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Applications of gas chromatography for air and food quality assessment, and for monitoring health-related biomarkers

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INDEX

Abstract……..……3

Chapter 1: Study of the volatile organic compounds (VOCs) produced during cooking and development of a new method to test the efficiency of odor filters……8

Chapter 2: Study of volatile organic compounds as markers of shelf-life of organic and conventional chicken meat stored in different packaging……..…….102

Chapter 3. Development and application of a rapid and simple method to analyze squalene in different food matrices…….....……156

Chapter 4: Development and application of a GC-FID method for the analysis of short chain fatty acids in rat and human faeces and in fermentation fluids……..……...….202

Abstract

Gas chromatography has been applied in a wide range of fields: from environmental analysis to culture heritage sector, from biochemical field to food analysis, because of its high versatility and its availability in many laboratories. In this work, gas chromatography (GC) coupled to flame ionization detector (FID) or mass-spectrometry (MS) was exploited in various sectors related to food chemistry, demonstrating its high performance and versatility.

In the first study, GC-MS has been exploited for the assessment of the efficiency of kitchen hoods filters, and to monitor volatile organic compounds (VOCs) development in different cooking systems and foods, thus contributing to assess air quality in indoor environments. In particular, a new method involving the sampling of the air during a cooking process by a polyethylene terephthalate (PET, Nalophan) bag and the subsequent analysis by solid-phase microextraction (SPME) and GC-MS, was applied for the first time to the study of VOCs developed in different cooking systems (deep-frying of French fries, grilling of a hamburger, boiling of cauliflower and heating of sunflower oil). The same method was applied for the efficiency test of odor filters used in domestic kitchen hoods and also to develop and validate an alternative method to the one proposed by current legislation EN IEC 61591.

The second study exploited again a new SPME-GC-MS, but in a different field: the study of food shelf-life. The first objective was the study of the efficiency of a new biopackaging (BP), based on biopolymers, to preserve the quality of organic chicken meat under modified atmosphere (MAP) in comparison with a polyethylene terephthalate (PET) material, during storage at 2°C. The second purpose regarded the comparison of the shelf-life of organic and traditional chicken meat (of the same species) in a cellophane packaging under aerobic conditions at 2°C. The results were promising and this study contributed to encourage the use of new biodegradable materials and the consumption of sustainable and organic products.

In chapter 3, the presented study applied GC-FID, for the valorisation of food products and by-products, by analysing a bioactive compound, squalene, in vegetable oils and apple by-products (seeds and peels), after developing and validating a new, rapid and simple analytical method. Squalene has been demonstrated to have several beneficial properties; in the present study, its content in different extra virgin olive oils and olive oils were investigated. Squalene was also monitored during the refining process of different vegetable oils: olive, soybean, grapeseed, sunflower, sunflower with high oleic content and maize oils, in order to evaluate possible losses during the process. The proposed method was applied also to the study of squalene in two apple

by-products (peels and seeds) to evaluate their possible exploitation in food, cosmetical or pharmaceutical fields. In the future, the method could be exploited in the study of squalene in many different food matrices, reducing time, solvents and costs respect other analytical methods present in literature.

In the study presented in the last chapter, GC was exploited for a different purpose again. The aim was the determination of analytes, short chain free fatty acids (SCFAs), in biological samples as faeces and fermentation fluids, where their measurement gives an indication of the effect of a special diet or environmental condition, where higher content is in general associated with positive effects. A new GC-FID method was developed and exploited in two different projects in collaboration with the School of Biosciences and Veterinary Medicine of the University of Camerino for the determination and quantification of eight SCFAs (acetic, propionic, *i*-butyric, butyric, *i*-valeric, valeric, *i*-caproic and caproic acids) in different biological samples (rat, mice and human faeces and in fermentation fluids samples). Indeed, in the last decades SCFAs were recognized for their beneficial effects on the host health status and their analysis contributed to the to investigate for instance the effect of diet supplementation in the people health or to study the adaptation of gut microbiota during geographical, habits and diet changes.

These works represent some examples of the possible exploitation of gas chromatography in food chemistry and in close fields. Important information and analytical tools have been obtained, that contributed to assess the quality of a food, or air quality, or the effect of a diet, or environmental conditions, on the overall health status of an individual.

INDEX

Abstract……..……3

Chapter 1: Study of the volatile organic compounds (VOCs) produced during cooking and development of a new method to test the efficiency of odor filters……8

1.1 Introduction…….……9

1.1.1 Indoor Air Quality (IAQ) …….……9

1.1.2 IAQ: The European and Italian legislation……..…….…….……13

1.1.3 IAQ and COVID-19……..……...…….…..……17

1.1.4 Cooking emissions……..……...…….…….…18

1.1.4.1 Volatile Organic Compounds (VOCs) from cooking activities……....….20

1.1.5 Air purification……..……...…….……...……21

1.1.5.1 Air filtration……..……...…….……...……..…24

1.1.5.1.1 Air filtration mechanisms……...…….……...……26

1.1.5.1.2 Air filter media……..…….....…….……...……27

1.1.6 Cooking hoods……..……...…….……...……30

1.1.7 Air sampling methods……..……...…….……...……31

1.1.7.1 Air sampling and analysis of volatile organic compounds with solid phase micro-extraction……..…….....……..……34

1.1.7.2 Olfactometric bags……..……...…….…..……35

1.1.8 Aim of the work……..……...…….……...……37

1.2 Materials and Methods……..……...……...……38

1.2.1 Reagents and standards……..……...…...……...……38

1.2.2 Study of VOCs development from different cooking systems……..…..……38

1.2.2.1 Samples and sample preparation……..……..……...……38

1.2.2.2 Headspace SPME-GC-MS and qualitative analysis..……...……40

1.2.3 Development and validation of a new method for assessing odor filters efficiency..…..41

1.2.3.1 Hood aspiration filters……..……...……...……..…43

1.2.3.2 Headspace SPME-GC-FID analysis……..……46

1.2.4 Statistical analysis……..……...……...……...46

1.3 Results and Discussion……...……...……...47

1.3.1 Development of VOCs from different cooking systems…….....47

1.3.1.1 Deep fat frying of French fries…….…….....48

1.3.1.2 Grilling of a beef hamburger…….....53

1.3.1.3 Boiling of a cauliflower and heating of sunflower oil…….....72

1.3.2 Development and validation of a new method for assessing odor filters efficiency…...81

1.3.2.1 Application of the method to real kitchen odor filters……..88

1.4 Conclusions……....92

References…….....94

Abstract

There is nowadays more awareness on the impact on health of pollutants emitted during cooking both from commercial as well as from domestic activities. In this study, a method consisting of sampling of the air during a cooking process by a polyethylene terephthalate (PET, Nalophan) bag and the subsequent analysis by solid-phase microextraction and gas chromatography coupled to mass spectrometry (SPME-GC-MS) was applied for the first time to the study of volatile organic compounds (VOCs) developed by different cooking systems. In particular, VOCs found in the cooking fumes released from deep fat frying of French fries, grilling of beef hamburger, boiling of cauliflower and heating of sunflower oil at 120°C were investigated. The proposed method allows to perform the analysis, even on samples produced in sites far from the instrument location, in an easy way and with instrumentations available in most of the laboratories. After this screening, molecules common to the investigated cooking activities were identified and among them acetic acid was chosen as a standard molecule for the efficiency test of odor filters used in domestic kitchen hoods. In this study, an alternative method to the one proposed by current legislation EN IEC 61591, which involves the use of toxic methyl ethyl ketone, was developed and validated. The method makes use of acetic acid as standard molecule; sampling of the air is performed by PET olfactometric bags and the analysis by solid-phase microextraction and gas chromatography coupled to flame ionization detector. After its development, the proposed method was applied to three different filters, resulted to provide similar filtering performance toward acetic acid.

Chapter 1.

Study of the volatile organic compounds (VOCs) produced during cooking and development of a new method to test the efficiency of odor filters

1.1 Introduction

1.1.1 Indoor Air Quality (IAQ)

Outdoor air pollution has been intensively studied for many decades. In fact, it has been always considered one of the world's largest health and environmental problems. Epidemiological studies have investigated health effects of air pollution [1,2].

Anyway, people spend around 90% of their time indoor [3], so indoor environments impact for the highest part on the exposure to air pollutants. In particular, it has been observed that people spend around 60% of their day at home and about 25% in working environment. **Table 1** shows the results obtained by two different studies on the time spent by people in indoor environments in USA and in some European countries.

Table 1. Percentage of time spent in different indoor environments.

Home (%)	Work (%)	Other indoor (%)	Population	Country	Ref.
68.7	18.2	-	Children and Adults	USA	Klepeis et al. (2001) [3]
58.1	28.0	7.0	Adults	Europe (Finland, Greece, Italy, Switzerland, Czech Republic, France, UK)	Schweizer et al. (2007) [4]

For these reasons, over the past 30 years there has been a greater interest on characterizing indoor air pollution. Many studies focused their attention on the sources and the parameters that affect the so called "indoor air quality" (IAQ). According to EPA (United States Environmental Protection Agency) IAQ refers to "the air quality within and around buildings and structures, especially as it relates to the health and comfort of building occupants"[5]. IAQ is influenced by different sources, such as infiltration of outdoor air pollutants or particulate matters (PM) and gases emitted indoors by human activities (e.g. cleaning, cooking, smoking, using fireplaces) or released by building surfaces and supplies [6]. The most common indoor pollutants (**Table 2**) include nitrogen oxides (NO_x), carbon oxides (CO and CO_2), volatile organic compounds (VOCs) and particulate matters ($PM_{2.5}$ and PM_{10}) [7]. In particular, $PM_{2.5}$ and PM_{10} (defined as thin particles having diameter of 2.5 µm or 10 µm or less, respectively) are commonly used as indicators of air quality respect to the total suspended particular matter

(TSPM), because these particles are small enough to deep inside the lungs inducing reaction of the surface and defense cells [8].

Table 2. Indoor air pollution sources and pollutants [9].

Type	Sources	Pollutants
Occupant-related sources	Human breath Skin metabolism	carbon dioxide ammonia sulfured hydrogen carbon monoxide volatile organic compounds
Building-related sources	Building materials Paints/adhesives/solvent furnishing Commercial products	formaldehyde volatile organic compounds radon ozone ammonia
Outside-related sources	Motor vehicles Industry Public work Agriculture Ground Sewage	sulfur dioxide nitrogen oxides carbon monoxide volatile organic compounds ozone lead particulates

Poor IAQ has been associated to several negative health effects, which undergo to the name of "sick building syndrome" (SBS). It consists of a group of nonspecific symptoms that are temporally related to staying in particular buildings [10]. The typical symptoms include headache, eye, nose, or throat irritation, dry cough, dry skin, dizziness and nausea, difficulty in concentrating, fatigue or sensitivity to odors. On the contrary, the term "building related illness" (BRI) is used for more serious health problems and indicate diagnosable illnesses attributed directly to indoor building contaminants. These building-related illnesses are asthma, inhalation fever, hypersensitivity pneumonitis, rhinosinusitis or infections [11]. IAQ is highly influenced by many factors, such as temperature of the air, the ventilation of the room or the presence of dust and biological contaminants [12]. Different studies revelead that a temperature higher than 22°C in closed environments tend to increase dryness sensation and other SBS symptoms [13,14]. Also a poor ventilation, which occurs primarily when heating, ventilating and air conditioning (HVAC) systems do not work properly, is an important factor in SBS. For this reason, the American Society of Heating, Refrigerating and Air-Conditioning Engineers

(ASHRAE) recommends a minimum of 15 cubic feet per minute (cfm) of outdoor air per person, in particular 20 cfm per person in office spaces [15]. Biological contaminants are mainly bacteria, molds, pollen, and viruses that can come from outdoor air or can be present in stagnant water in ducts and humidifiers or in water-damaged carpet, ceiling tiles, walls, furniture wallpaper and window coverings. The main physical symptoms related to the presence of biological contaminants in the air are cough, allergies, fever and mucous membrane irritation [16]. All these factors, if left unchecked, could weaken IAQ. Consequently, national organizations and worldwide committees, such as World Health Organization (WHO) have issued several standard and guideline values to manage IAQ. These dispositions help to limit exposure of humans to certain breathing and contaminants. It is important to underline the difference between the two terms: standard and guideline. An air quality standard is a description of air quality that is adopted by a regulatory authority as enforceable. On the other hand, air quality guidelines are designed to offer guidance to reduce adverse health impacts of air pollution based on expert evaluation of current scientific evidence. The international organizations involved in air quality guidelines and standards are listed in **Table 3**. They include for example the American Society of Heating, Refrigerating and Air–Conditioning Engineers (ASHRAE), the Hong Kong Environmental Protection Department (HKEPD), the World Health Organization (WHO), and the National Health and Medical Research Council (NHMRC) of Australia [17].

Table 3. International bodies involved in setting air quality guidelines and standards [17].

Country	Organization	Organization's Acronym
Australia	The National Health and Medical Research Council	NHMRC
Australia	The National Occupational Health and Safety Commission	NOHSC
Belgium	Air Infiltration and Ventilation Centre	AIVC
Canada	Health Canada	Health Canada
China	Administration of Quality Supervision, Inspection and Quarantine	AQSIQ
China	State Environment Protection Agency	SEPA

Hong Kong	Hong Kong Environmental Protection Department	HKEPD
Hong Kong	Hong Kong Indoor Air Quality Objectives	HKIAQO
Hong Kong	Government of the Hong Kong Special Administrative Region	HKSAR
Denmark	Danish Society of Indoor Climate	DSIC
Europe	European Commission	EC
Finland	Finnish Society of Indoor Air Quality and Climate	FiSIAQ
Germany	Deutsche Forschungs Gemeinschaft	DFG/MAK
Japan	Ministry of Health, Labor and Welfare	MHLW
Kuwait	Kuwait Environmental Protection Agency or Kuwait Environment Public Authority,	Kuwait EPA
Korea	Korea Environmental Industry and Technology Institute	KEITI
Malaysia	Department of Occupational Safety and Health	DOSH
Singapore	Singapore Indoor Air Quality Guideline	SIAQG
Singapore	Institute of Environmental Epidemiology, Ministry of the Environment	Institute of Environmental Epidemiology
UK	Health and Safety Commission	HSC
US	American Conference of Governmental Industrial Hygienists	ACGIH
US	American Society of Heating, Refrigerating and Air–Conditioning Engineers	ASHRAE
US	Illinois Department of Public Health	IDPH
US	Occupational Safety and Health Administration	OSHA
US	Office of Environmental Health Hazard Assessment (California EPA)	OEHHA
US	Texas Department of Health	TDH

US	The National Ambient Air Quality Standards	NAAQS
US	The National Institute for Occupational Safety and Health	NIOSH
US	US Environmental Protection Agency	U.S.EPA
Worldwide	World Health Organization	WHO

1.1.2 IAQ: The European and Italian legislation

The continuous and growing attention on IAQ has been evidenced by the huge quantity of scientific literature, such as articles, reviews, letters, conference papers or books, focused on chemical pollutants in indoor spaces. A search on the Scopus literature database, texting the keyword “indoor air quality”, led to a total of 8562 publications between 2000 and 2021 in the European Union, including UK, Norway, Turkey and Switzerland (search made on 24 March 2021). UK and Italy resulted to be the major contributors to these research topics, accounting for 12.4% and 10.7% of the total European publications respectively.

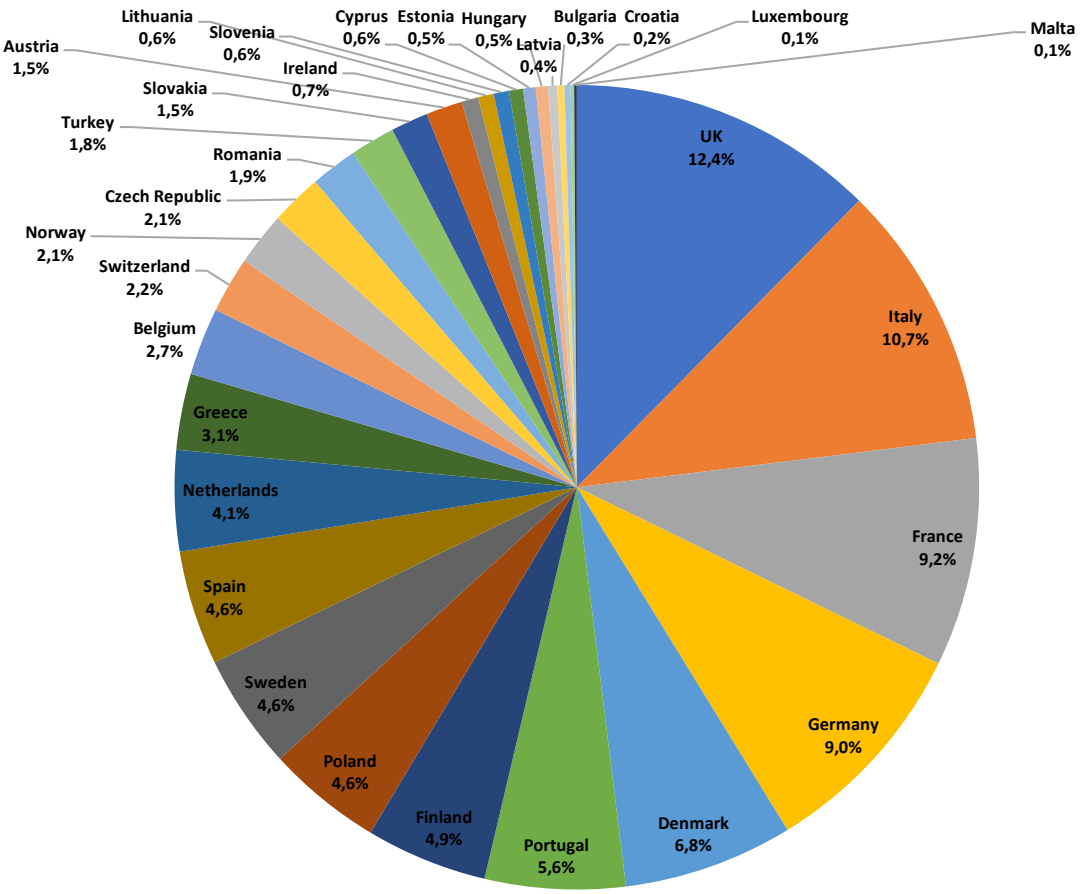


Figure 1. Country contributions (%) to the total publications regarding IAQ in the EU from 2000 to 2021 (Source: Scopus, search: 24 March 2021) including Norway, Turkey, Switzerland, and UK.

The WHO has indicated guidelines for IAQ, relating to a group of indoor contaminants, known to have some adverse effects against human health. These pollutants are: benzene, polycyclic aromatic hydrocarbons (PAHs), nitrogen dioxide, naphthalene, carbon monoxide, radon, tri- and tetrachloroethylene. Despite this, there is no specific directives on IAQ in the European legislation. Only few countries (France, Portugal, Finland, Austria, Germany, Belgium, UK, Poland, Lithuania and Netherlands) have begun to adopt some specific guidelines and reference values for IAQ, sometimes accomplished by legislative acts [18]. In this context, it is important to underline the work made in 2006 by institutions as the International Organization of Standardization (ISO) and the European Committee for Standardization (CEN) in the production of the specific standard "EN ISO 16000: Indoor air" [19]. The document contains the procedures for sampling and analysis of the main indoor pollutants, increasing the

possibility of a valid comparison between the different data obtained at the European level. **Table 4** summarizes the 40 parts of the ISO 16000 standard.

Table 4. Structure of ISO 16000 standard [19].

EN ISO 16000: Indoor Air	
Part 1	General aspects of sampling strategy
Part 2	Sampling strategy for formaldehyde
Part 3	Determination of formaldehyde and other carbonyl compounds—active sampling method
Part 4	Determination of formaldehyde—di_usive sampling method
Part 5	Sampling strategy for volatile organic compounds (VOCs)
Part 6	Indoor air Determination of volatile organic compounds in indoor and test chamber air by active sampling on Tenax TA sorbent, thermal desorption, and gas chromatography using MS or MS-flame ionization detector (FID)
Part 7	Sampling strategy for determination of airborne asbestos fiber concentrations
Part 8	Determination of local mean ages of air in buildings for characterizing ventilation conditions
Part 9	Determination of the emission of volatile organic compounds from building products and furnishing—emission test chamber method
Part 10	Determination of the emission of volatile organic compounds from building products and furnishing—emission test cell method
Part 11	Determination of the emission of volatile organic compounds from building products and furnishing—sampling, storage of samples, and preparation of test specimens
Part 12	Sampling strategy for polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polycyclic aromatic hydrocarbons (PAHs)
Part 13	Determination of total (gas and particle-phase) polychlorinated dioxin-like biphenyls and polychlorinated dibenzo-p-dioxins/dibenzofurans—collection on sorbent-backed filters with high resolution gas chromatographic/mass spectrometric analysis
Part 14	Determination of total (gas and particle-phase) polychlorinated dioxin-like biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins/dibenzofurans (PCDDs/PCDFs)—extraction, clean up, and analysis by high-resolutions gas chromatographic and mass spectrometric analysis)
Part 15	Sampling strategy for nitrogen dioxide (NO2)
Part 16	Detection and enumeration of molds—sampling of molds by filtration

Part 17	Detection and enumeration of molds—culture-based method
Part 18	Detection and enumeration of molds—sampling by impaction
Part 19	Sampling strategy for molds
Part 20	Detection and enumeration of molds—determination of total spore count
Part 21	Detection and enumeration of molds—sampling from materials
Part 22	Detection and enumeration of molds—molecular methods
Part 23	Performance test for evaluating the reduction of formaldehyde concentrations by sorptive building materials
Part 24	Performance test for evaluating the reduction of volatile organic compound (except formaldehyde) concentrations by sorptive building material
Part 25	Determination of the emission of semi-volatile organic compounds by building products—micro-chamber method
Part 26	Sampling strategy for carbon dioxide (CO2)
Part 27	Determination of settled fibrous dust on surfaces by SEM (scanning electron microscopy) (direct method)
Part 28	Determination of odor emissions from building products using test chambers
Part 29	Test methods for VOC detectors
Part 30	Sensory testing of indoor air
Part 31	Measurement of flame retardants and plasticizers based on organophosphorus compounds—phosphoric acid ester
Part 32	Investigation of buildings for pollutants and other injurious factors—inspections
Part 33	Determination of phthalates with gas chromatography/mass spectrometry (GC/MS)
Part 34	General strategies for the measurement of airborne particle
Part 35	Measurement of polybrominated diphenylether, hexabromocyclododecane, and hexabromobenzene
Part 36	Test method for the reduction rate of airborne bacteria by air purifiers using a test chamber ISO 16000
Part 37	Strategies for the measurement of $PM_{2.5}$
Part 38	Determination of amines in indoor and test chamber air—active sampling on samplers containing phosphoric acid impregnated filters
Part 39	Determination of amines—analysis of amines by (ultra-)high-performance liquid chromatography coupled to high resolution or tandem mass spectrometry
Part 40	Indoor air quality management system

Even if Italy plays an important role in the research on IAQ (10.4% of the total publications in EU in the last twenty years) (**Figure 1**), there is no reference legislation in the country.

However, some commissions and working groups are at work to provide technical documents to coordinate specific actions at national level and to improve the containment of indoor air pollution. In 2010, the National Study Group (GdS) on Indoor Air Pollution was created by the Italian Institute of Health (IIS). The GdS-IIS are composed by various ministerial institutions, regions and local authorities and research institutes and until now it has published many documents for the definition of a national plan on indoor air quality (Rapporti ISTISAN) [18].

All these results obtained at European level may lead to valid public health strategies to reduce the exposure to indoor air with poor quality.

1.1.3 IAQ and COVID-19

SARS-CoV-2 (COVID-19) spread globally in the past year and is still pandemic. Many countries, after China, took lockdown policy in order to decrease the virus spread. The implementation of stringent lockdown in many countries all over the world led to less industrial emissions and less traffic, reducing drastically air pollution [20]. Apart from outdoor air quality, IAQ should get more and more attention during lockdown, because people stay indoors for about 93% and sometimes for 100% of their time during lockdown, increasing their exposure to indoor contaminants [21]. Each year, 3.8 million people die from illnesses related to indoor air pollution [22]. Stay-at-home orders, smart-working and remote learning concern all people, but there are some vulnerable populations (children, elderly people, individuals with pre-existing pathologies, households of low socioeconomic conditions, such as those in multifamily buildings) that suffer most this situation and the exposure to indoor pollutants. So, it is important to focus on poor house indoor air quality as a public health problem during the pandemic. There are many sources of indoor pollutants, such as fireplaces, kitchens, furniture personal care and cleaning products. The use of these products has been implemented, because many people may be cleaning more often and with stronger disinfectants to decrease the chance of viral infection. Then, households prepare their food at home instead of eating out and subsequently cooking emissions would be higher. Another concern regards smoking; people in closed spaces suffer most from second-hand smoke. But all the human activities would be more frequent since families stay together at home almost of their time, increasing indoor air pollution [20]. Also the indoor microbiota (bacteria, molds and virus) is becoming an important contributor to IAQ. As the occupancy load increases, a proper ventilation, and other precautions (use of kitchen ventilator, not smoking at home, use clean fuels, exc.) are necessary. In the future, more investigation should be performed to estimate the impact of COVID-19 lockdown on human health caused by indoor air pollutants.

1.1.4 Cooking emissions

Gas and air fumes generated during cooking activities will be referred to as cooking emissions. Cooking emissions are produced from two main sources: the emissions from the stove used for cooking and the emissions produced by cooking food itself. Characteristics of both stove and food being cooked, influence cooking emissions type and concentration levels. The contribution of cooking emissions to the overall ambient aerosol was estimated to be between 12% and 20% [23]. The impact on human health of pollutant emissions from domestic or commercial cooking activities is often underrated. In fact, not many people are aware of the risks associated with cooking activities. Furthermore, as the use of fire became part of human culture, all populations have become exposed to these cooking-related risks, disregarding their race, age, food preferences or culture. In fact cooking oil fumes (COFs) represent a significant source of indoor and outdoor pollutants in both urban and rural areas. Visible COFs are usually due to submicrometer sized particles, which consist of oil droplets, combustion products, steam from water and condensed organic pollutants [24]. Many of them can be hazardous compounds deriving from the incomplete combustion of organic components in food material. The commonest pollutants are reduced sulphur compounds (RSC), volatile organic compounds (VOCs), particulate matters (PM), polycyclic aromatic hydrocarbons (PAHs), carbon monoxide (CO) and nitrogen oxides (NOx) [25]. It was seen that in the indoor residential environments the main sources of fine particulate matter (PM with a diameter less or equal to 2.5 μm) with non-smokers are cooking activities [26]. These particles can be a real problem, because they are associated with pulmonary, cardiac and renal toxic effects. For example, some studies revealed that some respiratory diseases and the increased lung cancer mortality among restaurants and hotels workers are linked to a high exposure to COFs [27,28], as a combination of the indoor air pollutants such as PAHs and some carbonyl compounds. Also, the International Agency for Research on Cancer (IARC) declared COFs from deep frying as “possible human carcinogens” [29]. It is also important to underline that the potential hazards of COFs are strictly dependant on the exposure concentration and time. Nevertheless, food cooking is a source not only of indoor air pollutants, but also of non-hazardous odor compounds (pleasant or unpleasant). Different factors could influence the concentration, the chemical composition and the diffusion of cooking emissions, such as fuel type, the edible oil used, cooking temperature and cooking method, but also ventilation conditions.

Cooking fuel has a high effect on the contribution of particles in environment. The most common used cooking fuels are charcoal or wood, coal, kerosene, liquefies petroleum gas

(LPG), natural gas or electricity. Several studies revealed that using clean fuels (energy or gas) could greatly reduce indoor air pollution. In particular, Xu et al. [30] observed that using electricity for cooking can reduce by half exposure to $PM_{2.5}$ respect to solid fuels. Combustion of biomass, coal, diesel and gasoline is the main source of indoor PAHs [31] and the burning of carbon-based fuels is also responsible for aldehydes release [32].

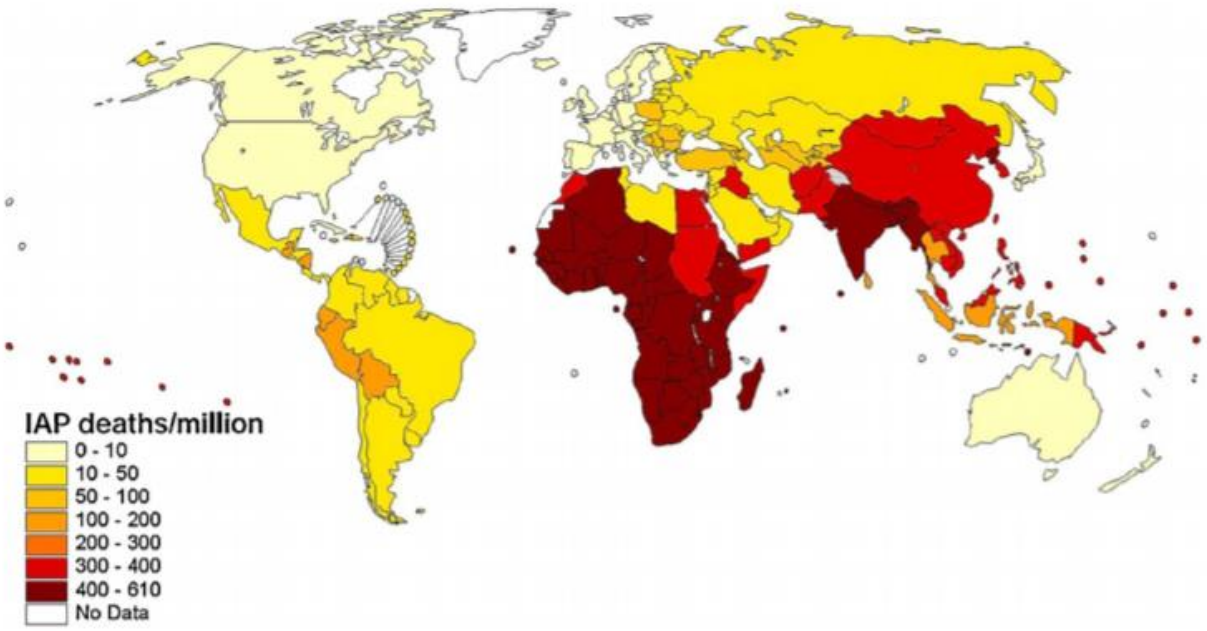


Figure 2. Global distribution of deaths caused by indoor smoke from solid fuels by sub-region for 2000 [20].

Globally indoor fumes from solid fuels rank as the eighth risk factor of mortality [20], even if it varies significantly on a regional basis (**Figure 2**).

Also temperature has a significant impact on the rate and the concentrations of emitted particles during cooking activities. A high cooking temperature increases PM number, mass concentration, diameter, and emission factor. Then, with the rising temperature, also the emission of other pollutants, such as PAHs, VOCs and inorganic particles increases. Therefore, high cooking temperatures should be avoided [33].

Also the cooking method is one of the most important factors that affects pollutants emission. Several researches indicated that heavy cooking methods, such as deep-frying, stir-frying or barbecuing, produce more PM, PAHs, toxic VOCs or inorganic substances than others (boiling, steaming, etc.) [28]. For example, **Table 5** shows the measured PAHs by Zhu and Wang [34] and See and Balasubramanian [35] corresponding to different cooking methods.

Table 5. Results showing influence of cooking methods on PAHs emissions [34,35].

Reference	Zhu and Wang 2003						See and Balasubramanian 2008				
Cooking method	Boiling fish	Frying fish	Broiling fish	Boiling short pork chops	Frying short pork chops	Broiling short pork chops	Steaming tofu	Boiling tofu	Stir-frying tofu	Pan-frying tofu	Deep-frying tofu
Oil type									Corn oil		
Kitchen type	Domestic kitchen						Domestic kitchen				
Phase	Particle and gas						Particle				
Unit	μg/ (m^3·kg)						ng/m^3				
Naphthalene	0.028	0.25	1.1	0.045	0.53	1.3	0.17±0.01	0.19±0.01	0.3±0.03	0.37±0.04	0.5±0.04
Acenaphthylene							0.13±0.03	0.42±0.05	0.57±0.07	0.98±0.1	1.17±0.14
Acenaphthene	Nd	0.080	0.10	0.0013	0.57	2.4	0.56±0.06	0.75±0.09	1.55±0.13	1.73±0.18	1.8±0.26
Fluorene	Nd	0.042	0.13	Nd	0.071	0.34	0.84±0.07	0.89±0.07	1.53±0.14	1.68±0.12	2.62±0.33
Anthracene	0.0030	0.048	0.043	0.0022	0.033	0.13	0.12±0.01	0.16±0.03	0.31±0.03	0.36±0.05	0.43±0.07
Pyrene	0.0090	0.25	0.15	0.013	0.17	0.55	0.8±0.1	0.96±0.14	1.3±0.18	1.35±0.13	1.45±0.16
Phenanthrene	0.0021	0.045	0.043	0.0031	0.069	0.18	3.18±0.23	3.97±0.32	5.11±0.86	5.87±0.87	8.98±0.68
Fluoranthene	0.011	0.25	0.15	0.015	0.31	0.43	0.86±0.1	1.05±0.14	1.61±0.16	1.64±0.18	1.64±0.13
Benzo[a]anthracene	0.0082	0.18	Nd	0.012	0.39	1.4	0.21±0.03	0.3±0.02	0.38±0.07	0.4±0.06	0.44±0.07
Chrysene	0.0022	0.033	0.028	0.0031	0.049	0.14	0.56±0.04	0.7±0.09	1.05±0.11	1.07±0.11	1.19±0.15
Benzo[b]fluoranthene							0.29±0.04	0.7±0.06	1.3±0.1	1.3±0.12	1.38±0.13
Benzo[k]fluoranthene	Nd	0.082	0.037	0.0011	0.14	0.26	0.29±0.02	0.37±0.04	0.48±0.04	0.78±0.1	0.94±0.07
Benzo[a]pyrene	Nd	0.055	0.040	Nd	0.075	0.089	0.2±0.03	0.23±0.02	0.38±0.05	0.49±0.05	0.56±0.05
Benzo[e]pyrene	0.0021	0.011	0.010	0.0031	0.079	0.080					
Indeno[1,2,3-cd]pyrene							0.42±0.04	0.87±0.07	1.09±0.14	1.38±0.19	3.47±0.3
Dibenzo[a,h]anthracene							0.046±0.008	0.21±0.02	0.52±0.05	0.62±0.06	1.18±0.15
Benzo[ghi]perylene							0.36±0.05	0.79±0.06	1.29±0.17	1.62±0.19	4.33±0.47

Nd: not detected.

Deep-frying resulted to emit the largest mass of PAHs, followed by pan-frying, stir-frying, boiling, and steaming. The release of odor nuisance and toxic pollutants from each food is difficult to predict, because it reflects a combination of many different factors.

1.1.4.1. Volatile Organic Compounds (VOCs) from cooking activities

Volatile organic compounds (VOCs) are organic chemical compounds that evaporate under normal indoor atmospheric conditions of temperature and pressure. Since the volatility of a compound is generally higher, the lower its boiling point temperature is, the volatility of organic compounds is sometimes defined and classified according to the boiling point.

The WHO classified VOCs as indoor organic pollutants with a boiling point range between 50/100 °C and 240-260°C [36]. WHO categorized also indoor VOCs as:

1. Very volatile organic compounds (VVOCs)
2. Volatile organic compounds (VOCs)
3. Semi-volatile organic compounds (SVOCs)

The higher the volatility (lower the boiling point), the more likely the compound will be emitted from a product or surface into the air. Very volatile organic compounds are so volatile that they are difficult to measure and are found almost entirely as gases in the air rather than in materials or on surfaces. The least volatile compounds found in air constitute a smaller fraction of the total present indoors. Common VOCs sampled in indoor air are aliphatic and aromatic hydrocarbons, alcohols, aldehydes, ketones, acids, terpenes, and esters. They are mainly generated via chemical reactions occurring during cooking activities: thermal oxidation and decomposition of lipids, Maillard reactions of some chemical species or secondary reactions of the intermediates or final products. Many VOCs are not dangerous to human health and are only responsible for food aromas. Others are known to be toxic and still others are considered carcinogenic, mutagenic or teratogenic. Eye and respiratory tract irritation, headaches, dizziness, visual disorders, allergic skin reactions, fatigue, and memory impairment are usually the primary symptoms that some people have experienced after exposure to some of these organic compounds. The most important parameters affecting VOCs toxicity are the number of carbons, if they are saturated or unsaturated, their chemical configuration, the presence of functional groups, their concentration and the length of exposure time [37]. For all these reasons, the monitoring of these substances in private and public environments is important to safeguard health of people.

1.1.5 Air purification

The air we breathe is made up by a mixture of gases, containing 78% nitrogen, 21% oxygen, 1% argon, 0.04% carbon dioxide, 0.5% water vapour and other gases in traces (**Figure 3**).

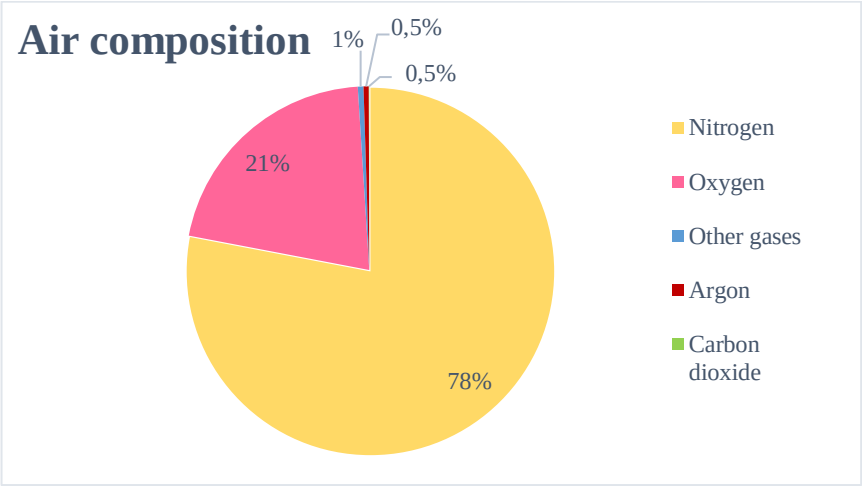


Figure 3. Air composition.

Air also includes dirt and pollutants (VOCs, PM, gases) generated by human activities, natural phenomena, industrial processes, building materials and finishes. As can be seen from **Figure 4**, the impurities present in the atmospheric air are very different in size; many of them are usually invisible to the naked eye (the human vision limit is about 20 µm).

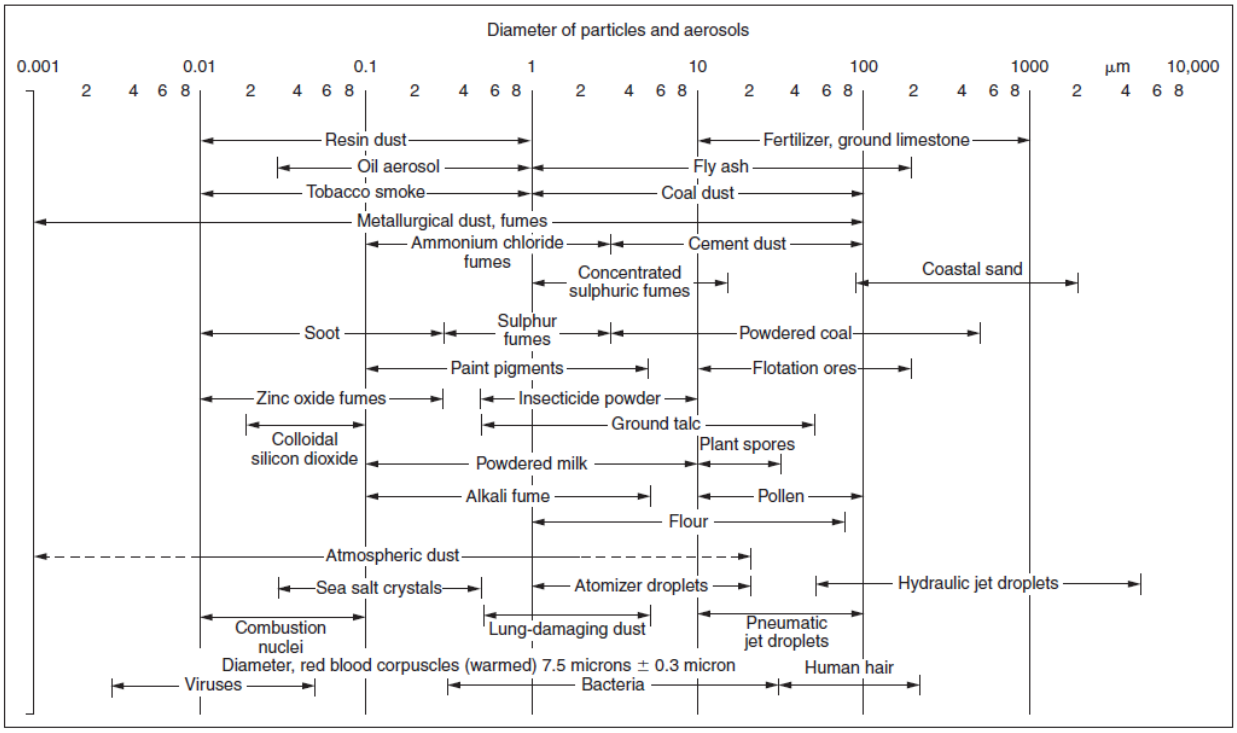


Figure 4. Impurities in air and their diameter [38].

For these reasons, it is very important to take some control over the air we breathe outdoor but also indoor. Air-conditioning is defined as "the process used to create and maintain certain temperature, relative humidity and air purity conditions in indoor spaces". This process is typically applied to maintain a level of personal comfort. An air-conditioning system must be effective regardless of outside climatic conditions and involves control over four fundamental variables: air temperature, humidity, movement, and quality. Comfort air-conditioning, as well as needing to satisfy personal temperature and humidity requirements, involves also other fields such as architectural design, weather forecasting, energy consumption and sound emissions to recreate the ideal conditions for human psychophysiological well-being [39].

Air filtration is one of the best technology to protect the health and safety of the building occupants by removing contaminants from the air. It is widely used to remove particles and gases from an air stream due to its relative ease and flexibility [40]. Also ventilation plays an important role in mantaining a clean indoor environment. However, ventilation systems can be

possible sources of airborn pollutants. The main pollutants in air can be divided into three categories: suspended particles, VOCs and microorganisms. For the first ones, the main purification technologies are water washing purification, filtration, electrostatic precipitation and anion technology. In particular, filtration is the most used purification technique for PMs. Then, the most efficient purification method for harmful gases is adsorption. Because of the simplicity, effectiveness and low cost, activated carbon (AC) is one of the most common adsorption material. Also photocatalysis and plasma cleaning are effective for VOCs purification. To eliminate microorganisms, the most powerful method is UV light, followed by photocatalytic and plasma purification. Many times a single purification technique is not suitable in an environment rich of different air pollutants. Sometimes it is necessary to combine some of the above purification systems [39]. The characteristics of the typical used air purification technologies are listed in **Table 6**.

Table 6. Characteristics of different purification technologies in building environment [34].

Technology	Target	Advantages	Disadvantages	Efficiency
Fibre filtration	Particles, microorganisms, Rn	Low cost	Resistance related to the purification efficiency, mild and high efficiency filters of high resistance	Can achieve 99.99999%
Electrostatic dust removal	Particles, microorganisms	High efficiency and wide range of particle size, small pressure loss	High investment, efficiency decline after dust discharge, electric field easy to breakdown	50 % (some only 20%)
UV sterilization	Microorganisms	High efficiency, safe and convenient, no residual toxicity, no pollution, small resistance	Poor dynamic sterilization effect	82.90%
Activated carbon adsorption	All the pollutants (except biological pollutants)	Wild sources, bigger pollutant purifying range, not easy to cause secondary pollution	Saturated regeneration problems, resistance is bigger,	-

			mineral processing not good	
Plasma	All indoor pollutants	Big range of pollutants	Cannot completely degrade pollutants and produce by-products	66.70%
Negative ions	Particles, microorganisms	Accelerate metabolism, strengthen cell function, effective to some diseases	Produce ozone, cause second pollution, deposition of dust damage the wall	73.40%
Photocatalysis	Total VOC, microorganisms and other inorganic gaseous pollutants	Wide range of purification, mild reaction conditions, no adsorption saturation phenomenon, long service life	Campared to AC adsortpion technology, slower purification process, easy to cause secondary pollution if response is not complete	75% (some may only 30% or even negative)

1.1.5.1 Air filtration

The term “filtration” refers to the act of separating a mixture into one or more distinct phases in a process which uses physical differences between the phases (such as particle size) [38].

Nowadays, filtration is applied in almost any human activity: commercial, industrial and domestic. The global filters market size was estimated at USD 70.38 billion in 2020 and is expected to grow at a compound annual growth rate (CAGR) of 4.7% from 2021 to 2028 [41]. Motor vehicles represent the biggest sector of the market, accounting for more than 47% of the global income in 2020. But also the use of filtration devices in many consumer goods (water filters, air filters, air conditioners) is expected to enhance this market over the coming years. **Figure 5** shows the global filters market share, by application, in 2020 [41]. The imposition of many environmental legislations is an important driver for the filtration market and for the development of filtration devices and technologies.

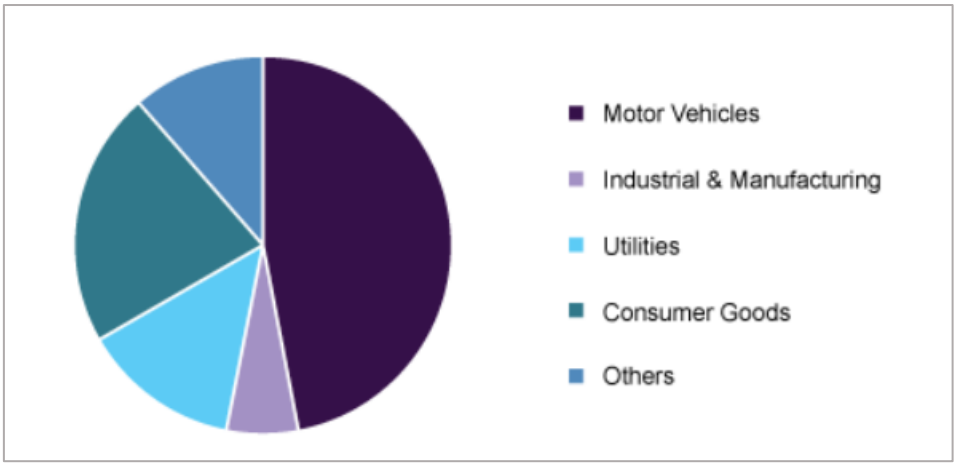


Figure 5. Global filters market share, by application, in 2020 (%) [39].

A filter can be defined as a device that separates a phase from another. The most common phase separation processes are the solid/fluid ones (solid/gas and solid/liquid separation).

The filtration system is defined by many different factors, established by the Standard International (SI) system of measurements (**Table 7**).

Table 7. SI units for filtration measurements.

Measurement	Symbol	Description
Air volume flow rate [a]	m^3/s	Cubic meters per second
Filter pressure drop	Pa	Pascal
Face area	m^2	Square meters
Filter dimensions	mm	Millimeters
Particle size	µm	Micrometers [b]
Velocity	m/s	Meter/second
Temperature	°C	Celsius Degrees [c]

[a] Air volume flow is also given in liters per second (L/s).

[b]"Micron" is not used in the SI system to represent the dimension of very small dust particles, instead the word "micrometer" is used.

[c]The temperature in Celsius degrees can be calculated by subtracting 273.15 from Kelvin degrees.

The filter is placed in the way of the fluid flow and it needs a pressure difference across the filter medium to operate. What really influence the type of filter chosen are the mean particle size and the particle size distribution. Indeed, according to the size of separated particles, the term "macrofiltration" refers to the separation of particles in the range of 1 mm down to 5 µm,

"microfiltration" for the particles from 5 µm to 0.1 µm, then "ultrafiltration" for the particles smaller than 0.1 µm.

1.1.5.1.1 Air filtration mechanisms

There are many mechanisms involved in air filtration of particles and microorganisms. These contaminants retention is affected by many factors, such as contaminant properties (mass, chemical nature, size), air properties (flow velocity, humidity), filter media characteristic and the filtration mechanism. The main air filtration mechanisms (**Figure 6**) comprehend the direct interception, inertial impaction, diffusional interception and electrostatic attraction.

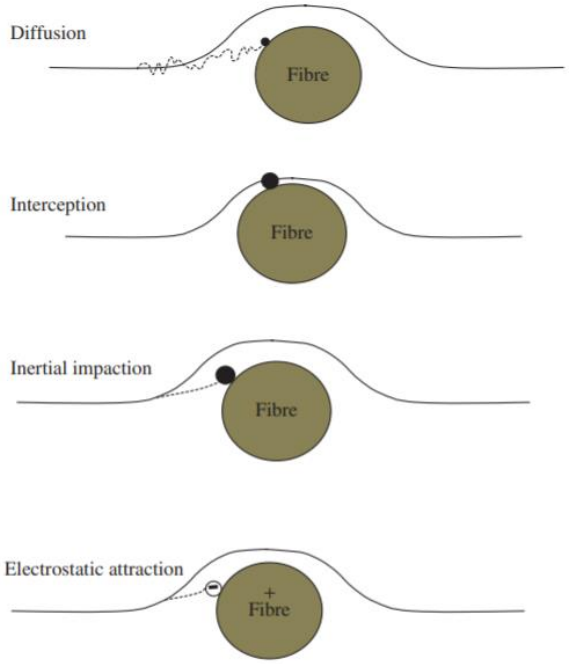


Figure 6. Filtration mechanisms [42].

- *Direct Interception or Size Exclusion* occurs with medium-sized particles that are not large enough to leave the flow path due to inertia or not small enough to diffuse. The particles will follow the flow stream where they will touch a fiber in the filter media and be trapped and held. Direct interception is the only filtration mechanism which is correlated to the filter pore size. Most of the contaminants in air filtration are removed by other means.
- *Diffusion or Diffusional Interception* occurs because air molecules are in random motion. As air molecules impact contaminants in the air stream, they displace them in different directions (Brownian motions). When the contaminants collide the filter media, they can be retained because of the molecular attraction. A filter pore larger also

up to 5-10 times the contaminant can intercept it. This filtration mechanism is very efficient, especially in dry air and for small particles (less than 0.1-0.3 µm).

- *Inertial Impaction* is one of the commonest filter mechanisms in air filtration. As air containing a particle flows toward a filter fiber or other collecting surface, the particle does not follow the air streamlines because of its inertia, caused by its mass and velocity. Instead it moves in straight line colliding with the filter fiber or surface to which it may become attached. This type of filtration mechanism is effective in high-velocity filtration systems and for contaminants greater than 0.5-1 µm.
- *Electrostatic Attraction* can be both active or passive. In the case of active electrostatic attraction, the synthetic filter fibers can be charged during manifacture to be either positively and/or negatively charged. This technology can be classified by the method used to create the electrostatic charge in:

– Triboelectric charging
– Corona charging
– Charging by induction

In the case of passive electrostatic attraction, the fiber media is charged by the air flow through it (passive electrostatic filters). In electrostatic attraction, contaminants which are naturally charged (e.g., negatively charged bacteria and yeasts) are attracted and retained by oppositely-charged filter media. Humidity of the air and the high air velocity can lower the efficiency of the electrostatic attraction, that lasts when all the charged sites are exhausted.

1.1.5.1.2 Air filter media

"A filter medium is any material that, under the operating conditions of the filter, is permeable to one or more components of a mixture, solution or suspension, and is impermeable to the remaining components" [42]. The number and multiplicity of materials than can be used as filter medium are really wide; in fact any material which can be made permeable is a filter. The most common filter media are:

- Minerals
- Glass
- Metals
- Charcoal and carbon
- Metal oxides and ceramic materials
- Natural or synthetic organic fibers

- Synthetic sheet materials

All these materials can be fabricated into a huge variety of forms: sheet, bar, fibres, granules, wire or monofilament. **Table 8** shows the main media types and their common formats.

Table 8. Types of filter media by material and their common formats.

Material	**Format**
Natural fibre	Felt
Natural filament (silk)	Wove yarn Knitted yarn Wound yarn
Processed natural fibre: cellulose	Wet-laid (paper, filter sheets)
Man-made organic: regenerated cellulose synthetic polymers	Granules Fibres and Filaments Foam Extruded mesh Sheet Tubular
Metals: ferrous and non-ferrous	Rod or bar Granules or powder Fibres Sheets Wire Expanded mesh Foam
Glass	Fibre Porous tube
Carbon: natural activated	Granules or powder Fibres Porous block
Ceramics: metal oxides and others	Granules or powder Formed blocks Fibres Foam
Other minerals: mineral wools sand, anthracite, garnet	Fibres Granules
Various materials (paper, metal, plastic)	Solid fabrications
Paper-like materials	Pleated sheet
Inert granules of all kinds	Packed beds
Mix of inert and active materials	Combination media

A filter medium should combine different properties, such as chemical resistance, mechanical strength, wettability or dimension, according to its specific application. These properties can be divided into three main categories (**Table 9**):

1- Application-related properties, that control the compatibility of the medium with the process environment;
2- Machine-oriented properties, which correlate the medium use only to defined types of filter;
3- Filtration properties, that determine the capability of the medium to obtain specific filtration tasks.

Table 9. Properties of filter media.

Application-related properties
Chemical stability
Thermal stability
Biological stability
Dynamic stability
Absorptive characteristics
Adsorptive characteristics
Wettability
Health and safety aspects
Electrostatic characteristics
Disposability
Suitability for reuse
Cost
Machine-oriented properties
Rigidity
Strength
Resistance to creep/stretch
Stability of edges
Resistance to abrasion
Stability to vibration
Dimension of available supplies
Ability to be fabricated
Sealing/gasketing function
Filtration properties
Smallest particle retained
Retention efficiency (structure of filter media, particle shape, filtration mechanisms)
Resistance to flow (porosity of media, permeability)
Dirt-holding capacity
Tendency to blind
Cake discharge characteristics

According to the particle filtration efficiency air filters are generally divided into four categories: pre-filters, medium filters, high efficiency particulate air (HEPA) filters and ultra-low particulate air (ULPA) filters [34].

1.1.6 Cooking hoods

VOCs are among the main pollutants in kitchen environments. The occurrence and temporal profile of VOCs is highly dynamic in nature. Adsorption may lead to lower peak concentrations of VOCs, while the subsequent desorption process prolongs the presence of indoor air pollutants. Cooking activities are a typical example of an intermittent and recurrent (Intermittent-Type 1) source of VOCs (**Figure 7**) [43].

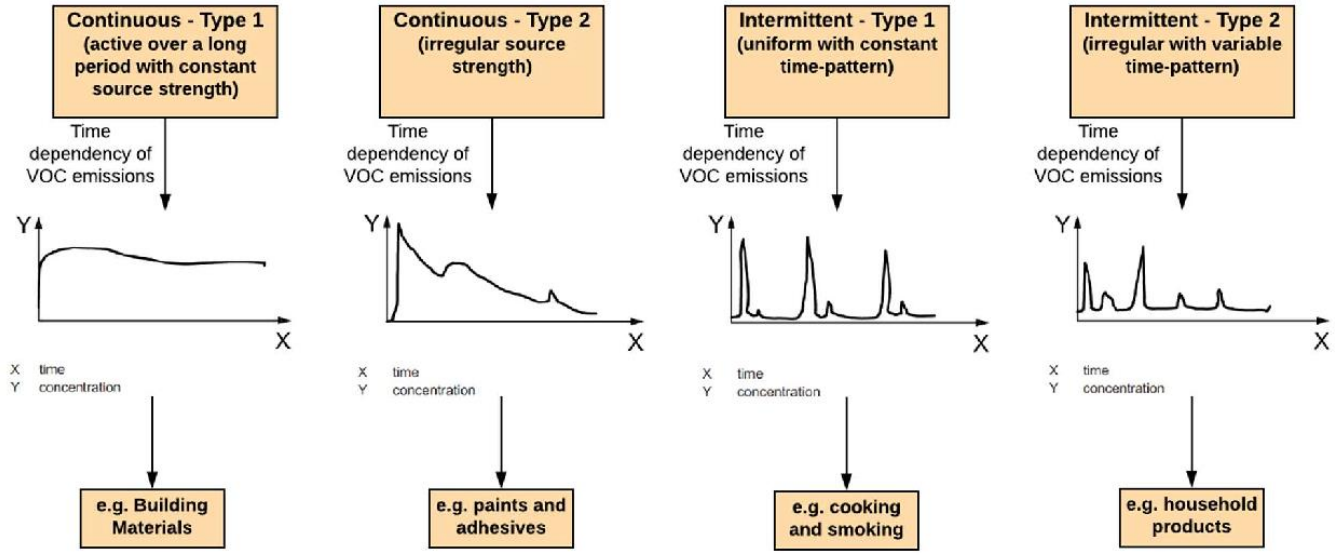


Figure 7. Emission characteristics and time dependency of VOC sources [43].

An efficient ventilation system is quite necessary to provide a comfortable and healthy kitchen environment. One significant element in the creation of a salubrious environment in the kitchen is the exhaust hood, that could significantly reduce the personal COFs exposure. So, kitchen hoods represent a local indoor air filtration device. It contains a mechanical fan that hangs above the stove. It helps to remove grease particles, combustion products, fumes, smoke and steam by evacuation of the air or filtration. In most kitchen hoods, a filtration system is used to remove grease (grease filter) and other particles. Some vent hoods exhaust air to the outside: they are the so called **aspiration hoods** (**Figure 8**). The fluid is directly discharged into the external environment by means of suitable drain pipes that connect the engine to the environment.

Other exhaust hoods recirculate the air to the kitchen. In a recirculating system, filters are used to remove odors and grease. So, in **filtering hoods** (**Figure 9**) the aspired air after filtration is further treated to eliminate odors with a technology based mainly on activated carbon. The purified air is returned to the kitchen again.

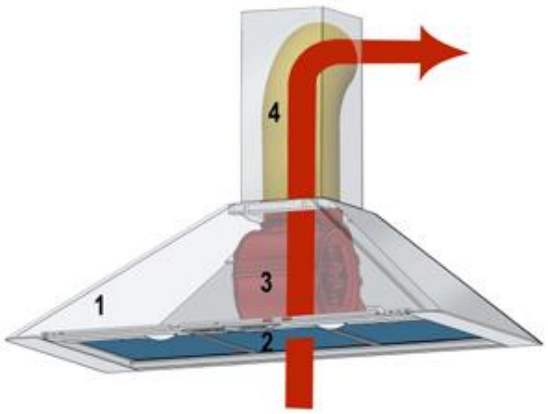


Figure 8. Aspiration hood**.**

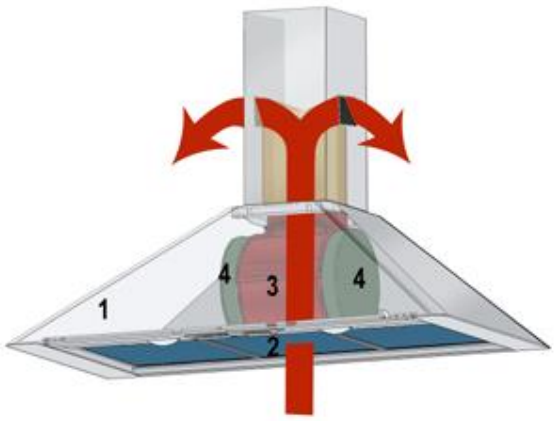


Figure 9. Filtering hood.

Kitchen hoods are designed to capture cooking fumes, which consist of a mixture of vapors and grease particles. To perform this duty, the hood is equipped with two different filtering systems:

- *Mechanical filters* to trap grease particles that can contaminate the environment and damage the hood itself;
- *Odor filters* to adsorb odorous fumes in order to avoid high concentrations of unpleasant odors in the kitchen and possible toxic compounds.

It is important to underline that efficiency of exhaust hoods to capture cooking-related pollutants can vary widely based on a number of factors, including equipment type, size and location, exhaust flow rate, exhaust ducting, installation details and use behavior [44]. In fact, use behavior is an important factor to maximize effectiveness, especially for those who are not able to purchase a higher performance unit.

1.1.7 Air sampling methods

The assessment of IAQ is a demanding task. Many parameters can affect indoor air, for this reason it is important to use standardized methods that can provide a comprehensive evaluation. The monitoring approach has to be adapted to the single case study, considering the choice of target pollutants, the adequate sampling and analytical method, sampling times and location,

number of samples and sampling preservation. The ideal method for indoor air sampling and analysis should consist on a direct and integrated automated scanning in real time for various pollutants at different locations without any interferences to the sampled microenvironments. Unfortunately, this method is not possible, so the air sampling methods need some compromises.

Depending on their transportability, personal, portable and stationary samplers can be found. Personal samplers can be carried or worn; they are usually used to define workplace exposure to different contaminants and for time-weighted average (TWA) measurements. Then, portable devices can be hand-carried during the sampling and the stationary units operate only from a fixed location. Depending on the operation mode, the air sampling can be active or passive [45]. The first one needs a power source, such as pump or vacuum, to constrain the air to an analyzer or collector. Indeed a defined air volume is pumped through an adsorbent tube where the contaminants are retained at a specific flow rate (0-5-1400 L/min). Sampling times are usually from a few minutes to several hours. On the contrary, passive sampling counts on diffusion or permeation of the analytes through a diffusive surface onto an adsorbent. After sampling, the analytes are desorbed off the adsorbent by thermal or solvent desorption. However, there is also another possible choice, named whole sampling. Whole sampling is the simplest way to collect air samples, using gas-tight syringes, glass or stainless-steel canisters or polymeric bags. A total air sample is collected, eliminating the need for thermal or solvent desorption [46]. Thus, sampling techniques for organic pollutants from indoor air can be classified into: whole, active or passive sampling techniques (**Table 10**).

Table 10. Advantages and disadvantages of air sampling methods [46].

Sampling method	Advantages	Disadvantages
Whole sampling	Simple total sampling No breakthrough No degradation No moisture effect Low blank levels Long storage Allows reinjection for multi-replicate analysis	Needs a preconcentration step to achieve acceptable detection limits. Possible contamination by the inner surfaces of the vessel. Needs careful pretreatment and preconditioning. Possible irreversible losses due to wall adsorption. Possible losses during water removing before analysis.

		Severe clean-up procedures between samples.
Active sampling	Short-term exposures Suitable for a wide volatility range of analytes Easy calibration Reutilization of sorbents High preconcentration efficiency	Careful sorbent selection. Not suitable for long-term exposures. Not integrating concentrations over longer periods. Requires the measurement of breakthrough volumes. Needs pumps and flow meters. Expensive. Possible degradations. Interferences with moisture. Collection of particles is possible by adding filters.
Passive sampling	Simple Cheap Long-term exposures Simultaneous deployment in several locations Pumps and flow meters not needed Time-weight average concentrations	Unstable flow-rates. Influenced by meteorological conditions. Not suitable for short-term exposures. Long sampling times. Low preconcentration capacity compared with active sampling. Difficult calibration. Traditional methods do not allow the collection of particles.

1.1.7.1 Air sampling and analysis of Volatile Organic Compounds with Solid Phase Micro-Extraction

Many conventional methods for VOCs sampling require a sorbent or impinger trap. The sampling is followed by thermal or solvent desorption into a detection instrument for the analysis. In many cases these methods require costly, noisy and non-reusable equipment. Solid phase micro-extraction (SPME) shows many advantages with respect to the traditional methods, providing a rapid extraction and preconcentration steps and permitting also to combine sampling and preconcentration in a single step. SPME has been usually applied in various environmental, food, pharmaceutical, flavor and forensic applications and in the last years many studies involved the SPME technique for the study of air composition, especially for the assessment of total volatile organic compounds (TVOCs), formaldehyde and volatile organic sulphur compounds in air. There are two main components of the SPME device: the SPME holder and the SPME fiber assembly (**Figure 10**). The fiber assembly consists of the extracting polymer coated on a fused silica fiber placed in a needle, while the holder guides the polymer into and out of the needle. SPME relies on the partitioning between the sample matrix and the fiber polymer.

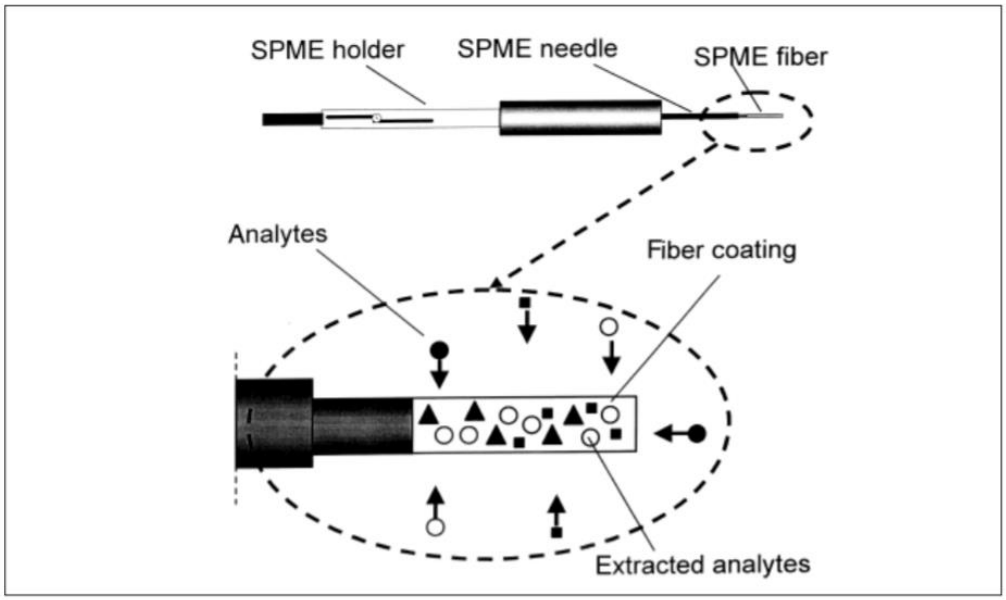


Figure 10. SPME device (fiber coating exposed to the sample matrix) [47].

Nowadays, there are different fiber coatings and each one has a different sensitivity toward certain analytes, such as polar or non-polar, semi-VOCs or VOCs.

There are several strategies in which SPME can be used for sampling of volatile compounds and their analysis:

- Test sampling of laboratory air, performing the sampling in a very short time and without the need of preserving the sample;
- As a field sampler before the analysis in the laboratory, choosing the suited coating polymer for the selected analytes;
- Using SPME as a sample preconcentration device in the laboratory, after the air sampling with other conventional methods;
- For field sampling associated to on-site analysis with portable GC.

1.1.7.2 Olfactometric bags

Collection of whole air using containers of a fixed volume has been exploited in many studies for assessment of trace VOCs in the atmosphere. Usually, the commonest used containers for whole air sampling are stainless steel canisters, glass bulbs or flexible plastic bags, such as olfactometric bags.

A sampling material is any material that is in contact with the gas sample, from the time of sampling until the analysis into laboratory, and it should meet the following characteristic:

- Inertia: the material should minimize the interactions with the air sample;
- Smooth surface;
- Absence of odors;
- Low permeability, to avoid sample losses and contamination of outside air.

The European Committee of Normalization (CEN) standard EN 13725 (EN 13725, 2003) [48] defined three materials to be allowed for olfactometric sampling bags: tetrafluoroethylene hexafluoropropylene copolymer (FEP); Tedlar™ (polyvinyl fluoride, PVF), and Nalophan (polyethylene terephthalate, PET). Moreover, European Standard set a maximum storage time of 30 hours allowed, during which the risk of sample modification during storage is minimized. Polymer bags (**Figure 11**) are very popular and commonly used containers in different types of analysis because of their low cost, inertness and good durability and reproducibility.

Figure 11. Olfactometric bag.

In literature, there are different studies showing the use and the application of olfactometric bags, also in combination with SPME and subsequent GC-MS analysis. For instance, this combination was applied to analyse fragrances from live plants [49] or to quantify siloxane levels in biogas [50]. It was exploited also for the measurement of VOCs from swine facilities [51], to assess the emissions of VOCs and carbonyl compounds during the combustion of barbecue charcoal [52] and to analyze PM and trace metals emitted from charcoal combustion during cooking time [53]. The analysis of VOCs emissions from historic plastics and rubbers was assessed placing the samples inside olfactometric bags and extracting and analyzing them performing SPME-GC-MS [54]. The same method was exploited for the analysis of sulfur compounds relevant in breath [55] or for the analysis of acetone in breath as a diagnosis for diabetes [56] and for the quantification of isoprene in breath again as a marker of cholesterol synthesis [57].

1.1.8 Aim of the work

This research project comes from a collaboration between University of Camerino and Elica S.p.A. company of Fabriano (AN, Italy). The final purpose of this collaboration is to improve the quality of odor filters used in kitchen hoods and to develop a new method for testing their efficiency. Cooking processes are one of the main contributors to emissions of pollutants inside home and restaurants, where, in many cases, the area is also poorly ventilated. Many pollutants, in particular VOCs, can be responsible for adverse health effects for humans and generate bad smells in closed environments, such as kitchen. Generally, aspiration or filtering systems are employed to minimize this discomfort. This is the central objective of this research project, aimed at planning innovative filtering systems capable to remove compounds having bad odors and that can be dangerous or toxic to humans. Another purpose of this work is to develop a new, simpler, more rapid and safer method to assess the efficiency of the filtering systems produced by the company. In order to achieve these purposes, it is necessary to perform a qualitative and quantitative study of the main VOCs emitted during different cooking processes. SPME-GC-MS has been applied for the determination of VOCs in air, combined with a previous air sampling in olfactometric bags. In fact, these plastic bags allow to transport the air sample from the location of sampling to the laboratory for the analysis. Despite several applications in different fields, this sampling system coupled to SPME-GC-MS has never been exploited to study the VOCs emissions during cooking activities. Firstly, different cooking processes and different food matrices have been analysed in order to find a pool of common compounds that emitted during cooking. The cooking models investigated were deep-fat frying of potatoes, roasting of hamburgers, boiling of cauliflowers and heating of sunflower oil. The definition of a set of pollutants common to different cooking models can bring to the development and validation of a new analytical method to test the efficiency of odor filters, alternative to the current legislation EN IEC 61591 which involves the use of the toxic methyl ethyl ketone.

1.2 Materials and Methods

1.2.1 Reagents and standards

A standard mixture of alkanes from C5 to C18 was purchased from Agilent Technologies (Milan, Italy). The analytical standard acetic acid (C2, purity ≥ 99%) was purchased from Sigma–Aldrich (Milan, Italy).

1.2.2 Study of VOCs development from different cooking systems

1.2.2.1 Samples and sample preparation

The SPME fiber assembly was purchased by Supelco (Bellefonte, PA, USA) and had a 50/30 μm thickness divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating with 2 cm length stationary phase. The extraction of the analytes was performed inserting the fiber into an 8L olfactometric PET bag (LOD s.r.l., Udine, Italy) using a glass cut vial with a pierceable septum replaced directly to the side Teflon tube of the bag (**Figure 12**).

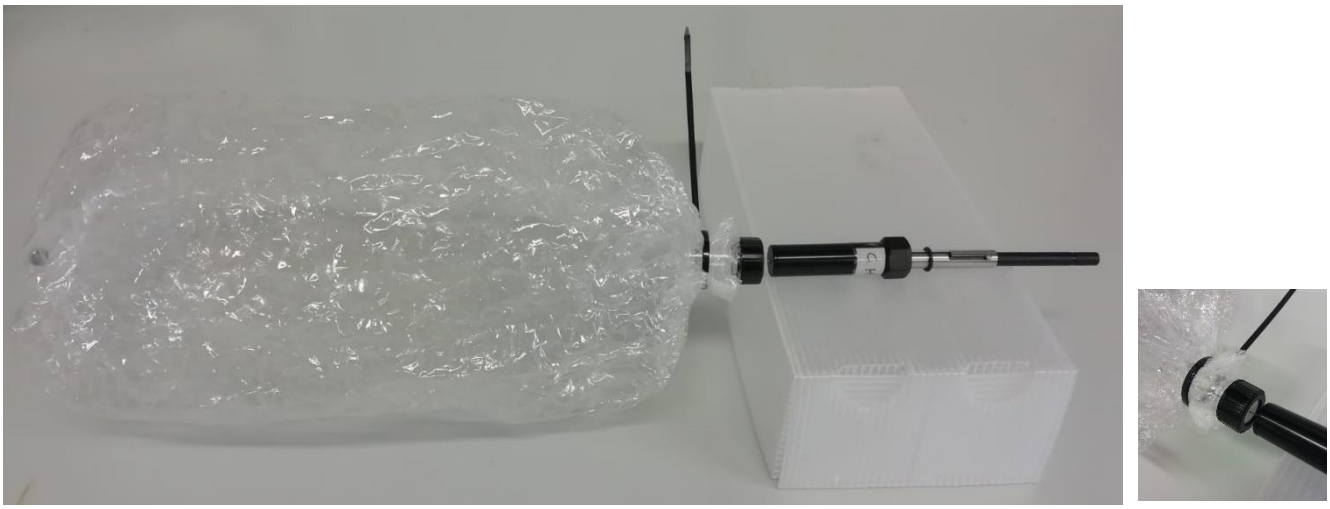


Figure 12. System used with SPME fiber exposed inside the PET bag using a modified glass cut vial (substituted to the Teflon tube) with a screw cap with pierceable septum to insert the needle.

The comparison of the efficiencies of different SPME procedures was performed inserting a mixture of alkanes (from *n*-C5 to *n*-C18) inside the olfactometric bag [58]. The procedure exploited in this study (SPME fiber inserted inside the cut vial) resulted to be the most efficient, because it allowed to avoid substantial losses of volatile compounds once the bag is open and

it permitted the entire exposure of the fiber to the analytes, remaining protected inside the PET bag. The best extraction time was evaluated using a deep fat fries frying at a temperature of 200 °C as a cooking model. The experiment was performed using a non-stick pan and an induction hob, which could be kept on at different power level (from 0 to 9). One liter of unused sunflower oil was placed in the pan and the hob was switched on at a high-power level (level 9). The temperature of the oil was monitored using a cooking thermometer until it reached 100°C. Then 250 g of frozen fries were put in the oil. After 10 minutes of cooking, the air above the cooking panel was sampled connecting the bag side tube near the hood. The analyses of VOCs were performed after SPME extraction from the air samples carried out for 1,8,16, and 24 hours (of exposure of the fibre) [58]. Then, the bag was opened, the holed protecting cap quickly removed from the holder and the fiber exposed inside the GC injector for the analysis of extracted VOCs. The best SPME extraction time resulted to be 24 h, so the method was applied to study the development of VOCs from different food matrices using different cooking methods. The first studied cooking system was a deep fat fries frying using sunflower oil at a temperature of 200 °C. Then the cooking of hamburger was monitored, sampling the air at different cooking times (5, 12, 16 and 19 minutes), passing from raw to overcooked meat. A whole beef hamburger was cooked in a non-stick pan using an induction hob. At the beginning the meat was placed in the pan and the hob was turned on at an intermediate power (level 5). After 5 minutes the air was sampled using a pump and placing the tube of the olfactometric bag under the kitchen hood. The air sampling was repeated always in the same place. Then the hamburger was flipped and the power of the hob was decreased a bit (level 4). After 12 minutes from the beginning of the cooking process the air was sampled for the second time. After 14 minutes the power was increased (level 6) and after 16 minutes the third air sampling was performed. At minute 18 the hob power was increased again (level 9) to accelerate the overcooking effect. After 19 minutes the air was sampled for the last time. The optimal cooking time was 12 minutes. Then, other three different systems were evaluated: the boiling of cauliflowers, the heating of sunflower oil at 120 °C and the combination of these two. A fresh cauliflower was weighted (1 kg) and placed in a pot and 4 L of cold water were added. The pot was placed in the induction hob and it was switched on at high-power (level 9). After 10 minutes, the power was decreased and set at level 7 until the air sampling, which was performed after 20 minutes from the beginning of the cooking process. Instead, the sunflower oil (2 L) was placed in a non-stick pan and placed on the induction hob, set at level 9. The oil temperature was monitored using a cooking thermometer. When the oil reached 100°C the power of the hob was decreased (level 4) and the

temperature was maintained at 120°C until the air sampling (after 20 minutes from the hob ignition).

For each sampling, four replicates were performed. Furthermore, due to the high sensitivity of the SPME fiber, a thermal cleaning before its use was necessary to avoid the presence of interferences. Therefore, before the samples analysis, the fiber was inserted into the hot injector of the GC-MS at 260 °C for 10 minutes after which cleaning run is performed. From the chromatogram obtained, it was possible to verify the condition of the fiber and to proceed with the samples analysis.

1.2.2.2 Headspace SPME-GC–MS and qualitative analysis

Volatile organic compounds were analysed employing a 6890 Network GC System coupled to a 5973 Network Mass Selective Detector both Agilent Technologies. The column was a capillary column coated by polyethylene glycol (60 m × 0.25 mm × 0.25 μm film thickness, DB-WAX, "Agilent Technologies". The carrier gas was helium at an initial flow rate of 1.2 mL/min. The injector was mantained at 260 °C and the splitless time was 4 min. The initial oven temperature was 35 °C for 4 min, then raised to 120 °C at 2.50 °C/min and then increased to 250 °C at 15 °C/min. This final temperature was held for 3.33 min, resulting in a total run time of 50 min. Mass analysis was carried out in scan mode in the range of 25-400 Da. The transfer line was held at 260 °C, the ion source at 230 °C and the quadrupole at 150 °C. The SPME fiber was left exposed into the GC injector for 10 min to be cleaned and reactivated after the desorption. The retention indices of the detected compounds were calculated using straight chain alkanes. The analytes in the air samples were identified by obtaining structural information from the mass spectrum and by comparison of their mass spectra and retention indices with those of standards from NIST-USA US National Institute of Standards and Technology (http://webbook.nist.gov). Blank tests were carried out to verify the absence of contaminants released: by the bag film (bag blanks), by the fiber after the analysis of real samples (carry over tests) and by the fiber after the cleaning (cleaning status check), by the different components of the instrument such as injector or column (instrument blanks). The only detected compounds were siloxanes, that are ubiquitous compounds not taken into consideration. For the bag blanks the olfactometric bag was filled up with nitrogen and the SPME extraction was carried out as for the real air samples (room temperature and 24 h of exposure). Bag blanks revealed that the detected analytes are negligible respect to the analytes

in real samples. Carryover experiments showed the absolute absence of this type of effect, probably due to the simple matrix and to the cleaning step of the fibre.

1.2.3 Development and validation of a new method for assessing odor filters efficiency

From the previous analysis, eighteen compounds resulted to be common to the different food matrices and cooking methods investigated (deep frying of potatoes, roasting of hamburger, boiling of cauliflower and heating of sunflower oil). Among them, acetic acid was chosen to develop and validate a new method for assessing odor filters efficiency, alternative to the current legislation (EN IEC 61591), because of its relative stability at high temperature, low toxicity and moderate low cost. The experiments were carried out in an insulated and certified room with a volume of 22±1 m^3 and under controlled conditions: temperature of 20±5 °C, relative humidity between 40 and 70% and the pressure of 860-1060 mbar. The first step was to evaluate the linearity of the method and to choose the best concentration for the use of the acetic acid. A heating plate was placed above the cooking panel in the room. Different quantities of acetic acid (10 µL, 25 µL, 50 µL and 100 µL) were evaporated in water bath at 60°C for 20 min (**Figure 13**).

Figure 13. Sampling device inside the odor room.

After this time, the air inside the room was sampled using olfactometric bags, placed in the same place and position each time. Each experiment was carried out four times. A calibration curve was obtained for acetic acid: the peak areas was plotted against standard concentrations. Linearity of response was determined by calculating the linear correlation coefficient (R^2) from the calibration curve. The volume of 25 µL was chosen for all the following experiments. Then the sealing of the room was verified. A fixed quantity of acetic acid (25 µL) was evaporated at the same conditions as before and the air was sampled after different time (20, 30, 40 and 50 min) at four different days. For each time of evaporation four replicates were performed.

Once the sealing of the room was verified and the evaporation time of the acetic acid in the room was chosen (20 min), a simulation of the sampling procedure was performed, to verify undesirable losses of the analyte. For this purpose, 25 µL of acetic acid were evaporated at 60°C for 20 min, then the door of the room was opened and rapidly closed to simulate the sampling of the air before the ignition of the hood. After 10 min, the door was opened and closed again and the air inside the room was sampled. The analysis was performed in quadruplicate and no significant losses of acetic acid were detected.

The last step was the evaluation of the best hood operation time. The developed sampling method was applied to a real kitchen odor filter, made of a polyurethane foam, coated with granular activated charcoal and inserted in black polyamide sock. Again, 25 µL of acetic acid were evaporated at 60°C in water bath inside the room. After 20 min the air was sampled and the hood was switched on. Then the second sampling was performed after different times: 10, 20 and 30 min. For each analysis an unused filter was tested. The sampling after 20 min were performed in three consecutive days to verify also the interday repeatability of the method. The dejection percentage for acetic acid was calculated for each hood operation time. The best one resulted to be 20 min.

Once all the operation conditions were evaluated the analytical method was applied to other real kitchen odor filters, to test their efficiency.

1.2.3.1 Hood aspiration filters

Three different filters were tested:

1-Washable filter MHGS (Figures 14 and **15)** is an open cell polyurethane foam, coated with granular activated charcoal and inserted in black polyamide sock (Nylon).

• Thickness [mm]: 11±0.5

• Weight per unit area [g/m^2] (DIN EN 12127): 2000±200

• Content of activated carbon [g/m^2]: 1400±200

Figure 14. Washable filter MHGS.

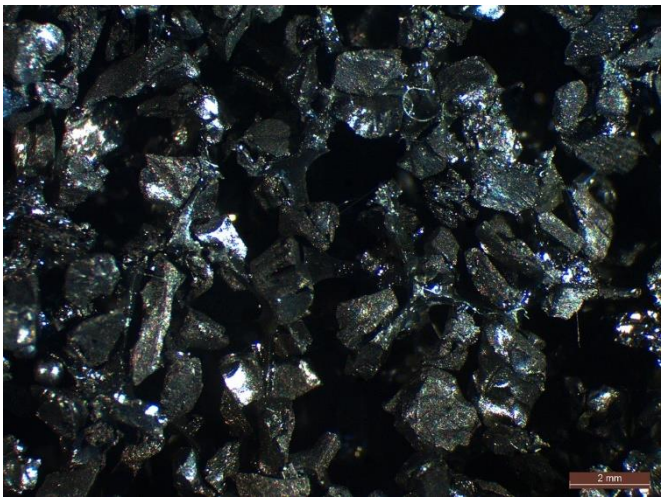


Figure 15. SEM-EDX image of the inner layer of the MHGS filter.

2-Washable filter SARATECH 2700 (Figures 16 and **17)** is an open cell polyurethane foam, coated with granular activated charcoal and inserted in black polyamide sock (Nylon).

• Thickness [mm]: 10±1

• Weight per unit area [g/m^2] (DIN EN 12127): 2700±250

• Content of activated carbon [g/m^2]: 2250±250

Figure 16. Washable filter SARATECH 2700.

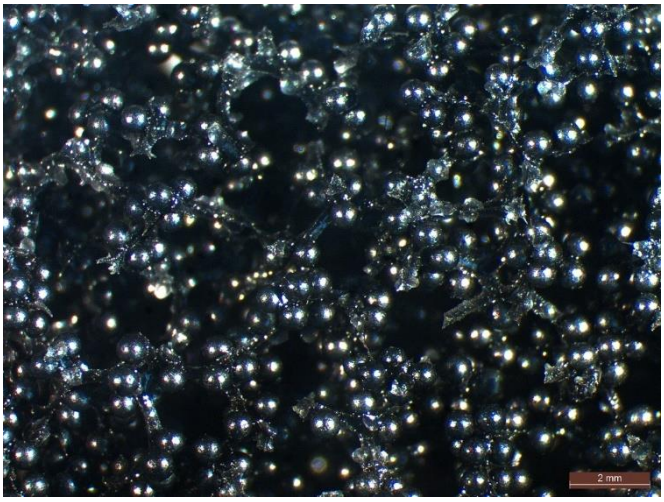


Figure 17. SEM-EDX image of the inner layer of the SARATECH 2700 filter.

3- Helsa-Sorbexx-CS filter (**Figures 18** and **19**) is a composite of activated charcoal reinforced by ceramic.

• Dimensions of single honeycombs [mm]: 48x48x40

• Quantity of honeycombs: 12

• Density of cells [cpsi]: 200

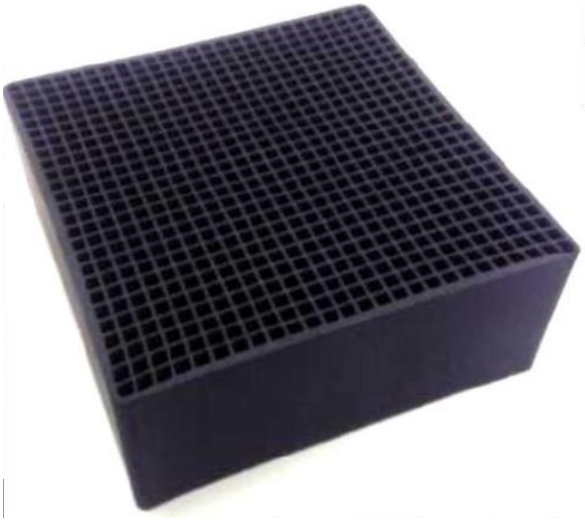


Figure 18. Helsa-Sorbexx-CS filter.

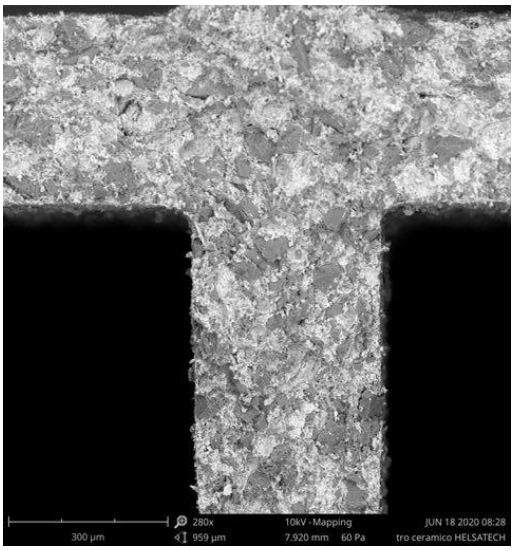


Figure 19. SEM-EDX image of the Helsa-Sorbexx-CS filter.

1.2.3.2 Headspace SPME-GC–FID analysis

The SPME fiber assembly was purchased by Supelco (Bellefonte, PA, USA) and had a 50/30 µm thickness divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating with 2 cm length stationary phase. The extraction of acetic acid was performed exposing the fiber inside the olfactometric bag for 24 h with the help of the glass cut vial and the analysis was made using a gas cromatograph Agilent Technologies 6850 GC (Santa Clara, CA, USA) equipped with a split/splitless injector and a flame ionization detector (FID). The capillary chromatographic column was covered by nitroterephthalic acid modified polyethyleneglycol (PEG) (DBFFAP, 25 m, 0.25 mm i.d., 0.25 µm film thickness, purchased from Agilent

Technologies, Santa Clara, CA, USA). The injector was set at 280 °C and the injection was carried out in splitless mode (splitless time 3 min). The oven was mantained at 40 °C for 3 min, then raised to 160 °C at a rate of 20 °C/min and again to 245 °C at 40 °C/min. The final temperature was held for 1.87 min, resulting in a total run of 13 min. The hydrogen was used as carrier gas at a flow rate of 3.70 mL/min. The FID temperature was maintained at 250 °C. The identity of the acetic acid was confirmed using a standard solution.

The extraction of the acetic acid was performed inserting the fiber into a 8L olfactometric PET bag (LOD s.r.l., Udine, Italy) using a glass cut vial with a pierceable septum replaced directly to the side Teflon tube of the bag, exposing the fiber for 24 h.

1.2.4 Statistical analysis of data

Significant differences between the content of the different classes of compounds and some selected compounds in the air sampled during the grilling of the hamburger at different time (5, 12, 16 and 19 minutes) were evaluated by one-way analysis of variance (ANOVA).

The same statistical analysis was employed for the evaluation of significant differences between the content of the acetic acid in the sampling room under different operating conditions. Differences with $P < 0.05$ were estimated significant. Data were elaborated using PAST software package [59]. Each experiment was performed four times.

1.3 Results and Discussion

1.3.1 Development of VOCs from different cooking systems

The aim of this work was to determine the composition of the main VOCs formed during cooking activities, from different food matrices and different cooking methods. The study was specifically aimed at evaluating possible common molecules, that can be used as standards to test the efficiency of real kitchen odor filters. In a previous study [58] the conditions to perform the SPME extraction and the best extraction time (24 h) were selected. Then, the method was applied to study the VOCs developed by different cooking methods and different cooking matrices. In particular, the studied cooking models were: the deep fat fries frying with sunflower oil at 200°C, the roasting of a beef hamburger and lastly the boiling of a cauliflower, the heating of sunflower oil at 120 °C and the combination of these two.

From each chromatogram the identification of the different peaks was performed. Firstly, the identification of the peaks relative to alkanes was performed and then the identification of the compounds in the real samples. This was possible comparing the data from the NIST library and retention indexes. The Kovats retention index (I or RI) refers to where an analyte elutes respect to straight chain alkanes. The retention index of a *n*-alkane is equal to 100 times the number of carbon atoms and the value is identical by definition for any chromatographic system. The following expression was applied to determine the value of the retention indexes of the analytes:

$$RI_X = 100 \cdot Z + 100 \cdot (T_X - T_Z)/(T_{Z+1} - T_Z)$$

where Z is the number of carbons of the *n*-alkane having a retention time T_Z, T_X is the retention time of the analyte of interest, T_Z is the retention time of the *n*-alkane which elutes before of the analyte and T_{Z+1} is the retention time of the n-alkane which elutes immediately after the analyte. Then, the detected compounds were divided according to their chemical nature into aldehydes, aromatic compounds, ketones, alcohols, esters, aliphatic hydrocarbons, organic acids and other compounds. The qualitative composition were evaluated for each examined cooking model system and for the examined filter.

Once the peaks recognition was completed, it was possible to do the relative quantification. The peaks in the chromatogram were integrated manually to obtain the corresponding area. Then, the areas obtained were processed using the Excel program.

1.3.1.1 Deep fat frying of French fries

The first cooking model investigated was deep fat frying of French fries in sunflower oil at 200 °C. In this section the results obtained from this system are reported. An example of the chromatogram obtained for the air sampled at the end of this cooking activity is shown in the figure below (**Figure 20**).

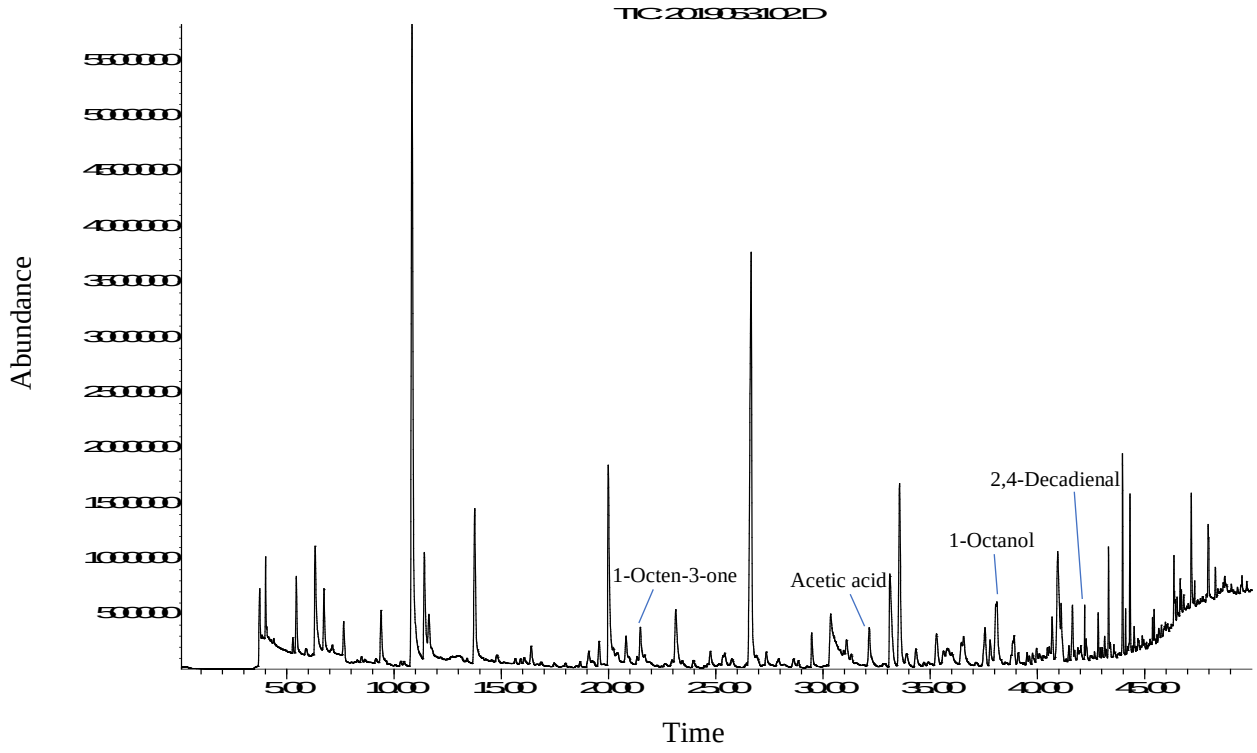


Figure 20. Chromatogram obtained from VOCs in the air sampled during deep fat frying of fries.

Table 11 shows the detected VOCs in the samples, divided into aldehydes, aromatic compounds, ketones, alcohols, esters, aliphatic hydrocarbons, organic acids and other compounds.

Table 11. Volatile compounds developed during deep fat frying of fries detected by HS-SPME-GC-MS, their experimental linear retention indices (LRI) on a DB-WAX column and values reported in literature on DB-WAX columns. Their abundances are reported in terms of peak areas and % relative standard deviation (RSD, *n*=4).

Compound detected[a]	LRI[b] (exptl)	LRI[c] (lit)	Area	RSD %
Aldehydes				
Pentanal	966	974	1.46E+07	20.6
Hexanal	1070	1079	1.16E+08	47.2
Heptanal	1173	1183	1.65E+07	21.3
2-Hexenal	1206	1212	1.81E+07	25.8
Octanal	1279	1287	1.03E+07	18.3
(Z)-2-Heptenal	1315	1320	2.65E+08	11.7
Nonanal	1386	1396	7.52E+07	13.4
(*E*)-2-Octenal	1421	1425	2.00E+07	15.2
Benzaldehyde	1511	1515	3.61E+07	13.5
(*E*,*E*)-2,4-Nonadienal	1707	1706	2.72E+06	33.1
2-Dodecenal	1766	1776	1.60E+07	39.1
(*E*.*E*)-2,4-Decadienal	1812	1821	2.79E+07	31.5
Vinylbenzaldehyde	1832	nf	1.04E+06	7.3
Ketones				
Acetone	807	814	3.51E+07	37.9
2-Butanone	891	900	1.37E+07	3.1
3-Hydroxy-2-butanone	1276	1286	3.20E+06	12.5
1-Octen-3-one	1292	1302	1.07E+07	14.0
6-Methyl-5-hepten-2-one	1329	1339	9.28E+06	14.9
α-Isomethylionone	1859	1869	5.75E+06	7.7
(*E*)-6,10-Dimethyl-5,9-undecadien-2-one	1868	1860	9.52E+05	0.7
Organic acids				
Acetic acid	1438	1447	1.05E+08	42.3
Propanoic acid	1535	1537	3.05E+07	26.4
Butanoic acid	1624	1624	1.61E+07	78.3
Isovaleric acid	1668	1676	2.80E+07	18.0
Pentanoic acid	1734	1743	1.17E+07	24.6
Hexanoic acid	1842	1847	3.47E+07	4.9
Heptanoic acid	1946	1939	7.63E+06	5.3
Octanoic acid	2052	2050	1.48E+07	16.7
Nonanoic acid	2160	2170	2.18E+07	11.7
Decanoic acid	2267	2266	1.92E+07	20.9
Alcohols				
1-Butanol	1144	1150	6.58E+06	18.7
1-Pentanol	1250	1256	6.12E+07	46.7
1-Butoxy-2-propanol	1340	1350	7.65E+06	29.3
2-Butoxyhexanol	1400	1410	2.05E+07	17.9
1-Octen-3-ol	1449	1456	8.97E+07	7.3
1-Heptanol	1456	1461	9.97E+06	27.8
4-Ethylcyclohexanol	1484	nf	2.15E+07	17.2
2-Ethyl-1-hexanol	1490	1492	1.31E+07	37.2
1-Octanol	1569	1566	2.82E+07	21.5

1,2-Ethanediol	1637	1635	1.30E+08	39.2
Benzyl alcohol	1874	1870	4.25E+06	10.0
2-Pentadecanol	1927	1935	3.59E+06	5.9
1-Dodecanol	1946	1950	4.38E+06	2.6
2,5,8,11,14-Pentaoxahexadecan-16-ol	1959	nf	1.21E+06	10.2
3-Methoxy-1-propanol	1980	nf	2.76E+06	15.3
Phenol	1994	1996	3.66E+06	10.1
Aromatic compounds				
Toluene	1023	1033	4.37E+07	27.5
Ethylbenzene	1116	1118	3.87E+06	28.7
p-Xylene	1122	1125	9.33E+06	23.9
o-Xylene	1165	1159	4.64E+06	31.0
Pyridine	1178	1181	7.49E+06	12.0
2-Pentylfuran	1218	1220	2.44E+07	27.6
1-Methyl-2-(1-methylethyl)-benzene	1258	1268	6.87E+06	16.2
1,2,4-Trimethylbenzene	1264	1268	6.24E+06	23.7
1-Ethyl-2,3-dimethylbenzene	1344	1352	1.10E+06	39.6
4-Ethyl-1,2-dimethylbenzene	1353	1362	8.77E+06	48.7
2-Ethyl-1,4-dimethylbenzene	1357	1364	2.32E+06	65.9
Butylated hydroxytoluene	1910	1909	1.31E+06	22.5
Aliphatic hydrocarbons				
Hexane	600	600	2.14E+07	19.5
Heptane	700	700	4.88E+06	13.5
Octane	800	800	5.59E+06	53.1
2-Octene	831	843	1.01E+07	9.2
Cycloheptane	880	883	1.10E+07	22.2
1-Nonene	917	923	3.93E+06	21.6
α-Pinene	1015	1013	2.28E+06	24.9
β-Pinene	1096	1097	4.81E+06	14.7
3-Carene	1130	1138	1.39E+07	74.8
D-Limonene	1182	1192	1.42E+07	25.0
1-Tridecene	1352	1343	5.00+06	55.6
Tetradecane	1400	1400	8.72E+06	13.1
3-Ethyl-2-methyl-1,3-hexadiene	1404	nf	5.25E+06	26.9
Pentadecane	1500	1500	1.30E+07	21.1
(E)-1,9-Tetradecadiene	1574	nf	6.04E+06	30.6
4-Ethylcyclohexene	1595	nf	3.93E+06	17.9
(Z)-3-Tetradecene	1661	nf	4.04E+06	0.9
Heptadecane	1700	1700	5.96E+06	30.5
Esters				
Ethyl acetate	875	884	2.28E+07	9.3
Isobornyl acetate	1584	1583	8.28E+06	8.5
Acetic acid phenylmethyl ester	1716	1726	3.79E+06	19.0
Methyl salicylate	1771	1763	6.23E+06	22.1
Dihydro methyl jasmonate	2405	nf	6.15E+06	7.7
Other compounds				
Methylene chloride	913	914	9.79E+06	33.8
2-Methyl-1,3-dioxolane	948	953	1.19E+07	12.7
N-Ethyl-1,3-dithioisoindoline	958	nf	4.81E+06	27.2
Trichloromethane	1006	1014	5.56E+08	50.4
6-Methoxy-1-methyl-5-phenyl-4,7-indoloquinone	1541	nf	1.05E+07	5.3

Abbreviation: nf, not found. [a]Compounds reported are those with peak area values higher than 500.000 units. [b]Experimental linear retention indices. [c]Linear retention indices reported in literature (NIST 2017).

Deep fat frying is one of the commonest cooking operations involved in the preparation of variety of foods, such as French fries, fish sticks, donuts and fried chicken. The method consists of cooking food by immersing it in hot edible oil or fat at a temperature higher than 180°C and it gives foods with a distinctive flavors and texture [60, 61]. During frying process all of the food compounds take part in chemical and physical changes, leading to some dissociation reactions of many components and to the interactions between these compounds. VOCs emission during deep-fat frying is affected by many factors, such as temperature, time, the type of edible oil and the fried product [61]. The typical reactions taking place during deep-fat frying are oxidation, polymerization, hydrolysis and isomerization. These reactions lead to the formation of hydrocarbons, aldehydes, free fatty acids, alcohols, ketones, lactones, acids or epoxy- and cyclic compounds [61].

In the present study, aldehydes were the most abundant volatile species (29%), followed by "other compounds" (26), alcohols (18%), organic acids (13%), aliphatic hydrocarbons (5%), aromatic compounds (4%), ketones (3%) and esters (2%) (**Figure 21**).

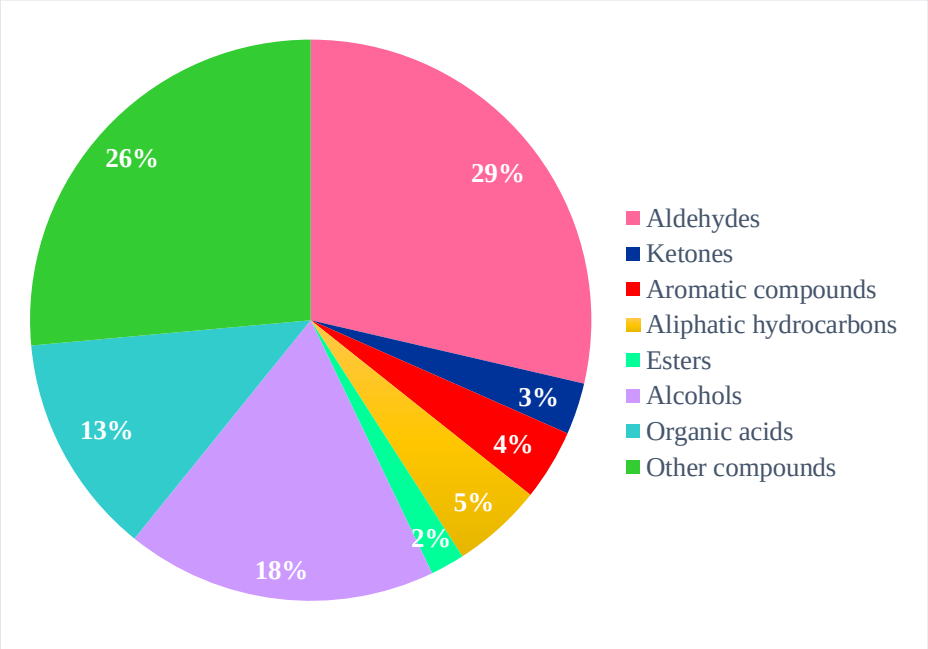


Figure 21. Percentage composition of the different classes of VOCs detected in the air sampled after the deep-frying of potatoes.

The high presence of aldehydes is due to lipids oxidation. The high temperature reached during the cooking process promotes the oxidation of fatty acid chains, especially the unsaturation sites, increasing the radicals number. These radicals can react with other fatty acids, developing the aldehydes formation with their characteristic aromas [62]. Pentanal shows a pungent and almond aroma, hexanal is fatty and fruity, heptanal is rancid, octanal has a green and fruity aroma, nonanal has a grassy and citrus scent [62]. The types and concentration of the aldehydes in the analyzed samples is mainly due to the sunflower oil composition, with its 62% of linoleic acid and 28% of oleic acid [58]. The formation of the different aldehydes during the cooking process depends mainly on the position and the number of double bonds along the fatty acids chain. The major quantities of the detected aldehydes arise from linoleic acyl groups (e.g. for hexanal, pentanal, heptanal, 2-heptenal, 2-octenal and 2,4-decadienal) or from oleic acyl groups (e.g. for octanal and nonanal) [58]. Some aldehydes can derive also from other bigger aldehydes, as for hexanal, which can derive also from the degradation of 2,4-decadienal [63].

The detected alcohols formed about the 18% of the total VOCs and many of them are again the products of the thermal degradation of lipids. For instance, 1-octen-3-ol was one of the most abundant alcohols and it could be generated from the enzymatic pathways coming from an intermediate of the linoleic acyl groups (linoleic acid 10-hydroperoxide). Pentanol can derive again from linoleic acid degradation; 1-octanol could be a cleavage product of methyl oleate hydroperoxide [58]. Because of their usual high odor thresholds, alcohols do not contribute conspicuously to the aromas of cooked food [62].

The detected organic acids were linear acids with chains from two to ten carbon and also a branched short chain fatty acid (isovaleric acid) was detected, probably mainly deriving from the oxidation of the corresponding aldehyde. Short chain fatty acid are known for their rancid flavour and contrarily to long chain fatty acids, they are perceivable at low concentrations. The odor thresholds of fatty acids increase with the number of the carbons of the fatty acid chain, while the volatility decreases [58].

Aliphatic hydrocarbons can be the products of linoleic and oleic acyl groups oxidation; they do not contribute so much to the total food aroma because of their high odor thresholds. Among aromatic compounds, alkylbenznes and toluene were found at highest amounts. Their development is due to the presence of linoleic and linolenic acids in the sunflower oil. A possible pathway for the alkylbenzenes formation is the thermal degradation of linoleic and linolenic acyl groups and the subsequent formation of (*E*,*E*)-2,4-decadienal, followed by its cyclization and water loss [64].

The less abundant class of compounds were ketones, which represented about 3% of the total VOCs, that could derive from the oxidation of unsaturated fatty acids, and esters (2%). Esters already present in food may undergo to a thermal degradation and new esters could be formed by reactions between alcohols and acids [58].

1.3.1.2 Grilling of a beef hamburger

The second investigated cooking model was the grilling of a hamburger, at different time of cooking, from raw to overcooked meat. Indeed, the air above the cooking plate was sampled at different time: after 5, 12, 16 and 19 minutes of cooking (**Figure 22**). The ideal cooking time of the meat was evaluated as 12 minutes.

5 min 12-16 min 19 min

Figure 22. Hamburger after 5 minutes (on the left), 12 and 16 minutes (in the middle) and after 19 minutes of cooking (on the right).

The results obtained from this system are presented in this section. The figures below (**Figures 23, 24, 25, 26** and **27**) show the chromatograms obtained from the air sampled during the cooking activity at different times (5,12,16 and 19 minutes).

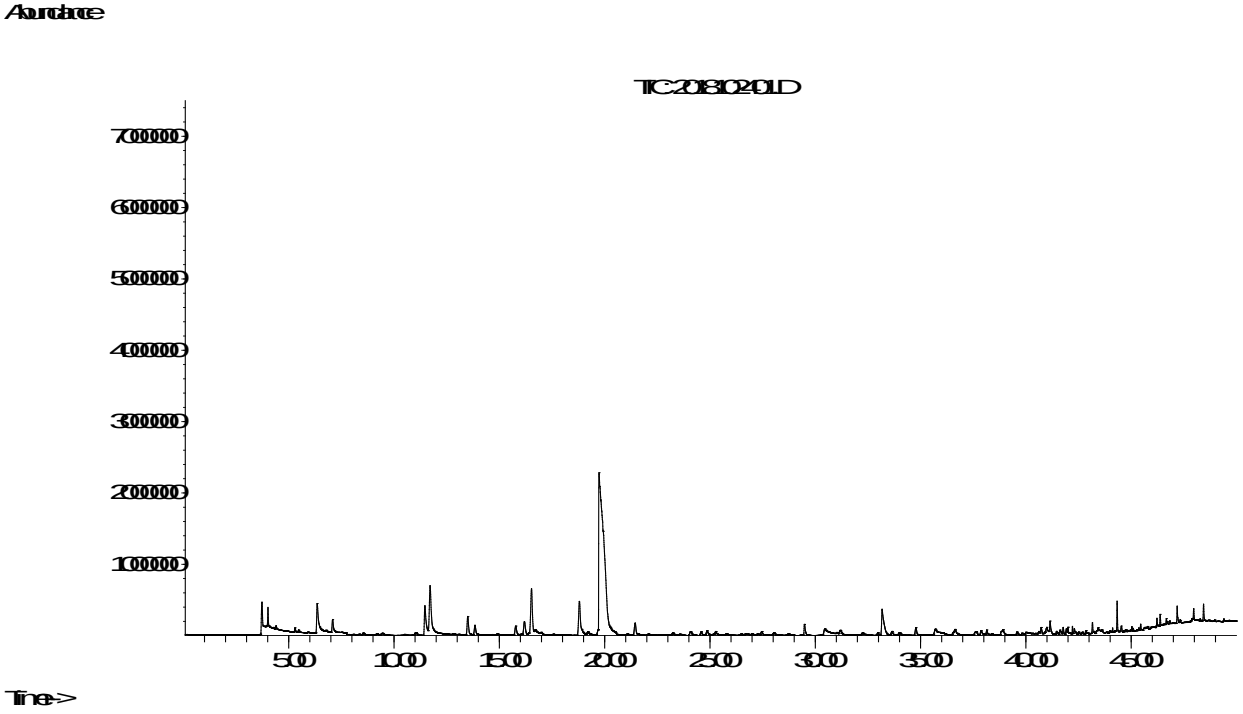


Figure 23. Chromatogram obtained from the analysis of VOCs in the air sampled after 5 minutes of grilling of the hamburger.

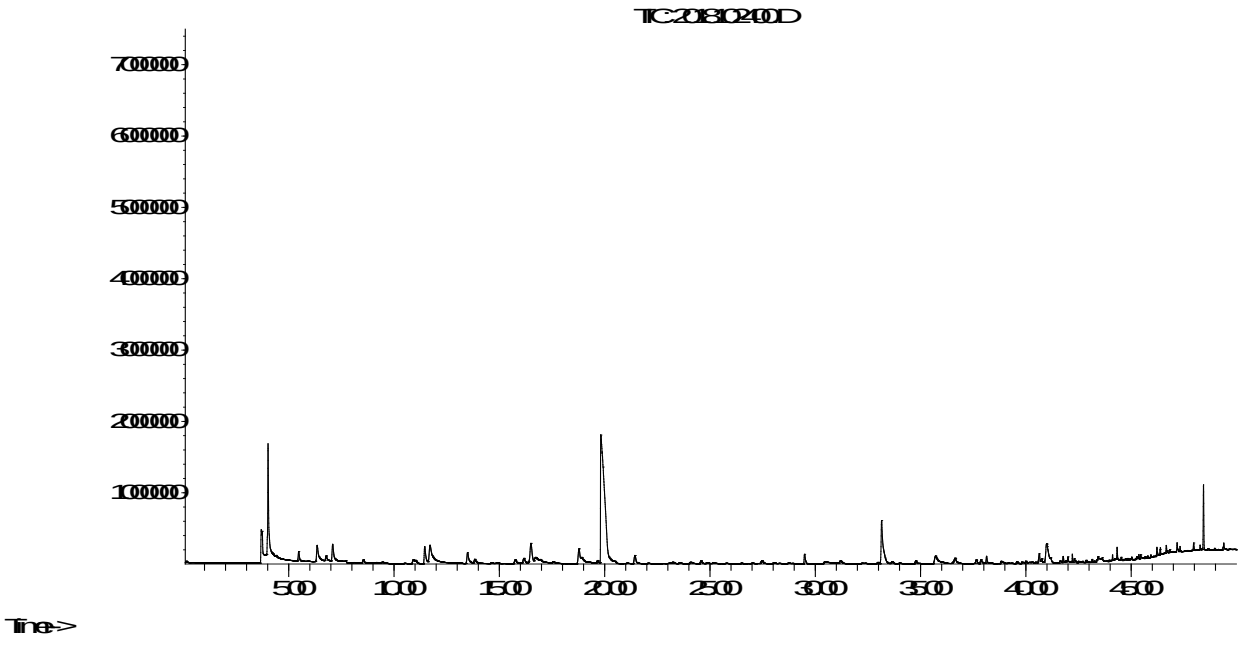


Figure 24. Chromatogram obtained from the analysis of VOCs in the air sampled after 12 minutes of grilling of the hamburger.

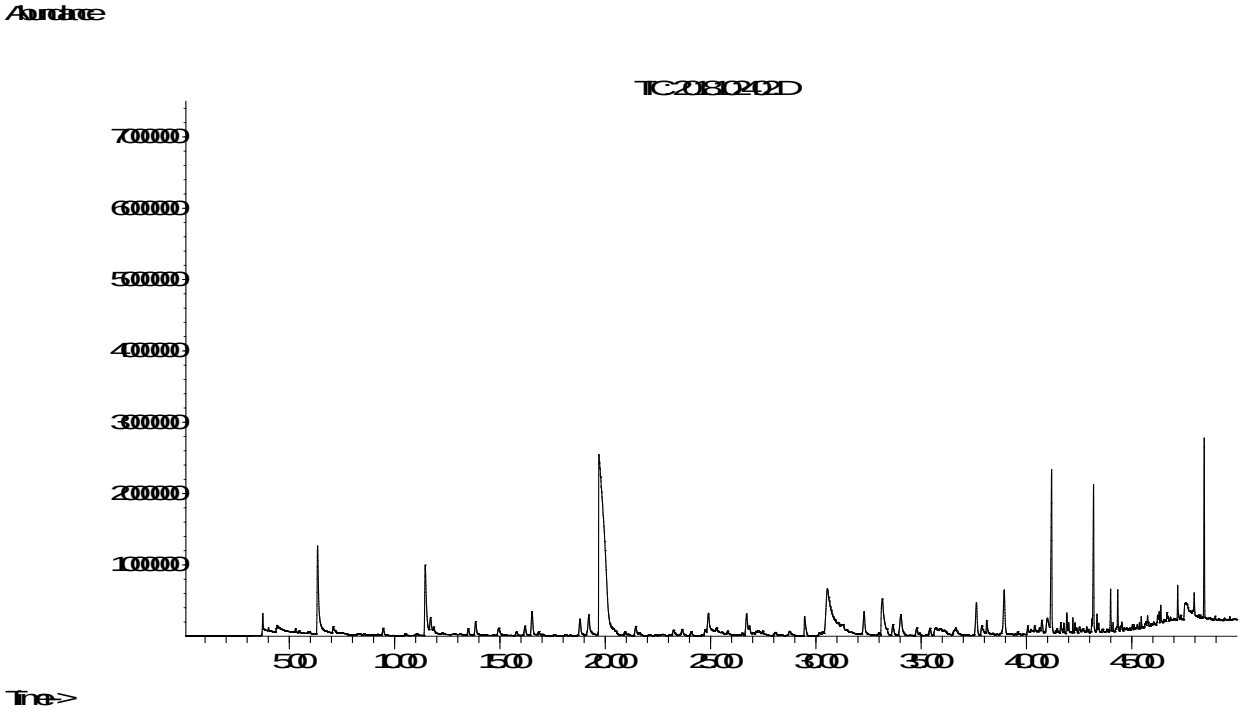


Figure 25. Chromatogram obtained from the analysis of VOCs in the air sampled after 16 minutes of grilling of the hamburger.

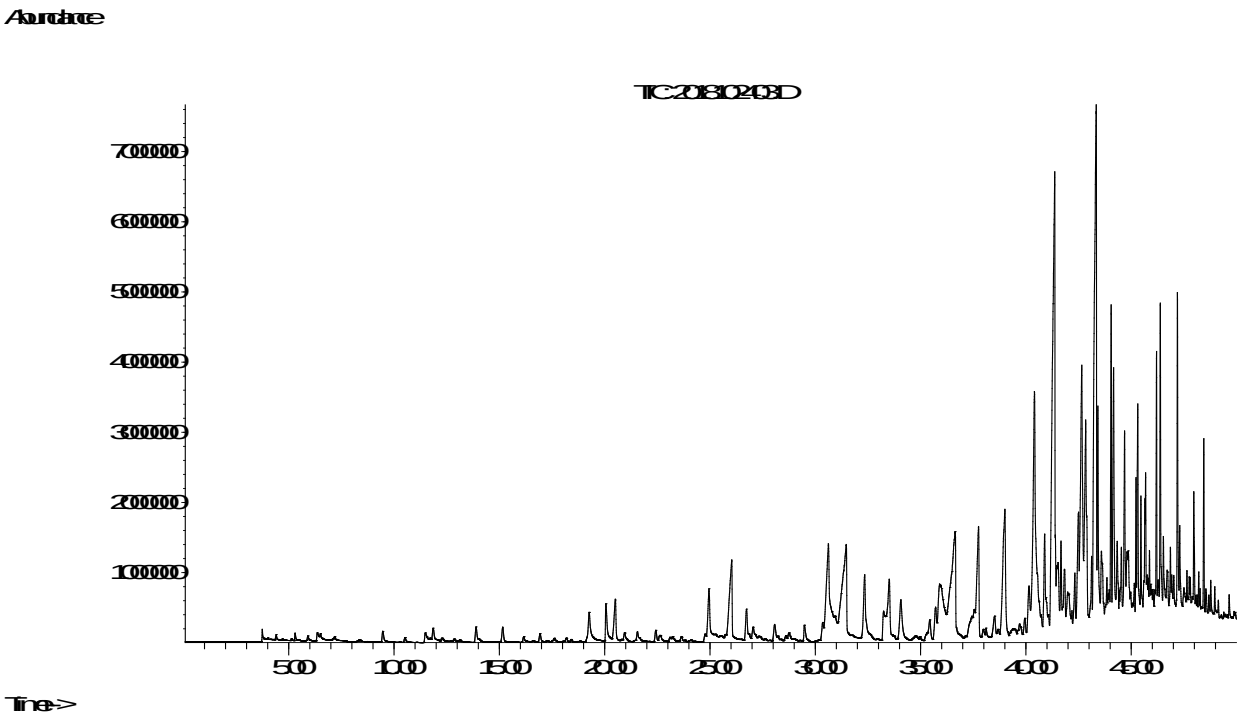


Figure 26. Chromatogram obtained from the analysis of VOCs in the air sampled after 19 minutes of grilling of the hamburger.

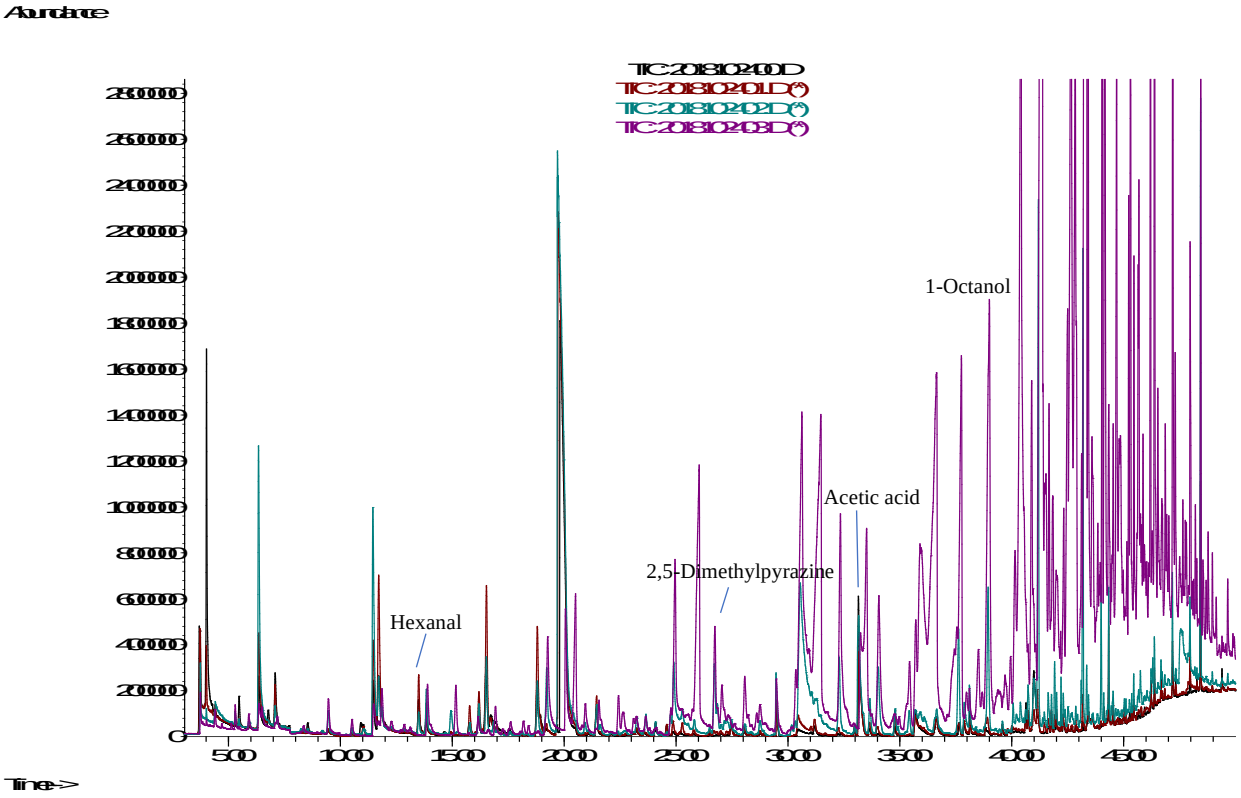


Figure 27. Chromatograms obtained from the analysis of VOCs in the air sampled after 5 minutes (black line), 12 minutes (red line), 16 minutes (blue line) and 19 minutes (purple line) of grilling of the hamburger.

The detected VOCs in the samples after different times of cooking were classified according to their chemical nature into aldehydes, aromatic compounds, ketones, alcohols, esters, aliphatic hydrocarbons, organic acids and other compounds and the results in terms of absolute area are reported in **Table 12**.

Table 12. Volatile compounds detected during grilling of an hamburger by HS-SPME-GC-MS, their experimental linear retention indices (LRI) on a DB-WAX column and values reported in literature on DB-WAX columns. Their abundances are reported in terms of peak areas and% relative standard deviation (RSD, *n*=4).

Compound detected[a]			Absolute area							
	LRI[b] (exptl)	LRI[c] (lit)	5 minutes	RSD%	12 minutes	RSD %	16 minutes	RSD %	19 minutes	RSD%
Aldehydes										
Propanal	783	784							2.59E+06	42.51
Butanal	859	890							2.36E+06	39.85
Pentanal	968	974					4.17E+06	31.46	5.69E+06	35.54
Hexanal	1073	1079	4.06E+06	19.86	5.67E+06	26.90	9.55E+06	18.43	8.63E+06	40.28
(*E*)-2-Pentenal	1119	1125							3.03E+06	27.46
Heptanal	1177	1183	8.97E+05	30.80	1.97E+06	42.16	1.85E+07	32.79	2.69E+07	38.22
(*E*)-2-Hexenal	1208	1212					2.21E+06	26.26	4.82E+06	29.45
(Z)-2-Heptenal	1313	1320			8.95E+05	21.87	1.27E+07	42.51	1.85E+07	20.66
Nonanal	1384	1396							8.47E+07	12.67
(*E*)-2-Octenal	1417	1425					1.47E+07	45.88	4.26E+07	19.72
(*E*,*E*)-2,4-Heptadienal	1477	1480					4.58E+06	43.29	1.72E+07	13.85
Benzaldehyde	1512	1512	8.67E+06	33.14	3.90E+06	18.32	2.77E+06			
(*E*)-2-Nonenal	1531	1540					1.99E+07	38.25	8.93E+07	15.66
(*E*)-2-Decenal	1651	1649					6.29E+07	35.78	3.88E+08	21.77
Dodecanal	1712	1709							1.62E+08	13.32
2-Undecenal	1763	1755							3.79E+08	22.93
Aromatic compounds										
5-Methyl-2-phenyl-1H-indole	898	nf							5.19E+06	23.85
Toluene	1026	1033	3.11E+07	49.00	4.42E+07	36.29	1.08E+07	33.28		
Ethylbenzene	1110	1118	2.33E+06	43.84	3.63E+06	57.09	1.51E+06	44.30		
p-Xylene	1127	1125	2.57E+06	48.97	4.91E+06	24.84	3.61E+06	55.73		
m-Xylene	1139	1143	9.08E+06	48.56	1.57E+07	52.81			1.40E+06	
2-Butyltetrahydrofuran	1157	nf							2.45E+06	26.09
1-Methylethylbenzene	1159	1168			3.76E+05	48.21				
o-Xylene	1168	1171	5.14E+06	26.67	1.26E+07	40.64				
2-Pentylfuran	1221	1220			5.96E+05	36.54	1.64E+06	30.69	1.74E+06	32.82
1,3,5-Trimethylbenzene	1230	1228			8.95E+05	17.64	8.69E+05	4.45		
Styrene	1243	1248	9.75E+05	6.78	1.01E+06	31.53	1.02E+06	22.51		
Methylpyrazine	1260	1270					3.67E+06	30.08	3.72E+06	10.58
2,5-Dimethylpyrazine	1320	1318					4.22E+06	36.16	3.30E+06	15.73
2-Hexylfuran	1319	1323							9.49E+06	10.16
2,6-Dimethylpyrazine	1326	1333					9.39E+05	47.91		
1-Ethyl-3,5-dimethylbenzene	1356	1350			1.46E+06	12.48				
2-Chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl)pyrimidine	1363	nf							1.06E+07	9.95
2-Ethyl-6-methylpyrazine	1383	1381					1.82E+06	26.83		
2-Heptylfuran	1418	1416							6.32E+06	20.57
2-Benzo[1,3]dioxol-5-yl-8-methoxy-3-nitro-2H-chromene	1549	nf					4.63E+06	31.46		
4-(4-Chlorophenyl)-2,6-diphenylpyridine	1591	nf	1.01E+06	7.76						

Benzonitrile	1593	1591	7.08E+05	35.30						
2-[(2-Furanylmethoxy)methyl]tetrahydro-2H-pyran	1861	nf							1.63E+07	15.16
Ketones										
2-Butanone	894	900	9.45E+06	41.64	5.03E+06	22.29	2.99E+06	37.26		
1-(1-Cyclohexen-1-yl)-ethanone	1105	nf					9.05E+05	43.87		
3-Heptanone	1146	1148							2.00E+06	44.81
4-Octanone	1219	1224							3.53E+06	38.17
3-Octanone	1247	1240					9.74E+05	15.45	3.03E+06	41.56
3-Hydroxy-2-butanone	1277	1286	3.12E+06	25.92	2.17E+06	41.90	8.02E+05	10.28		
2-Octanone	1280	1278					3.79E+06	24.43	4.59E+06	22.94
1-Hepten-3-one	1295	nf					1.06E+06	22.87		
2-Nonanone	1386	1389					1.82E+06	45.03	1.01E+07	22.11
2-Decanone	1482	1492							2.60E+07	3.04
3-Butylcyclopentanone	1538	nf							5.98E+06	5.97
3-Methyl-1-phenylthiobutan-2-one	1586	nf							1.28E+07	7.04
2-Undecanone	1599	1606					4.63E+06	31.83	3.59E+07	6.41
2-Dodecanone	1639	1650					1.59E+06	6.29		
4,6,6-Trimethyl-(1S)-bicyclo[3.1.1]hept-3-en-2-one	1713	nf			1.31E+06	9.07				
4,4,5-Trimethyl-2-cyclohexen-1-one	1728	nf							3.36E+07	8.78
2(5H)-Furanone	1742	1746					2.76E+06	43.28		
5-Tridecanone	1747	nf							1.85E+07	18.70
6,10-Dimethyl-(*E*)-5,9-undecadien-2-one	1860	1859			2.95E+06	7.71	4.57E+06	18.93		
2-Tetradecanone	1925	1915							3.91E+07	9.09
5-Butyldihydro-2(3H)-furanone	1933	1937							5.96E+07	14.14
2-Pentadecanone	2031	2041							7.87E+07	15.19
Tetrahydro-6-pentyl-2H-pyran-2-one	2119	nf							1.32E+07	8.73
2-Hexadecanone	2140	2150							1.12E+07	22.55
2-Heptadecanone	2210	2218							5.79E+06	12.13
Alcohols										
1-Butanol	1144	1150	1.34E+06						5.45E+05	44.17
1-Pentanol	1249	1256	1.64E+06	20.41	1.09E+06	36.65	3.13E+06	34.79		
1-Butoxy-2-propanol	1343	1350	2.25E+06	32.04	2.58E+06	5.20	3.18E+06	9.53		
1-Hexanol	1356	1359					3.12E+06	25.94	4.11E+06	26.07
2-Butoxy-ethanol	1403	1410	4.07E+06	20.40	3.54E+06	20.06				
3,7-Dimethyl-3-octanol	1438	1428	1.91E+06	41.84	2.52E+06	39.00				
1-Octen-3-ol	1452	1456					5.72E+06	37.34		
Heptanol	1459	1461					1.93E+07	27.88	3.61E+07	25.83
2,6-Dimethyl-7-octen-2-ol	1474	1470	3.81E+06	35.89	5.87E+06	14.33	6.87E+06	17.00		
2-(1,1-Dimethylethyl)-cyclohexanol	1556	nf			9.51E+05	19.81				
3,7-Dimethyl-1,6-octadien-3-ol	1567	1559	6.46E+05	34.89						
1-Octanol	1569	1566	2.37E+06	38.54	5.02E+06	8.88	2.97E+07	21.68	9.31E+07	36.89
3-Furanmethanol	1660	1670			1.34E+06	36.19	1.03E+06	49.45		
1-Nonanol	1668	1665					5.75E+06	34.22	2.73E+07	13.54
p-Menth-1-en-8-ol	1700	1700					2.08E+06	26.85		

1-Undecanol	1829	1839							6.53E+07	8.63
3,6,6-Trimethyl-2-norpinanol	1967	nf							1.77E+07	13.69
Esters										
Ethyl acetate	877	884	1.98E+06	30.53						
Acetic acid, butyl ester	1064	1072	4.98E+06	35.82	5.49E+06	22.29	1.81E+06	42.83		
Propanol, methoxy acetate	1218	nf	3.27E+06	46.06	3.93E+06	45.00				
Formic acid, octyl ester	1567	1560			2.03E+06	10.84				
Pentafluoropropionic acid, tridecyl ester	1593	1590							1.13E+07	5.72
Hexanoic acid, 3-tridecyl ester	1662	nf					6.62E+05	52.48		
4-Ethylbenzoic acid, 2-butyl ester	1681	nf					7.79E+06	8.43		
4-*tert*-Butylcyclohexyl acetate	1686	nf	2.02E+06	12.75	2.32E+06	8.83				
Acetic acid, phenylmethyl ester	1726	1726	6.27E+06	21.10	6.33E+06	34.54				
Methyl salicylate	1774	1763	9.61E+06	19.63	9.92E+06	44.80	1.50E+06	29.71		
Acetic acid, chlorohexadecyl ester	1841	nf							1.17E+07	11.80
3-Cyclopentylpropionic acid-3-pentadecyl ester	2128	nf							6.07E+06	15.17
2-Ethylhexyl salicylate	2415	nf	9.59E+06	26.55	1.03E+07	25.90	3.45E+07	30.72	4.37E+07	37.05
Organic acids										
Acetic acid	1443	1447	1.85E+07	16.57	1.29E+07	1.16	3.48E+07	23.29		
Butanoic acid	1615	1624			3.43E+06	7.44	6.40E+06	11.63		
Pentanoic acid	1736	1743			1.85E+06	15.74	2.39E+06	36.29		
Hexanoic acid	1844	1847			7.48E+06	18.96	1.05E+07	13.13		
Heptanoic acid	1943	1939					4.46E+06	47.62	2.45E+07	7.38
Decanoic acid	2269	2266					7.31E+06	18.72	4.75E+07	13.76
Undecanoic acid	2370	2365							1.04E+07	3.27
Aliphatic hydrocarbons										
1-Heptene	727	740							3.96E+05	36.38
1-Nonene	935	929							1.26E+06	17.94
Decane	1000	1000	8.13E+05	36.95	1.36E+06	40.51	1.73E+06	31.83	2.73E+06	27.98
α-Pinene	1012	1013			3.79E+06	13.92	2.15E+06	40.81		
β-Pinene	1090	1097			8.16E+05	27.26	5.89E+05	23.44		
Undecane	1100	1100							1.03E+07	19.60
3-Carene	1133	1138	2.20E+06	19.08	1.78E+06	46.59	1.33E+06	31.90	1.46E+06	25.50
Dodecane	1200	1200							3.36E+07	10.42
2,2,4,4,6,8,8-Heptamethylnonane	1290	1290	3.00E+06	27.24	4.17E+06	40.38	3.59E+06	33.54		
(*E*)-2-Tridecene	1324	1343							3.34E+06	10.59
(*Z*)-3-Tridecene	1329	1328							1.45E+06	17.77
5-Undecene	1337	nf							1.37E+07	3.26
1,1'-Oxybis-hexane	1359	nf					1.75E+06	48.34		
(*E*)-2-Tetradecene	1440	nf							6.77E+07	8.73
1,3-Dimethyl-cis-cyclopentane	1467	nf	7.12E+05	42.98	1.21E+06	38.65				
Nonyl-cyclopentane	1486	1478							1.97E+07	35.48
1-Pentadecene	1541	1550							1.98E+07	7.93
5-Butyl- 4-nonene	1558	nf							4.18E+06	16.80
6-Methyl-1-octene	1564	nf							6.47E+07	14.34

Cyclopentadecane	1580	1572		3.42E+06	17.03
(*Z*)-7-Hexadecene	1630	nf		5.82E+07	8.14
1-Hexadecene	1652	1644		1.78E+07	35.16
Cyclohexadecane	1658	nf		4.29E+07	4.47
Cyclododecene	1671	nf		3.07E+07	4.86
Heptadecane	1700	1700		7.40E+07	7.91

Abbreviation: nf, not found. [a]Compounds reported are those with peak area values higher than 500.000 units. [b]Experimental linear retention indices. [c]Linear retention indices reported in literature (NIST 2017).

From the chromatograms obtained from the analysis of VOCs in the air sampled after different times and the results showed in **Table 12,** it is clear the increase of the VOCs with the cooking time of the meat. Cooking activities can generate particulate emissions, PAHs and VOCs and their concentrations in the air are strongly related to cooking procedures, such as cooking temperatures and duration [65]. Ahn et al. [66] investigated the odor and VOCs emissions from pan frying of mackerel at three stages: raw, well-done, and charred. They found that the degree of fish frying increased both the odorants and VOCs emissions. Hildemann et al. [67] indicated that also the fine particle emission rate per unit of meat charbroiled increased proportionally to the cooking time of the hamburger on the grill. The differences between VOCs detected at different sampling times are strictly related to the cooking time and the temperature reached during the process.

The figure below (**Figure 28**) shows the evolution of the different classes of detected VOCs during the cooking process.

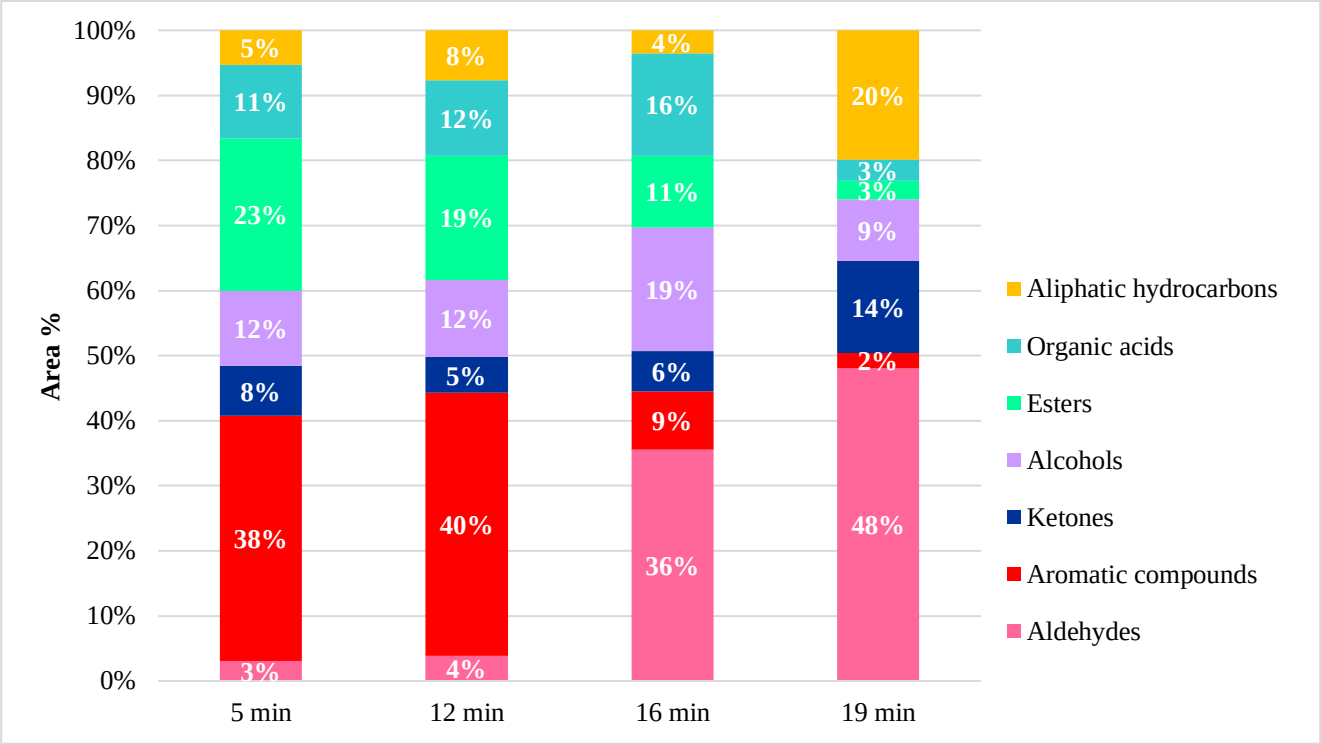


Figure 28. Percentage composition of the different classes of VOCs in the air sampled at different time during the grilling of a hamburger.

The graphs below (**Figures 29, 30, 31, 32, 33, 34** and **35**) represent the change of the percentage of each class of compounds during the cooking of hamburger (5,12,16 and 19 minutes).

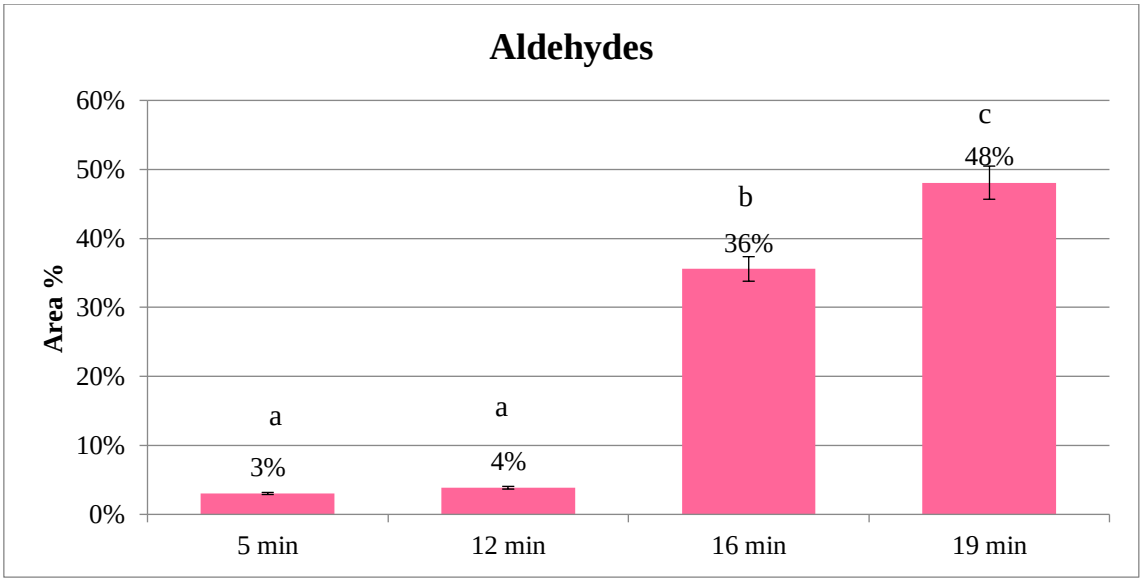


Figure 29. Percentage area of the aldehydes after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between aldehydes concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

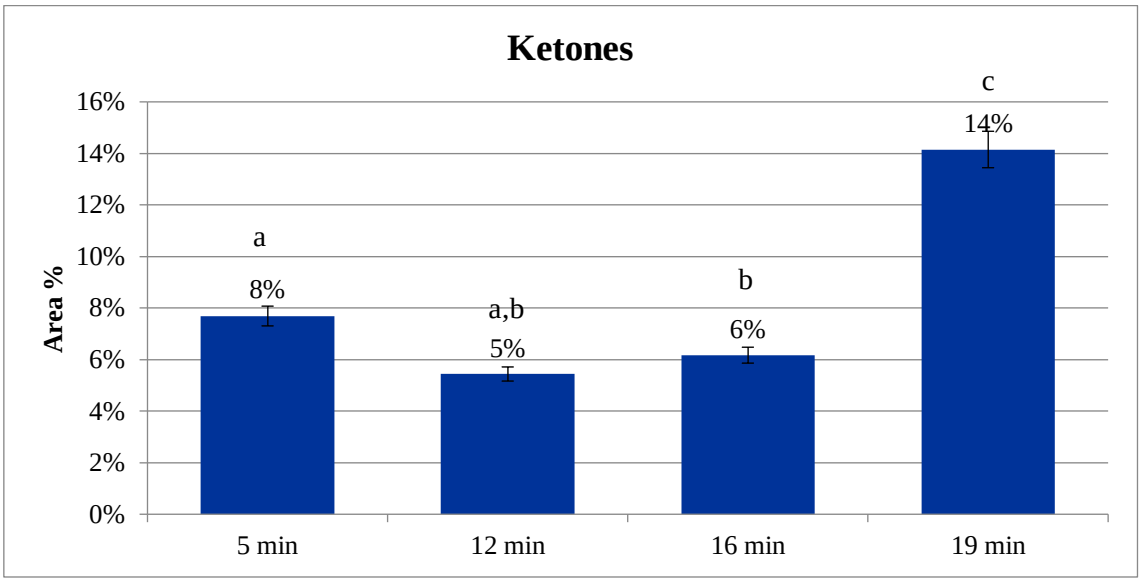


Figure 30. Percentage area of the ketones after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between ketones concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

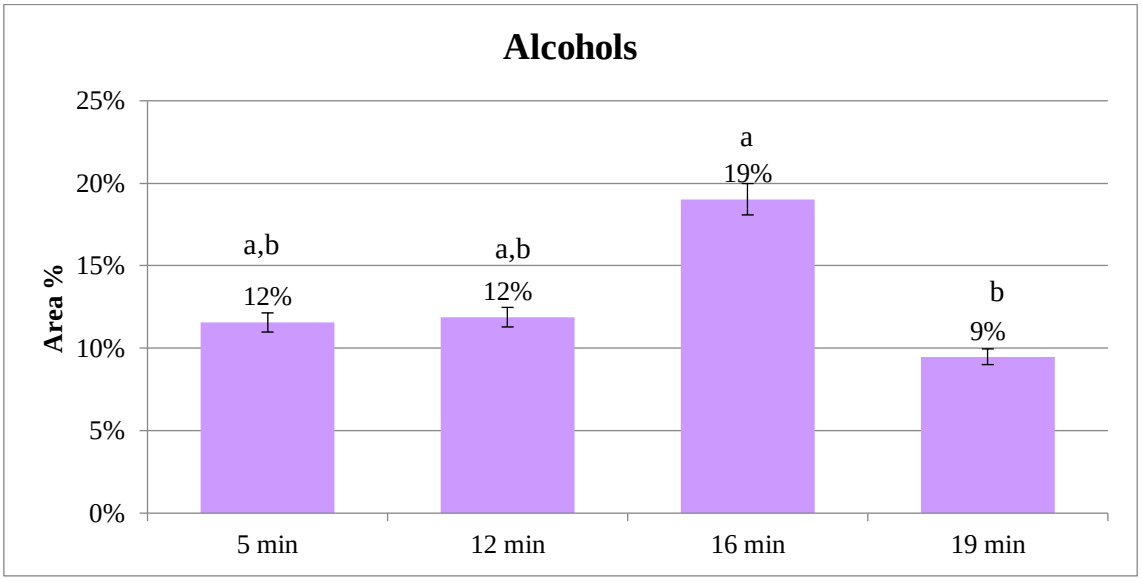


Figure 31. Percentage area of the alcohols after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between alcohols concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

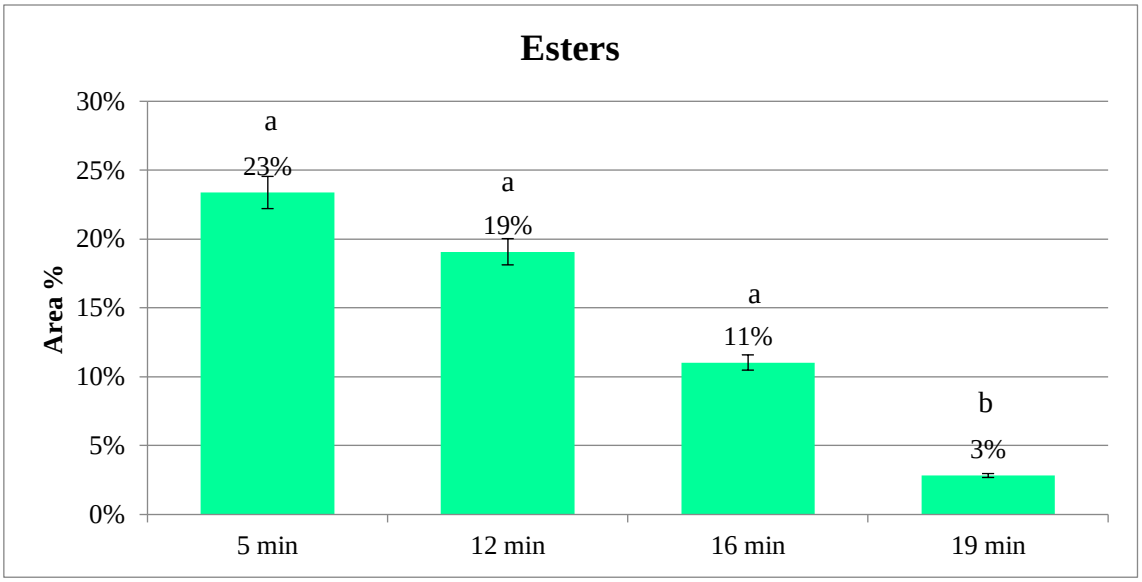


Figure 32. Percentage area of the esters after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between esters concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

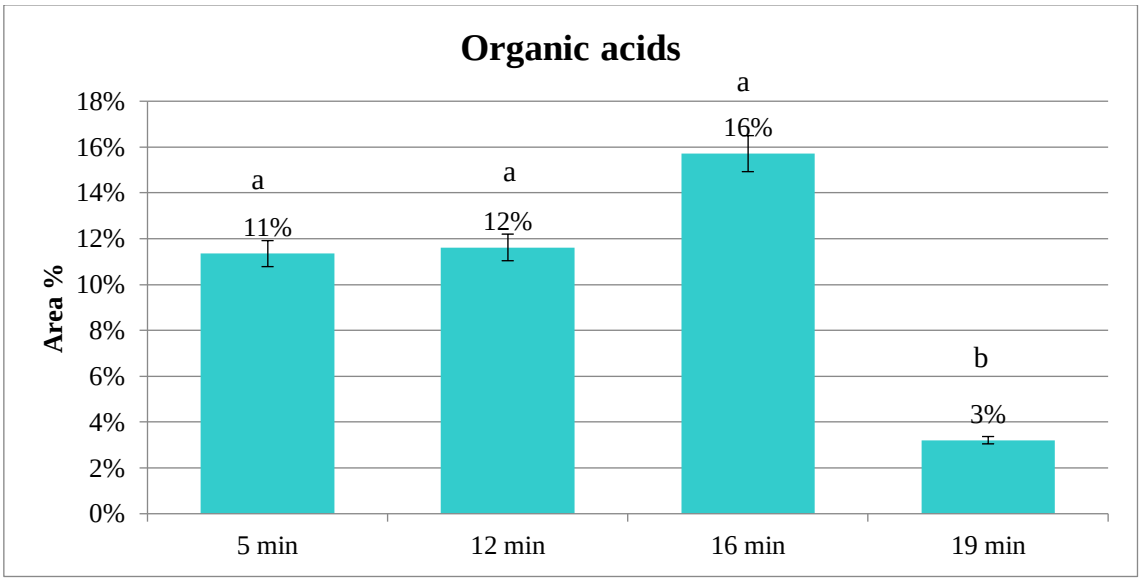


Figure 33. Percentage area of the organic acids after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between organic acids concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

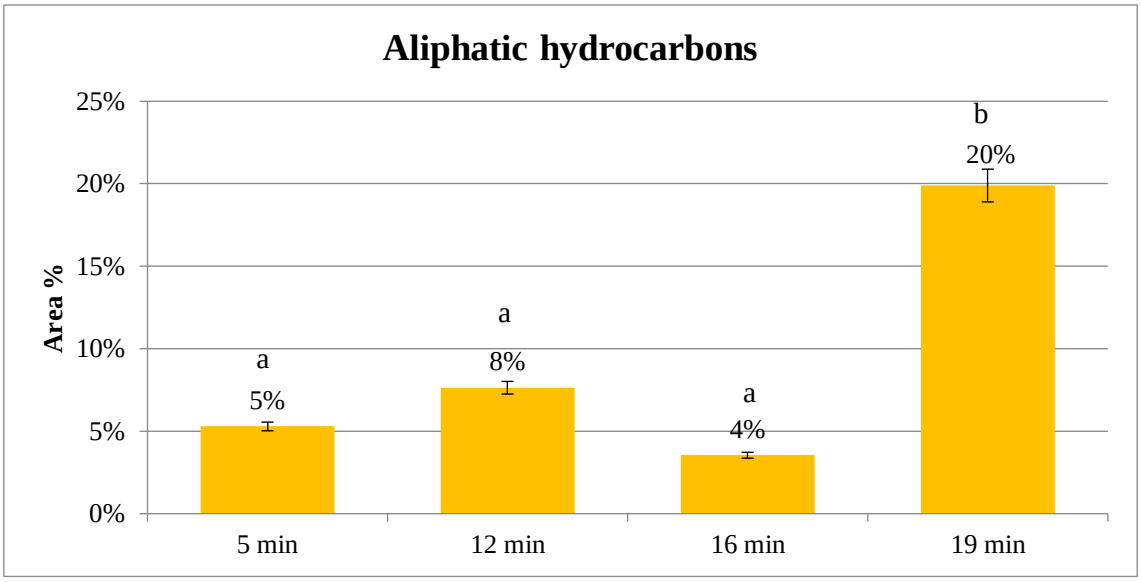


Figure 34. Percentage area of the aliphatic hydrocarbons after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between aliphatic hydrocarbons concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

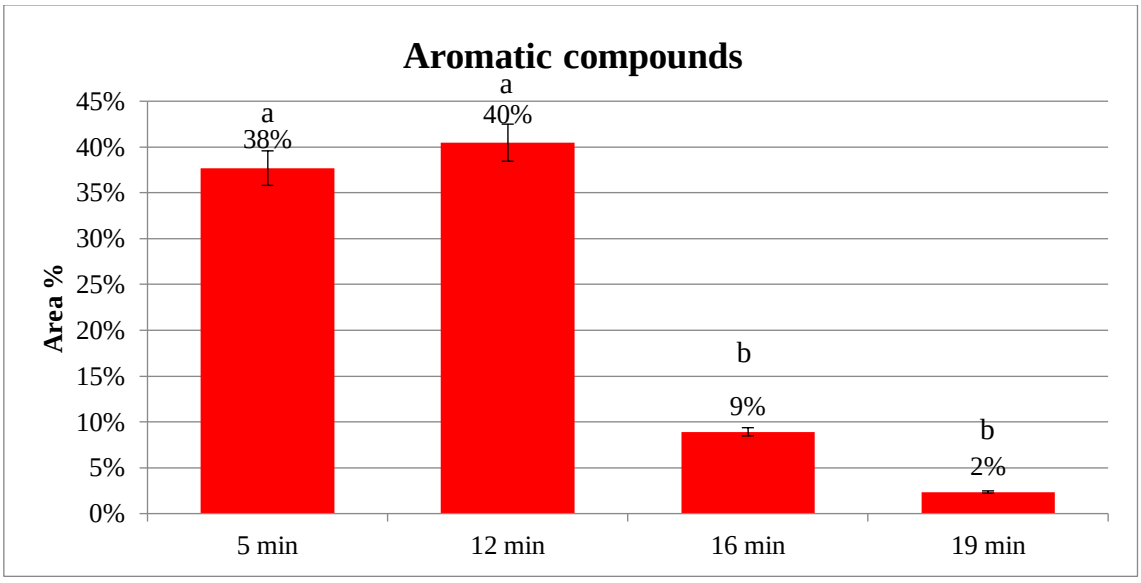


Figure 35. Percentage area of the aromatic hydrocarbons after 5, 12, 16 and 19 minutes of cooking. Bars indicated standard deviations. Different letters indicate significant differences between aromatic compounds concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

The class of the aldehydes was the most abundant and they increased during the cooking process, reaching the 48% of the total VOCs after 19 minutes of cooking of the meat. Two examples of specific molecules (hexanal and heptanal) are reported below (**Figure 36** and **37**).

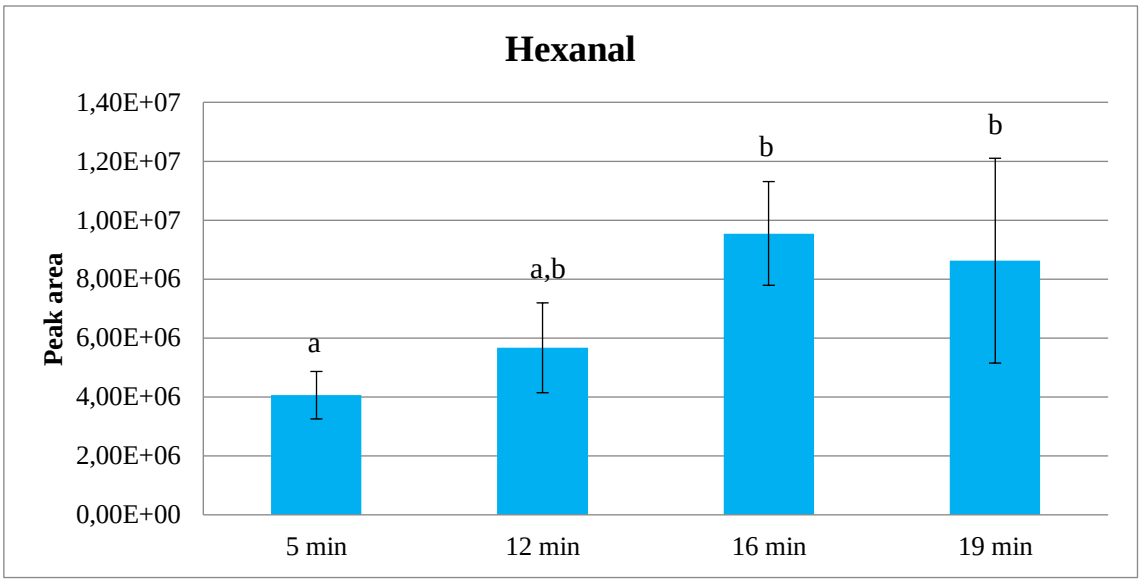


Figure 36. Concentration of the hexanal after 5,12,16 and 19 minutes of cooking of the hamburger. Bars indicated standard deviations. Different letters indicate significant differences between hexanal concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

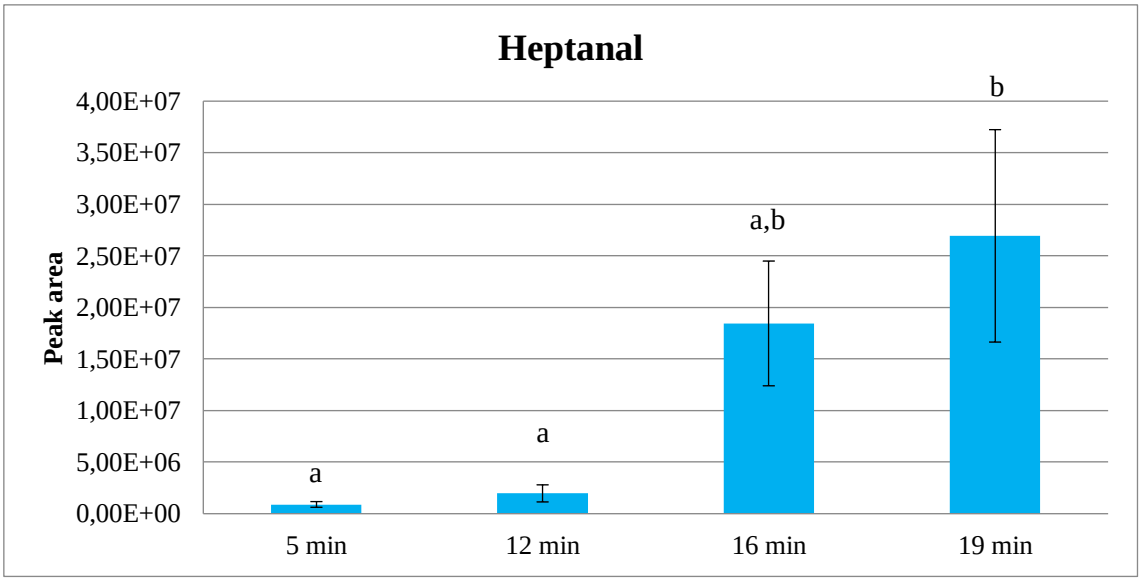


Figure 37. Concentration of the heptanal after 5,12,16 and 19 minutes of cooking of the hamburger. Bars indicated standard deviations. Different letters indicate significant differences

between heptanal concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

In both cases there is a high increasement of their absolute areas increasing the cooking time. In particular, hexanal, after 12 minutes of cooking, which was considered the ideal cooking time of the hamburger, represented the 66% of the total aldehydes, being the most abundant one.

Some carbonyl compounds are known to be toxic compounds derived from incomplete combustion, because they can be precursors of free radicals, peroxyacyl nitrates (PANs) or ozone. Some previous studies [68, 69] reported low molecular weight aldehydes (formaldehyde and acetaldehyde) as the most abundant carbonyl compounds from cooking processes, followed by hexanal, butanal and propanal. Formation of aldehydes may be due to the oxidation of the meat lipids. High temperatures used for grilling favour the rapid oxidation of the unsaturated and polyunsaturated fatty acids, increasing the number of free radicals in the middle. These radicals attack other less susceptible fatty acids, such as oleic acid, leading to the formation of aldehydes and ketones [69]. Aldehydes were reported as the most abundant volatile compounds in cooked foal meat by Dominguez et al. [62], with hexanal representing between 87.3-90.8 % of the total aldehydes. Other studies indicated hexanal as the major compound in cooked meat samples from goat [70], lamb [63], pork [71] and beef [72]. The high presence of hexanal can be attributed to the high number of its synthetic pathways. It can be formed from oxidation of oleic, linoleic and arachidonic acids and through the degradation of other unsaturated aldehydes (e.g. 2,4-decadienal) [62]. When the hamburger was overcooked (after 19 minutes), also the heavier aldehydes reached high concentrations. For example, nonanal, with its fatty and grassy odor, was found at high levels in cooked beef meat also in other studies [73]. Also Schauer [74] reported significant concentrations of heavier aliphatic aldehydes in the meat cooking exhaust, such as nonanal, 2-decenal and 2-undecenal, which were found also in our samples. A study conducted by Grosjean et al. [75] showed that food preparation could be a great contributor to the high molecular weight aldehydes in the air and that their chemical reactivity as a group was very similar to that of acetaldehyde and formaldehyde separately. The lipid thermal oxidation could bring also to the formation of other carbonyl compounds, such as ketones. In fact, in this study their concentration increased significantly with cooking time, reaching the 14% of the total VOCs at the end of the cooking process. In particular, the high-carbon-number ketones (with more than 9 carbons) are the most abundant ones after 19 minutes of cooking. Ramìrez et al. [71] indicated that ketones with small number of carbons were in lower amounts in fries loin

samples respect to the heavier ketones. Ketones, especially 2-ketones, are considered to have great influence in the overall aroma of meat [62]. The grilling of hamburger generated also high levels of aliphatic hydrocarbons after 19 minutes (20% of the total VOCs); at this sampling time they showed significant differences with respect to the other times of cooking. High emissions of aliphatic hydrocarbons, in particular alkanes, during meat grilling and charbroiling were reported also in other studies [24, 76]. Their high concentrations can be spelt out by the incomplete combustion of grease and meat [77]. Alcohols are another consistent group of VOCs detected in our samples. In particular, at the latest stages of the cooking process (16 and 19 minutes) they represent 19% and 9%, respectively. The behaviour of alcohols during cooking process is interesting with the shortest alcohols decreasing with time, and the heaviest ones showing opposite trend. **Figures 38** and **39** show an example for alcohols differing in carbon chain length, i.e., 1-pentanol and 1-octanol.

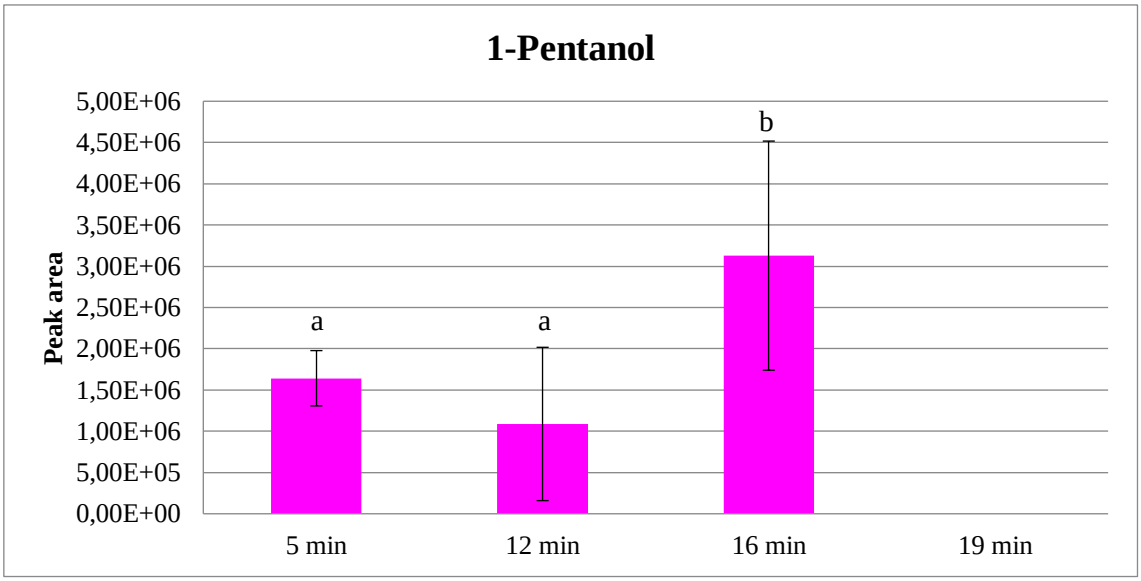


Figure 38. Concentration of 1-pentanol after 5,12,16 and 19 minutes of cooking of hamburger. Bars indicated standard deviations. Different letters indicate significant differences between 1-pentanol concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

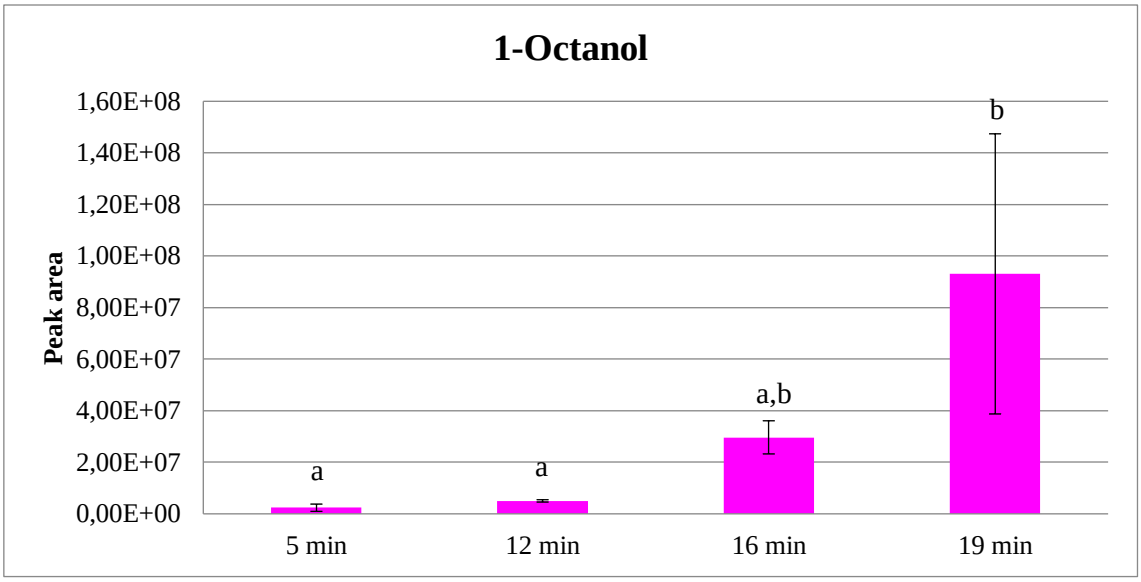


Figure 39. Concentration of 1-octanol after 5,12,16 and 19 minutes of cooking of hamburger. Bars indicated standard deviations. Different letters indicate significant differences between 1-octanol concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

This trend could be explained by the fact that the heaviest compounds are produced during the cooking process because of the thermal treatment, while the lightest alcohols could be degraded or could disperse because of their high volatility after 19 minutes of cooking. The development of alcohols during the cooking process are in accordance with literature. Domìnguez [62] found 1-pentanol, 1-hexanol and 1-octen-3-ol only in the cooked foal meat; Ramìrez [71] indicated an increase of the alcohols from the pork meat only with the heat treatment. As for the hydrocarbons, alcohols did not contribute to meat aroma, because of their high odor thresholds [62]. Organic acids showed a trend similar to the alcohols. Short chain fatty acids (having from 2 to 6 carbons) developed during cooking time, but they were not detected after 19 minutes of cooking, while the heaviest ones (heptanoic, decanoic and undecanoic acids) were found only at the latest stages of cooking (16 and 19 minutes). The acetic and decanoic acids (**Figures 40** and **41**) are presented as examples of this behaviour.

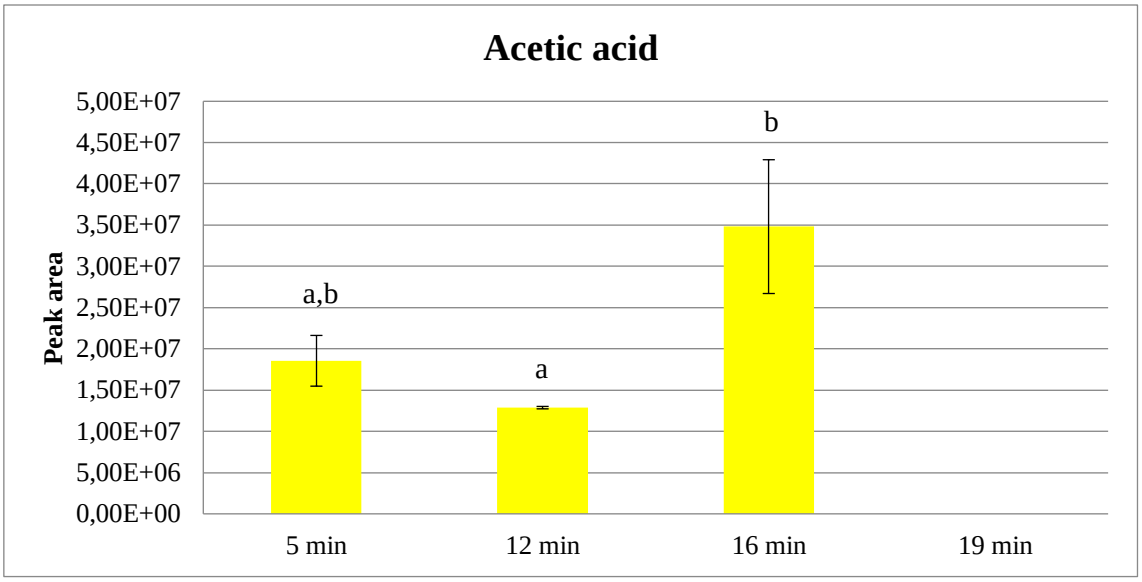


Figure 40. Concentration of acetic acid after 5,12,16 and 19 minutes of cooking of hamburger. Bars indicated standard deviations. Different letters indicate significant differences between acetic acid concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

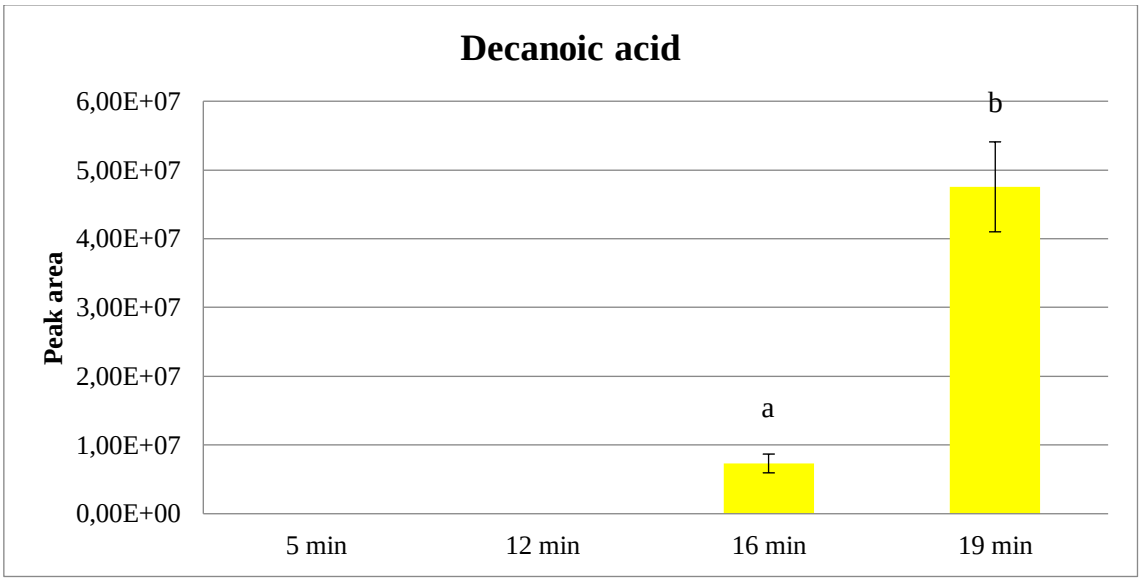


Figure 41. Concentration of decanoic acid after 5,12,16 and 19 minutes of cooking of hamburger. Bars indicated standard deviations. Different letters indicate significant differences between decanoic acid concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

The absence of short chain fatty acids at the end of the cooking processes could be due to their high volatility, while the heaviest ones are formed during the meat cooking. Organic acids are important constituents of the air sampled after 12 and 16 minutes and they are present in high concentration in the emissions from meat cooking also in previous studies [65,74]. High temperatures reached during cooking are responsible for the degradation of lipids in meat, which results in the production of free fatty acids, free glycerol and mono- and diglycerides. Among the organic acids *n*-alkanoic acid were generally the most abundant ones in the meat emissions [65], in accordance with the results of this study. Esters accounted for 23% of the total VOCs after 5 minutes of cooking and their concentrations decreased significantly with the cooking time, reaching 3% after 19 minutes. Esters arise from the esterification of some carboxylic acids and alcohols in meat. Domìnguez et al. [62] observed the highest amount of esters in raw foal meat and they highly decreased in the roasted meat samples. This suggests that thermal treatment could degrade this class of compounds. Regarding the aromatic compounds, their total concentration decreased during time, even if two different behaviours of these compounds were observed. Aromatic hydrocarbons such as benzene, toluene, ethylbenzene or xylenes tended to decrease in the air sampled after 19 minutes (**Figure 42**), because of their possible degradation. Another possible reason of the decrease of some classes of VOCs (esters or aromatic compounds) is the possible displacement effect of the DVB/CAR/PDMS fiber. Indeed, a disadvantage of this coating material is displacement effect of analytes with a lower affinity to the coating [78].

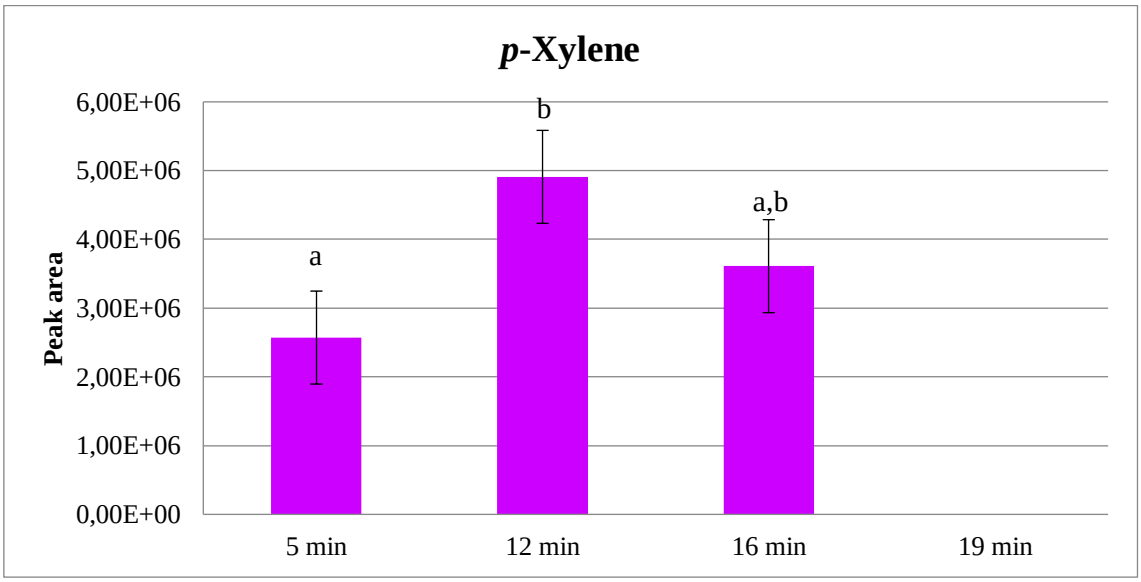


Figure 42. Concentration of *p*-xylene after 5,12,16 and 19 minutes of cooking of the hamburger. Bars indicated standard deviations. Different letters indicate significant differences

between *p*-xylene concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

However benzene, toluene, ethylbenzene and xylenes (BTEX) were found in considerable concentration at the early times of cooking. They are considered as HAPs (hazardous air pollutants) by US EPA [65] and they were found at significant levels during commercial cooking processes and in restaurant emissions [74, 79]. For this reason, it is important to apply control technologies to reduce their emissions and condensation.

On the contrary, the nitrogen-containing aromatic compounds tended to increase at the end of the meat cooking. The case of 2,5-dimethylpyrazine is reported in **Figure 43** as example.

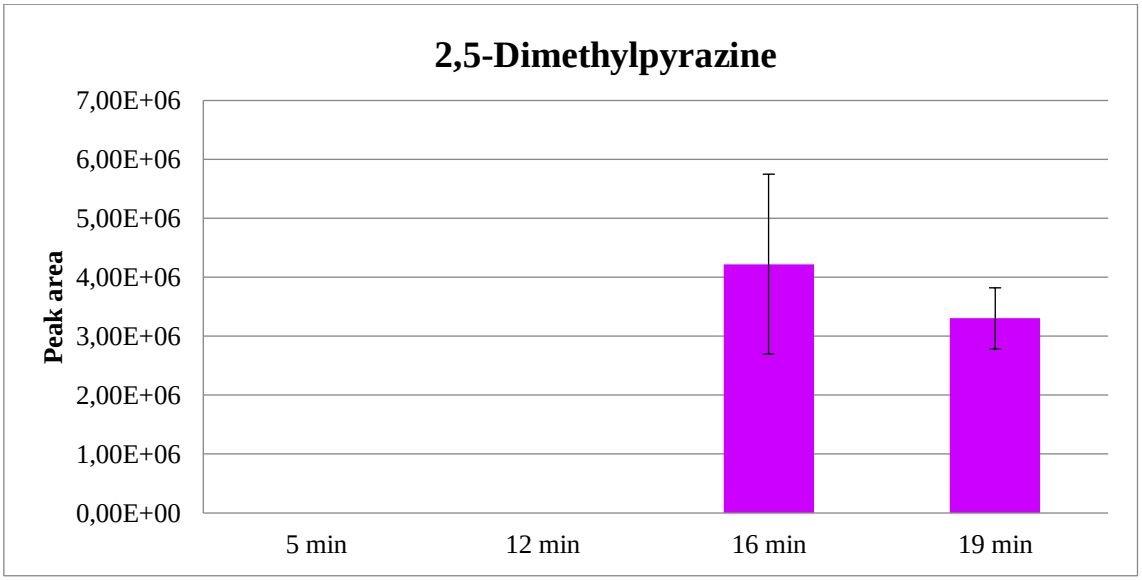


Figure 43. Concentration of 2,5-dimethylpyrazine after 5,12,16 and 19 minutes of cooking of the hamburger. Bars indicated standard deviations.

Pyrazines were formed at the end of the cooking process because the high temperature and the long cooking time favoured the Maillard reaction, that occurs between reducing sugars and free amino acids. They are very aromatic compounds, which confer pleasant odor notes such as roasted and caramel-like with low odor threshold. In particular, 2,5-dimethylpyrazine is known for its burned meat aroma [80].

1.3.1.3 Boiling of cauliflower and heating of sunflower oil

The last investigated cooking model system is the boiling of cauliflower and the heating of sunflower oil. In particular the analysis was performed using three different models: the boiling of cauliflower, the heating of sunflower oil at 120 °C and the combination of these two (**Figure 44**).

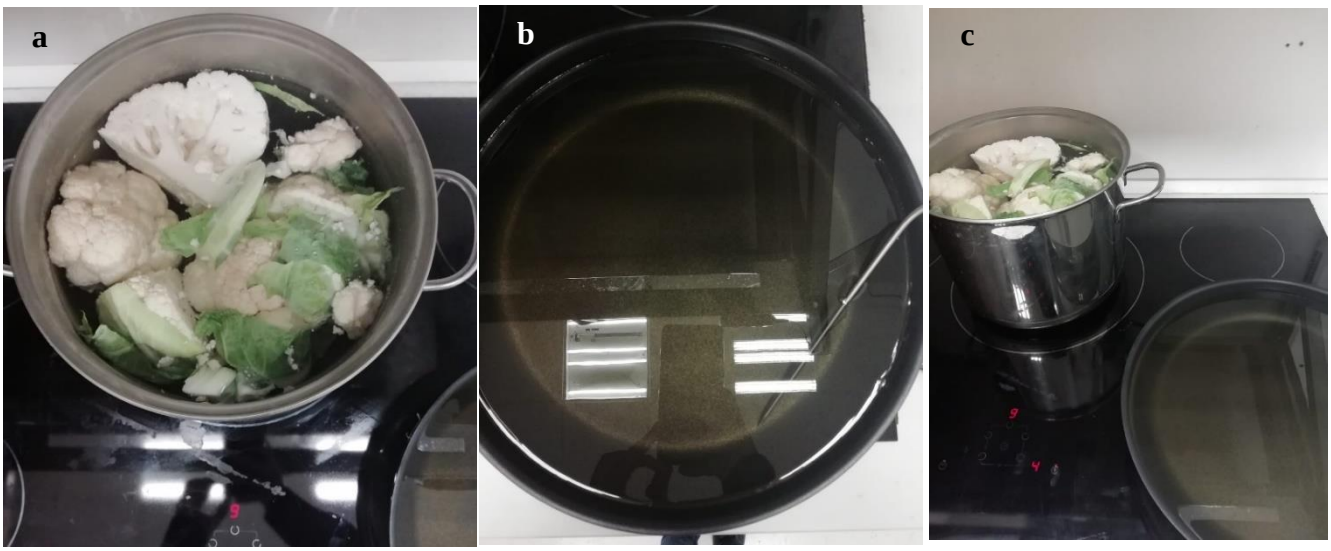


Figure 44. Cooking model systems used for the study of VOCs development from: boiling of cauliflowers (a), heating of sunflower oil at 120 °C (b) and a combination of these two (c).

Boiling of cauliflower was chosen because of its typical and distinctive aroma, while sunflower oil is one of the edible oils most used for cooking or frying in domestic kitchens in Western countries. They were analysed individually to investigate the contribution of each cooking system to the total VOCs and in combination to simulate a possible condition in domestic kitchens. **Figures 45, 46** and **47** show the chromatograms obtained from the air sampled during the boiling of the cauliflower, the heating of the oil and the combination of the two models, respectively.

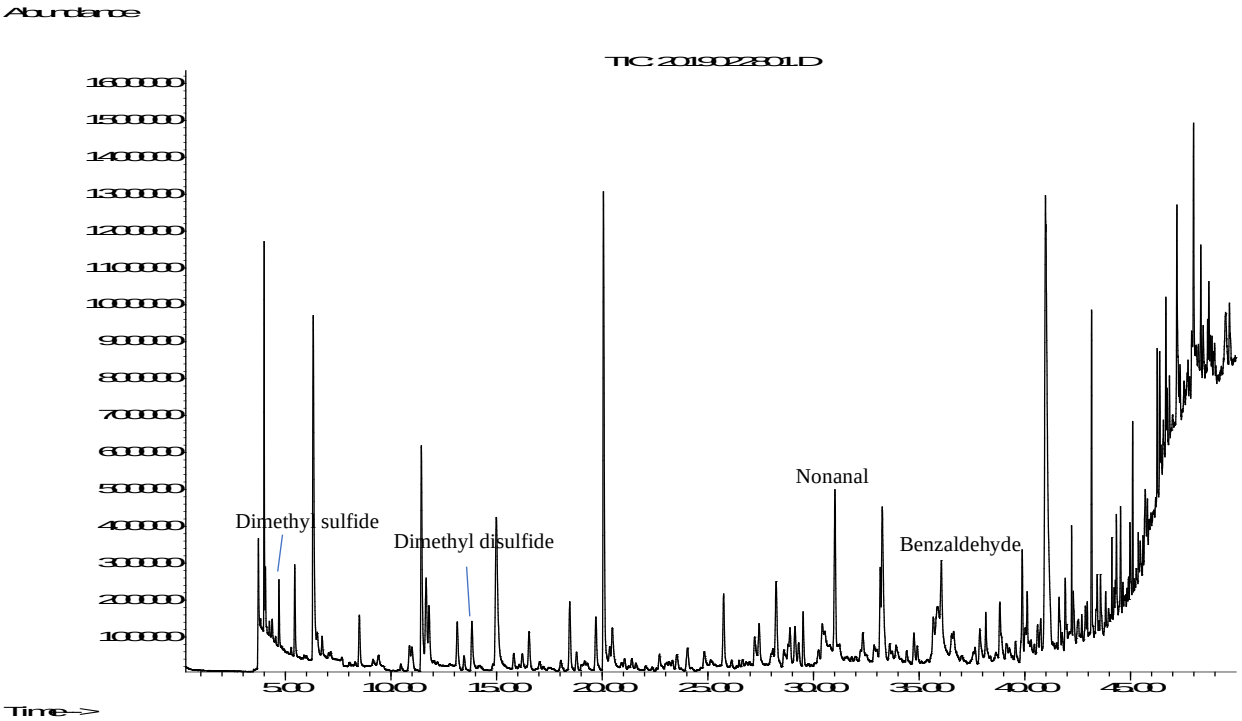


Figure 45. Chromatogram obtained from the analysis of VOCs in the air sampled after the boiling of cauliflower.

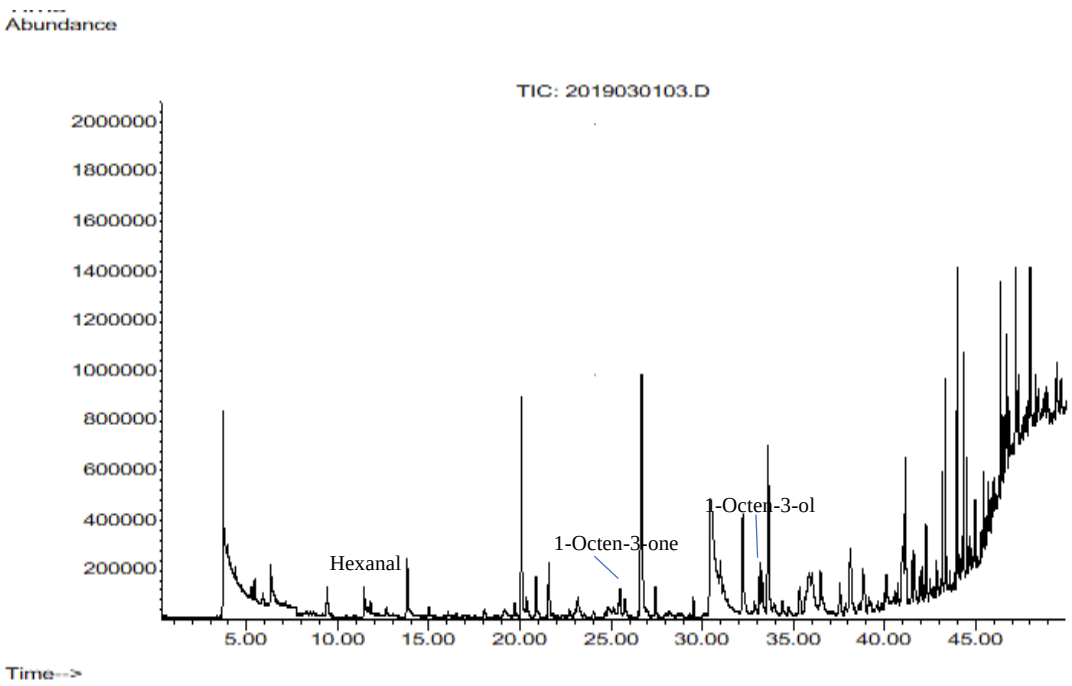


Figure 46. Chromatogram obtained from the analysis of VOCs in the air sampled after heating the sunflower oil.

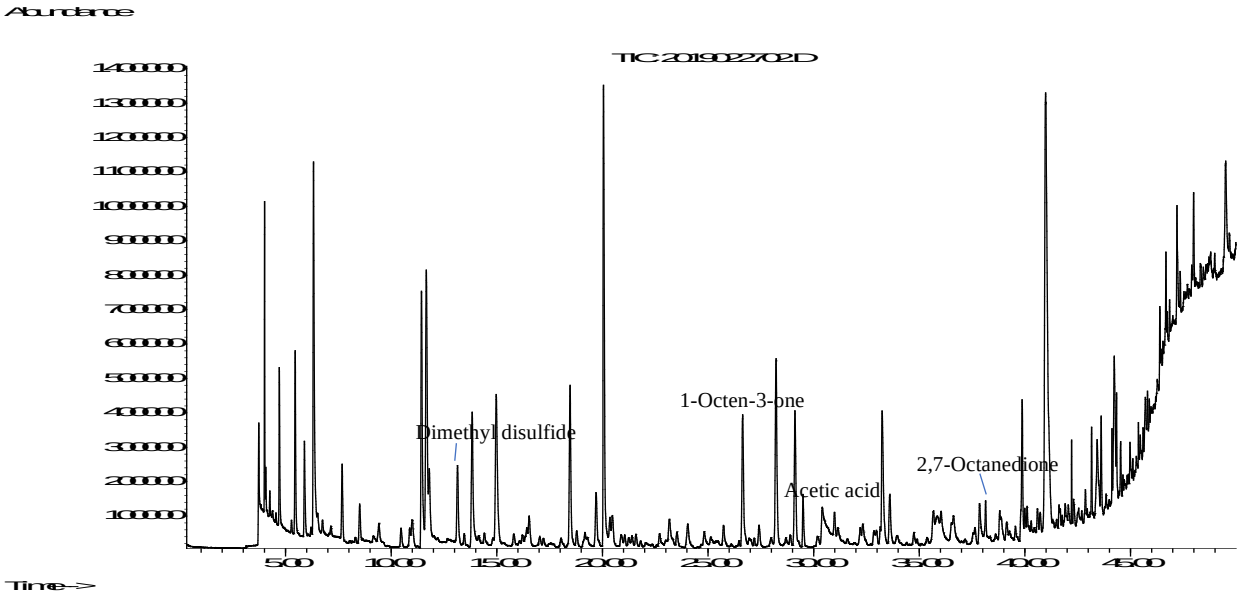


Figure 47. Chromatogram obtained after the anaysis of the VOCs in the air sampled after the boiling of cauliflower simultaneously with sunflower oil heating at 120°C.

The analytes were classified again according to their chemical nature into: aldehydes, ketones, alcohols, aromatic hydrocarbons, organic acids, ketones, aromatic compounds, aliphatic hydrocarbons and into other compounds. The results are shown in **Table 13**.

Table 13. Volatile compounds developed from boiling of cauliflower, heating of sunflower oil at 120 °C and a combination of the two processes detected by HS-SPME-GC-MS, their experimental linear retention indices (LRI) on a DB-WAX column and values reported in literature on DB-WAX columns. Their abundances are reported in terms of peak areas and% relative standard deviation (RSD, *n*=4).

			Boiling of cauliflower		**Heating of sunflower oil**		**Boiling of cauliflower + heating of sunflower oil**	
Compound detected[a]	**LRI[b] (exptl)**	**LRI[c] (lit)**	**Area**	**RSD%**	**Area**	**RSD%**	**Area**	**RSD%**
Aldehydes								
Pentanal	968	967			3.17E+07	33.20	2.82E+06	33.32
2-Butenal	1028	1030	9.18E+06	9.64	6.61E+06	45.55	1.44E+07	23.36
Hexanal	1071	1080	6.81E+06	12.73	1.36E+08	42.72	1.81E+07	39.43
Heptanal	1177	1180			9.07E+06	26.13		
2-Hexenal	1210	1215			2.80E+07	25.96	1.88E+06	18.47
Octanal	1283	1291	3.30E+06	21.48	5.13E+06	35.88	3.80E+06	42.23
2-Heptenal	1317	1318	6.60E+05	25.46	2.43E+08	28.15	1.62E+07	41.49
Nonanal	1389	1385	9.48E+06	39.59	1.05E+08	31.04	1.89E+07	48.51
2,4-Heptadienal	1487	1497			1.61E+07	0.00	1.09E+06	6.76

Decanal	1497	1497	1.36E+07	29.57	1.91E+07	19.27	1.67E+07	23.57
Benzaldehyde	1515	1515	9.31E+06	35.79	1.21E+07	30.20	1.27E+07	35.56
4-Ethylbenzaldehyde	1737	1730	2.40E+06	19.86	5.36E+06	10.62	3.12E+06	33.70
Vinylbenzaldehyde	1835	nf	1.66E+06	33.35	1.80E+06	16.45	3.06E+06	76.55
Trans-cynnamaldehyde	1870	1872	1.82E+06	19.05	1.86E+06	11.32	1.91E+06	38.55
Lilial	2064	nf	2.67E+06		6.27E+06	15.27	1.77E+06	33.68
Aromatic compounds								
Toluene	1026	1021	1.32E+07	31.34	6.97E+06	24.74	5.85E+07	37.69
Ethylbenzene	1112	1125	2.32E+06	28.53	1.78E+06	25.32	1.85E+06	17.17
p-Xylene	1120	1125	1.61E+06	32.74	5.15E+05	11.62	8.49E+05	28.32
o-Xylene	1166	1163	5.61E+06	30.56	2.78E+06	9.74	4.08E+06	23.05
1,3-Dimethylbenzene	1170	1161	2.98E+06	34.89	1.74E+06	38.72	2.28E+06	9.85
1-Ethyl-3-methylbenzene	1213	1222	1.04E+06	28.56			1.32E+06	11.85
1-Ethyl-2-methylbenzene	1216	1229	9.03E+05	33.68			1.54E+06	24.45
3-Methylisothiazole	1217	nf	5.63E+05	11.15			8.49E+05	48.58
(E)-2-[(N-hydroxy-N-phenyl)-amino]-3-[N-(phenylimino)]-indole	1220	nf	1.80E+06	33.30			2.40E+06	31.91
1,3,5-Trimethylbenzene	1249	1253	1.46E+06	41.32			9.16E+05	11.91
Styrene	1255	1255	1.73E+06	24.01	2.72E+06	23.80	1.98E+06	21.16
2-Pentylfuran	1223	1228	6.96E+05	23.45	2.05E+07	16.22	2.11E+06	4.73
1-Methyl-3-(1-methylethyl)-benzene	1259	1263	7.62E+07	39.19	2.23E+07	32.18	2.42E+06	31.40
1,2,4-Trimethylbenzene	1269	1272			4.42E+06	32.69	4.98E+06	23.94
1,2,3-Trimethylbenzene	1332	1342	1.64E+06	30.77	3.98E+06	17.18	1.24E+06	32.33
1-Ethenyl-3-ethylbenzene	1427	1424	5.62E+06	11.41			4.38E+06	35.93
1-Ethenyl-4-ethylbenzene	1439	1424	3.29E+06	7.86	4.98E+06	23.63	2.85E+06	25.40
Diethenylbenzene	1579	nf	9.17E+05	38.38	7.40E+05	24.44	2.37E+06	1.58
9-Propylanthracene	2096	nf	6.60E+06	28.82	1.09E+07	14.87	5.57E+06	14.43
Ketones								
Acetone	807	814	1.02E+07	30.62	6.18E+06	32.88	2.00E+07	26.85
3-Octanone	1251	1252			2.97E+06	7.74		
2-Octanone	1281	1278			1.29E+06	18.43		
1-Octen-3-one	1296	1300			1.44E+07	17.43		
6-Methyl-5-hepten-2-one	1332	1336	1.31E+07	23.04	1.15E+07	23.19	3.26E+06	3.57
2-Amino-5-ethoxycarbonylbenzophenone	1545	nf	4.35E+06	36.91	5.32E+06	17.80	6.83E+06	3.18
2,7-Octanedione	1552	nf			4.65E+07	22.65	5.13E+06	39.02
Tetrahydro-2H-pyran-2-one	1811	nf	1.07E+06	15.60			1.67E+06	37.12
6-Methyl-γ-ionone	1861	nf	8.32E+06	30.89	8.29E+06	36.98	3.23E+06	17.56

Alcohols								
1-Butanol	1145	1148	2.19E+06	15.2	2.59E+06	36.5	2.13E+06	13.14
1-Pentanol	1252	1249	2.08E+06	19.4	3.70E+07	29.4	5.83E+06	38.3
1-Butoxy-2-propanol	1343	nf					1.82E+06	24.0
4-Methyl-1-pentanol	1356	1347	1.31E+06	36.8	4.70E+06	22.4	1.75E+06	29.7
2-Butoxy-ethanol	1389	1391	2.46E+06	31.5			4.69E+06	11.7
1-Octen-3-ol	1453	1451	1.75E+06	13.6	9.29E+07	22.3	9.27E+06	19.8
2,6-Dimethyl-7-octen-2-ol	1458	1450	4.30E+06	23.9	1.94E+06	5.3	1.59E+06	17.4
2-Ethyl-1-hexanol	1493	1491	6.66E+06	17.0	7.63E+06	8.6	6.05E+06	15.4
2-(1,1-Dimethylethyl)-cyclohexanol	1525	nf			6.50E+06	11.6	3.34E+06	22.6
1-Octanol	1570	1566			1.27E+07	22.1	6.03E+06	33.8
2-(2-Ethoxyethoxy)-ethanol	1629	1622	1.64E+06	24.8	3.89E+06	29.8	3.91E+06	40.3
1,2-Ethanediol	1640	1635	1.05E+08	26.4	8.75E+07	36.2	1.14E+08	23.2
2-(Ethenyloxy)-ethanol	1939	nf	1.43E+06	50.2	9.49E+05	31.1	1.36E+06	17.5
Esters								
Ethyl acetate	876	884	2.85E+06	45.05	1.62E+06	15.10	2.97E+06	20.93
Butyl acetate	1064	1070	1.82E+06	17.69			1.40E+06	25.46
Isobornyl acetate	1587	1584	1.58E+06	21.23	1.33E+06	12.87	1.54E+06	29.14
Benzyl acetate	1726	1726	1.68E+06	24.29			8.39E+05	15.75
Methyl salicylate	1774	1779	4.95E+06	24.51	2.96E+06	44.14	6.74E+06	24.27
Isopropyl myristate	2044	nf	1.05E+07	10.96	4.22E+06	30.36	4.90E+06	23.65
Dihydro methyl jasmonate	2406	nf	1.70E+07	32.03	6.89E+06	31.83	2.68E+06	25.46
Diethyl phtalate	2449	nf	1.95E+06	38.28			5.30E+06	15.25
Organic acids								
5-Hydroxy-1H-indole-3-carboxylic acid	1095	nf	2.42E+07	35.71	3.04E+07	16.78	1.17E+07	10.65
Acetic acid	1445	1447	2.93E+07	23.36	2.20E+07	18.58	2.34E+07	28.41
Propanoic acid	1539	1536	2.83E+06	45.56			4.31E+06	5.67
Hexanoic acid	1844	1850	8.15E+06	32.36	3.68E+07	25.79	5.63E+06	12.12
Octanoic acid	2055	2050	2.05E+07	17.69	1.33E+07	34.98	6.77E+06	33.20
Nonanoic acid	2164	2157	1.38E+07	36.96	2.89E+07	25.41	1.24E+07	30.63
Decanoic acid	2271	2278	1.06E+07	22.08	3.13E+07	64.34	1.07E+07	32.97
Aliphatic hydrocarbons								
Pentane	500	500			2.82E+06	17.06		
Hexane	600	600	3.38E+07	37.52	6.35E+06	22.45	4.81E+07	34.50
Octane	800	800			8.46E+06	37.84	1.36E+06	40.40
2-Octene	848	847			1.04E+06	26.64	8.41E+05	13.15
1,1,1-Trichloroethane	864	885	2.25E+06	31.40			3.27E+06	18.91

Methylene chloride	914	925	1.40E+06	25.83			7.06E+06	21.28
Decane	1000	1000	3.25E+06	12.69	9.58E+05	9.41	1.87E+06	26.04
Trichloromethane	1009	1010	7.80E+06	23.87	2.10E+06	10.05	4.65E+06	20.96
α-Pinene	1011	1015	1.46E+06	22.93	1.82E+06		9.10E+06	32.95
β-Pinene	1092	1091	1.33E+06	18.59	1.19E+06	20.27	1.00E+06	35.17
Undecane	1100	1100			3.91E+06	29.55		
3-Carene	1136	1142					1.60E+06	8.75
2-Butenenitrile	1159	1162					8.47E+05	9.78
3-Butenenitrile	1174	1186	7.97E+06	15.95			2.13E+07	9.78
D-Limonene	1188	1193	1.71E+07	26.38	9.51E+06	14.25	7.36E+06	21.67
Dodecane	1200	1200	2.22E+06	37.24	2.32E+06	28.37	3.50E+06	11.59
2,2,4,4,6,8,8-Heptamethylnonane	1289	nf	4.73E+06	15.16	1.97E+06	26.91	1.38E+06	23.45
Tridecane	1300	1300	2.96E+06	31.53	3.75E+06	53.40	2.79E+06	47.91
Tetradecane	1400	1400	8.77E+06	13.61	1.54E+07	40.17	4.66E+06	24.81
Pentadecane	1500	1500	1.29E+07	33.95	1.41E+07	14.82	9.92E+06	21.02
Hexadecane	1600	1600	4.97E+06	14.18	2.19E+06	24.29	3.19E+06	41.84
2-Tetradecene	1669	nf	5.57E+06	41.34	6.65E+06	11.04	4.75E+06	37.82
Heptadecane	1700	1700	5.26E+06	19.75	8.40E+06	22.13	4.92E+06	11.23
1,1'-Oxybis-octane	1756	1763	3.37E+07	37.71	9.82E+06	16.04	4.35E+06	28.67
4-(Methylthio)-butanenitrile	1786	1806	2.17E+06	27.74			7.29E+06	45.95
Octadecane	1800	1800	2.16E+06		8.26E+05	6.10	2.33E+06	6.67
Nonadecane	1900	1900	7.00E+06	36.49			3.71E+06	11.81
Other compounds								
Ethyl ether	615	616	1.12E+07	33.55	2.38E+06	18.64	3.01E+06	33.34
Thiourea	713	nf	4.56E+05				1.02E+06	47.66
Dimethyl sulfide	731	740	6.66E+06	42.64			1.34E+07	25.58
N-ethyl-1,3-dithioisoindoline	876	nf	1.44E+06	35.42			2.16E+06	13.16
2-Methyl-1,3-dioxolane	940	953	9.43E+06	33.80	2.68E+06	29.34	1.04E+07	35.79
Dimethyl disulfide	1057	1061	7.43E+06	22.30				
Dimethyl trisulfide	1364	1376	3.93E+06	25.87				
Dimethyl sulfoxide	1594	1582	1.18E+07	25.35	2.72E+06	15.28	1.74E+07	22.91
Butyrolactone	1622	1623	3.99E+06	20.41	4.04E+06	34.26	1.11E+07	

Abbreviation: nf, not found. [a]Compounds reported are those with peak area values higher than 500.000 units. [b]Experimental linear retention indices. [c]Linear retention indices reported in literature (NIST 2017).

The percentage compositions of each class of compounds in the air sampled from the three investigated cooking models were calculated, and the results are shown in **Figures 48, 49** and **50**.

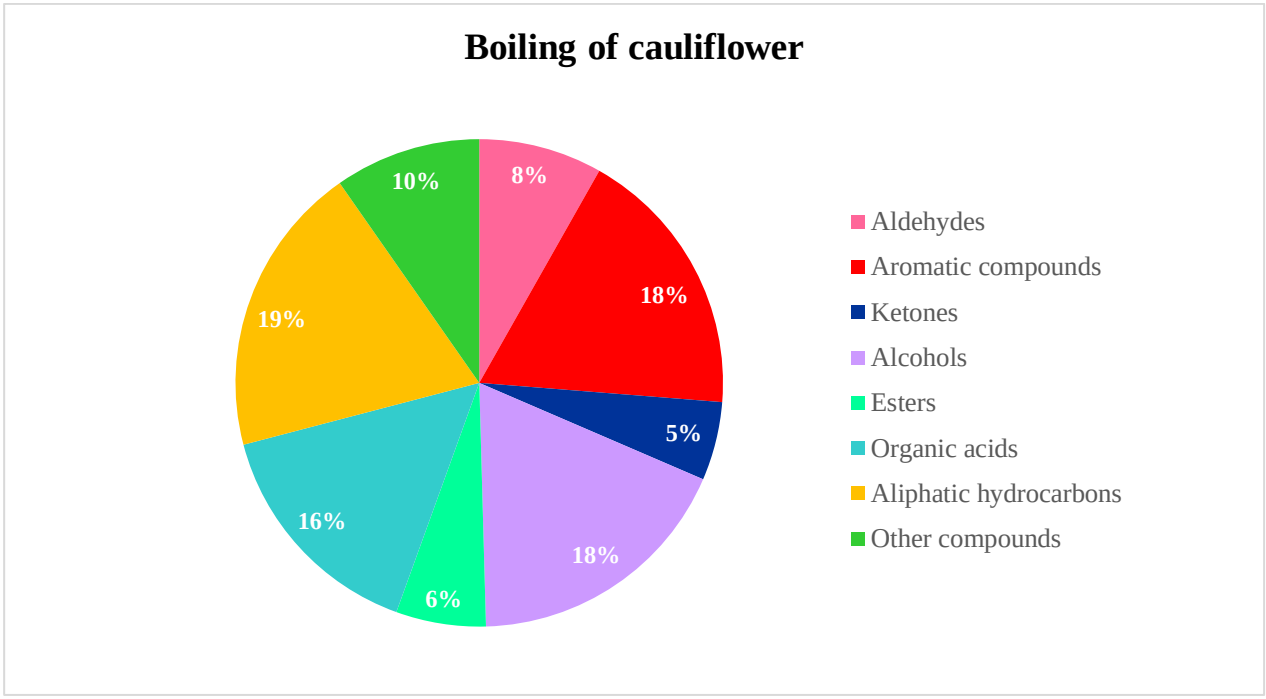


Figure 48. Percentage composition of the different classes of VOCs in the air sampled during the boiling of cauliflower.

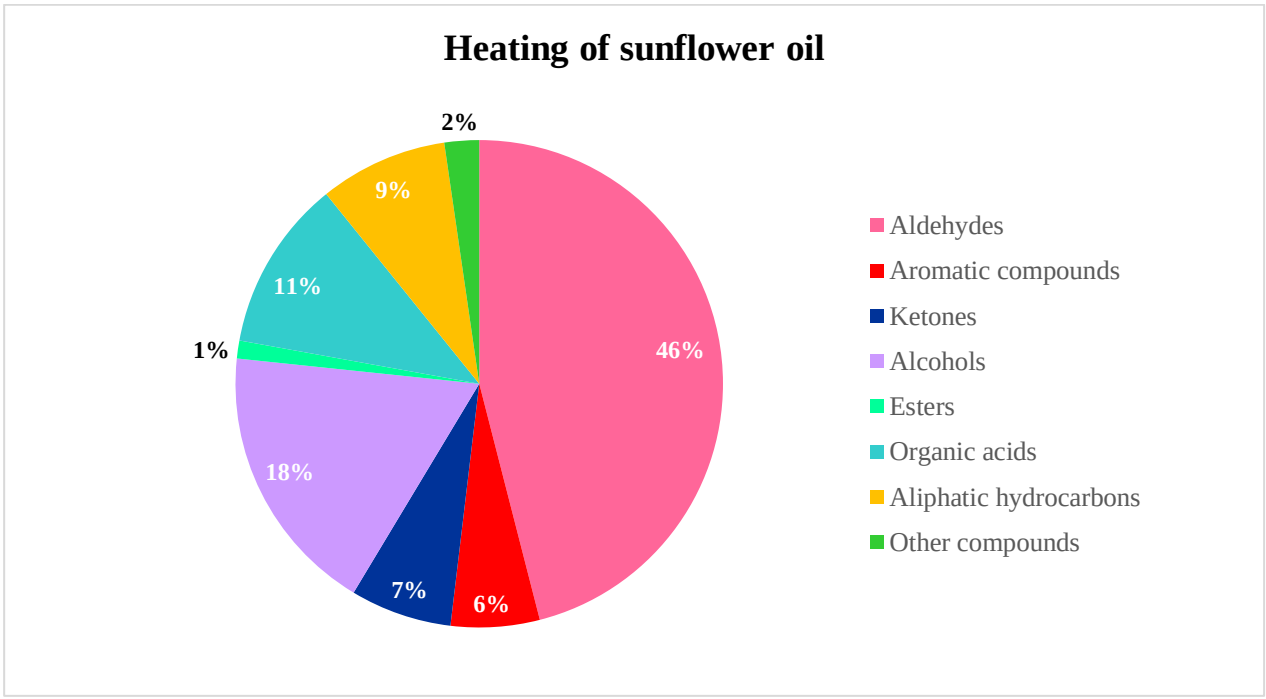


Figure 49. Percentage composition of the different classes of VOCs in the air sampled during the heating of sunflower oil (120 °C).

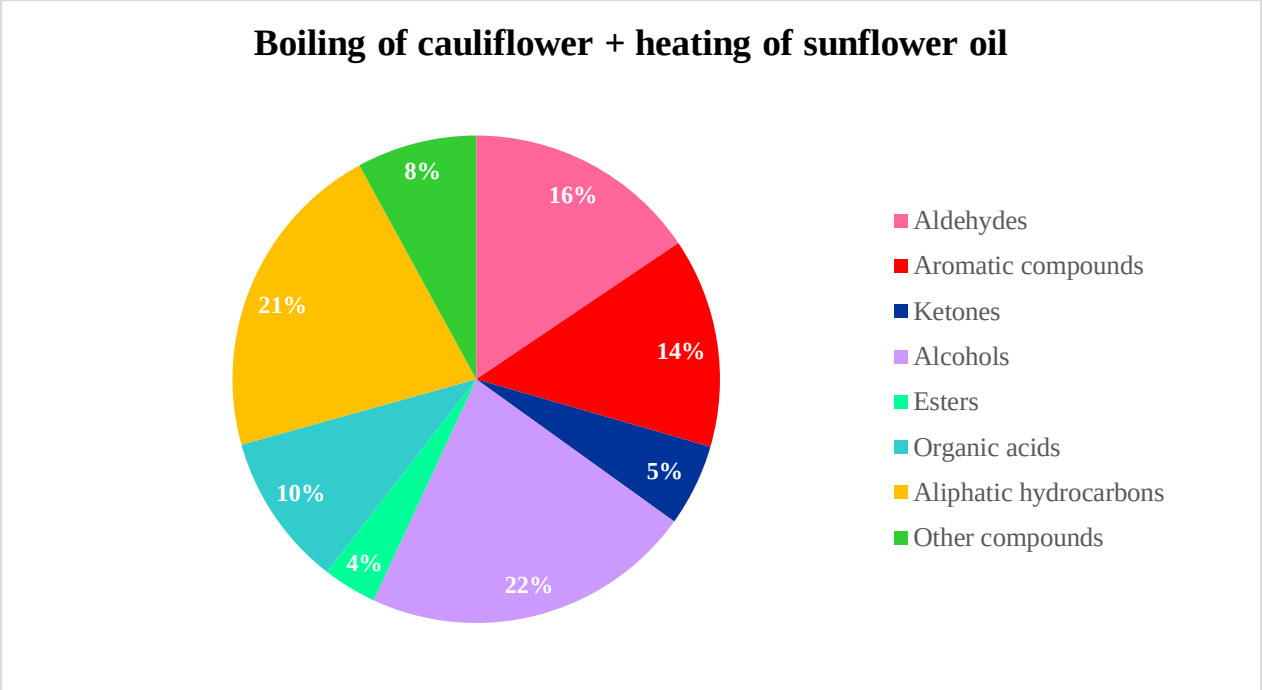


Figure 50. Percentage composition of the different classes of VOCs in the air sampled during the combination of boiling of the cauliflower and the heating of sunflower oil.

Brassica vegetables are a significant component of the human diet and their popularity is systematically increasing, because of their pro-health properties. However, some consumers reject these vegetables because of their specific bitter taste and sharp, sulphurous aroma. Their characteristic flavour is more perceptible after the thermal treatment. The analysis of the air sampled during the cauliflower boiling showed high concentrations of aliphatic hydrocarbons (19%), aromatic compounds (18%), alcohols (18%) and organic acids (16%). There were also consistent quantities of aldehydes (8%) and the class under the name of "other compounds" represented the 10% of the total VOCs, with sulfur containing compounds which formed the largest part. In the air sampled after the boiling of cauliflower sulphur compounds represented the 43% of the "other compounds class" and the 3.90% of the total detected VOCs. Similar results were obtained from the analysis of the air after the boiling of cauliflower and heating of sunflower oil together, where the sulphur compounds represented the 43% and the 3.9% of the "other compounds" and the total VOCs, respectively. These results indicated that the boiling of cauliflower contributed almost totally to the sulphur compounds content developed in the investigated cooking processes. In fact, after the heating of the oil alone, the sulphur compounds formed the 8.26% of the class of "other compounds" and only the 0.2% of the total VOCs. Indeed, sulphur compounds were not detected in the previous studies on the deep-frying of

potatoes and the grilling of the hamburger. A study carried out by Wieczorek and Jeleń [81] investigated the volatile composition of 15 *Brassica* cultivars, both raw and cooked. They found that the most abundant compounds were sulphur components, aldehydes and alcohols. They reported also a considerable amount of nitriles, which were detected also in the present study. Nitriles could be products of glucosinate hydrolysis and their concentration seemed to reduce in cooked cauliflowers [81]. Sulfides derive mainly from S-alk(en)yl-L-cysteine pathway and they are very abundant components of the volatile fraction of cruciferous vegetables, reaching also the average percentage content of 10% [81, 82]. Because of their very low odor threshold, which are detectable at levels as low as one part per trillion by the human nose, their role in the cauliflower characteristic and unpleasant flavor is obvious [81]. The same study indicated that for the species of cauliflower sulfides concentration was usually higher after cooking. On the contrary, a marked decrease in the contents of aldehydes and alcohols was observed in cooked samples [81]. Aldehydes contribute the "green" type aroma to the cauliflower fragrance. Decanal, nonanal, benzaldehyde, 2-butenal and hexanal were found at highest levels. Hexanal, benzaldehyde and nonanal were some of the dominant aldehydes also in previous studies [81] and 2-butenal, with its flower-type odor, was the most abundant aldehyde in the kohlrabi varieties [81]. Significant levels of sulfides and short chain fatty acids in the air sampled during steaming of cauliflowers were indicated also by Kabir et al. [83].

The VOCs composition of the air sampled during the heating of sunlfower oil at 120°C was very different, being characterized by a high percentage of aldehydes (46%), followed by alcohols (18%) and organic acids (11%). As already said, a common worldwide cooking method is immersion of the food in hot oil. In Western country sunflower and olive oils are edible oils commonly used for this purpose. The VOCs and sensory properties of the oil are altered after intense heating, mainly because of the lipid oxidation. This chemical reaction leads to the formation of alkyl radicals and alkylperoxyl radicals and the subsequent release of many flavor compounds (fatty acids, alkanes, aldehydes, ketones, alcohols, aromatic hydrocarbons), which can be pleasant or unpleasant. Some of them are associated to human health risks [84]. Sabbatini et al. [84] monitored in real time the changes in VOCs emission in sunflower oil upon heating (from room temperature up to 180°C). The study indicated that the production of volatiles increased enormously at 120°C. Also Katragadda et al. [85] demonstrated that total concentrations of aldehydes, hydrocarbons, alcohols and ketones are correlated to the increase of oil frying temperatures. Since the increase of oil temperature directly increases the VOCs production, including some pollutants, the accurate control of the temperature is very important

in cooking practices. Besides the aldehydes, heating of sunflower oil contributed a lot in the total amount of alcohols and ketones released in the atmosphere. In particular, among the alcohols, the most abundant were 1-octen-3-ol and 1,2-ethanediol, while 2,7-octanedione and 1-octen-3-one were the prevalent ketones. Both these classes of compounds derive from fatty acids degradation.

The fact that some compounds (e.g 3-octanone, heptanal, dimethyl sulfide and trisulfide) were detected only in the air sampled during boiling of cauliflowers or sunflower oil heating and not in the air sampled when both the cooking processes were performed can be due to the competition and displacement effect of the SPME fiber during the exposure to the samples.

1.3.2 Development and validation of a new method for assessing odor filters efficiency

The results obtained for the different cooking systems were compared to evaluate the presence of some common analytes. A total of 18 VOCs were found common to all the performed analyses and they are shown in **Table 14**.

Table 14. Common analytes developed from the investigated cooking model systems (deep-frying of potatoes, grilling of hamburgers, boiling of cauliflowers and heating of sunflower oil at 120°C).

Common detected compounds
Aldehydes
Hexanal
Benzaldehyde
Aromatic compounds
2-Pentyl-furan
Ethylbenzene
Styrene
Toluene
p-Xylene
Ketones
6-Methyl-5-hepten-2-one
Alcohols
1-Octen-3-ol

1-Pentanol
Esters
Ethyl acetate
Aliphatic hydrocarbons
2,2,4,4,6,8,8-Heptamethyl-nonane
α-Pinene
β-Pinene
Decane
Dodecane
Organic acids
Acetic acid
Hexanoic acid

The current legislation (EN IEC 61591) regarding assessment of odor filtration efficiency of filters used in kitchen hoods involves the use of methyl ethyl ketone (MEK) as standard. It is a colorless, fairly volatile liquid with a pungent odor. It is classified as hazardous compound in REACH regulation and according to the harmonised classification and labelling (CLP00) approved by the European Union, this substance is a highly flammable liquid and vapour, causes serious eye irritation and may cause drowsiness or dizziness [86]. It is not an analyte found in many types of food matrices and cooking processes. For all these reasons, the present study aimed to find an alternative standard for testing the efficiency of kitchen hood filters. This standard molecule should be a substance really present in the air in domestic and commercial kitchens, and released commonly by as much as possible types of cooking processes and food matrices. Furthermore, it should present a lower toxicity with respect to MEK.

Considering the 18 common VOCs detected in all the performed analysis of this study, the acetic acid was selected as alternative standard to MEK to develop and validate a new possible analytical method for assessing the efficiency of hood filters. Acetic acid was chosen because of its relative stability at high temperatures, low toxicity, moderate cost and easy accessibility.

The experiments were carried out in the same insulated and certified room used in the EN IEC 61591 legislation. To set the best concentration of acetic acid to be used during the analysis and to evaluate the linearity of the method, different increasing amounts of acetic acid (10 µL, 25 µL, 50 µL and 100 µL) were evaporated in water bath at 60°C for 20 minutes inside the room. **Figure 51** shows the chromatogram obtained from the analysis of VOCs in the air sampled after the evaporation of 25 µL of acetic acid (1.14 µL/m^3). The peak of the analyte of interest resulted well resolved with a rapid GC-FID analysis (13 min).

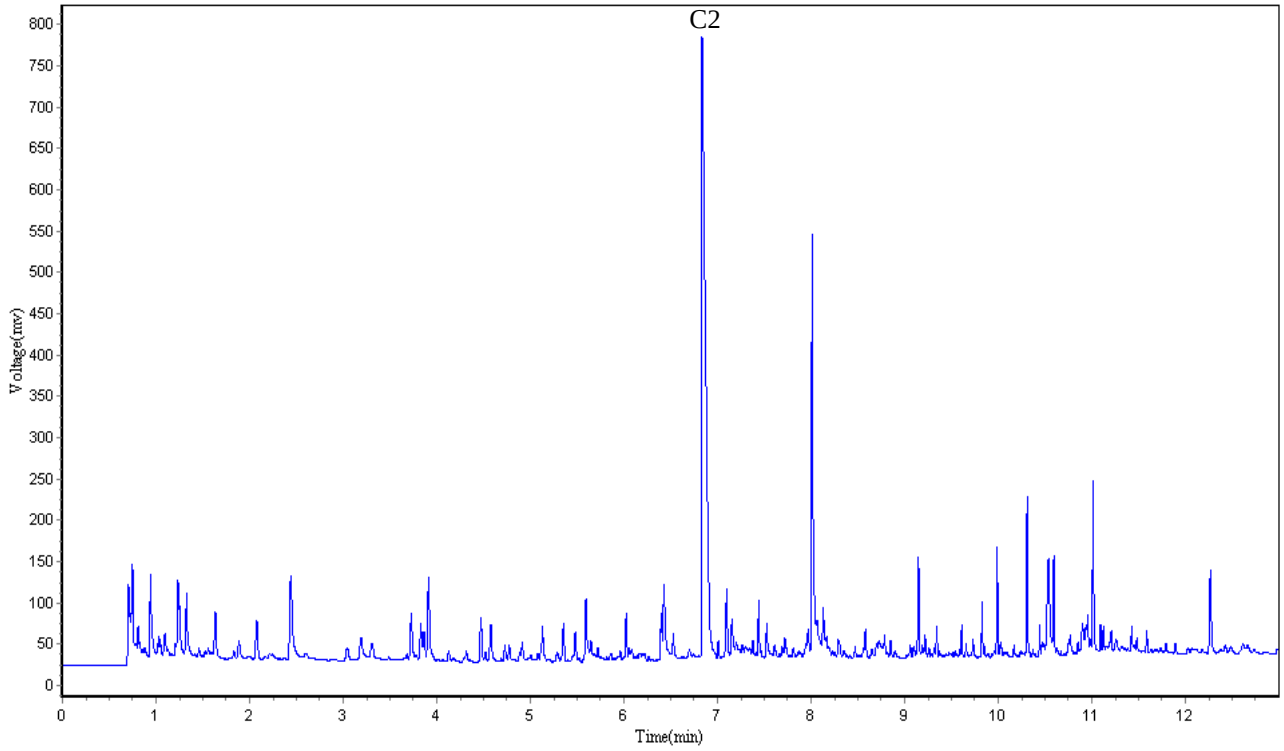


Figure 51. GC-FID Chromatogram obtained from the analysis of the air sampled after the evaporation of acetic acid inside the testing room.

To determine the linearity of the method a calibration curve (**Figure 52**) was obtained, plotting the peak areas was against standard concentrations (µL/m³). Considering the sampling conditions, linearity was found to be excellent with a calculated linear correlation coefficient (R^2) of 0.9917.

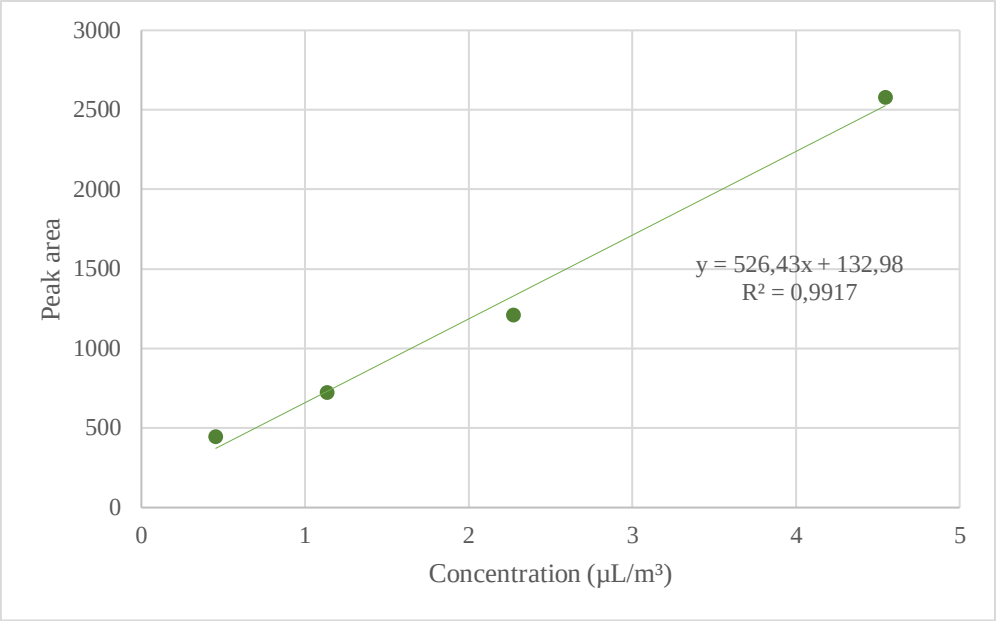


Figure 52. Calibration curve obtained for the acetic acid.

The second step of the validation of the sampling method was the verification of the sealing of the room. A fixed quantity of acetic acid (25 µL) was evaporated inside the room and the air sampling was performed after different times of evaporation (20, 30, 40 and 50 min) at four consecutive days.

The figure below (**Figures 53**) shows the chromatograms obtained after 20 minutes of evaporation (four replicates were performed for each time).

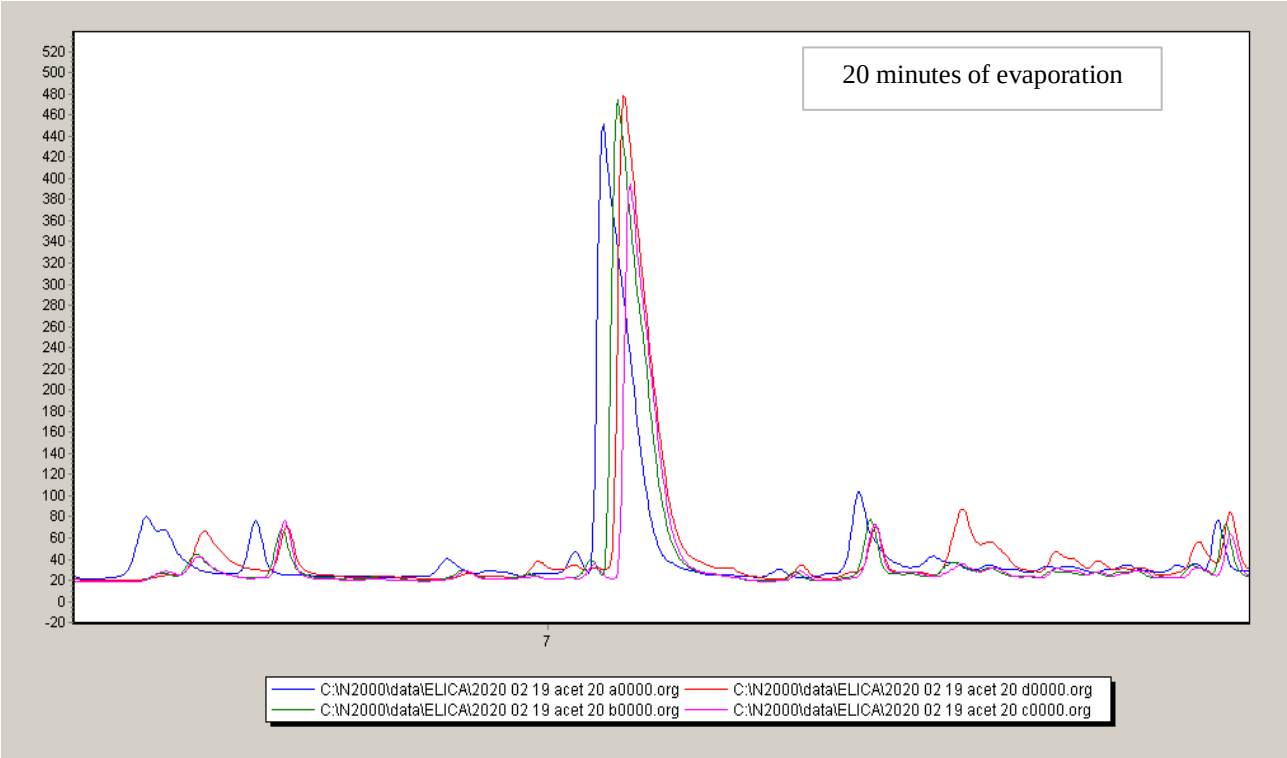


Figure 53. GC-FID chromatogram obtained from the analysis of the air in the room after 20 minutes of evaporation of the acetic acid.

The results obtained from the different evaluated evaporation times are presented in **Table 15**. Values are reported as the average area of the peak. The obtained RSD% (15.86%) was not high and also statistical analysis did not detected significant differences between the evaluated times. For these reasons the sealing of the room was verified.

Table 15. Concentration of acetic acid after different times of evaporation (20, 30, 40 and 50 min) in terms of mean area values.

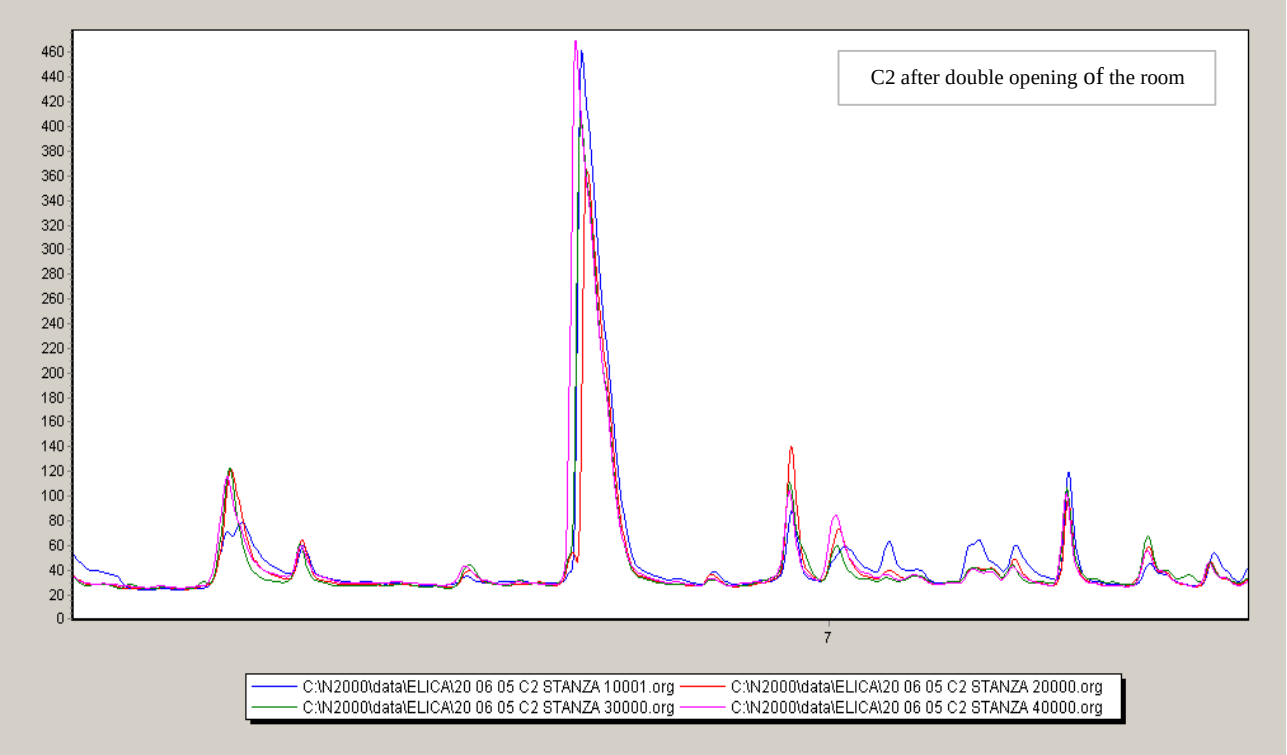
20 min of evaporation	**30 min of evaporation**	**40 min of evaporation**	**50 min of evaporation**	**Average peak area**	**SD**	**RSD%**
Peak area	Peak area	Peak area	Peak area			
773	689	625	662	687	102	15.9

SD: standard deviation; RSD: relative standard deviation (*n*=4).

Once the sealing of the room was verified and the evaporation time of the acetic acid in the room was estimated (20 min), a simulation of the sampling procedure was performed, to verify possible undesirable losses of the analyte. For this purpose, 25 µL of acetic acid were evaporated at 60°C for 20 min, then the door of the room was opened and rapidly closed to

simulate the sampling of the air before the hood functioning. After 10 min, the door was opened and closed again (for the sampling of the air after the hood operation) and the air inside the room was sampled. In fact, the two sampling of the air were performed by a person inside the room at two different times (before and after the hood functioning) and opening twice the door of the testing room was unavoidable.

Figure 54 shows the chromatograms (*n*=4) obtained after the simulation of the sampling method.



The mean absolute area obtained for acetic acid was 809 and again no significant differences were found between this result and the areas obtained from previous analysis, ascertaining that there were no acetic acid losses from the room during the sampling procedure.

The last step for the development of the sampling method was the evaluation of the best hood operation time. To do that, the developed sampling method was applied to a real kitchen odor filter (MHGS) made of a polyurethane foam, coated with granular activated charcoal and inserted in black polyamide sock. Again, 25 µL of acetic acid were evaporated at 60°C in water bath inside the room. After 20 min the air was sampled for the first time and the hood was switched on. Then the second sampling was performed after 10, 20 and 30 minutes (T10, T20 and T30, respectively). For each analysis a new filter was tested. Results are shown in **Figure 55**.

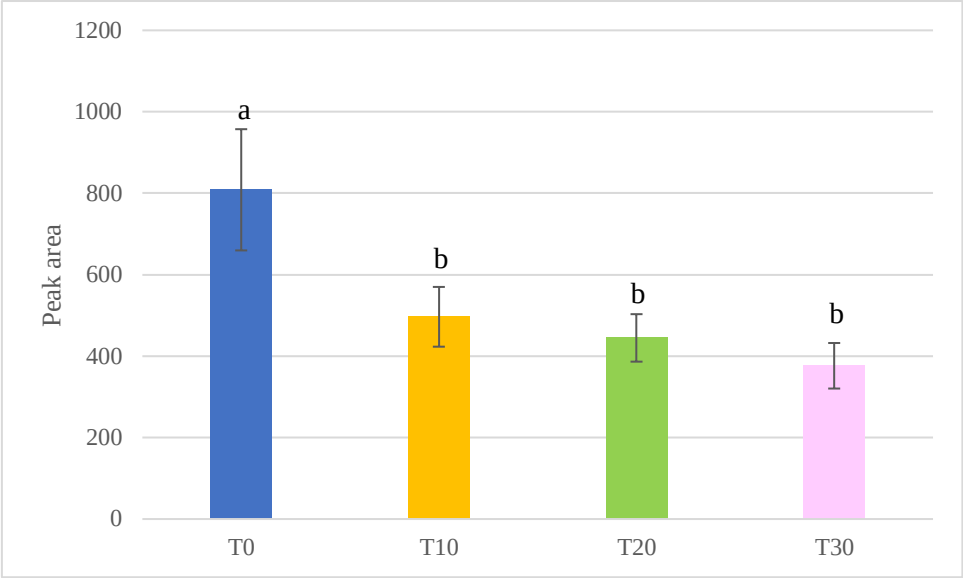


Figure 55. Concentrations of acetic acid (in terms of peak area) inside the testing room after different times of kitchen hood functioning (T0, T10, T20 and T30): 0, 10, 20 and 30 minutes. Bars indicated standard deviations. Different letters indicate significant differences between acetic acid concentrations at different sampling times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

The dejection made by the filter (in terms of percentage) (**Figure 56**) was calculated for the acetic acid for each hood operation time as indicated by the formula below.

$$\text{Dejection\%} = \frac{\text{Area before the hood functioning} - \text{Area after the hood functioning}}{\text{Area before the hood functioning}} \text{x100}$$

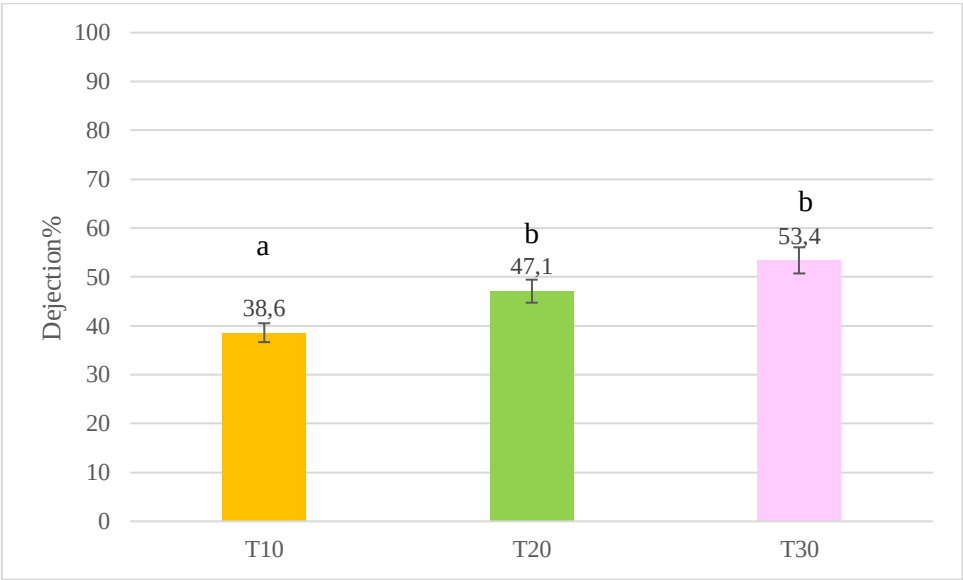


Figure 56. Dejection percentages of acetic acid after passage through the MHGS filter after different times of the functioning of the hood: 10, 20 and 30 minutes (T10, T20 and T30,

respectively). Bars indicated standard deviations. Different letters indicate significant differences between the filter operation times (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

The dejection of the acetic standard increased proportionally to the hood operation time, as expected. Statistical analysis did not reveal significant differences between the acetic acid dejection after 20 and 30 minutes. Because of economics of time, 20 minutes were chosen as the best time for hood operation time for the new efficiency testing method of the filters.

The analysis after 20 minutes of hood operation were used to determine also the intraday (n=4) and the interday (n=3) reproducibility of the method. In fact, the air sampling and analysis after 20 minutes were performed in three consecutive days and in quadruplicate each day. The intraday and interday repeatability resulted to be 5.98% and 2.42%, considering the RSD% calculated with the peak areas obtained after the analysis.
Once all the operation conditions were evaluated and the linearity and reproducibility were confirmed to be excellent, the analytical method was applied to other real kitchen odor filters, to test their efficiency.

1.3.2.1 Application of the method to real kitchen odor filters

The developed analytical method, making use of acetic acid as an alternative standard to the MEK as defined by the current legislation EN IEC 61591, was applied to three filters, that differed for their physical characteristics and economical value too.
The three evaluated filters, from the cheapest to the most expensive, are:

- Washable filter MHGS
- Washable filter SARATECH 2700
- Helsa-Sorbexx-CS ceramic filter

The first two are very similar: a polyurethane foam coated with activated carbon and inserted in a nylon sock and they differ primarily for the content of activated carbon. The last one is made by composite activated charcoal reinforced by ceramic.

The washable filter MHGS was used for the development of the analytical method and it showed a dejection of acetic acid after the passage through the filter and the functioning of the hood for 20 minutes of 47.1%. **Figure 57** shows the chromatograms obtained from the analysis of VOCs in the air in the room after the evaporation of the acetic acid before and after the

functioning of the hood (20 min) where the MHGS filter was installed. It is clear the reduction of the peak area and thus the concentration of acetic acid.

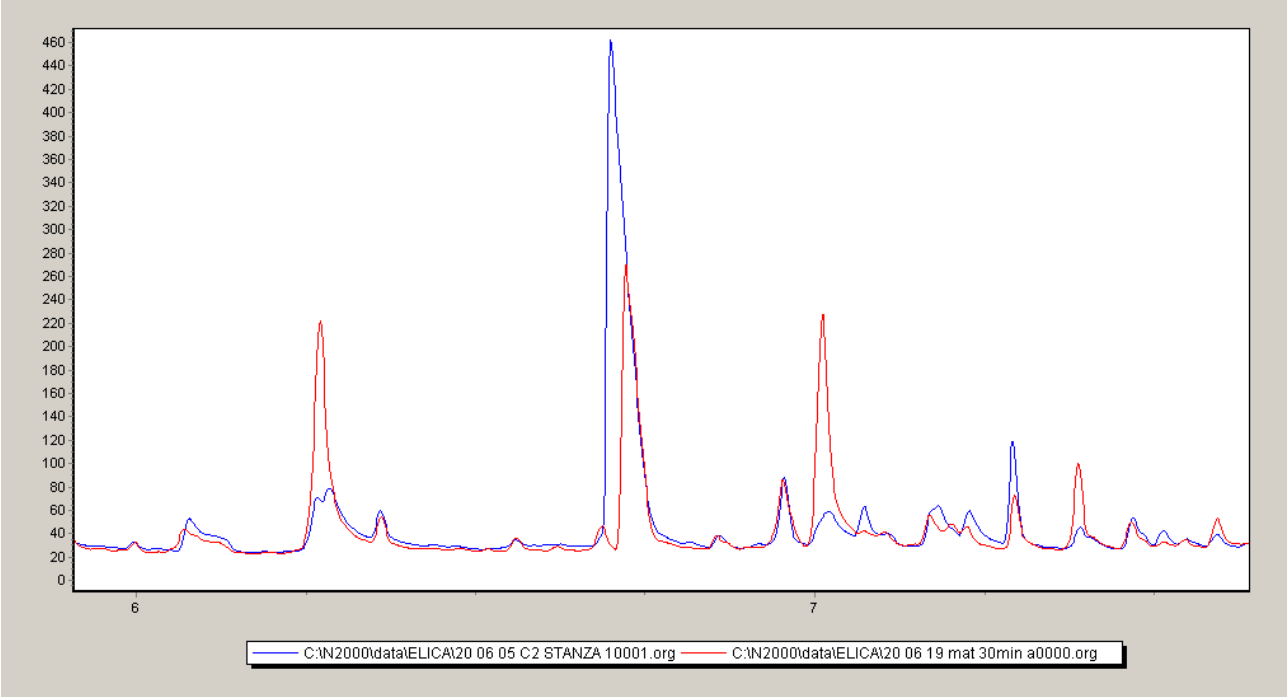


Figure 57. GC-FID chromatograms obtained from the analysis of VOCs in the air in the room before (blue line) and after (red line) 20 minutes of functioning of the hood with MHGS filter.

The washable filter SARATECH 2700 was used in the same conditions. The chromatograms obtained from the analysis after the evaporation of the acetic acid before and after the functioning of the hood (20 min), where the SARATECH filter was installed, are reported in **Figure 58**. Also in this case, the concentration of acetic acid in the room was highly reduced by the kitchen hood filter, showing a dejection percentage of 49.2%.

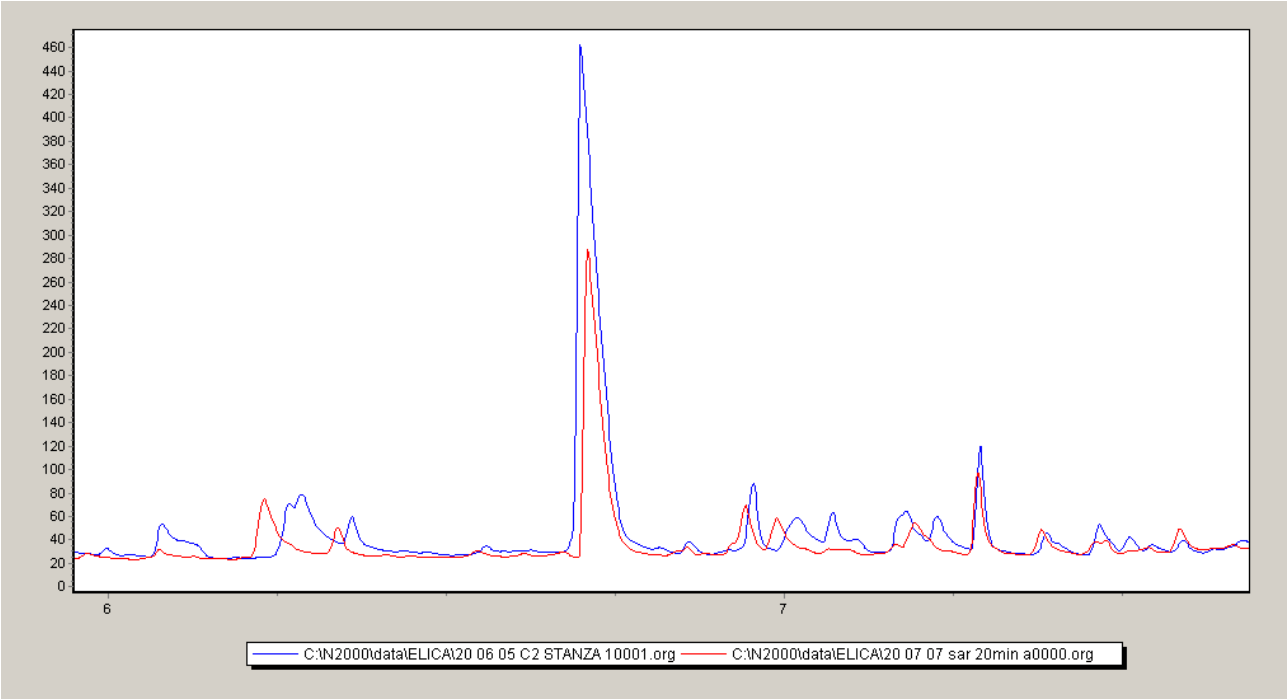


Figure 58. GC-FID chromatograms obtained from the analysis of VOCs in the air in the room before (blue line) and after (red line) 20 minutes of functioning of the hood with SARATECH 2700 filter.

The last investigated filter was Helsa-Sorbexx-CS filter. It is the filter with the best characteristics with respect to the others. It has a high cleaning performance, huge capacity, low energy consumption, low noise level during operation and a high ability to regenerate at high temperatures. It is highly customizable but it is more expensive than the other two washable filters. Therefore, it is possible to expect higher performances (in terms of filtering) than the other two filters.

The chromatograms obtained from the Helsa-Sorbexx-CS filter efficiency test are reported in **Figure 59.** The reduction in terms of peak area of the acetic acid is relevant. Also in this case the dejection percentage for the standard molecule was calculated, indicating the same dejection % as for the MHGS filter: 47.1%.

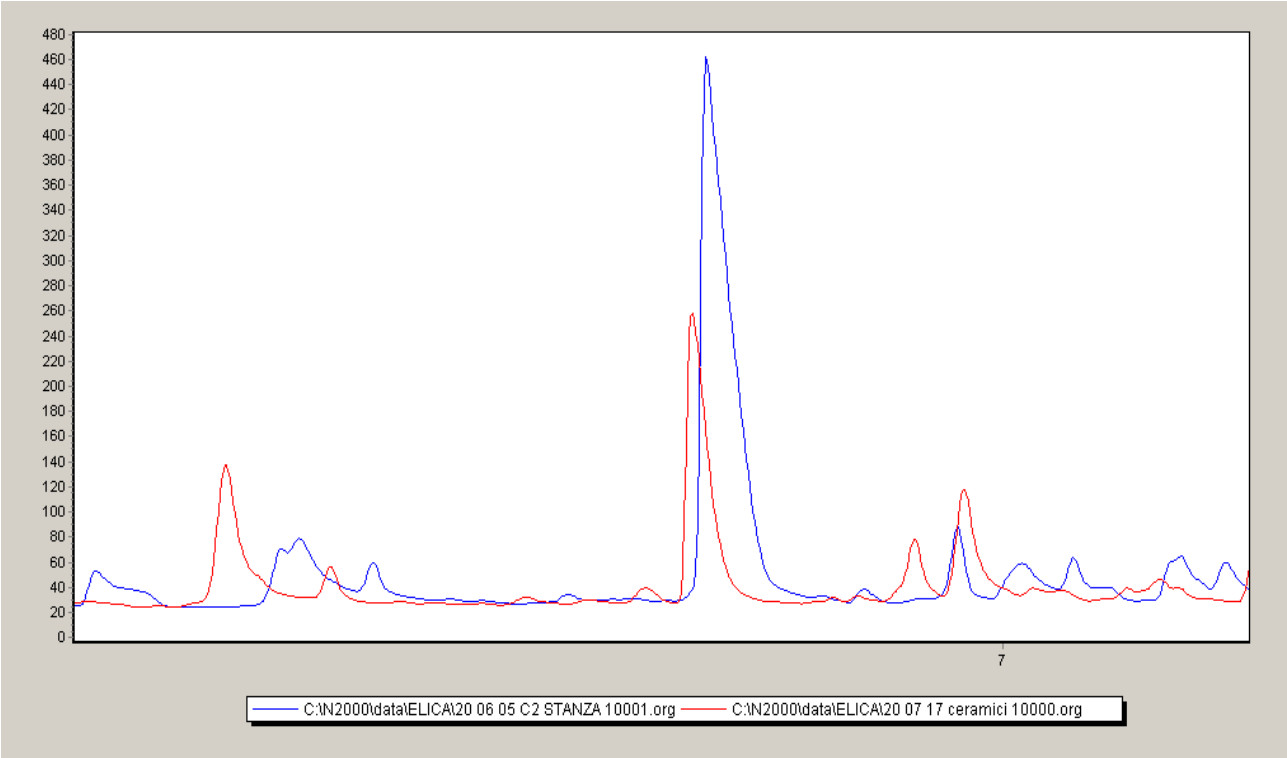


Figure 59. GC-FID chromatograms obtained from the analysis of VOCs in the air in the room before (blue line) and after (red line) 20 minutes of functioning of the hood with SARATECH 2700 filter.

The results obtained from the efficiency test of the three filters are summarized in **Figure 60.**

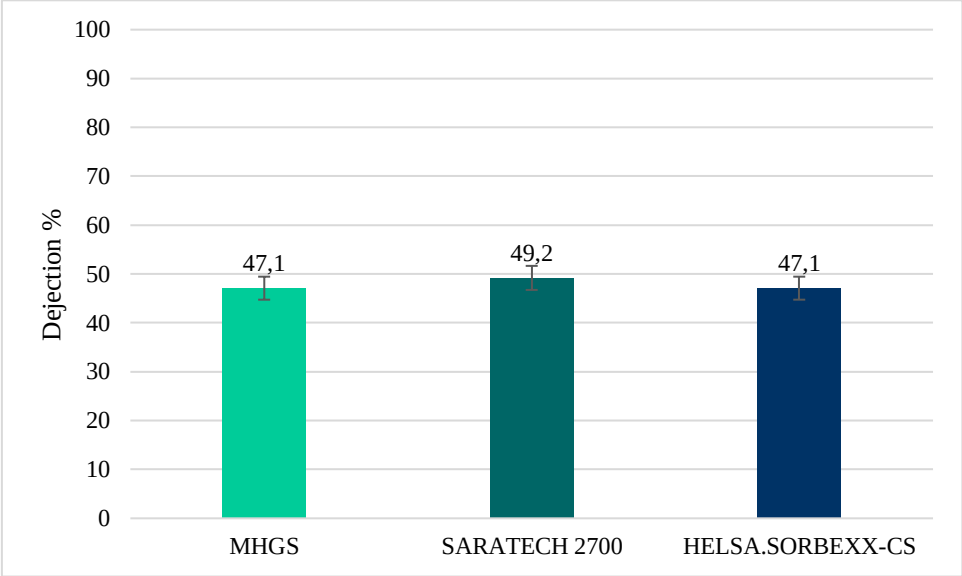


Figure 60. Dejection % of acetic acid given by the three investigated filters. Bars indicated standard deviations.

The obtained dejections toward the acetic acid were very similar and also statistical analysis did not find any significant differences between them. Only SARATECH 2700 washable filter showed a slightly higher performance with respect to the other two. It is possible that a filter media is more effective toward specific compounds or classes of compounds, so in the future the behaviour of these studied filters and others can be investigated toward other VOCs found in cooking fumes.

1.4 Conclusions

In conclusion, considering the purpose of this research work, we can summarize:

- The proposed system allows to analyze VOCs produced during cooking, making use of olfactometric bags to collect air samples, and SPME-GC-MS to extract and analyse VOCs. This procedure is simple, economic and exploits tools and analytical instrumentation readily available in many laboratories. In addition, it allows to analyse samples even collected far from the analytical instrument location to be used for the determination.
- Different cooking model systems were investigated: deep frying of potatoes, grilling of a beef hamburger, boiling of cauliflowers and heating of sunflower oil at 120°C. A total of 18 common volatile organic compounds were identified, that could be considered as possible markers of cooking processes.
- An alternative method to the current legislation (EN IEC 61591) for the testing of efficiency of odor filters was developed and validated. It exploits the use of acetic acid (one of the VOCs common to all the investigated cooking systems) instead of the more toxic MEK. The method provides good results in terms of reproducibility, linearity, costs and time consumption.
- Three different filters based on activated charcoal (washable filter MHGS, washable filter SARATECH 2700 and Helsa-Sorbexx-CS filter) were tested, analyzing their efficiency in terms of acetic acid dejection.
- SARATECH 2700 washable filter provides slightly better filtering performance of acetic acid than the other two.
- The behaviour of washable filter MHGS and Helsa-Sorbexx-CS filter toward acetic acid is the same, even if the second one has the highest market price.

Further studies are needed to better understand the behaviour of the filters, applying the developed testing method with other molecules. In fact, filters can show a certain selectivity for

particular classes of compounds. The VOCs to be tested could be chosen among the 18 common molecules selected from the presented study, in order to investigate real cooking fumes found in domestic kitchens.

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[86] www.echa.europa.eu

INDEX

Abstract……...101

Chapter 2: Study of volatile organic compounds as markers of shelf-life of organic and conventional chicken meat stored in different packaging……..…….102

2.1 Introduction……...103

2.1.1 Chicken meat….……...103

2.1.2 Organic and conventional chicken farming……...……...105

2.1.3 Shelf-life of chicken meat…….……...111

2.1.4 Packaging of poultry meat products……..……...112

2.1.4.1 Modified Atmosphere Packaging (MAP) ……..……...112

2.1.4.2 Active Packaging technologies……..113

2.1.4.3 Intelligent packaging……..……...114

2.1.4.4 Biodegradable packaging……..114

2.1.5 Volatile organic compounds as meat shelf-life indicators……...116

2.1.6 Aim of the work……...119

2.2 Materials and Methods……..……...120

2.2.1 Chemicals and reagents……120

2.2.2 Comparison of packaging materials……..……...120

2.2.3 Comparison of organic and traditional chicken meat……121

2.2.4 HS-SPME-GC-MS analysis of volatile organic compounds……...121

2.2.5 Statistical analysis……122

2.3 Results and Discussion……...……...123

2.3.1 Comparison of packaging materials……123

2.3.2 Comparison of organic and traditional chicken meat……133

2.4 Conclusions…….……...146

References……..148

Abstract

An intensive use of traditional packaging is a serious ecological problem, leading to a shift to compostable and biodegradable materials. In the last years, consumers are careful to environmental impact and to possible health hazards of intensive production systems. For this reason, unconventional and alternative production methods, such as organic farming, increased greatly, in particular the organic poultry production. This study has two main aims. Firstly, the objective is the study of the efficiency of a new biopackaging (BP), based on biopolymers, to preserve the quality of organic chicken meat under modified atmosphere (MAP) in comparison with a polyethylene terephthalate (PET) material, during storage at 2°C. The second study regards the comparison of the shelf-life of organic and traditional chicken meat (of the same species) in a cellophane packaging under aerobic conditions at 2°C. A HS-SPME-GC-MS method was applied to evaluate the volatile organic compounds (VOCs) development during time. Then, other chemical and biological parameters were investigated by other research groups: content of biogenic amines (BAs), pH measurement, microbiological counts and sensorial evaluation. Comparing the two packaging, the monitored VOCs and the BAs indices resulted to have a similar trend in both packaged meats, as well as the microbiological counts. The new BP showed similar properties of non-biodegradable material (PET) to preserve the shelf-life of organic chicken meat. Considering the two types of chicken meat (organic and conventional) 21 VOCs were monitored and they were detected mostly at the end of the storage period. After 10 days the total VOCs concentration was higher in conventional meat respect to the organic one. Also the total BAs content was highly richer in traditional meat. On the contrary, organic meat shows higher microbial counts and yellowness at the end of the storage with respect to the conventional one, even if the odor and the tenderness were more pleasant in the organic one. This study contributes to encourage the use of new biodegradable materials and the consume of sustainable and organic products.

Chapter 2. Study of volatile organic compounds as markers of shelf-life of organic and conventional chicken meat stored in different packagings

2.1 Introduction

2.1.1 Chicken meat

Poultry meat is one of the animal-source foods most widely eaten at global level, across different cultures, traditions and religions. Its consumption has rapidly increased in the past years and its demand is expected to increase further due to population growth. According to FAO, between 2000 and 2030, per capita demand for poultry meat is expected to increase by 271% in South Asia, 116% in Eastern Europe and Central Asia, 97% in the Middle East and North Africa and 91% in East Asia and the Pacific [1]. The choice of poultry meat is linked to its sensorial and nutritional properties, its low price, its extensive supply and varied assortment [2]. Then, this meat is suitable for simple and fast preparations, being a usual choice of consumers with modern lifestyle. Among poultry meat, chicken breast is one of the most used products of white meat category, showing less fat and more protein content than red one, thus making it a dietetic product. Furthermore, if it is eaten without skin it provides 2 or 3 times less fat than chicken with skin, ensuring high-quality protein intake without extra fat and calories [3]. A comparison between the nutritional values of chicken meat and other types of meat is shown in **Table 1** [3].

Table 1. Macronutrients content of different types of meat (per 100 g).

Nutrient	Chicken[a]	Pork[b]	Beef[c]	Lamb[d]
Energy/kcal	165	165	185	180
Water/g	65.26	65.75	64.83	64.92
Protein/g	31.02	28.86	27.23	28.17
Saturated fatty acids (SFAs)	1.010	1.451	2.661	2.380
Monounsaturated fatty acids (MUFAs)	1.240	1.878	3.214	2.920
Polyunsaturated fatty acids (PUFAs)	0.770	1.066	0.285	0.440

a Chicken, broilers or fryers, breast, meat only, cooked, roasted.
b Pork, fresh, leg (ham), rump half, separable lean only, cooked, roasted.
c Beef, round, bottom round, roast, separable lean only, trimmed to 0″ fat, choice, cooked, roasted.
d Lamb, domestic, leg, shank half, separable lean only, trimmed to 1/4″ fat, choice, cooked, roasted.

Chicken meat does not differ much in cholesterol content from other types of meat, but it has better nutritional value because of its higher protein content, lower total fat content, less saturated fatty acids and lower calories intake. For all these reasons, chicken meat is recommended for all consumers who takes care of diet and health. It contains also less collagen with respect the other meat, so chicken meat results more digestible than other types of meat [3].

Chicken meat is also a good source of important micronutrients, such as vitamins and minerals (**Table 2**).

Table 2. Content of minerals and vitamins in different types of meat (per 100 g).

Minerals	**Chicken**[a]	**Pork**[b]	**Beef**[c]	**Lamb**[d]
Calcium (mg)	15	16	6	8
Iron (mg)	1.04	0.97	2.40	2.06
Magnesium (mg)	29	27	18	26
Phosphorus (mg)	228	273	172	208
Potassium (mg)	256	425	222	342
Sodium (mg)	74	80	36	66
Zinc (mg)	1.00	2.48	4.74	5.02
Vitamins				
Vitamin C (mg)	0.0	0.0	0.0	0.0
Thiamin (mg)	0.070	0.523	0.057	0.110
Ribolfavin (mg)	0.114	0.408	0.170	0.280
Niacin (mg)	13.712	7.940	5.232	6.390
Vitamin B6 (mg)	0.600	0.538	0.380	0.170
Folate (µg)	4	0	9	24
Vitamin B12 (mg)	0.34	0.67	1.61	2.71
Vitamin A (µg)	6	1	0	0
Vitamin E (mg)	0.27	0.26	0.37	0.18
Vitamin D (D2+D3) (µg)	0.1	0.3	_	_
Vitamin K (µg)	0.3	0.0	1.3	_

a Chicken, broilers or fryers, breast, meat only, cooked, roasted.
b Pork, fresh, leg (ham), rump half, separable lean only, cooked, roasted.
c Beef, round, bottom round, roast, separable lean only, trimmed to 0″ fat, choice, cooked, roasted.
d Lamb, domestic, leg, shank half, separable lean only, trimmed to 1/4″ fat, choice, cooked, roasted

Respect to the other meat (except for pork meat) chicken breast meat contains more sodium, magnesium, phosphorus and calcium. Magnesium is known to be important in muscle activity and in the protein synthesis. Sodium is an essential electrolyte of human body. Calcium and phosphorus help to mantain bones and teeth healthy. Iron, instead, is fundamental in the hemoglobin synthesis, in the anemia prevention and in muscle activity. The iron content in chicken meat is very similar to the pork meat one. Among vitamins, niacin (vitamin B3) is the most abundant, followed by vitamins A and

B6, which are present at higher quantities respect the other meat types. Niacin is required for the proper function of fats and sugars in the body and to maintain healthy cells. Retinol (vitamin A) is fundamental in the well-being of the visual apparatus. It is useful for the skin, because it improves melanin production, protecting the skin from damages deriving from UV rays. Then, it is also important for protein synthesis and for the bones formation [4]. Pyridoxine (vitamin B6) is essential for the proper functioning of the central nervous system, participating in the production of the neurotransmitters serotonin and norepinephrine. Some studies indicated that vitamin B6 could prevent some degenerative diseases, such as Parkinson's and Alzheimer's [5]. Considering what has been mentioned above, chicken meat is an affordable source of important nutrients, minerals and vitamins essential for proper body functioning.

2.1.2 Organic and conventional chicken farming

For the last three decades, intensive farming has helped to enlarge this sector so fast. The animals have started to grow faster and also the number of animals per unit area has increased. This system aims to reach the highest productivity with the lowest cost, but it can generate some food safety problems to the consumers. In the last years, consumer expectations for meat production have changed, leading to the development of unconventional and alternative poultry production systems, such as organic farming [6]. Organic systems are production methods based on a holistic view of the ecosystem and the relationships between organisms, in which the use of synthetic products is minimised or omitted. Organic farming (OF) was born as a reaction to negative effects of modern industrialized agriculture in 20th century. OF uses environmentally friendly methods of pest, weed and disease control. It does not allow the use of synthetic pesticides and fertilizers, improves animal welfare, cares about the biological diversity and implements the renewable sources of energy taking care of recycling raw materials [7]. Modern organic farming grew as a response to the environmental harm and to safeguard people's health. Organic production has important social, economic and ecological functions (**Figure 1**), providing public goods that contribute to environmental protection, animal welfare and rural development and developing a specific market that responds to consumers' demand for organic products. Organic agriculture exploits the natural fertility of the soil and uses traditional cultivation techniques. Then, it only uses natural substances to eliminate potential parasites. The use of genetically modified organisms (GMOs) is not allowed in organic farming [7].

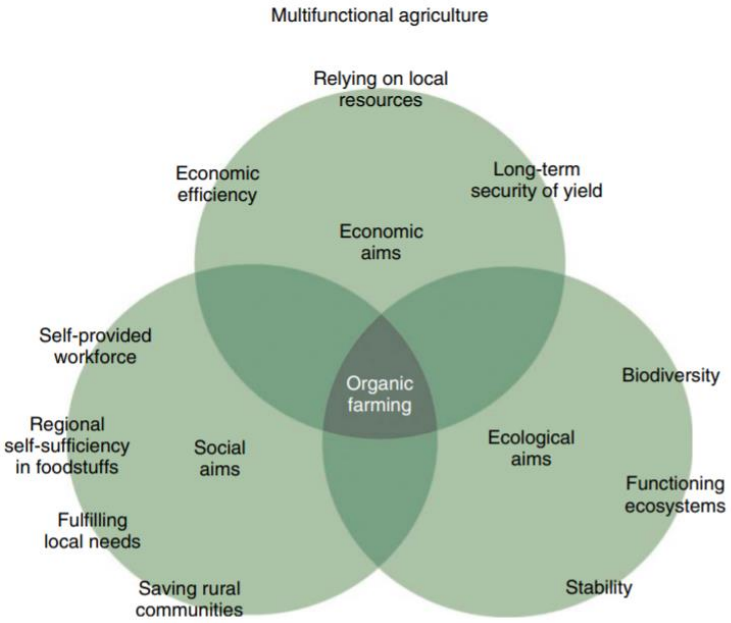

Figure 1. The importance of organic farming (OF) [7].

The organic movement was born 100 years ago; this first phase was called "Organic 1.0". Some visioners understood the connections between our health, the health of the soil, the food we eat and the way we produce it. The founding of the International Federation of Organic Agriculture Movements (IFOAM) in 1972 at Versailles coincided with the emergence of the second phase "Organic 2.0". This organization developed some detailed regulation for organic agriculture. In particular, IFOAM presented the four basic principles of organic agriculture [8]:

- Principle of ecology
- Principle of health
- Principle of fairness
- Principle of care

After the big development of organic farming, "Organic 3.0" phase started in 2016, with the purpose of allowing a greater diffusion of sustainable systems and markets based on IFOAM principles.

The first regulation issued by the European Union for organic agriculture was the EEC Regulation N° 2092/91 [9]. Regulation of animal production from organic farming dates to 1999, with the publication of EC Regulation N° 1804/99. These regulations were subsequently confirmed by the EC Reg. N° 834/2007, implemented by the Reg. (EC) N° 889/2009 containing the methods of application [10]. This regulation specifies the minimum percentages of agricultural ingredients that must be organic. For example, organic production methods should contain at least 95% of organic ingredients. Products that contain organic ingredients between 70 and 95% should refer to organic methods only

in the list of ingredients and not in the sales description. Organic content less than 70% do not refer to organic production systems [7]. All countries have introduced specific logos for indicating organic production. **Figure 2** shows the logo used in European Union.

Figure 2. Logo used in European Union for organic products.

Born in the 1990s, the global organic food market today's worth is 105.5 billion US dollars. According to a survey made by FiBL (German abbreviation for Research Institute of Organic Agriculture) amounted to 97 billion euros in 2019. The country with the largest market for organic food is the United States (44.7 billion euros), followed by Germany (11.9 billion euros), France (11.2 billion euros), China (8.5 billion euros). In fifth place there is Italy with 3.6 billion euros (**Figure 3**) [11].

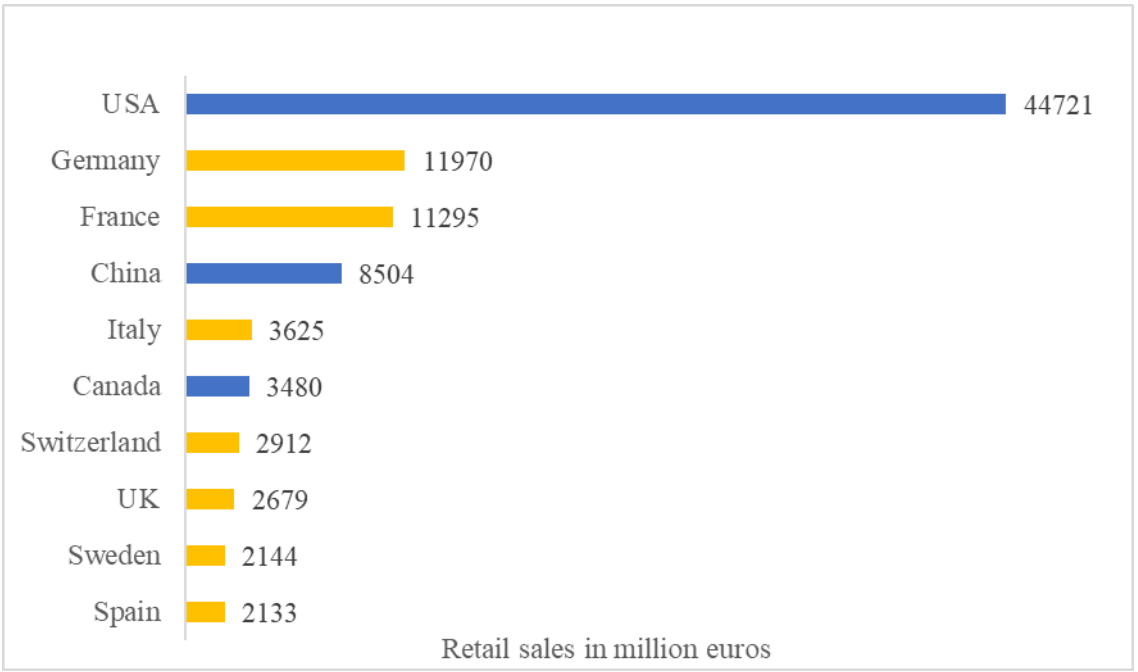

Figure 3. Countries with the largest markets for organic food 2019. (in yellow, European countries).

Not only sustainability, but also new marketing opportunities are offered to companies which support organic productions. According to EUROSTAT (European Statistical Office), in the last 20 years the

organic market value in Italy has grown greatly and today it is 10 times higher than 20 years ago (**Figure 4**). Since 2011, the Italian market value of organic food products has increased constantly to 4.3 billion euros as of 2020. [12].

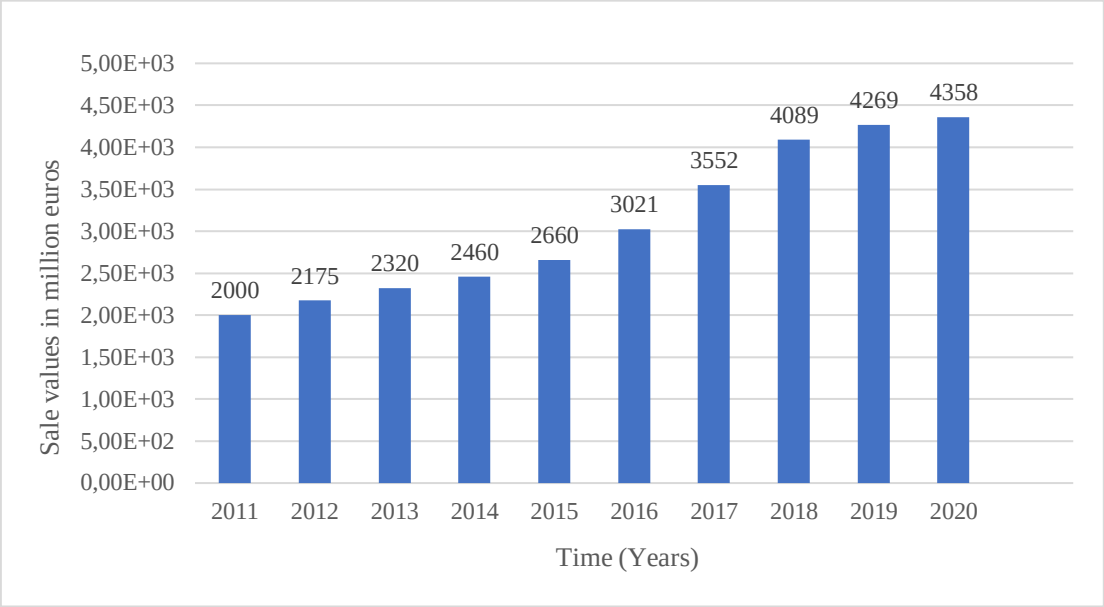

Figure 4. Organic food market value in Italy in selected years from 2011 to 2020 (in million euros).

Organic production has really increased in the last years especially because consumers have started to care a lot about the possible adverse health effects of food produced with intensive farming methods. The health benefits of organically grown fruit, vegetables, and animal products have sparked greater and greater interest. In the past, "Food Quality" was more related to safety, sensory and shelf-life aspects of food products. Nowadays it is associated with nutrition, well-being, and health. Furthermore, the industry faces other challenges in terms of consumer perception especially in the areas of health (nutrition), animal welfare (meat and dairy products) and convenience. For example, **Table 3** reports the most important attributes and qualities that push the consumer to purchase organic meat [13].

Table 3. Meat attributes researched by consumers.

Point of sale	Point of consumption	Major background cues
• Meat color	• Tenderness	• Safety
• Packaged meat color	• Flavor	• Nutrition
• Visible drip	• Juiciness	• Sustainability
• Visible fat	• Succulence	• Ethics

Considering the significant increase in consumer interest in this field, it is necessary to determine more in detail the effects of the organic production on the final products composition. Generally,

organic agriculture is more studied than organic breeding, even if the composition of animal feed is known to affect the quality of eggs, milk and meat [14]. For instance, a study by Średnicka-Tober et al. [15] revealed that organic milk has a better fatty acid composition than conventional milk. Moreover, organic eggs have shown a lower protein content, but with a better biological value and higher lecithin content than traditional eggs [16]. Based on the limited studies present in literature now, organic food appears to have higher nutritional value than traditional food. There are also other reasons to prefer organic food, such as the lower exposure to antibiotic resistant bacteria, that are a great warning to global health [17]. According to a consumer report made in 2017 [18], over the last 10 years, 43% of respondents said that they look for meat and poultry marked "antibiotic-free" when shopping [19]. Consequently, farmers have adopted alternative methods (organic and antibiotic-free methods) of producing meat to face the growing market demand. In Italy the consumption of organic products has increased at a rate of about 10% per year over the last five years; lots of these consumers believe that administering antibiotics to animals in feed can reduce the effectiveness of these drugs in humans [19]. Another important aspect that can lead the consumer to choose an organic product rather than a conventional one regards the environmental impact. Several recent studies confirm people belief in healthier properties of food from organic agriculture as consequence of the environmental-friendly management [20,21].

Nevertheless, even today there are some limitations to organic consumption, as high price of the products, lack of consumer confidence and insufficient marketing effort. The price is a very important factor for consumer; several studies have found out that high price is an important barrier to organic consumption, but at the same time some consumers tend to value organic food more than conventional one just because of higher prices and limited availability [22].

Going into detail to the chicken meat production systems, it is important to underline the main differences between organic and traditional farming. The main difference regards the space available to the animal. Conventionally reared poultry, grow in indoor pen. Here, humidity and temperature levels are constantly monitored and also cyclical rhythms are regulated (usually 6 hours of darkness per day). Also daily water and feed are controlled in order to verify also the weight of the animal. There are also ventilation systems to allow air circulation in order to limit risks for the animals. These expedients permit to ensure a good welfare state of animals even if chickens complete their life inside the shed. On the contrary, organically reared poultry stay in open air and can enjoy a grass paddock. In organic farms, animal welfare is guaranteed not only inside the shed, but also by the possibility of going outside. The organic chicken can choose how long it wants to stay outside or inside. Moreover,

in order to avoid the use of intensive rearing methods, organic poultry are reared until they reach a minimum slaughter age of 81 days [23].

The different housing methods determine the animals welfare and as consequence also the quality of the meat. A free-range (extensive) system results in more favourable broiler meat quality traits, primarily in chemical composition [24]. Unfortunately, there are only few studies about the comparison between organic and conventional chicken meat. The most investigated parameters regard water holding capacity (WHC), pH, color, shear force, fatty acid composition and cooking loss. In a normal living muscle, pH is approximately 7.2. Glycogen is broken down to lactic acid after slaughter, so ultimate pH (pHu) is an important parameter determined by pH decline during 24 hours after slaughter. A low pHu decreseas water holding capacity by proteins and determines lighter color of the meat (**Figure 5**).

Figure 5. Examples of chicken breast fillets having different pH [25].

Castellini et al. [26] found that organic chicken meat has a slightly lower pHu value than conventional chicken meat. This could be due to a lower consumption of glycogen because of reduced pre-slaughter stress of animals kept in organic farmings. Another important quality parameter that could influence consumer decisions is the color of the meat. Skin and breast color can be different according to housing system: organic meat seems to be less red but more yellow than the others [27]. Shear force is an indicator of meat tenderness. Organic meat seems to show higher shear force than traditional one. The muscular tissue could be more difficult to tenderize during post-mortem storage because of the slower daily growth rate of the organic chickens. By the way, also other factors (genetics, slaughter age and slaughter procedures) can interfere in determining the final meat tenderness [28]. At last, fatty acid composition influences flavor, color, shelf-life and organoleptic characteristics of meat. High consumption of roughage and fresh grass, typically of organic feeding, leads to higher content of unsaturated fatty acids in muscle [29], as it has been observed in different types of organic meats: beef cattle [29], lambs [30], free-range reared pigs [31], broilers [26] and rabbits [32].

2.1.3 Shelf-life of chicken meat

Shelf-life is defined as "the time, under defined storage conditions, during which food remains safe, retains desired sensory, chemical, physical and biological characteristics as well as it complies with any label declaration" [33]. Shelf-life of food products highly depends on different factors: packaging (air, vacuum or modified atmosphere), product composition (fat content, sodium chloride content, nitrites, water activity, pH), storage temperature and other extrinsic factors (antibacterial substances or biopreservatives) [33].

Oxidative reactions are the most frequent processes that lead to the end of food shelf-life. The shelf-life highly depends on the oxidation rate and the acceptability limit of food. For this reason, it is essential to identify oxidative indices easily measurable and correlated to sensory perception. **Table 4** shows the principal indicators to monitor the oxidation development during food storage.

Table 4. Main indicators of oxidative reactions in foods potentially correlating with consumer rejection [33].

Compounds undergoing oxidation	**Indicator**
Unsaturated fatty acids	Peroxide value
	Conjugated dienes (CD)
	Volatile organic compounds (VOCs)
	Anisidine value
	Thiobarbituric acid index (TBA)
	Hydrocarbons and fluorescent products
	Sensory attributes (off-flavor, off-odours)
Pigments	Color
	Selected compounds (i.e., carotene, lycopene)
	Sensory attributes (color fading, off-color)

The techniques for lipid oxidation monitoring permit the evaluation of both primary and secondary oxidation products. Aldehydes, ketones, alcohols and hydrocarbons are odorous and aromatic compounds that can be good predictors of consumer acceptability. Consumer rejection could be also related to some compounds deriving from other compounds than fat and lipids, such as pigments. Food color, in fact, is the quickest and easiest indicator of consumer acceptability [33].

Meat, in particular poultry meat, is one of the most perishable food, because of its high water, protein and fat content, relative high pH and the consequent possibility of microbial growth [34]. Shelf-life is also the results of poultry management, processing, distribution and storage conditions both on the market and in the consumers' homes [35]. Microbial spoilage is actually the most common cause of alterations in food quality and meat spoilage is usually due to microorganisms proliferation. Usually *Brochothrix thermosphacta*, *Lactic acid bacteria*, *Enterobacteriaceae Pseudomonas* spp. and *Aeromonas* spp. are the species responsible for poultry meat spoilage [36]. The preservation of raw meat depends on hygiene, type of packaging and the temperature during processing, storage and distribution of the product. These three factors are equally important and they are strictly correlated.

2.1.4 Packaging of poultry meat products

Packaging plays a significant role in enhancing the quality and safety of food. With the need from retailers to extend the shelf-life in cost-effective way and to meet the consumers expectations, the food packaging industry has been tremendously growing with many innovations.

The most commonly used primary packaging for poultry meat is a polymer film wrap or overwrap or a composite layer containing paper, foil, and cellophane [37]. Because of the higher and higher demand of ready food products, the cutting and wrapping of meat in paper or waxed paper by butchers have been replaced by store cutting and the packages in refrigerated self-service display cases [36].

Some of the current methods for fresh poultry packaging are trays wrapped using flexible packaging film for whole birds or portioned poultry or flexible packaging films wrapped around the meat without a tray [38]. In case of ready to eat chicken meat products, some paper bags and folding cartons with windowed portions (in addition to trays and plastic films) are widespread. However, trays with overwrap packaging is the most used method for fresh chicken meat packaging [38].

Modern packaging techniques for meat products includes also vacuum packaging (VP) or modified atmosphere packaging (MAP), which permit to control the gaseous environment within the package and the growth of certain type of microorganisms. Other innovative types of packaging are active (for example antimicrobial packaging) or intelligent packaging [38].

2.1.4.1 Modified Atmosphere Packaging (MAP)

MAP technology consists in the removal and/or replacement of the atmosphere surrounding the product before sealing, using primarily vapor barrier polymers. The MAP can be vacuum packaging, so the removal of most of the air before the sealing of the product in a barrier polymer, and controlled

atmosphere packaging (CAP), where the internal atmosphere is modified and controlled to maintain a stable level of gases, temperature and humidity within the packaging [38]. MAP technology in the preservation of meat products has been widely studied and significant advance in shelf-life extensions have been observed with respect to normal air packaging [38]. Nitrogen (N_2), carbon dioxide (CO_2) and oxygen (O_2) are the three mostly used gases in MAP, even if some studies investigated the use of CO [39,40] and argon as an alternative to N_2 [41]. Nitrogen is an inert gas and its primary function is to replace oxygen. It has no antimicrobial activity and when it is used together with CO_2, nitrogen prevents package from collapse. On the contrary, carbon dioxide is important for its antimicrobial activity; the fact that CO_2-enriched atmosphere extends shelf-life of food has been well established [38]. Oxygen highly influences the shelf-life of food products including meat. For example, it is used to maintain red color of the fresh meat through the formation of oxymyoglobin. Color is not so important in poultry meat as in beef meat. Some studies revealed that the color of poultry meat is not so stable in atmosphere rich in O_2 [42]. So, packaging in high oxygen atmosphere is appropriate for myoglobin rich meat to get an attractive color. Comparing the high oxygen MAP and the oxygen-free MAP, the second one has a higher efficiency in the protection of freshness characteristics of chicken meat, preferable for the packaging of chicken meat [43].

2.1.4.2 Active Packaging technologies

Active packaging (AC) is one of the most innovative packaging systems that allows the product to interact with the surrounding environment in order to extend the shelf-life and to ensure microbial safety and quality of the product. AC technology modifies the gas environment by removing or adding gases in the packaging. The internal atmosphere is controlled by specific substances, that absorb or release gases or vapours. AC is usually used in sachets and pads placed inside the package and active ingredients (oxygen, carbon dioxide, moisture, ethylene, and flavor absorbers or ethanol, carbon dioxide, and preservative emitters) are incorporated directly into packaging materials. There are also self-heating, self-cooling packages and UV- and surface-treated packages [44]. Among AC, antimicrobial packaging is an excellent method in achieving the safety of food products. In this type of technology, agents may be coated or incorporated onto a packaging material. Different types of antimicrobial agents such as silver ions, sorbates, nitrites, organic acids or phytochemicals have been investigated for their efficacy. The objective of the last years is to develop “green” packaging by incorporating bioactive antimicrobial substances into bio-based polymers to enhance sustainability of food packages [45].

2.1.4.3 Intelligent Packaging

Intelligent packaging is a packaging system able to detect any deterioration inside the food package. It permits to enhance food safety and quality and to warn about possible problems during transport and storage. Specific indicators are placed inside and outside the package to monitor the interaction between the food and the material. The most used data carriers are bar code labels and RFID tags, while the commonest package indicators are biosensors, gas or time-temperature indicators [46]. Since poultry meat is a highly perishable product, the application of intelligent packaging system in this field can have an important impact.

2.1.4.4 Biodegradable packaging

By 2050, around 2.2 billion additional people will need to be fed and there will be also an increase in living standards. It results in food demand of more than 50%. This situation needs a better use of agricultural lands and also an optimization of the conservation of raw and finished food products [47]. It is important the use of a packaging capable of protecting food, ensuring long shelf life and together preserving the environment and health. Plastic materials have become predominant in the food packaging sector, from fresh to frozen products, from dairy to meat products, fruit and vegetables too. Indeed, the production of packaging plastic polymers has been growing for 40 years, passing from 50 million tons in the Eighties to more than 360 million tons in 2019 [48]. The packaging waste produced per capita has increased during the last decades, rising to 32.7 kg per capita in 2017 in Europe. On the other side, the amount of plastic packaging waste destined to recycling has increased. Unfortunately, recycled plastic amount is still much less than the amount produced during the same period [47]. There are two main directions for reducing the impact of packaging on the environment: reusable packaging or biodegradable packaging. Different compounds from waste recovery (especially agro-food wastes) can be exploited as packaging materials, such as biopolymers, fibers, nanoparticles, inorganic compounds or bioactive components. Biopolymers are the most used and basic component for packaging, forming its matrix. The other constituents are usually considered as additives (active agents, plasticizers, etc.) or fillers (fibers). **Table 5** summarizes the most common compounds recovered from agro-food waste, their origin and their function in packaging.

Table 5. Potential compounds recovered from agro-food sectors for packaging applications.

Packaging components	Properties for packaging applications	Nature	Origin
Polymers and biopolymers	Structural properties, continuous structure, and network Transfer and migration control	PHA, PHB, PHBV PLA Cellulose Chitin/Chitosan Starch Collagen/Gelatins Caseins Corn zein, etc.	Seafood and fish, milk and dairy, cereal, meat, sugar industrie. Exhaust water from food industry
Fibers	Fillers, structural reinforcer, barrier properties, encapsulation matrix, moisture and fogging control	Cellulose Lignin Powders of fruits stones, pits, or shells Bran, husk	Cereal crops, Sugar cane Fruits
Nanoparticles	Fillers, barrier and mechanical properties, active properties, encapsulation support	Cellulose nano whiskers Lignin Protein or polysaccharide nano- and micro-objects or beads (gelatin, pectin, chitosan, etc.)	Cereal industry, crop productions Seafood, fish, and meat industries Paper industry
Bioactive compounds	Antimicrobial, antifungal, antioxidant, antibrowning, antitumoral properties	Phenolic compounds, Lignin Essential oils Enzymes, peptides, protein hydrolysates	Food, agro, seafood and fish, meat, and milk industries Paper and wood industries
Additives	Plasticizers Antistatic, wetting agent	Polyols, fatty esters, emulsifiers	Oil and soap industry Seafood and fish industries

Inorganic compounds	Light or oxygen barrier, Inert fillers	Calcium, sodium, potassium carbonates, calcite, zeolites	Husks from cereals Fish scales and bones Mammalian bones

Biodegradable food packaging was the first commercialized bioplastic product which is certified as compostable. Flexible packaging uses biodegradable polymers for the largest part [49].

Biopolymer based packaging materials are classified into three main categories depending on their method of production and origin:

1. Biopolymers derived by concentration from biomass (polysaccharides, e.g., starch, cellulose, chitin, chitosan and pectin or proteins such as collagen, gelatin, casein and gluten). It is the most promising group and many starch-based films are already on the market.
2. Biopolymers obtained by chemical synthesis from monomers, such as carbohydrate fermentation. The most developed and commercialized biopolymer is polylactide (PLA).
3. Biopolymers produced by the activity of microorganisms. They are mainly polyesters such as polyhydroxyalkanoates (PHAs), polyhydroxybutyrate (PHB), polyhydroxybutylvalerate (PHBV). Most materials based on bacterial cellulose are under development and their cost is a limit for their industrial production.

Biodegradable packaging is increasingly used for modified atmosphere packaging, active packaging systems and edible packaging for many different food products (fruits, vegetables, raw meat, etc.) to enhance their shelf life, increasing the overall environmental sustainability [49]. Before adopting any packaging for food preservation it is important to make proper studies on the interaction between biopolymers and food components during the production and the storage period.

2.1.5 Volatile organic compounds as meat shelf-life indicators

Fat, proteins, carbohydrates, minerals and water are the main constituents of meat. Meat quality deteriorates mainly due to microbial spoilage or fat oxidation. In particular, there are three main pathways for meat spoilage after slaughtering and during processing and storage: lipid oxidation, microbial spoilage and autolytic enzymatic spoilage. All these reactions result in the production of new compounds, causing some modifications of meat flavor, juiciness, tenderness, texture and odor. For these reasons, VOCs are known to be good indicators of the spoilage state of food and in particular of meat products. For instance, Zareian et al. [50] evaluated VOCs released in MAP by minced raw

pork meat in relation to its shelf-life. Other studies investigated VOCs for the evaluation of shelf-life of poultry meat [51,52,53]. The typical VOCs associated with off-odors in poultry meat have been reported by Casaburi et al. [54]. The most common compounds are aldehydes (hexanal, heptanal or octanal), alcohols (ethanol, 2-propanol and butanol), ketones (acetoin and diacetyl), short chain fatty acids (acetic and butyric acid) and sulfur containing compounds (dimethyl sulfide, dimethyl disulfide, cardon disulfide or hydrogen sulfide). The generation of sulfur compounds is related with the growth of bacteria, mainly with the microbial decomposition of sulphur containing amino acids (cysteine and methionine). Mikš-Krajnik et al. [55] suggested alcohols (ethanol and 3-methyl-1-butanol) and short chain fatty acids (acetic acid) as possible markers for early spoilage detection of chicken meat, while sulphides for secondary stage spoilage. Alcohols and fatty acids can derive from catabolism via oxidation or via glycolytic pathway of the primary products of the microbial degradation, such as glucose and lactic acid. Most of the studies present in literature focused on the investigation of VOCs released from raw poultry meat stored in air [55, 56, 57, 58, 59] or under vacuum packaging [60]. Only few studies investigated VOCs developed by poultry meat stored in MAP [61, 62]. The study of Casaburi [54] is interesting from this point of view, because it evaluated the volatilome composition of meat stored in air, under vacuum and in modified atmosphere packaging. **Figure 6** reports the main VOCs and related odors developed by raw meat under vacuum conditions and during air storage, according to the days of storage [54]. VOCs found during aerobic chill storage of naturally contaminated or artificially inoculated fresh meat are: esters, ketones, aldehydes, branched chain alcohols, organic acids and sulphur containing compounds and branched chain alcohols. Among them the most abundant were ethyl esters, sulphur compounds (dimethylsulphide, dimethyldisulphide, dimethyltrisulphide and methyl thioacetate) and alcohols (3-methyl-1-butanol, 1-octen-3-ol, 1-hexanol and 2-ethyl-1-hexanol). In meat in VP it is possible to detect the same classes of compounds, even if at different concentration. Esters in VP were lower compared to air storage. Alcohols were the most abundant species and they are present at higher levels respect to the number detected in air. Aldehydes usually found in VP are hexanal, nonanal, decanal, tetradecanal and benzaldehyde, while the major ketones are acetoin, 2-butanone, propanone and 2-decanone. The presence of organic acids (butanoic, hexanoic and nonanoic) are related to the presence of specific bacteria in VP (*Clostridia*, *Serratia spp*., *Pseudomonans spp*., *Carnobacterium spp*. and other *Lactobacilli*), while sulfur compounds are more abundant at the first days of spoilage in VP.

There is less information on VOCs associated with storage in MAP, even if the most abundant compounds detected during MAP storage are acetoin, hexanal, butanoic acid, hexanoic acid, ethyloctanoate, dimethylsulfide, 1-octen-3-ol and 3-methyl-1-butanol [54].

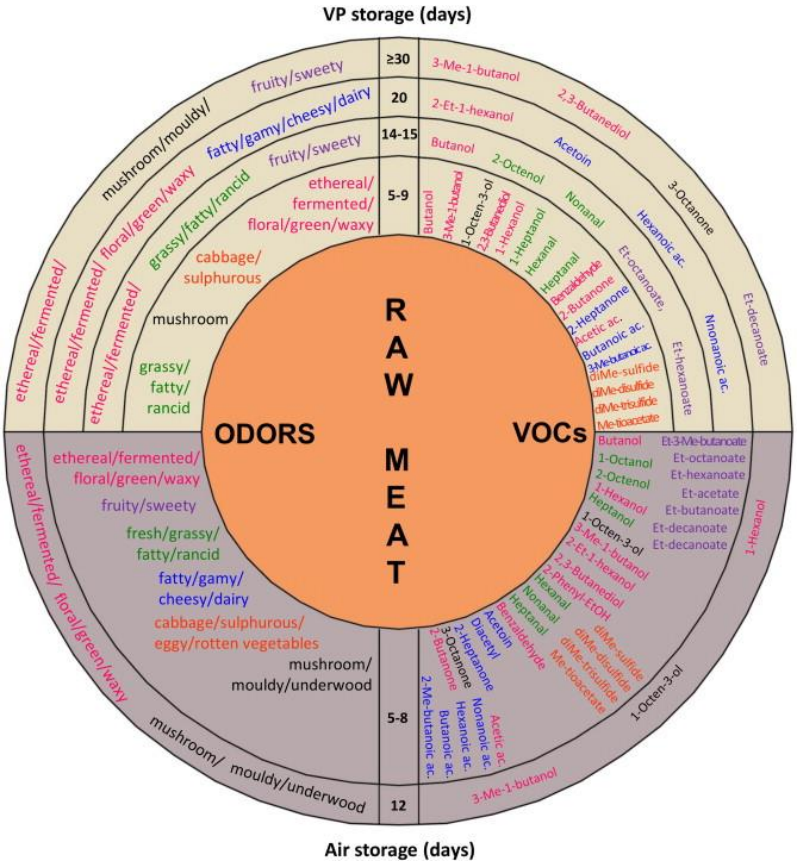

Figure 6. Meat spoilage aroma wheel: schematic representation of volatile fraction evolution during storage of meat in air (lower part of the wheel) and VP (upper part of the wheel). On the right side of the wheel VOCs occurring at different times of storage are reported in the corresponding sections. Accordingly, on the left side, the odor descriptors corresponding to the VOCs at different times of storage are represented. In the early storage times, all the detected VOCs are listed, whereas only VOCs whose concentration usually rises during storage are reported at later times. Different colors are used to associate molecules with their corresponding odor descriptors [54].

Analysis of VOCs may provide important information about the hygienic and sensory quality of packaged food. As seen in many studies, they can be exploited for the identification of microorganisms in packaged meat, such as *Escherichia Coli*, *Enterobacteriaceae*, *Pseudomonas* and *Lactic acid bacteria* (LAB). LAB were found to be prevalent in MAP, causing "souring", which is less invasive than aerobic putrefaction [52]. Under vacuum packaging some typical spoilage bacteria, such as *Pseudomas*, are highly reduced, because the oxygen supply is restricted and this change has a selective effect on the microbial population [63].

Currently, one of the most common methods for the determination of meat spoilage is the analysis of total viable bacteria counts (TVC), but this technique needs an incubation time of 72 h for colony formation. On the contrary, the analysis of VOCs in meat sample is a more rapid and easy method. It

can be performed by identification and quantification of VOCs by GC-MS. The previous extraction step is often performed by SPME, because it is rapid, inexpensive and solvent-less technique. It enables VOCs extraction and concentration in the headspace of the sample into one step, without affecting the chemical composition of the food matrix [64]. Thus, SPME sampling followed by GC-MS analysis can be a powerful tool to assess the spoilage level of food.

2.1.6 Aim of the work

This study comes from a collaboration between University of Camerino and Fileni® S.p.A. (Cingoli, Italy). The company was born in 1965 with the founder Giovanni Fileni. He started working at the first farmyard animal husbandry system in Marche region countryside. In 1967 he opened the first shop for direct sale of chickens. The first slaughterhouse was built in 1968, starting also the sale of ready-to-eat products. At the end of the Eighties the company made a transition from retail to large-scale distribution. In 1989 the first factory for ready-made products was opened in Cingoli. Today, Fileni is the third national player in the poultry meat industry and the first Italian producer of organic agriculture white meat.

The enormous use of traditional packaging is a serious ecological concern leading to a shift to biodegradable and compostable materials. The first aim of this work is to study the efficiency of a new biopackaging (BP), made of biodegradable and compostable polyesters to preserve the quality of chicken breast meat. The meat was analyzed during 14 days of storage and the biopackaging's performance was compared with that of a conventional polyethylene terephtalate (PET) packaging. After slaughtering, the meat quality can deteriorate because of microbial spoilage, the presence of digestive enzymes and lipid oxidation. The reactions occurring, can lead to pH and appearance changes, slime formation, structural component degradation and volatile organic compounds (VOCs) development. For this reason, VOCs were exploited as indicators of meat spoilage. HS-SPME-GC-MS analysis was exploited to assess the spoilage level of meat. Over the last decades, also the attention towards organic production has notably increased. Consumers demand products with high levels of quality and safety. In literature, there is only little information about possible differences between organic and conventional chicken meat. The most investigated parameters are WHC, pH, color, shear force, cooking loss and fatty acid composition. For these reasons, this work aimed also to compare chicken breast meat reared with organic methods (OC) with conventional chicken meat (CC). The 10-day shelf-life of the two types of meat was monitored analyzing the development of characteristic VOCs. The study of shelf-life of chicken meat was completed through the monitoring of biogenic amines (BAs), microbiological markers, pH and sensorial parameters performed by

research groups of the School of Pharmacy and the School of Bioscience and Veterinary Medicine of University of Camerino, participating in the project.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Acetone (≥99,5% CAS No. 67-64-1) and 3-octanol (≥ 97%, CAS No. 589-98-0) were supplied by Sigma-Aldrich (Milano, Italy).

2.2.2 Comparison of packaging materials

The studied biopackaging (BP) includes a completely biodegradable and compostable tray and film (**Figure 7**). The material used for BP is provided by Novamont (Novara, Italy). It is obtained by means of Novamont's proprietary technologies, using bio polyesters obtained by polycondensations of diacids and diol. As a result, a compostable multilayer structure constituted by Novamont's bio-polymers is obtained. The geometry of the product is:

- film: weight 1,5 g, thickness 39 µm, 23 cm x 14,5 cm;
- tray: weight 23,5 g, thickness 600-700 µm; 23 cm x 14,5 cm x 4 cm.

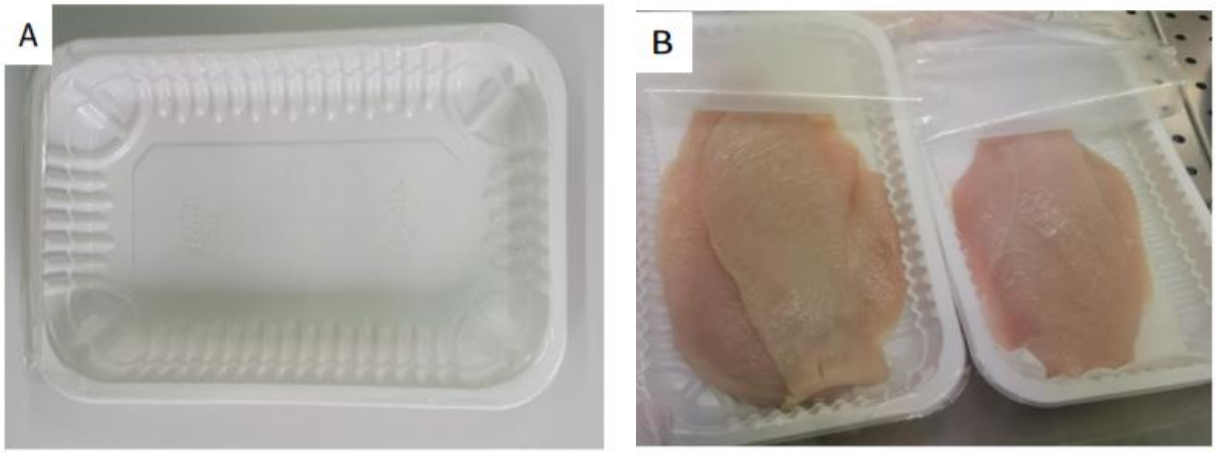

Figure 7. Biopackaging (BP) without (A) and with chicken meat (B).

The conventional packaging used for comparison consists of a traditional polyethylene terephthalate (PET) packaging (PET-EL L1523-27 TR1160000 O2WH, Linpac Packaging Pravia, Spain) combined with a cryovack film (LID 830X, Sealed Air Food Care, Charlotte, USA).

The analyzed samples were provided by Fileni® industry (Cingoli, Italy). They consisted of fresh organic chicken breast meat packaged with new BP composed by biodegradable and compostable

tray and film. The same fresh organic chicken breast meat was wrapped with PET packaging combined with a cryovack film. All samples were wrapped in modified atmosphere (MAP) (70% O_2, 20% CO_2, and 10% N_2). Analyses were performed on chicken breast meat at different days: 0, day 3, day 6, day 10, and day 14; every time a new package was opened. During the storage period, samples were held at 4 °C to simulate the consumer storage conditions. All analyses were performed in triplicate.

2.2.3 Comparison of organic and traditional chicken meat

Organic (OC) and conventional (CC) *Aviagen Ross 308* breast meat were provided by Fileni® industry (Cingoli, Italy). Each chicken breast was wrapped in common sheet, which is a composite layer containing paper, foil and cellophane. Analysis were performed on chicken breasts after 0, 3, 6 and 10 days of storage. Every day two new breasts for each type were minced together and analyzed. During the analysis period, samples were held at 4 °C in the dark. All analysis were performed in triplicate.

2.2.4 HS-SPME-GC-MS analysis of volatile organic compounds

An aliquot of 5 g of chicken breast meat was finely minced and homogenized for 30 sec in an analytical blender (Tube Mill Control, IKA-Werke GmbH & Co. KG, Germany). Then, 2 g of the sample was weighted in 10 mL vial with a perforable septum and conditioned at 40 °C for 20 min (**Figure 8**).

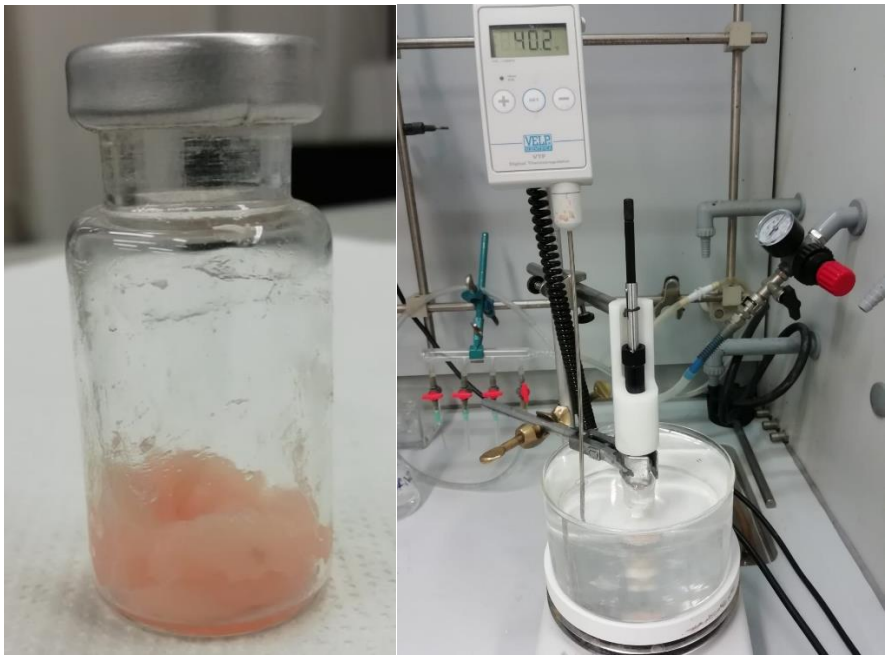

Figure 8. Head-space-SPME-GC-MS (HS-SPME-GC-MS) system for the analysis of VOCs developed during the storage of chicken breast meat.

The SPME fiber assembly was from Supelco (Bellefonte, PA, USA) and had a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coating with 1 cm length stationary phase. The fiber was chosen after the study of the literature. In fact, it showed higher sorption capacity compared to PDMS/DVB and PA fibers; it was very sensitive also to small molecules and possess greater affinity for aromatic compounds, aliphatic hydrocarbons and alcohols than CAR/PDMS and PDMS [65]. Then, the fiber was exposed in the headspace of the vial containing the sample for 30 min. Volatile organic compounds (VOCs) were analyzed by GC-MS using a 6890N Network GC System coupled to a 5973 Network Mass Selective Detector both from Agilent Technologies (Santa Clara, CA, USA). A capillary column coated with polyethylene glycol (60 m x 0.25 mm x 0.25 µm film thickness, DB-WAX, Agilent Technologies, Santa Clara, CA, USA) was used. The initial carrier gas (helium) flow rate was 1.2 mL min^{-1} . Injector temperature was 260 °C, splitless time was 4 min. The oven temperature was held at 35°C for 4 min, then raised to 120 °C at 2.5°C min^{-1} and then went up to 250°C at 15 °C min^{-1} and held at this final temperature for 3.3 min, for a total run time of 50 min. Mass analysis was performed in scan mode in the range of 25-400 Da. The transfer line was maintained at 260°C, ion source at 230°C, and quadrupole at 150°C. The SPME fiber was left exposed in the injector for 10 min to be cleaned after desorption and reactivated. Straight chain alkanes were used to calculate retention indices. Thus, the detected VOCs were identified by comparing their retention indices and mass spectra with those of standards from the US National Institute of Standards and Technology database (NIST-USA, http://webbook.nist.gov). An external standard (3-octanol in acetone, 0.5 mg mL^{-1}) was used to control the repeatability of the method. A proper aliquot of the standard solution (10 µL) was put in 10 mL vial and analyzed in duplicate each day of analysis under the same conditions reported above.

2.2.5 Statistical analysis

Significant differences between the storage in the two different packagings (BP and PET) and between the different times of analysis were evaluated by one-way analysis of variance (ANOVA). Also significant differences between the two types of chicken meat (organic OC and conventional CC) at different storage days were investigated. Differences with $P<0.05$ were considered statistically significant. Data elaboration was carried out using the PAST software package [66]. Each experiment was performed in triplicate.

2.3 Results and Discussion

2.3.1 Comparison of packaging materials

The use in food packaging of compostable and biodegradable materials, such as biopolymers, represents a good opportunity for reducing the amount of plastic wastes. Biopolymers are produced from renewable resources and are characterized by a relatively high permeability to water and oxygen. For this reason, their utilization in food packaging has to be deeply investigated. In this study the shelf-life of chicken breast meat packaged in biopackaging (BP) was compared to the same meat stored in conventional packaging (PET). The development of VOCs was monitored during storage time, in conjunction with microbiological and sensorial changes. Also some chemical metabolites produced in the microbiological deterioration of the food products, such as biogenic amines (BAs) were considered as quality indicators of chicken meat.

In this section, the results obtained from VOCs analysis are shown. The figures below show the chromatograms obtained from the analysis of the chicken meat during time stored in PET and in biopackaging (**Figures 9** and **10**, respectively).

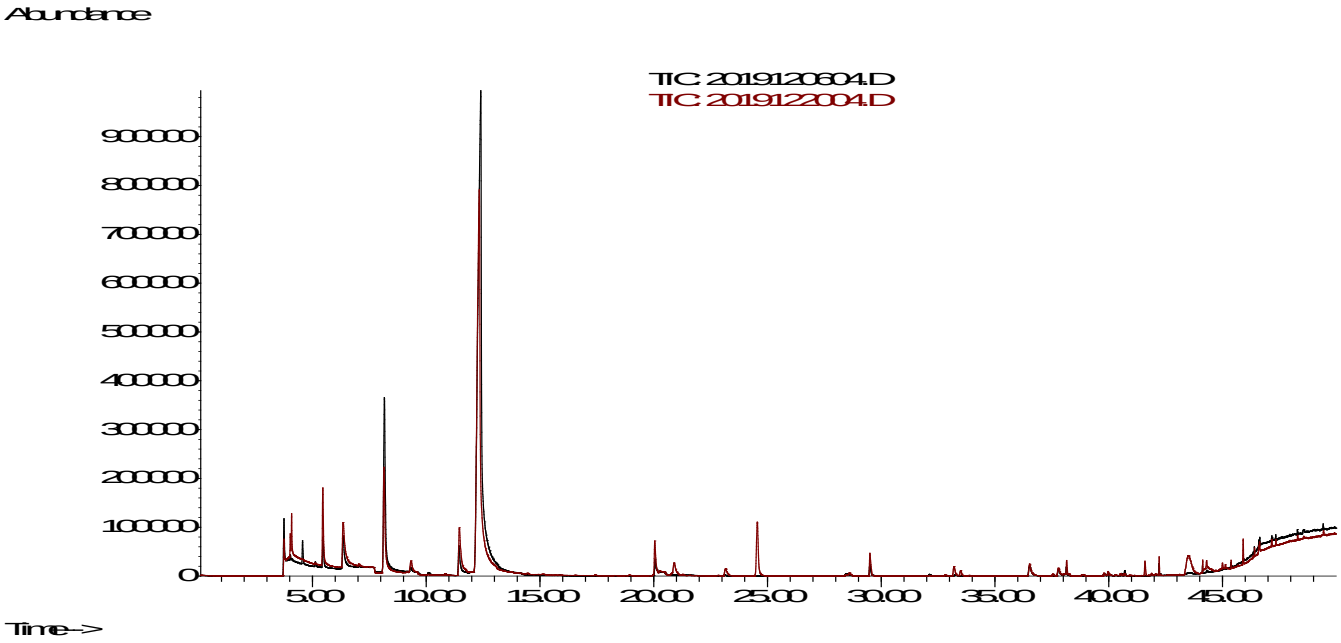

Figure 9. Chromatograms obtained from the analysis of chicken breast meat stored in conventional packaging after 0 (black line) and 14 (red line) days of storage.

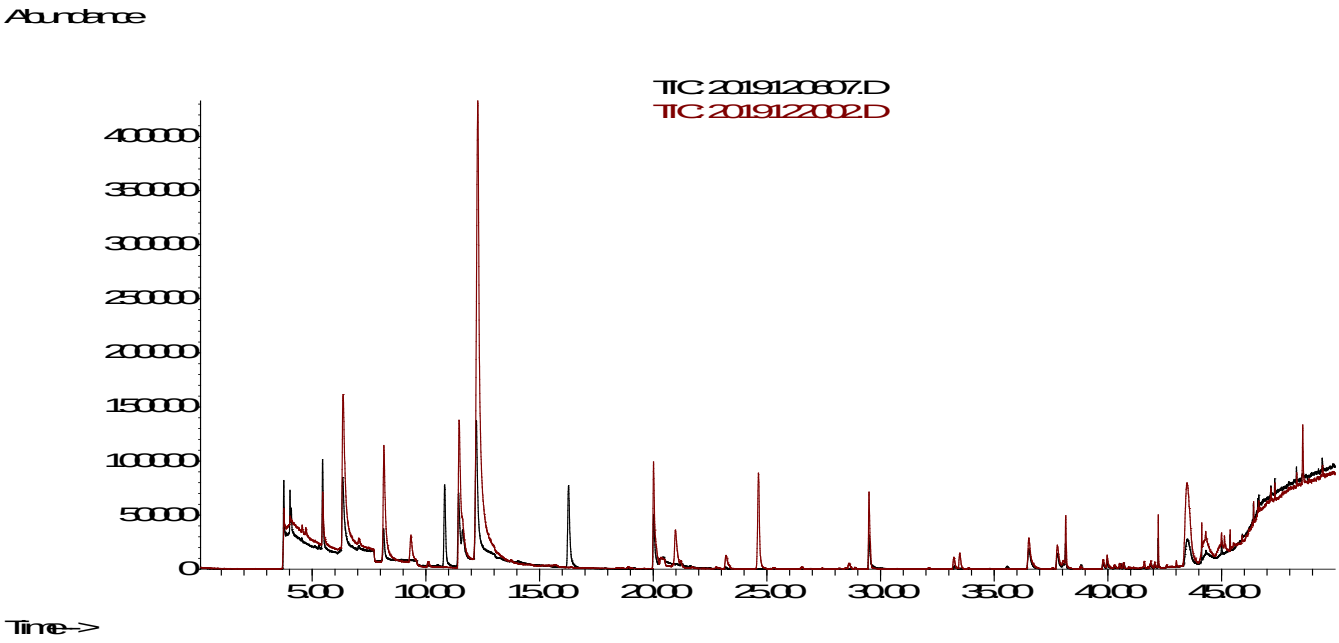

Figure 10. Chromatograms obtained from the analysis of chicken breast meat stored in biopackaging after 0 (black line) and 14 (red line) days of storage.

A total of 18 VOCs were identified in chicken meat stored in BP and PET packaging. The analytes were divided according to their chemical nature into: alcohols and phenols, organic acids, ketones and sulfur containing compounds. During the storage period meat was held at 4°C to simulate the consumer storage conditions.

The peak area of each compound in both types of packaging was determined during time and the results are listed in **Table 6**.

Table 6. Volatile compounds detected by HS-SPME-GC-MS during the storage of chicken breast meat in BP and PET packaging, their experimental linear retention indices (LRI) on a polyethyleneglycol coated column, their odor attribute, their abundances in terms of peak areas and % relative standard deviation (RSD, *n*=2). Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk. Significant differences ($P < 0.05$) between the different days for each of the two packaging are indicated by different letters in the same row.

Compound	Odor attribute	LRI[1] (exptl)	LRI[2] (lit)		Day of storage 0		3		6		9		14		SI[3] (%)
					Area	RSD%	Area	RSD%	Area	RSD%	Area	RSD%	Area	RSD%	
Alcohols and phenols															
Isopropyl alcohol	Musty	929	926	BP	5,04E+06^{a} *	8.0	4,73E+06^{a} *	4.3	2,14E+06^{b} *	7.8	2,38E+06^{b} *	0.3	7,22E+06^{c} *	3.0	80
				PET	1,93E+07^{a}	4.5	2,07E+07^{a}	1.6	6,47E+06^{b}	8.9	1,48E+07^{c}	14.8	1,18E+07^{c}	4.3	
1-Propanol	Musty	1038	1036	BP	2,86E+07^{a} *	8.5	5,13E+07^{b} *	0.7	1,49E+07^{c} *	10.1	1,20E+07^{c} *	11.0	4,25E+07^{d} *	2.3	86
				PET	1,24E+08^{a}	1.1	1,27E+08^{a}	0.8	5,96E+07^{b,d}	11.9	9,04E+07^{c,d}	10.5	8,08E+07^{d}	2.1	
3-Methyl-1-butanol	Roasted	1213	1208	BP	nd	nd	nd	nd	nd	nd	4,70E+05^{a}	27.6	2,08E+06^{b}	17.9	80
				PET	nd	nd	nd	nd	nd	nd	8,46E+05	29.3	1,63E+06	6.4	
1-Pentanol	Fusel	1254	1252	BP	1,08E+05^{a}	10.5	1,57E+05^{a} *	1.7	6,64E+05^{b}	9.7	8,68E+05^{c}	1.6	9,56E+05^{c}	5.2	88
				PET	1,58E+05^{a}	12.1	6,97E+05^{b}	18.3	1,02E+06^{b}	11.4	7,72E+05^{b}	14.2	1,03E+06^{b}	6.6	
1-Hexanol	Fruity	1356	1354	BP	nd	nd	nd	nd	2,72E+05^{a}	2.0	1,77E+05^{b}	2.0	2,92E+05^{a}	11.4	82
				PET	nd	nd	3,33E+05	11.1	2,61E+05	0.9	2,97E+05	22.3	3,66E+05	14.7	
1-Octen-3-ol	Earthy	1451	1449	BP	nd	nd	nd	nd	1,92E+05^{a}	20.1	3,63E+05^{b}	5.4	7,66E+05^{c} *	2.1	83
				PET	nd	nd	1,57E+05^{a}	26.0	3,25E+05^{a,b}	17.9	4,76E+05^{b}	15.9	5,27E+05^{b}	14.7	
1-Octanol	Waxy	1566	1565	BP	nd	nd	nd	nd	nd	nd	nd	nd	3,15E+05*	33.3	85
				PET	nd	nd	nd	nd	nd	nd	nd	nd	9,48E+05	7.8	
Phenol	Sweet	1998	1996	BP	1.6E+05	10.4	nd	nd	1,29E+05	2.1	1,08E+05	16.8	1,30E+05 *	22.3	83
				PET	1,23E+05^{a}	28.7	2,41E+05^{a}	21.8	nd	nd	2,82E+05^{a}	30.9	1,02E+06^{b}	2.5	
Ketones															
2-Butanone	Fruity	892	894	BP	3,76E+05^{a}	22.7	2,82E+05^{a}	13.8	8,70E+05^{b} *	3.7	7,89E+05^{b} *	0.5	2,99E+05^{a}	18.8	82
				PET	nd	nd	2.75E+05^{a}	5.1	3,64E+05^{a,b}	8.4	3,96E+05^{b}	11.9	1,31E+05^{c}	4.4	

2,3-Butanedione	Buttery	966	970	BP	nd	nd	7,87E+05[a]	25.3	3,25E+05[b]	1.5	6,64E+05[a,b]	5.1	1,54E+06[c]	4.3	81
				PET	3.75E+05[a]	30.1	1,45E+06[b]	21.6	2,70E+05[a,c]	30.2	1,04E+06[a,b]	23.4	1,71E+06[b,d]	7.6	
3-Hydroxy-2-butanone	Creamy	1280	1278	BP	nd	nd	nd	nd	7,88E+05[a]	1.2	1,80E+06[b] *	6.7	4,96E+06[c] *	3.2	84
				PET	nd	nd	nd	nd	nd	nd	1,11E+06[a]	6.7	6,41E+06[b]	3.8	
	Acids														
Acetic acid	Vinegar	1446	1453	BP	1,84E+05[a]	25.3	1,52E+05[a]	4.7	4,02E+05[a,b]	23.5	2,64E+05[a]	14.6	7,36E+05[b]	23.3	90
				PET	nd	nd	1,66E+05[a]	7.0	2,82E+05[a,b]	6.6	3,08E+05[b]	17.1	1,12E+06[c]	0.8	
Propanoic acid	Pungent	1539	1538	BP	nd	nd	7,68E+05[a] *	6.3	6,96E+04[b]	23.8	nd	nd	8,75E+04[b] *	35.2	89
				PET	nd	nd	9,55E+05[a]	2.2	6,33E+04[b]	13.3	2,98E+05[c]	5.6	2,22E+05[d]	3.6	
Isovaleric acid	Cheesy	1670	1670	BP	nd	nd	nd	nd	6,22E+05	28.0	6,43E+05 *	12.6	2,41E+05 *	16.8	83
				PET	nd	nd	nd	nd	1,41E+05[a]	4.7	4,00E+06[b]	8.5	9,29E+05	4.0	
Hexanoic acid	Sour	1843	1839	BP	1,62E+05[a,b]	4.5	1,49E+05[a,b]	2.3	2,84E+05[a]	12.5	1,33E+05[b]	12.8	1,79E+05[a,b]	34.3	80
				PET	1,55E+05[a]	16.7	nd	nd	2,12E+05[a,b]	1.3	2,63E+05[a,b]	22.3	3,32E+05[b]	11.6	
Nonanoic acid	Rancid	2161	2168	BP	2,25E+05	28.3	1,86E+05	30.1	3,32E+05	21.9	2,19E+05	14.2	2.74E+05	21.9	81
				PET	2,19E+05	31.1	2.25E+05	12.8	2,85E+05	9.3	2,06E+05	17.8	2,76E+05	18.2	
	Sulfur containing compounds														
Carbon disulfide	Ether-like	714	710	BP	2.27E+05[a]	31.8	2,05E+05[a]	8.9	2,00E+06[b]	33.0	8.57E+05[a,b]	16.1	2,05E+05[a]	28.9	94
				PET	1,15E+06[a,b]	11.0	1,95E+05[a]	1.7	2,14E+06[b]	38.9	7,66E+05[a,b]	22.6	nd	nd	
Dimethyl sulfone	Cabbage-like	1904	1911	BP	4,11E+05[a] *	8.6	9,78E+04[b]	14.2	1,16E+05[b,c]	39.3	1,72E+05[b,c]	11.7	2,40E+05[c]	13.6	96
				PET	5,38E+04[a]	7.1	1,14E+05[a]	26.7	3,68E+05[b]	27.4	8,17E+04[a]	33.0	2,42E+05[a,b]	0.6	

[1] Experimental linear retention index;
[2] Linear retention indices reported in literature (NIST 2017);
[3] Similarity index.
nd: not detected (peak area value below 5E+04).

Most of the 18 detected VOCs in this work were detected during fresh meat storage in MAP also in other studies [54, 67]. Changes in VOCs composition during time were observed, indicating chemical, enzymatic and microbial deterioration in samples. Most of the detected VOCs were found at all storage times in both the packaging types, although generally in different amounts. In fact, some of these compounds (e.g. 3-methylbutanol, 1-pentanol, 3-hydroxy-butanone and acetic acid) seemed to increase during the storage period in both BP and PET packaging. On the contrary, other volatiles (e.g. 1-octanol, 1-octen-3-ol) could only be found at the end of the storage period (after 9 or 14 days). A selection of identified compounds is plotted in **Figures 11, 12, 13, 14, 15** and **16** for a better understanding of the VOCs development in the analyzed samples.

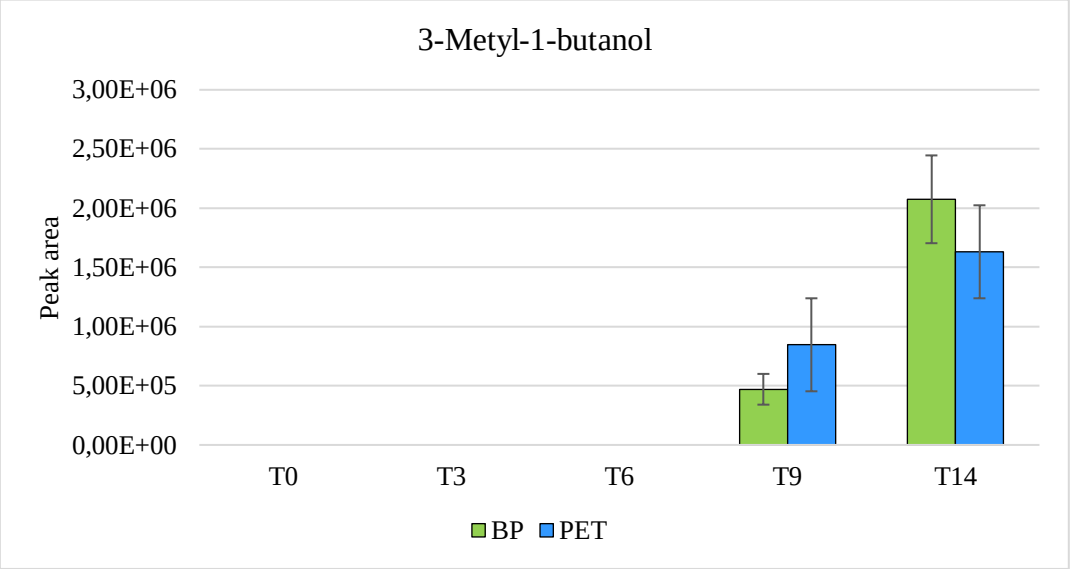

Figure 11. Comparison of the average peak areas (± standard deviation) of 3-methyl-1-butanol in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk.

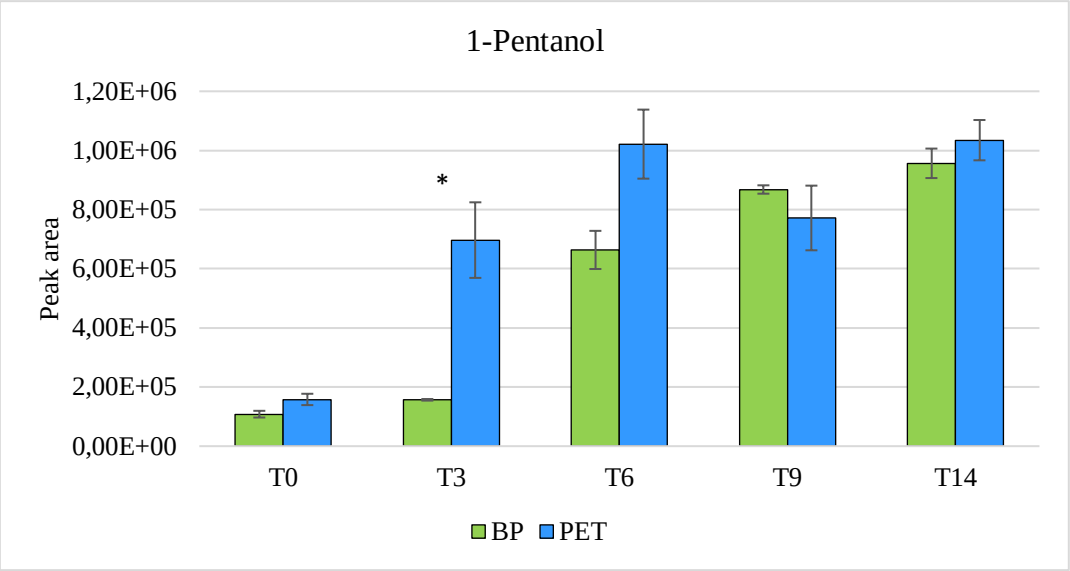

Figure 12. Comparison of the average peak areas (± standard deviation) of 1-pentanol in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk.

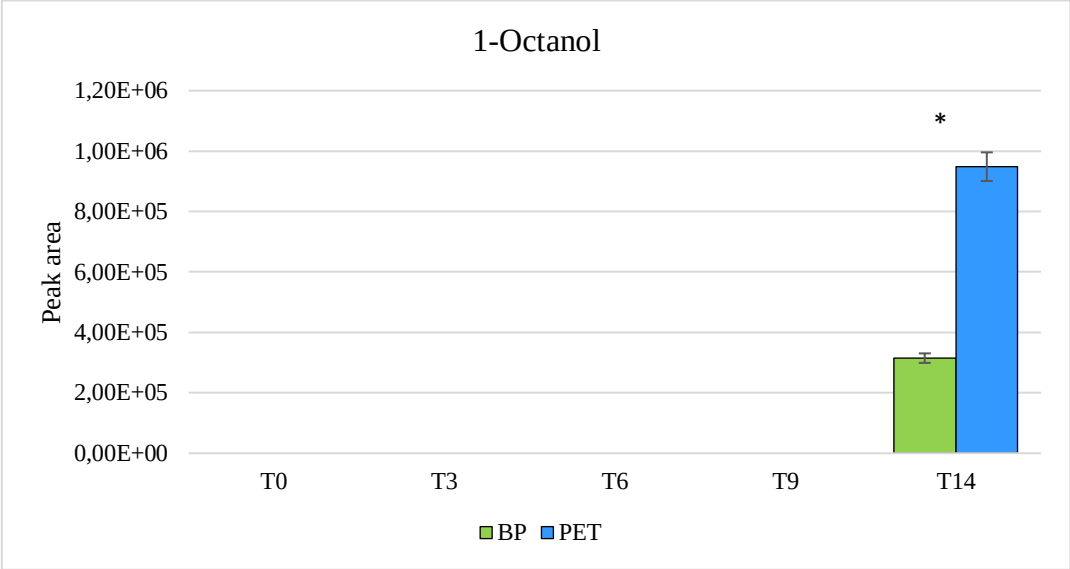

Figure 13. Comparison of the average peak areas (± standard deviation) of 1-octanol in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk.

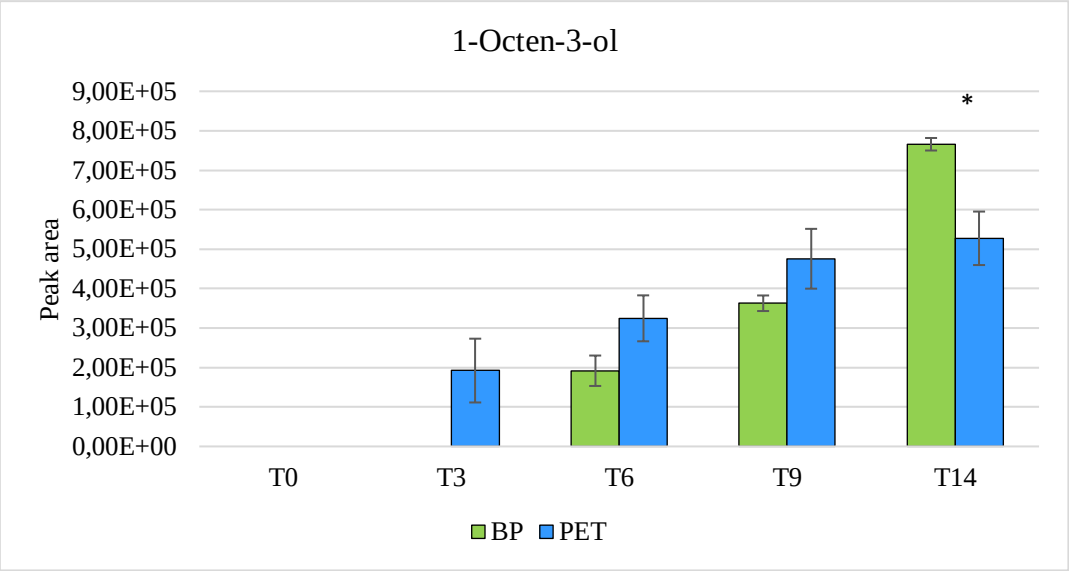

Figure14. Comparison of the average peak areas (± standard deviation) of 1-octen-3-ol in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk.

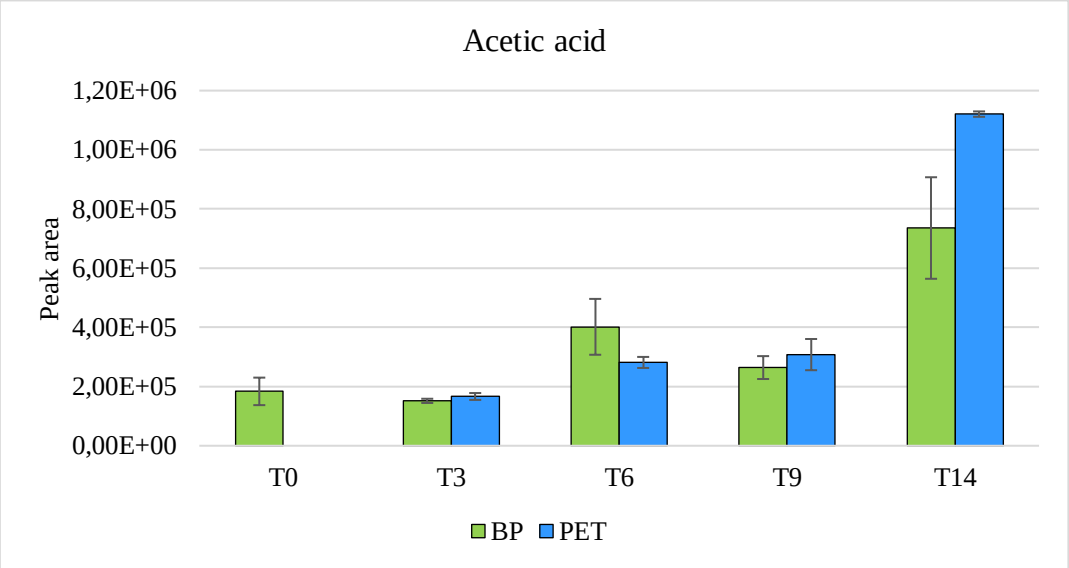

Figure 15. Comparison of the average peak areas (± standard deviation) of acetic acid in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk.

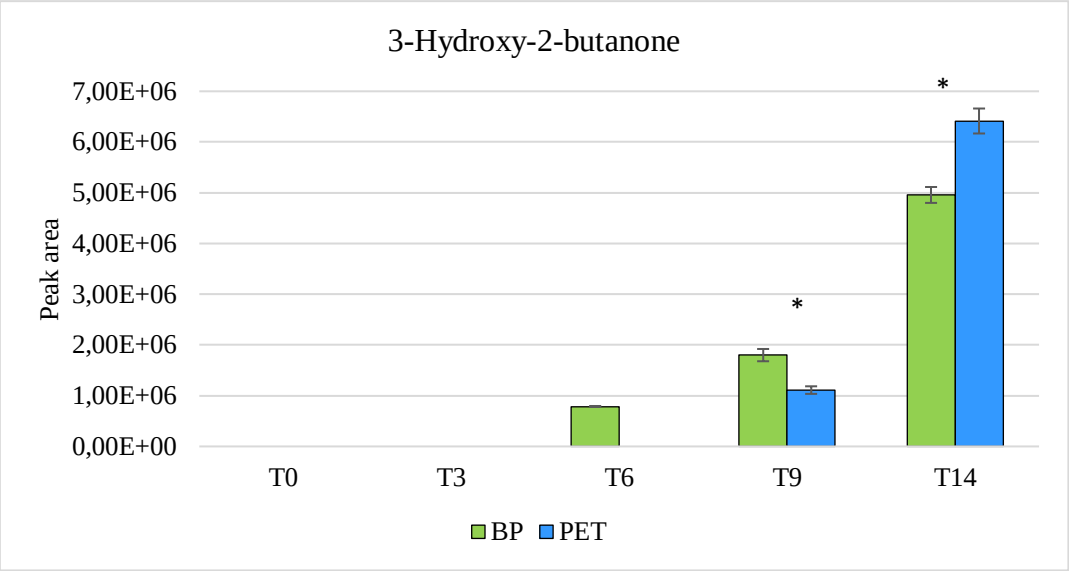

Figure 16. Comparison of the average peak areas (± standard deviation) of 3-hydroxy-2-butanone in chicken breast meat in the two types of packaging (BP and PET) during the storage period. Significant differences ($P < 0.05$) between the two packaging in each day are indicated by the asterisk.

Alcohols were the most abundant species found in the analyzed chicken breasts. The principal metabolic pathways involved in the synthesis of alcohols found in raw meat are: methyl ketones reduction, reduction of aldehydes deriving from lipid oxidation or proteolytic activity. During the storage, they are mostly produced by specific spoilage microorganisms, such as *Pseudomonas spp.* and *Carnobacterium spp.* 1-Octen-3-ol and 3-methyl-1-butanol are the most frequently detected alcohols in raw meat stored at air and in VP, and 1-octen-3-ol was found in abundance also in MAP packaging [54]. 1-Octen-3-ol is mainly a product of the oxidation of linoleic and linolenic acids [68], its persistent mushroom and mouldy-like notes and its low odor threshold (1 µg/L), are known to contribute significantly to the aroma of meat [54]. It was detected after day 3 in the PS packaging and after day 6 in MB packaging, showing an increasement in both packaging during the storage period. At day 14, the quantity of 1-octen-3-ol is significantly higher in MB respect to the conventional packaging. 3-Methyl-1-butanol, with its whiskey-like odor, is a possible product of the proteolytic pathway of leucine and it has been considered as a chemical marker for poultry meat spoilage in other studies [54,59]. In the present study, it was detected after 9 days in both BP and PET packaging; reasonably it could be considered a marker of the latter stages of the spoilage process. The same consideration can be done for 1-octanol, which was detected only at the end of the storage period, after 14 days of storage, in both packaging. It is a well-known lipid oxidation product, as 1-pentanol, that

was determined in both fresh and aged samples, with a higher presence in the latter for both the packaging [69].

Ketones detected in the present study are 2-butanone, 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin). They could originate from fatty acid oxidation, both chemical auto-oxidation and enzymatic β-oxidation. Ketones presence in stored fresh meat is mostly related to the activity of *Pseudomonas spp., Carnobacterium spp*. and *Enterobacteriaceae*. Acetoin is the ketone mostly detected in fresh meat stored at different conditions, being important in terms of flavor (odor threshold of 800 µg/L). Acetoin and diacetyl together release dairy, creamy and cheesy odors, which indicates that the meat is not fresh [70]. Being the acetoin the ketone most commonly produced by different microbial species and under different storage conditions in meat, it was reported as a meat aging indicator also in previous studies [50, 55]. Acetoin was detected at day 6 in MB packaging and at day 9 in the conventional one, showing a significant increase in 14-day old samples. In particular, at day 14 the quantity of acetoin was significantly higher in conventional packaging as compared to MB packaging. The presence of 2-butanone was associated to the activity of yeasts and moulds, while the formation of 2,3-butanedione derived from the degradation of proteins and carbohydrates, via enzymatic β-oxidation [71]. Diacetyl produces butter, sweet, creamy and pungent caramel aroma, while 2-butanone is related to acetone-like flavor [54].

Volatile fatty acids detected in the analyzed samples are acetic, propionic, isovaleric, hexanoic and nonanoic acids. Short chain fatty acids (with 2 to 6 carbon atoms) generally come from the degradation of amino acids, but also from esters, ketones and aldehydes oxidation. Usually, *Br. thermosphacta* and *Carnobacterium spp*. are the major responsible of organic acids formation during meat storage. Casaburi et al. [54] indicated that straight-chain fatty acids (e.g. acetic or hexanoic) were present in meat stored under different conditions, while branched-chain fatty acids (e.g. isovaleric acid) were typical of meat stored aerobically. In detail, in the present study, the most abundant detected SCFA in meat was acetic acid. It was found in both fresh and aged samples, showing a significant increase for both the types of packaging after 14 days of storage. Acetic and hexanoic acids appear frequently in meat stored in different conditions and they are related to vinegar and pungent odor, and sweaty and cheesy aroma, respectively. Acetic acid was considered a good indicator for meat spoilage also in previous studies [50, 55].

Sulphur compounds have a major role in the off-flavor of spoilaged meat, because of their putrid and sulfury odor, also at the earlier stages of storage [70]. Sulphur compounds are usually produced by meat microorganisms (e.g. *Pseudomonas spp*.), responsible for the degradation of

sulphur containing amino acids (cysteine and methionine) [54]. In the present study, two sulfur containing VOCs (carbon disulfide and dimethyl sulfone) were detected and they reached their highest concentration after 6 days of storage. Carbon disulfide was detected in spoiled Irish chicken breast by Alexandrakis et al. [59], while dimethyl sulfone was detected in chicken breast fillets under modified atmosphere packaging by Klein et al. [67].

In general, VOCs development during meat storage was not significantly more pronounced in the BP packaging, thus contributing to demonstrate BP packaging suitability for meat chilled storage.

However, the results obtained from the VOCs study were enriched with the other monitored parameters: biogenic amines, selected microorganisms, pH and sensorial qualities. The studied indices of BAs indicated a similar trend for both types of packaging. Also the counts of total aerobic mesophile, mesophilic lactic acid bacteria, *Enterobacteriaceae*, presumptive *Pseudomonas spp.*, and coagulase-positive staphylococci did not show significant differences between BP and PET packaging. The same results were observed for the sensorial evaluation of the meat in the two packagings. Moreover, pH values remained approximately constant during the 14 days of storage. Concluding, BP packaging showed similar performances in fresh chicken meat preservation compared to conventional packaging (PET). The optimal performances of compostable packaging in fresh meat conservation were recently reported also by Cheng et al. [72], even if further improvements could be done through the incorporation of antimicrobial bioactive additives [73]. Hassan et al. [74] proposed a new starch-based biopackaging with rosehip extract to limit lipid oxidation occurring during chicken breast storage. The studied biopackaging has a multi-layers structure developed by exploiting the qualities of Novamont's bio-polymers, such as barrier and HDT (heat deflection temperature) properties, which overcome the main concerns related to storage temperature. After these hopeful and excellent results, further developments will be done to assess the preservation of other foods in the same packaging in order to promote a green and circular economy, that can be reached only with the exploitation of compostable and low environmental impact materials.

2.3.2 Comparison of organic and traditional chicken meat

The same HS-SPME-GC-MS method was applied for the study of the VOCs released by two types of chicken breast meat (organic and conventional) over 10-days of aerobic storage. In particular, VOCs were monitored at specific days: after 0, 3, 6 and 10 days (T0, T3, T6 and T10). Example of the chromatograms obtained from the analysis of the conventional and organic chicken breast meat are shown in **Figure 17** and **18**, respectively.

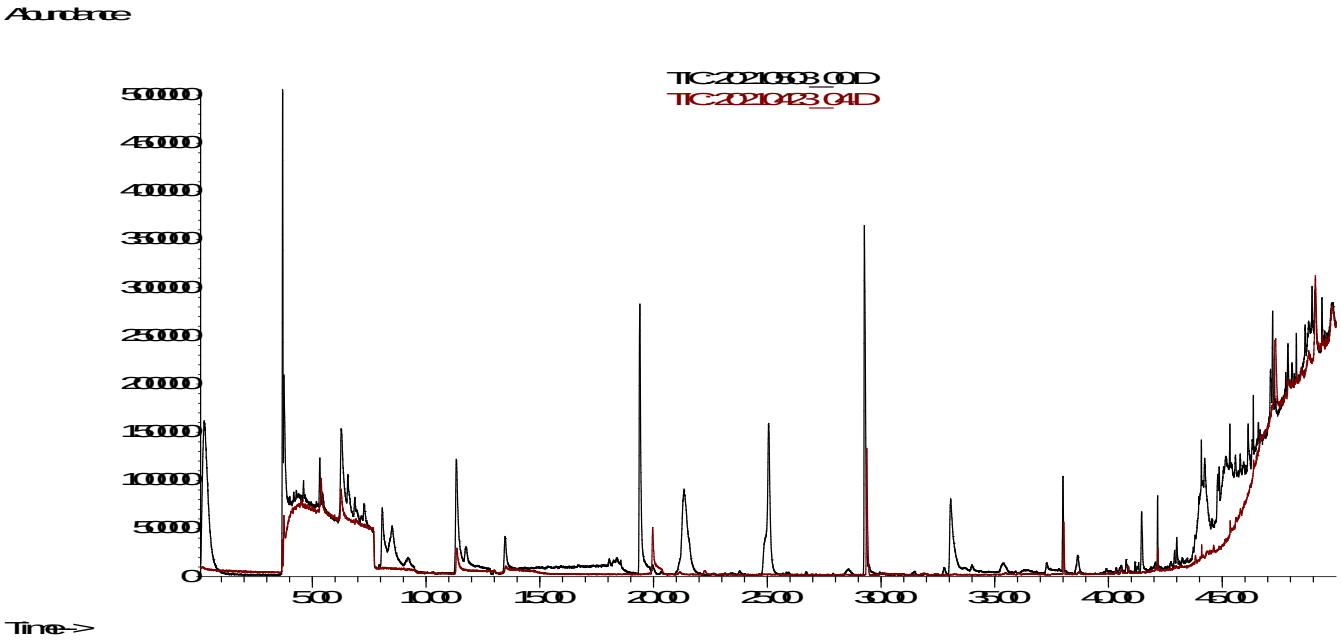

Figure 17. Chromatograms obtained from the analysis of conventional chicken breast meat after 0 (red line) and 10 (black line) days of storage.

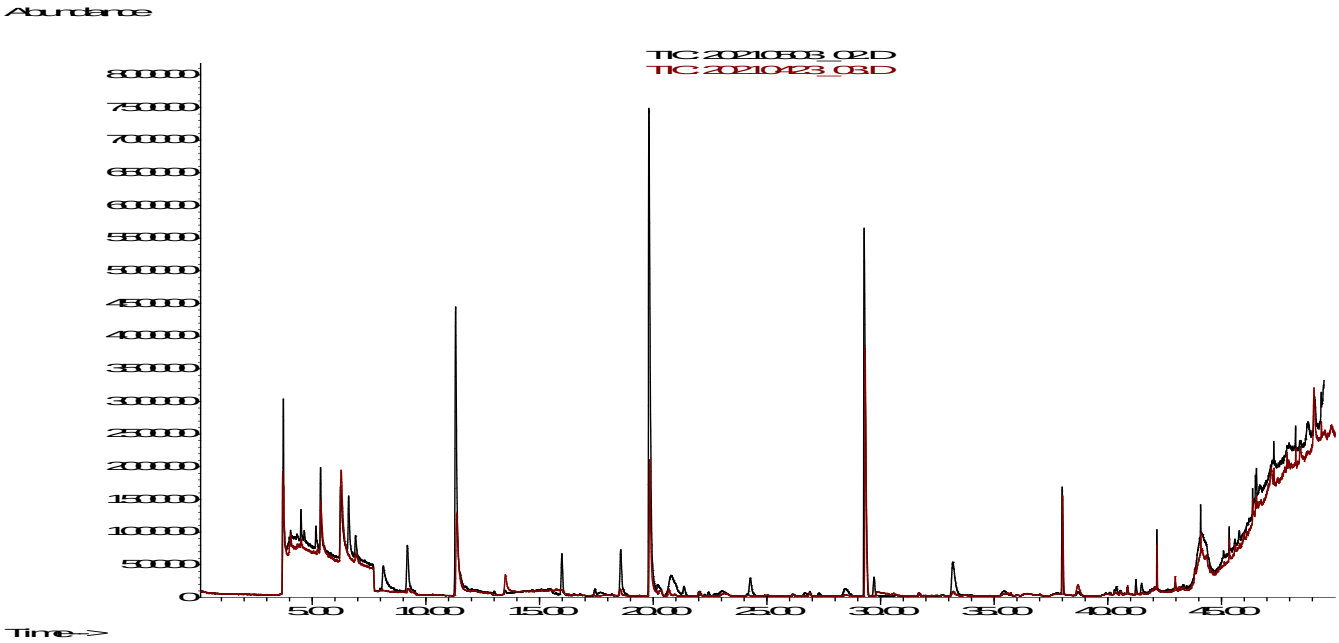

Figure 18. Chromatograms obtained from the analysis of organic chicken breast meat after 0 (red line) and 10 (black line) days of storage.

A total of 21 VOCs were identified in both kind of samples and they are listed in **Table 7**; according to their chemical nature they were divided into: organic acids, ketones, aldehydes, alcohols, phenols and sulfur containing compounds.

Table 7. Volatile compounds detected by HS-SPME-GC-MS during the storage of chicken breast meat packaging without modified atmosphere, their experimental linear retention indices (LRI) on a polyethyleneglycol coated column, their odor attribute, their abundances in terms of peak areas and % relative standard deviation (RSD, *n*=2). Significant differences ($P < 0.05$) between the two types of meat (biological and traditional) in each day are indicated by the asterisk. Significant differences ($P < 0.05$) between the different days for each of the two meats are indicated by different letters in the same each row.

Compound	Odor attribute	LRI[1] (exptl)	LRI[2] (lit)		Day of storage								SI[3] (%)
					0		3		6		10		
					Area	RSD%	Area	RSD%	Area	RSD%	Area	RSD%	
Alcohols and phenols													
Ethanol	Vinous	929	930	OC	nd	nd	nd	nd	nd	nd	3.36E+06	13.91	80
				CC	6.29E+05[a]	3.37	nd	nd	1.30E+06[a,b]	23.78	3.30E+06[b]	25.86	
3-Methy-l-butanol	Roasted	1213	1208	OC	nd	nd	nd	nd	nd	nd	5.96E+06*	4.78	86
				CC	nd	nd	nd	nd	1.00E+06[a]	30.33	2.27E+07[b]	37.93	
1-Pentanol	Fusel	1254	1252	OC	5.25E+05[a]	2.16	6.72E+05[a]	30.22	7.42E+05*[a]	8.85	1.39E+06[b]	4.76	82
				CC	nd	nd	nd	nd	1.70E+06	20.2	nd	nd	
1-Hexanol	Fruity	1356	1354	OC	nd	nd	3.55E+05[a]	36.38	4.76E+05[a]	1.31	2.01E+06*[b]	2.72	78
				CC	nd	nd	nd	nd	8.75E+05	74.29	7.73E+05	14.99	
1-Octen-3-ol	Earthy	1451	1449	OC	7.70E+05[a]	4.59	1.27E+06[b]	9.05	1.45E+06*[b]	7.73	5.78E+06*[c]	2.57	86
				CC	nd	nd	nd	nd	2.45E+06[a]	7.50	2.53E+06[b]	8.53	
1-Octanol	Waxy	1566	1565	OC	1.48E+06[a]	9.53	3.49E+05*[b]	16.98	8.68E+05[c]	0.09	6.99E+05[c]	6.22	80
				CC	1.23E+06	11.49	7.86E+05	13.67	1.97E+06	25.44	1.87E+06	27.88	
2-Octen-1-ol	Green	1615	1620	OC	nd	nd	nd	nd	3.14E+05	42.95	6.03E+05	4.67	80
				CC	nd	nd	nd	nd	nd	nd	nd	nd	
Phenol	Sweet	1985	1987	OC	nd	nd	nd	nd	nd	nd	nd	nd	86

				CC	nd	nd	nd	nd	1.23E+06	22.04	1.31E+06	18.00	
Ketones and aldehydes													
2-Butanone	Fruity	892	894	OC	9.21E+05*	3.07	9.21E+05	35.03	9.84E+05	18.45	1.35E+06*	4.87	79
				CC	1.05E+06[a]	1.61	4.50E+05[b]	18.16	4.76E+05[b]	18.16	6.03E+05[b]	5.25	
2-Pentanone	Fruity	961	970	OC	nd	nd	nd	nd	5.34E+05[a]	32.25	4.44E+06*[b]	1.78	72
				CC	3.25E+05[a]	4.35	nd	nd	6.08E+05[a]	23.17	1.80E+06[b]	0.13	
Hexanal	Green	1064	1065	OC	2.37E+06*[a]	8.96	nd	nd	5.53E+05*[b]	13.60	3.53E+05*[b]	24.17	90
				CC	1.59E+06[a]	8.88	nd	nd	1.33E+06[a]	13.56	2.65E+06[c]	10.20	
2-Heptanone	Cheesy	1166	1173	OC	nd	nd	nd	nd	nd	nd	4.17E+06	4.50	80
				CC	nd	nd	nd	nd	nd	nd	9.84E+05	8.90	
3-Hydroxy-2-butanone	Creamy	1280	1278	OC	nd	nd	6.21E+05	11.13	2.45E+06	5.67	3.37E+06	8.79	90
				CC	nd	nd	nd	nd	2.70E+06[a]	24.62	1.25E+07[b]	5.71	
2-Nonanone	Fruity	1377	1380	OC	nd	nd	nd	nd	nd	nd	1.59E+06*	9.92	84
				CC	nd	nd	nd	nd	nd	nd	5.23E+05	8.90	
Acids													
Acetic acid	Vinegar	1446	1453	OC	nd	nd	nd	nd	nd	nd	nd	nd	91
				CC	nd	nd	nd	nd	nd	nd	4.53E+06	35.59	
Propanoic acid	Pungent	1539	1538	OC	nd	nd	nd	nd	nd	nd	nd	nd	82
				CC	nd	nd	nd	nd	nd	nd	6.89E+05	36.39	
2-Methyl-butanoic acid	Cheesy	1665	1668	OC	nd	nd	4.21E+05[a]	11.26	4.52E+05[a]	7.48	8.02E+05[b]	2.14	76
				CC	nd	nd	nd	nd	nd	nd	nd	nd	
Hexanoic acid	Sour	1843	1839	OC	nd	nd	nd	nd	nd	nd	nd	nd	83
				CC	nd	nd	nd	nd	nd	nd	1.15E+06	38.30	
Sulfur containing compounds													
Carbon disulfide	Sulfurous	707	710	OC	4.43E+05	1.60	nd	nd	1.60E+06	33.24	1.05E+06	3.16	83

				CC	1.12E+06	12.59	4.55E+05	50.01	3.73E+05	23.40	nd	nd		
Dimethyl sulfide	Sulfurous	724	720	OC	nd	nd	nd	nd	nd	nd	5.16E+05	29.03	94	
				CC	nd	nd	nd	nd	nd	nd	4.09E+05	2.85		
Dimethyl sulfone	Cabbage-like	1904	1911	OC	nd	nd	nd	nd	nd	nd	nd	nd	87	
				CC	nd	nd	nd	nd	4.20E+05[a]	0.53	9.15E+05[b]	11.55		

[1] Experimental linear retention index;

[2] Linear retention indices reported in literature (NIST 2017);

[3] Similarity index.

nd: not detected (peak area value below 3E+05).

Some compounds were detected at the different times of storage in both types of meat (e.g. 1-octen-3-ol, 2-methyl-butanoic acid, 2-pentanone and hexanal), while other analytes, such as acetic acid, dimethyl sulfide, 2-heptanone and propanoic acid could be found only at the latter days of storage of the samples, usually after 10 days. Some selected compounds were reported in **Figures 19, 20, 21, 22, 23, 24, 25, 26, 27, 28** and **29** for a better understanding.

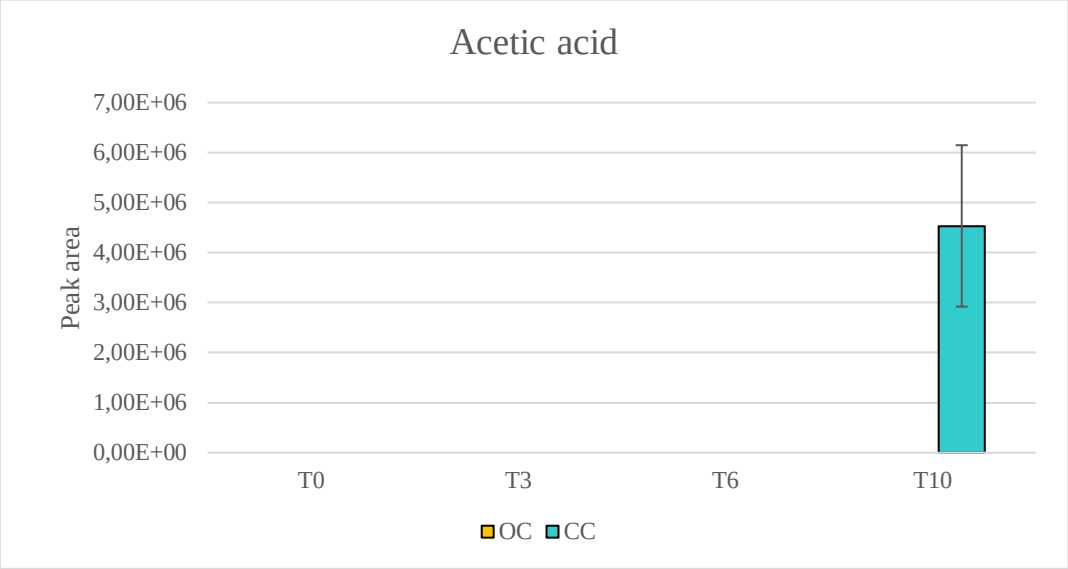

Figure 19. Comparison of the average peak areas (± standard deviation) of acetic acid in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

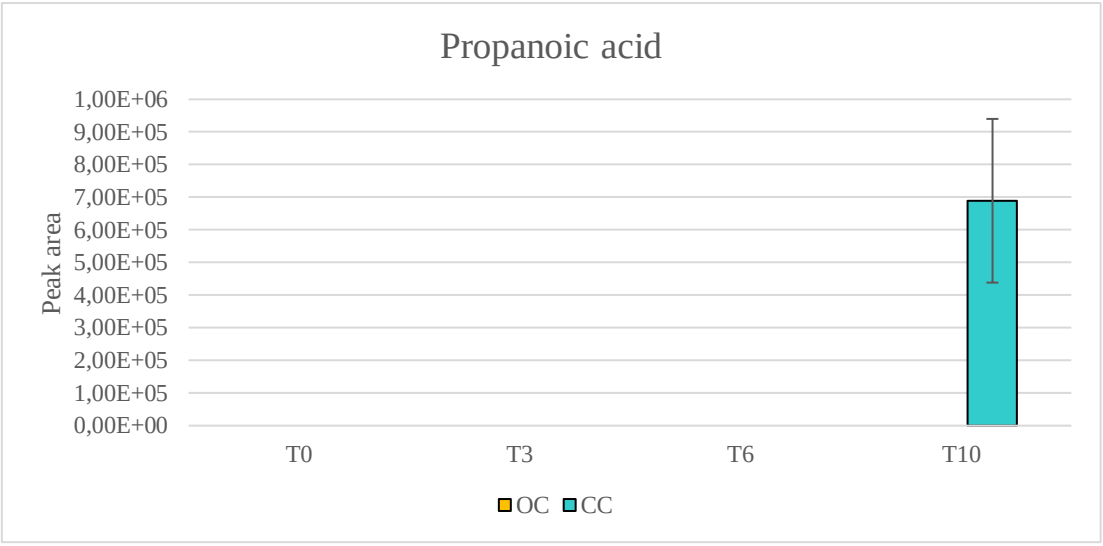

Figure 20. Comparison of the average peak areas (± standard deviation) of propanoic acid in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

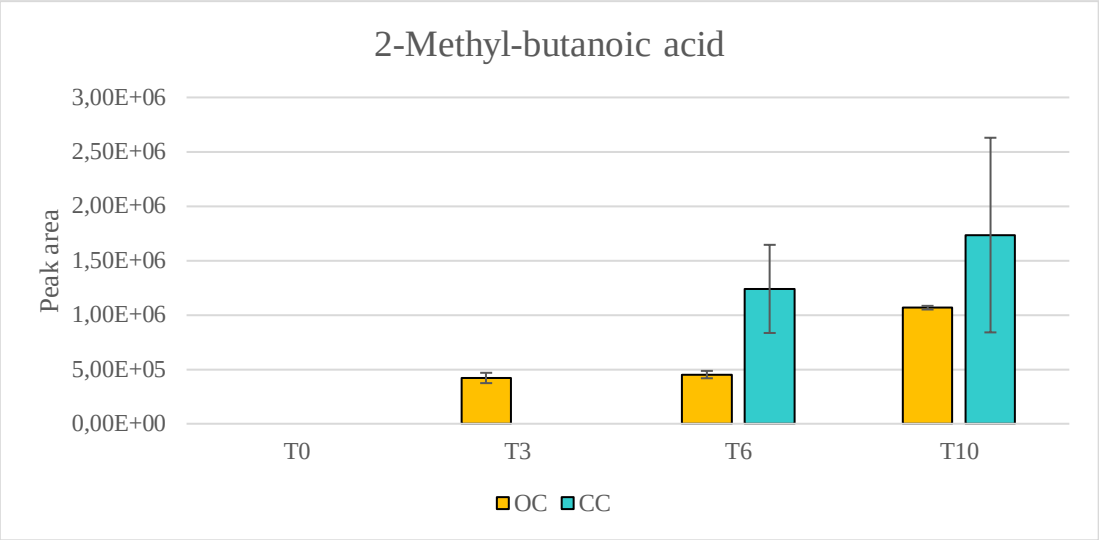

Figure 21. Comparison of the average peak areas (± standard deviation) of 2-methyl-butanoic acid in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

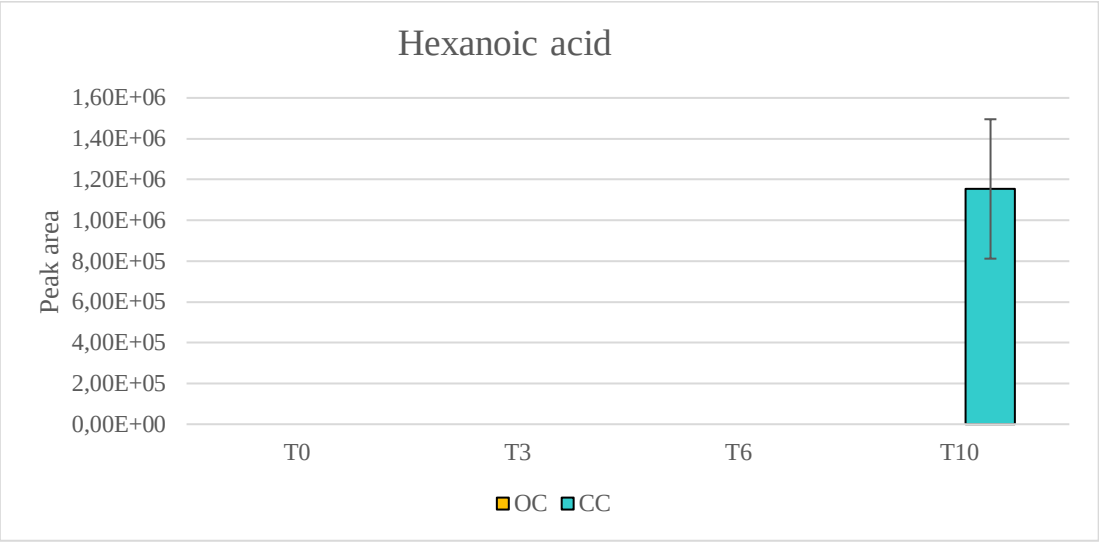

Figure 22. Comparison of the average peak areas (± standard deviation) of hexanoic acid in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

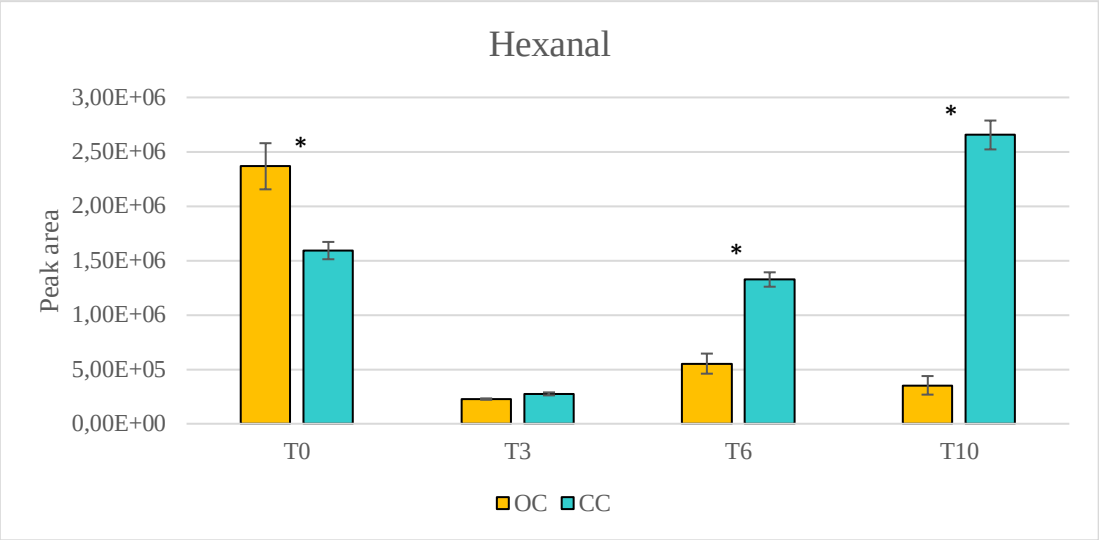

Figure 23. Comparison of the average peak areas (± standard deviation) of hexanal in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

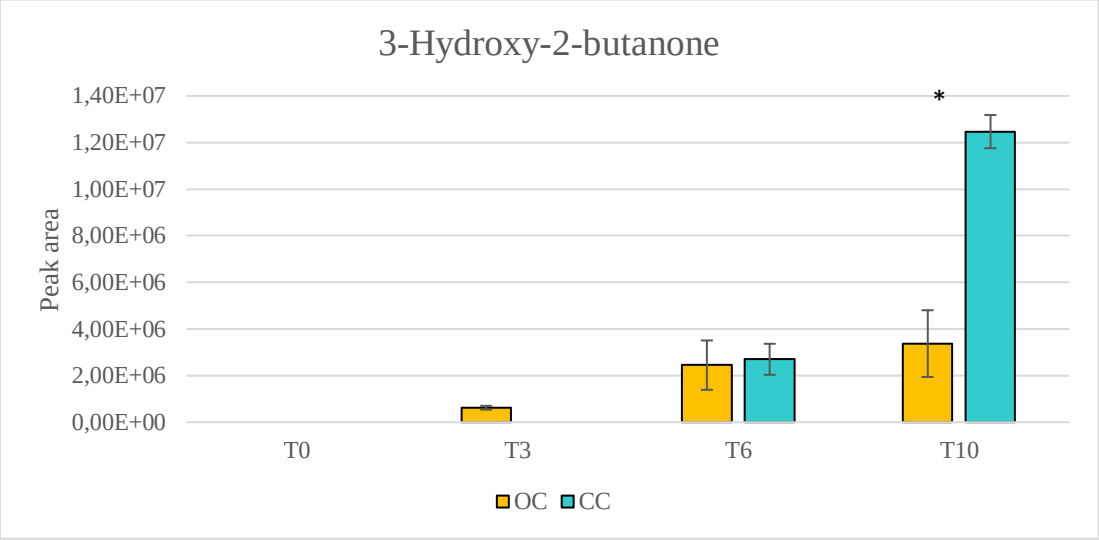

Figure 24. Comparison of the average peak areas (± standard deviation) of hexanal in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

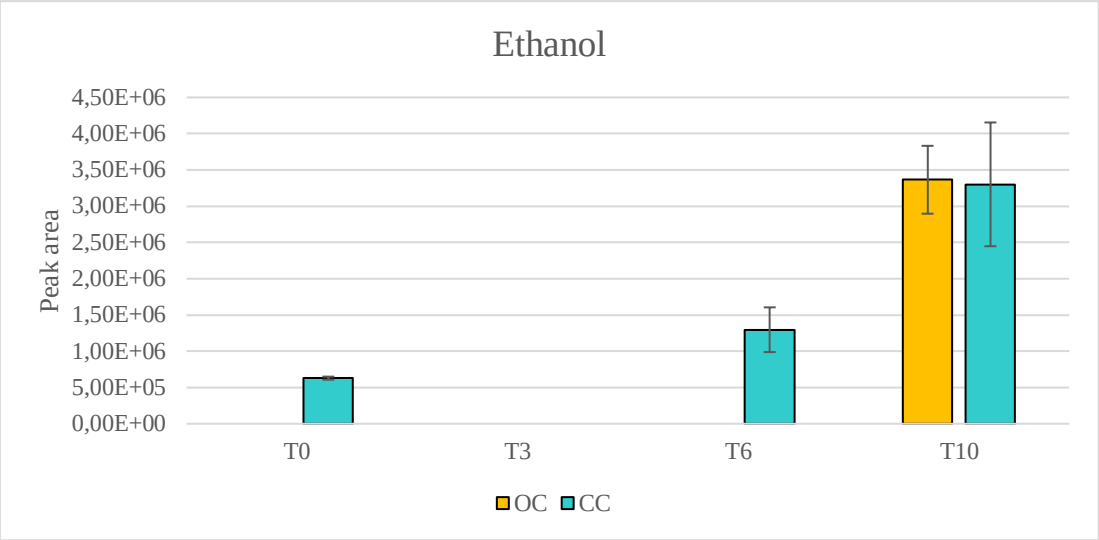

Figure 25. Comparison of the average peak areas (± standard deviation) of ethanol in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

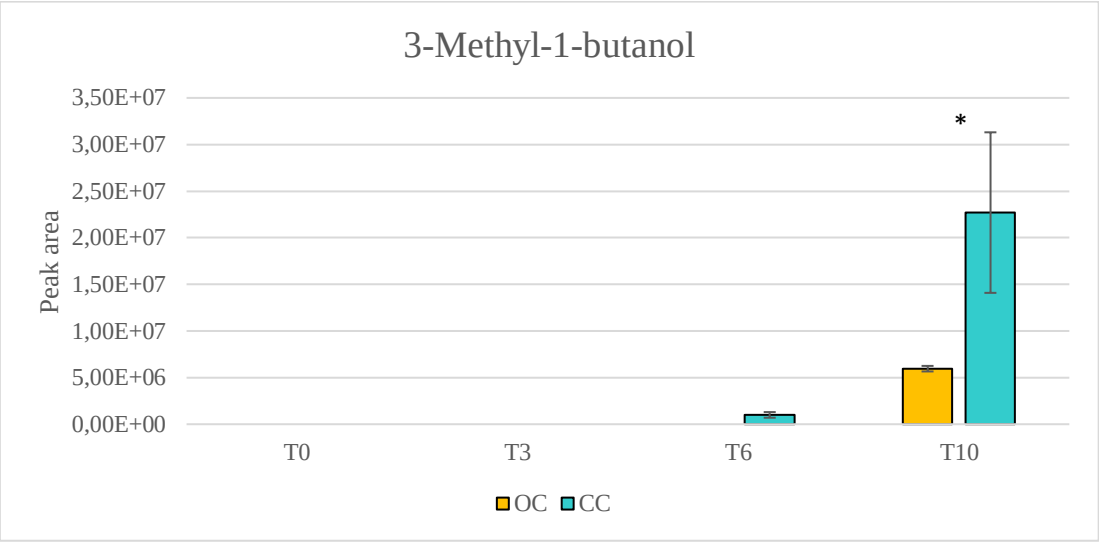

Figure 26. Comparison of the average peak areas (± standard deviation) of 3-methyl-1-butanol in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

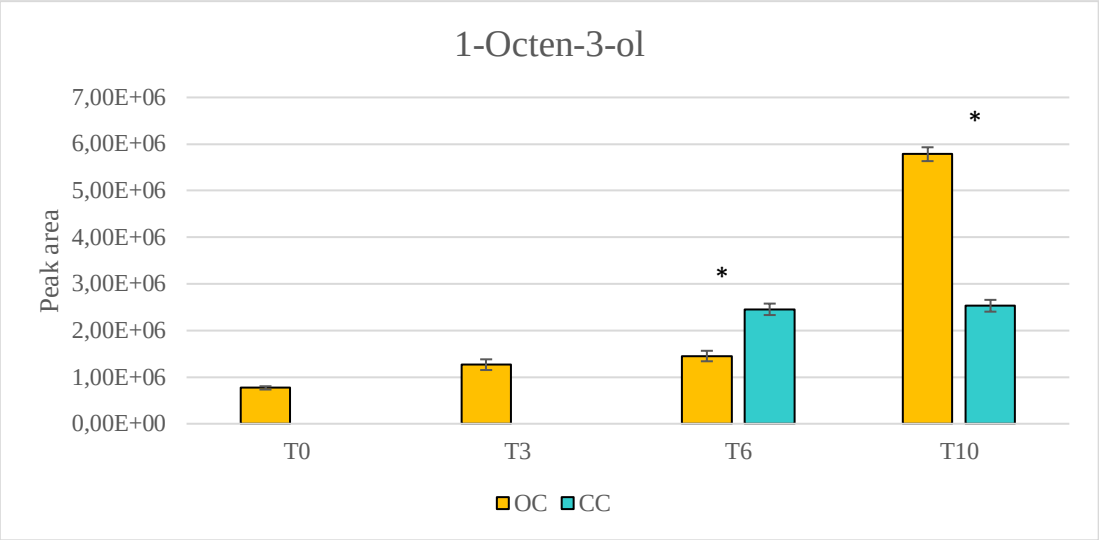

Figure 27. Comparison of the average peak areas (± standard deviation) of 1-octen-3-ol in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

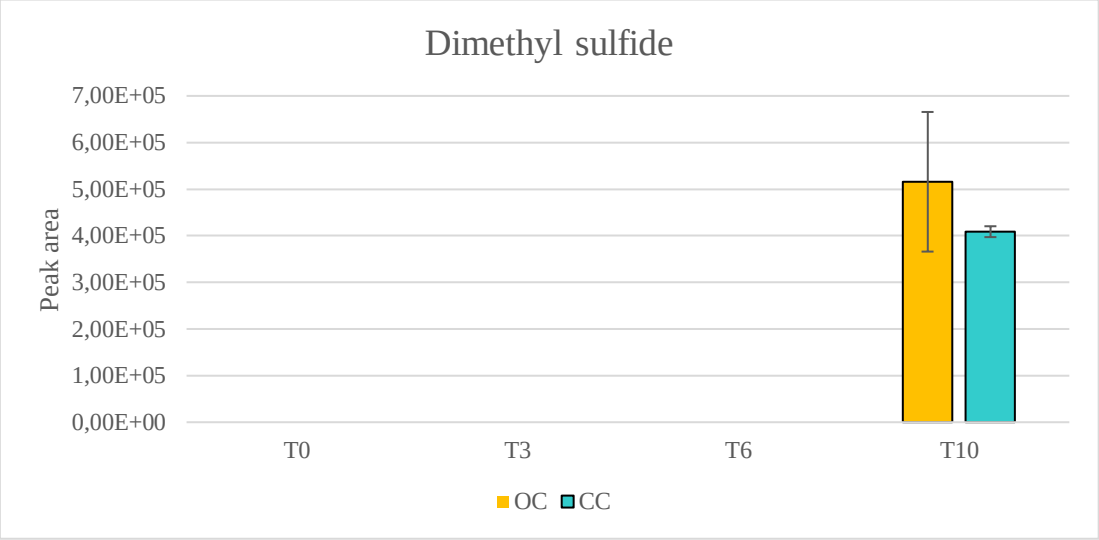

Figure 28. Comparison of the average peak areas (± standard deviation) of dimethyl sulfide in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

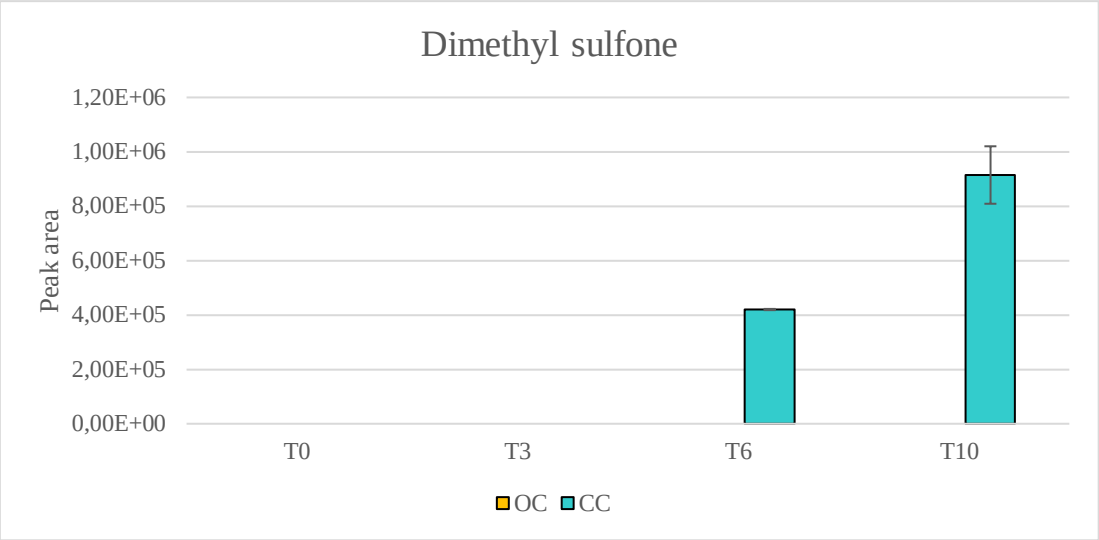

Figure 29. Comparison of the average peak areas (± standard deviation) of dimethyl sulfone in traditional and organic chicken breast meat (CC and OC) during the storage period. Significant differences ($P < 0.05$) between the two types of meat in each day are indicated by the asterisk.

Most VOCs identified in both organic and conventional meat were detected in the previous study and also in the head space of both spoiled and unspoiled chicken meat in many studies present in literature [51, 54, 55]. As previously said, there are little information about the VOCs profile of organic chicken meat. The main analyzed parameters regard pH, color, shear force, water holding capacity, cooking loss and fatty acid composition. Mancinelli et al. [75] investigated the VOCs content in raw and cooked meat of different chicken strains, differing in their growth rates (slow-growing, medium-growing and fast-growing). The study indicated that the genotype of the poultry and the farming method were responsible for the major differences detected in VOCs profiles. The reason is highly dependent on the fatty acid composition of the meat. High grazing and forage diets (similar to those of organic farming) reduce total fat and/or saturated fatty acids content, increasing the PUFAs concentration, especially *n*-3 PUFAs, respect to a concentrate-based diet (typical of intensive and conventional farming) [15]. Slow-growing and organic poultry meat is characterized by highest *n*-3 PUFAs content and so the developed VOCs could show some differences respect the conventional one [75].

Alcohols were the most abundant class of compounds detected after 10 days of storage in both conventional and organic meat. The alcohol found at highest amount is 3-methyl-1-butanol, which was detected also in meat stored in MAP. It derives mainly from the proteolytic pathway of leucine. In this study, it was detected at day 10 in both samples (at higher concentration in the conventional meat), confirming that it could be exploited as a marker of the latter stages of the meat spoilage process. Another interesting and abundant alcohol developed during storage time in both samples is

ethanol. It was found at significantly higher amount in traditional meat. With its vinegar and vinous aroma, it was considered a meat spoilage indicator also in other studies [55, 59]. Then, Zareian [50] indicated both ethanol and 3-methyl-1-butanol as possible markers of chicken meat spoilage under aerobic condition. Not all the alcohols were found at higher level in conventional meat. For instance, 1-octen-3-ol, found also in chicken meat under MAP conditions, were detected at significant higher concentration in biopackaging. It is probably due to the higher concentration of PUFAs in organic meat, in fact 1-octen-3-ol is mainly a product of the linoleic and linolenic acids oxidation [68].

Another abundant class of compounds were the ketones, with the acetoin (3-hydroxy-2-butanone) being the second most abundant compound in conventional meat. In fact, it was detected at significant highest quantity in CC meat respect to the OC. It was an interesting compound, because it was found at high levels in poultry meat stored under different conditions (air storage, VP and MAP) [54]. It is commonly produced in meat by many different microbial species and it is responsible for the development of cheesy odor in spoiling meat [50].

In the meat stored under aerobic conditions (both conventional and traditional samples) an aldehyde, the hexanal, was detected. It was reported also by other studies [75], as a product of unsaturated fatty acids oxidation. In the present study it was found at higher significant amount in conventional meat respect to the organic one.

The detected organic acids were short chain fatty acids: acetic, propionic, 2-methylbutanoic and hexanoic acids. Acetic, propionic and 2-methylbutanoic acids were found also in the headspace of aerobically stored raw chicken breast by Zareian et al. [50]. All the detected SCFAs were present after 10 days of storage in the traditional meat, except for 2-methylbutanoic acid, that developed during the storage period only in organic meat. Organic acids could derive from the degradation of amino acids and/or the oxidation of aldehydes [50]. Acetic acid was the prevalent fatty acid detected in the traditional meat sample. It can be also synthetized from glucose in glycolytic pathway by LAB or *B. thermosphacta* [54]. In many studies it has been considered an optimal indicator of meat spoilage [50, 55], with its characteristic vinegar and pungent odor.

Another important class of detected compounds were the volatile sulphur containing compounds, which are responsible for the sulphury and putrid odor of meat at advanced storage. Alexandrakis [60] found high levels of dimethyl sulfide in samples of Irish chicken breast muscle under aerobic conditions only after 8 days of storage. Also in the present study, it was detected after 10 days in both samples, in greater quantity in the organic one. Dimethyl sulfone was found instead only in the traditional meat after 6 and 10 days of storage. They are important indicators of advanced meat

spoilage, especially dimethyl sulfide, which is one of the most common volatile sulphur compounds in meat [54].

Considering the total content of volatile compounds detected at the end of the evaluated storage period (**Figure 30**), it was found that the traditional meat released the highest amount of VOCs, even if there are no statistical differences with the organic meat. Also Mancinelli et al. [75] reported a lower VOCs content in slow-grown animals with respect to fast-grown ones (244.1 ppb vs 1771.3 ppb, respectively).

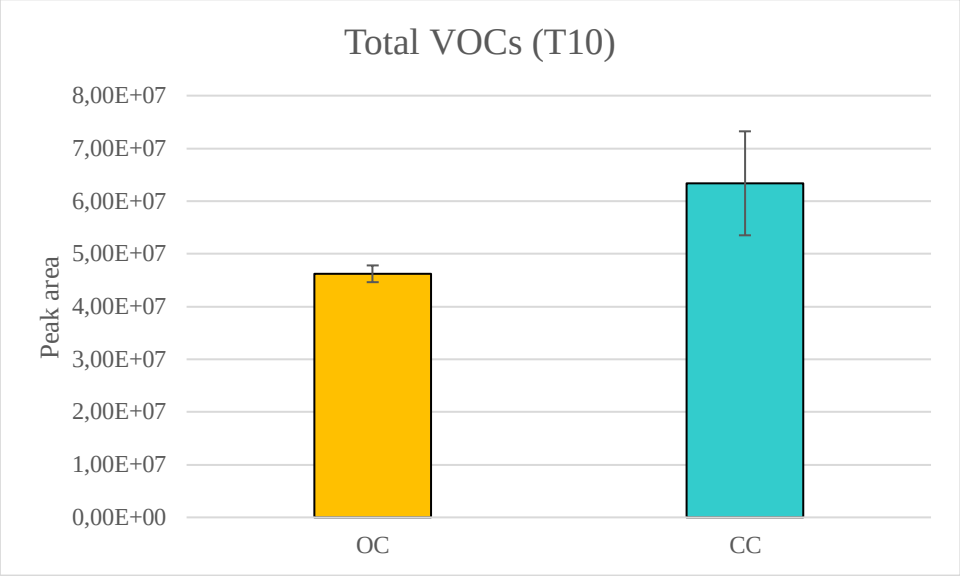

Figure 30. Comparison of the total VOCs content (in terms of average peak areas) in traditional and organic chicken breast meat (CC and OC) after 10 days of storage. Significant differences ($P < 0.05$) between the two types of meat are indicated by the asterisk.

As previously said, the poultry genotype is one of the main factors affecting the volatilome composition of chicken meat, but every detected compound is strictly dependent also on animal species/age, feeding, processing and storage [54]. Even if there are not much information about the differences in VOCs composition between the traditional and organic meat, some studies indicated some sensorial and nutritional differences. The most important one, which was already described, is the higher content of *n*-3 PUFAs in organic chicken meat [75]. Then sensory panellists indicated organic broiler meat as tougher and tastier than traditional one, demonstrating a slight superior quality of organic chicken meat. The differences found in the lipid composition of organic and traditional chicken meat are highly important, because VOCs development is linked to the contents of PUFAs, antioxidants and microrganisms too [76].

For an overall comparison of the shelf-life of organic and traditional chicken breast meat, also in this case an evaluation of other chemical and microbiological parameters was performed. Total BAs concentration increased during the shelf-life of both analyzed meats. In the first 6 days both meats showed low BAs levels, then conventional meat presented an important increase, with a statistically significant difference compared to organic chicken at day 10. Total BAs concentration values on day 10 were more than two times higher in CC than in OC (893.8 mg/Kg and 344.7 mg/Kg respectively).

From the microbiological analysis the results indicated a good quality for both samples, with low initial microbial load, especially OC sample, in which a delayed microbial growth was observed. The growth of total mesophiles was observed after 6 days storage and 10 days for the other bacterial groups with exception of lactic acid bacteria that was under detection limit during study period. Despite the delayed microbial growth, the level of total aerobic mesophiles and *Pseudomonas spp.* – meat spoilage indicators and β-glucuronidase *E. coli* was higher in organic than conventional chicken at day 10, but the overall microbial content was lower respect to other studies [77], suggesting the good hygiene processing conditions in the current study. The pH evaluation did not show statistically significant differences between the two meats from *Aviagen Ross 308* (CC and OC). The pH values during the whole shelf-life period were all in the range of 5.76-5.94.

From a sensorial point of view, organic chicken exhibited more yellowness respect to the conventional one. However, OC meat was more elastic than CC meat as well as the odor resulted to be better at the end of the study. The fact that the odor of OC meat was not as unpleasant and pungent as the CC one is in accordance with the results from the VOCs study.

2.4 Conclusions

Poultry meat is widely eaten at global level. In the last years public interest toward a sustainable economy contributed to a greater demand for organic and natural animal products. Also the global awareness of the environmental problems associated to the use of synthetic and non-degradable packaging led to an increased interest in biopackaging, which are biodegradable and natural polymers.

It is important to investigate the shelf-life of this new packaging materials and also to evaluate the characteristics of organic meat, which has not been deeply studied until now. This study firstly investigated the shelf-life performances of two different packagings (biopackaging and conventional packaging in PET), evaluating different important parameters: VOCs content, BAs concentration, pH and microbial and sensorial quality. In particular, our research group focused on the study of VOCs development during storage time. 18 principal VOCs were identified in the samples stored in biopackaging (BP) and conventional packaging (PET). Many of them (acetoin, acetic acid, 1-octen-

3-ol, 3-methyl-1-butanol, 1-octanol and sulphur compounds) are already known meat spoilage indicators. After 14 days of storage of the meat in MAP conditions, the VOCs development resulted to be not significantly more pronounced in the BP packaging. Also the other evaluated parameters contributed to demonstrate BP packaging suitability for meat chilled storage, with the great advantage of its biodegradability and sustainability for the environment.

The second objective of this study was the evaluation of organic and conventional chicken breast meat stored under aerobic conditions. Chemical and biological parameters were evaluated for 10 days. A total of 21 VOCs were detected in both samples and many of them were found also in the meat stored in MAP conditions. The majority of the analytes developed in the latter days of storage (after 6 and 10 days). Considering the total content of VOCs after 10 days, the traditional chicken meat developed higher quantity of off-odors compounds respect to organic meat. It was confirmed also by sensorial evaluation, which indicated a more intense flavor of conventional meat. Total BAs content was more than two times higher in CC than in OC meat after 10 days of storage. There were no differences between the pH values of the two kinds of meat, while the yellowness and the microbial content were higher in organic meat respect to traditional chicken. The differences found in the two samples are strictly dependent on the rearing and farming methods, but in conclusion organic and conventional chicken meat presented a similar shelf-life performance.

Further studies will assess the preservation of other foods in the same biopackaging to promote a circular and green economy. New studies will be done also on organic poultry meat, stored also in different conditions (VP or MAP), because of its huge consumption and because there is only little information about its chemical and sensorial changes during shelf-life.

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INDEX

Abstract……...……154

Chapter 3. Development and application of a rapid and simple method to analyze squalene in different food matrices……...……156

3.1 Introduction……...……...……157

3.1.1 Squalene……...……...……157

3.1.2 Properties and applications of squalene……...……...163

3.1.3 Aim of the work……...……...……166

3.2 Materials and Methods……...…….……167

3.2.1 Chemicals and reagents……...……....…167

3.2.2 Determination of squalene in olive oils and extra virgin olive oils……...….167

3.2.2.1 Quantification and method validation…….……....168

3.2.3 Determination of squalene in refined vegetable oils…….....168

3.2.4 Determination of squalene in apple peels and seeds ……...……...……171

3.2.4.1 Quantification and method validation……...……...……172

3.2.5 GC-FID analysis of squalene……...…...……173

3.2.6 Statistical analysis ……..……173

3.3 Results and Discussion..……...……174

3.3.1 Determination of squalene in olive oils and extra virgin olive oils……174

3.3.2 Determination of squalene in refined vegetable oils…….....180

3.3.3 Determination of squalene in apple peels and seeds……..…189

3.4 Conclusions……...……...……195

References……...……...……196

Abstract

Squalene is a triterpene with several interesting properties and applications, present in many different vegetable sources, especially amaranth oils and extra virgin olive oils. A rapid and easy method was developed and validated to quantify squalene in vegetable oils and apple by-products (seeds and peels) by gas chromatography (GC) coupled to flame ionization detector (FID). A transmethylation of the samples (oils and lipid fractions of apple peels and seeds) was exploited to allow the direct GC analysis without the separation of the unsaponifiable fraction. The method provides high sensitivity, short time (10 minutes for the preparation of the sample and 5 minutes of chromatographic run), low quantity of sample and reagents and involves instruments and materials usually available in many laboratories. The validation was performed assessing linearity, intraday and interday repeatability, recovery and sensitivity in terms of limit of detection and quantification. Firstly, different kinds of olive oils were investigated: monovarietal EVOOs and niche blend EVOOs, EVOOs and OOs from large scale distribution retail. Within EVOOs investigated, the niche blend EVOO contained significantly higher amount ($P < 0.05$) of squalene (0.81–1.02 g/100 g) respect to all the other EVOOs investigated with a squalene concentration in the range 0.31–0.65 g/100 g. As expected, OOs showed the lowest squalene content (0.17–0.27 g/100 g), significantly lower ($P < 0.05$) as compared to EVOOs. Then, seven different vegetable oils were analyzed with the same analytical method. To be consumed, vegetable oils (except for EVOOs) need to be refined, causing losses of several bioactive compounds to different extent. In order to evaluate the losses of squalene during refining, its content was monitored during the refining process of seven oils: olive, soybean, grapeseed, sunflower (sample 1 and sample 2), sunflower with high oleic content and maize oils. The squalene level decreased for all the oils during the refining process, except for olive and soybean oils, where the content did not show significant differences between crude and refined samples. For the other oils squalene decreased from 25 to 58 %, for maize and grapeseed oil respectively. A high difference between both crude and refined olive oil and the other seed oils in terms of squalene level was found: olive oil contained 10 to 30 more squalene compared to the other investigated vegetable oils. Apple is a very consumed fruits worldwide. Its manufacturing produces huge quantity of waste every day. The proposed method was applied to the study of squalene in two apple by-products (peels and seeds) as a contribution to evaluate their possible exploitation in food, cosmetical or pharmaceutical fields. The method exhibited good linearity, sensitivity, recovery and repeatability also in this case. Eleven apple varieties (seven ancient and four commercial cultivars) were evaluated. The results showed a squalene content between 1.7-24.7 mg/100 g in fresh seeds and 0.9-2.9 mg/100 g in fresh peels. Apple seed oils resulted to have a squalene concentration comparable to maize, rapeseed and sunflower oil,

contributing to exploit this by-product in different sectors decreasing also environmental impacts and enhancing economic profits.

Chapter 3. Development and application of a rapid and simple method to analyze squalene in different food matrices

3.1 Introduction

3.1.1 Squalene

Terpenes (or terpenoids) are a group of molecules with highly different chemistry, structure and function. They include more than 40.000 compounds, constituting the largest class of natural products. Their name derived from the turpentine oil, from which the first monoterpenes were isolated in 1850s. Ourisson [1] suggested that the most primitive membranes were formed only by polyterpenoids, because their lipophilic character and their large presence in all living organisms. All terpenes derive from repeated isoprene units (**Figure 1**). Under suitable chemical conditions, the isoprene units can be polymerised to form different terpenoids.

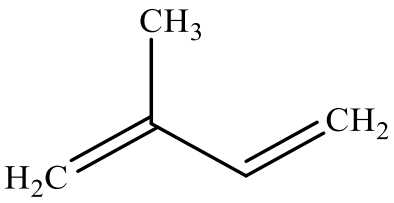


Figure 1. Isoprene unit.

Terpenes are classified according to the number of isoprene units and carbon atoms (**Table 1**); they are identified by the notation a:b. For instance monoterpenes have the notation 2:10, diterpenes 4:20, triterpenes 6:30 or carotenoids 8:40 [2].

Table 1. Classification of terpenes according to the number of isoprene units and carbon atoms [2].

	Number of isoprene units	**Number of carbon atoms**
Monoterpenes	2	C_{10}
Sesquiterpenes	3	C_{15}
Diterpenes	4	C_{20}
Sesterterpenes	5	C_{25}
Triterpenes	6	C_{30}
Tetraterpenes	8	C_{40}
Polyterpenes	>8	>C_{40}

Many plant terpenoids are used as toxins, repellents or antibiotics; other have found applications as flavors or supplements food. An important part of terpenes are known for their pharmacological qualities, such as antimicrobial, anticancer, analgesic, antiviral or antihyperglycemic properties [3].

Squalene is a polyunsaturated triterpene with chemical formula $C_{30}H_{50}$, formed by six isoprene units. Its IUPAC name is (6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene. Its name is due to the fact that it was originally identified in the shark (squalus in Latin) liver oil extract by Tsujimoto Mitsumaru in 1916. Shark liver oil contains about 60% wt of squalene and it has been used as a source of this substance by the traditional medicine for ages. At room temperature, squalene is a colorless liquid with a bland taste. Some of its physical properties are reported in **Table 2**.

Table 2. Physical properties of squalene [4].

Properties	**Values**
Viscosity	~ 11 cP
Octanol/water partitioning coefficient (log P)	10.67
Solubility of squalene in water	0.124 mg/L
Surface tension	~32 mN/m
Density	0.858 g/mL

The presence of many double bonds permits squalene to be present in several conformations: stretched, coiled or "sterol-like" form (**Figure 2**).

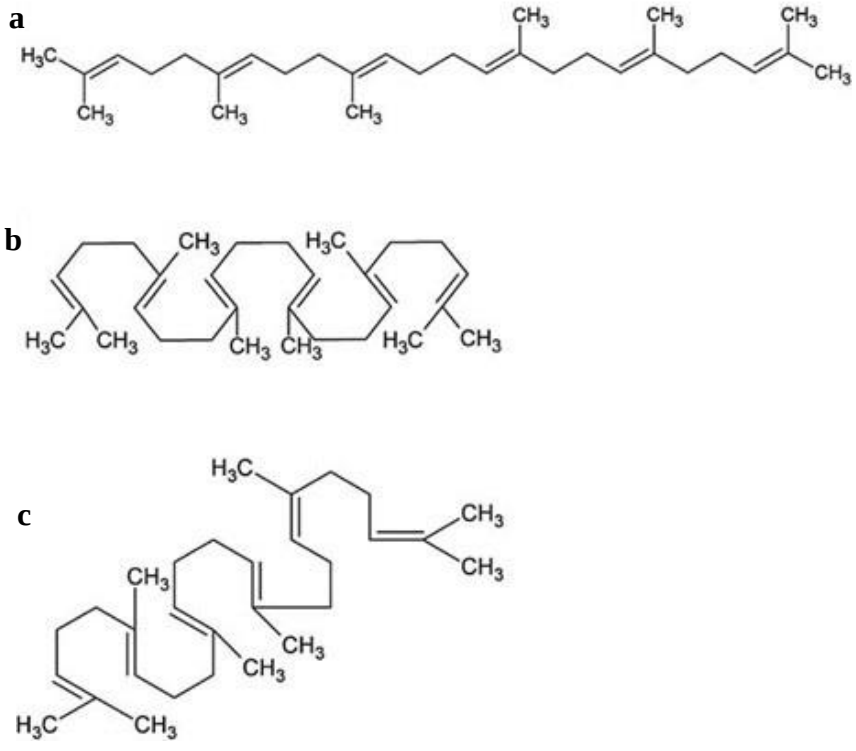


Figure 2. Chemical structure of squalene: stretched form (a), coiled form (b) and "sterol-like" form (c) [4].

The sterol-like conformation may allow squalene to be in cell membranes. In human body squalene is at its highest content in sebum (about 13%) and in skin (**Table 3**).

Table 3. Squalene levels in different human tissues [5].

Human tissues (dry weight)	Squalene concentration (µg/g)
Skin	210–1100
Adipose tissue (subcutaneous)	270-560
Adipose tissue (abdominal)	110-310
Adrenal gland	49-96
Liver	56-92
Cerebrum	16-31

Squalene is synthetized in the skin and in the liver, then transported in the blood by low and very low density lipoproteins (LDL and VLDL) and secreted mainly by sebaceous glands. Squalene, in turn, is a starting material for the synthesis of several steroids, mainly cholesterol in human body [3]. **Figure 3** shows the biosynthetic pathway of cholesterol with squalene as intermediate in human cells.

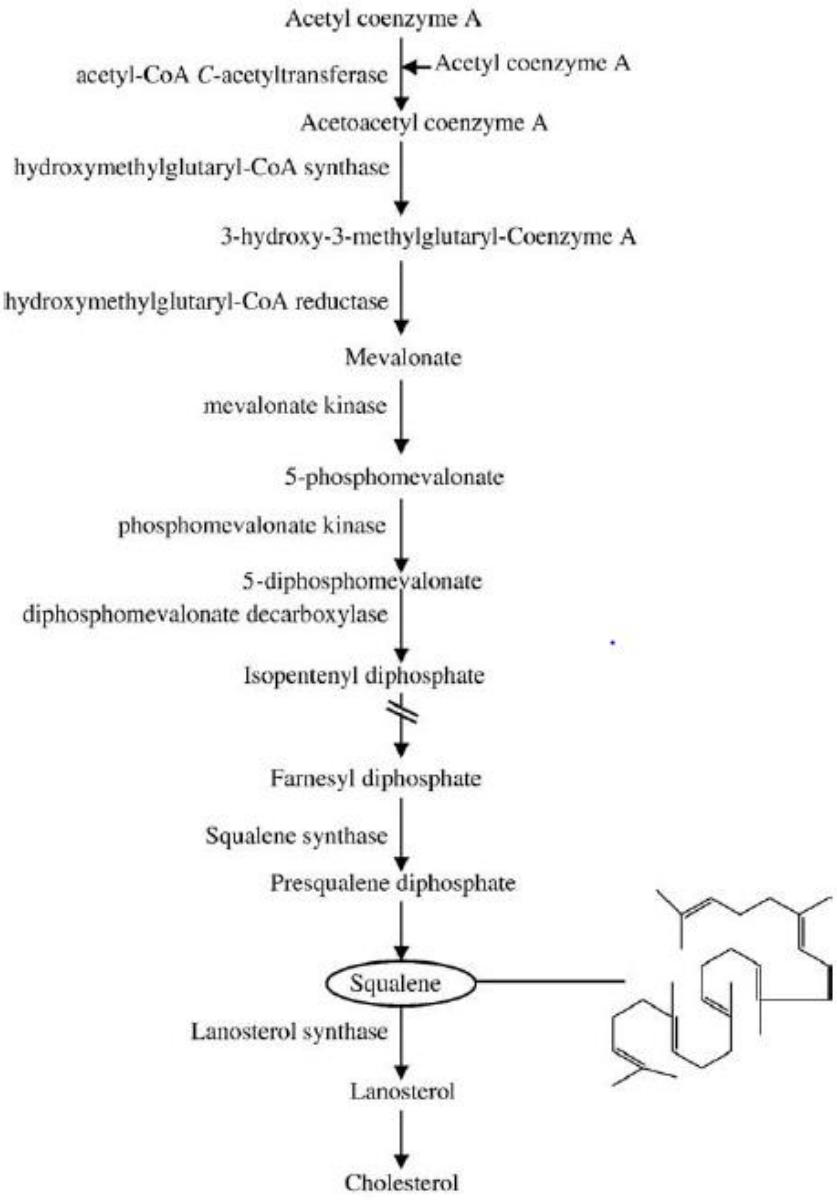


Figure 3. Biosynthetic pathway of cholesterol and the intermediate squalene [3].

In other species squalene is the precursors of different terpenoids, for instance ergosterol in fungi, β-sitosterol in plants, hopanoids in bacteria and 24-methylenecholesterol in protists (**Figure 4**).

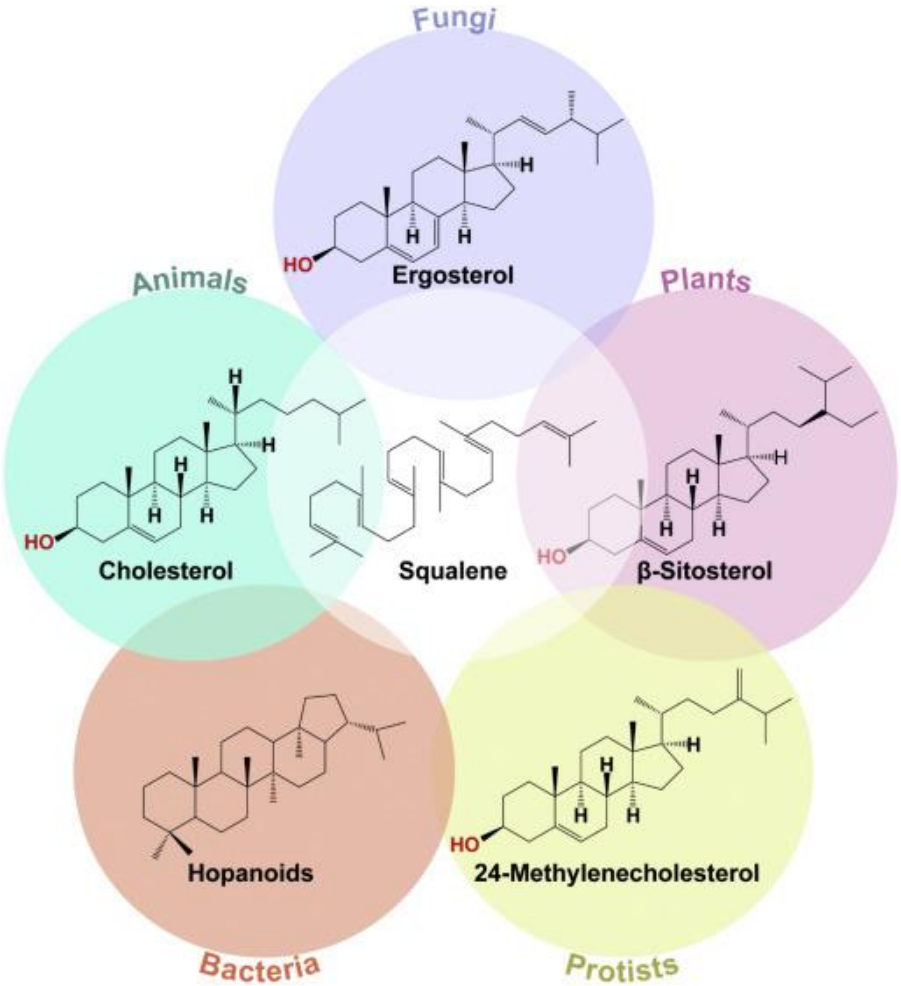


Figure 4. The different fates of squalene: precursor of cholesterol (animals), ergosterol (fungi), β-sitosterol (plants), 24-methylenecholesterol (protists) and hopanoids (bacteria) [5].

As already said, the principal source of squalene is the liver of marine animals rich in lipids and unsaponifiable component, such as sharks. Squalene is important for their survival in deep-water, because of the scarce supply of oxygen. In the last years the use of shark liver oil as a source of squalene has been limited by animal protection regulations. Also the presence of possible carcinogenic substances in the shark liver oil, such as organochlorine pesticides, polycyclic aromatic hydrocarbons, dioxins or heavy metals, led to find new natural sources for squalene, especially vegetal ones. Squalene was found in different plant oils at various concentrations. The first vegetable source in which it was identified was the olive oil, where the squalene content is about 564 mg/100 g. Then, it is present in grape seed oil (14.1 mg/100 g), in corn oil (27.4 mg/100 g), in peanuts oil (27.4 mg/100 g), in hazelnuts oil (27.9 mg/100 g) and in soybean oil (9.9 mg/100 g) [6]. In Europe squalene production on a large scale derives mainly from: sunflower oil (0–0.19 g/kg), corn oil (0.1–0.17 g/kg), soybean oil (0.03–0.2 g/kg) [7] and olive oil (1.7–4.6 g/kg) [8].The best plant source of squalene is the pseudograin *Amaranthus sp.*, about 4.16 g/kg of seeds. A study conducted on 104

genotypes of amaranth indicated squalene concentrations between 10.4 and 73.0 g/kg of oil [9]. **Table 4** shows the contents of squalene in different vegetables species. The yield is strictly dependent on the extraction and quantification methods, but also by biological and geographical factors, such as harvesting time, variety and geographical area [6].

Table 4. Content of squalene in different vegetable sources [6].

Vegetable source	Squalene concentration (mg/100 g oil)
Olive	150-747
Amaranth	6000-8000
Grape seeds	2.7-14.1
Pistachio	1.1-2.2
Walnuts, macadamia	0.9-18.6
Peanuts	9.8
Maize oil	10-27
Sunflower oil	2.2-2.6
Palm oil	0.1-1300
Soybean	1.2-180

Many studies have proved the health benefits of squalene in many aspects: nutritional, pharmaceutical and medicinal ones. Because of its potential uses, the statistics of Global Market Insights for 2016 revealed that the global production of squalene accounted for 5900 tons for a total value of USD 111.9 million. This production would increase by 2022 for about 9%. Asia and Europe are the most appealing markets for squalene, in particular Germany, France, UK, Italy, China and India have the

highest squalene demand [6]. The world need for squalene is covered by three main sources: animal (sharks), vegetable and synthetic methods. The squalene from shark liver oil is appreciated for the high yields, but plant sources are becoming important because of their environmental impact. Only 10% of world production of squalene is obtained by biotechnology techniques from *Saccharomyces cerevisiae, E. Coli, Aurontiochytrium sp.* and *Botrycoccus braunii* [10]. In vegetable sources the squalene concentration is highly dependent on the extraction method. Oils are commonly extracted by mechanical pressure or organic solvents, but refining processes are necessary to eliminate undesiderable components (pigments, free fatty acids, phospholipids). Refining methods can reduce the squalene yield to less than 80%. Then, solvent methods, even the high oil yield (98%), are not so suitable techniques because it is difficult to obtain high-purity squalene. Another promising method for oil processing is supercritical fluid extraction, that facilitates the separation of squalene without any traces of organic solvents. Another interesting source of squalene is the distillate of deodorization stage during oil refining. Squalene resulted to increase up to 30% because the distillate contains more unsaponifiable fraction than the distillates of other refining steps [6].

Squalene is mainly destined to food, cosmetic and medicinal industries that require high purity and quality. These two characteristics are strictly related to extraction and analytical methods. The most spread techniques for squalene determination are gas chromatography (GC) and high-resolution liquid chromatography (HPLC). These methods need some pretreatments of the samples, such as saponification. For these reasons they are usually time needing and could cause some modifications of squalene, e.g. during saponification [11]. Simpler and faster preparation techniques for subsequent GC analysis involve transmethylation of acylglycerols with KOH in MeOH and extraction with an organic solvent, having the sample ready for the analysis without the interference of triglycerides. The identification and quantification of squalene is mostly done by gas chromatography coupled to mass spectroscopy (GC-MS), or GC coupled to a flame ionization detector (GC-FID) and mass spectroscopy coupled to liquid chromatography (LC-MS) [11,12].

3.1.2 Properties and applications of squalene

Squalene has attracted the interest of the scientific world because of the beneficial effects of some natural sources containing it. From ancient times, fishermen used the oil extracted from shark liver to cure a wide range of conditions. Fishermen in Micronesia called shark liver oil “miraculous oil”, locals from Japanese peninsula Izu referred to it as “Samedawa”, that means “cure-all” [13]. In the last years different studies proved the beneficial effects of squalene, in different fields: cosmetical, pharmaceutical or nutraceutical. **Table 5** summarizes the principal health properties and applications of squalene.

Table 5. List of squalene properties and applications [6].

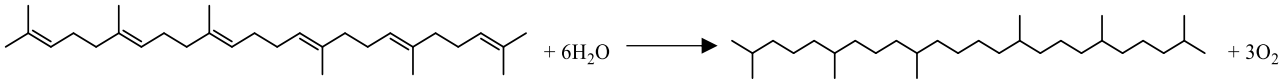
Biactive property	Application
Cardioprotector	Intravenous injection, oral consumption to cholesterol control
Antioxidant	Topical emulsions, oral administration
Antibacterial and antifungal	Cream topical, oral medication
Anticancer	Preventive and chemiotherapeutic substances
Detoxifying	Food supplement
Skin care	Cream topical
Drug delivery agent	Drugs and vaccines (emulsions, conjugates)

As previously said, olive oil is another important natural source of squalene. The lower incidence of CHD (cardiovascular heart diseases) and some cancers in the Mediterannean area, where the olive oil is highly used, encouraged on the possible protective effects of the minor components of olive oil. The daily squalene uptake from olive oil in Mediterranean region is about 200-400 mg/person, while in US the squalene intake reaches 30 mg/person per day [14]. In Greece the breast cancer incidence is 65% lower than in USA [14]. Newmark [15] suggested that this protective effect of olive oil utilization is connected to the high concentration of squalene. It is important to underline that the high content of this triterpene in shark liver could be responsible for the absence of cancer in this animal species [3]. Squalene alone is a weak inhibitor; on the contrary, when it is in conjunction with anticancer drugs it seems to prevent and arrest tumor cells proliferation [3]. Squalene appears to contribute more to prevention than treatment, as well. It exhibited antitumor properties against skin, sarcoma, colon and lung cancer in rodents in many studies [16,17]. Squalene exhibits its anticancer activities following these three possible mechanisms:

1. inhibition of Ras oncoprotein farnesylation;
2. regulating biosynthesis and function of xenobiotic meatabolizing enzymes;
3. acting as a free radical scavenger.

In anticancer drugs, squalene emulsions contributed to the potentiation of co-administered anticancer substances, such as adriamycin, bleomycin, cisplatin and 5-fluorouracil [18]. Squalene seemed to show not only antitumor but also chemoprotective effects. Cancer chemotherapy produces free radicals, which are responsible for many side effects (tissues damages, organ toxicity). Squalene is an effective antioxidant and it proved to be non-toxic, well-tolerated and a good cytoprotective

compound [14]. Its recognized antioxidant activity can be explained by its chemical structure: the six unsaturation sites along the isoprenoid chain. Due to the double bonds in the structure, this isoprenoid hydrocarbon plays as a strong antioxidant, being highly reactive to get into the oxidized structure. The unsaturated carbons react with the hydrogen ions from water and release 3 unbound oxygens, becoming the saturated form squalene (**Scheme 1**).



Scheme 1. Hydrogenation of squalene to squalane.

After this reaction, the free oxygen molecules reach the cells, the metabolism is improved and also the vital function of some organs (kidneys or liver) [14]. Squalene and other phenolic compounds in olive oil are also known for their protection against coronary and cardiovascular diseases. Even if squalene is an intermediate in the biosynthesis of cholesterol, its consumption does not increase the cholesterol content in human body. A study conducted by Chan [19] on patients with hypercholesterolemia, indicated that squalene supplementation decreased total cholesterol, LDL cholesterol and TAGs and increased HDL cholesterol. In addition, a supplementation of the diet with a high dosage of squalene seemed to decrease the blood level of glucose and body fat in rats and dogs [20, 21].

At the early 1950s, squalene was discovered as a component of human sebum and it explains its important role in the skin health. It is fundamental in skin repairing and hydration and in rejuvenating of aged skin. The study of Cho et al. [22] indicated that high squalene dosage (>13.5 g/day) decreased a lot wrinkles, increased type I procollagen and reduced the incidence of DNA damages by ultraviolet radiation in vivo experiments. Squalene, in fact, is a potent oxygen-scavenging agent. After an oxidative stress (for instance sunlight exposure), it acts as a quencher of singlet oxygen and helps to avoid the corresponding lipid oxidation on the skin surface [22]. Kohno et al. [23] revealed that its quenching rate constant is higher than those of other lipids and it is similar to the action of 3,5-di-*t*-butyl-4-hydroxytoluene. Squalene is not very susceptible to peroxidation and acts as a quencher of singlet oxygen, protecting human skin surfaces from peroxidation caused by exposure to UV light and other oxidative sources [22]. The hydration and emollient qualities of squalene and its compatibility with human skin make squalene one of the principal components of many cosmetics, such as creams, makeup, hair and nail products or lipsticks [24]. Squalene is quickly and deeply absorbed into the skin, restoring smoothness and elasticity without leaving oily residues. For these reasons, it is usually administered to cure atopic dermatitis, xerosis or skin lesions. The moisturizing effect of squalene was demonstrated using vernix oleosa, which is a fatty material present in the

newborns skin made by squalene, cholesterol, fatty acids, triglycerides and ceramides. its application was successful, expecially in the psoriasis treatment [24].

Many lipids are considered excellent carriers for their ability to permeate the cell and their non-toxicity. Squalene is one of these lipids; it is efficient in the emulsions and conjugates preparations for the release of drugs, prolonging also their shelf-life. Squalene is mostly used as adjuvant in vaccines. The oil in water emulsions with squalene favour solubilization and modifies the release and cell uptake of many adjuvants, drugs or vaccines. The most known today adjuvant containing squalene is the emulsion known as MF59, formulated by Novartis® with oil in water containing squalene (4.3% dispersed phase), surfactant span85, tween 80 and citrate in continuous phase. It is used in many vaccines, such as hepatitis B and C, herpes simplex virus, HIV virus and influenza virus [6]. According to World Health Organization (WHO) squalene was used in 22 million flu vaccines administered to patients in Europe in the last 24 years and no side effects were registered [25]. Another important application of squalene emulsions regards the carry and supply of poorly soluble drugs, reducing the toxicity of the drug. For instance, these lipid conjugates have acquired importance in the market with paclitaxel (Taxoprexin®) and cardiolipin conjugated with gemcitabine. The process of carrying the drug is known as “squalenylation”. It exploits the ability of squalene to coat the antiviral and anticancer compounds, to transport them into the cell and to induce their cytotoxic activity. In the last years squalenylation has been employed in the formation of nanoparticles (nanostructured lipid carriers) (100-300 nm), which are attracting attention as novel colloidal drug carriers [6].

3.1.3 Aim of the work

Squalene is a triterpene with demonstrated several beneficial properties. Because of its importance in many fields, this work aims to develop and validate a rapid and easy method to quantify squalene in different food matrices. Firstly, squalene content is evaluated in different types of extra virgin olive oils (EVOOs) (monovarietal EVOOs, niche blend EVOOs and EVOOs from supermarkets) and olive oils (OOs) provided by large distribution retail market. The purpose is to find possible differences in squalene level between these groups of oils in order to exploit this triterpene also as an adulteration marker. Squalene in also known to decrease after the refining process of vegetable oils. The second objective of this work is to investigate squalene losses during the refinement in different vegetable oils, also to identify the best source of squalene among different oils commonly used in food preparations. At the end, squalene is evaluated also in other food matrices, namely apple pomace (peels and seeds) of eleven different varieties (seven ancient cultivars and four commercial apples) in order to exploit these by-products as sources of interesting bioactive compounds. Apples are one

of the most abundant consumed fruits worldwide, generating large amount of wastes. Their appropriate reuse in cosmetical, food supplementation, nutraceutical and pharmaceutical sectors could contribute to environmental sustainability and economic profits too.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

The solvents chloroform and *n*-hexane were purchased from Carlo Erba Reagents (Cornaredo, Milan, Italy), while methanol was purchased from Fischer Chemicals (Hampton, New Hampshire, USA). Squalane (CAS number 501-94-0), squalene (CAS number 111-02-4) and all the standards mixture were purchased from Sigma-Aldrich (Milan, Italy). Supelco 37 Component FAME Mix certified reference material was purchased from Supelco (Bellefonte, Pennsylvania). Anhydrous sodium sulfate, sodium chloride and sodium hydroxide were purchased from Panreac Quimica SA (Barcelona, Spain). Deionized water (resistivity above 18 MΩ cm) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

3.2.2 Determination of squalene in olive oils and extra virgin olive oils

A total of 27 samples of extra virgin olive oils (EVOOs) and olive oils (OOs) were analyzed. A number of 11 oils were supposed to be high quality EVOOs: 6 Italian monovarietal Leccino EVOOs, 3 Italian niche EVOOs produced with a blend of Piantone di Mogliano and Orbetana (olives varieties cultivated in the province of Macerata, Marche region, Italy) and 2 Italian monovarietal Frantoio EVOOs. All these oils were produced by local companies in Marche region (Italy) and they were furnished by the producers. The other 10 EVOOs and 6 OOs were industrially produced, they are commonly found in retail market and they were purchased from supermarkets.

Transmethylation was carried out for each oil sample in order to perform the direct GC analysis together with FAMEs. An aliquot of 15 mg of oil was weighted in a 4 mL screw cap vial. Then, 1 mL of hexane and 10 μL of internal standard solution (squalane in hexane, 10 mg/mL) were added. After the addition of 0.1 mL of methanolic potassium hydroxide 2 N, the solution was vigorously stirred for 2 minutes with the help of a vortex device. After that time, the transmethylation reaction was quenched by adding 1.5 mL of saturated brine and the solution was stirred again for 2 minutes. Then, the organic and aqueous phases were stratified with the help of a centrifuge (5000 rpm) for 5 minutes. The upper hexane phase was separated and directly analyzed.

3.2.2.1 Quantification and method validation

The method was validated assessing linearity, interday and intraday repeatability, recovery and method sensitivity in terms of limit of detection (LOD) and limit of quantification (LOQ). Squalene in oils was quantified using the linear calibration curve obtained dividing the peak area of squalene for that of the internal standard area (squalane), analyzing six standard solutions at different squalene concentrations (0.01–0.50 mg/mL) and at fixed internal standard concentration (0.10 mg/mL). Repeatability was performed by analyzing five replicates of one of the EVOOs sample in the same day and five replicates in 5 different days and then calculating the % relative standard deviation (RSD %) for the squalene concentration found in each replicate. Recovery was assessed by spiking with squalene an EVOO sample containing squalene at 0.31 g/100 g, at two concentration levels: 0.05 and 0.10 mg/mL (0.33 and 0.66 g/100 g in the oil, respectively). LOD and LOQ were calculated on the basis of 3 and 10 times the signal to noise ratio, respectively. Each sample and each standard solution was prepared in triplicate and each replicate was analyzed twice.

3.2.3 Determination of squalene in refined vegetable oils

The squalene concentration was determined also in 7 different vegetable oils at different refining steps (**Figures 5, 6, 7, 8, 9, 10** and **11**), to investigate the effects of refining process on squalene content. In particular 3 oils were purchased from Casa Olearia Italiana S.p.a.-Marseglia Group (Monopoli, BA, Italy):

- Sunflower oil (crude, bleached and deodorised);

Figure 5. Sunflower oil samples from Marseglia Group.

- Grape seed oil (crude, bleached and deodorised);

Figure 6. Grape seed oil samples from Marseglia Group.

- Olive oil (crude and refined)

Figure 7. Olive oil samples from Marseglia Group.

Other 4 oils were furnished by Oleificio Zucchi S.p.a. (Cremona, Italy):

- Soybean oil (crude, neutralized, bleached and refined);

Figure 8. Soybean oil samples from Oleificio Zucchi.

- Maize oil (crude, dehydrated, bleached and refined);

Figure 9. Maize oil samples from Oleificio Zucchi.

- Sunflower oil (crude, neutralized, bleached and refined);

Figure 10. Sunflower oil samples from Oleificio Zucchi.

- High oleic (HO) sunflower oil (crude, dehydrated, bleached and refined).

Figure 11. HO sunflower oil samples from Oleificio Zucchi.

Before the analysis the samples were subjected to the transmethylation as described above for EVOOs and OOs. The same quantification and analytical method were applied.

3.2.4 Determination of squalene in apple peels and seeds

Squalene was determined in the peels and seeds of 11 apple varieties (7 ancient varieties and 4 commercial ones). The fruits of the 7 different ancient apple cultivars (*Mela Rosa di Pietra, Mela Rosa, Mela Rosa di Pietra Locale, Mela Rosa di Fogliano, Mela Rosa Fragola, Mela Carella* and *Mela Limoncella)* were picked at ripening in an orchard of the educational agricultural company "Istituto Tecnico Agrario Giuseppe Vivarelli" sited in Fabriano (GPS coordinates: N 43°20'29.76"; E 12°54'29.52", altitude 325 m a.s.l.), Marche region, Italy, while the commercial apples *Granny Smith, Golden Delicious, Royal Gala* and *Fuji* were purchased in local supermarkets.

The fruits were stored at 4°C in a refrigerator until analysis. Apples of individual varieties were separated to obtain the skin and the seeds. All the peel was removed using a peeler and scraping off the uppermost 2 mm of peel (upper skin including epidermis and small part of sub-epidermis), making attention to avoid scraping also the pulp. The seeds and the peels destined for lipid extraction were homogenized using a laboratory mill and stored in freezer at - 20°C. While collecting the fruits and preparing the samples, special attention was addressed to limit degradation of their different components.

Lipid fraction was extracted from apple peels and seeds according to Folch method [26]. In brief, apple seeds (0.5 g) and apple peels (10 g) were grinded with the help of an analytical mill (IKA® Tube Mill control), supplemented with a solvent mixture of chloroform/methanol 2:1 and homogenized by Ultraturrax (YellowLine DI 25 basic immersion-type homogenizer). The solution was filtered and washed with KCl in water 0.88% *w*/*v*. The organic phase was recovered and dried over anhydrous sodium sulfate. The solvent was removed by a rotavapor until constant weight. Lastly, the lipid extract was recovered with chloroform and stored in a refrigerator at - 20°C. Also, moisture content of peels and seeds was determined after oven drying the samples until a constant weight at 105°C in duplicate.

For the determination of squalene content, 15 mg of lipid extract (both from apple peels and seeds) was dissolved with 0.5 mL of hexane and added with 25 μL of internal standard solution (squalane in hexane at 1 mg/mL). An aliquot of 0.1 mL of methanolic KOH 2N was added and the sample was stirred for 2 min with the help of a vortex device. Then, the reaction was quenched by adding saturated brine (1.5 mL) and after vigorous vortex stirring for 2 min, the two layers were stratified with the help of a centrifuge (5000 rpm) for 5 min. The upper hexane phase was directly analyzed.

3.2.4.1 Quantification and method validation

Method validation has been performed by assessing linearity, intraday and interday repeatability, recovery and method sensitivity in terms of LOD and LOQ. The quantification was performed by using the linear calibration curve obtained dividing the peak area of squalene for that of the internal standard area (squalane), analysing six standard solutions at different squalene concentrations (0.005-0.250 mg/mL) and at fixed internal standard concentration (0.05 mg/mL). Recovery and repeatability were assessed for both apple peels and seeds samples. The intra-assay precision was determined by analysing three aliquots of the same apple peel sample and three aliquots of the same seeds sample on the same day. The inter-assay repeatability was assessed by three replicates of the same apple peels and apple seeds samples on three different days. In between, the samples were stored at −20°C. The

repeatability of the method was expressed as precision (RSD, %). For the recovery test, a solution containing squalene at 0.10 mg/mL was added to an apple peel sample and to an apple seeds sample at three different and known concentration levels: 50, 100 and 200 μL (spike 1, 2, and 3, respectively). Then, the spiked samples were subjected to treatment as described above and analyzed by GC-FID. LOD and LOQ were determined on the basis of 3 and 10 times the signal to noise ratio, respectively. Each sample and each standard solution were prepared in triplicate and each replicate was injected twice.

3.2.5 GC-FID analysis of squalene

After the pretreatment of the samples, the hexane phase was directly analyzed for the determination of squalene using a GC coupled to flame ionization detector (6850, Agilent Technologies, Santa Clara, CA, USA). An aliquot of 1 μL was injected in split mode (20:1 split ratio). The injector was at 300 °C. The carrier gas was hydrogen, produced by a generator (PGH2-250 from DBS Analytical Instruments, Vigonza, Italy). The chromatographic column coating was 5% phenyl 95% methylpolysiloxane (HP-5, length 30 m, 0.32 mm i .d., 0.25 μm film thickness; Agilent Technologies). At the beginning the gas flow rate in the column was 3.7 mL/min. The oven temperature was held at 260 °C for 3 min, then raised at 50 °C/min until 350 °C and held at 350 °C for 0.2 min, for a total run time counting for 5.0 min. The FID temperature was set at 360 °C, while the hydrogen and air flows were 40 and 400 mL/min, respectively. Identity of the different FAMEs, squalane and squalene was also confirmed by GC-MS.

3.2.6 Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) and to Tukey's test for pairwise comparison in order to assess significant differences ($P<0.05$) between the squalene content in the different EVOOs and OOs groups, in the vegetable oils at different refining steps and in the peel and seeds between the different apple varieties. The software used was PAST [27].

3.3 Results and Discussion

The principal aim of this study was the optimization and validation of an easy and rapid method for the analysis of squalene in different food matrices. The basic idea was the analysis of this terpene together with FAMEs after the transmethylation of the lipid fraction, avoiding the time-needing saponification reaction. The same approach has been performed in other studies [11, 28, 29, 30], but in this case the amount of reagents and samples was reduced and also the chromatographic analysis time. The use of a 5% phenyl-polydimethylsiloxane coated capillary column and the most suitable GC conditions provided a good separation of squalene from the other analytes and a total chromatographic run of 5 minutes. So, this method is very convenient especially when there are high number of samples to process. During this study the method was applied to the evaluation of squalene content in EVOOs and OOs, in refined edible oils and in apple peels and seeds.

3.3.1 Determination of squalene in olive oils and extra virgin olive oils

The proposed method was firstly applied to the analysis of squalene in 27 different EVOOs and OOs of different qualities. Some of them were purchased from large retail distribution market and some EVOOs were provided by little Italian producers. **Figure 12** shows a typical chromatogram obtained from the analysis of squalene in an EVOO sample. As it can be seen, the squalene peak is well resolved and separated from the FAMEs that eluted earlier and also from the peak of squalane, which was used as internal standard.

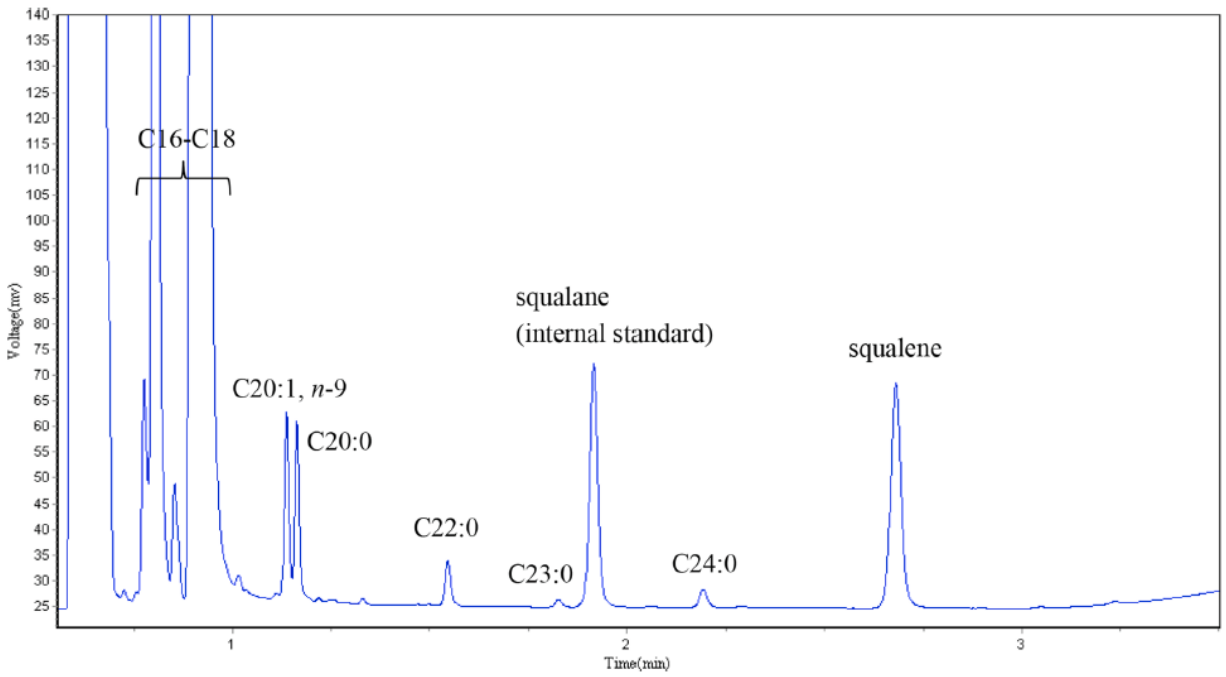


Figure 12. Chromatogram obtained from the analysis of squalene in an EVOO sample (niche blend). FAMEs are also indicated, specifying the number of carbons along their fatty acid chain. For resolved FAMEs also the number and the position of double bonds are described (Cx:y, x is the carbons number and y the number of double bonds).

Before the application to all the samples the method was validated by assessing linearity, intraday and interday repeatibility, sensitivity (LOD and LOQ) and recovery. Calibration curve (**Figure 13**) was forced through zero because the intercept was not significantly different than zero.

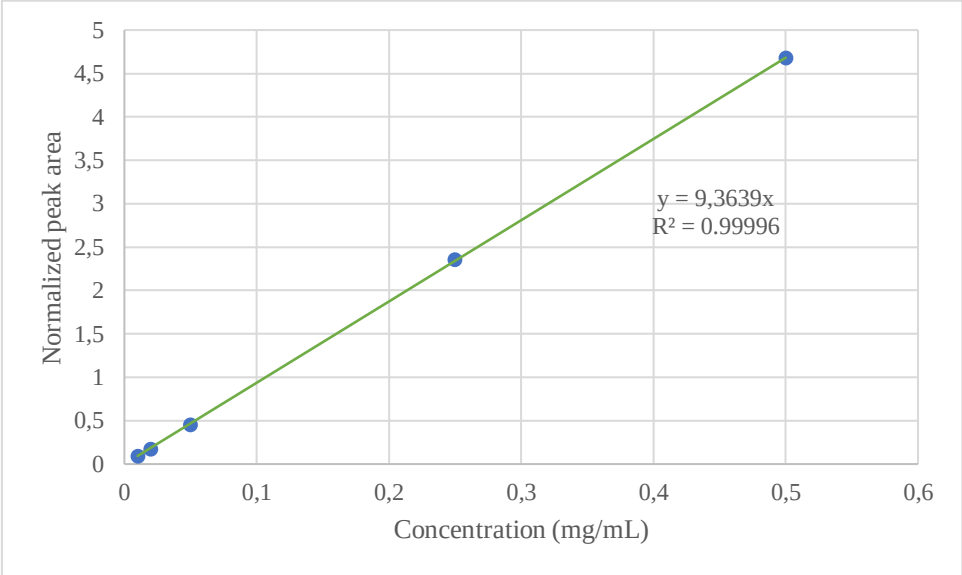


Figure 13. Calibration curve of squalene. The equation and the R^2 value are indicated on the graph.

Linearity was excellent with a calculated R^2 equal to 0.99996. Also the other validation parameters are shown in **Table 6**.

Table 6. Method validation parameters.

Linearity[a] (mg/mL)	R^2	LOD (g/100 g of oil)	LOQ (g/100 g of oil)	Repeatability[c] RSD[b] % (*n*=5)		Recovery[d] %	
				Intraday	Interday	Spike 1	Spike 2
0.01-0.5	0.99996	0.007	0.022	1.1	1.6	95.2	97.4

a.Range of concentration values of squalene standard solution in hexane, used for the calibration curve;

b.RSD: relative standard deviation

c,d: determined using the EVOO sample n.7 (Table 7)

The method provided good results in terms of sensitivity (LOQ equal to 0.022 g). It has been possible to quantify squalene in all the EVOOs, where the usual range is about 0.1-1.2 g/100 g [29] and OOs, where the concentration is lower because it is significantly influenced by oil refining process [14]. The lowest squalene concentration detected in one of the OOs samples was 0.17 mg/100 g, ten times higher respect to the LOQ value. Regarding the obtained recovery values, Budge and Barry [30] reported higher values respect the present study: 100-103% versus 95.2% and 97.4% for spike 1 and 2 (respectively). However, the obtained values were higher respect the data of Nenadis and Tsimidou [31] (81% and 92%), who performed fractioned crystallization and rather higher than those obtained by Seçmeler and Ustüdang [32] after the saponification of the oil (92 and 95%). The method was validated also in terms of intraday and interday repeatability (1.1% and 1.6% respectively, as relative standard deviation of the squalene concentration in 5 replicates). De Leonardis et al. [29] and Budge and Barry [30] reported higher values for repeatability (4.5% and 2.7%, respectively), while Lanzón et al. [11] obtained similar results (1.37%-1.97%). Despite all, the most important advantage of the proposed method is the very fast sample preparation and the short chromatographic run. Our method employed smaller quantity of sample and reagents with respect to other methods employing transmethylation for the sample preparation [11, 29] and very short time for sample preparation (10 minutes). It also provided shorter chromatographic times: 5 minutes with respect to 15, 30 and 33 minutes needed in other studies employing GC-FID analysis [11,29,33].

The rapidity and easiness of the method allowed to analyze a quite high number of samples in relative short time. A total of 27 oils were studied, composed by EVOOs and OOs industrially produced and sold in supermarkets and by EVOOs produced by little companies: Italian Leccino and Frantoio

monovarietal and niche blend produced using olives from Orbetana and Piantone di Mogliano varities.

The obtained results are reported in **Table 7** for all the analyzed samples, indicating also the squalene content range for all the evaluated oil category.

Table 7. Squalene concentration (g/100 g of oil) in olive oils (OOs) and EVOOs industrially produced and purchased from supermarkets and in EVOOs locally produced. SD indicates standard deviation. Different letters reported in the mean values column indicate statistically significant differences (One-way ANOVA, Tukey's test for pairwise comparison, $P < 0.05$) between the oil groups in term of squalene content.

Sample (n)	Commercial category	Market distribution	Squalene concentration (g/100 g of oil)		
				Mean ± SD	Range
1	OO	supermarket	0.17	0.23 ± 0.03 [a]	0.17-0.27
2	OO	supermarket	0.23		
3	OO	supermarket	0.23		
4	OO	supermarket	0.24		
5	OO	supermarket	0.24		
6	OO	supermarket	0.27		
7	EVOO	supermarket	0.31	0.44 ± 0.10 [a,b,c]	0.31-0.65
8	EVOO	supermarket	0.31		
9	EVOO	supermarket	0.35		
10	EVOO	supermarket	0.41		
11	EVOO	supermarket	0.41		
12	EVOO	supermarket	0.46		
13	EVOO	supermarket	0.47		
14	EVOO	supermarket	0.49		
15	EVOO	supermarket	0.51		
16	EVOO	supermarket	0.65		
17	EVOO	Little producer (Leccino)	0.35	0.56 ± 0.25 [c]	0.35-1.02
18	EVOO	Little producer (Leccino)	0.35		
19	EVOO	Little producer (Leccino)	0.40		
20	EVOO	Little producer (Leccino)	0.43		
21	EVOO	Little producer (Leccino)	0.46		
22	EVOO	Little producer (Leccino)	0.52		
23	EVOO	Little producer (Frantoio)	0.43		

24	EVOO	Little producer (Frantoio)	0.43
25	EVOO	Little producer (niche blend: Piantone di Mogliano and Orbetana)	0.81
26	EVOO	Little producer (niche blend: Piantone di Mogliano and Orbetana)	0.97
27	EVOO	Little producer (niche blend: Piantone di Mogliano and Orbetana)	1.02

Olive oils showed the lowest amount of squalene, in the range 0.17-0.27 g/100g. In industrial EVOOs it was higher: 0.31-0.65 g/100g and in EVOOs from little producers squalene was found in the broader range of 0.35-1.02 g/100 g, with the niche blend EVOO containing the highest values (0.81-1.02 g/100 g). **Figure 14** shows the general trend of squalene in the investigated samples.

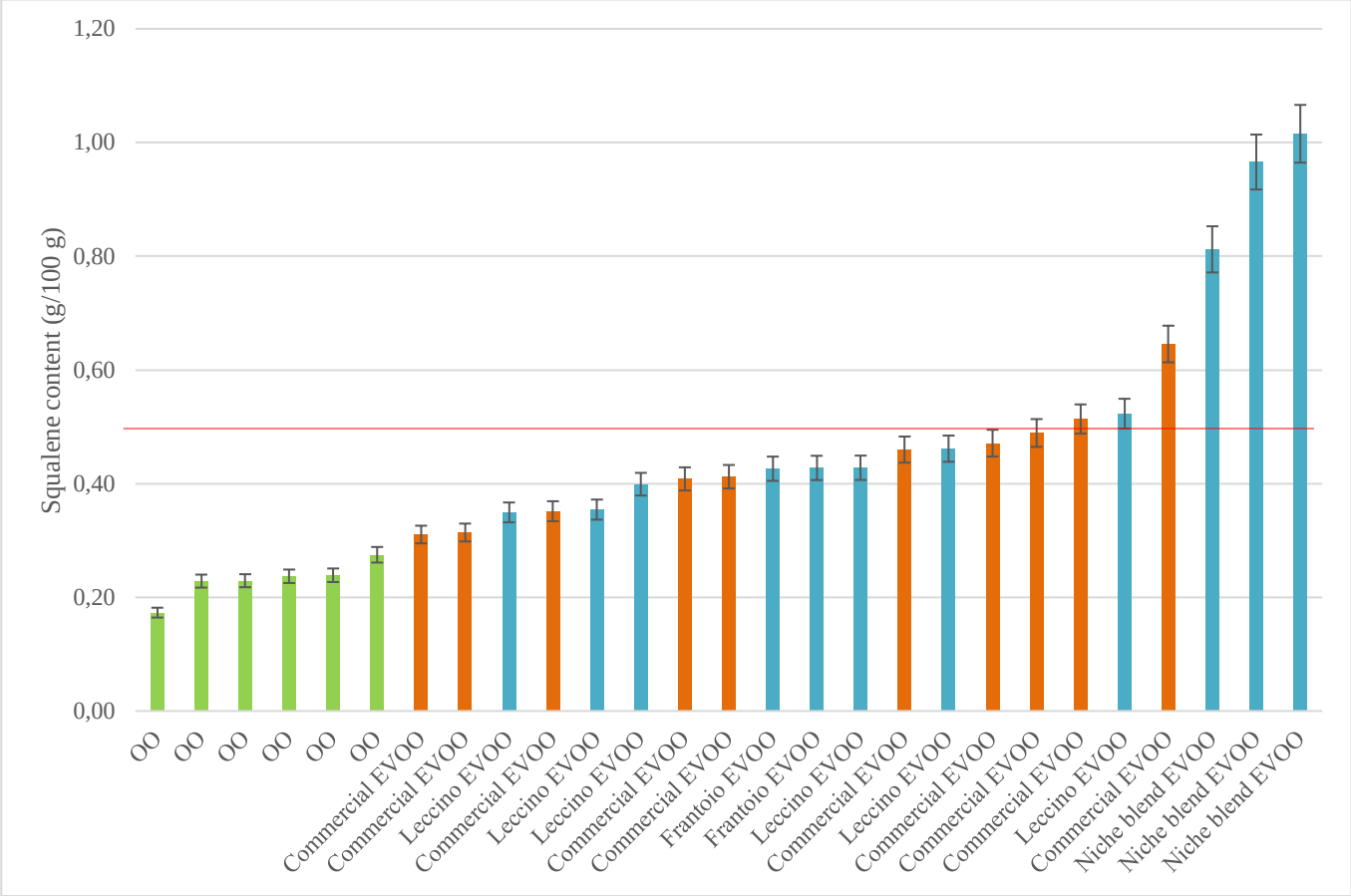


Figure 14. Squalene content in each investigated oil sample. OO indicates olive oils. Extra virgin olive oils from large scale distribution are indicated as "Commercial EVOOs", while EVOOs from little producer are indicated as "Leccino EVOOs", "Frantoio EVOOs" and "niche blend EVOOs". Bars indicate ± standard deviations. The red line indicates the average content of squalene between all the samples (0.44 g/100 g).

As shown, olive oils are grouped at the beginning of the graph because their lowest squalene content, while niche blend EVOOs at the end, because of the highest concentrations. All the 3 investigated niche blend EVOOs presented squalene contents much higher than the average amount of all the samples (0.44 g/100 g), indicated by the red line. On the contrary, commercial EVOOs and the monovarietal EVOOs Frantoio and Leccino reported similar squalene content (0.31-0.65 g/100 g).

The differences in terms of squalene content between the investigated oils groups are reported in **Figure 15**.

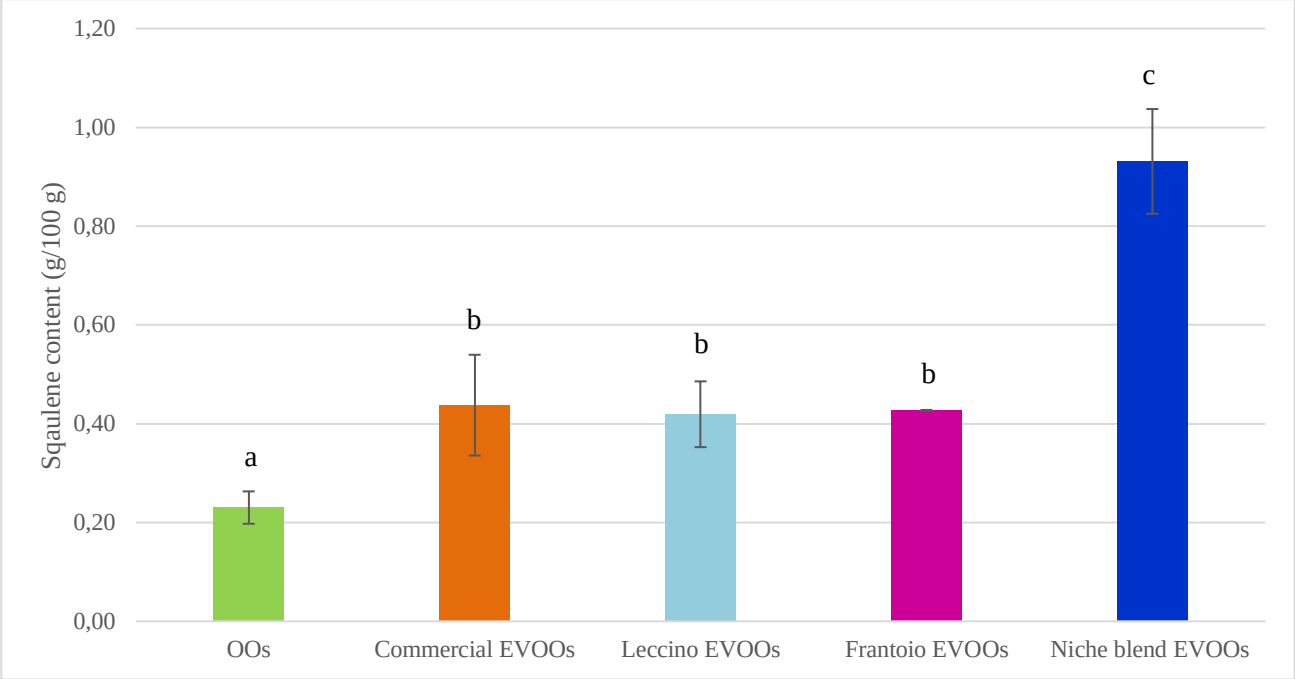


Figure 15. Average squalene content (g/100 g) in olive oils (OOs), commercial extra virgin olive oils (commercial EVOOs) and EVOOs from little producers (Frantoio EVOOs, Leccino EVOOs and Niche blend EVOOs). Bars indicate ± standard deviations. Different letters indicate significant differences between the oils groups (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison).

Statistical analysis indicated that all the investigated EVOOs had significantly higher amount of squalene respect to OOs. A similar situation was reported also by Owen et al. [34], who compared the squalene content of extra virgin olive oils and refined olive oils (424 mg/100 g and 340 mg/100 g, respectively). The lower concentrations in refined oils can be due to the losses occurring during the refining steps. Squalene content in oils is highly influenced by many factors, such as the methods of olive growing, oil extraction techniques, olives variety, refining process and possible adulteration of EVOOs with seed oils. The squalene amount in EVOOs is in fact used as an indicator of adulteration [35]. Among all these factors, refining process is crucial, because all the crude vegetables

oils need to be refined before their consume, except good quality virgin olive oils. Nergiz et al. [35] reported an average squalene content in crude olive oil samples of 491 mg/100 g, similar to the average value found in the present study (0.44 g/100 g). This content decreased for all the refining steps, especially after the deodorization process. The total reduction reported by Nergiz et al. [35] was 40.94%, in fact at the end of the refining the squalene content in the olive oils was on average 290 mg/100 g. Also in this case, significant differences were found before and after the refining process in terms of squalene reductions ($P < 0.05$).

Comparing the different EVOOs groups, statistical differences were found between the niche blend EVOOs and the other commercial and monovarietal EVOOs. Squalene variability can be explained with a large number of factors, such as climatic and geographical conditions, stage of maturity of the fruits, agricultural methods, harvest period, extraction and process technology [36], but many studies revealed that squalene content is mainly related to genetic factors [37, 38]. In particular, Beltràn et al. [37] investigated EVOOs from 28 olives cultivars grown in the same orchard and under identical growing conditions. Harvest and ripening periods, and extraction method were the same, thus, the variability was represented largely by genetic factors. When ANOVA was performed, the variety accounted for 95.6%. The study indicated that VOOs squalene content is affected by the genetic factor and it can be used as an indicator to discriminate oils from different cultivar and to certify monovarietal oil authenticity. Commercial EVOOs and the monovarietal Frantoio and Leccino EVOOs did not show significant differences between them, much lower as compared to the niche blend EVOOs investigated. Considering the classification made by Beltràn et al. [37], Leccino and Frantoio EVOOs can be considered as “low-medium” squalene concentration (0.15-0.6 mg/100 g), while niche blend EVOOs as “very high” squalene content (> 0.75 g/100 g), indicating these oils as optimal source of this bioactive compound. Besides the importance of squalene in the diet and its use in pharmaceutical or cosmetical formulations, squalene seems to contribute to the oxidative stability of olive oil. Psomiadou and Tsimidou [39] suggested that the protective and antioxidant effect of squalene increased with concentration. EVOOs with high squalene content could have also a longer shelf-life, being less susceptible to oxidation.

3.3.2 Determination of squalene in refined vegetable oils

As previously said, refining process affects the squalene content in edible oils. Since crude vegetable oils can’t be consumed without refining (except for EVOOs), it is important to investigate the changes in the amount of squalene in different vegetable oils during refining process. The typical characteristics for refined vegetal oils are bland taste, good oxidative stability and a light colour. In order to become suitable for human consumption refining aims to remove undesired substances with

unpleasant effects on taste, aspect, smell and stability, such as free fatty acids, waxes, metals, color pigments, oxidation products and odorous components. By refining, the oil stability and organoleptic properties are improved, but many minor and beneficial compounds are removed, such as antioxidants, vitamins, phytosterols or squalene. Two different processes (**Figure 16**) can be performed: chemical and physical refining. The main difference between physical refining and chemical refining of edible oil lies in the way that how to remove free fatty acids: a chemical neutralization and a physical refining by distillation, respectively. In the chemical refining process a degumming step removes phospholipids by washing with water or acid treatment. Then free fatty acids are neutralized by a weak alkali solution and washed out of the oil as soaps. During the bleaching step, residual phospholipids, metals and soaps are removed using bleaching earth pigments. Then, deodorization permits to remove odorous components and residual free fatty acids. It is made by a steam vacuum distillation. During this last step, free fatty acids and many other minor compounds (squalene, phytosterols, tocopherols) are distilled and recovered in the deodorized distillate (DD) [40]. Especially the loss of these minor and functional compounds negatively influences the nutritional quality of refined oil. Nergiz et al. [35] reported the decrease of squalene in olive oil during the different refining steps: 13% by physical refining, 7% after the discolouring step and 15.6% by deodorization. Due to the low content of squalene in vegetable oils, this decrease could become relevant and considerable.

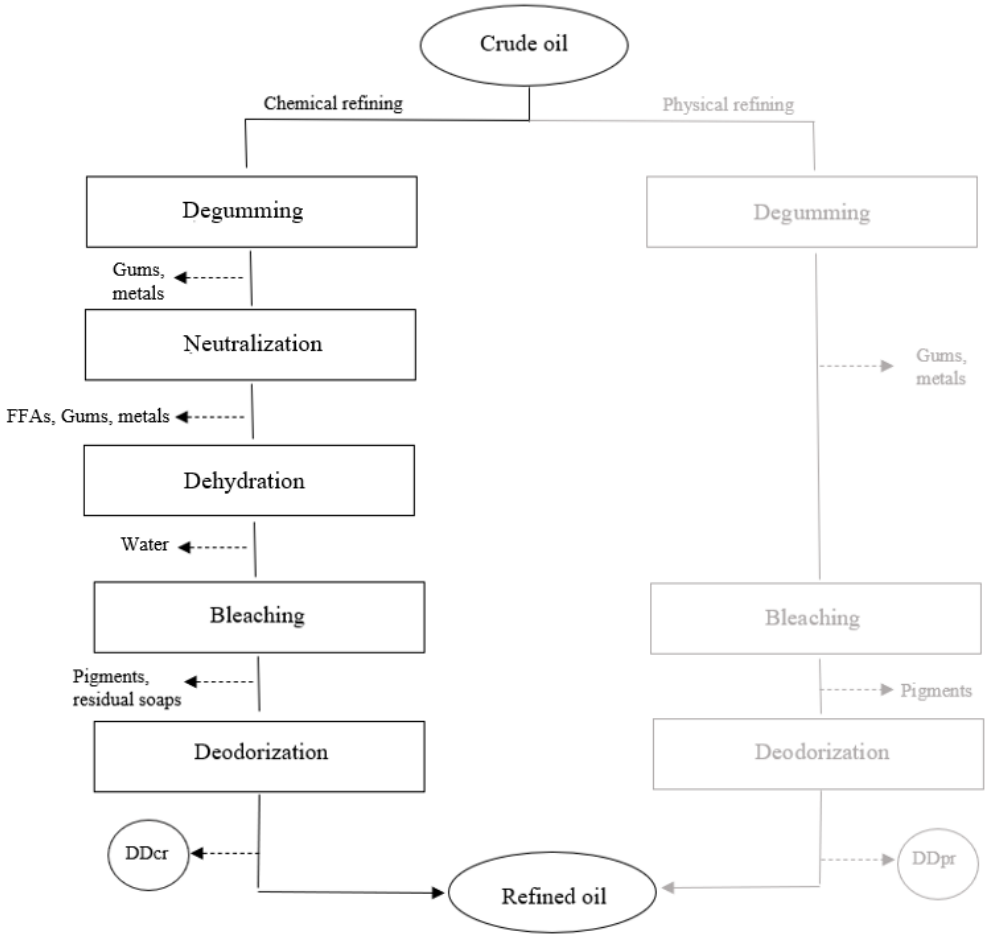


Figure 16. Chemical and physical process of refining crude vegetal oils. DDcr indicates "deodorization distillate from chemical refining" and DDpr "deodorization distillate of physical refining" [14].

A promising source of squalene is the deodorizer distillate, which results as by-products rich in many valuable compounds. It contains 15%-30% of unsaponifiable fraction, with a squalene content up to 80%. Olive oil DD has the bigger concentration of squalene with respect to other distillates obtained from other vegetal oils.

It is also a source of other valuable components, such as fatty acids, sterols and vitamins (**Table 8**), even if the need for squalene from vegetal sources can not be completely covered by oil by-products from distillation. It is important to find new valuable vegetal sources and new easier and cheaper ways to obtain squalene.

Table 8: Chemical composition (% wt) of most common edible oil deodorizer distillates (DD) [14].

Oil DD	Free fatty acids (% wt)	Sterols (% wt)	Hydrocarbons (% wt)	Other compounds (% wt)
Olive oil	34.2	4.6	31.5 (squalene 28%)	5.6
Soybean oil	30-60	10-35	10-30	7
Palm oil	20.34	4.77	3.94 (squalene 100%)	62.12

In the present study, the proposed GC-FID analytical method was applied for the evaluation of squalene content in refined vegetal oils, studying the influence of different refining processes on its concentration. The oils at different refining steps were purchased by two Italian vegetable oil refineries and the results in terms of squalene content are showed in **Table 9**. Also the reduction of squalene content at each step, when occurred, was calculated, by the formula:

$$\%\ Reduction = \frac{Squalene\ content\ in\ previous\ step - Squalene\ content\ in\ following\ step}{Squalene\ content\ in\ previous\ step} x100$$

The total reduction was calculated in similar way:

$$\%\ Total\ reduction = \frac{Squalene\ content\ in\ crude\ oil - Squalene\ content\ in\ refined\ oil}{Squalene\ content\ in\ crude\ oil} x100$$

Table 9. Changes in squalene content (mg/100 g) ± standard deviation (*n*=3) of vegetable oils during refining steps. The average percentage reduction, when occurred, at each step is indicated in brackets. The average percentage total reduction is also indicated.

Refining step	Olive oil (mg/100 g)	Grapeseed oil (mg/100 g)	Sunflower oil 1[a] (mg/100 g)	Sunflower oil 2[b] (mg/100 g)	HO Sunflower oil[b] (mg/100 g)	Soybean oil (mg/100 g)	Maize oil (mg/100 g)
Crude oil	497.96±18.82	45.45±2.71	40.54±3.35	32.39±0.55	38.69±2.79	18.33±0.49	36.74±1.99
Neutralization	nr	nr	nr	32.32±3.19 (0.22 %)	nr	22.87±0.32	nr
Dehydration	nr	nr	nr	nr	33.25±0.88 (14.06 %)	nr	35.07±1.81 (4.54 %)
Bleaching	nr	36.46±2.48 (19.77 %)	29.06±1.39 (28.31 %)	27.96±0.28 (13.49 %)	28.32±2.17 (14. 83 %)	21.96±2.26 (4.14 %)	37.42±0.53
Deodorization	nr	19.18±0.70 (47.39 %)	20.75±1.05 (28.59 %)	nr	nr	nr	nr
Refined oil	529.62±12.57	nr	nr	27.21±2.55 (2.68%)	23.50±2.07 (17.02 %)	24.27±0.95	27.69±1.85 (26.00 %)
Total reduction	No reduction	57.80 %	48.81 %	25.99 %	39.26 %	No reduction	24.63 %

a. purchased by Casa Olearia Italiana S.p.a.-Marseglia Group
b. purchased by Oleificio Zucchi S.p.a.
nr. Not received

For a better understanding **Figures 17, 18, 19, 20, 21, 22, 23** indicate the results for each analyzed oil.

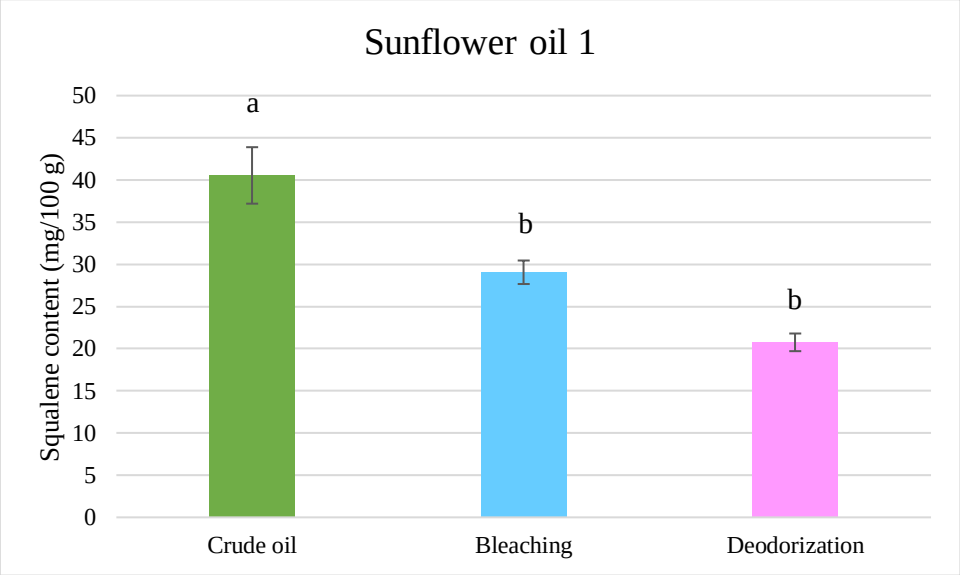


Figure 17. Squalene content of sunflower oil 1 during refining steps. Bars stand for standard deviation (SD). Different letters indicate significant differences ($P < 0.05$) between the different refining steps.

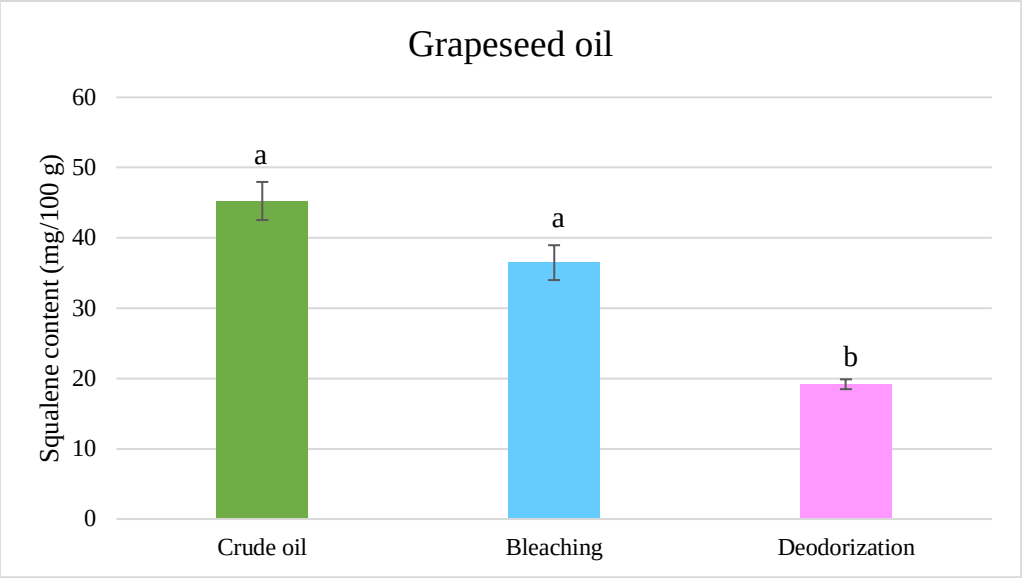


Figure 18. Squalene content of grapeseed oil during refining steps. Bars stand for standard deviation (SD). Different letters indicate significant differences ($P < 0.05$) between the different refining steps.

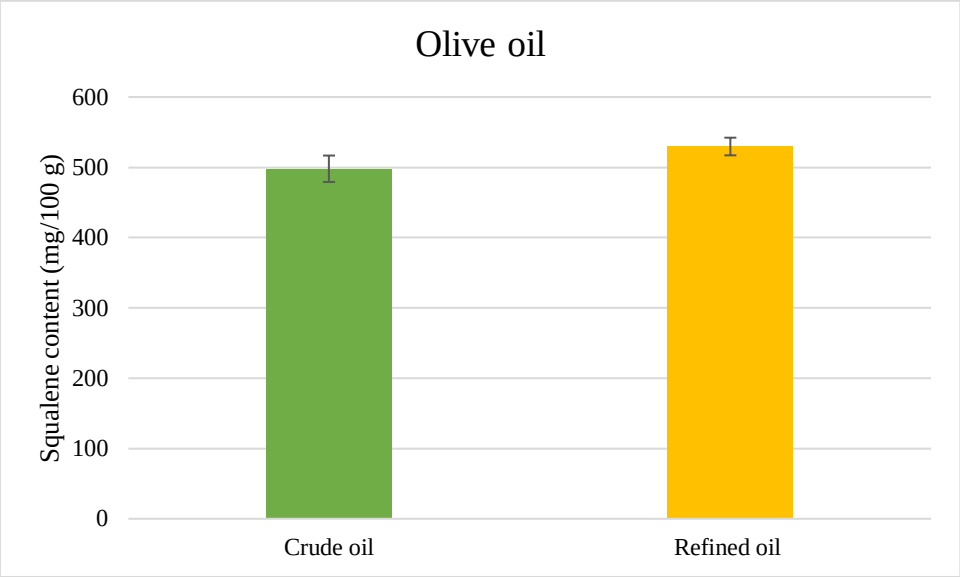


Figure 19. Squalene content of olive oil during refining steps. Bars stand for standard deviation (SD).

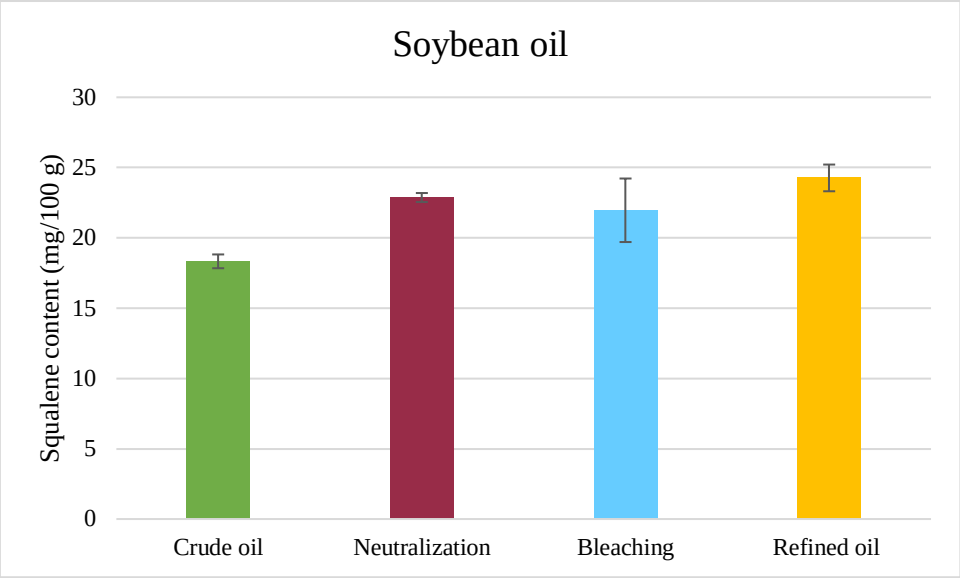


Figure 20. Squalene content of soybean oil during refining steps. Bars stand for standard deviation (SD).

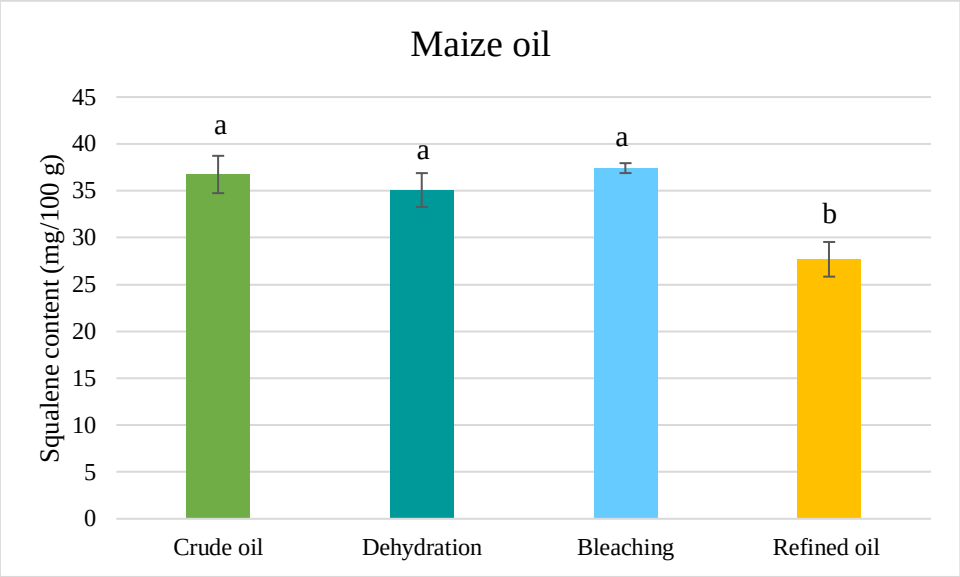


Figure 21. Squalene content of maize oil during refining steps. Bars stand for standard deviation (SD). Different letters indicate significant differences ($P < 0.05$) between the different refining steps.

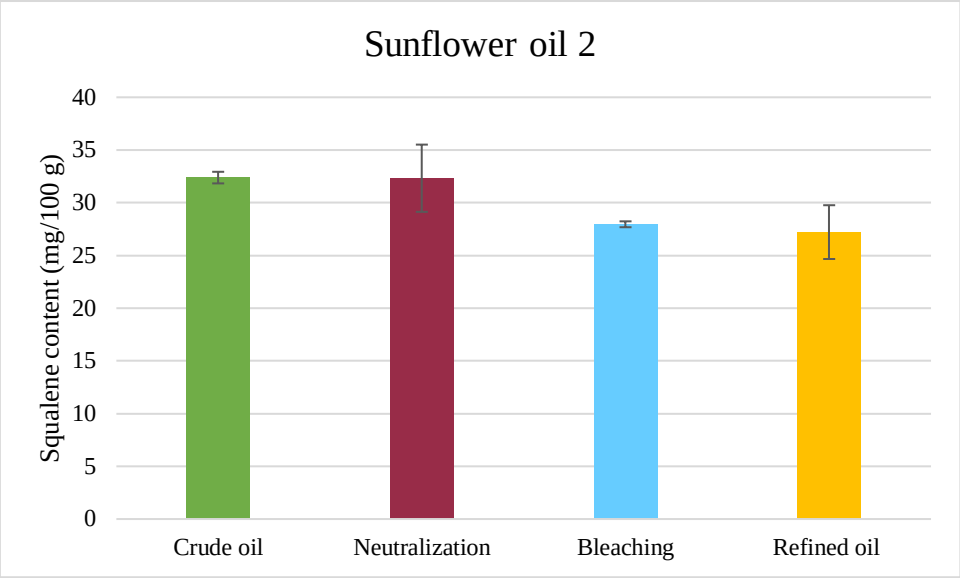


Figure 22. Squalene content of sunflower oil 2 during refining steps. Bars stand for standard deviation (SD).

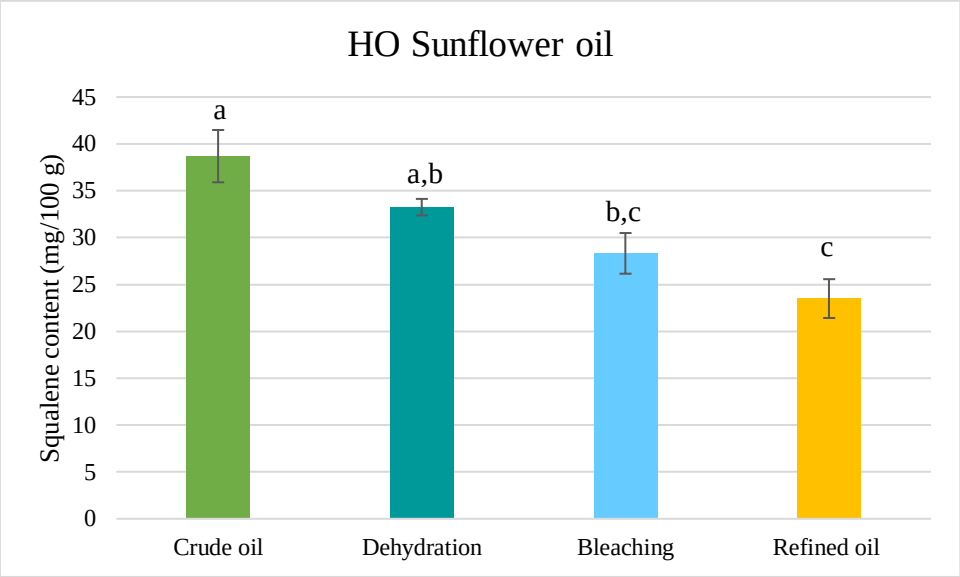


Figure 23. Squalene content of HO sunflower oil during refining steps. Bars stand for standard deviation (SD). Different letters indicate significant differences ($P < 0.05$) between the different refining steps.

Squalene content of crude oils was the lowest in soybean oil, with a value of 18.33 ± 0.49 and the highest in olive oils, with a value of 497.96 ± 18.82 mg/100 g. It was in accordance with the values reported by other studies related to squalene content of olive and seed oils [34, 35]. The average concentration of squalene in olive oils was found to be 497.96 ± 18.82 mg/100 g and it did not decrease after the refining process. The squalene content in refined olive oil did not show any

significant differences with the content in crude oil, even if it is in contrast with the results reported by other studies. Nergiz et al. [35] reported a total reduction of 40.9% and a significant decrease was found also by Vazquez et al. [41].

Three different samples of sunflower oil were analyzed: two conventional oils and one high oleic acid sunflower oil. The average amount of squalene in these oils was 37.21 mg/100 g, quite lower with respect to olive oil. The obtained value was found to be higher with respect to the squalene content in sunflower oil indicated by Nergiz et al. [35] (13.8 mg/100 g). Table 9 shows a high squalene reduction for all the three analyzed sunflower oils, in particular for the sample 1 (48.8%), followed by HO sunflower oil (39.3%) and sunflower oil 2 (26.0%). The highest reduction has occurred during deodorization for sample 1 (28.59 %), as reported also by Nergiz et al. [35]. Statistical analysis showed significant difference (P<0.05) between the crude and refined sunflower oil 1 and HO sunflower oil. For the sample 2 no statistical differences were recorded among the different refining steps.

In the refining process the squalene amount of maize oil was reduced marginally. The squalene content remained almost unchanged during all the refining process. In fact, there were no significant differences between the squalene amount in crude (36.74 mg/100 g), dehydrated (35.07 mg/100 g) and bleached (37.42 mg/100 g) maize oil. Total lowering was 24.6% in maize oil at the end of the refining, similarly to the 25.9% reduction indicated by Nergiz et al. [35].

Crude soybean oil contained 18.33±0.49 mg/100 g squalene as average (**Table 9**). Similar values have been reported by Nergiz et al. [35] (18.1 mg/100 g). Squalene did not show any reduction during the refining steps, as found for olive oils and its content remained almost the same (no significant differences between the refining steps). This different behaviour of soybean and olive oils respect the other investigated vegetables oils may be due to both differences in nature of oils and refining conditions [35] and more samples should be analyzed to investigate the possibility of some contaminations of these oils during the refining process.

Grapeseed oil exhibited the highest total reduction of squalene (57.8%) during the refining process. Crude grapeseed oil contained 45.45±2.71 mg/100 g squalene as average. Lower values were reported both by Zhao et al. [42] (17.81 mg/100 g) and Wen et al. [43] (10.20-16.29 mg/100 g). These differences could be due to the different extraction methods applied during the sample pretreatment, in fact both Zhao et al. [42] and Wen et al. [43] performed saponification for the analysis of squalene. Afinisha Deepam and Arumughan [44] indicated a reduction of squalene from 0.36 mg/g to 0.15 mg/g

in crude rice bran oil analyzed without saponification and after saponification, respectively. However, differences due to the grape varieties could also explain the difference. As for sunflower oil (sample 1), also for grapeseed oil the highest squalene reduction occurred after the deodorization step (47.4%). Also Shahidi and Wanasundara [45] indicated that the highest losses of squalene (63%) occurred after the deodorization step for sea bubbler oil refining. This could be explained by the fact that high temperature reached during deodorization caused both evaporation and degradation of squalene.

During the refining of the different vegetable oils, the decrease in the squalene amount varied from 24.6% to 57.8%, with a considerable difference between crude and refined seed oils in terms of squalene level, with the exceptions of olive and soybean oils where there were no losses of squalene after the refining process. Moreover, olive oil, even if it is refined, contains 10 to 30 times more squalene compared to seed oils. Considering that only 60% of squalene taken through the diet could be absorbed by human body [35], the consumption of foods rich in squalene such as olive oil may offer considerable quantity of squalene to human body and substantial health benefits.

3.3.3 Determination of squalene in apple peels and seeds

Apples are one of the most consumed fruits worldwide; according to FAOSTAT the global per capita consumption of apples and apple products was 8.6 kg in 2017 [46]. These fruits are so widespread because of their easiness in cultivation, high adaptability to different climates and their healthy nutritional composition, with a low-calorie intake and a well-balanced acids and sugar content too, conferring a pleasant and sweet taste. The largest part of apple production is destined to table purposes, but a high portion is processed into different food products, such as jam, vinegar, juice, forming significant volumes of residues, known as apple pomace. It represents about 25 % of the fresh fruit, that consists of skin and flesh (95%), seeds (2-4%) and stalks (1%) [47]. The valorization and exploitation of these by-products can both reduce environmental impact and give economic advantages. Apple pomace has been used for pectin extraction, fuel purposes, livestock feed and biotransformation until now [48], but it could be exploited better considering its application in pharmaceutical, cosmetic and food fields. Many studies [48, 49, 50] indicated apple peel and seeds as potential sources of bioactive compounds, such as unsaturated fatty acids, polyphenols, essential oils, carotenoids, vitamins, triterpenes, phytosterols and squalene too.

The rapid and easy GC-FID method developed for the analysis of squalene in vegetable oils and presented in the previous sections was applied also for the study of squalene in apple peels and seeds of different apple varieties: seven ancient cultivars (*Mela Rosa, Mela Rosa Fragola, Mela Rosa di*

Fogliano, Mela Rosa di Pietra, Mela Rosa di Pietra Locale, Mela Carella and *Mela Limoncella*) and four commercial varieties (*Royal Gala, Golden Delicious, Fuji* and *Granny Smith)* for comparative purposes.

At the beginning of the new millennium, many efforts were made by local public authorities to save as many of these ancient cultivars as possible from total extinction. All these ancient apples belong to the genus *Malus x domestica* Borkh, Maloideae subfamily, a branch of Rosaceae family. In particular, the *Mela Rosa* is traditionally cultivated in the pre-Apennine zone of Sibillini mountains, Marche region, central Italy, between 400 and 900 m of altitude. The typical characteristics of this fruit are the small size, the pink peel, flat shape, the intense aroma and the sweet taste [51]. *Mela Limoncella* is a variety typically cultivated in south-central Italy since Roman times. The fruit has a yellow-green skin with lentils; the flesh is white, juicy, aromatic and lightly acidic, a characteristic responsible for its name [52]. *Mela Carella* has been cultivated since the 14th century, mainly in the territory of Cerreto D'Esi, Marche region. The little fruit has a rusty peel and a tender pulp with a high sugar content, characterized by a sweet taste [53]. Despite the longstanding use of these apple varieties in central Italy, there are very limited information about their chemical and nutritional profile and even lower on the chemical composition of the by-products (peel and seeds) of these fruits, that could represent sources of precious substances to be used in several fields.

Before the application of the analytical method to the samples, it was validated by assessing linearity, intraday and interday repeatability, recovery and method sensitivity in terms of limit of detection (LOD) and limit of quantification (LOQ). **Table 10** reports the evaluated validation parameters.

Table 10. Method validation parameters for squalene analysis of apple peels and seeds.

	Linearity (mg/100 g of sample)	R^2	LOD (mg/100 g of sample)	LOQ (mg/100 g of sample)	Repeatability[a] RSD[b] % (n=3)		Recovery[a] (%)		
					Intraday	Interday	Spike1	Spike 2	Spike 3
Peels	0.25-219.64	0.9996	0.23	0.77	1.92	1.74	92.8	87.4	90.4
Seeds					1.07	1.75	82.7	90.7	85.1

a.Determined using the *Granny Smith* sample
b.RSD: relative standard deviation

Linearity range covered all the squalene concentrations in the samples and it was very good considering the calculated square correlation coefficient (R^2) of 0.9996. LOD and LOQ values permitted to quantify squalene in the peels and seeds of almost all the apple varieties. The method was validated also considering interday and intraday repeatability: 1.71% and 1.92% for the apple

peel samples and 1.75% and 1.07% for the seeds samples (as relative standard deviation of the squalene content in 3 replicates). In this case, the results are very similar to the interday and intraday obtained in the previous method validation (1.6% and 1.1%, respectively). Recovery was acceptable for both apple peels and seeds (above 82% in both samples), even if it was lower for the seeds.

After its validation, the method was applied to the study of squalene content in peels and seeds of eleven different apple varieties. Firstly, the moisture and lipid contents in the analyzed samples were determined (**Table 11**).

Table 11. Moisture and lipid content of apple peels and seeds of the different analyzed apple cultivars. Values are expressed as g/100g of fresh samples ± standard deviation (SD).

Apple variety	Moisture content (g/100 g of fresh sample)		Lipid content (g/100 g of fresh sample)	
	Peel	Seeds	Peel	Seeds
Mela Carella	71.66±5.56	29.87±2.31	0.86±0.06	15.36±0.79
Mela Limoncella	70.95±1.15	28.76±1.67	1.46±0.11	17.50±0.42
Mela Rosa di Pietra	71.47±0.19	30.13±2.01	0.91±0.03	16.67±0.76
Mela Rosa di Pietra Locale	79.67±1.66	28.87±1.56	1.25±0.02	15.85±0.91
Mela Rosa	74.06±0.73	32.91±2.03	1.12±0.06	19.78±0.31
Mela Rosa di Fogliano	79.56±0.73	29.65±1.55	0.90±0.06	18.26±0.92
Mela Rosa Fragola	77.00±0.04	27.90±1.85	1.71±0.13	16.00±1.03
Fuji	77.46±0.05	29.77±1.98	1.45±0.03	15.00±0.42
Golden Delicious	82.27±0.27	30.15±2.65	1.47±0.05	15.85±0.64
Granny Smith	76.07±0.59	31.55±2.11	1.10±0.03	13.00±1.01
Royal Gala	80.98±0.58	32.55±3.02	1.10±0.03	14.60±0.57

Oil yield is one of the most important goals of management of apple pomace, because of economic motivations [54]. The lipid fraction obtained by the peels was in the range 0.86-1.71 g/100 g of fresh sample (3.03-8.29 g/100 g of the dry sample) and in the seeds, between 13.00 and 19.78 g/100 g of fresh sample (18.99-29.48 g/100 g of the dry sample). Fromm et al. [55] indicated a lipid content in apple seeds of 21.7 g/100 g, Pieszka et al. [56] of 20.2 g/100 g and Górnaś et al. [54] of 22.0 g/100 g of dry matter. The average seed oil content could be different between different cultivars, but also within the same variety [55], because of varietal, geographical and abiotic factors (cultivation

temperature, water availability, sunlight, farming techniques). In the case of apple peel lipid content, Feumba et al. [57] reported an average value of 9.96±1.52 g/100 g of dry matter. Also for the peels, their composition is dependent on varietal and abiotic factors.

Then squalene was determined in the lipid fraction of both seeds and peels of the eleven investigated apple varieties. The chromatogram obtained from the analysis of *Granny Smith* seeds oil is shown in **Figure 24** and the results of this study are reported in **Figure 25** and **26**, for the peels and the seeds, respectively.

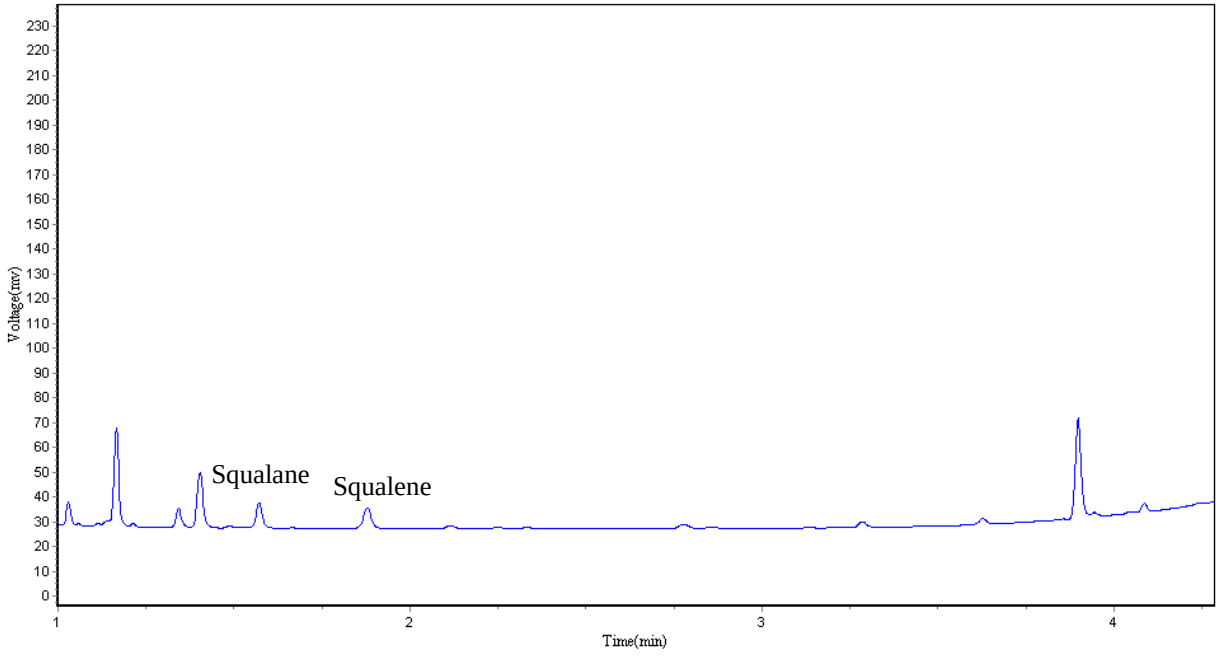


Figure 24. GC-FID chromatogram obtained from the analysis of squalene in *Granny Smith* apple seeds oil.

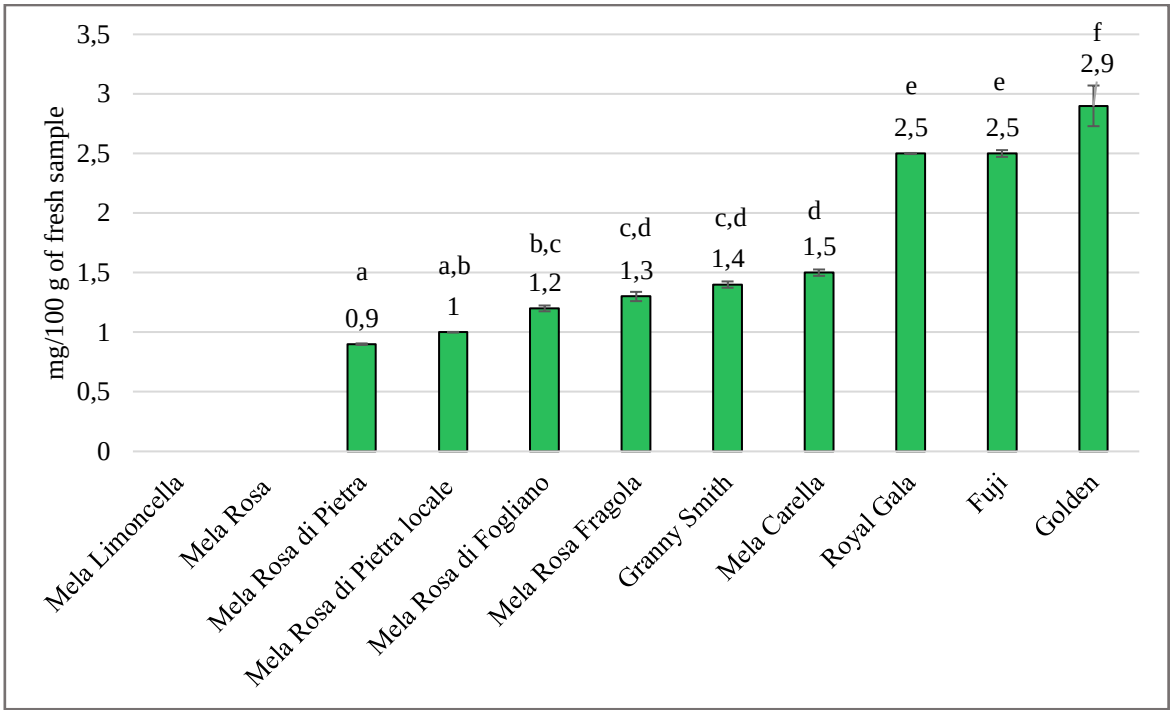


Figure 25. Squalene content (mg/100g of fresh sample) in peels of the analyzed apple cultivars. Bars indicate standard deviation. Significant differences (One-way analysis of variance, $P < 0.05$, Tukey's test for pairwise comparison) are indicated by different letters.

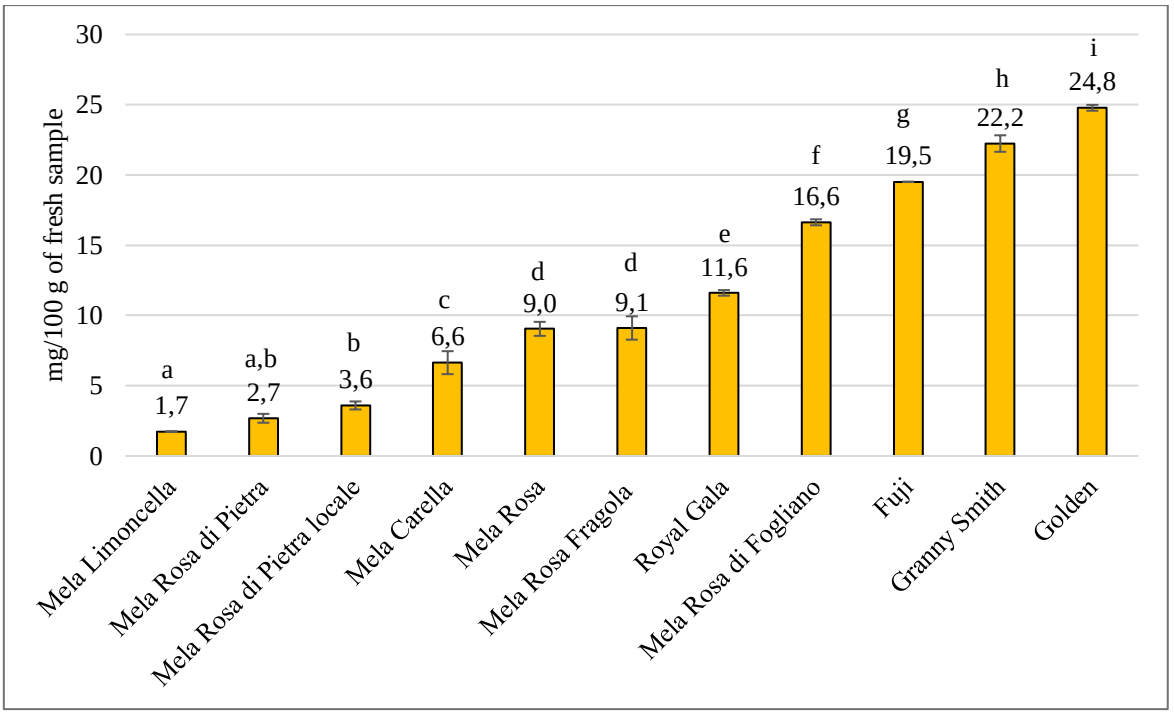


Figure 26. Squalene content (mg/100g of fresh sample) in seeds of the analyzed apple cultivars. Bars indicate standard deviation. Significant differences (One-way analysis of variance, $P < 0.05$, Tukey's test for pairwise comparison) are indicated by different letters.

Seeds contained higher squalene content, in the wide range of 1.7-24.8 mg/100 g of fresh sample for *Mela Limoncella* and *Golden Delicious* respectively. Ancient varieties, except for *Mela Rosa di Fogliano,* contained a lower squalene content with respect to the commercial ones, showing also statistical differences. Peels instead showed a lowest squalene content in the range 0.9-2.9 mg/100 g of fresh samples of *Mela Rosa di Pietra* and *Golden Delicious*, respectively. The squalene amount in the peels of Mela Limoncella and Mela Rosa cultivars was not quantified, because below the LOQ (**Table 10**).

Together with Golden Delicious, also the two commercial varieties Royal Gala and Fuji presented the highest concentration of squalene in their peels, confirmed also by statistical analysis. Considering both seeds and peels, ancient cultivars (except for the seeds of *Mela Rosa di Fogliano* with 16.6 mg of squalene per 100 g of fresh sample and for the peels of *Mela Carella* with 1.46 mg of squalene per 100 g of fresh sample) proved to have lower squalene with respect to the commercial ones. These differences could be due to varietal factors and also to other conditions (sunlight exposure, water availability, temperature, horticultural measures). There are only few information in literature about squalene content in apple seeds and peels in literature. An interesting study by Górnaś et al. [50] investigated the lipophilic composition of different apple seed oils (six crab apples and five dessert apples), indicating a squalene content in the range 0.01 to 0.34 mg/g of oil. These results are similar to the ones obtained in the present study. Arain et al. [58] indicated squalene as the most abundant hydrocarbon present in the unsaponifiable fraction of apple seed oils (5.7-6.7%). Lu and Foo [59] studied the composition of hexane extract of *Royal Gala* seeds and reported squalene and nonacosane as the two major constituents of the non-fatty acid fraction of the extract. Apple peels resulted to contain low amount of squalene, but they represent the highest portion of apple pomace; despite all their exploitation could give considerable yield of squalene. In conclusion, apple pomace has great potential to be considered as a valuable source of oil destined to cosmetic and pharmaceutical sectors, to functional foods or edible purposes.

3.4 Conclusions

The proposed rapid and easy analytical procedure to analyze squalene in different food matrices (olive oils, vegetable oils and apple peels and seeds) permits to reduce time, solvents and reagents amounts. The method validation provided good results in terms of linearity, repeatability, sensitivity and recovery in both olive oils and apple by-products. The application of the method to different olive oils: monovarietal EVOOs, niche blend EVOOs, EVOOs and OOs provided from large retail distribution market allowed to quantify squalene in all the samples and permitted to find significant difference between the different types of oils, proving that squalene content is strictly dependant on genetic factors.

The same analytical method permitted to analyze squalene content in seven different vegetable oils subjected to refinement. During the refining treatment the squalene level decreased for all the oils, except for olive oil and soybean oil, where its content remains almost the same in the crude and refined sample (no statistical differences were found). For the other oils the decrease in squalene amount varied from 24.6 to 57.8 %, for maize and grapeseed oil respectively. Significant differences between natural and refined oils were found for maize, grapeseed and sunflower (sample 1 and HO) oils, highlighting considerable losses of squalene during the refining process. It was proved that there is a considerable difference between crude and refined olive oil and seed oils in terms of squalene level: olive oil contained 10 to 30 more squalene compared to the other investigated vegetable oils, being one of the most important sources of squalene by diet.

The proposed method was applied also to the study of squalene content in peels and seeds, to evaluate their possible re-use in supplementary food, cosmetical or pharmaceutical sectors. Eleven apple varieties (seven ancient and four commercial cultivars) were evaluated. The results showed a squalene content between 1.7-24.7 mg/100 g in fresh seeds and 0.9 and 2.9 mg/100 g in fresh peels. Between the two analyzed by-products, apple seed oils have the most abundant concentration of squalene, comparable to maize, rapeseed and sunflower oils thus contributing to destine this product to interesting exploitation. In the future, other bioactive substances could be evaluated in apple pomace, in order to make a more complete reuse of this by-products, contributing to environmental sustainability and economic profits.

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INDEX

Abstract……..……201

Chapter 4: Development and application of a GC-FID method for the analysis of short chain fatty acids in rat and human faeces and in fermentation fluids……..……...….202

4.1 Introduction……..……...…203

4.1.1 Gut microbiota……..……...……...….203

4.1.1.1 Factors affecting GI microbiota……..……207

4.1.1.2 Role of gut microbiota in health and disease……..…..……211

4.1.2 Short chain fatty acids (SCFAs)…..…….....213

4.1.2.1 Mechanisms of SCFAs production...…….....215

4.1.2.2 Biological functions of SCFAs)…..……..…….....217

4.1.2.3 Methods of analysis of SCFAs in faeces and fermentation fluids……...222

4.1.3 Aim of the work……..225

4.2 Materials and Methods……..……226

4.2.1 Standards, reagents and solvents…...…….226

4.2.2 Standard solutions preparations…...…….226

4.2.3 SCFAs extraction from faecal samples…...……..…….226

4.2.4 SCFAs extraction from fermentation fluids…...……227

4.2.5 GC-FID analysis of SCFAs…...…….227

4.2.6 Method validation…...……..…….228

4.2.7 Statistical analysis…...……..…….229

4.3 Results and Discussions…...……..…….229

4.3.1 Method procedure…...……..…….229

4.3.2 Method Validation…...…….235

4.3.2.1 Linearity, limits of detection and limits of quantification……..235

4.3.2.2 Recovery and repeatability……..236

4.3.3 Application of the method to real samples……...……..237

4.3.4 Application of the method in “Probiosenior” and “Gut microbiota in mobility” projects……....241

4.4 Conclusions…...……...245

References…...……...246

Abstract

Short chain fatty acids (SCFAs) are important gut microbiota metabolites. In the last years they were recognized for their beneficial effects on the host health status. In this study a new method for the determination and quantification of eight SCFAs (acetic, propionic, *i*-butyric, butyric, *i*-valeric, valeric, *i*-caproic and caproic acids) in different biological samples (rat, mice and human faeces and in fermentation fluids samples) has been developed and validated. The method consists in a rapid and easy extraction by ethyl ether after acidification of the sample. Then, the SCFAs are analyzed by direct injection and gas chromatography coupled with flame ionization detection (GC-FID). The number of extractions has been evaluated in order to obtain a satisfactory yield for all the analyzed SCFAs. There was a significant increase of the extracted analytes passing from 1 to 2 and from 2 to 3 extractions, confirmed also by statistical analysis, while there were no significant differences performing 3, 4 or 5 extractions. The extracted SCFAs are directly analyzed by GC-FID without any time-consuming derivatization step and separated on a polyethylene glycol nitroterephthalic acid modified coated capillary column, with a chromatographic run time of 13 min. The method was also validated, showing a good sensitivity, linearity and repeatability. It resulted to be suitable also for the quantitative analysis of SCFAs in very small amount of faecal sample (20 mg).

The method has been applied to two projects in collaboration with the School of Biosciences and Veterinary Medicine of the University of Camerino. Indeed, its easiness and rapidity permitted to investigate SCFAs also in high numbers of samples in a short time. The SCFAs analysis is one of the parameters considered to investigate the effect of probiotics in the health status of elderly people (Probiosenior project) and to study the adaptation of gut microbiota of healthy young people during geographical and diet changes (Gut microbiota in mobility).

Chapter 4. Development and application of a GC-FID method for the analysis of short chain fatty acids in rat and human faeces and in fermentation fluids

4.1 Introduction

4.1.1 Gut microbiota

According to Hippocrates “death sits in the bowels” and “bad digestion is the root of all evil”. The importance of the intestine in human health has been known since 400 B.C. However, there has been a huge increase in the study of the relationship between gut microbiota and human health and disease (**Figure 1**).

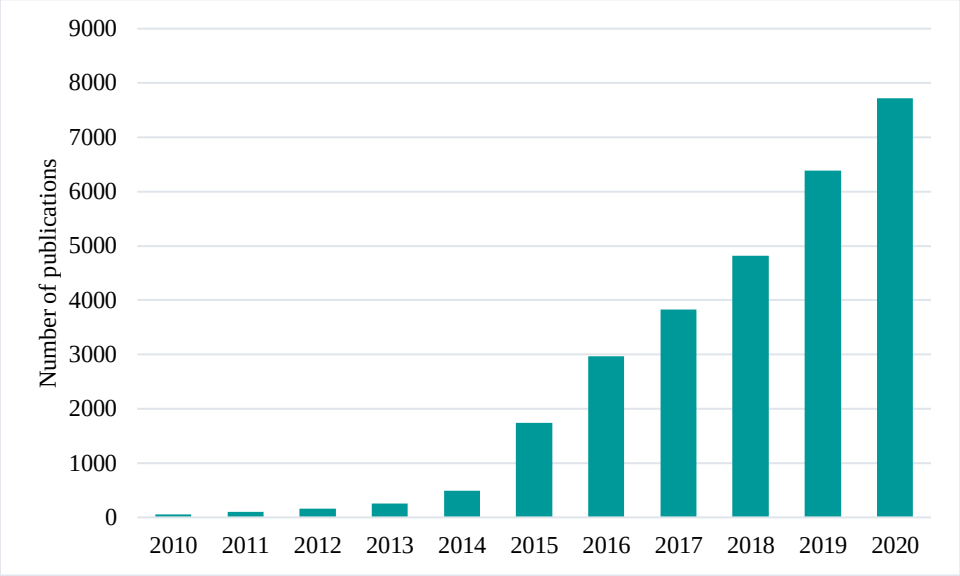


Figure 1. Number of publications related to the intestinal microbiota in the last decade, per year. Data were obtained by searching in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) the following terms: intestinal microbiota, gut microbiota, intestinal flora, gut flora, intestinal microflora, and gut microflora.

Human gastrointestinal (GI) tract is one of the largest interfaces between the human body and antigens, accounting for 250-400 m^2. The term “gut microbiota” refers to the collection of bacteria, eukarya and archaea living in the GI tract. They have co-evolved with the host over thousands of years, forming a complex and mutually beneficial relationship. Gut microbiota is formed by 10^{14} microorganisms, ten times more than the number of human cells. Because of the huge number of bacterial cells in the body, the host and the microrganisms are referred as “superorganism”. The microbiota is responsible of many benefits for the host, such as the maintenance of gut integrity, the harvesting of energy, the regulation of host immunity and the protection against patogens [1].

The composition of human gut microbiota has been deeply studied, especially thanks to the advent of culture-independent approaches, such as the targeting of the bacterial 16S ribosomial

RNA gene, which is present in all bacteria and archaea. The combination of different studies identified 2172 species in the human gut microbiota, classified into 12 phyla, with Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes representing the 93.5%. The composition of gut microbiota is not so different from microbial communities of other sites of the body and reveals a high degree of functional redundancy. A study of Li et al. [2] identified some country-specific microbial signatures, indicating that gut microbiota is affected by environmental factors, such as diet and host genetics. **Table 1** shows the dominant species of human colonic bacteria and their principal fermentation products.

Table 1. Main species of human colonic bacteria and their major fermentation products [1].

Genus/species	**Phylum (family)**	**Products**
Eubacterium rectale	Firm *(Lach)*	Bu, Fo, La
Roseburioa inulinivorans	Firm *(Lach)*	Bu, Fo, La
Eubacterium hallii	Firm *(Lach)*	Bu, Fo, But
Anaerostipes hadrus	Firm *(Lach)*	Bu
Coprococcus catus	Firm *(Lach)*	Bu
Ruminococcus obeum	Firm *(Lach)*	Ac, La
Blautia wexlerae	Firm *(Lach)*	Ac, Su
Faecalibacterium prausnitzii	Firm (*Rum)*	Bu, Fo, La
Ruminococcus bromii	Firm (*Rum)*	Ac, Eth
Bacteroides thetaiotaomicron	Bact (*Bac*)	Ac, Su, Pr
Bacteroides vulgatus	Bact (*Bac*)	Ac, Su, Pr
Bifidobacterium adolescentis	Actin (*Bif*)	Ac, La, Fo
Collinsella aerofaciens	Actin (*Cor*)	Ac, La

Firm: Firmicutes, *Lach: Lachnospiraceae, Rum: Ruminococaceae,* Bact: Bacteroidetes, *Bac: Bacteroidaceae,* Actin: Actiobacteria, *Bif: Bifidobacteriaceae, Cor: Coriobacteriaceae*, Bu: butyrate, Fo: formate, La: lactate, But: butanol, Ac: acetate, Eth: ethanol, Su: succinate.

Microbiota starts to develop from birth, although some studies indicated the presence of microbes in womb tissues, such as the placenta [3, 4]. After birth, the GI tract is quickly colonised because of many changes in diet and also some illness or antibiotic treatments. Also the delivery mode could affect the microbiome composition. Vaginally delivered infants microbiota contains higher quantity of Lactobacilli [5], while the microbiota of caesarean section delivered infants is richer of facultative anaerobes, such as Clostridium species [6]. In the first stages of development the microbiota is not so complex and it is dominated by

Actinobacteria and Proteobacteria. On the contrary, during the first year the diversity increases and the microbiota composition starts to assume the characteristics of the adult-like microbial profile with transitional patterns typical of each baby. By 2.5 years of life, the composition and the functions of the infant microbiota are very similar to those of adult microbiota (**Figure 2**).

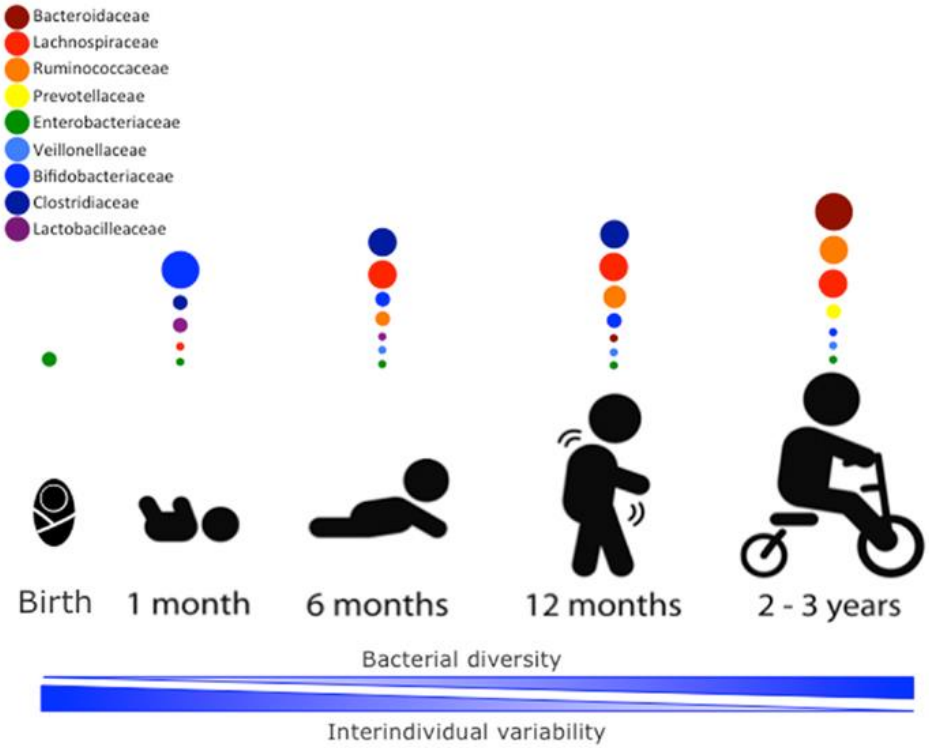


Figure 2. Stages of microbial development of the infant and child intestine [4].

In adulthood the gut microbiota composition remains relatively stable, but it is subjected to many life events and conditions. In people over the age of 65 Bacteroidetes phyla and *Clostridium* cluster IV increase, while in younger individuals *Clostridium* cluster XIV is more abundant [7]. The main difference between young and elderly people microbiota is the capacity to carry out metabolic processes. For instance, the production of short chain fatty acid (SCFAs) and the amylolysis are reduced in the elderly, while proteolytic activity is enhanced [8], resulting in higher risk of "inflamm-ageing" process in the intestine of old people.

Microbiota composition changes along the GI tract, because of the physiological properties of each region and the chemical and nutritional gradients along the tract (**Figure 3**). Firstly, in the small intestine there are more acids, oxygen, antimicrobials and a short transit time, limiting the bacterial growth except for the facultative anaerobes [9]. On the contrary, in the colon there are a dense and heterogeneous bacteria community, mainly anaerobes (*Prevotellaceae*, *Lachnospiraceae* and *Rikenellaceae*) that are able to use undigested complex carbohydrates [9].

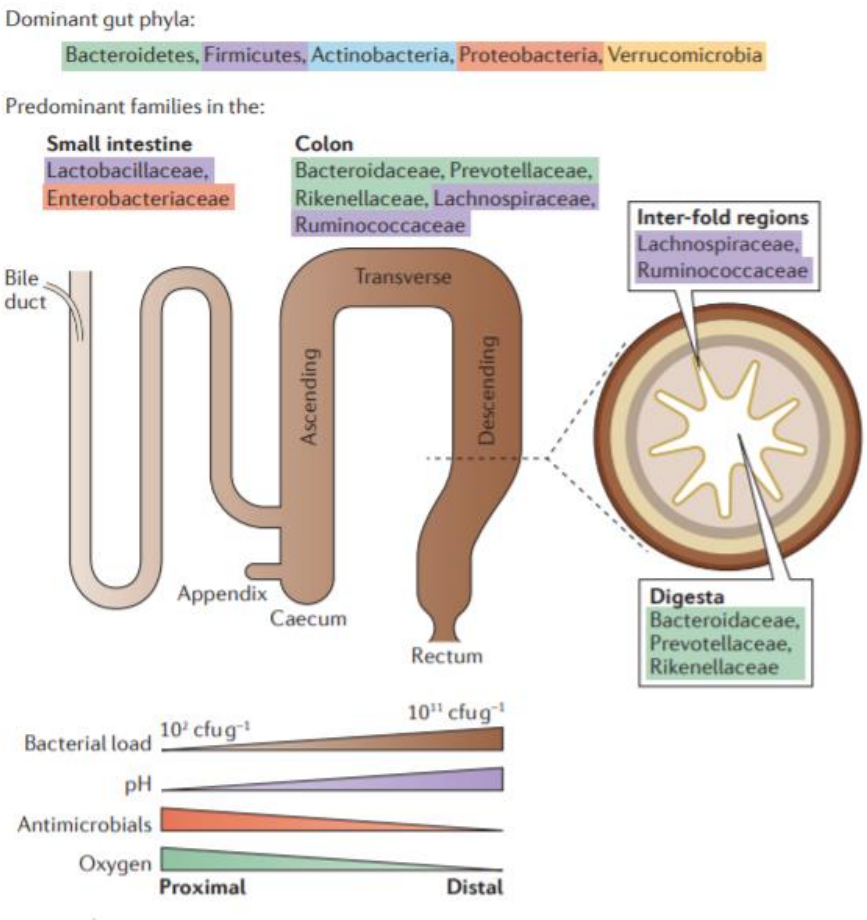


Figure 3. Microbial habitats in the human lower GI tract. Colours correspond to the relevant phyla. A cross-section of the colon shows the digesta and the inter-fold regions of the lumen. (cfu: colony-forming units) [9].

The microbial composition changes longitudinally within the gut but also over the cross-sectional axis. The wall of the colon is characterised by compartments between folds (inter-fold regions), different from the central lumen compartment. The Firmicutes families *Lachnospiraceae* and *Ruminococcaceae* are most abundant in folds, while the Bacteroidetes families *Prevotellaceae*, *Bacteroidaceae* and *Rikenellaceae* are richer in the digesta [9].

The microbiota composition changes between the different GI organs, while the microbiome of diverse colorectal mucosal regions remains almost the same within the same individual, also in the period of inflammation. On the contrary, the faecal/luminal and mucosal compositions present huge differences, with Bacteroidetes higher in the faecal/luminal samples and Firmicutes more abundant in the mucus layer [10] (**Figure 3**).

4.1.1.1 Factors affecting GI microbiota

Gut microbiota is an unstable organ, affected by many factors which result in many changes in microbial composition during lifespan. Many studies have focused on the relationship between microbiota and various disease conditions. Gut microbiome is associated to many metabolic disorders, such as obesity and diabetes. Diet plays an important role in stabilizing gut microbiota and changes in diet are related to gut microbial alteration (dysbiosis). Also other factors (**Figure 4**), such as age, environmental factors, geography, hygiene conditions and antibiotic treatments are important factors in the composition of gut microbial population [11].

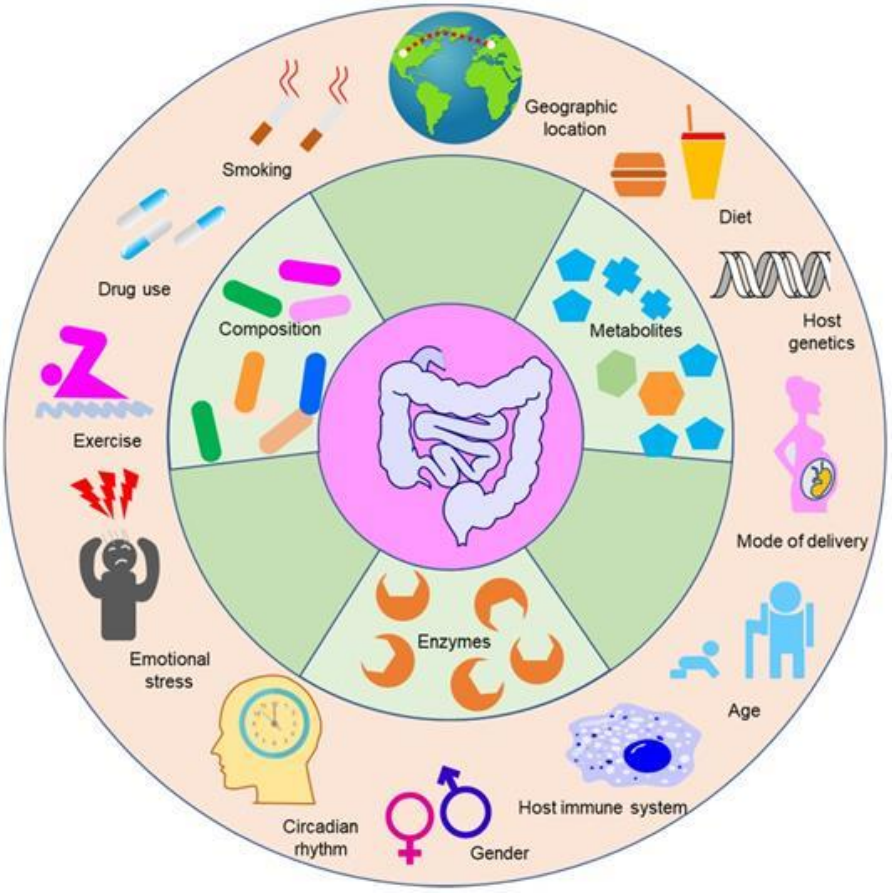


Figure 4. Factors influencing the functions and composition of gut microbiota [12].

A study conducted by Ley et al. [13] indicated that bacterial richness is linked to Body Mass Index (BMI), dyslipidaemia, obesity and insuline resistance. Then, gut microbiota influences the homeostatis of the intestinal epithelium and favours the development of immune systems, protecting the host by pathogens. Intestinal dysbiosis leads to changes in energy metabolism and immune response, increasing the risk of some diseases, such as infections, obesity, inflammatory bowel disorders (IBD) or allergies [11]. However, the mechanisms involved in the gut microbial alteration are not fully understood.

As previously said, maternal microbiota and mode of delivery influence the infant's gut microbiota and stimulate the immune system. Also variations in genes linked to immune response could shape gut microbiota, leading to health disorder. For instance, genetic modifications affecting the signalling events in inflammatory response or metabolic traits could importantly affect the microbial population [14].

Another important factor affecting physiological, metabolic and immunological functions is aging. Gut microbiome is diverse and intricate community in the gastrointestinal tract and could change through all the stages of the life. After 3-4 years of life, the microbial composition transformed into the community specific to adults. During the adolescence stage gut microbiome could be altered because of hormonal and metabolic changes. Then, during adulthood gut microbiota alters because of stress conditions, changes in lifestyle, geographical location and metabolic disorders. It is during elderly stage that there is the most evident shift in the composition of GI microbiota, due to drug uses, food habits, psychological and physical disorders [15].

After birth, diet is the major responsible in the regulation of gut microbiota. In the last years, many studies investigated the role of food habits in the landscape of gut microbiota. Different types of diets were investigated (high carbohydrate, high fat, western diet or vegetarian diet), showing a high alteration of the gut microbiome within 24 hours from food intake [16]. According to the type of diet, the phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria are the most altered in the GI tract. Also the quantity, the nature and the type of the major macronutrients (lipids, proteins and carbohydrates) highly alter the composition and the metabolism of gut microbiota [11]. When food is ingested, it is digested in stomach and pass through the small intestine. At last, undigested food reaches large intestine. Here gut microbiota contributes to host health by fermenting undigested food, generating metabolic by-products responsible for bio-signalling pathways (regulation of energy homeostasis) [17]. Diets are mainly divided into beneficial and non-beneficial diet, according to the nature and composition of food and the caloric value (**Figure 5**). Many inflammatory and autoimmune diseases are linked to diet and the correlated beneficial and non-beneficial microrganisms, but also physical activity and lifestyle are important factors to be considered. High caloric, high sugar and fat and western diets stimulate more non-beneficial microbes respect to beneficial diets (pre/probiotic and fiber rich diets), which seem to rejuvenate beneficial microbiota [18]. Moreira et al. [19] indicated that a diet rich in saturated fatty acids (SFAs) increases the quantity of Gram-negative bacteria, stimulating the production of lipopolysaccharides (LPS) that

enhance the gut permeability and metabolic endotoxemia. Also the high consumption of sugar (desserts, white bread, sweet drinks) alters gut microbiota, increasing the risk of diabetes, obesity and cardiovascular problems [20].

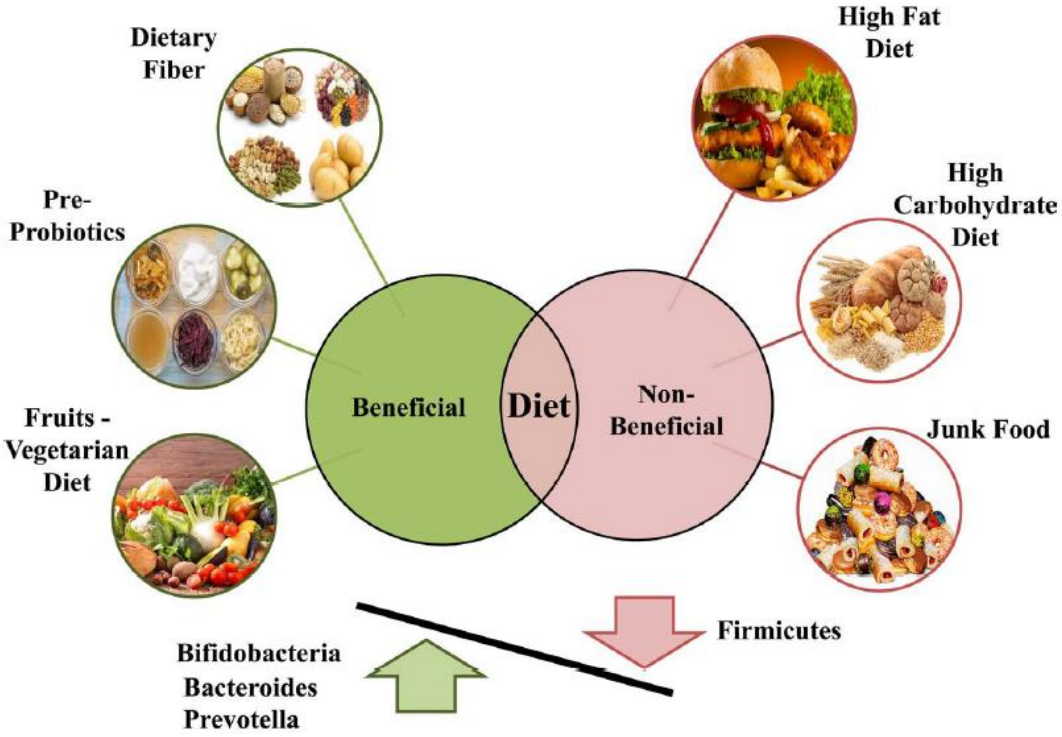


Figure 5. Beneficial and non-beneficial diets and their influence in the composition of gut microbiota [11].

Diets rich in fiber, prebiotics, probiotics and vegetarian favour the growth of beneficial microbes which compensate the dangerous effects of other extrinsic factors in the host body. In particular, probiotics are living organisms able to control directly or indirectly the gut microbiota and enhance the host health status. Instead, prebiotics are non-digestible food components that favours the fermentation process of beneficial gut microbes. The most common prebiotics are inulin, lactulose, fructooligosaccarides, galactooligosaccharides and resistant starch are considered to enhance the production of *Lactobacillus* and *Bifidobacterium* that produce short chain fatty acids (SCFAs), enhancing the host health status [21].

Beyond diet, non-dietary lifestyle (smoking, alcohol consumption, stress, physical exercise, environmental factors) is fundamental for gut microbiota composition [11].

GI microbiota is susceptible to changes due to smoking and inhalation of nicotine components. Savin et al. [22] reported that the genera of *Clostridium*, *Prevotella* and *Bacteroides* and the

phyla of Bacteroidetes and Proteobacteria were increased, while the genera *Lactococcus* and *Bifidobacteria* and the phyla Firmicutes and Actinobacteria were decreased in faecal samples of smokers. It could lead also to changes in tissues integrity, gut pH alteration and oxidative stress, responsible for IBD and obesity.

Alcohol consumption is considered as an important dietary factor that influence dysbiosis of GI microbiota. In alcohol dependant individuals the risks of gut functions depression, leaky gut syndrome, anxiety and relapse in gut-vein-liver interactions are highly enhanced [23].

Physical exercise, together with diet, is an important mean of protection against many diseases, such as obesity, diabetes and IBD. Matsumoto et al. [24] indicated that exercise influences gut microbiota diversity in rats, increasing butyrate production and cecum diameter, reducing colon disorders. Evans et al. [25] reported also that physical exercise is important in the high-fat diet induced obesity mice model; their gut microbial composition is similar to that of lean mice.

Stress is a physical and emotional condition linked to many physical disorders (depression, anxiety, hypertension, immune disturbances). It can also induce problems in gastrointestinal tract (peptic ulcer, IBD and ulcerative colitis). The study of Van de Wouw et al. [26] indicated that SCFAs could alleviate the stress related problems and also the consumption of probiotics could reduce the production of cortisol, a stress hormone [27].

Another important factor affecting GI microbiota shaping is the use of drugs, especially antibiotics. Antibiotic treatment affects beneficial gut microbial population, leading to many metabolic disorders and also the development of antibiotic resistant bacteria creates several health problems [28]. On the other hand, also non-antibiotic drugs have shown adverse effects on the host, changing again the gut microbial population [29].

Some taxonomical variation of the GI microbiota are related to geographical location and origin of the person, even if there are only few studies focused on the relationship between microbial diversity and socio-economic and geographic factors [30]. The research of Suzuki and Worobey [31] suggested that there is an important association between gut microbiota properties and geographical location of the individual and the ancestors. In fact, the type of food consumption is strictly related to cultural and geographical characteristics of the country.

4.1.1.2 Role of gut microbiota in health and disease

Gut microbiota has a crucial role in health and disease in human body, even if sometimes it is indicated as "a forgotten organ". It is involved in energy storage and many metabolic functions, such as fermentation and absorption of undigested carbohydrates. Furthermore, GI microbiota interacts with the immune system, transmitting signals for the maturation of immune cells [32]. To better understand the state of disruption of gut microbiota in metabolic disorders is important to define the composition of the GI microbiota of metabolically healthy people. It is not possible to exactly define a healthy human gut microbiota, because of diversities in microbial growth and composition, the host genetics and external environmental factors. But, it is possible to define the core functions of mature healthy intestinal microbiota: genes encoding glycosaminoglycan degradation, the biosynthesis of many essential amino acids and vitamins, the production of SCFAs via fermentation of undigestible polysaccharides and the synthesis of specific LPS [32].

Urbanization, higher standards for housing and a better hygiene respect to people living traditional lifestyles (for example in the Amazonas or Africa) lead to a decrease of the abundance of many genera in the gut microbiota (*Bacteroides, Prevotella, Desulfovibrio, Lactobacillus* and *Oxalobacter*). This situation is related to the spread of common chronic metabolic disorders, such as obesity [32]. The low microbial diversity and richness increase adiposity, inflammation, insulin resistance and dyslipidaemia. Also the use of antibiotics before and during pregnancy and in early childhood stage could affect the microbial composition, increasing the early-onset obesity [33]. Obesity is not the only health problem related to the composition of GI microbiota. The classical approach in the study of a disease was "one microbe-one disease" thinking. Nowadays, this viewpoint has changed: many diseases might derive from dysbiosis rather than the presence of a single disease-causing microbe. Some common diseases linked to GI microbial dysbiosis are allergic and autoimmune diseases, diabetes, inflammatory bowel disease and the already mentioned obesity [34]. **Table 2** reports the most common diseases linked to gut microbial dysbiosis and the most important changes in gut microbiota linked to each health disorder.

Table 2. Changes in gut microbiota and their relationship with the most common metabolic diseases [34].

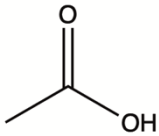
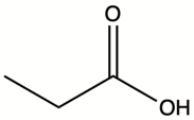
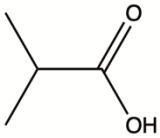
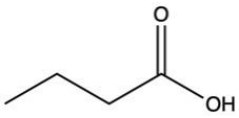
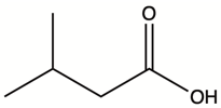
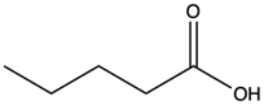
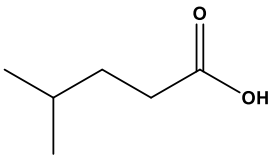
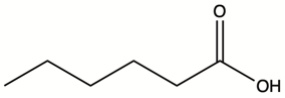
Implicated microbiota	Changes in microbiota presence and/or function
Allergies	
Lactobacillus spp. ↓ *Bifidobacterium adolescentis* ↓ *Clostridium difficile* ↓ *Helicobacter pylori* ↓	Early colonization with Lactobacillus linked to decreased allergies
Celiac's disease	
Bacteroides vulgatus ↑ *Escherichia coli* ↓ *Cloostridium coccoides* ↓	Higher diversity in Celiac's disease patients versus controls
Gastric cancer	
H. pylori ↑	Important element in the development of gastric adenocarcinomas
Autism	
Bacteroidetes ↑ *Proteobacteria* ↑ *Actinobacteria* ↓ Firmicutes ↓	Increased microbial diversity in faeces of autistic children
Obesity	
Bacteroidetes ↓ *Lactobacillus* ↑ Firmicutes/bacteroidetes ratio ↓ *Methanobrevibacter smithii* ↓	Significant changes in GI microbiota are linked to obesity
IBD-Crohn's disease	
Bacteroides ovatus ↑ *Bacteroides vulgatus* ↑ *Bacteroides uniformis* ↓	Less diversity in patients with Crohn's disease
IBD (general)	
Bacteroidetes ↓ *Lachnospiraceae* ↓ *Actinobacteria*↑ *Proteobacteria* ↑ *Clostridium leptum* ↓ *Clostridium coccoides* ↓	IBD associated with overall community dysbiosis

Faecalibacterium prasnitzii ↓ Firmicutes/Bacteroidetes ratio ↓ *Bifidobacteria* ↓	
Type 2 Diabetes	
Firmicutes ↓ *Clostridia* ↓ *Bacteroides-Prevotella* ↑ versus *Clostridia coccoides-Eubacterium rectale* ↓ *Betaproteobacteria* ↑ Bacteroidetes/Firmicutes ratio ↑	Changes in gut microbiota associated with increased level of glucose in plasma

4.1.2 Short Chain Fatty Acids (SCFAs)

Short chain fatty acids (SCFAs) are organic monocarboxylic acids with a chain length from 1 to 6 carbon atoms. They are the principal products of the anaerobic fermentation of indigestible carbohydrates (resistant starch and dietary fibers) by the microbiota in the large intestine. They are mostly represented by acetate (C2), propionate (C3) and butyrate (C4) in the molar rate of 60:20:20. About 500-600 mmol of SCFAs are produced in a healthy gut per day, according to the fiber content, gut transit time and microbiota composition [35]. Anaerobic fibers fermentation is the principal source of SCFAs, but they can derive also by amino acids metabolism. Protein fermentation takes place in the distal large intestine. Here the amino acids valine, leucine and isoleucine can be converted into branched-chain SCFAs (BSCFAs), such as isobutyrate, isovalerate and 2-methyl butyrate, that represent a very little amount of the total SCFAs content (about 5%) [36]. **Table 3** shows the most abundant SCFAs in gut microbiota and their principal physical and chemical properties.

Table 3. Principal SCFAs in gut microbiota and their main physico-chemical characteristics.

Traditional name	IUPAC name	Structure	Molecular Mass	Density at 25°C (g/cm^3)	Boiling point (P=1 atm)	pka
Acetic acid	Etanoic acid		60	1.05	118	4.76
Propionic acid	Propanoic acid		74	0.99	141	4.88
Isobutyric acid	2-Methylpropanoic acid		88	0.95	155	4.86
Butyric acid	Butanoic acid		88	0.96	164	4.82
Isovaleric acid	3-Methylbutanoic acid		102	0.93	177	4.77
Valeric acid	Pentanoic acid		102	0.94	186	4.84
Isocaproic acid	4-Methylpentanoic acid		116	0.92	200	5.09
Caproic acid	Hexanoic acid		116	0.93	206	4.88

4.1.2.1 Mechanisms of SCFAs production

The principal products of the CHO catabolism of gut microbiota are acetate, propionate and butyrate (**Figure 6**). Lactate is not classified as a SCFA, but it is also produced by some species, such as lactic acid bacteria, bifidobacteria or proteobacteria. It does not accumulate in the colon because usually some species (as *Eubacterium hallii*) convert it into the different SCFAs [36].

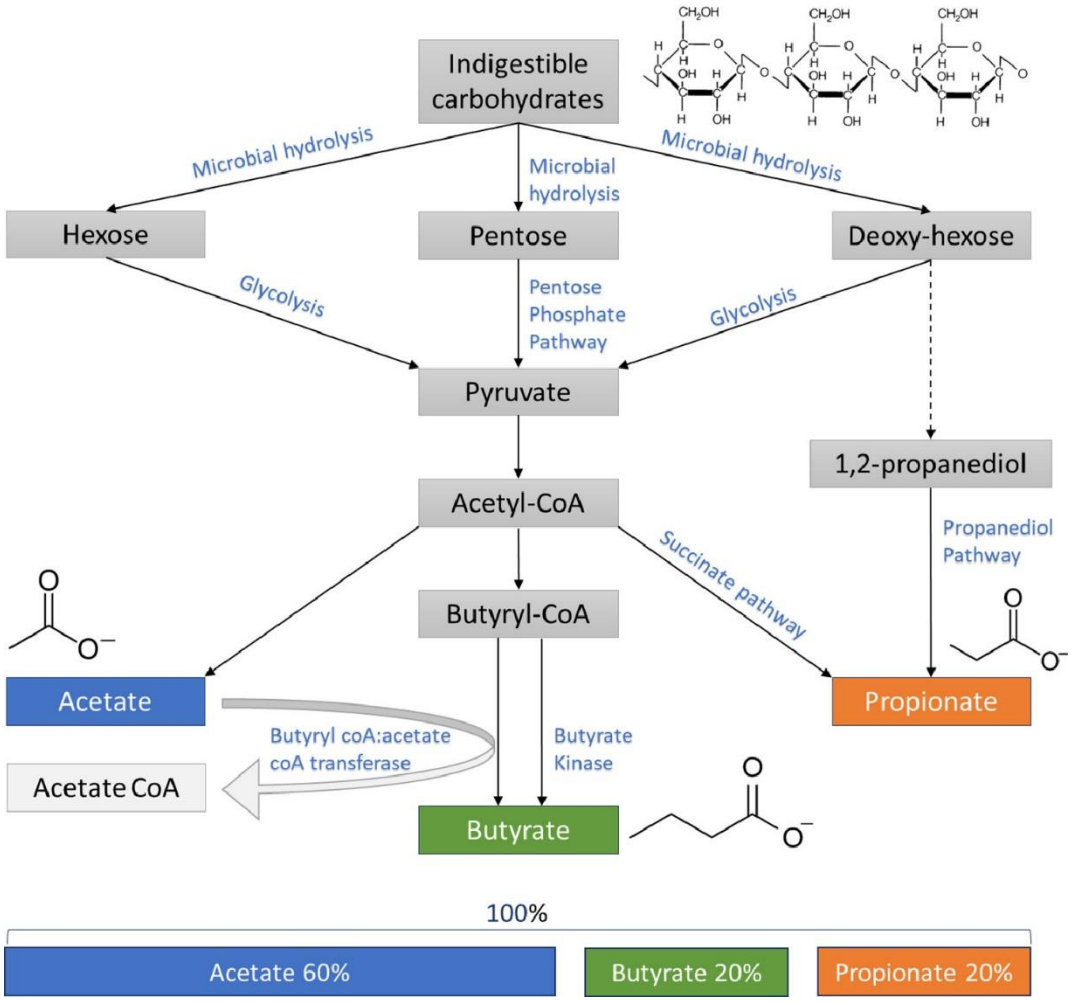


Figure 6. Principal pathways of bacterial fermentation for the production of the SCFAs hydrolysis, including molar ratios of the principal SCFAs (acetate, propionate, butyrate) in the colon on a total of 100% [37].

As previously said, acetic acid is the principal SCFA in the colon and the most abundant SCFA detected in faeces (around 50%). The largest quantity of acetate is produced by enteric bacteria from CHO fermentation. One third is produced by acetogenic bacteria, through the synthesis from hydrogen and carbon dioxide or formic acid through the Wood-Ljunddahl mechanism [36].

After acetic acid, propionic and butyric acids are the most abundant SCFAs. They have received big attention in the last years, because of low concentration of propionate and butyrate bacterial producers are linked to some inflammatory diseases. For instance low levels of propionate producers were found in children with asthma [38] and butyrate producers are low in ulcerative colitis [39].

Propionate is formed by colonic bacteria with three different mechanisms: succinate pathway, acrylate pathway and propanediol pathway. Firmicutes and Bacteroidetes use the succinate route, which exploits succinate as a substrate and involves the decarboxylation of methylmalonyl-CoA to propionyl-CoA. The acrylate route involves *Veillonellaceae* and *Lachnospiraceae*: lactate is converted into propionate by the activity of the lactoyl-CoA dehydratase and some enzymatic reactions [40]. The last route (propanodiol pathway) is characterized by the conversion of deoxy-sugars to propionate by CoA-dependent propionaldehyde dehydrogenase and it involves mainly proteobacteria and members of the *Lachnospiracea*e family [36]. The propionate concentration is linked to the relative abundance of Bacteroidetes, proving that succinate pathway is the principal route within GI microbiota [41].

Two pathways are involved in the butyrate production. The first one, the butyrate kinase, is limited to some Coprococcus species and it employs phosphotransbutyrylase and butyrate kinase enzymes to convert butyryl-CoA into butyrate. The second one is the butyryl-CoA: acetate CoA-transferase pathway and it is used by most of the buyrate-producers species (*Faecalibacterium, Eubacterium,* and *Roseburia)*. In this mechanism butyryl-CoA is converted to butyrate in a single step enzymatic reaction [36].

Other important mechanisms involved in the SCFAs production are the so called bacterial cross-feeding. These pathways consist in the use of end products from the metabolism of a microorganism by another one (metabolic cross-feeding) and the exploitation of the energy rich CHO breakdown products derived by another one (substrate cross-feeding). Cross-feeding interactions are favoured by anoxic conditions, with are common in the large intestine [36].

The composition and abundances of SCFAs and BSCFAs are highly affected by diet, which influences gut microbiota characteristics and activity. Wu et al. [42] published a study linking long-term diet with the human microbiota (“enterotypes”) for the first time in 2011, but many researches demonstrated the correlation between short-term diets and gut microbiome [43]. The concentration and relative abundances of SCFAs are interesting markers of the healthy status of an individual. For instance, high fiber and low-fat intake favour the presence of higher

amounts of faecal SCFAs respect to a low fiber intake [30]. Lower quantitities of butyrate were detected in the faeces of patients with colorectal adenocarcinoma, while obesity tends to increase the total SCFAs, which tend to decrease anti-obesity treatments [36]. Then the use of prebiotic substrates favours the growth of beneficial bacteria and induce changes in SCFAs production not only in patients with some gastrointestinal disease (such as IBD), but also in healthy individuals [36]. Moreover, the intake of dairy products obtained by beneficial bacteria modifies the intestinal microbiota producing more butyrate than acidified milk products [44] and dietary intervention seem to be effective in lowering the high amounts of faecal SCFAs in different obese populations [45].

4.1.2.2 Biological functions of SCFAs

In the last few decades, it became clear that SCFAs have an important role in the interplay between diet, gut microbiota and regulation of host energy metabolism. They have been considered important also in the prevention and treatment of some diseases (metabolic syndrome, IBD, some cancer types) [46] (**Figure 7**).

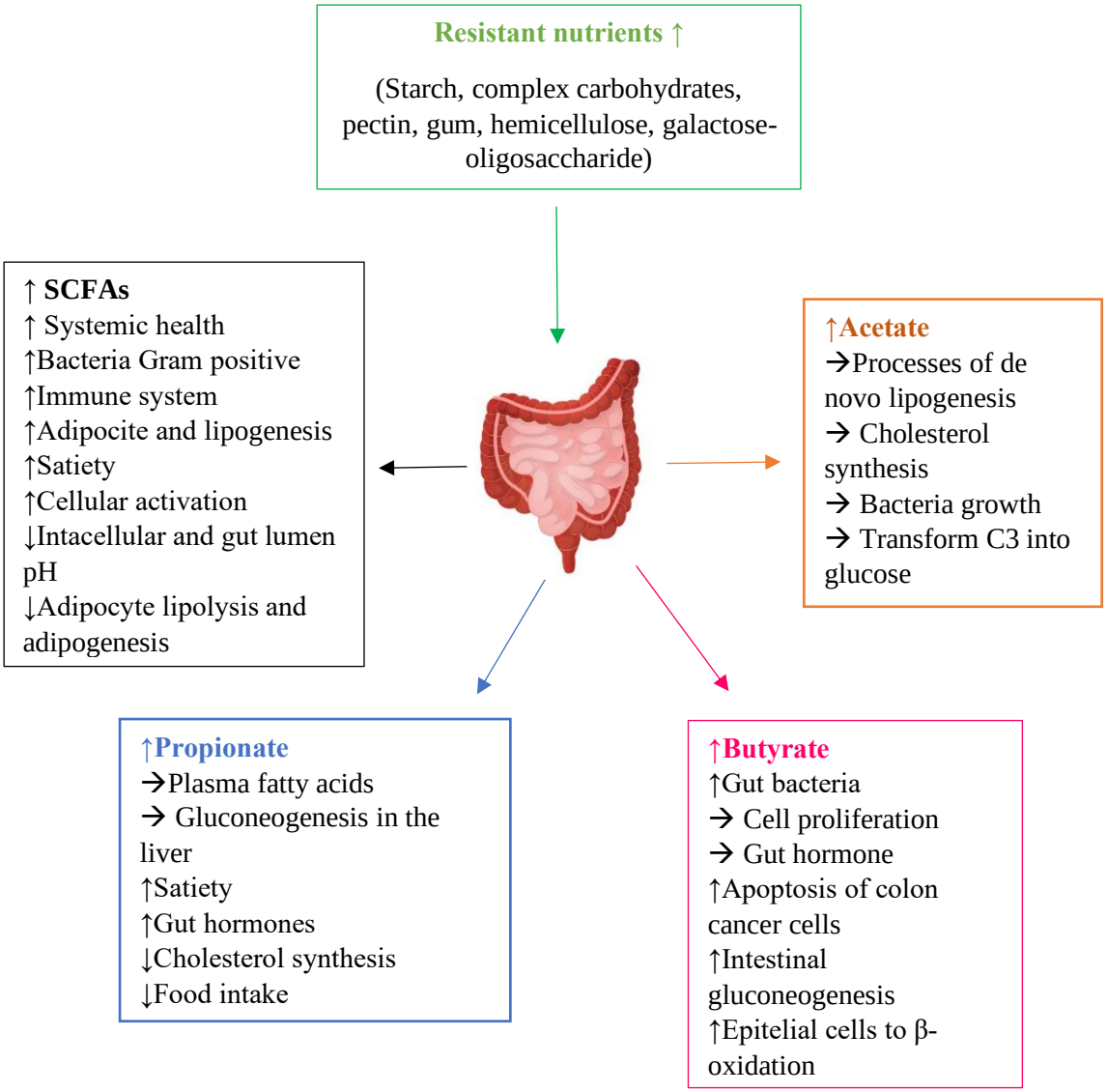


Figure 7. Role of the gut microbiota in SCFAs production and their benefits to human physiology regulation. (↑ = significant increase; ↓ = significant decrease; → = stable performance, C3 = propionate; CVD = cardiovascular diseases) [47].

One of the effects related to the SCFAs production is the reduction of the luminal pH, which increases the nutrients absorption and reduces the presence of pathogenic microorganisms [36]. Most SCFAs are absorbed by the host in exchange for bicarbonate, so the luminal pH is a consequence of microbial SCFAs and bicarbonate neutralizing capacity. The SCFAs concentration lowers from the proximal to the distal colon, so the pH increases from cecum to rectum. The pH reduction from the ileum to the cecum, because of the higher SCFAs quantity, determines two consequences. Firstly, a lower pH changes the gut microbiota composition. Studies of human faecal microbiota indicated that at pH 5.5 butyrate-producing bacteria (*Roseburia spp.* and *Faecalibacterium prausnitzii*, both of the Firmicutes phylum) represent about 20% of the total population. In the distal large intestine the luminal pH increases to 6.5

and the butyrate-producing bacteria almost disappear, while acetate- and propionate-producing Bacteroides species become the largest part. Secondly, a lower pH prevents the growth of pH sensitive and pathogenic bacteria, such as Clostridia and Enterobacteriaceae [46]. In particular, acetate has been considered a key player in the capacity of Bifidobacteria to inhibit the growth of enteropathogens. Butyrate instead is the major energy source for intestinal epithelial cells and favours mucin production, increasing bacterial adhesion and improving tight-junction integrity [36]. SCFAs are absorbed and employed in different biosynthetic pathways after their production.

SCFAs as a source of energy

A big part of absorbed SCFAs is used as a source of energy. In humans about 10% of the daily caloric intake is provided by SCFAs. In particular, colonocytes derive 60-70% of their energy by SCFAs oxidation. They prefer butyrate respect to acetate and propionate, oxidizing it to ketone bodies and CO_2 [46].

Exogenous acetate produced by colonic bacterial fermentation is mixed in the blood with endogenous acetate released by organs and tissues. Then about 70% of the acetate is used by the liver as an energy source and for the synthesis of long-chain fatty acids and cholesterol. It is also a co-substrate for glutamine and glutamate synthesis. The remainder acetate is metabolized by hearth, kidneys, muscles and adipose tissues [48].

The liver clears also the biggest part of butyrate and propionate from portal circulation. Propionate is especially a precursor for gluconeogenesis, while butyrate is mostly used a fuel for colonocytes as discussed above. The rest of butyrate is oxidized by hepatocytes, preventing toxic systemic concentrations [49].

Regulation of fatty acid metabolism by SCFAs

Another important role of SCFAs is the regulation of the equilibrium between fatty acids synthesis, their oxidation and lipolysis in the body. SCFAs activate fatty acid oxidation and inhibit their synthesis and lipolysis [46]. The consequence is the reduction of fatty acids in plasma and a decrease of body weight. The lipolysis reduction was proved with data where intravenous administration of acetate and propionate was submitted to humans [50]. Concluding, the prevention of dietary-induced obesity by SCFAs could be due to an increase fatty acid oxidation in different tissues and a reduction of fat storage in white adipose tissue.

Regulation of glucose and cholesterol metabolism

The plasma glucose concentration can be attributed to uptake by the food, by multiple organs and by gluconeogenesis. SCFAs seem to beneficially affect glucose metabolism by normalizing plasma glucose and enhancing glucose handling. For instance, the studies of Sakakibara et al. [51] and Boillot et al. [52] showed that oral administration of acetate and propionate, respectively, reduce glycemia in diabetic hyperglycaemic KK-A(y) mice and normal rats.

SCFAs, especially propionate, have an important impact also in the reduction of cholesterol in rodents and humans. Fushimi et al. [53] indicated that also acetate affect serum cholesterol level and the study of Kondo et al. [54] confirmed that acetate supplementation decreases human hypercholesterolemia.

SCFAs role in immunity

Human microbiota and the immune system have evolved together and regulated each other. Microorganisms participate in the regulation of the host's immune system by producing metabolites, SCFAs for the largest amount. Firstly, SCFAs are the most abundant energy source for colon and ileum cells; they affect the intestinal epithelial barrier and the defence factors by regulating related gene expression. Then, they regulate the functions of innate immune cells (Neutrophiles, macrophages, dendritic cells). Lastly, SCFAs regulate the differentiation of B and T cells and the antigen-specific adaptive immunity controlled by them [55].

SCFAs role in inflammation

Immunity and inflammation are strictly related and the important role of SCFAs in the host's inflammation response has been deeply discussed. Adding dietary fibers to diet could improve cardiovascular health and reduce systemic inflammation. In particular, after two weeks of a diet implementation of soluble fibers the amounts of circulating pro-inflammatory mediators decreased [56]. When a certain part of the body is attacked by pathogens, immune cells secrete pro-inflammatory/anti-inflammatory cytokines. When this balance is broken, systemic inflammation and pathological diseases can occur. SCFAs regulate inflammation by controlling the production of cytokines in immune cells (macrophages, neutrophils, T cells, B cells, dendritic cells) [55].

SCFAs role in the pathogenesis of COVID-19

Corona virus SARS-CoV-2 infection and COVID-19 pandemic is a worldwide concern of public health. An interesting and central question concerning COVID-19 is why most infected

people do not develop severe diseases, while others have critical symptoms. This situation can be addressed to age, gender, comorbidities and immunosuppression, but also many young people succumb to the virus. One of the COVID-19 risk factors is the dysbiosis of gut microbiota and the related low-grade inflammation and loss of epithelial barrier function (**Figure 8**). As described above, SCFAs is linked to the integrity of intestinal barrier and diet and age may influence their production, affecting barrier function and severity of COVID-19 [57]. COVID19 patients receive antibiotics and have drastically changed their diet, critically influencing gut microbiota. So, it is important to evaluate the potential influence of microbiota on COVID19 symptoms. Uninfected subjects at risk and also infected people can take preventive measures to enhance the status of their microbiota (use of prebiotics, probiotics, supplements, higher fiber intake) to lower the risk of developing severe complications of COVID19 and also of other viruses.

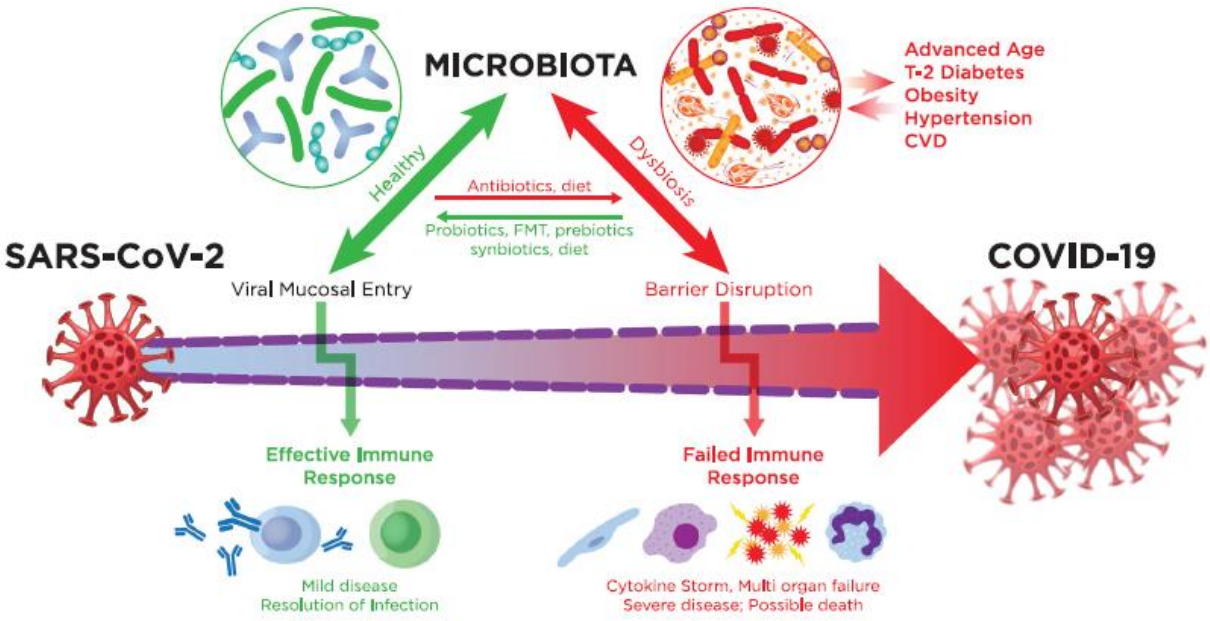


Figure 8. The potential microbiota role in COVID-19 [57].

SCFAs role in tumor cells proliferation and apoptosis

After their importance in immunity, metabolism and inflammation, SCFAs play a great role on tumor cell proliferation and apoptosis. Gupta et al. [58] demonstrated that the addition of 10 mM of butyrate in bovine kidney epithelial cells can cause cell cycle arrest and cell growth inhibition. Also Kim et al. [59] suggested that the treatment of SCFAs, in particular propionate and butyrate, inhibit the proliferation, migration and invasion of colon cancer cells. The role of SCFAs (butyrate) depends mainly on their concentration in tissues and cells. At low

concentration (<0.5 mM) butyrate is used for energy supply, at higher concentration (0.5-5 mM) butyrate induces apoptosis and cell cycle arrest. So, an adequate concentration of SCFAs may be a promising candidate for future cancer treatments, especially intestinal cancer [55].

4.1.2.3 Methods of analysis of SCFAs in faeces and fermentation fluids

There is a need for an effective qualitative and quantitative SCFAs assessment in laboratories on a daily routine. SCFAs have been determined in different biological matrices, such as serum, blood plasma, brain, fermentation fluids and also in various environmental samples or food. For instance, volatile fatty acids affect the aromas and quality of food. Because acetate, propionate and butyrate are generated by fermentation during food storage, their concentration give important indications. Acetic acid in wine is important in the formation of various acetate esters, producing fruity flavors. But, when it is found at concentration higher than 0.05 g per 100 mL of wine, it is responsible of the vinegar defect. Moreover, butyric acid in cheese at high concentration is responsible for rancidity defect [60].

SCFAs have become common targets to link gut microbiota to pathological conditions and potential beneficial effects in humans. Methods for their assessment have improved a lot in the last years, even if gas chromatography (GC) remains the most exploited, especially for SCFAs quantification. Other methods include liquid chromatography (LC), especially high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and nuclear magnetic resonance (NMR) [61]. SCFAs are very volatile compounds, especially the ones with the shortest carbon chain (acetic and propionic acids). Then faeces and fermentation fluids contain high quantities of microbes, so it is extremely important to prevent sample deterioration during its storage. Samples are usually kept at -80°C or -20°C. Another important factor to be considered is the samples pretreatment, such as the extraction. SCFAs are partially hydrophilic, so it is not so easy to perform a quantitative extraction with organic solvents. A sample acidification is usually applied to keep acids protonated and less hydrophilic, facilitating a better extraction with organic solvents [62].

GC analytical methods

The first direct detection of fatty acids with GC was first performed in 1952 by James and Martin [63]. For an appropriate GC determination of SCFAs an adequate pretreatment, chromatographic column and detection system are essential.

The pretreatment of biological matrices, such as faeces, is very important for the SCFAs detection. The commonest physical pretreatment methods without any extraction are filtration,

ultrafiltration, sample dilution or centrifugation. They are simple and fast, but many impurities could overload the system, bringing to a shorter column life span [61]. Then, steam distillation is a separation technique for temperature-sensitive samples. It preserves the sample quality but it shows some disadvantages, for example a very high variability in recovery coefficients for SCFAs [64]. On the contrary, vacuum distillation, also coupled with acid driven protonation of SCFAs, is a precise method, but very time-consuming and not suitable for routine practice [61]. Instead of the time-consuming distillation, a direct sample acidification is usually performed, mostly with hydrochloric acid, phosphoric acid, formic acid, sulfuric acid and oxalic acid. After acidification, SCFAs are usually extracted by organic solvent, using chloroform, ethyl acetate or the most popular ether. Simple acidification may result in some disadvantages, such as a much quicker degradation of the column [61].

Many studies described the use of another step in the sample pretreatment: derivatization (especially silylation). Silyl derivatives, such as trimethylsilyl (TMS) and tert-butyldimethylsilyl (TBS/TBDMS) derivatives are very volatile, less polar and thermally more stable. However, possible evaporation of more volatile derivatives during the pretreatment procedure could occur with a potential loss of SCFAs [61].

Besides solvent extraction another important solventless extraction has become popular: the solid-phase extraction (SPE), especially the solid-phase microextraction (SPME). It is faster and more selective and sensitive technique, even if fibres are expensive and the technique requires supplementary instrumentation in order to perform an automated analysis [61]. Also a purge and trap technique coupled to GC-MS has been developed, obtaining a higher number of volatile compounds respect to SPME, but worse recovery of volatile with higher molecular mass [65].

Advantages and disadvantages of the different described sample pretreatment methods for the SCFAs detection are summarized in **Table 4**.

Table 4. Advantages and disadvantages of the principal pretreatment techniques using GC methods for SCFAs analysis [61].

Pretreatment	Advantages	Disadvantages
Centrifugation/Filtration/Ultrafiltration (direct injection)	• Speed	• Column overload • Unspecific results
Steam distillation	• Separation with low temperature	• Decomposition of sample components • Unspecific results

		• Low recovery rate
Vacuum distillation	• Separation with very low temperature • Sensitive	• Time consuming • Not practical for routine analysis • Loss of SCFAs
Simple acidification	• Direct and fast method	• Column overload
Acidification and organic solvent extraction	• Good purification	• More steops • Possible loss of SCFAs • Occupational exposure to solvents
Derivatization (silylation)	• Very good purification	• Time consuming • Loss of SCFAs • Large quantities of reagents • Occupational exposure
SPME	• High purity of sample • Longer lifespan of the system	• High cost (fibres, supplementary instrumentation and knowledge)
Purge and trap	• Higher number of volatile compounds respect to SPME	• Extraction capacities of volatiles decrease with a higher molecular mass

Another important issue in SCFAs analysis is the choice of the GC column. The more effective are the capillary columns, containing silica as the most used supporting material. Then, the stationary phase are usually polysiloxanes or polyethylene glycol (PGE). The polysiloxanes columns used by different authors for SCFAs analysis are highly polar (for the FAMEs separation), but mostly non-polar/polar of low bleed and high temperature limits. Among PGE columns, Zhao et al. [62] described the use of a "free fatty acid phase" (FFAP) column, which permits the direct SCFAs detection from water solutions without any derivatization. FFAP columns are PGE type modified with terephthalic acid, that are highly polar and commonly used for the analysis of acidic compounds and free fatty acids [61].

Regarding the choice of the detector, the flame ionization type (FID) is the most used for the SCFAs detection in GC. GC could be coupled also to mass spectrometry (MS), resulting usually in better sensitivity and selectivity of the analysis.

However, it is important that research considers the instrumentation availability, costs, time and sensitivities needs when choosing a detection method for SCFAs in biological complex matrices.

4.1.3 Aim of the work

SCFAs are gut microbiota metabolites known for their beneficial effects on the host body. The first scope of this work was the development and validation of a rapid and simple method for the SCFAs analysis by direct injection and GC coupled with FID (GC-FID), in particular for the quantification of eight SCFAs (acetic, propionic, *i*-butyric, butyric, *i*-valeric, valeric, *i*-caproic and caproic acids) in rat, mice and human faeces and in fermentation fluids samples. The method involved the extraction of the SCFAs by ethyl ether after the sample acidification. Also the number of extractions was evaluated in order to obtain a satisfactory yield for all the analyzed SCFAs.

After its validation, the method was applied to two different projects in collaboration with two research groups of the School of Biosciences and Veterinary Medicine of University of Camerino. The first project, named "Probiosenior Project", consisted in the evaluation of the impact of a probiotic diet in the health status of the intestine of elderly people. The faecal SCFAs were monitored together with other biological parameters in collaboration with other research groups. The age influences the microbiota status, inducing sometimes inflammation ("inflammaging") and other possible diseases. Probiosenior project aims to use the diet as a way to enhance the health status of the elderly people and to prevent the development of many diseases.

The present method was applied also to a second project, "Gut microbiota in mobility". The objective was the investigation of gut microbiomes of young people from different cultures, adapted to different diets and subjected to drastic diet changes. In this project, the developed GC-FID method was exploited to analyse SCFAs in the faeces of Chinese students in mobility in Italy. The aim was to evaluate possible biochemical relationships within and between microbial species and potentially to predict the effect of ecosystem-wide perturbations, such as diet or environmental changes.

4.2 Materials and methods

4.2.1 Standards, reagents and solvents

The analytical standards acetic, propionic, *i*-butyric, *n*-butyric, *i*-valeric, *n*-valeric, *i*-caproic and *n*-caproic acids (C2, C3, *i*C4, C4, *i*C5, C5, *i*C6 andC6 respectively), were purchased from Sigma–Aldrich (Milan, Italy). Sulfuric acid was purchased from Carlo Erba (Milan-Italy) and ethyl ether from J.T. Baker (Phillipsburg-New Jersey- USA). Water was deionized (resistivity above 18 MΩ cm) using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA).

4.2.2 Standard solutions preparation

A diethyl ether stock standard solution was obtained for each acid at a concentration of 7.00×10^3 μM for the more abundant SCFAs in the samples (C2, C3 and C4) and at 1.00×10^3 μM for the less abundant SCFAs (*i*C4, *i*C5, C5 and C6). Two stock standard solutions were prepared for the two internal standards, dissolving 24 μL of *i*C6 (IS1) in 10 mL of diethyl ether and 120 μL of C5 (IS2) in 10 mL of the same solvent. All the stock standard solutions were stored at 4 °C until used.

4.2.3 SCFAs extraction from faecal samples

Rat, mouse and human faeces were collected after deposition and frozen at -20°C until further analysis. All the samples were purchased by research groups of the School of Biosciences and Veterinary Medicine of University of Camerino.

The samples are thawed and well homogenized with the help of a spatula. An aliquot of 100 mg is weighted in a 2 mL vial and acidified with 0.25 mL of aqueous sulfuric acid 50% w/v. The solution is shaken for 3 min with the help of a vortex device. Then, the internal standard solution (IS1) is added (50 μL of *i*C6, 2.4 μL/mL in ethyl ether) and an extraction with 1 mL of ethyl ether is performed. The solution is centrifugated for 5 min at 2800 x g. The organic phase is collected into a 4 mL vial and the extraction procedure is repeated three times, collecting a total of 3 mL of organic phase. At the end, 0.5 μL of the solution are injected into the GC for the analysis.

The same extraction method is applied to smaller amount of sample (20 mg), minimizing the reagents quantity: 50 μL of sulfuric acid, 10 μL of IS1 and 0.2 mL of ethyl ether in each extraction. The procedure is performed into a 400 μL glass insert and collecting the organic phase into a 2 mL vial, obtaining a total extract volume of 600 μL.

4.2.4 SCFAs extraction from fermentation fluids

The fermentation fluids were purchased by a research group of the School of Biosciences and Veterinary Medicine of University of Camerino. They were obtained from an *in vitro* fermentation cycle, using a culture system which simulates physiological processes, digestion and colonic fermentation, exploiting a human faecal inoculum (from 3 healthy donors). The fermentation system is a pilot fermenter (Applikon Fermentation System, Applikon Biotechnology) under anaerobic and semi-continuous culture conditions fitted with a 2 L culture vessel. Temperature, pH, stirrer speed and gas are controlled and after 24 h of fermentation samples were collected and stored at -20°C until the analysis. Then, 250 mg of the fluid sample is collected and acidified with 200 µL of sulfuric acid 50% w/v and mixed with the mean of a vortex device for 1 min. Then, an aliquot (10 µL) of the internal standard solution (IS2) (C5, 12 µL/mL in ethyl ether) is added and SCFAs are extracted with 800 µL of ethyl ether. At the end the solution is centrifugated for 5 min at 2800 x g. The organic phase is transferred into a 4 mL glass vial and the extraction is repeated for three times, collecting a total volume of 2400 µL. Also in this case, 0.5 µL of the collected solution are injected into the GC for the analysis, without any derivatization step of the SCFAs.

4.2.5 GC-FID analysis of SCFAs

The analysis was performed using a gas chromatograph Agilent Technologies 6850 GC (Santa Clara, CA, USA) equipped with a split/splitless injector and FID. A GC 6890N equipped with a mass spectrometer detector 5973 (both from Agilent Technologies, Santa Clara, CA, USA) was also used to confirm the identity of the analytes. The capillary column was a nitroterephthalic acid modified polyethyleneglycol (PEG) column (DBFFAP, 25 m, 0.25 mm i.d., 0.25 µm film thickness, purchased from Agilent Technologies, Santa Clara, CA, USA). The GC injector was set at 280 °C and the injection was performed in splitless mode (splitless time: 3 min). The oven temperature started from 40 °C for 3 min, then raised at 20 °C/min to 160°C and finally at 40 °C/min until 245 °C and maintained for 1.87 min, resulting in a total run time of 13 min. Hydrogen was used as carrier gas at a flow rate of 3.70 mL/min. The detector temperature was maintained at 250 °C. MS operational parameters were: electron ionization (EI) at 70 eV; transfer line and ion source temperature: 250 °C; quadrupole temperature: 150 °C; and mass range: *m/z* 29–300. The SCFAs identity in real samples was confirmed by comparison of their retention times and their mass spectra with those of authentic

standards and with reference spectra from the US National Institute of Standards and Technology (NIST, 2008).

4.2.6 Method validation

Single calibration curves were obtained for each analyte using SCFAs standard mixture. A solution was prepared starting from the stock solutions of all the individual SCFAs and adequately diluted to get seven calibration standard solution spiked with the internal standard *i*C6 (IS1). The concentrations of the calibration standard solution were comprised between 55 and 6500 μM for the most abundant SCFAs and between 8 and 850 μM for the less abundant ones. These ranges were chosen to cover the concentrations usually found for each SCFA in the biological matrices investigated. Then, seven calibration standard solutions were used to obtain the calibration curves: the normalized response (ratio between each SCFA peak area and the internal standard peak areas) was plotted against standard concentrations. Linearity was determined by calculating the linear correlation coefficient (R^2) from the calibration curves for each analyte. Limit of detection (LOD) and limit of quantification (LOQ) were set considering the the normalized peak areas corresponding to 3 and 10 times the signal to noise ratios, respectively.

Recovery and repeatibility of the method were defined for both faecal and fermentaion fluids samples, starting from 20 mg in the case of a rat faecal sample and from 250 mg of a fermentation fluid sample.

For the recovery test, the standard mixture was added to the sample at three different and known concentration levels. The rat faecal sample was spiked with 5, 15 and 25 μL (spike 1, 2, and 3, respectively) of a standard mixture containing all the analytes at these concentrations: C2, 262.50×10^3 μM; C3, 26.76×10^3 μM; C4, 21.82×10^3 μM; iC4, 3.27×10^3 μM; iC5 and C5, 2.82×10^3 μM; C6, 2.48×10^3 μM. The fermentation fluid sample was spiked with 2.5, 5 and 10 μL (spike 1, 2 and 3, respectively) of the same standard mixture used for the faecal sample. Also the spiked samples were subjected to the same treatment of the real samples and analysed by GC-FID. The recovery percentages were calculated by the following equation:

$$\%\ Recovery = \frac{Css - Cus}{Csp} x100$$

Css= concentration of the spiked sample;
Cus= concentration of the unspiked sample;
Csp= concentration spiked to the sample.

Then, intra and inter-day repeatibility were defined for both faecal and fermentation fluid samples and expressed as precision (RSD, %). The intra-assay precision was calculated by analyzing five aliquots of the same rat faecal sample and three aliquots of the same fermentation fluid sample on the same day; the inter-day repeatibility by analyzing five aliquots of the same rat faecal sample on five different days and five aliquots of the same fermentation fluid sample on five different days. Validation was performed applying the procedure to both 20 mg and 100 mg of faecal sample, giving similar results.

4.2.7 Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) and to Tukey's test for pairwise comparison, in order to determine significant differences ($P < 0.05$) between the different number of extractions applied to the samples, using the software PAST [66].

4.3 Results and Discussion

4.3.1 Method procedure

The composition and the quantity of free fatty acids (FFAs) are important in food and in other biological matrices from many points of view. In many food products, such as cheese, FFAs give the characteristic aromas and flavors, mostly related to the production process and product quality [67]. On the contrary in milk, cream or butter FFAs are negative compounds, because they impart a cheese-like off-flavor [68]. Then, acetic acid above a certain limit in wine is related to the presence of acetic bacteria, causing spoilage [69]. So, the analysis of SCFAs play an important role in the evaluation of food quality. For these reasons, our research group started to study SCFAs in different food products (wines and cheeses) developing a HS-SPME-GC-MS method [70]. Then, the need to study the presence of SCFAs in different biological matrices, such as rat and human faeces and fermentation fluids, and the high amounts of samples to be analyzed has encouraged the development of a more rapid and easier GC-FID analytical method for SCFAs determination. In the proposed study the sample pretreatment suspends the sample in aqueous sulfuric acid and performs three subsequent extractions with diethyl ether. The SCFAs collected in the organic phase are directly analysed by gas chromatography without any derivatization. The acidification is an important step, because the SCFAs in the undissociated forms are incremented in the organic phase [62, 71]. The same approach was exploited by several studies, as reported by Primec et al. [61]. As regard the extracting solvent, the good performances provided by ethyl ether are recognised, even if other

solvents behave in similar way and can show some advantages. Garcia-Villaba [72] compared the use of diethyl ether, ethyl acetate and dichloromethane and indicated that diethyl ether together with ethyl acetate gave the best results, even if ethyl acetate was chosen because of its easier handling. The research by Lotti et al. [73] compared different solvents and tert-butyl ether (MTBE) was selected due to the improved sensitivity, selectivity and accuracy provided to the method. In the present study ethyl ether was chosen because of its good performance combined to a lower toxicity respect to MTBE and because of its higher inertness as compared to ethyl acetate, that can undergo hydrolysis.

As regards the number of extractions to be performed during the pretreatment of the sample there is no homogeneity in the methods reported in literature, being one, two or three extractions [61] and in many cases the effect of the number of extractions has not been previously studied. Indeed, this parameter is important on the overall method efficiency in this specific application. In fact, the SCFAs show great differences in polarity and so the number of extractions could affect differently the extraction extent of each analyte. The increase of the carbon chain length brings to a high decrease of the molecule polarity. The carbon chain is relatively short for all the SCFAs, so an increase of even only one carbon strongly affects the polarity and so the partition between the aqueous and organic phases. Maybe for the acetic acid, which is the most polar compound, a single extraction would result in a lower relative extraction respect to the heaviest SCFAs (i.e. hexanoic acid). A factor for the first time investigated in this study is the number of extractions to be performed in this application, to understand the impact of the number of extractions and to optimize the extraction procedure of all the eight analytes. The number of extractions (from 1 to 5) was studied by employing the same homogenized human faecal sample divided into different aliquots (100 mg). In each one a different number of extractions were performed, bringing all the solutions to the same final volume (5 mL), making a direct comparison. The results are shown in **Figure 9.**

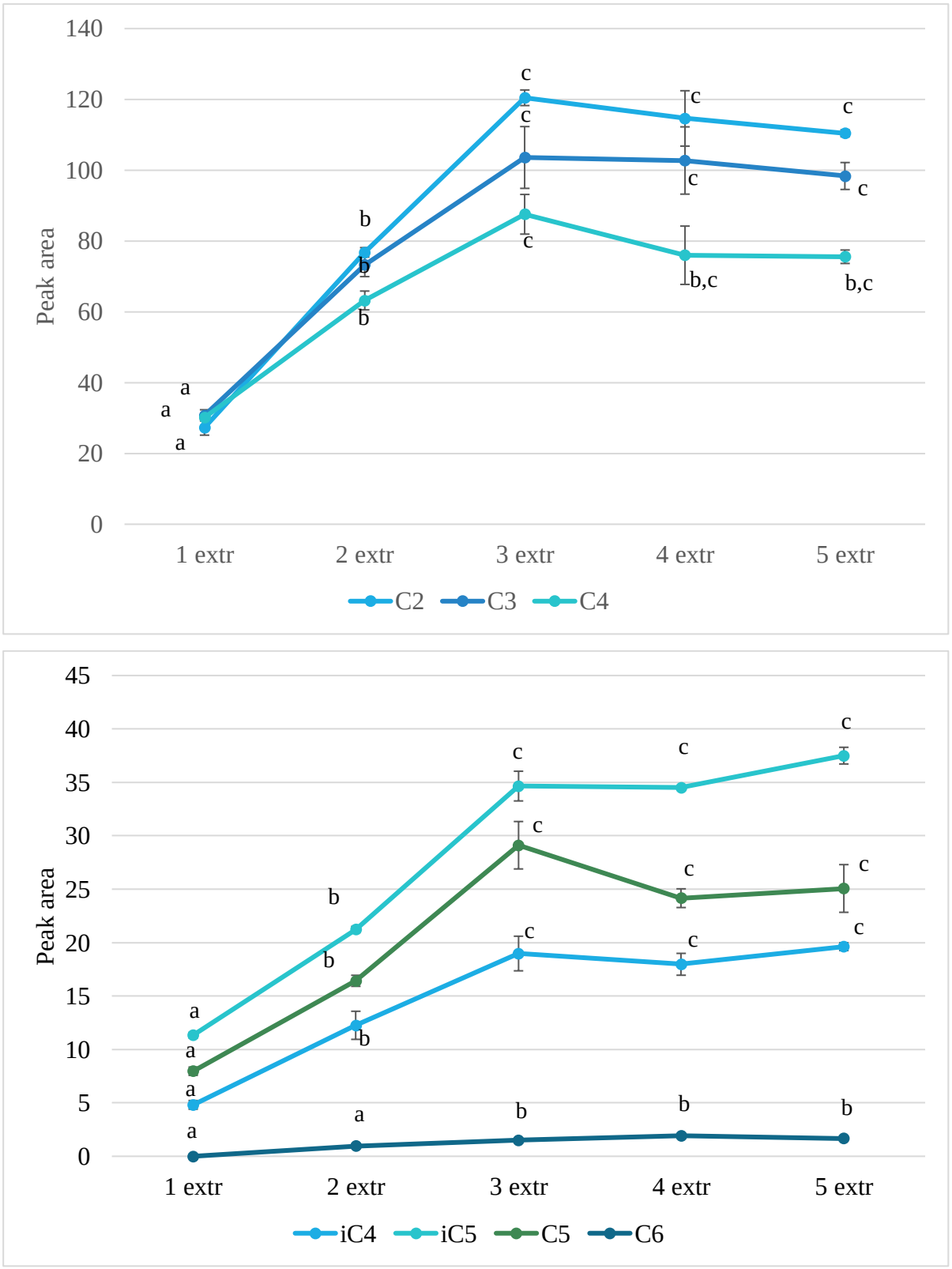


Figure 9. Quantity of short chain fatty acid (SCFA) extracted, in terms of peak area units, performing 1, 2, 3, 4 or 5 extractions (extr). Bars indicate ± standard deviations. Statistics compares the quantity of a same SCFA obtained with a different number of extractions. Different letters indicate significant differences between the SCFA quantity obtained

performing a different number of subsequent extractions (One-way ANOVA, $P < 0.05$, Tukey's test for pairwise comparison). C2: acetic acid; C3: propionic acid; C4: butyric acid; *i*C4: *iso*-butyric acid; *i*C5: *iso*-valeric acid; C5: valeric acid; C6: caproic acid.

As indicated also by the graphs, there is a significant increase of the extracted amount for the the investigated SCFAs passing from 1 to 2 to 3 extractions. On the contrary, passing from 3 to 4 to 5 extractions the content of the extracted SCFAs remains almost the same ($P>0.05$). These results indicated that the minimum and necessary number of subsequent extractions to be performed is 3.

Another purpose of this study was to develop a rapid and easy extraction procedure appropriate also when only few mg of sample are available, as in the case of mice faeces. For this reason, after the determination of an extraction procedure to be applied on 100 mg of faecal sample, the method was downscaled to 20 mg and the method was validated also in this case.

Another strong point of the present method is the short time of the analysis. The total chromatographic run (separation of the analytes and final thermal cleaning of the column) was 13 min. Many of the other methods reported in literature reported an average total run of 30 min [62, 74,75]. Nevertheless, the procedure described by Garcia-Villaba et al. [72], using a GC-MS analysis, needs a similar time of analysis (14 min).

So, in summary, the present method is simple, rapid and inexpensive, needing only a small quantity of organic solvent (3 mL for 100 mg of sample) and aqueous sulfuric acid for the sample pretreatment. Then, the method is suitable also when the sample quantity available is very scarce (i.e. 20 mg in the case of mouse faecal samples).

It is also useful the use of FID detector instead of a MS spectrometer, that could be not easily available in all laboratories. The use of multiple extractions and the splitless injection permit to achieve higher sensitivity also employing a FID detector. **Figure 10** shows the typical chromatogram obtained after the injection of a standard mixture containing all the eight SCFAs of interest. Their elution follows the order: (1) C2, (2) C3, (3) *i*C4, (4) C4, (5) *i*C5, (6) C5, (7) *i*C6, (8) C6.

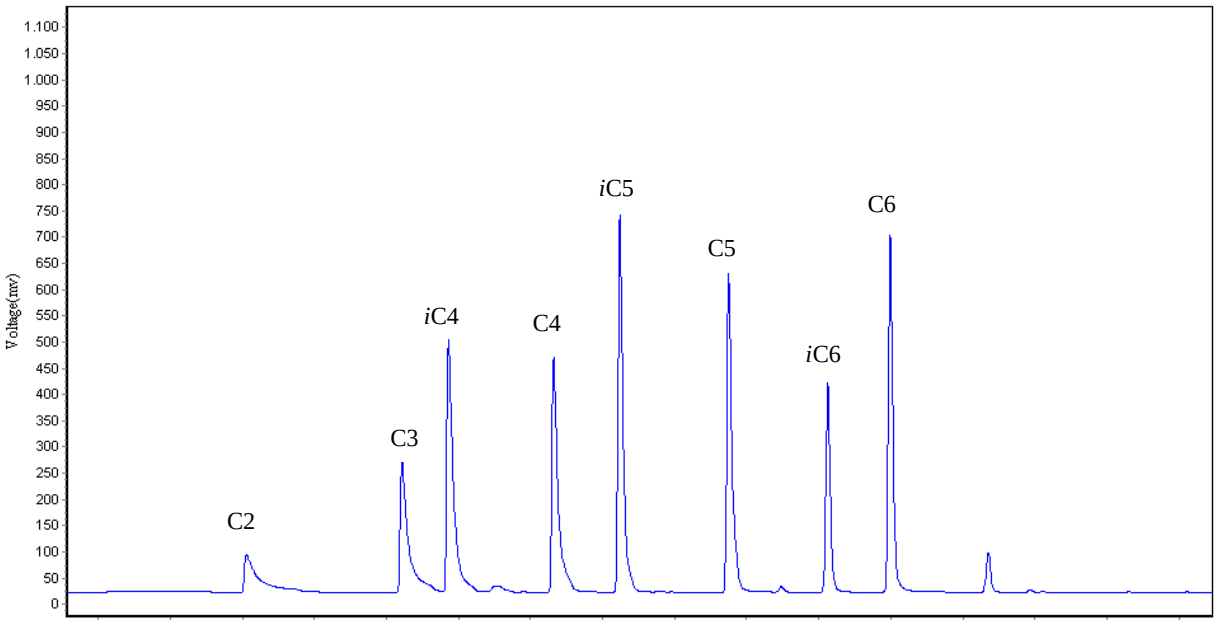


Figure 10. Chromatogram obtained from the analysis of short chain fatty acids (SCFAs) in a standard mixture.

The present experimental conditions allowed to obtain clean chromatograms and well resolved and separated peaks for all the analytes. After the analysis of the standard mixture also real samples were analyzed. **Figures 11** and **12** show the chromatograms from the SCFAs analysis in a rat faecal and in a fermentation fluid sample, respectively.

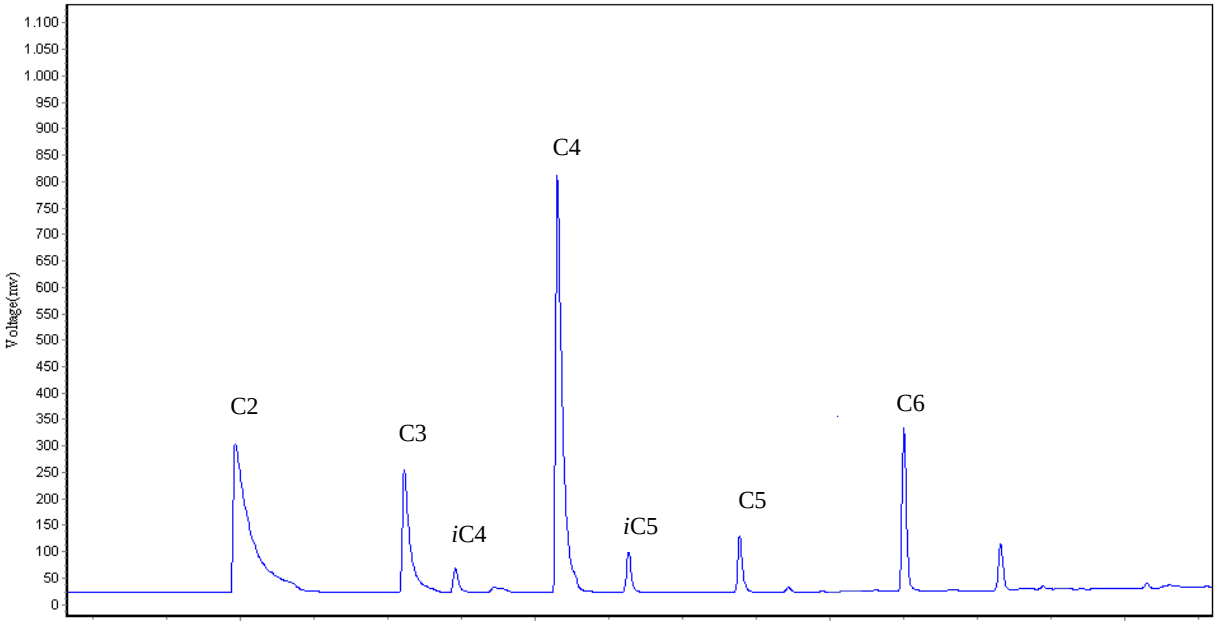


Figure 11. Chromatogram obtained from the analysis of short chain fatty acids (SCFAs) in a rat faecal sample.

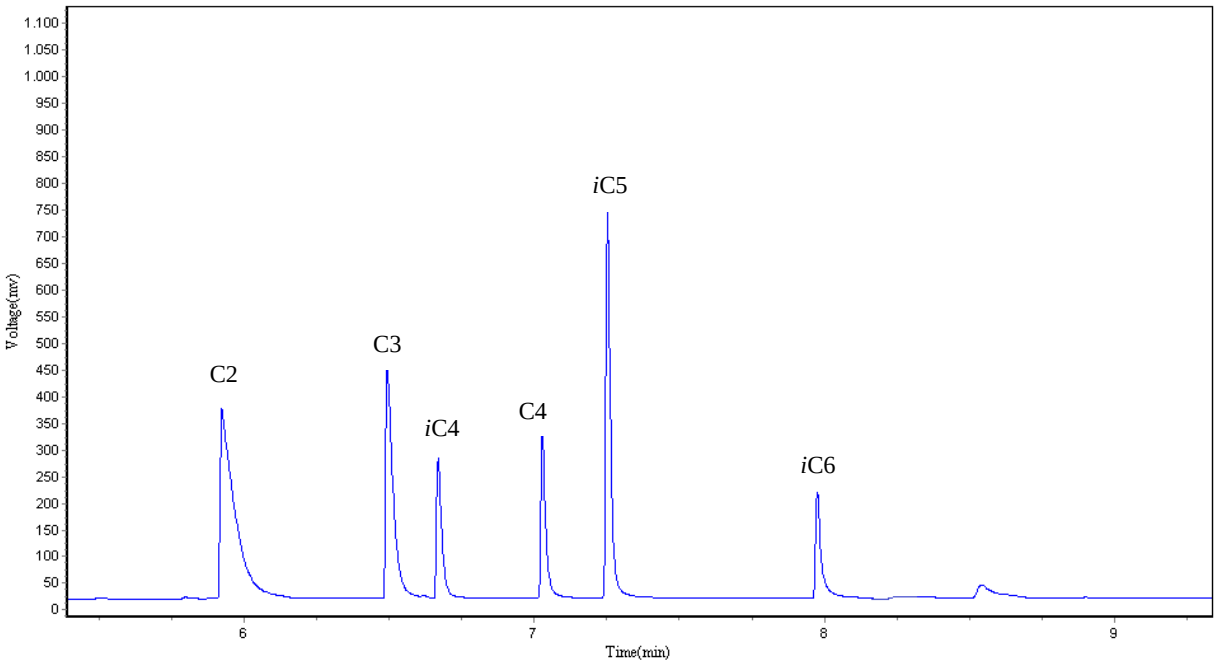


Figure 12. Chromatogram obtained from the analysis of short chain fatty acids (SCFAs) in a fermentation fluid sample.

Comparing the three chromatograms (**Figure 10, 11** and **12**) *i*C6 is not detected in the faecal samples, while C5 and C6 are not present in fermentation fluid samples. As a result of this, *i*C6 was selected as internal standard for faecal samples and C5 for the analysis of the fermentation fluids. Between different analyses, repeated blank tests were performed in order to verify the absence of possible contaminants. They always gave results below the limits of detection (LOD).

4.3.2 Method validation

4.3.2.1 Linearity, limits of detection and limits of quantification

The values regarding the limit of detection (LOD), limit of quantification (LOQ) and linearity are shown in **Table 5**.

Table 5. Limit of detection (LOD), limit of quantification (LOQ), linearity range and correlation coefficient (R^2) for the eight short chain fatty acids (SCFAs).

SCFA	LOD	LOQ	Linearity range	R^2
	(µM)			
Acetic acid	0.64	2.12	2.12-6487.50	0.9994
Propionic acid	0.14	0.48	0.48-3010.12	0.9991
i-Butyric acid	0.04	0.14	0.14-817.55	0.9998
Butyric acid	0.05	0.18	0.18-2454.60	0.9989
i-Valeric acid	0.08	0.27	0.27-690.55	0.9994
Valeric acid	0.05	0.18	0.18-690.55	0.9991
i-Caproic acid	0.08	0.25	0.25-594.83	0.9989
Caproic acid	0.08	0.26	0.26-594.83	0.9992

The calculated R^2 are in the range of 0.9989-0.9998, indicating an excellent linearity for all the investigated SCFAs. Moreover, the linearity ranges enclose all the concentrations of the analytes in both kinds of samples (faeces and fermentation fluids).

As indicated in **Table 5**, LOD values vary from 0.04 to 0.64 µM and LOQs from 0.14 to 2.12 µM.

The use of splitless injection, the low total amount of the extracting solvent and the good chromatographic peaks shape permitted to have lower LOD values respect to other previous studies. For instance, Zhao et al. [62] indicated LODs from 0.72 to 9.04 µM, performing an aqueous extraction of SCFAs in faeces followed by a GC-FID analysis. The method proposed by Han et al. [76] explained a method similar to the present one, using diethyl ether as extracting solvent and GC-FID analysis and indicated LOD values in the range of 0.03-0.26 µg mL^{-1} (corresponding to 0.26-4.33 µM).

The proposed method seemed to be more sensitive also than other methods which used different extraction techniques, such as SPME. For example, the reported LOD values are lower (except

for C6) respect to the method proposed by Fiorini et al. [77] consisting in the exploitation of SPME, known to provide high sensitivity.

The present analytical method was more sensitive also respect to some GC-MS methods in literature. LOD values of all the analytes (except for C2) were lower than the ones indicated by Garcia-Villaba [72] (0.49-4.31 µM); LOQ values were highly lower than the ones showed by Lotti et al. [73], included in the range 10-100 µM.

The validity of the method was investigated also when the procedure is applied to very small amount of faecal sample. The validation was performed starting from 100 mg and 20 mg of sample, providing very similar results. The results obtained with 20 mg of sample are here presented (**Table 5**).

4.3.2.2 Recovery and repeatability

The recovery and repeatability values were reported for rat faeces and fermentation fluid samples in **Table 6** and **7**, respectively.

Table 6. Repeatability (interday and intraday) as precision (RSD, %) and recovery of the short chain fatty acids (SFCAs) in rat faecal samples. Results obtained with the procedure applied to different amounts of starting sample (20 mg and 100 mg).

SCFA	Recovery (%) [a]			Repeatability [a] (RSD, % *n*=5)		Recovery (%) [b]			Repeatability [b] (RSD, % *n*=5)	
	Spike 1	Spike 2	Spike 3	Intraday	Interday	Spike 1	Spike 2	Spike 3	Intraday	Interday
Acetic acid	93.4	92.5	93.4	2.2	1.4	82.9	91.2	90.3	1.2	3.7
Propionic acid	99.2	106.4	106.8	2.2	3.3	79.0	105.2	104.8	2.4	2.4
i-Butyric acid	86.6	98.7	96.6	2.7	4.1	108.9	103.7	99.6	3.0	3.0
Butyric acid	97.9	108.7	108.2	1.8	1.2	73.7	103.5	103.0	1.9	1.2
i-Valeric acid	80.8	94.2	103.5	0.6	4.6	97.6	103.5	98.1	4.3	2.8
Valeric acid	82.6	101.5	102.4	4.2	4.5	98.4	103.0	101.6	3.7	4.7
Caproic acid	95.2	106.8	108.8	3.9	2.7	91.7	99.9	105.7	3.0	5.0

a Procedure applied to 20 mg of sample.
b Procedure applied to 100 mg of sample.

Table 7. Repeatability (interday and intraday) as precision (RSD, %) and recovery of the short chain fatty acids (SFCAs) in fermentation fluid samples.

SCFA	Recovery (%)			Repeatibility (RSD, % *n*=5)	
	Spike 1	Spike 2	Spike 3	Intraday	Interday
Acetic acid	78.4	77.9	76.7	0.4	4.7
Propionic acid	96.1	96.7	98.7	1.1	4.5
i-Butyric acid	105.6	100.9	103.0	1.0	4.0
Butyric acid	100.4	101.4	103.4	0.6	3.9
i-Valeric acid	97.1	98.5	105.8	1.3	3.7
Valeric acid	98.7	103.4	104.8	1.3	2.5
Caproic acid	99.7	103.7	104.6	nd	nd

The recovery of the method was determined by spiking a rat faecal sample and a fermentation fluid sample with a standard solution at three known levels of concentrations. In particular, for the rat faecal samples, the recovery was assessed also for 20 mg of sample, giving values in the range 80.8-108.8 %, indicating good reliability of the method for all the analytes. Good results were achieved also with fermentation fluid samples (starting from 250 mg), obtaining values from 77.9 % to 105.8 %.

Intraday repeatability was obtained for both the biological samples, performing five subsequent analyses a day. The intraday assay RSD values were in the range of 0.6-4.2 % for the faecal sample and 0.4-3.2 % for the fermentation fluids. Interday repeatability was evaluated on five different days, obtaining RSD values comprised between 1.2 % and 4.6 % for the faeces and 3.7 % and 4.7 % for the fermentation fluids. All the relative standard deviations were low enough to consider the procedure acceptable and they are similar to the data reported by other studies [71, 72, 73].

4.3.3 Application of the method to real samples

After its validation, the proposed method was applied for the study of SCFAs content in rat, mouse and human faeces and in fermentation fluids. Seven SCFAs (C2, C3, *i*C4, C4, *i*C5, C5 and C6) were detected in faecal samples and six (C2, C3, *i*C4, C4, *i*C5 and *i*C6) in the fermentation fluid samples. All the analytes were quantified and the obtained results are

summarized in **Table 8**. The values are reported as the mean values of five replicates ± standard deviation (SD) for all the samples.

Table 8. Concentration (µmol/g) of the SFCAs in fermentation fluid samples and in rat, mice and human faecal samples. Mean concentration values ± standard deviation (SD), range and percent relative standard deviation (RSD, %) from rats (*n*=5) mice (*n*=5) and human (*n*=5) faecal samples and from fermentation fluids (*n*=5) are indicated.

		Acetic acid	Propionic acid	*i*-Butyric acid	Butyric acid	*i*-Valeric acid	Valeric acid [a]	*i*-Caproic acid [b]	Caproic acid
Fermentation fluids	Mean (µmol/g) ±SD	48.6±8.3	11.3±3.3	4.2±0.8	2.0±0.8	5.1±1.0	IS2	2.9±0.8	nd
	Range (µmol/g)	39.0-59.1	7.9-16.4	3.1-5.0	1.4-3.3	3.9-6.1	IS2	1.9-3.6	
	RSD, %	17.2	29.3	18.2	37.0	19.7	IS2	27.3	
Rat faeces	Mean (µmol/g) ±SD	135.0±16.5	15.7±2.2	1.8±0.5	34.9±7.9	2.0±0.2	2.5±0.5	IS1	2.3±0.6
	Range (µmol/g)	118.1-161.6	13.3-19.4	1.0-2.2	26.0-47.8	1.6-2.2	1.7-2.9	IS1	1.4-3.1
	RSD, %	12.2	14.2	26.4	22.8	12.1	18.5	IS1	27.1
Mice faeces	Mean (µmol/g) ±SD	47.2±5.9	6.9±2.7	nd	4.4±1.5	0.6±0.0	1.2±0.2	IS1	nd
	Range (µmol/g)	36.6-55.2	2.6-10.7		2.4-6.6	0.5-0.6	1.1-1.4	IS1	
	RSD, %	10.7	24.9		23.4	2.7	12.1	IS1	
Human faeces	Mean (µmol/g) ±SD	24.3±1.0	9.7±2.7	1.6±0.5	7.2±1.9	2.7±0.2	3.5±0.5	IS1	1.1±0.4
	Range (µmol/g)	21.3-26.3	6.2-12.9	1.2-2.4	4.5-9.6	2.4-3.2	2.5-4.1	IS1	0.9-1.4
	RSD, %	4.9	43.8	35.6	42.4	7.1	18.1	IS1	43.3

a. Internal standard for fermentation fluids samples (IS2).
b. Internal standard for faecal samples (IS1).
nd: not detected (below the limit of detection).

As expected, acetic, propionic and butyric acids were the most abundant SCFAs in faecal samples, in accordance with the data found in literature [78, 79]. The concentrations of the minor SCFAs (*i*C4, C5 and C6) were similar to the results showed by Høverstad et al. [79] and Garcia Villaba et al. [43]. On the contrary *iso*-butyric and caproic acids were not detected in mice faeces.

All the analytes showed a quite high variability of their concentration within the different samples (RSD between 12% and 43%). The reason is that the SCFAs content is strictly related to many factors, such as the fiber consumption, the gut microbiota composition, the gut transit time and the overall health status of each individual [46].

The differences in the SCFAs composition between the different biological samples were shown more efficiently in **Figures 13** and **14**, for the most abundant fatty acids (C2, C3 and C4) and less ones (*i*C4, *i*C5, C5, *i*C6 and C6), respectively.

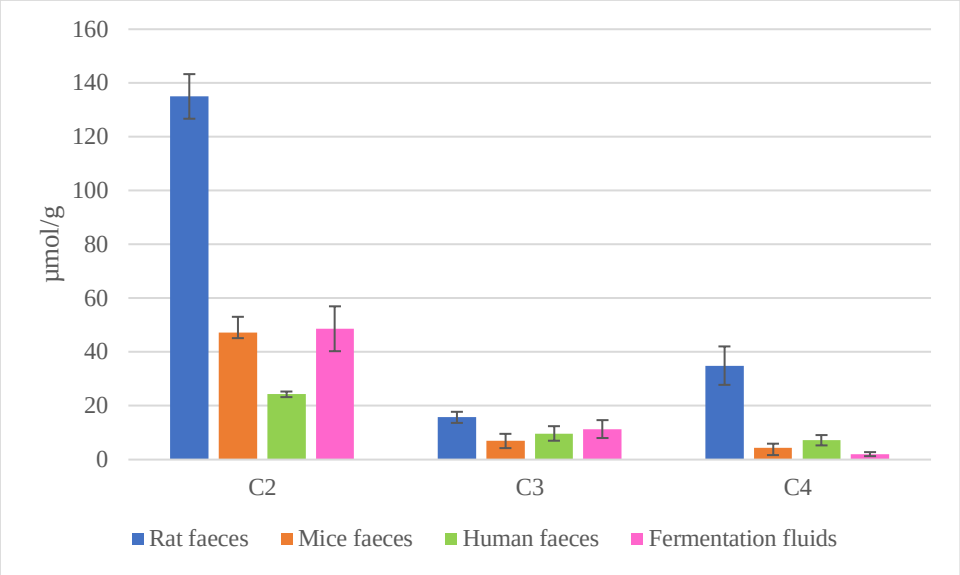


Figure 13. Comparison between the average content (µmol/g) of the most abundant short chain fatty acids (C2: acetic acid; C3: propionic acid; C4: butyric acid) in rat (blue), mice (orange), and human faecal sample (green), and in fermentation fluids (pink). Bars indicate standard deviation.

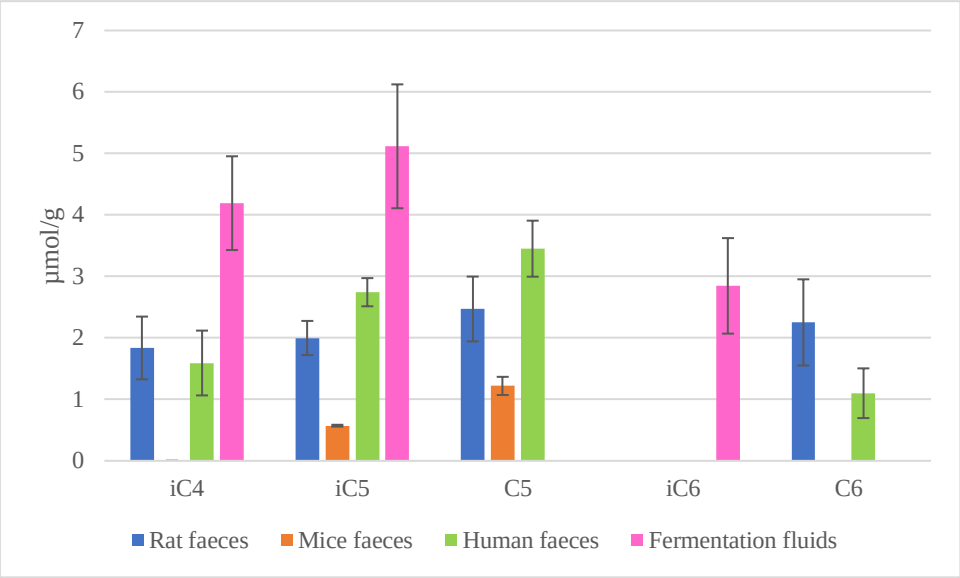


Figure 14. Comparison between the average content (µmol/g) of the less abundant short chain fatty acids (*i*C4: *iso*-butyric acid; *i*C5: *iso*-valeric acid; C5: valeric acid; *i*C6: *iso*-caproic acid; C6: caproic acid) in rat (blue), mice (orange), and human faecal sample (green), and in fermentation fluids (pink). Bars indicate standard deviation.

Fermentation fluids presented a lower content of the principal SCFAs (acetic, propionic and butyric acids) and a higher quantity of the branched SCFAs (BSCFAs). These acids usually are the products of the metabolism of branched amino acids, such as valine, leucine and isoleucine. Their presence is due to some particular fermenting bacteria species, such as some Eubacteria, *Megasphaeraelsdenii*, saccharolytic and asaccharolytic Bacteroides and many anaerobic Gram-positive Coccis [56].

Also the fermentation fluids samples presented a quite high variability (RSD 17-37%) in the concentration of each SCFA, due to the type of food used as substrate for the fermentation process and by the faecal inoculum nature, that is characteristic for each individual. Also McBurney and Thompson [80] indicated that the substrate is fundamental for the rate, the amount and the types of SCFAs produced by microbial fermentation.

4.3.4 Application of the method in "Probiosenior" and "Gut microbiota in mobility" projects

The proposed analytical method for the study of SCFAs in different biological samples, such as human faeces, has been applied in two real projects in collaboration with two research groups of the School of Biosciences and Veterinary Medicine of the University of Camerino.

Probiosenior project

The first one is called "Probiosenior project" and it investigates the impact of a probiotic diet on wellbeing of healthy senior. The relationship between diet and the diversity of the intestinal microbiota has been demonstrated by different studies. Many of them confirmed also the beneficial effect of probiotic microorganisms on the health of gut microbiota and the balance of produced metabolites, such as SCFAs [81]. Many researches suggested that aging has a significant effect on the microbiota. These alterations in the intestinal microbiota of elderly people could be caused not only by aging, but also by the decline of the general state of health or malnutrition or increased use of medication. Some differences in microbiota composition have been noticed also between healthy and hospitalized or institutionalized elderly people [82]. The studies present in literature indicate that the use of probiotics is safe and it could help to prevent some disease, such as antibiotic-associated diarrhoea, to reduce the severity of other symptoms or to avoid constipation [82].

The aim of Probiosenior project is to study the impact of new probiotic formulation in the health status of the intestine of elderly people. Several parameters have been considered for each patient:

- Questionaries: Mini Nutritional Assessment (MNA), medical history, evaluation of the diet and lifestyle, Psychological General Well-Being Index (PGBWI);
- Faecal samples analyses: microbiological composition, SCFAs content, cytokines;
- Blood samples analyses: cytokines, CBC, differential leucocyte count;
- Urine samples analyses

The project was a randomised, double-blind, placebo-controlled study assessing the effect of daily consumption of SYNBIO® (SYNBIOTEC Srl, Camerino, Italy), which consists in a mixture 1:1 of *Lactiplantibacillus rhamnosus* IMC 501® and *Lactiplantibacillus paracasei* IMC 502® by probiotic-enriched foods or by dietary supplement on healthy seniors' status. Specifically, the supplementation should improve the parameters related with the aging process, such as intestinal inflammation.

All the samples were given by the patients of six different boarding homes and several private homes of senior in Marche Region, Italy. The subjects were randomly assigned to one of two parallel groups, to receive either probiotic-enriched foods and capsules or the respective placebo.

In detail, the participants were all senior healthy female and male with more than 65 years. Exclusion criteria were based on the physical status and history of health conditions, such as chemotherapy medications or use of anti-inflammatories in the previous four months, malnutrition (BMI <18.5 kg/m^2 or weight loss > 10% in the last 6 months). Then, antibiotic treatment in the previous week was not allowed and the people having daily habits to consume probiotics were excluded.

Regarding the functional foods, six different products were used as carriers for delivering probiotic bacterial strains (SYNBIO®, lyophilized powder containing 5 billion live cells per gram): yogurt, mozzarella cheese, fruit smoothies, ricotta cheese, primo sale cheese and chocolate. Also SYNBIO® in capsules (containing $5x10^9$ CFU/capsule) was also provided (Synbiotec Srl, Camerino, Italy). For the placebo control group, the food used as carriers of the placebos were the same for the probiotics. In this case the capsule were identical capsules containing maltodextrin, instead of probiotics. Maltodextrin is completely digested before reaching the colon, so it does not affect the gut microbiota. Also the placebo capsules were provided by Synbiotec Srl. The study was conducted with a 4-week run-in period followed by a 24-week intervention period. All the volunteers received six different probiotic or placebo food products for 2, 3 or 6 months, to be consumed one per day.

All the chemical and biological parameters were determined at time zero (T0) and after the supplementation period (2, 3 or 6 months) (T1), when it was possible, to evaluate possible differences and changes during time. The results obtained for the SCFAs composition in the faceal samples (**Table 9**) are under investigation and they will be considered together with all the other parameters, in order to know the effect on the composition of the microbiota after administration of probiotics and the efficacy of probiotics intake on symptoms of major gastrointestinal diseases in elderly people.

Table 9. Concentration ranges (μmol/g) of the SFCAs in the faecal samples of elderly patients at time zero (T0) and after the placebo or probiotic supplementation (T1). The samples are divided according to the supplementation period (6 months or less than 6 months).

SCFA	Probiotic supplementation (6 months)		Probiotic supplementation (< 6 months)		Placebo supplementation (6 months)		Placebo supplementation (< 6 months)	
	T0	T1	T0	T1	T0	T1	T0	T1
Acetic acid	12.18-77.83	8.15-70.14	13.81-114.57	9.26-90.49	8.83-79.83	7.40-61.48	27.18-102.82	11.65-82.12
Propionic acid	2.32-17.63	1.95-13.97	2.81-28.55	2.20-24.42	0.85-12.93	0.93-32.31	7.80-34.21	3.23-23.12
i-Butyric acid	0.78-3.91	0.70-3.39	0.84-6.89	0.68-5.01	0.53-4.59	0.63-6.35	1.67-11.92	0.68-1.80
Butyric acid	1.73-26.41	1.20-22.91	1.41-32.89	0.77-24.00	1.02-16.46	0.33-22.23	4.59-42.58	2.19-29.53
i-Valeric acid	1.57-6.74	1.17-6.51	1.27-10.22	0.91-8.37	0.90-8.17	1.07-10.05	2.29-20.13	0.90-2.82
Valeric acid	0.93-7.09	0.63-4.76	1.03-6.42	0.68-5.14	0.37-5.81	0.73-6.62	1.13-11.40	0.57-3.76
Caproic acid	0.31-1.96	0.38-2.49	0.34-3.12	0.27-2.99	0.32-1.28	0.33-2.54	0.51-5.64	0.34-0.80

Gut microbiota in mobility

Gut microbiota has been extensively studied in relation to different geographical areas, ethnic groups, food habits and age. It has been demonstrated that dietary products and the associated eating habits and geographical provenance of individuals could influence the gut microbiota composition [83].

However, the gut microbiota changes by temporary full immersion in different environments and dietary habits are still not well investigated, considering the high level of global mobility.

The aim of this project is to investigate the plasticity/adaptation of the gut microbiota in healthy young people during geographical changes, in order to discover microbiota modulations by a drastic change of circadian clock, diet, and environment.

The main target are Chinese students (24 enrolled volunteers) from Jilin Agricultural University in mobility at Camerino (Italy) for one academic period. Firstly, they were interviewed about their diet.

Then, they provided stool samples at different times:

1. In China
2. At their arrival in Italy
3. After one week in Italy
4. After one month in Italy

Also in this case, different biological and chemical parameters were considered for each sample, for a better understanding of possible gut microbiota modulations:

- 16S rDNA sequence and bioinformatic analysis;
- Short chain fatty acids analysis (SCFAs);
- Cultivation in aerobic and anaerobic media and analysis by MALDI-TOF Biotyper (BRUKER);
- Selection and characterization of putative probiotics to be further analysed;
- Metagenomic analysis of selected samples to detect yeasts and other eukaryotic microbes.

Preliminary results indicated that some differences in bacterial relative abundances were observed among the samples given at the arrival in Italy, after one week and one month respect to the samples taken in China at phylum and genus levels.

Then a possible correlation was found between bacteria composition and the diversity of SCFAs in the stool samples provided at the arrival in Italy and after one month.

All the results are under investigation and the values obtained for the SCFAs (**Table 10**) will be considered together with all the other parameters in order to find more interesting biochemical relationships within and between microbial species and potentially to predict the effect of ecosystem-wide perturbations, such as diet or environmental changes.

Table 10. Concentration ranges (μmol/g) of the SFCAs in the faecal provided by the volunteers when in China, at their arrival in Italy and after one week and one month.

SCFA	In China	Arrival in Italy	After one week in Italy	After one month in Italy
Acetic acid	17.25-116.80	25.85-192.25	21.25-103.68	24.85-109.78
Propionic acid	8.89-52.58	5.71-55.84	9.00-45.79	8.37-56.11
i-Butyric acid	0.99-5.60	0.86-3.88	0.25-5.70	0.79-3.82
Butyric acid	4.34-33.18	4.10-36.23	4.03-35.52	4.04-45.97
i-Valeric acid	1.19-7.92	0.97-5.86	0.57-6.91	0.83-5.92
Valeric acid	1.06-5.54	0.59-4.91	0.34-6.05	0.34-6.00
Caproic acid	0.29-0.57	0.27-2.18	0.26-3.20	0.30-2.92

4.4 Conclusions

This study aimed to develop and validate a new method for the identification and quantification of a total of eight SCFAs in different biological matrices (faecal samples and fermentation fluids), performing the analysis with a relatively short time (about 25 min) with an easy and rapid pretreatment of the sample (acidification with aqueous sulfuric acid and extractions with diethyl ether) and exploiting commonly available instrumentation (GC-FID). The extraction method has been applied successfully also to very small amount of sample (20 mg), when the starting material is present in low quantity.

For the first time, the number of extractions to be performed in order to obtain good extractions for all the analytes has been evaluated. The results highlighted significant increase in the extraction extent passing from one to two extractions and from two to three extractions. In this way the best conditions to obtain the highest sensitivity were identified.

The validation of the method indicated good linearity, precision, high sensitivity and good repeatability. The proposed procedure is suitable to investigate both linear and branched SCFAs, giving useful information about physiological state of an organism and providing information about the effect of supplementation treatment or specific diet.

The method was applied in two real projects in collaboration with the School of Biosciences and Veterinary Medicine of University of Camerino. The SCFAs analysis was only one of the parameters involved in the study of the effect of probiotics in the healthy status of elderly people (Probiosenior project) and in the investigation of the plasticity of gut microbiota in healthy young people after geographical and diet changes (Gut microbiota in mobility). Until now only preliminary results have been collected and deeper investigation will be done in this sense.

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