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DEVELOPMENT OF NEW ANALYTICAL METHODS AND APPLICATION OF CHEMOMETRIC TOOLS IN FLAVOUR RESEARCH

Relatore

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“What we observe
is not nature in itself,
but nature under our
method of questioning”
- W. Heisenberg -

“We live in a
multivariate world,
thus our sight
must be multivariate”

“Everything should be made
as simple as possible,
but not simpler”
- A. Einstein -

Table of Contents

Chapter 2: Materials and methods

Chapter 3: Analytical surveys in the FACET context

Chapter 4: Post-harvest factors effect on tomato flavour

List of Publications

1. A. Raffo, A. D'Aloise, A. D. Magrì, and C. Leclercq – "*Quantification of allyl hexanoate in pineapple beverages and yogurts as a case study to characterise a source of uncertainty in dietary exposure assessment to flavouring substances*" – Food Additives & Contaminants: Part A (2012) 29, 43

2. A. Raffo, S. Nicoli, N. Nardo, I. Baiamonte, A. D'Aloise, and F. Paoletti – "*Impact of different distribution scenarios and recommended storage conditions on flavour related quality attributes in ripening fresh tomatoes*" – Journal of Agricultural and Food Chemistry (2012) 60, 10445

3. A. Raffo, A. D'Aloise, A. L. Magrì, and C. Leclercq – "*Quantitation of tr-cinnamaldehyde, safrole and myristicin in cola-flavoured soft drinks to improve the assessment of their dietary exposure" – Submitted to* Food and Chemical Toxicology

List of Congresses

1. Poster presentation at the *First International Conference on ORGANIC FOOD QUALITY AND HEALTH RESEARCH*, 18th-20th May 2011, Prague (Czech Republic): *"Commercial fertilizers and organoleptic quality of organically grown apple fruits (cv. Golden Delicious)"* - I. Baiamonte, E. Moneta, A. Raffo, A. D'Aloise, N. Nardo, M. Kelderer, E. Lardschneider, F. Paoletti – *Book of Abstracts, p. 70*

2. Poster presentation at the *XIII Weurman Flavour Research Symposium*, 27th-30th September 2011, Zaragoza (Spain): *"Effect of soil nutrition on aroma compound formation in organically grown apples (cv. Golden Delicious)"*- A. Raffo, A. D'Aloise, E. Lardschneider, F. Paoletti, F. Marini, R. Bucci, M. Kelderer – *Book of Abstracts, pp. 258-259*

3. Poster presentation at the *XIII Weurman Flavour Research Symposium*, 27th-30th September 2011, Zaragoza (Spain): *"Variability of allyl hexanoate concentration in pineapple-flavoured beverages and yogurts"* - A. Raffo, A. D'Aloise, A. D. Magrì, A. L. Magrì & C. Leclercq – *Book of Abstracts, pp. 262-263*

4. Poster presentation at the 5th *International Symposium on Recent Advances in Food Analysis (RAFA 2011)*, 1st-4th November 2011, Prague (Czech Republic): *"Comprehensive PTR-MS/tribologic study on aroma release from dairy emulsions: the influence of friction and fat level"* – A. D'Aloise, R. Bucci, F. Marini, K. Burseg – *Book of Abstracts, p. 188*

5. Presentation at the European Commission, October 2012, Brussels (Belgium): *"Assessment of variability of cinnamaldehyde, safrole and myristicin concentration levels in cola-flavoured soft drinks*" – A. Raffo, A. D'Aloise, A. L. Magrì, C. Leclercq - *EU Project FACET, Final Conference* -

6. Poster presentation at the *IV Convegno SISS*, 22nd-23rd November 2012, Trieste (Italy): *"Effetto di condizioni di estrazione e gramolatura sul profilo organolettico dell'olio extravergine d'oliva"* - A. D'Aloise, R. Bucci, E. Moneta, G. Pastore, M. Peparaio, A. Raffo, F. Sinesio -

7. Accepted abstract for poster presentation at the *10th Wartburg Symposium on Flavour Chemistry & Biology*, 16th-19th April 2013, Eisenach (Germany): *"Impact of reduced oxygen levels during malaxation on the organoleptic quality of extra virgin olive oils obtained by means of different separation systems"* - A. Raffo, A. D'Aloise, A. D. Magrì, M. T. Maldini, E. Moneta, G. Pastore, M. Peparaio, F. Sinesio -

Chapter 1: Introduction

1.1 Flavour: a very old story

Aroma and taste are more than sensory experiences: they are signals of nutritional value or poison. It has evolved as a vital survival mechanism in mammals and driven epic periods of human history: it was, after all, the quest for spices that helped launching the age of exploration.

The origin of using odorous substances simply for enjoyment or medicinal reasons is as old as mankind: people have used perfume oils and unguents on their bodies for thousands of years.

The centrality of flavour to human culture has driven scientists, chefs and the food industry to experiment with new ways of producing familiar and novel tastes, as well as to create a scientific style of experimental cooking.

In the 19th century, the commercialization of flavors and fragrances (for perfumery) on an industrial scale started with the isolation of single chemicals responsible for the characteristic aroma of natural products (e.g. cinnamaldehyde isolated from cinnamon oil and benzaldehyde from bitter almond oil).

Over the time, due to the increasing demand, the high cost and lack of availability of natural flavor extracts, most of the commercial flavourants became "nature-identical", which means that they are the chemical equivalent of natural flavors but are chemically synthesized mostly from petroleum-derived precursors, rather than being extracted from the source materials.

Although industrial production of flavours is about 150 years old, the use of synthetic materials started only about 60 years ago, after World War II.

Today, the total market for flavors and fragrances is estimated at US$18 billion, with market shares between the flavor and fragrance businesses being almost equal[1]; the largest markets are Europe, Africa and the Middle East Region (36%) and North America (32%), followed by the Asian Pacific region (26%)[2].

The big era of analytical flavour research started in the 50s: many scientific publications and patents in subsequent years were achieved; the next big change happened in the 90s, when research started to become a lot more application-driven.

In 1955, the first commercial gas-chromatograph was introduced, and 15 years later the full power of capillary *GC-MS* became practical; then, it took 15 years more to have the first mass spectral databases available, to support in compound identifications[3].

Nowadays, research carried out by flavour and fragrance companies is generally for the purpose of understanding, designing or improving the sensory characteristics and/or the functionality of new products; formulated flavors are then used by the food and beverage, tobacco and pharmaceutical industries, mostly.

While it seems obvious for most consumers why pharmaceuticals are needed and beneficial, the use of flavours for foods and beverages as well as fragrances for various applications is not so easily

[1] M. Guentert – "The flavor and fragrance industry - past, present and future". In *Flavours and Fragrances* (Berger R.G. Ed.) - Berlin: Springer (2007) pp. 1

[2] http://www.leffingwell.com

[3] J.C. Leffingwell -"Reflections on a half century of flavour chemistry" - Speech at the 50th anniversary flavour symposium of the Society of Flavour Chemists (2004)

understood by consumers: therefore, it is important to inform them about the safety of flavour and fragrances and the benefits for their use in consumer products.
One of the important tasks of a marketing department in the flavour and fragrance industry is to study lifestyle trends to help research to work on the appropriate long-term projects, and force sales to target the right customers and product categories.
In the modern, affluent food marketplace, the key to success is delivering food that pleases the consumer's palate. Even the most nutritious foods are not routinely accepted and regularly consumed if they have poor sensory properties for the individual consumer choosing them. Therefore, in building a knowledge base of food choices and particularly the role of flavour, it is necessary to study and understand olfactory preferences. Food perception is more than the simple volatile compounds in food biomaterials capable of binding to olfactory receptors.
Understanding flavour means first understanding the individual responses to olfactory stimuli and subsequently building an understanding of how those responses lead to preferences[4].

[4] M. Griep, T. Mets, D. Massart – British Journal of Nutrition (2000) 83, 105

1.2 Volatile compounds (VOC) in foodstuffs

When food is consumed, the interaction of taste, odor, and textural feeling provides an overall sensation, which is best defined by the English word "*flavour*".
Flavour results from compounds that are divided into two broad classes: those responsible for taste and those responsible for odors, the latter often designated as aroma substances; however, there are compounds which provide both sensations[5].
Compounds responsible for taste are generally nonvolatile at room temperature: therefore, they interact only with taste receptors located in the taste buds of the tongue.
Aroma substances are *volatile organic compounds* (VOC) which are perceived by the odor receptor sites of the olfactory tissue of the nasal cavity: they reach the receptors when drawn in through the nose (orthonasal detection) and via the throat after being released by chewing (retronasal detection).
It is worth mentioning that there is no general classification into "good" or "bad" flavours, since a substance might contribute to the typical (and desirable) odor or taste in one food, while it might cause a faulty odor or taste (off-flavour) in another food.
The amount of volatile substances present in food is extremely low (10-15 mg/kg): however, they comprise a large number of components, in some cases even more than 800; all known volatile compounds are classified according to the food and the class of compounds, and published in a constantly updated database[6].
Of all the volatile compounds, only a certain number is important for aroma: these are the ones present in food in concentrations higher than the odor/taste thresholds; among all, those volatiles that provide the characteristic aroma of the food are called key odorants, or character impact aroma compound[7].

1.2.1 VOC in fresh fruits

Various types of fresh fruits produce distinct volatile profiles; these volatiles are mainly comprised of diverse classes of chemicals, including esters, alcohols, aldehydes, ketones, lactones, and terpenoids[8].
Fruit flavour, and therefore the volatile composition, is a very important aspect of quality: a complete understanding of the flavour chemistry and biochemistry cues is important in order to improve the quality of fresh and processed products.
It is characteristic for many of the compounds responsible for the aroma of fruits that they have strong penetration odours, with low threshold values.
Many climacteric fruits, e.g. apples, pears, peaches, nectarines, apricots and plums, have a green note when unripe, and this note disappears during ripening[9]: thus, the typical flavour of most fruits is not present during early fruit growth and development, but develops after a ripening process[10].

[5] "Aroma compounds". In *Food chemistry* - H. D. Berlitz, W. Grosch, P. Schieberle – Berlin: Springer (2009) pp. 340
[6] L. M. Nijssen, C. A. Visscher, H. Maarse, L. C. Willemsens, M. H. Boelens – "Volatile compounds in food. Qualitative and quantitative data" – 7th Edition (1997), TNO Nutrition and Food Research Institute, Zeist, The Netherlands
[7] R. G. Buttery – "Flavour chemistry and odor thresholds" – In *Flavour chemistry. Thirty years of progress* (R. Teranishi, E. L. Wick, I. Hornstein Ed.) – New York: Kluver Academic/Plenum Publ. (1999) pp. 353
[8] J. Song, C. F. Forney - Canadian Journal of Plant Science (2008) 88, 537
[9] G. R. Takeoka, R. A. Flath, T. R. Mon, R. Teranishi, M. Guentert – Journal of Agricultural and Food Chemistry (1990) 38, 471

Volatile compounds formed by anabolic or catabolic pathways include fatty acid derivatives, terpenes and phenolics.
In contrast, volatile compounds formed during tissue damage, such as during production of olive oil[11], are typically formed through enzymatic degradation and/or autoxidation reactions of primary and/or secondary metabolites, including mainly lipids, amino-acids, terpenoids and phenolics.

1.2.1.1 Compounds formed by degradation of fatty acids (FAs)

Fatty acids originate from triglycerides, phospholipids or glycolipids that are important parts of the cell membranes: they are precursors for a large number of volatile compounds of which many are important character-impact aroma compounds responsible for the fresh, green and fruity notes of fruits and vegetables.
Degradation of fatty acids occurs mainly by three different oxidative routes: (1) β-oxidation, (2) oxidation by the lipoxygenase (LOX) pathway and (3) autoxidation; however, fatty acids do not accumulate in healthy plant tissue and therefore the initial phase in the oxidative degradation is their liberation by acyl hydrolases[12].
β-oxidation is the classical biochemical pathway involved in fatty acid degradation that typically occurs in intact tissue during ripening of fruits and vegetables[13]; the breakdown can also be stopped, resulting in the liberation of medium-chain-length or short-chain-length volatile compounds.
These metabolites can exit the pathway between β-oxidation cycles or inside the sequence: the entire process can lead at the end to a variety of volatile compounds such as saturated and unsaturated lactones, esters, alcohols, ketones and acids.
The volatiles produced by the LOX pathway and autoxidation are typically volatile aldehydes and alcohols responsible for fresh and green sensorial notes.
In the LOX pathway these volatile compounds are produced in response to stress, during ripening or after damage of the plant tissue, as in the case of olive oil (Fig. 1)[14].
Precursors of the LOX catalyzed reactions are C18-polyunsaturated fatty acids with a (*Z*,*Z*)-1,4-pentadiene moiety, such as linoleic (LA) and α-linolenic (LnA) acids that are typically oxidized into 9-, 10- or 13-hydroperoxides depending on the specificity of the LOX catalyst.
These compounds are then cleaved by hydroperoxide lyase (HPL) into mainly C_5, C_6, C_9 and C_{10} aldehydes, which can then be reduced into the corresponding alcohols by alcohol dehydrogenase (ADH); the mediation of alcohol acetyl transferase (AAT) produces esters.
The production of volatile compounds by the LOX pathway depends on the plants, as they have different sets of enzymes, pH in the cells, fatty acid composition of cell walls, etc.
Hexanal and 2,4-decadienal are the primary oxidation products of linoleic acid, whereas autoxidation of linolenic acid produces 2,4-heptadienal as the major product; further autoxidation of these aldehydes leads to the formation of other volatile products.

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[10] L. P. Christensen, M. Edelenbos, S. Kreutzmann – "Fruits and Vegetables of Moderate Climate". In *Flavours and Fragrances* (Berger R.G., Ed.) - Berlin: Springer (2007) 135
[11] C. M. Kalua, M. S. Allen, D. R. Bedgood Jr, A. G. Bishop, P. D. Prenzler, K. Robards - Food Chemistry (2007) 100, 273
[12] F. A. Tomas-Barberan, R. J. Robins – "Phytochemistry of fruits and vegetables" – Oxford: Clarendon (1997)
[13] I. A. Graham, P. J. Eastmond – Progress in Lipid Research (2002) 41, 156
[14] F. Angerosa, M. Servili, R. Selvaggini, A. Taticchi, S. Esposto, G. Montedoro - Journal of Chromatography A (2004) 1054, 17

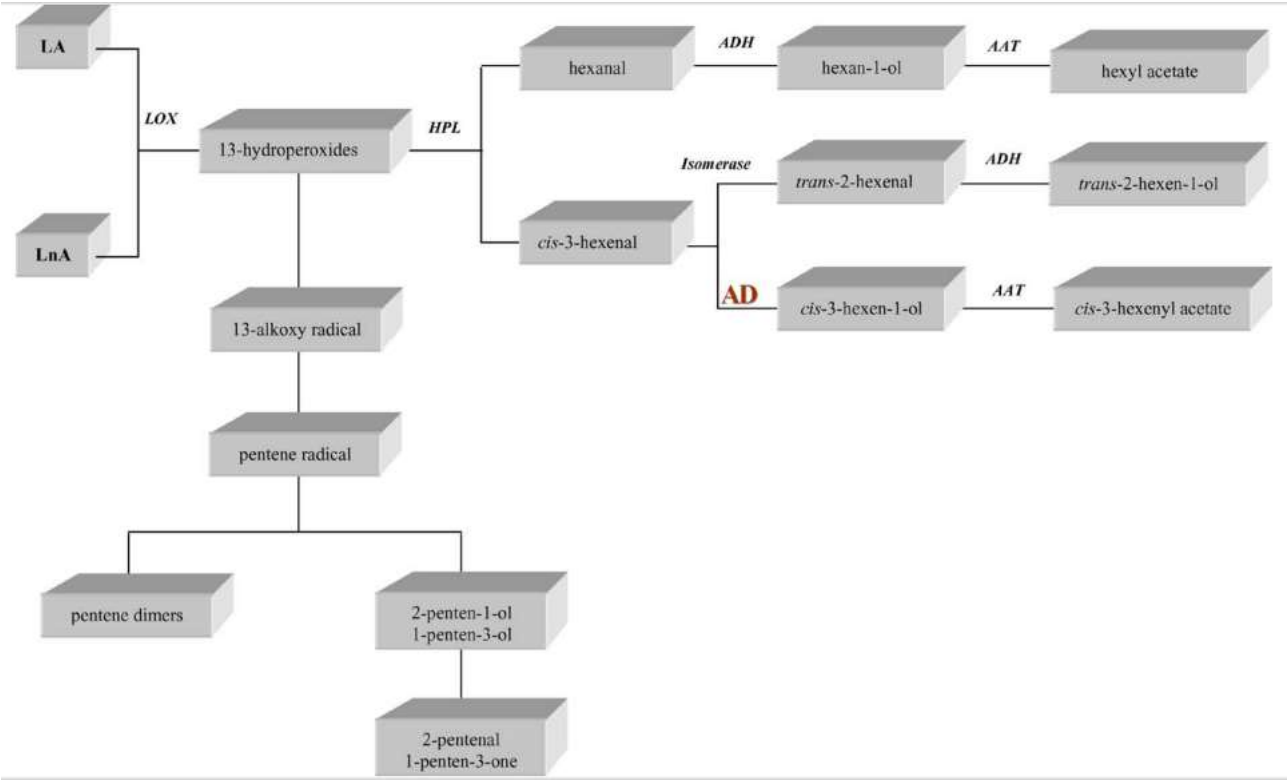

Figure 1. Lipoxygenase pathways involved in the production of C6 and C5 volatile compounds (from Servili et al., J. Chrom. A (2004) 1054, 17)

1.2.1.2 Compounds formed from amino-acids

Amino-acid metabolism generates aromatic, aliphatic, and branched-chain alcohols, acids, carbonyls, and esters that are important to fruit flavor; some volatile compounds can be produced by the action of enzymatic systems on amino acids.

The major types of volatile compounds formed from the interaction of amino-acids and sugars include aldehydes, alkyl pyrazines, alkyl thiazolines and thiazoles, and other heterocycles from the Strecker degradation[15].

Amino-acids are precursors for some branched aliphatic compounds such as 2-methyl-1-butanol and 3-methyl-1-butanol; these compounds can be further synthesized to form esters, which are important volatile compounds in many fruits with distinct "fruity" odor.

As they share the same precursor "pyruvate", which is generated from glycolysis, the interaction between FAs and branched amino-acids is another important factor in the volatile biosynthesis of fruits: as apple fruits ripen, there is a great production of volatile compounds from the branched amino-acid pathway[16].

A large class of secondary metabolites in plants is derived from phenylalanine, the so called "phenylpropanoid" and "benzenoid" compounds.

The phenylpropanoid compounds are nonvolatile, but when reduced at the C_9 position to form an aldehyde, alcohol, or alkene/alkane, or when they contain alkyl additions to the hydroxyl group of

[15] H. Maarse – "*Volatile Compounds in Food and Beverages*" - 1st Ed., New York: CRC (1991)
[16] J. Song, F. Bangerth - Acta Horticulturae (1994) 368, 150

the phenyl ring or to the carboxyl group, these compounds are volatiles, and they are common constituents of the scent of many plant species[17].

Phenylpropanoid-related compounds such as 2-phenylacetaldehyde and 2-phenyl-ethylalcohol have an important contribution to tomato flavor: at low concentration, both compounds have pleasant fruity or floral odors; however, at high levels, the pungent aroma of 2-phenylacetaldehyde is unpleasant[18].

Other benzenoids such as methyl salicylate and methyl benzoate are common components of floral scent (e.g. vanillin), and have trans-cinnamic acid as the precursor.

Other phenylpropenes, such as isoeugenol or eugenol and related compounds, are associated with pleasant aromas and flavors; however, the biochemical pathways for their synthesis have not been completely elucidated yet (Fig. 2).

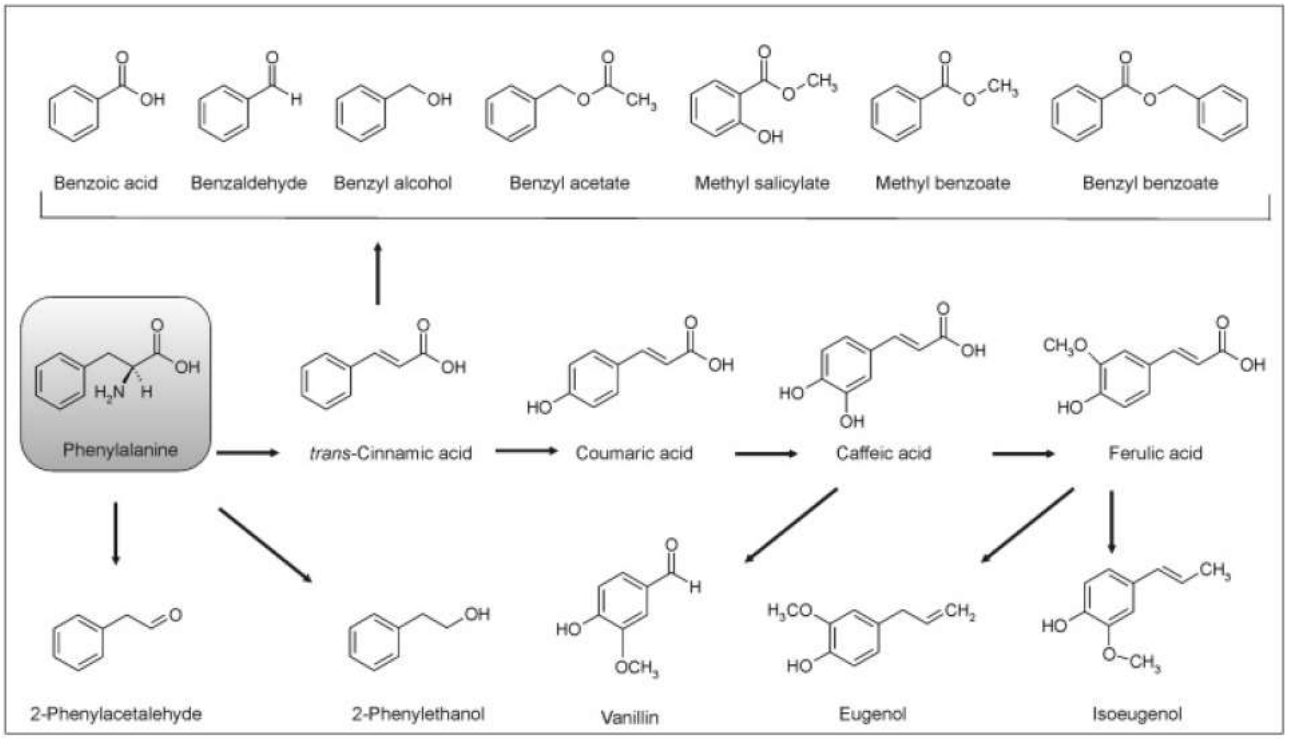

Figure 2. Schematic representation of the "shikimate" pathway that leads "benzenoid" and "phenylpropenes" volatiles (from Verdonk et al., Plant Cell (2005) 17, 1612)

Other groups of flavor compounds are sulfur molecules: the sulfur-containing flavor compounds are synthesized from methionine and cysteine; in onion and garlic, a series of volatile sulfur compounds responsible for the typical flavors and odors of these *Alliums* have been described[19].

Amino acids such as alanine, leucine, isoleucine, and valine are also involved in volatile synthesis. In tomatoes, valine is a reported precursor for 1-N-2-methylpropane, 3-methylbutylnitrile, 1-N-3-methyl butane, and 2-isobutylthiazole.

[17] N. Dudareva, E. Pichersky – "*Biology of Floral Scent*" - Boca Raton: CRC (2006) pp.23

[18] R. G. Buttery – "Quantitative and sensory aspects of flavor of tomato and other vegetables and fruits" - In "*Flavour Science: Sensible Principles and Techniques*" – (T. E. Acree, R. Teranishi, Eds.) - Washington, DC: American Chemical Society (1993) pp.259

[19] M. G. Jones, J. Hughes, A. Tregova, J. Milne, A. B. Tomsett, H. A. Collin – Journal of Experimental Botany (2004) 55, 1903

Isoleucine is a precursor of 2-methylbutanol and 2-methylbutyric acid; compounds derived from leucine such as 3-methylbutanal, 3-methylbutanol, and 3-methylbutanoic acid are abundant in various fruits such as strawberry, tomato, and grape varieties[20].
In addition, amino-acids, as well as their alcohol and acid derivatives, can be esterified to compounds with a large impact on fruit odor, such as 3-methylbutyl acetate and 3-methylbutyl butanoate in banana, by deamination, decarboxylation, several reductions, and esterification[21].

1.2.1.3 Phenols and Related Compounds

A large number of volatile phenols and related compounds occur in vegetables and fruits, and some of them are potent aroma compounds.
They are formed mainly through the shikimic acid pathway (Fig. 2), and are present in intact plant tissue either as free aglycones or bound as glycosides that can be liberated by enzymatic hydrolysis[22].
Although many of the phenols and related compounds, in particular the phenylpropanoids, originate from some of the "building blocks" of lignin such as ferulic acid and *p*-coumaric acid, these compounds are not breakdown products of lignin.
Generally the volatile phenols and related compounds are substituted benzene derivatives with methoxy and phenolic groups with often an allyl, a vinyl or an aldehyde group; common flavour compounds of this group are eugenol, myristicin, apiole, elemicin and benzaldehyde.

1.2.1.4 Compounds of terpenoid origin

Terpenoids are widely distributed among vegetables and fruits, and in some vegetables, e.g. carrots, they are the major contributor to the flavour; the monoterpenes and sesquiterpenes are mainly formed by anabolic processes and are therefore present in intact plant tissue (Fig. 3)[23].
Tissue disruption therefore does not normally alter the profile of monoterpenes and sesquiterpenes significantly in the raw product, although changes in the concentration of some monoterpenes and sesquiterpenes may occur owing to oxidation and release of glycoside-bound oxygenated terpenoids.
There are two main types of terpenoids that may contribute significantly to the flavour of vegetables and fruits: (1) monoterpenes and sesquiterpenes and (2) irregular terpenes, mainly produced by catabolistic pathways and/or autoxidation; there are also glycoside-bound oxygenated terpenoids that enzymatically release volatile oxygenated terpenoids, especially during ripening or cell disruption[24].
The formation of some irregular terpenes cannot be explained by anabolic pathways in plants: these are primarily oxidative degradation products of the carotenoids, which seems somewhat related to the oxidative breakdown of unsaturated fatty acids previously discussed.
As with fatty acids, carotenoid oxidation occurs whenever the plant tissue is damaged and/or during senescence, and the volatile degradation products generated obviously depend on the carotenoids present in the different vegetables and fruits[25].

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[20] C. Aubert, S. Baumann, H. Arguel – Journal of Agricultural and Food Chemistry (2005) 53, 8881
[21] A. G. Perez, J. J. Rios, C. Sanz, J. M. Olias – Journal of Agricultural and Food Chemistry (1992) 40, 2232
[22] E. Stahl-Biskup, F. Inert, J. Holthuijzen, M. Stengele, G. Schulz – Flavour and Fragrance Journal (1993) 8, 61
[23] W. D. Loomis, R. Croteau – "*The biochemistry of plants*" – London: Stumpf PK Academic Ed. (1980) vol 4, pp.363
[24] E. Stahl-Biskup, F. Inert, J. Holthuijzen, M. Stengele, G. Schulz – Flavour and Fragrance Journal (1993) 8, 61
[25] M. Aguedo, M. H. Ly, I. Belo, J. A. Teixeira, J-M. Belin, Y. Waché - Food Technology and Biotechnology (2004) 42, 327

For example, the tomato volatiles 6-methyl-5-hepten-2-one, geranyl acetone and farnesyl acetone may result from the oxidative cleavage of acyclic carotenoids; similarly, α-ionone, β-ionone and β-damascenone probably result from the oxidative breakdown of cyclic carotenoids and as for other terpenoids may exist in intact plant tissue bound as glycosides.

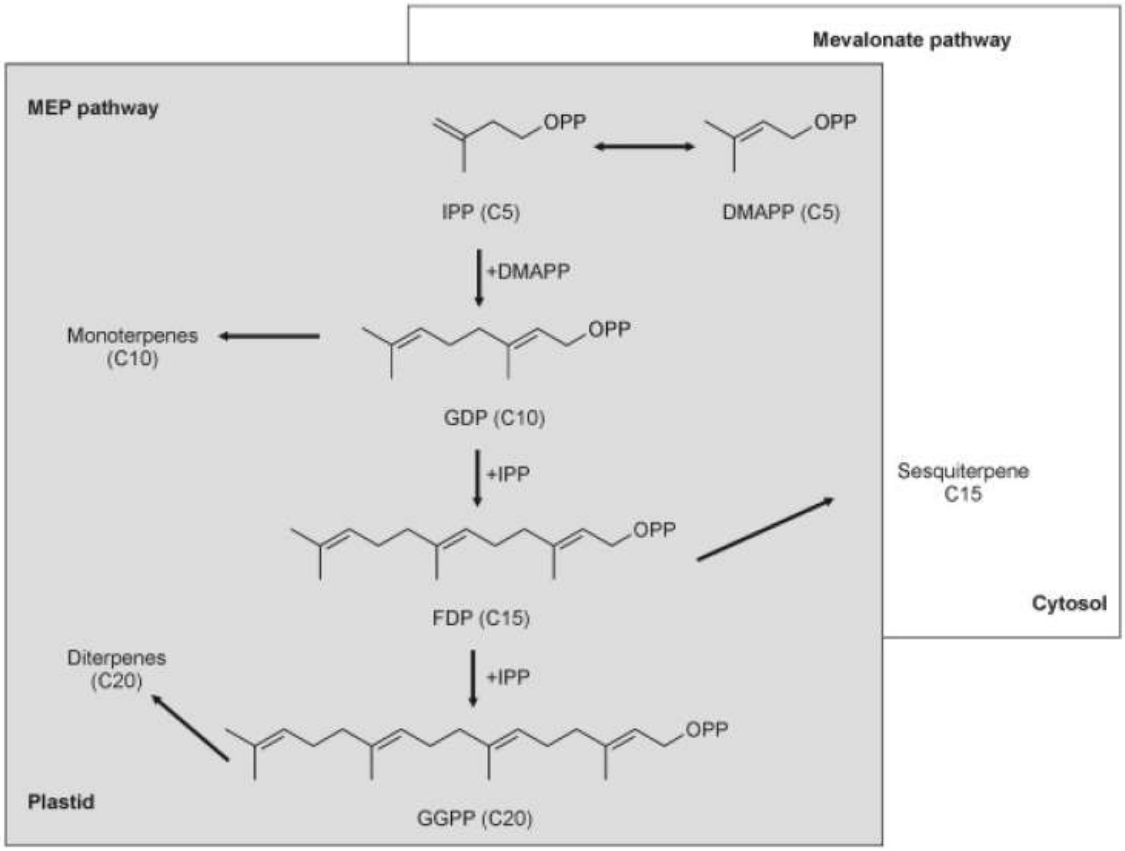

Figure 3. Terpenoid pathway for volatile in plants. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GDP, geranyl diphosphate; FDP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate (from *Handbook of fruit and vegetable flavors* (2010) - John Wiley & Sons)

1.3 Volatiles and flavour perception: hedonic and health benefits

1.3.1 Flavour perception

Both the release and the perception of flavor are complex processes involving different physicochemical and physiological phenomena[26]: flavor perception begins when suitable molecules are released from the food matrix and travel to either the taste or the odor receptors located in the mouth and nose, respectively.

The nonvolatile compounds are transported in the saliva to the taste receptors in the mouth, while the volatile ones are carried in the air to the odor receptors located in the nose; then, a process of transduction of the signals detected by the corresponding receptors through the brain occurs, where the flavor sensation experienced is generated.

Perhaps, one of the best definitions of flavor is that proposed by Heymann and others[27]: "*Flavour is the biological response to chemical compounds (the physical stimuli) by the senses, interpreted by the brain in the context of human experience*".

Indeed, flavour is not simply the result of the sum of both volatile and nonvolatile compounds of a certain fruit or vegetable: in fact, it depends on the interactions between those compounds and possibly with other ones, and also on the physicochemical characteristics of the food matrix it needs to be liberated from.

It is well-known that the characteristics and the structure of the matrix exert a decisive influence on the effective concentration of the chemical stimuli and on their rate of transport to the receptors. The theoretical basis of the transport phenomena governing the release of chemical stimuli out of the food matrix, such as phase partition, mass transport, and diffusion are well defined[28].

However, the study of these processes in the actual mouth conditions during mastication is far more complex: the food surface area in contact with saliva and the concentration of both volatile and nonvolatile stimuli present in the aqueous and air phases undergo continuous changes, mainly due to the continuous movements of the mouth components during mastication and deglutition[29].

This type of study gets even more complicated, especially in the case of solid foods, due to the individual differences in respiration rates, in salivation, and in mastication patterns, all of them affecting the transport of the stimuli to the receptors.

In the second phase of the flavor perception process, beside the type and concentration of stimuli reaching the receptors, other factors like psychological and cognitive phenomena have to be taken into consideration.

1.3.2 Sensory quality: a challenge

Plants produce many volatile metabolites: a small subset of these compounds is sensed by animals and humans, and the volatile profiles are defining elements of the distinct flavours of foods[30].

The organoleptic (appearance, aroma, taste, and texture) quality of fruit and vegetables plays an important role in consumer satisfaction, and it can influence their choice at the moment of purchase

[26] P. Overbosch, W. G. M. Afterof, P. G. M. Haring - Food Reviews International (1991) 7, 137

[27] H. Heymann, D. L. Holt, M. A. Cliff – "Measurement of flavor by sensory descriptive techniques". In *Flavour Measurement* (C. T. Ho, C. H. Manley, Eds.) - New York: Marcel Dekker (1993) pp. 113

[28] A. J. Taylor - International Journal of Food Science & Technology (1998) 33 (1), 53

[29] M. Hodgson, R. S. T. Linforth, A. J. Taylor – Journal of Agricultural and Food Chemistry (2003) 51, 5052

[30] W. Schwab, R. Davidovich-Rikanati, E. Lewinsohn – The Plant Journal (2008) 54, 712

and modify the degree of pleasure they experience when consuming these products: these characteristics contribute differently to the acceptability of various fruits and vegetables[31].
For the last decades, the genetic improvement of plants was mainly focused to increase yield or to get more illness-resistant genotypes; sensory quality control is generally considered at most as a secondary activity, usually based on the opinion and expertise of a reduced number of judges[32].
The study of food flavor requires a multidisciplinary approach combining information on the following: concentration of both volatile and nonvolatile compounds within the food, physical characteristics of the food matrix, physicochemical mechanisms governing release of volatile and non-volatile compounds, adequate sensory techniques to ascertain how flavor is perceived, and how this perception affects the final acceptance of the product.
Flavour perception measurement and control present serious problems: the establishment of relationships between the physical stimuli and the physiological reaction of humans and between the latter and the sensation experienced by people upon consuming is a tricky task.
The application of sensory analysis to the evaluation of fruits and vegetables flavor is not straightforward: it requires a wide knowledge of the different experimental methods and of the various possible statistical treatments to be used in order to choose the most adequate ones.
It constitutes also the base of the development and setup of efficient systems to measure and control fruit and vegetable flavor, as required by the particular characteristics of any product[33].
This is why it is very difficult to make predictions as to the possible perceptible differences between products differing in composition or structure, as a result of genetic manipulation, pre-harvest, harvest, or post-harvest factors[34].
In the context of fruit and vegetable breeding programs, sensory analysis plays instead an important role as a selection tool[35]; sensory analysis provides, in fact, the adequate methodology to investigate, for example, how different tomato genotypes[36] affect flavor or how flavor of peaches is affected by storage conditions[37].

1.3.3 Volatiles: not only a sensory cue

The predominance of volatiles derived from essential nutrients and health-promoting compounds suggests that these volatiles provide important information about the nutritional makeup of foods: for example, in tomatoes, almost every important volatile is derived from an essential nutrient[38].
Of course, not all desirable volatiles are expected to be derived from essential nutrients, nor will all volatiles derived from essential nutrients be viewed as desirable across all populations.
Whereas the taste sensory system provides information on major nutrients such as carbohydrates, proteins and lipids, the olfactory sensory system and the food volatiles with which they interact provide the basis for the diversity of flavours found in the human diet.

[31] W. V. Wismer, F. R. Harker, F. A. Gunson, K. L. Rossiter, K. Lau, A. G. Seal, R. G. Lowe, R. Beatson – Euphytica (2005) 141, 93
[32] P. Ranalli, J. I. Cubero - Field Crops Research (1997) 53, 69
[33] E. Costell - Food Quality and Preference (2002) 13, 345
[34] G. Bartoszewski, A. Niedziela, M. Szwacka, K. Nienirowicz-Szczytt - Plant Breeding (2003) 122, 347
[35] S. R. Jaeger, K. L. Rossiter, K. Lau – Journal of the Science of Food and Agriculture (2005) 85, 480
[36] F. Sinesio, E. Moneta, M. Peparajo - Journal of Food Quality (2007) 30, 878
[37] A. Raffo, N. Nardo, M. R. Tabilio, F. Paoletti - European Food Research and Technology (2008) 226(6), 1503
[38] K. L. Mueller et al. - Nature (2005) 434, 225

Odorants must possess certain molecular properties in order to produce a sensory impression: they must have a certain degree of lipophilicity and sufficiently high vapour pressure, so they can be transported to the olfactory system; some water solubility to permeate the thin layer of mucus; finally, they must be present at sufficiently high concentrations to be able to interact with one or more olfactory receptors.

It should be pointed out that plant volatiles are involved in species-specific ecological interactions, and being restricted to specific lineages, they have been considered to be associated with defensive and attractive roles[39]. It is also believed that they are not essential for plant survival but provide adaptive characteristics under strong environmental selection.

For example, isoprene, a ubiquitous volatile hydrocarbon, acts to increase the tolerance of photosynthesis to high temperature by stabilizing the thylakoid membranes or quenching reactive oxygen species[40].

Compounds emitted by flowers most probably serve to attract and guide pollinators: this function of fruit volatiles as a signal of ripeness and as an attractant for seed-dispersing organisms, is supported by the fact that some substances are specifically formed in ripe fruits, but are absent in vegetative tissues and non-ripe fruit.

Volatiles showing anti-microbial and anti-herbivore activity serve to protect reproductive parts of plants from enemies[41]; however, volatiles also act as direct repellents or toxicants for herbivores and pathogens, and some have the potential to eliminate reactive oxygen species. For humans, volatiles in fruits have a considerable economic impact, as parameters of food quality and consumer preference: indeed, volatiles released during fruit ripening are sensed as principal flavour constituents that signal the ripeness of the fruit, and therefore the highest nutrient bioavailability. Unlike ripening fruits, vegetables produce most of the volatiles sensed as flavours only after their cells are disrupted.

In conclusion, a robust correlation exists between health and the volatiles that contribute to the positive perception of foods, and behavioural research supports more and more a connection between sensory perception, flavour preferences and health benefits.

[39] E. Pichersky, J. P. Noel, N. Dudareva – Science (2006) 311, 808

[40] N.Dudareva, E. Pichersky, J. Gershenzon - Plant Physiology (2004) 135, 1893

[41] S. A. Goff, H. J. Klee – Science (2006) 311, 815

1.4 Aroma release

1.4.1 Interactions between flavours and food matrix

Food matrix macro-components, such as proteins, polysaccharides, and lipids are known to interact with flavor compounds: modifications of the formulation of a product will thus induce a modification of the flavor perception by changing the nature of the interactions involved.

A systematic development of products with desired flavoring properties will only be possible if the flavor-binding behaviour of food ingredients has been elucidated[42].

Knowledge of flavor–ingredient interactions is important to understand flavor perception[43], to provide the information necessary to select suitable fat replacers for developing healthier foods, and to adapt flavor cocktails optimally to the ingredients used in newly developed products.

Proteins are often-used food ingredients because of their functional properties, such as emulsifying and stabilizing capacities in lipid-dispersed food systems.

Proteins are known to interact with flavor compounds by reversible or irreversible binding, mainly via hydrophobic interactions[44].

Data from model systems illustrate that several factors, e.g., the chemical nature of the flavor compound, temperature, pH[45], and the structure and processing history of the food protein determine the extent of interactions between proteins and food flavors[46].

Retention of flavor compounds in protein solutions is more pronounced in the presence of lipids; interactions between proteins and flavor compounds may induce retention in emulsions for the compounds with higher binding constants.

Addition of polysaccharides can modify flavor release and flavor perception: main effects are due to modifications of the viscosity[47].

Among the polysaccharides, starch is widely used in food technology: it consists of linear amylose and branched amylopectin, which in the native state are packed in well-organized starch granules; as shown in binary model systems, amylose binds flavor compounds by formation of inclusion complexes.

For flavor compounds belonging to the same chemical class, retention by carbohydrates increased with molecular weight and decreased with increasing polarity and volatility of the aroma compound[48].

Lipids influence the flavor of foods through their effects on flavor perception (mouth-feel, taste, and aroma), flavor stability, and flavor generation; in systems containing lipids, flavor compounds are distributed between the lipid and the aqueous phase, following the physical laws of partition.

The solubility of flavor compounds in aqueous and oil phase depends on their hydrophobicity, expressed by *logP* (logarithm of the octanol-water partitioning coefficient) value: hydrophobicity plays an important role in the thermodynamic behaviour of flavor compounds[49].

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[42] E. Guichard – Food Reviews International (2002) 18(1), 49
[43] H. Plug, P. Haring - Food Quality and Preference (1994) 5, 95
[44] H. A. Gremli – Journal of the American Oil Chemists' Society (1974) 51, 95A
[45] E. Jouenne, J. Crouzet – Journal of Agricultural and Food Chemistry (2000) 48, 1273
[46] S. Lubbers, P. Landy, A. Voilley - Food Technology (1998) 52, 68–74, 208–214
[47] M. A. Godshall - Food Technology (1997) 51, 63
[48] I. Goubet, J. L. Le Queré, A. J. Voilley – Journal of Agricultural and Food Chemistry (1998) 46, 1981
[49] C. Druaux, M. Le Thanh, A. M. Seuvre, A. Voilley – Journal of American Oil Chemists' Society (1998) 75, 127

The kinetic behaviour of flavor compounds in liquid and vapour phases is not only determined by the affinity of the volatiles for the liquid phase, but also by solute–solvent interactions; for example, the strength of hydrogen bonds between lipids and aroma compounds is an additional parameter influencing liquid–liquid partition coefficients of aroma compounds in biphasic systems[50].
It is important to take into account not only the volatility of compounds, but also their rate of release; small changes in fat content have been shown to have significant effects on the vapour pressure of fat-soluble flavor compounds (limonene), whereas the vapour pressure of more polar compounds decreased only slightly[51].
The volatility of flavor compounds in oil depends on the chain length and degree of unsaturation of the fatty acids contained in the triglycerides.
Considering a mixture of flavor compounds exhibiting different polarities, the relative percentages of these volatiles in the headspace will significantly change by changing the oil ratio and inducing a different overall flavor perception.
Although there is the need to study simplified systems in order to gain more insights, real foods are complex multi-phasic systems: the organization of the different phases will determine structure and texture of the food product, and thus influence the diffusion of flavor compounds in the different phases of the food and in the saliva during mastication.
Studies on real foods will have to take into account the breakdown of the matrix during mastication, should follow flavor release in real time, and they should consider the behaviour of aroma compounds in mixtures.

1.4.2 In-vivo flavour release and sensorial experience

Our understanding of how taste works has lagged behind the other senses; moreover, taste is revealed to be a whole-body experience: taste receptors are found in the gut and the airways, but the function of many of these receptors remains unclear[52].
Studies are showing that the way we experience food is influenced by all five senses, although scientists still debate whether taste is an inherent attribute of food or a personal psychological construct.
Unlike smell, in taste perception there is only consensus about the existence of five basic taste qualities (and their dedicated receptors): sweet, sour, salty, bitter and umami.
But there are hundreds of receptors for odours: the possibilities for mixing and matching taste and odour are immense, and lead to the wide variety of flavours we perceive, that subjectively bear little resemblance to any of those basic tastes[53].
Smell and taste are linked neurologically in a way that no other human senses are: both taste and retronasal odours arrive via the same pathway (mouth), and there are several so called "multi-modal" brain areas identified (e.g orbitofrontal cortex, amygdala) where aroma and taste derived sensory information is integrated, to form an overall perception of flavour.

[50] M. Le Thanh, I. Goubet, J. L. Le Queré, A. Voilley – Journal of American Oil Chemists' Society (1998) 75, 441
[51] J. P. Schirle-Keller, G. A. Reineccius, L. C. Hatchwell – Journal of Food Science (1994) 59, 813
[52] H. Brody – Nature (2012) 486, S1
[53] N. Bakalar – Nature (2012) 486, S5

Oral processing is the initial step that releases volatile and non-volatile flavour compounds from food; these compounds are then transported to their respective receptors, via processes that are affected by a wide range of physiological and physical factors[54].

The proposed sequence of events starts in the mouth with the release of aroma compounds from the food into the mouth liquid phase; partition from the liquid phase to the gas phase then occurs, and portions of the mouth gas-phase are transferred to the throat during swallowing[55].

Delivery from the throat to the olfactory receptors is achieved by two mechanisms: the first is a rapid, direct transfer of gas-phase aroma compounds to the olfactory receptors caused by the swallowing action.

The second mechanism involves a slower partition of aroma compounds from the liquid phase, which now lines the throat, into the tidal air stream and subsequent delivery to the olfactory receptors during exhalation[56].

The latter is thought to be responsible for the persistence of aroma delivery after swallowing: a high concentration of aroma is seen on the first breath, followed by a much lower concentration on subsequent breaths, which show an exponential decrease.

The first breath concentration is very variable, presumably because it depends on the exact way the person swallowed the solution as well as factors such as different mouth volumes.

Next to a number of physiological (mastication, swallowing, breath, etc.), biochemical (enzymatic and biological reactions), physicochemical (partition, heat, and mass transfers) and physical (product fragmentation, dissolution and/or dilution) processes occur and interact within the mouth during food consumption.

Indeed, it is well known that individual tasters often perceive different flavours from the same food, and some of this variation may be due to differences in release of flavour compounds in the mouth (Fig. 4)[57].

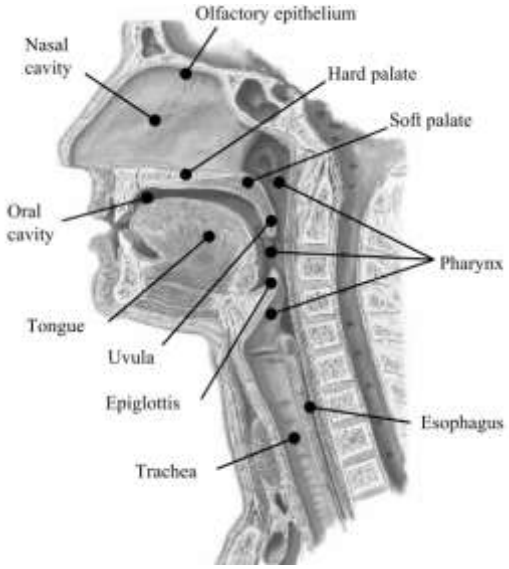

Figure 4. The human olfactory apparatus
(from K. G. C. Weel - Wageningen University & NIZO Food Research (2004): PhD Thesis)

[54] M. D. Hodgson, J. P. Langridge, R. S. T. Linforth, A. J. Taylor – Journal of Agricultural and Food Chemistry (2005) 53, 1700

[55] K. M. Wright, B. P. Hills, T. A. Hollowood, R. S. T. Linforth, A. J. Taylor – Journal of Food Science and Technology (2003) 38, 343

[56] V. Normand, S. Avison, A. Parker - Chemical Senses (2004) 29, 235

[57] S. M. van Ruth, K. Buhr - International Journal of Mass Spectrometry (2004) 239, 187

1.5 Added flavours: UE legal issues

From a legal point of view, flavourings are regarded as a major category of ingredients intentionally added to food and feeding stuffs in small amounts, with the primary purpose to impart flavour, except for substances that have an exclusively sweet, sour or salty taste[58].
The previous EU Flavour Directive 88/388/EEC[59] dictated the categories in which they were divided:

Flavouring substances, chemically defined substances in the definitions of:

1. Natural flavouring substances
2. Nature-identical flavouring substances
3. Artificial flavouring substances

Flavouring preparations: natural complexes, obtained by appropriate physical, microbiological or enzymatic processes from foodstuffs or other material of vegetable or animal origin, either in the raw state or after processing for human consumption by traditional food-preparation processes (e.g. drying, fermentation).

Process flavourings: products which are obtained according to good manufacturing practices by heating a mixture of ingredients to a temperature not exceeding 180°C for a period not exceeding 15 min , the ingredients themselves not necessarily having flavouring properties, and at least one of which contains nitrogen (amino) and another is a reducing sugar.

Smoke flavourings: smoke extracts used in traditional foodstuff smoking processes.

Flavouring adjuvants: foodstuffs, food additives, other food ingredients or processing aids which are necessary to ensure the safety and quality of flavourings and to facilitate the production, storage and intended use of flavourings.

The new Regulation (EC) N°1334 of the European Parliament and of the Council on flavourings was adopted on 16th December 2008, and applied as from 20th January 2011[60]; from 20th January 2009, the industry had 24 months to comply, and the new Regulations have been enforced from 20th January 2011.
The definitions of flavouring preparations, process flavouring, smoke flavourings and the categories of flavouring substances has changed, except for "natural flavourings".
The terms "nature identical" or "artificial flavourings" no longer exists, and they are defined as "flavouring substances"; process flavourings have "thermal" added; "smoke flavourings" can now be declared as being from a source, i.e. "smoke flavouring from *x*"; "flavour precursor" is a new

[58] D. Muller – "Flavours: the Legal Framework". In *Flavours and Fragrances* (Berger R.G., Ed.) - Berlin: Springer (2007) 15
[59] "Council Directive of 22 June 1988 on the approximation of the laws of the Member States relating to flavourings, for use in foodstuffs and to source materials for their production (88/388/EEC)" - Official Journal of the European Communities n°184, 15 July 1988
[60] J. C. R. Demyttenaere – Flavour and Fragrance Journal (2012) 27, 3

category and covers process type flavourings, which when added to a product reacts with or breaks down to add or modify the flavour in the application; "other flavourings" will include other substances which do not fall into any other categories[61].
About the labeling, requirements for flavourings in the EU are laid down in EU Flavour Directive 88/388/EEC for the flavourings themselves and in EU Directive 91/72/EEC, concerning their designation in the list of ingredients of the final foodstuff.
The Regulation stipulates requirements both for flavouring manufacturers and (final) food manufacturers; these include:

- e.g. packaging labelling of flavourings for downstream manufacturers and consumers must include details about the presence of food allergens and date of minimum durability.
- e.g. labelling as "natural flavouring substance(s)" may only be used for flavourings which contain exclusively natural flavouring substances.

Together with the amending Directive 91/71/EEC regulating the labelling of flavourings for end consumers, the New Regulation defined the categories of flavouring ingredients, purity criteria and maximum levels for certain "biological active principles" (BAPs)[62].
Following article 5 of the EU Flavour Directive, EU Regulation 2232/96 defined the basic rules for the use of flavouring substances for foodstuffs in the EU.
In addition, it lays down a procedure for establishing a positive list for flavouring substances in the EU, and the procedure for evaluation was published as Commission Regulation 1565/2000.
In 1998 the EU Commission within the Commission Decision 199/217/EEC published an inventory of flavouring substances used in or on foodstuffs in the EU.
Since then, flavourings reported in the list have been subjected to evaluation, leading to a positive list of substances to be used in foodstuffs in the EU[63], reported in the recently adopted Regulation EU 872/2012[64].
It enters into force on 22 October 2012 and will apply as of 22 April 2013; the EU food industry will only be able to use flavourings that are on the EU list, whereas compounds that are not on the list will be banned after an 18-months phasing-out period.

[61] http://www.uk.synergytaste.com/

[62] Commission Directive 91/71/EEC of 16 January 1991 completing Council Directive 88/388/EEC on the approximation of the laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production" - Official Journal of the European Communities n°*42, 15 February 1991*

[63] Commission Decision 1999/217/EC of 23 February 1999 adopting a register of flavourings substances used in or on foodstuffs, drawn up in application of Regulation (EC) n°2232/96 of the European Parliament and of the Council of 28 October 1996" - Official Journal of the European Communities n°*84, 27 March 1999*

[64] Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC

1.6 FACET

The Project *Flavourings, Additives and food Contact materials Exposure Task*, or in short *FACET*, was conceived with a view to improve current methods for assessment of dietary exposure to flavours, additives and food contact materials across Europe.

FACET was funded under the *European Commission 7th Framework programme Theme 2 food*[65], Agriculture and Fisheries and Biotechnology, as a large collaborative project.

This project started on the 1st September 2008, and lasted for 4 years, till the 31st October 2012.

FACET was developed in response to a call by the European Commission to produce a risk management tool consisting of a database containing information on the levels of food additives, flavourings and food packaging migrants and corresponding food consumption data[66].

The concept behind was the creation of a food chemical exposure surveillance system, sustainable beyond the life of the project, which covers representative regions of the EU and which will meet, to the highest possible standard, the needs of the EU regulatory authorities in the protection of consumer health.

In the context of the project the INRAN research group was responsible for the Work Package 2, covering "flavourings".

The general objective of this Work Package was to improve current methods for the assessment of dietary exposure to flavouring substances, and to reduce the uncertainty in this which is at present very large.

One of the specific tasks in this study was the assessment of the uncertainty associated to occurrence and concentration data on flavouring substances in foods currently available; to achieve this goal analytical surveys on products purchased from the European market have been carried out. Different food matrices have been selected to cover representative uses of the flavourings, and special emphasis has been given on those present in foods and beverages particularly consumed by children.

[65] http://www.ec.europa.eu/research/fp7/

[66] http://www.ucd.ie/facet/aboutfacet/#food_additives

1.7 State-of-the-art for VOC analysis

1.7.1 Dealing with flavours

Flavour research has largely meant studying the volatiles in a food; identification and quantification of aroma compounds are among the most challenging tasks faced by an analytical chemist.

The first obstacle is that analytical instruments are less sensitive to many aromas than the human nose: in fact, the nose has detection limits far higher than the most sensitive methods[67].

Moreover, natural systems are composed of several hundred volatile components that have an exceedingly broad range of chemical and physical properties, usually present in very low quantities (parts per million or parts per billion).

It is also to take into account that many volatile compounds are not flavour-active, i.e. they cannot be detected by the olfactory system, while others may even in trace amounts have significant effects on the overall flavour quality owing to their low odour-threshold values; consequently, the most abundant volatiles are not necessarily the most important contributors to flavour.

When considering the difficulties in the analytical methods for volatile compounds in food matrices, it has to be kept in mind that the instrumental determination is carried out on extracts that are obtained by submitting the original food products to several sample preparation treatments like grinding, homogenization, clean-up, extraction, that are essential to achieve the required reliability of the method: on the other hand, these treatments undoubtedly decrease representativeness of the data with respect to the actual conditions of the presence of such compounds in the analyzed item.

Hence, it is important to remember that no method of obtaining an aroma isolate from a food gives a complete quantitative or qualitative picture of the aroma compounds actually present in the food: every method used in aroma isolation has biases in isolation, and may produce artifacts[68].

Thus, the task of choosing isolation methods must be approached in a thoughtful manner.

In addition, in recent years miniaturization has become one of the dominant trends in analytical chemistry, and typical examples are Solid-Phase Micro-Extraction (SPME)[69], Single Drop Micro-Extraction (SDME)[70], and Stir Bar Sorptive Extraction (SBSE)[71].

These new extraction methods, combined with state-of-the-art instrumental techniques for final determination, can result in faster analysis, higher sample throughput, and lower solvent consumption, while maintaining or even improving sensitivity.

In particular, reduction of solvent consumption in analytical laboratories is expected to significantly contribute to the reduction of costs; in most instances, miniaturized sample preparations can also be automated and coupled on-line to the analysis.

Sensorial analysis is complementary to the chemical information; it considers the foodstuff as a whole and gives an overall impression, although some of its techniques such as quantitative descriptive analysis[72] (QDA) can provide a description of the main attributes contributing to the overall flavour of the food.

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[67] M. Stuiver – "*Biophysics of the sense of smell*" – Thesis (1958), Groningen University

[68] G. A Reineccius – In *Flavour measurement* (C. T. Ho, C. J. Manley Eds.) – New York: Dekker (1993) pp. 61

[69] C. L. Arthur, J. Pawliszyn – Analytical Chemistry (1990) 62, 2145

[70] E. Psillakis, N. Kalogerakis – Journal of Chromatography A (2001) 938, 113

[71] A. Prieto, O. Basauri, R. Rodil, A. Usobiaga, L.A. Fernández, N. Etxebarria, O. Zuloaga – Journal of Chromatography A (2010) 1217, 2642

[72] H. Stone, J. H. Siedel, J. Bloomquist - Cereal Foods World (1980) 25, 642

Obviously, as the measuring instrument is a group of tasters, it is subjective, even though it works with standardized and precise techniques; however, using a good analytical (trained) panel the subjectivity is reduced, and it is possible to obtain reproducible results[73].
Especially new methods for measuring flavour release in complex matrices and sensory techniques, combined with novel chemometric tools, may give answer to still open questions.

1.7.2 Chemical analysis

In general, studying the volatile fraction of foods requires several steps: (1) separation and concentration, (2) fractionation of the different compounds, and (3) identification; all these steps must be properly chosen[74].
Separation techniques may be grouped in those that take advantage of either solubility or volatility of the target compounds.
Volatility is an essential requirement for a compound to contribute to the perceived aroma, so it is the basis of a number of techniques to separate them from the food matrix. On the other hand, most of aroma compounds are more soluble in an organic solvent than in water, which is the main component of many food matrixes, and consequently many isolation techniques are based on solvent extraction principles.

1.7.2.1 Separation and concentration of the volatile fraction

Sample preparation is an essential step in analysis, greatly influencing the reliability and accuracy of resulted the time and cost of analysis.
There are two great groups of methods: those not involving concentration and those involving concentration (Tab. 1).

Methods without Concentration	Methods with Concentration	Others
Direct injection	Distillation	Extraction with solvents
Static headspace	Simultaneous distillation–extraction	
	Dynamic headspace/purge and trap	Membrane dialysis
	Supercritical fluid extraction	
	Solid phase microextraction	

Table 1 Methods of separation and concentration of volatiles

1.7.2.1.1 Without concentration

Direct injection. This is the simplest technique for the analysis of volatile compounds, and requires the least manipulation of the sample; it was developed for the analysis of lipids[75].
It consists of the introduction of a small amount of sample into a tube filled with glass wool at the injector inlet: then, the sample is heated to a specific temperature and purged with gas to the gas-chromatograph column.

[73] M. A. Drake – Journal of Dairy Science (2007) 90, 4925
[74] R. V. Golovnya – Journal of Chromatography (1982) 251, 249
[75] W. H. Morrison, B. G. Lyon, J. A. Robertson – Journal of the American Oil Chemists' Society (1981) 58, 23

The technique of direct injection is not very sensitive because the amounts of sample have to be small, and having to use high temperatures between 180 and 200°C, decomposition products can appear in the volatile fraction.
A further disadvantage is the need for a thorough cleaning of the chromatograph between samples, to prevent the "memory effect".
Static headspace. This method consists of analyzing an aliquot of the vapour phase, which is in equilibrium with the sample in a sealed vial, then subjected to a specific temperature for a certain time. The temperature and sampling have to be strictly controlled[76].
The greatest disadvantage of this technique is poor sensitivity, and often, there is a large amount of water in the headspace; thus, it is unsuitable for the analysis of traces, and of compounds with low vapour pressure.

1.7.2.1.2 With concentration

Distillation. This is one of the techniques most used for the isolation of volatile compounds in foodstuffs; distillation under reduced pressure enables working at low temperatures, thereby minimizing possible formation of artifacts due to sample heating.
Generally, the vapours from the distillation are condensed on a refrigerant, or are trapped in different cryogenic traps or on absorbent materials.
The distillate can be injected directly into the chromatograph, although a concentration step is usually necessary, because some of the compounds may be in trace amounts; such concentration is normally carried out by several evaporative techniques that take advantage of differences in boiling point between the target volatiles and the solvent.
Simultaneous distillation–extraction. This widely used method was introduced by Likens and Nickerson in 1964[77]: the sample volatiles dissolved in water and the organic solvent distill separately, and condense in the same zone of extraction; the two phases are then separated and recycled several times.
The method uses small amounts of solvents, thus reducing contamination, it yields a high concentration of volatile compounds in a short time, and minimizes thermal degradation by working at reduced pressure; its disadvantage is that it is not suitable for thermolabile volatile compounds.
Dynamic headspace. This method is used to determine volatiles dissolved in aqueous samples; it consists of purging the sample with an inert gas at a specific temperature, passing this controlled flow through a trap where the compounds are first retained and subsequently desorbed, and then injected into the chromatograph for their separation.
The method has two forms: (1) real dynamic headspace, which consists of sweeping the surface of the sample under shaking; (2) purge and vapour, in which the purge gas bubbles through the sample.
The process can be affected by various factors, such as the diameter and length of the traps, the size and shape of the container used in the isolation, and the particle size of the absorbent[78].
For the concentration step, adsorbent (Tenax, Porapak, Chromosorb)[79] and/or cryogenic traps are used[80].

[76] A. Nunez, L. F. Gonzalez, J. Janak – Journal of Chromatography (1984) 300, 127
[77] J. Rujks – Journal of Chromatography (1983) 279, 395
[78] L. Senf, H. Frank – Journal of Chromatography (1990) 520, 131
[79] M. T. Morales, R. Aparicio - Grasas Aceites (1993) 44, 113

Supercritical Fluid Extraction (SFE). In the last few years, SFE has been applied in the extraction of seed oils, but rarely in studying the volatile fraction of other food products; recently, extra-virgin olive oil and olives have been analyzed using supercritical carbon dioxide for isolating the volatile components[81].

Solid Phase Microextraction (SPME). Pawliszyn's group developed this solvent-free method in 1989 and applied it to environmental analysis; since then, it has become a widely used technique for the analysis of volatiles in foods[82].

The technique uses a fiber coated with an adsorbent material, which forms the retractable needle of a modified chromatographic syringe, to isolate and concentrate the target compounds; the fiber is then positioned in the headspace of the sample or dipped in a liquid sample for a fixed time.

The volatile compounds are concentrated on the polymer coating depending on their coefficients of distribution and adsorption mechanisms; then, the fiber is removed from the sample and placed in the GC injector, where the compounds are thermally desorbed.

Stir Bar Sorptive Extraction (SBSE). This technique was developed by Baltussen and colleagues in 1999[83]; it is a technique for the sorptive extraction of aqueous samples offering an improved sensitivity with respect of SPME, due to the higher amount of the adsorbent phase which is placed on an inert glass stir bar.

The Stir Bar is introduced in the aqueous sample and sorptive extraction occurs whilst stirring; then, the Stir Bar is removed from the sample, dried and introduced into a glass desorption tube inserted in a desorption unit on top of the chromatographic column.

Thereafter, the analytes are thermally desorbed and transferred to a GC-MS instrument; a variant of SBSE is headspace sorptive extraction (HSSE), where sampling takes place in the headspace above a sample, similar to headspace SPME[84].

1.7.2.2 Fractionation and identification of the volatiles

The analytical method used to analyze an aroma isolate depends on the purpose.

To determine the amount of one or more aroma compounds in a food, GC may be a suitable option: if one is looking for odorous compounds in a food, then gas-chromatography-olfactometry (GC/O)[85] should be used; to identify the aroma compounds, GC/MS is now mandatory[86].

Another technique, Ion Mobility-Mass Spectrometry (IMS-MS) is a method that is potentially able to rapidly separate volatile components in a gaseous mixture.

First, the ion mobility spectrometer separates ions according to their mobilities; in a second step, the MS separates ions according to their m/z ratio: such a combination is often referred to as a hyphenated or multi-dimensional fractionation[87].

Electronic noses and MS-based sensors are particularly attractive for certain applications of quality control, where a decision of acceptable/unacceptable food is often necessary, on the basis of the detected volatile compounds[88].

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[80] M. Servili – Journal of the Science of Food and Agriculture (1995) 67, 61

[81] M. T. Morales, A. J. Berry, P. S. McIntyre, R. Aparicio – Journal of Chromatography A (1998) 819, 267

[82] J. Pawliszyn – *Solid Phase Microextraction: Theory and Practice*. Wiley-VCH (1997)

[83] E. Baltussen, P. Sandra, F. David, C.Cramers – Journal of Microcolumn Separation (1999) 11, 737

[84] B. T. Weldegergis, A. G. J. Tredoux, A. M. Crouch – Journal of Agricultural and Food Chemistry (2007) 55, 8696

[85] C. M. Delahunty, G. Eyres, J-P. Dufour - Journal of Separation Science (2006) 29(14), 2107

[86] R. J. Molyneux, P. Schieberle – Journal of Agricultural and Food Chemistry (2007) 55, 4625

[87] Z. Karpas, A. V. Guaman, D. Calvo, A. Pardo, S. Marco – Talanta (2012) 93, 200

Real time techniques such as atmospheric pressure chemical ionization mass-spectrometry (APCI-MS) and PTR-MS have been applied to the measurement of in vivo volatile release; indeed, in-vivo methods allow the most accurate indication of food interaction with the human olfactory system[89].

[88] D. Hodgins, D. Simmonds - Cereal Foods World (1995) 40(4), 186
[89] M. Mestres, N. Moran, A. Jordan, A. Buettner – Journal of Agricultural and Food Chemistry (2005) 53(2), 403

1.9 Aim of the Thesis

In food science, there are several ways of defining *quality*, and perhaps there is no single universal definition that adequately satisfies all situations; in general terms, *quality* is defined as "*the combination of attributes or characteristics of a product that have significance in determining the degree of acceptability of that product by the consumer*".

The aim of this Thesis lies mostly on food quality field: indeed, one of the most important criteria for consumer acceptance of food is *flavour*.

Due to the increasing demand of high quality and enjoyable foods, the need for methodologies enabling a better understanding of flavour has arisen: chemical analysis is a valuable way of studying volatile composition of different food matrices, and very sophisticated instrumentations are available nowadays, but almost always there is the need of clean-up/concentration steps before such analysis.

Moreover, when volatile compounds are present in traces (ppb or even ppt), their detection and identification require the development of analytical tools which can tackle these difficult tasks: in the past, they were largely detected with insufficient performances or even undetected, due to the inadequacies in old-fashioned analytical techniques.

Hence, in this work novel, simple, rapid, and "environmental friendly" sample preparation methods were implemented, which coupled really well with *Thermal Desorption – Gas Chromatography – Mass Spectrometry (TD-GC-MS)*, following also the *Green Chemistry* requirements.

It was investigated the use of *Stir Bar Sorptive Extraction (SBSE)* technique, showing that it is extremely suited for troublesome analytical challenges such as isolation of volatile compounds from several food matrixes, including both processed (beverages, yogurts) and fresh foods (fruits and vegetables); the application of this technique was thus broaden in new complex matrices (e.g. yogurts).

This sorptive sample preparation showed good performances for practical, real-life analytical problems, which cannot easily be solved by alternative and time-consuming approaches: it allows an effective extraction of volatiles in the trace range, and a rich aromatic profile may be pursued using optimized conditions.

SBSE has proven to be a technique that can offer high and reproducible extraction recoveries for the complex matrices under study; it was also characterized by ease of use, good repeatability and robustness.

In addition, *Headspace Solid Phase Micro-Extraction (HSPME)* was used for sampling volatile from the headspace of olive oil samples: the point is that enrichment for gaseous samples is more difficult; particularly the analysis of polar compounds is an analytical problem that cannot be addressed adequately by conventional techniques, i.e. distillation, dynamic headspace, etc.

Using this other solventless extraction technique, a reliable analytical method for the determination of a broad range of volatiles in olive oil was developed.

Then, looking also at volatile release, which is a complementary task for a better understanding of interactions among food components, volatiles and in-vivo behavior, a real-time instrumentation, *Proton Transfer Reaction - Mass Spectrometry (PTR-MS)*, was used to follow these rapid changing phenomena, without any need of sample preparation or calibration.

The application of these analytical methods was then combined with the use of suitable chemometric techniques such as *PCA (Principal Component Analysis), ASCA (Analysis of Variance*

combined to Simultaneous Component Analysis), *MIXTURE DESIGN MODELING*, and *PLSR (Partial Least Squares Regression)*.
We truly think that nowadays chemometrics is unavoidable and of utmost importance, when scientists have to deal with multivariate datasets, and when robust "chemical-driven" modeling of complex phenomena is needed.

More in detail, the experimental work was focused on the following research topics:

1. Investigation on the effects of pre- and post-harvest factors on the development of flavour profile in apples and tomatoes, respectively (**Chapters 4-5**)
2. Study of innovative processes for the extraction of extra virgin olive oil, unravelling their effects on the organoleptic properties (**Chapter 6**)
3. Application of a mixture design approach to the modeling of *in-vitro* and *in-vivo* aroma releases from dairy emulsions; this is important for the development of healthier products such as low-fat foods, and new flavour formulations with the desired behaviour (**Chapter 7**)
4. Analytical surveys on flavoured products purchased from the market (yogurts, fruit juice-based and cola-beverages) within the activities of the EU Project *FACET* (*Food Additives and Food Contact Material Exposure Task*), addressed to the reduction of the uncertainty in the assessment of dietary exposure of the EU population to flavouring substances.
   In this context, developed analytical methods were also validated (**Chapter 3**)

Most of the experimental work was conducted at the former *National Institute for Food and Nutrition* (*I.N.R.A.N.*) which is now part of the *C.R.A.* (*Agricultural Research Council*) in Rome, under the supervision of Dr. Antonio Raffo and Prof. Andrea Magrì; in that Institute, a fruitful collaboration was established with the sensory analysis group, led by Dr. Fiorella Sinesio.
The study on aroma release was the outcome of a 6 months-traineeship, held at *NIZO Food Research*, a research centre situated in Ede (NL), under the supervision of Dr. Kerstin Burseg.
Statistical analysis and chemometric elaborations were performed at *SAPIENZA University*, whitin the research group led by Dr. Federico Marini.
Given the amount of data collected, the multidisciplinary approach followed covering analytical chemistry, chemometrics, food science and biochemistry, and being this Thesis the result of a collaboration between different research groups, there is the need of more time to be aware of all the insights pursuable: our sake and hope is to get published on top-level Journals all the achievements got, in the next future.

Antonio D'Aloise

Chapter 2: Materials and methods

2.1 Introduction

Increased awareness by consumers regarding the quality and safety of the foodstuffs they consume has necessitated the development of fast, accurate and sensitive analytical methods for the determination of compounds linked to safety and quality.
Especially concerning safety issues, governmental regulatory bodies are responsible for determining maximum levels at which certain substances, for instance carcinogens, may be present in products intended for human consumption.
Flavour, being a combination of taste and olfaction, is a crucial factor in consumer acceptance of foods, and is sensitive to compositional alterations.
Therefore, important flavour compounds often need to be monitored for quality control purposes; due to the different chemical nature and levels of these compounds in a variety of sample matrices, efficient sample extraction methods and suitable instrumental analysis is required.
In general, the analytical method involves processes such as sampling, sample preparation, separation, detection and data analysis, and more than 80% of the analysis time is spent on sampling and sample preparation steps such as extraction, concentration, fractionation and isolation of analytes.
It is not an exaggeration to say that the choice of an appropriate sample preparation method greatly influences the reliable and accurate analysis of volatile compounds; traditional methods such as steam distillation, extraction with organic solvents, surfactants and supercritical fluids, and solid-phase extraction involve some drawbacks, such as being tedious, time-consuming, prone to loss of analytes, and requiring large volumes of samples and solvents[90].
Analytical methods used for the experimental work, and suitable chemometric tools used for data analysis and interpretation will be discussed in the present chapter:

- *Headspace-Solid Phase Micro-Extraction (HS-SPME) and Stir Bar Sorptive Extraction (SBSE)* among the novel and fast extraction methods of volatiles, having also the requirements for being classified in the "Green Chemistry", a way of research that encourages the design of processes that minimizes the use of hazardous substances, such as solvents
- *Gas-chromatography Mass Spectrometry (GC-MS)*, the election technique for volatiles identification and analysis
- *Proton Transfer Reaction Mass Spectrometry (PTR-MS)*, a real-time instrumental technique particularly suitable for studies on aroma release
- *Principal Component Analysis (PCA)*, "the mother of all methods in multivariate data analysis"

[90] H. Kataoka, H. L. Lord, J. Pawliszyn - Journal of Chromatography A (2000) 880, 35

- *Analysis of variance combined to Simultaneous Component Analysis (ASCA)*, a well-suited technique to evaluate the potential effect of different factors underlying an experimental design-based study
- *Partial Least Squares Regression (PLSR)*, a regression method able to handle high correlated *X*-variables and relate them to *Y*-dependent variables, as it occurs when linking chemical composition of foods to their organoleptic properties (sensory descriptors)
- *Mixture Design Modeling*, a robust statistical approach for relating measured properties of mixtures to their composition, and useful for building either descriptive or predictive models

2.2 Analytical methods

2.2.1 Headspace-Solid Phase Micro-Extraction (HSPME)

Solid-Phase Micro-Extraction (SPME) is a very simple and efficient, solventless sample preparation method, invented by Pawliszyn in 1989.

It has been widely used in different fields of analytical chemistry since its first applications to environmental and food analysis, and is ideally suited for coupling with mass spectrometry[91], because SPME has a low recovery thus requiring a sensitive detection.

SPME reduces the time necessary for sample preparation, decreases purchase and disposal costs of solvents and can improve detection limits, integrating into a single solvent-free step sampling, extraction, concentration and sample introduction.

It has been routinely used in combination with GC-MS and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semi-volatile organic compounds from environmental, biological and food samples.

The concept of SPME may have been derived from the idea of an immersed GC capillary column, and the SPME apparatus is a very simple device.

It looks like modified syringe (Fig. 5) consisting of a fiber holder and a fiber assembly, the latter containing a 1–2 cm long retractable SPME fiber.

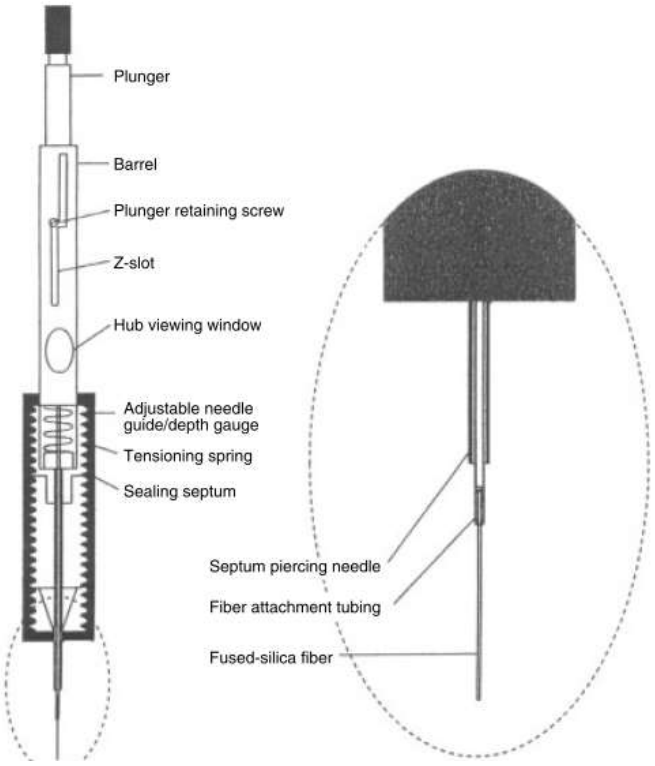

Figure 5. Schematic diagram of a commercial SPME device (from Kataoka et al., J. Chrom. A (2000) 880, 35)

The SPME fiber itself is a thin fused-silica optical fiber, coated with a thin polymer film, conventionally used as a coating material in chromatography; as shown in Fig. 6, seven kinds of fibers are commercially available.

[91] G. Vas, K. Vekey – Journal of Mass Spectrometry (2004) 39, 233

The polymer coating acts like a sponge, concentrating the analytes by absorption/adsorption processes; extraction is based on a similar principle to chromatography[92], based on gas–liquid or liquid–liquid partitioning.

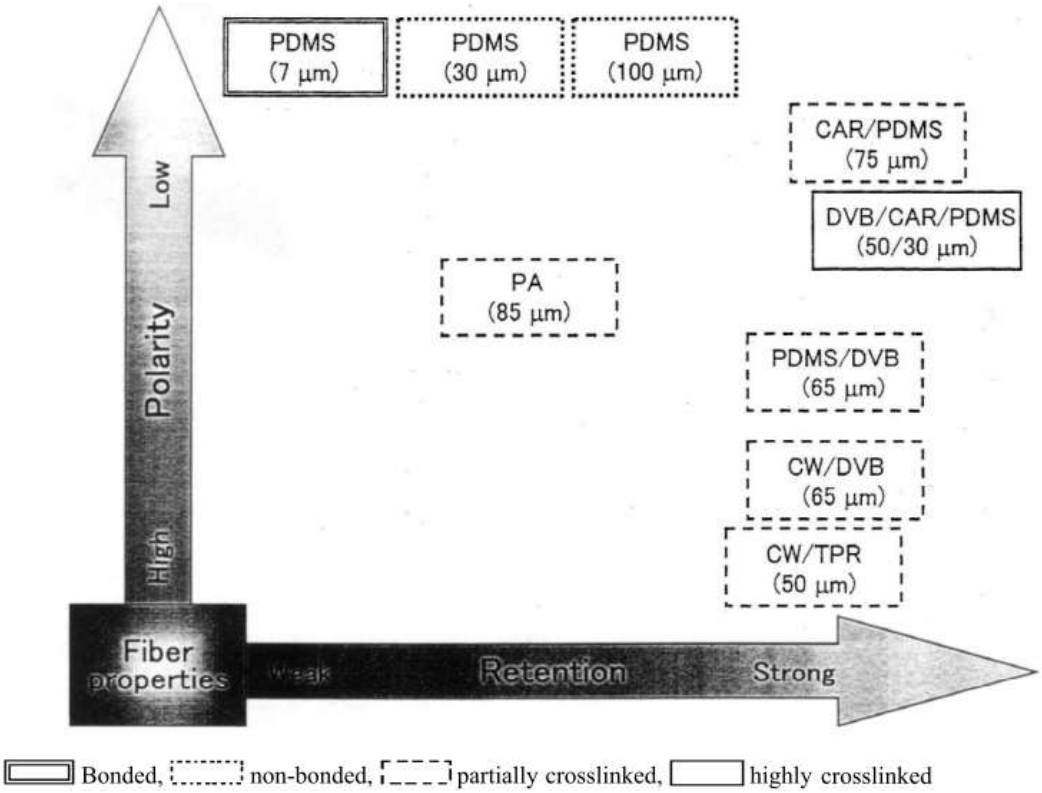

Figure 6. Properties of commercially available SPME fibers
(from Kataoka et al., J. Chrom. A (2000) 880, 35)

The more-polar polyacrylate (PA) fiber is preferred for the extraction of more-polar analytes; mixed coating fibers, containing divinylbenzene (DVB) copolymers, templated resin (TPR) or Carboxen (CAR: a porous activated carbon support), increase retention capacity due to the mutually potentiating effect of adsorption and distribution to the stationary phase.
PDMS–DVB, CAR–DVB, Carbowax (CW: polyethylene glycol)–DVB and CW–TPR can be used for the extraction of volatile low-molecular-mass and polar analytes.
Kinetics of the SPME extraction process depends on a number of parameters (e.g. film thickness, agitation of the sample); sampling times are typically in the order of a few minutes.
After sampling, the fiber is retracted into the metal needle (for mechanical protection), and the next step is transfer of the analyte from the fiber into the gas chromatograph: thermal desorption of the analytes takes place in the hot GC injector; after inserting the needle into the injector, the fiber is pushed outside the metal needle.
The other common option is analysis by HPLC: in this case, the needle is placed into a modified Rheodyne or Valco valve, where the fiber is exposed and the analytes are eluted by the mobile phase.

[92] S. Ulrich – Journal of Chromatography A (2000) 902, 167

Two types of fiber SPME techniques can be used to extract compounds: headspace HS-SPME and direct immersion DI-SPME; in either case, the SPME needle is inserted into the appropriate position (e.g. through a septum into the headspace), the needle protecting the fiber is retracted and exposed to the environment.
Volatiles are most conveniently studied by headspace analysis, followed by GC-MS: as there is no solvent (only that absorbed by the fiber), usually splitless injection is used, and analysis is very sensitive; sampling fibers can be used multiple times, hundreds of analyses in the case of HS analysis and dozens of times in the case of immersion analysis.
The time of extraction is independent of the concentration of analyte in the sample and the relative number of molecules extracted at a distinct time is also independent of the concentration of analyte; probably, the most important feature determining the analytical performance of SPME is the type and thickness of the coating material.
The time of extraction (until equilibrium) may be decreased with use of any type of agitation method (stirring, ultrasonics, etc.), and in the case of perfect agitation the extraction time depends only on the geometry of the fiber and the analyte diffusion coefficients.
Efficient thermal desorption of the analyte in a GC injection port is dependent on the analyte volatility, the thickness of the fiber coating, injection depth, injector temperature and exposure time.
The needle exposure depth should be adjusted to place the fiber in the centre of the hot injector zone; generally, the optimal desorption temperature is approximately equal to the boiling-point of the least volatile analyte, but in practice the extraction temperature should be 10–20°C lower than the temperature limit of the fiber.
To prevent peak broadening, the initial GC column temperature should be kept low or possibly even cooled (cryo-focusing); in this way, concentration of analytes at the head of the column is achieved; desorption time depends on the injector temperature and the linear flow-rate around the fiber.
HS-SPME involves multi-phase equilibrium processes, so careful consideration must be given to the physicochemical properties of the candidates for internal standards.
With SPME, the amount of analyte removed by the fiber (or extraction capillary) is proportioned to the concentration of the compounds in the sample; the ability to use SPME quantitatively before reaching equilibrium permits much shorter sampling times, producing a fast economical and versatile technique.
The reproducibility and precision can be improved with fiber SPME through careful control and monitoring of time and temperature (which should be precisely constant) during sample extraction; the extraction time is a critical parameter in the SPME sampling process.
Although SPME has a maximum sensitivity at the partition equilibrium, a proportional relationship is obtained between the amount of analyte adsorbed by the SPME fiber and its initial concentration in the sample matrix before reaching partition equilibrium; therefore, full equilibration is not necessary for quantitative analysis by SPME[93].

2.2.2 Stir Bar Sorptive Extraction (SBSE)

In some cases, the applicability of adsorptive sample preparation falls short, particularly for the enrichment of polar and/or high-molecular-weight compounds, especially in combination with thermal desorption.

[93] J. Ai - Analytical Chemistry (1997) 69, 1230

Because of the very strong retention of adsorbent materials, undesired effects such as incomplete desorption and artifact formations are observed: polar solutes are easily adsorbed, but readily undergo surface-catalyzed reactions, and on desorption yield compounds different than those originally sampled; moreover, high molecular-weight compounds cannot be desorbed because of extremely strong interactions with the adsorbent and their low volatility.

To overcome some of these problems sample preparation techniques based on sorption have been developed over the past 15 years; in contrast with adsorptive trapping, sorption is based on dissolution of the analytes in a liquid polymeric material.

This is a much more inert means of solute retention, which overcomes some of the limitations encountered when working with adsorbents[94].

In contrast with adsorbents, sorption (dissolution or partitioning) materials are a group of polymeric materials that are above their glass transition point (T_g) at all the temperatures employed; in this temperature range sorbents are in a gum-like or liquid-like state, and behave similarly to organic solvents.

The most commonly used sorbent is the apolar polydimethylsiloxane (PDMS), commonly used as a stationary phase in gas chromatography: this material is so popular because it is very inert; its degradation products are, moreover, very well-known and can easily be identified by mass spectrometry.

Sorbents are, in principle, homogeneous, non-porous materials in which the analytes can dissolve; therefore, the analytes do not undergo real (temporary) bonding with the material but are retained by dissolution; since the solutes migrate into the sorbent, the total amount of extraction phase is important in sorptive extraction.

An important breakthrough was made with the previously mentioned SPME: however, the amount of extraction medium (i.e. the amount of PDMS coated on the fiber) is very limited.

For a typical 100 µm PDMS fiber, which is the most widely used, the volume of extraction phase is approximately 0.5 µL: consequently, the extraction efficiency for solutes that are partially water soluble can be quite low[95].

For very apolar compounds, on the other hand, competition can occur between the aqueous phase, the SPME fiber, the glass of the extraction vessel, and the surface of the polytetrafluoroethylene Stir Bar used to stir samples[96].

Based upon these observations, a new extraction method was developed: Stir Bars of 1 or 2 cm long, coated with a 0.5 or 1 mm layer have been made commercially available (Twister™, Gerstel GmbH, Mullheim a/d Ruhr, Germany).

A magnetic rod is encapsulated in a glass jacket, on which a PDMS coating is placed; after extraction, either thermal desorption or liquid desorption can be used: the technique was named Stir Bar Sorptive Extraction (SBSE)[97].

The basic principles of SBSE are thus identical to SPME using PDMS coated fibers, but the volume of extraction phase is 50–250 times larger.

Sorptive extraction by nature is an equilibrium technique, and for water samples the extraction of solutes from the aqueous phase into the extraction medium is controlled by the partitioning

[94] E. Baltussen, C. A. Cramers, P. J. F. Sandra – Analytical and Bioanalytical Chemistry (2002) 373, 3

[95] C. L. Arthur, K. Pratt, S. Motlagh, J. Pawliszyn, R. L. Belardi – Journal of High Resolution Chromatography (1992) 15, 741

[96] E. Baltussen, P. Sandra, F. David, H. G. Janssen, C. Cramers - Analytical Chemistry (1999) 71, 5213

[97] E. Baltussen, P. Sandra, F. David, C. A. Cramers – Journal of Microcolumn Separation (1999) 11, 737

coefficient of the solutes between the silicone phase and the aqueous phase; studies have correlated this partitioning coefficient with the octanol–water distribution coefficients ($K_{o/w}$)[98].
It is very important to realize that the sorption equilibrium is also dependent upon the phase ratio, and thus on the amount of polydimethylsiloxane applied; this relationship is shown in the following equation:

$$K_{o/w} \approx K_{PDMS/\,w} = C_{PDMS} / C_w = (m_{PDMS} / m_w) \cdot (V_w / V_{PDMS}) = (m_{PDMS} / m_w) \cdot \beta$$

The recovery (under full equilibrium conditions), expressed as the ratio of the extracted amount of solute (m_{PDMS}) over the original amount of solute in the water ($m_0 = m_{PDMS} + m_w$) thus is determined by the distribution coefficient $K_{PDMS/w}$ and by the phase ratio β:

$$m_{PDMS} / m_0 = (K_{PDMS/w} / \beta) / (1 + (K_{PDMS/w} / \beta))$$

It is clear that the extraction efficiency increases with increasing $K_{PDMS/w}$: indeed, extraction efficiency decreases with increasing polarity; besides the $K_{PDMS/w}$ factor, phase ratio β also is important: the higher the PDMS amount, the lower the β and the higher the extraction efficiency.
Fig. 7 shows the influence of $logK_{o/w}$ and phase ratio on extraction efficiency: for a given β, an "S-shape" curve is obtained, whereby the position of the curve depends on the β ratio.

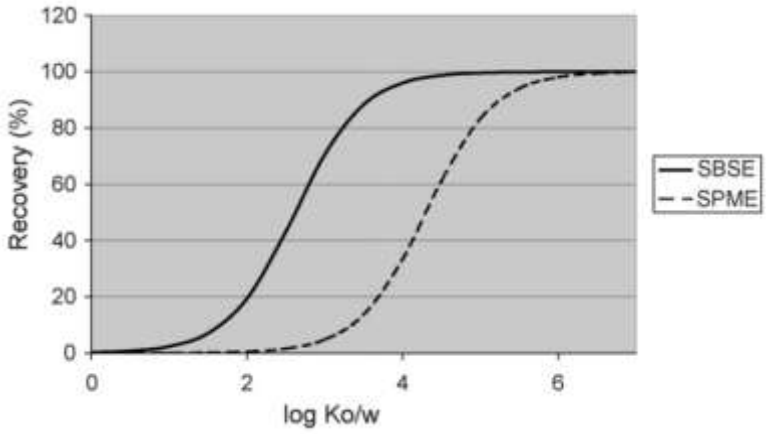

Figure 7. Theoretical recovery (%) in function of solute $logK_{o/w}$ for SPME (100 µm fiber, 0.5 µL PDMS) and SBSE (1 cm × 0.5 mm d_f, 25 µL PDMS) and 10 mL sample volume; equilibrium sampling is assumed (modified from A. G. J. Tredoux - Stellenbosch University (2008): PhD Thesis)

For SPME, the volume of PDMS is approximately 0.5 µL: for a sample of 10 mL, β is thus 20,000, and this results in poor recoveries for solutes with low $K_{o/w}$ values; a solute with $logK_{o/w} = 3$, is only recovered for 4.8%.
Using SBSE with a 1 cm Stir Bar coated with 0.5 mm phase (film-thickness 0.5 mm), the PDMS volume is 25 µL and the β 417 (also for a 10 mL sample volume): for a solute with $logK_{o/w} = 3$, the recovery increases to 71%.
Hence, it is clear that in SBSE quantitative extraction (100%) is reached at much lower $logK_{o/w}$ values than in SPME[99].

[98] C. Bicchi, C. Cordero, P. Rubiolo, P. Sandra – Journal of Separation Science (2003) 26, 1650
[99] F. David, P. Sandra – Journal of Chromatography A (2007) 1152, 54

It can be calculated that for apolar solutes ($logK_{o/w}$ > 3) the extracted amount increases proportionally with increasing sample amount, thus resulting in higher sensitivity; for more polar compounds, no proportional increase is obtained, since the effect of sample volume increase will be counteracted by a decreased recovery.

In addition to these thermodynamics, controlling the extraction under equilibrium conditions, the kinetic aspect is also important, and determined by sample volume, stirring speed, and Stir Bar dimensions: it must be optimized for any given application.

This relationship is quite complex, but it is clear that the time needed to reach equilibrium drastically increases with increasing sample volume and PDMS volume; as in SPME, often non-equilibrium conditions are used in practical SBSE.

Using an appropriate internal standard and calibrated condition, excellent quantification is possible.

The extraction of a liquid sample is performed by placing a suitable amount of sample in a headspace vial or other container: the Stir Bar is added and the sample is stirred, typically for 30–240 min.

After extraction, the Stir Bar is removed, dipped on a clean paper tissue to remove water droplets, and introduced in the thermal desorption unit of the gas chromatograph; in some cases, it is recommended to rinse the Stir Bar slightly with distilled water to remove adsorbed sugars, proteins, or other sample components.

This step will avoid the formation of non-volatile material during the thermal desorption step; rinsing does not cause solute loss, because the sorbed solutes are present inside the PDMS phase.

Alternatively, liquid desorption can be used; typically, the Stir Bar is placed in a small vial (2 mL, or vial with insert) and desorption is performed with apolar solvents followed by GC analysis, or with polar solvents (methanol, acetonitrile), followed by LC analysis.

After thermal or liquid desorption, the Stir Bars can be reused, and typically the life-time of a single Stir Bar is often more than 50 extractions; headspace sampling is also possible (*HSSE – Headspace Sorptive Extraction*), and the Stir Bar can be placed above a liquid or solid sample: special devices to hold the Stir Bars are commercially available.

For complete transfer of the sorbed fraction into the analytical system, thermal desorption is preferred: temperatures between 150 and 300°C are typically used.

In contrast to SPME, in which desorption is performed in the inlet (typically split/splitless inlet) of a gas chromatograph, SBSE is used in combination with a thermal desorption system for optimum desorption, re-concentration and GC analysis.

Special fully automated thermal desorption units are available (Gerstel GmbH, Mullheim a/d Ruhr, Germany), and a programmed-temperature vapourizing (PTV) injector is operated as a cryotrap for cryogenic refocusing of the thermally desorbed analytes: temperatures as low as −150°C are used along with liquid nitrogen cooling.

Because more sorptive extraction phase is used, the desorption process is slower than for a SPME fiber: longer desorption times (5-10 min) in combination with desorption flows between 10 and 100 mL/min are typically used.

The applied thermal desorption system should thus preferably allow independent control of desorption and injection flow if complete (splitless) transfer of the extracted solutes to the GC–MS is needed (highest sensitivity).

The required cryofocusing temperature depends on the solute volatility: for semi-volatiles with boiling points above 200°C, trapping at ambient temperature is often sufficient for focusing.

A drawback of SBSE is that only PDMS is available as an extraction phase on commercial Stir Bars; attempts have been made to apply other coatings, but problems such as irreproducible coating and excessive bleeding in thermal desorption have hindered the commercial introduction.
Moreover, by using in situ derivatization, SBSE with PDMS-coated Stir Bars can be applied to relatively polar solutes[100]; it is however clear that further developments in Stir Bar coatings and designs will extend the applicability of the method.

2.2.3 Gas-chromatography - Mass Spectrometry (GC-MS)

Gas chromatography is a separation tool in which separation is achieved by interactions between solutes in the gas phase and either a solid adsorbent (adsorption), or a liquid phase (partitioning). Thus GC separations can be subdivided into gas liquid chromatography (GLC), which is most widely used nowadays, and gas solid chromatography (GSC)[101].
Golay illustrated in the late 50's the vast increase in resolution that could have been obtained by using an open tube with a small inside diameter coated with a stationary phase; this improvement is mainly caused by the fact that a capillary column has much less resistance to mass transfer due to a significantly shorter diffusion distance.
Furthermore, the low pressure drop across the capillary column makes it possible to increase the column length significantly, thus improving resolution[102].
For these reasons, GLC employing wall coated open tubular columns is the preferred technique nowadays; in addition, these columns have the availability of numerous highly specific liquid polymers.
Modern capillary GC is characterized by high sensitivity, efficiency and versatility: it is therefore the method of choice for the analysis of relatively volatile and thermally stable organic molecules; molecules not directly amenable for GC are either derivatised, or are analyzed by liquid-based separation methods such as HPLC or CE.
In this Thesis, GLC with fused silica open tubular columns were used throughout, and therefore the following discussion will only deal with capillary columns.
Any chromatographic instrument consists of a sample introduction device, column, detector and data collecting system; in addition modern GC versions include accurate electronically regulated pneumatic and temperature control, providing extremely reproducible chromatographic results.
A wide range of capillary columns are available nowadays, differing in length, inner diameter, film thickness and type of stationary phase, this being determined by the analytes of interest.
The most common dimensions used in CGC are 30m × 0.25mm i.d., 0.25 µm d_f, but for very complex samples such as found in food and flavour industries, columns of up to 60–100 m in length are not uncommon.
Considering stationary phase selection, a phase with a polarity similar to the analytes of interest is generally selected, and in GC two separation mechanisms may be exploited.
The first is separation according to boiling point of the solutes, and is most relevant when using apolar stationary phases; separation based on selective partitioning (interactions with the stationary phase) is most prevalent when using polar columns.

[100] M. Kawaguchi, R. Ito, K. Saito, H. Nakazawa – Journal of Pharmaceutical and Biomedical Analysis (2006) 40, 500
[101] A. G. J. Tredoux – PhD Thesis (1998) – University of Stellenbosch (South Africa)
[102] M. J. E. Golay - Analytical Chemistry (1957) 29, 928

A variety of stationary phases ranging from apolar to polar are available for utilizing the optimal combination of these two mechanisms to achieve the desired separation; the most widely used stationary phases are polydimethylsiloxane (PDMS) and polyethelene glycol (wax) phases.
In GC, temperature control plays a crucial role: for this reason, the column is housed in a thermostatted oven, which allows running analysis by using a temperature program.
In order to sweep the analytes through the column, a carrier gas at a certain pressure is applied to the inlet of the column: hydrogen, helium, or to a lesser extent nitrogen are used for this purpose.
To obtain reproducible retention times, it is critical that the carrier gas pressure is regulated with high accuracy: for this, a high-precision electronic pneumatic control permits to vary the pressure while keeping the flow constant, as the oven temperature increases during the analysis.
Since GC is a gas phase technique, compounds need to be vapourized before entering the column, thus a heated injector is most commonly used; the classical split/splitless injector, in which the sample is introduced in a hot injector and almost instantaneously vapourized, is the oldest and still the most widely used injector[103].
An alternative to the split/splitless injector is the programmed temperature vapourizing injector (PTV), illustrated in Fig. 8.

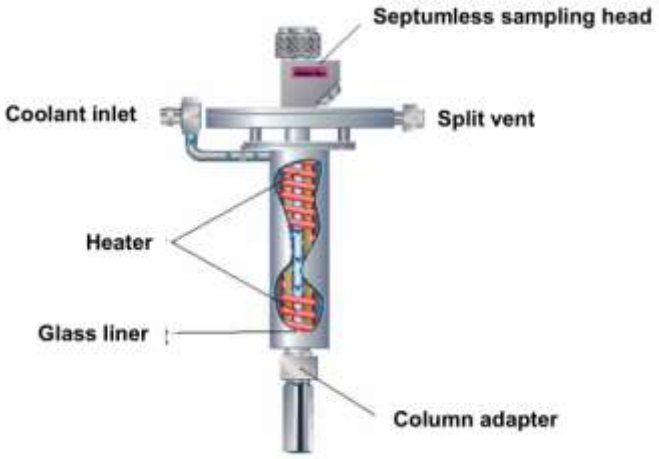

Figure 8. A programmed temperature vapourization (PTV) injector (CIS-4)
(from A. G. J. Tredoux - Stellenbosch University (2008): PhD Thesis)

The PTV is essentially a split/splitless injector with a much lower injector volume and the possibility to introduce the sample at low temperature; this is followed by rapid heating of the injector to transfer the sample to the column.
Heating is performed either by direct or indirect resistive heating while for cooling compressed air, carbon dioxide or liquid nitrogen is employed.
A PTV offers the possibility of large volume injection (LVI), allowing a relatively large amount of sample to be injected at low temperature (close to the boiling point of the solvent but not the analytes): with the split vent open initially, the sample solvent is vented from the injector before closing the split vent and heating the injector to introduce the sample to the column.
Since the sample is concentrated before injection, this leads to an increase in sensitivity and is beneficial in trace analysis; analogously, a PTV can be used to cryogenically trap analytes originating from a thermal desorption (TD) process or headspace sampling prior to injection.

[103] R. L. Grob – In *Modern Practice of Gas Chromatography* – NewYork: Wiley-interscience (1995)

A modern thermal desorption system (TDS-2) used in combination with a PTV, as depicted in Fig. 9, consists of a sealed tube holder that can be heated or cooled as in the case of the PTV.
A solid sample or a sorptive or adsorptive sampling device is placed in a glass sample tube: upon heating of this tube, volatiles and semi-volatiles are released and transferred by gas flow via a fused silica transfer line to the PTV for cryo-trapping.
The pneumatics of a TDS resembles those of a PTV injector, offering split, splitless or solvent venting modes; during thermal desorption, the PTV is typically operated in solvent vent mode, while being cooled to trap desorbed analytes, whit the TDS set in splitless mode.
After desorption, the PTV will be switched to splitless mode for injection, while the TDS will be in solvent vent mode to flush out impurities remaining after desorption and prevent them from entering the column[104].

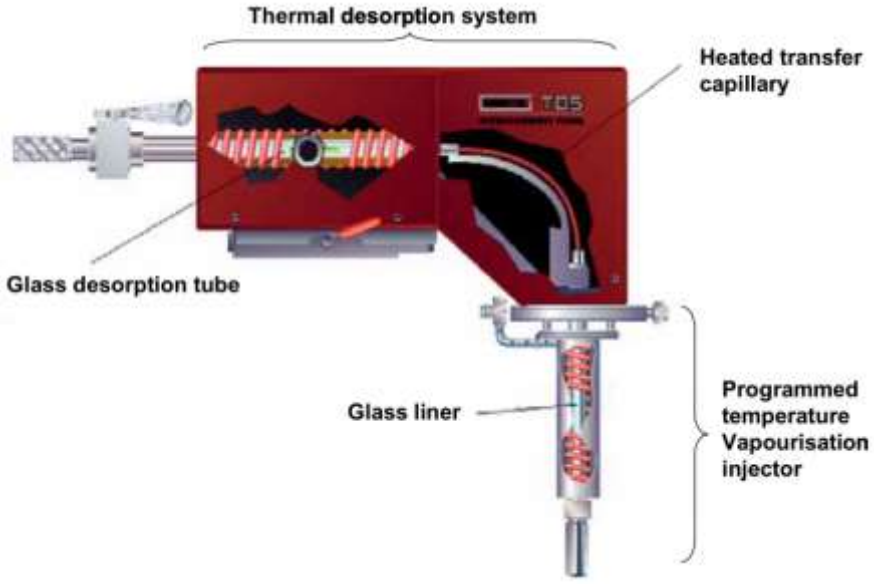

Figure 9. The TDS-2 thermal desorption system
(from A. G. J. Tredoux - Stellenbosch University (2008): PhD Thesis)

Several detectors have been developed for use in GC, varying significantly in terms of detection limits, linear range and specificity: the flame ionization detector (FID) is the most widely used, regarded as a universal detector; a number of specific detectors have also been developed, for example an electron capture detector (ECD) specific for electronegative species (e.g. halogenated compounds), and the nitrogen phosphorus detector (NPD) for nitrogen and phosphorus containing molecules (pesticides).
The mass spectrometer (MS), when coupled to GC, can be used as a selective or universal detector; due to its versatility, robustness and sensitivity, MS is nowadays one of the most common and valuable detectors available.
As this is the detector exclusively used in this study, the MS is discussed in more detail.
Coupling of GC with MS was first demonstrated by Holmes and Morrell in 1957[105]; it offers not only good sensitivity, but provides structural information, in the form of a mass spectrum.

[104] Courtesy of Gerstel GmBH, Germany (2007)
[105] J. C. Holmes, F. A. Morrell - Applied Spectroscopy (1957) 11, 86

A mass spectrometer essentially consists out of four parts: a sample inlet system, an ion source in which ionization and fragmentation of molecules takes place, a mass analyzer for separation of the ions according to their mass to charge ratio (m/z), and an ion detector, e.g. an electron multiplier.
In GC-MS, the sample is introduced into the MS[106] by positioning the outlet of the GC column, after being transferred to the MS via a heated transfer line, in the ion source as close as possible to the path of an electron beam.
The ion source consists of a filament providing high energy electrons for ionization, and various lenses for guiding the ions into the analyzer; the electron beam in the source is created by a heated filament and ionization can occur either directly by using electron impact ionization (EI) or indirectly, by chemical ionization (CI).
In CI, a reaction gas such as methane, ammonia or isobutane is ionized by electrons from the filament and the resulting ions ionize sample analytes by charge transfer processes; the result is a softer ionization technique with less fragmentation, and a higher possibility of obtaining molecular ions, indicative of a compounds molecular weight.
Several mass analyzers have been developed, the most common being the quadrupole (Fig. 10), which consists of four parallel rods around the flight path of the ions.

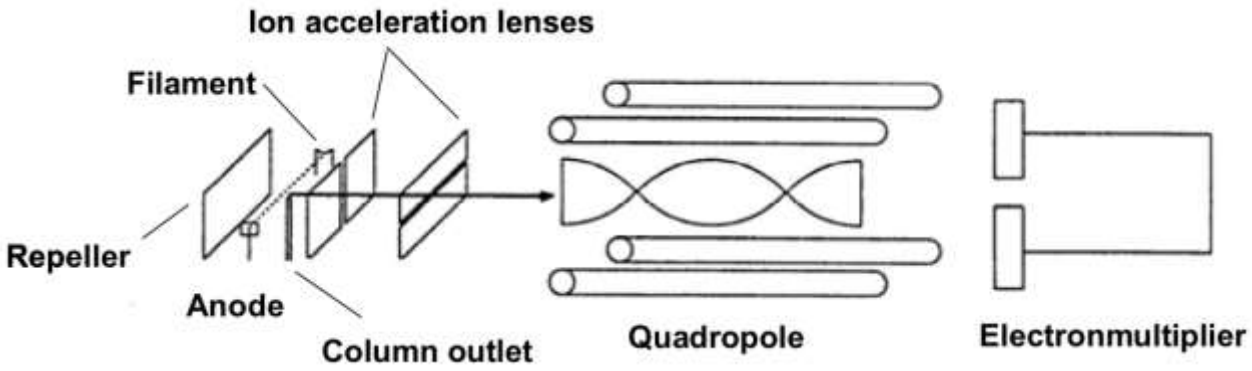

Figure 10. Basic components of a quadrupole mass spectrometer
(from A. G. J. Tredoux - Stellenbosch University (2008): PhD Thesis)

By applying a radio frequency on two of the opposite rods and a direct current voltage on the other two, a magnetic field is created between the rods: this field alters the resonance of all ions, in such a way that only one ion of a specific mass to charge ratio (m/z) will have a stable resonance and pass through the quadropole, while all other ions will collide with it and be lost.
Therefore, only ions of a specific m/z pass through the quadrupole at a specific time to be detected by the ion detector; by altering the voltages on the rods, it is possible to continuously select different ions.
If the voltages are changed in such a way that ions of sequentially increasing m/z ratios are allowed through the quadrupole, the instrument is being operated in the scan mode; in selected ion monitoring (SIM) mode, only the selected ions are monitored, thus sensitivity increase can be as much as 1000 fold.

[106] C. F. Poole, S. K. Poole – In *Chromatography Today* – Amsterdam: Elsevier (1991)

2.2.4 Proton Transfer Reaction - Mass Spectrometry (PTR-MS)

Volatile organic compounds (VOCs) are a major topic in food science and technology: they play an important role in food appreciation; furthermore, their fast, non-invasive detection helps to control product quality and to monitor fundamental and industrial processes[107].

Indeed, over the past few decades the need for quantitative and fast determination of a variety of VOCs in complex air matrices at ultra-low concentrations (pptv) has continuously pushed the limits of analytical chemistry: for a wide variety of problems , mass spectrometry has provided unique and competitive solutions.

The traditional solution to this problem has been to separate the compounds with a gas chromatograph (GC) before they enter a mass spectrometer (MS); unfortunately, GC analysis are inherently slow, thus while GC-MS is suitable for analyzing a single sample or monitoring slowly changing situations, it cannot usually be regarded as a "real time" or on-line technique.

In order to overcome these problems, a new technique called "Proton Transfer Reaction Mass Spectrometry" (PTR-MS) was developed in 1997 by Lindinger et al.[108]: this novelty enables a variety of volatile organic species in complex matrices to be monitored in real-time (within ms). PTR-MS links the idea of chemical ionization (CI) introduced by Munson and Field in 1966[109] with the swarm technique of flow-drift-tube type (FDT), invented by Ferguson and his colleagues in the early seventies[110].

CI is a versatile method for the identification/quantification of mixtures of organic molecules[111]: it exploits ion-molecule reactions rather than electron impact or photoionization. As a result, only little fragmentation is observed and the, protonated molecules (M+1) are the predominant species in the mass spectrum.

In PTR-MS, the CI system is based on proton-transfer reactions, thus using preferentially H_3O^+ as the primary reactant ion, even if there are other precursor ions available such as NO^+ and O_2^+.

H_3O^+ is a most suitable primary reactant ion when complex air samples are to be analyzed: in particular, H_3O^+ ions do not react with any of the natural components of air such as O_2 or N_2, as they have proton affinities lower than that of H_2O.

Moreover, most VOCs of interest in, for example, the food industry have proton affinities larger than that of H_2O; therefore, proton-transfer to the volatiles under study occurs with high efficiency[112].

Most PTR-MS set-ups (Fig. 11) us a hollow cathode ion source to produce an intense, pure H_3O^+ ion beam that is driven by a homogeneous, relatively high electrical field through a drift-tube reactor:

[107] F. Biasioli, C. Yeretzian, F. Gasperi, T. D. Mark - Trends in Analytical Chemistry (2011) 30(7), 968-977

[108] W. Lindinger, A. Hansel, A. Jordan - International Journal of Mass Spectrometry and Ion Processes (1998) 173, 191

[109] M. S. B. Munson, F.H. Field – Journal of the American Chemical Society (1966) 88, 2621

[110] M. McFarland, D. L. Albritton, F. C. Fehsenfeld, E. E. Ferguson, A. L. Schmeltekopf – Journal of Chemical Physics (1973) 59, 6620

[111] A. G. Harrison - *Chemical Ionization Mass Spectrometry* – London: CRC Press (1992)

[112] A. Hansel, A. Jordan, R. Holzinger, P. Prazeller, W. Vogel, W. Lindinger - International Journal of Mass Spectrometry Ion Processes (1995) 149-150, 605

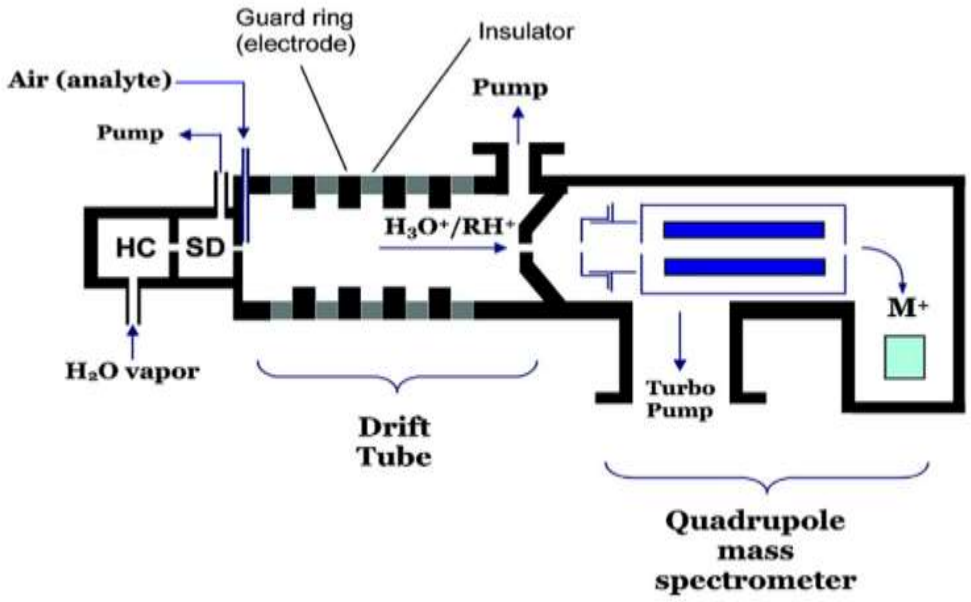

Figure 11. Simplified representation of a proton-transfer reaction mass spectrometer utilizing a quadrupole mass filter. HC = hollow-cathode discharge source; SD = source drift region; RH^+= protonated volatile compound (from Biasioli et al., Trends Anal. Chem. (2011) 30, 968)

Here, the gaseous mixture to be measured is directly admitted, and all volatile compounds *R* with a proton affinity higher than H_2O will react with H_3O^+ according to the reaction:

$$H_3O^+ + R \rightarrow H_2O + RH^+$$

With a reaction coefficient *k*, the resulting product ions, with no or minor fragmentation, are then detected via a Quadrupole mass analyzer (Q), Ion Trap (IT) or Time of Flight (TOF) analyzer.
Absolute volatile concentrations [R] in the original mixture can be derived by the experimentally determined quantities via the following equation:

$$[R] = (k \cdot t)^{-1} \cdot [RH^+] / [H_3O^+]$$

with *t* (s) being the average transit time of the ions in the drift tube; the kinetics of the proton-transfer reaction is controlled by the ratio between electric field and gas density in the drift tube (E/N).
Values in the range 120–140 Td (1Td = 10^{-17} V·cm^2) are usually chosen as a trade-off between too high a fragmentation at higher E/N, and increased formation of water/hydronium ion clusters $(H_2O)_n \cdot H_3O^+$, that might induce other ion-molecule reactions.
PTR-MS does not use primary ion filtering, thus simplifying the apparatus, and this also enhances sensitivity by increasing the denominator in the previous equation.
Moreover, air can be used as carrier and buffer gas, because air constituents do not undergo protonation, and the formation of adduct products in the drift-tube reactor is limited by the non-thermal conditions induced by the electrical field.

The possibility to determine absolute concentrations directly, even with some caution[113], and the high time resolution are other positive features of PTR-MS.
Especially high time resolution is of advantage for food scientists, when dealing with relatively fast processes (i.e. formation of volatile compounds during food processing, breath analysis and nose–space measurements during and after food consumption).
On the other side, negative aspects used to be the impossibility to distinguish isobaric compounds and some residual fragmentation that is not always avoidable: indeed, PTR-MS is a one-dimensional analytical method, and unambiguous identification is less straightforward, compared to a two-dimensional technique (e.g. GC-MS).
To overcome this, some strategies for improving compound identification were suggested[114], the simplest one being the decrease of the drift tube voltage; like this, fragmentation can be reduced, resulting in a higher contribution of the protonated molecule or of a characteristic fragment ion.
This voltage decrease, however, also results in increased hydration of reactant and product ions (cluster formation), which in turn can also lead to more complex mass spectra.
It was also pointed out that, in order to differentiate between isobaric or isomeric compounds, it might be useful to change the PTR-MS settings (in essence the E/N parameter) as a function of the VOC to be monitored; in this respect, there is a clear need for systematic studies of product ion distributions of various classes of VOCs, as a function of instrumental parameters (E/N)[115].
Considered as a whole, it is clear that PTR-MS is of great value in food science and technology in a wide range of situations: from the possibility of exploiting complete mass spectra as characteristic fingerprints, all the way to fast process monitoring, including identification and quantification of single compounds within a complex food matrix, also for in-vivo flavour release studies.

[113] J. Degouw, C. Warneke, T. Karl, G. Eerdekens, C. Vanderveen, R. Fall - International Journal of Mass Spectrometry (2003) 223–224, 365
[114] M. Demarcke, C. Amelynck, N. Schoon, F. Dhooghe, H. Van Langenhove, J. Dewulf - International Journal of Mass Spectrometry (2009) 279, 156
[115] A. Tani, S. Hayward, C.N. Hewitt - International Journal of Mass Spectrometry (2003) 223–224, 561

2.3 Chemometrics

2.3.1 Some history and its relevance in flavour science

In 1974, as a young aspiring assistant professor, Svante Wolde wrote the following: "The art of extracting chemically relevant information from data produced in chemical experiments is given the name of *chemometrics* in analogy with biometrics, econometrics, etc."[116].

Hence, the term "chemometrics" was used to describe a new way of analyzing chemical data, in which elements of both statistical and chemical thinking are combined; since then, it has developed into a legitimate technical field of its own, and is rapidly growing in popularity within a wide range of chemical disciplines.

Anyway, it must be pointed out that chemometrics should not be separated from chemistry, or even be allowed to become a separate branch of chemistry: it must remain its complementary part.

Despite its successes in other fields, chemometrics was not introduced in analytical chemistry until the late 60's and 70's; somewhat surprisingly, the first appearance for analytical chemistry in the literature came from the food industry, rather than from academia[117].

Through the 80's and 90's, the development of chemometrics for analytical chemistry was largely driven by the increasing popularity and utility of non-specific NIR spectroscopy technology.

At the same time, increased efficiency and accessibility of computing power allowed the power of chemometrics to be used by any scientist or engineer with access to a personal computer: these forces combined to generate a boom in the publication of chemometrics applied to analytical chemistry starting in the 90's.

A reasonable definition of chemometrics still holds as "how to get chemically relevant information out of measured chemical data, how to represent and display this information, and how to get such information from data".

There appear to be three elements that are consistently used in historical applications of chemometrics: (1) empirical modeling; (2) multivariate modeling; (3) chemical data.

The empirical modeling element indicates an increased emphasis on data-driven rather than theory-driven modeling of data: this is not to say that prior chemical knowledge is ignored, but that it is not relied upon completely to model the data; the multivariate element indicates that more than one response variable of the analyzer is used to build a model.

Chemometrics solves both descriptive and predictive problems, including experimental design[118], exploratory data analysis[119], and calibration models[120].

In descriptive applications, properties of chemical systems are modeled with the intent of learning the underlying relationships and structure of the system; in predictive applications, properties of chemical systems are modeled with the intent of predicting new properties or behaviour of interest.

In food science, scientists are faced with many different quality control tasks, such as making sure that flavours meet certain standards, identifying changes in process parameters that may lead to a

[116] S. Wolde - Chemometrics and Intelligent Laboratory Systems (1995) 30, 109
[117] K. H. Norris, R. F. Barnes, J. E. Moore, J. S. Shenk – Journal of Animal Science (1976) 43, 889
[118] R. Leardi - Analytica Chimica Acta (2009) 652, 161
[119] S. Kallithraka, I. S. Arvanitoyannis, P. Kefalas, A. El-Zajouli, E. Soufleros, E. Psarra - Food Chemistry (2001) 73(4), 501
[120] P. Geladi, B. R. Kovalski - Analytica Chimica Acta (1986) 185, 1

change in quality, detecting adulteration in any ingredient and identifying the geographical origin of products[121], i.e. by using aroma profile.
Many of these quality control issues have traditionally been assessed by experts, who are able to determine a product's quality by observing its color, texture, taste, aroma, etc.; however, it takes years of experience to acquire these skills[122].
It would therefore be advantageous if there were a way for food scientists to measure the quality of a product by instrumented means.
Unfortunately, quality is a difficult parameter to quantify: it is difficult to find direct sensors for quality parameters such as aroma, freshness or expected shelf life; therefore we are forced to measure an indirect set of parameters which, individually, may be only weakly correlated to the properties of interest.
In that quest, chemometrics is a powerful approach to the interpretation of patterns in multivariate data, which are related to crops or final products quality and can be recognized: for example, a chromatogram or spectral profile can be thought of as a fingerprint, which could be related to cultivar, production methods, shelf-life, etc.
One of the major thrust in the food and beverage industry is to bring analytical instrument techniques to play in sensory evaluation, because traditional sensory panels are expensive to maintain and might lead to inconsistent conclusions, due to subjectivity.
Taste and smell are related to fats, proteins, sugars, minerals, and volatile compounds present in food: many of these components can be profiled, correlated to sensory information by chemometric methods[123], and the resultant statistical model can be used in on-line or routine applications to predict flavour characteristics of unknown samples.

2.3.2 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) can be considered as the founder of all methods in multivariate data analysis: its aim is dimensionality reduction, by computing linear latent variables, the so called principal components.
PCA was invented in 1901 by Karl Pearson: now it is mostly used as a tool in exploratory data analysis and for making predictive models in applied science[124].
It can be seen as a method to compute a new coordinate system formed by the latent variables, which are orthogonal, and by which only the most informative dimensions are kept and then used for: (1) visualization of multivariate data by scatter plots; (2) transformation of highly correlated *m* variables into a smaller set of uncorrelated latent variables; (3) separation of relevant information from the noise.
PCA can be performed by eigenvalue decomposition of the data covariance matrix or singular value decomposition of a data matrix, usually after mean centering the data matrix for each attribute.
The results of a PCA are usually discussed in terms of component scores, sometimes called factor scores (the transformed variable values corresponding to a particular data point), and loadings (the

[121] M. Bevilacqua, R. Bucci, A. D. Magrì, A. L. Magrì, F. Marini – Analytica Chimica Acta (2012) 717, 39
[122] E. Riverside – "Chemometrics in Food and Beverage" – In *Chemometrics Applications Overview* - Bothell, WA: Infometrix, Inc. (1996)
[123] M. T. Morales, M. V. Alonso, J. J. Rios, R. Aparicio – Journal of Agricultural and Food Chemistry (1995) 43, 2925
[124] K. Pearson - "On Lines and Planes of Closest Fit to Systems of Points in Space" – In *Philosophical Magazine* (1901) 2 (11), 559

weight by which each standardized original variable should be multiplied to get the component score)[125].
Hence, each component is defined by a loading vector $\boldsymbol{p_n}$:

$$\mathbf{p_1} = (p_1, p_2, \ldots, p_m)$$

and loading vectors are normalized to unit length:

$$\mathbf{p_n}^T \mathbf{p_n} = 1$$

The scores (projection coordinates of the objects) are linear combinations of the loadings and the corresponding variables; for instance for object *i*, defined by a vector $\boldsymbol{x_i}$ with elements x_{i1} to x_{im}, the score $\boldsymbol{t_{i1}}$ of *PC1* is:

$$\mathbf{t_{i1}} = x_{i1}\, p_1 + x_{i2}\, p_2 + \ldots. + x_{im}\, p_m = x_i^T\, p_1$$

For all *n* objects arranged as rows in the matrix *X* the score vector t_1 of *PC1* is obtained by:

$$\mathbf{t_1} = \mathbf{X} \cdot \mathbf{p_1}$$

PC1 represents the direction in space that contains the maximum variance of the original data; *PC2* is defined as an orthogonal direction to *PC1*, and captures the maximum possible remaining variance, and so on.
Further *PCs* can be computed up to the minimum between the number of variables *m* and the number of objects *i*; this transformation is defined in such a way that each succeeding component in turn has the highest variance possible, under the orthogonality constraint (which assures uncorrelation) with the preceding ones.
Because the loading vectors of all *PCs* are orthogonal to each other, this data transformation is a rotation of the coordinate system[126].
For many practical data sets, usually the first two to three *PCs*, containing the main amount of variance (potential information), are used for scatter plots.
All loading vectors are collected as columns in the loading matrix ***P***, and all score vectors in the score matrix ***T***:

$$\mathbf{T} = \mathbf{X} \cdot \mathbf{P}$$

The X matrix can be reconstructed from the PCA scores T: usually, only a few *PCs* are used, thus resulting in an approximated X-matrix with reduced noise, and error matrix **E** (Fig. 12):

$$\mathbf{X_{appr}} = \mathbf{T} \cdot \mathbf{P}^T \qquad \mathbf{X} = \mathbf{T} \cdot \mathbf{P}^T + \mathbf{E} \qquad \mathbf{E} = \mathbf{X} - \mathbf{X_{appr}}$$

[125] S. Wold, K. Esbensen, P. Geladi - Chemometrics and Intelligent Laboratory Systems (1987) 2, 37
[126] I. T. Jolliffe - *Principal Component Analysis, Second Edition* – New York: Springer-Verlag (2002)

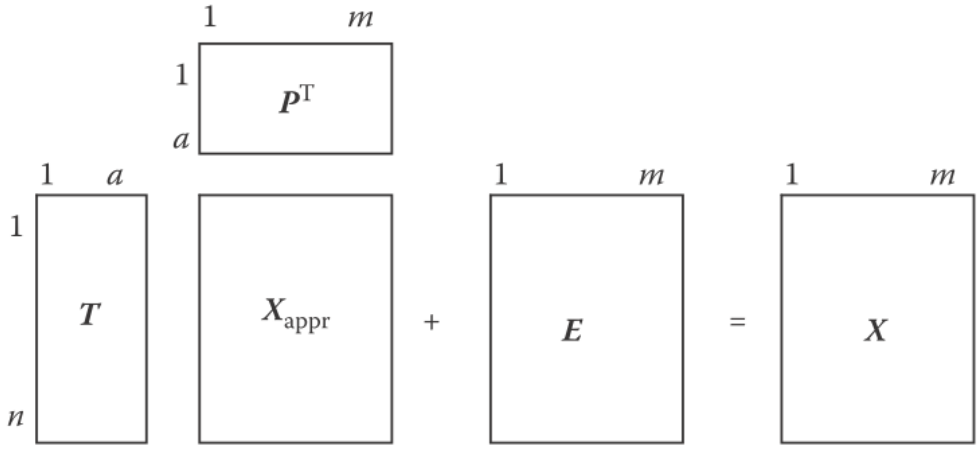

Figure 12. Approximate reconstruction X_{appr} of the X-matrix from PCA scores matrix *T* and loading matrix *P* using *a* components; *E* is the error (residual) matrix
(from *Introduction to multivariate statistical analysis in Chemometrics* (2009) - Taylor & Francis Group)

PCA is sensitive to the scaling of the original variables; mean-centering and auto-scaling are the most chosen pre-treatment of data.
After computing the *PCs*, there is the need to decide how many components should have to be kept: the cumulative variance shows how much of the total variance is preserved by a set of *PCs*; as a rough rule of thumb, *PCs* should explain at least 80% of the total variance.
Cross validation techniques can be applied for a statistically based estimation of the optimum number of *PCs*[127]: the idea is to randomly split the data into training and test set; then PCA is applied to the training data, and the observations from the test data are reconstructed, computing the prediction error on the test set.
Repeating this procedure many times indicates the distribution of the prediction errors when using 1 to *a* components, which then allows deciding on the optimal number of components based on the minimum error in cross-validation.

2.3.3 Analysis of variance combined to Simultaneous Component Analysis (ASCA)

Designed experiments with a single dependent variable were typically analyzed with ANOVA; today, such single-variable experiments are rather the exception than the rule; this is particularly true in metabolomics, and proteomics, where routinely hundreds or thousands of variables are measured simultaneously.
The effect of the experimental factors on each dependent variable can be the same quantified with ANOVA: however, such an analysis does not take the interrelation between variables into account.
Moreover, Multivariate ANOVA (MANOVA)[128] breaks down when the number of measurements is smaller than the number of variables, which is commonplace nowadays, and assumption of multi-normality in order to obtain interpretable results are only very seldom fulfilled.
Two recently developed methods that overcome the limitations of MANOVA are Analysis of variance combined to Simultaneous Component Analysis (ASCA) and ANOVA–PCA[129]: both

[127] G. Diana, C. Tommasi - Statistical Methods and Applications (2002) 11(1), 71
[128] L. Ståhle, S. Wold - Chemometrics and Intelligent Laboratory Systems (1990) 9, 127
[129] P. B. Harrington, N. E. Vieira, J. Espinoza, J. K. Nien, R. Romero, A. L. Yergey - Analytica Chimica Acta (2005) 544, 118

methods use ANOVA to decompose the data matrix into effect matrices that contain the level averages for the experimental factors, and a matrix with residuals not explained by the model[130].
The two methods differ in the way the effect matrices are analyzed: instead of ASCA, ANOVA–PCA adds the residual matrix to the effect matrices before PCA, and score plots obtained immediately show grouping of data points for the different levels of the independent variables.
ASCA is a chemometric tool that can deal very well with complex multivariate datasets with an underlying experimental design, allowing for easy interpretation of the variation induced by the different factors.
By that way, it is possible to isolate the variation induced by a factor, revealing the relations between samples and measured properties under the constraint of orthogonality (independence); thus, ASCA is successfully applied in NMR, X-ray fluorescence, GC and LC-MS[131].
We consider an experimental design with J-dependent variables, two independent variables, α and β, also called experimental factors or design variables, and the interaction between both factors; factor α has A levels, factor β has B levels, and the design must be balanced, with each measurement repeated I times for each combination of factor levels.
In the model, the data matrix X with dimensions $N \times J$ is split into effect matrices X_α and X_β, containing the level averages for each factor and a matrix $X_{\alpha\beta}$ that describes the interaction between the two factors.
The variation that cannot be represented by the model is collected in the residual matrix E; thus, the decomposition of the data matrix is:

$$X = 1m^T + X_\alpha + X_\beta + X_{\alpha\beta} + E$$

where the first term represents the overall mean for the data, 1 is a column vector of length N, m^T is a row vector of size J with the averages over the data for each variable.
Thus, for each effect matrix, we can write the ASCA decomposition in principal components as:

$$X_k = T_k P_k^T$$

where T_k and P_k are the scores and loadings for the effect matrix k.
As in PCA, loadings give information about the contribution of each dependent variable to the principal components of the effect matrix: thus, the loadings provide an interpretation of the factor levels in terms of the dependent variables.
Being ASCA applied on the factor matrices directly, no assessment of the significance of the results obtained is then made: however, a permutation test can be implemented to assess the statistical significance of the effects and of their interactions; randomizing or permutation is the uncoupling of the data from the group labels[132].

[130] G. Zwanenburg, H. C. J. Hoefsloot, J. A. Westerhuis, J. J. Jansen, A. K. Smilde – Journal of Chemometrics (2011) 25, 561
[131] I. Stanimirova, K. Michalik, Z. Drzazga, H. Trzeciak, P. D. Wentzell, B. Walczak - Analytica Chimica Acta (2011) 689, 1
[132] A. K. Smilde, J. J. Jansen, H. C. J. Hoefsloot, R. A. N. Lamers, J. van der Greef, M. E. Timmerman – Bioinformatics (2005) 21, 3043

The null hypothesis H_0 assumes that there is no effect of the factor: the permutation test is done by randomly permuting the labels in the original data matrix (i.e. 50.000 times) and recalculating the type I sum of squares of the factors[133].
The p-value is then calculated dividing the number of cases on which the sum of squares is larger than the original one by the number of performed permutations: depending on whether p_k is smaller or larger than a predetermined probability value, the null hypothesis H_0 is accepted or rejected.
By the permutation procedure, it is also possible to compute confidence intervals for the loadings and their statistical significance, thus establishing the variables affected by the several factors.

2.3.4 Partial Least Squares Regression (PLSR)

Partial Least Squares Regression (PLSR) is the PLS approach in its simplest, most used form[134].
PLSR is gaining importance in many fields of chemistry; analytical, physical, clinical chemistry and industrial process control can benefit from the use of the method.
The pioneering work in PLS for chemical applications was carried out by the groups of S. Wold and H. Martens in the late seventies, after an initial application by Kowalski et al[135].
PLSR is a method for relating two data matrices, *X* and *Y*, by a linear multivariate model, but goes beyond traditional regression in that it models also the structure of *X* and *Y*.
The regression problem, i.e., how to model one or several dependent *Y* variables (PLS2), by means of a set of predictor variables, *X*, is one of the most common data-analytical problems in science and technology.
Examples in chemistry include relating *Y* properties of chemical samples to their chemical composition *X*, or relating *Y*, being the quality of manufactured products, to the conditions of the manufacturing process.
PLSR derives its usefulness from its ability to analyze data with many, noisy, and collinear variables in both *X* and *Y* (unlike multiple linear regression), as like as in NIR spectroscopy; it has also the desirable property that the precision of the model parameters improves with the increasing number of relevant variables and observations[136].
It consists of finding a subspace common to both the predictor (*X*) and the dependent variable (*Y*) blocks, by decomposing them in a single process[137]:

$$X = TP' + E$$
$$Y = UQ' + F$$

where *T* and *U* are the *X*- and *Y*-block score matrices, *P'* and *Q'* are the *X* and *Y* loadings, and *E* and *F* are the residuals.
Then, a linear inner relationship is modeled between the projections of the dependent and independent variables (*U* and *T* respectively), according to:

[133] D. J. Vis, J. A. Westerhuis, A. K. Smilde, J. van der Greef - BMC Bioinformatics (2007) 8, 322
[134] S. Wold, M. Sjostrom, L. Eriksson - Chemometrics and Intelligent Laboratory Systems (2001) 58, 109
[135] P. Geladi, B. Kowalski - Analytica Chimica Acta (1986) 185, 19
[136] S. Wold, M. Sjöström, L. Erikson, Chemometrics and Intelligent Laboratory Systems (2001) 58, 109
[137] F. Marini, R. Bucci, I. Ginevro, A. L. Magrì - Chemometrics and Intelligent Laboratory Systems (2009) 97, 52

$$U = TB$$

Even if the linear model might appear an oversimplification of the actual relationship between the two blocks of variables, in many cases it leads to a high accuracy and predictive ability, as widely reported in the literature.

2.3.5 Mixture Design

Many real problems, in the most different fields of application, can be successfully solved when studied according to the techniques of experimental design: among them, a great part involves finding the best composition of a mixture of more than two components, i.e. when a pharmaceutical company must find the best formulation for a tablet.

When planning a design, all the knowledge about the specific problem has to be taken into account, in order to detect all the potentially relevant variables, to postulate a reasonable model and to define the experimental domain.

A mixture experiment is a special type of response surface experiment in which the factors are the components of a mixture and the response is a function of the proportions of each ingredient.

In these cases, the "classical" and most known designs such as Fractional Factorial Design or Central Composite Design cannot be applied, since the constraint that the sum of the components must be 1 has to be valid, and the components cannot be varied independently[138].

Another relevant difference with the design for independent variables is that the object of the study in these problems is not the effect of the variation of the absolute quantity of the variables, but the effect of the variation of the ratios among the variables[139].

In many mixture designs, there are also restrictions on the component proportions X j that prevent the experimenter from exploring the entire simplex region: these additional constraints take the form of lower (L_j) and upper (U_j) constraints on the component proportions, the effect of which is to limit the feasible space to a sub-region of the original simplex.

Fig. 13 shows the graphical representation of a three-component mixture: it is an equilateral triangle, in which the vertices correspond to the pure components, the sides to the binary mixtures and the internal points to the ternary mixtures.

For any number of components of a mixture, the domain will be the regular figure having as many vertices as components, lying in the space having dimensionality equal to the number of components minus one.

For more than four components, we cannot visualize the whole domain since it lies in a space having more than three dimensions, but this is just a problem of our limited mind: from the mathematical point of view, the number of dimensions does not make any difference[140].

In mixture problems, the purpose of the experimental program is to model the blending surface with some form of mathematical equation, so that predictions of the response for any mixture of the ingredients can be made empirically; in general a polynomial model is chosen, which takes a canonical form because of the restriction $\Sigma j\ X_j = 1$.

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[138] S. Cafaggi, R. Leardi, B. Parodi, G. Caviglioli, G. Bignardi - Chemometrics and Intelligent Laboratory Systems (2003) 65, 139

[139] G. E. P. Box, W. G. Hunter, J. S. Hunter – In *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building* – New York: John Wiley & Sons (1978)

[140] R. Leardi - Analytica Chimica Acta (2009) 652, 161

The collected data are then analyzed as in the response surface methodology (RSM)[141], using the results to: (1) fit the empirical model; (2) test the adequacy of the fitted model; (3) visualize the shape of the three-dimensional response surface; (4) plot the contours of the predicted responses; (5) determine the optimal settings of the component proportions or understand the roles played by separate mixture components.

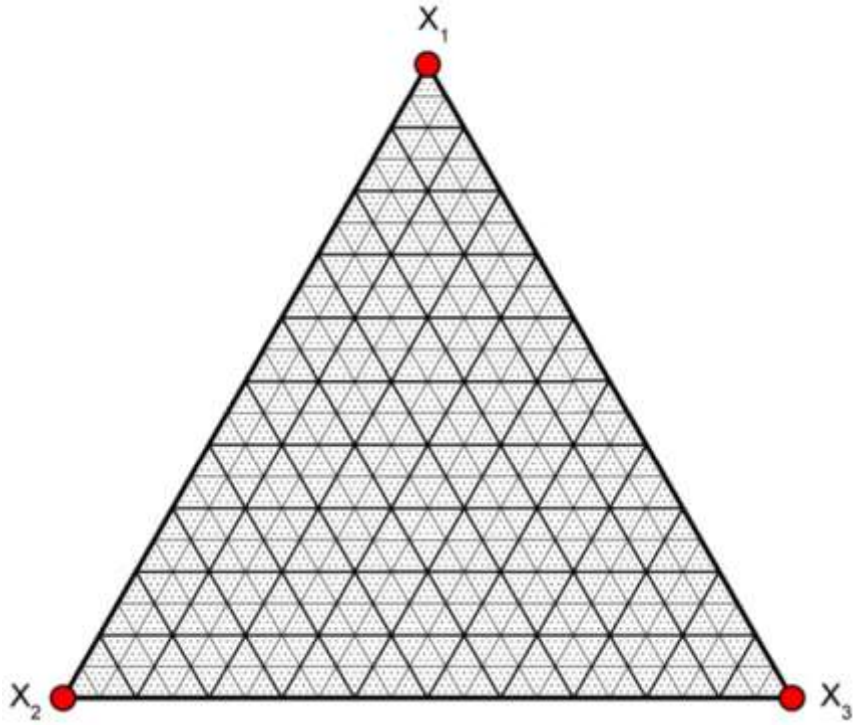

Figure 13. The representation of a 3-component mixture, with the lines of the grid drawn at steps of 10% (dark solid lines), 5% (light solid lines) and 1% (dotted lines)

The linear model for a three-component mixture is:

$$Y = b_1 X_1 + b_2 X_2 + b_3 X_3$$

where X_i is the fraction of component *I*, and the coefficients b_i correspond to the response obtained with the pure component *I*, thus having nothing to do with the effects of the components.
Comparing it with the model for independent variables, it can immediately be seen that the constant is not present: this appears quite logical if we think that the constant corresponds to the response when all the variables have level 0, and in the case of mixtures, since the sum of all the components must be 1, it is not possible to have a condition in which all the variables are at level 0.
The quadratic model for a three-component mixture is the following:

$$Y = b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3$$

The coefficients b_{ij} are contrasts comparing the response at the midpoint of the edge connecting the vertices of components *i* and *j* with the responses at the vertices *i* and *j*.

[141] R. H. Myers, D. C. Montgomery – In *Response Surface Methodology: Process and Product Optimization Using Designed Experiments* – New York: Wiley (1995)

These three terms are said to represent measures of departures from the plane of the second-degree surface resulting from the non-additive blending of the components; with this model, synergic or antagonistic effects of binary mixtures can therefore be taken into account[142].
To consider also the effect of ternary blending among the components, a term $b_{123}\ X_1\ X_2\ X_3$ is added, obtaining what is called a "special cubic model":

$$Y = b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{123} X_1 X_2 X_3$$

The magnitude of the synergic effects of the two-component mixtures is given by the coefficients divided by 4; in the same way, the coefficient of the three-terms interaction divided by 27 corresponds to the magnitude of the synergic effect of the three components (this is the reason why the coefficients of the higher interactions are usually very large).
While in experimental designs for independent variables, the magnitude and significance of the effects can be directly deduced by the coefficients of the model, this is not true at all in the case of mixture designs.
Generally speaking, it can be said that the effect of a variable corresponds to the variation of the response generated by the increase or decrease of the variable itself.
When applying this definition to a mixture, it can be said that the effect of a component corresponds to the variation of the response generated by the introduction or removal of a certain amount of the component itself.
Of course, since the sum of all the components must be 1, when one component is added its fraction increases, while the fractions of all the remaining components correspondingly decrease, with their ratios unchanged; this can be more easily understood when thinking at the real action of pouring some ingredient into a mixture.
Therefore, it can be defined as the effect of component *i* the difference between the responses at two points, centered on the barycenter of the experimental domain, having a pre-established difference in the content of component *I*, while the remaining components maintain the corresponding ratio at the barycenter of the experimental domain.
To allow experimental error estimation, one should prepare independent replications at least of one mixture, usually at the central point of the domain; data are fitted to all available mixture regression models of increasing complexity, starting from the linear one, and the selected model is then reduced (if any), leaving only significant terms.
Once the estimated model equation for each response is obtained, contour and surface plots are generated; model significance, significance of lack-of-fit, and adjusted R^2 value are used to judge the adequacy of model fitness.
Finally, if the experimental effort is not so high, the model is validated by comparing the mean responses of newly prepared emulsions (not already included in the model) with the respective value predicted by the model equations[143].

[142] J.A. Cornell – In *Experiments with Mixtures: Designs, Models, and the Analysis of Mixture Data* - New York: Wiley (1990) pp. 32
[143] N. H. Ibrahim, Y. B. Che Man, C. Ping Tan, N. A. Idris - World Academy of Science, Engineering and Technology (2010) 43, 1000

Chapter 3: Analytical surveys in the FACET context

3.1 Introduction to the specific problem

Food consumption data and chemical concentrations in foods are the two types of information required to assess dietary exposure to chemical substances and, among them, to flavouring substances[144].
Both data on food consumption and on the concentrations of flavourings are affected by some degree of uncertainty that generates an overall uncertainty in the exposure assessment and, as a consequence, in the risk assessment when the results of the exposure assessment are compared with the relevant health-based guidance value for the food chemical of interest.
An accurate characterization of the extent of uncertainty in the risk assessment is needed by risk managers in order to take appropriate measures[145].

According to the principle of a stepwise approach for the assessment of dietary exposure to chemical substances, both screening methods and refined methods have been developed in order to assess dietary exposure to flavourings.
For most of these methods, the concentrations in foods are generally approximated by use level data provided by industry in a number of food categories that may be more or less refined.
On the other hand, the probability of the presence and the concentrations of a flavouring substance in a food are known to vary with the product formulation and, as such, may vary from brand to brand of every single food item.
However, data on use levels for single products are kept confidential and only average or maximum use levels provided by industry surveys are available, the relationship between such levels and actual concentrations in food products being very uncertain.
A more detailed knowledge of this relationship, in some selected case studies, could allow for a more precise evaluation of the uncertainty associated with the estimation of flavouring concentrations in foods and, in turn, with the overall estimation of dietary exposure.
In that quest, two case-studies were chosen:

1) Analytical determinations on pineapple-flavoured yogurts and beverages, in which allyl hexanoate, the target analyte is added as a character impact compound, conferring pineapple-like taste and aroma[146];

2) Analytical determinations for safrole, myristicin and trans-cinnamaldehyde, which are compounds present in cola-flavoured soft drinks: the first two are part of the natural extract

[144] World Health Organization (WHO) – Chapter 6: Dietary exposure assessment of chemicals in food. In *Principles and methods for the risk assessment of chemicals in food* – Geneva (2009)
[145] European Food Safety Authority (EFSA) – "Guidance of the Scientific Committee on a request from EFSA related to uncertainties in dietary exposure assessment" - EFSA Journal (2005) 438, 1
[146] R. J. McGorrin - "Character impact compounds: flavours and off-flavours in foods" – In *Flavour, fragrance, and odor analysis* - (R. Marsili Ed.) - New York: Marcel Dekker (2002) pp. 375

from nutmeg and mace; the latter is present as natural flavouring (Cassia oil) or nature identical compound.

In this case, safrole[147] and myristicine[148] have also toxicological concerns, because they are capable of genotoxic effects.

Analytical methods based on the Stir Bar Sorptive Extraction (SBSE) technique for the isolation of the target analyte, and on GC-MS analysis for final determination, were developed and validated for the two studies[149].

[147] S. Y. Chiang, P. Y. Lee, M. T. Lai, L. C. Shen, W. S. Chung, H. F. Huang, K. Y. Wu, H. C. Wu – Mutation Research (2011) 726(2), 234

[148] C. Martins, C. Doran, A. Laires, J. Rueff, A. S. Rodrigues – Food and Chemical Toxicology (2011) 49(2), 385

[149] A. Raffo, S. Nicoli, C. Leclercq - Food and Chemical Toxicology (2011) 49, 370

3.2 Quantification of allyl hexanoate in pineapple beverages and yogurts as a case study to characterize a source of uncertainty in dietary exposure assessment to flavouring substances

3.2.1 Overview

The aim of this study was to assess the variability of concentration levels of allyl hexanoate, considered as a case study, in two main food categories to which it is often added: pineapple juice-based beverages and yogurts.

The main focus was the potential exposure to flavourings in children; fruit based beverages and yogurts were identified as potential important sources of exposure in this age class.

Thirty-four beverages and 29 yogurts, with pineapple fruit or juice and added flavourings declared as ingredients on the package, were purchased from the local market in Rome, and analyzed.

Allyl hexanoate is a flavouring substance characterized by rare natural occurrence in foods, at levels too low to exert a sensory effect per se in pineapple[150], and it has rarely been reported in more than trace levels in other foods such as mango and mushrooms.

As an added flavouring it is a relatively high poundage substance, and it is used mainly in pineapple flavouring formulations: it can be considered a character impact compound, conferring pineapple-like taste and aroma, even though minor uses in peach, apricot, apple, tropical, rum and liqueur flavours have been reported[151].

Use levels of allyl hexanoate in food have been reported in the rough range of 0.1–10.0 mg kg^{-1}; based on that, it can be concluded that dietary exposure to allyl hexanoate is essentially due to its presence in foods as an added flavouring rather than as a naturally occurring compound. Consequently, in this case, uncertainty in dietary exposure related to natural occurrence of allyl hexanoate in foods can be considered negligible.

In the context of the European evaluation programme of flavourings by EFSA, allyl hexanoate has been assigned to the Flavouring Group Evaluation 19, including α,β-unsaturated aldehydes or ketones and their precursors, which are substances containing a structural alert for genotoxicity.

At present it has been stated that allyl hexanoate could not be put through the Procedure established for the evaluation of chemically defined substances on the basis of the available data and that there is a need for additional toxicological information before conclusions on the safety of this substance can be reached[152].

3.2.2 Experimental section

Allyl hexanoate was determined in beverage and yogurt samples by two similar but distinct methods.

The relatively high value of the octanol–water partition coefficient ($logK_{o/w}$ = 3.183) for allyl hexanoate, estimated by means of the KowWIN software (Syracuse Research Corp., Syracuse, NY, USA), suggests that relatively high yields can be achieved by SBSE in the extraction of this compound from aqueous samples.

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[150] R. G. Berger, F. Drawert, H. Kollmannsberger, S. Nitz, B. Schraufstetter – Journal of Agricultural and Food Chemistry (1985) 33, 232

[151] http://www.leffingwell.com/flavbase.htm/

[152] http://www.efsa.europa.eu/en/events/event/afc071127-m.pdf/

A key point in the development of a reliable method for quantification using SBSE for the extraction step is the selection of a suitable internal standard capable of compensating for the matrix effect: for this purpose n-propyl hexanoate, isobutyl hexanoate, butyl hexanoate and hexyl propanoate were tested, the last one resulting to be the best choice.
Hexyl propanoate ($logK_{o/w}$ = 3.32) has not been reported to naturally occur in pineapple[153], whereas the low annual volume of production reported for Europe (40 kg $year^{-1}$), indicates an infrequent use as an added flavouring substance.
It was not detected in any of the beverage and yogurt samples tested with the purpose of method development; a further reduction of the matrix effect in the SBSE extraction process was obtained by diluting the sample in water[154].
The extent of dilution of the sample in water (1:200 for beverage and 1:400 for yogurt) was set in order to obtain a good accuracy by minimizing the matrix effect and guaranteeing at the same time the needed sensitivity.
The beverage sample was firstly filtered with Whatman filter paper (*No. 113*) by percolation; a total of 1mL of the filtered beverage was added with 100 mL of a methanolic solution (17.42 mg L^{-1}) of hexyl propanoate, and with deionized water to 200 mL.
Isolation was performed on 15 mL of this working solution, by stirring at 700 rpm a PDMS-coated Stir Bar (1.0 mm thickness, 10 mm length) for 90 min, at room temperature, in hermetically closed vials.
In the case of yogurt analysis, the content of a yogurt pot was firstly homogenized by a Waring blender for 1min; then 0.5g of the homogenized yogurt sample were added with 100 mL of a methanolic solution of hexyl propanoate (69.68 mg L^{-1}) and with deionized water to 200 mL. Isolation was performed on 15 mL of this working solution, by stirring at 1000 rpm a PDMS-coated Stir Bar for 60 min, at room temperature, in hermetically closed vials.
After the extractions, stir bars were rinsed with distilled water, dried with filter paper and transferred into a thermal desorption tube, which was inserted into the thermal desorption unit mounted onto the GC injector, where the extracted compounds were thermally desorbed.
A Gerstel CIS-4 PTV injector cooled at -30°C with liquid CO_2 was used for cryogenic focusing of the compounds thermally desorbed from the Stir Bar; a liner filled with Tenax was used within the PTV injector.
Capillary GC-MS analyses were performed by using a DB-1 column (30 m; 0.25 mm i.d., 0.25 mm film thickness) and split injection.
All extractions were performed in triplicate; before each analysis the twisters were conditioned at 300°C, for 20 min with a carrier gas flow of 100 mL min^{-1}.
The mass spectrometric detection (quadrupole) was performed in the selected ion monitoring (SIM) mode, using mass fragments 99, 71 and 113 m/z for allyl hexanoate (for quantification and as two qualifier ions, respectively), 75, 84 and 69 m/z for hexyl propanoate, and selecting a dwell time of 100 ms for all of them.
AOAC Guidelines 2002 for validation of methods for dietary supplements and botanicals[155] were followed; for the method for beverage analysis, a calibration curve was built by performing

[153] http://www.vcf-online.nl/VcfHome.cfm/
[154] N. Ochiai, K. Sasamoto, M. Takino, S. Yamashita, S. Daishima, A. C. Heiden, A. Hoffmann – Analytical and Bioanalytical Chemistry (2002) 373, 56
[155] http://www.aoac.org/Official_Methods/slv_guidelines.pdf/

duplicate determinations on standard solutions containing the internal standard (at 8.71 mg l^{-1}) and the target analyte at eight different concentration levels, ranging from 0.44 to 133.05 mg l^{-1}.

The repeatability of the method was evaluated by analysing six aliquots (15 mL) of the same working solution obtained from a sample of one of the analyzed beverages, according to the above described procedure, the SBSE being carried out with six distinct PDMS-coated Stir Bars.

Accuracy was evaluated by a recovery study: working solutions from a sample of beverage, spiked with the analyte at two different concentration levels (8.87 and 17.74 mg l^{-1}), corresponding approximately to 1× and 2× the expected concentration, were analyzed in triplicate.

Regarding the method for yogurt analysis, a calibration curve was obtained by preparing standard solutions containing 0.5g of plain non-flavoured yogurt, the internal standard (at 34.84 mg l^{-1}) and the target analyte at eight different concentration levels, ranging from 0.11 to 155.05 mg l^{-1}, and performing duplicate determinations on them.

Repeatability was determined similarly to what done for the beverage method, whereas the recovery study for accuracy assessment was performed by analyzing in triplicate working solutions obtained from a sample of yogurt and spiked with the analyte at two different concentration levels (22.15 and 44.30 mg l^{-1}).

When developing the method for yogurt, based on the supposedly stronger matrix effect due to the higher fat content, the calibration curve was built by using model standard solutions that were prepared by also adding an amount of plain yogurt equal to that of flavoured yogurt added in working solution for analyses; of course, the absence of allyl hexanoate in the plain yogurt was verified.

Data on performance characteristics show good repeatability and accuracy, and linearity over a range of concentrations larger than two orders of magnitude for both methods (Tab. 2).

Parameter	*Beverages method*	*Yogurts method*
Calibration		
Concentration range	0.088 − 26.61[a]	0.042 − 61.20[b]
r^2	0.9998	0.9979
Repeatability		
Relative Standard Deviation (%) n=6	0.63	1.43
Accuracy		
Recovery (%) at 1× level	103.7	98.6
Recovery (%) at 2× level	99.2	99.5

[a] expressed as mgL^{-1} and referred to concentration levels in the beverage sample, and not in the diluted working solution used for SBSE extraction.
[b] expressed as $mgkg^{-1}$ and referred to concentration levels in the yogurt sample, and not in the diluted working solution used for SBSE extraction.

Table 2. Performances of methods used to determine allyl hexanoate in pineapple beverages and yogurts

3.2.3 Conclusions

For having more information on refined exposure assessment based on the levels found in this survey, which is beyond the scope of this Thesis, please refer to the related article[156].

The present study shows that the average observed concentration of allyl hexanoate in yogurts and beverages containing pineapple as the main fruit and available on the Italian market are in fair agreement with refined use levels reported by industry for the food categories to which these products belong.

In a single yogurt product, a level more than ten-fold higher than the average reported use level has been systematically found: assessment of dietary exposure to flavourings provide estimates that are in the order of magnitude of the exposure in regular consumers who would be loyal to pineapple yogurts and beverages that contain this highest observed concentration.

Exposure in children who are high consumers of pineapple yogurt or beverages containing the highest observed level of allyl hexanoate could exceed by up to 2.4 and 1.3 times, respectively, the ADI established for this substance.

Therefore, the uncertainty in the results of exposure assessment obtained with the use of standard screening techniques based on industry reported use levels is low.

[156] A. Raffo, A. D'Aloise, A.D. Magrì, C. Leclercq - Food Additives & Contaminants: Part A (2012) 29(1), 43

3.3 Quantification of *tr*-cinnamaldehyde, safrole and myristicin in cola-flavoured soft drinks

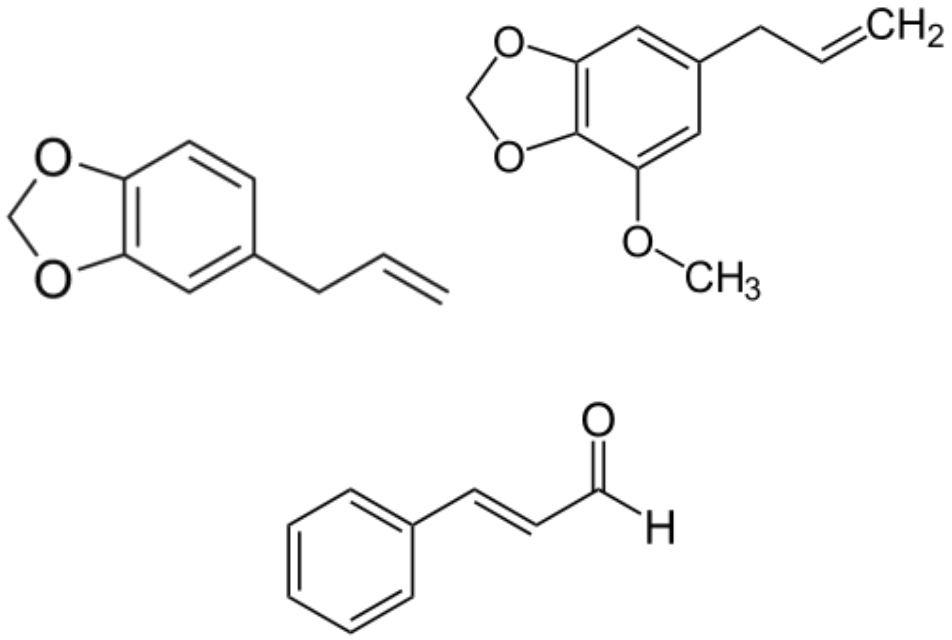

Figure 14. Molecular structures of safrole (top left), myristicin (top right) and tr-cinnamaldehyde (bottom)

3.3.1 Overview

In the present study, the variability of concentration levels of tr-cinnamaldehyde, safrole and myristicin in cola-flavoured soft drinks has been assessed in order to gather information about the variability of levels of these flavouring substances present in food or beverages, mainly as added natural flavourings.

70 samples of cola soft drinks were purchased from local sales points in 8 European Countries: 36 from two major brand leaders and 34 from private brands.

An analytical method based on Stir Bar Sorptive Extraction (SBSE) for analyte isolation and GC-MS for final determination was developed for quantitation of the three substances in cola-flavoured soft drinks.

3.3.2 Collection of samples

Samples of cola-flavoured soft drinks were purchased from local sales point in 8 member states of European Union: France (n=10), Hungary (n=8), Ireland (n=8), Italy (n=13), Poland (n=8), Portugal (n=9), Sweden (n=6), United Kingdom (n=8).

A total of 70 cola-flavoured soft drink samples were collected: 61 of the normal cola formulation (original version) and 9 of diet versions of the product.

Thirty-six samples came from two major brand leaders and 34 from private brands; products with different types of packaging, such as cans, small or large plastic bottles, were purchased.

According to declarations on product labels, some products were added with “natural flavourings”, whereas other products were added with “flavourings”, denoting the possible presence of ingredients other than natural extracts in the flavouring formulation.

3.3.3 Method development

Physico-chemical properties of the analytes considered in this study seemed to be suitable to the application of the SBSE: $logK_{o,w}$ values of 1.82, 3.45 and 3.53 calculated for *tr*-cinnamaldehyde, safrole and myristicin by the KowWIN software (Syracuse Research Corp., Syracuse, New York, USA), gave place to theoretical recovery at equilibrium of 19.4, 91.2 and 92.6%, respectively, in the conditions of our procedure.

Both stable isotopic isomers and molecules similar in physico-chemical properties have been used as internal standard: stable isotopically labelled analogues represent the best choice for the quantitative determination of this kind of substances using coupled gas chromatography/mass spectrometry[157].

Whenever compounds with similar physico-chemical and structural properties tested as candidate internal standards prove to fulfil method performance needs, they may serve as such a second option.

Being not commercially available isotopically labelled analogues, the following compounds were examined as candidate internal standard, the selection of which was based on similarity in physico-chemical properties (boiling point, $logK_{o,w}$) and in functional group: 3-vinylbenzaldehyde and α-methyl-*tr*-cinnamaldehyde for *tr*-cinnamaldehyde; 3,4-methylenedioxytoluene and 3,4-diethoxytoluene for safrole; 3,4-diethoxytoluene and 1,3-diisopropoxybenzene for myristicin (3,4-methylenedioxytoluene was early discarded on the basis of the large distance from the analyte in terms of retention time).

In the early stages of method development, the absence of compounds having ions in common with the internal standards in the area of their elution was checked in a group of cola-flavoured soft drinks.

With regard to the possible occurrence of these compounds in the considered beverage products, none of them, except for α-methyl-*tr*-cinnamaldehyde, are reported in the register of flavourings substances used in or on foodstuff in Europe, nor are mentioned in the Fenaroli's Handbook of Flavour Ingredients[158], while a search performed on the literature revealed their very rare occurrence in the plant kingdom.

On the contrary, α-methyl-*tr*-cinnamaldehyde is present in the European register of flavourings substances and it is reported in the Fenaroli's Handbook as a medium to low poundage added flavouring, found in nature in peppermint oil and sherry; in a previous work, in which about 60 volatile compounds were identified in cola-flavoured beverages, α-methyl-*tr*-cinnamaldehyde was not reported.

For method optimization a thorough exploration of the experimental space was not pursued, but some key factors impacting mainly precision and accuracy were investigated in some detail: factors examined in order to optimise method performances included, among others, sample dilution rate, salt addition, extraction time and GC injection parameters.

The solvent vent mode was selected for the CIS injector because splitless injection resulted in poor peak shape for some analytes; purge flow to split vent, which determines the injection split ratio in the solvent vent mode, was set at the lowest value (5 mL min^{-1}) maintaining good peak shape.

[157] IOFI (International Organization of the Flavour Industry) Working Group on Methods of Analysis - Flavour and Fragrance Journal (2012) 27, 224

[158] G. A. Burdock - *Fenaroli's Handbook of Flavour Ingredients* – CRC Press: Fifth Edition (2005)

Sample dilution in water is an effective way to minimize matrix effects; consequently, different dilution rates were tested: *20*-, *80*-, and *100*-fold dilutions, and the higher dilution rate was chosen, providing at the same time the required sensitivity.
CIS temperatures from -30° to 0°C were tested and effective analyte trapping within the liner was ascertained at a temperature as high as 0°C.
The evaluation of the effect of extraction time on peak area response for analytes and internal standards was carried out on a cola-flavoured soft drink with medium analyte levels, with the following extraction times: 30, 60, 90, 120, 210 and 300 minutes.
Results of the experiment (Fig. 15) showed relatively long equilibrium times for *tr*-cinnamaldehyde, and to a lesser degree for 3-vinylbenzaldehide, α-methyl-*tr*-cinnamaldehyde, 3,4-diethoxytoluene.
As with all equilibrium based techniques, it is not required to attain chemical equilibrium for accurate quantitation, and with the aim to optimise the trade-off between precision and sample throughput an extraction time of 90 minutes was selected.
On the basis of these observations a lower accuracy and precision might be expected for *tr*-cinnamaldehyde with respect to the other two analytes.
Addition of NaCl (15%) to water used for sample dilution was tested in order to increase extraction efficiency: as expected, only for the more polar *tr*-cinnamaldehyde a significant increase (+70%) in response was observed, whereas only a slight increase (+6%) for myristicin and a significant decrease (-23%) for safrole were found.
Taking also into account that twisters exposed to salt are more prone to degradation than those used in water, it was decided to dilute samples in water.

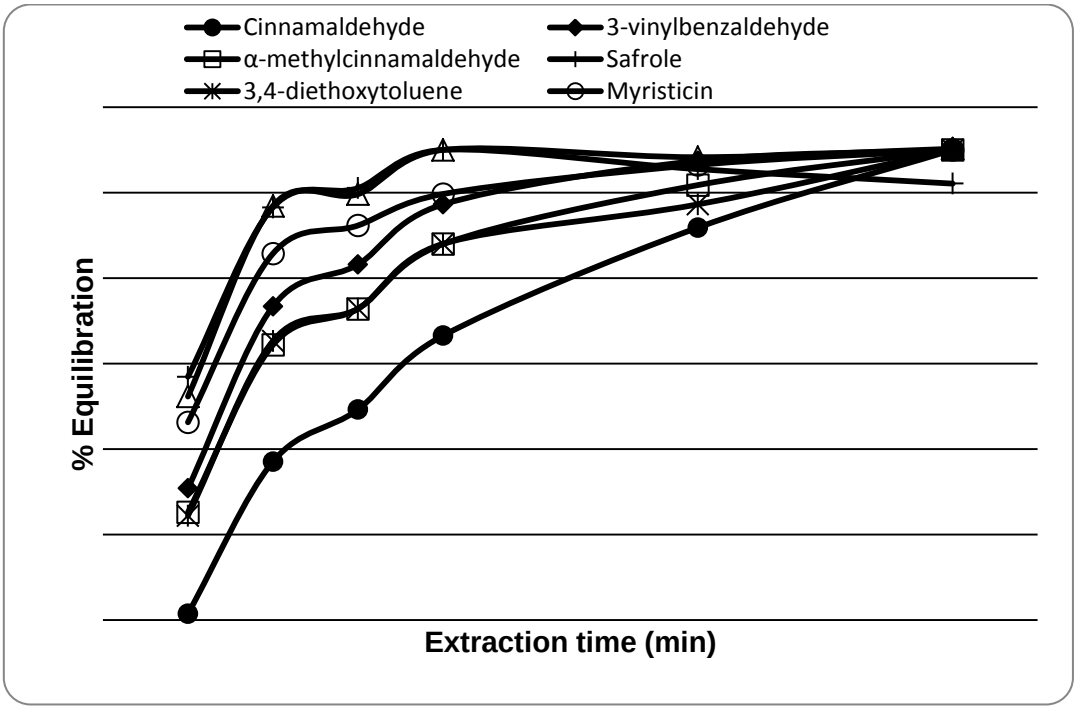

Figure 15. %Equilibration vs. extraction time for the several compounds under study

For method validation experiments, a flavoured soft drink purchased from the market was used as blank matrix: the formulation of this soft drink was quite similar to that of normal cola-flavoured soft drinks (carbonated water, sugar, caramel, flavourings (including caffeine), phosphoric acid and

preservative) but it contained all of the target substances studied in the present work at very low levels (approximately 20-fold lower).
A matrix matched eight-point (in duplicate) calibration curve was obtained by analyzing standard solutions prepared as follows: 2 mL of the blank matrix was added with 100 μL of the internal standards solution, spiked with the target analytes to get the levels in the range reported in tab. 3, and then added with water to 200 mL.
Any response from target analytes determined in the blank matrix (mean of triplicate) was subtracted to produce the calibration plot.
To evaluate method accuracy and precision recovery tests were performed: the blank matrix was spiked at the three levels reported in tab. 3, preparing and analyzing four replicates at each fortification level.
Accuracy was assessed by recovery from the fortified samples, whereas precision was assessed by relative standard deviation and expanded uncertainty[159].
Due to the presence of trace levels of the analytes in the blank matrix, LODs were determined in water: the LODs were calculated as three times the standard deviation (for six replicates) obtained for an analyte concentration no higher than ten times the LOD, as described in the paper by Ochiai et al[160].
To improve the quality of the calibration curves, weighted least squares linear regression was used in addition to the more common not weighted regression: weighting factors considered for calculation were $1/x^{0.5}$, $1/x$ and $1/x^2$, and results were evaluated to select the best choice (tab. 4).
For all the combinations analyte/internal standard, the weighted least squares linear regression obtained by using the factor $1/x^2$ proved to be the best regression, on the basis of the lowest sum of the absolute values of the relative error *RE* (computed as the %relative difference between the nominal concentration and the concentration found with the regression curve)[161].
Some performance characteristics of the optimised method are reported in tab. 3; linearity ranges covered more than two orders of magnitude of concentrations.
By comparing results of recovery tests, it came out that for *tr*-cinnamaldehyde accuracy and precision were a little worse than for the other two analytes, particularly at the lowest level of fortification, as expected on the basis of its lower extraction rate.
Between the two internal standards for *tr*-cinnamaldehyde, 3-vinylbenzaldehyde was selected for quantitation in view of the better recovery at the highest level of spiking, which is more relevant to the aim of this study.
Performances of the two internal standards for myristicin are similar, and 3,4-diethoxytoluene was selected due to slightly better recovery scores, the same as for safrole.

[159] EURACHEM/CITAC Guide - *Quantifying Uncertainty inAnalytical Measurement* – S. L. R. Ellison, M. Rosslein, A. Williams Eds. – Second Edition (2000)
[160] N. Ochiai, K. Sasamoto, M. Takino, S. Yamashita, S. Daishima, A. C. Heiden, A. Hoffmann – Analytical and Bioanalytical Chemistry (2002) 373, 56
[161] A. M. Almeida, M. M. Castel-Branco, A. C. Falcao - Journal of Chromatography B (2002) 774, 215

Analite	Internal standard	Linear range (μgL^{-1})/ Correlation coefficient (r^2)	LOD (μgL^{-1})	Recovery tests			
				Fortif. level (μgL^{-1})	Average recovery (%)	RSD (%)	Uncertainty (μgL^{-1})
tr-cinnamaldehyde	3-vinyl-benzaldehyde	10.5-2100 0.99088	0.7	105	117	10.5	±41.2 (33.6%)
				945	98	3.1	±92.1 (9.9%)
				1890	96	1.4	±80.4 (4.4)
tr-cinnamaldehyde	α-methyl-cinnamaldehyde	10.5-2100 0.99193	0.6	105	109	4.6	±16.8 (14.6%)
				945	96	1.4	±39.7 (4.4%)
				1890	91	2.8	±156.4 (9.1%)
safrole	3,4-diethoxy-toluene	1.1-164.6 0.99654	0.06	11.0	97	2.7	±0.9 (8.5%)
				43.9	105	0.6	± 0.9 (1.9%)
				99.0	94	1.4	±4.1 (4.4%)
myristicin	3,4-diethoxy-toluene	2.9-572 0.99824	0.11	57	109	1.9	±3.8 (6.0%)
				172	99	1.2	±6.6 (3.9%)
				458	98	1.2	±17.8 (4.0%)
myristicin	1,3-diisopropoxy-benzene	2.9-572 0.99745	0.10	57	112	1.9	±3.8 (6.0%)
				172	98	0.9	±4.8 (2.9%)
				458	97	1.7	±24.6 (5.5%)

Table 3. Performance characteristics of the analytical method

Analyte	Internal standard	type of regression	slope	intercept	r^2	Σ *%RE* [1]
tr-cinnamaldehyde	3-vinyl-benzaldehyde	linear	0.0007885	0.0368	0.99587	1228
		$1/x^{0.5}$	0.0008119	0.0137	0.99739	547
		1/x	0.0008362	0.0037	0.99580	261
		$1/x^2$	0.0009291	-0.0015	0.99088	136
tr-cinnamaldehyde	α-methyl-cinnamaldehyde	linear	0.0006635	0.0316	0.99146	1296
		$1/x^{0.5}$	0.0006839	0.0115	0.99582	556
		1/x	0.0007050	0.0028	0.99455	264
		$1/x^2$	0.0007801	-0.0013	0.99193	142
safrole	3,4-diethoxy-toluene	linear	0.01300	0.0367	0.99821	639
		$1/x^{0.5}$	0.01326	0.0154	0.99901	269
		1/x	0.01347	0.0068	0.99856	143
		$1/x^2$	0.01428	0.0022	0.99654	103
myristicin	3,4-diethoxy-toluene	linear	0.003437	0.0144	0.99943	415
		$1/x^{0.5}$	0.003467	0.0058	0.99965	213
		1/x	0.003503	0.0016	0.99932	119
		$1/x^2$	0.003695	-0.0012	0.99745	86
myristicin	1,3-diisopropoxy-benzene	linear	0.01285	0.0372	0.99950	294
		$1/x^{0.5}$	0.01292	0.0169	0.99972	166
		1/x	0.01301	0.0049	0.99950	101
		$1/x^2$	0.01359	-0.0037	0.99824	73

[1]Note: computed as the %relative difference between the nominal concentration and the one found with the regression curve

Table 4. Parameters related to different least squares linear regression schemes

3.3.4 Analytical determination settings

Soft drinks were initially degassed at ambient temperature for 5 minutes in an ultrasonic bath; then, 2 mL of the soft drink were added with 100 μL of the internal standards solution (3-vinylbenzaldehyde 10.4 μgmL^{-1}, and 3,4-diethoxytoluene 1.60 μgmL^{-1} in methanol) and then diluted 100-fold in deionized water.

Isolation by SBSE[162] was performed on 15 mL of this working solution by stirring at 1100 rpm a PDMS-coated stir bar (1 mm thickness, 10 mm length, Gerstel GmbH, Mülheim and der Ruhr, Germany) for 90 min, at room temperature, in hermetically closed vials. The stir bar was then carefully removed from the solution, rinsed with deionized water, patted dry on a clean filter paper and placed in a desorption tube for analysis.

After each extraction the stir bars were reconditioned by desorption at 280°C for 10 minutes under a carrier gas flow of 100 mL min^{-1}; to minimise carry over, it is important to put in the desorption tube not more than two bars at a time.

Analysis were carried out on an Agilent 6890 GC gas chromatograph fitted with a 5973N mass selective detector, based on a quadrupole mass filter (Agilent Technologies Inc., Palo Alto, CA); thermal desorption of compounds extracted by the stir bars were performed by a thermal desorption unit (TDU, Gerstel GmbH) mounted onto the GC injector.

Desorption conditions were: temperature program from 35 °C to 270 °C (5 min) at 720 °C min^{-1}, with a flow rate of the carrier gas (He) of 50 mL min^{-1}; a Gerstel CIS-4 PTV injector was used for cryogenic focusing of the compounds thermally desorbed from the stir bar.

The PTV injector was cooled at 0 °C using liquid CO_2, and at the start of the GC run the injection temperature was raised to 280 °C (3 min) at 12 °C s^{-1}, and a liner filled with Tenax was used within the PTV injector.

Capillary GC-MS analyses were performed by using a DB-1MS (Agilent Technologies Inc.) column (30 m x 0.25 mm i.d., 0.25 μm film thickness).

Chromatographic conditions were as follows: split injection (by setting the solvent vent mode, and purge flow to split vent at 5 mL min^{-1} after 0.01 min); temperature program: from 70 °C (1 min) to 180 °C at 6 °C min^{-1}, and then to 280 °C (5 min) at 30 °C min^{-1} (total run time of 27.7 min); linear velocity of the He carrier gas was 37 cm s^{-1}.

Mass spectra were generated in the electronic impact ionization mode at 70 eV; transfer line, source, and quadrupole temperatures were set, respectively, at 300 °C, 230 °C and 150 °C.

The mass spectrometric detection for the whole set of substances was performed in the selected ion monitoring (SIM) mode, using the following mass fragments: 131 and 103 *m/z* for 3-vinylbenzaldehide and *tr*-cinnamaldehyde, 162 and 131 *m/z* for safrole, 145 and 117 *m/z* for α-methyl-*tr*-cinnamaldehyde, 124 and 180 *m/z* for 3,4-diethoxytoluene, 110 and 194 *m/z* for 1,3-diisopropoxybenzene, 192 and 165 *m/z* for myristicin.

The first fragment was used for quantification and the second as qualifier, and for all of them a dwell time of 100 ms was selected.

[162] K. Ridgway, S. P. D. Lalljie, R. M. Smith - Analytica Chimica Acta (2010) 677, 29

3.3.5 Conclusions

The level of the three considered substances was fairly constant in samples of brand leader products purchased approximately at the same time (within a period of 2 months) in 8 distinct European Countries.

A higher level of variation was observed, as expected, in the group of private brands products, representing a more heterogeneous collection of commercial products, with differing formulations.

In the case of safrole and myristicin, the highest contents were found in brand leader products, whereas the contrary was observed for tr-cinnamaldehyde, where the highest concentration found in private brand products was approximately twice the maximum level observed in brand leader products.

The observed differences may be partly due also to differences in storage time between samples.

From the exposure point of view, the higher variability observed in private brands products with respect to brand leader products, widens the range of concentration found in the category of cola drinks, on the side of low concentrations, for safrole and myristicin, and on the side of high concentrations for tr-cinnamaldehyde, by doubling the maximum value in this latter case.

Extensive quantitative data, toxicological considerations (for safrole and myristicin) and related insights on exposure estimates will be hopefully found soon in the paper we recently submitted to *Food and Chemical Toxicology* for publication; a summary statistics describing distribution of concentration levels (μg L^{-1}) within different groups of samples is shown in tab. 5.

Group of samples	Parameter	*tr*-cinnamaldehyde	safrole	myristicin
All samples	mean	608.8	23.0	168.3
(n=70)	minimum	2.1	0.6	0.4
	10^{th} percentile	52.6	6.1	21.6
	90^{th} percentile	1119.0	37.3	285.9
	maximum	1926.5	43.9	325.6
Brand leader 1	mean	810.0	22.2	164.4
(n=16)	minimum	636.2	15.3	141.8
	10^{th} percentile	676.2	15.5	142.9
	90^{th} percentile	964.3	30.3	185.1
	maximum	1009.0	34.5	200.1
Brand leader 2	mean	689.5	28.9	261.3
(n=14)	minimum	332.8	14.9	188.8
	10^{th} percentile	449.5	15.9	198.2
	90^{th} percentile	814.8	39.9	309.1
	maximum	1073.5	43.9	325.6
Private brands	mean	394.6	19.8	125.9
(n=34)	minimum	2.1	0.6	0.4
	10^{th} percentile	21.4	3.7	3.7
	90^{th} percentile	1167.6	38.0	285.8
	maximum	1926.5	40.3	297.3
Diet versions				
Brand leader 1 (n=4)	mean	1095.6	28.7	177.6
Brand leader 2 (n=2)	mean	698.6	36.6	279.0
Private brands (n=3)	mean	662.1	11.5	53.3

Table 5. Summary statistics describing distribution of concentration levels (μg L^{-1}) within different groups of samples

Chapter 4: Post-harvest factors effect on tomato flavour

4.1 Overview: effect of distribution scenarios and storage conditions

In recent years, in many Western countries consumer demand for food that is locally produced and marketed is generating a growing interest in some forms of short food supply chains characterized by a more direct connection between the food consumer and producer.
Investigations on the attitudes of consumers who purchase local foods have highlighted that freshness and quality are among the main factors in determining their preferences; however, in the debate on local food systems it has been pointed out that quite poor scientific evidence supports the claimed higher quality of locally produced fresh foods versus non locally produced foods[163].
In this context, fresh tomato may represent an appropriate and significant case study, based not only on the economic value of this crop, but also on the several implications of the postharvest handling and marketing system for the flavour quality of this product[164].
Detrimental effects on flavor formation are ascribed, in particular, to the practice of harvesting fruits at early ripening stages as well as to improper temperature management during postharvest handling.
In a few tomato cultivars, cold storage of fruits, both within and above the chilling range, has been observed to result in a significant alteration of the fresh fruit flavor, even before any visual symptoms of injury could be seen[165].
On the whole, a thorough understanding of the influence of commercial postharvest handling practices on fresh tomato flavour quality is still lacking.
In the present study, the impact of different distribution scenarios and of recommended commercial storage conditions on some flavor related quality attributes in fresh tomatoes have been investigated, by simulating in the laboratory distribution chain conditions occurring in the current postharvest handling system in Italy, as well as reproducing recommended storage conditions.
The flavor quality of fruits exposed to these conditions was evaluated by chemical determination of organic acids, sugars, and volatile compounds, and by physical measurement of texture properties.

[163] G. Edward-Jones et al. - Trends in Food Science and Technology (2008) 19, 265
[164] M. Dorais, A. P. Papadopoulos, A. Gosselin - Horticultural Reviews (2001) 26, 239
[165] A. A. Kader, L. L. Morris, M. Allen Stevens, M. Albright-Holton – Journal of the American Society of Horticultural Science (1978) 103, 6

4.1 Tomato flavour

The tomato (*Lycopersicon esculentum*), a member of the potato (Solanaceae) family, is very important as a food source.

The tomato's important role in nutrition can be explained by its hedonic value and versatility; its consumption in the raw state is increasing all over the world, and in addition it is widely used in soups, meat and pasta dishes, and in the manufacture of soft drinks as the main raw material or as a flavouring additive[166].

Tomato flavour is mainly attributed to its aroma volatiles, sugars (fructose and glucose), acids (mainly citric acid) content, and their interactions; it is determined by cultivars[167], stage of ripeness, practice management during cultivation[168] and postharvest treatments[169].

Of the minerals, potassium (by influencing the free acid content) and phosphate (due to its buffering capacity) indirectly affect the taste.

More than 400 volatile compounds have been identified in tomato[170], of which 34 or so have odour threshold values that indicate that they contribute to tomato flavour; no character-impact compound has been identified in tomatoes, although 2-isobutylthiazole is unique to tomato flavour.

The nature and relative amount of volatiles in tomato seem to depend on species, maturity and preparation of the product more than in any other vegetable.

The most important compounds in tomatoes are 3-methylbutanal, hexanal, (Z)-3-hexenal, (E)-2-hexenal, 3-methyl-1-butanol, 1-hexanol, (Z)-3-hexen-1-ol, 1-penten-3-one, 6-methyl-5-hepten-2-one, β-ionone, β-damascenone, 2-phenylethanol, methyl salicylate, furaneol and 2-isobutylthiazole, and of these, (Z)-3-hexenal and β-ionone have the highest odour units[171].

This pool of substances is the main responsible of the tomato-like flavour: they develop partly during ripening, partly during the comminution of the ripe fruit, as an effect of the enzymes activated, and they are predominantly derived from fatty acids and amino-acids.

[166] M. Petrò-Turza – Food Reviews International (1986) 2(3), 309

[167] E. A. Baldwin, M. O. Nisperos-Carriedo, R. Baker, J. W. Scott – Journal of Agricultural and Food Chemistry (1991) 39, 1135

[168] M. Oke, A. TaeHyun, A. Schofield, G. Palivath – Journal of Agricultural and Food Chemistry (2005) 53(5), 1531

[169] F. Maul, S. A. Sargent, C. A. Sims, E. A. Baldwin, M. O. Balaban, D. J. Huber – Journal of Food Science (2000) 65(7), 1228

[170] C. Maneerat, Y. Hayata, H. Kozuka, K. Sakamoto, Y. Osajima – Journal of Agricultural and Food Chemistry (2002) 50, 3401

[171] R. G. Buttery, G. R. Takeoka, L. C. Ling – Journal of Agricultural and Food Chemistry (1995) 43, 1638

4.2 Experimental section

Fresh tomatoes (*Solanum lycopersicum cv. Caramba, Nerina, and Rebelion*) were grown according to organic cultural practices in an unheated greenhouse in a commercial farm located at Maccarese, in proximity to Rome, during fall 2010 and spring 2011.

4.3.1 First post-harvest experiment

For the first experiment, Caramba and Rebelion fruits were harvested at the following ripening stages, defined on the basis of external color: Breaker and Pink, for samples representing tomatoes marketed at an intermediate stage of ripeness; Light Red and Red, for samples corresponding to tomatoes marketed at full ripeness.

Fruits were picked early in the morning, immediately transported to the laboratory, washed, dried, and sorted to eliminate defects; then, they were grouped (25 fruits each group) and subjected to the conditions reported in tab. 6, simulating postharvest conditions corresponding to short, medium, and long distribution chains.

In the case of tomatoes marketed at an intermediate stage of ripeness, fruits picked at the Pink stage were used for the simulation of short chain conditions, whereas fruits harvested at the Breaker stage were selected for the medium/long chain conditions; similarly, in the case of tomatoes marketed at full ripeness, fruits picked at the Red and Light Red stages were subjected, respectively, to short and medium/long chain conditions.

For the postharvest experiment, to mimic cold storage and transport, fruit samples were kept at a temperature of 6±1°C, the relative humidity ranging from about 55% to 80%; tomato samples exposed to conditions of medium/long distribution chain were analyzed at the end of the simulated cold storage/transport period, 2, 4, and 7 days after the harvest.

To evaluate the combined effect of harvesting at early ripening stages and refrigeration on fruit ability to develop the typical tomato flavor profile following ripening at room temperature, a group of fruits harvested at the Breaker stage and exposed to conditions of extended long chain was transferred afterward to room temperature (21±1°C), allowed to achieve full ripeness (Red stage), and then analyzed.

4.3.2 Second post-harvest experiment

A second experiment was designed to also evaluate the effect of the duration of cold storage (combined with harvesting at an early ripening stage, Green) on fruit ability to develop the flavor profile of vine ripened fruits, when they were allowed to ripen at room temperature after the refrigeration period; for this experiment, cv. Nerina fruits were used.

Fruits harvested at the Green stage were subjected to medium/long chain conditions or to recommended storage conditions for extended times, then allowed to fully ripen at room temperature, and finally evaluated at the Red stage (tab. 6); these fruits were compared to tomatoes harvested at the Red stage and exposed to short chain conditions.

To also consider the case of tomatoes marketed at an intermediate stage of ripeness, cv. Nerina fruits harvested at Turning and subjected to short, medium, and long chain conditions, as well as to

recommended storage conditions for extended times, were evaluated just at the end of the simulated postharvest handling process, without following ripening at room temperature.

Fruit group	Ripeness stage at harvest	Post-harvest conditions Days in simulated cold storage/transport and ripening at room temperature 0 1 2 4 6 7 9 12 Full ripeness
1st Experiment: Caramba and Rebelion tomatoes		
Short chain (harvest day)	P/R	H-**A**
Short chain (1 day)	P/R	H RT **A**
Medium/Long chain at harvest	B/LR	H-**A**
Medium chain	B/LR	H 6°C **A**
Long chain	B/LR	H 6°C **A**
Extended long chain	B/LR	H 6°C **A**
Extended long chain + ripening	B	H 6°C | RT **A**
Recommended commercial storage conditions	B/LR	H 13°C Breaker – 10°C Light Red / 95% R.H. **A**
Recommended commercial storage conditions (extended time)	B/LR	H 13°C Breaker – 10°C Light Red / 95% R.H. **A**
2nd Experiment: Nerina tomatoes evaluated at full ripeness		
Short chain (harvest day)	R	H-**A**
Short chain (1 day)	R	H RT **A**
Medium/Long chain at harvest	G	H-**A**
Medium chain + ripening	G	H 6°C | RT **A**
Long chain + ripening	G	H 6°C | RT **A**
Extended long chain + ripening	G	H 6°C | RT **A**
Recommended commercial storage conditions + ripening	G	H 13°C / 95% R.H. | RT **A**
Recommended commercial storage conditions(extended time)+ripening	G	H 13°C / 95% R.H. | RT **A**
2nd Experiment: Nerina tomatoes marketed an intemediate ripening stage		
Short chain (harvest day)	T	H-**A**
Short chain (1 day)	T	H RT **A**
Medium chain	T	H 6°C **A**
Long chain	T	H 6°C **A**
Extended long chain	T	H 6°C **A**
Recommended commercial storage conditions	T	H 10°C / 95% R.H. **A**
Recommended commercial storage conditions (extended time)	T	H 10°C / 95% R.H. **A**

NOTES: H: harvest. A: analyses. P: Pink. R: Red. B: Breaker. LR: Light Red. G: Green. T: Turning. RT: room temperature (21±1°C).

Table 6. Conditions Adopted in the Postharvest Experiments[a]

4.3.3 Analytical determinations for volatile compounds

Tomato sample treatment was carried out following the procedure developed by Buttery et al.[172], because most of the tomato volatile published data have been obtained by this method.

The whole tomato sample (200 g), formed by pieces cut from 8 different fruits, was blended for 30 s; the blended mixture was allowed to stand at room temperature for 180 s longer, then a saturated $CaCl_2$ solution (200 mL) was added and the mixture blended for 10 s.

A standard solution (1 mL) containing 2-octanone (24.51 mg L^{-1}), 3-pentanone (24.45 mg L^{-1}), and trans-anethole (9.98 mg L^{-1}) in water was then added and the mixture blended again for 10 s.

Then the mixture was centrifuged for 15 min at 12000 rpm and 4°C, and the resulting supernatant collected and filtered by Whatman filter paper n.113.

Isolation of volatile compounds from the obtained aqueous mixture was carried out in duplicate by the Stir Bar Sorptive Extraction technique (SBSE)[173]: 15 mL of the mixture were stirred at 800 rpm with a PDMS-coated Stir Bar (1.0 mm thickness, 10 mm length) for 90 min at room temperature, in hermetically closed vials.

Thermally desorbed compounds were cryogenically focused by means of a Gerstel CIS-4 PTV injector cooled at −50°C using liquid CO_2 during the desorption step, and a liner filled with Tenax was used within the PTV injector.

Capillary GC-MS analyses were performed by using a DB-1MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness), with split injection; a mass spectrometer with a quadrupole mass filter was used for detection.

Identification of compounds was carried out by comparing mass spectra, obtained by the full scan mode (m/z range 40−400 amu) and Kovats linear retention indices determined on chromatograms of tomato sample isolates with spectra and retention indices obtained from authentic standards.

When authentic standards were not commercially available, compounds were tentatively identified based on a comparison with spectra and retention indices reported in the NIST/EPA/NIH Mass Spectra Library 2005 (tab. 7).

The two nitro-compounds were tentatively identified based on their reported presence in tomato volatile fraction[174], the retention index of structurally related compounds, and on mass spectra characteristics: 1-nitro-3-methylbutane was identified on the basis of diagnostic signals (m/z 71 and 55) also present in the mass spectra of the structural isomer 2-nitro-pentane.

1-Nitro-2-phenylethane was tentatively identified based on similarity in the fragmentation pattern with 2-nitro-1-phenylethene: in both spectra were present prominent signals at m/z (M - 47), 77 and 91.

(E,Z)-2,4-Decadienal was tentatively identified based on its reported presence in tomato volatiles[175], retention indices, and mass spectra reported on a freely accessible commercial database[176].

For the semiquantitative determination of volatiles, spectrometric detection in the selected ion monitoring (SIM) mode was used: mass fragments (m/z) selected for each detected compound are reported in tab. 7.

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[172] R. G. Buttery, R. Teranishi, L. C. Ling, R. A. Flath, D. J. Stern – Journal of Agricultural and Food Chemistry (1988) 36, 1247

[173] A. Raffo, M. Kelderer, F. Paoletti, A. Zanella – Journal of Agricultural and Food Chemistry (2009) 57, 915

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[175] F. Mayer, G. R. Takeoka, R. G. Buttery, L. C. Whitehand, M. Naim, H. D. Rabinowitch – Journal of Agricultural and Food Chemistry (2008) 56, 3749

[176] http://www.pherobase.com

Concentration levels were expressed as μ/g equivalents of internal standard per kilogram of fruit fresh weight, and are to be considered as relative data because response factors related to the internal standard were not determined.

Compound name/ analytical parameter	Abbreviation	identification	*m/z* fragment
acetaldehyde	AcAl	ref. comp.[1]	44
ethanol	Eth	ref. comp.[1]	45
acetone	Ace	ref. comp.[1]	58
ethylacetate	EtAc	ref. comp.[1]	61
3-methylbutanal	3MeBAl	ref. comp.[1]	58
2-methylbutanal	2MeBAl	tentatively[2]	58
isopropylacetate	IsAc	ref. comp.[1]	61
1-penten-3-one	PeOne	ref. comp.[1]	84
1-penten-3-ol	PeOl	ref. comp.[1]	57
1-pentanal	PeAl	ref. comp.[1]	58
3-pentanone	Int. std.		57
2-methyl-2-butenal	2MeBEAl	ref. comp.[1]	84
3-methyl-1-butanol	3MeBOl	ref. comp.[1]	70
2-methyl-1-butanol	2MeBOl	ref. comp.[1]	57
(E)-2-pentenal	PeEAl	ref. comp.[1]	84
1-pentanol	PeOl	ref. comp.[1]	55
(3Z)-3-hexenal	ZHexe	ref. comp.[1]	80
hexanal	Hexa	ref. comp.[1]	72
(E)-2-hexenal	EHexe	ref. comp.[1]	69
(Z)-3-hexenol	HeEol	ref. comp.[1]	67
1-hexanol	HeOl	ref. comp.[1]	56
1-nitro-3-methylbutane	NiBu	tentatively[3]	55
benzaldehyde	Ben	ref. comp.[1]	106
2-heptenal	Hept	ref. comp.[1]	83
1-octen-3-one	OcOne	tentatively[2]	70
6-methyl-5-hepten-2-one	6MeOne	ref. comp.[1]	108
2-octanone	Int. std.		58
6-methyl-5-hepten-2-ol	6MeOl	ref. comp.[1]	95
5-ethyl-2(5H)-furanone	EtFu	tentatively[2]	83
phenylacetaldehyde	PhAc	ref. comp.[1]	91
2-isobutylthiazole	IsTh	ref. comp.[1]	99
2-methoxyphenol	MePh	ref. comp.[1]	109
2-phenylethanol	PhEt	ref. comp.[1]	91
linalool	Lin	ref. comp.[1]	93
camphor	Cam	ref. comp.[1]	152
furaneol	Fur	ref. comp.[1]	128
methylsalicylate	MeSa	ref. comp.[1]	120
β-cyclocitral	BCyc	ref. comp.[1]	137
neral	Ner	ref. comp.[1]	69
geranial	Ger	ref. comp.[1]	69
1-nitro-2-phenylethane	NiPh	tentatively[3]	104
tr-anethol	Int. std.		148
(E,Z)-2,4-decadienal	Zdec	tentatively[3]	81
(E,E)-2,4-decadienal	Edec	ref. comp.[1]	81
eugenol	Eug	ref. comp.[1]	164
vanillin	Van	ref. comp.[1]	151
β-damascenone	BDam	ref. comp.[1]	69
geranylacetone	GeAc	ref. comp.[1]	69
β-ionone	BIon	ref. comp.[1]	177
fructose	Fru	ref. comp.[1]	
glucose	Glu	ref. comp.[1]	
malic acid	Mal	ref. comp.[1]	
citric acid	Cit	ref. comp.[1]	

firmness	Firm
deformation	Def
stiffness	Stif

NOTES: [1] based on reference pure compound; [2] based on comparison with MS spectra reported in the NIST/EPA/NIH Mass Spectra Library 2005; [3] based on other spectral and chemical information as reported in Materials and Methods section of the related paper

Table 7. Analytical Parameters and Abbreviations Used in PCA Plots[a]

4.3.4 Statistical and chemometric analyses

Chemometric analysis was performed by using PLS Toolbox 5.2 (Copyright 1995-2008, Eigenvector Research, Inc.) in MATLAB 7.5.0.342 environment (The MathWorks Inc., Natick, MA), while univariate statistical analysis was carried out by SPSS 16.0.2 (SPSS Inc., Chicago, IL) software package.

To look for significant effects associated with the postharvest conditions within all samples from the same cultivar, one-way ANOVA analysis was performed on volatiles, acids, sugars, and texture data sets, and means were compared by the Tukey multi-comparison test, considering a significance level of $p < 0.05$.

Data sets used for principal component analysis (PCA) were obtained by first averaging replicate determinations and then applying auto-scaling as a pretreatment; by merging the same data sets used for PCA, a Pearson's correlation matrix was built, choosing a significance level of $p < 0.01$, to highlight possible metabolic relationships across the three cultivars.

For detailed information, on organic acids, sugars and texture properties determinations also, please refer to the related paper[177].

[177] A. Raffo, S. Nicoli, N. Nardo, I. Baiamonte, A. D'Aloise, and F. Paoletti – Journal of Agricultural and Food Chemistry (2012) 60, 10445

4.4 Results

4.4.1 First post-harvest experiment

Cultivar Caramba Tomatoes. PCA was applied to all chemical and physical data collected, and the resulting biplot can be seen in Fig. 16; PC1 and PC2 explained 39% and 17% of the variation in the data, respectively.

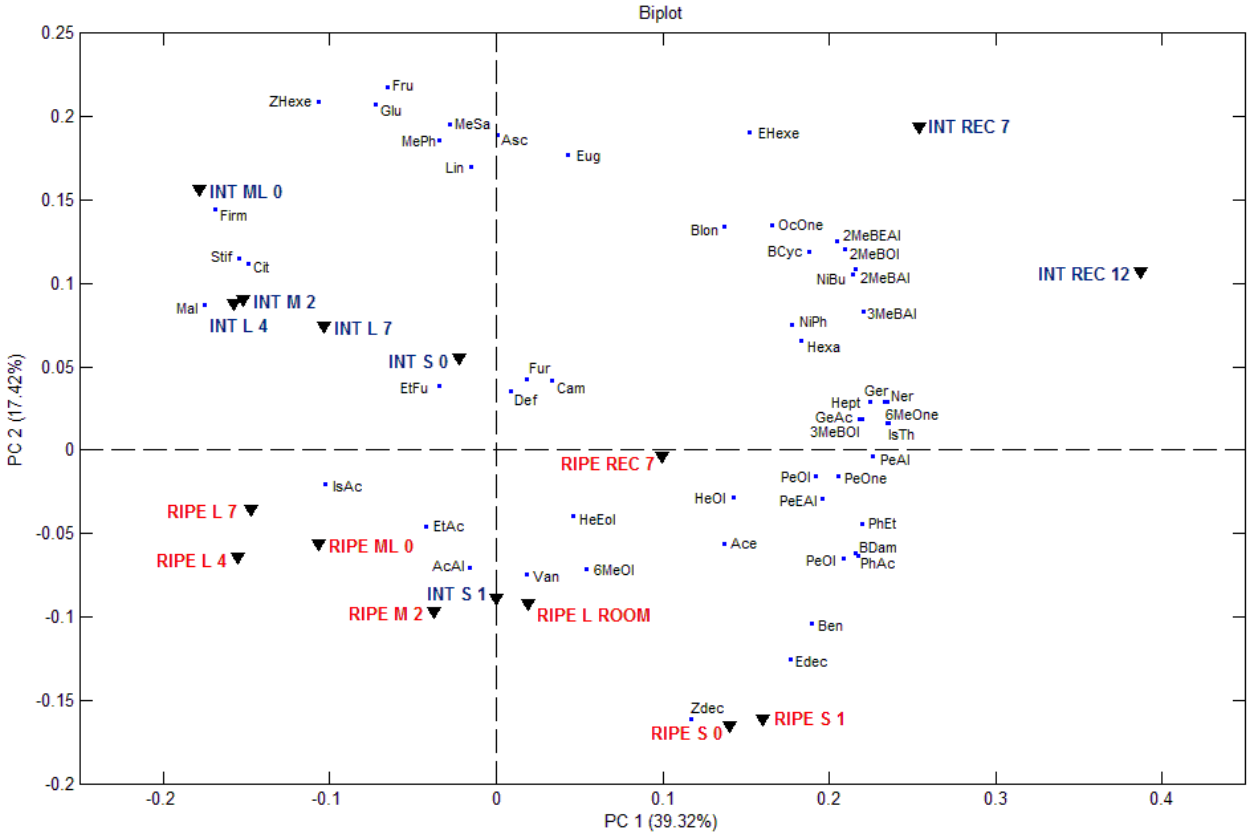

Figure 16. PCA biplot of cv. Caramba tomato samples. Tags for tomato samples indicate fruits marketed at full (RIPE) or intermediate (INT) stage of ripeness; S, M, and L stand for short, medium, and long distribution chain, whereas REC denotes recommended commercial storage. The final number indicates the number of days between harvest and evaluation, whereas L ROOM denotes samples exposed to the long chain and ripened at room temperature

In the group of tomato samples to be marketed at full ripeness (denoted by RIPE), the two samples of fruits exposed to short chain conditions (RIPE S 0 or 1) were very similar and well separated from all the other fruit samples subjected to medium and long chain conditions.

In the case of tomatoes to be marketed at an intermediate stage of ripeness (INT), differentiation between samples exposed to short and medium/long chain conditions was not as clear.

Interestingly, tomatoes harvested at the Breaker stage, exposed to extended long chain conditions and then allowed to achieve full ripeness at room temperature (RIPE L ROOM), did not develop the same profile observed on fruits fully ripened on the vine (RIPE S 0), even though they showed external color corresponding to full ripeness.

The overall profile of fruits to be marketed at both ripening stages and exposed to recommended commercial storage conditions (INT REC, RIPE REC) followed a different pattern of variation, mainly described by the sole PC1.

The PCA biplot suggests also the main drivers of differentiation among groups of tomato samples: significantly enhanced levels of some volatile compounds, such as the lipid-derived decadienals, and three volatiles derived from phenylalanine (benzaldehyde, phenylacetaldehyde, and 2-phenylethanol) were found in fruit exposed to short chain conditions.

β -damascenone, and the group of C5 volatiles (1-penten-3-one, 1-penten-3-ol, (E)-2-pentenal, 1-pentanol, and 1-pentanal), C6 aldehydes ((Z)-3-hexenal, hexanal, and (E)-2-hexenal) and the corresponding alcohols ((Z)-3-hexenol, hexanol), which are key contributors to the fresh tomato top-note, seemed not to be strongly affected by the distribution chain conditions, as it happens for sugar contents and texture parameters.

A reduced level in the main organic acids, citric and malic, contributed to the differentiation of Red harvested tomatoes exposed to the short chain.

Fruit samples exposed to recommended commercial storage conditions were associated with increased levels of important tomato volatiles derived from the two aminoacids isoleucine (2-methylbutanal, 2-methylbutanol, and 2-methyl-2-butenal) and leucine (3-methyl-butanal and 3-methylbutanol); another aminoacid-derived compound, 2-isobutylthiazole, showed a similar trend.

A similar increase was also observed on some linear carotenoid-derived volatiles, such as geranylacetone, 6-methyl-5-hepten-2-one, and geranial.

Cultivar Rebelion Tomatoes. Results of the PCA are reported in Fig. 17: the first two PCs explained 52% and 15% of the variation.

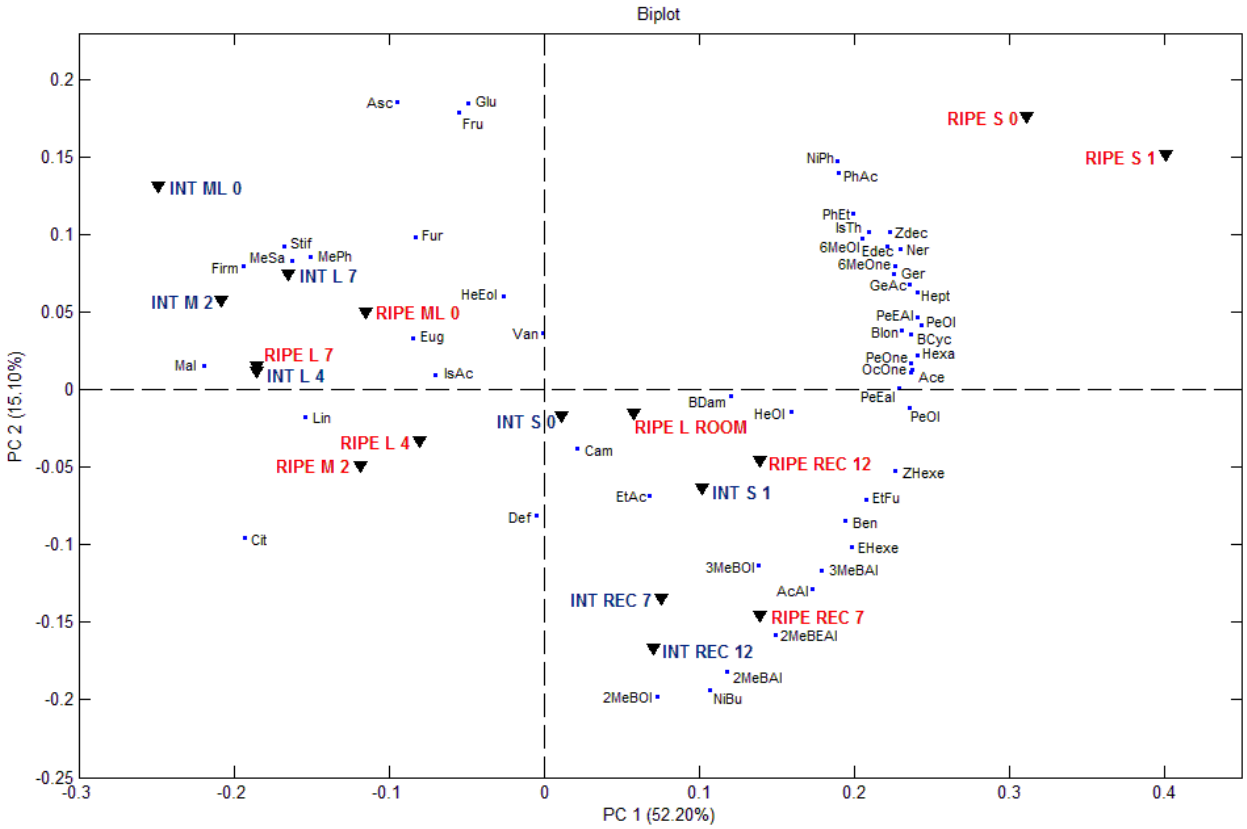

Figure 17. PCA biplot of cv. Rebelion tomato samples. Tags for tomato samples indicate fruits marketed at full (RIPE) or intermediate (INT) stage of ripeness. S, M, and L stand for short, medium, and long distribution chains, whereas REC denotes recommended commercial storage. The final number indicates the number of days between harvest and evaluation, whereas L ROOM denotes samples exposed to the long chain and ripened at room temperature

Also in this case, tomatoes harvested at the Red stage and exposed to short chain (RIPE S) were well separated from all of the other fruits to be marketed at full ripeness and exposed to medium or long chain conditions (RIPE M or L).
Also in the group of tomato samples to be marketed at an intermediate stage of ripeness, fruit samples exposed to short chain (INT S) were separated from fruit samples subjected to medium-long chain conditions (INT M or L).
Similar to what was observed on Caramba tomatoes, fruits exposed to recommended commercial storage conditions (INT REC, RIPE REC) seemed to follow a different pattern of variation when compared to fruits exposed to the more commonly occurring cold storage/transport conditions, i.e., stored within the chilling range (INT M or L, RIPE M or L).
A number of volatiles were strongly associated with the fruits fully ripened on the vine and subjected to short chain conditions, contributing to its marked differentiation from all the other samples, such as phenylalanine-derived compounds 1-nitro-2-phenylethane, phenylacetaldehyde, and 2-phenylethanol.
Interestingly, fruits harvested at the Breaker stage and exposed to extended long chain conditions almost completely lose the ability to synthesize these compounds when allowed to ripen at room temperature at the end of the refrigeration time.
The formation of the two lipid-derived aroma compounds (E,E)- and (E,Z)-2,4-decadienal was affected by medium and long chain conditions in a quite similar way than in Caramba tomatoes.
Many of the other lipid-derived volatiles, the groups of C_5 and C_6 volatiles, tended also to accumulate in higher amounts in fruits exposed to short than to common medium or long chain conditions.
Also in this cultivar, fruits exposed to recommended storage conditions showed enhanced levels of isoleucine and leucine derivatives when compared to fruits cold stored at a lower temperature.
Similar to what was observed on Caramba tomatoes, organic acids also contributed to the differentiation of tomatoes subjected to short chain when compared to medium/long chain conditions, and changes in sugar contents and texture parameters were not clearly linked to the examined postharvest handling conditions.

4.4.2 Second post-harvest experiment

Cultivar Nerina Tomatoes. A less clear picture about the influence of the postharvest conditions resulted from the PCA biplot of experimental data on Nerina fruits (Fig.18).
The first two PCs explained 42% and 15% of the variation. Fruits harvested at full ripeness and subjected to short chain (RIPE S) were similar between them, but differentiation with respect to samples harvested at the Green stage, exposed to medium or long chain conditions and then allowed to ripen at room temperature (GREEN M or L), was not as clear as for the other two cultivars.
Green harvested fruits subjected to recommended storage conditions for 7 days (GREEN REC 7) were similar to fruits exposed to common long and extended long chain (GREEN L 6 or 9), whereas after 12 days (GREEN REC 12) their profile showed a drift away from these samples and from those fully ripened on the vine.
A different trend was observed here for the two compounds derived from phenylalanine, phenylacetaldehyde, and 2-phenylethanol.

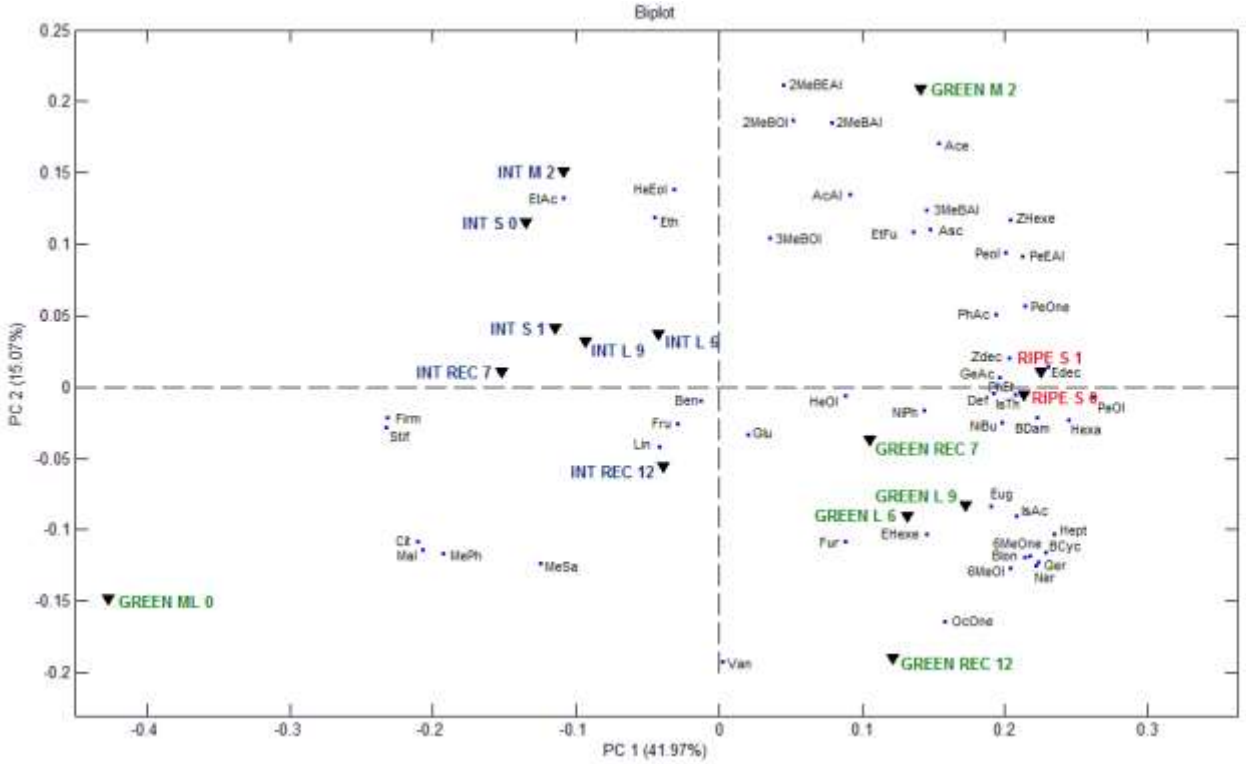

Figure 18. PCA biplot of cv. Nerina tomato samples. Tags for tomato samples indicate fruits harvested at the green (GREEN), turning (INT), or red (RIPE) stage. S, M, and L stand for short, medium, and long distribution chains, whereas REC denotes recommended commercial storage. The final number indicates the number of days between harvest and evaluation for the short chain and the time of the cold storage/transport step in the other cases.

In this experiment, the formation of these compounds was markedly enhanced in samples harvested at the Green stage, exposed to medium chain and allowed to ripen at room temperature, and not significantly altered in the samples exposed to longer chain or to recommended storage conditions when compared to fruits fully ripened on the vine.

Enhanced levels of the isoleucine derivatives contributed also to the differentiation of the overall profile of the Green harvested fruits exposed to the medium chain with respect to fruits exposed to longer chain conditions.

Interestingly, in this cultivar harvesting at the Green stage and refrigeration for an extended time at both 6°C and 13°C did not cause a significant suppression of C_6 volatiles in fruits allowed to ripen at room temperature afterward with respect to fruit ripened on the vine.

Different from what was observed on the other two cultivars, accumulation of isoleucine and leucine derivatives was not enhanced in fruits exposed to recommended storage conditions; organic acids did not show marked effects associated with the postharvest conditions.

In most cases, no significant effects of the distribution chain conditions were observed on sugar content and firmness parameters.

4.4.3 Correlations between volatile compounds

Pearson's correlation coefficients (r) between volatile compounds obtained from the whole data set have been analysed; only variables showing correlation coefficients higher than 0.8 and significant at the 0.01 level were considered.

Strong linear correlations ($r > 0.9$) were observed between some biochemical related compounds: between the two isoleucine derivatives 2-methylbutanal and 2-methylbutanol, among the open chain carotenoid derived compounds 6-methyl-5-hepten-2-one, neral, and geranial, between 1-pentanol and, separately, 2-heptenal and (E,E)-2,4-decadienal (lipid derived compounds), and among the phenylpropanoid metabolism related compounds 2-methoxy-phenol, methylsalycilate, and eugenol. Less strong linear correlations ($0.9 > r > 0.8$) were observed between other biochemical related compounds: in particular, among 2-methyl-butanal, 3-methylbutanal, 1-nitro-3-methylbutane, and 2-methylbutanol (isoleucine and leucine derivatives) and among 1-penten-3-one, 1-penten-3-ol, 1-pentanol, and (E)-2-pentanal (C_5 lipid derivatives).

4.5 Discussion and conclusions

Previous studies have examined the effects of different standardized postharvest treatments on tomato flavor quality, but contrasting results have been obtained for some aroma compounds.
In particular, the adoption of different protocols for the deactivation of enzymes during tomato sample preparation, before volatile isolation, is expected to profoundly influence the formation of some important volatile compounds, such as, for instance, the C_6 compounds, thus changing the analytical output.
Significant differences between the three cultivars were observed in the effects of the considered postharvest factors on the overall profile and on many of the evaluated individual quality attributes: in two out of three cultivars, Caramba and Rebelion, the length of the distribution chain significantly affected the overall fruit profile, the effect being more pronounced in tomatoes marketed at full ripeness than in those marketed at an intermediate stage.
Cold storage at 6°C up to 7 days, slowing down all the main biochemical processes associated to flavor formation, provided at the end of the storage/transport process a fruit quite similar to that at harvest; consequently, the significant differences in the profile between these fruits subjected to medium or long chain conditions and the counterparts exposed to short chain conditions were due to differences in the ripening stage at harvest.
Early harvesting combined with cold storage has the potential to affect the biosynthetic pathways of flavor formation when the fruit is brought back to room temperature after exposure to chilling; moreover, the effects due to cold storage at chilling temperatures, for instance 5−6°C, could be significantly different from those produced by cold storage above the chilling range, at about 10°C for red tomatoes and 13°C for green tomatoes, and at optimal relative humidity[178].
Among all volatile compounds, those related to the metabolism of aminoacids seemed to be the most strongly affected by the considered postharvest handling factors: three compounds derived from phenylalanine, 1-nitro-2-phenylethane, phenylacetaldehyde, and 2-phenylethanol, all of these reported as major contributors of tomato aroma, were markedly affected in the considered cultivars.
The formation of 1-nitro-2-phenyl-ethane, which apparently took place in the later stages of vine ripening, was strongly inhibited in Nerina and Rebelion fruits exposed to every evaluated cold storage condition.
Similarly, the formation of phenylacetaldehyde and 2-phenylethanol, which share the same immediate precursor with 1-nitro-2-phenylethane, phenylethylamine, was also inhibited in Caramba and Rebelion fruits by postharvest conditions involving refrigeration but not in Nerina fruits, where, on the contrary, enhanced levels were produced in the sample exposed to cold storage for 2 days.
2-Isobutylthiazole, which has been proposed to be formed from leucine[179] and reported to contribute a pungent−bitter note, was also strongly influenced by the considered postharvest conditions.
Formation of isoleucine and leucine derivatives appeared to be highly sensitive to cold storage temperature: it was remarkably promoted by cold storage above the chilling range in Caramba and Rebelion fruits, whereas their content was unaffected or reduced in fruits kept at a chilling temperature.

[178] http://postharvest.ucdavis.edu/pfvegetable/Tomato/
[179] S. Mathieu, V. Dal Cin, Z. Fei, H. Li, P. Bliss, M. G. Taylor, H. J. Klee, D. M. Tieman – Journal of Experimental Botany (2009) 60, 325

Regarding lipid-derived volatile compounds, the most remarkable alterations due to the distribution chain conditions were observed on (E,E)- and (E,Z)-2,4-decadienal, recently identified as important odorants in fresh tomatoes[180] and associated with sweet and floral notes.
In our experiment, their formation was significantly inhibited in Caramba and Rebelion fruits subjected to cold storage; the other important lipid-derived volatile compounds, the C_5 and C_6 groups, on average appeared to be less affected by the considered postharvest conditions[181].
Among nonvolatile compounds contributing to tomato flavor, long distribution chain conditions were associated with higher levels of organic acids, in particular in Caramba and Rebelion fruits, presumably as a result of cold storage.
On the contrary, effects on sugars levels and on measured texture properties were not significant or not clearly associated with the considered distribution chain conditions.
Correlations observed in the present study confirmed the existence of some of these metabolic relationships also in fruits exposed to a range of postharvest handling conditions, indicating that coordinated biosynthesis of certain groups of volatile compounds takes place not only during ripening on the vine, but also under the considered postharvest conditions: this was found to be true for the formation of leucine and isoleucine derivatives, for a group of volatiles sharing the same precursor lycopene (6-methyl-5-hepten-2-one, neral, and geranial), and for the group of C_5 lipid-derived volatiles.
Results of this study may represent the basis for further investigation designed to establish the extent to which these effects can be generalized to other tomato cultivars, and to find out whether they can reflect significant sensory differences and influence consumer quality perception.

[180] F. Mayer, G. R. Takeoka, R. G. Buttery, L. C. Whitehand, M. Naim, H. D. Rabinowitch – Journal of Agricultural and Food Chemistry (2008) 56, 3749
[181] F. Boukobza, A. J. Taylor - Postharvest Biology and Technology (2002) 25, 321

Chapter 5: Pre-harvest factors effect on apple flavour

5.1 Overview: effect of soil nutrition

Increased consumer demand for healthier fruit and more environmentally sustainable farming has driven international growth in the number of producers using organic management systems in orchards.

Organic fruit production systems offer alternatives to conventional ones that use regular applications of broad-spectrum insecticides, which have the potential to adversely affect the surrounding environment and the health of consumers[182].

Little is known about the influence of soil nutrition on the biogenesis of volatile compounds in fruits and vegetables; some studies have explored the effects of nitrogen nutrition on production of aroma compounds in apple, but no conclusive statements were made[183].

When using organic fertilizers the mineralization process, needed to provide the plants with the required amount of available nitrogen, tends to be slower than in the synthetic counterparts and this might affect aroma compounds formation in the fruit.

Italy is the main producer of organic apples in Europe (about 42,000 MT on 115,000 MT in 2006), and more than 60% of the organic apples produced are grown in Alto Adige region.

In this region, problems with nitrogen supply to apple plants in spring are common: in spring the demand of nitrogen of the apple trees in Alto Adige is high, but the weather conditions are often bad and the temperature of the soil is low.

In these conditions, the mineralization of the organic fertilizers starts slowly, the trees own reserves may become exhausted, the nitrogen supply from the mineralization of the soil organic matter is not yet sufficient: therefore, it is important to supply the plants timely in spring with fertilizer nitrogen.

The aim of the present study was, indeed, to assess the influence of the use of different organic fertilizers on the formation of aroma compounds in *cv. Golden Delicious* apples, by ANOVA-Simultaneous Component Analysis[184].

[182] G. M. Peck, P. K. Andrews, J. P. Reganold, J. K. Fellman – Horticultural Science (2006) 41(1), 99

[183] J.K. Fellman, T. W. Miller, D. S. Mattinson, J. P. Mattheis - Horticultural Science (2000) 35(6), 1026

[184] M. Farrés, M. Villagrasa, E. Eljarrat, D. Barceló, R. Tauler – Analytica Chimica Acta (2012) 731, 24

5.2 Apple flavour

When one bites into an apple (*Malus domestica* Borkh.), the olfactory sensations received from volatile molecules are responsible for the perception of "apple flavour", hence it is pertinent to examine factors that can influence production of these molecules, in the hope of having a better product available in the marketplace.

Flavour typical to apples develops during ripening, and the greatest concentration of volatiles is produced at the climacteric peak in ethylene production; the influences of genome, growing conditions, harvest maturity and storage regime on compounds that serve as precursors for volatile formation are critical factors that determine their levels in fresh and stored apples.

Dimick and Hoskin reported that nearly 300 volatiles have been isolated from apple[185]: these compounds include alcohols, aldehydes, carboxylic esters, ketones, and ethers; about 20 of them are "character impact" compounds, having a broad range of aroma thresholds[186].

Volatiles important for aroma and flavour are synthesised from amino acids, membrane lipids and carbohydrates; in apple aroma, esters formation is dependent on the availability of C_2-C_8 acids and alcohols[187].

Some are present in very low concentrations and contribute potent aroma characteristics typical of apple aroma/flavour, e.g., ethyl-2-methyl butanoate, others contribute to aroma intensity, e.g., (E)-2-hexenal[188].

The majority are esters (78-92%) and alcohols (6-16%); the most abundant compounds are even numbered carbon chains including combinations of acetic, butanoic, and hexanoic acids with ethyl, butyl, and hexyl alcohols; higher molecular weight volatiles, often with one or two hydrophobic aliphatic chains, are likely to be trapped by skin waxes and are generally not found.

Three esters (butyl acetate, 2-methylbutyl acetate, and hexyl acetate) are considered major contributors to the characteristic apple-like aroma and flavour in most cultivars, and especially in the "Golden Delicious" cultivar, which our study is focused on.

As volatiles are comprised of at least five chemical classes, there are several pathways involved in volatile synthesis; these have not been fully unraveled, but appear to be common for different fruits.

5.2.1 Fatty acids catabolism

Aroma volatiles in intact fruit are formed via the β-oxidation biosynthetic pathway, whereas when fruit tissue is disrupted, volatiles are formed via the lipoxygenase pathway.

β-oxidation is the primary biosynthetic process providing alcohols and acyl co-enzyme A (CoA) for ester formation[189]; substrates for ester biosynthesis may also be formed via α-oxidation[190]; varietal differences in volatile composition of apples depend on the specific activities of β-oxidation enzymes.

[185] P. S. Dimick, J.C. Hoskin - Critical Reviews in Food Science and Nutrition (1983) 18, 387
[186] J. Dixon, E. W. Hewett - New Zealand Journal of Crop and Horticultural Science (2000) 28, 155
[187] M. Knee, S. G. S. Hatfield - Journal of the Science of Food and Agriculture (1981) 32, 593
[188] R. A. Flath, D. R. Black, D. G. Guadagni, W. H. McFadden, T. H. Schultz - Journal of Agricultural and Food Chemistry (1967) 15, 29
[189] I. M. Bartley, P. G. Stoker, A. D. E. Martin, S. G. S. Hatfield, M. Knee - Journal of the Science of Food and Agriculture (1985) 36, 567
[190] R. Tressl, F. Drawet - Journal of Agricultural and Food Chemistry (1973) 21, 560

Ethyl and hexyl ester synthesis was stimulated by ethanol and hexan-1-ol at the expense of butyl esters, indicating that ester formation in apple fruit is a competitive reaction[191]; the steps in biosynthesis of acetate esters, catalyzed by alcohol acetyl-CoA transferase (AAT), are summarized in Fig. 19.

In intact fruit, enzymes in the lipoxygenase (LOX) biosynthetic pathway and their substrates have different subcellular locations, preventing formation of volatile compounds; during ripening, cell walls and membranes may become more permeable, allowing the LOX pathway to become active without tissue disruption.

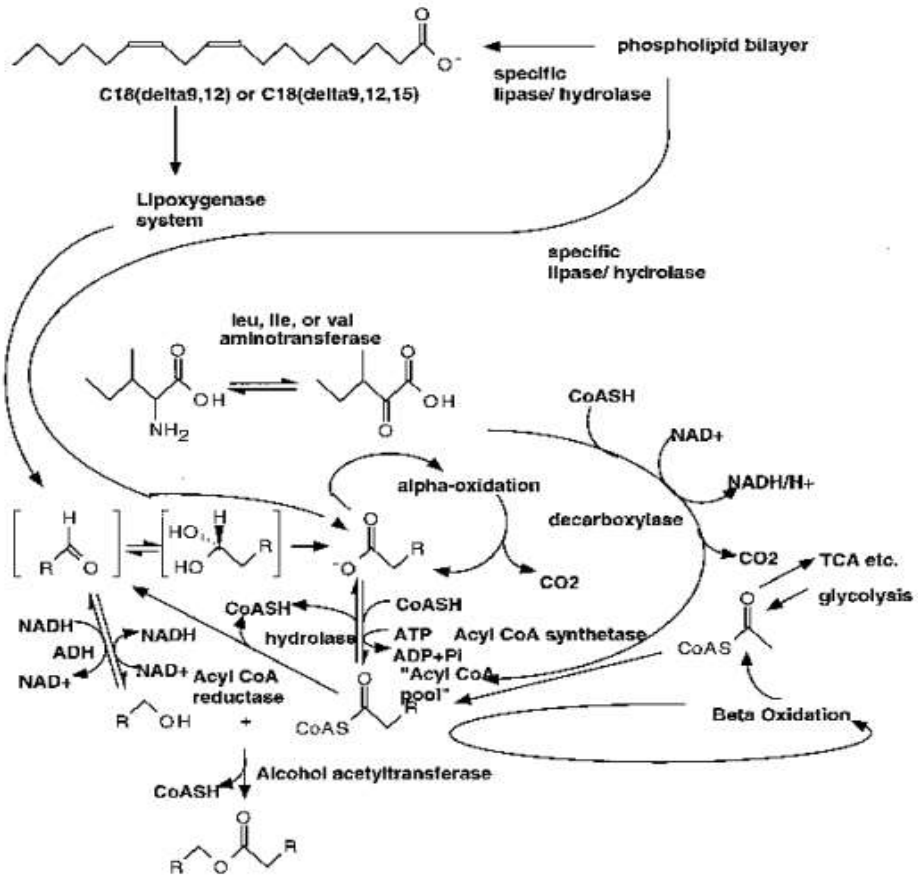

Figure 19. Pathways of acetate ester formation in apple
(from Fellman et al., Hort. Science (2000) 35, 1026)

5.2.2 Amino-acids catabolism

Branched chain alcohols, carbonyls, and esters are produced by metabolism of the amino-acids valine, leucine, iso-leucine, alanine, and aspartic acid; varying concentrations of free amino acids could account for different concentrations of branched chain volatiles in fruit.

Amino-acids are converted to branched chain alcohols and esters by three biosynthetic pathways: aminotransferase, decarboxylase, and ADH; iso-leucine is considered to be the biosynthetic precursor of 2-methyl butanoic acid and its esters in apples.

Different enzyme activity and selectivity, rather than substrate availability of the amino-acid degradation pathway, determines the concentration of branched chain esters for each cultivar.

[191] H. Kollmannsberger, R. G. Berger - Chemie Mikrobiologie Technologie Lebensmittel (1992) 14, 81

5.3 Experimental section

5.3.1 Field experiment: fertilization products and their application

The fruits were grown in the experimental fields of the "*Centro di Sperimentazione Agraria e Forestale Laimburg*", in the Alto Adige region, by applying 3 different organic fertilizers:

- Azocor 105 (both animal and vegetal origin) – treatment *cod. 3, 4, 5*
- Compost + Biogas slurry – treatment *cod. 6*
- Agrobiosol (from fungal biomass) – treatment *cod. 7*

In addition, fruits from a plot without the use of fertilizers ("control" – treatment *1*) and from a plot where a mineral synthetic fertilizer (ammonium sulphate – treatment *2*) have been considered for comparison; a single application was carried out in all fertilized plots, except for treatments *4* and *5*, for which 3 applications were repeated.

The rate of N application was normalized for all plots (24 g N/tree/year), except for "control" (no fertilization) and treatment *5*, where a higher rate (32 g N/tree/year) was applied in order to evaluate the effect of different rates of the same fertilizer.

For each fertilization product/condition of use, 4 field replicates were collected for the analysis of volatile compounds, for 2 years (2 analytical replicates for each sample).

5.3.2 Analysis of apple aroma compounds

200 g of apples, sampled from ten fruits, were homogenised after the addition of 400 mL of deionised water containing NaCl (20%) for enzyme inactivation, and 500 µL each of two internal standards, 4-methyl-2-pentanol (0.802 mg mL^{-1}) and allyl hexanoate (0.887 mg mL^{-1}), to cover at best the entire chromatographic range.

Isolation of volatile compounds was carried out on 15 mL of the obtained filtered solution (on Whatman filter paper n°113) by the Stir Bar Sorptive Extraction (SBSE) technique: a "twister" (10 mm x 1 mm) was stirred for 90 min in hermetically closed vials, at room temperature.

Before each analysis, the twisters were conditioned at 280°C, for 10 min with a carrier gas flow of 75 mL min^{-1}; thermal desorption of the volatile compounds from the twister was accomplished by the Gerstel TDU unit installed on an Agilent 6890 GC 5973N MS system.

Desorption conditions were as follows: temperature program from 30 to 250°C (5 min) at 720°C min^{-1}, with a flow rate of the carrier gas (He) of 50 mL min^{-1}.

Cryofocalization of the analytes was achieved within a CIS-4 PTV injector, hold at -50°C using liquid CO_2 during the desorption step; then, at the start of the GC run, the PTV injector temperature was raised to 270°C (3 min) at 12°C s^{-1}, and a liner filled with Tenax was used within the PTV injector.

GC separation was performed by a DB-1 MS (Agilent) column (0.25mm x 30m x 0.25mm). Chromatographic conditions were as follows: split injection (by selecting the solvent vent mode and setting the purge flow to split vent at 20 mL min^{-1}; temperature program from 40°C (2 min) to 160 at 4°C min^{-1}, and then to 270°C (5 min) at 20°C min^{-1} (total run time of 42.50 min); linear velocity of the He carrier gas was 36 cm s^{-1}.

A mass spectrometer with a quadrupole mass filter was used for detection, and mass spectra were generated in the electronic impact ionization mode at 70 eV; transfer line, source, and quadrupole temperatures were set, respectively, at 290, 230, and 150°C.

Identification of compounds was carried out by comparing mass spectra, obtained by the full scan mode (m/z range 40−400 amu), and Kovats linear retention indices determined on chromatograms of an apple sample with spectra and retention indices obtained from authentic standards.
When authentic standards were not commercially available, compounds were tentatively identified based on a comparison with spectra and retention indices reported in the NIST/EPA/NIH Mass Spectra Library 2005 (tab. 8).
A TIC chromatogram is shown in Fig. 20:

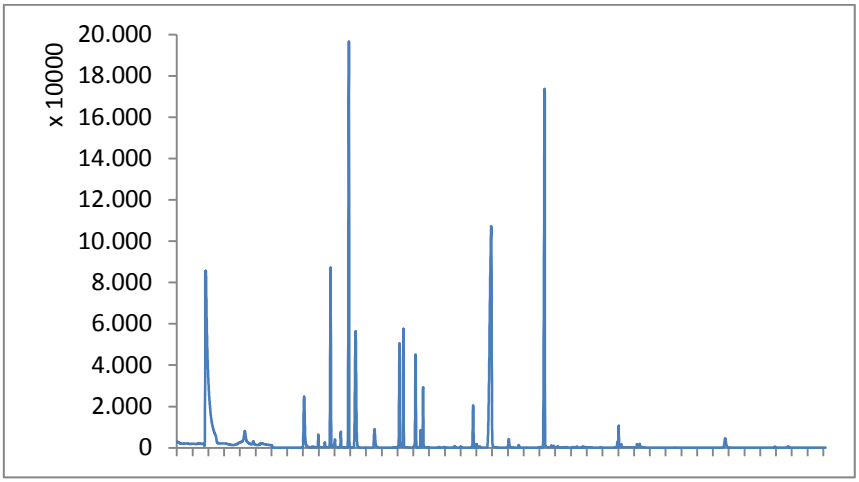

Figure 20. TIC chromatogram of an apple sample (total run time = 42.5 min)

For the relative determination of volatiles, spectrometric detection in the selected ion monitoring (SIM) mode was used: mass fragments (m/z) selected for each detected compound are also reported in tab. 8.

Compound name	identification	*m/z* fragment
1-butanol	ref. comp.[1]	56
propyl acetate	ref. comp.[1]	61
2-methyl-1-butanol	ref. comp.[1]	57
isobutyl acetate	ref. comp.[1]	56
hexanal	ref. comp.[1]	72
propyl propanoate	ref. comp.[1]	57
4-methyl-2-pentanol	int.std.	45
butyl acetate	ref. comp.[1]	56
(E)-2-hexenal	ref. comp.[1]	69
1-hexanol	ref. comp.[1]	55
2-methyl-butyl acetate	ref. comp.[1]	70
propyl butanoate	ref. comp.[1]	71
butyl propanoate	ref. comp.[1]	57
pentyl acetate	ref. comp.[1]	70
butyl butanoate	ref. comp.[1]	71
hexyl acetate	tentatively[2]	56
allyl hexanoate	int.std.	99
butyl-2-methyl butanoate	ref. comp.[1]	103
estragole	ref. comp.[1]	148
hexyl butanoate	tentatively[2]	89
benzothiazole	tentatively[2]	135
hexyl-methyl butanoate	tentatively[2]	103
butyl-3-hydroxy butanoate	tentatively[2]	87

NOTES: [1] based on reference pure compound; [2] based on comparison with MS spectra reported in the NIST/EPA/NIH Mass Spectra Library 2005.

Table 8. List of ions used to detect each volatile in the SIM mode

5.3.3 Chemometric analysis

Chemometric analysis were performed by using a custom-made routine (Copyright: Prof. Gooitzen Zwanenburg – Amsterdam University) in MATLAB 7.5.0.342 (The MathWorks Inc., Natick, MA) environment, considering a significance level of $p < 0.05$; analysed data matrix were built by introducing the ratio between the areas of the analytes and the suitable internal standard, respectively.

5.4 ANOVA combined to Simultaneous Component Analysis (ASCA)

Different chemometric techniques have been used to evaluate the effect of distinct experimental conditions and factors explored by an experimental design approach.
The data collected are often multivariate in nature, and their structure is difficult to visualize and interpret directly, if no suitable tools are implemented.
Therefore ASCA, which is suitable also for the analysis of highly correlated data, and is able to incorporate information about the underlined experimental design, is greatly valued.
In this study, the ASCA methodology was used to investigate the impact of different organic fertilization on the volatile compounds profile developed by apple cv. *Golden* Delicious; the effects of the several field replicates, and the harvesting years (2) were also evaluated (level of significance $p = 0.05$).
Each sample was described by 21 volatiles; mean-centering was chosen as a pretreatment of raw data.

5.4.1 ASCA results and discussion

The analysis of the data was done assuming the general three-factor ANOVA model with interactions for balanced data, i.e. using the type I sums of squares analysis: this choice was motivated by the fact that each treatment group does contain the same number of samples.
The permutation test was used to check the statistical significance of the "treatment", "field replicate" and "harvest year" factors, together with the three possible interactions; two replicates for all samples were performed to allow the evaluation of natural variability and interaction effects.
A comparison of the type I sum of squares of a given factor using the true experimental data with the distribution of type I sum of squares obtained from the original data with a permuted order of objects was performed; in this study, the null distributions of the type I sum of squares for each of the factor matrices were generated from 50.000 permutations.
It is important to emphasize that while the objects of a given factor are permuted among the levels, the order of the objects of the other factors is fixed; as an example, the distribution of the null hypothesis for the "treatment" factor is presented in fig. 21:

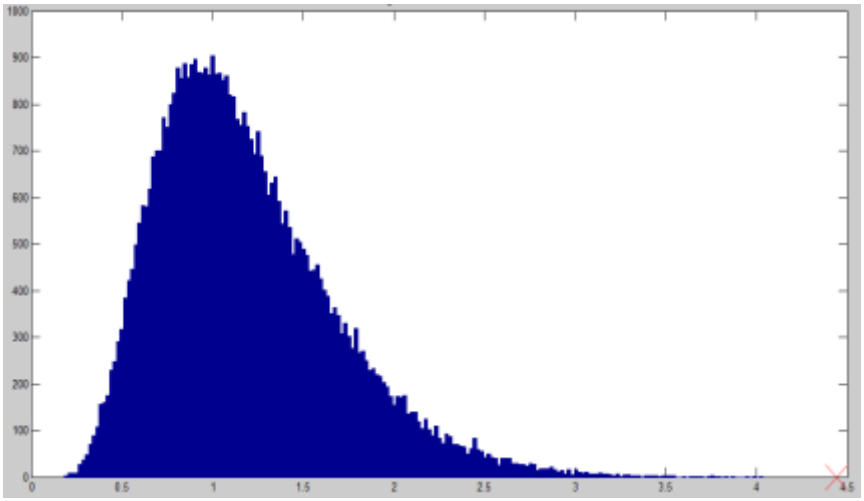

Figure 21. Null hypothesis distribution for the "treatment" factor

From fig. 21, the factor can be claimed as significant because the type I sum of squares (red cross) for the experimental data falls outside the bell-shaped null hypothesis distribution.
Indeed, the null hypothesis (defined as that in which the factor has no effect) is true for the investigated factor when the type I sum of the squares for the experimental data is smaller than the one for permuted data.
The level of significance ($p = 0.05$) is estimated as the proportion of cases in which the type I sum of squares from the distribution generated from the permutations is larger than the experimental type I sum of squares.
In other words, there is no difference in the group means in the original data in comparison to the ones estimated after the random assignment of objects to these groups, i.e. the null hypothesis can be rejected if $p < 0.05$.
Permutations tests gave these outputs, in terms of probability levels: "treatment" ($p < 0.001$), "field replicate" ($p > 0.05$), "harvesting year" ($p < 0.001$), "interactions" ($p > 0.05$), thus only "treatment" and "harvesting year" factors had statistical significant effects on the development of volatiles in apples.
Factors effects can be well verified, when visualizing the distribution of individual samples in the plots spanned by the first two PCs computed by the ASCA model.
The effect of the "treatment" factor is shown in the score plot (fig. 22), where averages of the different modalities of fertilization and surrounding samples dispersions are plotted; PC1 and PC2 explained 53.6% and 42.8% of the data variation, respectively:

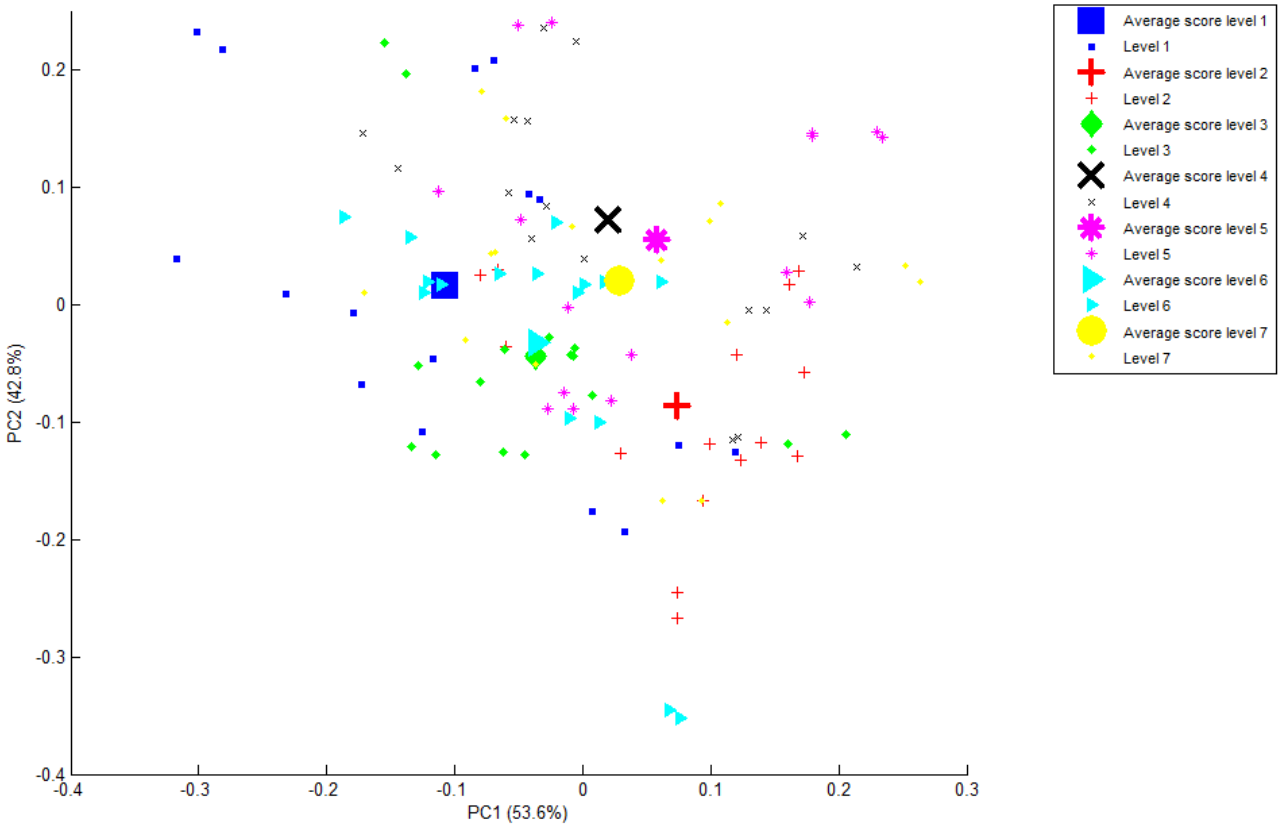

Figure 22. ASCA model: score plot for "treatment" factor

From this plot, it can be seen that along PC1, "control" apples (level *1*) *and* "synthetic fertilized" ones (level *2*) constitute the extremes of the space spanned by the two PCs, thus differing in terms of aroma content from the "organic fertilized" apples.
The different modalities of organic fertilization lie in between these two extremes: more in detail, a cluster is composed by treatments *4*, *5* and *7* (fertilization with *Azocor 105* at lowest and highest rates of N supply, and *Agrobiosol*, respectively), which are quite similar in terms of volatiles developed; the same happened for treatments *3* and *6* (one application of *Azocor 105* at a lowest rate of N supply, and fertilization with *Compost + Biogas slurry*, respectively).
Anyway, treatments *4*, *5* and *7* were more different from the "control", and the "synthetic" fertilization is close to those treatments.
The relative importance of different variables (volatile compounds) in this discrimination was determined by analyzing the loading plot obtained from the ASCA model (fig. 23); variables that contribute mostly to a particular component are associated with large negative or positive coefficients in the loading plot.

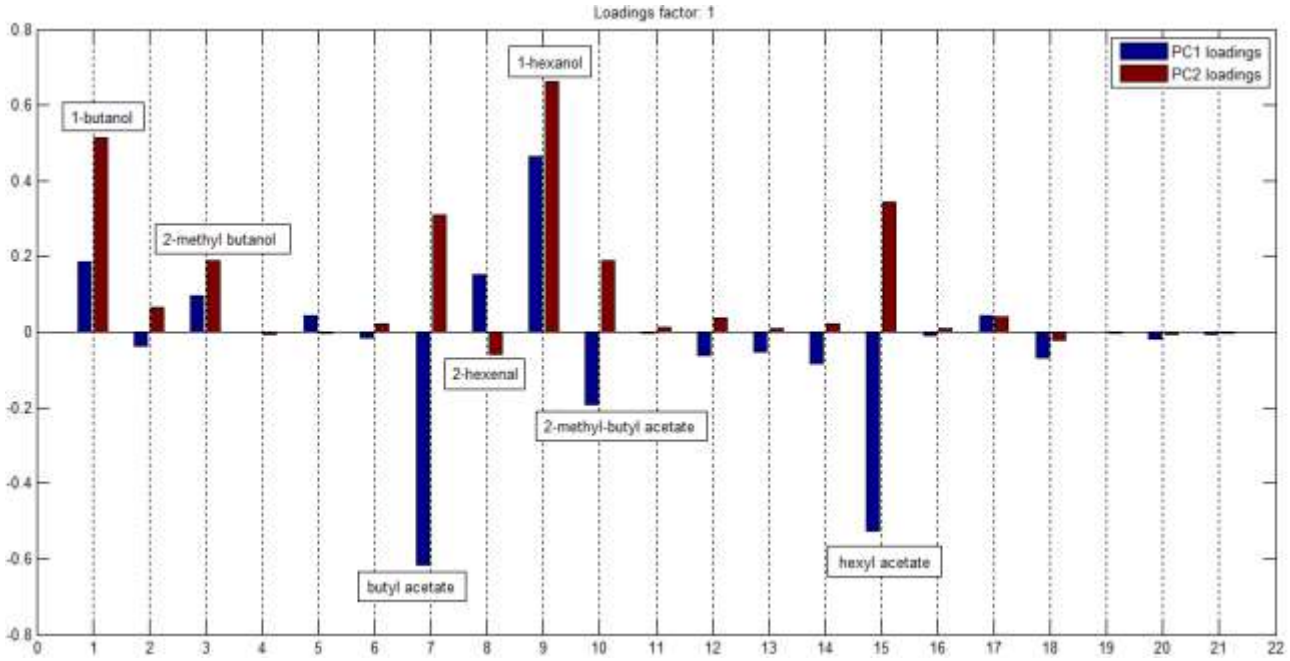

Figure 23. ASCA model: loading plot for "treatment" factor

From this plot, it can be easily seen that "control" fruits, which lie on the negative side of PC1, are characterized by higher levels of the esters butyl acetate, 2-methyl butyl acetate, and hexyl acetate: these are among the major contributors to the pleasant fruity apple flavour.
Treatments *4*, *5* and *7*, lying on the positive side of PC1, are instead characterized by higher levels of the esters precursors' 1-butanol, 2-methyl butanol, and 1-hexanol, and 2-hexenal, which gives a fresh, green aroma to the fruits.
The absence of any N application to the soil in the "control" plot, correspond to a lower level of N in the soil and in turn to a lower concentration of N in the fruits at harvest; hence, this appear to be associated to the relatively high level of butyl, 2-methyl-butyl and hexyl acetate found in the "control" fruits.

The change in the rate of N application, from treatments *4* to *5*, seems to have minor effects on the main acetate formation, whereas the partition of N dose in three applications did not produce significant changes; moreover, these evidences seem to be not dependent on the field replicates.
As already said, "harvest year" had an effect on the flavour developed by fruits: generally, the same compounds were affected.
In some samples from the second year, levels of estragole, which is the character impact compound for fennel flavour, were found to be higher (data not shown): this was confirmed by the sensory panel that perceived a fennel note in those fruits.

5.5 Conclusions

Combining the statistical properties of ANOVA to identify different sources of variation caused by experimental factors with the possibility for analysis of the covariance structure of several variables by SCA, makes it possible to evaluate the effect of fertilization treatment on the flavour developed by apples cv. *Golden Delicious.*

ASCA allowed a better determination of the significant factors through a statistically sounder validation procedure, and an easy visual interpretation of the variability by loading and score plots.

Effects of the fertilization were observed: the level of three of the main apple esters, butyl, 2-methyl-butyl, and hexyl acetate, tended to be higher in fruits from trees that had been subjected to no fertilization.

Organic-fertilized plants tended to be associated with a lower concentration of the same esters, and to a higher level of their alcohol precursors; the use of different organic fertilizers, characterised by different efficiency in N mineralization, seemed to have the potential to significantly affect the formation of aroma compounds in fruits.

An higher rate of N supply for the same organic fertilizer (Azocor 105) seemed to have no effect on volatile compounds development, while changing the number of applications (with constant N supply) might affect flavour in apples.

Of course, chemical and chemometric analysis need to be confirmed by the sensory study (still in progress) for resulting in an effective influence on apple flavour; moreover, new insights on metabolic pathways modifications and relationships can be obtained in a further step.

Chapter 6: Effect of extraction conditions on the organoleptic quality of extra virgin olive oil (EVOO)

6.1 Overview: impact of reduced oxygen atmosphere concentration during malaxation on the flavour quality of extra virgin olive oils obtained by different separation systems

Extra virgin olive oil (EVOO) may be considered a functional food, because of its demonstrated antithrombotic, anti-inflammatory, anti-hypertensive and anti-atherogenic properties: indeed, a regular consumption of olive oil can provide a good protection against oxidative damage, due to the intake of oleic acid and phenolic compounds[192].

EVOO is a major component of the Mediterranean diet, and especially in Italy, Greece and Spain, the consumption of this vegetable fat is significant.

Besides its health benefits, it is well known that olive oil is often preferred by consumers for its palatable taste and pleasant aroma, attributes which are given by minor components like phenols and volatiles[193].

The International Olive Oil Council (IOOC, 2001) and the EEC (EC, 1991) have defined the quality of olive oil, based on parameters that include free fatty acid (FFA) content, peroxide value (PV), UV specific extinction coefficients (K_{232} and K_{270}) and sensory score[194]; the general classification into different commercial grades is based on FFA and also sensory characteristics (taste and aroma).

However, stability to oxidation is an important requirement excluded in the regulation; indeed, some parameters are not included in the IOOC and EC standards, such as phenolic content, even if they are known to have a significant effect on the stability and sensory characteristics of olive oil[195].

The absence of sensory defects in olive oil is necessary for the oil to be classified as "extra virgin", whereas the presence and intensity of sensory defects is used to categorise oils of other qualities, and volatile and phenolic compositions are key determinants of sensory quality of EVOO.

The levels of these substances in the freshly pressed olive oil are dependent on various endogenous enzymatic activities (polyphenoloxydases, peroxydases, and lipoxygenases) that are activated during processing.

In addition, they may undergo further changes during storage[196].

These enzymatic activities are, in turn, critically affected by several technological parameters implicated in the extraction process, which consists of three operations.

In the first step, called milling, olive fruits are crushed to obtain a paste; in the second step, called malaxation, the paste is kneaded to promote coalescence of oil drops; during the final step, the centrifugation, a decanter is used to separate water and oil phases.

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[192] A. Vezzaro, A. Boschetti, R. Dell'Anna, R. Canteri, M. Dimauro, A. Ramina, M. Ferasin, C. Giulivo, B. Ruperti – Analytical and Bioanalytical Chemistry (2011) 399, 2571

[193] C. M. Kalua, M. S. Allen, D. R. Bedgood Jr, A. G. Bishop, P. D. Prenzler, K. Robards - Food Chemistry (2007) 100, 273

[194] D. Boskou - *Olive Oil Chemistry and Technology* - USA: AOCS Press (1996)

[195] E. Monteleone, G. Caporale, A. Carlucci, E. Pagliarini - Journal of the Science of Food and Agriculture(1998) 77, 31

[196] M. Migliorini, M. Mugelli, C. Cherubini, P. Viti, B. Zanoni – Journal of the Science of Food and Agriculture (2006) 86, 2140

Several studies have attempted to identify the effects of reducing the oxidation processes during olive oil extraction by using an inert gas such as nitrogen (N_2) or by changing the malaxation conditions, e.g. by applying different regimes of temperature and duration time[197].

These studies have investigated the role played by oxygen on the development of olive oil aroma components during milling and malaxation, showing only a slight effect of oxygen; nevertheless, systematic studies on this are still lacking[198].

It may be assumed that an optimisation of malaxation/extraction operating conditions may be able to selectively control both sensory and nutritional quality of virgin olive oil, by either inhibiting or activating the above enzymes.

Indeed, it can be said that the control of sensory defects in olive oil is achieved through good management practices, including post-harvest fruit handling procedures that control the exogenous enzyme activity.

An understanding of the pathways that produce the volatile compounds is important in enhancing the quality of olive oil; promotion of certain stages of the lipoxygenase pathway can be used to enhance some desired volatile compounds.

For instance, conditions that promote hydroperoxyde liase (HPL) and inhibit alcohol dehydrogenase (ADH) and alcohol acetyl transferase (AAT) activities can be applied to elevate the "green" aroma; similarly, conditions that promote AAT activity can be applied to enhance the fruity aroma[199].

The aim of this study was to evaluate the effect of reducing oxygen levels in the headspace of sealed malaxation chamber on phenolic and volatile compositions, and on sensory profiles of extra virgin olive oils extracted by means of two different centrifugal decanters, in order to define technological parameters aimed at improving product quality.

[197] M. Servili, A. Taticchi, S. Esposto, S. Urbani, R. Selvaggini, G. F. Montedoro – Journal of Agricultural and Food Chemistry (2008) 56, 10048

[198] A. Sánchez-Ortiz, C. Romero, A. G. Pérez, C. Sanz – Journal of Agricultural and Food Chemistry (2008) 56, 4681

[199] J. J. Salas - Journal of Agricultural and Food Chemistry (2004) 52(10), 3155

6.2 Extra virgin olive oil flavour

Although a complete description of the organoleptic characteristics of olive oil is only obtainable through sensory analysis, the qualitative and quantitative determination of the volatiles can give very useful information on product quality.

The composition of the headspace of an extra virgin olive oil is highly complex: it is composed of over 100 components, most of which are present in very low concentrations, just a few ppm or less.

The aroma of olive oil is attributed to low molecular weight aldehydes, alcohols, esters, hydrocarbons, ketones, furans and, other yet unidentified volatile compounds; the major volatile compounds reported in virgin olive oils are the C_6 and the C_5 volatile compounds, and among them hexanal, (E)-2-hexenal, hexan-1-ol and 3-methylbutan-1-ol are found in most virgin olive oils[200].

Volatile compounds are not produced in significant amounts during fruit growth, but some arise during the last stages of ripening; most of them are formed during fruit milling through the action of enzymes released by tissue disruption and continue to form during malaxation of the olive paste.

Several pathways are involved in their formation, although they are mainly formed by chemical and enzymatic oxidation: through the so called lipoxygenase pathway (LOX), C_6 and the C_5 compounds are produced from polyunsaturated fatty acids (Fig. 24); their final concentrations depend on the level and the activity of each enzyme involved in the LOX pathway.

Initially, 9- and 13-hydroperoxides of linoleic and linolenic acids are formed; these products are then cleaved by very specific hydroperoxide lyases, giving C_6 aldehydes, where unsaturated species can isomerize from (Z)-3 to the more stable (E)-2 form.

Alcohol dehydrogenases can reduce aldehydes to the corresponding alcohols, which in turn can give esters thanks to the activity of alcohol acetyl transferases; in addition, volatiles formed by chemical oxidation are mainly responsible for off-flavours of the oil, such as rancidity.

But an additional branch of the LOX pathway is active when the substrate is LnA: LOX would catalyse, besides the hydroperoxide formation, also its cleavage via an alkoxy radical giving rise to the formation of stabilized 1,3-pentene radicals.

These last can dimerize leading to C_{10} hydrocarbons (known as pentene dimers) or couple with a hydroxyl radical present in the medium producing C_5 alcohols, which can be enzymatically oxidated to corresponding C_5 carbonyl compounds[201].

Although volatile compounds are also formed from amino-acids, the action of amino acids in olive oil volatile generation has been paid little attention: it is established that valine and leucine are converted to volatile compounds, including methyl-branched alkyl and acyl compounds of esters, and into methyl-branched alcohols, which have the potential to adversely affect the sensory quality[202].

With olive oil, relationships between aroma and volatile compounds have emphasised the role of C_5 to C_9 compounds: the most abundant compounds contributing favourably to the aroma of virgin olive oil are the C_6 aldehydes and alcohols, which relate to sweetness.

C_5 aldehydes and alcohols also contribute to the positive attributes of olive oil, providing pungent sensations and correlating with bitterness; small amounts of C_5 ketones, pentene dimers or

[200] G. Flamini - Current Analytical Chemistry (2007) 3, 149

[201] F. Angerosa, L. Camera, N. D'Alessandro, G. Mellerio – Journal of Agricultural and Food Chemistry (1998) 46, 648

[202] R. Tressl, F. Drawert - Journal of Agricultural and Food Chemistry (1973) 21(4), 560

monoterpenes affect the aroma, and most of the smaller ketones, with five to seven carbon atoms, are linked to positive sensory characteristics[203].

The esters are predominantly linked to the positive fruity aroma of olive oil, while carboxylic acids are linked to sour and pungent sensations; chain length also influences flavour perception, and volatile compounds with 7–12 carbon atoms are important contributors to aroma, as the oil ages.

It has also been reported[204] that the main factors that characterise off-flavours are the low abundance of the C_6 aldehydes, C_6 alcohols and esters from the lipoxygenase pathway and the presence of many C_7–C_{12} aldehydes and other volatile compounds with low odour thresholds.

There are volatile compounds that are formed in oxidised olive oil regardless of the external conditions: hexanal, octanal and nonanal are the major compounds in this group; other volatile compounds detected in the late oxidation stages are 2-pentylfuran and 2-ethylfuran, which might be useful in distinguishing oxidation at late stages.

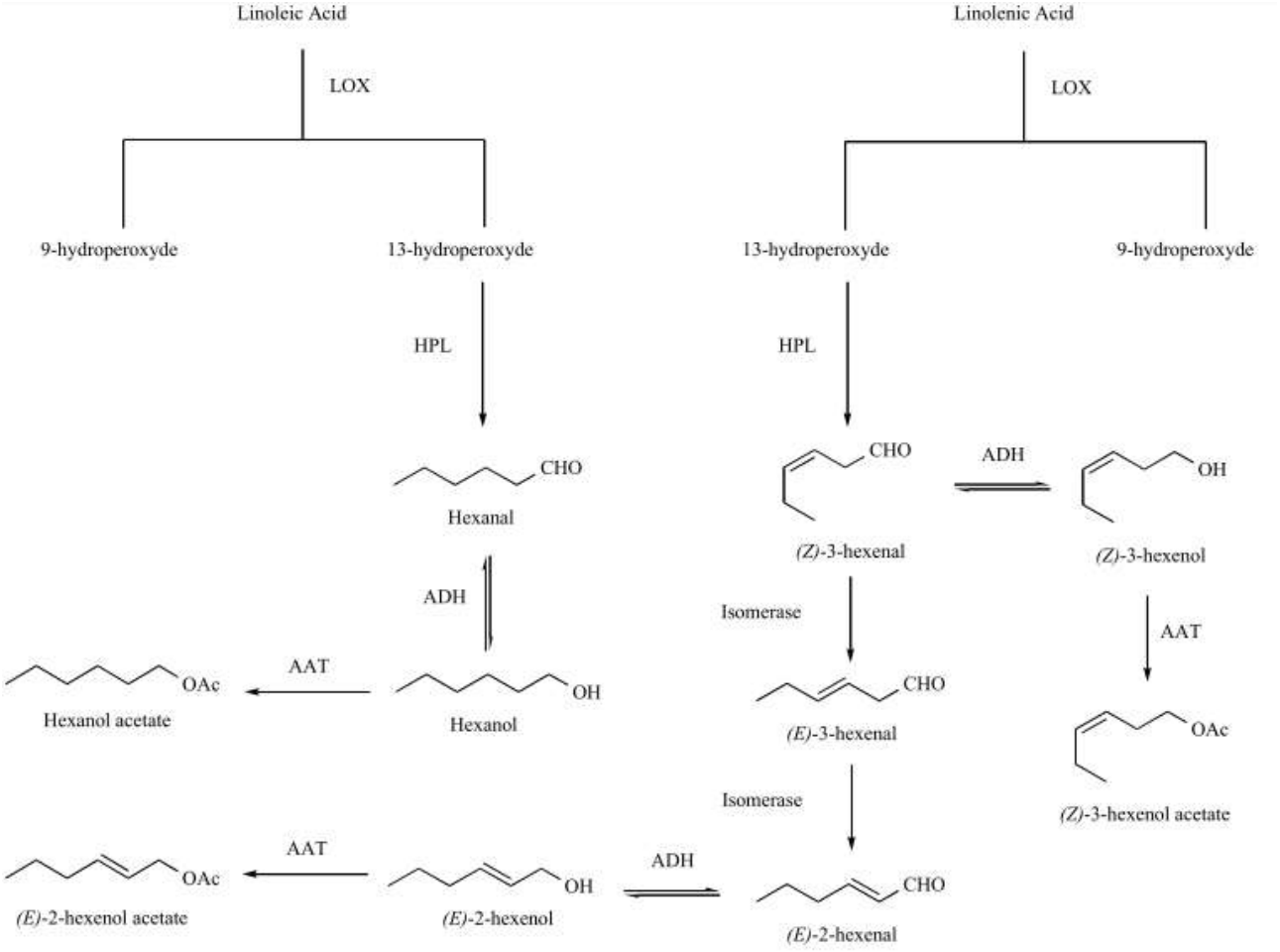


Figure 24. Main volatiles of lipoxygenase pathway (LOX: lipoxygenase; HPL: hydroperoxyde lyase; ADH: alcohol deidrogenase; AAT: alcohol acetyl transferase)
(from Flamini, Curr. Anal. Chem. (2007) 3, 149)

[203] C. M. Kalua, M. S. Allen, D. R. Bedgood Jr, A. G. Bishop, P. D. Prenzler, K. Robards - Food Chemistry (2007) 100, 273
[204] F. Angerosa - European Journal of Lipid Science and Technology (2002) 104(9–10), 639

6.3 Experimental section

6.3.1 Extraction/Malaxation process conditions: Experimental Design

Extra virgin olive oils were obtained from cv. *Carboncella* drupes in two different industrial scale oil mills, the first using for the extraction a centrifugal decanter able to separate the oily phase from the pastes without requiring any addition of warm water ("two phases" process), and the second operating at "two and a half phases" extraction conditions, with water addition.

In both oil mills, malaxation was performed within top-covered chambers, equipped with a valve for N_2 gas entry, in order to obtain reduced oxygen levels (three trials at, respectively, 11%, 6%, 3% O_2 and one control trial with normal atmosphere composition) in the malaxer headspace.

In addition, changes in quality traits were followed throughout 9 months of storage, in sealed dark-glass bottles: analyses were performed as soon as possible after production, and then at the 1st, 3rd, 6th and 9th month of storage.

6.3.2 Volatile compounds analysis

Being a solvent-free sample preparation technique, HSPME implementation was really fast and simple, and it couples well with GC-MS systems; despite the popularity and advantages of the technique, SPME has limitations.

SPME fibers having either absorbent- or adsorbent-coatings were used, even if it is ascertained that the absorbent-type fibers are insufficiently sensitive and thus inadequate for the analysis of olive-oil compounds present at trace levels; consequently, only the adsorbent or at least mixed fibers, such as DVB/CAR/PDMS ones, are suitable for a global analysis of the headspace of olive oils[205].

Adsorbent-type fibers are characterised by their marked ability to capture small quantities of analytes (particularly indicated for the analysis of trace components) but they may be easily saturated and have a shorter linear range compared to absorbent fibers[206].

When adsorbent SPME fibers are utilized, fiber capacity and displacement effects need to be carefully evaluated, because they can prejudice the quantitative determination of compounds: i.e., shorter sampling times may be useful to avoid these drawbacks[207].

Limitations include also difficulties in inter-fiber comparisons, because there is usually good intra-fiber reproducibility on a day-to-day basis, whereas inter-fiber reproducibility is poor especially when different batches are used[208].

Thus, use of several fibers in a study may affect the results, and the possibile lack of comparativeness may limit the use of SPME in long-term experiments that might require change of fibers[209]; the other disadvantage of SPME in comparative long-term studies is the signal drift with the approach of fiber death, due to loss of adsorption capacity[210].

[205] F. Doleschall, K. Recseg, Z. Kemeny, K. Kovart - European Journal of Lipid Science Technology (2003) 105, 333
[206] M. Contini, M. Esti - Food Chemistry (2006) 94, 143
[207] D. D. Roberts, P. Pollien, C. Milo - Journal of Agricultural and Food Chemistry (2000) 48, 2430
[208] P. A. Martos, J. Pawliszyn – Analytical Chemistry (1997) 69, 587
[209] C. M. Kalua, D. R. Bedgood Jr., P. D. Prenzler – Analytica Chimica Acta (2006) 556, 407
[210] M. Steinhaus, H. T. Fritsch, P. Schieberle – Journal of Agricultural and Food Chemistry (2003) 51, 7100

This study considers also the flavour quality during the shelf-life of EVOO, thus fibers performances and stability were tested using a sample kept in freezer at -20°C since the beginning of the experimental work.
From it, aliquots were taken at each time point, and the averages (of 3 replicates) for the major volatiles were compared by the means of a *t* test, to check whether they were equal or not along the whole study.
30 g of olive oil, sampled from a sealed bottle, were additioned of 100 µL of internal standard, 4-methyl-2-pentanol (0.2 mg mL^{-1}), and thoroughly stirred for homogenisation.
From this sample, 3 aliquotes of 2 g each were taken, placed in 15 mL vials for SPME, and hermetically closed with a silicon septum.
Each sample was placed in a water bath at 40°C, and a DVB/CAR/PDMS fiber (50/30 µm, 2 cm long from Supelco Ltd., Bellefonte, PA) was maintained for 60 min in the sample headspace.
The volatile compounds were then desorbed for 2 min at 260°C in the gas chromatograph split-splitless injection port, via splitless injection (purge flow 20 mL min^{-1}); before each extraction, fibers were cleaned and conditioned at 270°C, for 10 min with a carrier gas flow of 75 mL min^{-1}.
GC separation was performed by a DB-Wax column (0.25mm x 30m x 0.5mm) on an Agilent 6890 GC 5973N MS system; chromatographic conditions were as follows: temperature program from 40°C (10 min) to 190 at 4°C min^{-1}, and then to 200°C (5 min) at 30°C min^{-1} (total run time of 52.8 min); linear velocity of the He carrier gas was 36 cm s^{-1}.
A mass spectrometer with a quadrupole mass filter was used for detection, and mass spectra were generated in the electronic impact ionization mode at 70 eV; transfer line, source, and quadrupole temperatures were set, respectively, at 220, 230, and 150°C.
A TIC chromatogram is shown in Fig. 25:

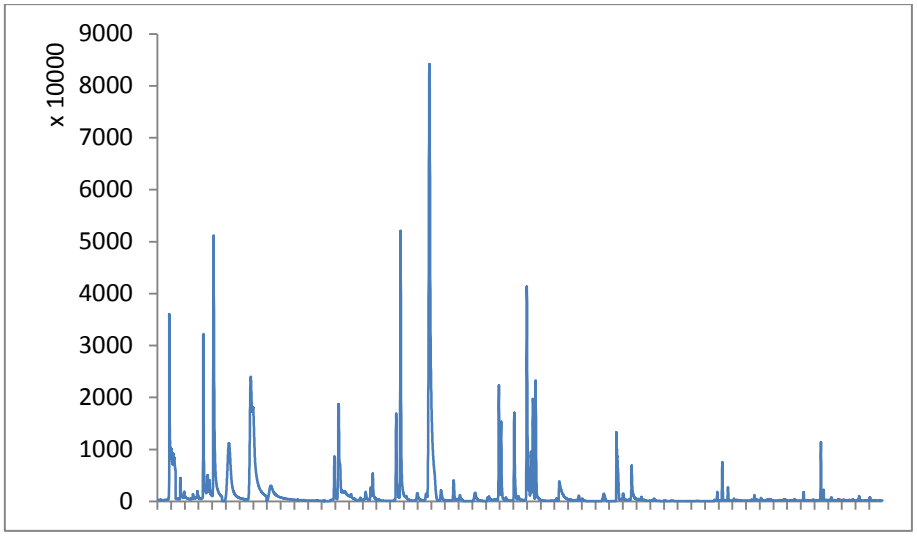


Figure 25. TIC chromatogram of an olive oil sample (total run time = 52.8 min)

Identification of compounds was carried out by comparing mass spectra, obtained by the full scan mode (m/z range 40−400 amu) and Kovats linear retention indices determined on chromatograms of an olive oil sample, with spectra and retention indices obtained from authentic standards.
When authentic standards were not commercially available, compounds were tentatively identified based on a comparison with spectra and retention indices reported in the NIST/EPA/NIH Mass Spectra Library 2005 (tab. 9).

For the relative determination of volatiles, spectrometric detection in the selected ion monitoring (SIM) mode was used: mass fragments (m/z) selected for each detected compound are also reported in tab. 9.

Compound name	identification	*m/z* fragment
pentane	ref. comp.[1]	57
hexane	ref. comp.[1]	57
1,3-pentadiene	tentatively[2]	67
octane	ref. comp.[1]	85
acetone	ref. comp.[1]	58
methyl acetate	tentatively[2]	74
ethyl acetate	ref. comp.[1]	61
2-methyl butanal	tentatively[2]	57
3-methyl butanal	tentatively[2]	58
ethanol	ref. comp.[1]	45
4-methyl-2-pentanol	int. std.	45
3,4-diethyl-1,5-hexadiene	tentatively[2]	69
meso-3,4-diethyl-1,5-hexadiene	tentatively[2]	69
3-ethyl-1,5-octadiene (1)	tentatively[2]	69
1-penten-3-one	ref. comp.[1]	55
3-ethyl-1,5-octadiene (2)	tentatively[2]	69
toluene	ref. comp.[1]	91
decadiene (1)	tentatively[2]	69
decadiene (2)	tentatively[2]	69
hexanal	ref. comp.[1]	56
decadiene (3)	tentatively[2]	69
2-methyl propanol	tentatively[2]	43
3-pentanol	tentatively[2]	59
isoamyl acetate	ref. comp.[1]	70
(Z)-3-hexenal	ref. comp.[1]	69
1-butanol	ref. comp.[1]	56
1-penten-3-ol	ref. comp.[1]	57
1-methyl-3-(hydroxyl-ethyl)-propadiene	tentatively[3]	69
3-methyl butanol	tentatively[2]	56
(E)-2-hexenal	ref. comp.[1]	69
6,10-dimethyl-1-undecene	tentatively[3]	55
o-cimene	tentatively[2]	93
1-pentanol	ref. comp.[1]	55
hexyl acetate	ref. comp.[1]	56
(Z)-3-hexenyl acetate	ref. comp.[1]	67
(Z)-2-pentenol	tentatively[2]	67
6-methyl-5-heptenone	tentatively[2]	108
1-hexanol	ref. comp.[1]	56
(Z)-3-hexenol	ref. comp.[1]	67
nonanal	ref. comp.[1]	57
2,4-hexadienal (1)	tentatively[2]	81
2,4-hexadienal (2)	tentatively[2]	81
(E)-2-hexen-1-ol	tentatively[2]	57
1-octen-3-ol	tentatively[2]	57
2-ethyl-hexanol	tentatively[2]	57
acetic acid	ref. comp.[1]	60
benzaldehyde	ref. comp.[1]	106
5-ethyl-2-furanone	tentatively[2]	83
4-hydroxy-butanoic acid	tentatively[2]	86
5-ethyl-dihydro-2(3H)-furanone	tentatively[2]	98
α-farnesene	ref. comp.[1]	93
unknown furanone	tentatively[3]	83
methyl salicilate	tentatively[2]	120

NOTES: [1] based on reference pure compound; [2] based on comparison with MS spectra reported in the NIST/EPA/NIH Mass Spectra Library 2005; [3] based on comparison with MS data reported in literature

Table 9. List of ions used to detect each volatile in the SIM mode

6.3.3 Phenolic compounds analysis

Determination of phenolic compounds was carried out using the method described by Mulinacci et al.[211] with minor changes, by the co-operating group of Dr. Gianni Pastore.

Target compounds were extracted with a mixture ethanol:water 70:30 (v/v); HPLC separation was carried out with a Kinetex PFP column (100×4.6 mm, 2.6 µm), and a DAD was used for detection at a fixed wavelength of 278 nm.

6.3.4 Sensory analysis

Sensorial profiles of olive oils were defined by a panel composed of 8 panellist with high expertise in the organoleptic evaluation of olive oils, using the approach described by Lawless & Heymann (1998)[212]; tests were executed in triplicate for each sample/panellist combination.

The data matrix, on which chemometric analyses were carried out, was built by keeping the three "overall" replicates: these were computed by averaging sensory scores of the replicates coming from each panellist.

6.3.5 Chemometric analyses

ASCA analyses were performed by using two custom-made routines (developed by: Dr. Federico Marini, Marta Bevilacqua, and Raffaele Vitale – SAPIENZA University / Prof. Gooitzen Zwanenburg - Amsterdam University); PCA and PLS2 analyses were carried out using PLS Toolbox 5.2 (Copyright 1995-2008, Eigenvector Research, Inc.).

Both elaborations were performed in MATLAB 7.5.0.342 (The MathWorks Inc., Natick, MA) environment, considering a significance level of $p < 0.05$.

Analysed data matrices were built by introducing the ratio between the areas of the analytes and the suitable internal standard, respectively.

[211] N. Mulinacci, C. Giaccherini, M. Innocenti, A. Romani, F. F. Vincieri, F. Marotta, A. J. Mattei – Journal of the Science of Food and Agriculture (2005) 85, 662

[212] H. T. Lawless, H. Heymann – *Sensory evaluation of food: Principles and practices* – New York: Chapman & Hall (1998)

6.4 PCA exploratory analysis of sensory data

A first exploratory data analysis on the whole matrix (containing all samples collected during the study) of sensory data was carried out using PCA, after column auto-scaling: four components were chosen according to cross-validation, explaining about 80% of the original variances.

By inspecting the score plot on the first two PCs, it is possible to observe a clear separation of the samples according to the oil mill along the first principal component (fig. 26); anyway, a good separation between the two oil mills can still be observed on PC1 vs. PC3.

Inspection of the related loadings shows that this difference is mainly due to the fruity odour and flavour, prevalent in the "two phases and a half" mill *1*, and the spicy-bitter sensations, prevalent in the "two phases" mill 2 (less significant loadings were removed from the plot for simplicity).

Atmosphere during malaxation was found to be less influent on the overall sensory profile (data not shown); this was confirmed in more detail with the subsequent ASCA model, which found a statistical significant effect of the oil mills on the main organoleptic quality of olive oils.

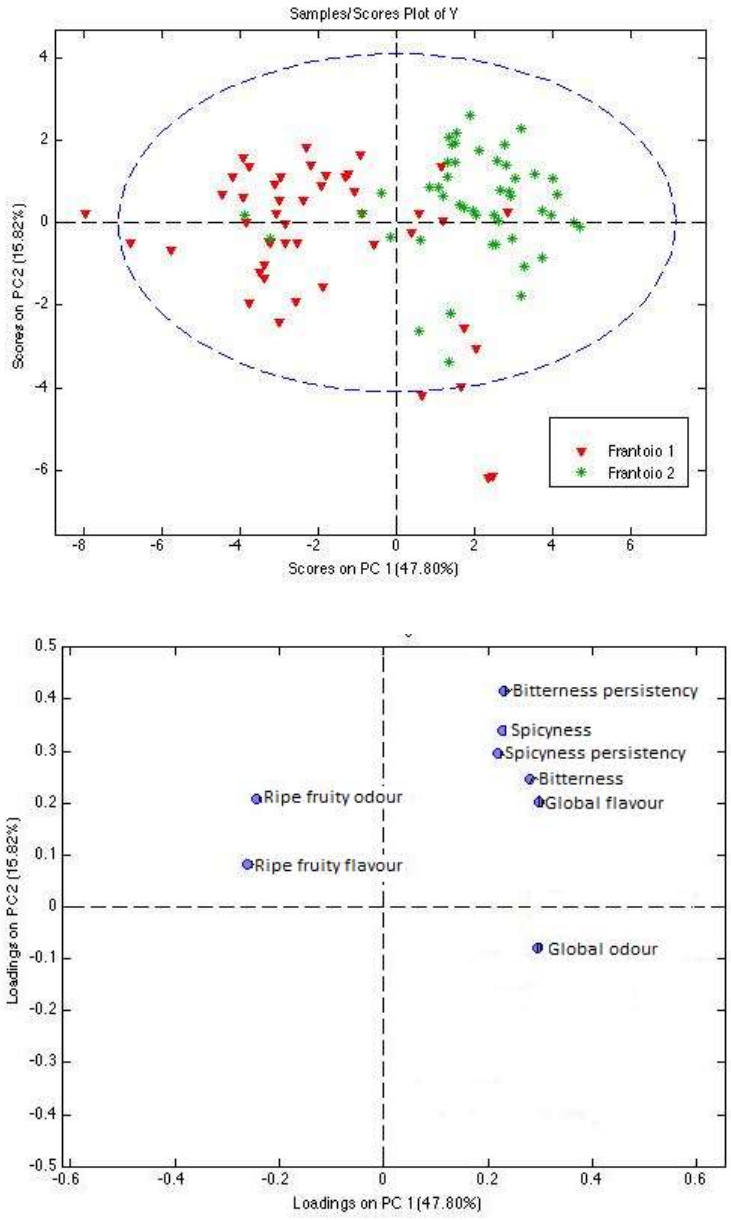


Figure 26. PCA model on the whole sensory dataset: score plot (top) and loading plot (bottom)

6.5 ANOVA combined to Simultaneous Component Analysis (ASCA)

6.5.1 Fresh extra virgin olive oils (t_0)

ASCA analysis of the three datasets (volatiles, phenolic compounds and sensory data) was done assuming the general two-factor ANOVA model with interactions for balanced data, i.e. using the type I sums of squares analysis (level of significance $p = 0.05$), on mean-centered data.

The permutation test (50.000 permutations) was used to check the statistical significance of the "separation system (oil mill)" – factor 1, and "atmosphere during malaxation" – factor 2, together with the interaction.

Three replicates for all samples were performed to allow the evaluation of natural variability and the interaction.

Results from the ASCA model on sensory data show how the separation system exerted a marked effect ($p < 0.001$) on the organoleptic properties, and especially on those associated to the phenolic fraction such as bitterness, spicyness, their persistence, and the astringency (fig. 27):

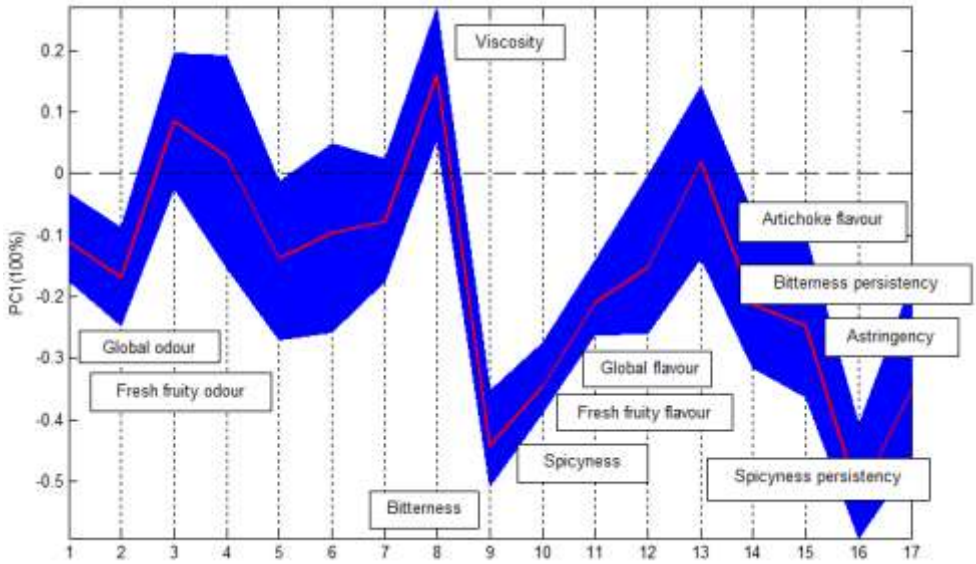


Figure 27. ASCA model on sensory data: loadings on PC1 (with their own confidence interval in blue) for factor 1

A significant effect is expressed also on descriptors associated to the volatiles content of olive oils, such as global odour intensity, fresh fruity odour and flavour, artichoke flavour, and global flavour intensity, which is due to both phenolic and volatiles contents.

The main drivers of the perceived differences in bitterness, spicyness, and astringency (ASCA plots not shown, $p = 0.001$), were the phenolic alcohols tyrosol and hydroxytyrosol[213], and the secoiridoid compounds such as the di-aldehydic form of the oleuropein-aglycone bound to hydroxytyrosol (3,4-DHPEA-EDA) or tyrosol (p-HPEA-EDA), the mono-aldehydic form of the same aglycone bound to hydroxytyrosol (3,4-DHPEA-EA) or tyrosol (p-HPEA-EA).

The tyrosol-derived secoiridoids seems to be the main carriers of the spicyness sensation[214].

[213] M. Esti, M. Contini, E. Moneta, F. Sinesio – *Food Chemistry* (2009) 113, 1095

[214] P. Andrewes, J. L. Busch, T. de Joode, A. Groenewegen, H. Alexandre – Journal of Agricultural and Food Chemistry (2003) 51, 1415

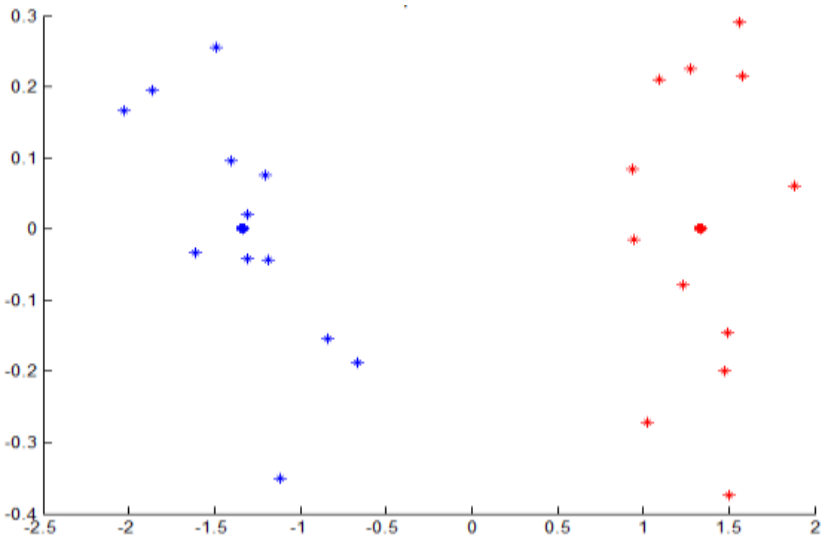


Figure 28. ASCA model on sensory data: score plot for factor 1
("two phases" mill – blue; "two phases and a half" mill – red)

The score plot (factor 1) from the ASCA model on sensory data (fig. 28) shows the perfect separation between the two mills, operating with different extraction methods.
In particular, olive oils produced in the "two phases" mill (negative scores) were more characterized by the "phenolic" sensations, whose loadings were found to be the lowest (fig. 27).
Indeed, this separation system uses no addition of water, thus avoiding washout of phenolic compounds.
Modifications of the oxygen level in the atmosphere during malaxation (factor 2) influenced a less number of descriptors ($p < 0.001$) associated to the content of volatiles, such as artichoke flavour, global odour intensity, fresh and ripe fruity odours, green cut grass odour (fig. 29).
Oxygen concentration during malaxation seemed not to influence phenolic compounds levels ($p = 0.6133$), except for hydroxytyrosol, for which the univariate analysis of variance showed an effect ($p = 0.041$): this could partly explain the differences in astringency, and persistency of spicy-bitter sensations perceived by the panellists (fig. 29).
Looking at the score plot (factor 2), it comes out (fig. 30) that level *1* (highest oxygen concentration) and *2* lied on the negative side of PC1, while level *3* and *4* (lowest oxygen concentration) were not separated, and they lied on the opposite side of PC1.
Hence, decreasing oxygen levels promotes the production of olive oils for which "phenolic sensations" are predominant, while fruity sensation characterized oils produced at higher oxygen concentrations.
Indeed, higher oxygen concentrations might cause depletion of phenolic compounds by oxidation processes, while lipoxygenase pathway, and thus volatiles biosynthesis, are enhanced in these atmosphere conditions of malaxation.
This picture seemed to be coherent to what comes out from PCA analysis (after mean-centering) on sensory data, which confirmed a differentiation of the two oil mills, driven by descriptors associated to the phenolic content, at an higher level when a "two phases" centrifugation system is used, as expected (results not shown).

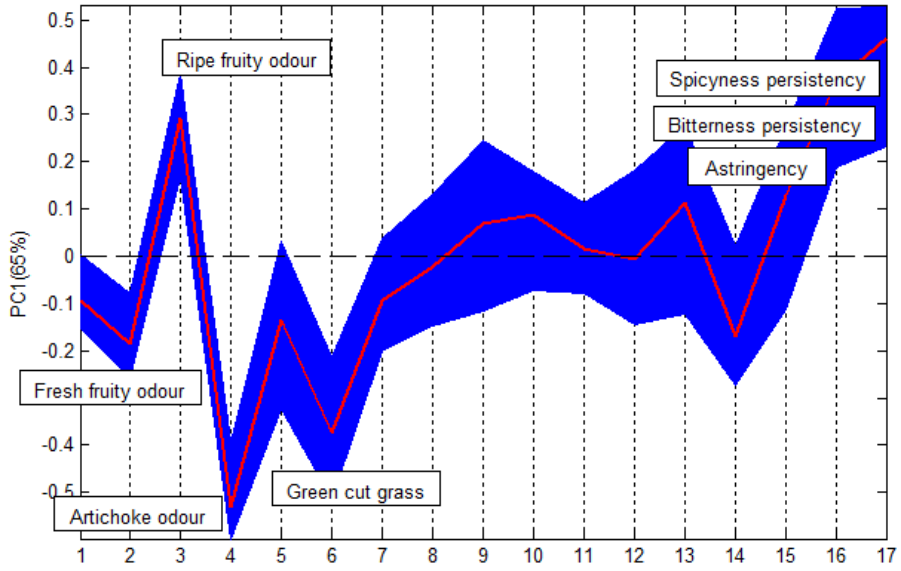


Figure 29. ASCA model on sensory data: loadings on PC1 (with their own confidence interval in blue) for factor 2

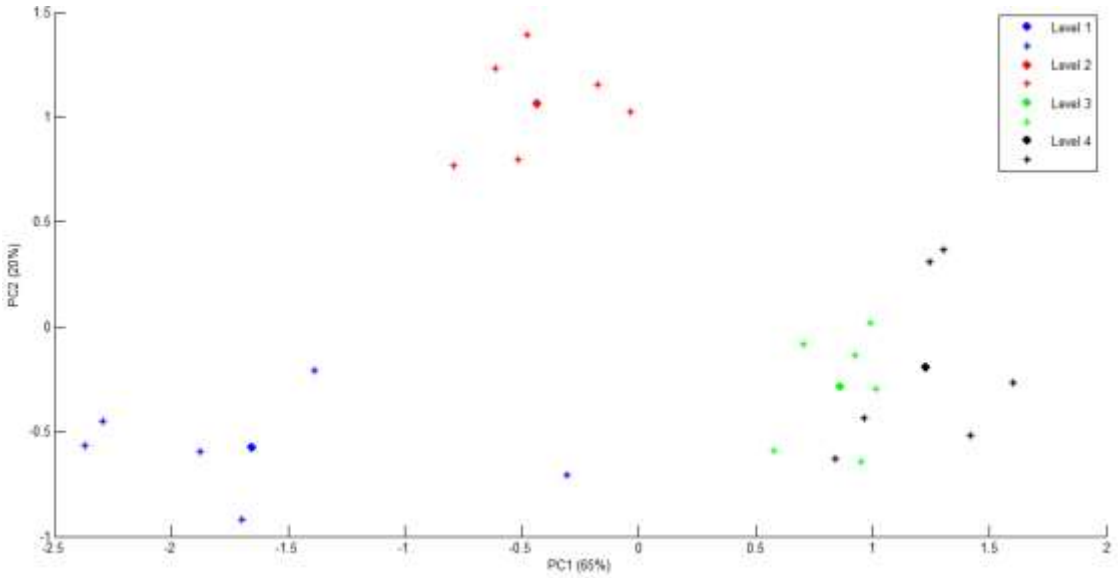


Figure 30. ASCA model on sensory data: score plot for factor 2
(decreasing oxygen concentrations going from level 1 → level 4)

Confirmation of these insights arose from ASCA analysis on volatile compounds dataset.

The obtained ASCA model globally explained how modifications of oxygen levels during malaxation (factor 2) exerted a strong influence ($p < 0.001$) on the "key" aroma compounds of olive oils.

Keeping in examination both ASCA loading plot (fig. 31) and score plot (fig. 32) for factor 2, it is clear that olive oils obtained at higher oxygen levels (*1, 2*) are associated with higher concentrations of C_6 alcohols, C_6 aldehydes, 2,4-hexadienals, 3-hexenyl acetate, penten-3-one, and two furanones, thus differing from olive oils produced at lower oxygen levels *3, 4*.

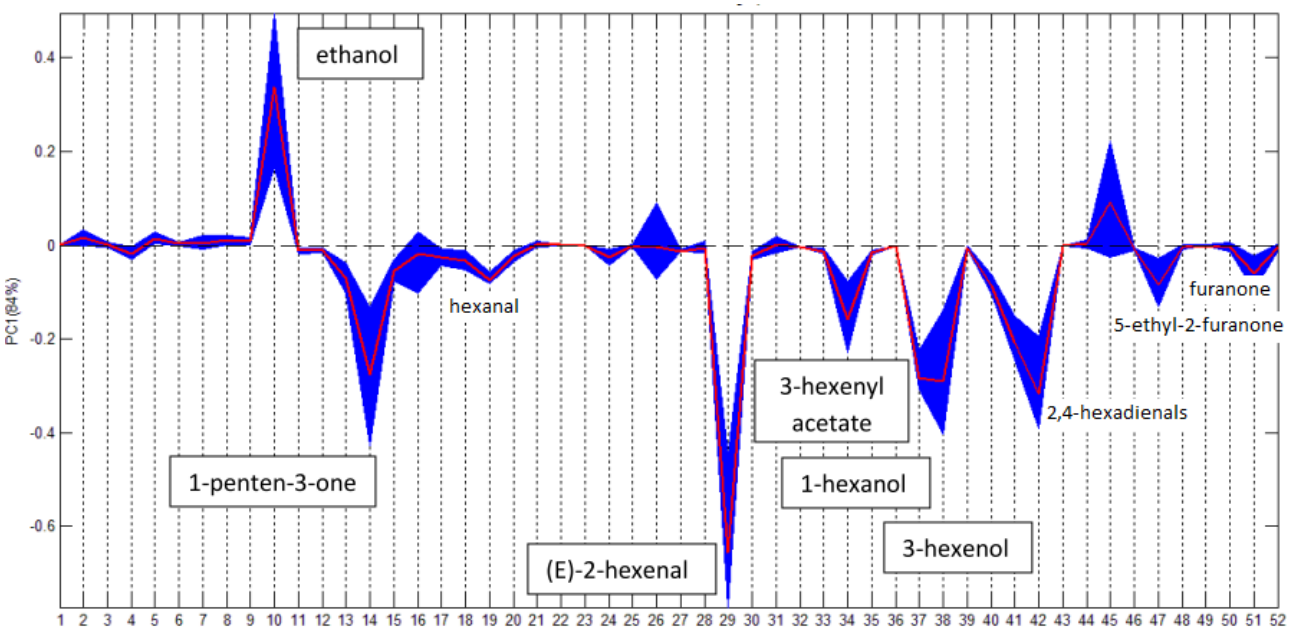


Figure 31. ASCA model on volatiles data: loadings on PC1 (with their own confidence interval in blue) for factor 2

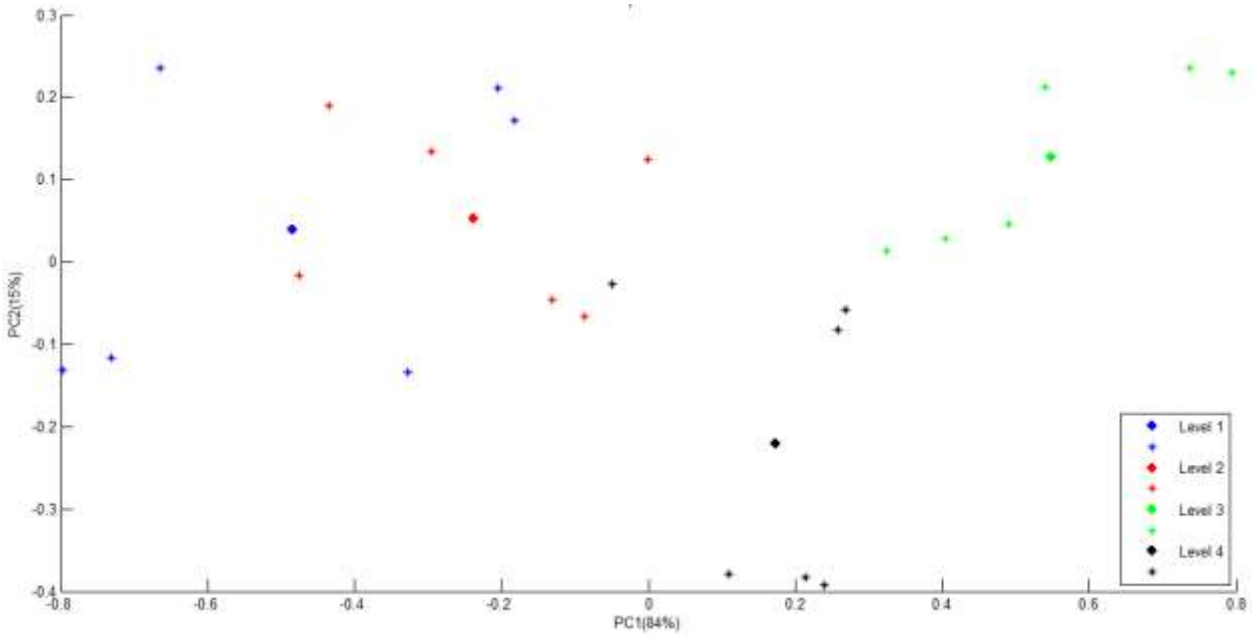


Figure 32. ASCA model on volatiles data: score plot for factor 2
(decreasing oxygen concentrations going from level 1 → level 4)

The centrifugation system (factor 1) seemed to be responsible of a significant effect on a few volatiles, such as ethanol, 3-hexenol and acetic acid (plots not shown, $p < 0.001$).

6.5.2 Shelf-life of olive oils: effect of storage time

Changes in quality traits of olive oils were followed throughout 9 months of storage, in sealed dark-glass bottles; analyses were performed in correspondence of the 3rd, 6th and 9th month of storage.

When PCA analysis was carried out on the whole matrix of the GC-MS volatile profiles, 5 components are chosen as the optimal complexity (again explaining about 80% of the original variance): it is clear that the PC1 is driven by the temporal variation, for both oil mills (t_0 → 9 months, corresponds to increasing scores over PC1), shown in fig. 33:

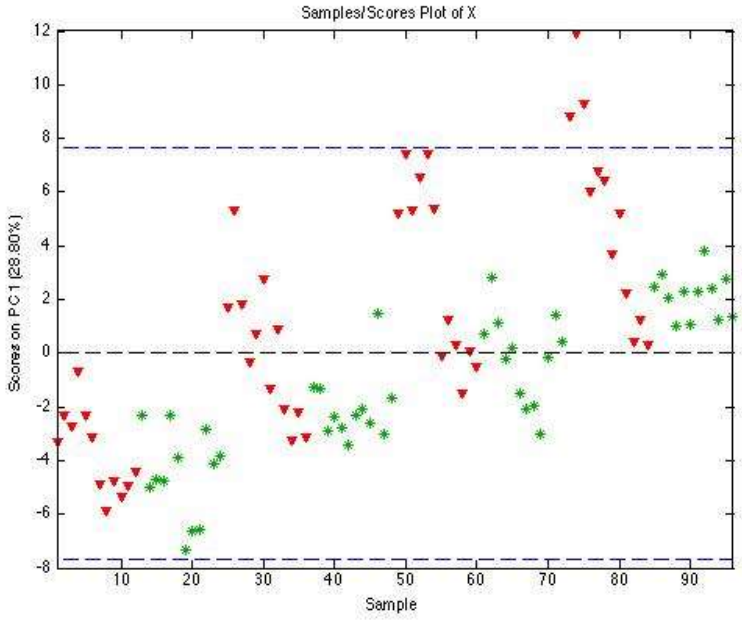


Figure 33. PCA model on the whole aroma data matrix (red: mill 1 – green: mill 2): score plot showing the time trend, from t_0 (left) to the 9th month of storage (right)

The effect of storage on extra virgin olive oil quality was investigated in more detail by ASCA performed on the three datasets, considering three factors: "mill", "atmosphere", "month of storage", and their interactions; related plots are not shown.

Volatile content ($p < 0.001$) seemed to not change until three months of storage, and after this point, a little change occurred, which continued till the 6th month; ASCA on separate datasets for the two mills may confirm this evidence or show different behaviours.

Main contributors to this effect were the increasing levels of octane and hexanal (common oxidation indexes), the two decadienes, 1-hexanol, 3-hexenol, (E)-2-hexen-1-ol, and acetic acid, and the decreasing content in 1-penten-3-one, and (E)-2-hexenal over time.

Phenolic compounds were also affected by storage time ($p < 0.001$): especially the di-aldehydic form of the oleuropein-aglycone bound to hydroxytyrosol (3,4-DHPEA-EDA) decreased over time, while the mono-aldehydic form (3,4-DHPEA-EA) showed increased levels in stored olive oils.

Giving that, also sensory properties of olive oils were expected to change over time ($p < 0.001$).

Indeed, three months of storage were enough to change the organoleptic properties related to most descriptors: stored olive oils received minor scores in the sensory evaluations, which were more characterized respect to fresh olive oils only by the ripe fruity flavour and odour.

6.6 Correlating volatile compounds and sensory properties: a Partial Least Squares Regression (PLSR) approach

Unraveling relationships between volatile profiles and sensory descriptors, thus linking chemical to sensory analysis, is still a tricky goal to achieve at best, in a meaningful and objective view.
In that quest, first attempts were carried out by using Partial Least Squares Regression (PLSR).
Since both X (GC-MS profiles) and Y (sensory descriptors) matrices were multivariate, a PLS2 model with 8 components was chosen, basing this decision on a 10-fold cross-validation (CV) with all replicates kept.
In fig. 34, the loadings for the X- and Y- blocks on the first two latent variables are reported. Analysis of this plot is useful to understand the global correlation among the variables in each block and, at the same time, to highlight the relations among the variables in different blocks.
The presence of clusters of variables within the plot is an index of the fact that many variables in the two blocks are correlated.
In particular, the pairs of variables Y13 (ripe fruity flavour) and Y3 (ripe fruity odour), Y2 (fresh fruity odour) and Y12 (fresh fruity flavour), and Y1 (global odour intensity) and Y14 (artichoke flavour) appear to be highly related to one another.
The same can be affirmed for variables X17 and X18 (two decadienes), for the group of variables X7 (ethyl acetate), X8 (2-methyl butanal), X9 (3-methyl butanal) and X27 (1-methyl-3-(hydroxy-ethyl)-propadiene), for the pair X36/X39 (6-methyl-5-heptenone/nonanal) and for other clusters observed in the plot.
As far as the relationships between the two blocks are concerned, the couple of Y3 (ripe fruity odour) and Y13 (ripe fruity flavour) are positively correlated with the variables 17, 18, 20 (three decadienes), 52 (methyl salicylate), 46 (benzaldehyde), and 30 (6,10-dimethyl-1-undecene) in the X block.
On the other hand, variable Y15 (astringency) is positively correlated to X14 (1-penten-3-one), X7 (ethyl acetate), X8 (2-methyl butanal), X9 (3-methyl butanal) and X27 (1-methyl-3-(hydroxy-ethyl)-propadiene), and negatively correlated to Y3 (ripe fruity odour), Y13 (ripe fruity flavour), and the variables in the X block correlated to them.
Root mean squares errors in cross-validation (RMSECV) were found to be in between 0.3-0.7; by now, only previsions corresponding to lower RMSECVs were analysed in more detail (best models).
In particular, the results for one generic sensory descriptor, clearly associated to the volatile content, i.e. global odour intensity, are shown as an example in the following.

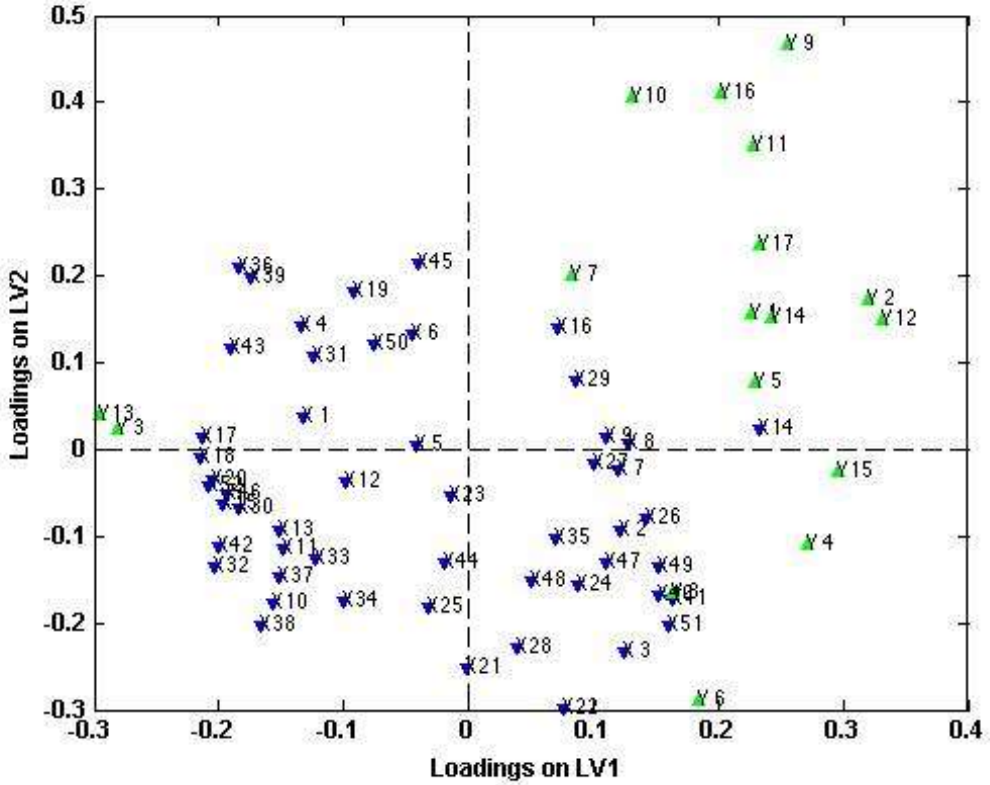


Figure 34. PLS2 model: loadings for both X (blue) and Y (green) blocks for latent variables *1* and *2*

For the global odour intensity, in fig. 35 predicted in CV vs. observed values are reported:

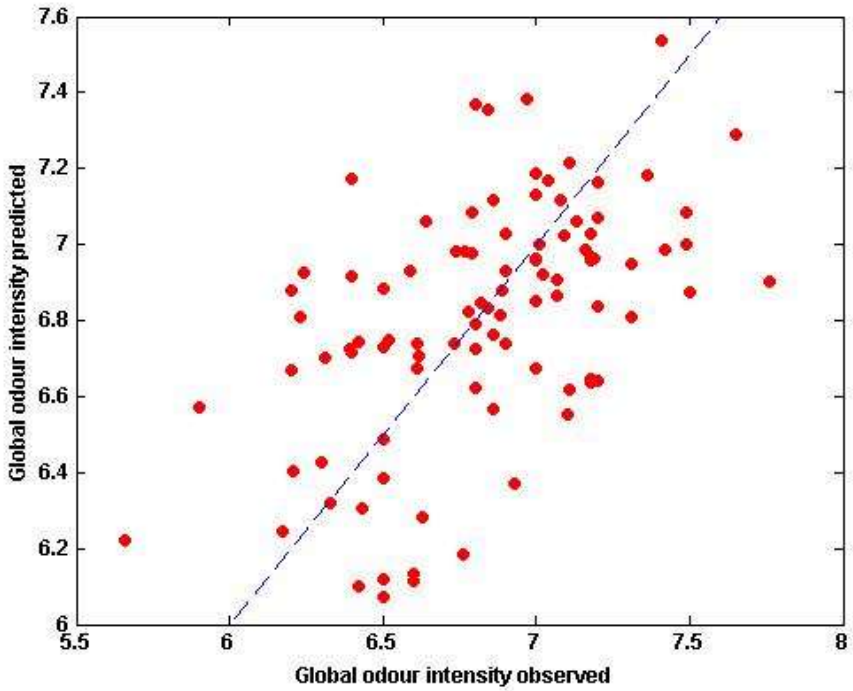


Figure 35. PLS2 model on "global odour intensity" descriptor: predicted in CV vs. observed values

The related VIP score plot (fig. 36) is important to understand which X-variables contribute more to the prediction of the descriptors:

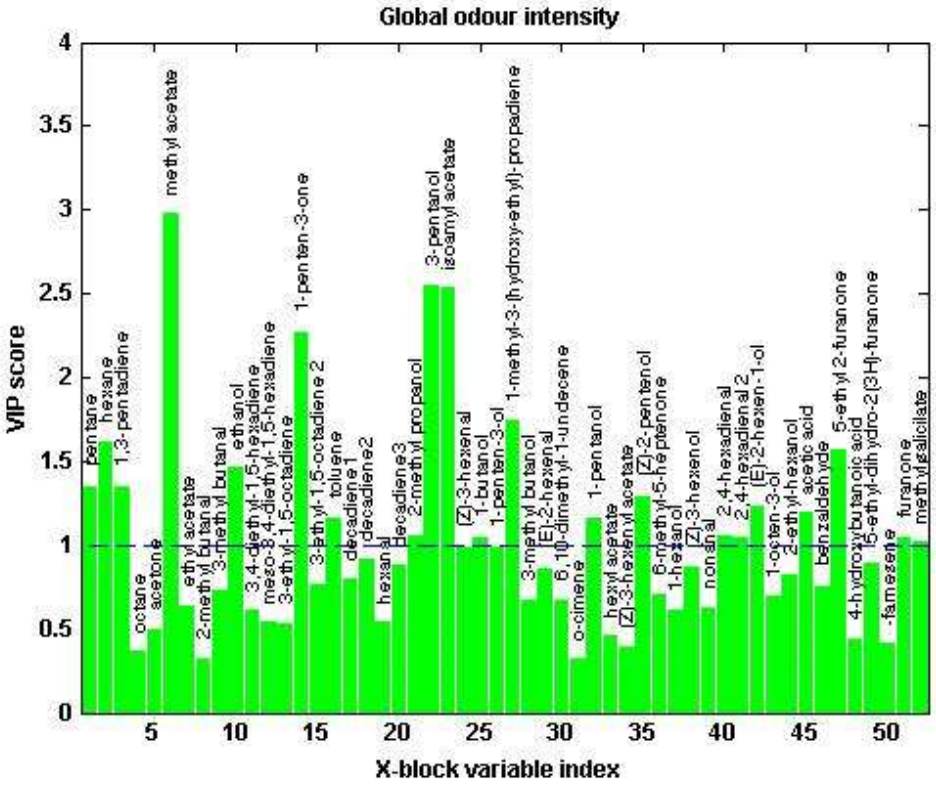


Figure 36. PLS2 model on "global odour intensity" descriptor: VIP score plot

In the previous plot, variables having scores > *1* are effectively correlated to the selected Y-variable: this is the case for the hydrocarbons pentane, hexane, and 1,3-pentadiene, toluene, methyl and isoamyl acetate, ethanol, 2-methyl propanol, 1-penten-3-one, 1- and 3-pentanol, (Z)-2-pentenol, (E)-2-hexen-1-ol, 1-methyl-3-(hydroxyl-ethyl)-propadiene (whose presence in olive oils is seldom reported in literature), the two 2,4-hexadienals, acetic acid, and 5-ethyl-2-furanone.
Regression coefficients are shown in the next plot (fig. 37); important X variables for VIP scores computation are coloured in red:

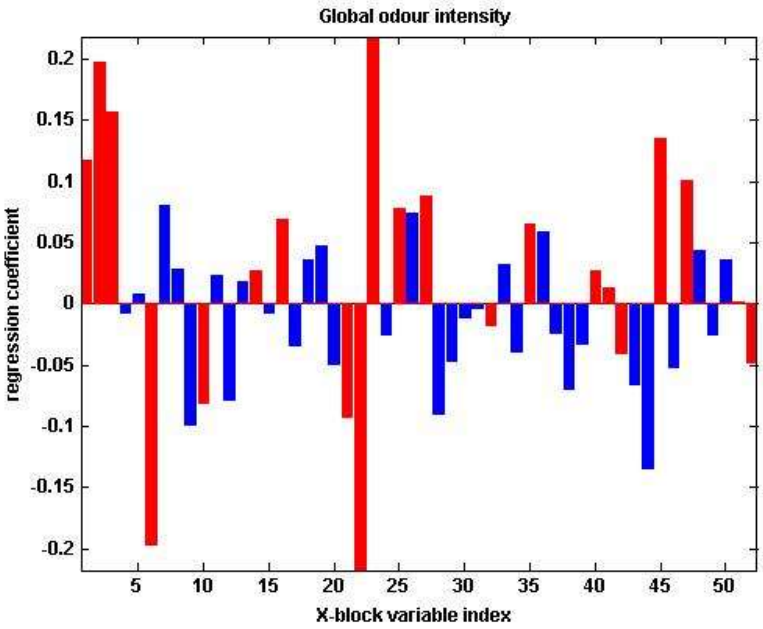


Figure 37. PLS2 model on "global odour intensity" descriptor: regression coefficients for the several X variables

Hence, especially hydrocarbons contribute positively to the global odour intensity, together with isoamyl acetate, acetic acid and 5-ethyl-2-furanone; a negative contribute was brought by methyl acetate, 2-methyl propanol, and 3-pentanol.

6.7 Conclusions

The use of a “two phases” mill had a relevant impact on the phenolic fraction and thus on the related sensory descriptors: olive oil produced were perceived to be bitter, spicy and astringent, more than those produced in the old-fashioned “two phases and a half” mill.

Modification of the atmosphere during malaxation seemed to be able to modulate mainly the formation of volatile compounds and their sensory contribution.

The reduction of oxygen levels weakened the perceived global odour, the artichoke odour, the green and fresh fruity sensations, by partially inhibiting the formation of several “key” aroma compounds of extra virgin olive oils.

Given the multivariate nature of collected data, ASCA was thus proved to be a powerful chemometric tool, which gave a coherent picture on the phenomena under study.

It allowed separation of the different effects, their individual significance testing, and the most contributing variables; moreover, easy visualization and interpretation were achieved by the generated loading and score plots.

Studying the underlying effects on metabolic pathways, and the relations between sensory notes and compounds determined, will be the first aims in the next future.

In our advice, all the insight collected will be useful to obtain extra virgin olive oils with the desired organoleptic quality.

Moreover, the deep investigation on the effect of storage on olive oils, obtained by different processes, may serve to understand which combination of production methods gives rise to olive oils whose change over time in organoleptic quality is limited.

Hopefully, in a further step improved PLS regression models might help unravelling correlations between volatile compounds and sensory descriptors.

Chapter 7: Modeling of volatile compounds release from model system emulsions

7.1 Overview: the pursuit for aroma release unraveling

Apart from the absolute quantity of volatile compounds in a food, sensory impressions during eating and consumer preferences are greatly determined by the amount and timing of volatile compounds released from the food matrix.

In foods, volatile compounds are distributed between the lipid and the aqueous phases, following the physical laws of partition.

The strength of hydrogen bonds between water, fat and volatile compounds is an additional parameter influencing liquid–liquid partition coefficients of volatile compounds, and emulsifiers interact with volatiles mainly by hydrophobic forces.

Thus, in a mixture of compounds with different polarities, changing the oil-water ratio vapour will change the volatile release profile with the change depending on the polarity of the volatile compound.

Proteins, polysaccharides, and lipids are known to interact with volatile compounds, and a deep knowledge is important (1) to understand flavor perception, (2) to provide the information necessary to select suitable fat replacers, and (3) to adapt flavouring mixtures optimally to the ingredients used in newly developed products[215].

Indeed, one of the major issues in the food industry is that the unique textural and mouth-feel characteristics of fat are associated with a high hedonic value[216], but overconsumption of pleasurable high-fat foods is a factor contributing to the increase in global obesity.

Therefore, creating low-fat food products with full-fat flavor still remains a considerable challenge.

Moreover, in order to adapt the texture and release of volatile compounds to obtain the desired organoleptic characteristics of the final (low fat) product, understanding of the physicochemical interactions between volatile molecules and the non-volatile constituents of a food matrix is mandatory.

Interactions between non-volatile food ingredients and volatile molecules have been widely studied, and they are influenced mainly by the hydrophobicity ($logK_{o/w}$) and volatility (Henry's law constant, k_H) of the latter[217]; it is also well-known that especially fat can have an effect on the temporal release of volatile compounds and their subsequent perception, acting as an "aroma sink"[218].

As for the presence and type of emulsifiers in fat-water based liquid products, there are contradicting findings showing either suppression or enhancement of volatile release.

Consequently, also with respect to emulsifier-volatile interactions there is need for a better understanding.

[215] M. Brauss, R. Linforth, I. Cayeux, B. Harvey, A. Taylor – Journal of Agricultural and Food Chemistry (1999) 47, 2055

[216] D. Bowen, P. Green, N. Vizenor, C. Vu, P. Kreuter, B. Rolls – Physiology Behaviour (2003) 78(2), 247

[217] A. M. Seuvre, E. Philippe, S. Rochard, A. Voilley - Food Research International (2007) 40(4), 480

[218] K. Weel, A. Boelrijk, J. Burger, M. Jacobs, H. Gruppen, A. Voragen, G. Smit – Journal of Agricultural and Food Chemistry (2004) 52(21), 6572

However, the knowledge of the distribution of volatile compounds between the different phases of the food product at equilibrium, i.e. the thermodynamic component, is not enough to describe the phenomena involved either during release or mastication.
Before being released from the food matrix into the oral cavity prior to their transport to the olfactory receptors and, consequently, sensorial perception, vapour, volatiles have to migrate through several interfaces, so mass transfer at each step in a multi-component system must be taken into account.
The kinetic component (mass transfer) is of utmost importance, and several physiological (mastication, swallowing, breath, saliva dilution, etc.) and biochemical (enzymatic and biological reactions) processes occur during food consumption.
Real time techniques such as atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and more recently proton transfer reaction – mass spectrometry (PTR-MS)[219] have been applied to measure both *headspace* (*in-vitro*) and *in-vivo* volatile release from foods.
In-vivo methods allow the most accurate indication of food interaction with the human olfactory system, because perception of a food is not a static experience during the course of eating: indeed, it is based on initial impact, perception during chewing, and the perception of residual volatiles (dynamics).
In order to measure these dynamics in real-time, fast analysis without losing analytical performances is required: indeed, this is possible with PTR–MS as it shows a typical time resolution of less than 0.1 s per compound.
Hence, in this study PTR-MS and a robust mixture design were used to investigate relationships between fat and emulsifier (whey protein) levels, *in-vitro* (for the thermodynamic contribution) and *in-vivo* (for the kinetic contribution) volatile release from model system emulsions at different compositions.
The main objective of this study was to use the mixture design approach with subsequent graphical and statistical analysis of PTR-MS data, to model and predict volatile release from model systems with changing fat and emulsifier levels.

[219] C. Siefarth, O. Tyapkova, J. Beauchamp, U. Schweiggert, A. Buettner, S. Bader - Food Research International (2011) 44, 3202

7.2 Experimental section

7.2.1 Samples preparation

Volatile release from complex food matrices is difficult to predict: thus, interactions between molecules and food ingredients are often investigated in simplified binary and ternary model systems (e.g., water, fat, carbohydrates) to characterize specific release mechanisms[220].

Emulsified systems are dispersions of droplets of one liquid (i.e. butter fat) in another liquid with which it is immiscible (i.e. water): milk, mayonnaise and ice cream are examples of food emulsions.

Whey protein isolate (WPI; Bipro, The Netherlands), used as emulsifier, consisted (based on dry weight) of β-lactoglobulin (74%), alpha-lactalbumin (12.5%), bovine serum albumin (5.5%), and globulins (5.5%).

Butter fat (anhydrous; Viv Buisman B.V. The Netherlands) was purchased; sucrose was used at a constant concentration, to impart a pleasant taste to the samples (with regard to the in-vivo analysis, see below).

Sample compositions were established by following a mixture (optimal) experimental design[221] (tab. 10), choosing reasonable lower and upper constraints for the *%fat* and *%WPI* (*%water* and fixed *%sugar* were the complement to 100%): first, suitable amounts of sugar and WPI were weighted and dissolved in the pre-defined amount of water (tap water) under stirring, while slowly melting the right quantity of butter milk in a microwave.

When complete dissolution was achieved, melted butter was added to the water-sugar-WPI solution.

Exp. N°	Sugar (%)	Fat (%)	WPI (%)	Water (%)
1	3,0	0,0	1,6	95,4
2	3,0	38,8	1,6	56,6
3	3,0	0,0	2,5	94,5
4	3,0	38,8	2,5	55,7
5	3,0	0,0	2,0	95,0
6	3,0	38,8	2,0	56,2
7	3,0	19,4	1,6	76,0
8	3,0	19,4	2,5	75,1
9	3,0	9,7	1,7	85,6
10	3,0	9,7	2,3	85,0
11	3,0	29,1	1,7	66,2
12	3,0	29,1	2,3	65,6
13	3,0	19,4	2,0	75,6
14	3,0	19,4	2,0	75,6
15	3,0	19,4	2,0	75,6

Table 10. Samples composition established by the optimal design

Pre-homogenization of the two phases (water-sugar-WPI and melted milk fat) was performed by Ultra-Turrax stirring; this was followed by two-stage homogenization at 50 bar (1st stage) and 200

[220] C. Siefarth, O. Tyapkova, J. Beauchamp, U. Schweiggert, A. Buettner, S. Bader - Food Chemistry (2011) 129, 1462

[221] G. F. Piepel – Journal of Quality Technology (1988) 20, 125

bar (2^{nd} stage): homogenizer was pre-heated to 65 °C with hot water, and the pre-emulsion warmed up at the same temperature (to avoid solidification of the milk fat.
650 mL of each sample (fat range: 0 – 38,8%; WPI range: 1,6 – 2,5%) were prepared: the total quantity was divided in such a way to be able to run headspace and in-vivo experiments; emulsion did not undergo phase separation before running experiments.
Sauter droplets mean diameter, $D_{(3,2)}$, were measured by laser diffraction, and no differences were found among non-zero fat samples via ANOVA analysis.
Volatiles commonly found in foods and beverages (ethyl propanoate, ethyl-2-methyl butanoate, ethyl hexanoate, I-2-hexen-1-ol, 1-hexanol, D-limonene) were selected and purchased from Sigma-Aldrich.
They presented a broad range of physicochemical parameters, such as $logK_{o/w}$, boiling point (*bp*), and vapour pressure (tab. 11):

COMPOUND	MW	m/z	$logK_{o/w}$	bp (°C)	vapor pressure at 25°C (mm Hg)
ethyl propanoate	102	75	1,21	99	35,9
€-2-hexenol	100	83	1,76	159	0,87
hexanol	102	85	1,86	157	0,95
ethyl 2-methyl butanoate	130	131	2,12	132	7,85
ethyl hexanoate	144	145	2,83	167	1,66
D-limonene	136	137	4,45	176	1,54

Table 11. Physico-chemical properties of the volatiles under study

Volatile compounds were dissolved in commercial coffee cream serving as food-grade solvent (4% of fat), to have a final food grade 10 mL stock solution, by adding: 200 mg each of ethyl propanoate, benzaldehyde, ethyl-2-methyl butanoate and ethyl hexanoate (20 mg/mL); 600 mg of I-2-hexen-1-ol and D-limonene (60 mg/mL); 400 mg of 1-hexanol (40 mg/mL).
The coffee cream was previously tested for the absence of volatile compounds with the same m/z as the volatiles under study.
A literature study was conducted to verify that there was no concentration-effect whether the the volatile compounds were added before or after homogenization to the samples.
For *dynamic-headspace* and *in-vivo* experiments, 400 µL of the stock solution were added to 400 g of each sample; for the zero-fat headspace samples, an 8-times lower concentration (50 µL in 400 g sample) was used.
Like this, depletion of precursor ions ($[H_3O^+]$), and thus the loss of signal linearity, were avoided as volatile release was much higher in these samples.
Final samples (except for the zero-fat headspace samples) had the following concentrations of volatile compounds: ethyl propanoate (20 ppm), ethyl-2-methyl butanoate (20 ppm), ethyl hexanoate (20 ppm), I-2-hexen-1-ol (60 ppm), 1-hexanol (40 ppm), limonene (60 ppm).
After the addition of volatile compounds, samples were left under agitation in a "rolling-machine" overnight at room temperature, and PTR-MS measurements were performed the day after.
To reduce the experimental error, in-vivo and headspace measurements were conducted with the same batch of samples. Samples were analysed in triplicate.
To achieve this, only 5 samples were prepared at a time and all experiments were conducted on the same day.

7.2.2 Proton transfer reaction – mass spectrometer settings

Volatile release was measured using a high-sensitivity PTR-MS equipped with a quadrupole MS detector (IONICON Analytik GmbH, Innsbruck, Austria): this technique is based on gas-phase chemical ionization with H_3O^+ as the primary reactant ion.

Primary reactant ions are generated from water vapour and passed into a drift tube, together with volatile compounds which may undergo a proton transfer reaction, producing a detectable RH^+ ion. Normal air components do not react; however, proton transfer occurs readily for compounds with a proton affinity greater than that of H_2O; this is the case for most organic volatile compounds commonly present in foods and beverages.

Moreover, most volatiles undergo ionization with minimal fragmentation, thus usually molecular ions (M+1) can be easily measured in real time and at a ppb level: these features and the fast response, make PTR-MS ideal for measurement of volatiles, and especially for in-vivo breath measurements.

15 mL/min of sampled air were drawn into the reaction chamber of the PTR-MS; the transfer tubing and the inlet were held at 120°C to avoid condensation of the less volatile compounds, while the reaction chamber was kept at 60°C (2,12 mbar), and the drift tube voltage set at 600 V.

PTR-MS was set in the multiple ion detection (MID) mode, and the following ions were measured: m/z 21 (protonated water isotope (H_3O^{18+}) to check [H_3O^+] constancy; m/z 59 (acetone) to follow panellists breathing pattern; ions listed in tab. 11 for monitoring volatiles release, assuring that ion signals were unique for each volatile under study.

All volatiles were measured with a dwell time of 50 ms (besides H_3O^+ with a dwell time of 100 ms), so the instrument measured the selected m/z signals every 500 ms, that is, two scans per second. Periodically, the water cluster ($H_3O^+*H_2O$ – m/z 37) and oxygen^{34} ($O^{16}O^{18+}$ - m/z 34) were monitored and kept both below 3% of the total ambient air signal, to have reproducible reaction conditions in the PTR-MS reaction chamber.

7.2.3 PTR-MS dynamic-headspace measurement

5 mL of each sample were put in a 20 mL vial and closed hermetically, and allowed to equilibrate for 90 min at 37°C (to mimic body temperature) in a water-bath; then, the sample's headspace was drawn through into PTR-MS inlet tubing at a flow rate of 100 mL/min for immediate ionization and MS-analysis.

7.2.4 PTR-MS in-vivo measurement

Each 112panellist (n=6) tasted 10 mL of each sample, using a syringe to load the mouth.

PTR-MS sampling of exhaled air (inlet flow: 400 mL/min) was performed during oral processing using a strict breathing protocol (pre-swallow phase: 3 breathing cycles – 3 sec in, 3 sec out; post-swallow phase: 10 breathing cycles – 3 sec in, 3 sec out), to standardize panellists behaviour.

Samples were tested in a randomized order (randomized over subject and sample) with using panellist in an alternating fashion, to avoid volatile-carry-over between samples.

For the same reason, panellists were asked to clean their mouth with plain crackers and tap water.

7.2.5 Data processing and statistical analysis

Volatile concentrations measured by the PTR-MS were scaled by dividing raw data by the concentration of volatiles added to the samples to obtain unit concentration scaled data, on which statistical analysis was performed.

After a background subtraction, *Imax* (the maximum intensity of the PTR-MS headspace ion/signal) extrapolation, and *AUC* (the area under the PTR-MS in-vivo ion/signal) were performed on PTR-MS data, using *MATLAB 7.5.0.342 2007b (The Matworks, Inc.) BioInformatic Toolbox 3.0*, and no smoothing treatment was applied to the ion signals.

Mixture Design experimental planning and subsequent statistical analysis were performed using *STATISTICA 7* (*Copyright StatSoft, Inc*. 1984-2004), at a significance level $p \leq 0.05$.

7.3 Mixture Design

7.3.1 Introduction

The use of a well-designed data collection method can lead to experiments with high success rates: Design of Experiments (DoE) is the best systematic approach to problem solving that could be applied to data collection and statistical analysis.

The main purpose of designing experiments statistically is to ensure that valid results are obtained with minimum effort, time, and resources.

In a mixture design, it is desirable to fit the response variable Y (here: volatile release concentration, expressed as I_{max}) to the experimental values of the independent factors X_i (e.g. fat level, WPI level): thus, finding a suitable approximation for the true functional relationship is required[222].

It is worth mentioning that, compared to a factorial design, in mixture designs data analysis becomes more challenging, because the mixture factors are correlated: this means that the classical multiple linear regression (MLR) method is not directly applicable, and that a special model form is required.

In this study, there were restrictions on the levels of X_i, which limited the feasible space of variables between the lower (L_i) and upper (U_i) constraints[223]: in this case, a simplex design could not be used, and an algorithm based on the optimality criteria was selected to build the design.

Constrained mixture region was delimited by the following ranges of compositions (leaving out sugar content):

$$0 \leq \%fat \leq 40.0$$

$$1.6 \leq \%WPI \leq 2.6$$

$$57.4 \leq \%water \leq 98.4$$

As with all good experimental practice, experiments are conducted randomly, in order to prevent any occurring systematic time trend to become a random unsystematic variation.

It is now useful to advocate the use of four model evaluation tools, for giving guidance of how to formulate the most valid model: the first one is goodness of fit, R^2, which as usual it has to be as close to 1 as possible.

The second tool is analysis of variance, suitable to check the adequacy of the model in terms of a lack of fit test; this test implies that the residual response sum of squares is separated into the components *model error* and *pure error*, and their size compared by an F-test: it should turn out statistically insignificant ($p < 0.05$).

The third tool is the evaluation of the model residuals using a normal probability plot for detecting deviating experiments; finally, the fourth tool is the residual plot, by which it is possible to check whether the residuals are randomly distributed or not.

When the assumption of normality is not verified, data transformation is needed; this step is also essential when the error (residual) is a function of the response magnitude (predicted values), the so called "heteroscedasticity".

[222] L. Eriksson, E. Johansson, C. Wikstrom – Chemometrics and Intelligent Laboratory Systems (1998) 43, 1

[223] Z. Jeirani, B. M. Jan, B. Si Ali, I. M. Noor, S. C. Hwa, W. Saphanuchart – Chemometrics and Intelligent Laboratory Systems (2012) 112, 1

All these diagnostics were executed on the created models, together with an internal validation procedure: for both *in-vitro* and *in-vivo* experiments the three replicates for each sample/volatile were randomly split into two subsets, one comprising two replicates by which models were built, and the one including the excluded replicate, on which the models were validated.
In this final step, goodness of fit of the models was checked by comparing residuals obtained in the building phase with the ones resulting from prediction (data not shown).
It has to be pointed out that it is not the best procedure to use for model validation, because this should be performed on samples not considered in model building: anyway, it is a good compromise when it is not possible to run other experiments, and the design has only a descriptive goal.

7.3.2 Dynamic-headspace (in-vitro) modeling

Data for model building were transformed by using the fourth square root operator, mainly to have uniformly distributed values along the explored numerical range, which led to an improved robustness.
Models of increasing complexity were tested for all volatile release data, and "*full cubic*" models for three-component mixtures (sugar is not considered because the content is constant in all samples) were chosen.
This is shown in tab. 12 for ethyl propanoate; it is clear that fit improves by adding more terms to the model:

Model	ANOVA; Var.:ethyl propanoate HS data set 3 Factor mixture design; Mixture total=100., 45 Runs Sequential fit of models of increasing complexity								
	SS Effect	MS Effect	SS Error	df Error	MS Error	F	p	R-Sqr	R-Sqr Adjusted
Linear	1093799	546899.5	631955.4	42	15046.56	36.34715	0.000000	0.633809	0.616372
Quadratic	357230	119076.7	274725.4	39	7044.24	16.90412	0.000000	0.840809	0.820399
Special Cubic	8143	8143.4	266581.9	38	7015.31	1.16081	0.288090	0.845527	0.821137
Cubic	209426	69808.6	57156.2	35	1633.04	42.74774	0.000000	0.946880	0.896764
Total Adjusted	1725754	39221.7							

Table 12. Statistical parameters of the several models fitted for ethyl propanoate

The canonical form of the full cubic model is:

$$Y = \sum_{i=1}^{3} \beta_i X_i + \sum \sum_{i<j}^{3} \beta_{ij} X_i X_j + \sum \sum_{i<j}^{3} \delta_{ij} X_i X_j (X_i - X_j) + \sum \sum \sum_{i<j<k}^{3} \beta_{ijk} X_i X_j X_k$$

where Y is a predicted response, β_i is a linear coefficient, β_{ij} is a quadratic coefficient, and β_{ijk} is a cubic coefficient, and δ_{ij} is the highest order non-linear blending term.
B_i represents the linear blending contribute, while parameters β_{ij} and β_{ijk} represent either synergistic or antagonistic blending between components, depending on the sign, positive or negative, respectively; finally, one can think of 'δ_{ij}' as a symbol for a difference: it depicts a very unusual

response surface for three components (here: water, fat and WPI), a wave superimposed on the other blending terms.
The reason for this rather unusual model has its origin in the fact that this model is a better descriptor of the data than more common higher order models; in this particular case, asymmetries in the response surface are better described as it was confirmed by the lower lack of fit (data not shown).
All these terms together give the resultant shape of the response, on the basis of their magnitude.
However, the utmost prudence must be given to draw conclusions from this mathematical description of the data, because this level of mathematical detail does not need to have a physical meaning.
In the following, validated regression equations for the headspace data are listed per volatile (with the legend referring to the level of significance); all models were significant at a level $p < 0.001$:

Legend
p < 0.001 p < 0.01 p < 0.05 insignificant

ETHYL PROPANOATE

$Y^{\wedge 0.25}$ = **- 74***FAT + **4239821***WPI – **44***WATER – **6474493***FAT*WPI + **99***FAT*WATER +
+**6503378***WPI*WATER + **4509037***FAT*WPI*WATER + **2239066***FAT*WPI*(FAT-WPI) +
+**59***FAT*WATER*(FAT-WATER) – **2270301***WPI*WATER*(WPI-WATER) $R^2_{adj} = 0.8967$

2-HEXEN-1-OL
$Y^{\wedge 0.25}$ = **- 41***FAT + **1495738***WPI – **16***WATER – **2286903***FAT*WPI + **57***FAT*WATER -
+**2295104***WPI*WATER + **1594609***FAT*WPI*WATER + **793181***FAT*WPI*(FAT-WPI) +
+**31***FAT*WATER*(FAT-WATER) – **801819***WPI*WATER*(WPI-WATER) $R^2_{adj} = 0.9085$

1-HEXANOL
$Y^{\wedge 0.25}$ = **- 28***FAT + **929252***WPI – **10***WATER – **1419728***FAT*WPI + **41***FAT*WATER -
+**1427103***WPI*WATER + **990857***FAT*WPI*WATER + **491674***FAT*WPI*(FAT-WPI) +
+**25***FAT*WATER*(FAT-WATER) – **499488***WPI*WATER*(WPI-WATER) $R^2_{adj} = 0.9772$

ETHYL-2-METHYL BUTANOATE
$Y^{\wedge 0.25}$ = **- 115***FAT + **2596001***WPI – **27***WATER – **3961920***FAT*WPI + **190***FAT*WATER -
+**3984778***WPI*WATER + **2761131***FAT*WPI*WATER + **1369007***FAT*WPI*(FAT-WPI) +
+**116***FAT*WATER*(FAT-WATER) – **1393176***WPI*WATER*(WPI-WATER) $R^2_{adj} = 0.9704$

LIMONENE
$Y^{\wedge 0.25}$ = **- 92***FAT + **1689210***WPI – **24***WATER – **2555322***FAT*WPI + **194***FAT*WATER -
+**2601057***WPI*WATER + **1780966***FAT*WPI*WATER + **865921***FAT*WPI*(FAT-WPI) +
+**129***FAT*WATER*(FAT-WATER) – **915440***WPI*WATER*(WPI-WATER) $R^2_{adj} = 0.9622$

ETHYL HEXANOATE
$Y^{\wedge 0.25}$ = **- 98***FAT + **1561797***WPI – **19***WATER – **2371150***FAT*WPI + **189***FAT*WATER -
+**2401444***WPI*WATER + **1652172***FAT*WPI*WATER + **810131***FAT*WPI*(FAT-WPI) +
+**120***FAT*WATER*(FAT-WATER) – **842664***WPI*WATER*(WPI-WATER) $R^2_{adj} = 0.9689$

The R^2_{adj} values were found to be high (> 0.89) showing that the variances found in all responses were explained well by the models; moreover, the F-statistic on individual model terms indicated that several terms in the equations appeared to have no significance (p>0.05).
In these cases, data can be described with simpler equations (that is: without the non-significant model terms) without losing descriptive and predictive value: however, in order to evaluate the effect of all components, it was decided to use the full equations.
As an example, the normal probability plot obtained from the modeling of limonene release is reported in fig. 38; residuals follow a normal distribution, since they fall along the straight red line:

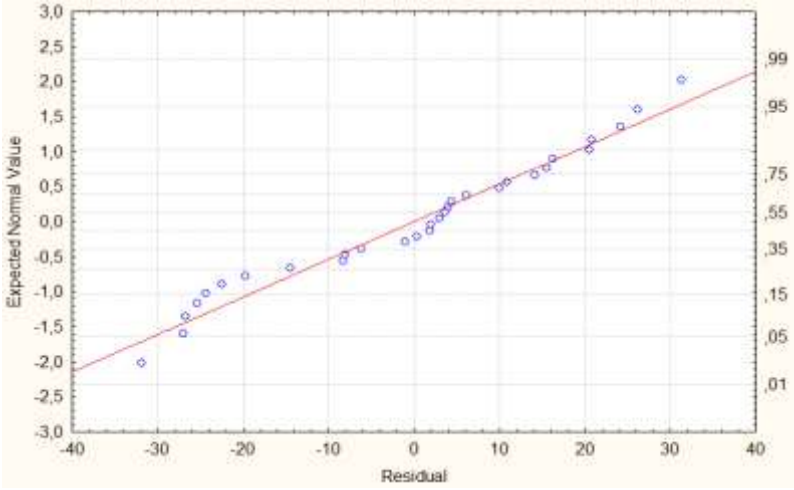


Figure 38. Model for limonene release: normal probability plot of residuals

In fig. 39, the plot of residuals vs. observed values for the same model can be checked; residuals are quite homogeneously distributed on both positive and negative sides of *y-axis*:

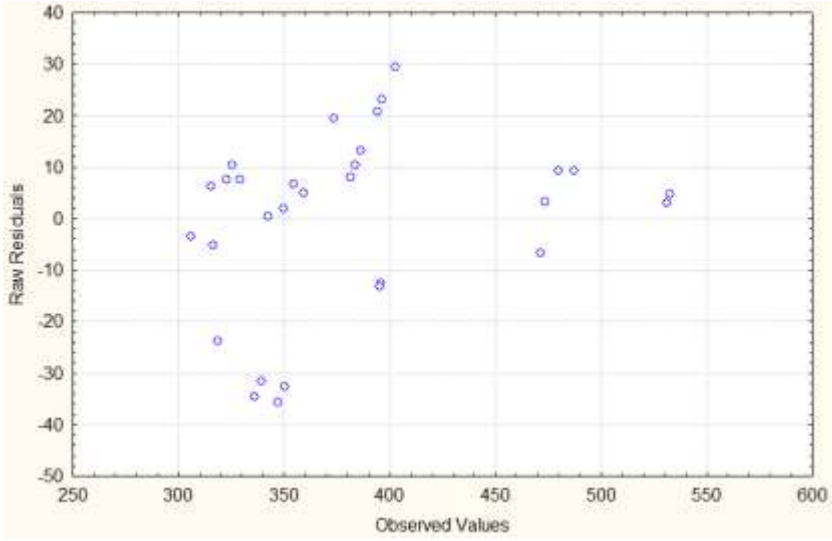
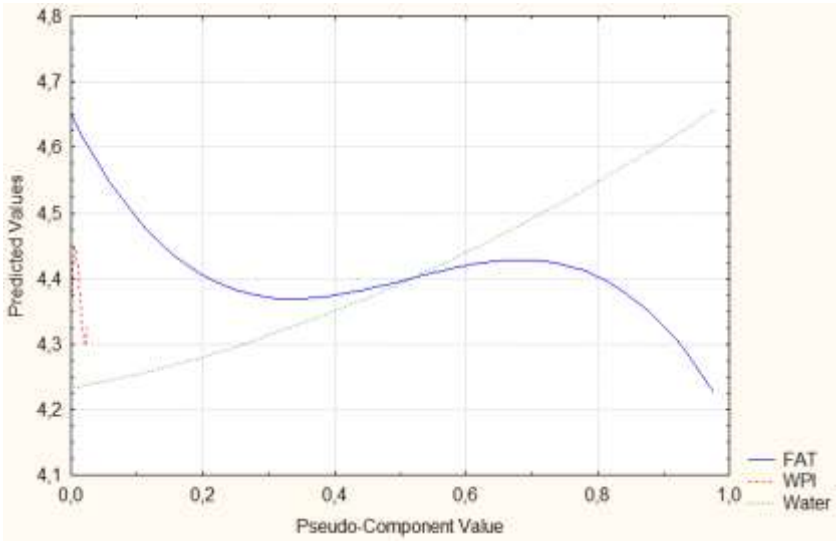


Figure 39. Plot of residuals vs. observed values from the model for limonene

As expected, each of the 6 volatile compounds investigated in this study displayed a different release pattern, which may be attributed to their chemical structure and thus different physicochemical characteristics (tab. 11), in relation to the matrix composition.

As already mentioned earlier, it is not possible to directly relate coefficients to the effects of components, even more when dealing with an unequally constrained region.
Still, it can be concluded that WPI had a significant effect on the release of ethyl propanoate and 1-hexanol: thus, the presence of the emulsifier influenced the headspace concentration of these volatiles, also for its interaction effects with water (WPI*WATER) and with fat (WPI*FAT).
Moreover, for the remaining volatiles, apart from the significant negative effect of fat on volatile headspace release, there is an influence of the interaction between fat and water.
In order to evaluate the contribution of each of the three components, an easier visualization is given by the response trace technique: it measures changes in the estimated response that are brought about by changing the proportion of a single component, while keeping the relative proportions of the other components fixed[224].
In practice, trace plots show the estimated response values as we move away from a reference mixture S (in general, the centroid of the experimental region).
Two examples are given in fig. 40, which shows the response trace plots of the headspace release for ethyl propanoate (top), the most hydrophilic compound, and limonene (bottom), the most hydrophobic one, using as reference mixture the centroid of the constrained domain (fat = 0.2; WPI = 0.021; water = 0.779).
In this figure, the vertical axis is the predicted response and the horizontal axis is the incremental change in each pseudo-component, obtained by rescaling the original factor values on the basis of the established constraints.



[224] A. Kamoun, M. Chaabouni, M. Sergent, R. Phan-Tan-Luu - Chemometrics and Intelligent Laboratory Systems (2002) 63, 69

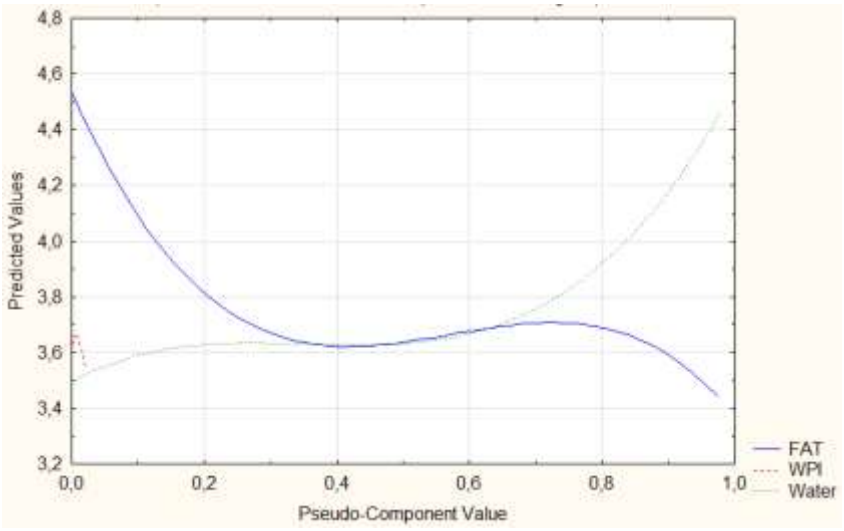


Figure 40. Trace plots for ethyl propanoate (top) and limonene (bottom)

This graph is readily interpreted: for example, the same variation in pseudo-component 'fat' results in a higher depletion of headspace release for limonene if compared to ethyl propanoate; indeed, more hydrophobic volatiles such as limonene are expected to be much stronger affected by a change in fat level than less hydrophobic ones (e.g. ethyl propanoate).
For ethyl propanoate, it is confirmed a small WPI effect on the headspace concentration.

7.3.3 *In-vivo* modeling

There is no correlation at all between *in-vivo* data and headspace data[225], confirming that *in-vivo* volatile release cannot be predicted by experiments in thermodynamic equilibrium conditions; indeed, kinetic factors such as mass transfer, physiological factors and in-mouth conditions play a prominent role on *in-vivo* volatile release.
Modelling the *in-vivo* data set using the data obtained from all six panellists did not result in good models (very low R^2): this can be explained by inter-individual differences in mouth geometries, saliva flow-rates, exhalation volumes, etc.
It was hoped that at least the analysis of PTR-MS data of individual panellists (trying several data pre-treatments) would have led to an improved models quality: unfortunately, this was not the case; indeed, R^2 were still very low (< 0.45).
By looking at these outcomes, it can be said that for *in-vivo* data, mixture design regression doesn't represent a good choice for figuring out the underlying behaviour.

[225] R. Linforth, F. Martin, M. Carey, J. Davidson, A. J. Taylor – Journal of Agricultural and Food Chemistry (2002) 50, 1111

7.4 Discussion and conclusions

Although more complex from a data analysis point of view, using a mixture-design approach allowed building descriptive models to estimate the effects of mixture components water, fat and WPI on *in-vitro* release of volatiles with different physicochemical properties; if statistically validated with a sounder procedure, these models can also be used as a predictive tool.
Conversely, given that attempts on *in-vivo* dataset did not result in good models, a different chemometric tool should be used to get robust insights.
Hence, in the next future *Anova Simultaneous to Component Analysis (ASCA)* will be performed on this dataset, hoping to reach solid conclusions.
Flavours/fragrances used in the food industry can be divided into "top notes", "heart notes", and "base notes" (ranging from high vapour pressure to low vapour pressure).
Assuming that current model emulsions were representative of actual dairy emulsions, models built from these data can be used to get an insight in the desired concentrations of the different notes in emulsion based products for a desired release profile.
It is worth bearing in mind that in real products, there are more ingredients that might influence that volatile release behaviour, but the primary objective of the present work was to "paint a picture" of the situation, which is the first guide in the development of new products in the reality.
Hence, the current approach allows for a prediction to what composition of ingredients is more suitable for a "top note" formula (fast release profile), and what composition is more suitable for a "base note" formula (sustained release profile): this is the specific "tuning" of formulations, towards their desired volatile release behaviour.
Of course, conclusions got from the modeling should be verified by sensory analysis, to see whether the analytical differences can be perceived or not: this could make the chemometric study more fundamental and real.

Chapter 8: Final conclusions

In this Thesis, the development of new analytical methods for volatile (aroma) compounds determination, and the subsequent application of chemometric tools for interpreting the analytical results were carried out, in order to pursue the objectives set at the beginning.
Preliminary results of the whole experimental work are shown: new purposes and deeper insights will be hopefully achieved soon.
The main goal of the research activity was a better understanding of the concept of food quality, focusing the attention on the organoleptic properties, and thus, on aroma compounds.
Headspace Solid Phase Micro-Extraction (HSPME) and Stir Bar Sorptive Extraction (SBSE) were the techniques used for volatiles pre-concentration prior to their chromatographic analysis, utilizing Thermal Desorption-Gas Chromatography-Mass Spectrometry (TD-GC-MS).
HSPME was confirmed to be a well-suited option for the determination of aroma compounds in olive oils; especially SBSE could provide with extremely low detection limits even for the analysis of quite polar and volatile analytes, in complex matrices such as fatty foods (e.g. yogurts), fruits (apples) and vegetables (tomatoes).
Both solventless techniques showed good performances with minimal experimental effort, allowing the determination of a broad range of volatiles through the direction of the Green Chemistry.
Proton Transfer Reaction – Mass Spectrometry (PTR-MS) was the instrumental tool to investigate in-vitro and in-vivo volatiles release from model system emulsions: this is useful to help in understanding flavour perception.
PTR-MS allowed the "real time" monitoring of gaseous species; in fact, it is nowadays the election technique, when fast processes need to be followed.
By this Thesis, as a complementary task, we wanted to highlight the impact of the chemometric "philosophy" on several field covering analytical chemistry, such as exploratory analysis (PCA); investigations on the effect of factors underlying an experimental design (ASCA); Mixture Design planning and related statistical analysis; correlation studies between chemical and sensory properties (PLSR).
Chemometrics allowed an easy visualization and interpretation of complex phenomena, on which multivariate datasets were collected.
Indeed, with modern analytical instrumentations, a huge quantity of variables can be determined on lots of samples: hence, the "multivariate thinking" is nowadays unavoidable, and the use of chemometric tools is the unique solution in this sense.
I truly believe in following this direction, and scientists should look at chemometrics with trustful eyes, without any fear: it's not a matter of complex mathematics, but only of experience and good sense.
I definitely do not want to assert that chemometrics is a "panacea", since a solid chemistry background and good data are always needed; anyway, a beautiful sentence depict at best my persuasion: "*We live in a multivariate world, thus our sight must be multivariate*".