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UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN

Pharmaceutical, Nutraceutical and Food Sciences

XXXV Cycle

STUDY OF MEAT SHELF-LIFE MARKERS AND FOOD QUALITY THROUGH DIFFERENT INSTRUMENTAL ANALYTICAL METHODS

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Mustafa, A. M., Angeloni, S., Abouelenein, D., **Acquaticci, L.**, Xiao, J., Sagratini, G., Maggi, F., Vittori, S., Caprioli, G. (2022). A new HPLC-MS/MS method for the simultaneous determination of 36 polyphenols in blueberry, strawberry and their commercial products and determination of antioxidant activity. *Food Chemistry*, 367, 130743.

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CHAPTER 1

INTRODUCTION: MEAT SHELF-LIFE

1.1. Shelf-life

Shelf-life is defined as the time during which a food product remain safe, comply with label declaration of nutritional data and retain desired sensory, chemical, physical and microbiological characteristics when stored under the recommended conditions [1].

Shelf-life is a function of time, environmental factors and susceptibility of product to quality change [2].

The study of food shelf-life is becoming increasingly important for two main reasons: the need to furnish safe food to a continuously growing population and the need to reduce the food waste. In fact, an increase of food production to supply the needs of the population is not sustainable from an ecological point of view [3] and it is also inevitably correlated to food loss and waste.

The Food and Agriculture Organization (FAO) reports that the food waste in Europe and in North America is 95–115 kg/year per capita, and the extent of food loss and waste globally reaches approximately 1.3 billion tons per year [4]. While food losses are mainly related to food-processing [5], food waste is related to food shelf-life as it occurs during distribution and consumption processes in the food-chain [6]. In fact, the distribution of food products under inadequate conditions, such as high temperature or humidity, that is one of the causes of the shortening of shelf-life [7-9], or the delay in the consumption after the expiring date, that gives to the food product an uncertain safety [10, 11], are responsible for the wasting of a percentage of perishable foods that ranges from 15% (mainly for damage and spoilage) [12] to 35% (for inadequate temperature) [13]. By the consequence, monitoring food shelf-life means monitoring the degree of food spoilage and therefore reducing food waste [14].

However, monitoring food shelf-life is not simple as deterioration processes can be evidenced by several attributes that are product, consumer and market specific [15, 16].
In foods with long shelf-lives, the end of the acceptability for the consumption is usually determined through the evaluation of nutritional and organoleptic properties [17], while that of high perishable foods is mainly detected through the loss of sensorial properties or changes in the organoleptic characteristics (flavour, colour) [18, 19].
The study of food spoilage degree can be carried on through sensorial analyses, which are based on the use of survival analysis methodologies [20] to evaluate the consumer rejection probability level, or through instrumental measurements, which are often related to sensory attributes. As reported in ***Table 1***, several attributes related to food shelf-life have been regulated and used as markers of shelf-life in diverse food products.
Most times, the study of microbial spoilage is the most reliable attribute related to food spoilage and it is directly connected to a loss of sensorial attributes [21].

Table 1: Examples of sensory attributes identified as markers of shelf life in several food products, and regulatory/instrumental measurements used to evaluate their acceptability [22].

Food Category	Food product	Sensory attribute	Regulatory/instrumental measure associated with acceptability	References
Meats	Beef (fresh)	Off flavours	Spoilage microorganisms ≥ 10^7 CFU/g	[23, 24]
	Poultry (cooked)	Smell, taste, slime inside package	Spoilage microorganisms ≥ 10^7 CFU/g	[25]
Fruits	Bananas (fresh)	Discoloration	Colour changes (image and spectral analysis)	[26]
	Orange juice	Discoloration	Volatile organic compounds Increase in oxides and sulphur compounds Decrease in aldehydes	[27, 28]
	Mango juice	Discoloration	Markers for browning (ascorbic acid, 5-hydroxymethylfurfural, furfural)	[29]
Seafood	Shrimp	Odour and texture scores	Spoilage microorganisms ≥ 10^7 CFU/g	[30, 31]
Vegetables	Frozen leafy greens (spinach)	Overall acceptability (score for the acceptability 5/9)	70% vitamin C loss 60% chlorophyll retention	[32]
	Fresh leafy greens (rocket)	Overall appearance	Ascorbic acid content (50% of recommended daily intake)	[33]
	Carrot juice	Taste	Spoilage microorganisms ≥ 10^6 CFU/g (regulatory limit)	[34]

As food is a complex matrix, it is difficult to select just one marker (called also limiting factor) to monitor the shelf-life. For this reason, several approaches have been developed to study more than one limiting factor to have an overall idea of spoilage process.

With regard to this, MathematicaR (Wolfram Research, Champaign, IL) is a new interactive program, developed by Peleg and Normand (2015) that combine two limiting factors simultaneously to evaluate food shelf-life [35]. Another approach concerns the use of a single index called global stability index (GSI), developed by Achour (2006),

which combine sensory, chemical and microbiological attributes to evaluate food shelf-life [36]. Even if GSI has been used to study the shelf-life of fresh products and fish [37-40], its limitation is related to the lack of standard procedures to assign the values for each limiting factor. One more strategy used to study food shelf-life is based on fingerprinting kinetics and it was developed by Grauwet et al. (2014) [41]. This approach allows to identify the most important markers of shelf-life for a specific food product by screening the modifications in the food product, due to degradation processes, and selecting the most important markers involved in the degradation process. Then, it allows to connect the selected markers to specific spoilage reactions though a multivariate analysis. This technique allows to have an overall idea of food spoilage process and it has been used to study the shelf-life of fruit/vegetable juices and purees [42-46].

The study of food shelf-life, the identification of a marker of shelf-life and the regulation on the minimum level to be respected for that specific marker is essential to establish useful information for the consumer, such as the expiration date. The expiration date represents the time needed for the chosen quality attribute to reach its unacceptable level under specific storage conditions. The expiration date is clearly visible on the packaging of the food product and reported as “use by date” or “best before” [47]. An accurate match between the expiration date and the end of the shelf-life of the food product is found, if the storage conditions have been respected from the dealers and the consumers [48-50]. However, adequate storage conditions are not often respected, especially regarding the temperatures of refrigerators [51-53] or improper packaging which slows the air-flow with subsequent increase of the temperature [52, 54]. This leads to a mismatch between the expiration date and the effective spoilage degree of the food product. Moreover, these evidences have pointed out the ongoing process according to which the expiration dates

will have always less importance [55], giving way to new technologies that are able to provide a real-time shelf-life estimation of food products.

1.2. Real-time shelf-life devices

Real-time shelf-life devices should be integrated or substituted to the expiration date to give a realistic idea about the spoilage degree of the food product.

The most studied alternative devices are time-temperature indicators (TTIs) and sensors coupled to data collection/storage devices.

Indicators and sensors have differences [56] mainly because indicators provide a visible signal (such as colour change), while sensors detect an input (such as temperature, moisture, pressure) from the environment and send the signal to a network for the processing [47, 57, 58]. As an example, a sensor continuously monitors the temperature during storage, while indicators point out the cumulative effect of the time-temperature history of a marker of shelf-life [55].

Time-temperature indicators (TTIs) have been used from 1970s and they have been used as potential substitutes of conventional labelling [48, 59-62] to better reproduce the spoilage degree of food products. TTIs are attached on food packaging and the shelf-life of the food product is monitored through an irreversible chromatic change. For this reason, the development of a TTI needs the study of the relation between the degradation kinetics of the shelf-life marker and the chromatic change related to the temperature change. TTIs can be classified on the basis of their operating principles in chemical, biochemical, biological, nanoparticle-based and diffusion-based. The chemical TTIs are projected on the basis of polymerization, photochromic, or oxidation reactions such as Fresh-CheckR or OnVuTM [63, 64]; the biochemical ones are based mainly on enzymatic

hydrolysis of a substrate [65-67]; the biological ones measure the growth of microorganisms, such as yeast or lactic acid bacteria, whose metabolites are responsible for the change of pH, detected with the change of the colour of a pH indicator [50, 68, 69]; the nanoparticle-based ones are based on changes in nanoparticles' size, shape and morphology caused by temperature variations, which are responsible for changes in visual attributes [55]; the diffusion-based ones work through the diffusion of coloured fatty acids at high temperature (i.e. Monitor MarkTM) [70].

However, these indicators have some limitations in terms of toxicity of the components or reagents, short stability, lack of specificity and the limited correspondence between the colour change and changes in the selected marker of shelf-life [49, 50]. Some of these limitations have been overcame by replacing toxic components with natural ones and/or general recognized as safe (GRAS) indicators, such as anthocyanins [71] or a combination of laccase, guaiacol, and cysteine as O_2 concentration indicators [72].

Sensors and data collection/storage devices are another alternative to have a real-time idea on the shelf-life of a food product. The use of the radio-frequency identification (RFID) as data collection/storage device is continuously increasing [73]. It is usually coupled with sensors and connected to a wireless sensor network (WSN). In this way, the sensor-RFID unit perceives changes in the environment of food product during storage and distribution and the WSN records the signals sent from the sensor-RFID unit.

Sensors can be coupled to GPS (global positioning system) devices, too. Sciortino et al. (2016) developed a device formed by a GPS module, three types of sensors (temperature, humidity and gas), a remote connection for communication of quality parameters and a real-time shelf-life prediction algorithm executed in a WebGIS platform to study the shelf-life of strawberries [16]. However, the use of these devices is limited by the high

cost [58], even if the benefits in terms of reduction of food waste will reward the investment.

The use of both indicators and sensors involves the use of monitoring devices. The most used one are ultraviolet/visible (UV/VIS) [74], near-infrared (NIR), or luminescence spectroscopy, fluorescence fingerprinting and e-nose fingerprinting.

Small devices provided with these analytical tools can be used to have a real-time idea on the shelf-life of a food product. These approaches need a robust virtual infrastructure to easily communicate and analyze data for the real-time monitoring and adequate visualization platform that can be easily used by the consumers.

1.3. Meat spoilage and shelf-life

Dietary habits have been changed worldwide over the years. In particular, the consumption of meat has been increased in the last century, especially from 1960s-80s to today. Some studies have suggested an increment of meat consumption of 204% (from 1960 to 2010) [75], while other studies have reported an increment of meat consumption of 500% (from 1992 to 2016) [76]. In 1960s the protein intake came from plant-derived products, such as wheat, while nowadays meat products furnished more than 58% of available proteins, becoming the most important source of proteins for people (28g of proteins/person/day) [77]. The most consumed types of meat are poultry and pig meats, mainly in North America and Southeast Asia respectively [75, 78].

However, about 3.5 billion kg of poultry and meat product are wasted every year [79]. In fact, meat and meat products are high perishable foods, due to oxidation and deterioration processes [80-82].

Several factors can influence the meat spoilage: pre/post slaughtering, intrinsic and extrinsic factors (***Table 2***).

Table 2: Intrinsic and extrinsic factors affecting meat shelf-life [83].

Type	Factors
Intrinsic	Type of animal (bovine, porcine) Breed and feed regime Age of animal at time of slaughter Initial microflora Chemical properties (peroxide value, pH, acidity, redox potential) Availability of oxygen Processing conditions and control Hygiene (standard of personnel and equipment cleaning)
Extrinsic	Quality-management system Temperature control Packing system (materials, equipment, gases) Storage types

One of the most significant is the pre and post-slaughter stress, that can influence the meat spoilage mainly in terms of glycogen content which in turn influences the pH of the meat, with subsequent changes in colour and tenderness [83-85]. In fact, the content of glycogen is related to the production of lactic acid via an anaerobic glycolytic pathway as shown in **Figure 1** that will influence the pH of meat.

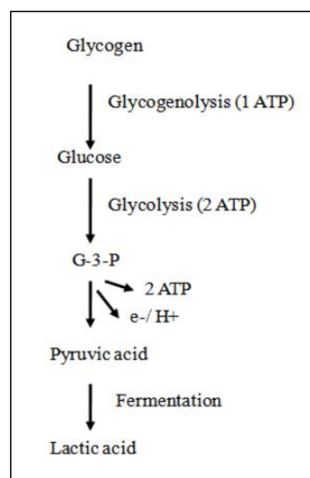


Figure 1: Scheme of the production of lactic acid via glycolytic pathway [86].

In particular, a high value of pH (6.4-6.8) result in Dark, Firm and Dry (DFD) meat, while a low one in Pale, Soft and Exudative (PSE) meat (**Figure 2**).

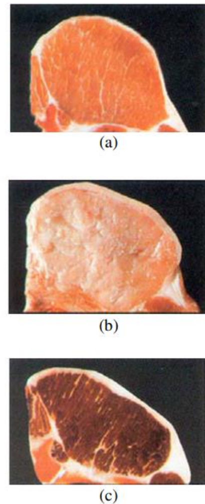


Figure 2: Meat texture and colour (Chambers and Grandin, 2001) (a) Normal meat; (b) Pale soft and Exudative (PSE) meat; (c) Dark Firm and Dry (DFD) meat [87].

DFD meat is the result of a long term stress and it has a shorter shelf-life [84, 85], while the low pH of PSE meat favours the bacterial growth [83-85].

Three factors are the main mechanisms responsible for meat spoilage after slaughtering: lipid oxidation, microbial spoilage and autolytic enzymatic spoilage.

In particular, lipid oxidation is the main factor responsible for meat spoilage as it is a complex process that leads to the formation of several compounds, negatively related to meat quality in terms of sensory and nutritional properties [88]. Lipid oxidation, in fact, occurs in fatty acids in tissues after slaughtering, when the blood circulation and metabolic processes stopped [89, 90]. This reaction involves the oxygen with double bonds of fatty acids [91] and leads to the production of free radicals through the typical three steps (initiation, propagation and termination) [92-94]. The oxidation of fatty acids is responsible for the formation of secondary reaction products such as pentanal, hexanal, 4-hydroxy-nonenal, malondialdehyde (MDA) and other aldehydes, ketones and acids

[94-96], which can cause the loss of organoleptic (colour) and nutritional properties [97], and also carcinogenic and mutagenic effects [98].

With regard to the microbial spoilage, meat and meat products provide a fertile media for the growth of a wide range of microflora species (bacteria, molds and yeasts) [99], whose composition is influenced by several factors, such as preslaughter practices, age of the animal and time of slaughtering, handling during slaughtering/evisceration/processing/distribution, preservation methods and type of packaging, handling and storage by consumer [100].

Among the bacteria, the most common are *Pseudomonas*, *Micrococcus*, *Streptococcus*, *Sarcina*, *Lactobacillus*, *Salmonella*, *Escherichia*, *Clostridium* and *Bacillus* [101-103]. The most common mold species are *Cladosporium*, *Sporotrichum*, *Geotrichum*, *Penicillium* and *Mucor,* while yeasts ones are *Candida spp*., *Cryptococcus spp*. and *Rhodotorula spp*. [104]. The importance of studying the microflora of meat products relies in monitoring the presence of potentially harmful pathogens [99] and, by the consequence, also the shelf-life of the meat product.

The autolytic enzymatic spoilage is the result of the natural action of enzymes, that have the ability of catalyzing reactions responsible for deterioration [105]. In fact, complex compounds (carbohydrates, fats and proteins) in tissues are transformed in simpler ones during the autolysis process, leading to a softening and greenish discoloration of meat. Instead, the post-mortem action of proteases on polypeptides is responsible for flavour and textural changes in the meat product [106].

1.4. Food packaging technology to prolong shelf-life

Packaging influences food shelf-life, quality and safety and, for this reason, several studies have been conducted in the last years to develop new packaging materials or new packaging technologies to improve food shelf-life, quality, and safety.

Traditional packaging have different functions: holding food during storage, transportation and distribution; protecting food from physical damage, chemical and biological contamination; informing costumers about nutritional properties and branding [107-110].

Over the past years, plastic films have been the best candidates used in food packaging to preserve food from heat, moisture, microorganisms, dust and dirty particles, thanks to their low weight, easy handling and low cost [110]. However, in recent years, the growing attention of consumers for eco-friendly packaging [111-113] drives the research through new solutions, such as the use of bioactive films [114-116], the development of smart [117, 118], active [119, 120] and intelligent packaging [117, 121, 122].

Regarding polymers, they are used on food packaging thanks to their easy production, strong molecular networks and crosslinking, and technological properties (strength, barrier to moisture and oxygen, and resistance to food component attack) [123-125]. Polymers can be biodegradable (mainly cellulose, chitosan, starch, agar, gelatine, soy protein, and whey protein [124, 126]) or non-biodegradable (mainly polypropylene (PP), polyethylene (PE), polyethylene-co-vinyl acetate (EVA), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) [126]). The biodegradable ones are the most interesting from an environmental point of view for packaging application. In particular, cellulose and chitosan are polysaccharide-type polymers and they have a good film and gel-forming ability, recyclability and antimicrobial properties that make them good candidates for

food packaging applications [126, 127]. Starch, instead, is a polysaccharide-type polymer and its use in packaging application is mainly as adhesive and additive [126]. Among the protein-type polymers, gelatine has strong film-forming ability [110, 128], while soy protein is used thanks to its intermolecular binding potential via covalent bonds [124]. Finally, polylactic acid blend (PLA) is used as an environmental-friendly polymer combined with antimicrobial agents to form films for packaging to preserve perishable food products such as fruits, vegetables and meat [110, 124, 129].

Smart packaging is mainly used as an alternative packaging thanks to its ability of monitoring physicochemical influences from environment or microbiological changes [121, 130]. Active and intelligent packaging re-enter in the class of smart packaging and they are widely used in food and beverages, healthcare products and personal care.

Active packaging is a new advanced technology based on the incorporation of active compounds, such as antioxidants, on a polymeric matrix [82, 131] to prolong food shelf-life. In fact, the active compounds are released naturally from the polymeric matrix to the food or the environment inside the packaging, slowing the deterioration processes thanks to its action [132]. Among the most used active compounds, there are antimicrobials, antioxidants, carbon dioxide emitters/absorbers, oxygen scavengers, and ethylene scavengers [130, 133] with a wide range of application, as reported in ***Table 3***.

Table 3: Active agents for food packaging [134].

Type	*Active component*	*Applications*
Antimicrobial	Chitosan Essential oils Gallic acid Lactoferrin Lysozyme Metals Nisin	Fruits [135], bread [136], meat [137-141], fish [142, 143].
Antioxidant	Essential oils Lignin Plant extracts Phenolic compounds α-Tocopherol	Cereals, nuts, meat, meat products[137, 139, 144].
Carbon dioxide absorber/emitter	Citric acid Ferrous carbonate Sodium bicarbonate	Meat, fruits, vegetables [145].
Oxygen scavenger/absorber	Ascorbic acid Gallic acid Glucose oxidase Iron Laccase Palladium Pyrogallol	Most baked products and nuts [146], meat [133, 146], fish [146], fruits [147].
Ethylene scavenger	Activated carbon Potassium permanganate Metal oxides Metal organic frameworks (MOFs) Titanium dioxide	Fruits, e.g., kiwifruit, banana, vegetables [148].

The technology of an intelligent packaging is based on the ability of this system to monitor the physicochemical conditions of the food product (such as the freshness degree) and the influence of environmental factors (such as temperature, pH, gas) during transportation and storage [122, 149, 150]. In fact, the intelligent packaging is provided of a device which is able to detect changes and inform the costumer about the status of the food product [148, 149, 151]. The devices used for this purpose can be sensors, indicators or radio frequency identification systems (RFID) for several food packaging applications, as reported in ***Table 4***.

Table 4: Different types of intelligent packaging [134].

Type	*Active component*	*Applications*
Sensor	Bio-sensor Gas sensor Fluorescence-based oxygen sensor	Meat [152], fish, fruits [149, 153], beverages, vegetables [154, 155].
Indicator	Time/temperature indicator	Food stored under chilled and frozen conditions (e.g., meat [133], milk [156]), pharmaceutical products [157].
	Oxygen indicator	Food stored in MAP (e.g., meat [133]).
	Carbon dioxide indicator	Food stored in MAP or CAP [158, 159].
	Color indicator	Fish [160], milk [161].
	Pathogen indicator	Meat [159], fish [162, 163].
	Breakage indicator	Canned baby foods [117].
	Leak indicator	Perishable foods [153].
	Freshness indicator	Fish [162, 163], poultry [164], fruits [153].
Radio frequency identification tag (RFID)		Cheese [165], meat [148], vegetables [155].

MAP: Modified atmosphere packaging; CAP: Controlled atmosphere packaging.

1.5. Food shelf-life markers: biogenic amines (BAs) and volatile organic compounds (VOCs)

Biogenic amines (BAs) are low molecular weight nitrogenous compounds, formed by the decarboxylation of amino acids or amination/transamination of aldehydes and ketones during metabolic processes in cells [166]. They are physiologically present in plants, animals and humans and they are involved in important functions, such as neurotransmission, regulation of blood pressure and immune functions in the intestine [167].

However, high amounts of BAs in food are related to a certain degree of spoilage [71] and they can be toxic for humans, causing adverse effects on nervous, respiratory, cardiovascular systems or allergic reactions [168]. In fact, one of the most common

intoxications is that caused by histamine, whose main symptoms are nausea, vomiting, diarrhea, headache, urticaria, tachycardia, and even death, while those by tyramine intoxication are mainly headache, palpitations, nausea and vomiting, and an increment of blood pressure [169-171].

The most common BAs occurring in food during deterioration are cadaverine (CAD), putrescine (PUT), spermidine (SPD), spermine (SPM), β-phenylethylamine (PHE), tyramine (TYR), and histamine (HIS) [172].

The formation of BAs is influenced by several factors, such as pH, chemical composition, handling/manufacturing operations, temperature/time of storage [173] and the presence of microorganisms [174]. In fact, all these factors influence the concentration of free amino acids, which are the substrates for the formation of BAs, as shown in **Figure 3**.

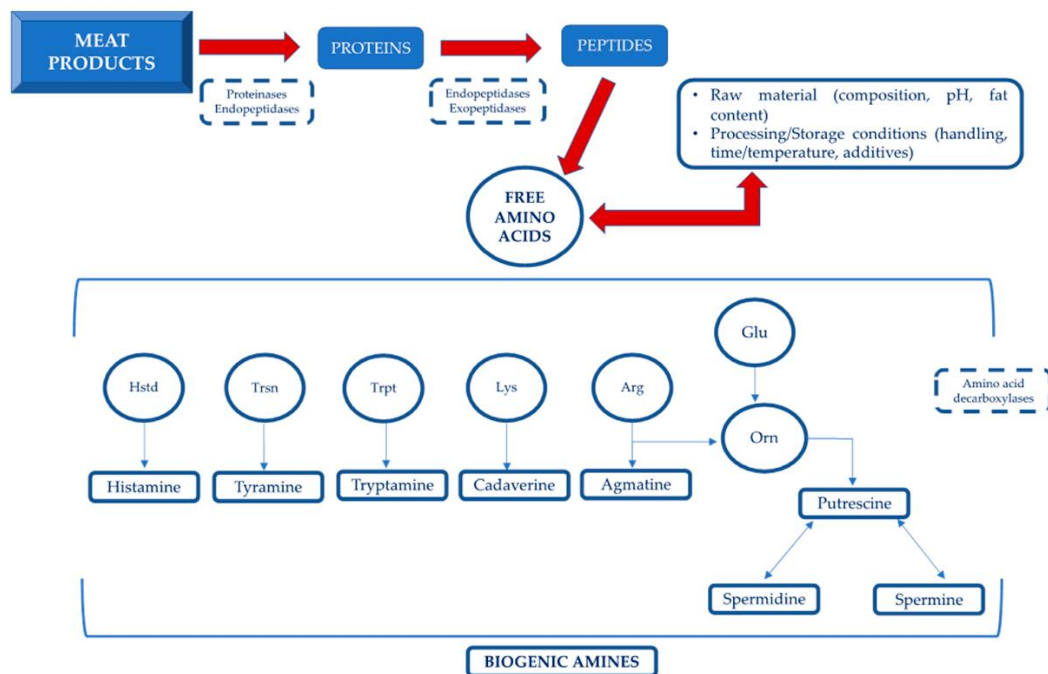


Figure 3: Mechanisms of BAs formation [175].

Meat and meat products are high-protein foods and for this reason, they are subjected to the production of BAs during spoilage. SPM (20 and 60 mg/kg) and SPD (10 mg/kg) are naturally present in fresh meat [176], while the other BAs are produced during spoilage, mainly in relation to the proteolysis reaction that favours the degradation of proteins into free amino acids [169]. In particular, the proteolysis reaction is influenced by acidity, dehydration, the presence of sodium chloride in meat or microbial activity [177].

For all these reasons, BAs can be considered important markers of shelf-life and indicators of freshness and quality for meat products [176].

Two are the most used indexes to evaluate the presence BAs in foods: the Biogenic Amine Index (B.A.I.) and the ratio between SPM and SPD (SPM/SPD). B.A.I. was calculated by the sum ($mg \cdot kg^{-1}$) of PUT, CAD, HIS and TYR and if its value is less than 5 $mg \cdot kg^{-1}$ the meat is fresh and has a good quality, between 5 and 20 $mg \cdot kg^{-1}$ the meat product is still acceptable and more than 20 $mg \cdot kg^{-1}$ the meat product has a high degree of spoilage [178].

Two are the main analytical methods for the detection of BAs in food products: UV-vis spectrophotometry and Liquid Chromatography (LC). As BAs lack a chromophore group, most of the studies conducted to detect BAs provide a derivatization step before the analysis, which allows to bind a chromophore group to the amino structure and gain sensitivity for the analysis.

Even if it’s important monitoring the concentration of BAs to keep it as low as possible, just the maximum level of histamine in fish and fish products is regulated by the European Legislation (Commission Regulation EC No 2073/2005 and further amendments).

Further studies are needed to evaluate the possible influence of new packaging materials in reducing BAs content in foods. Sirocchi et al. studied the influence of a new active

packaging, containing 4% *Rosmarinus Officinalis* essential oil, in prolonging meat shelf-life by reducing the concentration of BAs [179]. Other strategies to reduce the concentration of these compounds are the use of additives, hydrostatic pressure, irradiation, pasteurization, smoking, starter culture and temperature [180, 181].

Volatile organic compounds (VOCs) are responsible for odour and taste of food, and they give an indication of the meat quality and freshness [182]. In fact, changes in the organoleptic characteristics in food products, e.g., odours and colour, are usually related to a certain degree of spoilage and by consequence in an unsuitability of the product for the human consumption [183, 184]. Meat spoilage can be the consequence of the bacterial metabolism, which increases during the storage with the increase of bacterial growth and produces undesirable VOCs [185]. In particular, exogenous enzymes of bacterial origin result to be responsible for the degradation of protein and fats, associated with the formation of specific VOCs [186]. Moreover, volatile and non-volatile compounds are formed from the metabolization of sugars and amino acids by *Pseudomonas*, lactic acid bacteria (LAB) and *Enterobacteriaceae*, mainly present in refrigerated meats [187].

Meat spoilage can be also the consequence of some metabolic changes in meat constituents, such as lipid autoxidation (which leads to the formation of aldehydes, hydrocarbons, alcohols and ketones), microbial esterification (which leads to the formation of esters), amino-acids catabolism (which leads to the formation of aldehydes and alcohols such as 2-methylbutanal, 2-methyl-1-butanol) [188-192].

The major VOCs in meat products related to a certain degree of deterioration are odorous sulphur derivatives, ammonia, acetone, methane, and organic acid-based components [193].

As explained above, the degree of spoilage is influenced by many factors such as the environment inside the packaging; for this reason, one of the strategies to reduce the formation of undesiderable VOCs and by consequence meat spoilage is the use of innovative packaging able to prolong the shelf-life of meat. With this regard, the use of modified atmosphere packaging (MAP) results to prolong the shelf-life of meat products by reducing the bacterial proliferation (aerobic pseudomonads mainly) in favour of the growth of gram-positive aerobic bacteria such as LAB. Moreover, as MAP contains 70-80% of oxygen, the red colour of meat is maintained for a longer period of time, giving to the consumer the idea of a good quality product [194].

VOCs can be used not only as markers of meat spoilage degree to predict the suitability of the product for the human consumption [195-198], but also to identify specific microorganisms such as *Enterobacteriaceae*, *Pseudomonas* and LAB in meat [199].

CHAPTER 2

ANALYSIS OF BIOGENIC AMINES AND VOLATILE ORGANIC COMPOUNDS AS INDICATORS OF MEAT SHELF-LIFE.

2.1 **RESEARCH ACTIVITY:** QUANTIFICATION OF BIOGENIC AMINES THROUGH AN HPLC-DAD ANALYSIS TO COMPARE THREE TYPES OF PACKAGING FOR THE PRESERVATION OF RAW AND BAKED HAMS.

Abstract

Food packaging contributes to the preservation and shelf-life of food.

In literature, several studies demonstrate that active packaging, enriched of bioactive compounds like the essential oil of Rosmarinus Officinalis [179], and modified atmosphere packaging (MAP) can positively influence the preservation of food. The study of new types of packaging is continuously increasing, mostly in terms of environmental impact and food preservation.

In this study, three types of packaging provided by Esseoquattro company were tested in order to assess their capability in the preservation of food. *Ideabrill*® packaging, a three layers pack of polyethylene high-density layer, metallic layer and cellulose with long fiber layer, combined with *Ideabrill*® Salvafreschezza bag was compared to paper coupled with wings combined with *Ideabrill*® Salvafreschezza bag and paper coupled with wings alone. The study was conducted on raw and baked hams preserved in the packaging described above through the quantification of biogenic amines (BAs) at day 0, 3, 5 and 7. BAs can be considered markers to evaluate the freshness and the quality of food. In particular, a higher concentration of BAs is related to a higher deterioration degree of food. BAs were extracted, derivatized with dansyl chloride, purified with a SPE C-18 and then analyzed with an HPLC-DAD method. This study demonstrated that *Ideabrill*® packaging combined with *Ideabrill*® Salvafreschezza bag showed the best conservation capability for raw and baked hams when compared to the others. Moreover, from an eco-

friendly point of view, *Ideabrill*® packaging layers can be easily separated in order to encourage recycling.

2.1.1. Materials and methods

2.1.1.1. Chemicals

BAs, named cadaverine dihydrochloride (CAD, $C_5H_{14}N_2 \cdot 2HCl$, >98%), histamine dihydrochloride (HIS, $C_5H_9N_3 \cdot 2HCl$, >99%), 2-phenylethylamine hydrochloride (PHE, $C_8H_{11}N \cdot HCl$, >98%), putrescine dihydrochloride (PUT, $C_4H_{12}N_2 \cdot 2HCl$, >98%,), spermine tetrahydrochloride (SPM, $C_{10}H_{26}N_4 \cdot 4HCl$, >98%), spermidine trihydrochloride (SPD, $C_7H_{17}N_3 \cdot 3HCl$, >98%), tryptamine hydrochloride (TRY, $C_{10}H_{12}N_2 \cdot HCl$, >99%) and tyramine hydrochloride (TYR, $C_8H_{11}NO \cdot HCl$, >98%) for standard solutions preparation were supplied by Sigma-Aldrich (Milano, Italy). 1,7- diaminoheptane (98%) as the internal standard was supplied by Sigma-Aldrich (Milano, Italy). Hydrochloric acid (HCl, 37%), trichloroacetic acid (TCA, ≥99.0%), acetone (≥99,5%), sodium hydroxide anhydrous (NaOH, ≥98%), sodium carbonate anhydrous (Na_2CO_3, ≥99,5%), acetonitrile (CH_3CN, HPLC gradient grade, ≥99.9%), methanol (CH_3OH, HPLC gradient grade, ≥99.9%) and dansyl chloride ($C_{12}H_{12}ClNO_2S$, 98%) for extraction and derivatization were from Sigma-Aldrich (Milano, Italy).

Individual stock solutions of biogenic amines (BAs) were prepared by dissolving 10 mg of each compound in 10 mL of HCl 0,1 M (Merck Darmstadt, Germany) and were then stored in glass-stoppered bottles at 4 °C. Standard working solutions, at various concentrations (1.25, 2.5, 5, 10, 25 and 50 $mg \cdot kg^{-1}$), were prepared daily by appropriate dilution of different aliquots of the stock solutions with deionized water (<8MΩ cm resistivity) obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Derivatization solution was prepared with dansyl chloride in acetone (10%).

2.1.1.2. Packaging and meat samples

In this study, three packaging were compared (**figure 4**): a multilayer packaging named *Ideabrill*® coupled with a multilayer packaging bag named *Ideabrill*® Salvafreschezza (P1), a paper coupled with wings combined to a multilayer packaging bag named *Ideabrill*® Salvafreschezza (P2) and paper coupled with wings (P3).

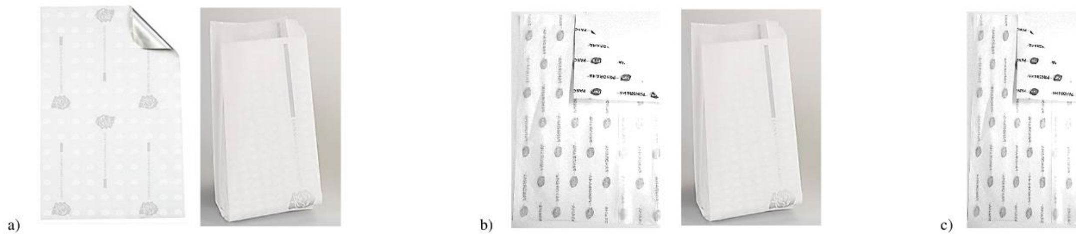


Figure 4: a) *Ideabrill*® coupled with *Ideabrill*® Salvafreschezza bag (P1); b) paper coupled with wings combined to *Ideabrill*® Salvafreschezza bag (P2); paper coupled with wings (P3).

Ideabrill® packaging was furnished by ESSEOQUATTRO s.p.a. (Carmignano di Brenta, Italy). *Ideabrill*® is a European patented packaging (EP 1584464 A1) constituted of 3 layers (**figure 5**): an external layer made of pure cellulose coupled to an internal layer made of high density (HD) polyethylene (PE) with a third aluminum layer fixed between them.

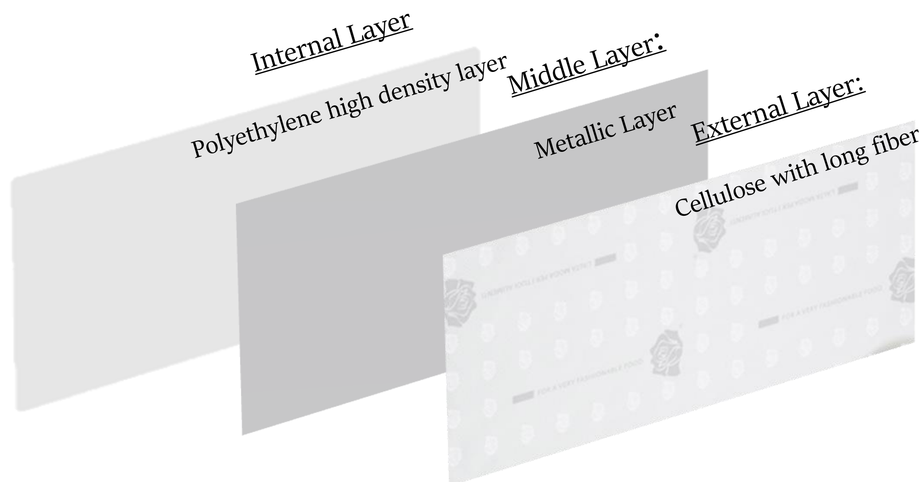


Figure 5: Structure of *Ideabrill*® packaging.

Ideabrill® is suitable for direct contact with food and it is used to preserve cured meats, cheeses, meats and also bakery products. Moreover, *Ideabrill®* packaging has a low environmental impact as its layers can be easily separated and recycled according to separate waste collection.

In this study, raw and baked hams preserved in these three types of packaging have been analyzed. The meat products were bought in a local supermarket and preserved for 7 days at 4 °C. The analyses were performed at days 0, 2, 5 and 7 and experiments were performed in triplicates.

2.1.1.3. Analysis of BAs

The analysis of BAs was performed following a procedure developed previously by Sirocchi et al. [179] slightly modified. Briefly, 5 g of ham were weighted in a centrifuge tube, added with 25 mL of 5% TCA, homogenized by Ultra-Turrax S 18 N-10G homogenizer (IKA-Werke Gmbh & Co., Germany) to favour the extraction process and centrifuged at 5000 rpm for 10 min. Then, 1 mL of supernatant solution was mixed with 200 μL of a 10 $mg \cdot L^{-1}$ 1,7-diaminoheptane solution, used as internal standard, 300 μL of $NaHCO_3$ saturated solution and 50μL NaOH 2N. The dansylation reaction was performed adding 2 mL of dansyl chloride solution (10 $mg \cdot mL^{-1}$ acetone) and conducted in the dark at 45 °C for 45 minutes. Afterwards, the excess of dansyl chloride was removed by adding 100 μL of 28% NH_4OH and evaporated to 1.5 mL under flow of N_2. The purification step was performed using SPE STRATA X 33 μ Cartridges, 200 mg/6 mL (Phenomenex, Bologna, Italy). The cartridges were activated with 5 mL of CH_3CN and conditioned with 5 mL of Milli-Q water. Then, samples were purified through the cartridge and eluted with 4 mL of CH_3CN. The eluate was filtered on a 0.45 μm PTFE filter (Supelco Bellefonte, PA, USA) before the analysis. BAs were analyzed using a 1260 HPLC coupled to a 1260

diode-array detector (DAD) from Agilent Technologies. The separation was performed using a Gemini C18 analytical column (250 × 4.6 mm I.D., particle size 4 μm) from Phenomenex (Torrance, CA, USA). The column was thermostated at 25 °C. The mobile phase for HPLC analysis was MilliQ water (A) and CH_3OH/CH_3CN 70:30 v/v solution (B), at a flow rate of 0.5 $mL \cdot min^{-1}$. The gradient program was: 0 min 60% B, 10 min 70% B, 20 min 90% B, 26 min 100% B, 29 min 100% B and 32 min 60% B until 40 min. The injection volume was 20 μL. High-performance liquid chromatography (HPLC) system was coupled with a Diode Array Detector (DAD). Peak responses were measured at 254 nm.

2.1.1.4. Statistical analysis

Statistical analysis was performed using a three-factor analysis of variance (ANOVA). The statistical package STATISTICA 8.0 software (StatSoft, Tulsa, UK) was used.
Tukey's HSD (Honestly Significantly Different) test was computed for post hoc analysis, in order to identify means that are significantly different from each other at 95% confidence interval.

2.1.2. Results and discussion

Eight BAs were monitored in this study: tryptamine, 2-phenylethilamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine. **Figure 6** showed the chromatogram of BAs mixture at 25 $mg \cdot L^{-1}$ and 1,7-sdiamoheptane, used as internal standard.

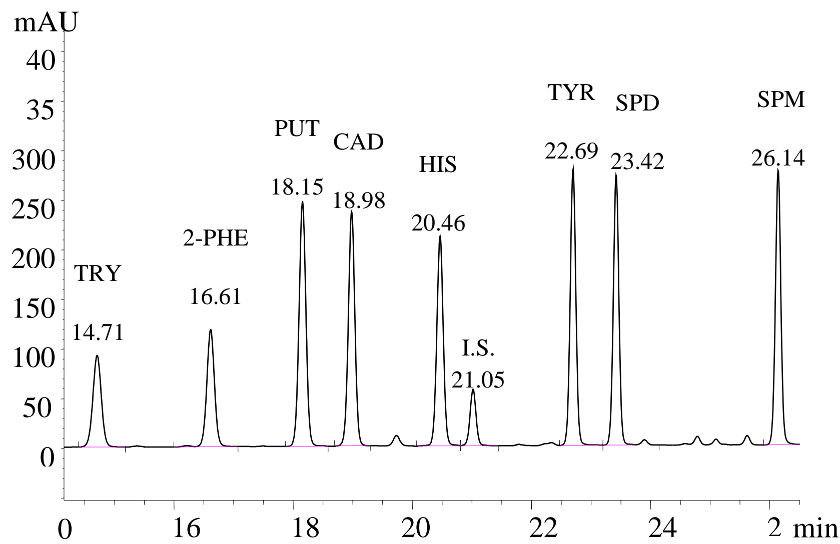


Figure 6: HPLC-DAD chromatogram of a 25 mg L^{-1} standard solution of all analysed BAs and Internal Standard. Tryptamine (TRY), 2-Phenylethylamine (2-PHE), Putrescine (PUT), Cadaverine (CAD), Histamine (HIS), 1,7- Diaminoheptane Internal Standard (I.S.), Tyramine (TYR), Spermidine (SPD), Spermine (SPM).

The method was previously validated and the concentration of each BA was calculated using the calibration curves. The calibration curve of each BA was calculated using the response factor, which is the ratio between the BA peak area and the internal standard peak area. All calibration curves of BAs showed good linearity ($R^2 > 0.9956$).

The performances of three packaging in preserving raw and baked hams have been tested by monitoring the degree of deterioration of meat products over the time through two indices: the total content of BAs (Total BAs) that is the sum of the concentration (mg·kg^{-1}) of the eight BAs quantified and the Biogenic Amine Index (B.A.I.) that is the sum (mg·kg^{-1}) of putrescine, cadaverine, histamine and tyramine. If B.A.I. value is less than 5 mg·kg^{-1} the meat is fresh and has a good quality, between 5 and 20 mg·kg^{-1} the meat product is still acceptable and more than 20 mg·kg^{-1} the meat product has a high degree of spoilage.

Figure 7 reports the shelf-life study of raw and baked ham preserved in P1, P2 and P3 for 7 days taking into account the Total BAs and the B.A.I. Each sample was analyzed in duplicate.

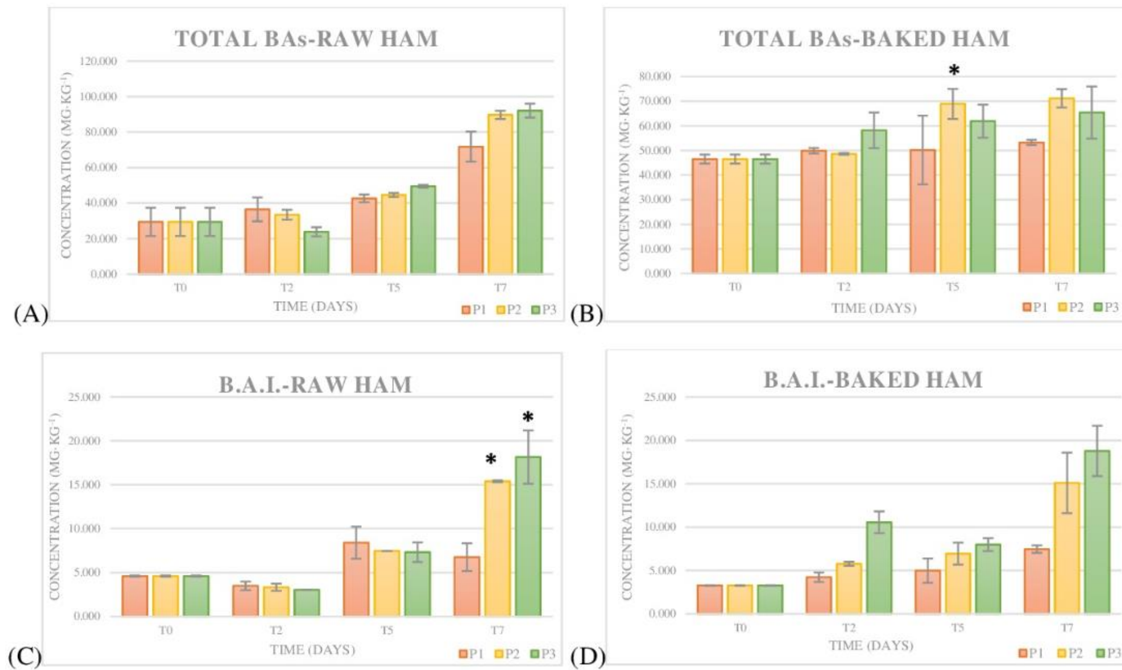


Figure 7: Total BAs in raw (A) and baked (B) hams; B.A.I. in raw (C) and baked (D) hams.
* means statistically significative differences ($p<0.05$).

BAs are considered indicators of meat freshness, as reported in several studies [200-202]. Regarding Total BAs (Figure 7A and 7B), the values were lower or similar in P1 with respect to P2 and P3, with a statistically significative difference in baked ham preserved in P2 at t5.

Concerning B.A.I. index (Figure 7C and 7D), the values were lower or similar in raw and baked hams preserved in P1 with respect to P2 and P3, with statistically significative differences for raw ham in t7. However, none of the samples overcame the 20 $mg \cdot kg^{-1}$ limit, even if raw and baked hams preserved in P2 and P3 have values between 15 and 20 $mg \cdot kg^{-1}$ at t7, while those for hams preserved in P1 were around 5 $mg \cdot kg^{-1}$.

2.1.3. Conclusions

This study demonstrated that *Ideabrill* ® formula results to be useful to prolong the shelf-life of raw and baked hams. In particular, *Ideabrill* ® combined with *Ideabrill*® Salvafreschezza bag gave the best results in terms of preservation of raw and baked hams. In fact, both Total BAs and B.A.I. resulted to be lower in raw and baked hams preserved in P1 with respect to P2 and P3 over the time. Differences in samples are more evident after 5 and 7 days of storage with statistically significative differences.

In conclusion, the three-layers *Ideabrill* ® packaging, especially when combined with with *Ideabrill*® Salvafreschezza bag, results to slow the deterioration of raw and baked hams with a consequent extension of shelf-life.

2.2. RESEARCH ACTIVITY: A NEW HS-SPME-GC-MS ANALYTICAL METHOD TO IDENTIFY AND QUANTIFY SHELF-LIFE MARKERS FOR FIVE TYPES OF MEAT PRODUCTS.

Abstract

Nowadays, it is important to monitor the freshness of meat during storage to protect consumers' health. Volatile organic compounds (VOCs) are responsible for odour and taste of food, and they give an indication of the meat quality and freshness, as they are the products of bacterial metabolism or oxidation processes which increase over the time. This study had the aim to seek and select hypothetical new markers of meat spoilage through a semi-quantitative analysis in different types of meat (beef, raw ham, baked ham, pork sausage and chicken) and then to the develop a new quantitative analytical method that is able to detect and quantify hypothetical markers on five types of meat simultaneously.

Firstly, a new headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) method was developed to evaluate the volatile profile of five types of meat, preserved at 4 °C for 5 days. The analyses were performed at day 0, 2 and 5 and, among the 40 compounds identified, 15 were chosen and selected as potential markers of shelf-life: hexanal, 1-butanol, 3-methylbutanol, 2-pentylfuran, 1-pentanol, acetoin, octanal, 1-hexanol, 2-nonanone, nonanal, 1-octen-3-ol, 1-heptanol, linalool, 1-octanol and 2-octen-1-ol on the basis of their presence in most of meat samples or/and for their constant increasing/decreasing concentration within the samples.

Afterwards, a new quantitative HS-SPME-GC-MS analytical method was developed to identify and quantify VOCs in meat samples and to confirm which VOCs can be

considered markers of shelf-life for these meat products. The analyses were conducted on five types of meat stored at 4 °C for 12 days (day 0, 3, 6, 9 and 12). Results confirmed that these compounds can be considered markers of shelf-life for some of the meat products analyzed. Some of them attracted attention as they can be considered markers of shelf-life for at least 4 types of meat: 1-butanol for beef, baked ham, pork sausage and chicken; 3-methylbutanol for all types of meat; 1-hexanol for beef, raw ham, pork sausage and chicken; 2-nonanone for beef, raw ham, baked ham and chicken; nonanal for beef, raw and baked hams and chicken; 1-octen-3-ol for beef, baked ham, pork sausage and chicken; linalool for beef, raw ham, pork sausage and chicken.

In conclusion, in this study a new quantitative HS-SPME-GC-MS analytical method to quantity 15 VOCs in five types of meat was developed and it was demonstrated that some of the compounds quantified can be considered markers of shelf-life for some of the meat products analyzed.

2.2.1. Materials and methods

2.2.1.1. Chemicals

Sodium sulphate (Na_2SO_4) and sodium hydrogen carbonate ($NaHCO_3$) were purchased by J. T. Baker. Sodium chloride (NaCl) was purchased by chem-lab. Sodium phosphate monobasic (Na_2HPO_4), the alkane mixture (C_7-C_{30}) and pure standards of hexanal (CAS 66-25-1), 1-butanol (CAS 71-36-3), 3-methylbutanol (CAS 123-51-3), 2-pentylfuran (CAS 3777-69-3), 1-pentanol (CAS 71-41-0), acetoin (CAS 513-86-0), octanal (CAS 124-13-0), 1-hexanol (CAS 111-27-3), 2-nonanone (CAS 821-55-6), nonanal (CAS 124-19-6), 1-octen-3-ol (CAS 3391-84-4), 1-heptanol (CAS 111-70-6), linalool (CAS 78-76-6), 1-octanol (CAS 111-87-5), 2-octen-1-ol (CAS 18409-17-1) and 2-methypentanal, used as internal standard (I.S.), (CAS 123-15-9) were purchased by Sigma Aldrich (Milano, Italy).

2.2.1.2. Meat samples

Meat samples were bought in a local supermarket, stored in the packaging furnished by the store at 4 °C. Five types of meat were chosen: beef, raw ham, baked ham, pork sausage, and chicken. The day of the analysis 3 g of sample were cut, triturated and weighted in the vial and immediately analyzed. The analyses were performed at days 0, 2 and 5 for the semi-quantitative analysis and at days 0, 2, 6, 9 and 12 for the quantitative analysis.

2.2.1.3. Semi-quantitative analysis through HS-SPME-GC-MS

2.2.1.3.1. HS-SPME extraction conditions

The extraction was performed using a HS-SPME method and the analysis was conducted on a GC-MS instrument. Briefly, 3 g of sample were put in a 20 mL vial, added with 5 mL of NaCl saturated solution and it was tightly clapped with a PTFE/silicon septum.

HS-SPME conditions were optimized: quantity of sample, salt addiction, incubation time, extraction time and temperature, type of fiber. 1 and 3 g of samples were weighted in the vial to choose the optimum quantity for the analysis. After that, the addiction of 5 mL of salt (i.e., Na_2SO_4, NaCl, Na_2HPO_4 and $NaHCO_3$) saturated solution were evaluated to determine the optimum starting conditions for the analysis. Then, samples were incubated at three different temperatures (40 °C, 50 °C and 60 °C) and at three different times (20, 30 and 40 min). Samples were stirred during incubation at 250 rpm with 5 s of on-time and 2 s of off-time. Afterwards, samples were extracted and three different extraction times were evaluated (20, 30 and 40 min). Two different fiber coatings were compared: a Divinylbenzene/Polydimethylsiloxane DVB-PDMS (65 µm) and a Divinylbenzene/Carbon-Wide Range/Polydimethylsiloxane DVB/C-WR/PDMS (80 µm) fibers from Supelco (Bellefonte, PA, USA). The fibers were conditioned for 10 min at 250 °C and then inserted inside the headspace of sample vial with a speed of 20 $mm \cdot s^{-1}$ and a penetration depth of 35 mm. The extraction was performed and then the fiber was inserted into the injector port at a speed of 100 $mm \cdot s^{-1}$ and a penetration depth of 40 mm. The desorption occurred at 250 °C for 2 min. After desorption, the fiber was conditioned at 250 °C for 5 min.

2.2.1.3.2. GC-MS analysis

GC-MS system was composed of an Agilent 8890 GC coupled to an Agilent 5977B MSD quadrupole detector with an electron ionization (EI) source (Santa Clara, CA, USA). The system was equipped with an autosampler PAL RTC 120 System. The injector temperature was set at 250 °C, and the liner used was recommended for SPME injection, namely, Inlet liner, Ultra Inert, splitless, straight, 0.75 mm id, from Agilent. The gas carrier was helium at flow rate of 1 $mL \cdot min^{-1}$. A DB-WAX UI capillary column (60 m x

0.25 mm x 0.25 μm) was used. Thermal desorption was carried out at 250 °C in a splitless mode for 2 min. Oven temperature was set at 35 °C held for 3 minutes, increased up to 70 °C at 3 °C/min, increased up to 210 °C at 5 °C/min, increased up to 250°C at 15°C/min held for 10 min. The temperatures of the ionization source and the mass analyzer were set at 230 and 150 °C, respectively. The acquisition was carried out in SCAN mode (35–450 m/z).

Volatile compounds were identified through the comparison of their mass spectra with those of NIST library (US National Institute of Standards and Technology) in combination with the calculation of their experimental linear retention indexes, which have been compared to those reported in literature. Compounds abundances were determined using the relative percentage of the area (%) of each peak, that was calculated by dividing the area of each component by the total area of all separated components. Data results were managed using MSD ChemStation Software (Agilent, Version G1701DA D.01.00, Santa Clara, CA, USA).

2.2.1.4. Quantitative analysis through HS-SPME-GC-MS

2.2.1.4.1. Sample preparation and HS-SPME extraction conditions

The extraction was performed using a HS-SPME method and the analysis was conducted on a GC-MS instrument. Briefly, 5 g of samples were put in a 50 mL centrifuge tube and 15 mL of distilled water was added. Then, samples were homogenized with an Ultra Turrax S 18 N-10G homogenizer (IKA-Werke Gmbh & Co., Germany). After that, 1500 μL of water added with 25% of NaCl and 40 μL of I.S. 50 $mg \cdot mL^{-1}$ were added to 500 μL of supernatant in a 20 mL vial which was tightly clapped with a PTFE/silicon septum. The incubation of the sample was performed at 40 °C for 40 min under agitation (250 rpm, 5 s of on-time and 2 s of off-time). The grey fiber (DVB/C-WR/PDMS) from

Supelco (Bellefonte, PA, USA) was selected for this work. The fiber was conditioned for 10 min at 250 °C and then, it was inserted inside the headspace of sample vial with a speed of 20 mm·s^{-1} and a penetration depth of 40 mm for 20 min. The desorption occurred at 250 °C for 1 min. After desorption, the fibre was conditioning at 250 °C for 10 min.

2.2.1.4.2. GC-MS analysis

The GC-MS was the same used for the semi-quantitative analysis. The separation of target molecules was established on DB-WAX capillary column (60 m, 250 µm i.d., 0.25 µm film thickness) with the following ramp of temperature: 35˚C at 3°C/min, increased up to 70˚C at 3˚C/min, increased up to 210˚C at 5˚C/min, increased up to 250˚C at 15˚C/min, maintained for 10 min. The run time was about 44.3 min. The transfer line was set at 250 °C and the temperature of the ionization source and the mass analyser were set at 230 and 150 °C, respectively. The ion species selection and optimization for all the volatile compounds were carried out by injecting a standard solution (10 µg·mL^{-1}) in SCAN mode (35-450 m/z) and the three most abundant ions were selected for each analyte. The acquisitions were carried out in 'Selected Ion Monitoring' (SIM) mode and detection was divided into time windows to enhance the sensitivity. The most abundant ions were used for quantitation, while the others to confirm the presence of the analytes. The GC-MS parameters including the retention time (Rt) and time windows are reported in ***Table 5***. Data results were managed by MSD ChemStation Software (Agilent, Version G1701DA D.01.00). Samples were analysed in triplicate. Acceptable relative standard deviation (% RSD) were set up below 20%.

Table 5. GC-MS parameters used for the analysis.

Compounds	Time window (min)	Ions (m/z)	Retention time (min)
2-methypentanal	0-9	58[a], 71, 100	7.10
Hexanal	0-9	56[a], 100, 57	8.48
1-butanol	9-11	43, 56[a], 74	10.06
3-methylbutanol	11-12.5	55[a], 70, 87	12.18
2-pentylfuran	12.5-14.5	81[a], 82, 138	13.09
1-pentanol	12.5-14.5	42[a], 55, 70	13.97
Acetoin	14.5-17	45[a], 43, 88	15.79
Octanal	14.5-17	84[a], 57, 128	15.94
1-hexanol	17-20.5	56[a], 69, 101	19.67
2-nonanone	20.5-24.5	58[a], 71, 142	22.08
Nonanal	20.5-24.5	57[a], 98, 141	22.35
1-octen-3-ol	24.5-30	57[a], 72, 127	26.87
1-heptanol	24.5-30	70[a], 55, 115	27.38
Linalool	30-38	71[a], 93, 154	35.44
1-octanol	30-38	56[a], 69, 129	36.53
2-octen-1-ol	38-66	57[a], 81, 128	41.48

[a] These ions were used for quantitation, the other without any superscript letters for qualification.

2.2.1.5. Statistical analysis

The results were reported as the mean value ± standard deviation. Significant differences between data were calculated by unidirectional analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test, with a significance level of $p < 0.05$. The statistical package STATISTICA 8.0 software (StatSoft, Tulsa, UK) was used.

2.2.2. Results and discussion

2.2.2.1. HS-SPME-GC-MS semi-quantitative analytical method optimization

HS-SPME technique is one of the most common analytical approaches for the VOCs analysis because it is simple, cheap, solvent-free, easy to handle and very sensitive.

Moreover, this technique best represents what happens inside the packaging due to the use of low extraction temperatures and the lack of preparation steps.

Chicken samples were used to optimize parameters before the analysis. 1 g and 3 g of sample were weighted into a 20 mL vial and analyzed to evaluate the best results. 1 g of chicken resulted to give less than 50% of peaks when compared to 3 g. Moreover, the total area also resulted to be higher for 3 g with respect to 1 g. Amount higher than 3 g led to a fibre saturation with important loss of time for its cleaning. Statistical analysis performed through the ANOVA system revealed that values of total peak area were statistically significative. Then, 5 mL of satured solutions of sodium sulfate (Na_2SO_4), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4) and sodium bicarbonate ($NaHCO_3$) were added to 3 g of chicken. The addiction of sodium sulphate gave the highest total peak area, but sodium chloride was chosen as the best salt to be addicted because this resulted to give the highest number of peaks, even if with no statistically significative differences. Two fibers were tested: a divinylbenzene-polydimethylsiloxane fiber (DVB/PDMS) and a divinylbenzene/carbon-wide range/polydimethylsiloxane fiber (DVB/C-WR/PDMS). Results showed there were no statistically significative differences between the two fibers, anyway the DVB/C-WR/PDMS fiber was chosen because of its triple coating which generally makes it a broad spectrum one. The temperature was also optimized and chosen among 60 °C, 50 °C and 40 °C. The highest value of peak number was reached using a temperature of 40 °C with the highest corresponding total peak area value, which is statistically different when compared to the others. Three incubation times were evaluated: 20, 30 and 40 minutes. The highest values of number of peaks and total peak area were reached with an incubation time of 40 minutes with statistically significative differences. Finally, the

extraction time was optimized, testing samples with 20, 30 and 40 minutes of extraction. The highest values of number of peaks and total peak area were reached with an extraction time of 20 minutes suggesting the equilibrium realization. ***Table 6*** summarizes all the parameters monitored and optimized.

These results indicated that 3 g of meat sample, added with 5 mL of NaCl saturated solution, incubated for 40 min at 40 °C and extracted for 20 min with a DVB/C-WR/PDMS fiber were adequate conditions for this type of analysis.

Table 6. Summary of monitored parameters for the optimization of HS-SPME technique with values of number of peaks and total peak areas.

Parameters		Number of peaks	Total peak area
Quantity	1 g	65	4404715[a]*
	3 g	134	6444721[b]
Salt addiction	Na_2SO_4	81	4650747[a]
	NaCl	109	2992152[a]
	Na_2HPO_4	84	2466213[a]
	$NaHCO_3$	50	11768923[a]
Fiber	DVB/C-WR/PDMS	109	2992152[a]
	DVB/PDMS	111	3681447[a]
Temperature	60 °C	119	4292656[a]
	50 °C	137	5436620[b]
	40 °C	169	6004650[c]
Incubation time	20 min	162	10252836[a]
	30 min	50	4678962[b]
	40 min	176	12628200[c]
Extraction time	20 min	177	12528137[a]
	30 min	49	2377225[b]
	40 min	71	2661980[c]

* Different letters indicate significant differences at $P \leq 0.05$.

2.2.2.2. Semi-quantitative analysis of VOCs in five types of meat through HS-SPME-GC-MS

Firstly, a semi-quantitative analysis was performed to select the main compounds that can be considered potential markers of shelf-life and to subsequently include in the quantitative analytical method.

The analysis was performed on five types of meat at day 0, 2 and 5. ***Table 7*** summarizes the abundance of VOCs in meat samples.

Table 7: Peak areas of VOCs in five types of meat analyzed on t0, t2 and t5.

n.d. means "not detected".

Class of compounds	No	***Beef***			***Raw Ham***			***Baked Ham***			***Sausage***			***Chicken***		
		Peak Area			**Peak Area**			**Peak Area**			**Peak Area**			**Peak Area**		
		T0	**T2**	**T5**	**T0**	**T2**	**T5**	**T0**	**T2**	**T5**	**T0**	**T2**	**T5**	**T0**	**T2**	**T5**
Esters	**1**	n.d.	1.1*10^4 ±11.47	6.4*10^ 3 ±8.41	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.1*10^4± 11.47	6.4*10^3 ±8.41	n.d.	n.d.	n.d.
	2	n.d.	n.d.	7.1*10^ 3 ±9.45	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.1*10^3 ±9.45	n.d.	n.d.	n.d.
	3	n.d.	n.d.	n.d.	1.1*10^ 4±1.87	1.3*10^4± 2.56	2.6*10^ 4±3.84	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.9*10^ 4±15.95	1.3*10^4± 12.41	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	n.d.	n.d.	n.d.	3.4*10^ 4±39.84	4.2*10^4± 41.29	3.3*10^ 4 ±37.51	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hydrocarbons	**6**	3.9*10^4 ±557.14	3.5*10^4 ±512.84	3.6*10^ 4±520.1 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.9*10^4 ±557.14	3.5*10^4 ±512.84	3.6*10^4± 520.10	n.d.	n.d.	n.d.
	7	8.1*10^4 ±619.35	7.4*10^4 ±594.76	7.7*10^ 4±557.7 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.1*10^4 ±619.35	7.4*10^4± 594.76	7.7*10^4± 557.79	n.d.	n.d.	n.d.
	8	2.6*10^4 ±284.44	2.2*10^4 ±266.11	1.8*10^ 4±216.5 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.6*10^4 ±284.44	2.2*10^4± 266.11	1.8*10^4± 216.55	n.d.	n.d.	n.d.
	9	1.2*10^5 ±34.74	9.9*10^4 ±12.55	8.9*10^ 4 ±28.79	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2*10^5 ±34.74	9.9*10^4± 12.55	8.9*10^4 ±28.79	n.d.	n.d.	n.d.

	10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.4*10^4±124.61	4.0*10^4±138.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	11	6.8*10^5±446.21	6.6*10^5±428.11	6.9*10^5±657.52	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.8*10^5±446.21	6.6*10^5±428.11	6.9*10^5±657.52	n.d.	n.d.	n.d.
	12	3.6*10^4±351.54	3.4*10^4±317.24	3.5*10^4±345.31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.6*10^4±351.54	3.4*10^4±317.24	3.5*10^4±345.31	n.d.	n.d.	n.d.
Alcohols	**13**	4.7*10^4±194.75	5.5*10^5±395.14	5.1*10^6±373.52	1.1*10^5±325.48	1.3*10^5±360.12	5.3*10^6±451.95	4.5*10^4±125.75	1.9*10^5±341.84	4.9*10^6±516.95	4.7*10^4±194.75	5.5*10^5±395.14	5.1*10^6±373.52	6.3*10^4±254.61	1.2*10^5±349.86	5.3*10^6±579.16
	14	n.d.	n.d.	1.5*10^4±111.74	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5*10^4±111.74	n.d.	n.d.	n.d.
	15	n.d.	n.d.	n.d.	n.d.	1.1*10^4±8.73	3.0*10^4±18.73	n.d.	3.5*10^4±15.94	1.7*10^4±13.46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	16	6.7*10^3±11.79	3.5*10^4±35.45	8.1*10^4±67.11	n.d.	n.d.	n.d.	n.d.	1.1*10^4±9.68	3.6*10^5±21.75	6.7*10^3±11.79	3.5*10^4±35.45	8.1*10^4±67.11	1.3*10^4±9.68	4.3*10^4±12.57	1.8*10^5±12.62
	17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.8*10^4±39.52	1.4*10^4±32.85	1.2*10^4±31.43
	18	1.6*10^4±134.11	4.2*10^4±193.44	2.9*10^4±169.84	6.8*10^3±178.86	6.8*10^3±170.50	7.0*10^3±192.30	n.d.	1.7*10^4±124.97	7.9*10^3±113.51	1.6*10^4±134.11	4.2*10^4±193.44	2.9*10^4±169.84	3.4*10^4±174.85	7.2*10^4±310.49	1.2*10^5±432.41
	19	2.8*10^5±323.88	2.8*10^5±365.40	1.5*10^5±299.75	n.d.	n.d.	n.d.	4.6*10^4±57.84	6.9*10^4±68.71	3.7*10^4±51.12	2.8*10^5±323.88	2.8*10^5±365.40	1.5*10^5±299.75	3.6*10^4±45.84	3.5*10^4±43.91	3.6*10^4±47.12
	20	n.d.	n.d.	8.1*10^3±24.16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.1*10^3±24.16	n.d.	n.d.	1.3*10^4±21.31

	21	n.d.	n.d.	n.d.	1.6*10^4 ±762.35	2.0*10^4± 793.80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7*10^5± 758.48	1.5*10^5 ±715.14	1.2*10^5± 729.46
	22	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1*10^4 ±23.75	2.0*10^4± 21.68
	23	3.6*10^4 ±147.81	2.6*10^4 ±135.75	2.3*10^4±131.55	n.d.	n.d.	n.d.	2.4*10^4±27.88	3.3*10^4±32.75	2.5*10^4± 29.51	3.6*10^4 ±147.81	2.6*10^4± 135.75	2.3*10^4± 131.55	n.d.	n.d.	n.d.
	24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.1*10^4± 29.52	3.1*10^4± 36.75
	25	2.4*10^4 ±77.54	1.8*10^4 ±75.11	1.4*10^4±65.95	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4*10^4 ±77.54	1.8*10^4 ±75.11	1.4*10^4± 65.95	n.d.	n.d.	n.d.
	26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.2*10^4± 39.08	3.3*10^4± 41.64	1.0*10^4± 21.67
	27	1.2*10^5 ±84.56	9.8*10^4 ±75.65	7.1*10^4±71.99	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.2*10^5 ±84.56	9.8*10^4± 75.65	7.1*10^4± 71.99	n.d.	n.d.	n.d.
Aldehydes	**28**	n.d.	n.d.	n.d.	n.d.	2.0*10^4± 127.59	2.3*10^4±138.75	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	29	2.5*10^4 ± 284.11	2.3*10^4 ± 271.65	n.d.	2.2*10^4± 397.50	6.3*10^4± 425.20	2.2*10^4 ± 390.30	n.d.	n.d.	n.d.	2.5*10^4 ± 284.11	2.3*10^4± 271.65	n.d.	1.8*10^5± 364.50	8.3*10^4± 284.51	n.d.
	30	n.d.	n.d.	n.d.	8.5*10^3 ±52.86	1.5*10^4± 71.20	9.3*10^3 ±61.24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	31	2.3*10^4 ±469.54	1.6*10^4 ±448.45	n.d.	3.4*10^4 ±515.27	4.6*10^4 ±575.90	3.8*10^4±528.84	5.8*10^4±486.14	4.3*10^4±437.95	n.d.	2.3*10^4 ±469.54	1.6*10^4± 448.45	n.d.	7.4*10^4 ±538.46	4.2*10^4± 476.64	n.d.

	32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7*10^4±127.11	3.3*10^4±139.14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	33	n.d.	n.d.	n.d.	2.1*10^4±95.47	2.0*10^4±93.75	1.7*10^4±85.49	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ketones	**34**	1.6*10^4±133.61	1.4*10^5±125.84	5.0*10^4±176.55	n.d.	n.d.	n.d.	n.d.	5.2*10^4±154.52	5.1*10^5±148.69	1.6*10^4±133.61	1.4*10^5±125.84	5.0*10^4±176.55	4.5*10^4±187.22	2.8*10^5±296.47	4.4*10^5±379.51
	35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5*10^4±56.15	1.2*10^4±62.75	2.8*10^4±84.91
	36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.3*10^4±68.81
	37	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5*10^5±12.75	1.6*10^5±15.41	1.2*10^5±31.75	n.d.	n.d.	n.d.	n.d.	n.d.	1.0*10^4±10.76
Ethers	**38**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.1*10^5±7.12	1.1*10^5±5.92	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Organic acids	**39**	1.1*10^5±50.11	9.5*10^4±43.84	1.6*10^5±65.49	n.d.	n.d.	n.d.	3.2*10^4±54.69	3.0*10^4±52.15	7.1*10^4±75.42	1.1*10^5±50.11	9.5*10^4±43.84	1.6*10^5±65.49	n.d.	1.8*10^4±61.24	8.0*10^4±70.49
Furans	**40**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.9*10^4±79.40	2.2*10^4±82.75	6.4*10^3±61.42
Total identified (%)		93.33	70.24	92.46	70.93	90.48	96.12	84.98	86.17	93.16	75.4	72.14	94.29	90.11	83.07	96.18

1. Ethyl acetate (R.I. lit.: 884; R.I. calc.: 883); **2.** Ethyl hexanoate (R.I. lit.: 1239; R.I. calc.: 1231); **3.** Ethyl octanoate (R.I. lit.: 1429; R.I. calc.: 1429); **4.** Isorbornyl acetate (R.I. lit.: 1582; R.I. calc.: 1588); **5.** Ethyl decanoate (R.I. lit.: 1637; R.I. calc.: 1638); **6.** β-pinene (R.I. lit.: 1108; R.I. calc.: 1106); **7.** 3-carene (R.I. lit.: 1146; R.I. calc.: 1148); **8.** α-phellandrene (R.I. lit.: 1177; R.I. calc.: 1164); **9.** Limonene (R.I. lit.: 1198; R.I. calc.: 1195); **10.** Dodecane (R.I. lit.: 1200; R.I. calc.: 1197); **11.** Caryophyllene (R.I. lit.: 1610; R.I. calc.: 1610); **12.** Humulene (R.I. lit.: 1682; R.I. calc.: 1686); **13.** Ethanol (R.I. lit.: 933; R.I. calc.: 933); **14.** 2-methylpropanol (R.I. lit.: 1110; R.I. calc.: 1100); **15.** 1-butanol (R.I. lit.: 1150; R.I. calc.: 1149); **16.** 3-methylbutanol (R.I. lit.: 1208; R.I. calc.: 1207); **17.** 1-pentanol (R.I. lit.: 1248; R.I. calc.: 1248); **18.** 1-hexanol (R.I. lit.: 1344; R.I. calc.: 1345); **19.** 2-butoxyethanol (R.I. lit.: 1402; R.I. calc.: 1397); **20.** 1-heptanol (R.I. lit.: 1440; R.I. calc.: 1447); **21.** 1-octen-3-ol (R.I. lit.: 1446; R.I. calc.: 1441); **22.** 2-ethyl-1-hexanol (R.I. lit.: 1484; R.I. calc.: 1482); **23.** Linalool (R.I. lit.: 1540; R.I. calc.: 1540); **24.** 1-octanol (R.I. lit.: 1555; R.I. calc.: 1552); **25.** Terpinen-4-ol (R.I. lit.: 1601; R.I. calc.: 1606); **26.** 2-octen-1-ol (R.I. lit.: 1612; R.I. calc.: 1612); **27.** α-terpineol (R.I. lit.: 1698; R.I. calc.: 1701); **28.** 3-methylbutanal (R.I. lit.: 912; R.I. calc.: 913); **29.** Hexanal (R.I. lit.: 1083; R.I. calc.: 1083); **30.** Octanal (R.I. lit.: 1287; R.I. calc.: 1283); **31.** Nonanal (R.I. lit.: 1389; R.I. calc.: 1387); **32.** Decanal (R.I. lit.: 1496; R.I. calc.: 1495); **33.** Benzaldehyde (R.I. lit.: 1528; R.I. calc.: 1523); **34.** Acetoin (R.I. lit.: 1277; R.I. calc.: 1278); **35.** 3-octanone (R.I. lit.: 1252; R.I. calc.: 1252); **36.** 2-nonanone (R.I. lit.: 1386; R.I. calc.: 1383); **37.** Camphor (R.I. lit.: 1526; R.I. calc.: 1526); **38.** Eucalyptol (R.I. lit.: 1209; R.I. calc.: 1209); **39.** Acetic Acid (R.I. lit.: 1435; R.I. calc.: 1437); **40.** 2-pentylfuran (R.I. lit.: 1228; R.I. calc.: 1228).

In beef 23 compounds were identified, among which there are esters, hydrocarbons, alcohols, ketones, aldehydes, furans, ethers and organic acids. Ethanol, 1-butanol, 3-methylbutanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, 1-octanol, 2-octen-1-ol and phenylethyl alcohol were the alcohols identified in beef. Peak areas of ethanol, 3-methylbutanol and phenylethyl alcohol increased from day 0 to day 5 and this was in agreement with their production. In fact, alcohols are the products of lipid oxidation [203], which is an increasing process with the degradation. In particular, linear alcohols are the products of the oxidative decomposition of fatty acids [71, 204]. The branched alcohols, instead, can be the product of the reduction of branched aldehydes, of the catabolism of aminoacids by Streker degradation [205, 206] or of the microbial activity that leads to an increased formation of branched aldehydes [204]. For what concern the compounds identified, 1-hexanol comes from the reduction of hexanal by enzymatic reaction [207] and 1-octen-3-ol is the product of the autoxidation of polyunsaturated fatty acids, like linoleic acid [203]. The aroma given by the presence of alcohols is due to their low odour threshold values [208] and it is mainly characterized by herbaceous, woody and fatty notes [208, 209] and sweet, fruity or onion and mushroom like odours [210]. In particular, 1-octen-3-ol gives mushroom aroma [211-213] and earth, dust, fatty, sharp and rancid odours [214, 215]. Moreover, the peak area of 1-hexanol had an overall increase, even if its increase was not constant. Among the ketones, 3-methyl-2-butanone, acetoin and 2-nonanone have been identified. The peak areas of acetoin and 2-nonanone increased from day 0 to day 5. This behaviour was in agreement with their production which involved microorganism or oxidation processes [210, 213, 216]. Among the aldehydes, hexanal, heptanal, octanal, nonanal have been identified and their peak areas decreased from day 0 to day 5. Aldehydes, in general, are the products of lipid oxidation or amino

acid degradation and this means that their concentration should increase with the degradation of food. Aldehydes can be the result of lipid oxidation of fatty acids (linear aldehydes); in particular octanal and nonanal come from oleic acid autoxidation [207], hexanal comes from linoleic, linolenic and arachidonic fatty acids oxidation [207, 212, 217]. Branched aldehydes, instead, are the products of proteolysis and amminoacids degradation [204, 210, 217] or of the metabolism of microorganisms, like Staphylococci [218, 219]. In particular, 3-methylbutanal comes from the oxidative-deamination of leucine [204, 211, 216], while benzaldehyde is the product of Strecker degradation of some amino acids as leucine or phenylalanine [212]. Their presence is related to sweet, floral, grassy and fruity aroma for linear aldehydes [220] and to fruity, acorn-like, salty and cheesy aroma for branched aldehydes [216, 221]. In particular, hexanal gives unpleasant rancid aroma at high concentration and pleasant grassy aroma at low concentration [213, 217, 222], octanal gives meat-like, green, fresh, grass and fruity aroma [214, 223], nonanal gives sweet and fruity aroma [224], benzaldehyde gives floral, acorn and bitter almond notes [214, 225] and 3-methylbutanal gives fruity, acorn-like, salty and cheesy aroma [216, 221].

Acetic acid, whose peak area increased from day 0 to day 5, is an organic acid, which contribute to give a vinegar note to the meat product and it contributes to the ripened aroma [226]. Its production could be related to the Maillard reaction probably during meat processing [188, 219].

In raw ham a total of 11 compounds were identified: alcohols, aldehydes and esters. Ethanol, 1-butanol, 1-hexanol and 1-octen-3-ol are the alcohols identified in raw ham. Their peak areas increased from day 0 to day 5, as they are products of lipid oxidation, as described above, suggesting their possible involvement as markers of raw ham shelf-life.

Among the aldehydes, 3-methylbutanal, hexanal, octanal, nonanal and benzaldehyde have been identified in raw ham. 3-Methylbutanal peak area increased from day 0 to day 5, that of octanal increased from day 0 to day 2, then it decreased from day 2 to day 5, while peak areas of hexanal, nonanal and benzaldehyde decreased from day 0 to day 5. Aldehydes, in general, are the products of lipid oxidation or amino acid degradation and this means that their concentration should increase with the degradation of food. In this case we found a different behaviour among the aldehydes and this is probably due to the oxidation of some aldehydes to carboxylic acids, of which only those with short-chain can be detected with a HS-SPME-GC-MS method [227]. Ethyl octanoate and ethyl decanoate are the esters identified in raw ham and their peak areas increased from day 0 to day 5, suggesting their possible involvement as food shelf-life markers. This behaviour is in agreement with their production. In fact, esters are mainly produced by the esterification of carboxylic acids and alcohols [213], which can be promoted by the action of some microorganisms such as lactic acid bacteria or Micrococcaceae thanks to their esterase activity [204, 228]. The aroma given by esters formed by short-chain acids is characterized by fruity notes, while those formed by long-chain acids is characterized by fatty odour [213, 229]. Moreover, ethyl esters give to fermented sausages the proper flavour and mask rancid odours [188, 230].

In baked ham 15 compounds were identified: hydrocarbons, alcohols, ethers and ketones. For what concern alcohols, ethanol, 1-butanol, 3-methylbutanol, 1-hexanol, 2-butoxyethanol and linalool were identified. The peak areas of ethanol, 1-butanol, 3-methylbutanol and linalool increased from day 0 to day 5 and this is in agreement with their production, as described above. Decanal and nonanal are the two aldehydes identified and they had an opposite behaviour. In fact, the peak area of decanal increased

from day 0 to day 2, then it decreased, while that of nonanal decreased from day 0 to day 5. This had probably the same explanation given above. Acetic acid is the main organic acid found in cooked ham and this was in agreement with other works that showed the presence of this VOC in meat samples [207]. It contributes to the ripened aroma with its vinegar notes [226]. Acetoin is a ketone and its peak area increased from day 0 to day 5 suggesting its possible involvement as food shelf-life marker. In fact, its production could be related to the Maillard reaction [231] or to the microbial carbohydrate metabolism [210, 213, 216]. Acetoin gives a characteristic buttery and sweet odour [232]. Camphor is a cyclic ketone and its peak area increased from day 0 to day 5. Its use in food is mainly as a flavouring agent. Eucalyptol levels decreased from day 0 to day 5. It could be derived from spices used in the preparation of the product and its decrease could be associated in a reduction of flavouring characteristics of the product with the time.

In pork sausages 23 compounds were identified, among which there were esters, hydrocarbons, alcohols, nitriles, aldehydes, ketones, ethers and organic acids. 3-Carene, caryophyllene, humulene peak areas increased from day 0 to day 5, while β-pinene peak area decreased from day 0 to day 5. They were all involved in the flavour characteristics of the product. Ethanol, 2-methylpropanol, 3-methylbutanol, 1-hexanol, 1-heptanol, linalool, terpinen-4-ol and α-terpineol were the alcohols identified in pork sausages. The peak areas of ethanol, 3-methylbutanol, 1-hexanol and 1-heptanol increased from day 0 to day 5 and this was in agreement with their production, as described above. Among the aldehydes, hexanal and nonanal have been identified and whose peak areas decreased from day 0 to day 5. This behaviour has been explained before and involved the oxidation of aldehydes to carboxylic acids. Acetoin was the main ketone identified in this meat product and its peak area increased from day 0 to day 5. This behaviour has been

explained before and involved microorganism activity. Acetic acid, whose peak area increased from day 0 to day 5, is an organic acid, whose presence and production have been described above.

In chicken 19 compounds were identified, among which there were hydrocarbons, alcohols, aldehydes, furans, ketones, ethers and organic acids. Ethanol, 3-methylbutanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, 1-heptanol, 1-octanol, 2-octen-1-ol and 1-hexanol-2-ethyl were the alcohols identified in chicken. Their peak areas increase from day 0 to day 5 and this was in agreement with their production, as described above. Hexanal and nonanal were the aldehydes identified in chicken, whose peak areas decreased from day 0 to day 5 and this behaviour has been explained above. Among the furans, furan, 2-pentyl has been identified in chicken and its peak area increased from day 0 to day 5. 2-pentylfuran likely came from the oxidation of fatty acids, like linolenic and other n-6 acids [208, 233] and its presence in meat was confirmed by other authors [212, 217, 234, 235]. 2-pentylfuran is responsible for the pleasant aroma [236] that is characterized by sweet, green, fruity, vegetable aromatic notes and roasted nuances [214, 217]. 3-Octanone, acetoin, 2-nonanone and camphor were the ketones identified in chicken. Their peak areas increased form day 0 to day 5. Acetoin production has been described above, while 2-nonanone production was related to the oxidation of free fatty acids [204, 236] and this should explain the increment of its concentration over the time. In fact, as the oxidation processes increase over the time, also the concentration of their products increases with the time. Among the organic acids, the acetic acid was found in chicken and its levels increased from day 0 to day 5. This could be related to its production, which is induced by microorganisms though a carbohydrate fermentation process [188]. Since microorganism proliferation increases with the time, it is reasonable to explain the

increasing concentration of this compound. However, this reaction is mainly involved in products that have a fermented stage during the elaboration process. Instead, in other meat products, the production of acetic acid could be related to the Maillard reaction [188, 219]. Acetic acid has the feature of giving a vinegar note to the meat product and it contributes to the ripened aroma [206, 226].

The semi-quantitative analysis allowed to select some compounds that can be considered potential markers of shelf-life for these five types of meat.

Compounds were chosen on the basis of their presence in most of meat samples or/and for their constant increasing/decreasing concentration within the sample.

Among the 40 compounds identified, 15 were chosen and selected as potential markers of shelf-life: hexanal, 1-butanol, 3-methylbutanol, 2-pentylfuran, 1-pentanol, acetoin, octanal, 1-hexanol, 2-nonanone, nonanal, 1-octen-3-ol, 1-heptanol, linalool, 1-octanol and 2-octen-1-ol.

The chosen compounds and their presence/trend in samples were summarized in ***Table 8***.

***Table 8**:* List of the selected compounds.

↑ means that the concentration increased in the sample; ↓ means that the concentration decreased in the sample. n.d. means "not detected".

Compounds	Beef	Raw Ham	Baked Ham	Sausage	Chicken
Hexanal	↓	↑t0-t2 ↓t2-t5	n.d.	↓	↑t0-t2 ↓t2-t5
1-butanol	n.d.	↑	↓	n.d.	n.d.
3-methylbutanol	↑	n.d.	↑	↑	↑
2-pentylfuran	n.d.	n.d.	n.d.	n.d.	↑t0-t2 ↓t2-t5
1-pentanol	n.d.	n.d.	n.d.	n.d.	↓
Acetoin	↑t0-t2 ↓t2-t5	n.d.	↑	↑t0-t2 ↓t2-t5	↑
Octanal	n.d.	↑t0-t2 ↓t2-t5	n.d.	n.d.	n.d.
1-hexanol	↑t0-t2 ↓t2-t5	↑	↓	↑t0-t2 ↓t2-t5	↑t0-t2 ↓t2-t5
2-nonanone	n.d.	n.d.	n.d.	n.d.	↑
Nonanal	↓	↑t0-t2 ↓t2-t5	↓	↓	↓
1-octen-3-ol	n.d.	↑t0-t2 ↓t2-t5	n.d.	n.d.	↓
1-heptanol	↑	↓	n.d.	n.d.	↓
Linalool	↓	n.d.	↑t0-t2 ↓t2-t5	↓	n.d.
1-octanol	n.d.	n.d.	n.d.	n.d.	↑
2-octen-1-ol	n.d.	n.d.	n.d.	n.d.	↑t0-t2 ↓t2-t5

2.2.2.3. HS-SPME-GC-MS quantitative analytical method validation

A new analytical method for the quantification of 15 VOCs in five types of meat, stored at 4 °C for 14 days, was developed. HS-SPME is one of the most used techniques for the analysis of VOCs because it's simple, economic, solvent-free, easy to handle and very sensitive. For these reasons, this technique has been chosen to quantify 15 VOCs. The chromatographic separation was characterized by a good resolution for all peaks and **figure 8** showed a mix standard chromatogram of 15 VOCs quantified in this study.

The quantification was performed by injecting different concentrations of standard solutions added with the internal standard.

The new analytical method was validated studying linearity, LOQ and LOD (***Table 9***). Linearity was studied by injecting five different concentrations of 15 VOCs and plotting the calibration curves with the respective determination coefficients (R^2). All compounds showed a good linearity as R^2 was equal or greater than 0.990. The calculation of LOQs and LODs was conducted by injecting serial dilutions of the standard solutions, taking the signal-to-noise ratio 3:1 for the LOD and 10:1 for the LOQ, respectively.

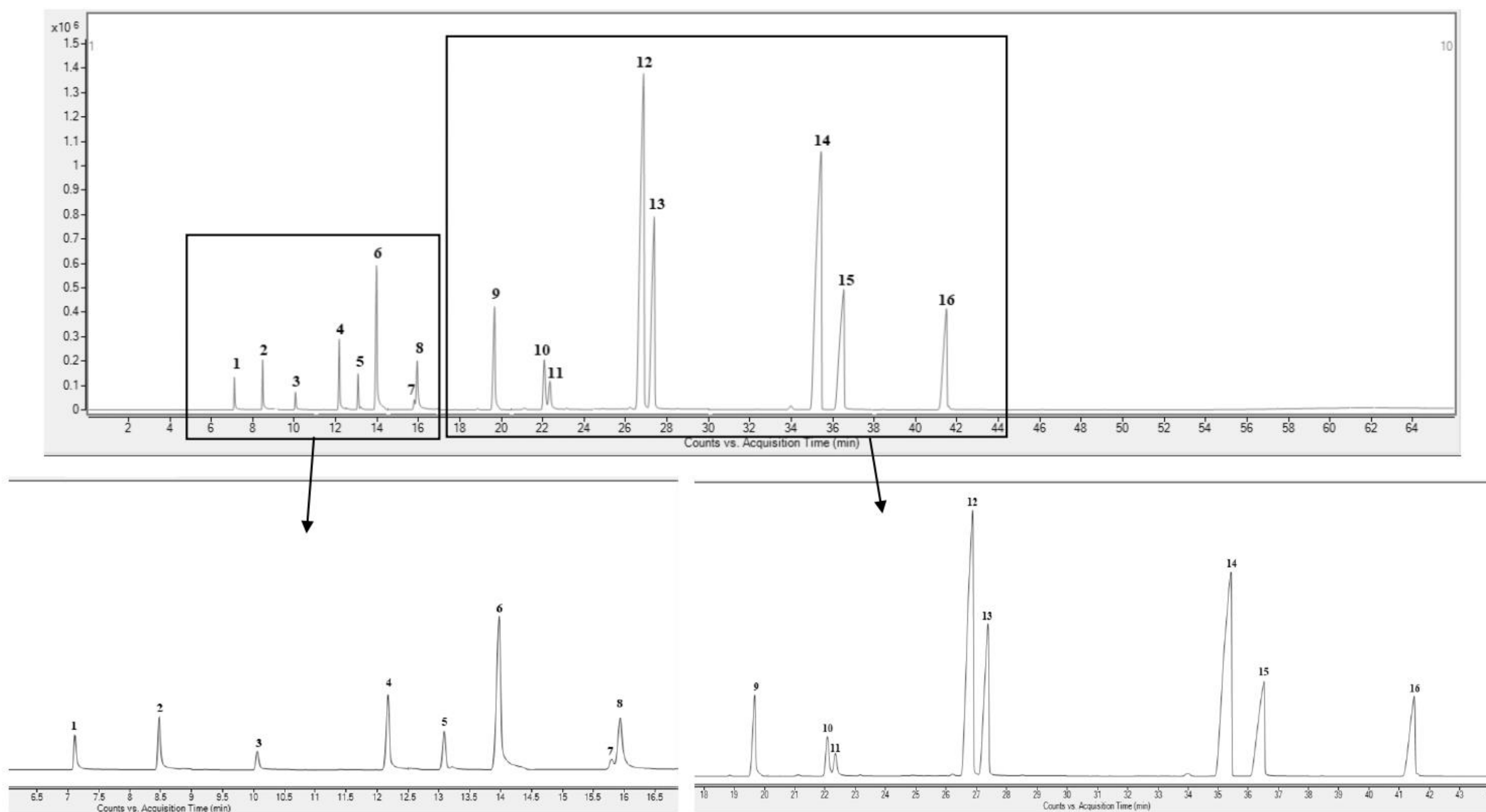


Figure 8: Chromatogram of all monitored compounds acquired in Selected Ion Monitoring (1: 2-methylpentanal (S.I.); 2. Hexanal; 3. 1-butanol; 4. 3-methylbutanol; 5. 2-pentylfuran; 6. 1-pentanol; 7. Acetoin; 8. Octanal; 9. 1-hexanol; 10. 2-nonanone; 11. Nonanal; 12. 1-octen-3-ol; 13. 1-heptanol; 14. Linalool; 15. 1-octanol; 16. 2-octen-1-ol).

Table 9. Validation parameters: equation of the calibration curve, linearity, LOQ and LOD.

Compounds	Equation of the calibration curves	Linearity (R^2)	LOQ ($mg \cdot L^{-1}$)	LOD ($mg \cdot L^{-1}$)
Hexanal	y = 0,4525x + 0,001	1	0.03	0.010
1-butanol	y = 0,0394x + 0,00003	0.9998	0.03	0.010
3-methylbutanol	y = 0,1606x + 0,0042	0.9972	0.02	0.007
2-pentylfuran	y = 0,0085x – 0,00007	0.9954	0.09	0.030
1-pentanol	y = 0,2492x + 0,0046	0.9900	0.01	0.003
Acetoin	y = 0,9276x + 0,0079	0.9985	0.01	0.003
Octanal	y = 4,077x + 0,0565	0.9988	0.05	0.017
1-hexanol	y = 1,253x + 0,0241	0.9940	0.04	0.010
2-nonanone	y = 35,966x + 0,5983	0.9943	0.005	0.002
Nonanal	y = 10,496x - 0,0448	0.9939	0.02	0.007
1-octen-3-ol	y = 8,0119x + 0,1518	0.9960	0.02	0.007
1-heptanol	y = 3,2736x + 0,0411	0.9946	0.01	0.003
Linalool	y = 5,5562x + 0,0636	0.9976	0.03	0.010
1-octanol	y = 6,4219x + 0,0295	0.9960	0.03	0.010
2-octen-1-ol	y = 4,135x - 0,0145	0.9970	0.04	0.013

2.2.2.4. Quantitative analysis of VOCs in five types of meat through HS-SPME-GC-MS

2.2.2.4.1. Beef

The concentrations of all monitored compounds in beef are summarized in ***Table 10.***

Table 10: Concentration of monitored VOCs in beef (μg·kg^{-1}).
n.d. means "not detected".

		t0	t2	t6	t9	t12
Aldehydes	Hexanal	1836.0±0.33^{a}	762.9±0.04^{b}	650.0±0.13^{bc}	47.9±0.01^{c}	250.0±0.04^{bc}
	Octanal	14.1±0.00^{a}	8.6±0.00^{a}	7.2±0.00^{a}	6.7±0.00^{a}	5.7±0.00^{a}
	Nonanal	120.4±0.00^{a}	120.8±0.00^{a}	133.7±0.02^{a}	125.0±0.01^{a}	131.0±0.02^{a}
Sum		1970.5	892.3	790.9	179.6	386.7
Alcohols	1-butanol	n.d.	33.1±0.01^{a}	37.2±0.00^{a}	44.0±0.01^{a}	532.5±0.08^{a}
	3-methylbutanol	n.d.	n.d.	53.0±0.01^{a}	938.3±0.08^{b}	1115.5±0.22^{b}
	1-pentanol	120.5±0.02^{ab}	240.7±0.02^{b}	143.2±0.00^{a}	231.8±0.04^{b}	93.9±0.02^{a}
	1-hexanol	n.d.	59.5±0.00^{a}	485.3±0.05^{b}	230.5±0.05^{c}	307.5±0.06^{c}
	1-octen-3-ol	173.7±0.03^{a}	190.0±0.01^{a}	285.6±0.01^{b}	163.0±0.03^{a}	129.3±0.01^{a}
	1-heptanol	75.9±0.01^{a}	90.0±0.00^{a}	101.9±0.01^{a}	59.0±0.01^{b}	n.d.
	Linalool	n.d.	n.d.	5.1±0.00^{b}	7.7±0.00^{c}	n.d.
	1-octanol	29.3±0.00^{a}	30.0±0.00^{a}	39.3±0.01^{a}	n.d.	n.d.
	2-octen-1-ol	89.0±0.01^{a}	101.0±0.00^{a}	109.5±0.00^{a}	99.6±0.01^{a}	87.9±0.00^{a}
Sum		488.4	744.3	1260.1	1773.9	2266.6
Ketones	Acetoin	40.8±0.01^{a}	60.0±0.00^{ab}	625.0±0.11^{bc}	963.5±0.14^{c}	2320.8±0.4^{d}
	2-nonanone	5.0±0.00^{a}	6.0±0.00^{a}	11.5±0.00^{b}	5.5±0.00^{a}	3.7±0.00^{a}
Sum		45.8	66.0	636.5	969.0	2324.5
Furans	2-pentylfuran	64277.1±11.67^{ab}	70250.0±7.44^{a}	81500.0±14.85^{b}	20709.8±3.77^{c}	17753.5±0.53^{c}

[a]Values in the same line that don't share the same letters are statistically significative different ($p<0.05$).

Hexanal concentration decreased from t0 (1836 $\mu g \cdot kg^{-1}$) to t9 (47.9 $\mu g \cdot kg^{-1}$) with statistically significative differences for t2, t6 and t9. This result has been observed in other studies [237], where the concentrations of the most important VOCs, that are responsible for the spoilage of beef, are monitored over the time and ranged between 49.3 $\mu g \cdot kg^{-1}$ and 1459.4 $\mu g \cdot kg^{-1}$. Octanal and nonanal concentrations had an oscillatory trend from t0 to t12 with no statistically significative differences and their values coincided with those reported by Argyri et al. (26.3-157.4 $\mu g \cdot kg^{-1}$ for octanal and 37.0-181.7 $\mu g \cdot kg^{-1}$ for nonanal) in almost 7 days of storage at 4 °C [237].

The concentration of aldehydes usually increases with the time as they are the products of oxidation processes; however, in this study the sum of the concentration of aldehydes decreased and this should be due to their conversion to carboxylic acids.

Concerning alcohols, 1-butanol concentration increased from t0 (<LOQ) to t12 (532.5 $\mu g \cdot kg^{-1}$), 3-methylbutanol from t0 (<LOQ) to t12 (1115.5 $\mu g \cdot kg^{-1}$) and 1-pentanol from t0 (120.5 $\mu g \cdot kg^{-1}$) to t2 (240.7 $\mu g \cdot kg^{-1}$) with statistically significative differences. 1-hexanol, 1-octen-3-ol, 1-heptanol, 1-octanol and 2-octen-1-ol concentrations increased from t0 to t6 with statistically significative differences for 1-hexanol and 1-octen-3-ol. Linalool concentration increased from t0 (<LOQ) to t9 (7.7 $\mu g \cdot kg^{-1}$) with statistically significative differences for t6 and t9.

The concentrations of some alcohols, such as pentanol, hexanol and octanol, are in the same range of those found in the study by Argyri et al. [237].

The sum of the concentrations of the monitored alcohols increased with the time (from t0 to t12) and this is in agreement with their formation, as they are formed from the lipid oxidation, which is an increasing process over the time.

Acetoin concentration increased from t0 (40.8 $\mu g \cdot kg^{-1}$) to t12 (2320.8 $\mu g \cdot kg^{-1}$) with statistically significative differences. 2-nonanone concentration increased from t0 (5.0 $\mu g \cdot kg^{-1}$) to t6 (11.5 $\mu g \cdot kg^{-1}$) with a statistically significative difference.

The sum of the concentrations of acetoin and 2-nonanone increased from t0 to t12 and this agrees with their production, as they are formed by the microbial carbohydrate metabolism and the oxidation of free fatty acids, respectively. Also in this case, the range of concentrations agrees with the concentrations found in the study of Argyri et al. [237].

2-Pentylfuran concentration increased from t0 (64277.1 $\mu g \cdot kg^{-1}$) to t6 (81500.0 $\mu g \cdot kg^{-1}$), then, it decreased from t6 (81500.0 $\mu g \cdot kg^{-1}$) to t12 (17753.5 $\mu g \cdot kg^{-1}$) with statistically significative differences.

2.2.2.4.2. Raw Ham

The concentrations of all monitored compounds in raw ham are summarized in ***Table 11.***

***Table 11*: Concentration of monitored VOCs in raw ham ($\mu g \cdot kg^{-1}$).**
n.d. means "not detected".

		t0	t2	t6	t9	t12
Aldehydes	Hexanal	2199.7±0.01[a]	1217.6±0.00[b]	1171.8±0.19[b]	1513.6±0.12[b]	1282.0±0.21[b]
	Octanal	86.8±0.01[a]	70.0±0.00[a]	62.5±0.01[ab]	53.2±0.00[b]	33.5±0.01[c]
	Nonanal	20.5±0.00[a]	24.7±0.00[a]	25.0±0.00[a]	26.3±0.00[a]	27.8±0.01[a]
Sum		2307.0	1312.3	1259.3	1593.1	1343.3
Alcohols	1-butanol	295.0±0.06[a]	43.2±0.00[bc]	44.9±0.00[bc]	61.1±0.01[bc]	88.7±0.00[c]
	3-methylbutanol	n.d.	68.7±0.00[b]	71.2±0.00[b]	76.0±0.02[b]	72.5±0.01[b]
	1-pentanol	197.3±0.02[ad]	112.9±0.00[bc]	90.1±0.01[b]	167.1±0.03[c]	116.1±0.01[bc]
	1-hexanol	n.d.	n.d.	n.d.	n.d.	170.0±0.03[b]
	1-octen-3-ol	102.1±0.02[a]	64.9±0.00[a]	85.0±0.01[a]	75.9±0.02[a]	88.2±0.01[a]
	1-heptanol	157.6±0.02[a]	67.6±0.00[b]	54.9±0.01[b]	73.3±0.01[b]	n.d.
	Linalool	n.d.	0.1±0.00[a]	1.2±0.00[a]	4.5±0.00[b]	6.2±0.00[c]
	1-octanol	83.9±0.01[a]	50.7±0.00[bc]	67.8±0.00[bc]	41.5±0.01[b]	40.0±0.01[b]
	2-octen-1-ol	32.0±0.01[a]	24.3±0.00[a]	39.4±0.00[a]	25.0±0.00[a]	36.0±0.01[a]
Sum		867.9	432.4	454.5	524.4	617.7
Ketones	Acetoin	n.d.	n.d.	n.d.	n.d.	n.d.
	2-nonanone	3.4±0.00[a]	3.4±0.00[a]	3.4±0.00[a]	3.6±0.00[a]	4.8±0.00[a]
Sum		3.4	3.4	3.4	3.6	4.8
Furans	2-pentylfuran	20726.5±1.49[a]	8808.0±0.00[bc]	7185.5±0.93[b]	7000.0±0.16[b]	6523.7±0.81[b]

[a]Values in the same line that don't share the same letters are statistically significative different ($p<0.05$).

Hexanal and octanal concentrations decreased from t0 to t6 and from t0 to t12 respectively, with statistically significative differences, while nonanal concentration increased from t0 (20.5 $\mu g \cdot kg^{-1}$) to t12 (27.8 $\mu g \cdot kg^{-1}$) with no statistically significative differences.

In this meat product, both hexanal and octanal concentrations decreased probably because they are transformed in the corresponding carboxylic acids, while nonanal concentration increased for the oxidative processes. The sum of the concentrations of the monitored aldehydes decreased over the time from t0 to t6 probably because of their transformation in carboxylic acids.

Regarding alcohols, 1-butanol and 1-pentanol concentration decreased from t0 to t6 with statistically significative differences and 1-octen-3-ol concentration underwent an overall decrease from t0 (102.1 $\mu g \cdot kg^{-1}$) to t12 (88.2 $\mu g \cdot kg^{-1}$) with no statistically significative differences. The concentration of 1-heptanol and 1-octanol also decreased from t0 to t12 with statistically significative differences. 3-methylbutanol concentration increased from t0 (<LOQ) to t9 (76.0 $\mu g \cdot kg^{-1}$), while 1-hexanol and linalool increased from t0 to t12 with statistically significative differences. 2-octen-1-ol concentration did not vary among the samples. The sum of the concentrations of the monitored alcohols underwent a slight decrease (from t0 to t2) and then a slight increase (from t6 to t14).

Acetoin was not present in all samples at all times, while 2-nonanone concentration increased from t0 (3.4 $\mu g \cdot kg^{-1}$) to t12 (4.8 $\mu g \cdot kg^{-1}$) with no statistically significative differences.

2-pentylfuran concentration decreased from t0 (20726.5 $\mu g \cdot kg^{-1}$) to t12 (6523.7 $\mu g \cdot kg^{-1}$) with statistically significative differences.

2.2.3. Baked Ham

The concentrations of all monitored compounds in baked ham are summarized in ***Table 12.***

Table 12**:** Concentration of monitored VOCs in baked ham (μg·kg^{-1}).
n.d. means "not detected".

		t0	t2	t6	t9	t12
Aldehydes	Hexanal	0.0±0.00[a]	18.0±0.00[bc]	16.4±0.00[bc]	14.1±0.00[b]	25.0±0.00[c]
	Octanal	6.6±0.00[a]	22.0±0.00[bc]	14.4±0.00[bd]	14.4±0.00[bd]	22.5±0.00[c]
	Nonanal	65.3±0.01[a]	84.5±0.02[ab]	131.7±0.00[bc]	106.1±0.00[ac]	140.6±0.03[c]
Sum		71.9	124.5	162.5	134.6	188.1
Alcohols	1-butanol	53.0±0.01[a]	164.5±0.03[a]	217.6±0.03[a]	250.86±0.01[a]	618.5±0.11[b]
	3-methylbutanol	57.4±0.00[ad]	70.0±0.00[ad]	311.9±0.05[b]	350.0±0.04[b]	552.4±0.00[c]
	1-pentanol	9.4±0.00[a]	19.5±0.00[ab]	32.5±0.00[b]	35.7±0.00[b]	72.2±0.01[c]
	1-hexanol	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]
	1-octen-3-ol	0.0±0.00[a]	1.6±0.00[b]	2.5±0.00[b]	1.9±0.00[b]	2.0±0.00[b]
	1-heptanol	0.0±0.00[a]	15.4±0.00[b]	30.5±0.00[c]	26.6±0.00[c]	2.9±0.00[a]
	Linalool	217.0±0.02[a]	195.4±0.01[a]	228.1±0.03[a]	203.2±0.02[a]	171.4±0.02[a]
	1-octanol	7.2±0.00[a]	8.2±0.00[ac]	12.3±0.00[b]	11.0±0.00[bc]	0.0±0.00[d]
	2-octen-1-ol	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]
Sum		344.0	474.6	835.4	628.4	1419.4
Ketones	Acetoin	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]	0.0±0.00[a]
	2-nonanone	10.6±0.00[a]	13.5±0.00[a]	22.2±0.00[b]	22.9±0.00[a]	24.0±0.01[b]
Sum		10.6	13.5	22.2	22.9	24.0
Furans	2-pentylfuran	1766.4±0.03[a]	2970.9±0.27[a]	27661.7±3.00[bc]	26770.4±3.57[bc]	3067.9±0.06[a]

[a]Values in the same line that don't share the same letters are statistically significative different ($p<0.05$).

Hexanal, octanal and nonanal concentration increased from t0 to t12, from t0 to t2 and from t0 to t6 respectively, with statistically significative differences.

In this type of meat, the sum of the concentrations of monitored aldehydes increased from t0 to t12, as they are the products of oxidative process that increases over the time.

The concentrations of 1-octen-3-ol and 1-octanol increased from t0 to t6 with a statistically significative difference for 1-octen-3-ol and 1-octanol, while 1-butanol, 3-methylbutanol and 1-pentanol concentrations increased from t0 to t12 with statistically significative differences. 1-heptanol concentration increased from t0 (0.0 $\mu g \cdot kg^{-1}$) to t6 (30.5 $\mu g \cdot kg^{-1}$), with statistically significative differences.

Linalool concentration remained quite the same in all samples, while 1-hexanol and 2-octen-1-ol were not present in all samples at all times.

The sum of the concentrations of the monitored alcohols increased from t0 to t12 (with a slight decrease on t9) and this agrees with their production.

Regarding ketones, acetoin was not present in all samples at all times, while 2-nonanone concentration increased from t0 (10.6 $\mu g \cdot kg^{-1}$) to t12 (24.0 $\mu g \cdot kg^{-1}$) with no statistically significative differences and this is in agreement with their production from oxidation processes.

2-pentylfuran concentration increased from t0 (1766.4 $\mu g \cdot kg^{-1}$) to t6 (27661.7 $\mu g \cdot kg^{-1}$) with statistically significative differences.

2.2.4. Pork Sausage

The concentrations of all monitored compounds in pork sausages are summarized in ***Table 13*.**

***Table 13*:** Concentration of monitored VOCs in pork sausage (μg·kg^{-1}).
n.d. means "not detected".

		t0	t2	t6	t9	t12
Aldehydes	Hexanal	28.9±0.00^{a}	137.7±0.03^{b}	1.8±0.00^{a}	7.5±0.00^{a}	3.4±0.00^{a}
	Octanal	8.4±0.00^{a}	72.7±0.01^{ab}	365.9±0.07^{b}	450.0±0.03^{b}	1095.1±0.20^{c}
	Nonanal	9.3±0.00^{a}	2.1±0.00^{b}	1.9±0.00^{b}	1.7±0.00^{b}	6.9±0.00^{c}
Sum		46.6	212.5	369.6	459.2	1105.4
Alcohols	1-butanol	27.6±0.00^{a}	32.9±0.00^{a}	33.3±0.00^{a}	34.4±0.00^{a}	88.6±0.02^{b}
	3-methylbutanol	95.6±0.01^{a}	324.5±0.04^{b}	982.6±0.04^{c}	1050.0±0.11^{c}	1212.2±0.05^{d}
	1-pentanol	2.8±0.00^{a}	9.7±0.00^{b}	10.4±0.00^{b}	3.4±0.00^{a}	9.5±0.00^{b}
	1-hexanol	3.8±0.00^{ade}	28.2±0.00^{b}	14.1±0.00^{c}	7.3±0.00^{a}	n.d.
	1-octen-3-ol	1.1±0.00^{a}	3.2±0.00^{abc}	4.0±0.00^{bc}	4.3±0.00^{bc}	6.2±0.00^{c}
	1-heptanol	0.6±0.00^{ac}	2.2±0.00^{ab}	2.8±0.00^{b}	3.2±0.00^{b}	3.9±0.00^{b}
	Linalool	0.9±0.00^{a}	1.5±0.00^{a}	3.5±0.00^{b}	3.8±0.00^{b}	7.6±0.00^{c}
	1-octanol	n.d.	n.d.	14.1±0.00^{b}	4.4±0.00^{c}	n.d.
	2-octen-1-ol	0.5±0.00^{a}	n.d.	0.2±0.00^{a}	n.d.	n.d.
Sum		132.9	402.2	1065.0	1110.8	1328.0
Ketones	Acetoin	5879.0±1.05^{a}	27535.0±3.65^{ab}	71483.7±7.90^{b}	73541.0±11.64^{b}	202052.5±37.20^{c}
	2-nonanone	0.2±0.00^{a}	0.4±0.00^{a}	0.1±0.00^{a}	0.1±0.00^{a}	0.0±0.00^{a}
Sum		5879.2	27535.4	71483.8	73541.1	202052.5
Furans	2-pentylfuran	1.9±0.00^{a}	27.6±0.01^{b}	8.7±0.00^{a}	8.2±0.00^{a}	8.3±0.00^{a}

[a]Values in the same line that don't share the same letters are statistically significative different ($p<0.05$).

Hexanal and octanal concentrations increased from t0 to t2 and from t0 to t12, respectively with statistically significative difference. The concentration of nonanal decreased from t0 (9.3 $\mu g \cdot kg^{-1}$) to t12 (6.9 $\mu g \cdot kg^{-1}$) with statistically significative differences.

The sum of the concentrations of the monitored aldehydes increased from t0 to t12 and this agrees with their production.

1-Butanol, 3-methylbutanol, 1-octen-3-ol, 1-heptanol and linalool concentrations increased from t0 to t12 with statistically significative differences. 1-Pentanol and 1-octanol concentrations increased from t0 to t6 with statistically significative differences.

The concentration of 1-hexanol increased from t0 (3.8 $\mu g \cdot kg^{-1}$) to t2 (28.2 $\mu g \cdot kg^{-1}$), with statistically significative differences, while 2-octen-1-ol was not present in the sample.

The sum of the concentrations of all monitored alcohols increased from t0 to t12 and this agrees with their production.

Acetoin increased from t0 (5879.0 $\mu g \cdot kg^{-1}$) to t12 (202052.5 $\mu g \cdot kg^{-1}$) with statistically significative differences, while 2-nonanone concentration increased from t0 (0.2 $\mu g \cdot kg^{-1}$) to t2 (0.4 $\mu g \cdot kg^{-1}$) with no statistically significative differences.

2-pentylfuran increased from t0 (1.9 $\mu g \cdot kg^{-1}$) to t2 (27.6 $\mu g \cdot kg^{-1}$) with a statistically significative difference.

2.2.5. Chicken

The concentrations of all monitored compounds in chicken are summarized in ***Table 14.***

Table 14**:** Concentration of monitored VOCs in chicken (μg·kg^{-1}).
n.d. means "not detected".

		t0	**t2**	**t6**	**t9**	**t12**
Aldehydes	Hexanal	707.4±0.09^{a}	652.4±0.04^{a}	192.4±0.04^{c}	1364.6±0.20^{d}	1694.0±0.6^{d}
	Octanal	62.6±0.01^{a}	35.0±0.00^{a}	29.5±0.00^{a}	986.4±0.00^{b}	1025.7±0.02^{b}
	Nonanal	11.6±0.00^{a}	12.6±0.00^{a}	13.2±0.00^{a}	1257.9±0.21^{b}	161.5±0.03^{a}
Sum		781.6	700.0	235.1	2622.5	2881.2
Alcohols	1-butanol	23.3±0.00^{a}	59.1±0.01^{a}	104.0±0.01^{a}	110.0±0.00^{a}	1109.8±0.22^{b}
	3-methylbutanol	12.4±0.00^{a}	21.6±0.00^{a}	390.6±0.06^{a}	5995.4±1.90^{b}	11663.9±2.20^{b}
	1-pentanol	44.9±0.00^{a}	29.4±0.00^{ab}	14.9±0.00^{b}	0.0±0.00^{b}	169.9±0.02^{c}
	1-hexanol	8.5±0.00^{a}	9.0±0.00^{a}	9.1±0.00^{a}	239.8±0.04^{b}	259.9±0.05^{b}
	1-octen-3-ol	32.5±0.01^{a}	19.4±0.00^{ac}	5.8±0.00^{a}	91.3±0.01^{b}	107.7±0.02^{b}
	1-heptanol	4.8±0.00^{a}	3.9±0.00^{b}	0.0±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}
	Linalool	0.1±0.00^{a}	0.2±0.00^{a}	0.3±0.00^{a}	0.6±0.00^{b}	3.3±0.00^{b}
	1-octanol	11.3±0.00^{a}	9.7±0.00^{b}	0.0±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}
	2-octen-1-ol	14.9±0.00^{a}	8.3±0.00^{b}	2.9±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}
Sum		152.7	160.6	527.6	6437.1	13314.5
Ketones	Acetoin	1868.5±0.05^{a}	2015.8±0.02^{a}	2238.7±0.07^{a}	14522.0±28.3^{b}	8360.0±5.07^{c}
	2-nonanone	0.3±0.00^{a}	0.3±0.00^{a}	0.4±0.00^{a}	2.9±0.00^{b}	8.1±0.00^{c}
Sum		1868.8	2016.1	2239.1	14524.9	8368.1
Furans	2-pentylfuran	164.9±0.01^{a}	78.9±0.01^{a}	32.3±0.01^{a}	1167.4±0.21^{b}	830.7±0.16^{b}

[a]Values in the same line that don't share the same letters are statistically significative different ($p<0.05$).

The concentration of hexanal decreased from t0 to t6 with statistically significative differences, while octanal concentration decreased from t0 (14.6 μg·kg^{-1}) to t9 (0.00 μg·kg^{-1}) with a statistically significative difference. The concentration of nonanal increased from t0 (11.6 μg·kg^{-1}) to t9 (1257.9 μg·kg^{-1}) with statistically significative differences.

The sum of the concentrations of monitored aldehydes decreased from t0 to t6 and this should be due to their transformation to carboxylic acid.

1-butanol, 3-methylbutanol, 1-hexanol and linalool concentrations increased from t0 to t12 with statistically significative differences. On the contrary, 1-pentanol and 2-octen-1-ol concentrations decreased from t0 to t9 with statistically significative differences and 1-octen-3-ol, 1-heptanol, 1-octanol decreased from t0 to t6 with statistically significative differences for 1-heptanol and 1-octanol.

The sum of the concentrations of all monitored alcohols increased from t0 to t12 and this agrees with their formation from the oxidative processes.

The concentration of acetoin increased from t0 (1868.5 $\mu g \cdot kg^{-1}$) to t9 (14522.0 $\mu g \cdot kg^{-1}$) with statistically significative differences, while 2-nonanone increased from t0 (0.3 $\mu g \cdot kg^{-1}$) to t12 (8.1 $\mu g \cdot kg^{-1}$) with statistically significative differences.

The sum of the concentrations of acetoin and 2-nonanone increased from t0 to t9 and this agrees with their formation.

2-pentylfuran concentration decreased from t0 (164.9 $\mu g \cdot kg^{-1}$) to t9 (32.3 $\mu g \cdot kg^{-1}$) with statistically significative differences.

2.2.3. Conclusions

The semi-quantitative analysis of 5 types of meat allowed to select 15 volatile compounds as potential markers of shelf-life.

Then, the 15 selected VOCs were quantified in 5 types of meat over 12 days of storage to assess their reliability as meat shelf-life markers. ***Table 15*** summarizes the trend of the 15 VOCs quantified in 5 types of meat.

Table 15**:** Trend of 15 VOCs quantified in 5 types of meat.

Compounds	Beef	Raw Ham	Baked Ham	Sausage	Chicken
Hexanal	↓	↓	↑	↑	↓
1-butanol	↑	↓	↑	↑	↑
3-methylbutanol	↑	↑	↑	↑	↑
2-pentylfuran	↑	↓	↓	↑	↑
1-pentanol	↑	↓	↑	↑	↓
Acetoin	↑	n.d.	n.d.	↑	↑
Octanal	↑	↓	↑	↑	↓
1-hexanol	↑	↑	n.d.	↑	↑
2-nonanone	↑	↑	↑	↔	↑
Nonanal	↑	↑	↑	↓	↑
1-octen-3-ol	↑	↓	↑	↑	↑
1-heptanol	↑	↓	↑	↑	↓
Linalool	↑	↑	↔	↑	↑
1-octanol	↑	↓	↑	↑	↓
2-octen-1-ol	↑	↔	n.d.	↔	↓

Results showed that all the compounds monitored can be considered markers of shelf-life for one or more types of meat because their trend is constant over the time (increasing or decreasing), with the exception of acetoin for raw and baked ham (n.d.), 1-hexanol and 2-octen-1-ol for baked ham (n.d.), 2-nonanone and 2-octen-1-ol for sausage (remained constant over the time), linalool for baked ham (remained constant over the time) and 2-octen-1-ol (remained constant over the time).

Among the monitored compounds, some can be considered markers of shelf-life for at least 4 types of meat: 1-butanol, 3-methylbutanol, 1-hexanol, 2-nonanone, nonanal, 1-octen-3-ol and linalool. In particular, 1-butanol can be considered a marker of shelf-life for beef, baked ham, pork sausage and chicken; 3-methylbutanol for all types of meat; 1-hexanol for beef, raw ham, pork sausage and chicken; 2-nonanone for beef, raw ham, baked ham and chicken; nonanal for beef, raw and baked hams and chicken; 1-octen-3-ol for beef, baked ham, pork sausage and chicken; linalool for beef, raw ham, pork sausage and chicken.

In conclusion, in this study an HS-SPME-GC-MS semi-quantitative analysis allowed to select the most important VOCs (in terms of increasing/decreasing trends) in five types of meat, then a new quantitative HS-SPME-GC-MS analytical method to quantity the 15 VOCs selected in five types of meat preserved for 12 days was developed and it was demonstrated that all compounds quantified can be considered markers of shelf-life for at least 2 types of meat and 7 of them for at least for 4 types of meat products analyzed.

This study is innovative because to the best of our knowledge, there are no quantitative analyses of VOCs in different types of meat simultaneously to find possible markers of shelf-life.

2.3. **RESEARCH ACTIVITY:** APPLICATION OF THE NEW QUANTITATIVE HS-SPME-GC-MS METHOD TO EVALUATE THE PERFORMACES OF THREE TYPES OF PACKAGING FOR THE PRESERVATION OF RAW AND BAKED HAMS.

Abstract

At global level, the consumption of meat is increasing and for this reason the attention in studying its quality and safety is increasing. The aim of this study was to compare three packaging for the preservation of raw and baked hams preserved at 4°C for 9 days. Packaging were supplied by Esseoquattro S.p.A.: *Ideabrill*® coupled to *Ideabrill*® Imprigionagusto bag, *Ideabrill*® coupled to *Ideabrill*® Sacchetto Salvafreschezza, and paper coupled with wings.

The analysis was performed through a new HS-SPME-GC-MS analytical method to quantify 15 volatile organic compounds (VOCs) selected as potential markers of shelf-life for these meat products (hexanal, 1-butanol, 3-methylbutanol, 2-pentylfuran, 1-pentanol, acetoin, octanal, 1-hexanol, 2-nonanone, nonanal, 1-octen-3-ol, 1-heptanol, linalool, 1-octanol and 2-octen-1-ol). The analyses were performed at day 0 (T0), 2 (T2), 6 (T6) and 9 (T9).

Extraction was performed through HS-SPME using DVB/C-WR/PDMS fiber at 40°C for 20 minutes, after an incubation time of 40 minutes. The chromatographic separation of the volatile compounds was performed in GC-MS using a DB-WAX column (60 m x 0.25 mm x 0.25 μm) with a temperature gradient (35°C maintained for 3 minutes, increased up to 70°C at 3°C/min, increased to 210°C at 5°C/min, increased to 250°C at 15°C/min maintained for 10 min). Spectra were acquired in SIM mode.

9 VOCs have been monitored to study the performances of the packaging in raw ham: hexanal, nonanal, 3-methylbutanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, linalool, 1-octanol and 2-octen-1-ol.

12 VOCs have been monitored to study the performances of the packaging in baked ham: hexanal, octanal, nonanal, 3-methylbutanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, linalool, 1-

octanol, acetoin and 2 -pentylfuran. The concentrations of the VOCs mentioned above, for both matrices, were lower in hams preserved in P1 and P2 with respect to P3, highlighting a higher degree of deterioration of the meat product preserved in P3 with respect to that preserved in P1 and P2.

This study demonstrated that *Ideabrill®* coupled to Imprigionagusto bag (P1) and Salvafreschezza bag (P2) had a better ability in preserving raw and baked hams with respect to paper coupled with wings (P3).

2.3.1. Materials and methods

2.3.1.1. Chemicals

Sodium chloride (NaCl) was purchased by chem-lab. Pure standards of hexanal (CAS 66-25-1), 1-butanol (CAS 71-36-3), 3-methylbutanol (CAS 123-51-3), 2-pentylfuran (CAS 3777-69-3), 1-pentanol (CAS 71-41-0), acetoin (CAS 513-86-0), octanal (CAS 124-13-0), 1-hexanol (CAS 111-27-3), 2-nonanone (CAS 821-55-6), nonanal (CAS 124-19-6), 1-octen-3-ol (CAS 3391-84-4), 1-heptanol (CAS 111-70-6), linalool (CAS 78-76-6), 1-octanol (CAS 111-87-5), 2-octen-1-ol (CAS 18409-17-1) and 2-methypentanal, used as internal standard (I.S.), (CAS 123-15-9) were purchased by Sigma Aldrich (Milano, Italy).

2.3.1.2. Packaging and meat samples

Meat samples (raw and baked hams) were purchased in a local supermarket and preserved for 9 days at 4 °C. Meat samples were stored in three different packaging furnished by Esseoquattro S.p.a. (**figure 9**): *Ideabrill*® coupled to *Ideabrill*® Imprigionagusto bag, *Ideabrill*® coupled to *Ideabrill*® Sacchetto Salvafreschezza, and paper coupled with wings. Both Imprigionagusto and Salvafreschezza bag have been produced with the *Ideabrill* ® formula with slight differences. In fact, Imprigionagusto bag has a thicker cellulose and metallic layers with respect to the classic *Ideabrill* ® used in the Salvafreschezza bag, suggesting a possible increment in the preservation ability of foods.

The analyses were performed at day 0 (T0), 2 (T2), 6 (T6) and 9 (T9).

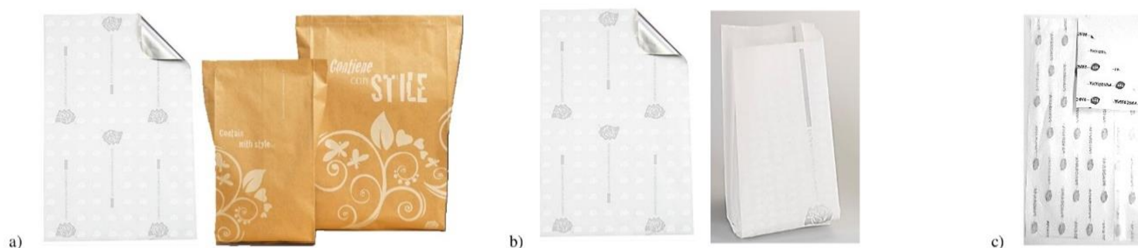


Figure 9: a) *Ideabrill*® combined with Imprigionagusto bag (P1), b) *Ideabrill*® combined to Salvafreschezza bag (P2) and paper coupled with wings (P3).

2.3.1.3. Quantitative analysis through HS-SPME-GC-MS

The analysis was performed following the procedure developed previously and described in the chapters 2.2.1.4 and 2.2.2.3.

Briefly, 5 g of sample were added with 15 mL of distilled water, homogenized and centrifuged. Then, 1500 µL of water added with 25% of NaCl and 40 µL of I.S. 50 mg·mL^{-1} were added to 500 µL of supernatant in a 20 mL vial which was tightly clapped with a PTFE/silicon septum. The vial was incubated at 40 °C for 40 min and extracted for 20 min with a DVB/C-WR/PDMS) fiber. The separation was performed on a DB-WAX capillary column (60 m, 250 µm i.d., 0.25 µm film thickness) with the following ramp of temperature: 35˚C at 3°C/min, increased up to 70˚C at 3˚C/min, increased up to 210˚C at 5˚C/min, increased up to 250˚C at 15˚C/min, maintained for 10 min. The acquisitions were carried out in ‘Selected Ion Monitoring’ (SIM) mode and detection was divided into time windows to enhance the sensitivity. The most abundant ions were used for quantitation, while the others to confirm the presence of the analytes. The GC-MS parameters including the retention time (Rt) and time windows are reported in ***Table 5***. Data results were managed by MSD ChemStation Software (Agilent, Version G1701DA D.01.00). Samples were analysed in triplicate. Acceptable relative standard deviation (% RSD) were set up below 20%.

2.3.2. Results and discussion

3.1.1. Quantitative analysis of VOCs in raw ham

The concentrations (µg·kg^{-1}) of VOCs in raw ham have been reported in ***Table 16***.

Table 16: Concentrations (μg·Kg^{-1}) of VOCs in raw ham. ^{a}Different letters indicate statistically significative differences ($p<0.05$). P1: *Ideabrill®*+Imprigionagusto bag; P2: *Ideabrill®*+Salvafreschezza bag; P3: paper coupled with wings.

	T0			**T2**			**T6**			**T9**		
COMPOUNDS	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Aldehydes												
HEXANAL	750.0 ±0.14^{a}	750.0 ±0.14^{a}	750.0 ±0.14^{a}	332.0±0.05^{a}	925.0±0.18^{b}	1095.0±0.15^{b}	300.0±0.06^{a}	470.0±0.07^{a}	589.0±0.12^{b}	525.0±0.10^{ab}	463.0±0.09^{a}	692.0±0.06^{b}
OCTANAL	53.0±0.01^{a}	53.0±0.01^{a}	53.0±0.01^{a}	29.0±0.00^{a}	82.0±0.02^{b}	41.0±0.01^{a}	31.0±0.01^{a}	13.0±0.00^{b}	50.0±0.01^{c}	45.0±0.01^{a}	83.0±0.02^{b}	13.0±0.00^{c}
NONANAL	20.0±0.00^{a}	20.0±0.00^{a}	20.0±0.00^{a}	11.0±0.00^{a}	24.0±0.01^{b}	26.0±0.00^{b}	24.0±0.00^{a}	25.0±0.00^{a}	30.0±0.00^{a}	18.0±0.00^{a}	18.0±0.00^{a}	20.0±0.00^{a}
Alcohols												
1-BUTANOL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-METHYLBUTANOL	52.0±0.00^{a}	52.0±0.00^{a}	52.0±0.00^{a}	11.0±0.00^{a}	20.0±0.00^{b}	32.0±0.00^{b}	5.0±0.00^{a}	8.0±0.00^{a}	52.0±0.01^{b}	55.0±0.01^{a}	54.0±0.01^{a}	69.0±0.01^{a}
1-PENTANOL	100.0±0.01^{a}	100.0±0.01^{a}	100.0±0.01^{a}	49.0±0.01^{a}	105.0±0.01^{b}	107.0±0.02^{b}	65.0±0.01^{a}	102.0±0.02^{b}	128.0±0.02^{b}	92.0±0.02^{a}	84.0±0.01^{a}	107.0±0.01^{a}
1-HEXANOL	30.0±0.01^{a}	30.0±0.01^{a}	30.0±0.01^{a}	21.0±0.00^{a}	42.0±0.01^{b}	53.0±0.00^{c}	29.0±0.01^{a}	56.0±0.01^{b}	60.0±0.01^{b}	58.0±0.02^{a}	77.0±0.02^{a}	83.0±0.01^{a}
1-OCTEN-3-OL	31.0±0.01^{a}	31.0±0.01^{a}	31.0±0.01^{a}	26.0±0.01^{a}	64.0±0.01^{b}	103.0±0.00^{c}	41.0±0.01^{a}	91.0±0.02^{b}	105.0±0.02^{b}	60.0±0.01^{a}	139.0±0.03^{b}	144.0±0.02^{b}
1-HEPTANOL	88.0±0.02^{a}	88.0±0.02^{a}	88.0±0.02^{a}	32.0±0.01^{a}	72.0±0.01^{b}	45.0±0.01^{ab}	32.0±0.01^{a}	n.d.	61.0±0.01^{a}	60.0±0.01^{a}	48.0±0.01^{a}	47.0±0.01^{a}
LINALOOL	13.0±0.00^{a}	13.0±0.00^{a}	13.0±0.00^{a}	n.d.	n.d.	33.0±0.00	n.d.	n.d.	54.0±0.01	n.d.	n.d.	85.0±0.02
1-OCTANOL	27.0±0.00^{a}	27.0±0.00^{a}	27.0±0.00^{a}	25.0±0.01^{a}	48.0±0.01^{b}	59.0±0.01^{b}	37.0±0.01^{a}	42.0±0.01^{a}	55.0±0.01^{a}	45.0±0.00^{a}	88.0±0.02^{b}	94.0±0.01^{b}
2-OCTEN-1-OL	40.0±0.01^{a}	40.0±0.01^{a}	40.0±0.01^{a}	9.0±0.00^{a}	21.0±0.01^{a}	29.0±0.00^{b}	21.0±0.00^{a}	30.0±0.01^{ab}	40.0±0.01^{a}	24.0±0.00^{a}	64.0±0.01^{b}	66.0±0.00^{b}
Ketones												
ACETOIN	2.0±0.00^{a}	2.0±0.00^{a}	2.0±0.00^{a}	4.0±0.00^{a}	6.0±0.00^{b}	4.0±0.00^{a}	6.0±0.00^{a}	9.0±0.00^{b}	6.0±0.00^{a}	62.0±0.01^{a}	27.0±0.01^{b}	6.0±0.00^{b}

2-NONANONE	3.0±0.00[a]	3.0±0.00[a]	3.0±0.00[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Furans												
2-PENTYLFURAN	4571.0±0.40[a]	4571.0±0.40[a]	4571.0±0.40[a]	2495.0±0.20[a]	5150.0±0.91[b]	5515.0±0.75[b]	4856.0±0.36[a]	6906.0±1.31[a]	8928.0±1.04[b]	5003.0±1.00[a]	5593.0±0.82[a]	8156.0±0.42[b]

Results from the previous study (chapter 2.2) showed that among the 15 VOCs quantified in raw ham, 13 of them (excepted acetoin and 2-octen-1-ol) can be considered markers of shelf-life for this meat product on the basis of their constant trend over the time.

In this study, 15 VOCs were quantified to confirm that these compounds can be considered markers of shelf-life for this meat product and to compare the performances of three packaging in the preservation of this meat product.

Among the aldehydes, only hexanal and nonanal were used as markers of shelf-life to study the preservation ability of the packaging. In fact, even if their concentrations increased and decreased over the time because these compounds are continuously subjected to chemical transformations, their trend was constant among the samples. In particular, the concentration of hexanal was lower in P1 with respect to P2 and P3 from t2 to t6 with statistically significative differences, while in t9 the lower concentration of hexanal was found in P2 with respect to P1 and P3 with a statistically significative difference for P3. The concentration of nonanal was lower in P1 with respect to P2 and P3 from t2 to t9 with a statistically significative difference at t2. Among the alcohols, just 1-butanol and 1-heptanol were not considered in this study, while 2-octen-1-ol which was excluded in the previous study, has been considered in this one thanks to its constant trend over the time. The concentration of 3-methylbutanol decreased over the time (from t0 to t6) and then it increased again. In all meat samples, its concentration was lower in P1 and P2 with respect to P3, with statistically significative differences for P1 at t2 and P3 at t6. The concentrations of 1-hexanol and linalool increased over the time, as alcohols are the products of lipid oxidation, which in an increasing process over the time. In particular, the concentration of 1-hexanol was lower in P1 with respect to P2 and P3 with statistically significative differences, while the concentration of linalool increased over the time in raw ham preserved in P3 and it was zero in all other samples at all times. Moreover, 1-pentanol, 1-octen-3-ol, 1-octanol and 2-octen-1-ol were selected to have a trend that is typical of a marker of shelf-life. In fact, their concentration increased over the time and this is in accordance with their

production. In all meat samples, the concentration of these compounds was lower in raw ham preserved in P1 and P2 with respect to that preserved in P3, with statistically significative differences. Non-considered compounds have a trend that is not homogeneous in all meat samples and that's because they cannot be considered markers of shelf-life for raw ham.

Figure 10 reported some of the volatile compounds used as markers of shelf-life for raw ham to better visualize their trend over the time in the different packaging.

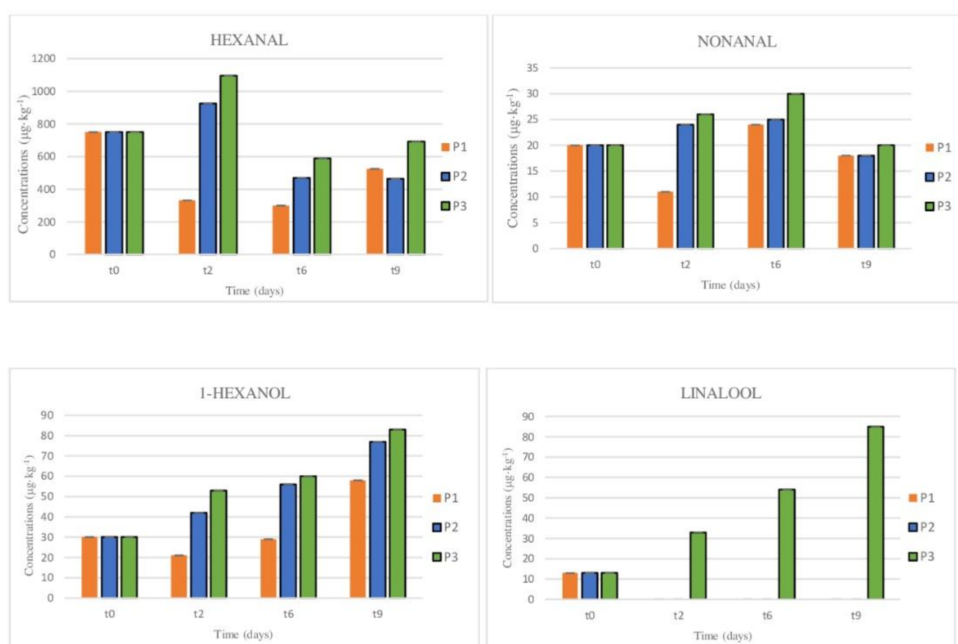


Figure 10: Concentrations (µg·Kg^{-1}) of some VOCs selected as markers of shelf-life for raw ham.

In conclusion, 9 VOCs have been considered as markers of shelf-life for raw ham: hexanal, nonanal, 3-methylbutanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, linalool, 1-octanol and 2-octen-1-ol. Results demonstrated that P1 and P2 have a higher ability in preserving raw ham with respect to P3. Regarding P1 and P2, the preservation ability of P1 was higher with respect to P2 for most of the compounds monitored, with the exception of hexanal, 3-methylbutanol and 1-hexanol, for which P2 resulted to have a better preservation ability with respect to P1.

3.1.2. Quantitative analysis of VOCs in baked ham

The concentrations (µg·Kg^{-1}) of VOCs in baked ham have been reported in ***Table 17***.

Table 17: VOCs concentration in baked ham (µg·Kg^{-1}). ^{a}Different letters indicate statistically significative differences ($p<0.05$).
P1: *Ideabrill®*+Imprigionagusto bag; P2: *Ideabrill®*+Salvafreschezza bag; P3: paper coupled with wings.

	T0			T2			T6			T9		
COMPOUNDS	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Aldehydes												
HEXANAL	1782.0±0.16^{a}	1782.0±0.16^{a}	1782.0±0.16^{a}	1700.0±0.18^{a}	2331.0±0.46^{a}	4147.0±0.53^{b}	1199.0±0.23^{a}	646.0±0.04^{a}	3200.0±0.64^{b}	418.0±0.02^{a}	180.0±0.03^{b}	7000.0±0.03^{c}
OCTANAL	7934.0±1.51^{a}	7934.0±1.51^{a}	7934.0±1.51^{a}	9016.0±1.01^{a}	14252.0±0.65^{b}	13917.0±1.41^{b}	9713.0^{a}	11196.0±1.68^{b}	11677.0±2.23^{b}	7640.0±0.45^{a}	7610.0±1.35^{a}	8976.0±0.22^{a}
NONANAL	771.0±0.08^{a}	771.0±0.08^{a}	771.0±0.08^{a}	564.0±0.11^{a}	773.0±0.12^{a}	720.0±0.10^{a}	486.0±0.01^{a}	514.0±0.00^{b}	680.0±0.01^{c}	585.0±0.09^{a}	399.0±0.01^{a}	925.0±0.18^{b}
Alcohols												
1-BUTANOL	800.0±0.14^{a}	800.0±0.14^{a}	800.0±0.14^{a}	825.0±0.13^{a}	1336.0±0.22^{b}	1304.0±0.20^{b}	904.0±0.04^{a}	1364.0±0.18^{b}	n.d.	972.0±0.19^{a}	610.0±0.11^{a}	817.0±2.38^{a}
3-METHYLBUTANOL	589.0±0.00^{a}	589.0±0.00^{a}	589.0±0.00^{a}	660.0±0.11^{a}	788.0±0.00^{a}	1507.0±0.19^{b}	n.d.	20931.0±2.50^{b}	42192.0±3.34^{a}	58317.0±11.10^{a}	59557.0±5.89^{ab}	92071.0±10.68^{b}
1-PENTANOL	4765.0±0.94^{a}	4765.0±0.94^{a}	4765.0±0.94^{a}	2500.0±0.43^{a}	6180.0±1.03^{b}	6527.0±0.74^{b}	5615.0±0.34^{a}	5951.0±0.67^{a}	7099.0±0.54^{b}	4710.0±0.50^{a}	3374.0±0.29^{a}	5783.0±0.46^{a}
1-HEXANOL	336.0±0.05^{a}	336.0±0.05^{a}	336.0±0.05^{a}	504.0±0.04^{a}	578.0±0.05^{a}	1840.0±0.30^{b}	918.0±0.06^{a}	733.0±0.02^{a}	5890.0±0.99^{b}	340.0±0.06^{a}	336.0±0.04^{a}	2360.0±0.34^{b}
1-OCTEN-3-OL	156.0±0.00^{a}	156.0±0.00^{a}	156.0±0.00^{a}	152.0±0.02^{a}	237.0±0.01^{b}	402.0±0.04^{c}	213.0±0.02^{a}	212.0±0.04^{a}	580.0±0.11^{b}	148.0±0.03^{a}	89.0±0.00^{a}	375.0±0.04^{b}
1-HEPTANOL	260.0±0.03^{a}	260.0±0.03^{a}	260.0±0.03^{a}	300.0±0.04^{a}	480.0±0.02^{b}	400.0±0.05^{ab}	414.0±0.07^{a}	676.0±0.08^{ab}	845.0±0.12^{b}	515.0±0.09^{a}	300.0±0.01^{a}	614.0±0.10^{a}
LINALOOL	88.0±0.00^{a}	88.0±0.00^{a}	88.0±0.00^{a}	95.0±0.01^{a}	179.0±0.03^{a}	1722.0±0.24^{b}	162.0±0.01^{a}	200.0±0.03^{a}	5500.0±0.85^{b}	127.0±0.02^{a}	105.0±0.02^{a}	4240.0±0.82^{b}
1-OCTANOL	278.0±0.00^{a}	278.0±0.00^{a}	278.0±0.00^{a}	315.0±0.05^{a}	588.0±0.12^{b}	706.0±0.07^{b}	261.0±0.02^{a}	372.0±0.06^{ab}	598.0±0.11^{b}	446.0±0.06^{a}	230.0±0.00^{b}	547.0±0.10^{a}
2-OCTEN-1-OL	n.d.	n.d.	n.d.	44.0±0.00^{a}	111.0±0.01^{b}	n.d.	n.d.	79.0±0.01	n.d.	57.0±0.01^{a}	46.0±0.01^{a}	40.0±0.00^{a}
Ketones												
ACETOIN	800.0±0.14^{a}	800.0±0.14^{a}	800.0±0.14^{a}	717.0±0.11^{a}	1135.0±0.02^{a}	2429.0±0.39^{b}	158038.0±15.05^{a}	253452.0±34.56^{a}	310344.0±60.73^{b}	448505.0±21.70^{ab}	269240.0±22.49^{a}	700273.0±123.15^{b}
2-NONANONE	27.0±0.00^{a}	27.0±0.00^{a}	27.0±0.00^{a}	24.0±0.00^{a}	37.0±0.01^{a}	23.0±0.00^{a}	21.0±0.00^{a}	23.0±0.00^{a}	18.00±0.00^{a}	16.00±0.00^{a}	8.0±0.00^{a}	10.0±0.00^{a}
Furans												
2-PENTYLFURAN	31898.0±2.68^{a}	31898.0±2.68^{a}	31898.0±2.68^{a}	45500.0±9.19^{a}	37209.0±1.08^{a}	51227.0±7.53^{a}	59271.0±9.37^{a}	45818.0±5.50^{a}	64975.0±12.99^{a}	16881.0±0.80^{a}	23543.0±4.52^{a}	42094.0±0.46^{b}

Results from the previous study (chapter 2.2) showed that among the 15 VOCs quantified in baked ham, 11 of them (excepted acetoin, 1-hexanol, linalool and 2-octen-1-ol) can be considered markers of shelf-life for this meat product on the basis of their constant trend over the time.

In this study, 15 VOCs were quantified to confirm that these compounds can be considered markers of shelf-life for this meat product and to compare the performances of three packaging in the preservation of this meat product.

Among the aldehydes, hexanal, octanal and nonanal have been considered as markers of shelf-life for baked ham. The concentrations of these three aldehydes have an oscillatory trend over the time because they are continuously subjected to chemical transformations. However, in all meat samples, the concentration of aldehydes was lower in baked ham preserved in P1 and P2 with respect to that preserved in P3.

Among the alcohols, just 1-butanol and 2-octen-1-ol which were excluded in the previous study, have been considered in this one thanks to its constant trend over the time. The concentration of these alcohols had an oscillatory trend over the time and this is probably due to their transformation in some other compounds. However, in all meat samples, the concentrations of these compounds were lower in baked ham preserved in P1 and P2 with respect to that preserved in P3, with statistically significative differences. Among the ketones, acetoin, which has been excluded in the previous study, has been considered as a marker of shelf-life for baked ham in this one because its concentration increased over the time in all meat samples. In particular, the concentration of acetoin was lower in P1 and P2 with respect to P3, with statistically significative differences for P3 at t2, t6 and t9. Finally, the concentration of 2-pentylfuran was lower in P1 and P2 with respect to P3, with statistically significative differences for P3 at t9.

Non-considered compounds have a trend that is not homogeneous in all meat samples and that's because they cannot be considered markers of shelf-life for baked ham.

Figure 11 reported some of the volatile compounds used as markers of shelf-life for baked ham to better visualize their trend over the time in the different packaging.

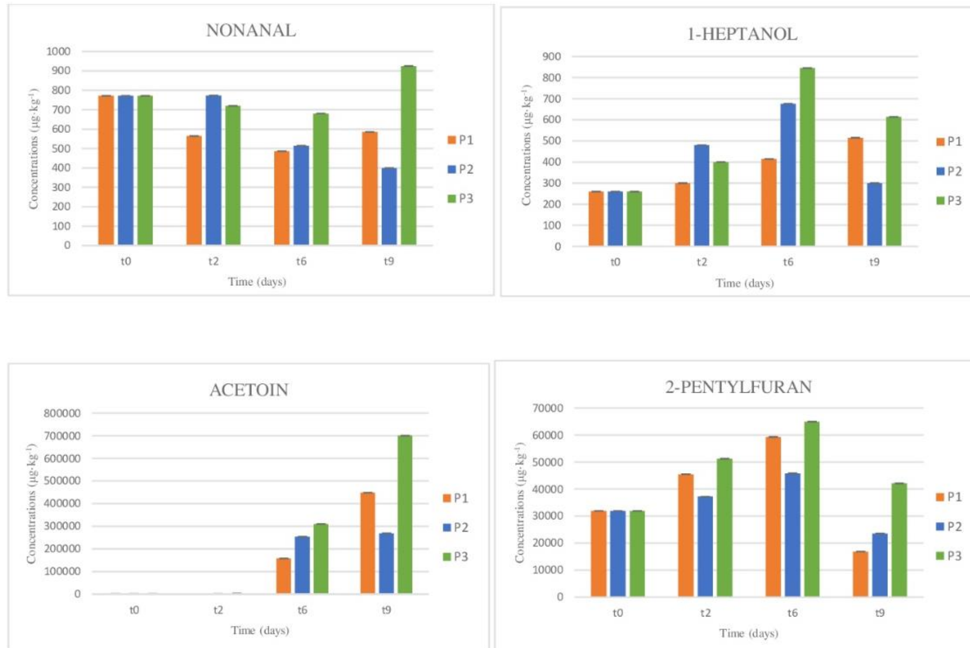


Figure 11: Concentrations (µg·Kg^{-1}) of some VOCs selected as markers of shelf-life for baked ham.

In conclusion, 12 VOCs have been considered in this study as markers of shelf-life for baked ham: hexanal, octanal, nonanal, 3-methylbutanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, 1-heptanol, linalool, 1-octanol, acetoin and 2-pentylfuran.

Results demonstrated that P1 and P2 have a higher ability in preserving baked ham with respect to P3. Regarding P1 and P2, the preservation ability of P1 was higher with respect to P2 for some of the compounds monitored.

2.3.3. Conclusions

This study demonstrated that P1 and P2 have a higher preservation ability for raw and baked hams with respect to P3. Regarding raw ham, the concentration of VOCs selected as markers of shelf-life was lower in P1 with respect to P2 suggesting a better preservation ability of P1 with respect to P2 until t6. Regarding baked ham, the concentration of VOCs selected as markers of shelf-life was lower in P1 with respect to P2 suggesting a better preservation ability of P1 with respect to P2 until t6 (with the exception of 2-pentylfuran).

In conclusion, *Ideabrill®* coupled to Imprigionagusto bag or Salvafreschezza bag turned out to be better than paper coupled with wings in the preservation of raw and baked hams.

The *Ideabrill®* formula resulted to be useful to prolong the shelf-life of raw and baked hams.

CHAPTER 3

INTRODUCTION: FOOD QUALITY AND SAFETY

3.1. The concept of food quality and safety

Worldwide, the interest in studying food quality and safety is continuously increasing [238, 239]. Food quality is related to internal (size, shape, colour, gloss and consistency) and external (chemical, physical and microbial) quality attributes of food, while food safety is related to handling, preparation and storage processes that can be responsible for foodborne illness [240].

Food quality and safety are monitored by human panel tests, chemical analytical measurements or mechanical methods. The human panel tests have been used for the study of wine-quality, tea-quality or diary-quality; however, this strategy is time consuming and has a certain degree of subjectivity due to the analyst [241]. For this reason, the identification and quantification of the compounds involved in food safety are performed through chemical analytical measurements, even if this strategy is expensive and lab-dependent because it needs instruments. The third strategy consists in the use of mechanical methods, that allow to detect foreign substances working on differences in mass (mechanical sieving), colour (optical methods) and surface density (ultrasonic detection) [240].

In this field, we focused the attention on the study of food quality and safety of coffee and bakery products (biscuits, wafer and panettone), using gas chromatography-mass spectrometry (GC-MS) analysis.

3.2. Study of quality and safety of coffee and bakery products

Coffee is one of the most popular beverages in the world because of its flavour and aroma and for its stimulating effects. As consequences, the interest in studying coffee quality is increasing not only to evaluate the flavour and aroma but also to assess its potential toxicological effects. During the roasting process, in fact, different thermal processes, such as Maillard reaction, caramelization of sugars, Strecker degradation, pyrolysis, occur in

coffee beans leading to the formation of several compounds responsible for the coffee sensory quality but also dangerous to human health [242].

Biscuits are one of the most consumed bakery products in the world thanks to their pleasant sensory properties and long shelf-life [243-245]. Their global consumption has increased by 31.6% during the COVID-19 outbreak [246]; thus, the importance of studying these products is constantly increasing.

Even though the preparation of biscuits is quite simple, as it consists of mixing the ingredients, shaping, baking and packaging [247, 248], several complex physical and chemical mechanisms occur during the baking process. In fact, it can be divided into three phases: expansion of the dough, drying of the surface and browning of the crust [249]. The baking process is mainly influenced by the time-temperature conditions and moisture of the system [250]. The major physical and chemical changes in biscuits [251] include volume expansion, evaporation of water and formation of a porous structure, heat and mass transfer processes, non-enzymatic browning, starch gelatinization, and protein denaturation [252-254]. All these changes are desirable and involved in the development of aroma, flavour, and colour of the final product. However, the two main non-enzymatic reactions, the Maillard reaction and caramelisation, also lead to the formation of some compounds that can be toxic to humans [255].

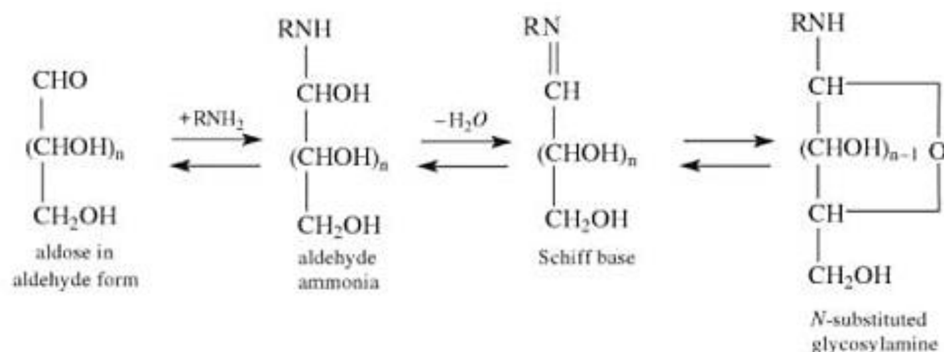
Among these, acrylamide and furanic compounds are some of the most studied: acrylamide is classified in the group 2A as a substance "probably carcinogenic to humans" and furan is classified as group 2B as a substance "possibly carcinogenic to humans, based on limited evidence of carcinogenicity in humans and sufficient evidence in experimental animals" by the International Agency for Research on Cancer (IARC). For example, 5-hydroxymethylfurfural (5-HMF) and furfuryl alcohol (FFA) are two of the most studied furanic derivatives and they result to be responsible for toxic effects on living organisms

because of their transformation in 2-sulfoxymethylfuran and 5-sulfoxymethylfurfural, respectively by sulfotransferases (SULT). These derivatives are considered as carcinogens because they can react with DNA or proteins and create mutations [256-258].

Acrylamide is formed mainly by condensation of amino group of amino acids (primarily asparagine) and carbonyl group of reducing sugars (e.g., glucose and fructose) during the Maillard reactions, at temperatures above 120 °C [259].

Furanic compounds are a class of heterocyclic organic compounds with five-membered-rings that are formed by the Maillard reaction that occurs during the roasting or baking processes. In fact, 5-HMF and furfural are formed by acid-catalysed dehydration of reducing sugars during heat processing [260], whereas the formation of furfuryl alcohol is related to different reactions, such as a retro-aldol cleavage of Amadori product formed in the Maillard reaction [261] or a α-dicarbonyl cleavage of 1,2-enaminol at low pH [262].

The Maillard reaction occurs during the cooking process and it can be divided under three steps: the early, the advanced and the final Maillard reaction [263]. The first step involves a carboxylic group of a reducing sugar with an N-terminal amino acid, leading to the formation of a glycosylamine whose bonds will rearrange to form an Amadori compound (**Figure 12a** and **b**). In this step, no coloured or perfumed compounds are formed, and lysine loss is the most important factor from a nutritional point of view.



a)

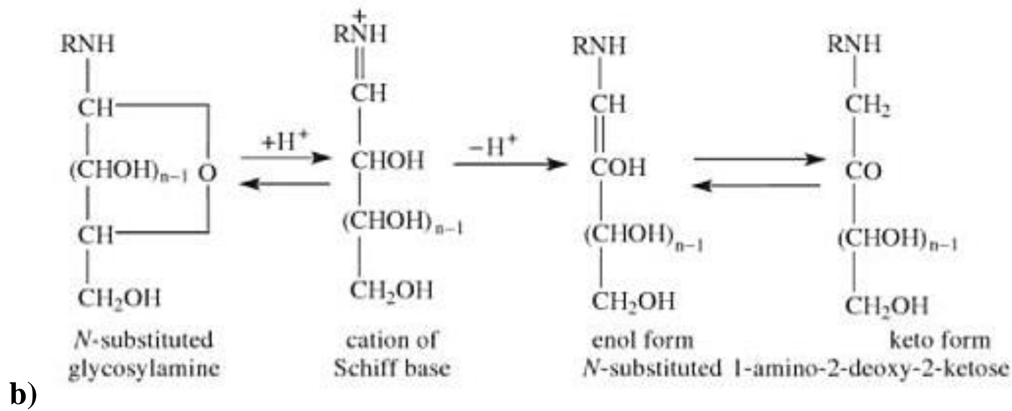


Figure 12: The first step of Maillard reaction: sugar amine condensation to form N-substituted glycosylamine (**a**) and Amadori rearrangement leading to the Amadori compound, the N-substituted 1-amino-2-deoxy-2-ketose (**b**). [264].

In the second step, several reactions may occur and lead to the formation of several compounds by splitting reactions of Amadori products. These reactions are influenced by the temperature and pH. The final step involves the compounds formed previously to form melanoidins, that are high molecular weight brown-yellow coloured compounds. Reactions are shown in **Figure 13**.

High temperature may lead to the formation of compounds like acrylamide and hydroxymethylfurfural from melanoidins.

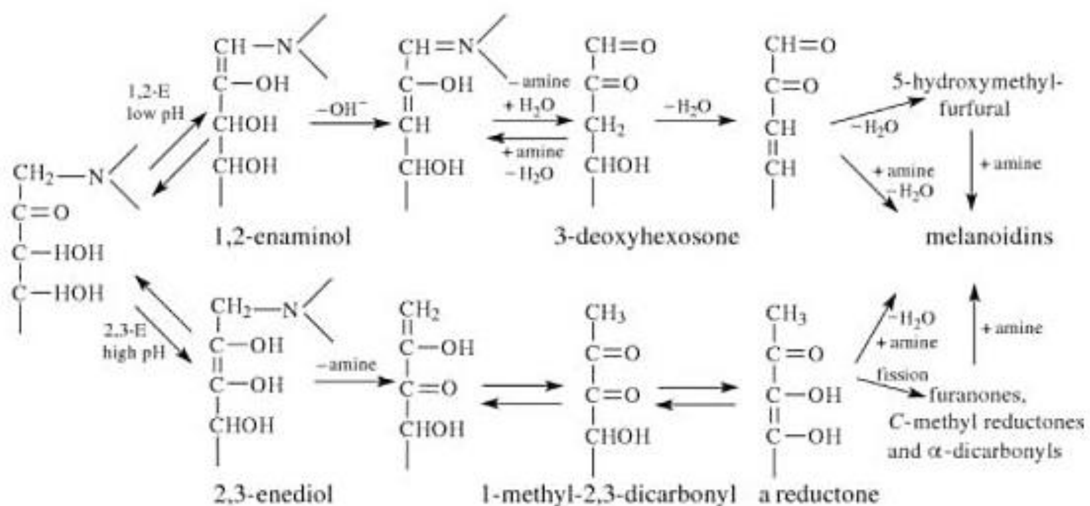


Figure 13: Maillard reactions: the two major pathways from Amadori compounds to melanoidins [264].

Furanic compouds form in various foods and drinks, such as coffee, fruit juices, baby foods, breakfast cereals, crackers, crispbreads, biscuits and they are responsible for the characteristic flavour of the final products as they have high volatility [265]. Interest in the study of this class of molecules is steadily increasing, as they are potentially toxic to human health.

Data on population exposure to furan and its derivatives have led researchers to focus the attention on these compounds. In fact, infants are exposed to the highest amount of furans, 70% of which come from ready-to-eat foods. On the other hand, coffee results to be the main source of responsible of exposure in adults (about 85%) [266].

In the Annex IV of the Commission Regulation (EU) 2017/2158 [267] are reported the benchmark levels for the presence of acrylamide in foodstuffs: a level of 400 µg kg^{-1} of acrylamide was established for roasted coffee. Although there is no specific reference level for furans to be respected in foods, it is important to maintain their concentration As Low as Reasonably Achievable (ALARA concept) [268].

In addition, the method of analysis for the determination of the furans in food should complies with specific criteria such as a limit of quantification (LOQ) not higher than 20 µg kg^{-1} (for coffee) (Commission Recommendation, 2022).

Several strategies have been studied in the last years in order to lower the levels of these chemical hazards so as to ensure the fitness of food for human consumption. As regards coffee, possible approaches to mitigate the concentration of these carcinogenic compounds are: selection of coffee varieties with a low precursor compounds content or optimization of roasting conditions to contain toxic compounds formation. Robusta coffee beans showed to have higher acrylamide content than Arabica due to higher levels of asparagine, the main limiting factor for the formation of acrylamide; the opposite trend took place for furanic derivates since it was noted that Arabica species contain higher levels than Robusta [269].

Instead, as far as roasting process, an increase in the intensity of roasting conditions led to a decrease of both acrylamide and furans concentrations: after reaching 175 °C their level quickly decreased [270, 271].

For what concern biscuits, instead, new strategies to reduce the production of harmful compounds (mainly acrylamide and 5-HMF) are referred to baking conditions, pre-treatments to improve baking and novel baking technologies.

Temperature, airflow and baking time during the baking process play a central role in the formation of toxic compounds. In fact, a higher temperature favours the formation of acrylamide and 5-HMF [272, 273]; while an increase in the airflow [274] or an increased baking time [275] reduces the production of acrylamide and 5-HMF because these strategies allows to reduce the baking temperature.

Ozone, plasma, pulsed electric field, high-pressure processing (HPP), UV irradiation, LED treatment and addition of phenolic compounds are the pre-treatments most used to improve the baking process. In fact, the pre-treatment of the wheat flour with ozone [276], plasma [277-280], pulsed electric field [281-283], HPP [284, 285], UV irradiation [286], LED treatment [287, 288], and phenolic compounds [276, 289, 290] reduce the formation of acrylamide and 5-HMF.

Finally, vacuum baking [291, 292], microwave baking [293], halogen lamp-microwave baking [294], infrared heating [71, 295, 296], power ultrasound baking [297, 298] and supercritical CO_2 extrusion process [299-301] are the novel baking technologies most used to reduce the formation of acrylamide and 5-HMF.

The aroma and the flavour of coffee and biscuits play a pivotal role in influencing consumers acceptability. Therefore, monitoring the development of compounds related to storage conditions and sensory quality, such as 3-methylbutanal and hexanal, is of crucial importance. Indeed, they are related to the food quality both for the incoming oxidative

processes and taste. In particular, 3-methylbutanal, which imparts a malty note, is a product of the oxidation of cholesterol and can be considered as a potential volatile oxidation marker for food products [302]. Hexanal is the product of lipid oxidation and is responsible for the rancid taste. It comes from the alteration of coffee during storage [303] and it is often used as a marker of secondary lipid oxidation [304, 305], as a food quality parameter for biscuits [306].

CHAPTER 4

STUDY OF QUALITY AND SAFETY OF COFFEE AND BAKERY PRODUCTS THROUGH GC-MS.

4.1. RESEARCH ACTIVITY: Impact of coffee variety, post-harvesting treatments and roasting conditions on coffee quality and safety related compounds.

Abstract

The aim of this study was to investigate the effect of coffee variety (Arabica and Robusta), post-harvesting methods (dry and wet process) and roasting conditions on concentrations of coffee safety-related compounds, such as acrylamide and furanic compounds, and volatile compounds related to coffee sensory quality, such as 3-methylbutanal and hexanal. Acrylamide content was determined through a HPLC-MS/MS, while as regards furanic compounds, 3-methylbutanal and hexanal, quantification was performed with a new HS-SPME-GC-MS method. Results showed that the concentration of acrylamide decreased with stronger roasting conditions, while the levels of 3-methylbutanal, hexanal and furanic compounds had an oscillatory trend when the roasting degree increased. The highest concentrations of acrylamide, hexanal and furanic compounds were found in dried Arabica coffee samples with respect to all other samples, while 3-methylbutanal was found in dried Robusta coffee samples. Moreover, wet-processing resulted to reduce the concentration of all monitored compounds. In conclusion, this study provided information about how to choose the proper variety, post-harvesting method and roasting degree in order to mitigate the formation of toxic compounds during coffee production, thus avoiding their high dietary intake, and to prevent high levels of off-flavours.

4.1.1. Materials and methods

4.1.1.1. Standards and chemicals

Sodium chloride (CAS 7647-14-5), sodium sulphate (CAS 7757-82-6), sodium hydrogen carbonate (CAS 144-55-8), sodium phosphate monobasic (CAS 7558-80-7), pure standards of 3-methylbutanal (CAS 590-86-3), hexanal (CAS 66-25-1), furfural (CAS 98-01-1), furfuryl acetate (CAS 623-17-6), 5-methylfurfural (CAS 620-02-0), furfuryl alcohol (CAS 90-00-0), 5-(hydroxymethyl)furfural (5-HMF) (CAS 67-47-0), 2-methylpentanal (CAS 123-15-9) and HPLC-grade ethanol were purchased from Sigma Aldrich (Milan, Italy). The stock solutions of target volatile compounds were prepared by mixing 10 mg of pure standards with 10 mL of HPLC-grade ethanol (1000 µg mL^{-1}). Standard working solutions at different concentration were prepared by diluting the stock solutions in ultrapure water. 2-Methylpentanal were used as internal standard (IS) and it was added in each standard solution at a concentration of 100 µg mL^{-1}. The calibration curve was prepared by plotting the standard solution concentrations by the respective Response Factor (RF). RF is the ratio between the peak area of analyte and the peak area of IS. Acrylamide (AA) (for molecular biology, ≥99% (HPLC), C_3H_5NO, molecular weight 71.08 g mol^{-1}, (CAS No 79–06-1) and 2,3,3-d_3-AA (AA-d_3, AA-d_3), standard solution 500 mg mL^{-1} in acetonitrile (analytical standard, CAS 122775-19-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of AA was prepared by dissolving the pure powder (10 mg) into 10 mL of ultrapure water and it was stored in a glass vial at −18 °C up to use. For AA quantitation AA-d_3 was used as internal standard (IS). Daily, standard working solutions at different concentrations were prepared by adequate dilution of the stock solutions in water and an exact aliquot of AA-d_3 at 5 µg mL^{-1} was combined with the standard working solutions of native AA. LC-MS grade acetonitrile was supplied by Sigma-Aldrich (Milano, Italy) and HPLC-grade formic acid (99%) was obtained from Merck (Darmstadt, Germany). Ultrapure

water was prepared by using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All other solvents and reagents were analytical grade. Two different cartridges for solid-phase extraction (SPE) were employed for sample clean-up such as Bond Elut-Accucat, 200 mg, 3 mL cartridges, from Agilent Technology (Santa Clara, CA, USA) and Oasis HLB 200 mg, 6 mL cartridges, from Waters (Milford, MA, USA). Before high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis each sample was filtered by Phenex™ RC 4 mm, 0.2 µm syringeless filter, Phenomenex (Castel Maggiore, BO, Italy). Captiva PTFE 13 mm, 0.45 µm syringeless filter was bought from Agilent Technology (Santa Clara, CA, USA).

4.1.1.2. Coffee samples

Arabica (*Coffea arabica* L., Brazil, Santos) and Robusta (*Coffea canephora* var. Robusta, India) coffee cherries were used in this study. Each coffee variety underwent two different post-harvesting treatments: dry (D) and wet (W). Green coffee beans were supplied by "Ing. Napoli & C. Industrie Riunite srl" (Lavello, Potenza, Italy) and roasted with a "Tostabar Genius K3" roaster, designed and built by the company. Coffee samples were roasted at five different roasting degrees: Cannella (A), City (B), Full-city (C), Italiana (D) and Napoletana (E). ***Table 18*** shows the information of twenty coffee samples used in this study and the roasting conditions adopted for each roasting degree. Moreover, eight different commercial ground coffee samples were purchased in a local store and their reported features are shown in ***Table 19***. The study of commercial ground coffee samples aimed also at monitoring the concentration of compounds responsible for coffee sensory quality and safety in real coffee samples.

Table 18. Composition and information of coffee samples.

Sample ID	Variety	Post-harvesting	Roasting degree	Final temperature	Roasting time (min)
1WA	Arabica	Wet-processed	Cannella	206 °C	11.26
1WB	Arabica	Wet-processed	City	208 °C	12.42
1WC	Arabica	Wet-processed	Full-City	210 °C	13.26
1WD	Arabica	Wet-processed	Italiana	220 °C	14.34
1WE	Arabica	Wet-processed	Napoletana	230 °C	16.47
1DA	Arabica	Dry-processed	Cannella	206 °C	11.26
1DB	Arabica	Dry-processed	City	208 °C	12.42
1DC	Arabica	Dry-processed	Full-City	210 °C	13.26
1DD	Arabica	Dry-processed	Italiana	220 °C	14.34
1DE	Arabica	Dry-processed	Napoletana	230 °C	16.47
2WA	Robusta	Wet-processed	Cannella	206 °C	11.26
2WB	Robusta	Wet-processed	City	208 °C	12.42
2WC	Robusta	Wet-processed	Full-City	210 °C	13.26
2WD	Robusta	Wet-processed	Italiana	220 °C	14.34
2WE	Robusta	Wet-processed	Napoletana	230 °C	16.47
2DA	Robusta	Dry-processed	Cannella	206 °C	11.26
2DB	Robusta	Dry-processed	City	208 °C	12.42
2DC	Robusta	Dry-processed	Full-City	210 °C	13.26
2DD	Robusta	Dry-processed	Italiana	220 °C	14.34
2DE	Robusta	Dry-processed	Napoletana	230 °C	16.47

Table 19. Information of eight commercial ground coffee samples (Medium± 215°C; Dark±246°C; NR: Not Reported).

Sample ID	Variety	Roasting degree
KIMBO	NR	Dark
LAVAZZA	Arabica	Medium
SEGAFREDO	Arabica	Medium
HAG	Arabica	Medium
ROMCAFFE’	Arabica/Robusta	Medium
COOP	Arabica	NR
ILLY	Arabica	NR
DON JEREZ	Arabica	NR

4.1.1.3. Sample preparation

Roasted coffee beans were stored in sealed containers in dark and at room temperature for 14 days. Before each analysis, roasted coffee samples were grounded with a “Rancilio Rocky” coffee grinder (Rancilio Group spa, Milan, Italy) consisting of a flat grinding blades of diameter 50 mm. The grinder had 40 levels of grinding, 0 for the coarsest point level and 40 for the finest.

4.1.1.4. Acrylamide quantification by HPLC-MS/MS system

The quantification of acrylamide in different coffee samples was performed following a previous developed and validated procedure [259]. Briefly, the instrument employed was an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source operating in positive ionization mode. The column used for the present analytical method was a Kinetex Hilic analytical column (100 mm × 4.6 mm i.d., particle size 2.6 µm) from Phenomenex (Torrance, CA, USA) preceded by a KrudKatcher ULTRA HPLC In-Line Filter (2.0 µm Depth Filter × 0.004 in I.D.). The mobile phase was composed of water (A) and acetonitrile (B) both with 0.1% of formic acid and the separation has been obtained at 0.8 mL min^{-1} in gradient elution mode. The mobile phase varied as following: 0–2.5 min, 85% B; 2.5–3.5 min, 85–70% B; 3.5–5.5 min, 70% B; 5.5–6.5 min, 70–60% B; 6.5–10 min, 60% B. The injection volume was 2 µL, the temperature of the drying gas in the ionization source was kept at 350 °C, the gas flow was 12 L min^{-1}, the nebulizer pressure was 45 psi and the capillary voltage was set at 4000 V. The acquisition was performed in “Selected Reaction Monitoring” (SRM) mode: ***Table 20*** reports the acquisition parameters optimized including precursor ion, product ion, fragmentor, collision energy and retention time.

Table 20. HPLC-MS/MS acquisition parameters optimized for acrylamide quantitation.

Compound	**Precursor Ion (*m/z*)**	**Product Ion (*m/z*)**	**Fragmentor (V)**	**Collision Energy (V)**	**Retention Time (min)**	**Polarity**
Acrylamide	72	55	45	8	1.52	Positive
Acrylamide-d_3	75	58	45	8	1.52	Positive

The extraction was performed following a previous work by Schouten et al. (2021) [259] with some adjustments. Briefly, 1 g of ground coffee was fortified with the internal standard, i.e., AA-d_3 (1 mL at 500 ng mL^{-1}), and the fortified coffee powder was extracted adding other 9 mL of water. Then, the sample was agitated with a vortex mixer for 30 s and the acrylamide extraction was carried out by keeping the sample in a water bath under magnetic stirring at 80 °C for 30 min. After centrifugation at 5000 rpm for 10 min, the sample was filtered with a 0.45 µm filter and purified using two different SPE cartridges. The first cartridge was the Oasis HLB. This was initially conditioned with 3.5 mL of methanol and then with 3.5 mL of water. 1.5 mL of sample were loaded onto cartridge followed by 0.5 mL of water. The sample was allowed to pass completely through the sorbent material. For AA elution, water (1.5 mL) was added onto the cartridge and the eluent was collected in a 3 mL glass vial. Before conditioning the second SPE column, a mark was placed on the outside of the cartridge at a height equivalent to 1 mL of liquid above the sorbent bed. The Bond Elut-Accucat column was conditioned with 2.5 mL of methanol followed by 2.5 mL of water. The solvents used for conditioning were then discarded. The eluent collected from the first cartridge was added to the Bond Elut-Accucat cartridge. The sample was allowed to eluate from the column up to the mark previously placed on the outside; the eluent was then collected into a 6 mL glass vial. Lastly, the samples were filtered with a 0.2 µm filter and injected into HPLC-MS/MS.

4.1.1.5. Headspace - solid phase microextraction (HS-SPME)

Furanic compounds were determined using a headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS). Immediately after the packaging opening, 1 g of ground coffee sample was weighted into a 20 mL vial and rapidly the internal standard 100 μg mL^{-1} was added into. Immediately after, the vial was sealed with a screw cap with a PTFE/silicon septum. The influence of the extraction temperature and time, incubation time, the type of fibre coating and the salt addiction on the adsorption of the VOCs were evaluated. In fact, three extraction temperatures (50, 60, 70 °C), three incubation times (10, 15 and 25 min), three extraction times (20, 30 and 40 min), three types of fibre coatings (DVB/C-WR/PDMS 80 μm, PDMS/DVB 65 μm and PDMS 30 μm) and four types of salt (Na_2SO_4, NaCl, NaH_2PO_4 and $NaHCO_3$) were evaluated. The incubation of the sample was performed at 60 °C for 15 min under agitation (250 rpm, 5 s of on-time and 2 s of off-time). The grey fibre (DVB/C-WR/PDMS) from Supelco (Bellefonte, PA, USA) was selected for this work. The fibre was conditioning for 10 min at 250 °C and then it was inserted inside the headspace of sample vial with a speed of 20 mm s^{-1} and a penetration depth of 40 mm. The extraction was performed at 60 °C for 30 min and then the fibre was inserted into injector port at a speed of 100 mm s^{-1} and a penetration depth of 40 mm. The desorption occurred at 250 °C for 1 min. After desorption, the fibre was conditioning at 250 °C for 10 min.

4.1.1.6. GC-MS analysis

The GC-MS analysis was carried out using an 8890 gas chromatograph (GC) from Agilent equipped with a PAL RTC 120 autosampler and a 5977B mass spectrometer (MSD) Agilent (Santa Clara, CA, USA). The ionization was obtained by using an electron ionization source (EI). The injector temperature was set at 250 °C and the liner used was recommended for SPME injection namely Inlet liner, Ultra Inert, splitless, straight, 0.75 mm id, (5190-4048)

from Agilent. The gas carrier was helium at flow rate of 1 mL min^{-1}. The separation of target molecules was established on DB-WAX capillary column (60 m, 250 µm i.d., 0.25 µm film thickness) with the following ramp of temperature: 35 °C held for 3 min, 35-200 °C at 5 °C/min and then 250 °C at 15 °C/min held for 5 min. The run time was about 44.3 min. The transfer line was set at 250 °C and the temperature of the ionization source and the mass analyser were set at 230 and 150 °C, respectively. The ion species selection and optimization for all the volatile compounds were carried out by injecting a standard solution (10 µg mL^{-1}) in SCAN mode (35-450 m/z) and the three most abundant ions were selected for each analyte. The acquisitions were carried out in 'Selected Ion Monitoring' (SIM) mode and detection was divided into time windows to enhance the sensitivity. The most abundant ions were used for quantitation while the other to confirm the presence of the analytes. The GC-MS parameters including the retention time (Rt) and time windows are reported in ***Table 21***. Data results were managed by MSD ChemStation Software (Agilent, Version G1701DA D.01.00). Samples were analysed in duplicate. Acceptable relative standard deviation (% RSD) were set up below 20%.

Table 21. GC-MS parameters used for the analysis.

Compound	Time window (min)	Ion (m/z)	Retention time (min)
3-methylbutanal	0-12.20	58[a], 44, 71	8.5
2-methylpentanal	0-12.20	58[a], 71, 43	11.0
Hexanal	12.20-20.00	56[a], 44, 72	13.2
Furfural	20.00-25.20	96[a], 95, 39	24.3
Furfuryl Acetate	25.20-26.60	81[a], 98, 140	26.2
5-methylfurfural	26.60-28.20	110[a], 109, 53	27.2
Furfuryl alcohol	28.20-35.00	98[a], 81, 41	29.1
5-HMF	35.00-44.33	97[a], 126, 41	43.3

[a] These ions were used for quantitation, the other without any superscript letters for qualification.

4.1.1.7. Colorimetric analysis

Colour was determined using a Konica-Minolta CR-400 model chromameter (Minolta Camera Co, Osaka, Japan). Colour was expressed by CIELab parameters (Commission Internationale l'Eclairage): L* in the brightness; a* is the green-red component; b* is the blue-yellow component. The measurements were made in triplicate placing 5g of ground coffee on a watch glass.

4.1.1.8. Statistical analysis

Statistical analysis was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France). Data were expressed as the mean ± standard deviation. A three-factor analysis of variance (ANOVA) was selected to investigate the effect of independent variables and interactions among them on dependent variables (acrylamide, furanic compounds, 3-methylbutanal and hexanal content). Coffee variety (Arabica and Robusta), post-harvest treatment (wet and dry process) and roasting degree (Cannella, City, Full-city, Italiana, Napoletana) were fixed as independent variables. Tukey's HSD (Honestly Significantly Different) test was computed for post hoc analysis, in order to identify means that are significantly different from each other at 95% confidence interval.

4.1.2. Results and Discussion

4.1.2.1. Impact of variety, post-harvesting method and roasting degree on acrylamide content

Acrylamide content is related to the presence of its two main precursors present in green coffee beans: free asparagine and sucrose. Previous studies demonstrated that there is a weak positive correlation between asparagine content of green coffee beans and acrylamide level in roasted samples. On the contrary, no significant correlation between sucrose and acrylamide content was proven. Therefore, asparagine content is the main limiting factor for acrylamide development during roasting process [259, 271, 307-309]. In general, Robusta variety has higher asparagine (and hence acrylamide content) and lower sucrose content rather than Arabica one. In contrast with earlier findings [271, 307], in this study Arabica samples showed a significantly higher acrylamide content ($p<0.05$) than Robusta ones (**figure 14A**). This unexpected result could be derived from a different reduced sugars content (mainly glucose and fructose) in green coffee beans, due to a different agricultural practices or different post-harvesting method.

Post-harvesting treatments are related to the cleaning and elimination of defective or immature beans and this step can affect the coffee quality. In dry processing, coffee cherries are spread out and sun-dried for 3-9 days and then the dried pulp is removed together with parchment skin by a peeling machine. In wet processing, instead, the pulp is mechanically removed and beans are subjected to a controlled water flow and left for the fermentation for 12-48 h and finally the parchment skin is removed by a peeling machine [310]. In general, dry-processed beans have higher acrylamide content than wet-processed ones [271, 309]. Wet process, in fact, consists in depulping of green coffee beans resulting in reduction in asparagine content and, as a result, in acrylamide content of final product [309]. In addition, wet and dry-processed coffee beans have different levels of reduced sugars both as a result

of a reduction in glucose and fructose content due to the fermentation and leaching process to which wet-samples are submitted [71, 311, 312] but also due to an increase in glucose and fructose content during the drying process [312, 313]. The impact of the post-harvesting method on the asparagine and sugars content of coffee beans could explain the difference in acrylamide content between wet and dry-processed samples. Results from this study are in line with previous findings, since wet-processed samples demonstrated a significantly lower acrylamide content ($p<0.05$) than dry-processed ones. In addition, as reported in **figure 14B**, the dry-processed Arabica samples showed a higher acrylamide content than the other samples highlighting the significant effect ($p<0.05$) of the interaction between variety and post-harvesting method on acrylamide content.

Roasting conditions, such as high temperatures and low moisture content, promote the development of compounds important to the flavour and colour of roasted coffee. However, some of them, including acrylamide, have a negative influence on human health. It was demonstrated that also the compound 3-aminopropionamide (3-APA) is a precursor for acrylamide development [314]. It is the biogenic amine of the asparagine, which originates by degradation in the presence of glucose. The conversion of asparagine in 3-APA during heating processes is more incisive and prevalent than enzymatic conversion. This is the reason why this compound is considered the main intermediate compound in the acrylamide formation during the roasting process of coffee [315]. In addition, chlorogenic acid improves the formation of acrylamide as a result of the development of 5-hydroxymetylfurfural (5-HMF), an acrylamide precursor [316]. As shown in **figure 14C**, acrylamide content decreased with the increase in the roasting temperature. This result confirmed previous findings in the literature [259, 307, 308, 317, 318]. Acrylamide content was higher under low-temperature and short-time roasting conditions (Cannella and City, for example). As the roasting temperature and time increased, the level of acrylamide decreased until no longer

detected. This is due to the fact that asparagine is thermally degraded very quickly without further transformation [307]. All experimental samples, except for the sample 1DB, had an acrylamide content below the safe threshold of 0.4 μg g^{-1} of acrylamide established by IARC for roasted coffee. Acrylamide content of twenty coffee samples is reported in ***Table 22***.

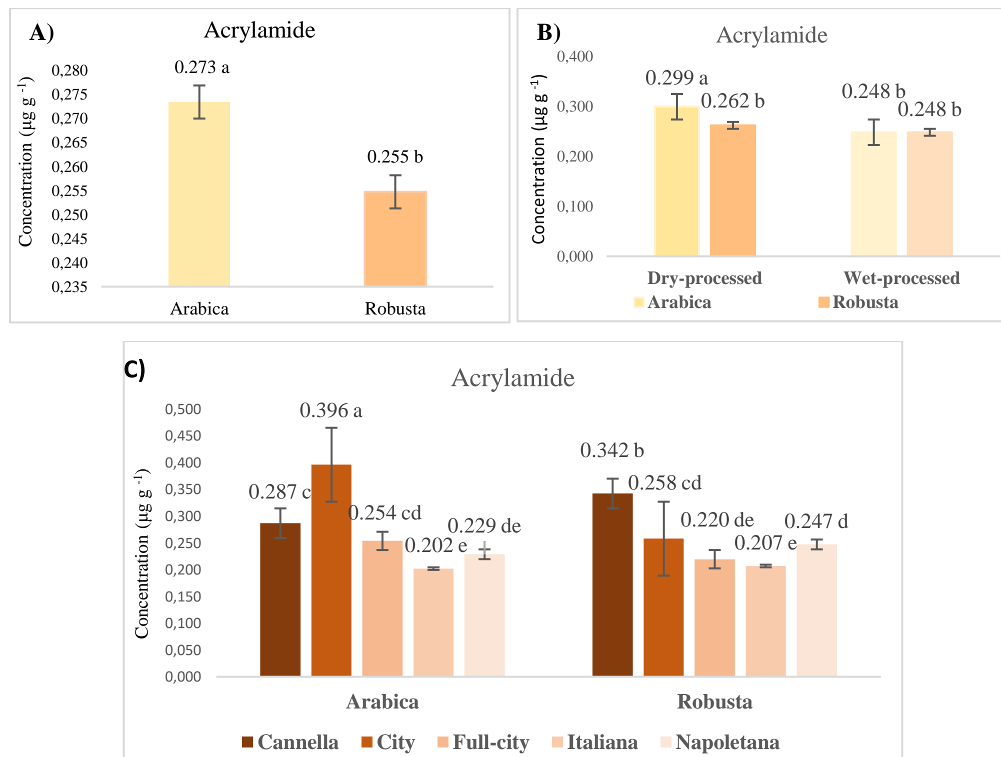


Figure 14: A) Acrylamide content in Arabica and Robusta ground coffee samples; **B)** Effect of the interaction between variety and post-harvesting method on acrylamide content; **C)** Acrylamide content (μg g^{-1}) in Arabica and Robusta sample for each roasting degree.
Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

Table 22. Acrylamide content of twenty experimental ground coffee samples.

SampleID	Acrylamide content (µg/g)
1DB	0.420±0.018^{a}
1WB	0.372±0.015^{b}
2WA	0.363±0.024^{b}
1DA	0.326±0.012^{c}
2DA	0.322±0.014^{c}
2DB	0.282±0.009^{d}
1DE	0.274±0.022^{de}
1DC	0.259±0.014^{def}
2DE	0.257±0.019^{defg}
1WC	0.248±0.015^{efgh}
1WA	0.248±0.014^{efgh}
2WE	0.237±0.010^{fghi}
2WB	0.234±0.017^{fghi}
2DC	0.226±0.015^{ghi}
2DD	0.221±0.023^{hij}
1DD	0.216±0.008^{hijk}
2WC	0.213±0.009^{ijkl}
2WD	0.193±0.016^{jkl}
1WD	0.188±0.008^{kl}
1WE	0.184±0.012^{l}

Data are expressed as mean ± standard deviation. Different letters in the same column indicate significant difference ($p < 0.05$), following pairwise comparison by Tukey's HSD test.
1=Arabica; 2=Robusta; D=dry-process; W=wet-process;
A= Cannella; B= City; C= Full-City; D= Italiana; E= Napoletana.

4.1.2.2. HS-SPME-GC-MS analytical method optimization and validation

A new analytical method for the quantification of 7 volatile organic compounds (VOCs) in ground coffee samples was developed using a HS-SPME-GC-MS system. HS-SPME technique is one of the most common analytical approaches for the VOCs analysis because it is simple, cheap, solvent-free, easy to handle and very sensitive. For these reasons, it was selected for the quantification of five furanic compounds together with hexanal and 3-methylbutanal in different coffee samples. Three fibres were evaluated in their capacity to extract target volatile compounds from coffee matrix: a divinylbenzene-polydimethylsiloxane fibre (DVB/PDMS), a divinylbenzene/carbon-wide range/polydimethylsiloxane fibre (DVB/C-WR/PDMS) and a polydimethylsiloxane

(PDMS) fibre. The use of DVB/C-WR/PDMS fibre gave the highest total peak area therefore it was selected for the present study. The effect of salt addiction was also evaluated by adding 3 mL of saturated solutions of four types of salt: sodium chloride (NaCl), sodium sulphate (Na_2SO_4), sodium hydrogen carbonate ($NaHCO_3$), and sodium phosphate monobasic (NaH_2PO_4). Results showed that the addiction of salts did not improve the extraction efficiency hence samples were analysed without salt adding. Three incubation times (10, 15 and 25 min), three extraction temperatures (50, 60, 70 °C) and three extraction times (20, 30 and 40 min) were tested. Results showed that 15 min of incubation at 60 °C and 30 min of extraction time were the best conditions for this analysis. ***Table 23*** summarizes all the monitored and optimized parameters.

Table 23. Summary of monitored parameters for the optimization of HS-SPME technique with values of total peak areas.

Parameters		**Total peak area**
Fiber	DVB/C-WR/PDMS	$1x10^{8\ a}$
	DVB/PDMS	$6.9x10^{7\ b}$
	PDMS	$2.6x10^{7\ c}$
Salt addiction	Na_2SO_4	$7.4x10^{7\ a}$
	NaCl	$9.6x10^{7\ b}$
	NaH_2PO_4	$7.7x10^{7\ c}$
	$NaHCO_3$	$5.3x10^{7\ d}$
Temperature	70 °C	$4.6x10^{7\ a}$
	60 °C	$1x10^{7\ b}$
	50 °C	$5.4x10^{7\ c}$
Incubation time	10 min	$5.8x10^{7\ a}$
	15 min	$1.1x10^{8\ b}$
	25 min	$4.7x10^{7\ c}$
Extraction time	20 min	$4.1x10^{7\ a}$
	30 min	$1.1x10^{8\ b}$
	40 min	$4.7x10^{6\ c}$

[a]Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

These results indicated that 1 g of ground coffee sample, incubated for 15 min at 60 °C and extracted for 30 min with a DVB/C-WR/PDMS fibre were the most performing conditions for this type of analysis. Chromatographic separation was characterized by good resolution for all nearby peaks and, as an example, **figure 15** shows the chromatogram of a standard mixture of the 7 VOCs quantified using HS-SPME-GC-MS technique.

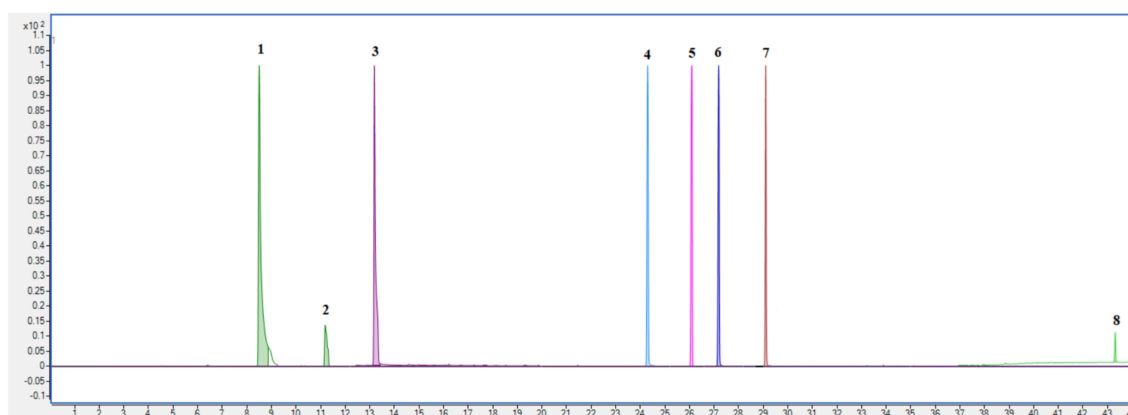


Figure 15: Chromatogram of all monitored compounds acquired in Selected Ion Monitoring (1: 3-methylbutanal, 2: 2-methylpentanal; 3: hexanal; 4: furfural; 5: furfuryl acetate; 6: 5-methylfurfural; 7: furfuryl alcohol; 8: 5-HMF).

The newly developed analytical method was validated by studying linearity, sensitivity and repeatability (***Table 24***). Linearity was assessed by injecting five different concentrations of 7 VOCs and plotting the calibration curves with the respective determination coefficients (R^2). All compounds showed a good linearity as the R^2 was equal to or greater than 0.990. Repeatability was expressed as relative standard deviation (% RSD) and intra-day repeatability was assessed by injecting 5 replicates of mix standards five times in a day while inter-day repeatability was 3 replicates of mix standards once a day for three days. The intra-day repeatability ranged from 3.9 to 18.3% while inter-day repeatability was 5.4−19.9% for all target volatile compounds.

Table 24. Validation parameters: equation of the calibration curve, linearity, LOQ, LOD, Intra-day and Inter-day repeatability.

Compounds	**Equation of the Calibration Curve**	**Linearity (R^2)**	**LOQ (µg L^{-1})**	**LOD (µg L^{-1})**	**Intra-day repeatability (RSD%)**	**Inter-day repeatability (RSD%)**
3-methylbutanal	y=0.0108x - 0.0237	0.9960	0.012	0.004	13.1	19.4
Hexanal	y=0.0136x + 0.0338	0.9956	0.016	0.005	5.4	5.4
Furfural	y=0.0169x + 0.0918	0.9980	0.005	0.001	18.3	19.9
Furfuryl acetate	y=0.0191x + 0.1115	0.9906	0.06	0.02	16.9	19.1
5-methylfurfural	y=0.0138x + 0.5735	0.9900	0.009	0.003	9.5	19.8
Furfuryl alcohol	y=0.0004x + 0.0581	0.9978	0.145	0.048	3.9	18.4
5-HMF	y=0.0002x - 0.0043	0.9959	0.155	0.052	16.9	18.4

4.1.2.3. Volatile organic compounds (VOCs) quantification by HS-SPME-GC-MS

Coffee samples were analysed through a HS-SPME-GC-MS method to quantify 7 VOCs: 3-methylbutanal, hexanal and 5 furanic compounds (furfural, furfuryl acetate, 5-methylfurfural, furfuryl alcohol and 5-HMF). Firstly, a content screening of these seven VOCs was also performed in eight commercially available coffee samples to evaluate the concentration of these compounds in real coffee samples. Concentrations of all the monitored volatile compounds in commercial coffee samples were reported in ***Table 25***.

Table 25. Summary of the concentrations of all volatile compounds in commercial coffee samples. Concentrations were expressed in mg kg^{-1}. Data are expressed as mean ± standard deviation.

Samples	3-methyl butanal	Hexanal	Furanic compounds					Sum of furanic compounds	Sum of total
			Furfural	Furfuryl acetate	5-methyl furfural	Furfuryl alcohol	5-HMF		
Kimbo	44.4± 4.0^{abf}	0.03±0.0^{ad}	509.9±9.6^{ag}	385.0±7.2^{ad}	753.4±9.5^{ae}	467.7±517.3^{ac}	350.0±51.0^{af}	2466.0 ±594.6	2510.4±598.7
Lavazza	63.4±0.5^{ace}	0.08±0.0^{bd}	695.4±25.7^{bf}	310.1±13.9^{ab}	899.0±34.4^{a}	396.6±1330.5^{a}	559.5±13.2^{b}	2860.6±1391.4	2924.1±1391.8
Segafredo	39.2±1.5^{abdf}	0.11±0.0^{bf}	725.6±16.9^{b}	264.1±5.8^{b}	903.2±25.6^{a}	395.7±994.0^{a}	553.4±65.9^{b}	2842.0±1108.2	2881.3±1106.7
Hag	77.7±9.4^{ce}	0.18±0.0^{c}	976.7±11.3^{c}	252.5±2.9^{b}	1126.4±6.9^{b}	462.2±522.2^{ac}	737.6±66.0^{c}	3555.4±471.4	3633.3±480.9
Romcaffè	20.0±1.4^{bdf}	0.10±0.0^{bf}	295.6±8.9^{dg}	210.3±0.7^{b}	444.1±8.6^{c}	351.9±1159.1^{a}	124.4±14.1^{de}	1426.3±1163.4	1446.4±1161.9
Coop	18.4±3.6^{df}	0.03±0.0^{a}	150.0±29.8^{e}	86.9±17.3^{c}	207.4±41.1^{d}	127.5±2544.4^{b}	103.9±20.8^{e}	675.7±2653.4	694.1±2656.9
Illy	71.2±13.9^{e}	0.06±0.0^{d}	570.6±63.3^{f}	424.6±53.7^{d}	918.4±107.2^{ab}	594.7±6771.9^{c}	274.4±15.0^{fd}	2782.7±7011.2	2854.0±7025.1
Don Jerez	22.0±0.1^{f}	0.12±0.0^{f}	412.2±59.1^{g}	391.8±51.7^{ad}	627.7±88.5^{ce}	585.9±8614.5^{c}	159.9±14.1^{ade}	2177.5±8827.8	2199.6±8827.7

Concentration of 3-methylbutanal in commercial samples was between 18.4-77.7 mg kg^{-1}, for hexanal between 0.03-0.2 mg kg^{-1}, for furfural between 150-976.7 mg kg^{-1}, for 5-methylfurfural between 207.4-1126.4 mg kg^{-1} and for 5-HMF between 103-737 mg kg^{-1}. The highest concentration of these compounds was found in Hag coffee sample. This result agrees with data obtained in other studies, in which the concentration of 5-HMF was between 23.3–4112 mg kg^{-1} in ground commercial Colombian coffee samples [319] and between 24-2186 mg kg^{-1} in ground Spanish coffee samples [320]. Concentration of furfuryl acetate was between 86.9-424.6 mg kg^{-1} and for furfuryl alcohol between 12746.9-59473.0 mg kg^{-1}. The highest concentrations of these compounds were found in Illy coffee sample. The effect of coffee variety, post-harvesting methods and different roasting degrees on concentration of these compounds in twenty experimental samples was evaluated. The concentrations of all monitored volatile compounds in experimental coffee samples were summarized in ***Table 26***.

Table 26. Summary of the concentrations (mg kg^{-1}) of all volatile compounds in twenty experimental coffee samples. Values that don't share the same letter in the same column indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.
1=Arabica; 2=Robusta; D=dry-process; W=wet-process; A= Cannella; B= City; C= Full-City; D= Italiana; E= Napoletana

			Furanic compounds					
Sample ID	**3-methyl butanal**	**Hexanal**	**Furfural**	**Furfuryl acetate**	**5-methylfurfural**	**Furfuryl alcohol**	**5-HMF**	**Sum of furanic compounds**
1 DA	14.24±0.67^{a}	0.10±0.01^{a}	594.2±66.8^{ab}	36.1±4.2^{ac}	350.2±42.6^{ab}	321.6±3617.5^{a}	2736.8±359.2^{ab}	4038.9±4.1
1 DB	16.87±1.27^{a}	0.14±0.00^{a}	807.7±32.7^{a}	41.5±1.6^{abc}	490.5±18.9^{a}	359.2±1059.9^{a}	3216.2±110.7^{a}	4915.1±1
1 DC	14.63±2.05^{a}	0.62±0.10^{a}	752.5±110.6^{ab}	63.7±9.9^{ab}	567.3±89.2^{a}	413.0±5621.3^{a}	1971.9±286.6^{b}	3768.4±6.1
1 DD	16.03±1.70^{a}	0.11±0.01^{a}	517.7±80.4^{b}	67.4±10.4^{b}	426.9±72.5^{a}	300.5±4595.9^{a}	1038.7±204.8^{c}	2351.2±4.9
1 DE	9.22±0.88^{a}	0.08±0.01^{a}	166.7±16.7^{c}	33.6±4.5^{c}	137.0±18.2^{b}	139.4±1472.3^{b}	270.5±32.2^{c}	747.2±1.5
Average value	14.2	0.21	567.7	48.5	394.4	306.7	1846.8	3164.2
1 WA	17.72±0.87^{a}	0.16±0.01^{a}	304.3±18.4^{a}	69.6±5.1^{ab}	189.0±15.1^{a}	234.8±1465.0^{ab}	574.4±72.9^{a}	1372.1±1.6
1 WB	12.40±2.47^{ab}	0.06±0.01^{b}	397.4±77.5^{a}	27.4±5.2^{a}	220.9±43.1^{a}	137.7±2687.3^{a}	1110.9±214.9^{b}	1894.3±3.0
1 WC	15.80±1.62^{ab}	0.08±0.02^{bc}	453.8±87.5^{a}	69.2±13.2^{ab}	368.6±71.5^{a}	229.7±4569.6^{ab}	666.2±128.7^{ab}	1787.5±4.9
1 WD	10.22±0.29^{b}	0.07±0.00^{b}	214.4±0.5^{a}	76.1±0.1^{b}	214.2±0.4^{a}	209.5±153.9^{ab}	229.0±10.2^{a}	943.2±0.2
1 WE	14.11±1.61^{ab}	0.13±0.02^{ac}	328.1±65.0^{a}	105.1±20.9^{b}	309.1±61.4^{a}	353.5±6851.05^{b}	315.6±61.9^{a}	1411.4±7.1
Average value	14.0	0.10	339.6	69.5	260.4	233.04	579.2	1481,7
2 DA	20.96±0.76^{a}	0.19±0.00^{a}	404.6±9.8^{a}	57.3±0.8^{a}	308.8±8.2^{a}	288.3±701.9^{a}	816.9±55.2^{a}	1875.9±0.8
2 DB	18.82±3.65^{a}	0.15±0.03^{a}	381.9±74.6^{a}	60.5±11.9^{a}	325.5±64.9^{a}	266.5±5203.3^{a}	582.8±116.3^{ab}	1617.2±5.5
2 DC	21.46±0.46^{a}	0.22±0.01^{a}	309.0±9.6^{ab}	52.7±0.9^{a}	311.5±9.7^{a}	327.5±709.6^{a}	323.1±49.9^{b}	1323.8±0.8
2 DD	24.44±4.74^{a}	0.15±0.03^{a}	290.7±57.9^{ab}	63.7±12.7^{a}	292.3±58.1^{a}	340.4±6752.2^{a}	226.9±44.8^{c}	1214.0±6.9
2 DE	17.95±3.59^{a}	0.04±0.01^{b}	166.5±33.2^{b}	44.5±8.8^{a}	160.8±28.5^{a}	210.6±4187.3^{a}	184.4±36.6^{c}	766.8±4.3
Average value	20.7	0.15	310.5	55.7	279.8	286.66	426.8	1359.5
2 WA	13.61±0.08^{a}	0.12±0.00^{ac}	286.1±0.6^{ab}	19.2±0.1^{a}	216.7±1.2^{ab}	165.8±121.7^{a}	521.6±20.1^{a}	1209.4±0.1
2 WB	13.39±0.02^{a}	0.08±0.00^{ab}	122.9±0.2^{a}	30.8±0.3^{a}	98.0±0.3^{a}	155.8±152.1^{a}	135.9±1.9^{b}	543.37±0.1
2 WC	11.86±1.04^{a}	0.07±0.01^{b}	180.8±34.7^{a}	25.7±5.1^{a}	172.8±39.8^{a}	161.5±2806.0^{a}	162.5±35.0^{b}	703.3±2.9
2 WD	16.57±1.03^{ab}	0.08±0.00^{ab}	208.3±16.5^{a}	57.2±4.6^{a}	206.9±20.1^{ab}	280.1±2098.1^{b}	148.3±2.6^{b}	900.8±2.1
2 WE	24.38±4.36^{b}	0.16±0.03^{c}	418.6±82.7^{b}	104.5±20.8^{b}	354.7±70.5^{b}	264.0±5103.8^{ab}	445.3±86.2^{a}	1587.1±5.4
Average value	16.0	0.10	243.3	47.5	209.8	205.4	282.7	988.8

Analysis on Arabica and Robusta coffee samples, both wet and dried, showed an oscillatory trend of 3-methylbutanal concentration in both varieties. When comparing Arabica dried and wet samples and Robusta dried and wet samples (**figure 16**), data showed that the concentration of 3-methylbutanal was higher in dried samples compared to wet. When comparing Arabica and Robusta varieties the concentration of 3-methylbutanal was higher in dried Robusta coffee samples (from 17.9 mg kg^{-1} to 24.4 mg kg^{-1}) with respect to dried Arabica coffee samples (from 9.2 mg kg^{-1} to 16.8 mg kg^{-1}) with statistically significative differences in roasting conditions Cannella and Full-City. In wet samples concentration of this compound was higher in wet Robusta coffee samples with respect to Arabica, with a statistically significative difference in sample Italiana.

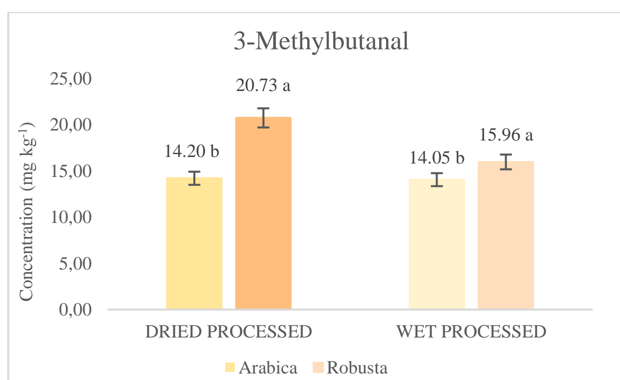


Figure 16: Effect of the interaction between variety and post-harvesting method on 3-methylbutanal content. Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

Concentration of hexanal had an oscillatory trend for both Arabica and Robusta varieties. When comparing dried and wet samples (**figure 17**), data showed that the concentration of this compound was higher in dried Arabica sample with respect to wet. When comparing Arabica and Robusta varieties the concentration of hexanal was statistically higher ($p<0.05$) in dried Arabica sample with respect to dried Robusta, while in wet Arabica and Robusta samples there is no significant difference ($p>0.05$) in the concentration of this compound.

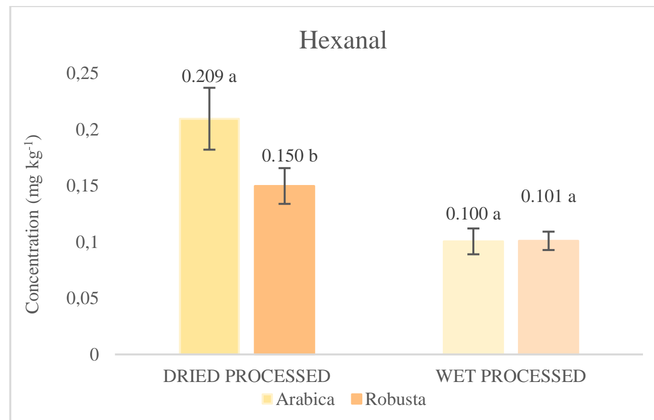


Figure 17: Effect of the interaction between variety and post-harvesting method on hexanal content. Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

The effect of the interaction between variety and post-harvesting method on general content of furanic compounds in coffee samples was reported in **figure 18**.

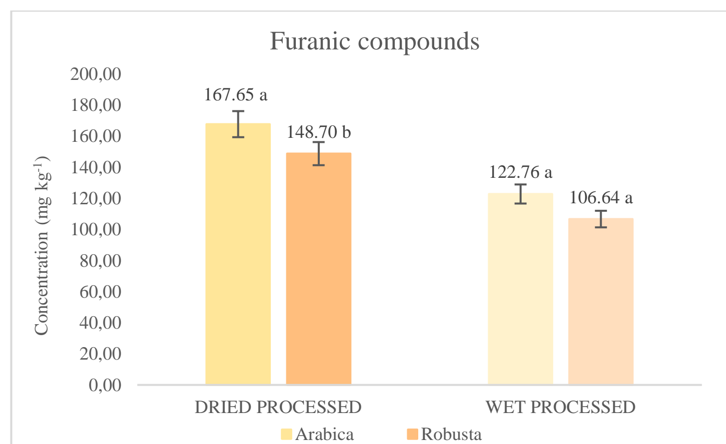


Figure 18: Effect of the interaction between variety and post-harvesting method on furanic compounds content. Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

Dried Arabica coffee samples had a significantly higher concentration of furanic compounds ($p<0.05$) with respect to dried Robusta and wet-processed coffee samples. This result agrees with data obtained by Gonzalez-Rios et al. (2007) [321], as the driest treatment used for coffee beans resulted in a higher content of volatile compounds, as furans, in ground coffee. The concentration of furanic compounds in Arabica and Robusta coffee samples had an

oscillatory trend when the roasting degree increased. This result was reported by Vignoli et al. (2014) [322] for furfural and 5-HMF, whose concentration decreased respectively of 67% and 100% when passing from light to dark roasted coffee samples. In this study a reduction of 80% and 92% of furfural and 5-HMF concentrations was found in ground coffee samples analysed when passing from Cannella to Napoletana. In dried Robusta coffee samples, the concentration of furanic compounds increased and then decreased with stronger roasting conditions, reaching the lowest values for Napoletana roasted coffee samples, with statistically significant differences for furfural and 5-HMF. In this case, also, the concentration of furfural and 5-HMF decreased of 59% and 78% in Napoletana roasted samples, respectively. In wet Arabica and Robusta coffee samples the concentrations of furanic compounds had an oscillatory trend, with a higher concentration in samples treated with lighter roasting conditions. When comparing dried and wet Arabica and Robusta samples, data showed that furanic compounds content was higher in Arabica coffee than Robusta ones, especially for furfural and 5-HMF, with statistically significative differences ($p<0.05$). In conclusion, the concentration of 3-methylbutanal was higher in dried Robusta coffee samples with respect to all other samples, hexanal content was higher in dried Arabica coffee samples with respect to Robusta ones and furanic compounds concentration was higher in dried ground Arabica coffee samples with respect to other coffee samples. In all wet samples the concentration of all monitored compounds was lower with respect to dried ones.

4.1.2.4. Colorimetric analysis

Colorimetric analysis was performed to evaluate the relationship between the degree of roasting process and the colour of the ground coffee samples by the measurement of three parameters: L^* (brightness), a^* (red to green colour) and b^* (yellow to blue colour).

In both Arabica (**figure 19**) and Robusta (**figure 20**) coffee samples L*, a* and b* values decreased with darker roasting conditions and these values were lower in wet coffee samples with respect to dried ones with statistically significative differences ($p<0.05$). Data obtained by analysis of commercial coffee samples showed L* values between 15.00 and 20.00, a* values between 10.00 and 12.00 and b* values between 14.00 and 18.00 (**figure 21**).

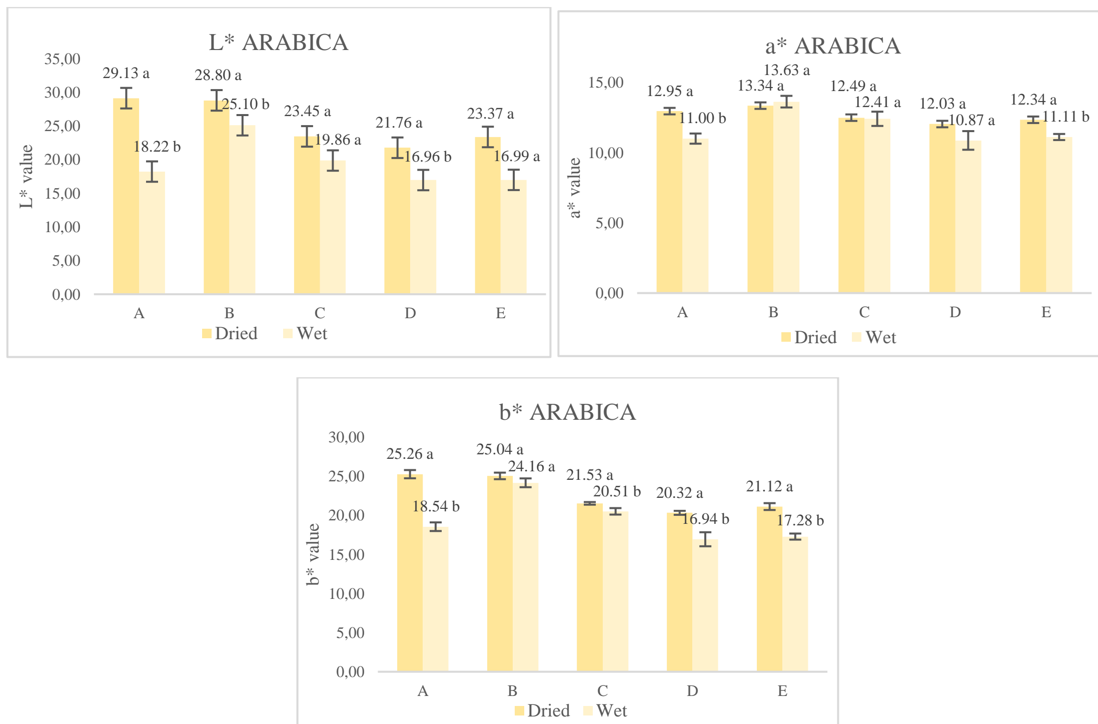


Figure 19: L*, a* and b* values for Arabica coffee samples. Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test. A=Cannella; B= City; C=Full-City; D=Italiana; E=Napoletana.

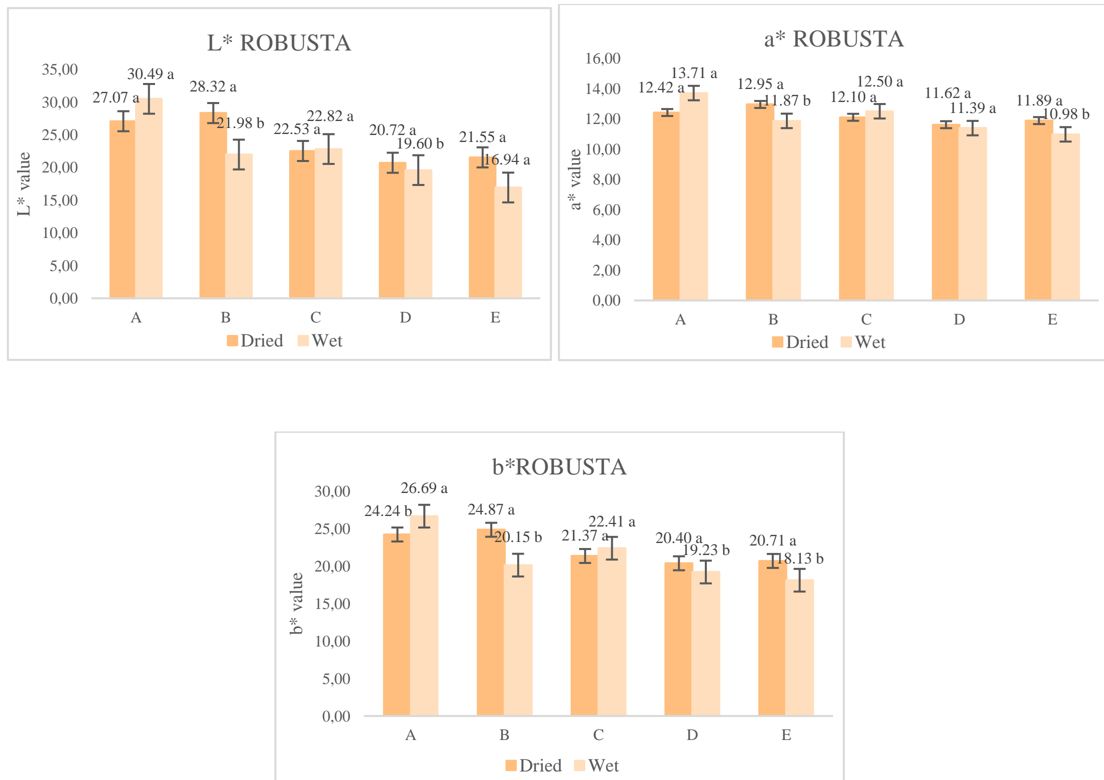


Figure 20: L*, a* and b* values for Robusta coffee samples. Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test. A=Cannella; B= City; C=Full-City; D=Italiana; E=Napoletana.

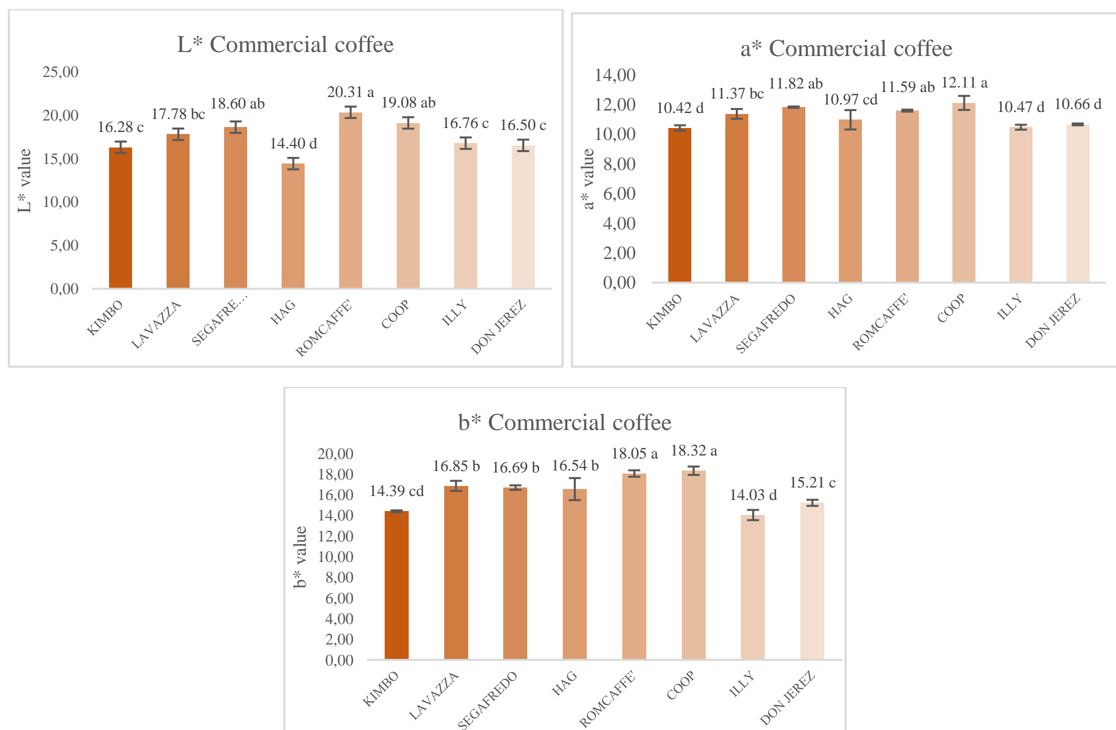


Figure 21: L*, a* and b* values for commercial coffee samples. Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

A comparison between coffee samples at different roasting degrees highlighted a decrease in all parameters (L*, a* and b*) with stronger roasting conditions. These results were in accordance with results found in literature: in fact, a negative correlation between the parameters L*, a* and b* and the roasting conditions was demonstrated by several studies [319, 323-325].

4.1.3. Conclusions

Since coffee is one of the most common beverages worldwide, consumed daily, it is important for coffee manufacturers carefully managing all steps with an impact on coffee quality from a safety and sensory point of view. Results of acrylamide content analysis, carried out on twenty experimental coffee samples, showed that only one sample (dried-process Arabica roasted according to the City roasting profile) exceeded the current limit of 0.400 μg/g set for acrylamide in roasted coffee by Commission Regulation (EU) 2017/2158 [267]. Data obtained in this study showed that the concentration of acrylamide was higher in Arabica coffee samples than Robusta ones. Moreover, acrylamide content decreased with the increase in the roasting temperature, confirming previous literature findings. In addition to acrylamide, furans content was monitored in all experimental coffee samples of this study, since they are considered as carcinogen compounds by IARC. Findings from this study highlighted an oscillatory trend of furans content as the roasting temperature increased. Taking into account the impact of variety and post-harvesting method on coffee safety-related compounds, wet-processed Robusta samples evidenced the lowest acrylamide and furans content. The new HS-SPME-GC-MS method developed in this study allowed not only to quantify furans content but also the amount of specific volatile compounds considered responsible for off-flavours in coffee, such as 3-methylbutanal and hexanal. In fact, this method showed a good linearity ($R^2 \geq 0.990$) and repeatability for all monitored

compounds. As for furanic compounds, 3-methylbutanal and hexanal had an oscillatory trend with stronger roasting conditions, globally decreasing when passing from light to dark roasting conditions for most of them. As regards the effect of the interaction between variety and post-harvesting method, the highest concentration of 3-methylbutanal was found in dried-process Robusta ground coffee samples, while the highest of hexanal in dried-process Arabica ground coffee samples. Therefore, it is possible to state that wet-process as post-harvesting method allowed to reduce the concentration of all monitored compounds monitored. **Figure 22** reports a graphical abstract to have an overview of this study and the most relevant results.

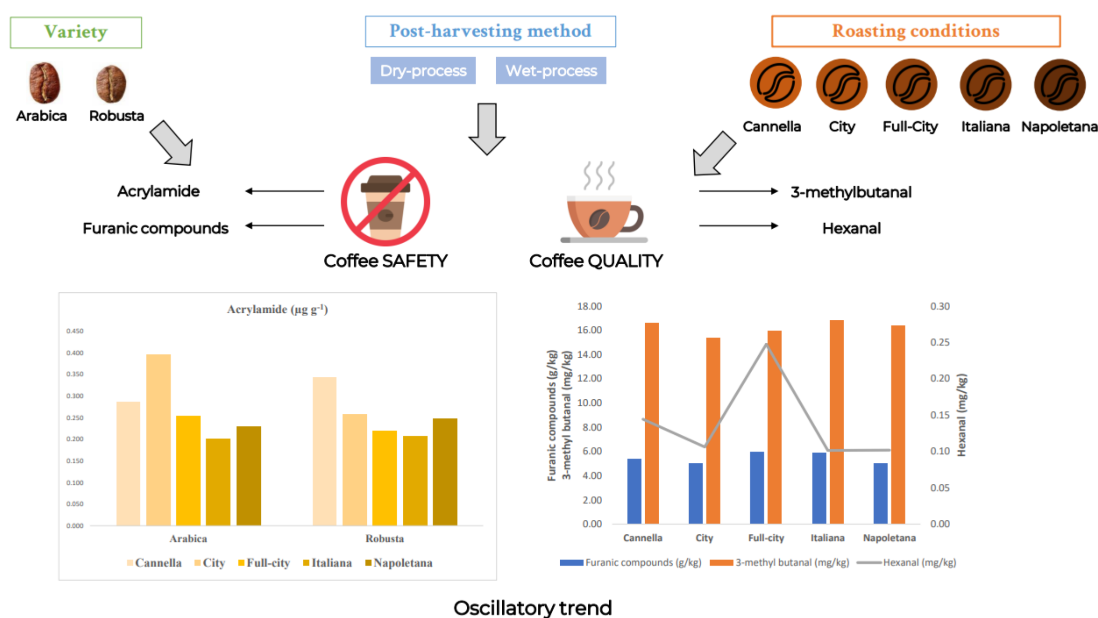


Figure 22: Overview of the study and most relevant results.

In conclusion, results from this study showed that not only roasting conditions had a significant impact on concentration of compounds responsible for coffee safety and aroma, but also the selection of the proper variety and post-harvesting method could be useful in ensuring a low dietary intake of carcinogenic compounds as well as limiting the development of volatile compounds which negatively influenced coffee sensory quality.

4.2. RESEARCH ACTIVITY: Evaluation of composition and cooking conditions of biscuits as potential strategies to improve food quality and safety

Abstract

Biscuits are one of the most consumed bakery products because of their pleasant odour and taste, which is why their consumption has increased over the years. For this reason, it is important to monitor the quality parameters, such as moisture, water activity, hardness, crispness, and the food safety in order to identify and quantify the compounds involved in the overall flavour and possible toxic compounds. Indeed, during the baking process, toxic compounds can be formed, such as furanic compounds, which are potentially carcinogenic to humans.

The aim of this work was to investigate the possible influence of baking conditions on the concentration of 3-methylbutanal, hexanal, furfural, furfuryl acetate, 5-methylfurfural, furfuryl alcohol and some physical quality attributes in lab-made biscuits formulated according to a standard recipe and baked under different heat-transfer modes for different time conditions. Then, the possible influence of the ingredient composition on the concentration of the above-mentioned volatile organic compounds (VOCs) and some physical quality attributes of 3 types of commercial biscuits (shortbread with eggs, shortbread with chocolate chips and dry petits) was evaluated.

A new analytical HS-SPME-GC-MS method was developed for the quantification of 3-methylbutanal, hexanal and 4 furanic compounds. Moreover, the quality parameters of moisture, water activity, colour and texture quality parameters were also monitored.

The results showed that in experimental biscuits, the concentration of all monitored compounds was lower in biscuits baked in a ventilated mode compared to those baked in a static mode. In this case, the values of moisture, water activity and texture quality were

almost the same static- and ventilate-baked biscuits. However, the values of moisture and water activity decreased with increasing baking time, while hardness and crispness parameters increased with increasing baking time.

The results in commercial biscuits showed that the composition could play a crucial role in the formation of 3-methylbutanal, hexanal and furanic compounds, as these compounds had a lower concentration in dry petit biscuits with respect to shortbreads. Moreover, dry petit biscuits have lower moisture, water activity and texture quality values than shortbreads.

In summary, this study demonstrated that both the baking conditions and the recipe of biscuits can influence the formation of furanic compounds and the main quality parameters. Therefore, in order to maintain the concentration of toxic compounds as low as possible while maintaining a high quality final product, it is important to consider both the baking conditions and the ingredients of biscuits to obtain the best food product in terms of quality and safety.

4.2.1. Materials and methods

4.2.1.1. Standards and chemicals

Pure standards of 3-methylbutanal (CAS 590-86-3), hexanal (CAS 66-25-1), furfural (CAS 98-01-1), furfuryl acetate (CAS 623-17-6), 5-methylfurfural (CAS 620-02-0), furfuryl alcohol (CAS 90-00-0), 2-methylpentanal (CAS 123-15-9) and HPLC-grade ethanol were purchased from Sigma Aldrich (Milan, Italy). The stock solutions of target volatile compounds were prepared by mixing 10 mg of pure standards with 10 mL of HPLC-grade ethanol (1000 µg mL^{-1}). Standard working solutions at different concentration were prepared by diluting the stock solutions in ultrapure water. The ultra-pure water was obtained through Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). 2-Methylpentanal were used as Internal Standard (IS) and it was added in each standard solution at a concentration of 100 µg mL^{-1}. The calibration curve was prepared by plotting the standard solution concentrations by the respective Response Factor (RF). RF is the ratio between the peak area of analyte and the peak area of IS.

4.2.1.2. Biscuit samples

4.2.1.2.1. Experimental biscuits

Experimental shortbread with eggs were prepared in lab using the formulation and preparation described by Schouten et al. (2022) [326] with ingredients purchased from the local market (Cesena, FC, Italy). The biscuits dough was characterized by a moisture content of 24.7% and an a_w of 0.92. The raw biscuits were placed on a baking tray covered with baking paper and baked in static mode (A) and in ventilated mode (B) in an electric oven (Procombi Plus, AEG-Electrolux, Berlin, Germany) at 175 °C for 18, 20, 22, 24 and 26 min. For each baking method, 3 batches of 10 biscuits were produced. The sample characteristics and abbreviation codes are reported in ***Table 27***.

Table 27: Sample code, heat-transfer mode and cooking time conditions of the experimental biscuits.

Sample code	Heat-transfer mode	Cooking time (min) at 175 °C
A18	Static	18
A20		20
A22		22
A24		24
A26		26
B18	Ventilate	18
B20		20
B22		22
B24		24
B26		26

4.2.1.2.2. Commercial biscuits

Commercial biscuits categorised by three different types, such as shortbread with eggs, shortbread with chocolate chips and dry petit biscuits, were purchased on the local market (Cesena, FC, Italy). Three different brands were analysed for each biscuit category, the samples characteristics and abbreviation codes are reported in ***Table 28***. For each brand, 30 biscuits were evaluated taking 10 biscuits from three different packages belonging to the same production batch.

Table 28: Category, sample code and labelled ingredients of the commercial biscuits.

Category	*Sample code*	*Labelled ingredients*
Shortbreads with Eggs	SE1	Wheat flour, sugar, sunflower oil, fresh eggs, raising agents (ammonium hydrogen carbonate, sodium hydrogen carbonate monopotassium tartrate), milk, salt, honey, milk protein, glucose-fructose syrup
	SE2	Wheat flour, sugar, sunflower oil, fresh eggs, raising agents (sodium hydrogen carbonate, ammonium hydrogen carbonate), milk, salt, milk protein, honey
	SE3	Wheat flour, sugar, sunflower oil, fresh eggs, glucose syrup, raising agents (sodium hydrogen carbonate, ammonium hydrogen carbonate), milk, salt, milk protein, honey
Shortbreads with Chocolate chips	SC1	Wheat flour, sugar, chocolate chips (cocoa paste, sugar, dextrose, soya lecithin), maize oil, butter, glucose-fructose syrup, raising agents (sodium hydrogen carbonate, ammonium hydrogen carbonate), wheat starch, dextrose, salt, sunflower lecithin, flavourings
	SC2	Wheat flour, sugar, chocolate (sugar, cocoa paste, low-fat cocoa powder, cocoa butter, soya lecithin), sunflower oil, butter, glucose-fructose syrup, wheat starch, raising agents (sodium hydrogen carbonate, ammonium hydrogen carbonate), salt, vanillin flavouring
	SC3	Wheat flour, sugar, chocolate chips (sugar, cocoa paste, low-fat cocoa powder, cocoa butter, soya lecithin), sunflower oil, butter, glucose-fructose syrup, raising agents (ammonium hydrogen carbonate, sodium hydrogen carbonate), salt, flavourings
Dry Petit	DP1	Wheat flour, sugar, maize oil, wheat starch, milk powder, glucose-fructose syrup, barley and maize malt extract, fresh eggs, raising agents (ammonium hydrogen carbonate, sodium hydrogen carbonate, monopotassium tartrate), salt, sunflower lecithin, flavourings
	DP2	Wheat flour, sugar, sunflower oil, glucose syrup, milk powder, raising agents (ammonium hydrogen carbonate, sodium hydrogen carbonate), dextrose, salt, flavourings
	DP3	Wheat flour, sugar, sunflower oil, raising agents (ammonium carbonate, sodium carbonate, potassium tartrate), wheat starch, salt, flavourings

4.2.1.3. Sample preparation

The preparation of biscuits followed the procedure by Schouten et al. (2022) [326]. Briefly, the followed procedure for the preparation of the dough of the biscuit was a standardised procedure by Canali et al. (2022) [244]. All the ingredients (500 g of wheat flour, 125 g of sucrose, 125 g of pasteurized eggs, 100 g of butter, 100 g of milk and 15 g of a leavening agent containing sodium diphosphate and carbonates) were purchased in a local market, weighted and mixed at a speed of about 3000 rpm (position 5) for 1 min and 30 s, reversing the direction of rotation after 80 s, in a household mixer (mod. Bimby Robot TM31, Vorwerk, Wuppertal, Germany). The dough resulted to have a moisture content and water activity of 23.53± 0.41% and 0.90 ± 0.01, respectively.

The dough was put in a refrigerator at 4 °C for 20 min and then sheeted to a thickness of 3.0 mm by a pasta filler machine (mod. SFSI 42040050 T, GAM International, Santarcangelo di Romagna, Italy) and cut in biscuits of 6 cm diameter.

4.2.1.3. Headspace solid phase microextraction (HS-SPME)

Furanic compounds concentration was determined using a headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS). Briefly, immediately after the packaging opening 1 g of sample was then weighted into a 20 mL vial and rapidly the internal standard 100 µg mL^{-1} was added into. Immediately after the vial was sealed with a screw cap with a PTFE septum. The analysis has been performed by using a PAL RSI 85 autosampler (Zwingen, Switzerland) which was able to yield strong analysis repeatability. The incubation of the sample was performed at 60 °C for 20 min under agitation (250 rpm, 5 sec of on-time and 2 sec of off-time). The grey fibre (DVB/C-WR/PDMS) from Supelco (Bellefonte, PA, USA) was selected for this work.

The fibre was conditioning for 20 min at 250 °C and then it was inserted inside the headspace of sample vial with a speed of 20 mm s^{-1} and a penetration depth of 40 mm. The extraction

was performed at 60 °C for 40 min and then the fibre was inserted into injector port at a speed of 100 mm s^{-1} and a penetration depth of 40 mm. The desorption occurred at 250 °C for 3 min. After desorption, the fibre was conditioning at 250 °C for 15 min.

4.2.1.4. GC-MS analysis

The GC-MS analysis was carried out by a 7890B gas chromatograph from Agilent equipped with a PAL RSI 85 autosampler and a 5977B mass spectrometer Agilent (Santa Clara, California, USA). The ionization source was a high efficiency electron ionization source (EI).

The injector temperature was set at 250 °C and the liner used was recommended for SPME injection namely Inlet liner, Ultra Inert, splitless, straight, 0.75 mm id, (5190-4048) from Agilent. The gas carrier was helium at a flow rate of 1 mL min^{-1}. The separation of target molecules was established on a DB-WAX capillary column (60 m, 250 µm i.d., 0.25 µm film thickness) with this ramp of temperature: 35 °C held for 2 min, 35-240 °C at 8 °C/min and 240 °C held for 3 min. The run time was about 32 min.

The transfer line was set at 250 °C and the temperature of the ionization source and the mass analyser were set at 230 and 150 °C, respectively. The gain factor was set at 0.1 that corresponded at 1413 V.

The ion species optimization for compounds were carried out by injecting a standard solution (10 µg mL^{-1}) in SCAN mode (35-450 m/z). The identification of compounds was performed by comparison with NIST library (US National Institute of Standards and Technology). The acquisitions were carried out in 'Selected Ion Monitoring' (SIM) mode and detection was divided into time windows. The most abundant ions were used for quantitation while the other to confirm the presence of the analytes. The GC-MS parameters including the retention time (Rt) and time windows are reported in ***Table 29***. Data results have been managed by

MSD ChemStation Software (Agilent, Version G1701DA D.01.00). Samples were analysed in triplicate. Acceptable relative standard deviation (% RSD) were set up below 20%.

Table 29: GC-MS parameters used for the analysis.

Compound	Time window (min)	Ion (m/z)	Retention time (min)
3-methylbutanal	0-12.20	58[a], 44, 71	8.5
2-methylpentanal	0-12.20	58[a], 71, 43	11.0
Hexanal	12.20-20.00	56[a], 44, 72	13.2
Furfural	20.00-25.20	96[a], 95, 39	24.3
Furfuryl Acetate	25.20-26.60	81[a], 98, 140	26.2
5-methylfurfural	26.60-28.20	110[a], 109, 53	27.2
Furfuryl alcohol	28.20-35.00	98[a], 81, 41	29.1

[a]These ions were used for quantitation, the other without any superscript letters for qualification.

4.2.1.5. Physical determinations

4.2.1.5.1. Moisture and water activity

The moisture content (%) and water activity (a_w) of commercial and experimental biscuits were evaluated on ground samples. Moisture content was determined gravimetrically by placing 3 g of the sample in an oven at 105 °C until the weight remained constant. The a_w was determined using an AQUALAB dew point hygrometer (Meter 4TE, Pullman, Washington, USA) at 25 °C. Three replicates per sample were performed for each commercial package and each experimental baking batch.

4.2.1.5.2. Colour and visual appearance

The colour of commercial and experimental biscuits was measured using a Colorflex Tristimulus Spectrophotocolorimeter (HunterLab, Sunset Hills Road Reston, Virginia, USA), illuminant D65 (6500 K) and geometry 45°/0°. Before each set of measurements, the instrument was properly calibrated with a standard white ceramic tile and a black glass. The colour was given in the standard scale CIE L* (lightness), a* (green-red) and b* (blue-

yellow). The colour measurements were made on the top side of 5 biscuits for each commercial package and each experimental baking batch.

To capture the visual/colour appearance of commercial and experimental biscuits a computer vision system (CVS) was used. The samples were placed inside a dark chamber over a black background in controlled lighting conditions with four daylight fluorescent lamps (TL-D Deluxe, Natural Daylight, 18W/965, Philips, USA) with a colour temperature of 6500 K. The RGB images of the samples were acquired using a colour digital camera (D7000, Nikon, Japan) equipped with 105 mm lens (AF-S Micro Nikkor, Nikon, Japan) and positioned vertically.

4.2.1.5.3. Texture and thickness

Texture measurement of commercial and experimental biscuits was carried out at room temperature using the TA -HDi500 texture analyser (Stable Micro System, Surrey, UK) equipped with three points bending ring, a horizontal stainless-steel probe and a 25 kg load cell. The pre-test speed was 5.00 mm/s, the test speed was 1.00 mm/s, the post-test speed was 10.00 mm/s, the probe distance 5 mm and the distance of the two beams was 30 mm. The recorded parameters were expressed as hardness (N), which was calculated from the maximum force values, crispness index, which was calculated from the linear distance between the first and the last recorded peak value. Force-distance curves and thickness were obtained from 5 biscuits for each commercial package and each experimental baking batch. As the commercial samples had different thicknesses (mm) for the different types examined, this parameter was evaluated using a vernier calliper. Thickness measurement was obtained from 5 biscuits for each commercial package.

4.2.1.6. Statistical analysis

The results were reported as the mean value ± standard deviation. Significant differences between data were calculated by unidirectional analysis of variance (ANOVA) followed by

Tukey's post-hoc comparison test, with a significance level of $p < 0.05$. The statistical package STATISTICA 8.0 software (StatSoft, Tulsa, UK) was used.

4.2.2. Results and discussion

4.2.2.1. HS-SPME-GC-MS analytical method optimization and validation

A new HS-SPME-GC-MS analytical method for the quantification of 6 volatile organic compounds (VOCs) was developed, optimized and validated. Three fibers, four types of salt, three incubation times and temperatures and three extraction times were evaluated. Results showed that 1 g of sample, without the addiction of salts, incubated for 15 min at 60 °C and extracted for 30 min with a DVB/C-WR/PDMS fiber were the best conditions for the analysis. ***Table 30*** showed the parameters optimized.

Table 30. Summary of monitored parameters for the optimization of HS-SPME technique with values of total peak areas.

Parameters		Total peak area
Fiber	DVB/C-WR/PDMS	$1x10^{8\ a}$
	DVB/PDMS	$6.9x10^{7\ b}$
	PDMS	$2.6\ x10^{7\ c}$
Salt addiction	Na_2SO_4	$7.4x10^{7\ a}$
	NaCl	$9.6x10^{7\ b}$
	NaH_2PO_4	$7.7x10^{7\ c}$
	$NaHCO_3$	$5.3x10^{7\ d}$
Temperature	70 °C	$4.6\ x10^{7\ a}$
	60 °C	$1\ x10^{7\ b}$
	50 °C	$5.4\ x10^{7\ c}$
Incubation time	10 min	$5.8\ x10^{7\ a}$
	15 min	$1.1\ x10^{8\ b}$
	25 min	$4.7\ x10^{7\ c}$
Extraction time	20 min	$4.1\ x10^{7\ a}$
	30 min	$1.1\ x10^{8\ b}$
	40 min	$4.7\ x10^{6\ c}$

[a]Different letters indicate significant differences at $p \leq 0.05$ following pairwise comparison by Tukey's HSD test.

The chromatographic separation was good for all peaks and **figure 23** showed an example of a chromatogram of a standard mixture of the 6 VOCs quantified with this method.

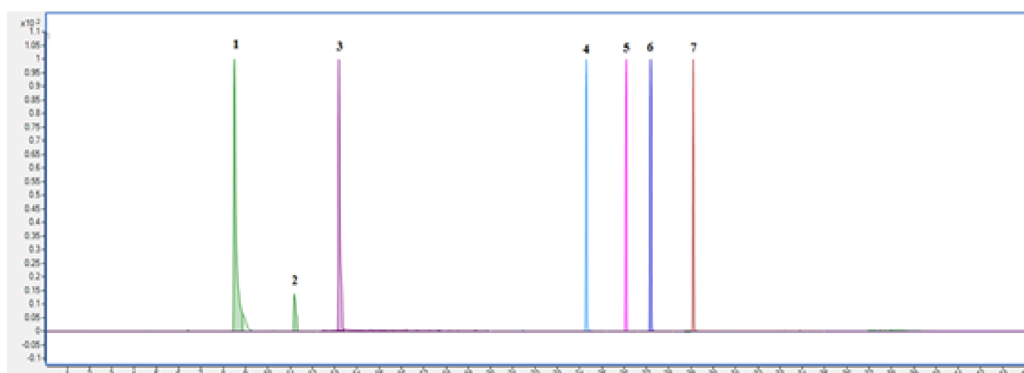


Figure 23: Chromatogram of all monitored compounds acquired in Selected Ion Monitoring (1: 3-methylbutanal, 2: 2-methylpentanal; 3: hexanal; 4: furfural; 5: furfuryl acetate; 6: 5-methylfurfural; 7: furfuryl alcohol).

This new method was validated by studying linearity, sensitivity and repeatability (***Table 31***). Linearity was studied by injecting five different concentrations of 6 VOCs and plotting the calibration curves with the respective determination coefficients (R^2). All compounds showed a good linearity as the R^2 was equal to or greater than 0.990. Repeatability was expressed as relative standard deviation (% RSD) and intra-day repeatability was assessed by injecting 5 replicates of mix standards five times in a day while inter-day repeatability was 3 replicates of mix standards once a day for three days. The intra-day repeatability ranged from 3.9 to 18.3% while inter-day repeatability was 5.4−19.9% for all target volatile compounds.

Table 31. Validation parameters: equation of the calibration curve, linearity, LOQ, LOD, Intra-day and Inter-day repeatability.

Compounds	Equation of the Calibration Curve	Linearity (R^2)	LOQ (μg L^{-1})	LOD (μg L^{-1})	Intra-day repeatability (RSD%)	Inter-day repeatability (RSD%)
3-methylbutanal	y=0,0108x - 0,0237	0.9960	0.012	0.004	13.1	19.4
Hexanal	y=0,0136x + 0,0338	0.9956	0.016	0.005	5.4	5.4
Furfural	y=0,0169x + 0,0918	0.9980	0.005	0.001	18.3	19.9
Furfuryl acetate	y=0,0191x + 0,1115	0.9906	0.06	0.02	16.9	19.1
5-methylfurfural	y=0,0138x + 0,5735	0.9900	0.009	0.003	9.5	19.8
Furfuryl alcohol	y=0,0004x + 0,0581	0.9978	0.145	0.048	3.9	18.4

4.2.2.2. Quantification of volatile organic compounds (VOCs) by HS-SPME-GC-MS of biscuit samples

4.2.2.2.1. Experimental biscuits

Biscuit samples were analysed through a HS-SPME-GC-MS method to quantify six different VOCs: 3-methylbutanal, hexanal and 4 furanic compounds (furfural, furfuryl acetate, 5-methylfurfural and furfuryl alcohol). The effect of static and ventilated mode for different time of baking on concentration of these compounds in nine experimental samples was evaluated. The concentrations of all the monitored compounds in experimental biscuit samples were reported in ***Table 32***.

Table 32. Summary of the concentrations of all compounds in experimental biscuit samples. Concentrations were expressed in $mg\cdot kg^{-1}$. Data are expressed as mean ± standard deviation (*n*=3).

Sample	**3-methylbutanal**	**Hexanal**	**Furfural**	**Furfuryl acetate**	**5-methylfurfural**	**Furfuryl alcohol**	**Total furanic compounds**
A18	8.1±0.34 $^{a, A}$	0.18±0.02 $^{a, A}$	2.7±0.07 $^{a, A}$	0.5±0.04 $^{a, A}$	1.1±0.04 $^{a, A}$	4.2±0.35 $^{a, A}$	8.6±0.24 $^{a, A}$
A20	3.7±0.16 $^{b, A}$	0.10±0.01 $^{b, A}$	2.4±0.24 $^{a, A}$	0.5±0.05 $^{a, A}$	0.9±0.1 $^{a, A}$	3.7±0.20 $^{a, A}$	7.5±0.20 $^{b, A}$
A22	2.6±0.26 $^{c, A}$	0.08±0.01 $^{b, A}$	1.9±0.23 $^{b, A}$	0.4±0.00 $^{b, A}$	0.7±0.1 $^{b, A}$	3.8±0.56 $^{a, A}$	6.7±0.42 $^{c, A}$
A24	2.0±0.37 $^{c, A}$	0.07±0.01 $^{b, A}$	2.1±0.16 $^{b, A}$	0.4±0.04 $^{b, A}$	0.7±0.07 $^{b, A}$	3.6±0.08 $^{a, A}$	6.8±0.21 $^{bc, A}$
A26	2.5±0.31 $^{c, A}$	0.06±0.00 $^{c, A}$	2.1±0.08 $^{b, A}$	0.4±0.02 $^{b, A}$	0.7±0.06 $^{b, A}$	3.9±0.24 $^{a, A}$	7.1±0.26 $^{bc, A}$
B18	2.7±0.37 $^{a, B}$	0.07±0.01 $^{a, B}$	1.7±0.03 $^{a, B}$	0.3±0.01 $^{a, A}$	0.6±0.02 $^{a, A}$	5.8±0.78 $^{a, B}$	8.3±0.77 $^{a, A}$
B20	2.7±0.50 $^{a, B}$	0.09±0.01 $^{a, A}$	1.7±0.14 $^{a, B}$	0.3±0.03 $^{a, A}$	0.5±0.05 $^{a, A}$	4.3±0.23 $^{b, A}$	6.8±0.29 $^{b, B}$
B22	2.3±0.25 $^{a, A}$	0.08±0.01 $^{a, A}$	1.7±0.11 $^{a, A}$	0.3±0.01 $^{a, A}$	0.5±0.03 $^{a, A}$	3.9±0.51 $^{c, A}$	6.5±0.66 $^{b, A}$
B24	1.7±0.17 $^{b, A}$	0.05±0.01 $^{a, A}$	1.7±0.06 $^{a, A}$	0.3±0.02 $^{a, A}$	0.5±0.03 $^{a, A}$	3.4±0.40 $^{d, A}$	5.9±0.49 $^{b, A}$
B26	1.5±0.12 $^{b, B}$	0.06±0.00 $^{a, A}$	1.7±0.15 $^{a, A}$	0.3±0.04 $^{a, A}$	0.5±0.07 $^{a, A}$	3.1±0.05 $^{e, A}$	5.7±0.27 $^{b, B}$

^{a}Different letters in the same column indicate significant differences among the samples belonging to the same biscuit category ($p < 0.05$).
^{A}Different letters between A and B at the same cooking time indicate significant differences among the samples ($p < 0.05$).

The highest average values of 3-methylbutanal and hexanal were found in biscuits baked in a static mode (A) with respect to those baked in a ventilate one (B).

The concentration of 3-methylbutanal in biscuits decreased in both static and ventilate baking when the time baking increased. The highest concentration was found in biscuits baked in a static mode for 18 and 20 minutes and the lowest for biscuits baked in a ventilate mode for 24 and 26 minutes, with statistically significative differences.

The concentration of hexanal in biscuits decreased in static baking when the time baking increased. Biscuits baked in a static mode for 18 minutes displayed the highest concentration of hexanal, with respect to others with statistically significative differences. No statistically significative differences among the concentrations of hexanal in biscuits baked in a ventilated mode were found.

3-Methylbutanal and hexanal concentrations decreased as time increased probably because of the evaporation process: 3-methylbutanal and hexanal have a boiling point of 94 °C and 129 °C degrees respectively [327]. This could also explain the lower concentration of both compounds in biscuits baked with a ventilate mode with respect to those baked in a static mode, as the ventilate mode removes the vapor from the surface favouring the evaporation process.

The average value of total furanic compounds was higher for biscuits baked in a static mode with respect to those baked in a ventilate mode and it decreased as the time baking increased. Ozolina et al. (2011) [328] established that the concentrations of furanic compounds in rye bread crust increased with prolonged baking times.

In particular, the concentration of furfural in biscuits had not an increasing or decreasing trend as it was quite the same at all baking times in both static and ventilate baking.

Biscuits baked in a static mode for 18 minutes had the highest concentration of furfural with respect to the others with statistically significative differences, while no statistical significative differences were found in those baked in a ventilate mode.

Biscuits baked in a static mode for 18, 20 and 26 minutes had a higher concentration of furfural with respect to those baked in a ventilate mode with statistically significative differences.

In conclusion, biscuits baked in a ventilate mode had a lower amount of furfural with respect to those baked in a static mode.

The concentration of furfuryl acetate in biscuits decreased in static baking when the baking time increased, while no statistically significative differences in samples baked in a ventilate mode were found. Biscuits baked in a static mode for 18 and 20 minutes had a higher concentration of furfuryl acetate with respect to some of the other samples with statistically significative differences.

Biscuits baked in a ventilate mode had a lower amount of furfuryl acetate regardless of baking time.

The concentration of 5-methylfurfural in biscuits decreased in both static and ventilate baking when the baking time increased. Biscuits baked in a static mode for 18 and 20 minutes had a higher concentration of 5-methylfurfural with respect to the others with statistically significative differences, while no statistically significative differences in biscuits baked in a ventilate mode were found.

Biscuits baked in a ventilate mode had a lower concentration of 5-methylfurfural with respect to those baked in a static mode.

The concentration of furfuryl alcohol in biscuits decreased in both static and ventilate baking when the baking time increased. Biscuits baked in a ventilate mode for 18 minutes had the highest concentration of furfuryl alcohol with statistically significative differences, while no

statistically significative differences in biscuits baked in a static mode were found. In this case, the average concentration of furfuryl alcohol in biscuits baked in a ventilate mode with respect to those baked in a static mode. This could be explained by the possible conversion of furfuryl alcohol to 4-hydroxycyclopent-2-enone (4-HCP) in water in acidic condition at high temperature [329]. This explains why furfuryl alcohol concentration in biscuits baked in ventilated mode is higher with respect to those baked in a static mode: the air removes water from the surface so furfuryl alcohol concentration is higher. Moreover, we can hypothesize that the decreasing concentration of this compound over the time is probably due to the evaporation process, as the boiling point of this compound is 171°C.

In depth, the concentration of furfuryl alcohol was lower in biscuits baked in a static mode until 22 minutes of baking time, with statistically significative differences for biscuits baked for 18 minutes. After 22 minutes of baking time, the concentration of this compound in biscuits baked in a static mode was higher with respect to those baked in a ventilate mode, with no statistically significative differences. The concentration of furfural was higher in biscuits baked in a static mode with respect to those baked in a ventilate mode, with statistically significative differences in biscuits baked for 18 and 20 minutes. The concentration of furfuryl acetate was higher in biscuits baked in a static mode with respect to those baked in a ventilate mode, with no statistically significative differences.

There was no statistically significative difference between the optimum baking conditions for furfuryl alcohol (22 minutes for static and 20 minutes for ventilate baking), while there were statistically significative differences between the optimum baking conditions for furfuryl acetate and furfural (20 minutes for static and 22 minutes for ventilate baking).

Overall, the average value of total furanic compounds was higher for experimental biscuits baked in a static mode with respect to those baked in a ventilate mode, with statistically significative differences for biscuits baked for 20 and 26 minutes. However, there was no

statistically significative difference between biscuits baked at the optimum baking conditions (22 minutes for static and 20 minutes for ventilate baking), as reported in **figure 24**. In both modes of baking the total furanic compounds decreased with the increasing of baking time with statistically significative differences, especially between 18 min of cooking time and the others. This result is in agreement with a study conducted on rye bread crusts by Ozolina et al. (2011) that showed a reduction in the concentration of furanic compounds as the baking time increased [328].

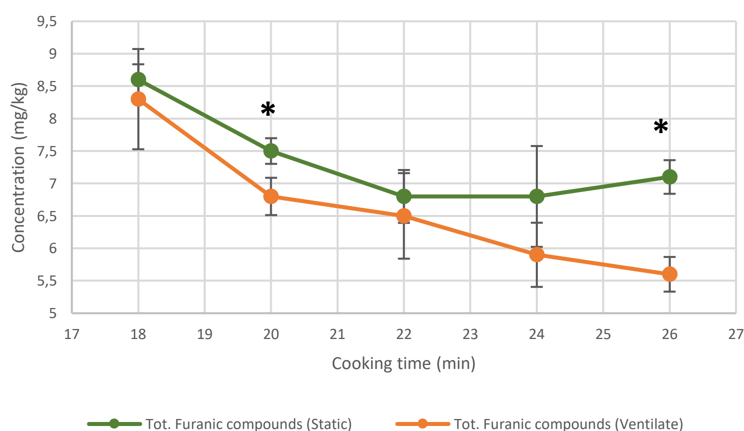


Figure 24: Concentration (mg·kg^{-1}) of total furanic compounds in experimental biscuits

4.2.2.2.2. Commercial biscuits

Biscuit samples were analysed through a HS-SPME-GC-MS method to quantify 6 VOCs: 3-methylbutanal, hexanal and 4 furanic compounds (furfural, furfuryl acetate, 5-methylfurfural and furfuryl alcohol). The concentrations of all the monitored compounds in commercial biscuit samples were reported in ***Table 33.***

Table 33. Summary of the concentrations of all compounds in commercial biscuit samples. Concentrations were expressed in mg kg^{-1}. Data are expressed as mean ± standard deviation (*n*=3).

	3-methylbutanal	**Hexanal**	**Furfural**	**Furfuryl acetate**	**5-methylfurfural**	**Furfuryl alcohol**	**Total furanic compounds**
SE1	1.3±0.25[a]	5.9±1.16[a]	2.6±0.47[a]	1.4±0.27[a]	1.4±0.28[a]	1.9±0.37[a]	7.3±1.40 [a]
SE2	0.9±0.05[a]	2.2±0.44[b]	1.6±0.14[b]	0.7±0.09[b]	0.8±0.09[b]	1.3±0.09[a]	4.4±0.41 [b]
SE3	1.1±0.15[a]	1.0±0.05[c]	0.9±0.02[c]	0.3±0.01[c]	0.5±0.01[c]	1.9±0.09[a]	3.7±0.06 [b]
Average	1.1±0.13 [A]	3.1±0.52 [A]	1.6±0.34 [A]	0.8±0.12 [A]	0.9±0.12 [A]	1.6±0.13 [A]	
SC1 D	1.5±0.02[a]	0.9±0.13[a]	1.3±0.17[a]	0.4±0.07[a]	0.6±0.09[a]	1.9±0.11[a]	4.2±0.43 [a]
SC2 E	3.8±0.75[a]	0.6±0.10[a]	1.2±0.10[b]	0.3±0.05[a]	0.5±0.04[a]	3.1±0.13[b]	5.2±0.06 [a]
SC3 F	8.9±1.71[b]	0.4±0.07[a]	0.9±0.02[c]	0.2±0.04[b]	0.4±0.01[a]	2.3±0.31[a]	3.9±0.29 [a]
Average	4.6±0.44 [B]	0.7±0.10 [B]	1.1±0.12 [A]	0.3±0.05 [B]	0.5±0.05 [B]	2.4±0.30 [B]	
DP1 G	1.1±0.22[a]	0.1±0.01[a]	0.4±0.08[a]	0.1±0.01[a]	0.3±0.06[a]	1.0±0.19[a]	1.8±0.34 [a]
DP2 H	1.1±0.09[a]	0.1±0.01[a]	0.6±0.02[a]	0.1±0.01[a]	0.3±0.01[a]	2.9±0.22[b]	4.0±0.22 [b]
DP3 I	0.7±0.03[a]	0.2±0.02[a]	0.4±0.01[a]	0.05±0.01[a]	0.2±0.00[a]	0.9±0.09[c]	1.7±0.08 [a]
Average	0.9±0.09 [A]	0.2±0.01 [B]	0.5±0.02 [B]	0.1±0.00 [C]	0.3±0.01 [C]	1.8±0.15 [A]	

[a]Different letters in the same column indicate significant differences among the samples belonging to the same biscuit category ($p < 0.05$).
[A]Different letters between the averages of SE, SC and DP indicate significant differences ($p < 0.05$).

The highest average concentration of 3-methylbutanal was found in shortbread with chocolate chips (4.7 mg kg^{-1}), followed by shortbread with eggs (1.1 mg kg^{-1}) and finally dry petit (0.9 mg kg^{-1}). This compound is responsible for malty aroma of buckwheat honey and it is a product of the Strecker reaction by deamination and decarboxylation of amino acids by dicarbonyls formed in the Maillard reaction [330]. This difference should be due to the presence of butter in SC, which is absent in the other two types of biscuits formulations and that give a higher fat contribution to the final product.

The highest average concentration of hexanal was found in shortbread with eggs (3.0 mg kg^{-1}), followed by shortbread with chocolate crisps (0.7 mg kg^{-1}) and finally dry petit (0.1 mg kg^{-1}).

Hexanal is the product of the oxidation of linoleic acid [304, 305] and it contributes to the buckwheat aroma [331]. It is an important indicator of food quality after a long storage period, as the aldehydes deriving from the action of lipoxygenase are responsible for undesirable odours. Regarding the composition of biscuits, SE and SC has almost the same composition, except for the presence of honey in SE. This ingredient should favour the oxidation processes increasing the production of this compound in SE biscuits.

The highest average concentration of furanic compounds was found in shortbread with eggs (5.1 mg kg^{-1}), followed by shortbread with chocolate crisps (4.5 mg kg^{-1}) and finally dry petit (2.5 mg kg^{-1}), except for furfuryl alcohol, whose concentration was higher in shortbread with chocolate crisps with respect to the others. The average concentration of furfural in dry petits (0.5 mg kg^{-1}) was in agreement with values reported in a previous study (0.65 mg kg^{-1}) [332]. The higher concentration of furanic compounds in shortbreads with respect to dry biscuits should be related to the composition of biscuits. In fact, the Maillard reaction, which occurs during the baking process and whose products are the furanic compounds, uses sugars

as starting substrates. For this reason, the different concentrations of furanic compounds should be related to a different content of sugars in biscuits.

Moreover, the different baking conditions of biscuits should be responsible for the different concentration of these compounds; in fact, shortbreads baking conditions are characterized by low temperatures/long times for shortbreads, while dry petits ones by high temperatures/short times. Ozolina et al. (2011) [328] established that the concentrations of furanic compounds in rye bread crust increased with prolonged baking times.

With regard to the concentrations of the most toxicologically interesting furanic compounds, furfural, furfuryl acetate and furfuryl alcohol were compared between the experimental biscuits baked under optimal conditions and similar commercial shortbread biscuits with eggs (**figure 25**). The most important difference was the higher concentration of furfuryl alcohol in experimental biscuits (4.1 mg·kg-1) with respect to SE biscuits (1.7 mg·kg-1) and this should be due to the different formulation or for the different baking conditions.

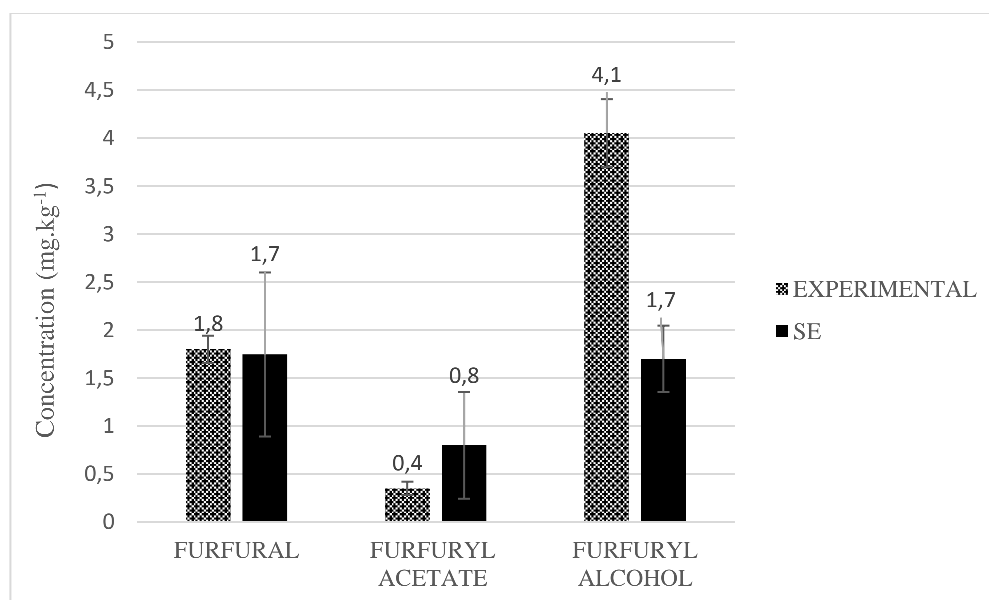


Figure 25: Comparison of the average values of furfural, furfuryl acetate and furfuryl alcohol between the experimental biscuits baked under optimal conditions (at 175 °C static for 22 minutes and ventilate for 20 minutes) and similar commercial shortbread biscuits with eggs.

In conclusion, the concentration of 3-methylbutanal, hexanal and furanic compounds was lower in dry petit biscuits with respect to the others, suggesting a possible involvement of both the composition of baked products in the formation of these compounds, as shortbreads are richer in fats and sugar with respect to dry petit, and the baking time.

4.2.2.3. Main physical and quality attributes of biscuit samples

4.2.2.3.1. Experimental biscuits

The physical properties analysed, measured in experimental biscuits formulated with a traditional shortbread recipe, are listed in ***Table 34***.

Table 34. Main physical quality attributes of experimental biscuits.

Sample	Moisture (%)	Water activity (a_w)	L*	a*	Hardness (N)	Crispness (linear distance)
Static mode						
A18	8.0 ± 0.2^b	0.55 ± 0.01^b	77.0 ± 0.8^a	8.9 ± 0.6^f	45.0 ± 3.1^f	59.1 ± 2.3^g
A20	5.8 ± 0.2^c	0.48 ± 0.02^c	74.7 ± 0.8^b	10.3 ± 0.6^e	46.7 ± 2.4^f	80.2 ± 3.0^e
A22	4.5 ± 0.1^e	0.38 ± 0.03^e	71.4 ± 0.8^c	12.1 ± 0.5^d	65.0 ± 3.0^c	106.3 ± 4.1^d
A24	3.7 ± 0.0^g	0.26 ± 0.02^g	70.0 ± 0.7^d	12.8 ± 0.4^{bc}	66.6 ± 2.5^c	127.1 ± 3.1^c
A26	2.8 ± 0.1^h	0.23 ± 0.01^h	68.0 ± 0.5^e	13.3 ± 0.3^{ab}	70.1 ± 4.1^b	169.2 ± 2.7^a
Ventilated mode						
B18	7.7 ± 0.2^b	0.56 ± 0.02^b	74.6 ± 0.7^b	10.3 ± 0.6^e	53.1 ± 1.2^e	69.1 ± 1.8^f
B20	5.1 ± 0.2^d	0.43 ± 0.02^d	70.0 ± 0.8^d	12.7 ± 0.5^{cd}	57.2 ± 3.1^d	108.0 ± 1.9^d
B22	4.1 ± 0.2^f	0.35 ± 0.04^f	68.6 ± 0.7^e	13.1 ± 0.3^{abc}	65.4 ± 2.1^c	129.3 ± 2.1^c
B24	3.6 ± 0.1^g	0.25 ± 0.03^g	67.9 ± 0.7^e	13.2 ± 0.4^{abc}	67.3 ± 2.1^{bc}	146.2 ± 3.1^b
B26	2.7 ± 0.0^h	0.21 ± 0.02^h	66.7 ± 0.4^f	13.5 ± 0.2^a	81.2 ± 3.2^a	170.9 ± 3.5^a

[a]Different letters in the same column indicate significant differences among the samples ($p < 0.05$).

The moisture content of the experimental biscuits baked under different conditions ranged from 2.7 to 7.7%; at 22 min baking time, all samples reached a moisture content of less than 5% within the standard range of commercial biscuits. As expected, the baking process promoted the water migration of the biscuits: however, the samples baked in static mode (A) for 20, 22 and 24 min had significantly higher moisture values than those baked in ventilated mode (B). This is because in static mode, heat is generated by electrical resistance on the top and bottom of the oven chamber (natural convection), whereas in ventilate mode, heat is generated by a fan and distributed by forced convection, heating the chamber faster and more evenly [333-335]. This was confirmed by the differences in the time-temperature profiles found in the previous study by Schouten et al. (2022) [326] that evaluated the same baking conditions in the same oven type.

Similar to moisture, up to 22 min of baking, the sample baked in the ventilated mode displayed lower a_w values than the sample baked in static mode. After 24 and 26 min of baking no significant a_w differences between the two heat-transfer methods were detected.

For the same baking time, the two heat-transfer modes also favoured the development of different colouration of the biscuit surfaces. When comparing the L* values, the samples baked in the ventilate mode consistently showed a significantly darker colour than those baked in the static mode. For both experimental biscuits, the parameter a* increased significantly as baking progressed. However, for the biscuits baked in static mode, the a* values increased more slowly compared to the biscuits baked in ventilate mode until 22 min. These differences in the colour parameters are again due to the different heat distribution in the oven for the two baking methods [326]. The slight differences found in biscuit samples colouration baked at different conditions were also perceptible from the visual appearance shown in **figure 26**.

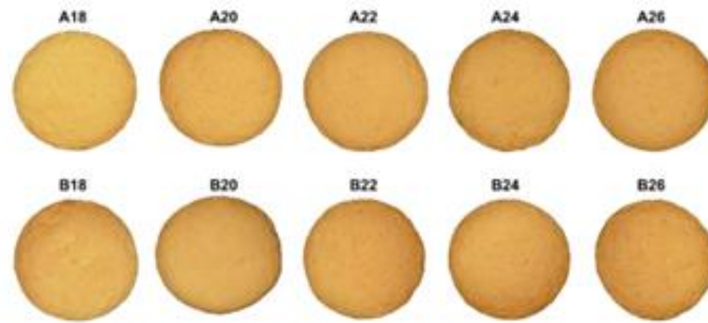


Figure 26: Visual appearance of commercial biscuit samples cooked in static mode (A) and ventilated mode (B) for different times.

As far as the textural properties are concerned, the hardness and crispiness of the experimental biscuits increased with increasing baking time for all samples; this positive correlation is explained by the higher degree of baking of the biscuits and the reduction in moisture content mentioned above. At all baking times, except for crispness at 26 min, where the values were not statistically different, the A biscuit samples had lower hardness and crispness values than the B samples. According to Palazoğlu et al. (2015) [336], the hot air flow circulating in the oven accelerates the formation of the crust on the surface when the oven is in convection mode.

4.2.2.3.2. Commercial biscuits

The analysed quality parameters such as moisture, a_w, texture and colour of the different tested commercial biscuits are reported in ***Table 35***.

Table 35: Main physical quality attributes of commercial biscuits.

Sample	**Moisture (%)**	**Water activity (a_w)**	**L***	**a***	**Hardness (N)**	**Crispness (linear distance)**
Shortbread with Eggs						
SE1	2.6 ± 0.1^b	0.23 ± 0.02^a	60.5 ± 1.1^a	14.6 ± 0.3^a	41.6 ± 6.1^b	54.0 ± 10.0^b
SE2	2.3 ± 0.1^c	0.19 ± 0.02^b	60.2 ± 1.5^a	13.9 ± 0.5^b	45.7 ± 4.6^b	52.5 ± 7.7^b
SE3	3.0 ± 0.2^a	0.24 ± 0.02^a	60.3 ± 1.9^a	13.3 ± 0.5^b	65.8 ± 8.7^a	76.1 ± 10.5^a
Shortbread with Chocolate chips						
SC1	2.5 ± 0.1^b	0.21 ± 0.02^b	59.6 ± 1.9^b	11.9 ± 0.4^a	95.1 ± 9.3^a	95.0 ± 9.3^a
SC2	2.7 ± 0.1^b	0.22 ± 0.01^b	55.7 ± 1.7^c	12.0 ± 0.4^a	59.9 ± 8.4^c	59.9 ± 8.5^c
SC3	3.2 ± 0.3^a	0.26 ± 0.03^a	63.1 ± 2.6^a	11.5 ± 0.9^b	73.0 ± 11.3^b	73.4 ± 11.4^b
Dry Petit biscuits						
DP1	0.8 ± 0.1^b	0.08 ± 0.02^c	68.1 ± 0.8^a	12.2 ± 0.4^b	30.7 ± 5.5^a	31.6 ± 5.6^a
DP2	2.3 ± 0.2^a	0.19 ± 0.02^a	58.9 ± 1.1^c	16.3 ± 0.5^a	28.1 ± 5.5^a	28.5 ± 5.7^{ab}
DP3	2.2 ± 0.2^a	0.15 ± 0.01^b	67.0 ± 1.7^b	11.3 ± 0.9^c	22.6 ± 4.5^b	25.5 ± 6.2^b

[a]Different letters in the same column indicate significant differences among the samples belonging to the same biscuit category ($p < 0.05$).

Moisture content of biscuits and bakery goods is a very important characteristic that identifies different products, the chemical reactions during baking (including VOCs formation) and influences their stability and storage [337, 338]. The moisture content of the different commercial biscuit samples tested ranged from 0.8 to 3.2%, which is within the standard range of 1-5% for this type of bakery product [339]. However, considerable variations were found between the moisture data of samples belonging to a different brand but to the same type of biscuits. The greatest differences were found in the dry petit biscuits, where sample DP1 had a significantly lower percentage moisture value than analogues DP2 and DP3. This result can be attributed to the use of the emulsifier lecithin, which was only used in the formulation of sample DP1; or to stronger time-temperature baking conditions.

Shortbreads with eggs (SE1, SE2, SE3) and shortbreads with chocolate chips (SC1, SC2, SC3) presented similar mean moisture values of 2.6 ± 0.3% and 2.8 ± 0.4%, respectively, and were always significantly ($p < 0.05$) higher than the mean moisture value of the dry petit biscuits (1.8 ± 0.7%). In general, shortbread biscuits, whose formulation is rich in fats and sugars, which are necessary to achieve the desired friability in the final product, require baking conditions characterised by low temperatures and long times. In contrast, dry petit biscuits require higher baking temperatures with shorter baking times, which, in addition to the different formulation, is due to the low moisture content characteristic of these products, which generally have a longer shelf-life [340, 341]. Furthermore, according to the literature, a higher amount of residual water after baking can be attributed to incomplete dissolution of the leavening agent used in relation to its purity and particle size [341]. Therefore, the use of different types and qualities of leavening agents can also influence the moisture content of the final product. According to the information on the labels, the leavening agents used were predominantly ammonium hydrogen carbonate and sodium hydrogen carbonate, and potassium tartrate was also added in three out of nine recipes (SE1, DP1 and DP3); however, the proportions and size characteristics of the agents used are not indicated.

As expected, the recorded a_w values were proportional and consistent with the percentage moisture values. Thus, the dry petit biscuit category had a significantly ($p < 0.05$) lower a_w of 0.14 ± 0.05 than the other biscuit types (0.22 ± 0.03% for SE and 0.23 ± 0.03% for SC). This result is probably still attributable to the different ingredients and baking processes between shortbreads and dry petit biscuits. For example, at the same concentration, salt contributes more than sugar to reducing water activity [342]. Furthermore, the presence of starch, proteins and hydrocolloids limits the availability and migration of water as they can act as 'binders' (water holding or water binding capacity). Comparing the ingredients, dry petit biscuits (DP1, DP2, DP3) had a higher glucose syrup content in the formulation (sample

DP1 and DP2) and the presence of starch (sample DP1 and DP3) or emulsifiers (sample DP1) compared to the formulation of shortbreads with eggs (SE1, SE2, SE3).

Another quality parameter that could depend on different biscuit formulations and process conditions is the surface colour of the product [340]. All commercial samples examined had lightness values (L*) higher than 50 with average of 66.5 ± 1.5, 64.5 ± 1.8 and 71.3 ± 0.9 for shortbreads with eggs, shortbreads with chocolate chips and dry petit biscuits, respectively. Generally, the lightest samples were the dry petit biscuits (DP1, DP2, DP3) ascribed to the lower fats and sugars content in the recipe of this category, which led to a lower development of Maillard and caramelisation reactions. In contrast, shortbreads with chocolate chips showed a significantly darker colouration, which can be attributed to a higher fats content in addition to the presence of chocolate chips. As for the colour parameter green-red (a*), positive values were obtained for all the biscuit samples tested, indicating that the colour red predominates over green leading to a browned colouring. The samples of the category shortbreads with chocolate chips (SC1, SC2, SC3) and dry petit biscuits (SB1, SB2, SB3) had significantly lower mean values for a* than the category shortbreads with eggs and showed a lower red and yellow colouration of the top biscuit side. This result could be attributed to the use of fresh eggs in the formulation of the shortbreads with eggs, an ingredient not used in the other categories, with the exception of sample DP1, which can however be assumed to contain a relatively small amount. The main described differences between the commercial biscuit types and brands were also recognisable from the visual appearance shown in **figure 27**.

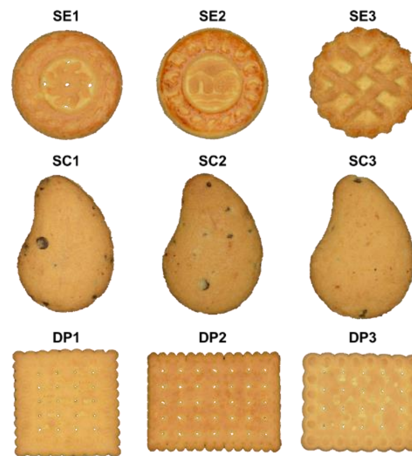


Figure 27: Visual appearance of commercial biscuit samples of the type Shortbreads with Eggs (SE), Shortbreads with Chocolate chips (SC) and Dry Petit biscuits (DP), and experimental biscuit samples.

The texture properties of biscuits are also closely influenced by the biscuits category. Hardness and crispness are useful structural properties in the assessment of biscuits quality and sensorial acceptability due to their close association with the perception of freshness [343, 344]. The commercial samples analysed revealed an average hardness and crispness of 50.9 ± 12.5 N and 60.7 ± 14.3, 75.5 ± 17.3 N and 75.6 ± 17.3, 27.3 ± 6.2 N and 28.6 ± 6.3 for shortbreads with eggs, with chocolate chips and dry petit biscuits, respectively. The hardest and crispiest samples were shortbreads with chocolate chips, particularly SC1 and SC3, followed by shortbreads with eggs, while the least hard and crispy were dry petit biscuits (DP). The low values of the consistency parameters of dry petit biscuits can be attributed to the lower moisture content of the samples and the lower presence of fats and sugars, which make the consistency of the latter more alveolate and less compact. Moreover, texture properties can also be influenced by other factors such as thickness, shape, and uniformity of the biscuits [345]. Also for this reason, the dry petit biscuit samples, which had a thinner thickness (average of 6.6 ± 6.6 mm) than the others (10.4 ± 0.9 mm for shortbreads with eggs and 11.7 ± 1.4 mm shortbreads with chocolate chips), presented lower hardness and crispness values. In contrast, for the biscuits with chocolate chips, the highest

hardness and crispness values can also be attributed to the presence of chocolate chips, which cause a more irregular structure of the biscuits.

4.2.3. Conclusions

In conclusion, it can be confirmed that both the baking conditions and the recipe of biscuits can influence the formation of 3-methylbutanal, hexanal and furanic compounds.

In fact, the concentration of all monitored compounds was lower in biscuits baked in a ventilate mode with respect to those baked in a static mode, with statistically significative differences for biscuits baked for 26 minutes. Moreover, results showed that a higher presence of sugar in the recipe should increase the concentration of furanic compounds, as sugar is the first substrate of the Maillard reaction.

4.3. RESEARCH ACTIVITY: Evaluation of the influence of filler cremes on the formation of furanic compounds to improve food quality and safety.

Abstract

The aim of this work was to investigate the possible influence of wafer filler ingredients on the concentration of furfural, furfuryl acetate, 5-methylfurfural, furfuryl alcohol.

The new analytical HS-SPME-GC-MS method, described in the chapter 4.2. and it was used to quantify furanic compounds in 14 samples of wafer filled with different cremes and belonging to different brands.

Results showed that the concentration of these compounds was higher in Cappuccino and cocoa cremes, demonstrating that the content of these compounds is influenced by the filler cremes.

4.3.1. Materials and methods

4.3.1.1. Standards and chemicals

Pure standards of furfural (CAS 98-01-1), furfuryl acetate (CAS 623-17-6), 5-methylfurfural (CAS 620-02-0), furfuryl alcohol (CAS 90-00-0), 2-methylpentanal (CAS 123-15-9) and HPLC-grade ethanol were purchased from Sigma Aldrich (Milan, Italy). The stock solutions of target volatile compounds were prepared by mixing 10 mg of pure standards with 10 mL of HPLC-grade ethanol (1000 µg mL^{-1}). Standard working solutions at different concentration were prepared by diluting the stock solutions in ultrapure water. The ultra-pure water was obtained through Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). 2-Methylpentanal were used as Internal Standard (IS) and it was added in each standard solution at a concentration of 100 µg mL^{-1}. The calibration curve was prepared by plotting the standard solution concentrations by the respective Response Factor (RF). RF is the ratio between the peak area of analyte and the peak area of IS.

4.3.1.2. Samples

Samples were provided by three companies (*A*, *B* and *C*) and they are numbered in ***Table 36.***

Table 36: Sample number and complete name.

No.	Sample description
1	*A* Fresh products
2	*A* Old products (T19)
3	*A* Wafer Cacao
4	*A* Wafer Vanilla
5	*A* Wafer Cappuccino
6	*A* Wafer Frutta-Raspberry-Yoghurt
7	*A* Wafer-chiari
8	*A* Wafer-scuri
9	*A* Wafer-neri
10	*B* Chocolate Wafers-baked with real cocoa
11	*B* Vanilla Wafers
12	*C* Wafer Chocolate
13	*C* Wafer Strawberry
14	*C* Wafer Vanilla

4.3.1.3. Headspace solid phase microextraction (HS-SPME)

Furanic compounds concentration was determined using a headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS). Immediately after the packaging opening the sample has been frozen with liquid nitrogen and has been finely triturated using pestle and mortar up to homogeneous powder. 1 g of sample was then weighted into a 20 mL vial and rapidly the internal standard was added into. Immediately after the vial was sealed with a screw cap with a PTFE septum.

The analysis has been performed by using a PAL RSI 85 autosampler (Zwingen, Switzerland) which was able to yield strong analysis repeatability.

The incubation of the sample was performed at 60 °C for 20 min under agitation (250 rpm, 5 sec of on-time and 2 sec of off-time). The grey fibre (DVB/CAR/PDMS) from Supelco (Bellefonte, PA, USA) was selected for this work.

The fibre was conditioning for 20 min at 250 °C and then it was inserted inside the headspace of sample vial with a speed of 20 mm s^{-1} and a penetration depth of 40 mm. The extraction was performed at 60 °C for 40 min and then the fibre was inserted into injector port at a speed of 100 mm sec^{-1} and a penetration depth of 40 mm. The desorption occurred at 250 °C for 3 min. After desorption, the fibre was conditioning at 250 °C for 15 min.

4.3.1.4. GC-MS analysis

The GC-MS analysis was carried out by a 7890B gas chromatograph from Agilent equipped with a PAL RSI 85 autosampler and a 5977B mass spectrometer Agilent (Santa Clara, California, USA). The ionization source was a high efficiency electron ionization source (EI).

The injector temperature was set at 250 °C and the liner used was recommended for SPME injection namely Inlet liner, Ultra Inert, splitless, straight, 0.75 mm id, (5190-4048) from Agilent. The gas carrier was helium at flow rate of 1 $mL \cdot min^{-1}$.

The separation of target molecules was established on DB-WAX capillary column (60 m, 250 µm i.d., 0.25 µm film thickness) with this ramp of temperature: 35 °C held for 2 min, 35-240 °C at 8 °C/min and 240 °C held for 3 min. The run time was about 32 min.

The transfer line was set at 250 °C and the temperature of the ionization source and the mass analyser were set at 230 and 150 °C, respectively. The gain factor was set at 0.1 that corresponded at 1413 V.

The ion species optimization for internal standard and furfuryl alcohol were carried out by injecting a standard solution (10 $\mu g \cdot mL^{-1}$) in SCAN mode (35-450 m/z). The identification of furfuryl alcohol and 2-methylpentanal were performed by comparison with NIST library

(US National Institute of Standards and Technology). The acquisitions were carried out in 'Selected Ion Monitoring' (SIM) mode and detection was divided into time windows. The most abundant ions were used for quantitation while the other to confirm the presence of the analytes. The GC-MS parameters including the retention time (Rt) and time windows are reported in ***Table 37***.

Data results have been managed by MSD ChemStation Software (Agilent, Version G1701DA D.01.00).

Table 37: GC-MS parameters used for the analysis.

Compound	Time window (min)	Ion (m/z)	Retention time (min)
2-methylpentanal	0-12.20	58[a], 71, 43	11.0
Furfural	20.00-25.20	96[a], 95, 39	24.3
Furfuryl Acetate	25.20-26.60	81[a], 98, 140	26.2
5-methylfurfural	26.60-28.20	110[a], 109, 53	27.2
Furfuryl alcohol	28.20-35.00	98[a], 81, 41	29.1

[a]These ions were used for quantitation, the other without any superscript letters for qualification.

4.3.1.5. Statistical analysis

The results were reported as the mean value ± standard deviation. Significant differences between data were calculated by unidirectional analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test, with a significance level of $p < 0.05$. The statistical package STATISTICA 8.0 software (StatSoft, Tulsa, UK) was used.

4.3.2. Results and discussion

Samples have been divided into 5 groups to better manage the results, as reported in ***Table 38*** that reports also the concentrations of all monitored compounds in 14 samples.

Table 38: Concentrations ($mg \cdot kg^{-1}$) of all monitored compounds in 14 samples of wafer.

Group	No		*Furfural*	*Furfuryl acetate*	*5-methylfurfural*	*Furfuryl alcohol*	*Total furanic compounds*
Group 1	**1**	*A* Fresh products	8.45 ± 1.63^{a}	2.55 ± 0.49^{a}	5.50 ± 0.71^{a}	3.20 ± 0.62^{a}	19.70
	2	*A* Old products (T19)	2.10 ± 0.38^{b}	0.21 ± 0.03^{b}	0.56 ± 0.09^{b}	2.98 ± 0.34^{a}	5.85
Group 2	**3**	*A* Wafer Cacao	5.12 ± 0.17^{ac}	1.84 ± 0.09^{ab}	2.95 ± 0.21^{a}	2.48 ± 0.49^{a}	12.39
	4	*A* Wafer Vanilla	4.36 ± 0.01^{b}	1.61 ± 0.10^{ac}	2.80 ± 0.07^{a}	1.51 ± 0.03^{a}	10.28
	5	*A* Wafer Cappuccino	5.36 ± 0.66^{b}	2.12 ± 0.21^{b}	3.00 ± 0.43^{a}	12.33 ± 0.91^{b}	22.81
	6	*A* Wafer Frutta-Raspberry-Yogurt	4.13 ± 0.45^{c}	1.01 ± 0.19^{c}	2.29 ± 0.33^{a}	2.71 ± 0.01^{a}	10.14
Group 3	**7**	*A* Wafer-chiari	0.76 ± 0.08^{a}	0.06 ± 0.01^{a}	0.16 ± 0.01^{a}	6.23 ± 0.01^{a}	7.17
	8	*A* Wafer-scuri	0.66 ± 0.00^{a}	0.05 ± 0.00^{a}	0.14 ± 0.00^{a}	4.53 ± 0.12^{a}	5.38
	9	*A* Wafer-neri	0.60 ± 0.04^{a}	0.05 ± 0.00^{a}	0.14 ± 0.01^{a}	5.17 ± 0.05^{a}	5.96
Group 4	**10**	*B* Chocolate Wafers-baked with real cocoa	4.00 ± 0.46^{a}	0.50 ± 0.04^{a}	1.14 ± 0.11^{a}	2.96 ± 0.14^{a}	8.60
	11	*B* Vanilla Wafers	2.36 ± 0.03^{a}	0.51 ± 0.04^{a}	1.17 ± 0.03^{a}	2.14 ± 0.28^{a}	6.18
Group 5	**12**	*C* Wafer Chocolate	20.37 ± 0.34^{a}	0.56 ± 0.02^{a}	1.37 ± 0.10^{a}	3.58 ± 0.39^{a}	25.88
	13	*C* Wafer Strawberry	5.96 ± 0.79^{b}	0.66 ± 0.02^{a}	1.38 ± 0.05^{a}	2.99 ± 0.07^{a}	10.99
	14	*C* Wafer Vanilla	3.89 ± 0.24^{b}	0.45 ± 0.01^{a}	1.08 ± 0.03^{a}	12.63 ± 2.29^{b}	18.05

[a]Values that did not share the same letters in the same group are statistically significative different ($p<0.05$).

The sum of the concentrations of furanic compounds in wafers varied between 5.39 $\mu g\ g^{-1}$ in sample 8 (*A* Wafer scuri) and 25.88 $\mu g \cdot g^{-1}$ in sample 12 (*C* Wafer Chocolate).

In the group 1, the highest concentration of furanic compounds was found in sample 1 (19.70 $\mu g \cdot g^{-1}$). In fact, the concentration of all furanic compounds was lower in sample 2 with respect with sample 1. In the group 2, the highest concentration of furanic compounds was found in sample 5 (22.81 $\mu g \cdot g^{-1}$). In fact, the concentration of all furanic compounds was higher for sample 5 (*A* Wafer Cappuccino), followed by sample 3 (*A* Wafer Cacao), sample 4 (*A* Wafer Vanilla) and sample 6 (*A* Wafer frutta- Raspberry-Yogurt). These results are reasonably related to the typical higher concentration of furans in coffee and chocolate as they are roasted products. Coffee, in fact, has higher concentrations of furfuryl alcohol (31-58 $\mu g \cdot mL^{-1}$) which vary on the based on roasting degree, as highlighted in literature [346, 347]. It would be useful to decrease the quantity of this compound using, for example, a light roasted coffee [347].

In the group 3, the highest concentration of furanic compounds was found in sample 7 (7.17 $\mu g \cdot g^{-1}$). In fact, the concentration of all furanic compounds was higher for sample 7 (*A* Wafer-chiari), followed by samples 8 (*A* Wafer-scuri) and 9 (*A* Wafer-neri) with slight differences for furfuryl acetate and 5-methylfurfural that have the same concentration in samples 8 and 9. In this case it could be useful try to optimize the cooking process of wafer to decrease as much as possible the concentration of furfuryl alcohol.

In the group 4, the total furanic compound concentration was higher for sample 10 (8.60 $\mu g \cdot g^{-1}$) with respect to sample 11 (6.18 $\mu g \cdot g^{-1}$). However, this difference was marked for furfural (4.00-2.36 $\mu g \cdot g^{-1}$) and furfuryl alcohol (2.96-2.14 $\mu g \cdot g^{-1}$), while the concentrations of furfuryl acetate and 5-methylfurfural were almost the same among the samples. Also in this case, the higher concentration of furans in the sample with coco with respect to that with vanilla could be explained with the previous roasting process of chocolate that favour the

formation of a higher amount of furans. In the group 5, the total concentration of furanic compounds was higher for sample 12 (25.88 $\mu g \cdot g^{-1}$), followed by 14 (18.05 $\mu g \cdot g^{-1}$) and 13 (10.99 $\mu g \cdot g^{-1}$). These results agreed with results obtained in the second group, with slight differences when we consider each compound. In fact, the concentration of furfural was statistically significative higher for sample 12 (20.37 $\mu g \cdot g^{-1}$), followed by sample 13 (5.96 $\mu g \cdot g^{-1}$) and 14 (3.89 $\mu g \cdot g^{-1}$) that are not statistically significative different each other. The concentration of furfuryl acetate was higher for sample 13 (0.66 $\mu g \cdot g^{-1}$), followed by 12 (0.56 $\mu g \cdot g^{-1}$) and 14 with no statistically significative differences (0.45 $\mu g \cdot g^{-1}$). The concentration of 5-methylfurfural was higher for sample 13 (1.38 $\mu g \cdot g^{-1}$), followed by samples 12 (1.37 $\mu g \cdot g^{-1}$) and 14 (1.08 $\mu g \cdot g^{-1}$) with no statistically significative differences. The concentration of furfuryl alcohol was statistically significant higher for sample 14 (12.63 $\mu g \cdot g^{-1}$), followed by samples 12 (3.58 $\mu g \cdot g^{-1}$) and 13 (2.99 $\mu g \cdot g^{-1}$) that are not statistically significative different each other. This result could be explained by the effect of the presence of Vanilla on the concentration of this compound. Vanilla, in fact, seemed to increase the level of this compound in espresso coffee as well [257].

It's important to highlight that the concentration of furfuryl alcohol varied between 1.443 and 20.160 $\mu g \cdot g^{-1}$ in final products with a media of 5.348 $\mu g \cdot g^{-1}$. This value agrees with values reported in literature, where the concentration of furfuryl alcohol in different biscuits was about 8.5 $\mu g \cdot g^{-1}$ [348].

4.3.3. Conclusions

This study showed that the wafer filling ingredients can influence the formation of furanic compounds. In fact, in all wafer samples the concentration of furanic compounds was higher for Cappuccino, followed by cocoa cream, vanilla cream and finally raspberry/strawberry creams. This is reasonably right as cappuccino comes from coffee, which is a food product subjected to previous roasting process, so as for cocoa. As explained in the chapter 3.2., furanic compounds are formed during roasting processes, so cremes with ingredients subjected to previous roasting processes have probably a higher amount off these toxic compounds.

4.4. **RESEARCH ACTIVITY:** COMPARISON OF TWO PACKAGING FOR THE PRESERVATION OF PANETTONE THROUGH THE STUDY OF VOCs AS INDICATORS OF FOOD QUALITY.

Abstract

In this study, two types of packaging provided by Esseoquattro company were tested in order to assess their capability in the preservation of Panettone. *Ideabrill®* packaging (called Sacchetto 19), a three layers pack of polyethylene high-density layer, metallic layer and cellulose with long fiber layer, was compared to the conventional packaging furnished by the bakery. The study was conducted on Panettone preserved in the packaging described above through the study of the volatile organic compounds (VOCs) through a new HS-SPME-GC-MS method at day 0, after 1 month, after 2 months and after 3 months. Moreover, microbiological and sensorial analysis were performed to better study the shelf-life of this bakery product. This study demonstrated, according to microbiological and sensorial analyses, that Ideabrill® packaging had a higher conservation ability for Panettone when compared to the conventional one.

4.4.1. Materials and methods

4.4.1.1. Chemicals

The alkane mixture (C7-30) and sodium chloride (NaCl) were purchased by Sigma-Aldrich, Co., St. Louis, USA.

4.4.1.2. Packaging and panettone samples

Two types of packaging were studied: Sacchetto 19 (produced with the patented formula Ideabrill ®) (ID) packaging and control (CTR) packaging. The ID packaging was a multilayer packaging bag provided by ESSEOQUATTRO s.p.a. (Carmignano di Brenta), while the CTR was furnished by the pastry chef supplier of panettone.

Ideabrill® is a European patented packaging (EP 1584464 A1) constituted of 3 layers: an external layer made of pure cellulose coupled to an internal layer made of high density (HD) polyethylene (PE) with a third aluminium layer fixed between them, as described in the chapter 2.1.2.2.

Samples of panettone were purchased in a local store and stored at 4°C until analysis. The analyses were performed in duplicate.

4.4.1.3. Headspace Solid Phase Microextraction (HS-SPME)

The extraction of VOCs in panettone was performed through a Headspace Solid Phase Microextraction (HS-SPME). Immediately after the packaging opening, the sample was put in a mortar and freezed with liquid nitrogen and finely powdered with a pestle to obtain an homogeneous powder. Then, 2 g of the powdered sample was weighted in a 20 mL vial and immediately sealed with teflonlined septum and screw cap. The vial was immersed in a water bath at 50 °C for 20 min under continuous agitation at 250 rpm with magnetic stirrer to promote the volatilization of compounds from the matrix to the headspace. After the equilibration, a DVB/CAR/PDMS fiber was inserted in the headspace of the vial for 30 min and then, it was inserted directly into the injection port of the gas-chromatographic system.

The desorption occurred at 260 °C for 10 min. A cleaning step of 30 min at 260 °C was applied between consecutive analyses to prevent cross-contamination.

4.4.1.4. GC-MS analysis

An Agilent 6890N gas chromatograph equipped with an Agilent 5973N mass selective detector (GC/MSD) (Santa Clara, CA, USA) was used. The chromatographic separation was performed using a DB-Wax capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA). The flow rate (He) was 1ml/min under splitless mode. The injector temperature was set at 260 °C. The oven temperature was set at 50 °C held for 3 min, then raised to 150 °C at 5 °C/min, raised to 250 °C at 10 °C/min held for 7 min.

The run time was about 40 min.

The transfer line was set at 250 °C and the temperature of the ionization source and the mass analyser were set at 230 and 150 °C, respectively.

The acquisitions were carried out in the electron impact (EI) mode, using the SCAN mode (29-450 m/z). The identification of compounds was performed by comparison with NIST library (US National Institute of Standards and Technology). The values were the mean of two replicates of each sample. Data were analysed by using MSD ChemStation software (Agilent, Version G1701DA D.01.00).

4.4.1.5 Microbiological analysis, moisture (%), water activity (A_w) and pH

Panettone microbiota was monitored by determining total aerobic mesophiles, β-glucosidase-positive Escherichia coli, presumptive Bacillus cereus, coagulase-positive staphylococci, total coliforms, moulds and yeasts. The analyzed parameters followed the criterion of CeIRSA (2017) and the applied procedures were in accordance with the respective ISO guidelines. Ten g of Panettone from each pack (CTR and ID) were aseptically weighted inside sample bag (Whirl-Pak®, Seward, UK) and 90 mL of sterile 0.9% sodium chloride solution (NaCl) (Sigma-Aldrich, Co., St. Louis, USA) were added into sample bags

and homogenized for 2 min by Stomacher. Then, 10-fold serial dilutions were prepared using a saline solution, and 0.1 mL of the corresponding dilutions was spread and inoculated into selective agar media. The aerobic mesophilic bacteria count was performed onto Plate Count Agar (PCA, Oxoid, Basinstoke, UK) under aerobic conditions at 30 °C for 72 h (ISO 4833). For the enumeration of mesophilic LAB (lactic acid bacteria), de Man, Rogosa, Sharpe agar (MRS Agar at pH 5.7, VWR, Leuven, Belgium) was used (ISO 15214:1998), while, Tryptone Bile X-glucuronide Agar (TBX, VWR) for the detection of β-glucosidase-positive E. coli was aerobically incubated for 18 h to 24 h at 44 °C (ISO 16649–2). The detection and enumeration of Enterobacteriaceae, were performed onto violet-red bile glucose agar (VRBGA, VWR) inoculated with samples and incubated at 37 °C for 24 h (ISO 21528–2). The enumeration of presumptive Pseudomonas spp. was carried out by aerobically inoculating samples onto Pseudomonas Selective Agar (CFC, Liofilchem s.r.l., Roseto degli Abruzzi, Italy) and incubated at 25 °C for 44 h ± 4 h (ISO 13720:2010[E]). The presence of coagulase-positive staphylococci was checked through the aerobic inoculation onto the Baird-Parker agar medium (VWR) after 24 to 48 h of incubation (ISO 6888–1:1999 [E]) at 35–37 °C. Sulfide-reducing bacteria were enumerated by using iron sulphite agar plates (Liofilchem) incubated under anaerobic conditions at 37 ± 1 °C for 48 h (ISO 15213:2003 [E]). Cl. perfringens count was on tryptose sulfite cycloserine (TSC) agar (VWR) after incubation under anaerobic conditions at 37 °C for 20 h ± 2 h (ISO 7937:2004 [E]).

The water activity of the Panettone was measured using an AQUA LAB 4TE Decagon Device (Hopkins Ct. Pullman, USA) calibrated each time before use with two mono use certified standards (Meter 0.250 aw LiCl 13.41mol/kg in H_2O - Meter 0.150 aw LiCl 17.18 mol/kg in H_2O). The measurement of water activity was done in duplicate, from T0 to T4 using two samples from each kind of Panettone. In addition, also the moisture was measured at the same time points by a thermobalance (Kern, DAB 100-3),

The pH of Panettone has been measured in triplicate, using a digital pH meter (Mettler Toledo, Columbus, UK) equipped with a probe for food through direct penetration in meat.

4.4.1.6. Sensorial analysis

Descriptive sensory analyses are the most sophisticated tools in the arsenal of the sensory scientist. These techniques allow the sensory scientist to obtain complete sensory descriptions of products, to identify underlying ingredient and process variables, and/or to determine which sensory attributes are important to acceptance. A generic descriptive analysis would usually have between 8 and 12 panelists that would have been trained, with the use of reference standards, to understand and agree on the meaning of the attributes used. They would usually use a quantitative scale for intensity which allows the data to be statistically analyzed. These panelists would not be asked for their hedonic responses to the products. However, there are several different descriptive analysis methods and, in general, these reflect very different sensory philosophies and approaches. Usually, descriptive techniques produce objective descriptions of products in terms of the perceived sensory attributes. Depending on the specific technique used, the description can be more or less objective, as well as qualitative or quantitative.

Descriptive analyses are generally useful in any situation where a detailed specification of the sensory attributes of a single product or a comparison of the sensory differences among several products is desired. These techniques are ideal for shelf-life testing, especially if the judges were well trained and are consistent over time. Descriptive analysis techniques should never be used with consumers because in all descriptive methods, the panelists should be trained at the very least to be consistent and reproducible.

Sensorial analyses were conducted on Panettone studying 16 descriptors, using a ten-point hedonic scale, ranging from a very low value for a specific descriptor (Score: 1) to a very high value for a specific descriptor (Score: 10) on the same time points of the analysis,

keeping the reference sample at a value of 5 or 2 for all the descriptors considered.. The sensory panel was composed of 10 panelists from the laboratory, they had been trained before performing tests on selected samples in order to familiarize with Panettone sensorial attributes and terminology. Each sample was assigned a code; the panelists gave scores based on the coded samples.

4.4.1.7. Statistical analysis

Significant differences between data were calculated by unidirectional analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test, with a significance level of $p < 0.05$. The statistical package STATISTICA 8.0 software (StatSoft, Tulsa, UK) was used.

4.4.2. Results and discussion

4.4.2.1. Qualitative analysis of VOCs in Panettone through a HS-SPME-GC-MS method

The analysis of VOCs in panettone was performed through a HS-SPME-GC-MS method to identify the most abundant compounds to evaluate the best conservation ability of two types of packaging (ID and CTR). The analyses were performed at t0 (day of purchase), t1 (after 1 month), t2 (after 2 months) and t3 (after 3 months). The identified compounds, the odour attributes and areas are reported in ***Table 39***.

Table 39: Identified compounds, odour attributes and areas.

Compounds	Odour attribute	LRI (exp)	LRI (lit)	0			1		2		3	
				Area	*RSD%*		*Area*	*RSD%*	*Area*	*RSD%*	*Area*	*RSD%*
<u>Ketones and Aldehydes</u>												
2,3-butanedione	Malty	965	970	$5.1*10^6$	7.15	CTR	$7.7*10^6$	19.83	$2.7*10^6$	0.96	$6.8*10^5$	16.38
						ID	$6.6*10^6$	8.37	$1.9*10^6$	11.78	$1.1*10^6$	0.58
Hexanal	Green	1087	1083	$3.3*10^6$	2.78	CTR	n.d.	n.d.	$6.3*10^5$	13.39	n.d.	n.d.
						ID	n.d.	n.d.	$8.8*10^4$	18.74	n.d.	n.d.
Acetoin	Buttery	1290	1287	$6.2*10^7$	16.84	CTR	$4.1*10^7$	0.95	$2.1*10^7$	12.47	$2.2*10^6$	12.18
						ID	$3.7*10^7$	9.92	$1.4*10^7$	0.19	$3.4*10^6$	13.77
1-hydroxy-2-propanone	Bread-like	1310	1317	$2.0*10^6$	11.41	CTR	$1.9*10^6$	2.81	$1.8*10^6$	18.01	n.d.	n.d.
						ID	n.d.	n.d.	$9.5*10^5$	1.69	$2.9*10^5$	18.33
2-nonanone	Fruity	1410	1408	$1.2*10^6$	14.95	CTR	$6.0*10^5$	13.27	$1.0*10^6$	19.83	n.d.	n.d.
						ID	$4.2*10^5$	19.11	$7.1*10^5$	3.48	n.d.	n.d.
Nonanal	Fatty	1390	1396	n.d.	n.d.	CTR	n.d.	n.d.	$1.5*10^6$	17.04	n.d.	n.d.
						ID	$1.6*10^6$	18.94	$1.2*10^6$	2.11	n.d.	n.d.
Decanal	Sweet, waxy, floral	1495	1500	n.d.	n.d.	CTR	n.d.	n.d.	$1.5*10^7$	19.26	n.d.	n.d.
						ID	n.d.	n.d.	$1.1*10^7$	0.15	$2.4*10^6$	7.86
Benzaldehyde		1530	1528	$2.0*10^6$	16.21	CTR	$1.1*10^6$	2.26	$3.0*10^6$	19.41	$1.7*10^6$	3.15

	Almond-like					ID	n.d.	n.d.	$1.3*10^6$	0.69	$6.1*10^5$	15.70
Esters											
Ethyl butanoate	Fruity, sweet, apple	1045	1041	$4.6*10^6$	12.95	CTR	n.d.	n.d.	$1.5*10^6$	13.35	$2.2*10^5$	18.81
						ID	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl hexanoate		1230	1239	$2.8*10^6$	12.54	CTR	$1.6*10^6$	10.72	$3.7*10^6$	12.45	$4.3*10^5$	11.81
						ID	$6.7*10^5$	8.82	$2.2*10^6$	3.10	$3.1*10^5$	4.61
Ethyl octanoate	Fruity, fatty	1440	1435	$1.1*10^7$	2.33	CTR	$6.5*10^6$	4.95	$9.2*10^6$	18.55	n.d.	n.d.
						ID	$5.8*10^6$	19.02	n.d.	n.d.	n.d.	n.d.
Alcohols											
2-methylpropanol		1090	1092	$2.4*10^6$	18.47	CTR	$1.7*10^6$	4,88	$1.6*10^6$	19.55	n.d.	n.d.
						ID	$1.3*10^6$	1.15	$1.8*10^5$	19.30	n.d.	n.d.
1-hexanol	Fruity	1363	1359	$3.5*10^6$	13.25	CTR	$1.6*10^6$	5.95	$8.0*10^5$	19.17	n.d.	n.d.
						ID	n.d.	n.d.	$5.1*10^5$	1.42	n.d.	n.d.
Linalool	Floral	1550	1552	$4.6*10^6$	8.57	CTR	$1.5*10^7$	1.36	$1.0*10^7$	19.63	n.d.	n.d.
						ID	$1.3*10^7$	5.37	$1.0*10^7$	0.12	$6.9*10^5$	16.66
1-octanol	Waxy	1570	1564	n.d.	n.d.	CTR	n.d.	n.d.	$6.1*10^6$	14.97	$1.2*10^6$	13.68
						ID	n.d.	n.d.	$6.1*10^6$	0.52	$1.4*10^6$	0.12
Terpenes											
β-myrcene	Resinous	1160	1167	n.d.	n.d.	CTR	$1.9*10^7$	18.15	$4.1*10^7$	14.33	$3.5*10^6$	15.33
						ID	$2.1*10^6$	19.48	$2.2*10^7$	1.85	$2.8*10^6$	7.99
D-limonene		1200	1198	n.d.	n.d.	CTR	$6.1*10^8$	1.74	$1.1*10^9$	14.63	$3.1*10^7$	14.63

	Citrus, lemon					ID	$3.8*10^8$	12.74	$6.8*10^8$	1.75	$3.6*10^7$	18.44
Terpinen-4-ol		1614	1616	n.d.	n.d.	CTR	n.d.	n.d.	n.d.	n.d.	$3.5*10^5$	0.01
						ID	n.d.	n.d.	n.d.	n.d.	$5.5*10^5$	18.99
α-terpineol	Lemon, piney, minty	1693	1695	n.d.	n.d.	CTR	n.d.	n.d.	n.d.	n.d.	$1.3*10^6$	18.77
						ID	n.d.	n.d.	n.d.	n.d.	$1.5*10^6$	15.29
<u>Hydrocarbons</u>												
Styrene		1249	1247	n.d.	n.d.	CTR	n.d.	n.d.	n.d.	n.d.	$2.3*10^5$	16.35
						ID	n.d.	n.d.	n.d.	n.d.	$2.6*10^5$	12.31
<u>Furans</u>												
2-pentylfuran	Fruity, sweet	1230	1235	$8.1*10^5$	15.95	CTR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
						ID	n.d.	n.d.	$1.2*10^5$	17.46	n.d.	n.d.
Furfural	Bred-like	1467	1466	$2.4*10^7$	10.14	CTR	$6.8*10^6$	0.51	$1.4*10^7$	9.28	$6.3*10^6$	4.14
						ID	$4.1*10^6$	4.35	$6.7*10^6$	2.95	$2.3*10^6$	3.19
Furfuryl alcohol	Biscuit, cake crust	1663	1665	$1.3*10^6$	11.95	CTR	$1.7*10^6$	3.94	$1.0*10^6$	10.96	$4.5*10^5$	8.59
						ID	$1.9*10^6$	5.32	$1.0*10^6$	0.18	$5.4*10^5$	17.14
<u>Organic acids</u>												
Acetic acid	Vinegar	1460	1461	$1.3*10^7$	12.83	CTR	$2.1*10^7$	5.51	$1.5*10^7$	16.90	$7.9*10^5$	13.13
						ID	$2.1*10^7$	18.54	$1.2*10^7$	0.62	$1.5*10^6$	15.09
Propanoic acid	Pungent	1537	1535	$4.5*10^5$	4.75	CTR	$6.4*10^5$	17.33	n.d.	n.d.	n.d.	n.d.
						ID	n.d.	n.d.	$2.8*10^5$	16.12	n.d.	n.d.
Butanoic acid	Sweaty	1625	1624	$7.7*10^6$	12.38	CTR	$5.5*10^6$	3.47	$3.9*10^6$	16.80	$9.2*10^5$	0.68

						ID	$5.1*10^6$	11.98	$3.6*10^6$	4.32	$1.5*10^6$	19.85
Pentanoic acid	Sweaty	1727	1729	$2.7*10^5$	4.99	CTR	$1.9*10^5$	1.72	$7.7*10^4$	18.14	n.d.	n.d.
						ID	n.d.	n.d.	$2.1*10^5$	17.99	n.d.	n.d.
Hexanoic acid	Sour	1863	1861	n.d.	n.d.	CTR	n.d.	n.d.	n.d.	n.d.	$1.4*10^6$	11.66
						ID	n.d.	n.d.	n.d.	n.d.	$1.5*10^6$	9.09
Octanoic acid	Fatty, soapy	2070	2072	$1.2*10^6$	17.66	CTR	$1.9*10^6$	8.42	$2.1*10^6$	16.28	n.d.	n.d.
						ID	$2.6*10^6$	15.40	$1.9*10^6$	3.35	n.d.	n.d.
Sorbic acid		2152	2150	$2.6*10^6$	8.84	CTR	$2.9*10^6$	3.17	$2.8*10^6$	11.63	$1.7*10^5$	17.16
						ID	$4.5*10^6$	7.13	$3.1*10^6$	2.09	$1.2*10^6$	18.56
Nonanoic acid	Waxy, dairy	2170	2169	n.d.	n.d.	CTR	n.d.	n.d.	$1.2*10^6$	19.11	n.d.	n.d.
						ID	n.d.	n.d.	$1.1*10^6$	2.14	n.d.	n.d.

As regards to the ketones and aldehydes, 8 compounds were identified.
Ketones are the products of microorganism metabolism or oxidation processes [210, 213, 216], while aldehydes are the products of lipid oxidation or amino acid degradation [207, 212, 217], so a higher concentration of these compounds suggested a higher degree of deterioration.

Most of them have a higher abundance in panettone preserved in CTR with respect to that preserved in ID, suggesting a faster degree of deterioration (mainly oxidation) in CTR packaging with respect to ID. To make some examples, hexanal is considered a negative marker as it comes from the lipid oxidation and it gives unpleasant rancid aroma at high concentration and pleasant grassy aroma at low concentration [213, 217, 222]. Its concentration was higher in panettone preserved in the CTR packaging with respect to that preserved in ID at t2. This demonstrated that the panettone preserved in ID packaging maintained a better sensory profile with respect to that preserved in CTR one. 2-Nonanone maintained a higher concentration in panettone preserved in CTR packaging with respect to that preserved in ID in both t1 and t2. Benzaldehyde is the product of Strecker degradation of some amino acids as leucine or phenylalanine [212] and its abundance was higher in panettone preserved in CTR packaging with respect to that preserved in ID at all times.

Esters are the products of the esterification of carboxylic acids and alcohols [213], that can be promoted by the action of microorganisms [204, 228], so a higher concentration of these compounds suggested a higher degree of deterioration. In fact, the abundance of ethyl butanoate, ethyl hexanoate and ethyl octanoate was higher in panettone preserved in CTR packaging with respect to that preserved in ID, suggesting the higher ability of ID packaging of preserving panettone for a longer period of time.

Alcohols are the products of lipid oxidation [203], which is an increasing process with the degradation so a higher concentration of these compounds suggested a higher degree of deterioration. The most important difference in the abundance of these compounds was

found for 2-methylpropanol and 1-hexanol. In fact, their abundance was higher in panettone preserved in CTR packaging with respect to that preserved in ID.

Terpenes are usually added in bakery products as flavouring agents, so their presence in the food product indicated that the sensory attributes are maintained. B-Myrcene, limonene, terpinene-4-ol and α-terpineol were the terpenes identified in panettone.

Furans are the products of the Maillard reaction which occur during the baking process and they are responsible for the bread-like odour. Although their odour is pleasant, they can be toxic for human health so their concentration should be maintained as low as possible.

For all the compounds considered, ID was found to be better or equal to the CTR as a lower concentration of negative compounds was demonstrated.

To have a more performing bag, in this case, the areas of Bag 19 should be lower than those of the control packaging, as we are talking about negative markers, i.e., compounds that negatively influence the characteristics of the product and which therefore should be present at the lowest possible concentration. This actually occurs for Bag 19.

4.4.2.2. Microbiological analysis, moisture (%), water activity (A_w) and pH

The microbial counts relating to aerobic mesophilic microorganisms, coagulase-positive staphylococci, moulds and yeasts, detected in the Panettone stored in the two types of packaging, showed values with an increasing trend in the first month and then a decrease to 2 log CFU/g of Panettone after 2 months. Finally, in the third month of storage the values went down below the limit of detection. After 1 month, the sample stored in the common CTR packaging, showed a total aerobic mesophilic bacteria (**figure 28**), moulds and yeasts (**figure 29**) counts higher than those of the sample stored in ID. The total aerobic mesophilic bacteria count of the CTR sample remained always higher until the 2nd month, when the values coincided in both samples up to the third month with a microbial count lower than 2.5 log CFU/g of Panettone.

On the other hand, the value of 3.4 log CFU/g in "panettone" stored in the CTR packaging for moulds and yeasts was reduced to undetectable levels after 1 month, while for the sample in ID packaging, it was lower (< 2 log CFU/g) already after one month.

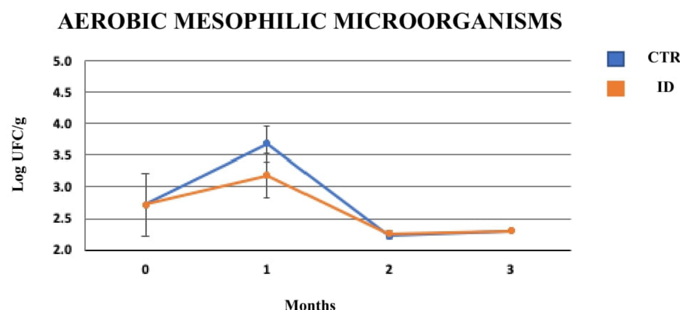


Figure 28: Microbial load (Log UFC/g) of aerobic mesophilic microorganisms in panettone, preserved for three months in two types of packaging at 25 °C.

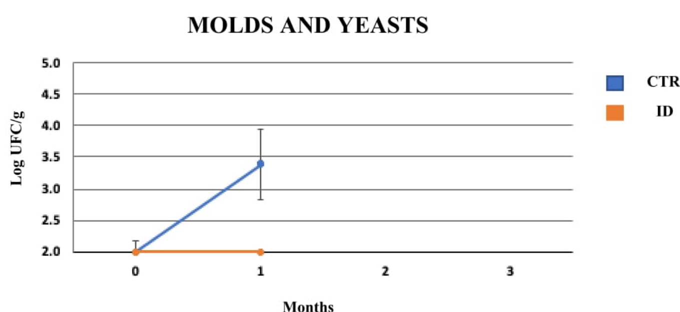


Figure 29: Microbial load (Log UFC/g) of molds and yeasts in panettone, preserved for three months in two types of packaging at 25 °C.

The coagulase positive staphylococci count (**figure 30**) was lower in Panettone preserved in the CTR packaging with respect to that kept in ID during the first two months of storage, then the bacterial level decreased during the first 2 months, until reaching levels below 2 log CFU/g, which is the limit of detection. The *E. coli* β-glucuronidase positive, the presumed *Bacillus cereus* and the total coliforms count are immediately below the detection threshold of the assay. In all cases the differences found between the Panettone preserved in the two packaging were not statistically significant.

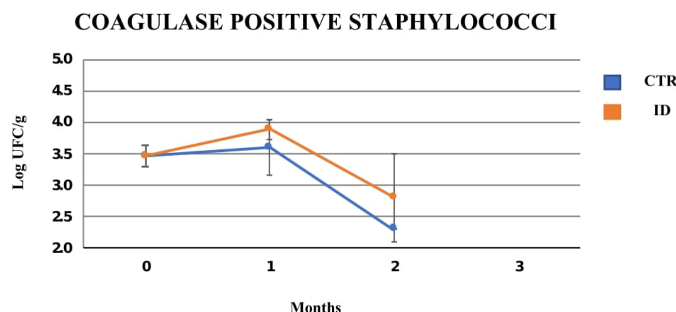


Figure 30: Microbial load (Log UFC/g) of coagulase positive *Staphilococci* in panettone, preserved for three months in two types of packaging at 25 °C.

The water activity (A_w) showed a reproducible trend from the day of production and insertion of the product into the respective packaging up to the 3rd month (**figure 31**). The sample stored in the CTR packaging showed a slightly higher A_w value with respect to that stored in ID. However, the difference between the two sample was not statistically significant.

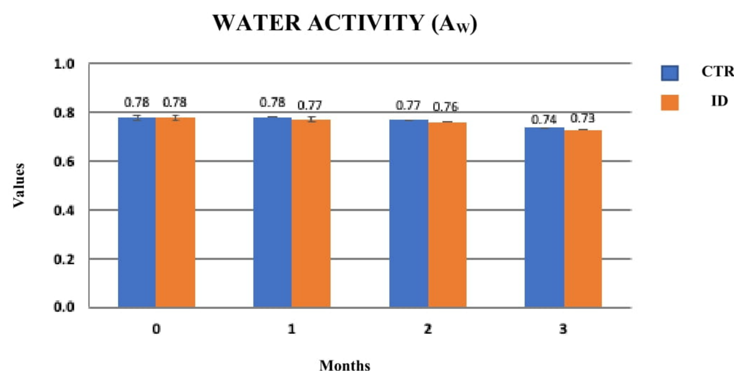


Figure 31: Water activity (A_w) in panettone, preserved for three months in two types of packaging at 25 °C.

The moisture of Panettone stored in the CTR packaging decreased from a value of 15.7% to 13.3% after 1 month, it remained stable throughout the second month of storage, and it decreased to a value of 10% after three months of storage (**figure 32**). The Panettone stored in CTR packaging showed a decrease in moisture content after 1 month (from 15.7% to 11.7%), but turned out to have the same initial moisture after 2 months of storage. After 3 months it reached a value of 14.4%, showing in both cases a statistically significant difference ($p<0.05$). In summary, ID packaging had the ability to maintain the moisture of

the panettone for longer (up to 3 months), even if with a small variation after 1 month of storage.

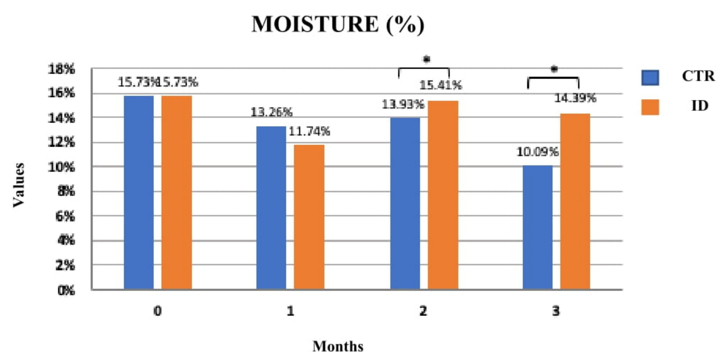


Figure 32: Moisture (%) in panettone, preserved for three months in two types of packaging at 25 °C.

In each sampling time, the pH of the panettone was measured. Small variations were detected in both samples with values not too different from the initial value (**figure 33**).

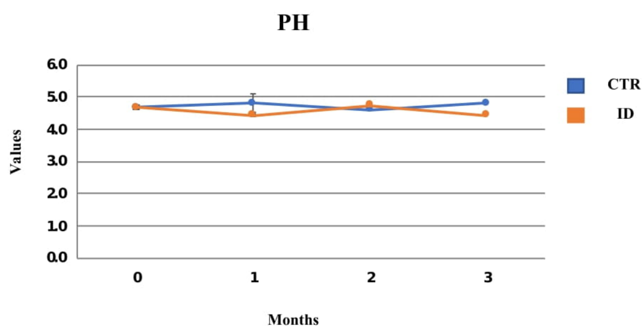


Figure 33: pH values in panettone, preserved for three months in two types of packaging at 25 °C.

4.4.2.3. Sensorial analysis

Sensorial analysis was performed in Panettone preserved in both packaging after 1 (t1) and 2 (t2) months of storage. The results obtained were summarized in ***Table 40***.

Table 40: Descriptors studied in the sensorial analysis.

	P value	Reference	Performance clusters	T1	Performance clusters	T2	Performance clusters
Smell of butter	0.3840	5.00	a	5.17	a	5..08	a
Egg smell	0.0552	5.00	a	5.00	a	5.42	a
Vanilla smell	0.0526	5.00	a	5.42	a	5.50	a
Toasty smell*	0.0007	5.00	b	5.25	b	6.08	b
Rancid smell*	0.0422	2.00	a	2.00	a	1.75	a
Sweet taste	0.3840	5.00	a	5.00	a	5.08	a
Bitter taste	0.3840	2.00	a	2.00	a	1.92	a
Butter flavour*	0.0076	5.00	b	5.08	b	5.58	a
Egg flavour*	0.0033	5.00	b	5.75	a	5.67	a
Vanilla flavour*	0.0023	5.00	b	5.25	b	6.00	a
Toasted flavour	0.5333	5.00	a	5.17	a	5.17	a
Rancid taste	0.2737	2.00	a	1.83	a	1.83	a
Moisture of the dough*	0.0011	5.00	b	5.67	b	6.83	a
Softness*	0.0340	5.00	b	5.50	ab	6.08	a
Airy/porous	0.2439	5.00	a	5.08	a	5.33	a
Dough elasticity*	0.0093	5.00	b	5.83	a	5.58	a

*The descriptors highlighted in yellow are statistically significative among the samples; the data highlighted in red represented statistically lower values, while data highlighted in green represented statistically higher values.

Results showed that there is a different performance in terms of maintaining sensorial attributes in the product preserved in CTR packaging with respect to that preserved in ID packaging. Statistically significative differences were found for these descriptors: toasty smell, rancid smell, butter flavour, egg flavour, vanilla flavour, moisture of the dough, softness and dough elasticity. **Figures 34** and **35** showed these descriptors and their pValue.

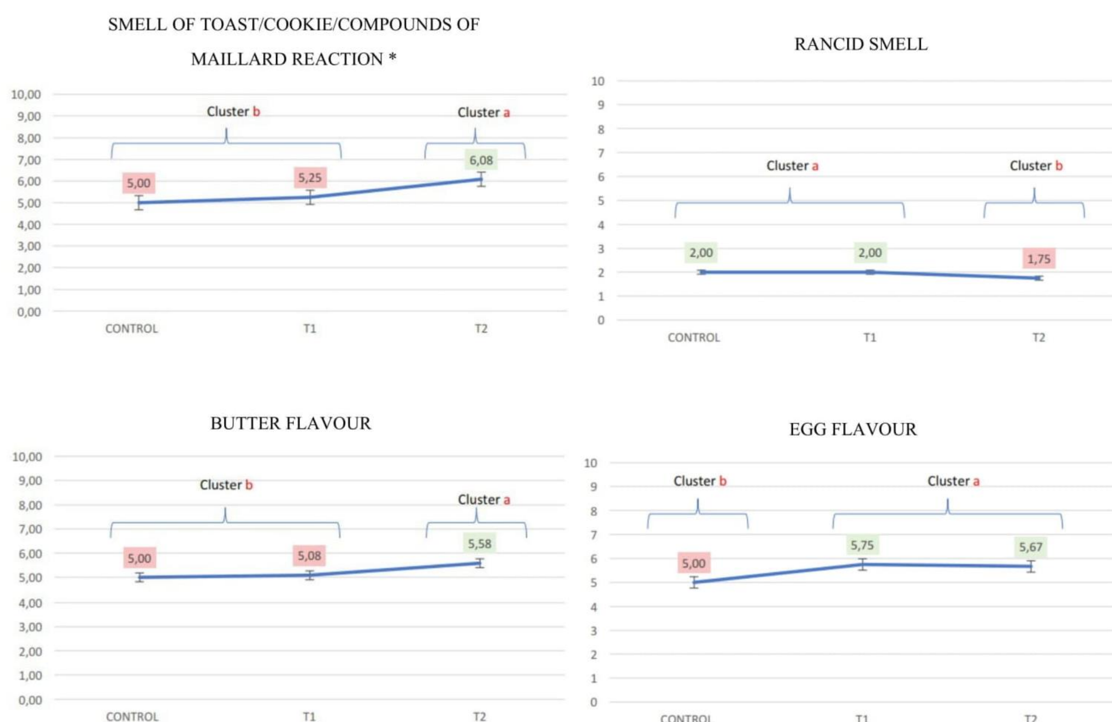


Figure 34: Smell of toast, rancid smell, butter and egg flavours descriptors.

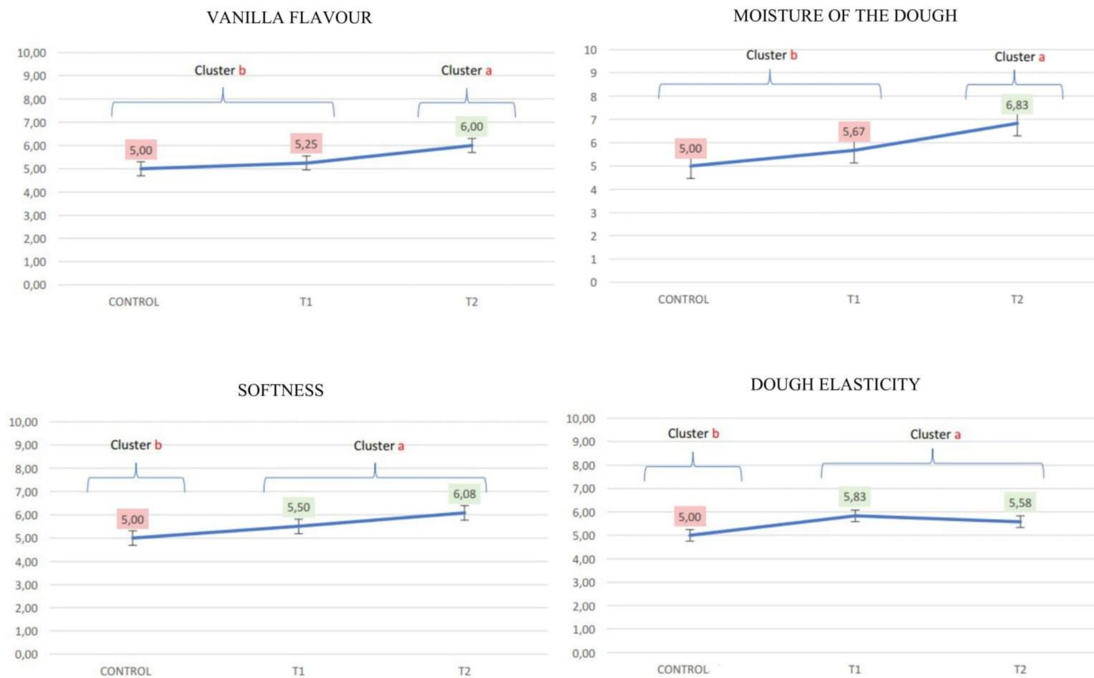


Figure 35: Vanilla flavour, softness, moisture and elasticity of the dough.

The results indicated a better ability to retain sensory characteristics in Panettone preserved in ID packaging with respect to that preserved in CTR packaging. In particular, all the yellow highlighted descriptors (with the exception of rancid smell) have a higher value at T2 in Panettone preserved in the ID packaging with respect to that preserved in CTR. It should also be noted that the gap between the Panettone preserved in the ID packaging and that preserved in CTR packaging increased with the shelf-life.

4.4.3. Conclusions

In conclusion, this study demonstrated that ID packaging have a higher ability in the preservation of Panettone with respect to CTR, according with chemical, microbiological and sensorial analyses. In fact, most of the VOCs identified in Panettone samples have a lower abundance (area%) in Panettone preserved in *Ideabrill* ® packaging with respect to that preserved in the conventional one and this was related to a lower degree of deterioration, as most of the compounds come from oxidation processes. Results from microbiological analysis demonstrated that aerobic mesophilic microorganisms, molds and yeasts proliferation were lower in Panettone preserved in *Ideabrill* ®, with respect to that preserved in the conventional. Moreover, the higher ability of preserving the moisture of Panettone of *Ideabrill* ® packaging with respect to the conventional one has been demonstrated. Regarding the sensorial analysis, 8 of the 16 descriptors considered resulted to be maintained better in Panettone preserved in *Ideabrill* ® packaging with respect to that preserved in the conventional one.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis had the aim of studying food shelf-life markers and food quality through analytical methods.

Regarding the studies on food shelf-life markers, results showed that *Ideabrill* ® packaging by Esseoquattro was able to prolong the shelf-life of raw and baked hams and this was demonstrated by the study of BAs through HPLS-DAD and VOCs through a new quantitative HS-SPME-GC-MS method.

Studies of the quality of coffee and bakery products resulted to be useful to find new strategies to improve food safety. In fact, the use of washed post-harvesting method in coffee, ventilated cooking and the use of filler cremes with ingredients that have not been subjected to previous roasting processes in wafers resulted to be useful to reduce the concentrations of furanic compounds that can be toxic for the human health. Finally, according to microbiological and sensorial analyses, the study of the volatile profile of Panettone resulted to be useful to determine the higher ability of *Ideabrill* ® packaging for the preservation of this food product with respect to the conventional ones.

In conclusion, this thesis demonstrated that the study of food shelf-life, food quality and safety play a central role in the field of food chemistry research. However, further studies are needed to better understand the processes involved in food degradation, the compounds involved in this process and to develop new strategies to improve food shelf-life in order to protect the health of the consumers.

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