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INVESTIGATIONS ON ODOUR-PATHOLOGY RELATIONSHIP IN HUMANS

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STUDIO DELLE RELAZIONI TRA I COMPOSTI VOLATILI EMESSI DALL'UOMO E LE PATOLOGIE

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Ai miei nonni

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Preface

Life quality has been improved in the last years especially by the technology development. Nonetheless, although mortality is decreasing for certain pathologies, but new diseases are emerging. New pathologies spreading is not so clear, but it is probably due to environment pollution and modifications which are a backward of the industrial development.

Human body can be represented as a complex machine whose internal mechanisms (as for example metabolic processes) are based on a delicate equilibrium. Several alterations of this equilibrium are due to the interactions of human body with the environment and the effects of these alterations may produce diseases. In this context research in medicine develops many effects not only to treat pathologies but also to prevent them. Prevention is the best healthcare methodology to decrease mortality, and to reduce money expenses. Research in this field requires a multidisciplinary approach. The prevention strategy is a new way to look to the patient.

We can define "prevention strategy" as a stream of techniques, methods and instruments for an early diagnosis. The use of non-invasive techniques is the main feature of this strategy. Generally, a technique is considered "non-invasive" when it doesn't cause pain to the patient. For example a pharmaceutical product may be defined as non-invasive if it doesn't produce side effects or a diagnostic methodology is not invasive if its interaction with the human body doesn't need to be cutted or needles in the skin etc. In this work, the volatile compounds surrounding the body and some biological liquids have been analysed. It is known that some pathologies modify a number of metabolic processes and their result in a modification of the chemical composition of headspace of some products as urine, breath, sweat. In

particular the aim of this work is to apply an artificial olfactory system based on gas sensor array to study some pathologies affecting the skin and induced by volatile compounds during the patient transpiration . In this study two pathologies have been investigated, the melanoma obviously directly involved in skin transpiration, and breast cancer which is not properly a skin disease, but from skin transpiration could be revealed. To better clarify these mechanisms in the last part of this work it has been described an experiment in which the growth of some cell lines, related to skin diseases, has been monitored; this experiment has been conducted with an electronic nose and a GC/MS firstly in cell coltures, and then in vivo on mice inoculated with the measured cells.

CHAPTER 1

Olfactory system for medical applications: scientific background and real potentialities

1.1 The importance of odours

In the past a lot of theories about odour were developed, controversies was burned about the transmission mechanisms of odors and about their classification [1].

Plato (ca 427-347 B.C.) considered the odour transmission as a physical movement of odours particles. In the *Timaesus* he states that "*smells always proceed from bodies that are dump, or putrefying, or liquefying, or evaporationg, and are perceptible only in the intermediate state, when water ids changing into air and air into water; and all them are either vapour or mist*." [2].

Aristotle, on the other hand, said that the transmission of odour did not require a movement of any physical matter " *the same account holds also of sound and smell; if the object of either of these senses is in immediate contact with the organ no sensation is produced. In both cases the object sets in movement only what lies between , and this in turn sets the organ in movement….What comes between in the case of sounds is air;the corresponding medium in the case of smell has no name*."[3].

Aristotle's theories influenced also Islamic scholars, such as Avicenna (980-1037) and Averroes (1126-1198). Thus he notes that, although they are more ingenious at smelling than other animals and can discover hidden odours by rubbing odoriferous bodies, humans do not have a strong sense of smell, and odours do not leave strong impressions in the imagination. Hence, for these philosophers the odors can be classified into two types. The first type depends, they said, by the pleasing or displeasing (for example redolent or fetid), while the second type is correlated with of taste names (for example, sweet or acid) [4].The medieval writers agreed with Aristotele that the human olfaction was not more developed than other animals. So they considered the sense of odour a minor sense. Another dispute regarded the way in which the odours were transmitted. For some philosophers a medium was necessary to transport the

odour. In particular it was inferred that the bodies odours arise from the evaporation of fumes. According to this theory Averroes proposed common medium both air and water as odour carries [5].

In the thirteenth century Albertus Magnus, discussing about the theories of Avicenna, accepted the idea that the odours need a medium to travel, but refused the theory of fumes. He started from the fact that, the bodies give off odour when they loose weight and that poisonous odours kill humans and animals. These evidences are inconceivable if only sensory qualities are transmitted[6].

Plato's theory of odour transmission was not generally adopted until well into the modern area. Robert Boyle (1627-1691), for example, believed into corpuscular nature of matter but he had difficulty with many of the phenomena noted in earlier times.

Although the mechanisms of odour transmission were not clear, it was known the importance of odour both for humans and animals, for example to distinguish between a bad or good food.

Regarding the odours released by the human body, for example, it can be inferred that a deviation respect to the "usual ones" can be interpreted as the manifestation of a state of illness. This sort of diagnostic methodology was often used by the physicians in the ancient Greece. Anyway, because of subjectivity of odour perception, some physician should have more sensitivity than other respect to certain pathology identification.

Avicenna himself used his sense of smell for the diagnosis of ailments sniffing the urines of patients. He believed that the changes in the smell of urine could show the presence of the pathology.

During the 17th and 18th century the odour was considered not only as a signal of an illness but also as a remedy to treat the pathology. Some physicians

promoted the use of perfumes to treat infection frequently referred to the therapeutic use of fragrances.

By the early 19th century, the use of fragrances for medicinal purposes was replaced by the chemical medicaments although nowadays some people still use contain aromatic product to treat some pathologies.

In the 20th century the findings about of olfaction mechanisms have contributed to stir up the interest around the odour potentialities, together with the development of novel instruments to detect odorous molecules.

1.2 Human olfactory system

Senses give to the humans the consciousness of the surrounding environment. The mechanisms through which the senses perceive the stimuli coming from outside seem very complicated. Their description can be articulated in different segments.

The first is the contact with the object to identify. The information sources coming from the ambient can be divided in two groups: physical sources and chemical sources. The physical sources are light , sound and body volumes and surfaces, while chemical sources are odours and other volatile compounds. The second is the elaboration of these signals performed through different types of filtering pre-processes. At the end of this elaboration process the resulting signals can be used for classification, storage etc. Light, sound and pressure are physical quantities and their recognition mechanisms are rather clear. On the other hand many studies have to be dedicated to the chemical interaction of the senses because their mechanisms are not still understood. The

large quantities of molecules with their interactions with receptors, and among them are of great interest for the researchers.

Olfactory systems are an ensemble of chemical sensors and their structure and mechanisms appear of a high complexity. Mammalian olfactory system has of course great potentialities among them. The olfactory system of human is less sensitive than the others mammalian animals, but it is able to discriminate large quantities of chemical molecules.

A brief description of natural olfaction (fig. 1.1) system is presented in the following, in order to have a general overview of the basic mechanisms. The odor perception is a result of different processes during an inhalation. If we imagine an ideal pathway of a molecule from environment to the nasal cavity we can consider it as a first phase in which a lot of volatile molecules reach the inside of the nose. In a second phase the molecules in the nasal cavity interact with the olfactory epithelium. In the epithelium the olfactory sensory neurons have non selective receptors and as consequence of the interactions with the different volatile molecules an electrical signal is transmitted to the brain. The described procedure is the way through which odour perception is generated by natural olfaction system as result of the interact with the environment [7] .

There are a lot of studies about both the role of the odorant receptors and the organization of the olfactory system. For their findings in this field Richard Axel and Linda Buck were awarded with a Nobel Prize in Physiology of Medicine in 2004.

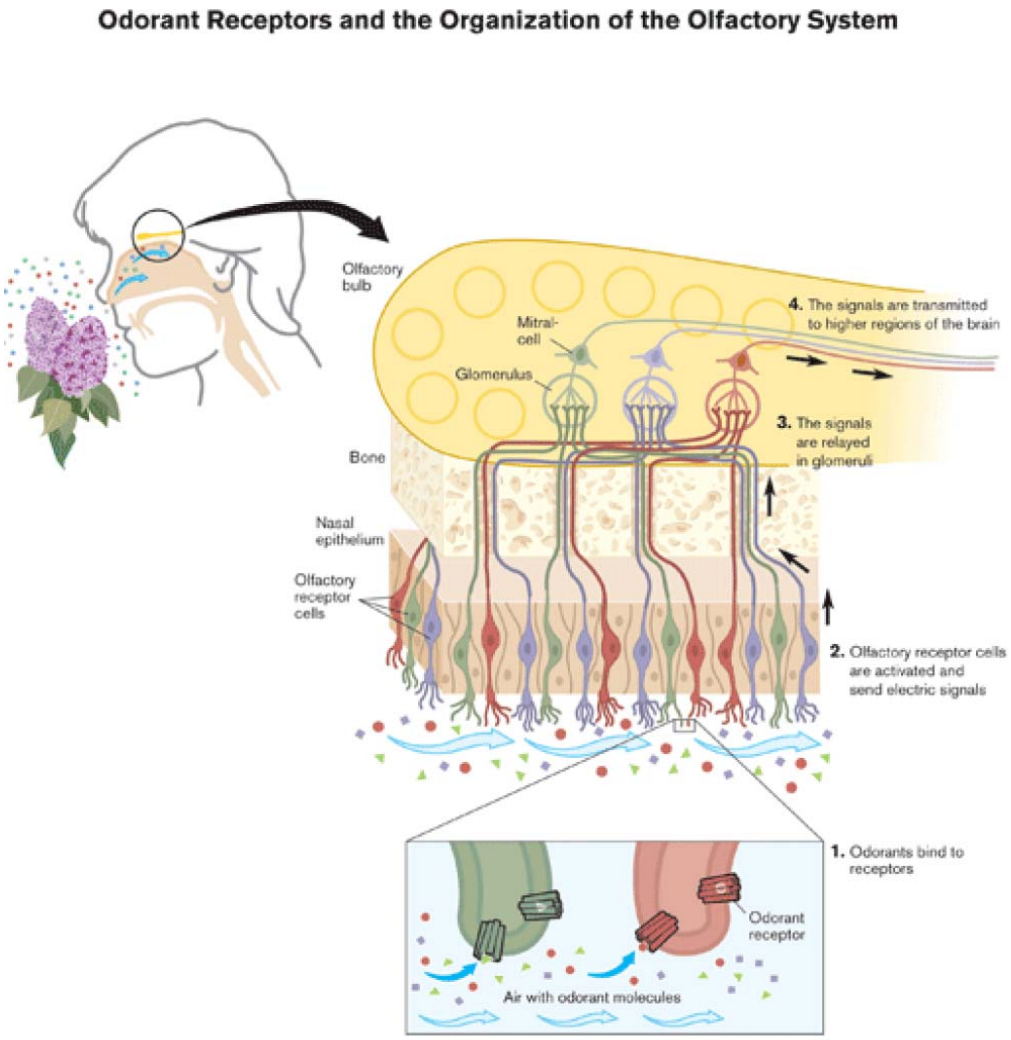

Figure 1.1: odorant receptors and the organization of the olfactory system.

In their research they studied the role of receptors. The receptors are proteins belonging to different families; the main class is rapresented by the G-protein linked with the receptors GPCRs (whose discovery produced the Nobel Price award in 1994). Axel and Buck in their work identified four group of these receptors deputed to odours recognition of pheromones, bitter taste and sweet taste. Their work was devoted, at first, to encode receptor proteins that

are expressed only in the olfactory epithelium. They discovered a large gene family comprising about 1000 different genes in human genome. They showed that each family of ORs (Odorant Receptors) belongs to the receptors GPCRs. These proteins(7TM) are localized in the olfactory epithelium and they contain about five million of olfactory neurons. The brain receives signals from each olfactory neurons through the cilia where receptors are distributed. Through ORs they are able to recognize and bind the odorant molecules stimulating the cell to sent signals to the brain. The G- protein actives the formation of cAMP (cyclic AMP) that opens the ion channel activating also the olfactory receptors. The mechanisms of OR choice are more complicated and four theories have been suggested.

The first method considers, according to deterministic model, that the choice of OR expression involves a unique combination of regulatory factors. In this model distinct sets of transcription factors recognize different cis-regulatory sequences contained in different OR genes.

The second is a stochastic model. In contrast with the deterministic model considers all the OR genes within a zone that contain the same cis-regulatory information and are controlled by the same set of transcription factors. For this model only a receptor gene is expressed in a given neuron. This choice must maintained during the cell life , because receptor switching after synapse formation would perturb odour discrimination.

According to the third model the choice of a specific receptor for an individual OR cell is obtained by a DNA arrangement.

The four model proposes that specialized transcriptional apparatus allows only one gene for each given cell. [8]

1.2.1 From olfactory perception to pattern discrimination

Previous paragraphs have shortly introduced the physical mechanisms of the perception. It is now worth to discern a crucial step in the elaboration of the perceived odour: from the recognition of a single odorant molecules to the discrimination of a mixture whose composition is different from one other.

About the recognition of odorant molecules, it is obtained by a large family of OR molecules. The discrimination of different mixtures is more complicated and consists of identifying the number of receptors that have been activated for a particular set of odorants. Each neuron, is able to express a receptor. This receptor converges information into two gromeruli present in the olfactory bulb. So in this way it is possible to define a pattern of projections spatially invariant providing a two dimensional representation of receptor activation in the brain.

Several studies on eukaryotes demonstrated that different odours define different patterns of glomerular activation. So, by mean of a sort of sensory map formed in the brain the quality of an odorant may be reflected by different spatial patterns [9].

In a work of *V.Jacquier et al.* the complete life cycle of the human odorant receptor OR17-40 was monitored in living cells [10].

Until now we have seen the importance and potentialities of human olfactory system, the mechanisms through which the molecules are detected and as the odour are discriminated. The next step is to understand the source of odorant molecules in the human body. Actually there is a relation between certain odorant molecules and particular odorants conditions (environment, pollution, food cooking); it will be useful to find a relation between some

volatile organic compounds released by human body and the genesis of a pathology.

1.3 Metabolic profile

In this paragraph the concept of the "metabolic profile" and its role in this work will be described. In the first decade of '900 Sir. A. Garrod, a physician, affirmed that the manifestation of a pathologic state could be reflected in characteristic changes in the profiles of constituents of a biological fluid[11]. Several years after Pauling defined "orthomolecular medicine" as a concept to preserve good health state. This concept was based on the possibility to treat a disease by varying the concentrations of some substances in the human body that are normally present and required to preserve a good health state [12] .

Jellum summarized the concept of metabolic profile as follows :

"*it seems reasonable to assume that if one were able to identify and determine the concentration of all compounds inside the human body, including high molecular weight as well as low molecular weight substances, one would probably find that almost every known disease would result in characteristic changes of the biochemical composition of the cell and the body fluids*" [13].

The metabolic profile may be defined as a fingerprint of the body fluid . The fluid is generally a mixture of substances in equilibrium with gas phase on its surface. This gas phase consists of volatile compounds as a "chemical image" of the fluid composition. This means that if the chemical composition of fluid changes also the chemical composition of the gas phase changes. Generally the organic volatiles consist of the nutrients, intermediates , contaminants and other substances of low molecular mass involved in the metabolism. They are composed of different groups of substances with diverse polarity as alcohols,

ketones, aldeydes, O- and N- heterocyclic compounds, isocyanates, sulfides and hydrocarbons. Generally containing 1 to 12 carbon atoms and with a boiling points ≤ 300 °C [14].

There are a lot of studies on metabolic profile fill the 80's in which the correlation between the modification of the body fluids composition and diseases looks interesting. The interest decreased in the following years even if several novel instruments and methodology of information extraction were developed.

1.3.1 Metabolic pathway

In the previous paragraph it was described the concept of metabolic profile as the "signature" of a body fluid or generally of a metabolic process. In fig. 1.2 it is represented a general scheme of a typical pathway of the compounds in a living being. It is obvious that the cell is an intermediate for hypothetic metabolic process. The compounds present in the environment are adsorbed in many different ways by human body. Inside the body they take part to the methabolic processes and conseguently they are transformed.

In the past two decades novel diagnostic criteria have been implemented for fatty acid oxidation disorders such as oxidation rate assay, urinary organic acid and acyglycine analysis by GC/MS. About the metabolic process inside the cell, several in *vitro* assays have been designed for example to detect disease-specific acylcarnitine profiles. Since the basis of mitochondrial fatty acid oxidation revolves around the requirement of L- carnitine, the discovery of disease-specific acylcarnitine metabolites in the urine, blood, and cells of patients with known branched chain amino acid deficiencies and fat oxidation disorders has facilitated the recognition of these disorders[15].

In this thesis these complex mechanisms of reaction will not be described, but it is important to remark the possibility to trace the compounds modification products, and to exploit them as a index of the presence of a pathology.

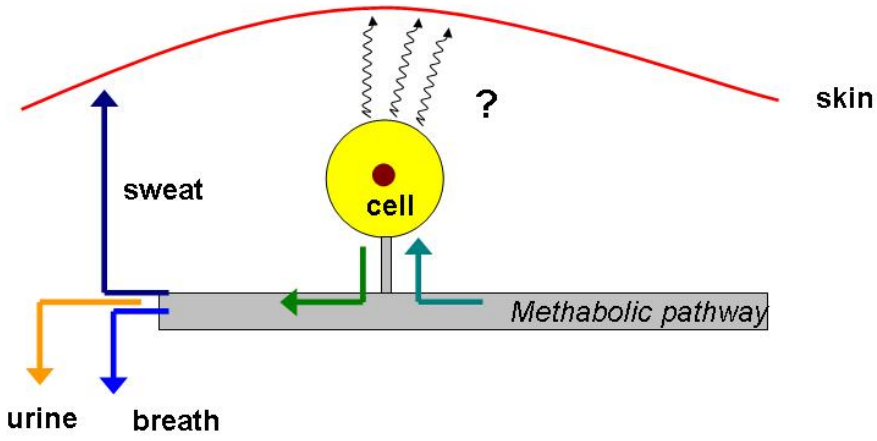

Figure 1.2: schematic view of the cell metabolic pathway with a focus on its possible outcomes (sweat,urine,breath).

In this way, we can consider the body fluids as a "mirror" of the state of health of the body. The metabolic profile is representative of the internal chemistry of the body, its external expression consists of a number of biological media: breath, blood, urine. These sources of information are only partially exploited by the current clinical chemistry where only the composition of human fluids such as blood and urines is analyzed, and correlation between a certain disease and chemical compounds are available for a large class of

pathologies. Moreover breath-test is also a well-accepted technique for routine analysis of bacterial overgrowth, fat malabsorption, pancreatic and liver function test, gastric emptying. The chemical composition of each of the biological solid and liquid listed above is reflected in its volatile part, and this process is mediated by physical and chemical proprieties of each molecule. As a consequence the way to access to the individual health state information is represented by the volatile organic compounds (VOCs) found in the air surrounding living beings. This air contains meaningful information about the internal chemistry of the body and then it can provide a vehicle for identification of diseases.

1.4 Breath analysis

The breath is compose by nitrogen, oxygen, carbon dioxide, water and inert gases. There are also other compounds that occur at concentrations of nmol/l-pmol/l (ppbv-pptv) range. In the last two decades several experiments were performed pointing out a number of volatile compounds probably connected with certain diseases[16]. Pauling in one of this first works about breath analysis by GC/MS showed that the number of these compounds is higher than 200. Some of these compounds could be markers of a specific pathology[17].

The correlation between VOCs and diseases seems to be due to the fact that several organs contribute to the composition of breath which is expected to be rich of information coming from biochemical processes and their alteration induced by pathologies[18].

The VOCs produced inside human body or in the environmental surrounding can have exogenous or endogenous origin with different informative content relative to different scopes. The exogenous compounds may be analyzed for environmental or expositional issues to detect compound

uptake and elimination into the body in order to study the metabolic or pathologic processes the endogenous substances are interesting.

The relationship between the presence of certain compounds and some pathologies in several experiments has been confirmed, because this relation is sometimes justified by a well-known biological process. In other cases it was observed the anomalous concentrations of some compounds for certain diseases.

In the following it will be described a series of works in which the compounds presented in the breath are associated to particular diseases.

Peroxidative activity [19] seems to be correlated to the presence of ethane and pentane: actually hydrocarbons are stable end products of lipid peroxidation. For their low solubility in blood, their exhalation into breath is an indication of the progress of oxidative stress damage[20]. Considering that protein oxidation and bacterial metabolism are other potential sources of hydrocarbons in the body, there could be some problems of interpretation, although these processes seems not to interfer with the breath test analysis [21].

Isoprene can be seen as cholesterol synthesis indicator [22], and it can give a measure of oxidative damage of the fluid lining the lung [23]. On this basis, cystic fibrosis [24] is a pathology related to this compound.

The concentration of acetone has high levels in patients with diabetes mellitus[25] while ethanol and methanol are indicators of alcohol addiction, and of potential source of endogenous short chain alcohols in the intestinal bacterial flora [26].

A high concentration of sulphur compounds is likely related to liver diseases. Compounds like ethyl mercaptane, dimethylsulfide are responsible for the characteristic odour in the breath of cirrhotic patients. Under normal conditions the concentrations of sulphur containing compounds in human blood and breath are rather low.

The characteristic odour of breath is due to elevated concentrations of dimethylamina and trimethylamine. The amines are typical products of putrefaction processes.

The ammonia is abundant in the breath of uremic patients and in cases of severe kidney failures [27].

1.5 Urine analysis

As the other body fluids also the urine is a product of a metabolic process and some desease can be revealed by mean of its chemical composition. It is possible to correlate some volatile compounds present in urine headspace with the diabetes mellitus. The deficiency in effective insulin that characterizes this disorder drastically alters the metabolism of carbohydrates, lipids, and proteins. The presence of this disease is characterized by the increase of glucose in the blood, intolerance to glucose, glucose excretion in the urine, ketosis, acidosis, and increased protein breakdown. In several works Zlatkis et al. and Leibich et al. studied the VOCs of urine both in normal and diabetic individuals [28].

From their studies it emerged that abnormal concentrations in urine of certain aliphatic alcohols, ethanol, n-prpanol, n-butanol, and isopentanol and ceratin ketones (4-hepatnone and cyclohexanone) reflect the metabolic disorders in patients with diabetes mellitus. The role of each compound changes with the form, stage or severity of this disease. The most important compounds detected , correlated with the diabetes mellitus, are ethanol and n-propanol. The combination of this technique with the monitoring of the glucose concentration in the blood provides an efficient diagnosis of this disease . To study renal insufficiently, the results obtained for serum and urine headspace were compared. It was known that some compounds, as the alcohols, are

present in serum with a higher concentration than in the urine. Probably the kidneys retain the alcohols. A similar behaviour is noted for the n-pentanol compound, found in the serum but not in the urine. In the patients with renal insufficiently less 4-haptanone is execreted [29].

Tab.1,tab.2 and tab3 show the VOCs detected from urine, Serum and Breast Milk identified with Gas Chromatography – Mass Spectrometry [14].

Table 1. Organic Volatiles in Urine, Serum, and Breast Milk Identified by Gas Chromatography–Mass Spectrometry

Compound class	No. of carbon atoms 1	2	3	4	5	6	7	8	9
Urine (14, 16, 17, 24, 34)									
Alcohols		ethanol	propanol 2-propanol	butanol 2-methyl-1-propanol 2,3-butanediol	pentanol isopentanol 2-methyl-1-butanol 3-methyl-1-butanol			octanol	
Ketones			acetone	2-butanone 2,3-butanedione	2-pentanone 3-methyl-2-butanone cyclopentanone 2,3-pentanedione 3-penten-2-one	2-hexanone 3-hexanone cyclohexanone 3-methyl-2-pentanone 4-methyl-2-pentanone 2-methyl-3-pentanone 4-methyl-3-penten-2-one cyclohexanone	2-heptanone 3-heptanone 4-heptanone 4-ethoxy-2-pentanone 5-methyl-3-hexanone	2-octanone 3-octanone 4-octanone 6(?)-methyl-3-heptanone	2-nonanone
Aldehydes		acetaldehyde	propionaldehyde	3-butanal 2-methylpropanal	2-methylbutanal 3-methylbutanal		benzaldehyde		
O-Heterocyclics					furfural 2-methyltetrahydrofuran-3-one	2,3-dimethylfuran 2,4-dimethylfuran 2,5-dimethylfuran 2-ethylfuran acetylfuran	2-methyl-5(?)-ethylfuran 2,3,5-trimethylfuran		2-*n*-pentylfuran
N-Heterocyclics				pyrazine	methylpyrazine	2,3-dimethylpyrazine	2,3,5-trimethylpyrazine	1-butylpyrrole	

(continued)

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Tab. 1

Table 1. *Continued*

Compound class	No. of carbon atoms 1	2	3	4	5	6	7	8	9
					pyrrole	2,6- or 2,5-dimethylpyrazine vinylpyrazine dimethylpyrrole picoline	2-methyl-6-ethylpyrazine 2-methyl-6(?)-vinylpyrazine		
S-Compounds	dimethyl sulfone, propylene sulfide, thiophene, butylisothiocyanate, dimethyl disulfide, allylisothiocyanate, 2,3-diethiabutane, thiolan-2-one								
Misc. compounds	acetic acid, hexane, benzene, phenol, toluene, chloroform, *p*-methylpropylbenzene, limonene, β-pinene, carvone, *p*-cresol, piperitone, 4-methyl-5-hydroxyhexanoic acid lactone, γ-valerolactone, α-terpineol, γ-hexalactone, δ-hexalactone								
Serum (28, 30, 31, 42, 44)									
Alcohols	methanol	ethanol	propanol 2-propanol	butanol isobutanol	pentanol	1-hexanol 2-hexanol		2-octanol	
Ketones[a]			acetones	2-butanone	2-pentanone 3-penten-2-one	2-hexanone cyclohexanone 4-methyl-2-pentanone	2-heptanone 3-heptanone 4-heptanone	2-octanone 4-octanone 6-methyl-2-heptanone 5-methyl-3-heptanone 6-methyl-5-acetophenone	5-nonanone trimethyl-2-cyclohexanone
Aldehydes[b]	formaldehyde	acetaldehyde	propanal propenal	butanal 2-butenal 2-methylpropenal	pentanal *trans*-2-methyl-2-butanal		heptanal benzaldehyde	octanal 2-ethylhexanal *o*-tolualdehyde	nonanal 2-nonenal nona-2,4-dienal
O-Heterocyclics				dioxane furan	2-methyltetrahydrofuran	2,3-dimethylfuran			
Sulfides		dimethyldisulfide							

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Tab. 2

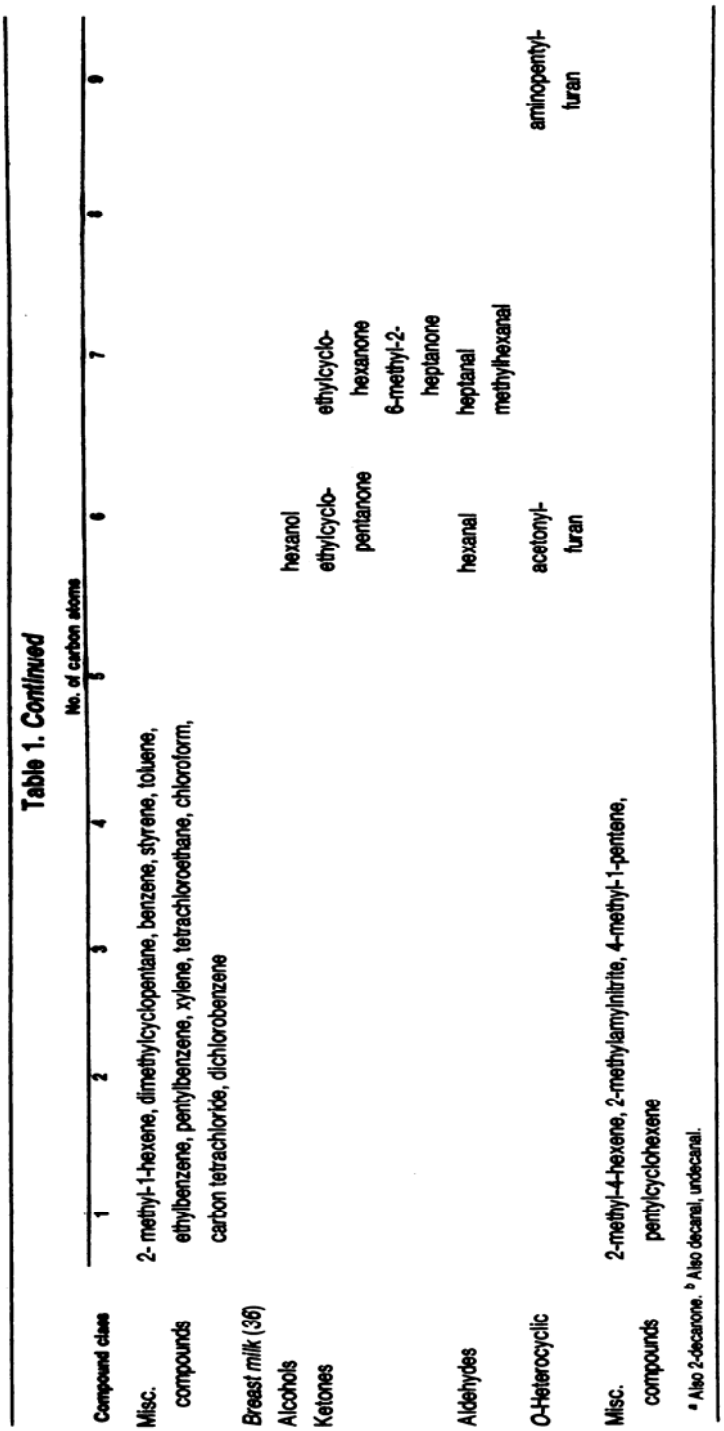

Table 1. *Continued*

Compound class	No. of carbon atoms								
	1	2	3	4	5	6	7	8	9
Misc. compounds	2- methyl-1-hexene, dimethylcyclopentane, benzene, styrene, toluene, ethylbenzene, pentylbenzene, xylene, tetrachloroethane, chloroform, carbon tetrachloride, dichlorobenzene								
Breast milk (36)									
Alcohols						hexanol			
Ketones						ethylcyclo-pentanone	ethylcyclo-hexanone		
							6-methyl-2-heptanone		
Aldehydes						hexanal	heptanal		
							methylhexanal		
O-Heterocyclic						acetonyl-furan			aminopentyl-furan
Misc. compounds	2-methyl-4-hexene, 2-methylamylnitrite, 4-methyl-1-pentene, pentylcyclohexene								

[a] Also 2-decanone. [b] Also decanal, undecanal.

Tab. 3

Tab. 1, Tab.2, Tab. 3: Organic Volatiles in Urine, Serum, and Breast Milk Identified by Gas Chromatography-Mass Spectrometry extracted by Albert Zlatkis et al., The Role of Organic Volatile Profiles in Clinical Diagnosis, *Clin. Chem.*, 27/6, 789-797 (1981).

1.6 VOCs released by Skin transpiration

The VOCs released by transpiration from skin result from metabolic processes also. In this case the production of a number of VOCs from skin headspace in different concentration can give information about alterations due to some pathologies. The starting point for the study of the skin from the odour point of view is the description of the basic pattern of bacteria colonization of a healthy human skin.

On the skin surface it is possible to distinguish between aerobic flora and anerobic flora.Gram-positive cocci of staphylococcus and micrococcus, and a variety of Gram-positive rods, mainly corynebacterium are present in the skin as resident aerobic flora. The main anaerobic residents are propionibacteria which are localized in the follicles of the sebaceous glands of adults. The role of microbial flora is to protect the skin from action against pathogenic bacterial and micotic infections.

Starting from this point of view it is clear that the skin surface is a source of VOCs giving to the human odor. In several studies the VOCs of human body odor have been investigated. Odour may be exploited for the discrimination between different individuals, but, until now, the lack of technology has hindered this kind of applications. The VOCs from human body have genetic and environmental origins . *Curran et al.* developed a terminology for these factors. They defined "primary odor" all compounds present in a individual that are stable and not influenced by the environment or diet. "Secondary odor" are related to the compounds correlated with the diet and environment while with the term "tertiary odor" defined all the compounds that have external origin (lotion, perfumes, soaps, etc)[30].

Several works in the last years have shown the role of some compounds related to the odor of axillary and plantar sweat. Through the development of

different methods for the GC/MS it was possible to study the compounds present both in the male and in the female. The analysis showed the presence of several C_6- C_{10} straight chains, branched and unsaturated acids. From these works it emerged main causes of odour generation are unsaturated acids, 2-methyl C_6- C_{10} acids, and 4-ethyl C_5- C_{11} acids, along with (E)- 3 – methyl-2-hexenoic acid. Only quantitative differences were observed among individuals without particular distinctions between male and female[31].

Body odours have been extensively studied but there is still a not exhaustive list of typical VOCs. In the other works the relation between age and odour was studied and also in this case hydrocarbons, alcohols, acids, ketones, and aldehydes seems to play a decisive role. An interesting result was obtained for a specific compound : 2-nonenal. This compound was detected in the odour of individuals over 40 yr of age. 2-nonenal as other aldehydes are produced during the oxidative degradation of monosaturated fatty acids, such as palmitoleic acid and vaccenic acid[32].

The relationship among particular compounds from the skin and certain pathologies have been investigated in the last years. The difference between schizophrenia and mental disorders, for example, is still not clear. Generally individual behaviour and emotional states of subjects are considered to discriminate between mental deseases . This studies require a different of dedicated protocols for each subject. In literature there are several works about novel approaches to identify schizophrenia[33]. These methods are based on objective and quantitative evaluations. It was noted that in schizophrenic patients a faulty gene that codes for the enzyme dopamine-b-hydroxylase operates an excessive biosynthesis of tyrosine (amino acid precursor of dopamine) and this excessive syntesis generate a high level of dopamine. This mechanism results in a general increase of trans-3- methyl hexenoic acid, which is a product of the auto-oxidation of dopamine excess[34]. On these basis trans-3-

methyl-hexenoic acid could be considered as a marker for schizophrenia patients. These considerations are presented in a work in which the presence of this compound was associated to schizophrenic individuals[35]. The possibility to identify the schizophrenia from the breath has also been investigated [36].

The volatile compounds from the skin can be used to monitor the damage of human skin induced by solar UVR. The damage of human skin produce free radicals . It is known that the pigmentation protects the skin by mean of scattering but this is not sufficient, so the continuous exposition to UVR is a cause of an increase in lipid peroxidation. This produces a reaction between polyunsaturated fatty acids and free radicals forming several products , including pentane, ethane and ethylene[37].

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CHAPTER 2

Non conventional approach to volatile compounds measurements in medicine: instruments overview

2.1 Analytical Chemistry instruments

In chapter 1 examples of the metabolism products possibly reflecting the presence of different human pathologies (in breath, skin, urine etc) have been described. The volatile organic compounds originated can be detected by several methodologies. In figure 2.1 the instruments related to specific analysis (qualitative, quantitative or specific) are shown.

The complexity of odours originated from human body requires the optimization of the sampling protocol for their extraction, but also the use of appropriate instruments for the analysis. It is possible to define several approaches starting from different strategies. Anyway the basical requirements are:

- Pre-treatment of the sample: often it is necessary to treat the sample before the analysis with solvents or by pre-concentrations techniques.
- A selective analysis for one or more compounds
- Low cost
- Fast responses

These conditions are not requested at the same time, but often the analytical techniques result from their combination. In the next section the most important features of some instruments will be described.

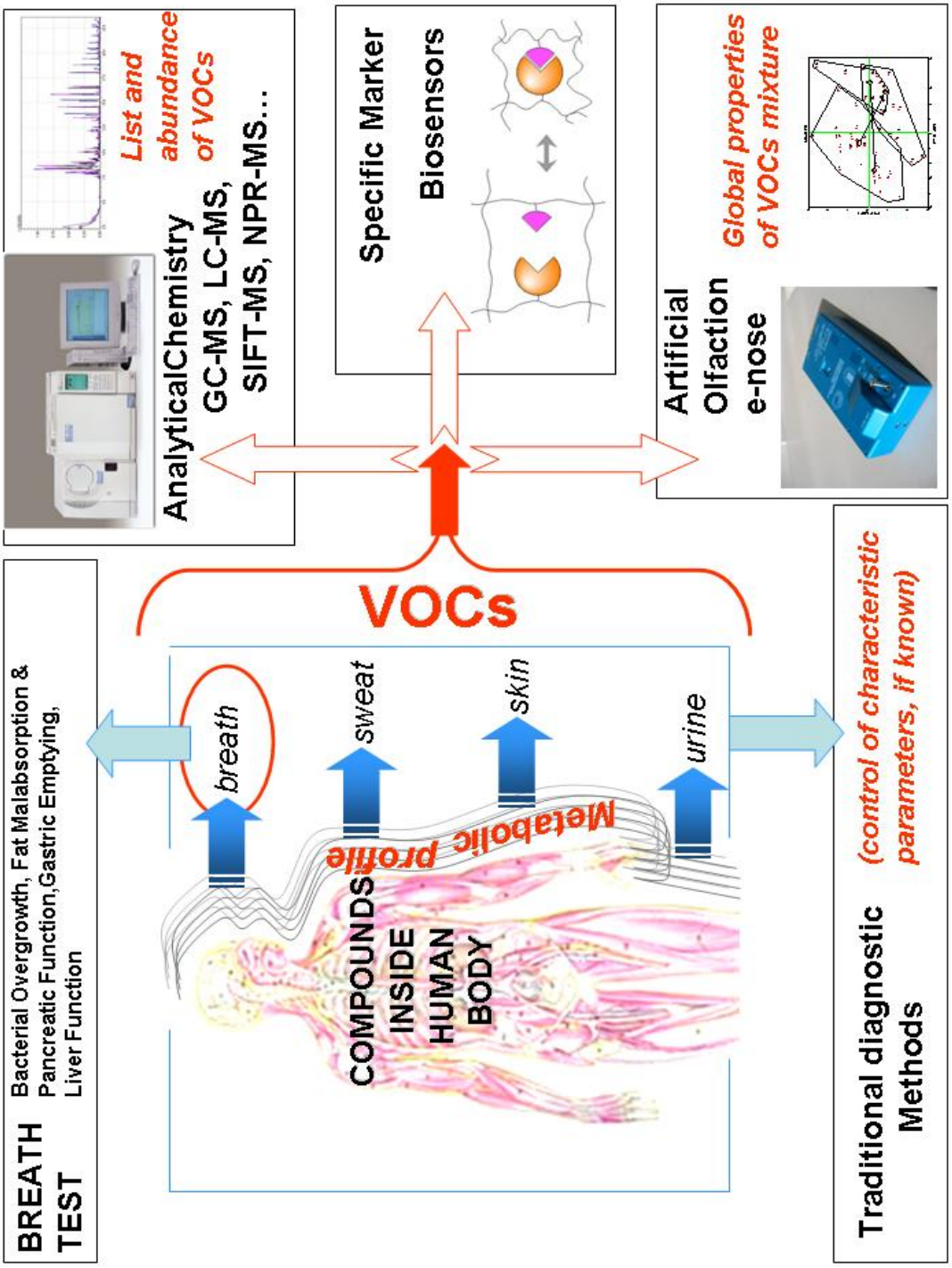

Figure 2.1: overview of the different instrumental approaches to metabolic profiles information.

The most representative instruments for a quantitative analysis of volatile compounds from a solid or liquid and of gaseous samples are GC-MS SIFT-MS and PTR-MS, while to detect liquid composition of a certain sample it is possible to use the LC-MS. These instruments can be used to detect a limited numbers of compounds in a first phase and to utilize specific sensors as biosensors in a second one.

A qualitative approach to measure the global properties of VOCs mixture is possible with non-selective sensors that are sensitive to different compounds. In the last decades the use of gas sensors arrays commonly called "electronic nose" has been deeply investigated.

In this thesis, in particular, GC/MS and electronic nose have been considered. In the following section ,an overview on the principal techniques used for references about their medical applications will be presented; then more attention will be given to GC-MS and E-Nose.

2.2 LC-MS

Liquid chromatography (LC) is an analytical chromatographic technique. Generally it is used to separate mixtures of less volatile or polar compounds dissolved in a solvent.

The mobile phase of the sample is sent through a column, coated with a stationary phase, with a high-pressure pump. The most common detector for liquid chromatography is the UV-detector. It utilises light in the ultraviolet area, and when a component in the sample passes the detector parts of the radiation are absorbed by the sample.

In order to analyse biochemical species such as amino acids and proteins, often ion chromatography is used. This is a form of high-pressure liquid chromatography which uses conductivity detectors.

Mass spectrometry is normally coupled to these systems to identify and measure several biological and chemical compounds. The mass spectrometer transforms the compounds into gaseous ions. The phase of ionization is obtained exposing the molecules to electrons (electron impact ionization (EI)). This is the first phase of fragmentation of each molecule. In the second moment the molecule is supplied with enough energy to eject its electrons becaming positively charged. From the fragmentation a number of ions with different mass-to-charge (*m/z*) ratios are obtained. The spectrum resulting from the fragmentation is unique for each molecule and it is used as a fingerprint to characterize the analyte.

To transfer the LC sample into the gas phase and to ionize the analytes soft ionization techniques are used. In this way protonated and deprotonated molecules are produced. Generally the interfaces used for the ionization are:

- Electrospray ionization (ESI): the sample is nebulized with a ion spray probe creating small charged droplets.
- Atmospheric-pressure chemical ionization (APCI): heating a stream of gas are used to vaporize the solvent and a corona discharge to provides the ionization of the compounds in the gas phase at atmospheric pressure.

Electrospray ionization(ESI) [1] is by far the most widely used LC–MS ionization method due to its superior sensitivity and extended mass range, enabled by the formation of multiply charged ions. However, ESI is supplemented with atmospheric pressure chemical ionization (APCI) [2]which, in some cases, shows better performance with relatively small and less polar compounds.

LC-MS has been used in medical applications to detect human carcinogens. For example, in a report of U.S. Environmental Protection Agency (EPA) it emerged that the exposition to 1,3-butadiene is a significant public health concern because it is a known human carcinogen, and traffic, which is a dominant source of ambient butadiene, is ubiquitous and it is in close proximity to residential communities[3]. In a pilot study the concentration of 1,3-butadiene was monitored by LC in urine of a group of people living in different areas of a city[4].

LC–MS has now become a leading method for the qualitative and quantitative analysis of DNA-adducts in vitro or in vivo[5].

2.3 SIFT-MS and PTR-MS

SIFT-MS (selected ion flow tube mass spectrometry) technology analyses the volatile compounds from the headspace in sorbent tubes by chemical ionization.

SIFT-MS can be used to provide, in real time, absolute concentrations for analytes. Generally combining gas phase ion chemistry and controlled reaction conditions concentrations of analytes can be detected in a mass range of 10-240 Da, although this range is increasing with the development of other technologies.

Figure 2.2 shows a general scheme of SIFT-MS system.

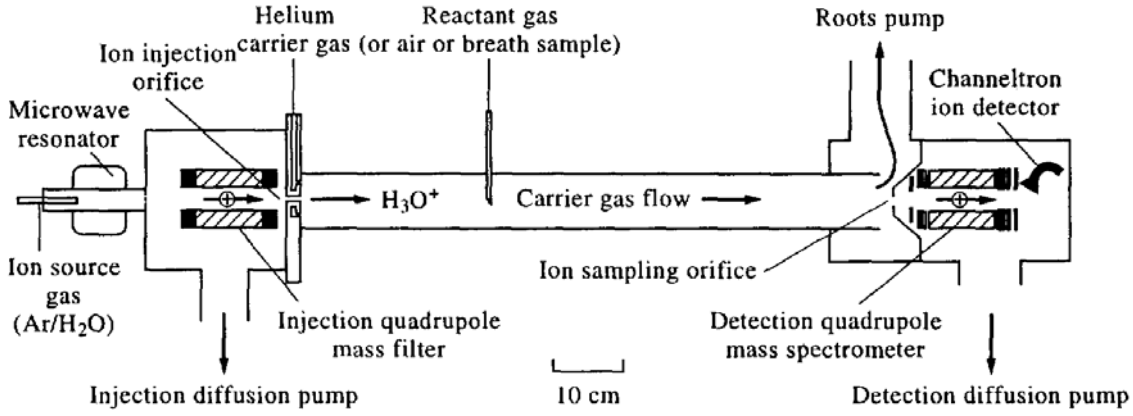

Figure 2.2 : A schematic diagram of the selected io flow tube (SIFT) apparatus (extracted from 'P. Spanel, P. Rolfe, B. Rajan and D. Smith ,The selected ion flow tube (SIFT)- a novel technique for biological monitoring, Pergemon PII:S003-4878 (96) 00028-2')

A microwave or radio frequency source is used to generate positive ions. The ions pass through a quadrupole mass filter that removes all but the preferred precursor ions, generally H_3O^+, NO^+ and O_2^+. Then the precursor ions pass through a venturi and reaches reaction chamber where they react with the sample, injected in the tube with a controlled rate. The products of this reaction are filtered by a second quadrupole mass filter. A particular multiplier detects it and gives information about the numbers of the selected products.

This system permits to give absolute quantitative information about the VOCs without chromatographic separation and pre-treatment of the sample. On the other hand it is necessary to know the dynamic of the tube and to thermalise the ions.

In this way it is possible to identify the analytes of interest and in particular to obtain their relative abundances but there is the need to know the chemistry of the analytes and their reactions with the precursor ions.

This method represents a system real time for a monitoring of the samples .To demonstrate this ability of the SIFT-MS systems, P.Spanel *et al.*

have monitored the partial pressure of acetone on a subject that expired directly in the system. In fig.2.3 it is shown the exhaled acetone increasing which follows two different slopes for the upper airways volume (first 2 seconds), and the alveolar part (from 2 seconds to the top)[6].

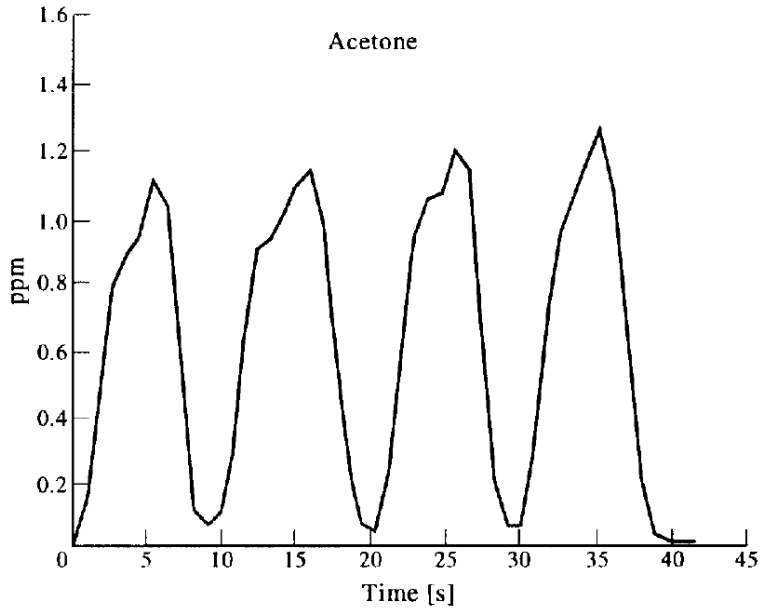

Figure 2.3: acetone concentration in the breath monitored by SIFT-MS system (figure extracted by P.Spanel *et al*. The selected ion flow tube (SIFT) a novel technique for biological monitoring, *Ann. Occup. Hyg.* 40/6, 615-626 (1996).

The SIFT-MS may be considered as derived from an other system: PTR-MS. The PTR-MS (proton transfer reaction-mass spectrometry) is a technique developed by *Lindiger et al.* used to analyse volatile compounds with concentrations as low as a few parts per trillion by volume. As for the selected ion flow tube mass spectrometry the PTR-MS is based on reactions of H_3O^+ ions, which perform nondissociative proton transfer to many of the common VOCs but do not react with any of the major components present in clean air. The chemical ionization of VOCs and the generation of H_3O^+ ions are separated

processes. This technique is based on the transfer of proton from H_3O^+ to all VOCs of the sample that have higher affinity with water. It consist of four components : an ion source , a drift tube , a quadrupole and an ion detector. The ions of H_3O^+ are produced at high concentrations from pure water vapour within a hollow cathode ion source and then they are sent in a venturi inlet in the drift tube . A lot of compounds of air with a low proton affinities do not react with H_3O^+ ions so they are considered as a buffer gas. On the other hand the components of gas with high proton affinity with water will be involved in a proton transfer reaction. The ions produced in this reaction are then analysed with quadrupole and measured with a electron multiplier counter[7].

A brief description of some medical applications of this system is reported in the following. The most important application is the detection of particular compounds in the breath.

In the previous paragraphs we have already said about the endogenous components of the human breath. In particular isoprene is important because it is still not clear both its origin and abundance in normal human subjects. Isoprene seems to be correlated with the metabolism since its concentrations changes during the day and in particular in different states of sleep. In several investigations small amounts of isoprene from breath may be a sign of the cholesterol peroxidation precursor, squalene[8]. Lindinger *et al* .applied the PTR-MS techniques to monitor the isoprene in the human breath obtaining that the concentrations were very different between adults (240 ±120ppbv) and children (100 ±50ppbv) while they were not age dependent.

In other applications the endogenous production of this volatile compounds in the breath was monitored with PTR-MS after consumption of fruits. In particular it was studied the release of methanol in the human body. The baseline concentration of methanol in the body reflects a balance between endogenous production and metabolic loss. It was seen that the metabolic loss

process is stopped when the human body contains high concentration of ethanol. For example it was seen that after the consumption of about 1Kg of apples, 0.5g of methanol is released in the human body. The same concentrations are present in a person after drinking 0.3 L of brandy containing 0.5% of methanol.

The major advantage PTR-MS and SIFT-MS is given by the possibility to detect the abundance of a volatile compound without the sample pre-treatment. On other hand the specific compounds to analyse and their chemistry binding with the precursor ions have to be known.

2.4 Biosensors

"A biosensor is a device" whose […] "recognition step involves a biological sensing element or receptor, on the surface that can recognize biological or chemical analytes in solution or in the atmosphere. The receptor way be an antibody, enzyme or a cell"[9]. The name electrochemical biosensors is applied to a molecular sensing device which intimately couples a bio-local recognition element to an electrode transducer[10].

Until now analytical chemistry instruments have been shown. These system had the properties to give both qualitative than quantitative information about the volatile compounds that must be detected. The biosensors, instead, can be used if a biochemical mechanism (mostly enzymatic catalysis, immuno-chemical reaction or complementary DNA hybridization) is used in a process of molecular recognition. These type of sensors are called biosensors.

The peculiarity of biosensors is to use biological sensitive material for a selective recognition. The main advantage of these sensors is the selectivity for a specific compounds. The disadvantage is their lifetime depending on the life of their used biological material. Describing the general principles of biosensors,

the analyte keeps in contact with the biological material deposed on the transducer surface. In this phase the compounds are captured by the specific material. This immobilization permits both to detect the compound and also to stabilize the biological material.

By mean of the transducer it is obtained an electric signal that can be amplified and analysed with signal processing techniques. The complexity of this sensors is due to the selection of the biological material. Generally enzymes, multienzyme systems, organelles, photosensitive membranes, prothoplasts, whole intact cells, tissue slice , antibodies, lectins, DNA are used.

When a biological material is chosen the second step is to define the transduction method in order to obtain a signal that will be elaborated with a signal processing techniques.

Generally the transducer is typical of the chemical interaction material deposed on its surface. So, in this way, different biosensors are obtained. In the tab 2.1 Extracted by *Chemical and Biochemical Sensors* of 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim are indicated the most important principles of transduction in the biosensors usually used.

Transuducer	Change of parameters	Interaction
Potentiometric electrodes	Ion concentration	Enzyme-catalyzed oxidation, hydrolysis, or cleavage of C-C, C-O, C-N, or other bonds
Calorimetric devices	Enthalphy	Enzyme –catalyzed reactions
Piezoelectric crystal	Mass	Bioaffinity reaction between an analyte and receptor protein or analyte and lectine
Fluorimetric fiber optics	Fluorescing concentration of enzyme substrate /products or immunolabels	Enzyme and bioaffinity reactions
Waveguides	Optical path	Bioaffinity reaction
Devices based on surface plasmon resonance	Resonance	Bioaffinity reaction
Amperometric electrodes	Electron transport	Enzyme-catalyzed oxidation or reduction of an analyte

Tab. 2.4: biosensors features: transduction principles and relative interactions.

Biosensors can be classified using two groups: biocatalytic (metabolic) sensors and bioaffinity sensors.

In the first case, the analyte reacts with the biological substrate and the signal is obtained from the product of reaction. In the second case, bioaffinity sensors, the surface of the sensors has a change in some properties (optical, light adsorption, or electrical charge). In this way the sensor must be reported to initial conditions. The responses of these kind of sensors are very fast.

Biosensors are able to detect compounds of several types such as hydrocarbon, heterocyclic compounds, alcohols etc. The range of detection is from 10^{-7} mol L^{-1} (biocatalytic sensors) to about 10^{-15} mol L^{-1} for the (affinity sensors).

The range of detection depends also on the structure of biosensor, which define its interaction principle. The specific interaction is given by the use of the biological material in the sensors. The receptor can be bound in covalent way on the surface of the transducer or directly to an electronic device as for example

the gate of a field effect transistors .Biosensors can be applied in several fields : for military purposes for the detection of dangerous compounds as nerve gases or viruses; in environmental protection to monitor the pollution, toxic compounds in water supplies; medical applications for pathologies study and diagnosis in the previous chapter the role of typical compounds for some diseases have been described . Specific sensors for these compounds permits the disease during a day or in a range of hours to be monitored. For example biosensors to detect the concentration of glucose in the blood prevention of problems due to diabetes have been already developed.

The biosensors are often used to analyze sample of small dimension as the labelling of the DNA chains. The possibility to immobilize the sample permits to define in a limited region the position and to perform their recognition. This technology was decribed by Lehamann in 2002[11](Fig.2.4).

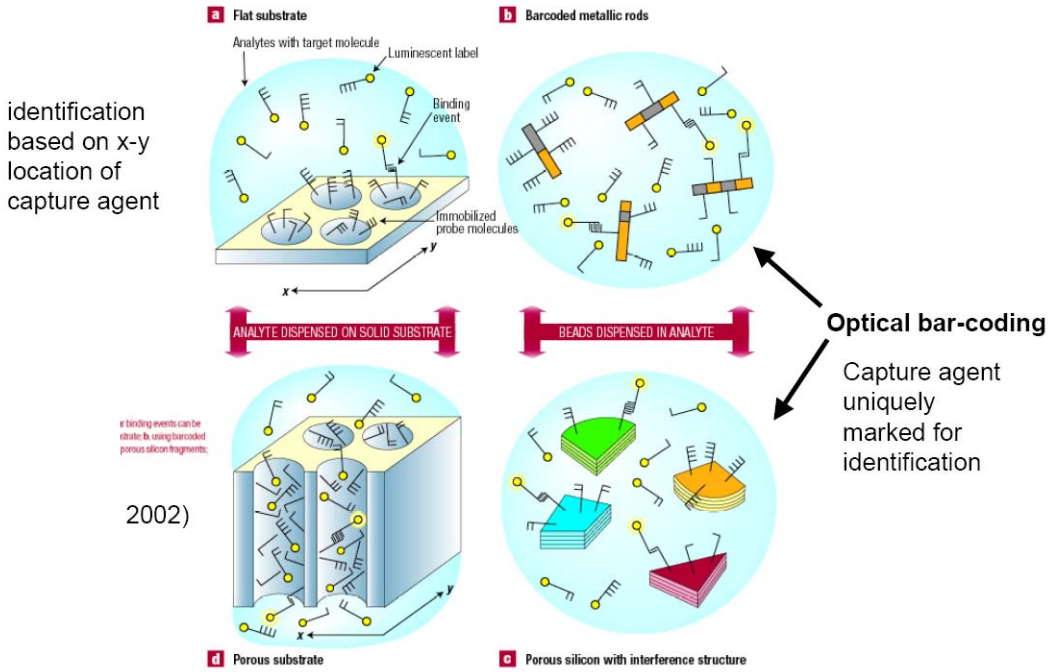

Figura 2.4: biosensor mechanism. Figure extracted by Lehmann, V. Biosensors: Barcoded molecules. ***Nat Mater*** **1, 12-3 (2002).**

The relative rapid responses of biosensors encourage to use them *in vivo* experiment to monitor dysfunctions of human body. The limitations are, until now, their life time and the biocompatibility.

2.5 GC-MS

Gas Chromatography mass spectrometry is a unique and versatile technique. As analytical tool , GC can be used for the direct separation and analysis of gaseous samples , liquid solution , and volatile solids. The detection is performed in two different phases. At first the sample passes through the GC devices in which it interacts with the column adsorbent phase and in the second phase the sample is detected by a mass spectrometer. We have already seen that mass spectrometry is also coupled with many other instruments (LC-MS, PTR-MS, SIFT-MS). By GC-MS is possible to detect chemical compounds of a complex mixture in low concentrations (ppb).

The detection is obtained with a separation of the sample: the separation depends from the volatility proprieties of the sample. Generally small molecules have more volatility than larger molecules. So the compounds can be trapped in a particular phase and can be released in different time in relation to their volatility.

A Chromatographic system is characterized by two different phases: a mobile phase and a stationary phase. The mobile phase is a inert gas as such as helium, argon , nitrogen and sometimes hydrogen. Helium is usually referred to as carrier gas which must be chemically inert to prevent interactions with the analytes. The stationary phase is a particular adsorbent material contained in a *column*, which can be glass or stainless steel of various dimension, and it is constituted by the coating of the column. Different column can be selected for

each specific application . The column can differ for length, thickness and as already said for the coating material (Fig.2.5).

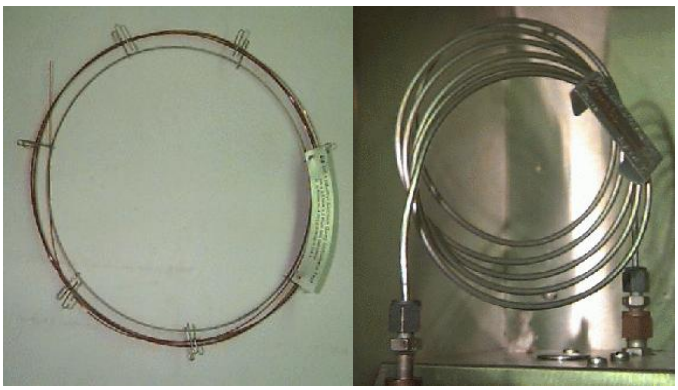

Fig. 2.5:GC/MS column: capillary column (left), packed column (right)

Generally for the capillary column, the coating material is a solid stationary phase (usually with 95% of dimethyl-siloxane polymer).

When the sample is carried by the mobile phase it passes through the column in which its components are differentially adsorbed by the stationary phase. The role of the column is then to adsorb and desorb the components with different rates . In a second phase with a specific temperature the components that interact quickly will elute from the column first while the compounds with a slowest interactions will leave the column late.

An example of the separation of chemicals by means of the mobile phase flow is shown in figure 2.6.

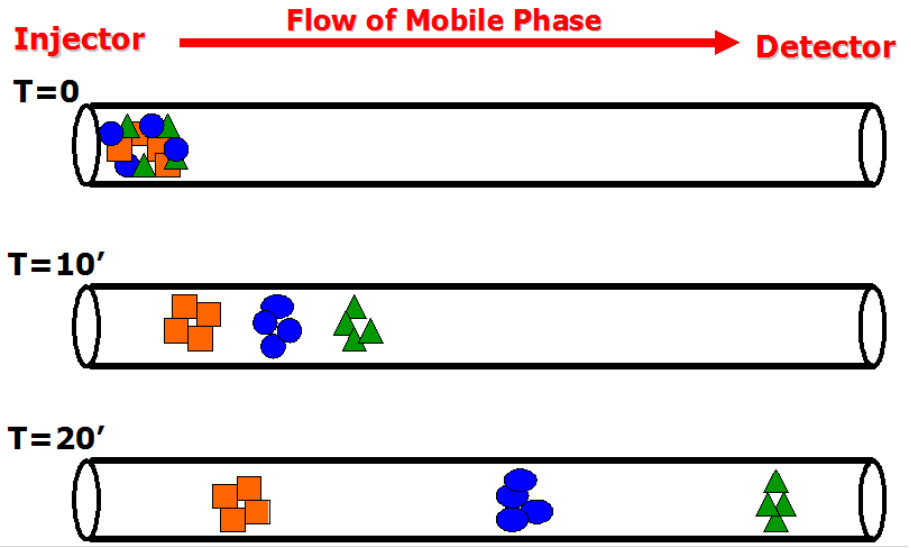

Fig. 2.6:example of the chemical separation by means of the mobile phase flow.

Changing some parameters as pressure, temperature and the velocity of flow of the inert gas it is possible to obtain different separation dynamics of the mixture.

In the following a brief description of the GC-MS system and of the sample treatment will be presented. The injection of the sample the GC is performed by an injection port called splitless at temperature also near 250 °C. Through a valve the volatile compounds of the sample are transferred in the capillary column. Either split, splitless, or on-column injections can be used in GC/MS. Split injections are usually avoided in cases of trace-level components analysis. Splitless or on column injections are preferred for trace component analysis. In this last case the sample is, generally, adsorbed with a polymeric fiber and injected in the splitless port (Fig 2.7). The split is set to high temperature so to perform fiber release of the volatile compounds of the sample. In this phase a valve (at first closed) is opened after sample vaporization. From the injection port the compounds are carried in the column. Column interactions mechanisms with the VOCs have been already described.

As the temperature of the column increases, compounds with low boiling points elute from the column sooner than that having higher boiling point.

When the compounds (with different rate time) exit from the column, they reach a detector which gives an electronic signal. The signal is proportional to the compound concentration.

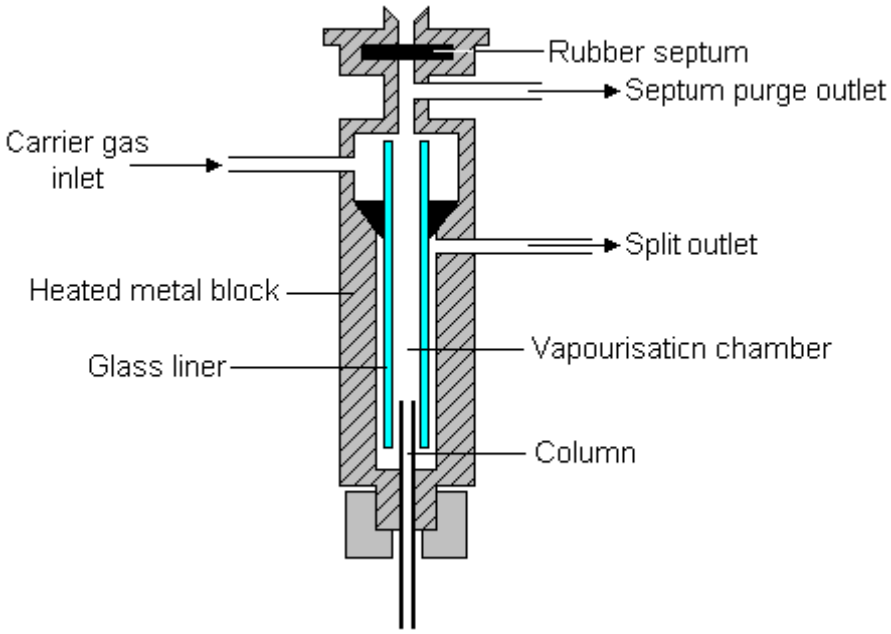

Fig. 2.7: split structure in a GC injector.

The retention time (RT) is defined as the interval time from injection (time zero) to elution . The obtained graph is called *chromatogram* and each peak in the chromatogram represents the signal occurred when a compound elutes from the GC column into the detector. By knowing the retention time of a given compound, it is possible to make assumption about the identity of the compound.

However it is possible to have similar compound with similar retention time. The final part of the column passes through a heated transfer line and

ends at the entrance of the ion source of the mass spectrometer. As eluted compounds exit from the GC column, they enter in the ionization source where they are bombarded by a high energy electron beam (70 eV), causing them to brake into fragments. This process is performed by an electron which strikes a neutral molecule and provides enough energy to remove another electron from the molecule, which becomes a charged particle. The fragments are charged particles with a certain mass. The mass of the particle divided by the charge is called the *mass to charge ratio m/z*. The purpose of the ion source, is to provide the energy necessary to ionize the analyte molecules, maintaining a high temperature to prevent analyte condensation.

The two types of ionization normally used in GC/MS are electron ionization (EI) and chemical ionization (CI).As the ions leave the source , they enter into the mass analyzer where they are separated depending on their mass-to-charge ratio. The mass range of interest is scanned causing separation of ions in space or time domains. The most common analyzers are the magnetic resonator and the quadrupole. The first utilizes an electromagnet to separate ions according to the radius of their trajectories. In a typical magnetic sector analyzer, the magnetic field is varied, directing the ion beam across a narrow slit through which ions of increasing or decreasing m/z are selected. In this way a full-range mass spectrum is obtained.

The second common mass analyzer is the quadrupole, which consists of four cylindrical rods oriented in a square arrangement. Generally radio frequency (RF) and direct current (dc) are applied to the rods , enabling ions with a specific m/z to have a stable trajectory and pass through to reach the detector. By simultaneously increasing the RF and dc potentials , ions of increasing m/z will pass through the analyzer and be detected. Others are drawn into the rods. Entire mass spectra are analyzed scanning over a mass

range, varying linearly the DC potential with the RF/DC ratio held constant. This occurs many times per second. Each cycle of ranges is referred to as a *scan*.

Figure 2.8 illustrates the three main part of the mass spectrometer: the ionization source, the mass analyzer and the mass detector.

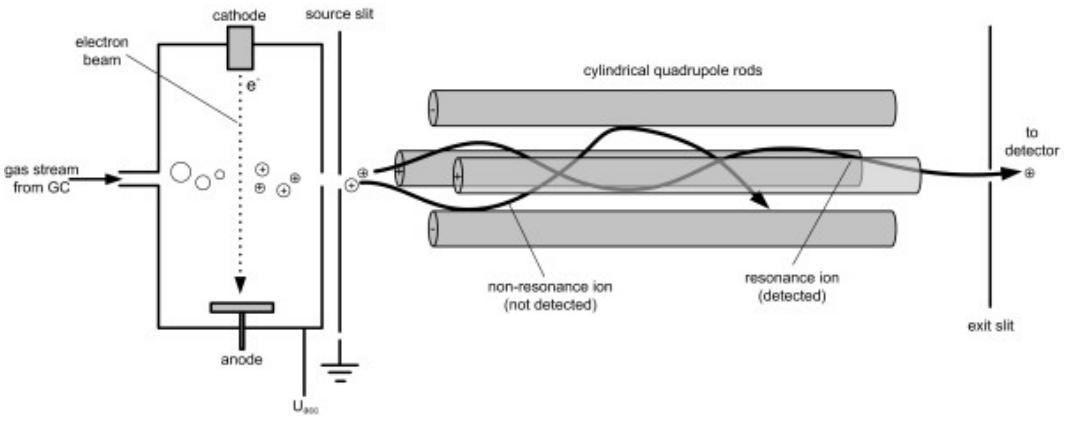

Fig. 2.8: Schematic illustration of a MS quadrupole

The quadrupole acts like a filter, separating the positively charged ions respect to their mass to charge ratio. Only ions with the proper mass to charge ratio can successfully traverse the entire filter. Uncharged molecules are pumped away. Of the four rods of the quadrupole, two are high pass filter and the other two are low pass filter. The high pass rods filter out ions with too low mass to charge ratio. The low pass rods filter out ions with too high mass to charge ratio.

Mass resolved ions travel from the analyzer to the ion detector. This is the last part of the MS, which sends information to the computer that records all the produced data. The detectors used in a MS system are required to have a fast response and a large gain to convert the small ion currents generated into recordable signals. These signals are represented in a graph in which the x-axis

represent the mass to charge ratio and the y-axis represents the abundance of each of the fragments detected during the scan. The mass spectrum can be considered as a fingerprint for the molecule, so a given chemical compound produces essentially the same mass spectrum every time.

2.5.1 Headspace analysis

Since the introduction of gas chromatography, it has been recognized the importance of sample handling, delivery and treatment for a successful analysis. Sample preparation is required in almost all GC analytical methods. Headspace analysis is generally defined as a vapour-phase extraction and it involves the partitioning of analytes between a non volatile liquid or solid phase and the vapour phase above the liquid or solid itself. The vapour phase contains fewer components than the liquid or solid phase and it is an aliquot of this mixture that is inserted into the GC for analysis. Usually the sample is placed in a sealed vial (and sometimes conditioned) until the volatile components partition into the vapour space above the sample and reach equilibrium. As a result of this process the components concentration in the vapor phase is proportional to the concentration in the original mixture. In this way is possible to obtain chemical information about the nature and composition of the condensed phase, by analyzing the gas phase.

Two different methods of headspace analysis can be carried on, depending on the conditions of the phase equilibrium: the static headspace analysis and the dynamic headspace analysis. The static method is performed when the equilibrium between the gas and the condensed phase forms a closed system.

On the contrary, when the contact between the two phases occurs in an open system, in which the gas is blown through a layer of liquid or solid phase, a dynamic headspace sampling is performed.

Static headspace sampling has been a primary tool for analysis of volatile organic compounds in a wide variety of field, such as environmental, pharmaceutical, clinical and biological analysis. This method has limited sensitivity and it is often employed for applications in the high-ppb to percent concentration range. Despite the rise of other techniques the static headspace remains the most validated of all the headspace sampling techniques [12].

The headspace to be measured is obtained form a sample, a dilution solvent and a matrix modifier contained in a vial, as shown in figure 2.9. Once the sample is introduced in the vial and the vial is sealed, volatile components diffuse into the gas phase. When the equilibrium is reached, the headspace is extracted from the vial. In a sampling procedure, it is important to maximize the concentration of volatile components in the headspace, while minimizing unwanted contamination from other compounds in the sample matrix.

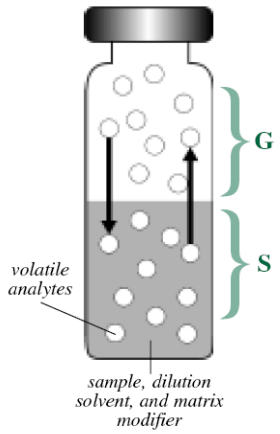

Fig. 2.9: Schematic illustration of headspace in a vial

There are two important parameters to determine the concentration of an analyte in gas phase: the partition coefficient and the phase ratio.

The *partition coefficient* is defined as the equilibrium distribution of an analyte between the sample phase and the gas phase:

$$K = C_s / C_g$$

where *Cs* is the concentration of the analyte in the sample phase and *Cg* is the concentration of the analyte in the gas phase. Compounds that have low K values will tend to partition more readily into the gas phase, and have relatively high responses and low limits of detection. Instead compounds that have high K values will tend to partition less readily into the gas phase and have relatively low response and high limits of detection.

However K can be changed varying the temperature at which the vial is equilibrated or the composition of the sample matrix. The partition coefficient depends on different parameters, such as :

- nature of the compound
- temperature
- equilibration time
- nature of the substrate
- pressure

Another important parameter to match with the partition coefficient is the *phase ratio* β, which is defined as the relative volume of the headspace compared to volume of the sample in the sample vial:

$$\beta = Vg/Vs$$

To obtain higher responses for volatile compounds β must have lower values. However, decreasing the β value will not always yield the increase in response needed to improve sensitivity. The final concentration of volatile compounds in gas phase depends on the partition coefficients and on the phase ratios. The concentration of volatile compounds in the gas phase is the result of a combination of the two contributes (*K* and *β):*

$$C_g = C_0 /(\mathrm{K} + \beta)$$

where *Cg* is the concentration of volatile analytes in the gas phase and *Co* is the original concentration of volatile analytes in the sample. Higher concentrations of analyte in gas phase result of lower values for K and *β.*

2.5.2 Solid Phase Micro Extraction

Solid Phase Microextraction (SPME) is a rapid, inexpensive and solvent less technique for the isolation of organic compounds from gaseous and liquid samples. Headspace SPME has been developed in 1993 and has been growing up in many research areas. SPME is generally applied to samples with concentrations in the low- ppb ppm range[13]. It is based on the enrichment of components on a polymer or adsorbent coated fused-silica fiber by exposing the fiber either directly to the sample or to its headspace.

It is possible to calculate the mass of analyte absorbed by coating as:

$$m = \frac{K_{fs} V_f C_0 V}{K_{fs} V_f + V_s}$$

where m is the mass of analyte absorbed by coating, C_0 is the initial concentration of analyte in sample, K_{fs} is the partition coefficient for analyte between coating and sample matrix while V_f and V_s are respectively the volume of coating and the volume of sample.

The coating used for SPME has a typical affinity for several organic compounds. Different coating materials have different selectivities to organic compounds and different values of K_{fs} can be obtained.

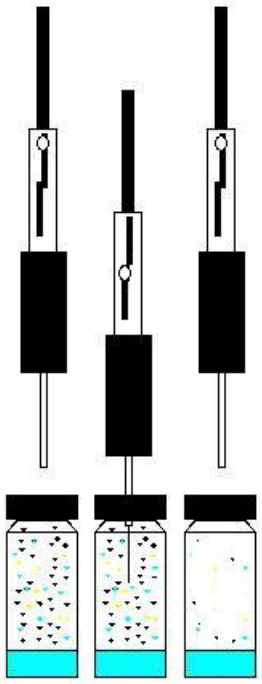

Fig. 2.10 Schematic illustration of the headspace SPME extraction method

Generally the fiber used for SPME are produced from Supelco with a several range of coatings. Figure 2.10 illustrates the SPME method, which uses a fine fused-silica fiber coated with a polymer, usually polydimethylsiloxane or polyacrylate, to extract organic compounds from their matrix. For gaseous and low molecular weight compounds for example, generally CAR/PDMS (carbon/

polydimethylsiloxane) fiber is used. SPME fiber combines sampling and preconcentration in a single step. After a well-defined adsorption time the fiber is removed and transferred to a standard split/splitless injector of GC/MS system, where the organic compounds are thermally desorbed from the polymeric phase, in the hot injector of the gas chromatograph.

The fiber can be used both in aqueous samples and in gas phase samples. The time of extraction is lower using headspace because the diffusion of analytes is many times greater in vapour phase than in the aqueous phase. The equilibrium of the analytes between the gas phase and the polymeric fiber is achieved in a few minutes, whereas in direct aqueous extraction, where the SPME fiber is directly introduced into the aqueous matrix, the sample must be stirred intensely in order to shorten the equilibration time.

A further advantage of the headspace SPME approach is that samples from virtually any matrix can be analyzed since the fiber is not in direct contact with the sample, although care should be taken to release analytes efficiently into the headspace [14,15].

2.6 Electronic noses

Instrumental methodologies, already described, for odours analysis, such as gas chromatography/mass spectrometry (GC/MS) are expensive and require for trained personnel. This complexity lead to ask for other methods to analyse the volatile compounds. A lot of applications need low response time and simple methods to analyse gas.

For this reason several kinds of sensors have been developed in the last decade and used in microarray sensor devices. In particular, the possibility to use chemical sensors was investigated. Chemical sensors interaction with the

volatile compounds have been studied together with the role of these sensors for the detection of molecules at relatively low concentrations. Devices using an array of these sensors with non-selective proprieties are generally called 'electronic noses'.

Persaud and Dodd first reported the design of an electronic nose using chemical sensors and pattern recognition in 1982[16]. The aim of their work was to mimic the mechanism of natural olfaction to detect and discriminate profiles of volatile compounds.We have described in the previous chapter the mechanisms of human odour perception. Natural olfaction is a complex sensorial system which leads to brain's odor perception. It is possible to define a parallelism between the human olfactory system and an artificial olfactory system (fig.2.11). Odour perception does not only consist of the interactions between volatile compounds and an olfactive receptor, but it is the result of pattern of different type of receptors response that are activated by odorous molecules which produce different olfactive maps in the brain.

Artificial olfaction wants to be the translation of these single steps into chemical, electronics and mathematical processes, in order to measure instead of perceive. An olfactory system includes as into the brain an appropriate pattern recognition system, capable of recognizing simple or complex odors. The most important applications were developed for the detection of microbiological, food safety and medical applications[17].

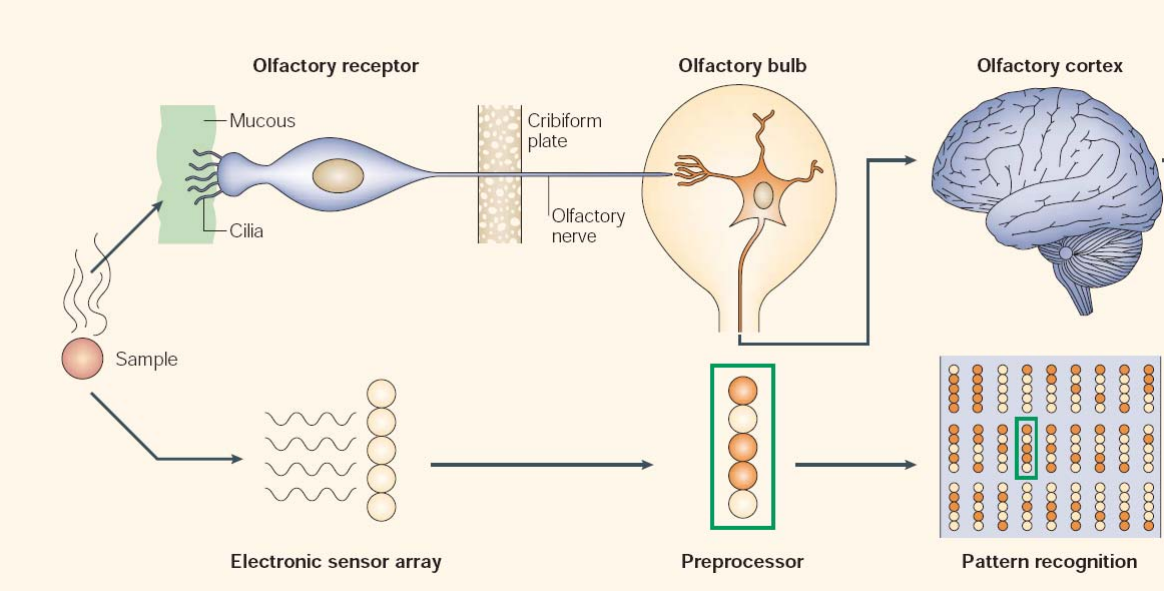

Fig 2.11: parallelism between the human olfactory system and an artificial olfactory system extracted from *A.P.F. Turner, N. Magan, Electronic noses and disease diagnostic, Nature review, Microbiology, vol.2, Feb.2004, pp. 161-166*

An e-nose is physically a gas sensor array, whose aim is the identification and quantification of volatile compounds. A gas sensor array, inspired to the olfactory system, has to be non-selective, enough sensitive and capable of responding to mixtures of gas. The definition of e-nose given by Gardner and Bartlett is: *an instrument, which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern-recognition system, capable of recognizing simple or complex odors*[18].

An electronic nose is composed of some fundamental parts. The sensing material deposed on sensors surface which catches the odorant molecules. In this phase a chemical signal is obtained and a transducer transforms the chemical information in a physical one. A basic device translates the physical quantity variation in an electric signal. Several electronic nose devices have been developed during the years, based on different implementation of the mentioned components. The first commercial e-nose used *conducting polymers sensors* . Different polymers are deposed on the sensors surface. Each polymer

reacts with the volatile compounds in a different way with respect to the others. In particular these sensors are based on a variation of conductivity when they interact with volatile compounds.

The *metal oxide sensors* are based on a variation of conductivity of the oxide when the sensing material interact with the volatile molecules. The oxide materials contain chemically adsorbed oxygen species.

In the *metal oxide silicon* field-effect sensors the output consists of a change of potential when the volatile compounds react with the catalytic surface. The electrical properties of the semiconductors are sensitive to the gases with which they are in contact. Taguchi were the first commercial sensors using the gas sensitivity of semiconductors[19].

In the *optical sensors* an interaction with the volatile compounds with a light source that excites the molecules results in a change of absorbance, reflectance or chemiluminescence. In this thesis an electronic nose based on mass sensors was used. These type of sensors are based on piezoelectric effect developed in two different modalities: bulk acoustic wave (BAW) and surface acoustic wave (SAW). BAW sensors are also known as Quartz Crystal Microbalances (QCM). These sensors consist of a thin slab of crystalline quartz, cut along a certain symmetrical axis in order to obtain a material able to sustain bulk electro-acoustical oscillation at certain frequencies.

The most commonly utilized for sensory applications is the so called AT cut which oscillate in a thickness shear mode around resonant frequencies in the range 5 - 30 MHz [20]. The AT-cut is obtained cutting the quartz with an angle of 35°25′ with respect to the crystallographic z axis. The most important advantage of this type of cut is that it permits to obtain a quartz with characteristics that are minimally sensible to the temperature, in the interval 10-50°C.

Saw sensors can be fabricated on a piezoelectric crystal by the use of an inter-digitated transducer (IDT). On 1979 the use of SAW as gas sensors was demonstrated.[21]The wave is propagated from the IDT, along the crystal. The area in which the wave travels is known as the delay line and it is the place in which there is the interaction with the sample. This interaction influences the parameters of wave transmission, resulting in a detectable signal.
There is a wide range of transducer available that differs from each other on the physical quantity analyzed (tab.2.12):

Principle of Transduction	**Transducers**
Fluorescence	Optical Fiber
Conductivity	Chemoresistance
Ionic Current	Amperometric Gas Sensor
Temperature	Thermopile
Mass	Cantilevers, Surface Acoustic Wave, Quartz Crystal Microbalance

Tab. 2.12: sensors features: transduction mechanism and relative transducers.

2.6.1 Piezoelectric sensors: quartz microbalance.

Piezoelectric materials oscillate if an alternate voltage is applied. This effect was already known and used in telecommunications system for frequency control applications, but the possibility to obtain information by frequency change was also investigated.

The properties of piezoelectric materials were used to develop mass sensors known as quartz microbalance (QMB)[22]. The Quartz Microbalance

Sensor (QCM) is a thin crystal quartz placed between two electrodes. The oscillation mode depends on the cut and on the geometry of the crystal. In 1959 Sauerbrey demonstrated that the mass can be calculated by the frequency change related to the mass load on crystal surface[23]. In particular he used the change in the frequency of a quartz resonator to measure the mass of a film adherently deposited on the quartz resonator surface. Mecea in his studies on quartz microbalance and mass sensors showed as the mass detected by these sensors is of 10^{-19} Kg while the mass detected with the commercial analytical microbalances is of 10^{-10} Kg[24].

Sauerbrey explored the possibility to use the QCM as quantitative mass measuring devices and demonstrated that for quartz crystal resonator (with AT or BT cut) the change of frequency was proportional to the added mass of the deposited film (Eq1):

$$\Delta f_0 = \frac{-2 f_0^2}{A\sqrt{\mu_q \rho_q}} \Delta m_s \qquad \textbf{Eq 1}$$

where f_0 is the fundamental resonant frequency of the quartz, ρ_q = 2.65 kg/dm^3 the quartz density and A is the surface area of the deposited film, the mass of which is m_s. An interesting aspect of these sensors was the dependence on the gravitational acceleration. In a tradition weight equilibrium balance the sensitivity depends on the gravitational acceleration. At this regard Mecea showed that the quartz microbalance working principle is independent on the gravitational acceleration. For a balance with arms of equal lengths the equilibrium is obtained when a mass (m_1) placed on a plate and the other mass (m_2) placed in a second plate are equal:

$$m_1 g = m_2 g$$

The equilibrium is obtained when $m_1 = m_2$, whatever the value of the acceleration is. So the two bodies with masses m_1 and m_2 will be in equilibrium both on the Earth and Moon. But generally the balance sensitivity for a beam balance can be calculate from the α angle obtained when the two mass are different (fig. 2.12):

$$\alpha = K(m_1 - m_2)a$$

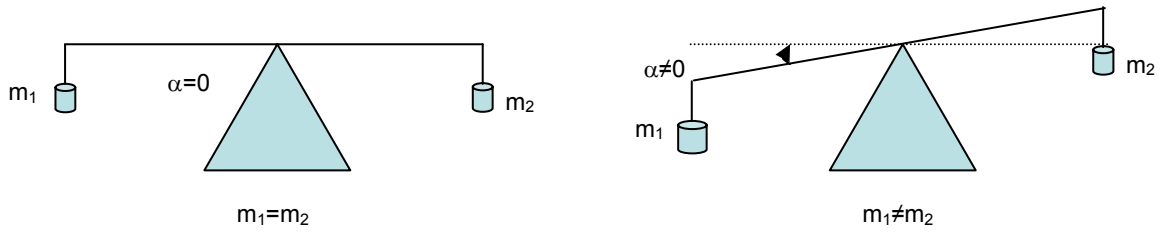

Fig. 2.12: schematic view of balance functioning for the calculus of its mathematical law.

α=0, whatever the acceleration is, when the two masses are equal. When the two masses are different α depends on the acceleration *a*. So if on the Earth α=6°, on the Moon α=1°. For this motive the common balances can't be used on a space laboratory orbiting around the Earth, because the gravitational acceleration is cancelled by the centrifugal acceleration. On the other hand the QCM can be used for space application because the mass deposited on the surface of a quartz crystal resonator is subjected to an acceleration caused by the vibration of the quartz resonator[25].

So a material deposed on a QCM doesn't depend on the gravitational acceleration *g*. But Mecea observed also as on the surface of a quartz crystal resonator the acceleration of the shear vibration varies from zero to several millions times the gravitational acceleration *g* over a distance of 2–3mm from

about the electrode edges to the electrode centre. In the same way the mass sensitivity varies with the distance from the QCM centre[26].

2.7 Chemical Interactive Material (CIM)

Until now we have described some examples of sensors used to build the electronic noses. Further the transduction techniques, the most important part of a chemical sensors is the material deposed on sensors surface with which the analyte interacts. The development of a chemical interactive material (CIM) requires the study of the adsorbent dynamic. Electronic noses are based on non-selective sensors arrays whose chemical interactions are generally reversible, weak and non specific.

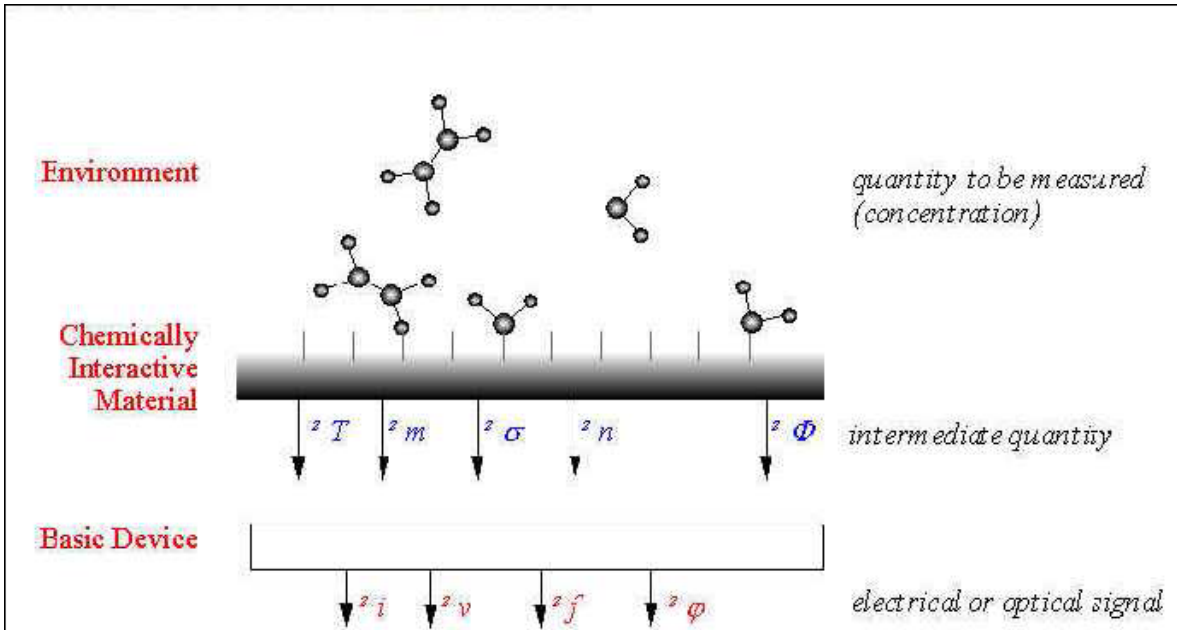

Fig. 2.13: schematic representation of the chemical intractive material as an intermediate between the environment and a sensor basic device.

Figure 2.13 illustrates the function of the CIM as an intermediary between the basic device and the environment.

The selection of a different CIM coating for each sensor composing the array is important to obtain a pattern of different responses. This difference in the responses reproduces what happens in natural olfaction, where each olfactory receptor gives a different response to the same stimulus and pattern of all receptors responses are activated simultaneously.The use of a non selective sensor arrays with different coatings, instead of a single sensor, grants the sensitivity to a wide range of analytes, and the capability to recognize complex mixtures of compounds. It has to be noted that, as in natural olfaction, the odor is not described as the sum of its individual components, but by qualitative representation, a sort of fingerprint or odor image.

Electronic nose strategy is based on the instrument sensitivities to the totality of the compounds, rather than to a specific one. In this viewpoint, an electronic nose is an array of individual sensors different one each other but globally selective according to the principle that each sensor senses more compounds and each compound is sensed by more sensors[27].

In the next paragraph a brief description of a chemical interactive material, the metallo-porphyrins, is presented. This kind of coating has been used as CIM for the electronic nose used in this work .

2.7.1 Metalloporphyrins

In the last decades the possibility to use different materials to capture the volatile compounds with different selectivities have been investigated. In particular a great interest has been devoted to non-selective materials for olfactory systems implementation. To distinguish gas mixture without separation selective sensors are needed as each of them can detect a particular

compound. From the seventies the non-selective materials have been investigated as a new approach to analyse the gasous mixture.

The possibility to sense more compounds opens the access to global information about the gas mixture. Some of these sensors began to be used in electronic noses.

Among the different materials with non-selective properties the metalloporphyrin will be now described . Porphirins are fundamental for life because of their functions as complexing ligands or redox catalysis essential for all organisms[28]. They are macrocycle and can be modified with simple changes on the basic molecular framework. The general structure of porphyrins consists of four pyrrole rings linked by methine bridges: the corresponding macrocycle is fully conjugated, containing an 18-electron aromatic π system and the molecule is planar. The porphyrins show higher absorbance for their aromatic system[29].

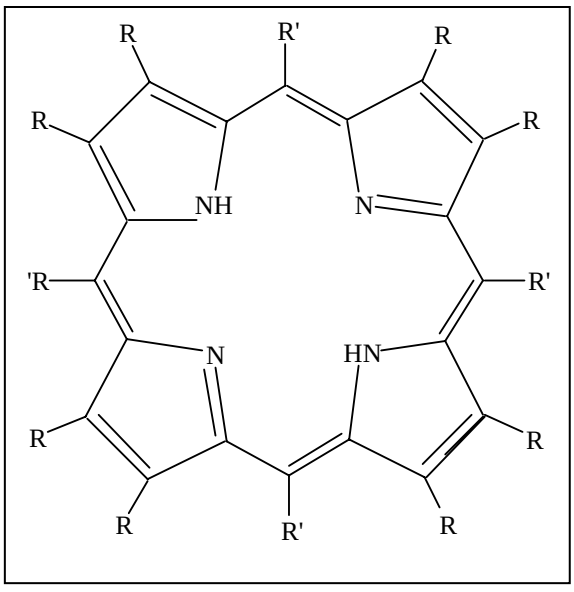

Fig. 2.14: general structure of a porphyrin

Its structure is a powerful coordinating system thanks to four nitrogen placed in the core of the molecule(fig.2.14). Almost all the elements present in the Periodic Table can coordinated to the porphyrin[30]. This basic structure is turned into metalloporphyrin when a transition metal atom replaces the two hydrogen atoms at the central core.The versatility, stability and reproducibility are the peculiar characteristics of this molecule. These properties have proposed the metalloporphyrins as a good material for chemical sensors.

In particular in this thesis the metalloporphyrins are used as coating material for QMB. As it has already been described the main feature of such sensors is the link between the sensing properties and the nature of the central metals and lateral goups. With small variations in the synthesis process it is possible to get sensors with different behaviors. To ensure the necessary porosity of the solid state for an optimal analyte diffusion through the absorbing molecules have been functionalized with four alkylic chains (O(CH2)3CH3). In particular the electronic nose used in the applications of this thesis is based on eight QMB coated with different metallo porphyrins:

Cu Butiloxy Tetra Phenyl Porphyrin
Co Butiloxy Tetra Phenyl Porphyrin
Zn Butiloxy Tetra Phenyl Porphyrin
Mn Butiloxy Tetra Phenyl Porphyrin
Fe Butiloxy Tetra Phenyl Porphyrin
Sn Butiloxy Tetra Phenyl Porphyrin
Ru Butiloxy Tetra Phenyl Porphyrin
Cr Butiloxy Tetra Phenyl Porphyrin

this is the sensors array of the last version of electronic nose developed at University of Rome "Tor Vergata". Since 1994 it has been used in several

applications such as medical, food industry, environmental monitoring, each of which has asked for ad hoc modification in the sampling procedures, in the chemistry of the sensing material and of course in the sensors themselves. The medical applications will be described in the next chapters. With regards to the gravitational acceleration for QMB, the more recent version of this kind of nose equipped with QMB sensors was optimized to work in microgravity conditions and aimed at measuring the air quality inside a spacecraft. The instrument has been successfully tested in April 2005 during the ENEIDE mission on board of the International Space Station[31].

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CHAPTER 3

The case of Melanoma

3.1 Introduction

The relationship between diseases and alterations of the airborne chemicals emitted from the body has been found in many different pathologies and in particular for various forms of cancer. Metabolism of cancer cells is greatly alterated during their life time; then modification of chemicals are supposed to be large around cancer tissues. Positive hints in this direction were provided, as an example, studying the breath composition of lung cancer affected subjects. Beside the conventional analytical approaches, in recent years sensor arrays was also applied to these researches considering the chemical composition changes as those occurring in other applications such as food quality.

In this chapter the first application of sensor arrays to study the differentiation between melanoma and nevi, namely malignant and benign affection of melanocytary cells respectively, is presented and discussed. The localization of lesions on the skin surface made possible the utilization of differential measurements aimed at capturing the differences between two adjacent skin regions. This approach strongly reduces the influence of skin headspace variability due to the peculiar subjective odour background and to the skin odour variability.

The measurement campaign involved 40 cases, ten of them were diagnosed melanomas referring to surgical intervention. Nine of these diagnoses were furtherly confirmed by histological examinations of the removed tissue and one was a false-positive. The differences in chemical composition of headspace were verified with a gas-chromatographic investigation, and the classification of electronic nose data provided an estimated cross-validated accuracy of the same order of magnitude of the currently employed diagnostic instruments.

3.2 Melanoma diagnosis: state of art

Chemical compounds exhaled from the skin surface are the result of a combination of several contributions. The most important sources are the skin glands emission, subjected to hormonal control, and bacterial populations at skin surfaces, which live metabolizing and transforming organic compounds. Any alteration of this equilibrium, for instance due to some pathologies, induces changes in both the nature and the amount of volatile compounds. Since the equilibrium is subjective in character, the chemicals can greatly change from individual to individual giving rise to a sort of personal chemical fingerprint.

Beside metabolism changes, any alteration of the skin surface is supposed to modify the quality and the quantity of the exhaled compounds. Such changes may include non usual micro-organisms, such as fungii, or more frequently skin dots more properly said nevi. Nevi can undergo further modifications such as melanoma that is a particular tumor generated from the melanocytary cells. This kind of tumor is not very frequent, nonetheless there has recently observed a clear increase in the number of cases: in the last thirty years in the white USA population melanoma has strongly increased in about all the age ranges but with a positive 5 times higher rate in male older than 65 (from 18,8 to 91.9/year in 100.000) and with lower rates also for the women of the same age range [1,2].

So far, surgery demonstrated to be the unique efficient therapy for melanoma and it can be considered resolutive when a melanoma is removed in its early stage; this is because, unfortunately, both medical oncological therapies and radiotherapy are unsuccessful on advanced and metastatic melanoma. In order to achieve an early diagnosis of melanoma, in the last years the clinical

ability of the dermatologists based on the visual inspection of supposed melanoma have been complemented by other "non invasive" methodologies based imaging.

3.2.1 Diagnostic techniques of melanoma

The more simple of them is dermatoscopy that coupling semeiologic criteria with image analysis algorithms, improves the clinic diagnosis accuracy from 5% to 30%[3]. It is a non invasive technique that uses a dermoscope equipped with a transilluminating light source and standard magnifying optics. A liquid (oil, water, or alcohol) interface is applied to the surface of the skin and the dermoscope lens is immersed into the fluid covering the lesion. The role of the liquid is to decrease light reflection, refraction and diffraction. The results is an image of the subsurface of the epidermis not discernible to the unaided eye[4].

A more sophisticated technique is the digital systems based on epiluminescence images. This procedure allows time monitoring of pigmented lesions suggesting surgical removals only for those lesions which show substantial modifications with time[5]. A recently introduced method is based on confocal laser microscopy that has been applied to study the pigmented lesions with encouraging results[6]. The accuracy of these methods is variable; the percentage of correct clinic diagnosis based on visual inspection are about 70%, this value is interesting because it indicates that about 30% of cases have not clearly visible features.

With dermoscopic test not diagnosed cases are reduced to 8%. The largest number of these cases comprise both "nevus-like" melanomas (those that have not yet showed the clinic and dermoscopic characteristics of the melanoma and thus resembling as nevus) and the "hypomelanotic" melanomas (in which only few characteristics of the vascular reticulum, if present, suggest the presence of the melanoma)[7].

In the last years ultrasounds have been applied in clinical dermatology. The images are obtained from different properties of tissues. High impulses are transmitted into the skin and then reflected, refracted or inflected when a tissue interface with different acoustic impedece is encountrated[8]. Finally, it is worth to remark a number of new technologies still oriented to non-invasive approaches and using techniques like Nuclear Magnetic Resonance[9] and based on the sentinel lymphnodes monitoring [10]. By this technique the tissues are exposed to a strong magnetic field .A proton in a strong magnetic field, when influenced by a radiofrequency pulse returns to a stable low energy state and gives off a weak radio signal that is detected by the coil, which acts as an antenna. Tissue contrast is the results of the differences in relative MRI signal intensity between the structures[11].

Eventually, none of the image-based diagnosis techniques offer a diagnosis guarantee of 100%. From the chemical activity point of view, in general tumor cells are characterized by an altered metabolism expected to give rise to an anomalous compositions of the emitted volatile compounds. In many cases, the measure of volatile compounds has been proven to provide sufficient information to identify tumors. For instance, lung cancer was studied for several years and a number of investigations evidenced the correlation between the anomalous concentration of a group of compounds and the presence of the disease [12]. In case of melanoma, an indirect demonstration of the possibility to identify a melanoma through the airborne chemicals has been given by trained dogs that used their olfaction to locate tumors[13].

Nonetheless, dog perception is rather complicated and largely based on sense integration then it is not completely clear if only olfaction is sufficient to detect the disease. In other word, it is still not clear if the volatile compounds emitted from a melanoma are sufficiently different from those emitted by a nevus or from a normal skin.

Beside this last example, most of the investigations about the relationship between exhaled chemicals and diseases were carried out developing analytical methods aimed at discriminating specific compounds directly related to the presence of the disease.

On the other hand, in recent years novel methodologies based on the use of arrays of partially selective chemical sensors for the classification of complex samples appeared. Such arrays are currently identified as electronic noses because of some similarity with the human sense of olfaction. Chemical sensor arrays were demonstrated to be able to identify a number of different diseases[14]. Among them also a tumor forms like lung cancer[15]. The analysis of skin was also investigated to study the evolution of ulcers and wounds [16] and the influence of metabolic alteration such as those supposed to take place in neurological disorders[17].

In this chapter the first investigation towards the identification of melanoma measuring the volatile compounds emitted from the lesion with a chemical sensor array is presented. The chapter is concerned with a crossectional study involving 40 individuals with suspect melanoma. 10 cases were diagnosed as melanoma according to conventional diagnostic criteria, including epiluminescence and for some of the individuals the histological inspection of the tissue. It has to be remarked that melanoma is a rather rare tumoral form, in Italy in the last year the rate of insurgence of new cases is around 5-7 cases each 10000 habitants per year, so in order to achieve a sufficiently large statistics may require a very long time. The data here reported shown in about 80% of correct discrimination of melanoma from nevi, a value not far from the performance of the more diffused diagnostic methods.

3.3 Experimental description

The experimental campaign took place for six months at the "Istituto Dermopatico dell'Immacolata" (IDI) in Rome. A total number of 40 patients presenting suspected lesions has been enrolled for this study. Each tested lesion was characterized by epiluminescence and for those cases requesting surgical removal the histological report was also available. A sub-group of 7 individuals who underwent surgical removal of melanoma were measured before and after the intervention. The experiment was approved by the local ethical committee and all the individuals adhering to the experiment were fully informed about the scope of the research.

The instrument used for this experiment is a last version of the electronic nose developed at the University of Rome 'Tor Vergata'. It has been already described in the previous chapter. The steady state frequency shifts of the electronic nose sensors gives rise to a pattern, and a collection of measurements produces a set of pattern that it is properly analyzed by some pattern recognition algorithm for classification purposes.

3.3.1 Measurements strategy

One of the most important, and often underestimated, steps in electronic nose analysis is the procedure of sample uptake. The sampling methodology has to ensure a sufficient reproducibility and it should contain enough concentration of those chemicals relevant to the investigated pathology. Figure 3.1 shows a schematic view of the sampling system developed to sample the headspace of small skin regions.

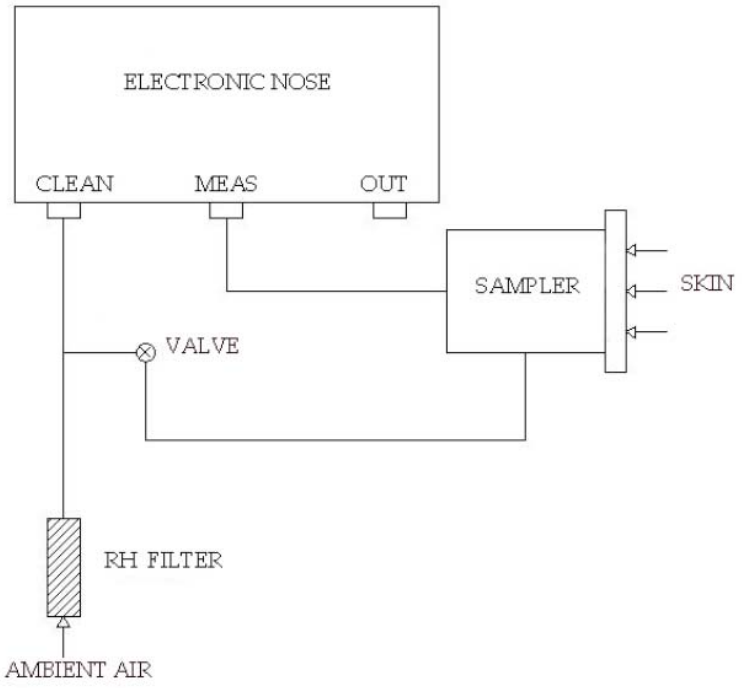


Fig. 3.1: Schematic overview of the measurement set-up elements and their pneumatic connections. RH filter is a standard CaCl filter mainly for ambient water vapour content attenuation.

The sampler is a stainless steel cylinder used to insulate a skin region of 4 cm of diameter. The area fitted with the size of all the cases here investigated. The use of the sampler was complemented by a differential measurement strategy aimed at enhancing the difference of chemicals emitted by melanoma with respect another portion of the skin used as reference. It is worth to point out that skin may be characterized by a large subjective variability in terms of quantity and quality of the emitted chemicals as it was evidenced by previous investigations [18]. On this basis it is rather likely to suppose that very close cutaneous regions are characterized by the same headspace, then for each measured lesion, a reference measure taken in correspondence of the closer free skin region was considered. Lesion and reference measurements were performed with respect to dry air which was chosen as the reference gas. The

final sensor response for each lesion was calculated as the difference of two frequency shifts calculated as the steady state frequency shift between lesion and dry air, and reference skin area and dry air respectively. The whole measurement protocol is graphically shown in figure 3.2.

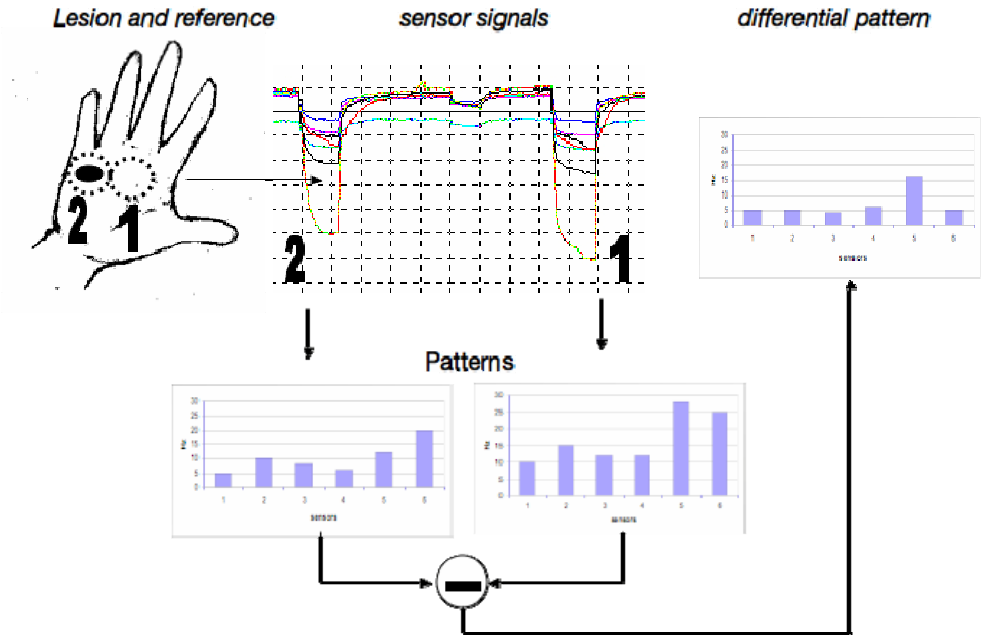


Fig. 3.2: Conceptual sketch of the differential measurement. A region closer as much as possible to the lesion to be measured is used as a reference. Sensors are then exposed to the air collected from the two surfaces and signals are subtracted to form the differential pattern used in the following data analysis.

The differential strategy used for the estimation of the final Δf response permits to separate as much as possible the contribution to the skin headspace due to the measured lesion disregarding the skin headspace composition peculiar of the individual and the skin region. The dry air flow during sensor flushing and measurements is schematically shown in figures 3.3 and 3.4.

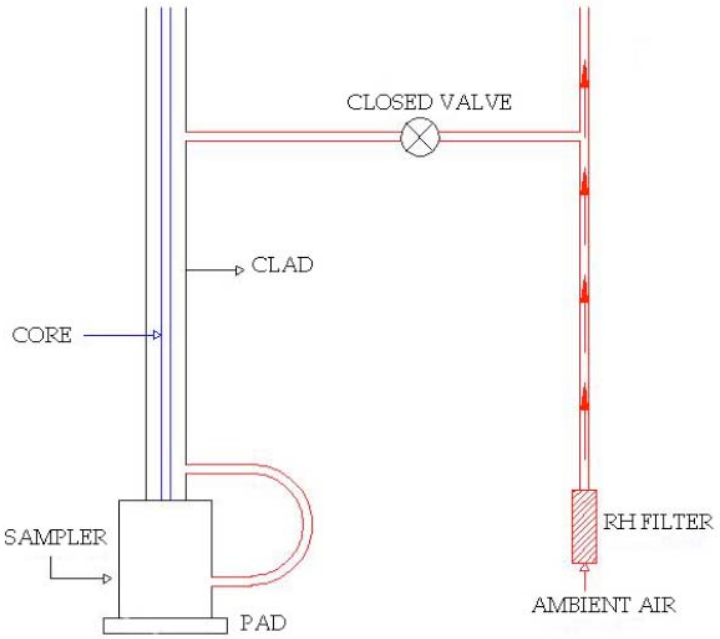


Fig. 3.3: During the flushing of sensors cell the ambient air, filtered by the CaCl cartridge is directly injected in the electronic nose inlet.

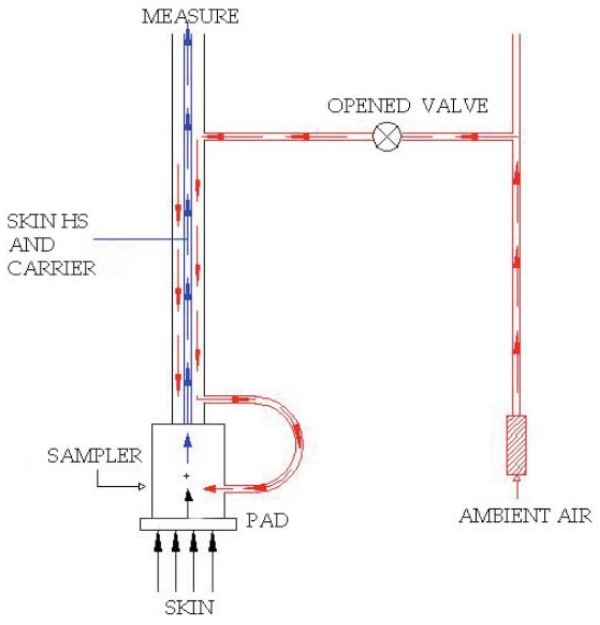


Fig.3.4: Skin odour is probed washing the skin surface with the filtered ambient air circulating in the external part of the coaxial tube of the sampler. Skin odour is then uptaken into the sensor cell by the electronic nose internal pump.

During the measurement, the valve switches the dry air flow inside the cladder of the coaxial tube. In this way the dry air works as a carrier gas for the sample. The skin headspace is finally up-taken inside the core of the cohaxial tube to reach the sensors cell. Although, strongly reduced by the differential measurement the use of perfumes and detergent may interfere with the measurement. For this reason, skin headspace sampling protocol was complemented by patients conditioning. Individuals adhering to the tests were requested to observe simple hygienic rules. The skin portion under analysis was lightly washed no less than two hours before the measurement with a neutral soap. For the scope, a single brand of a commercial soap was provided in advance to all the participants.

3.3.2 GC/MS measurements

Zhang et al. [19] developed a sampling device to analyze chemicals emitted from the skin of limbs using the Solid Phase Micro Extraction (SPME) with GCMS; this sampling device consisted of a canister enclosing the limb. Similar investigations, as described in the first chapter, were carried out for the identification of methane, ethylene, ethane[20], and ammonia [21] released by skin. Curran et al. (as seen in the first chapter) also tried to identify the volatile organic compounds present in human skin odor using SPME-GC/MS. They aimed at characterizing the various types of compounds present in the headspace above axillary sweat samples, and also at studying qualitative differences and similarities between male and female subjects. This last protocol is the one which best fitted the experimental conditions of the present work. The main modification is relative to the storage time of the sample at ambient temperature before SPME extraction.

Samples were collected wiping the skin surface with sterile 10x10 cm^2 gauze pad from Amukinmed, following the same procedure adopted by the Scent Transfer Unit-100 (STU-100) developed to aid US law enforcement with forensic tasks[22]. Gauze pads were stored and immediately sealed in 20 ml glass vials, crimp seal vials with PTFE/silicone septa (Supelco, Bellefonte, PS, USA). All samples were stored in ice to transport them, and were then stored in a freezer until analysis. Each sample was stored at room temperature for 9 hours prior to extraction.

Divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR on PDMS) 50/30 µm fiber (Supelco) was used to extract the volatile organic compounds from the headspace of the vials. Exposure was performed at room temperature for 15 hours inserting the fiber into the silicon septum of the vial. Shimadzu GCMS-QP2010 gas chromatograph mass spectrometer was used with an EQUITY-5 column, 30 m length, 0.25 µm thickness and 0.25 mm diameter, with helium as a carrier gas. The analytes, adsorbed in the fiber, were desorbed in the injection port of the GC, for 3 minutes at an inlet temperature of 250 °C, in splitless mode. The GC method was initiated with an initial oven temperature of 40°C for 5 min. The temperature was then ramped at 10°C/min until it reached 300°C, and then was held at 300°C for 2 min (total run time: 33 min). The mass spectrometer was used with a quadrupole analyzer in full scan mode (range: 50-550).

The interface and ion source temperatures were maintained at 250°C. The solvent cut time was 3 minutes. Mass spectra were obtained in TIC mode by electron impact. The compounds were identified by comparison with mass spectra from the NIST library database .

3.4 Results

The whole experiment consisted in 47 measured skin lesions corresponding to 40 different individuals. Seven lesions were measured before and after the surgical intervention. The lesions were classified according to the medical report of epiluminescence, and, in some cases, by histological analysis on removed tissues. The largest number of lesions were nevi while 10 lesions were diagnosed as melanoma. It is worth to point out that one case of the ten diagnosed as melanoma resulted negative to the histological screen, then it may be considered as a false positive. Figure 3.5 shows the classification of all the studied cases.

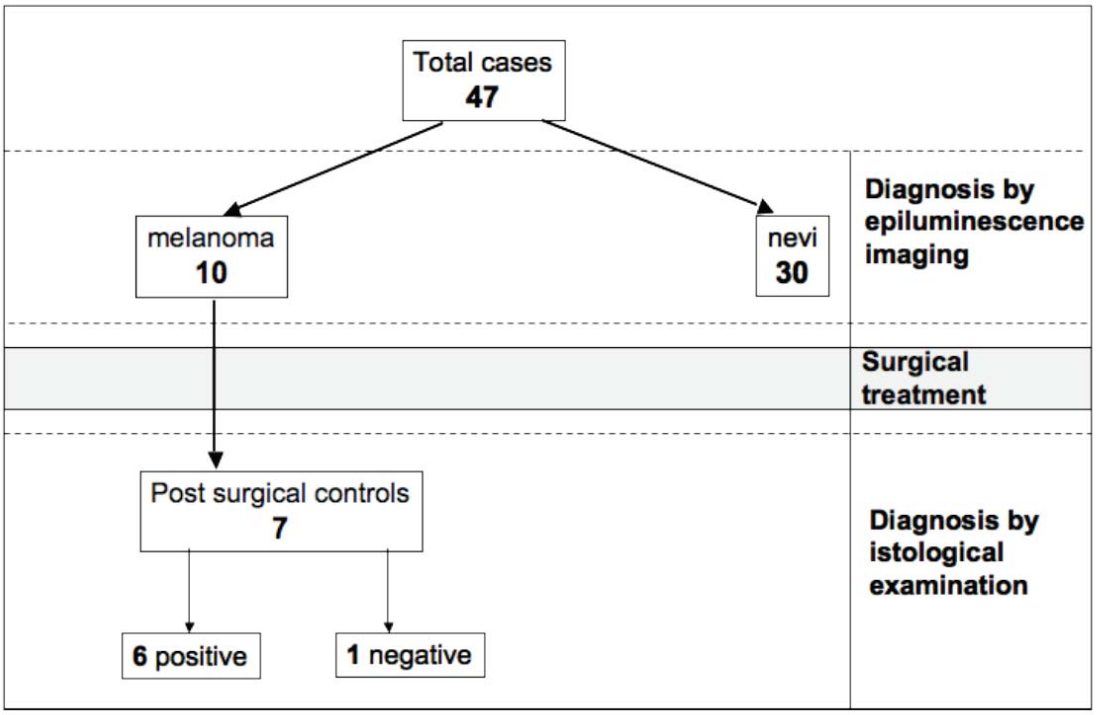


Fig. 3.5: Conceptual partition of the cases participating in the cross-sectional study.

The main differentiation is that provided by the epiluminescence image analysis. Only for seven cases a post-surgical diagnosis was available. For these

cases a histological report was also available. Six histological reports confirms epiluminescence diagnosis this fact is in agreement with the expected accuracy of epiluminescence based diagnostics.

The differential measurement protocol was tested on one subject sampling several nevi in different body regions. The amount of sensor signals is rather variable on the body surface, nonetheless differential patterns were qualitatively constant (same intensity ratio between the different sensors) in any parts of the body. Then, it is possible to suppose that the 'sensors pattern shape' is characteristic of the individual, while the intensity of the response is characteristic of the different region of skin sampled. These tests indicate that differential methods and sampler are efficient to guarantee repeatable measurements and to maintain the pattern characteristic for the individual and for the body part under test.

3.4.1 GC/MS measurements

The differences between the chemicals emitted by melanoma and nevi have been studied with the GCMS sampling the headspace of the lesions with the same method used for the electronic nose. The same differential methodology was applied; for each measurement two area were sampled: the one containing the lesion and the closest lesion-free area. In figure 3.6 two examples are shown, they were calculated subtracting the chromatogram obtained for the lesion free skin measure to the one relative to the lesion.

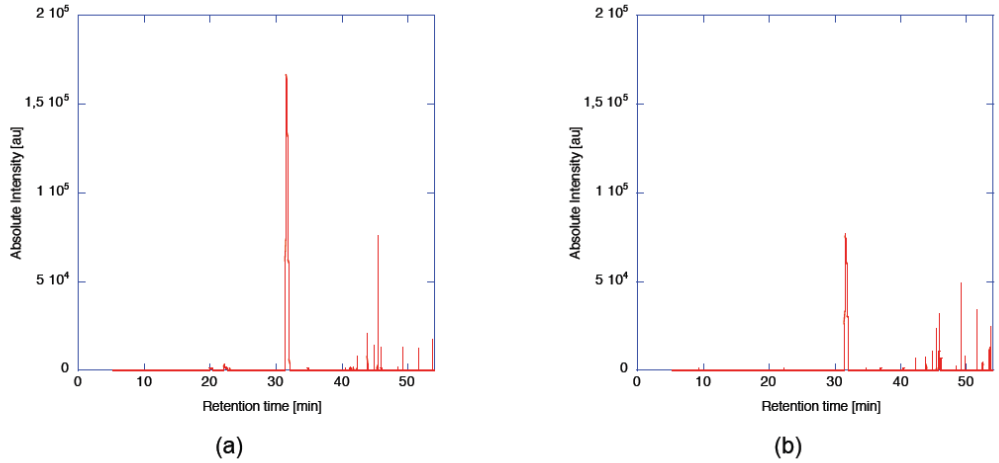


Fig. 3.6: In figure two differential chromatograms are shown. Differential chromatograms are calculated subtracting the recorded relative abundances of two chromatograms in correspondence of the same retention times. Curve a is the differential chromatograms between melanoma and a closer reference skin region and in curve b the same curve is obtained in the case of a nevus and its closer reference skin region.

Melanoma and nevus appears significantly different from both qualitative and quantitative points of view. The identification of the compounds marking the difference between the two cases is beyond the scopes of this thesis. It has to be remarked that the identification of specific compounds is necessary to connect these findings with the biochemical processes related to the presence of melanomas.

In this case two cases are decribed on two patients affected by melanomas at a different stage and located on right shoulder. The measurements have been performed on three points: skin cancer on the right shoulder, anearby portion of clean skin and nevus close to the same area. In Fig. 3.7 the three chromatograms relate to this measurements are reported.

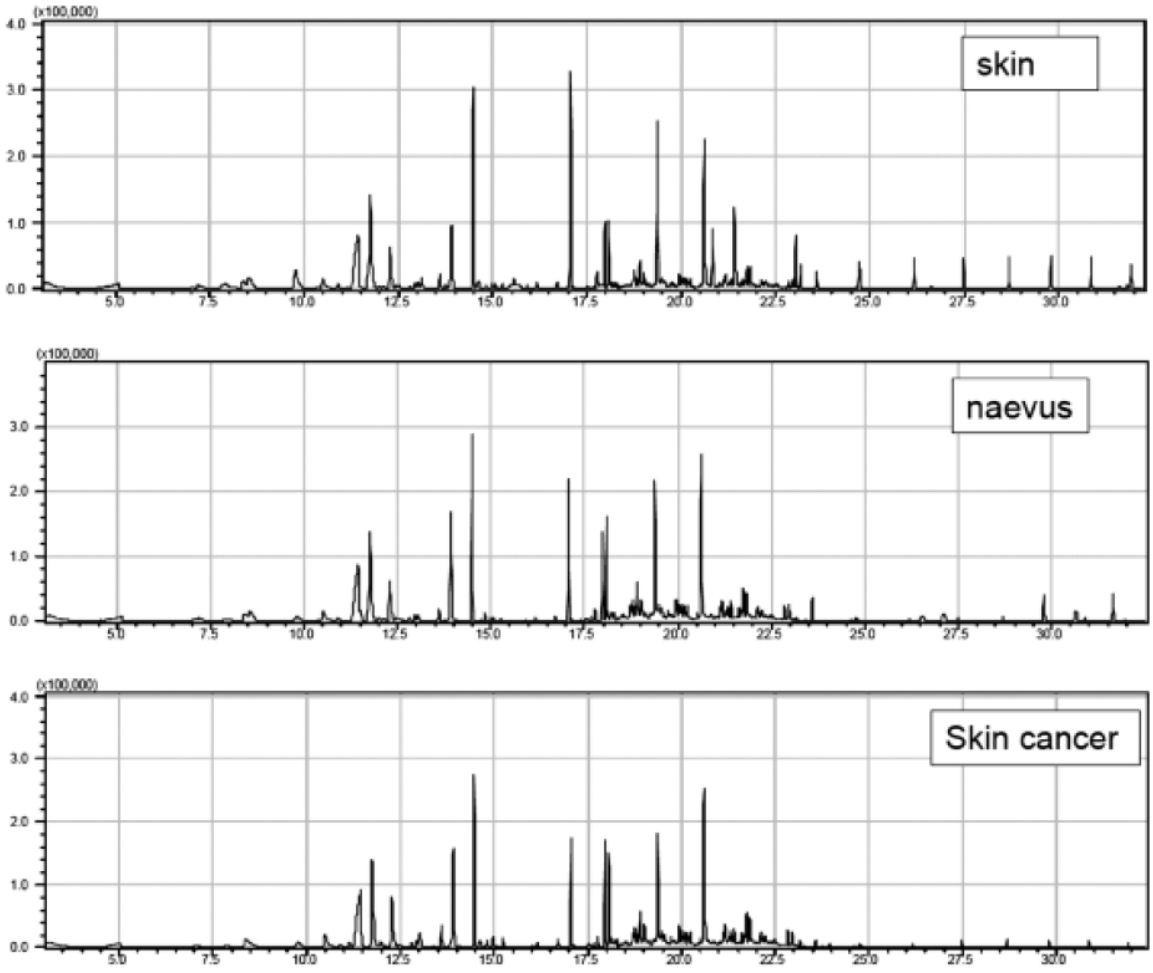


Fig. 3.7: chromatograms relate to three points of the same individual.

The chromatograms are very similar with little differences; nonetheless, a limited number of compounds appear only in the chromatogram related to skin cancer. The relative abundance of these peaks is low, and as a consequence the identification of the compounds is not reliable. A qualitative analysis of GC/MS data has been performed partitioning the chromatogram in pre-definitive set of retention time intervals and analysing the data with multivariate statistical methods.120 variables have been reduced to 12. In the Fig. 3.8 the score plot of the first two principal component analysis are shown. The most important

information is related to the discrimination between the cluster of skin and nevi grouped into a single cluster and skin cancer data.

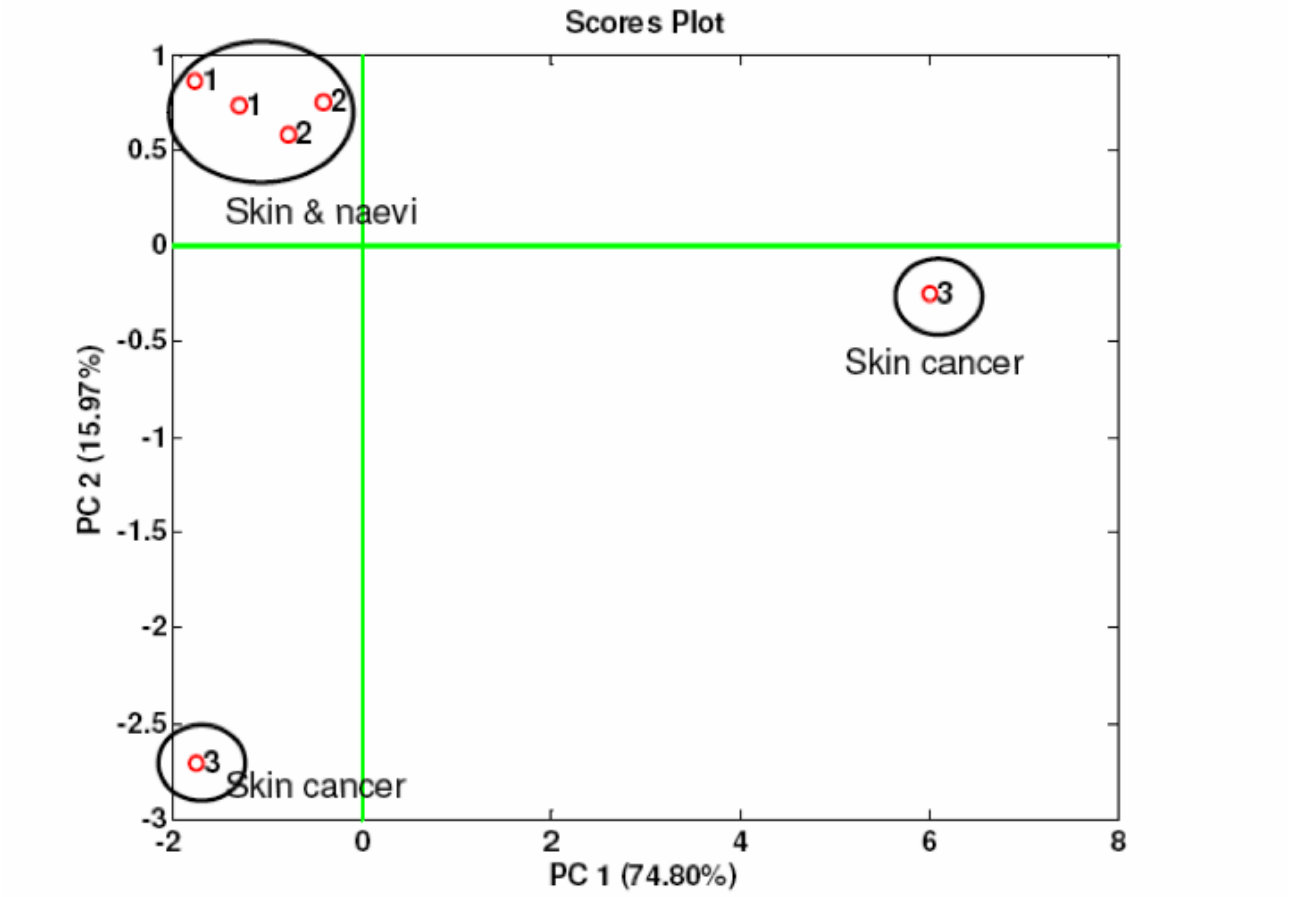


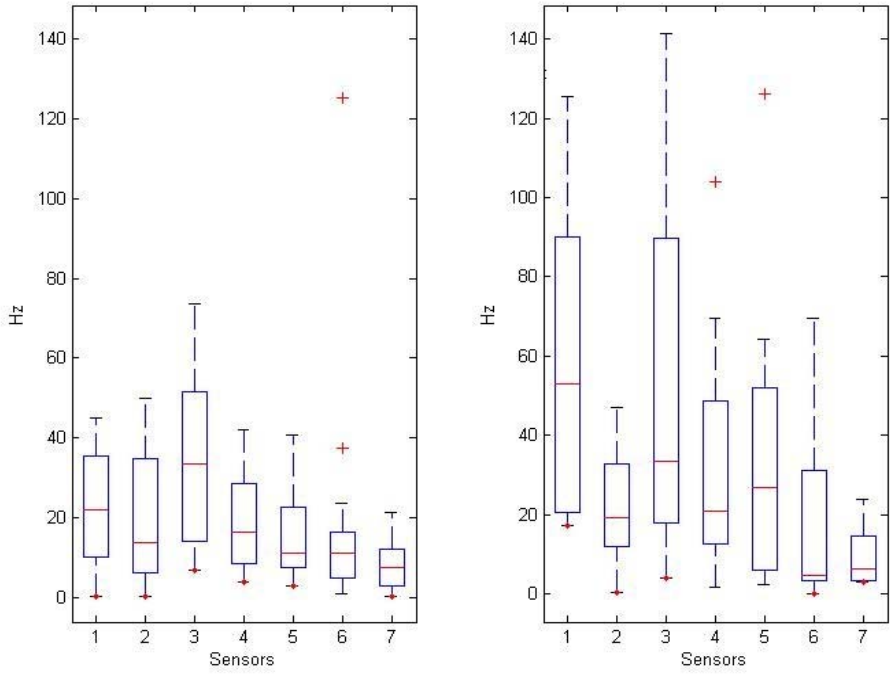
Fig. 3.8: scores plot of the first two component analysis model built on GC/MS data.

GC/MS analysis suggests that in case of melanoma, there is and anomalous composition of the volatile compounds released by the skin. On the other hands, the differences shown by GCMS, although biased by the affinities of GC solid phase and SPME fiber, corroborate the application of chemical sensor arrays to the identification of cutaneous lesions and, as a consequence, to the discrimination of melanoma from nevus.

3.4.2 Enose measurements

Sensor array provided, as a result of a single measurement, a pattern of seven values each corresponding to the difference between the frequency shift due to the exposure to the lesion and to the reference skin region respectively. The whole experiment then produced a data matrix with 47 rows (the samples) and 7 columns (the sensors). Before to apply to usual mutlivariate analysis it is interesting to study the behaviour of each sensor, in particular considering the distribution of each sensor output value with respect to the two largest classes of the experiment here illustrated: melanoma and nevi.

Figure 3.9 shows the distributions of nevi and melanoma data in a representation called boxplot.



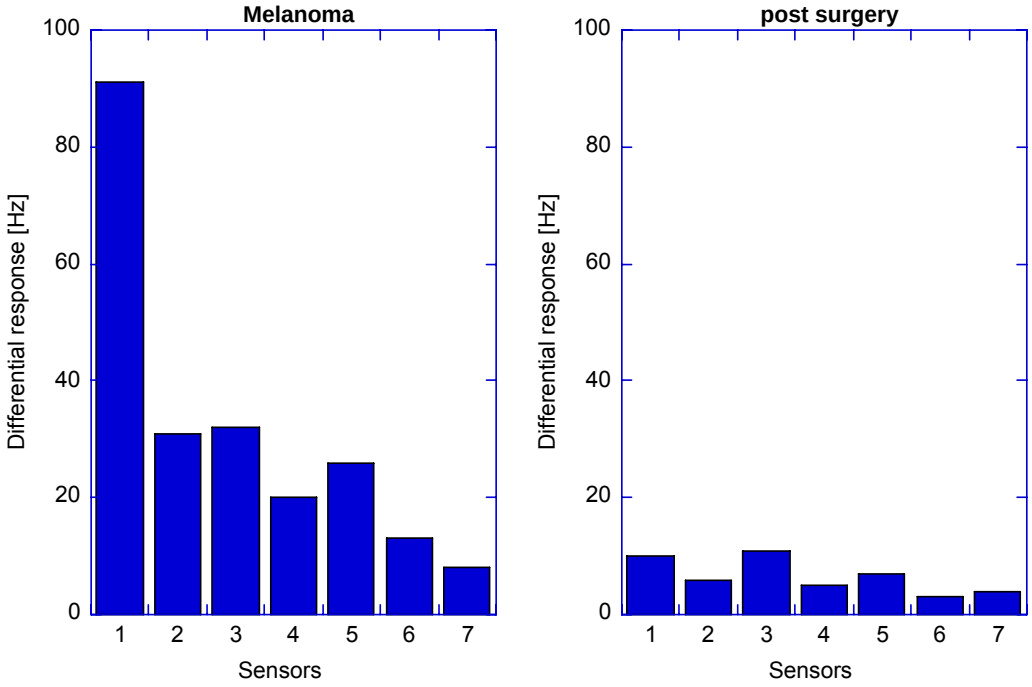
(a) **(b)**

Fig. 3.9: The response of sensors to all the cases are reassumed in the boxplots in figure. Data are separately considered: nevi (a) and melanoma (b). Although limited by the different population of the two distributions the boxplots display a largest variability of distribution is observed for melanoma. Details about the meaning of boxplot are given in the text.

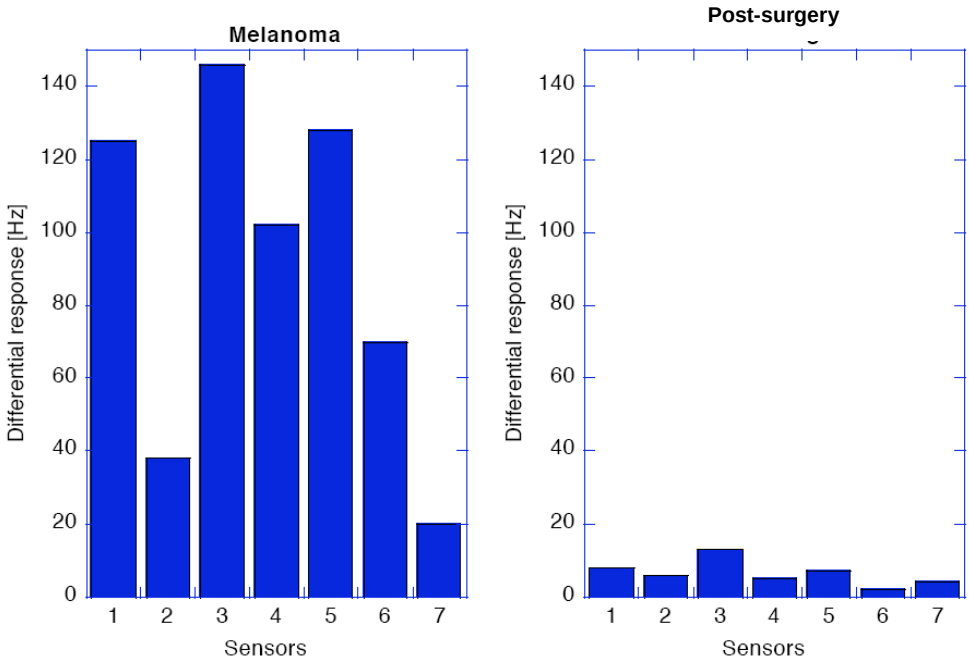
In a boxplot some of the synthetical descriptors of a data set are graphically reported: the extension of the box indicates the variance, the central line indicates the mean value, the dotted lines are the limits of the confidence interval, and the crosses indicate those measurements that exceeding the confidence interval may be considered as potential outliers. Box plots offer an immediate comparison between distributions indicating if two distributions are different or not. In figure 3.9 the boxplots of each sensor data related to nevi (9a) and melanoma (9b) distributions are respectively shown.

Figure 3.9 suggests that the variance of melanoma data are larger than that of nevi. The largest melanoma variability could be connected with several factors such as the typology of melanoma, its stadiation, and the surface conditions. Less clear is the behaviour of the distribution mean values. Only sensors 1 and 5 show a mean value significantly larger for melanoma while other sensors show a similar magnitude of response in the two cases. This behaviour is in agreement with the chromatogram comparisons of figure 3.6 which does not show a dramatic increase of abundance in melanoma headspace.

On these basis it is interesting to compare the patterns relative to the same patient but taken before and after the surgical treatment. These measurements were performed on seven patients. The second measurement was done fifteen days after surgery. With respect to other cases, for these patients the histological report was also available. Figure 3.10a and 3.10b show two cases of melanomas where a large difference is observed before and after surgery.



(a)



(b)

Fig. 3.10: Differential pattern the same patient examinated before and after the surgical complete removal of melanoma. The sensor response in the two cases is dramatically different and indicates the absence of tumor cells after surgery.

Figure 3.11 illustrates the case of the false positive for which a melanoma was diagnosed and surgery was required, but the istological inspection of the suspect tissue gave a negative result. In this case the sensor responses do not show a relevant change.

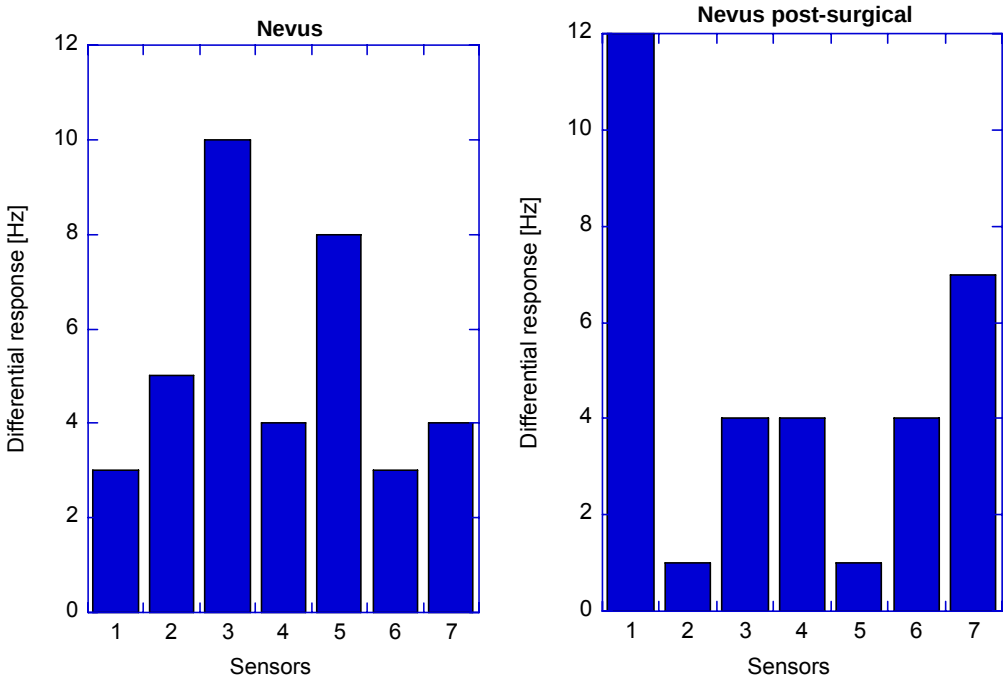


Fig. 3.11: Differential pattern of a false positive patient measured before and after the removal of nevus. Differential sensor signals are smaller with respect to melanoma, and the magnitude does not change after surgery. Electronic nose correctly identified this case as a nevus.

The analysis of pattern evidenced that sensors data of melanoma and nevi form two different distributions whose larger difference are in terms of variance instead of mean value. Furthermore evident different patterns characterize the headspace of the lesion before and after surgical intervention with a significant discrimination between melanoma and non melanoma also if this finding is obviously limited by the fact that only one false positive case was measured.

The results obtained in the pattern analysis have been confirmed by the multivariate discriminant analysis. There is a manifold of different algorithms performing discriminant analysis, and among them the partial least squares discriminant analysis (PLS-DA) is endowed with particularly interesting properties. PLS-DA is a particular way of use of PLS, an algorithm originally developed for quantitative regression, as a pattern recognition method[23]. One of the drawback of the application of multivariate techniques is the relationship between the number of variables and the number of samples. In general, an increase in the number of variables reduces the reliability of multivariate techniques based on covariance matrix estimation (such as PCA). Nonetheless to this regard it has been demonstrated that when the number of variables exceeds the number of samples and when the variables are highly correlated one each other, another typical occurrence for sensors in electronic noses, PLS-DA is more reliable than linear discriminant analysis[24].

A PLS-DA model was built in matlab. The model was aimed at discriminating nevi from melanoma. The model was properly optimized by the leave-one-out cross-validation method. In figure 3.12 the prediction error of the model versus the number of latent variables is reported, the percentage of correct classification in prediction is 87% largely comparable with other diagnosis methods currently in use for melanoma.

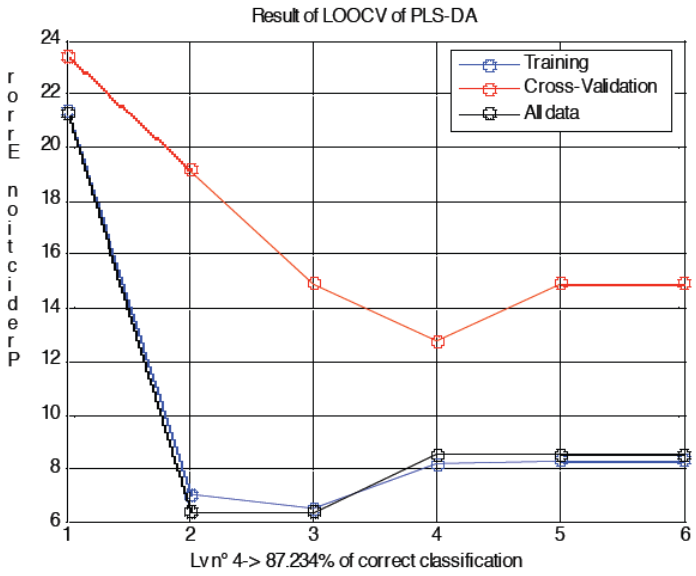


Fig. 3.12: Behaviour of the predicted error of classification as a function of the number of latent variables of the PLS-DA model. The cross-validation error achieves a minimum of 12%. This value is the estimated generalization error of classification. As usual the errors obtained in the training set and with the totality of the data are much smaller.

Nonetheless, this figure is biased by the different population in the two classes; indeed the benign lesions are correctly identified in 90% of the cases, while the correct prediction of the melanoma is only of 70%. The score plot of the first two latent variables of the PLS-DA model is reported in figure 3.13 where the separation between the distribution of nevi and melanoma is shown.

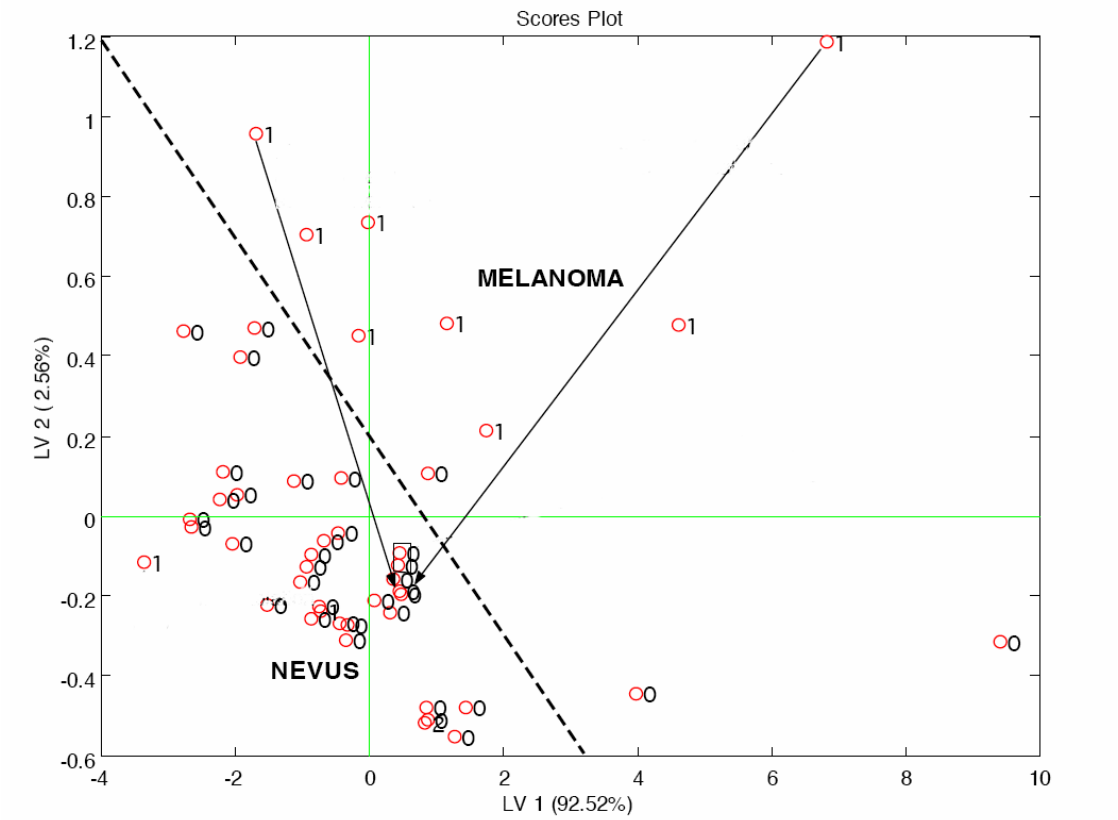


Fig. 3.13: Score plot of the first two latent variables of the PLS-DA model. The full PLS-DA model minimizes the error with four latent variables. Nonetheless, the plot of the first two variables provides a simple visualization of the classification properties.

The discrimination is far from perfection and a number of melanoma cases fall in the nevi region and viceversa. The large dispersions of melanoma data is in agreement with the boxplots behaviours, and as previously discussed, this spread can be explained by the differences between the tumoral form, different stages and different morphological states of the examined lesions. The temporal evolution of tumors introduces a further variable that necessitates to be taken into consideration since tumors become to emerge from the normality only at a certain development stage. The definition of this turning point is of paramount importance to evaluate the real potentiality of any early diagnostic method. As an example of the importance of stage in recognition it is worth to mention that one of the misclassified melanomas is a very early stadiation

malign lesion. In Figure 3.13 some cases measured before and after surgical intervention and the false positive (whose differential patterns are shown in figure 3.13)are shown.

This result is rather similar to a previously reported investigation about lung cancer where individuals measured after the surgical removal of tumor were classified in the control group class[15].

3.5 Conclusions

Experiences with trained dogs suggest that melanoma cells produce a different "bouquet" of volatile compounds. Nonetheless, the complexity of dogs perception does not allow for a straightforward attribution of the identification to the olfaction. As a consequence, these experiments did not univocally demonstrate the correlation between volatile compounds and melanoma. In this work, evidences that the gas chromatograms of the headspace of the skin surface are different in presence of melanoma or nevus have been shown. Such differences are large enough to be captured by a chemical sensor array; the application of linear pattern recognition was able to identify melanoma with an accuracy in prediction of about 80% comparable to that achieved by the diagnostic methods currently in use.

Sensor array result are rather underestimated because the array was composed by non optimized sensors and only a linear pattern recognition methods has been used. Concerning the sensor is worth to remark that the sensor coating was not chosen to maximize the sensitivity towards some particular compound basically because these compounds are still unknown. From the electronic nose measurement point of view melanoma identification is a favourable experimental condition because it is a very localized alteration that

can be insulated and measured with respect to a close unaltered skin region. The application of a differential strategy avoids in principle any contamination for the manifold of causes contributing to determine the skin headspace composition.

The results here presented have to be considered as a first step and many obscure points need to be furtherly clarified. First of all, GC findings have to be followed by an identification of the compounds occurring either in anomalous concentrations or appearing in melanoma headspace. This identification is of primary importance to correlate the findings to the biochemical processes occurring in the altered cells. Another important issue resulting from these studies is the scarce number of available cases. In the study here presented melanoma were only 25% of cases. The relative rarity of the disease does not allow a fully satisfactory characterization of the method according to the usual accumulation of experience typical in other electronic noses applications; the identification of the chemicals altered by the presence of melanoma will be then necessary to evaluate the actual sensitivity of the sensors and also for the optimization of the chemical sensors implementing in the array those more suitable for this particular scope.

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CHAPTER 4

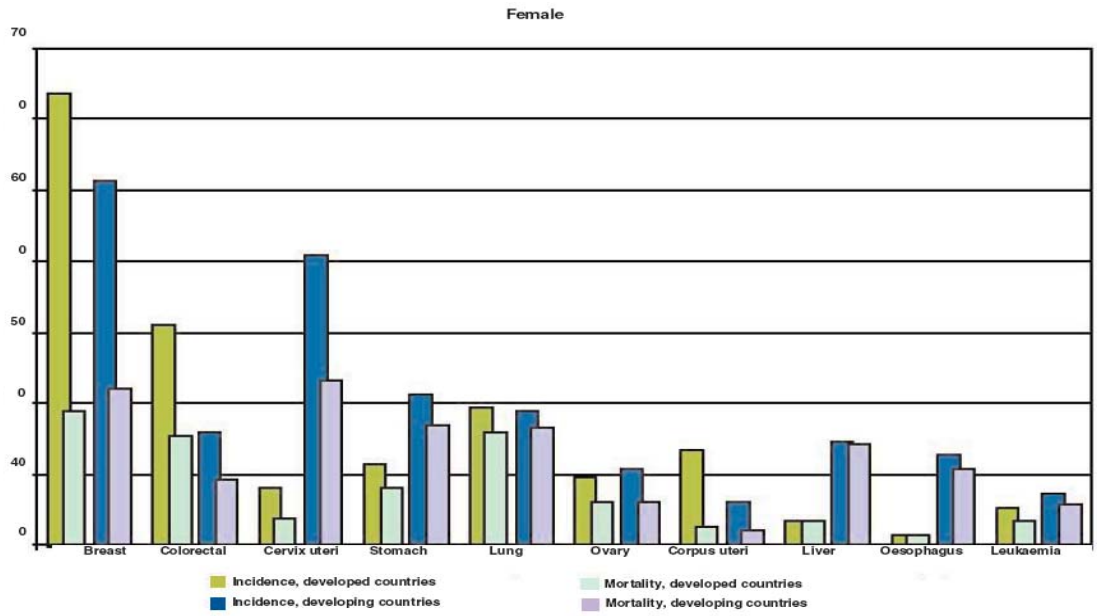
The case of Breast cancer

4.1 Cancer: statistics

IARC has estimated that in 2002, on the basis of the most recent available data (Ferlay *et al*, 2004; Parkin *et al*, 2005), there were in total 10.9 million new cases, 6.7 million deaths and 24.6 million persons alive with cancer (within five years of diagnosis). The most common cancers in terms of incidence were lung (1.35 million), breast (1.15 million) and colorectal (1 million). Because of its poor prognosis, lung cancer was also the most common cancer among causes of death (1.18 million), followed by stomach cancer (700,000 deaths) and liver cancer (598,000 deaths). In terms of prevalence, the most common cancers are breast cancer (4.4 million women surviving five years after diagnosis), colorectal cancer (2.8 million persons) and prostate cancer (2.4 million men). Overall, some 53% of the total number of new cancer cases and 60% of all cancer deaths occur in developing countries. In men, prostate cancer is now the most common form of cancer diagnosed in the developed regions (513,000 cases, 19% of all new cases), but only sixth in the developing countries (165,000 cases, 5.3%) where lung cancer ranks first (481,000 cases, 15%). In women breast cancer is by far the most frequent cancer worldwide, with an estimated 636,000 new cases diagnosed in the developed regions (27.4% of the total) and 514,000 in developing countries (18.8%).

Mortality reflects the fatality of the different cancers, and in men lung cancer remains the most common cause of death with an estimated 424,000 deaths in the developed regions (27.4% of the total number of deaths), and 423,000 in less developed countries (18.8%). About the incidence of cancer for women breast, lung and colorectal cancers account for 42.6% of the total deaths in developed countries. Figure 4.1 shows the ranking of cancers in terms of

incidence and mortality, for women in the developed (Europe, North America, Australia/New Zealand and Japan) and developing regions of the world.



From: annual report of IARC

Fig. 4.1: Cancer incidence and mortality (number per thousands) for men and women in Europe, North America, Australia/New Zealand and Japan and developing regions of the world

Breast cancer is considered an heterogeneous disease as it is much more aggressive in younger women, showing different characteristics in different group ages, and it has also different cell populations within the tumor itself. There are several types of breast cancer:

- ductal carcinoma in situ
- infiltrating ductal carcinoma
- medullary carcinoma
- infiltrating lobular carcinoma
- tubular carcinoma
- infiammatory breast cancer

The *ductal carcinoma in situ* is an early breast cancer confined to the inside of ductal system. Generally is divided into comedo or non-comedo type,of which the former is usually more aggressive and may show areas of microinvasion (small areas of invasion through the ductal wall into surrounding tissue).

The *infiltrating ductal carcinoma* is the most common type of breast cancer representing 78% of all malignacies. There are two kinds of lesion within this type, that are stellate lesions (as they appear as a star in the mammography) and rounded (or circumscribed) lesions. The stellate lesions generally have a poorer prognosis.

The *medullary carcinoma* comprise the 15% of breast cancer and is estrogen and progesteron receptor negative 90% of the times. As hormone receptor is a prognositic indicator, this implies that medullary carcinoma has better prognosis than other types of breast cancer. The *infiltrating lobular carcinoma* are usually difficult to diagnose with mammography. Microscopically, these tumors exhibit a linear array of cells and grow around the ducts and lobules. They can also be bilateral.

The *tubular carcinoma* is an orderly and well differentiated carcinoma of the breast and has a very favourable prognosis with a 90% 10 years survival. Finally *infiammatory breast cancer* is a particularly aggressive type of breast cancer, which presents changes in the skin of the breast including redness (erythema), thickening of the skin and prominence of the hair follicles. The diagnosis is made by a skin biopsy, which reveals tumor in the lymphatic and vascular channels 50% of the time.

4.2 Breast cancer diagnostic techniques

There are several breast cancer diagnostic tests available today. However an early diagnosis is the best strategy to defeat the disease. A preferable way to achieve an early diagnosis is a mass screening of the women population over 40 years. The standard procedure to perform this screening is composed of a triple testing: clinical examination tests, imaging (traditionally x-ray mammography, ultrasounds, Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET)) and needle biopsy (fine needle or core biopsy).

In the figure 4.2 is summarized the follow-up for a breast cancer screening.

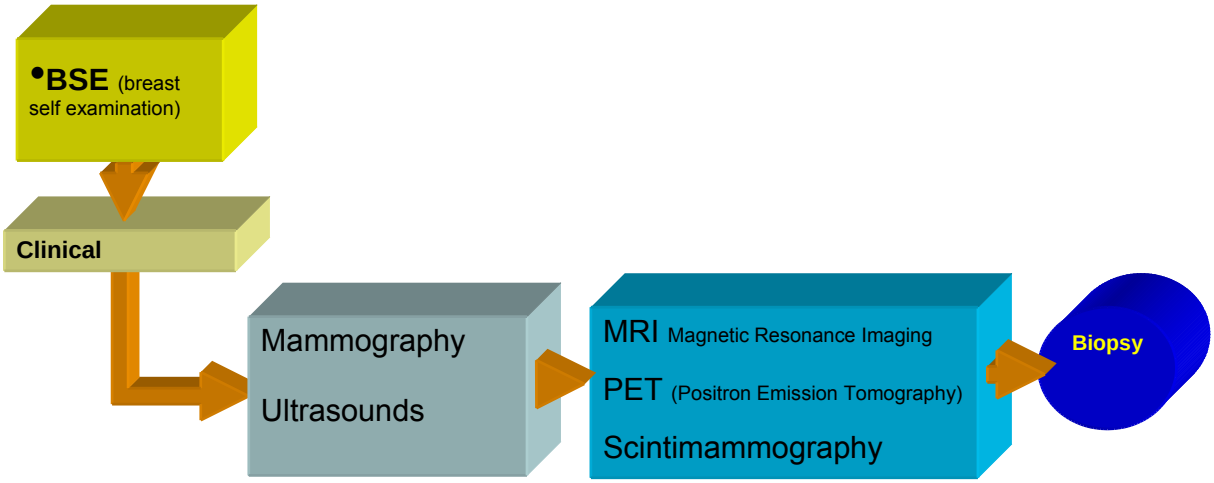


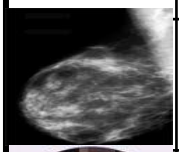
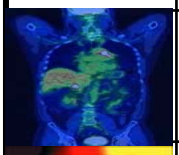
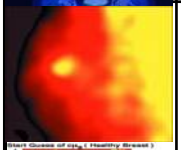
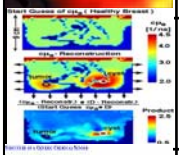
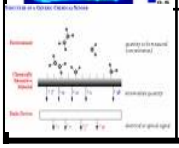
Fig. 4.2: schematic view of the Diagnostic procedure for breast cancer

Generally to the women a monthly *breast self-examination* (BSE) is frequently advocated. It has been demonstrated that this test doesn't reduce the

mortality and it is considered optional in all risk groups, but for the some subjects periodic and consistent breast self examination may facilitate self-awareness[1]. Moreover the BSE test have another advantage since it may detect interval cancers between routine screenings [2].

Following BSE test the *clinical breast examination* (CBE) is the most important step for an efficient breast cancer diagnosis. Doctor experience can advise for a deeper test. The goal of periodic BSE, as with CBE, is to detect palpable tumors. An additional role of BSE is to increase awareness of normal breast composition, so that there is an increased awareness of changes that may be detected during BSE. The importance of increased awareness, is commonly acknowledged for the of earlier treatment of both nonpalpable and palpable breast cancers. Anyway the possibility to use novel clinical instruments can reduce invasivity and lower the costs.

Actually invasively and low costs are two decisive parameters in the routinely clinical practice. Instruments interaction with the body defines the level of invasivity. In the tab 1 a summary of the mainly used technique is presented. A brief description of these techniques will be presented as introduction to the aim of this chapter: the detection of breast cancer by a gas sensors array.

	Technique	Interactions
	**Mammography**	Low dose X-ray
	**Ultrasound**	high-frequency sound waves
	**PET**	emission of positrons
	**Scintimammography**	Biological tracers
	**Magnetic resonance imaging**	radiofrequency waves and a strong magnetic field
	**gas sensors array**	Body emissions

Tab.1: an overview of the traditional techniques for breast cencer detection.

The *mammography* is an x-ray of the breast. An image produced by exposing compressed breast x-rays is obtained and this image is function of transmitted x-rays. Generally x-rays mostly adsorbed are described in the image with a dense regions while x-rays mostly transmitted are shown with a lucent regions. Screening with film-screen x-ray mammography remains the only single intervention shown in randomised controlled trials to significantly reduce mortality from breast cancer.

Ultrasound is accepted as a very effective technique for further assessment of mammographic abnormalities and for image guided breast

biopsy. There are some evidences of the benefit of ultrasound as a secondary adjunct to screening the mammographically dense breast, and also some evidence of its benefit in the primary screening of younger women at increased risk. Some studies have reported a significant increase in cancer detection using ultrasound in women whose mammograms show a level of background density that is likely to reduce sensitivity for the changes of early breast cancer. Breast ultrasound is widely available and is inexpensive compared with other imaging techniques such as nuclear medicine and magnetic resonance imaging. While ultrasound may improve invasive cancer detection in dense breasts, it has high false positive rates.[3]

Scintimammography uses standard biological tracers to locate the tumor. Biological tracers are specially prepared chemicals carrying a gamma-ray emitting radioactive isotope that can mark certain biological processes. Medical researchers have shown that several types of cancer cells uptake and accumulate these markers more readily than normal cells. The new device "senses" the gamma-rays emitted by the tumor and using those gamma rays, the device builds an image of the tumor. By this technique is possible to differentiate between benign and malignant tissue and detect small tumors in cases where mammograms are difficult or impossible to read. The most important advantage is the reducing of the use of biopsy.

Magnetic Resonance Imaging (MRI) is used to evaluate palpable breast masses between cancer and scar. The application of this type of technique is more indicate for cancer than mammography. MRI technique is based on radiofrequency waves and a strong magnetic field. Each of these techniques presents some drawbacks in term of invasivity and effectiveness, and the false negative percentage of each single test is in the range from 10% to 18%. Moreover it is worth to remark that the combination of the different imaging techniques increase the sensitivity respect to cancer up to 91%-98%[4]. X-ray

mammography is of course the most commonly used technology, but MRI is preferable for screening of high-risk cases because of its higher sensitivity, even if it is more expensive. Breast MRI is currently under investigation, but its application is of course very effective in some selected cases: highly suspicious findings, preoperative staging, women with lymphnode metastases and unknown primary cancer, monitoring of anti-cancer therapies [5].

Misclassified cancers are mainly very aggressive tumors that develops in one year period in women less than fifty years old, this supports the importance of a frequent monitoring strategy, which is achievable by mean of non-invasive and cheap diagnostic techniques. In summary, none of the reported tests has the necessary features and accuracy to be the exclusive diagnostic method to perform an early diagnosis of breast cancer, except for their combination [6].

All the techniques previous described interacts with the body of patients. The invasiveness of these techniques is very low and their use is limited only by the high costs. In tab. 1 the use of a gas sensors array to detect the breast cancer is suggested as novel non-invasive technique. The purpose of this study was to test the ability of a QMB based gas sensor array in the analysis of the volatiles present in the headspace surrounding the breast. The goal of the experiment was to use this instrument as support of other diagnostic tools, for an early and non-invasive diagnosis of breast cancer.

The effectiveness of 'imaging' tests suggests the use of a challenging technology which is non-invasive in principle: to perform an 'olfactive image' of the breast healthstate. VOCs emitted by human body are a powerful source of information on individual healthstate, and of course their examination does not need an invasive sampling procedure. Recent studies by Phillips et al.[7,8] proposed a new non-invasive technique to predict breast cancer by the identification of possible volatile biomarkers in the breath. The breath test developed was similar to that used by Phillips himself in the research on lung

cancer[9]. A fuzzy logic model was used to predict breast cancer, employing five different volatile organic compounds. Phillips achieved a sensitivity of 93.8% in the prediction set.

In the present experiment an electronic nose technology has been applied to the analysis of the headspace surrounding women breast. This technique allows to perform a measurement in situ instead of an indirect measure such as the case of breath analysis. The efficiency of this methodology is supported by other publications of the same group in the analysis of skin, sweat and breath [10,11,12], and also by promising researches about melanoma, bladder cancer and breast cancer performed by well-trained dogs [13,14,15].

4.3 Experiment description

A total number of 72 measurements were performed. The volunteers were recruited at the S.Eugenio Hospital in Rome. Twenty-eight measurements were performed on subjects affected by various forms of breast cancer and were hospitalized waiting for a surgical treatment and twelve patients were measured before and after the surgical treatment. Three volunteers were women whose breast had been replaced by a prosthesis. Twenty-six measurements were performed on subjects recruited among the nurse and medical staff of the Hospital as references. These controls were apparently not affected by any disease, and were not taking any drugs. The cancer patients did not show of any other pathology. Each person participating to the experiment was informed about the nature of the measurement to obtain an informed consent. In fig. 4.3 the experiment is shown.

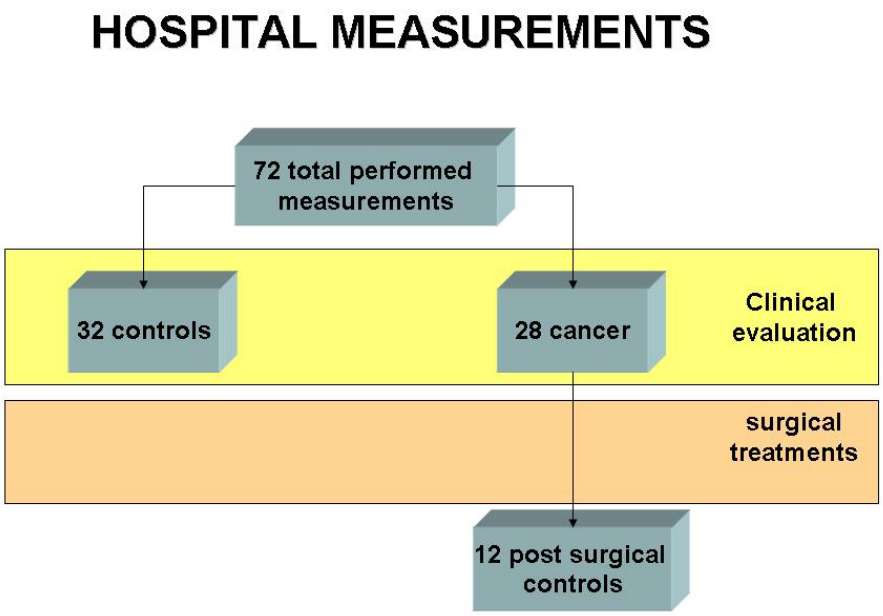


Fig. 4.3: total measurements performed at S. Eugenio Hospital in Rome. Different stages (clinical evaluation and surgical treatment) of clinical diagnosis are shown.

The purpose of this experiment was the analysis of the Volatile Organic Compounds (VOCs) present in the headspace surrounding the breast, in order to obtain information about the breast healthstate. To perform such an analysis a sampling protocol was developed to convey the VOCs to the measure chamber of the electronic nose. Each subject was required to discontinue the use of deodorants or perfumes on the day of the measurement, not to influence the volatile compounds measured. No other attempts were made to control the VOCs emission.

4.3.1 Sampling protocol

The sampling protocol required each subject to maintain a sampler over the breast during the time of the measurement. Measures from both the breasts

were taken into consideration in this analysis. An additional measure related to skin thorax was performed in order to obtain a reference measure regarding individual odor.

The sampler was designed to match the anatomy of the breast and it was provided with two openings: the first used to convey reference air into the sampler and the second to collect breast headspace air, which was then conveyed to the electronic nose chamber. An online measurement was thus performed. The scheme of the sampling methodology is identical to the system described in the previous chapter for melanoma detection (fig 4.4).

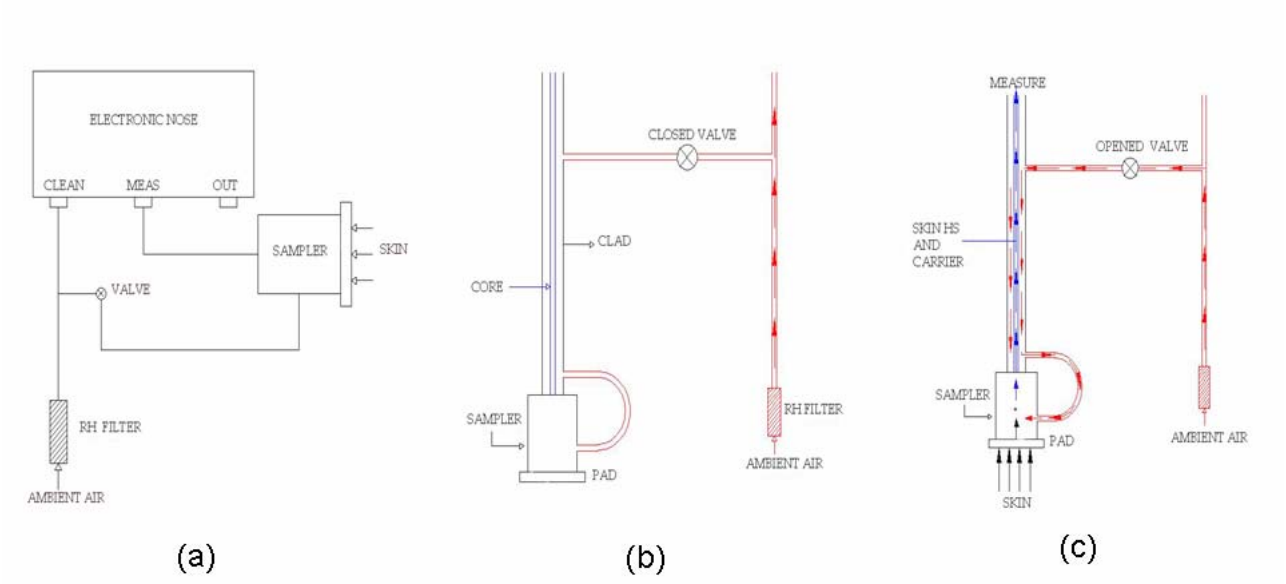


Fig. 4.4. :scheme of the sampling protocol used with the electronic nose

The headspace air was flown at the constant speed of 0.5 ml/s into the sensor chamber. A stabilization of the sensor response occurs when the thermodynamic equilibrium between adsorbed and desorbed molecules is achieved. The feature extracted from the sensor responses and utilized for the data analysis, were selected as the difference between the frequency shift registered between the cleaning and the measure phases. Data analysis was

performed in the MATLAB environment, by means of the PLS Toolbox. A partial least square discriminant analysis (PLS-DA) was performed, considering two classes (breast cancer disease and reference group). In order to evaluate the identification performances of the method, a leave-one-out validation criterion has been adopted.

4.4 Results

As the aim of this study was to test the capability of electronic nose to correctly classify groups of subjects (in this case cancer patients and controls), a supervised technique has been used. Figure 4.5 shows the statistics of the data of the four sensors utilized in the experiment, in the form of a box and whiskers plot, in which are reported the sensors on the x axis and the frequency values in Hertz on the y axis. It shows, for each group, the statistical distribution for each of the four sensors. In particular, the box has lines at the lower quartile, median, and upper quartile values. The whiskers are lines extending from each end of the box to show the extent of the rest of the data. Outliers are data with values beyond the ends of the whiskers.

This kind of representation of the data is useful to obtain the first information about the data that has been collected and about the effectiveness of the system employed, and it could also give important informations for the following multivariate data analysis to be performed. Actually the first observation that can be made, looking at the box and whiskers plot, is that the pattern of the prosthesis group seems to be very different from that of the other groups, as for the statistical distribution (in particular because of the mean value) of the four sensors. This is a good starting point that let us foresee that in the multivariate analysis the prosthesis group will be a well defined and

separated cluster. Even by the physiological point of view this assumption is reasonable, as a breast that has been deprived from all the glandular parenchyma, connective and adipose tissues, releases probably different compounds respect to a normal breast. The control group has also a distinctive pattern, especially because of the mean value of the four sensors. The values of the variance are very similar to that of the prosthesis group, and this is an important observation as the patients with prosthesis are however not affected by any manifested disease.

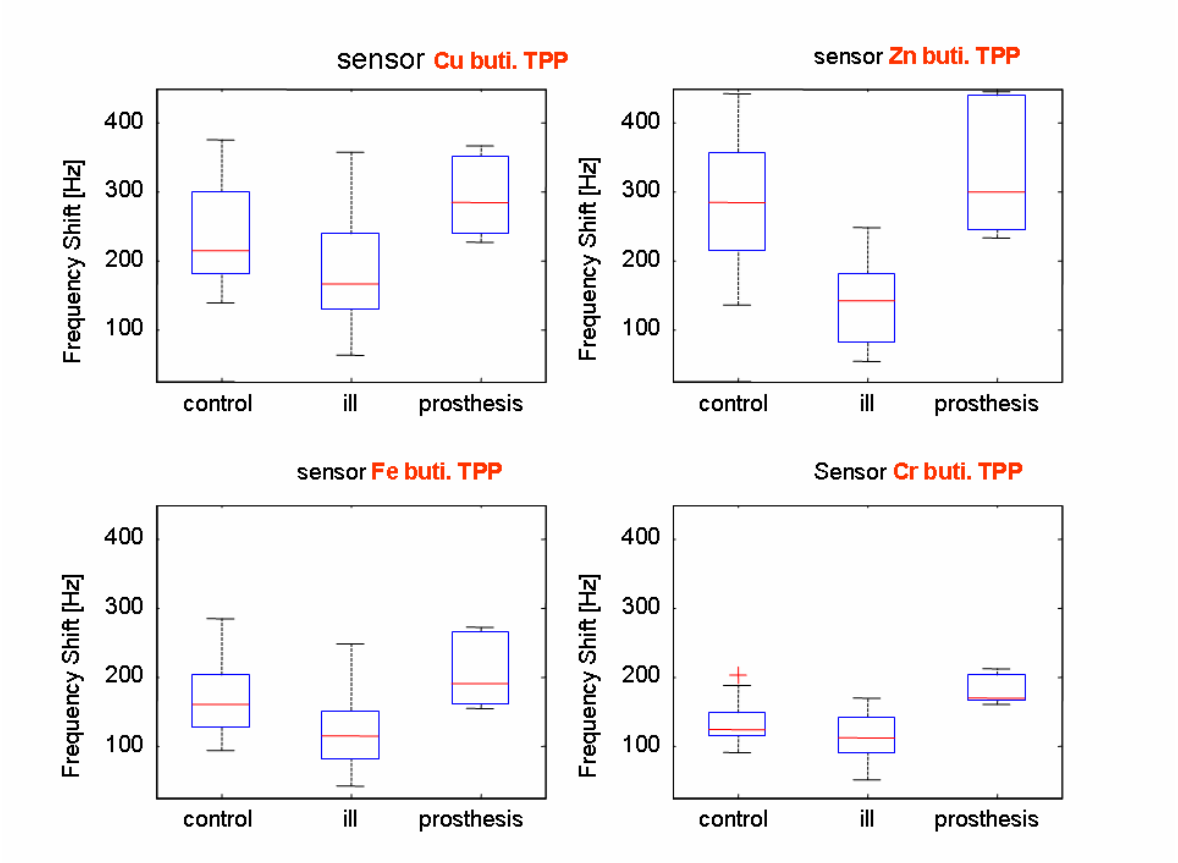


Fig. 4.5 : statistical distribution for each group (control, ill, prosthesis) of four sensors

The simplest possible analytical method is the discriminant analysis, where a linear model between sensor data and classes is built (Appendix). As in any supervised classification techniques, the classes has to be chosen a-priori. The natural classification for the samples in this experiment was to choose two

classes including the patients with breast cancer and the control group. The prediction error was minimized using the leave-one-out cross validation method. The best fitting method included two latent variables. Data have been scaled by mean center (zero mean). Figure 4.6 shows the score plot of the two latent variables. In the plot about 99% of the total variance of the data is represented. It can be observed a clear separation between the data related to the cancer patients (labeled as **1**) and the control group (labeled as **0**).

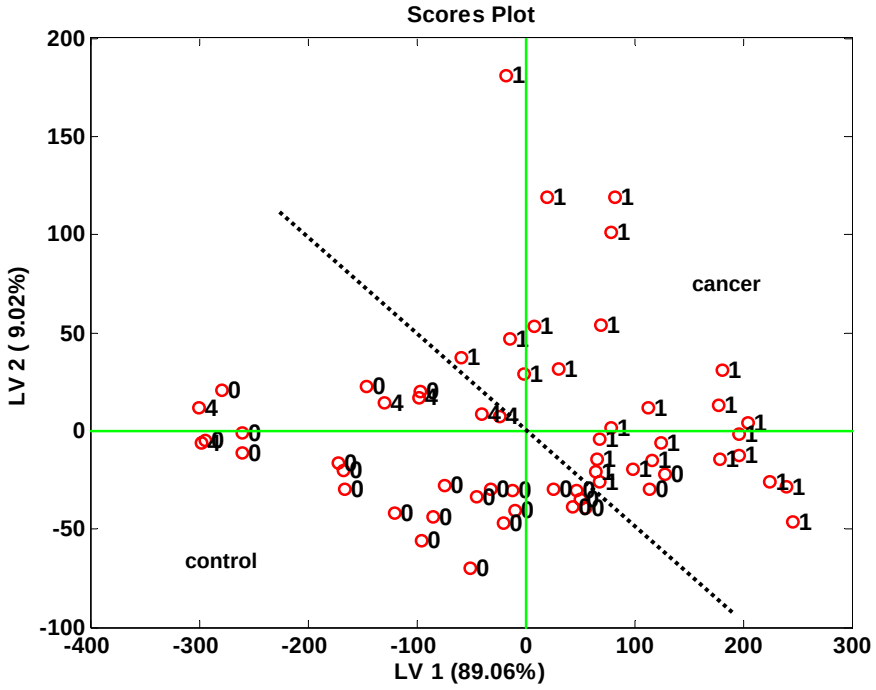


Fig. 4.6: scoreplot of the first two Latent Variables of the PLS-DA model built on e-nose data. (Labels: 0.controls; 1.breast cancer; 4.breast prosthesis).

Table 2 shows the confusion matrix of the PLS-DA model. The percentage of correct classification is about 93% for both the groups. The percentage of missed tumor identification is about 7%, of course less than 10-20% reported above for imaging diagnostic.

True/estimated	Control sample	Cancer disease
Control sample	**30**	**2**
Cancer disease	**2**	**26**

Table 2: Confusion matrix of the PLS-DA model built on e-nose data.

It can also be observed from Fig.4.6 that the measures related to women with prosthesis (labeled as 4), even though belonging to the control group, as they do not manifest any disease, made up a distinctive cluster. The electronic nose showed 86% sensitivity, 93% specificity, 14% false negative, 7% false positive, in the discrimination between the cancer patients and the control group. The next step has been to analyze all groups to test the ability of electronic nose to discriminate among three classes : controls, post surgery, cancer. In the fig. 4.7 the scores plot relative to the first two latent variables obtained with PLS-DA model is shown.

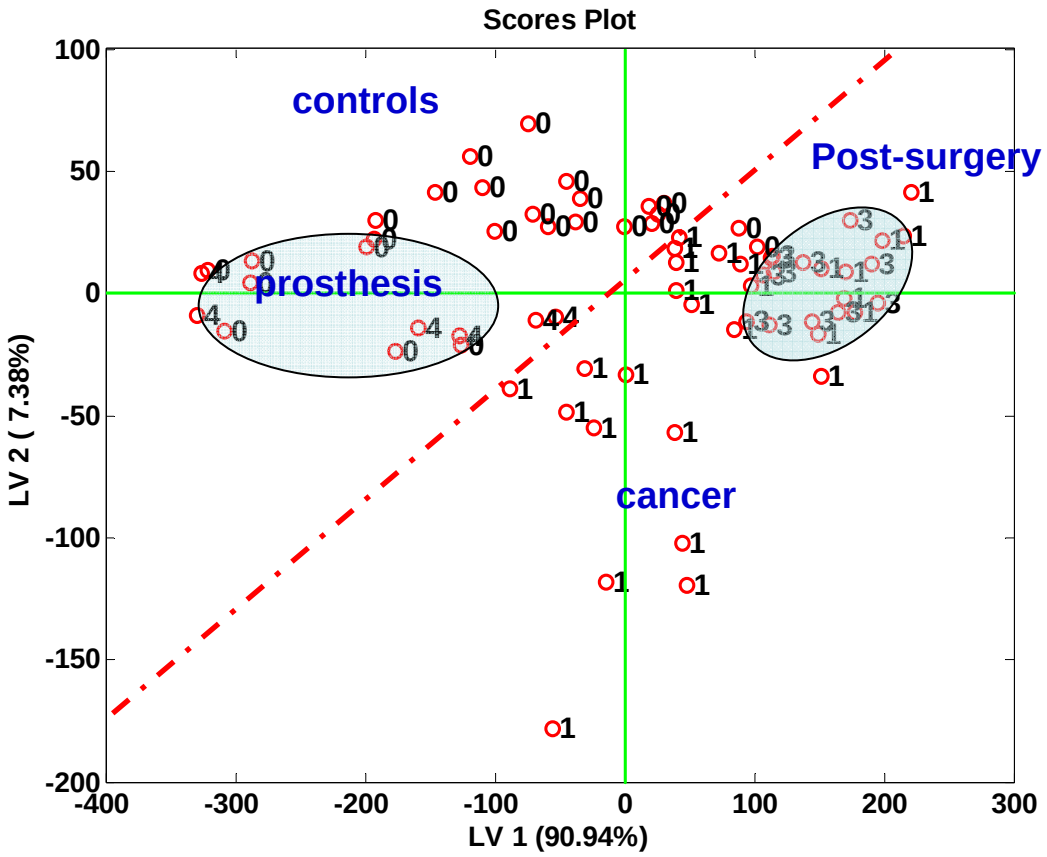


Fig. 4.7: score plot of the first two latent variables obtained with PLS-DA model built on the entire data set. In this case the classification has been obtained for three groups (controls, cancer, post-surgery).

The results obtained in this case are not so good in term of prediction. In this last study the percentage of correct classification is about of 73%. It is lower then the previous model. The confusion matrix (tab.3) shows twelve misclassified cases among the post-surgical treatment measurements that are classified as cancer patients. This anomaly has been investigated with the medical equipe. All patients after surgical treatment are treated with radiotherapy and chemotherapy. It is possible that these medical applications could influence the headspace surrounding the region of interest during the measure. This interesting aspect has to be better investigated.

	Controls	**Cancer**	**Post surgery**
Controls	**30**	**2**	**0**
Cancer	**4**	**23**	**1**
Post surgery	**0**	**12**	**0**

Tab. 3: confusion matrix relative to the PLS-DA model calculated on the entire data set with three class.

4.5 Conclusions

First evidences shows that electronic nose could be a suitable instrument to be used as an adjunct device for the diagnosis of breast cancer. The electronic nose will never replace other consolidated diagnostic techniques, but it can help to reach an early diagnosis of cancer. The non invasivity of the measurement and the low cost of the instrument are the main advantages of this technique. Several authors reported studies on the composition of volatile compounds emitted by the body for other kinds of cancer, and in particular for lung cancer, for which breath composition analysis is the simplest and more obvious way to obtain useful information about healthstate. The composition of the VOCs expected to be found in the breath is a problem quite well studied, even by the physiological point of view.

In the case of breast cancer, it is more difficult to have a-priori information about what to expect from breast skin emissions. For this reason it is also very difficult until now to give scientifics approach to discriminant capability obtained with these results.

At this moment, the investigation here reported does not allow to infer any explanation for the discrimination obtained with the e-nose. A deeper analytical investigation with GC-MS equipment will be necessary to explain these results. However the results here illustrated confirm that the headspace

air surrounding the breast of women with breast cancer is different from that of healthy people. These evidences suggest that the headspace surrounding the breast contains important information about breast healthstate, and the electronic nose is an efficient instrument to reveal and interpret these information.

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CHAPTER 5

The characterization of tumor cell lines in-vitro and in- vivo by a gas sensors array

5.1 Introduction

Volatile organic compounds (VOCs) released by melanoma cell lines *in vitro* were studied by a gas sensor array. The temporal behavior of volatile organic compounds were further studied by measuring the emission of implanted xenografts. Emissions of melanoma cells and growing tissues were compared with those of different tumors. Although qualitative differences between well-characterized human tumor cell lines of different embryonic origin were expected, consistent similarities were registered between odors from human melanoma cells and their respective proliferating xenografts *in vivo*. This suggests the existence of a 'volatile molecular signature' of malignancy with potential diagnostic and prognostic implications. Furthermore, proliferation experiments evidences the existence of a development time where the differences between the tumor lines, and the consequent identification, are maximised. The results of the non specific gas sensor array were corroborated by solid phase microextraction (SPME) gas chromatography-mass spectrometry (GC-MS) that evidenced the presence of various families of compounds in the volatiles spectra of tumors. In particular, ketones, aldehydes, esters, alcohols and benzene derivatives have been detected, nonetheless the complexity of the chromatograms does not allow the identification of compounds that can be clearly connected to the presence and the evolution of the tumor mass. This behavior has been rather hindering the possibility to utilize volatile compounds for diagnostic purposes.

Indeed, since the eighties different research groups have investigated the production of volatile compounds produced by different cancer forms aiming at defining a spectrum of organic compounds detectable as odors may likely provide a *volatile molecular signature* of fundamental biological processes including cell proliferation, tissue differentiation and cell death. The possibility

to distinguish between the 'normal scent of life' and anomalies arising during pathological human conditions, like tumor growth and progression, represents an interesting and useful field of research with potential diagnostic and prognostic implications.

Evidences about the relationship between volatile organic compounds and pathologies was firstly demonstrated since the eighties analyzing the exhaled breath with standard gas-chromatography equipments [1,2]. The methodology was soon oriented to determine the changes of normal concentrations in presence of various tumoral forms interesting the aerial view compartment and more recently also to identify anomalies in the exhaled breath in presence of tumors not directly exposed to the aerial views, such as breast cancer [3,4,5].

The above mentioned studies were mainly addressed to the definition of a list of marker compounds, whose presence is strongly correlated to the presence of a given pathology. This objective is challenging and extraordinary difficult for the current analytical instrumentation. The samples analyzed in these studies were very complex and their relation with a particular disease was often shadowed by an enormous quantity of other endogenous and exogenous biochemical processes; additionally the analytical complexity is increase by the peculiarity of the disease that also when it pertains to the same organ can appear in very different forms. On the other hand, fingerprinting techniques matched with pattern recognition algorithms were also studied in order to classify samples according to general similarities of the chromatograms were utilized in some cases showing the possibility to correctly identify the presence of the pathology [6].

These results have evidenced the possibility that a qualitative interpretation of the measured samples may provide a sufficently accurate distinction between positive and negative cases. The qualitative evaluation of

VOCs mixtures can be considered as a strategy aiming at perceiving a sort of 'quasi-odor' formed by the blend of many compounds. Obviously not all the volatile compounds are perceived by human olfaction, and in this case the term 'odor' is intended as a synthetical property, such as a fingerprint, univocally identifying a VOCs mixture with respect to any other of different composition. An unusual support to this approach is offered by the anedoctical reports about the identification by trained dogs of skin [7] and bladder tumors [8].

On these basis, an instrument mimicking the natural olfactory systems could provide objective measurements of complex VOCs mixtures reproducing the potentialities of animal odor perception. The artificial olfaction systems (widely known as 'electronic nose'), was introduced in the eighties, mainly considering the non selective character of olfactory receptors and then arguing that an array of non selective gas sensors could behave as the olfactory receptors [9]. This was also remarkable from the analytical point of view discovering that non-selective sensors, considered as the biggest defects of solid-state gas sensors, once properly treated are endowed of remarkable analytical properties.

Numerous researches conducted with electronic noses of different sensor technology enriches the literature about medical study with a 'VOCs fingeprinting approach'. The largest part of these studies regards in vitro and in vivo bacteria cultures and many different cancer typologies [10]. Many in-vivo studies were focused on breath analysis taking advantage of the many breath collection methods developed for various medical tests [11,12] as describe in the previous chapters.

The main negative feature of fingeprinting techniques is their cross-sectional character whose results are extremely sensitive to the experimental design and to hidden variables.

Therefore a convincing demonstration of the foundation of these research is still missing.

5.2 Electronic nose applications to cell lines monitoring

A case study in which the different variable could be controlled and many interferences could be avoided is the study of well selected and insulated tumoral cells cultures. To this regard, previous works demonstrated the use of electronic noses to follow cell cultures time behaviour [13]. Then assuming that an electronic nose can detect cancer in humans and monitor the activity of cells cultures, we can imagine to use it to monitor the evolution of cultured cancer cells and to follow the evolution of xenografts of human cancers cells on supporting animals such as mice

The possibility to monitor the development of an inoculated cancerous tissue in mice through the analysis of odorous emissions was demonstrated in mice inoculated with breast cancer [14]. The presence of these particular odors were also found when the tumor was present in the animals but the mice were not affected by cancer. The driven ideas of the research supporting this work was the potential uniqueness of the odor of proliferating tumor cells in vitro and in vivo.

The detection of mice odour with an electronic nose was demonstrated studying the different odour of mice urines as related to different expressions of the MHC genes [15]. The present chapter describes the results of a number of experiments on mice inoculated with three different cancerous cells. The inoculated cells have been previously measured 'in vitro' with the same instrument. In the present work the measurements were performed directly on the mouse skin, sampling the air from the headspace immediately above the inoculated skin regions. The underlying idea of the research was then to

demonstrate the existence of a potential uniqueness of the odour of proliferating tumor cells in vitro and in vivo.

5.2 In vitro experiment

The first experiment was devoted to the analysis of in-vitro cell coltures. The measured samples were coltures of: three melanoma cancers (labeled as LOIA, FIV, FORM), a synovial sarcoma (labelled as CME) and a thyroid cancer (labelled as FRO). Also in these case all the measurements were performed with an array of quartz microbalance (QMB) sensors functionalized with molecular films of different metalloporphyrins. Features and performance of arrays of metalloporphyrins coated QMB may be found elsewhere [16].

5.2.1 Sampling protocol

Different methodologies were used to follow the evolution of cells cultures headspace and to test the possibility of a discrimination between the diverse typologies. The optimized protocol for the measurement of tumor cell lines in vitro has been developed during eight months. In the first experiments headspace analysis of tumor cell lines in dry condition were analyzed. In this case the cell coltures were washed in a physiological solution (PBS) and measured. All the measurements were performed at room temperature and the cell coltures were placed in a Petri Dishe and in Falcon containers. The different strategies were considered to enrich the headspace of the sample. In both these methods , the short life time (about two hours) of the cell coltures in dry condition didn't permit to obtain good results.

In the second phase of the experiment the coltures were measured in wet condition, the cell coltures were measured in their growth medium. The most

encouraging results have been obtained measuring the sample in Petri Dishes at room temperature and with a Nafion filter to reduce the humidity effects (see appendix B). In Figure 5.1 the set-up for cell cultures headspace measurement is shown.

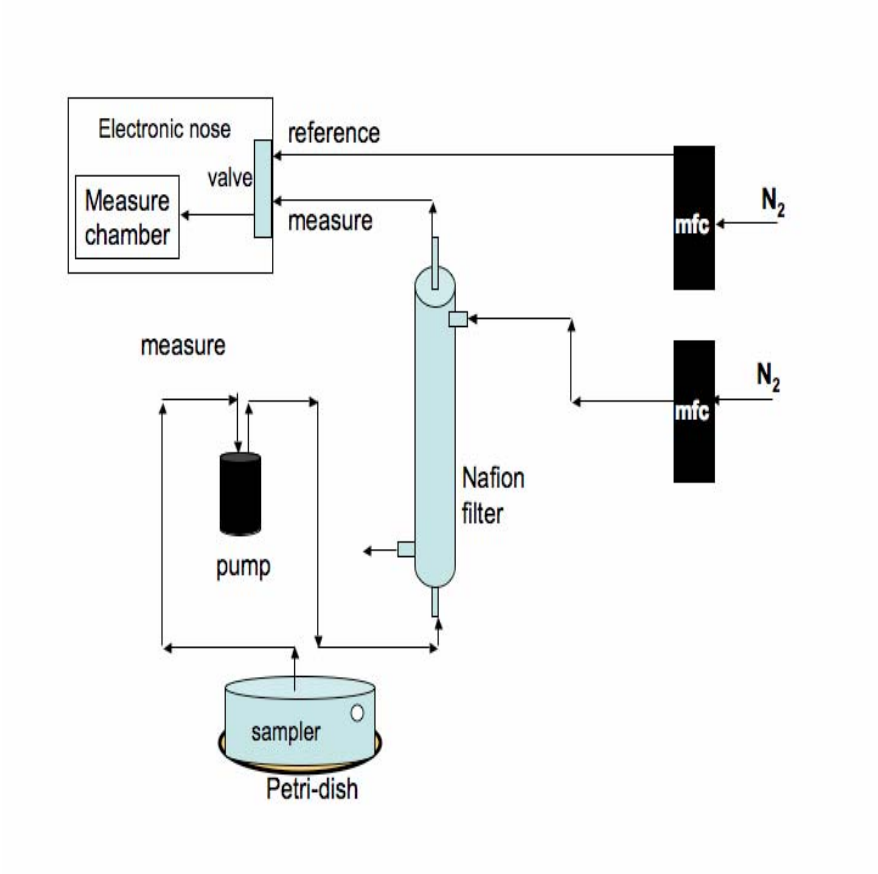


Fig. 5.1: set-up for cell cultures headspace measurements

A 7.5.cm Petri dish for cell culture is used and a metallic cylinder (vol. 35 cm^3) fitting the diameter of the Petri dish is held for 10 minutes on the sample at room temperature to allow the formation of an equilibrium headspace composition. The cylinder air volume is then sucked by a pump through a Nafion filter and then to the electronic nose chamber. Nitrogen flow, kept constant by a mass flow controllers (mfc) is used both for the electronic nose

reference and carrier and also as exchanger flow in the NAFION filter unit. The flow was fixed at 200 sccm. The NAFION filter is utilized to keep low and constant the humidity level in the cells headspace sample.

5.2.2 Results

The method described provided the best results which are presented in figure 5.2.

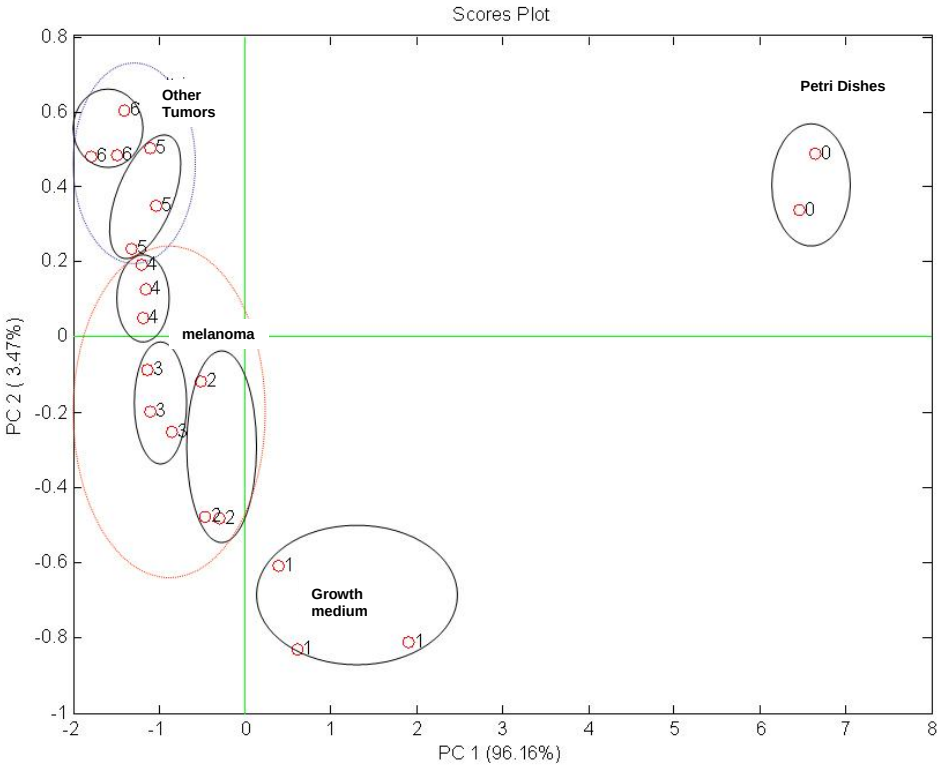


Fig. 5.2: scores plot of first two principal components of the PCA model built on the whole measurement data set. The figures shows a good discrimination among the empty Petri dishes, Petri dishes with growth medium and different tumor cell lines.

Figure 5.2 shows the scores plot of the first two principal components of the Principal Component Analysis (PCA) of the signals acquired by the array of six QMB sensors. PCA score plot are interpreted assuming that the distance in

the plane is measure of the similitude between samples, then clusters of them are identified as derived from similar samples.

Twenty samples were prepared and measured. The measure consisted in the exposure of the sensors to the atmosphere collected immediately over an open Petri plates. Two of them refer to empty Petri plates; three are Petri plate filled only with the nutrient substrate; and fifteen Petri plates were measured with five different types of cancer cells in triplicate:

- melanoma(labelled as FORM);
- melanoma (labelled as FIV);
- melanoma (labelled as LOIA);
- synovial sarcoma(labelled as CME);
- thyroid cancer (labelled as FRO).

A simple observation of figure 5.2 evidences the separation along the first principal component of clusters related to Petri Dishes and growth medium from the rest of the data set containing all the measurements related to the tumoral cells. A hint of separation between different tumors can be seen along the second principal component. Fig.5.3 shows the scores plot of first two component analysis of PCA model recalculated removing the empty plates and the nutrient substrates.

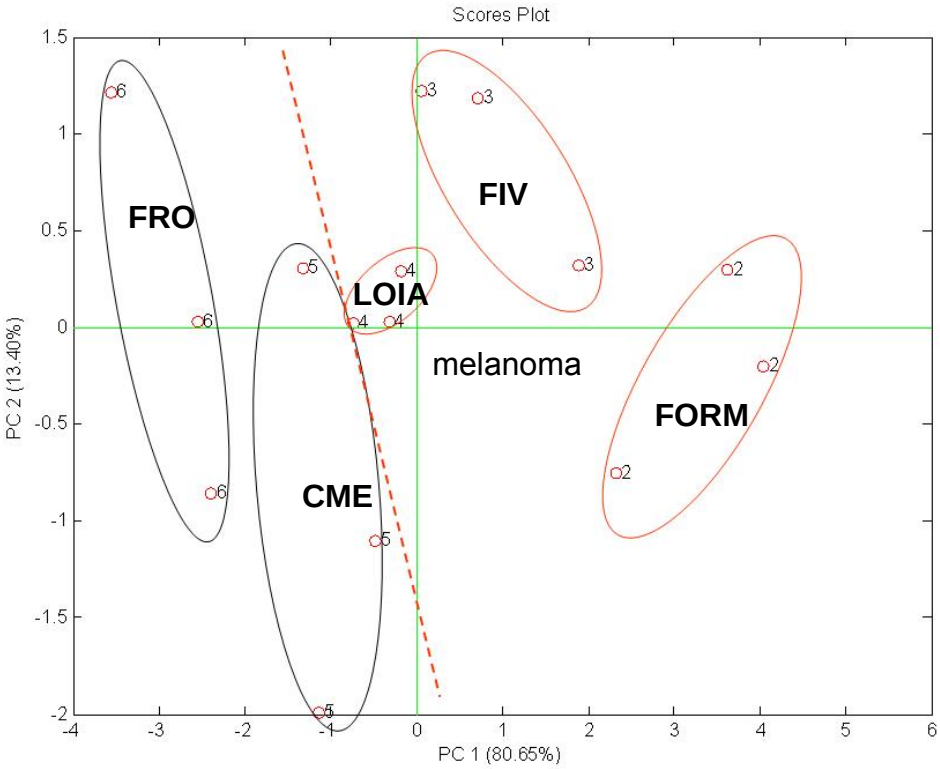


Fig. 5.3: scores plot of the first two component of the PCA model obtained from the tumor cell lines measurements. In this case the discrimination among different cell lines is shown.

The five different kinds of cancer cells are well separated and most interestingly the three melanomas cluster together separately from the other two kinds of tumors.

5.4 Headspace analysis of tumor cell line in vivo

5.4. 1 Experiment description

In vitro experiment results ,described in the previous paragraph, have suggested the possibility to discriminate different tumor cell lines from their headspace. It seems that the different metabolism of each tumor cell line

produces a different chemical composition of the headspace according to the works described in the first chapter.

The next step regarded the study of the correlation of a tumor mass and the relative headspace modification. For this experiment the same colture cell lines were injected in mice.

Pathogen-free 4-5-week-old nude *(nu/nu)* mice (Charles River Breeding Laboratories, USA) were used for establishing different tumor cell lines xenografts *in vivo*. Mice were maintained in cages of 4 animals each, in the animal facility at the National Cancer Institute of Rome, Italy, and fed with standard diet and water *ad libitum*. Cell lines were detached from tissue culture plates with Tripsin 0,05%-EDTA 0,02% (Gibco), washed in sterile phosphate buffered solution (PBS) pH 7.4 and injected subcutaneously at a concentration ranging from of $8x10^6$ - 10^7cells /0.2 ml saline solution.

Injection was performed by using a standard insulin needle, painless. No anaesthesia was necessary. 75 animals, in five different sets of experiments were xenografted (five animals for each cell line) and checked three times a week for signs of measurable tumor growth. In each experiment five xenograft-free mices were used as control.

Starting from two-three days after injection the animals were used for *in vivo* scent detection experiments as reported below. To determine the morphology and phenotype of the growing tumors, some of them were surgically excised and used for conventional histological analysis. This work was performed according to the specific guidelines provided by the Italian Ministry of Public Health for the animal experimentation. This study was approved by the Institutional Ethical Committee at the National Cancer Institute of Rome. The animal facility at the NCI of Rome is certified by the Italian Ministry of Public Health.

5.4.2 Enose sampling protocol

In fig 5.4 the sampling protocol designed to analyze tumor cell lines headspace in vivo by electronic nose is shown.

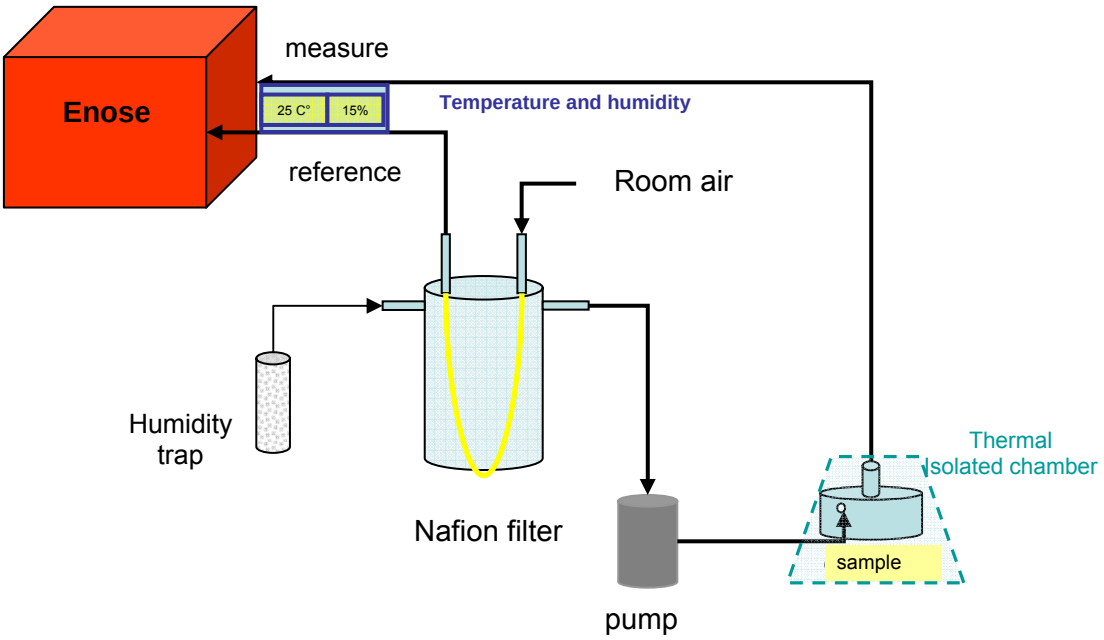


Fig. 5.4: sampling protocol for mice headspace measurements. A nafion filter for humidity effect reduction was used.

The protocol for the measurements on mice used dry air filtered by a NAFION membrane. In spite of a mass flow controller two pumps were used to control the cleaning and measure flow fixed at 0.5 l/min. A stainless steal sampler was fixed at the end of a adjustable arm used for the correct positioning on the tumoral mass to measure.

5.4.3 GC/MS method

The same protocol used for the melanoma study has been tested on nude mice previous introduced (see cap 3) [17,18]. A nude mouse is a genetic

mutant that has a deteriorated or removed thymus gland, resulting in an inhibited immune system due to a greatly reduced number of T cells. The phenotype, or main outward appearance of the mouse, is a lack of body hair, which gives it the "nude" nickname. The nude mouse is valuable to research because it can receive many different types of tissue and tumor grafts, as it mounts no rejection response.

In figure 5.5 are shown the overlapped chromatograms (from 10 to 25 minutes retention times) of three healthy nude mice, sampled on the back skin surface with the same protocol used for human skin. As they are inbred mice the chromatograms are very similar. Again this is a good sign of the quality of the method.

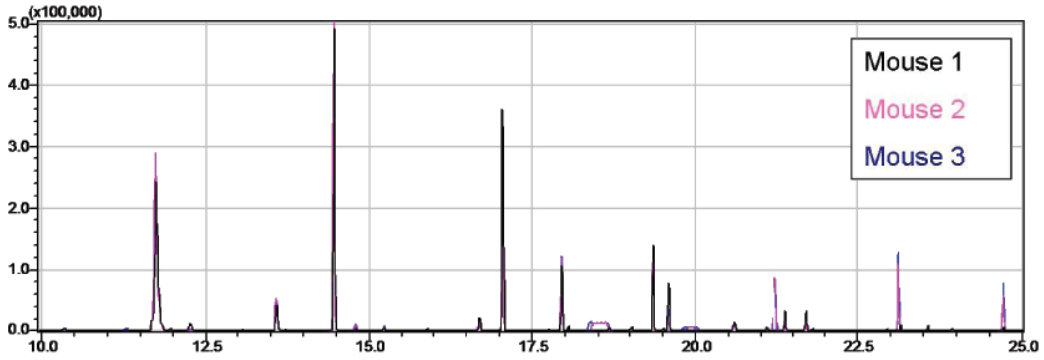


Fig. 5.5: chromatograms related to the headspace of 3 nude mice. The superposition of the three graph shows a good repeatability of the measurements..

5.5 In vivo experiment: first trial

In the first trial ten mice were made available. They were divided in four groups of three mice each. Mice of each group were injected with LOIA melanoma, FORM melanoma and CME synovial sarcoma. Additionally a non inoculated mouse was added to the experiment to provide a reference.

The results are indicated in fig5.6. It shows the plot of the first two principal components of the PCA model calculated on data collected the first day after the inoculation.

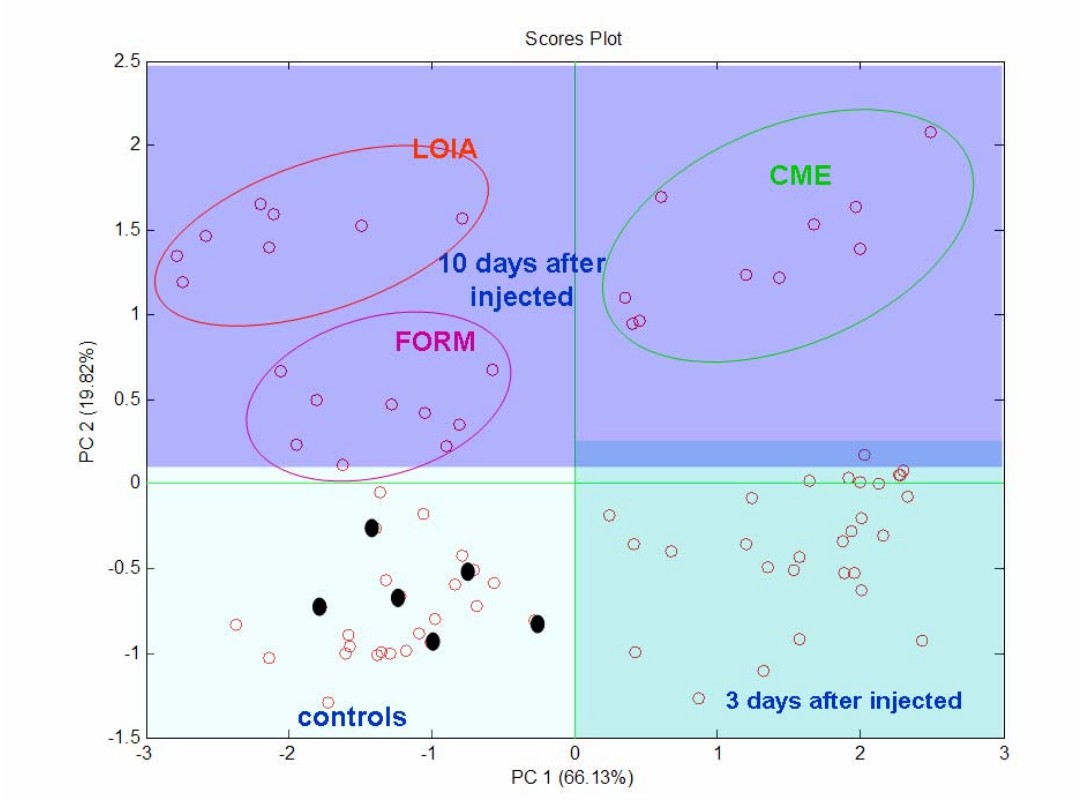


Fig. 5.6: scores plot of the first two components of the PCA model related to the measurements performed during the period of ten days. The control measurements are indicated by black points.

After three days a second set of data was taken and the score plot of the Principal Component Analysis is shown in figure 5.6. The data are mostly arranged along the first principal component, the data does not show a clear clustering and the measurement of the reference mouse falls in the controls region. Tumoral tissues emissions seem clearly different with respect to that of the normal mouse, and the subset of compounds peculiar of each tumoral form are not appreciable. Measurements were repeated at the tenth day after inoculation. At this stage of evolution the three tumoral forms becomes clearly

distinguishable one each other (see figure 5.6, upper part). The two melanoma forms are closer, in the score plot, with respect to the synovial sarcoma. It is worth to remark that the non-inoculated mouse is still plotted in the control region showing a substantial constancy of body odour of the animal and an optimal reproducibility of the measurement methodology. The data set of all measurements collected for thirty-one days and analyzed with the PCA model are shown in fig.5.7. The data have been normalized to control mice of each day.

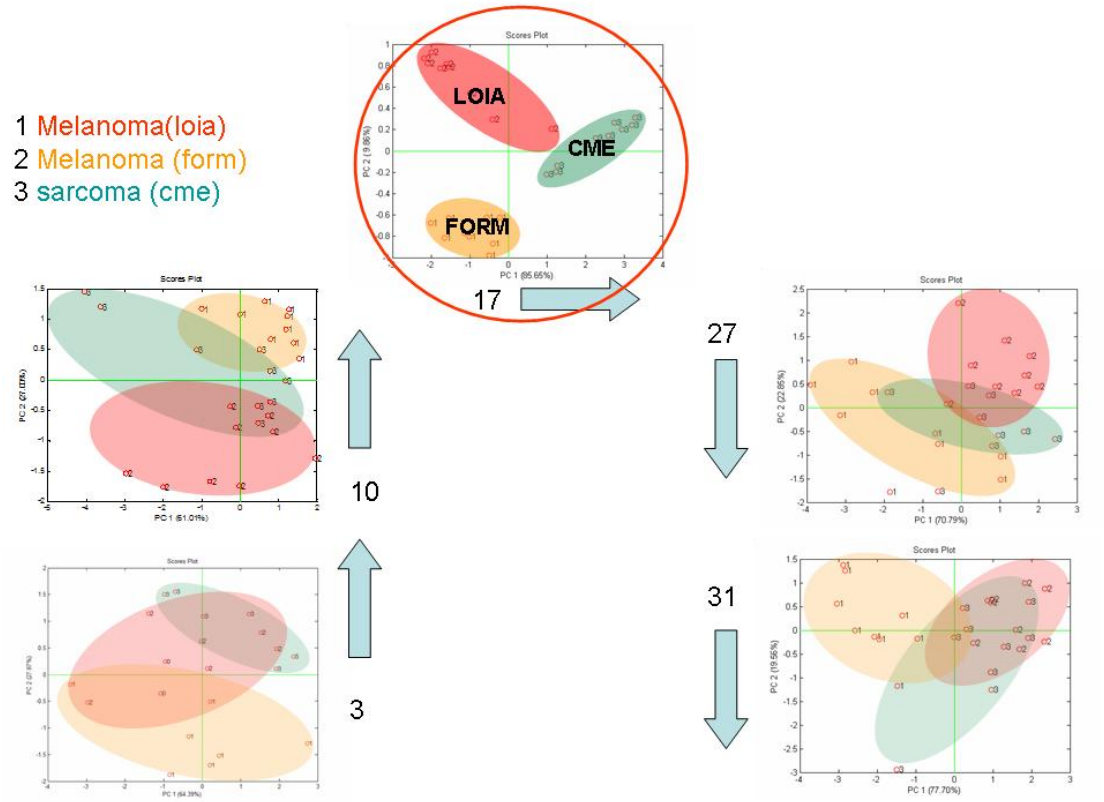


Fig. 5.7: time evolution of in vivo measurements. For each day it is reported the score plot obtained from the PCA model calculated on the data set relative to that day. Data are normalized to the reference measurements of the day. The better discrimination is obtained after 17 days.

In fig.5.7 it is clear the discrimination among the different tumor cell lines after seventeen days. After ten days since inoculation the clusters are mixed and it seems that the headspace of different cells are homogeneous. The peculiarity of this measurement day is also confirmed by the plot represented in fig.5.8 in which the response of one sensor has been considered.

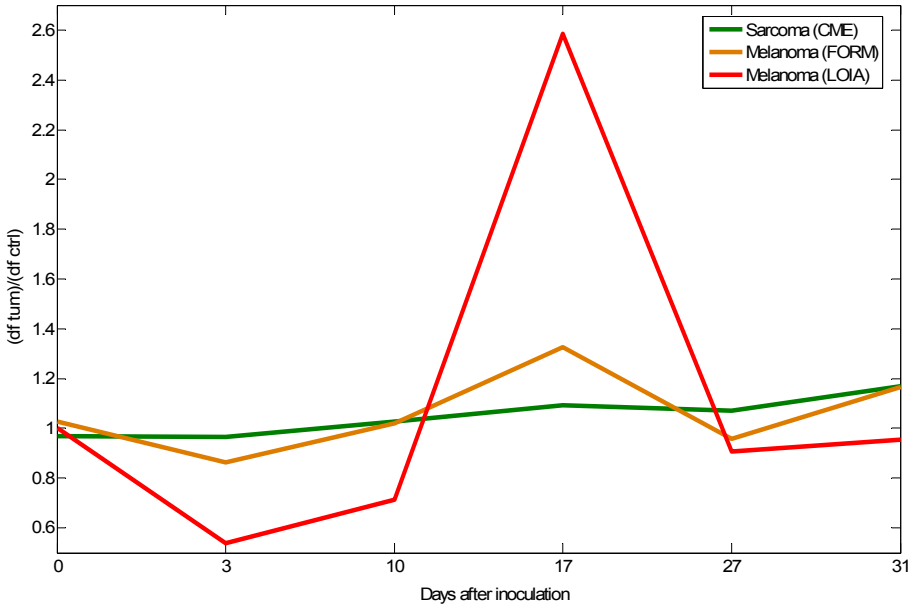


Fig.5.8: time evolution of one sensor response registered by the electronic nose in the monitoring of three different tumor growths. Each color corresponds to different tumor cells.

The responses related to three tumor cell lines are similar among them in the first days (before seventeenth day) and in the last days (after twenty-fifth day). This experiment shows that the difference between tumors evolves temporally so that the three investigated proliferated cells becomes distinguishable after ten days.

5.6 In vivo: second experiment

This result was confirmed in a second experiment that was performed on a larger population of mice and for a longer time. A total of 18 mice were included in the experiment. Three groups of five mice were formed, and mice of each group were inoculated with:

- melanoma (labelled as MULEN);
- melanoma (labelled as LOIA);
- renal cell carcinoma (labelled as KJ29);

A final group of three mice were not inoculated and held as control. The volatile compounds emitted from the inoculated skin region was measured twice a week. Since in the previous trial non inoculated mice were practically constant all along the experiment, the sensor data were normalized by dividing to the average signal recorded for the non inoculated mice. The data collected each day were then normalized and reduced by PCA. The progression of PCA score plots are shown in figure 5.8 where the data related to the three mice groups are evidenced.

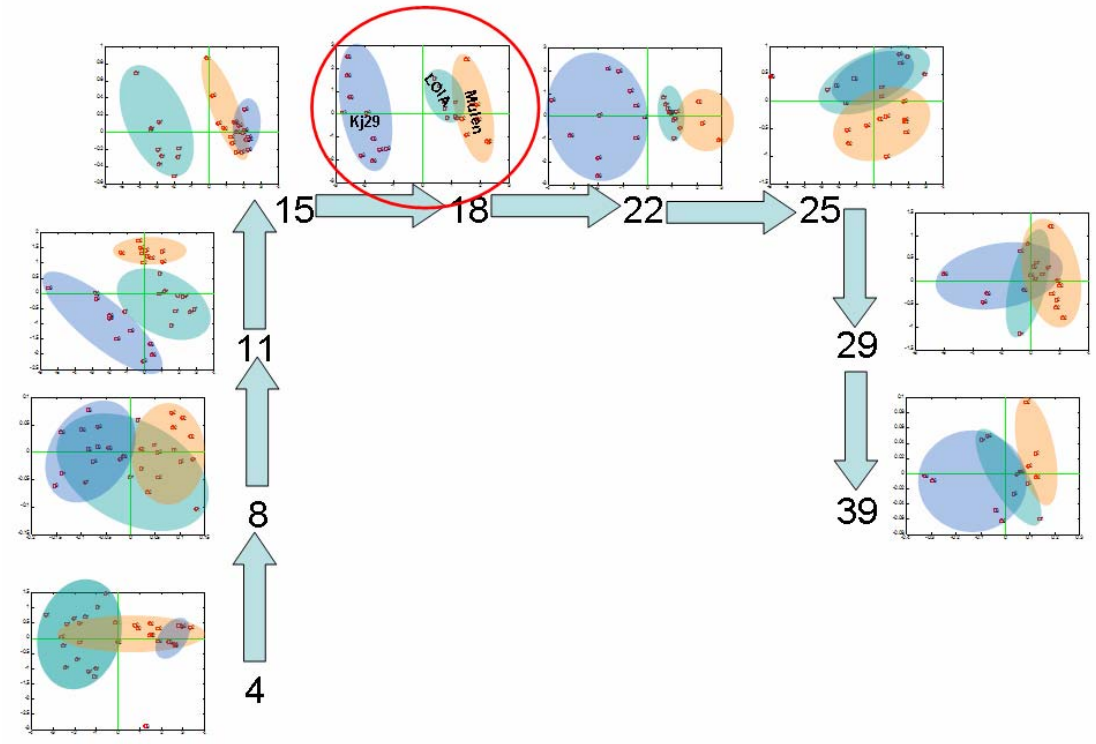
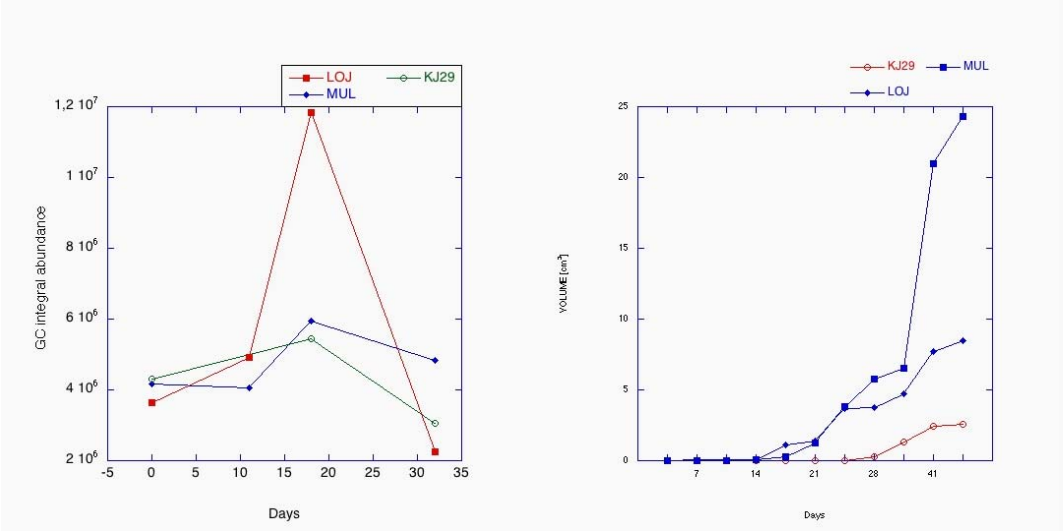


Fig. 5.9: time evolution of in vivo measuremets. For each day it is reported the score plot obtained from the PCA model calculated on the data set relative to that day. Data are normalized to the reference measurements of the day. The better discrimination is obtained after 18 days.

As in the previous experiment, the difference between groups becomes evident after eleven days from the inoculation. From 11 to 18 day the difference between the melanomas is attenuated but the difference between the LOIA sample and the melanomas grows. The 18th day is also a turning point after which the separation between tumors decreases and from the 25th day the three tumors are no more distinguishable like in the first stage of development. This behaviour is compatible with the expected development of the tumoral tissues when the necrotic processes exceeds the tumoral cells growth and the released volatile compounds are dominated by the typical products derived from living tissues spoilage processes that are rather similar disregarding the nature of the cells.

Chemical sensors behavior is partially correlated with the measure of the tumor volume onto the mice skin and by with gas-chromatographic measurement of the same headspace analyzed by the chemical sensors. In figure 5.10 the average sensor signal is compared with the total abundance of the headspace calculated as the integral of the gas-chromatogram and the tumor volume.



(a) (b)

Fig. 5.10: time evolution of mice headspace modification as revealed by GC/MS signal integration (a). It is confirmed a peak of compounds aboundance for all the three tumors round the eighteenth day. In fig. b the evolution of the tumor growth is presented in term of effective volume of the tumoral mass.

All these data are calculated as averages inside each group. The tumor volume growth follows the expected progression of proliferating tumor cells. It is rather interesting to note that the chemical signature of the tumor mass is detectable well before that the tumor mass becomes appreciable. Gas-chromatograms confirm the sensors signal peaks, even also if with different magnitudes in the two melanomas. The position of the chemical peak is not correlated with the geometrical growth of the tumor suggesting that the

chemistry express a quality on-set in the tumor tissue progression. If this is a mere effect of xenografts or it reveals something inherent in tumor progression will deserve further investigations.

5.7 Conclusions

Although the xenografts exhibits different growth rate, partially due to the different amount of implanted cells in the two experiments, the emission chemistry provides a unique signature of the kind of tumors showing a very strong similarity of the two melanomas with respect to the kidney cancer.

Among the different cell lines investigated the melanoma LOIA was included in the three experiments. This opportunity has offered the possibility to study the evolution of the same tumor in two different inoculation in two batches of xenografts. In figure 5.11 the temporal evolution of the signal of one of the sensors of the array is plotted.

In both cases the behaviour is characterized by a sharp signal peak occurring around 18^{th} day after the inoculation. The strong non linearity of the curves poses an intriguing question about the relationship between the sensor signals and the tissue growth.

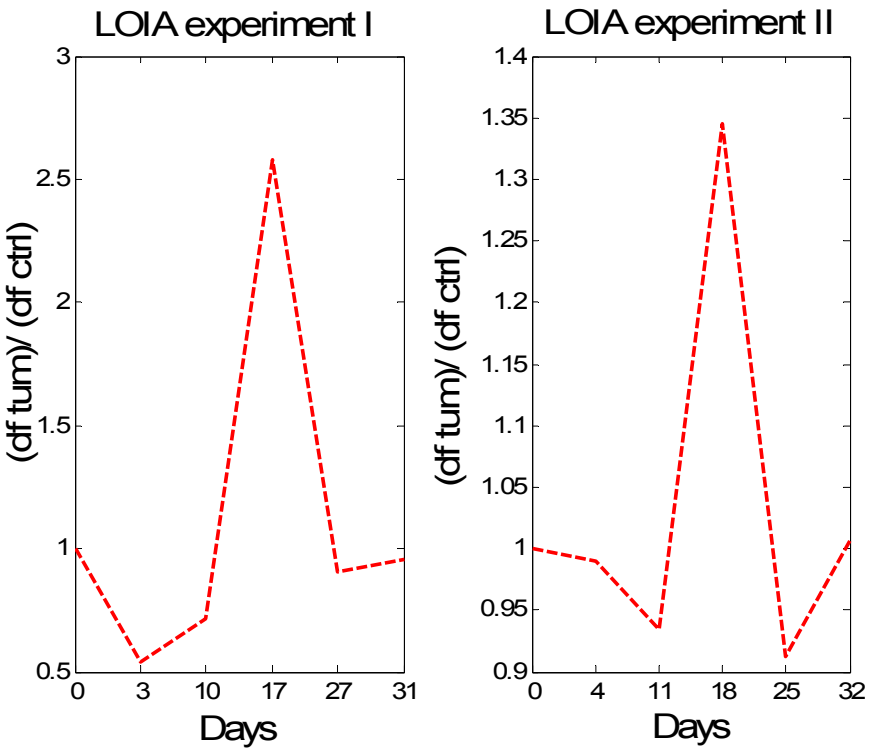


Fig. 5.11: time evolution of one sensor response registered by the electronic nose in the monitoring of the same tumor in the two exeperiments. It can be observed the repeatability of the curves shape and of the day of maximum response.The differences in magnitude depends on the number of cells injected.

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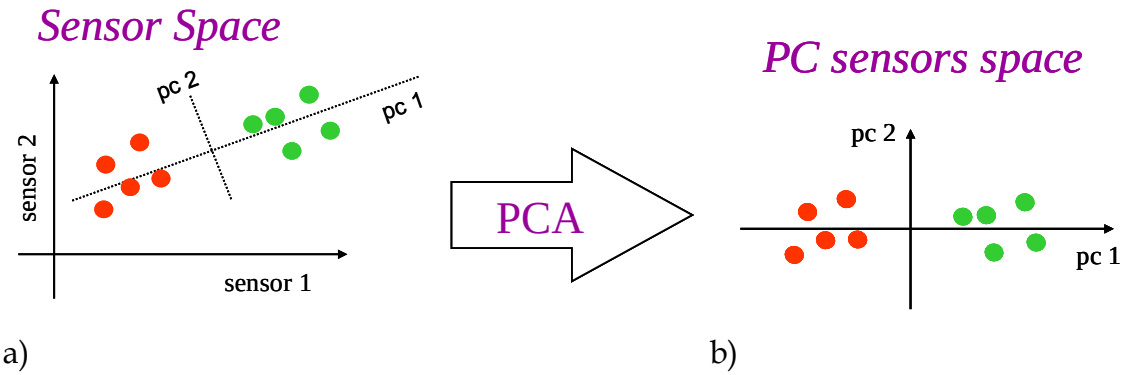
APPENDIX A

Data analysis methods

In this appendix a brief introduction on the techniques utilized in this thesis is given. The first one is the Principal Component Analysis (PCA) used as an unsupervised techniques but also as a standard pre-processing techniques. The Partial Least Square (PLS) is used as regression model and PLS Discriminant Analysis is applied in the case of classification problem. This linear technique has been preferred because datasets were not large enough to guarantee a reliable result when non linear techniques are used.

A.1 Principal Component Analysis (PCA)

PCA is a unsupervised techniques used to describe a dataset in a graphical representation carrying most of the data variance [1]. The axes of the new representation space, are given by a linear combination of the original axes and they are requested to be uncorrelated and orthogonal. The first axis, called first "Principal Component", is chosen in the direction of the largest variance, the second axis, called second "Principal Component", is taken along the orthogonal direction to the first PC with largest variance, and so on. This procedure is depicted in the figure A.1.



a) b)

Fig. A.1: A sketch of Principal Component procedure. PCA finds in the dataset the direction with maximum variance.

This technique allows to visualize, in two or three dimensions, a multi-dimensional datasets for a preliminary data exploration to study the intrinsic capability of the system to discriminate the data in clusters[2]. Considering an array of N non selective sensors, their outputs are always expected partially correlated because of the sensors cross sensitivities. This means that the real dimension of the data array is smaller than the numbers of feature extracted for the N sensors (see figure A.2). Then the Principal Component Analysis is an algorithm that searches the optimal representation of the data in a subspace of smaller dimension, maximizing the variance of the dataset. A scatter plot where the samples are represented as points in a space with coordinates the principal components (PCs) is called Scores Plot. Figure A.1b shows an example of Scores Plot where with only a first PC it is possible to discriminate the two classes.

The plot of the features on the plane spanned by the PCs is called 'loadings plot' and shows the contribution of the features to the calculation of the PCs.

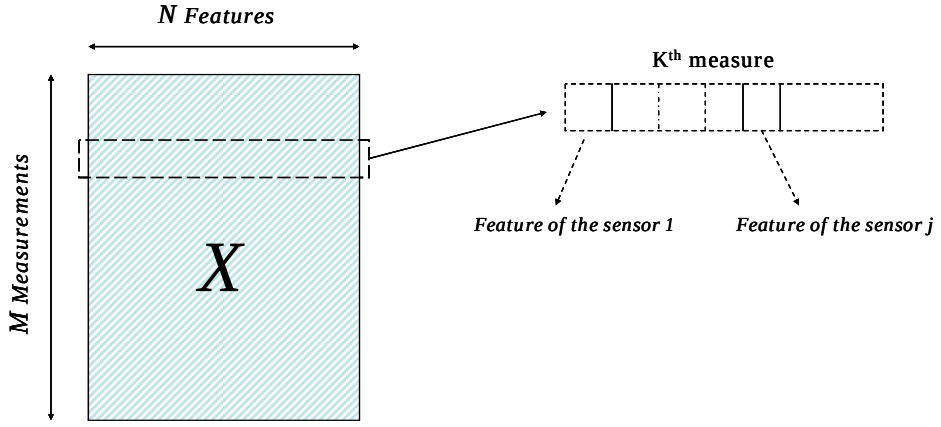


Fig. A.2: A data collected by the electronic nose measurements used as input of PCA

The loadings are concerned with the feature. They measure the contribution of each feature to the array. A high loading, for a feature, means that the principal component is aligned along the feature direction. On the other hand, features with a similar behaviour in all the PCs brings the alike information.

A.1.1 PCA calculus.

PCA can be calculated with several algorithm, among them the SVD is here presented.

Given a number of samples M, greater than the number of sensors N, the data matrix X (see figure A.2) can be written:

$$X_{MxN} = U_{MxN} D_{NxN} V^T_{NxN} = \tag{A.1}$$

$$= \begin{pmatrix} u_1 & u_2 & \cdots & u_N \end{pmatrix} \begin{vmatrix} \sigma_{11} & 0 & 0 & 0 \\ 0 & \sigma_{22} & \cdots & 0 \\ \vdots & \vdots & \ddots & 0 \\ 0 & 0 & 0 & \sigma_{NN} \end{vmatrix} \begin{pmatrix} v_1 & v_2 & \cdots & v_N \end{pmatrix}^T$$

where **U** the score matrix and the single term $\mathbf{u_{i,j}}$ represents the coordinate of the j[th] PCs for the i[th] measure; **D** NxN diagonal matrix of eigenvalues (it is a non singular matrix); the columns of V are the eigenvectors of the matrix XX^T associated with the eigenvalues $\sigma_{11}^2 \quad \sigma_{22}^2 \quad \cdots \quad \sigma_{NN}^2$. The amount of variance described by each eigenvector is determined by the magnitude of the associated eigenvalues. Since eigenvalues are sorted in descending order, the greatest amount of data variance will be described in the first PC. The Loadings of i[th] sensor for j[th] PC is defined as

$$Loadings\left(sensor_i, PC_j\right) = \sigma_{jj} \cdot v_{ij} \tag{A.2}$$

Given a Principal Component, an high loadings value for i[th] sensor indicates that the PC is aligned in a direction close to the original response of that sensor. Then, loading plots can be used to determine which sensor contributes mainly to the overall information and which sensors can be omitted. Scores and Loadings Plots can be plotted one over the other. With this representation it is possible to observe which sensors contribute to the classification problem. Figure A.3 shows an example of typical bi-plot. In this case the sensors 1 and 4 are more correlated with class 0 and the sensors 3 and 2 with class 1 and 2 respectively.

It is important to note that largest eigenvalues corresponds to components defining the directions of largest correlation, while the components characterized by smaller eigenvalues are mostly related to uncorrelated directions. Since sensor noises are uncorrelated representing the data using only the most meaningful components removes part of the noise.

Furthermore, observing that no a priori statistical knowledge is necessary to apply this techniques, PCA is also used a pre-processing techniques. Actually, a reduction of dimensionality and of noise to signal ratio

is possible to obtain . On the other hand, the use in a next step of a subset of PCs implies the assumption that the meaningful part of the data information is contained in the variance. This means that the data are considered Normally distributed.

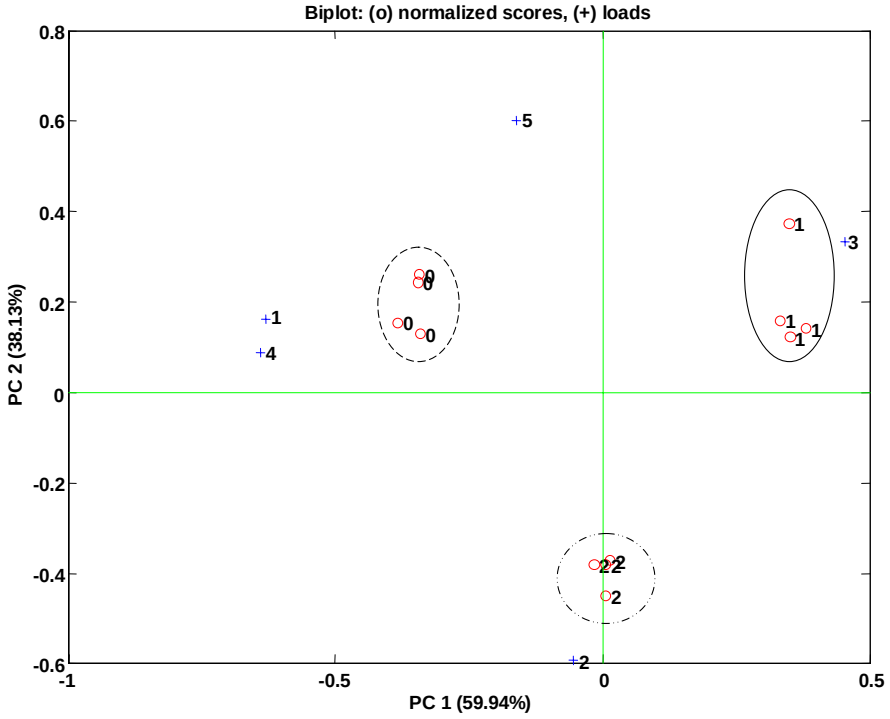


Fig. A.3: PCA biplot of the data matrix formed. Scores are labelled with circles while loadings are labelled with crosses. Three classes are present. The sensors 1 and 4 are strongly correlated with class 0 and the sensor 2 with the class 2; the sensor 3 with class 1.

In these cases, PCA application may not consider a part of the data information useful for the classification problem.

A.1.2 Partial Least Square (PLS).

Partial Least Square (PLS) regression is a data analysis technique that combines characteristics from principal component analysis and multiple

regression. It is mostly useful when we need to predict a set of dependent variables from a large set of independent variables (predictors). It originated in social sciences [2] but became popular in chemometrics due in part to Svante Wold, [3] and in sensory evaluation [4]. Let us consider M observations described by K dependent variables are stored in a MxK matrix denoted Y, the values of J predictors collected on these M observations are collected in the MxJ matrix X.

The goal of PLS regression is to predict Y from X and to describe their common structure. When Y is a vector and X is full rank, this goal could be accomplished using ordinary multiple regression [5]. When the number of predictors is large compared to the number of observations, X can be singular and the regression approach is no feasible (i.e., because of multi-collinearity). Several approaches have been developed to resolve this problem. One approach is to eliminate some predictors (trying to avoid the multicollinearity) another one, called principal component regression, is to perform a principal component analysis (PCA) of the X matrix and then use the PCs of X as regressors on Y. The orthogonality of the principal components eliminates the multi-colinearity problem. But, the problem of choosing an optimum subset of predictors remains. As example, the choice of the first PCs of X can not be relevant to find a correlation with the variables Y.

Instead, PLS regression finds components from X that are relevant for Y. In particular, PLS regression searches for a set of components, called latent vectors, that achieves a simultaneous decomposition of X and Y with the restriction that these components explain as much as possible of the covariance between X and Y. Then PLS algorithm searches the subset of X that show the maximum correlation with Y.

PLS regression decomposes both X and Y as a product of a common set of orthogonal factors and a set of specific loadings. So, the X matrix is decomposed in the following way

$$X = TP^T + \mathrm{E} \quad \text{with} \quad TT^T = I \tag{A.3}$$

with I the identity matrix, by analogy with PCA T is called the score matrix, P the loading matrix, E the residuals matrix. It is worth to note that in PLS regression the loadings are not orthogonal contrary to PCA where the loadings are orthogonal. In the same way, Y is decomposed as

$$Y = UC^T + \mathrm{G} \quad \text{with} \quad CC^T = I \tag{A.4}$$

where U is called the score matrix, C the loading matrix and G the residuals matrix. It is possible to estimate Y with

$$\hat{Y} = TC^T + F \tag{A.4}$$

where F is the residuals matrix.

Taking into account the eq A.12, the eq A.13 can be written as

$$\begin{aligned} Y &= XWC^T + F = XB + F \\ B &= WC^T \end{aligned} \tag{A.5}$$

where W is found with the relation $T = XW$.

The matrixes T and C have to find maximizing the covariance between X and Y. It is worth to remark that PLS is a projection method. Then, this technique can be seen as a projection of the X-matrix down on a K-dimensional plane in such way that the coordinates of the projection (t_a a=1,…,K given by the matrix T) are good predictors of Y. This indicated in figure A.4.

The prediction error of the model is strongly dependent by the number of variables (features) and by the number of measures . In the case of small dataset, in order to have a realistic error estimation a cross validation technique is applied. The Latent Variables, that minimizes the prediction error in the validation phase, are used to build a model for the further test phase . In this thesis, the Leave One Out Cross Validation has been considered.

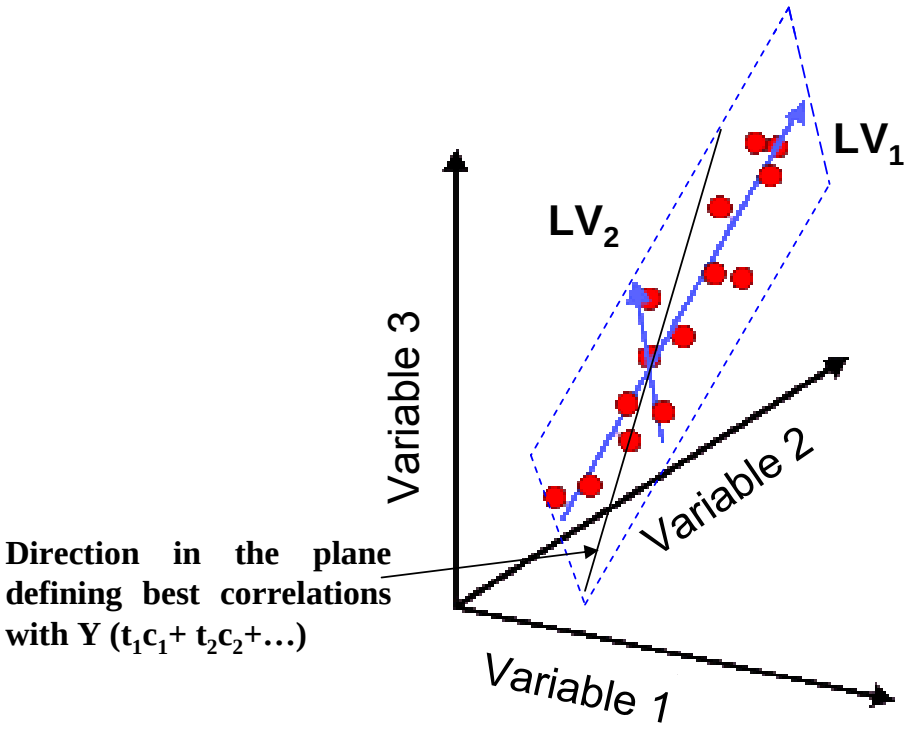


Fig. A.4: Geometrical representation of PLS regression

A.1.3 Leave One Out Cross Validation (LOOCV).

In literature, several techniques of cross validation have been introduced, here the Leave One Out Cross Validation is considered [1]. Since the number of samples is rather poor, the prediction error rate is computed using cross-validation by blocks or a leave-one-out cross-validation; the data set is divided into k subsets: the calibration is carried out on (k-1) blocks, and the prediction is made on the samples belonging to the kth subset. This is repeated k times with block permutation, in order to predict all the samples. The cross-validation is called leave-one-out cross-validation where each subset is composed of only one sample: in this case, there are as many subsets and models as samples (k=n).

At the end of validation phase the Predicted of Error Sum of Squares are calculated in the following way:

$$PRESS = \sum_{i=1}^{N} \left(\hat{y}_i - y_i \right)^2 \tag{A.6}$$

The number of latent variables that shows a minimum of PRESS corresponds to the optimal choice. This kind of choice avoids the model over fitting. Figure A.5 shows the prediction error in training phase (red line) considering all the sample, and the error in the validation phase (green line).
At the end of validation procedure, chosen the *k* optimal latent variables, the Root Mean Square Error Cross Validation (RMSECV) is calculated in the following way:

$$RMSECV_k = \sqrt{\frac{\sum_{i=1}^{N} \left(\hat{y}_i - y_i \right)^2}{N}} \tag{A.7}$$

where $\hat{y}_i$ is the estimated value and y_i the real value.
This value gives a generalization of the prediction error of the model. This result is relevant to have an idea of the real prediction performance of the model in presence of data not used in the training phase.

A.1.4 PLS Discriminant Analysis.

PLS regression is not perfectly suited to pattern recognition problems, i.e. for classification purposes. However, this technique can be adapted for classification, giving rise to the PLS-DA method.

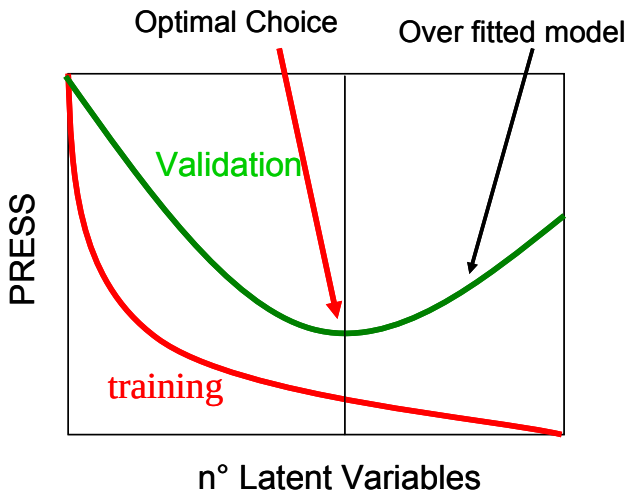


Fig. A.5: An example of the prediction error for the validation and training phase.

PLS-DA is carried out using an exclusive binary coding scheme with one bit per class, providing a triplet {a; b; c} if ones wants to discriminate between three classes. Each number represents a ''membership value'' for each class, e.g., a response encoded {0; 1; 0} means that the sample belongs to class 2. During the calibration process, the PLS-DA method is trained to compute the three ''membership values'', one for each class; the sample is then assigned to the class showing the highest membership value. Leave-one-out Cross- Validation was used to compare the performance of the various models. The prediction results are displayed in a confusion matrix, presenting the number of samples assigned to each class. So the classification rate of the system is given by the following relation

$$C_R(\%) = 100 * \left(\frac{M_{Samples}}{TOT_{Samples}} \right) \qquad \text{(A.8)}$$

where $M_{Samples}$ is the number of samples correctly classified and $TOT_{Samples}$ the total number of samples.

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APPENDIX B

NAFION filter

B.1 NAFION properties

In the experiments described in this thesis a NAFION filter to control humidity influence has been used. In this appendix a brief description of NAFION properties will be presented. It is a simple and efficient methods for removing water vapor from gases and it has many applications. There are several practised methods to achieved the mentioned objective but generally a membrane process is compact, modular, energy-efficient, and economically attractive. In recent years, some highly efficient membrane modules have been developed for water removal . Among them, NAFION membranes have attracted much attention because of their extraordinarily high water permeability and wide application in electrochemical processes.

The structure, properties, and water transport mechanism of NAFION membranes have been reviewed in Perma Pure's technical notes . NAFION, was developed by Dr. Walther Grot at DuPont in the late 1960's by modifying Teflon. NAFION combines the physical and chemical properties of its Teflon base material with ionic characteristics that give the final material the following properties. It is extremely resistant to chemical attack. According to DuPont, only metallic alkali metals (sodium in particular) can attack NAFION directly under normal conditions of temperature and pressure. This means NAFION does not release fragments or degradation products into the surrounding medium.

NAFION has relatively high working temperatures compared to many polymers and it is used in some applications at temperatures up to 190° C. One of the most important properties is to be highly ion-conductive. NAFION is a super-acid catalyst. The sulfonic acid groups attached to the Teflon backbone within NAFION function as an extremely strong proton donor due to the stabilizing effect of the large polymer matrix attached to the sulfonic acid.

The sulfonic acid groups in NAFION have a very high water-of-hydration, so they very efficiently absorb water. Interconnections between the sulfonic acid groups lead to very rapid transfer of water through the NAFION.

B.2 NAFION dryers

There are different ways to realize drying devices. Microporous hydrophobic filters are used to protect the devices from damage due to liquid water. They are based on microporous plastic materials with pores too small for liquids to pass but they will let gases of all types through. On the same principle the microporous tubing dryers work. In this case the microporous plastic material is placed in a tube. The sample is supplied to the inside of the tubing at an elevated pressure. In this way small molecules such as water vapour are forced through the pores in the tubing wall for pressure difference.

For NAFION tubing dryers a different principle from microporous materials is used. NAFION doesn't remove the gases for their molecular size but for their chemical affinity to sulfonic acid. NAFION membranes removes water with a particular mechanism: the rate of the binding of water as water-of-hydration and the rate of the passing of water through the sulfonic acid channels depend on the water content within the membrane, and thus on the partial pressures of water vapor in the sample and purge gases .

Since the mechanism of water transport is related to the interactions with the ionic groups. The ionomer consists of a fluorocarbon backbone substituted with a low molar concentration of fluoroether side groups terminated with the sulfonic acid residue. This corresponds to substitution with one fluoroether-sulfonic acid side-chain for thirteen CF_2 groups whereby the side-chain amounts to 33% by weight of the polymer. The main structural features of NAFION arise from the incompatibility of the ion containing fluoroether side group and the nonpolar fluorocarbon backbone .

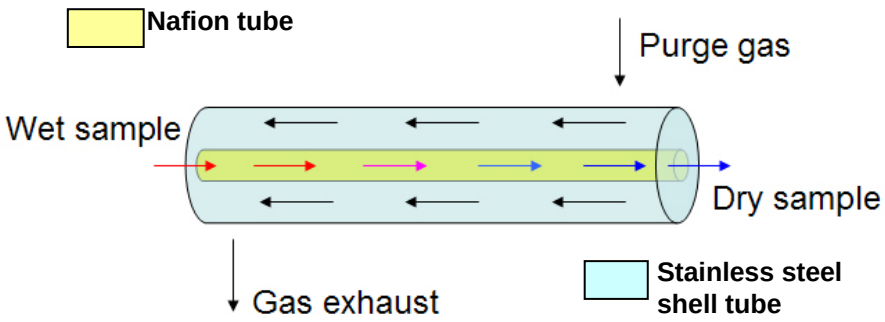

Fig. B.3: nafion filter mechanism. The mechanism of water vapour removal is based on chemical affinity of the sample to sulfonic acid.

In figure B.1 a scheme to use nafion tube is shown. Once absorbed into the wall of the nafion tubing, the water permeates from one sulfonic group to another until it reaches the outside wall of the tubing, where it perevaporates into surrounding gas (air or other gas). In the mechanism of nafion tube the water vapour pressure gradient are used and not the pressure difference. In this way it doesn't need to supply the sample under pressure such as microporous tubing dryers.

List of Publications

Papers

1) A. D'Amico, R. Bono, G. Pennazza, M. Santonico, G. Mantini, M. Bernabei, M. Zarlenga, C. Roscioni, E. Martinelli, R. Paolesse, C. Di Natale. Identification of melanoma with a gas sensor array. Skin Research and Technology, DOI: 10.1111/j.1600-0846.2007.00284.x, accepted 26 May 2007.
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