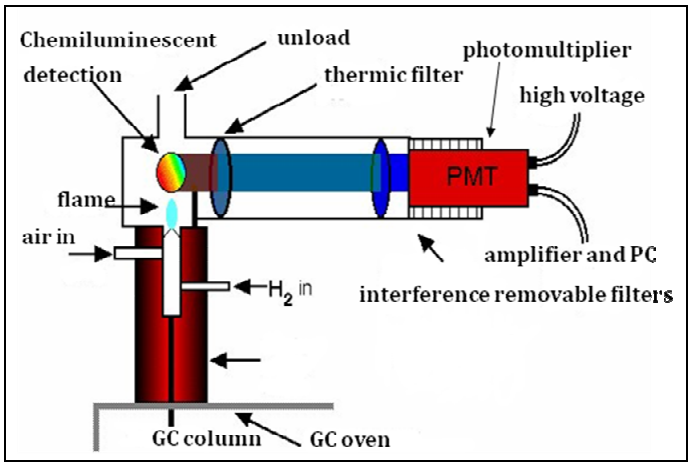


Figure 3.6 - Flame Photometric Detector

3.3 Mass Spectrometry

The mass spectrometry (MS) is an analytic technology able to analyze and identify known chemical compounds and trace levels of them. The first mass spectrometry instrumentation was invented by Joseph J. Thomson at the beginning of the 1900s (Thomson, 1913).

The mass spectrometer is an universally used analytical instrument, that allows to carry out qualitative and quantitative determinations of organic compounds belonging to several chemical classes. In detail, it provides informations about the molecular weight, elemental composition and structure for each chemical compound that is analyzed.

The mass spectrometer is principally composed by an ionization source, a mass analyzer and a detector.

The chemical compounds are at first invested by the gas-phase ions produced by the source and the originated ions travel through the mass analyser and arrive to the detector, according to their mass on charge (*m/z)* ratio. The new signals are then recordered by a computer system as peaks in the resulting mass spectrum together with a relative measure of their abundance. The mass spectral peak intensities are plotted as ordinates in arbitrary units or normalized to the most intense peak in the spectrum, which is assigned a value of 100.

Mass Spectrometry is usually coupled with Gas Chromatography MS (GC-MS) and Liquid Chromatography MS (LC-MS) (Moco et al., 2007; Dunn, 2008).

In GC-MS, the gaseous mobile phase carries the sample through a capillary chromatographic column and the GC output is connected to a mass spectrometer. In conclusion, each compound detected by GC-MS is characterized by a retention time or retention index, a relative intensity measure and a spectrum of peaks consisting of a set of fragment peaks sometimes accompanied by the molecular ion peak. The fragmentation of molecular ions complicates the resulting spectrum but also provides additional structural information about the molecule.

Several different types of ionization sources and mass analysers can be employed in experimental food chemistry. The most commonly applied ionization source in GC-MS is electron impact (EI), which produces positively charged radical ions with a reproducible fragmentation pattern. Ionization is induced by collisions of analyte molecules with a reagent gas followed by ion or charge transfer. Among the mass analyzers used in GC-MS, the quadrupole (figure 3.7) is the most widely used

analyzer due to its ease of use, mass range recovered, good linearity for quantitative work, resolution and quality of mass spectra.

Gas Chromatography-Mass Spectrometry system is the most popular technique used to identify volatile compounds in dairy products. Some of the main advantages of mass spectrometric methods concern the high sensitivity, high chromatographic reproducibility and resolution as well as reduction of matrix effects. The main drawback of this technique is that only volatile molecules can be separated by GC. Besides, this system is much more expensive than the FID.

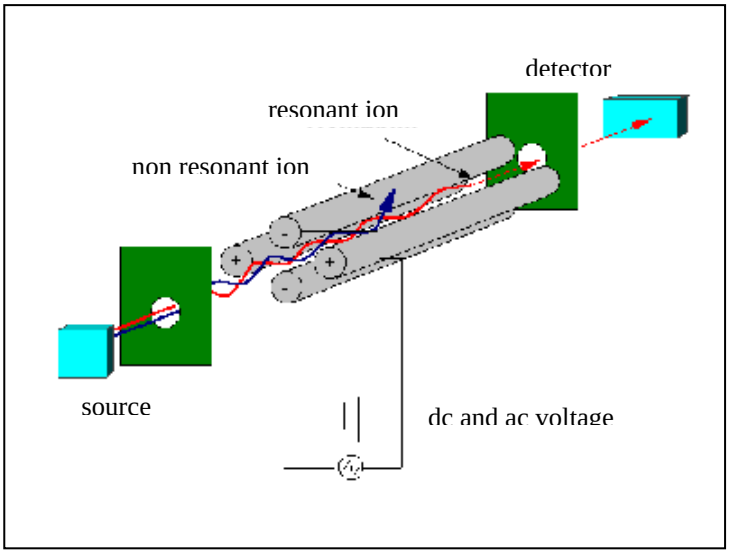


Figure 3.7 – Linear Quadrupole

3.4 Gas Chromatography Olfactometry

Gas Chromatography/Olfactometry (GC/O) is the combining techniques that unite olfactometry, the use of human detectors, to access odour activity in defined air streams, with the gas chromatographic (GC) separation of volatiles. The combination of the GC effluent with humid air under laminar flow dates from early 70s (Acree et al., 1976). The GC/O system is used in flavour research to determine the odour active compounds in raw matrix, combining the subjective sensorial analysis with the objective instrumental measurements.

During GC/O analysis, an extract or distilled sample from the food matrix is injected into a GC that has been modified with an olfactometer as detector (Figure 3.8). The human detector is the final sensor that uses the human nose (sniffer). It can be more sensitive than an instrumental detector, having in fact a detection limit down to 10-19 moles for certain odorants (Reineccius, 1994). The sniffer plays a key role as a unique interface with sensory analyses due to its specificity for flavour and off-flavour compounds.

3.4.1 Sniffer training method

Sniffers describe the nature of their perception. This usually involves the association of the percept with a word or group of words. It is well documented that training the sniffer with chemicals and standard vocabularies will result, at least for a short term (days to weeks), in a reproducible result (Cain, 1979). People can be trained to consistently identify smells if they are standardized periodically. The training for the sniffer consists in a specially formulated standard odorant mix of compounds to measure subjects' olfactory acuity, sensitivity and precision. The mix was formulated during research by Friedrich (2000) and Kittel (2006) at Cornell University and was designed to stimulate all the known classes of olfactory receptor proteins in the human olfactory system and is therefore an ideal medium to identify possible anosmias in prospective subjects. Overall sensitivity, being a different issue than anosmia, was measured by exposing subjects to a serial dilution set of the stimulus and determining his or her odour threshold for each component in the mix (Table 3.1)

Table 3.1 – chemical standards and chromatographic information for sniffer training.

Compound*	CAS	RI (OV1)a	RI (OV5)b	Character	Level (ppm)
hexanal	66-25-1	772	800	green, grass	600
butyl acetate	123-86-4	800	816	pear, fruity	5000
ethyl-2-methyl butyrate	7452-79-1	835	850	apple	2
isovaleric acid	503-74-2	877	877	cheese, rancid	32
(Z)-4-heptenal	6728-31-0	875	902	biscuit, rancid	3
benzaldehyde	100-52-7	920	960	almond	3400
1-octen-3-one	4312-99-6	956	976	mushroom	46
2-acetyl pyradine	1122-62-9	995	1034	popcorn	20
phenylacetaldehyde	122-78-1	1004	1049	floral, honey	20
acetophenone	98-86-2	1033	1070	floral, lavander	840
linalool	78-70-6	1085	1100	citrus , floral	26
cis- rose oxide	16409-43-1	1094	1112	rose, floral	60
isoborneol	124-76-5	1157	1158	dirt	0.3
2-secbutyl-3-methoxy-pyra	24168-70-5	1176	1186	bell pepper	0.2
benzothiozole	95-16-9	1188	1230	rubber	70
l-carvone	6485-40-1	1215	1248	carraway, mint	320
phenylacetic acid	103-82-2	1245	1262	floral, honey	20
eugenol	97-53-0	1330	1264	clove	2
beta-Damascenone	93726-93-4	1363	1386	sweet, rose	0.34

*Undiluted standard mix;a:OV1 column;b:OV5 column

3.4.1.1 Olfactory acuty

Olfactory acuity is defined as the ability to detect a wide range of odorants displaying widely varying odour qualities as well as chemical properties. The standard mix is formulated to test panelist's abilities to detect both high and low molecular weight compounds; a range of chemical family groups and compounds with various polarities. If responses to specific compounds are missing from the data set this warns on a possible specific anosmia indicating the subject may not have the ability to smell these specific compounds. Due to the genetic diversity in the general population, one specific anosmia would not be unexpected in a sample set of up to 20 compounds. More than one deficit could be indicative of a general impaired ability to smell, thus disqualifying the subject.

3.4.1.2 Sensitivity

Sensitivity is determined by the number of test mix compounds correctly detected by the subject over the course of the 3-sample GCO test set. Emphasis is to be placed

on the final 2 dilutions where correct responses to the more dilute samples demonstrate a level of sensitivity to the stimulus. A spreadsheet enumerating the number of correct responses in dilutions 3 and 4 will provide a good indication of the general level of sensitivity of the subject and whether they can be considered candidates for final testing. A score of (8-10) correct responses in the final (4) dilution would be indicative of acceptable sensitivity to the standard stimulus set.

3.4.1.3 Precision

Precision is the measure of consistency of response in both time and odour description during the sample set. To make a rough determination of the precision of the subject the retention index unit representing the start of each odour response over the 3 run set should be examined for consistency. Responses can be evaluated for precision in both response description and response timing. For time of response, the subject should show an ability to respond within 2-3 index units from the start of each odour detected over the course of the 3 runs. Ending response index units, while useful in evaluating the subjects ability to understand the process (as they could infer from their experience with the Simulator) should not be considered. Odour peak widths are problematic for the untrained analyst and cannot be measured for precision without experience. Odour descriptor response can be evaluated as a rough guide to the subject's ability to learn from the simulation run and also their innate ability to describe odour quality in an understandable manner. Responses described that match the descriptors from the simulation run show an ability to learn and agree with the quality of odours. However it is not necessary to put too much importance on this since this skill is, to a certain degree, experience dependent and can be much improved quickly with experience and further training.

3.4.2 Quantitative GC/O analysis

In the mid 1980's, Acree et al. (1984) in USA, and Ullrich and Grosch (1989) in Germany developed quantitative approaches identified as CharmAnalysis and Aroma Extract Dilution Analysis (AEDA), both based on serial dilutions until an odour is no longer perceivable to estimate the importance of a particular aroma compound in food.

In detail, the CharmAnalysis is based on sniffing of GC runs of serial dilutions, generated diluting the mother odour essence solution by a constant factor of 3 (1/3,

1/9, 1/27 etc.), in order to determine the presence of odour-active areas. Charm chromatograms are generated when the square waves produced by sniffing from all samples in a dilution series are added together, using the follow algotithm, to give the Charm value:

$$d_v = F^{n-1}\, d_i$$

$$\text{Charm} = \int_{peak} d_v$$

where d_v is the dilution value (or number of dilutions), F is the dilution factor used to prepare the dilution series, n is the number of dilutions in the series and d_i is the time or the retention index of each determined odour compound.

The AEDA analysis is a similar technique, where the aroma extract to be assayed is diluted by a factor of several times to form a series in which each member is 2 times as concentrated as the next most diluted sample. Aroma Extract Dilution Analysis analysis is based on the dilution factor (FD), which represents the highest dilution where an odour-active component may still be detected. Aroma Extract Dilution Analysis expresses the raw data as flavour dilution values. The data in both cases are comparable since they both express the relative number of dilutions until an odour is no longer detectable by the sniffer as it elutes from the column.

Gas Chromatography Olfactometry data is often expressed as a Charm or flavour dilution response chromatogram, an odour spectrum chromatogram, or odour activity value.

Both flavour dilution values and Charm values can be converted to an odour spectrum value (OSV) using Steven's law:

$$Y=kF^n$$

where Y is equal to the perceived intensity of a stimulant, k is a constant, F equals the stimulus level, and n is Steven's exponent. Steven's law exponents for odorants range between 0.3 and 0.8 (Stevens, 1958, 1960), and using a median value of 0.5 is adequate. Odour spectrum values are normalized to the most intense odourant. Plotting response against retention indices generates an odour spectrum chromatogram. The odour spectrum chromatogram is representative of the pattern of the odorants in the sample injected and is independent from concentration. Another way to express the results of GC/O analysis is through odour activity values which

can also be plotted as a chromatogram. An odour activity chromatogram is representative of both the intensity and pattern of the odour-active compounds since an odour activity value (OAV) is the ratio of the concentration of an odorant with its odour threshold determined in the food matrix (Friedrich and Acree, 1998).

Gas Chromatography Olfactometry analysis has some drawbacks, many of which are related directly to the use of a human as a detector. Although the olfactometric analysis may depend on subjective factors related to the psychophysical conditions of the sniffers, and the olfactory sensitivity of an individual changes throughout the day as well as over longer periods of time (Köster, 1965, 1968), it can, however, result in reproducible measurements if the sniffers are closely monitored, traning them with reference chemicals and agreed on the odour attributes. Recent studies have directly substantiated the odour relevancy of the results from dilution to threshold GC/O by demonstrating the similarity of standard solution, a mixture of identified potent aroma compounds, with an actual food product (Guth and Grosch, 1994; Grosch et al., 1995).

Figure 3.8 – GC-Olfactometer (CoRFiLaC Laboratory).

3.5 Smart Nose

SMart Nose, “the unsurpassed odour analyzer”, is the first artificial nose of a new generation of instruments based on mass spectrometry (Figure 3.9). It is sensitive to the volatiles present in the headspace of different kinds of samples and thus, in some way, mimic the mammalian nose (Ampuero and Bosset, 2003; Schaller et al., 1998). Smart Nose allows the direct characterization of volatile organic components components from liquid and solid samples without separation of the headspace constituents. It is a fully automated instrument, characterized by high sensitivity, stability and ease-of-use. The SMart Nose Mass spectrum represents a very reproducible and precise “fingerprint” of the headspace from each sample. The system is entirely controlled by software: the mass intensity lists generated are processed by the SMart Nose software to highlight the most discriminant ions between the different samples. Then, the statistical algorithms based on Principal Component Analysis (PCA) or Discriminant Function Analysis (DFA) convert the raw data into an interpretable data set.

SMart Nose has found applications in many areas such as the analysis of beverages, foods and polymers. In particular, it can be and have been used as rapid screening device for verifying sub-varietals (Jou and Harper, 1998), geography of production (Pillonel, et al., 2003; Belvedere et al., 2010), season of production (O’Riordan & Delahunty, 2003), ripening time (Drake et al., 2003).

In conclusion, SMart Nose system opens new perspectives in the analysis of odours and volatile compounds.

Figure 3.9 - SMart Nose system (CoRFiLaC Laboratory).

3.6 Conclusions

Several instrumental analyses have been applied to study the volatile compounds in raw matrix. In general, GC/MS is the method universally used to separate, identify and make qualitative or quantitative detection of the volatile compounds. By the GC/MS/O system, it is possible determine qualitative and quantitative (Charm and AEDA) analysis of many odour active volatile compounds. The final detector consists in human nose (sniffer). The training for the sniffer consists in a specially formulated mix of standard compounds to measure subjects' olfactory acuity, sensitivity and precision in order to detect trace levels of odourant. The other instrumental approach recently proposed is the so-called 'electronic' or 'artificial' nose (Smart Nose), which allows to investigate the fingerprint of the volatile compounds of a food matrix.

Due to focus to the major objects in the scope of the second part of this thesis, only gas chromatography/mass spectrometry/olfactometry and Smart Nose instruments mentioned in this chapter will be discussed in detail more in the experimental section.

PART II: EXPERIMENTAL APPLICATIONS

Chapter 4
Volatile Profile of Piacentinu Ennese Cheese

4.1 Traditional Sicilian Piacentinu Ennese Cheese

Piacentinu Ennese is a traditional Sicilian cheese produced in the province of Enna, usually consumed after 2 to 6 months of ripening. Piacentinu cheese is traditionally made from raw milk and is different from other Italian pecorino cheeses due to the addition of saffron to milk prior to coagulation, which gives the cheese a bright yellow colour. Saffron also contains a unique group of volatiles, including safranal and a set of methyl cyclohexenones, and may have antitumoral properties (D'Auria et al., 2004). Whole peppercorns are often added to the cheeses which contribute to their spicy aromas and flavours. Recently, new producers use pasteurized milk in an effort to eliminate pathogens and/or spoilage bacteria (Burton, 1986). While relatively little is known about the contribution of volatiles and other properties from saffron to Piacentinu cheese, previous results (Horne et al., 2005; Fallico et al., 2006) suggest that using raw or pasteurized milk to make Piacentinu produce different volatile, chemical, microbial, microstructure, and sensory characteristics. Other factors, such as season of production and the presence of certain pasture plants, influence the flavour increase during cheese ripening. Licitra et al., (2000) reported that traditional cheeses derive many of their desired and unique properties from their territory of production and from methods limited to the cheese-maker ability handed down from generations. When milk is pasteurized, non-native starter cultures need to be added or commercial rennet are used: some if not all of the uniqueness of traditional cheeses is lost.

Experimental work was carried out on Piacentinu Ennese cheese samples to determine whether traditional and non-traditional cheese-making process, using plastic tools or commercial saffron, alters the volatile fingerprints of this traditional cheese at different stages of ripening.

4.2 Differences in volatile fingerprint of Piacentinu cheese produced by traditional and non-traditional cheese-making process.

4.2.1 Extraction technique and analysis by SMart Nose

Forty-eight cheeses were produced by one farm in the province of Enna. The experimental cheeses were produced according to traditional cheese-making process from raw milk using artisan lamb rennet and no starter cultures. Local (L) or commercial (C) saffron was added to cheeses produced with traditional wood (L) and plastic (P) tool tina. In each cheese-making trial twelve cheeses were produced: three in a wood tina using local saffron (LL), three in a plastic tina using local saffron (PL), three in a wood tina using commercial saffron (LC), three in a plastic tina using commercial saffron (PC). Summarizing, four groups of cheeses were obtained: LL, PL, LC, PC. The experiment was replicated four times with 15 days interval between trials. The cheeses were aged at CoRFiLaC's experimental aging centre. All cheese samples were analysed at 0 days, 2 and 4 months of ripening. The analyses were performed with an electronic nose, SMart Nose system, which allows the direct analysis by MS of volatile organic components (VOCs) without separation of the headspace components. The SMartNose system (from LDZ, CH-2074 Marin-Epagnier) provided by a Combi Pal autosampler CTC Analytics AG (CTC Combi Pal with the Cycle Composer software) is a high-sensitivity quadrupole mass spectrometer (Inficon AG) with ionic mass detection ranging from 1 to 200 amu and a user-friendly multivariate analysis software (SMart Nose 1.51) for data processing. Four grams of cheese were filled into 10-ml vials (adapted for the Combi Pal autosampler) closed with a butyl/PTFE septum and a cap. The samples were randomly placed in the autosampler trays to avoid biases due to external factors. Three replicates were measured for each sample. The main operating conditions were as follow: incubation temperature at 60 ◦C; incubation time of 30 min; injection volume of 2.5 ml; syringe temperature at 100 ◦C; injector temperature at 160 ◦C; nitrogen as purge gas, with a purge flow of 200ml/min; EI ionization mode at 70 eV; mass spectrometer scan speed of 0.5 s/mass; mass range of 10–160 amu; Source Emission voltage at 1540. The total acquisition time was set to 170 s so that three cycles were measured for each injection. All data set transformations were carried out using the software supplied with the SMart Nose. The mean value of the three

cycles was calculated, and the processed data set was normalised using the atomic ion of argon (m/z = 40) fromair. This mass to charge ratio is subject to practically no contamination from other compounds and the concentration of this gas in the headspace can be considered as constant. Such a normalization makes it possible to correct the drift both within a single series of measurements and between different series. Then a Principal Components Analysis (PCA) was carried out. Though PCA is not a classification method, the program gives the possibility of making a group assignment by Euclidean distances in the multidimensional space created by the PCA. For each separation pattern, a new set of parameters was chosen to calculate the principal components scores.

4.2.2 Results and Discussions

The PCA two-dimensional plots show the data obtained for the analysis of the different Piacentinu cheese at 0, 2 and 4 months aging. Groups of samples at 0 days of ripening were not discriminated by SMart Nose; this result is likely due to the non pronounced aroma profile of fresh cheeses. In Figure 4.1 results for Piacentinu cheeses two-months aged produced in wooden tina with addition of local and commercial saffron, are shown. The score plot indicated a good separation with PC1 (92.15%) and PC4 (0.23%). In Figure 4.2, Piacentinu cheeses at 4 months aged, produced in the same conditions, showed a good separation with PC1 (80.26%) and PC2 (7.44%). These results indicate the important role of the wooden tina to determine the aroma quality of cheese. Previous studies (Lortal et al., 2009) carried out on wooden tina used for the Ragusano cheese-making, showed the presence of lactic acid bacteria in the wood as *biofilm,* responsible of the aromatic precursor release in to raw milk.

The same trend was found for Piacentinu cheese samples produced in plastic tina, adding the two different kinds of saffron. Aroma components of Piacentinu cheese are influenced by the different kinds of saffron, local or commercial added during cheese-making. When the tina is constant and the saffron is variable, SMart Nose is able to clearly differentiate between groups LL and LC, and PL and PC in both 2 and 4 months cheese samples. Principal Component Analysis elaboration showed a good separation between Piacentinu Ennese cheese samples produced using traditional wooden tina and plastic tina, added with the same kind of saffron: in Figure 4.3,

Piacentinu Ennese cheese samples, two-months aged added with local saffron, showed a clear separation with PC1 (61.19%) and PC2 (18,21%).

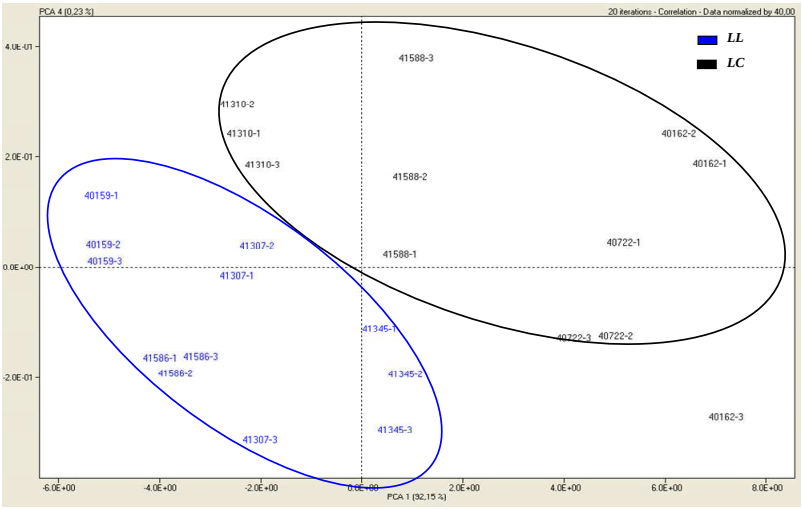


Figure 4.1 - Two months aged Piacentinu cheeses produced in wooden "tina" with local (LL) and commercial saffron (LC)—(PC1: 92.15%; PC4: 0.23%).

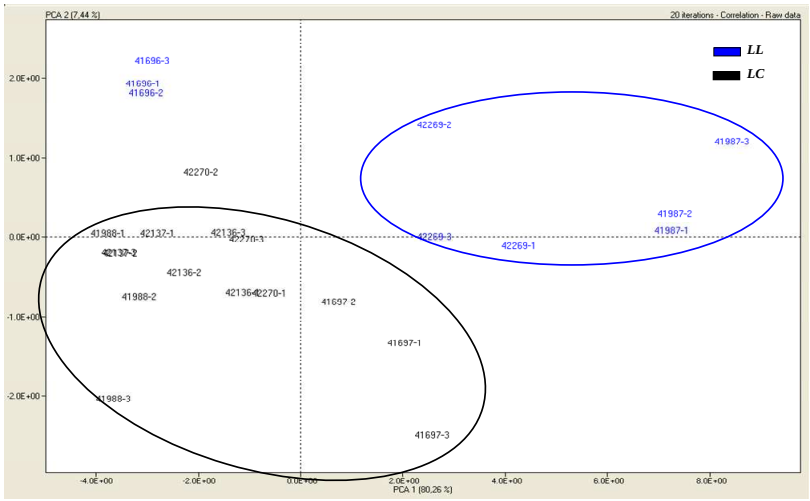


Figure 4.2 - Four months aged Piacentinu cheeses produced in wooden "tina" with local (LL) and commercial saffron (LC)—(PC1: 80.26; PC2: 7.44%).

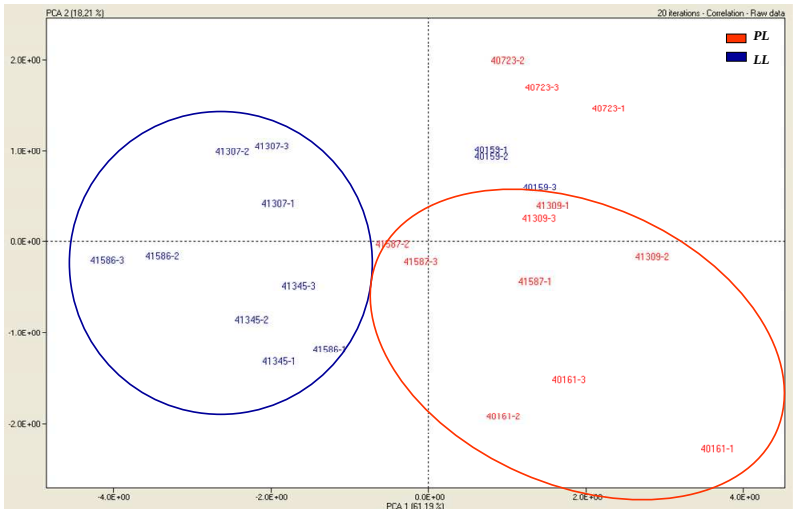


Figure 4.3 - Two months aged Piacentinu cheeses produced in plastic (PL) and wooden (LL) "tina" with local saffron—(PC1: 61.19%; PC2: 18.21%).

Piacentinu Ennese cheese samples, four months aged added with local saffron, showed a good separation with PC1 (64.99%) and PC2 (17.66%), like reported in Figure 4.4.

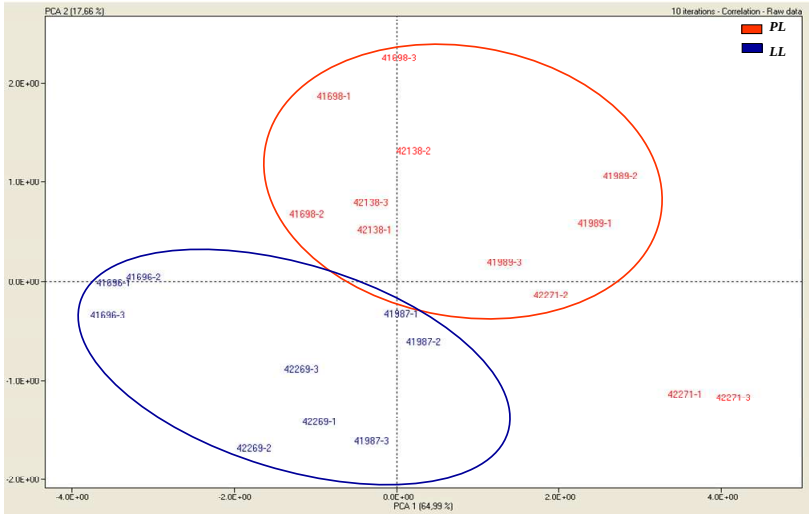


Figure 4.4 – Four months aged Piacentinu cheeses produced in plastic (PL) and wooden (LL) "tina" with local saffron—(PC1: 64.99%; PC2: 17.66%).

In Figure 4.5, Piacentinu Ennese cheese samples, two-months aged added with commercial saffron, showed an evident separation with PC1 (90.29%) and PC3 (1.76%). The same trend was found for Piacentinu Ennese cheese samples, four-months aged added with local saffron, with PC1 (84.62%) and PC2 (6.77%), as showed in Figure 4.6. The clearer separation between cheese samples at two and four months of aging compared to cheese samples not aged is likely due to the more advanced lipolytic and proteolytic processes releasing the aroma compounds that characterise the cheese.

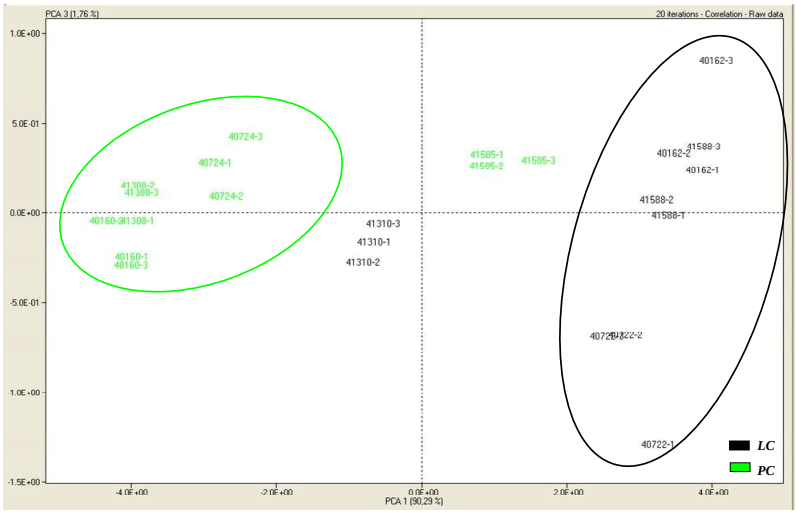


Figure 4.5 – Two months aged Piacentinu cheeses produced in plastic (PL) and wooden (LL) "tina" with local saffron—(PC1:90.29%; PC3: 1.76%).

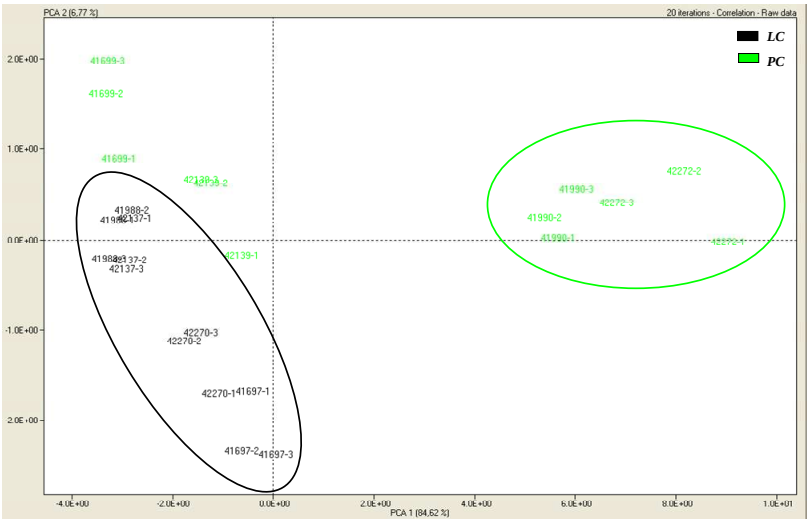


Figure 4.6 – Four months aged Piacentinu cheeses produced in plastic (PL) and wooden (LL) "tina" with local saffron—(PC1: 84.62%; PC2: 6.77%).

4.2.3 Conclusions

The more advanced lipolytic and proteolytic processes, responsible for the improvement of aroma profile in cheeses at 2 and 4 months of ripening, allowed SMart Nose to give a clearer separation between samples obtained with different cheese-making methods. Cheese samples at zero time of ripening, in fact, were not discriminated by SMart Nose. The wooden tina might play an important role in determining the aroma quality of cheese, mainly due to the lactic acid bacteria naturally present in the wood as *biofilm* and released to raw milk. The natural microflora is one of the most responsible factors on flavour development in cheese. Piacentinu Ennese cheese with added local saffron presented a richer aroma profile than Piacentinu Ennese cheese with added commercial saffron, showing how important the role of this kind of saffron has on aroma quality of cheese. In order to get high quality Piacentinu Ennese and considering these results, it would be important to define the technology of production and all the different parameters to produce consistent quality cheese.

Chapter 5
Volatile Organic Compounds and Palatability of Concentrates fed to lambs and ewes

5.1 Feed palatability of animal products

Despite the important influence of flavour on feed palatability (Burritt and Provenza, 1992; Villaba and Provenza, 1997a, 1997b; Arsenos et al., 2000a, 2000b; Atwood et al., 2001) and quality of animal feeds (Ha and Lindsay, 1991; Moio et al., 1993a, 1993b; Pulina et al., 2006; Carpino et al., 2004), only a few studies have been carried out on the effects of flavours present in concentrate feeds on ovine feeding behaviour (Cannas et al., 2009).

In particular, Quaranta et al. (2006) compared in Merino lambs the palatability (one feed by time in 30 min tests in experiments replicated in two periods) and then the preference (all feeds available together) of 11 concentrates and alfalfa pellets. The results of the palatability tests suggested a marked neophobia (i.e. refusal of unknown feeds) for several feeds in the first period and a quick learning process in the second, probably associated to strong post-ingestive effects caused by the length of the tests.

Recently, a feed palatability study was carried out on Sarda female lambs and multiparous dry ewes, by using 14 feeds, plus another for the preliminary period, chosen among the most commonly present in feed mixes for ruminants, in short-term palatability tests, so that post-ingestive effects could be minimized (Mereu, 2009). Results showed clear differences in palatability among feeds, and between lambs and ewes. Some feeds were accepted and other refused both by lambs and ewes, with no apparent relationship with possible post-ingestive effects. Therefore, it is likely that the palatability of feeds was associated with the presence of chemical compounds that affected their flavour. The importance of flavour, and in particular of odours, is indirectly confirmed by the fact that frequently sheep refuse concentrate mixes when one or more ingredients are changed. This occurs more frequently when they are supplied separately from forages, e.g. during milking.

Thus, the objective of this research was to investigate the volatile profile of fifteen feeds commonly used in small ruminants' diets by gas chromatography- olfactometry and mass spectrometry, and to associate the identified compounds with the palatability of feeds measured in a previous study (Mereu, 2009).

5.2 Measurement of palatability

A research was carried out, previously, by researcher of Sassari University, to test the short term palatability of 14 experimental feeds commonly used to prepare concentrate mixes or pellet for ruminants (Mereu, 2009). The feeds used were: dehydrated alfalfa (AD), beet pulps (BP), canola meal (CN), corn gluten meal (GL), corn grains (CG), corn middlings (CM), oat grains (OG), pea grains (PG), soybean hulls (SH), soybean meal 44 (SN), soybean meal 49 (HP), sunflower meal (SM), wheat brans (WB), wheat grains (WG). The palatability experiment is described in detail by Mereu (2009). In short, it was based on a 14 (feeds) x 14 (days) Latin Square design with 14 animals. Two trials were carried out in parallel on the same feeds, one with 14 Sarda female lambs and 14 Sarda multiparous dry ewes. The experiment consisted of an adaptation period (13 days) followed by an experimental period (14 days in total). During the adaptation period, a basal diet made by ryegrass hay and a concentrate mix, made by barley meal and urea, was fed. The animals had also access to water and to a block of minerals and vitamins *ad libitum*. The ration was designed to cover the requirements of both lambs and ewes. During this period the following daily routine was applied:

- at 7:00 a.m. concentrate mix and hay refusals were taken off from the two collective racks (one for lambs, one for ewes) in which the animals were fed;
- at 8:00 a.m. lambs and ewes, in sequence, were trained: 1) to spontaneously enter an individual pen with a rack containing two steel bowls with 100 g of barley meal each; 2) to stay there during 6 minutes for the palatability test; in this time the animals were left alone in order not to be disturbed, but they could see the other animals; 3) to leave the pen and go to an adjacent collective rack at the end of the palatability test, where the animals received ryegrass hay *ad libitum*, in order to limit the post-ingestive effects of barley;

– at the end of the routine described above, all animals were brought back to the original collective pen and fed rationed amounts of ryegrass hay and barley meal mixed with urea.

The order of entry of the two groups was inverted each day, in order to limit differences in fasting time between lambs and ewes. The adaptation period ended once the intake during the 6-min palatability test had become sufficiently stable for some days (day 13 of the adaptation period). Afterwards the 14th d experimental period started. During this period the same daily routine used during the adaptation period was followed, except that instead of barley meal the animals received, during the 6-min palatability test, 200 g (divided in two bowls) of one of the 14 different experimental feed ingredients (Table 5.1), all finely ground before the experiment to reduce the effect of their texture on sheep choices.

The 14 experimental feeds and the ingredients of the basal diet were analyzed for DM, ash, NDF, ADF, ADL (Van Soest et al., 1991), and CP (AOAC, 1990). The non-fiber carbohydrate (NFC) concentration was calculated as [100-NDF-CP-EE-ash], where EE = ether extract, estimated by feed tables.

5.3 Determination of Odour Active Compounds

5.3.1 Extraction methods

Subsamples of the 14 experimental feeds and of the barley meal (used in the adaptation period only), with a total of 15 feed samples, were collected during the experiment and stored at room temperature in sterilized containers. At the end of the experiment they were sent to the flavour laboratory of the Corfilac (Ragusa, Italy) to be analyzed.

Volatile organic compounds (VOCs) were extracted by a static headspace with a solid phase microextraction (SPME) fiber with a 50/30 µm divinylbenzene/carboxen/PDMS coating (Supelco, Bellefonte, PA). Fiber was pre-conditioned before initial use, by inserting them into the injector port of a gas chromatography-olfactometer for 3 h at 270 °C, and reconditioned between extractions at the same temperature for 5 min, followed by 10 min at room temperature. For each extraction, 5 g of grated concentrate sample were put into a 22 ml vial and conditioned in a bath for 1 h, at 37°C. During this time, the equilibrium

of the VOCs between the gas-phase and the sample was established. A syringe holding the SPME fiber was then fit into place, the stopcock opened and 1 cm of the fiber was exposed to the static headspace of the sample for further 30 min, to establish the equilibrium of the VOCs between the gas-phase and the solid-phase of the adsorbent. The syringe was then removed from the septum and the volatiles analyzed by gas chromatography olfactometry.

5.3.2 Gas chromatography and olfactometry

Gas chromatography and olfactometry (GC/O) analysis was performed by a single sniffer, previously trained using the procedure and the standard compounds described by Marin et al. (1988). These standards consisted of a group of 22 compounds used to evaluate olfactory acuity and to determine whether a sniffer has specific anosmia for certain odours. After extraction of volatiles, the fiber was desorbed into a modified Hewlett Packard 6890 gas chromatograph (Datu Inc., Geneva, NY) characterized by a fused-silica capillary column HP-1, 12 m, 0.32 mm i.d. and 0.52 μm film thickness. Splitless injection was performed at 250 °C; the oven temperature program was: 35 °C for 3 min, 6 °C/min to 190 °C, then 30 °C/min to 225 °C, and 225 °C for 3 min. He was used as carrier gas and the column flow rate was 1.9 mL/min. The eluted compounds were mixed with humidified air using the method described by Acree and Barnard (1994) and the sniffer was continuously exposed to this source for 30 min. The time of response to individual odours perceived by the sniffer was recorded by Charmware software (v.1.12, Datu Inc., Geneva, NY). Response times were converted into retention indices (RI) for each VOC and displayed by the software as a series of peaks in an aromagram. RI values were calculated relatively to a series of normal alkanes ($C_7 - C_{18}$) previously injected into the Flame Ion Detector port of the same gas chromatograph. Volatile organic compounds were also identified using the Flavornet internet database (Arn & Acree, 1997), which contains retention indices describing over 550 identified VOCs using GC/O techniques.

5.3.3 Gas chromatography mass spectrometry

The SPME fiber was re-exposed to the static headspace of the same sample and then desorbed into a HP 6890 Series gas chromatography mass spectrometer (NY, USA), with a cross linked methyl siloxane capillary column HP-1, 25 m, 0.20 mm i.d. and 0.11 μm film thickness, using the same GC/O temperature program. Retention times (RT) of volatile compounds were calculated relatively to the same series of normal alkanes ($C_7 – C_{18}$) used in GC/O, that had been previously injected into the GC/MS. This procedure permitted a direct comparison between RI values obtained from GC/O and RT values obtained from GC/MS.

5.3.4 Statistical analyses

The data derived from the 6 min palatability tests were processed through a statistical analysis by using the SAS Proc GLM (Version 9.1.3, SAS Institute, Inc., Cary, NC) on the basis of the following model:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_{ik} + \varepsilon_{ijkl}$

μ = overall mean,

α_i = fixed effect of feed,

β_j = fixed effect of animal,

δ_{ik} = fixed effect of time,

ε_{ijkl} = error term.

Treatment means differences were tested using the Tukey's test at a threshold of $P < 0.05$.

Comparisons between lambs and ewes eating data were carried out for each experimental feed by applying a one-way analysis of variance with two levels (lambs and ewes).

5.4 Results

The chemical composition of the experimental feeds (Table 5.1) was highly variable, especially in terms of content of CP (26.2 ± 18.5%), NDF (36.4 ± 14.9%) and ADF (19.4 ± 14.1%).

Table 5.1 - Chemical composition of the ingredients of the basal diet and of the experimental feeds.

	Chemical composition (% DM)							
	DM	CP	aNDFom	ADF	ADL	Ash	EE*	NFC**
Basal diet ingredients								
Ryegrass hay	86.7	10.3	61.1	35.0	7.1	10.0	2.0	16.6
Barley meal	87.2	11.8	11.9	7.2	0.3	2.1	2.0	72.2
Urea	99.0	281	-	-	-	-		
Experimental feeds								
Alfalfa, dehydrated	89.0	16.3	58.4	43.7	8.6	11.5	2.2	14.5
Beet pulps	88.9	10.0	50.6	26.2	6.9	4.2	0.7	31.8
Canola meal	87.1	38.3	35.2	26.0	14.4	8.0	2.8	9.3
Corn gluten meal	90.4	69.9	21.5	7.9	4.3	1.3	2.6	1.8
Corn grains	87.8	8.5	26.9	4.6	0.4	1.1	3.9	60.3
Corn middlings	87.3	17.8	27.7	7.6	1.7	4.1	3.7	49.1
Oat grains	89.3	11.0	43.3	24.9	3.4	6.3	5.2	37.1
Pea grains	86.7	22.3	19.1	7.5	1.5	4.3	1.9	55.3
Soybean hulls	88.7	14.9	60.9	44.9	9.5	5.1	2.1	12.6
Soybean meal 44	87.8	43.7	23.5	17.4	3.0	6.9	1.3	28.5
Soybean meal 49	87.6	51.5	23.5	9.6	2.0	7.3	1.8	21.2
Sunflower meal	89.4	32.6	52.6	34.4	11.7	7.1	2.9	0.2
Wheat brans	86.6	17.2	43.8	13.1	3.3	5.3	3.6	32.0
Wheat grains	86.3	12.6	22.4	3.8	0.6	1.7	1.9	62.5

* estimated from book values ** calculated as [100-NDF-CP-EE-ash], where EE = ether extract.

5.4.1 Palatability test

Feed palatability (expressed as level of dry matter intake, mg/kg Body Weight (BW), in the 6 minutes tests) by the lambs ranged between 0 mg/kg BW for oat grains and 1379 mg/kg BW for soybean meal 49, in a preference gradient that did not show clear cuts (Table 5.2). Ewes showed clear preferences, with higher ($P < 0.05$) LI for beet pulps, wheat grains, pea grains, and corn grains compared to the other feeds. The feeds with higher level of intake ($P < 0.05$) by lambs compared to ewes were: soybean meal 49 (1379 vs. 69 mg/kg BW, for lambs and ewes, respectively), soybean hulls (757 vs. 128 mg/kg BW, for lambs and ewes, respectively) and soybean meal 44 (611 vs. 142 mg/kg BW, for lambs and ewes, respectively).

The feeds with lower ($P < 0.05$) level of intake in lambs compared to ewes were corn gluten meal (92 vs. 504 mg/kg BW for lambs and ewes, respectively) and oat grains (0 vs. 203 mg/kg BW for lambs and ewes, respectively).

Oat grains, dehydrated alfalfa, sunflower meal, and canola meal were eaten in very small amounts by both sheep categories (Table 5.2). Low intake in both categories was also observed for corn gluten meal and corn middlings.

Table 5.2 - Dry matter level of intake of each feed, ranked in decreasing order of lamb preference, fed to lambs and ewes during the 6-min palatability tests.

Feed	Intake level, mg/kg BW	
	Lambs	Ewes
Soybean meal 49	1379 ^{Aa}	69 ^{Bb}
Wheat grains	1289 ^{a}	1130 ^{a}
Pea grains	941 ^{ab}	1138 ^{a}
Corn grains	820 ^{abc}	1049 ^{a}
Soybean hulls	757 ^{Aabcd}	128 ^{Bb}
Beet pulps	686 ^{abcd}	1267 ^{a}
Wheat brans	653 ^{abcd}	374 ^{b}
Soybean meal 44	611 ^{Aabcd}	142 ^{Bb}
Corn middlings	421 ^{bcd}	361 ^{b}
Canola meal	289 ^{bcd}	54 ^{b}
Sunflower meal	155 ^{cd}	122 ^{b}
Corn gluten meal	92 ^{Bcd}	504 ^{Ab}
Alfalfa, dehydrated	23 ^{d}	30 ^{b}
Oat grains	0 ^{Bd}	203 ^{Ab}
SEM	119	124
P (day) <	0.002	0.46
P (animal) <	0.001	0.001
P (feed) <	0.001	0.001
P (category) <	0.05	

^{A,B} Capital letter indicates differences between lambs and ewes on IL ($P < 0.05$).
^{a,b,c,d} Small letters indicate differences within columns ($P < 0.05$).

5.4.2 Gas chromatography olfactometry

The results of the GC/O qualitative analysis of the fifteen feed samples showed a quite large variability in the number of VOCs (Table 5.3). The total number of VOCs detected in the feeds was, in decreasing order: 27 in beet pulps, 24 in oat grains, 20 in dehydrated alfalfa, 19 in soybean hulls, 18 in soybean meal 44, 16 in sunflower meal, 15 in barley meal, 14 in corn gluten meal and soybean meal 49, 13 in wheat brans, 12 in corn middlings, 6 in canola meal and wheat grains, 5 in corn grains and pea grains.

Because of the complexity and the high number of chemical classes extracted from the fifteen feed samples by the SPME technique, VOCs were grouped in different tables. Aldehydes are shown in Table 5.4, amines and ketones in Table 5.5, esters, lactones and pyrazines in Table 5.6, sulphur compounds in Table 5.7, terpenes in Table 5.8 and heterocyclic compounds in Table 5.9.

Beet pulps

The aroma profile of beet pulps (BP) was mostly composed of aldehyde, terpene, ketone, lactone and sulphur chemical classes and, for a smaller part, by pyrazine (Table 5.3). In particular, beet pulps had two lactones (γ-heptalactone and γ-nonalactone; Table 5.6), which give peach fruity and nutty notes, and one pyrazine (ethyl dimethyl pyrazine; Table 5.6), which gives burnt nutty notes (Table 5.6). Two heterocyclic compounds (methyl cinnamate and safrole) with sweet and spice notes, and one alcoholic compound ((Z)-3-hexenol) with green notes, were detected in BP and were not present in the other feeds, except for safrole presented also in barley meal (Table 5.9). Among the tested feeds, BP had the highest number of aldehydes (11 VOCs; Table 5.4), followed by oat grains (10 VOCs). The two sulphur compounds found in the beet pulps were methylethyl sulfide and thiophene with unpleasant garlic odours (Table 5.7). Besides, three terpene compounds were present only in BP, (E)-linalool oxide and (Z)-dihydrocarvone giving pleasant notes of green, and geosmin giving pleasant notes of beet earth (Table 5.8).

Oat grains

The aroma profile of oat grains (OG) was characterized by very pleasant flavours like green, orange, nutty, hay, mushroom, peach, sweet, spice, pine, lemon and coconut notes, mainly due to their richness of aldehydes (10 VOCs) and terpenes (7

VOCs) and, to a lower extent, of ketones (3 VOCs) (Tables 5.3, 5.4 and 5.5). Three aldehydes were associated with oil notes, of which 2-decenal was unique of OG. Oat grains were the feed with the highest content of terpenes (Table 5.8). Among the terpenes, α-pinene (resine, pine notes), geranial (lemon note) and ρ-menthenethiol (sweet note) were found as unique compounds (Table 5.8). Only one sulphur compound, methyl ethyl sulfide, perceived like garlic note, conferred off-flavour, whereas another sulphur compound, ethyl dimethyl thiazole, gave a mushroom note (Table 5.7).

Alfalfa, dehydrated

The aroma profile of dehydrated alfalfa (AD) was mainly characterized by aldehyde, sulphur, ketone and terpene compounds (Table 5.3). The aldehydes in dehydrated alfalfa sample gave pleasant notes, except for the heptanal causing a bad rancid note to this feed (Table 5.4). The high number of sulphur compounds was responsible for unpleasant odours, like, garlic notes, due to the presence of methyl ethyl sulfide, thiophene and dimethyl trisulfide, cooked potato notes, due to the presence of the methional, and meat notes due to methyl dithio furane (Table 5.7). Dehydrated alfalfa presented three ketones characterized by pleasant note of butter, mushroom and geranium (Table 5.5), and finally, three terpenes, two of them (dihydro carvyl acetate and β-patchoulene; Table 5.8) were found as unique compounds. In addition, a note of popcorn originated from a pyrrole (2-acetyl-1-pyrroline) was also found in AD (data not shown).

Soybean hulls

Soybean hulls (SH) showed an aromatic profile (19 VOCs) richer than the one of soybean meal 49 and similar to the one of soybean meal 44. In particular, the aroma profile of SH was constituted mainly by eight aldehydes that gave to the sample pleasant notes, except for the heptanal (rancid odour); besides, 2-hexenal (green odour) was found as unique compound for this feed (Table 5.4).

The ketone class was also important to define the aroma profile of SH: four ketones responsible for pleasant odour like butter, mushroom and orange notes, were found in this feed sample (Table 5.5). Moreover, the SH presented three terpenes (Table 5.8), one unique, and two sulphur compounds (Table 5.7) that gave, respectively, pleasant (wood, nutty and green) and unpleasant notes (garlic). In

addition, one pyrazine with unpleasant burnt note, which was also present in soybean meals 49 and 44, in beet pulps and in dehydrated alfalfa (Table 5.6), one lactone with pleasant nutty, peach note, also present in the soybean meals 49 and 44 and in some other feeds (Table 5.6) were found.

Soybean meal 44

Soybean meal 44 (SN) was characterized by a rich aroma profile (18 VOCs), mainly represented by aldehyde, sulphur, and ketone compounds (Table 5.3). The aldehydes concurred to a "good" aroma profile of this sample, giving green, orange, hay and nutty notes (Table 5.4). Some sulphur compounds determined unpleasant notes, such as those of garlic, due to the presence of thiophene and dimethyl trisulfide, cooked potato notes from methional, and meaty notes from methyl dithiofurane (Table 5.7). The ketones gave "good" flavours to SN sample, except for the heptanone characterized by solvent note (Table 5.5). A negative aspect of the aroma profile of SN was due to the presence of methanamine (Table 5.5), which gave an off-flavour identified as rotted fish odour.

Sunflower meal

Sunflower meal (SM) mainly showed five aldehydes VOCs, 5 sulphur and three ketone VOCs. The aldehydes gave pleasant odours (Table 5.4) like green, orange, hay and nutty notes. At the same time, ketones concurred to pleasant flavours with butter, mushroom and orange note, on the other side methanamine with an off-flavour of rotted fish (Table 5.5) and sulphur compounds with unpleasant notes of garlic and cooked potato (Table5.7) were detected.

Barley meal

The GCO analysis made on barley meal (BA) showed an aroma profile characterized by 15 VOCs: eight aldehydes, three ketones, two lactones, one sulphur and one heterocyclic compound (Table 5.3). All chemical classes concurred to define a "good" aroma profile at this feed, except for the aldehyde, (E,E)-2,4-decadienal, with unpleasant fried oil note(Table 5.4).

Corn gluten meal and soybean meal 49

The aroma profile of corn gluten meal (GL) and soybean meal 49 (HP) showed the same number of volatile compounds (14 VOCs) (Table 5.3). However, the GL sample presented three aldehydes more than the HP sample, which gave the feed two bad odour of garlic and fried oil and a good odour of vanilla (Table 5.4). Another difference between the samples was the precence of a sulphur compound in HP, methyl dithio furane, with unpleasant, meat odour. The other sulphur compounds, common in both samples, gave unpleasant garlic, potato, and meat notes (Table 5.7), in addition a lactone, with pleasant nutty, peach note (Table 5.6) and a terpene compound, with a pleasant floral note (Table 5.8) were found, respectively, in HP and in GL.

Wheat brans

The aroma profile of wheat brans (WB) was characterized mainly by sulphur compounds, aldehydes and ketones (Table 5.3). The sulphur compounds (Table 5.7) were responsible for unpleasant notes of garlic, cooked potato and meat aroma profile, besides the aldehydes (Table 5.4), gave pleasant notes of green, hay and nutty. Two ketones (Table 5.5) were also found giving to this feed pleasant odour of butter and mushroom notes. In addition, a minimal contribution for the aroma profile of WB was do to a pyrazine (Table 5.6) and a terpene (Table 5.8) that gave, respectively an unpleasant burnt note and a good floral odour to this sample.

Corn middlings

Corn middlings (CM) showed in the aroma profile the same number of aldehydes and sulphur compounds (Table 5.3). The aldehyde chemical class (Table 5.4) gave pleasant odour like green, orange, nutty and hay notes, besides the sulphur chemical class (Table 5.7) was responsible for garlic, cooked potato and meat bad notes. In addition, two ketones (Table 5.5) were found in CM sample: 2,3-butanedione and 1-octen-3-one that gave, respectively, butter and mushroom pleasant notes.

Canola meal

The aroma profile of canola meal (CN) showed 7 VOCs (Table 5.3) mainly characterized by off-flavours due to the presence of three sulphur compounds: methyl furanthiol, dimethyl sulfone and dimethyl tetrasulfide (Table 5.7), which gave

an unpleasant garlic notes to this concentrate. Two aldehydes (Table 5.4), octanal (pleasant orange note) and (Z)-2-nonenal (pleasant hay note), a lactone (Table 5.6), γ-butyrolactone, a free fatty acid (pentanoic acid) and an alcohol (heptanol) were also found in CN sample, thus concurring to the off- flavour profile of this feed. The pentanoic acid and the heptanol are not report in this study.

Wheat grains

The aroma profile of wheat grains (WG) was similar to canola meal but showing 6 VOCs (Table 5.3) represented by five aldehydes (Table 5.4) giving, green, nutty, and hay pleasant note form (Z)-2-nonenal common with canola meal sample. One chetone, contributing with mushroom note (Table 5.5), was also detected.

Corn grains and pea grains

The corn grains (CG) and pea grains (PG) samples showed a very similar aroma profile with a total of five VOCs (Table 5.3). The aldehydes, (E)-2-nonenal (good green note) and (Z)-2-nonenal (good hay note), were found in both feeds (Table 5.4); 1-octen-3-one (Table 5.5), a ketone with pleasant mushroom odour and dihydro linalool (Table 5.8), a terpene with pleasant wood note, was also found in CG and PG samples;

5.5 Discussion

5.5.1 Palatability tests

The results of the palatability tests showed that the differences in intake between the most and the least preferred feeds were very large (Table 5.2).

The lambs showed a preference for several feeds, i.e., in decreasing order of preference, soybean meal 49, wheat grains, pea grains, corn grains, soybean hulls, beet pulps, wheat brans, and soybean meal 44 (Table 5.2). In reality their dry matter level of intake (DMI) during the tests varied from high to low values in a continuum. There was not a clear trend associable to protein, energy or fiber content of the feeds, as if the novelty of the feedstuffs under study pushed them to explore most feed options but also to refuse those feeds that induced negative, possibly innate, sensorial perceptions (Mereu, 2009).

In contrast, the ewes had a marked preference for 4 feeds often supplied as single ingredients (beet pulps, pea grains, wheat grains and corn grains) and low intake or complete rejection of the remaining feeds, including several commonly used in sheep feed mixes but rarely supplied alone (Mereu, 2009). This suggests that previous feeding experience had a major role in their sensorial perceptions and evoked a conservative behaviour. In other words, the ewes were not prone to eat novel feeds, even those generally considered very palatable, such as soybean meal.

Many animals refused even to taste certain feeds. In particular, dehydrated alfalfa, oat grains, canola meal, and sunflower meal were the most refused by both lambs and ewes (Mereu, 2009), indicating that these feeds were probably characterized by the presence of disagreeable aromatic compounds.

Based on these considerations, the association of VOCs profiles with the results of the palatability tests should focus more on the choices of lambs, with little previous experience, than that of ewes, whose choices were strongly influenced by previous experiences. Only the feeds with the most evident patterns will be considered in this discussion.

5.5.2 Relationship between palatability tests and VOCs content

Alfalfa, dehydrated, and sunflower meal

Both lambs and ewes refused almost completely dehydrated alfalfa and sunflower meal (Table 5.2). They were characterized by a high number of sulphur compounds (5 VOCs each) and had similar unpleasant notes, such as those of garlic, cooked potato and meat. Therefore, their low palatability was likely due to the prevalence of sulphur compounds.

Beet pulps

The aroma profile of the beet pulps, which were the most eaten feed by ewes and had intermediate intake by lambs (Table 5.2), was mostly composed of aldehyde, terpene, ketone, lactone and sulphur chemical classes and, to a smaller part, by pyrazine (Table 5.3). Beet pulps were the richest in aldehydes among the feeds studied. The origin of aldehydes is mainly due to the degradation of amino acids available in the feeds. Most of the feeds tested had higher content of Crude Proteine (CP) than beet pulps (Table 5.1), so the latter were expected to have a low content of

aldehydes compounds. Therefore, the aldehyde compounds present in beet pulps might be originated from the oxidation process of unsaturated free fatty acids (Ho and Chen, 1994; Hsieh, 1994). In general, aldehyde compounds gave pleasant notes to the feeds, such as green, orange, nutty, hay, fried oil, oil and vanilla notes, except for the mercapto acetaldehyde and heptanal which conferred, respectively, garlic and rancid notes. The 2-undecenal, characterized by fruity notes, was detected as a unique compound in beet pulps. Moreover, terpene compounds (Table 5.8) influenced positively the flavour of beet pulps, giving nutty, green, spice and characteristic beet notes to the sample. The terpenes found in beet pulps were likely originated from the degradation of the carotenoids precursors present in the feed (Lewinsohn et al., 2005). Beet pulps had two sulphur compounds characterized by negative garlic notes, which could be originated from the degradation of amino acids (Belitz and Grosch, 1986; McSweeney and Sousa, 2000) in the sample; however, the low number of these compounds probably did not hide the positive effect of aldehydes and terpenes in beet pulps. In summary, the general aroma profile of beet pulps was very pleasant and this fact might explain why both lambs and ewes showed a high level of intake of this feed.

Canola meal

Canola meal was eaten in small amounts both by lambs and by ewes. Canola meal is known to contain compounds perceived as unpalatable by the animals (Frederick et al., 1988). The aroma profile of canola meal was mainly characterized by off-flavours due to the presence of three sulphur VOCs which gave unpleasant garlic notes to this concentrate. A lactone, a free fatty acid and an alcohol (data not shown) were also found in the canola meal sample, thus concurring to the off- flavour profile of this feed. Naczk et al. (1998) suggest that the bitter taste and astringency of this feed is due to their content of phenolic acids and condensed tannins.

Corn gluten meal

In the palatability tests, the lambs showed a very low intake of corn gluten meal, while the ewes ate a significantly higher quantity of this feed. However, also for the ewes this feed was not included among the preferred ones (Table 5.2). The aroma profile of corn gluten meal was characterized by the presence of four sulphur compounds (Table 5.7), perceived high during the sniff runs (data not shown). These

compounds gave to the feed unpleasant notes of garlic and cooked potato (Table 5.7). Thus, these bad characteristics probably affected negatively its palatability.

Soybean meal 44, soybean meal 49 and soybean hulls

The palatability tests carried out on lambs showed that among the soybean by-products soybean meal 49 had the highest intake, being the highest among all feeds tested, soybean hulls were intermediate and soybean meal 44 had the lowest intake, being less than half of that of soybean meal 49. The results were markedly different for the ewes, which found markedly unpalatable all soybean by-products tested (Table 5.2).

The different behavior of lambs compared to ewes can be explained in two ways several ways.

First, the requirements for protein were probably higher in lambs than in ewes. Since the basal diets included as main protein source urea, it is possible that there was an higher essential amino acid requirement by lambs than by ewes, which might have stimulated in lambs the drive to eat high protein feeds. Second, the ewes had a marked preference for 4 feeds often supplied as single ingredients (beet pulps, wheat grains, pea grains, and corn grains) and low intake or complete rejection of the remaining feeds, including several commonly used in sheep feed mixes but rarely supplied alone, such as the soybean by-products. Thus the ewes probably were not able to associate the soybean by-product flavour with their post-ingestive effects. Thus, during the palatability tests these feeds were considered as novel feeds and refused. In contrast, as said before the lambs explored most of feed options, refusing those they found unpalatable.

The much lower intake in lambs of soybean meal 44 compared to soybean meal 49, and, to a lower extent, to soybean hulls, could be explained by the presence in the former but not in the other two soybean by-products of methanamine, which gave an off-flavour identified as rotted fish odour (Table 5.5). This compound had a high perception during the sniffing run of the soybean meal 44 reported in the aromagram (data not shown). Soybean meal 44 contained another unique VOCs, the terpene α-thujene, which gives a pleasant floral note was detected like a unique compound (Table 5.8). However, its perception during the sniffing run was low (data not shown), thus probably could not positively affect the palatability of this feed.

Soybean hulls showed a richer aromatic profile with respect to soybean meal 44 and 49. Their lower intake compared to soybean meal 49 could be explained by the much lower content of protein of the hulls and, possibly, by their more fibrous texture.

Oat grains

Both lambs and ewes found oat grains very unpalatable (Table 5.2). Oat grains had the highest number of terpens among all feeds (Table 5.3). Some studies demonstrated that terpenes negatively affect feed palatability in lambs (Estell et al., 1996; Villalba et al., 2006; Dziba and Provenza, 2008). Among the terpenes of oat grains, there was the α-pinene (Table 8), which was found to influence negatively and linearly the intake of alfalfa pellets by lambs (Estell et al., 1998b). Thus, even if in this analysis the terpenes of oat grains were generally associated with pleasant notes, it is not possible to exclude that α-pinene may have negatively affected the palatability of this feed.

In addition, Molteberg et al. (1996) reported the formation of rancid odours and flavour and the formation of bitter taste in oat grains physically processed with their hulls. Since the oats used in experiment were ground with their hulls, the same deterioration just described might have occurred.

Table 5.3 - Principal chemical classes of volatile organic compounds found in each feed sample.

Chemical Class	Identified compound *														
	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
Aldehyde	6	8	11	3	5	2	7	4	10	2	8	5	6	4	5
Amine	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Ketone	3	3	4	1	2	0	2	2	3	1	4	3	4	2	1
Ester	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0
Lactone	1	2	2	0	0	2	0	1	1	0	1	0	1	0	0
Pyrazine	1	0	1	0	0	0	0	2	0	0	1	0	1	1	0
Sulphur	5	1	2	0	5	3	4	5	2	1	2	5	4	5	0
Terpene	3	0	5	1	0	0	1	0	7	1	3	1	1	1	0
Heterocyclic comp.	0	1	2	0	0	0	0	0	2	0	0	0	0	0	0
Total	20	15	27	5	12	7	14	14	24	5	19	16	18	13	6

AD: alfalfa, dehydrated; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49 (high protein); OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44 (normal protein); WB: wheat brans; WG: wheat grains.
* Not identified compounds are not shown

Table 5.4 - Aldehyde compounds extracted from the feed samples by SPME technique.

Compound	Chemical class	Descriptor	LRI [a]	Ident [b]	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
mercapto acetaldehyde	aldehyde	garlic	674	PI							*								
hexanal	aldehyde	green	798	PI,MS	*	*	*		*		*	*	*			*	*	*	*
2-hexenal	aldehyde	green	890	PI											*				
heptanal	aldehyde	rancid	899	PI,MS	*		*								*				
(Z)-2-heptenal	aldehyde	orange	901	MS			*						*						
octanal	aldehyde	orange	1002	PI,MS	*	*	*		*	*			*		*	*	*		
(Z)-2-octenal	aldehyde	nutty	1058	PI,MS		*	*		*				*		*		*		*
nonanal	aldehyde	orange	1104	PI,MS			*						*						
(E)-2-nonenal	aldehyde	green	1147	PI,MS	*	*	*	*			*	*	*	*	*	*	*	*	*
(Z)-2-nonenal	aldehyde	hay	1157	PI	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2,4-nonadienal	aldehyde	nutty	1215	PI	*	*	*	*	*		*	*	*		*	*	*	*	*
2-decenal	aldehyde	oil	1250	PI									*						
(E,E)-2,4-decadienal	aldehyde	fried oil	1318	PI		*	*				*		*		*				
2-undecenal	aldehyde	fruit	1354	PI			*												
vanillin	aldehyde	vanilla	1384	PI		*					*								
Total					6	8	11	3	5	2	7	4	10	2	8	5	6	4	5

[a] LRI, Linear Retention Indices, capillary column HP -5. [b] Identification: MS (Wiley library); PI (Internet Database: flavornet)
AD: alfalfa, dehydrated; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49; OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44; WB: wheat brans; WG: wheat grains.

Table 5.5 - Amine and ketone compounds extracted from the feed samples by SPME technique.

Compound	Chemical Class	Descriptor	LRI[a]	Ident.[b]	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
methanamine	amine	rotted fish	618	MS												*	*		
Total					0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
2,3-butanedione	ketone	butter	640	MS	*		*		*		*	*			*	*	*	*	
heptanone	ketone	solvent	889	PI													*		
1-octen-3-one	ketone	mushroom	980	PI,MS	*	*	*	*	*		*	*	*	*	*	*	*	*	*
(Z)-1,5-octadien-3-one	ketone	geranium	983	PI	*														
octanone	ketone	orange	998	PI		*	*								*				
3,5-octadien-2-one	ketone	orange	1094	PI,MS		*	*						*		*	*	*		
undecanone	ketone	peach	1295	PI									*						
Total					3	3	4	1	2	0	2	2	3	1	4	3	4	2	1

[a] LRI, Linear Retention Indices, capillary column HP-5. [b] Identification: MS (Wiley library); PI (Internet Database: flavornet)
AD: alfalfa, dehydrated; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49; OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44; WB: wheat brans; WG: wheat grains.

Table 5.6 - Ester, lactone and pyrazine compounds extracted from the feed samples by SPME technique.

Compound	Chemical class	Descriptor	LRI [a]	Ident [b]	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
isobutyl acetate	ester	fruit	773	PI	*														
ethyl methylbutyrate	ester	fruit	844	PI												*			
ethyl octenoate	ester	fruit	1206	PI									*						
Total					1	0	0	0	0	0	0	0	1	0	0	1	0	0	0
γ- butyrolactone	lactone	garlic	874	MS						*									
γ- heptalactone	lactone	peach	1248	MS	*	*	*			*		*	*		*		*		
γ- nonalactone	lactone	peach	1365	PI		*	*												
Total					1	2	2	0	0	2	0	1	1	0	1	0	1	0	0
ethyl dimethyl pyrazine	pyrazine	burnt, nutty	1086	PI	*		*					*			*		*	*	
diethyl methyl pyrazine	pyrazine	nutty	1160	PI								*							
Total					1	0	1	0	0	0	0	2	0	0	1	0	1	1	0

[a] LRI, Linear Retention Indices, capillary column HP -5. [b] Identification: MS (Wiley library); PI (Internet Database: flavornet)
AD: dehydrated alfalfa; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49; OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44; WB: wheat brans; WG: wheat grains.

Table 5.7 - Sulphur compounds extracted from the feed samples by SPME technique.

Compound	Chemical Class	Descriptor	LRI [a]	Ident. [b]	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
methyl ethyl sulfide	sulphur	garlic	631	PI	*		*		*		*	*	*			*		*	
thiophene	sulphur	garlic	668	PI	*		*		*		*	*			*	*	*	*	
methyl furanthiol	sulphur	garlic, meat	865	PI						*						*			
methional	sulphur	cooked potato	908	PI	*				*		*	*				*	*	*	
dimethyl sulfone	sulphur	garlic	928	PI						*									
dimethyl trisulfide	sulphur	garlic	967	PI,MS	*				*		*	*			*	*	*	*	
ethyl dimethyl thiazole	sulphur	mushroom	1080	PI		*							*						
methyl dithiofurane	sulphur	meat	1175	PI	*				*			*		*			*	*	
dimethyl tetrasulfide	sulphur	garlic	1221	PI						*									
Total					5	1	2	0	5	3	4	5	2	1	2	5	4	5	0

[a] LRI, Linear Retention Indices, capillary column HP-5. [b] Identification: MS (Wiley library); PI (Internet Database: flavornet)
AD: dehydrated alfalfa; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49; OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44; WB: wheat brans; WG: wheat grains.

Table 5.8 - Terpene compounds extracted from the feed samples by SPME technique.

Compound	Chemical class	Descriptor	LRI [a]	Ident [b]	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
α-thujene	terpene	floral	931	PI,MS													*		
α-pinene	terpene	resin, pine	943	PI									*						
dihydro linalool	terpene	wood	1053	PI				*						*	*	*			
trans-sabinene hydrate	terpene	nutty	1110	PI			*						*		*				
menthone	terpene	green	1125	PI											*				
(E)-linalool oxide	terpene	green	1174	PI			*												
(Z)-dihydrocarvone	terpene	green	1193	PI			*												
geranial	terpene	lemon	1278	PI									*						
ρ-menthenethiol	terpene	sweet	1280	PI									*						
methyl geranate	terpene	floral	1305	PI							*							*	
dihydro carvyl acetate	terpene	solvent	1334	PI	*														
δ-elemene	terpene	coconut	1346	PI									*						
eugenol	terpene	spicy	1367	PI									*						
α-copaene	terpene	spice	1382	PI,MS	*		*						*						
geosmin	terpene	beet,earth	1410	PI,MS			*												
β-patchoulene	terpene	rose, plant	1412	MS	*														
Total					3	0	5	1	0	0	1	0	7	1	3	1	1	1	0

[a] LRI, Linear Retention Indices, capillary column HP -5. [b] Identification: MS (Wiley library); PI (Internet Database: flavornet)
AD: dehydrated alfalfa; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49; OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44; WB: wheat brans; WG: wheat grains.

Table 5.9 - Heterocyclic compounds extracted from the feed samples by SPME technique.

Compound	Chemical class	Descriptor	LRI [a]	Ident [b]	AD	BA	BP	CG	CM	CN	GL	HP	OG	PG	SH	SM	SN	WB	WG
phenyl acetic acid	heterocyclic comp.	sweet	1263	PI									*						
safrole	heterocyclic comp.	spice	1273	PI		*	*						*						
methyl cinnamate	heterocyclic comp.	sweet up	1374	PI			*												
Total					0	1	2	0	0	0	0	0	2	0	0	0	0	0	0

[a] LRI, Linear Retention Indices, capillary column HP-5. [b] Identification: MS (Wiley library); PI (Internet Database: flavornet)
AD: dehydrated alfalfa; BA: barley meal; BP: beet pulps; CG: corn grains; CM: corn middlings; CN: canola meal; GL: corn gluten meal; HP: soybean meal 49; OG: oat grains; PG: pea grains; SH: soybean hulls; SM: sunflower meal; SN: soybean meal 44; WB: wheat brans; WG: wheat grains.

5.6 Conclusions

In this preliminary trial, the feeds offered to lambs and ewes differed in their aroma profile for the total number of VOCs as well as for the chemical classes of volatile compounds present. Some interesting findings regarded especially the sulphur compounds, which in many cases seemed to negatively influence the palatability of the tested feeds both in lambs and ewes. Moreover, the class of terpenes seemed to negatively affect the palatability of oat grains. However, it was not possible to find for all feeds a clear and definite relationship between the intrinsic aromatic characteristics of the feeds and their palatability. In fact, the choice of lambs and ewes was also affected by their previous feeding experiences (especially for ewes) and their nutritional requirements (especially for lambs). Therefore, further studies are needed to deeply investigate the interactions among the flavours of concentrate feeds and their effects on feed intake in ruminants.

References

1. A.O.A.C. 1990. Official methods of analysis. 15th ed. Assoc. Off. Anal. Chem. Washington D.C., USA.

2. Acree, T.E., and J. Barnard. 1994. Gas chromatography-olfactometry using Charm analysis. In: Proc. 7th Weurman flavour research symposium, Amsterdam, Netherlands. pp, 211-220.

3. Acree, T.E. 1993. Bioassay for flavor. In: T.E. Acree and R. Teranshi (Eds), Flavor Science: Sensible principles and techniques, ASC Professional Reference Handbook, Washington, D.C.

4. Acree, T.E., J. Barnard, and D.G. Cunningham. 1984. A procedure for the sensory analysis of gas chromatographic effluents. Food Chemistry 14, 273-86.

5. Acree, T.E., R.M. Butts, and R.R. Nelson. 1976. Sniffer to determine the odour of gas chromatographic effluents. Anal. Chem. 48, 1821-1822.

6. Adda, J. 1986. Flavour of dairy products. In: G.G. Birch and M. G. Lindley (Eds), Developments in Food Flavours. Elsevier Applied Science Publishers, New York. pp, 151-172.

7. Adda, J., J.C. Gripon, and L. Vassal. 1982. The chemistry of flavour and texture generation in cheese. Food Chem. 9, 115–129.

8. Allen, C., and O.W. Parks. 1975. Evidence for methional in skim milk exposed to sunlight. J. Dairy Sci. 58, 1609-1611.

9. Alm, L. 1982. Effect of fermentation on volatile acids and ethanol in Swedish dairy products. J. Dairy Sci. 65, 186-190.

10. Ampuero, S., and J.O. Bosset. 2003. The electronic nose applied to dairy products: A review. Sensors and Actuators B. 94, 1-12.

11. Arn, H., and T.E. Acree. 1997. Flavornet. Worldwide Web resource: http://www.nysaes.cornell.edu/flavornet/index.html.

12. Arsenos, G., J. Hills, and B.J. Tolkamp. 2000b. Conditioned feeding responses of sheep towards flavoured foods associated with the administration of ruminally degradable and/or undegradable protein sources. Anim. Sci. 71, 597-606.

13. Arsenos, G., J. Hills, and I. Kyriazakis. 2000a. Conditioned feeding responses in sheep towards flavoured foods associated with casein administration: the role of long delay learning. Anim. Sci. 70, 157-169.

14. Aston, J.W, and K. Douglas. 1981. Detection of carbonyl sulphide in Cheddar cheese headspace. J Dairy Res 48, 473-478.

15. Aston, J.W., and K. Douglas. 1983. The production of volatile sulphur compounds in Cheddar cheeses during accelerated ripening. Aust. J. Dairy Technol. 38, 66–70.

16. Atwood, S.B., F.D. Provenza, R.D. Wiedmeier, and R.E. Banner. 2001. Changes in preferences of gestating heifers fed untreated or ammoniated straw in different flavours. J. Anim. Sci. 79, 3027-3033.

17. Badings, H.T. 1991. Milk. In: H. Maarse (Ed), Volatile Compounds in Foods and Beverages. Marcel Dekker, New York. pp, 91-106.

18. Badings, H.T., J.J.G. van der Pol, and R. Neeter. 1981. Aroma compounds which contribute to the difference in flavour between pasteurized milk and UHT milk. In: P. Schreier (Ed), Flavour '81. Walter de Gruyter, New York. pp, 683-692.

19. Barbeiri, G., L. Bolzoni, M. Careri, A. Mangia, G. Parolari, S. Spagnoli, and R.Virgili. 1994. Study of the volatile fraction of Parmesan cheese. J. Agric. Food Chem. 42, 1170–1176.

20. Barlow, I., G.T. Lloyd, E.H. Ramshaw, A.J. Miller, G.P. McCabe, and L. McCabe. 1989. Correlations and changes in flavour and chemical parameters of Cheddar cheeses during maturation. Aust. J. Dairy Technol. 44, 7–18.

21. Belitz, H.D., and W. Grosch. 1986. Food chemistry. In: D. Hadziyev (Ed), Aroma Substances. Translation from German, Springer-Verlag, New York, pp, 257–303.

22. Bemelmans, J.M.H. 1979. Review of isolation and concentration techniques. In: D.G. Land and H.E. Nursten (Eds), Progress in Flavour Research. Appl Sci Publ, London. pp, 79-98.

23. Benkler, K.F, and G.A. Reineccius. 1980. Flavor isolation from fatty foods via solvent extraction and membrane dialysis. J. Food Sci. 45, 1084-1085.

24. Bennett, G., B.J. Liska, and W.L. Hempenius. 1965. Effect of other flavor components on the perception of diacetyl in fermented dairy products. J. Food Sci. 30, 35-37.

25. Bosset, J.O., and R. Gauch. 1993. Comparison of the volatile flavour compounds of six European 'AOC' cheeses by using a new dynamic headspace GC-MS method. Int. Dairy J. 3, 359-377.

26. Bosset, J.O., B. Jeangros, T. Berger, U. Butikofer, M. Collomb, R. Gauch, P. Lavanchy, J. Scehovic, J. Troxler, and R. Sieber. 1999. Comparaison de fromages

à pate dure de type Gruyère produits en règions de montagne et de plaine. Revue suisse Agric. 31, 17-22.

27. Bosset, J.O., R. Gauch, R. Mariaca, and B. Klein. 1995. Comparison of various sample treatments for the analysis of volatile compounds by GC-MS: application to the Swiss Emmental cheese. Mitt. Geb. Lebensm. Hyg. 86, 672–698.

28. Bosset, J.O., U. Butikofer, R. Gauch, and R. Sieber. 1997. Ripening of Emmental cheese wrapped in foil with and without addition of Lactobacillus casei subsp. casei. II. Gas chromatographic investigation of some volatile neutral compounds using dynamic headspace. Lebensm. Wiss. u. Technol. 30, 464–470.

29. Brillat-Savarin, J.A. 1825. The Physiology of Taste. Harcourt Brace Jovanovich, New York.

30. Buchin, S., J.C. Salmon, A.P. Carnat, T. Berger, C. Bugaud, and J.O. Bosset. 2002. Identification de composès mono-terpèniques, sesquiterpèniques et benzèniques dans un lait d'alpage très riche en ces substances. Mitt. Lesbensm. Hyg. 93, 199-216.

31. Bugaud, C., S. Buchin, A. Hauwuy, and J. B. Coulon. 2001b. Relationships between flavour and chemical composition of Abundance cheese derived from different types of pastures. Lait 81, 757–773.

32. Bugaud, C., S. Buchin, J.B. Coulon, A. Hauwuy, and D. Dupont. 2001a. Influence of the nature of alpine pastures on plasmin activity, fatty acid and volatile compound composition of milk. Lait 81, 401–414.

33. Bugaud, C., S. Buchin, Y. Noel, L. Tessier, S. Pochet, B. Martin, and J.F. Chamba. 2001c. Relationships between Abundance cheese texture, its composition and that of milk produced by cows grazing different types of pastures. Lait 81, 593–607.

34. Burdge, J.R., and S.A. Farwell. 1994. Comparison of combustion sources for sulfur chemiluminescence detection. J. High Resol. Chromatogr. 17, 22-24.

35. Burritt, E.A., and F.D. Provenza. 1992. Lambs form preferences for non nutritive flavours paired with glucose. J. Anim. Sci. 70, 1133-1136.

36. Burton, H. 1986. Monograph on pasteurized milk. Microbiological aspects. Bulletin 200. Brussels: Int. Dairy Federation.

37. Burton, H. 1988. Ultra-High Temperature Processing of Milk and Milk Products; Elsevier Applied Science Publishers, New York.

38. Cain, W.S. 1979. To know with the nose: keys to odour identification. Science 203, 467-470.

39. Cannas, A., A. Mereu, M. Decandia, and G. Molle. 2009. Role of the sensorial perceptions in feed selection and intake by ruminants. Ital. J. Anim. Sci. 8 (suppl. 2), 243-251.

40. Careri, M, P. Manini, S. Spagnoli, G. Barbieri, and L. Bolzoni. 1994. Simultaneous distillation-extraction and dynamic headspace methods in the gas chromatography analysis of Parmesan cheese volatiles. Chromatographia 38, 386-394.

41. Carpino, S. 1997. Native Sicilian Pastures: Nutritional and Phytochemical Studies. Master Thesis.

42. Carpino, S**.**, Mallia S., Horne J., and Licitra G. 2002. Odor profile of typical Sicilian cheeses: Maiorchino, Pecorino, Provola dei Nebrodi and Ricotta Infornata. Caseus International 3, 33-37.

43. Carpino, S, T. Rapisarda, G. Belvedere, P. Papademas, M. Neocleous, I. Schadt, C. Pasta, and G. Licitra. 2010. Effect of dehydration by sun or by oven on volatiles and aroma compounds of Trachanas. Dairy Sci. Technol. 90, 715-727.

44. Carpino, S., E. Lavin, S. Mallia, S. La Terra, G. Licitra, and D.M. Barbano, 2004. Composition and aroma compounds of Ragusano cheese made from milk produced by cows fed native pasture and TMR diet. J. Dairy Sci. 87, 816-830.

45. Carpino, S., S. Mallia, S. La Terra, C. Melilli, G. Licitra, T.E. Acree, D.M. Barbano, and P.J. Van Soest. 2004. Composition and Aroma Compounds of Ragusano Cheese: Native Pasture and Total Mixed Rations. J. Dairy Sci. 87, 816-830.

46. Carpino, S., S. Mallia, G. Licitra, P.J. Van Soest, and T.E. Acree. 2004. Aroma compounds of some Hyblean pasture species. Flavour Fragr. J. 19, 293–297.

47. Carpino, S., L. Tuminello, T. Rapisarda, and G. Licitra. Piacentinu Ennese: Stato dell'arte. Sci. Tecnica Lattiero-Casearia 61, 187-217.

48. Casey, J., and R. Dobb. 1992. Microbial routes to aromatic aldehydes. Enzyme Microbiol. Technol. 14, 739–747.

49. Cogan, T.M. 1985. The Leuconostocs: milk products. In: S.E. Gilliland (Ed.), Bacterial Starter Cultures for Foods. CRC Press, Boca Raton, FL. pp, 25–40.

50. Cogan, T.M., and C. Hill. 1993. Cheese starter cultures. In: P.F. Fox (Ed.), Cheese: Chemistry, Physics and Microbiology. Chapman and Hall, London, 2nd ed. pp. 193–255.

51. Coulibaly, K., and I.J. Jeon. 1992. Solid-phase extraction of less volatile flavour compounds from ultra-high temperature processed milk. J. Agric. Food Chem. 40, 612-616.

52. Cronin, D.A. 1982. Techniques of analysis of flavours. ln: I.D. Morton and A.L. (Eds.), Food Flavours. Part A. Introduction Elsevier, Amsterdam. pp, 15-48.

53. D'Auria, M., G. Mauriello, and G.L. Rana. 2004. Volatile organic compounds from saffron. J. Flavour Frag. 19, 17–23.

54. Day, E.A. 1967. Cheese flavor. In: H.W. Schultz, E.A. Day and L.M. Libbey (Eds.), Symposium on Foods: The Chemistry and Physiology of Flavors. Avi Publ, Westport, CT. pp, 331-361.

55. de Frutos, M., J. Sanz, and I. Martinez-Castro. 1991. Characterization of Artisanal Cheeses by GC and GC/MS Analysis of Their Medium Volatility (SDE) Fraction. J. Agric. Food Chem. 39, 524–530.

56. Deibler, K.D., T.E. Acree, and E.H. Lavin. 1999. Solid Phase Microextraction in Gas Chromatography-Olfactometry Dilution Analysis. J. Agric. Food Chem. 47, 1616-1618.

57. Dimick, P.S., N.J. Walker, and S. Patton. 1969. Occurrence and biochemical origin of aliphatic lactones in milk fat and A.J. Miller. 1996. Changes in flavour and volatiles of full-fat and low-fat cheeses during maturation. Int. Dairy J. 6, 981–995.

58. Dougherty, R.W., W.F. Shipe, G.V. Gudnason, R.A. Ledford, R.D. Peterson, and R. Scarpellino. 1962. Physiological mechanisms involved in transmitting flavors and odours to milk. I. Contribution of eructated gases to milk flavor. J. Dairy Sci. 45, 472–476.

59. Drake, M.A., P.D. Gerard, J.P. Kleinhenz, and W.J. Harper. 2003. Application of an electronic nose to correlate with descriptive sensory analysis of aged Cheddar cheese Lebensmittel-Wissenschaft und Technol. 36, 13-20.

60. Dumont, J.P., J. Adda, and P. Rousseaux. 1981. Example de variation de l'arôme à l'interieur d'un même type de fromage: Le Comté. Lebensm. Wiss. Technol. 14, 198–202.

61. Dunn, H.C., and R.C. Lindsay. 1985. Evaluation of the role of microbial Strecker-derived aroma compounds in unclean-type flavours of Cheddar cheese. J. Dairy Sci. 68, 2859–2874.

62. Dunn, W.B. 2008. Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. Physical Biology 5.

63. Dziba, L. E., and F. D. Provenza. 2008. Dietary monoterpene concentrations influence feeding patterns of lambs. Appl. An. Beh. Sci. 109, 49-57.

64. Estell, R. E., E. L. Fredrickson, D. M. Anderson, K. M. Havstad, and M. D. Remmenga. 1996a. Tarbush leaf surface terpene profile in relation to mammalian herbivory. Pages 237–241 in Proc. Symposium on Shrubland Ecosystem Dynamics in a Changing Climate. Gen. Tech. Rep. INT-GTR-338, U.S., Ogden, UT.

65. Estell, R.E., E.L. Fredrickson, M.R. Tellez, K.M. Havstad, W.L. Shupe, D.M. Anderson, and M.D. Remmenga. 1998b. Effect of volatile compounds on consumption of alfalfa pellets by sheep. J. Anim. Sci. 76, 228–233.

66. Fabre, M., V. Aubry, and E. Guichard. 2002. Comparison of different methods: static headspace and solid-phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. J. Agric. Food Chem. 50, 1497-1501.

67. Fallico, V., L. Tuminello, C. Pediliggieri, J. Horne, S. Carpino, and G. Licitra. 2006. Proteolysis and microstructure of Piacentinu Ennese Cheese made using different farm technologies. J. Dairy Sci. 89, 37–48.

68. Fernández-Garcia, E. 1996. Use of Headspace Sampling in the Quantitative Analysis of Artisanal Spanish Cheese Aroma. J. Agric. Food Chem. 44, 1833-1839.

69. Ferretti, A., and V.P. Flanagan. 1972. Steam volatile constituents of state nonfat dry milk. The role of the Maillard reaction in staling. J. Agric. Food Chem. 20, 695-698.

70. Ferretti, A., and V.P. Flanagan. 1971. The lactose-casein (Maillard) browning system: volatile components. J. Agric. Food Chem. 19, 245-249.

71. Finney, D.A. 1978. Statistical Method in Biological Assay. Charles Griffin and Co., London.

72. Foda, F.A., E.G. Hammond, G.W. Reinbold, and D.K. Hotchkiss. 1974. Role of fat in flavor of Cheddar cheese. J. Dairy Sci. 57, 1137–1142.

73. Forss, D.A. 1981. Brief reviews of dairy and soy products. In: R. Teranishi and R.A. Flath (Eds), Flavor Research. Recent Advances; Marcel Dekker, Inc.: New York. pp, 335-372.

74. Forss, D.A. 1969. Flavors of dairy products: a review of recent advances. J. Dairy Sci. 52, 832-840.

75. Forss, D.A. 1971. The flavors of dairy fats - a review. J. Am. ail Chem. Soc. 48, 702-710.

76. Forss, D.A. 1972. Odour and flavor compounds from lipids. In: R.T. Holman (Ed), Progress in the Chemistry of Fats and Other Lipids. Perga mon Press, Oxford. 13, 177-258.

77. Forss, D.A. 1979. Review of the progress of Dairy Science: mechanisms of formation of aroma compounds in milk and milk products. J. Dairy Res. 46, 691-706.

78. Fox, P.F., J.A. Lucey, and T.M.Cogan. 1990. Glycolysis and related reactions during cheese manufacture and ripening. Crit. Rev. Food Sci. Nutr. 29, 237–253.

79. Frederick, G., J.M. Forbes, and C.L. Johnson. 1988. Masking the taste of rapeseed meal in dairy compound feed. Anim. Prod. 46, 518.

80. Friedrich, J.E. 2000. Formulation of a standard odourant mixture to test individuals for specific anosmia. Ph.D. dissertation, Cornell University.

81. Friedrich, J.E., and T.E. Acree. 1998. Gas Chromatography Olfactometry (GC/O) of Dairy Products. Int. Dairy J. 8, 235-241.

82. Belvedere, G. and T. Rapisarda. 2010. Il Pecorino Siciliano ha cinque cru. Caseus, Anno XV n.2, 38.

83. Galliard, T., and H.W.S. Chan. 1980. Lipoxygenases. In: P.K. Stumpf (ed), The Biochemistry of Plants. Vol. 4. Lipids: Structure and Function. Academic Press, New York, NY.

84. Genovese, A, P. Piombino, A. Gambuti, and L. Moio. 2009. Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. Food Chem. 114, 100-107.

85. Georgala A.I.K., E. Tsakalidou, I Kandarakis, and G Kalantzopoulos. 1995. Flavour production in ewes milk and ewe's milk yoghurt, by single strains and combinations of Streptococcus thermophilus and Lactobacillus delbrueckii subsp bulgaricus, isolated from traditional Greek yoghurt. Lait 75, 271-283.

86. Ghosh, S., and J.N. Coupland. 2007. Influence of food microstructure on flavours interactions. In: D.J. McClements (Ed), Understanding and controlling the microstructure of complex foods. Woodhead Publishing Limited, Abington, Cambridge, England, pp, 425-444.

87. Gómez-Ruiz, J.A., C. Ballesteros, M.Á. González Viñas, L. Cabezas, and I. Martínez-Castro. 2002. Relationships between volatile compounds and odour in Manchego cheese: comparison between artisanal and industrial cheeses at different ripening times. Lait 82, 613-628.

88. Gripon, J.C. 1993. Mould-ripened cheeses. In: P.F. Fox (Ed.), Cheese: Chemistry, Physics and Microbiology. Chapman and Hall, London, UK, Vol. 2. pp, 111–136.

89. Grosch, W. 1990. Analyse von Aromastoffen. Chem. Unserer Zeit 24, 82-89.

90. Grosch, W., M. Preininger, R. Warmke, and H.D. Belitz. 1995. Studies of the ßavour of Swiss cheese (Emmentaler). In: Aroma: Perception, Formation, Evaluation, eds. M. Rothe and H. P. Kruse. Deutsches Institut for Ernahrungsforschung, Potsdam-Rehbrucke, pp, 425-439.

91. Guth, H., and W. Grosch. 1994. Identification of the character impact odorants of stewed beef juice by instrumental analyses and sensory studies. J. of Agric. and Food Chem. 42, 2862-2866.

92. Guth, H., and W. Grosch. 1999. In: R. Teranishi, E.L. Wick, and I. Hornstein (Eds), Flavor Chemestry. New York:Kluwer Academic/Plunum. pp, 377-386.

93. Ha, J.K., and R.C. Lindsay. 1991. Contribution of cows, sheep, and goat milks to characterizing branched-chain fatty acids and phenolic flavours in varietal cheese. J. Dairy Sci. 74, 3267-3274.

94. Heyns, K., and M. Klier. 1968. Browning reactions and fragmentation of carbohydrates. IV. Comparison o-o-cladinosyl-5-o-desoaminyl-6,11,12-trihydroxy-2,4,6,8,10,12-trihydroxy-2,4,6,8,10,12-hexamethylpentadecane-13-o. Carbohydr. Res. 6, 436-448.

95. Heyns, K., R. Stute, and H. Paulsen. 1966. Browning reaction and fragmentation of carbohydrates. I. Volatile products from the thermal degradation of D-glucose. Carbohydr. Res. 2, 132-149.

96. Ho, C., and Q. Chen. 1994. Lipids in food flavors: An overview. In: ACS SymposiumSeries 558, Washington, DC. pp, 2–29.

97. Hodge, J.E. 1967. Origin of flavor in foods. Nonenzymatic browning reactions. In: H.W. Schultz; E.A. Day and L.M. Libbey (Eds), Symposium on Foods: The Chemistry and Physiology of Flavors. AVI Publishing Co., Westport, CN. pp, 465-491.

98. Horne, J., S. Carpino, L. Tuminello, T. Rapisarda, L. Corallo, and G. Licitra. 2005. Differences in volatiles, and chemical, microbial and sensory characterization between artisanal and industrial Piacentinu Ennese cheeses. Int. Dairy Sci. 15, 605-617.

99. Hsieh, J.R. 1994. Contribution of lipoxygenase pathway to food flavors. In: ACS Symposium Series 558, Washington, DC. pp, 30–48.

100. Hugenholtz, J. 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. 12, 165–168.

101. Hutton, J.T., and S. Patton. 1952. The origin of sulfhydryl groups in milk proteins and their contribution to "cooked" flavor. J. Dairy Sci. 35, 699-705.

102. Imhof, R., and J.O. Bosset. 1994. Relationships between micro-organism and formation of aroma compounds in fermented dairy products (Review). Z. Lebensm Unters Forsch. 198, 267-276.

103. Jaillais, B., V. Bertrand, and J. Auger. 1999. Cryo-trapping/SPME/GC analysis of cheese aroma. Talanta. 48, 747-753.

104. Jain, M.K., and O.P. Singhal. 1969. Chemistry of ghee flavours. II. Variation in carbonylic flavour compounds in ghee made from desi butter. Indian J. Dairy Sci. 22, 103-108.

105. Jennings, W.G., and M. Filsoof. 1977. Comparison of sample preparation techniques for gas chromatographie analysis. J. Agric. Food Chem. 25, 440-445.

106. Jeon, I.J, E.L. Thomas, and G.A. Reineccius. 1978. Production of volatile flavor compounds in ultra-high temperature processed milk during aseptic storage. J. Agric. Food Chem. 26, 1183-1188.

107. Jou, K.D., and W.J. Harper. 1998. Pattern recognition of Swiss cheese aroma compounds by SPME/GC and an electronic nose. Milchwissenschaft 53, 159-263.

108. Karahadian, C., and R.C. Lindsay. 1987. Integrated roles of lactate, ammonia, and calcium in texture development of mold surface-ripening cheese. J. Dairy Sci. 70, 909–918.

109. Keeney, P.G., and S. Patton. 1956. The coconut-flavor defect of milk fat. I. Isolation of the flavor compound from butter oil and its identification as delta-decalactone. J. Dairy Sci. 39, 1104-1113.

110. Keeney, P.G., and S. Patton. 1956. The coconut-flavor defect of milk fat. II. Demonstration of delta-decalactone in dried cream, dry whole milk, and evaporated milk. J. Dairy Sci. 39, 1114-119.

111. Kinsella, J.E. 1975. Butter flavor. Food Technol. 29, 82-98.

112. Kittel, K.M., and T.E. Acree. 2006. Using gas chromatography-olfactometry (GCO) to measure varying odourant-specific sensory deficits (OSDs). Dev. Food Sci. 43, 25-28.

113. Kosikowski, F.V. 1957. Cheese flavor. In: J.H. Mitchell et al. (Eds), Chemestry of Natural Food Flavors. Quartermaster Research and Engineering Center, Washington, D.C.

114. Koster, E.P. 1965. Olfactory sensitivity and the menstrual cycle. Inter. Rhinology 1, 57.

115. Koster, E.P. 1968. Olfactory sensitivity and ovulatory cycle duration. Olfactologia 1, 43.

116. Lacrampe, J.L., M. Dherbomez, and F. Weber. 1975. Problèmes d'extraction et d'analyse de certains composés aromatiques des produits laitiers. 1. Techniques de concentration et d'extraction. Lait 55, 517-529.

117. Laing, D., and A. Jinks. 1996. Flavour perception mechanisms. Trends in Food Sci. Technol. 7, 387-389.

118. Lamparsky, D., and I. Klimes. 1981. Cheddar cheese flavour - its formation in the light of new analytical results. ln: P. Schreier (Ed.), Flavour '81. 3rd Weumum Symp. Proc. lnt Conf Munich, W de Gruyter, Berlin. pp, 557-577.

119. Lawrence, R.C., and J.C. Hawke. 1966. The incorporation of acetate-1-14C into the methyl ketones that occur in steam distallates of bovine milk fat. Biochem. J. 98, 25-29.

120. Laye, I., D. Karleskind, and C.V. Morr. 1995. Dynamic headspace analysis of aceelerated storage commercial whey protein coneentrate using four different adsorbent traps. Milchwissenschaft 50, 268-272.

121. Lecanu, L., V. Ducruet, C. Jouquand, J.J. Gratadoux, and A. Feigenbaum. 2002. Optimization of Headspace Solid-Phase Microextraction (SPME) for the Odour Analysis of Surface-Ripened Cheese. J. Agric. Food Chem. 50, 3810-3817.

122. Lemieux, L., and R.E. Simard. 1992. Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition. Lait 72, 335–382.

123. Lenoir, J. 1984. The surface flora and its role in the ripening of cheese. Int. Dairy Fed. Bull. 171, 3–20.

124. Lewinsohn, E., Y. Sitrit, E. Bar, Y. M. Azulay, M. Ibdah, A. Meir, E. Yosef, D. Zamir, and Y. Tadmor. 2005. Not just colors carotenoids degradation as a link between pigmentation and aroma in tomato and watermelon fruit. Trends Food Sci. Tech. 16, 407-415.

125. Liardon, R., J.O. Bosset, and B. Blanc. 1982. The aroma composition of Swiss Gruyère cheese. 1. The alkaline volatile components. Lebensm Wiss Technol. 15, 143-147.

126. Licitra, G., G. Leone, F. Amata, and D. Mormorio. 2000. Heritage and Landscape: The Art of Traditional Ragusano Cheese-Making. F. Motta (Ed.). Ragusa, Italy: Consorzio Ricerca Filiera Lattiero-Casearia.

127. Lortal, S., Di Blasi A., Madec M.N., Pediliggieri C., Tuminello L., Tanguy G., Fauquant J., Lecuona Y., Campo P., Carpino S., and Licitra G. 2009. Tina wooden vat biofilm: A safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int. J. of Food Microb. 132, 1-8.

128. Lund, E.D. 1994. Determination of 2-methoxy-3- alkylpyrazines in carrot products by gas chromatography/ nitrogen-phosphorus detection. J. AOAC Int. 7, 416-420.

129. Manning, D.J., and C. Moore. 1979. Headspace analysis of hard cheeses. J. Dairy Res. 46, 539-545.

130. Manning, D.J., and J.C. Priee. 1983. Instrumental methods in determining flavour. J. Soc. Dairy Technol. 36, 33-35.

131. Mariaca, R., and J.O. Bosset. 1997. Instrumental analysis of volatile (flavour) compounds in milk and dairy products. Lait 77, 13-40.

132. Mariaca, R., M. Imhof, and J.O. Bosset. 2001. Occurrence of volatile chiral compounds in dairy products, especially cheese-A review. Eur. Food Res. Technol. 212-253.

133. Mariaca, R.G., T.F.H. Berger, R. Gauch, M.I. Imhof, B. Jeangros, and J.O. Bosset. 1997. Occurrence of volatile mono- and sesquiterpenoids in highland and lowland plant species as possible precursors for flavor compounds in milk and dairy products. J. Agric. Food Chem. 45, 4423–4434.

134. Marin, A.B., T.E. Acree, and J. Barnard. 1988. Variation in odour detection thresholds determined by Charm analysis. Chem. Senses 13, 435-444.

135. Marin, A.B., J. Barnard, R.B. Darlington, and T.E. Acree. 1991. sensory thresholds:estimation from dose-response curves. J. Sensory Studies pp, 205-225.

136. Martin, J.P., and R.L.M. Synge. 1941. A new form of chromatogram employing two liquid phases. Biochem. J. 35, 1358.

137. Mayol, A.R., and T.E. Acree. 2001. Advances in Gas Chromatography-Olfactometry. Chapter 1 in Gas Chromatography-Olfactometry the state of the art. ACS Symp. Series 782 Am. Chem. Soc. Washington DC. pp, 1-10.

138. McSweeney, P.H.L., and M.J. Sousa. 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening:A review. Lait 80, 293–324.

139. McSweeney, P.L.H., and P.F. Fox. 1997. Indices of Cheddar cheese ripening. Proc. 5th Cheese Symposium, Moorepark, Fermoy, Co. Cork, Ireland. pp, 73–89.

140. Meinhart, E., and P. Schreier. 1986. Study of flavour compounds from Parmagiano Reggiano cheese. Milchwissenschaft 41, 689–691.

141. Mereu, A. 2009. Palatability of concentrates fed to sheep. Ph.D Diss. University of Sassari, Sassari, Italy.

142. Meynier, A., A. Garillon, L. Lethuaut, and C. Genot. 2003. Partitioning of five aroma compounds between air and skim milk, anhydrous milk fat or full fat cream. Lait 83, 223-235.

143. Milo, C., and G.A. Reineccius. 1997. Identification and Qualification of Potent Odorants in Regular-Fat and Low-Fat Mild Cheddar Cheese. J. Agric. Chem. 45, 3590-3594.

144. Mistry, B.S., G.A. Reineccius, and B.L. Jasper. 1994. Comparison of gas chromatographie detectors for the analysis of volatile sulfur compounds in foods. ln: ACS Symp Ser 564. Sulfur Compounds in Foods (Mussinan CJ, Keelan ME, eds) Am Chem Soc, Washington DC, 8-21.

145. Moco, S., J. Vervoort, S. Moco, R.J. Bino, R.C.H. De Vos, and R. Bino. 2007. Metabolomics technologies and metabolite identification. TrAC - Trends in Analytical Chem. 26, 855-866.

146. Moio L., J. Dkimp, P. Etievant, and F. Addeo. 1993a. Neutral volatile compounds in the raw milks from different species. J. Dairy Res. 60, 199-213.

147. Moio, L., and F. Addeo. 1998. Grana Padano cheese aroma. Journal of Dairy Research, 65, 317-333.

148. Moio, L., D. Langlois, P. Etievant, and F. Addeo. 1993b. Powerful odorants in bovine, ovine, caprine and water buffalo milk determined by means of gas chromatography-olfactometry. J. Dairy Res. 60, 215-222.

149. Moio, L., P. Piombino, and F. Addeo. 2000. Odour-impact compounds of Gorgonzola cheese. J. Dairy Res. 67, 273-285.

150. Moio, L., P. Etievant, D. Langlois, J. Dekimpe, and F. Addeo. 1994. Detection of powerful odorants in heated milk by use of extract dilution sniffing analysis. J. Dairy Res. 61, 385-394.

151. Molimard, P., H.E. Spinnler. 1996. Review: compounds involved in the flavour of surface mold-ripened cheeses: origins and properties. J. Dairy Sci. 79, 169–184.

152. Molteberg, E.L., R. Solheim, L.H. Dimberg, and W. Frolich. 1996. Variation in oat groat due to variety, storage and heat treatment. II: sensory quality. J. Cereal Sci. 24, 273-282.

153. Moncrieff, R.W. 1967. the Chemical Senses. 2nd Ed. Leonard Hill Books, London.

154. Monnet, C., P. Schmitt, and C. Divies. 1994. Method for assaying volatile compounds by headspace gas chromatography and application to growing starter cultures. J Dairy Sci 77, 1809-1815.

155. Morgan, M.E. 1976. The chemistry of some microbially induced flavor defects in milk and dairy foods. Biotechnology and Bioengineering 18, 953-965.

156. Muller, C.J., R.E. Kepner, and A.D. Webb. Identification of 3-(methylthio)-propanol as an aroma constituent in Cabernet Sauvignon and Ruby Cabernet wines. Am. J. Enol. Vitic. 22, 953.

157. Naczk, M., R. Amarowicz, A. Sullivan, F. Shahidi. 1998. Current research developments on polyphenolics of rapeseed/canola: a review. Food Chemestry 62, 489-502.

158. Ney, K.H. 1973. Technik der Aromauntersuchung. Gordian 73, 380-387.

159. Ney, K.H. 1981. Recent advances in cheese flavor research. In: G. Charalambous and G. Inglett (Eds.), The Quality of Foods and Beverages. Chemistry and Technology, vol. 1, Academic Press, New York. 1, 385–435.

160. Ney, K.H., and I.P.G. Wirotama. 1978. Untersuchung der Aromabestandteile von Fontina, einem italienischen Käse. Fette Seifen Anstrichm. 80, 249-251.

161. Noble, R.C. 1981. Digestion, absorption, and transport of lipids in ruminant animals. In: W.W. Christie (Ed), Lipid Metabolism in Ruminant Animals. J. of Dairy Sci. Pergamon Press, New York, NY. 87, 57–94.

162. Noomen, A. 1983. The role of the surface flora in the softening of cheeses with a low initial pH. Neth. Milk Dairy J. 37, 229–232.

163. O'Riordan P.J., and C.M. Delahunty. 2003. Characterisation of commercial Cheddar cheese flavour. 1: traditional and electronic nose approach to quality assessment and market classification. Int. Dairy J. 13, 355-370.

164. O'Riordan, P.J., and C.M. Delahunty. 2001. Comparison of volatile compounds released during the consumption of Cheddar cheese with compounds extracted by vacuum distillation using gas chromatography-olfactometry. Flavour and Fragr. J. 16, 425-434.

165. O'Riordan, P.J., and C.M. Delahunty. 2001. Comparison of volatile compounds released during the consumption of Cheddar cheese with compounds extracted by vacuum distillation using gas chromatography-olfactometry. Flavour and Fragr. J. 16, 425-434.

166. Ohloff, G. 1972. Classification and genesis of food flavours. Flavor Ind. 3, 501-508.

167. Ohren, J.A., and S.L. Tuckey. 1967. Relation of flavor development in Cheddar cheese to chemical changes in the fat of the cheese. J. Dairy Sci. 52, 598–607.

168. Parks, O.W. 1965. Milk flavor. In: H.W. Schultz; E.A. Day and L.M. Libbey (Eds), Symp. on Foods: The Chemistry and Physiology of Flavors, AVI Publishing Co., Inc.: Westport, CN. pp, 296-314.

169. Parliament, T.H., M.G. Kolor, and D.J. Rizzo. 1982. Volatile components of Limburger cheese. J. Agric. Food Chem. 30, 1006-1008.

170. Parodi, P.W. 1996. Milk fat components: Possible chemopreventive agents for cancer and other diseases. Aust. J. Dairy Technol. 51, 24–32.

171. Patton, S. 1954. The mechanism of sunlight flavor formation in milk with special reference to methionine and riboflavin. J. Dairy Sci. 37, 446-452.

172. Pillonel, L., S. Ampuero, R. Tabacchi, and J.O. Bosset. 2003. Analytical methods for the determination of the geographic origin of Emmental cheese:Volatile compounds by GC/MS-FID and electronic nose. European Food Research and Tech. 216,179-183.

173. Povolo, M., and G. Contarini. 2003. Comparison of solid-phase microextraction and purge-and-trap methods for the analysis of the volatile fraction of butter. J. Chromatography A 985, 117-125.

174. Preininger, M, M. Rychlik, and W. Grosch. 1994. Potent odorants of the neutral volatile fraction of Swiss cheese (Emmentaler). ln: Trends in Flavour Research. (Maarse H, van der Heij DG, eds) Elsevier, Amsterdam, pp, 267-270.

175. Pulina, G., A. Nudda, G. Battacone, A. Cannas. 2006. Effects of nutrition on the contents of fat, protein, somatic cells, aromatic compounds, and undesiderable substances in sheep milk. Anim. Feed Sci. Technol. 131, 255-291.

176. Quaranta, A., A.G. D'Alessandro, A. Frate, G. E. Coltella, G. Martemucci, and D. Casamassima. 2006. Behavioural response towards twelve feedstuffs in lambs. Small Rumin. Res. 64, 60-66.

177. Rapisarda, T., S. Carpino, G. Belvedere, G. Azzaro, G. Licitra. 2010. Influenza del trattamento con essenze di citronella e geranio sull’aroma del latte. Sci. Tecn. Lattiero-Casearia. (Submitted).

178. Reineccius, G.A., and S. Anandaraman. 1984. Analysis of volatile flavors. In: J.F. Lawrence (Ed.), Food Constituents and Food Residues. M. Dekker, Basel. pp, 195-293.

179. Reineccius, G. 1994. Source Book of Flavors, New York. pp, 24.

180. Roberts, D.D., and T.E., Acree. 1995. Simulation and analysis of retronasal aroma. In: M. Rothe, and H.P. Kruse (Eds.), Aroma, 4th Wartburg aroma symposium pp, 619–626.

181. Rohan, T.A. 1970. Food flavor volatiles and their precursors. Food Technol. 24, 29-33.

182. Rohan, T.A. 1970. What constitutes flavour? Food Manuf. 45, 42-44.

183. Rychlik, M., and J.O. Bosset. 2001. Flavour and off-flavour compounds of Swiss Gruyèr cheese. Evaluation of potent odorants. Int. Dairy J. 11, 895-901.

184. Rychlik, M., and J.O. Bosset. 2001. Flavour and off-flavour compounds of Swiss Gruyèr cheese. Identification of key odorants by quantitative instrumental and sensory studies. 2001. Int. Dairy J. 11, 903-910.

185. Samuelsson, E.G. 1962. Experiments on sunlight flavour in milk - S35-labelled milk. Milchwissenschaft 17, 401-405.

186. Scanlan, R.A., R.C. Lindsay, L.M. Libbey, and E.A. Day. Heat-induced volatile compounds in milk. J. Dairy Sci. 51, 1001-1007.

187. Schaller, E.; J.O. Bosset, and F. Escher. 1998. Electronic noses and their application to food: a review. Lebensmittel -Wissenschaft und –Technol. 31, 305-316.

188. Schreier, P., and H. Idstein. 1985. High-resolution gas chromatography - Fourier transform infrared spectroscopy in f1avour analysis: limits and perspectives. Z Lebensm. Vnters Forsch 180, 183-188.

189. Schwartz, D.P., J. Shamey, C.R. Brewington, and O.W. Parks. 1968. Methods for the isolation and characterization of constituents of natural products. X. New and improved methods for the analysis of carbonyl 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylosazones. Microchem J. 13, 407-417.

190. Schwartz, D.P., O.W. Parks, and R.A. Yoncoskie. Quantitative studies on methyl ketone formatin in butteroil. J. Am. Oil Chem. Soc. 43, 128-129.

191. Self, R. 1967. Potato flavor. In: H.W. Schultz, E.A. Day and L.M. Libbey (Eds), Chemistry and Physiology of Flavors, AVI Publishing Co., Westport, Conn.

192. Shaw, P.E., J.H. Tatum, and R.E. Berry. 1968. Base-catalyzed fructose degradation and its relation to nonenzymatic browning. J. Agric. Food Chem. 16, 979-982.

193. Shibamoto, T. 1980. Flavor volatiles formed by heated milk. In: G. Charalambous (Ed), The Analysis and Control of Less Desirable Flavors in Foods and Beverages. Academic Press: New York. Pp, 241-265.

194. Shipe, W.F. 1980. Analysis and control of milk flavor. In: G. Charalambous (Ed.), The Analysis and Control of Less Desirable Flavors in Foods and Beverages. Academic Press: New York. pp, 201-239.

195. Shipe, W.F., R Bassette, G.M. Deane, W.L. Dunkley, E.G.. Hammond, W.J. Harper, D.H. Kleyn, M.E., Morgan, J.H. Nelson, and R.A. Scanlon. 1978. Off-flavors of milk: nomenclature, standards and bibliography. J. Dairy Sci. 61, 855-869.

196. Shiratsuchi H. Shimoda M. Imayoshi K. Noda K. Osajima Y (1994) Volatile flavor compounds in spray-dried skim milk powder. J Agric Food Chem. 42, 984-988.

197. Shooter, D., N. Jayatissa, and N. Renner. 1999. Volatile reduced sulphur compounds in butter by solid phase microextraction. J. of Dairy Res. 66, 115-123.

198. Shultz, H.W., E.A. Day, and R.O. Sinnhuber. 1962. Symposium on Foods: Lipids and their Oxidation. AVI Publishing Co., Westport, Conn.

199. Sloot, D., and H.J. Hofman. 1975. Alkylpyrazines in Emmental cheese. J. Agric. Food Chem. 23, 358.

200. Stark, W., G. Urbach, I.J. Cook, and J.R. Ashes. 1978. The effect of diet on the γ-and δ-lactone and methyl ketone potentials of caprine butterfat. J. Dairy Res. 45, 209-221.

201. Steffen, C., E. Flückiger, J.O. Bosset, and M. Rüegg.1987. Swiss–type varieties. In: P.F. Fox (Ed.), Cheese: Chemistry, Physics and microbiology. Major Cheese Groups, Elsevier Appl. Sci., London and New York. 2, 93–120.

202. Stevens, S.S. 1958. Measurement and man. Science 127, 383-389.

203. Stevens, S.S. 1960. The psychophysics of sensory function. American Scientist 48, 226-253.

204. Thomson, J.J. 1913. On the appearance of helium and neon in vacuum tubes. Nature, 90, 645-647.

205. Turner, K.W., H.A. Morris, and F.G. Martley. 1983. Swiss type cheese. II. The role of thermophilic lactobacilli in sugar fermentation. N.Z. J. Dairy Sci. Technol. 18, 117–124.

206. Ulberth, F., and D. Roubicek. 1995. Monitoring of oxidative deterioration of milk powder by headspace gas chromatogrography. Int Dairy J. 5, 523-531.

207. Ullrich, F., and W. Grosch. 1989. Identification of the most intense volatile flavor compounds formed during autoxidation of linoleic acid. Z. Lebensm. Unters. Forsch 184, 277-282.

208. Urbach, G. 1993. Relations between cheese flavor and chemical composition. Int. Dairy J. 3, 389–422.

209. Urbach, G. 1995. Contribution of lactic acid bacteria to flavour compound formation in dairy products. Int. Dairy J. 5, 877–903.

210. Urbach, G. 1996. The chemical and biochemical basis of chee se and milk aroma. In: B.A. Law (Ed.), Microbiology and Biochemistry of Cheese and Fermented Milk. Blackie Acad and Prof, imprint of Chapman and Hall, London. pp, 253-298.

211. Urbach, G., W. Stark, and D.A. Forss. 1972. Volatile compounds in butter oil. II. Flavour and flavour thresholds of lacrones, fatty acids, phenols, indole and skatole in deodourized synthetic butter. J. Dairy Res. 39, 35-47.

212. Vamos-Vigyazo, L., and N. Kiss-Kutz. 1974. Estimation of volatile acid in Emmental cheese by gas-liquid chromatography. Acta Aliment. 3, 309-325.

213. Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods of dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.

214. Van Straten, S., and H. Maarse. 1983. Volatile Compounds in Food. Qualitative Data, 5th ed. Krips Repro Meppel, The Netherlands.

215. Vandeweghe, P., and G.A. Reineccius. 1990. Comparison of flavour isolation techniques applied to Cheddar cheese. J. Agric. Food Chem. 38, 1549-1552.

216. Villalba, J.J., F.D. Provenza, and K.C. Olson. 2006. Terpenes and carbohydrate source influence rumen fermentation, digestibility, intake, and preference in sheep. J. Anim. Sci. 84, 2463-2473.

217. Villalba, J.J., and F.D. Provenza. 1997a. Preference for flavoured foods by lambs conditioned with intraruminal administration of nitrogen. Brit. J. Nutr. 78, 545-561.

218. Villalba, J.J., and F.D. Provenza. 1997b. Preference for wheat straw by lambs conditioned with intraruminal infusions of starch. Brit. J. Nutr. 77, 287-297.

219. Wache`, Y., A. Bosser-De Ratuld, and J.M. Belin. 2002. Production of aroma compounds by enzymatic cooxidation of carotenoids. In: P. Winterhalter and R. Rouseff (Eds), Carotenoid-Derived Aroma Compounds, ACS Symp. Series 802. Am. Chem. Soc. Washington, DC. pp, 102–111

220. Wallach, O. 1887. Zun KentiniB der Terpene und der atherischen Oele. Liebig's Ann. Chem. 239, 1-54.

221. White, S. and G. White. 1995. Dairy flavourings. In: P.R. Ashurst (Ed.), Food Flavorings; Blackie Academic & Professional, New York. pp, 240-275.

222. Wijesundera, C., L. Drury, K. Muthuku-marappan, S. Gunasekaran, and D.W. Everett. 1998. Flavour developmenet and distribution of fat globule size and shape in Cheddar–type cheeses made from skim milk homogenised with AMF or its fractions. Aust. J. Dairy Technol. 53, 107.

223. Woolley, C., and V. Mani. 1994. Schnelle und einfache Bestimmung von Aromastoffen mit Festphasen-mikroextraktion und Kapillar GC. Supleco Reporter. 13, 9-10.

224. Wu, Z., and D.S. Robinson. 1999. Co-oxidation of β-carotene catalysed by soybean and recombinant pea lipoxygenases. J. Agric. Food Chem. 47, 4899–4906.

225. Xanthopoulos, V., D. Picque, N. Bassit, C.Y. Boquien, and G. Corrieu. 1994. Methods for the determination of aroma compounds in dairy products: a comparative study. J. Dairy Res. 61, 289-297.

226. Zhang, Z., and J. Pawliszyn. 1995. Quantitative extraction using an internally cooled solid phase microextraction device. Anal. Chem. 67, 34-43.

227. Zhang, Z., M.J. Yang, and J. Pawliszyn. 1994. Solid-Phase Microextraction. Anal. Chem. 66, 844-853.