UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Agricultural Sciences and Veterinary Medicine

Second Cycle Degree (MSc)
in Italian Food and Wine

(Gas Chromatography on Italian Cheese Products)

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Abstract

In recent times, there has been a growing awareness among consumers regarding the authenticity and quality of local and traditional products. To meet the high-quality standards for food products, international regulations require certain criteria to be met, including the absence or presence within specific limits of certain ingredients. Gas chromatography (GC) is an analytical technique employed to separate the chemical components of food and determine their presence, absence, or quantity. In order to assess compliance with regulations and identify potential fraudulence or to better characterize products, a combination of gas chromatography with other systems has been utilized to evaluate Italian cheeses. The application of gas chromatography in this analytical method is preferred due to its ease of diagnosis, sensitivity, efficiency, cost-effectiveness, and short analysis time. The utilization of gas chromatography in this analytical method, including the combination with other systems, allows for both targeted and nontargeted approaches to assess the quality and authenticity of the Italian cheeses.

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Introduction

This dissertation is a collection of research based on practical experience gained during a six-month internship at Bilacon GmbH Berlin Laboratory. Gas chromatography is an essential method widely used to determine the quality and authenticity of valuable Italian cheeses. By combining gas chromatography with specific instruments, it's possible to identify volatile parts and tell different types of cheese apart. Moreover, gas chromatography plays a key role in understanding the diets of animals that produce milk by identifying specific markers. This helps evaluate and control the allowed ingredients in each cheese.

These articles discusses the importance of accurately confirming food and assessing quality. It highlights the significant role of the gas chromatography device. This method is used to tackle modern challenges of food tampering and dishonesty and has been thoroughly studied due to its vital role in setting and maintaining strict quality standards.

CHAPTER 1: FOOD AUTHENTICATION

1.1.What is Food Fraud

Food fraud denotes the purposeful maneuvers carried out by food suppliers with the intention of misleading their customers about the quality and composition of the items they are in the process of procuring. While financial gain often serves as the primary motivation behind food fraud, certain instances of such deception can also pose direct health risks to consumers. Detecting food fraud presents a formidable challenge, as consumers alone lack the ability to identify such fraudulent practices, and those perpetrating food fraud continually innovate methods to avoid detection. (FAO., 2021)

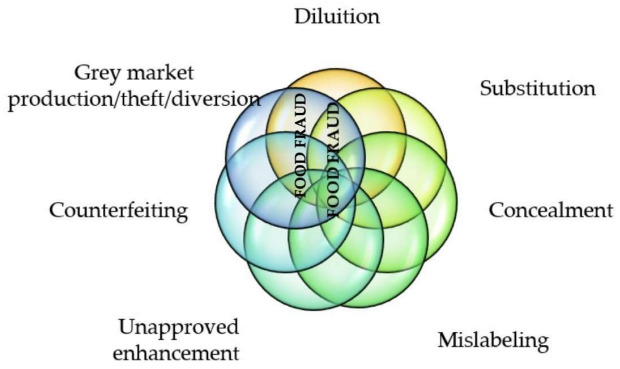

Figure 1:Types of food fraud
(EC, 2020)

Chemical, biological, and physical contamination can manifest at any point within the dairy supply chain, with various hazards introduced during both primary production and processing phases. (Figure 1)

- **Dilution:** The act involves blending a liquid ingredient of higher value with a liquid possessing lesser value.
- **Substitution:** This implies the exchange of a valuable ingredient or a portion of the product with a less valuable counterpart.
- **Concealment:** This revolves around veiling the subpar quality of food components or products.
- **Mislabeling:** The practice encompasses affixing false claims on packaging to achieve economic advantage.
- **Unapproved enhancement:** This pertains to introducing undisclosed and unknown substances into food items to augment their quality attributes.
- **Counterfeiting:** The action involves duplicating brand names, packaging concepts, recipes, processing methods, and more of food products with the intention of economic gain.
- **Grey market production/theft/diversion:** This involves the illicit sale of unreported surplus products. (EC, 2020)

1.2.Factors Contributing to Food Fraud

The rise in factors such as international trade, global markets, intricate food supply chains, and the rapid expansion of online commerce has opened up more avenues for food fraud. While some instances of food adulteration might not carry substantial health risks, such as misrepresenting the origin, others can be perilous due to the introduction of toxic or allergenic substances. Food fraud not only fosters unfair competition but also misleads consumers. Consequently, the significance of ensuring food authenticity and curbing food fraud has captured the attention of diverse stakeholders, including government bodies, policymakers, laboratories, producers, the industry, and researchers. Their collaborative endeavors are aimed at defining and effectively addressing these critical concerns (Hassoun et al., 2020).

1.3.Food Fraud in Dairy Products

Across the world, milk and milk products hold a crucial position in diets. Over the last three decades, production and consumption have risen due to their nutritional advantages. In order for this upward trend to persist, ensuring the safety and genuineness of dairy items becomes imperative, encompassing a substantial area of apprehension. Throughout the journey, spanning from the farm to the processing stage, various forms of contamination (be it biological, chemical, or physical) might arise either unintentionally or deliberately (Montgomery H et al., 2020).

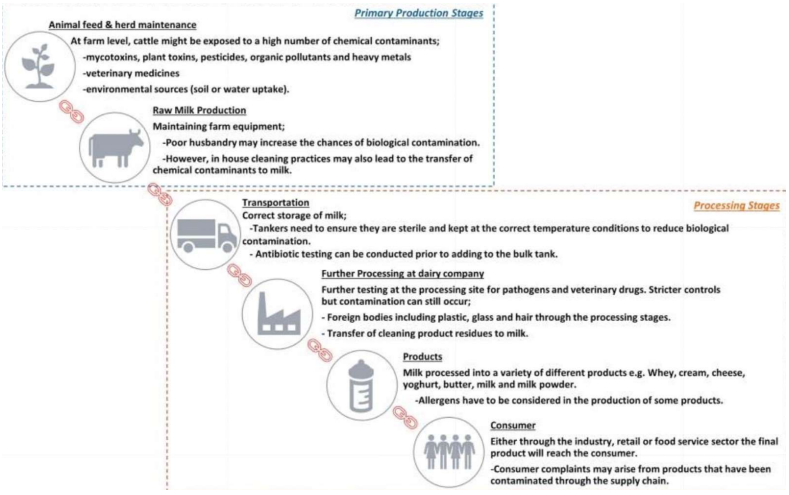

Figure 2: The dairy supply chain highlights zones of contamination during both the primary and processing phases. (van Asselt et al., 2017)

1.4.Food Authentication

Recently, heightened consumer awareness and concern regarding food quality and safety have spurred the quest for innovative solutions. The modern consumer's expectations extend beyond nutritional details during food purchases; they now seek comprehensive information encompassing origin, safety, traceability, and authenticity. Concurrently, environmental and ethical considerations have taken center stage, drawing attention to areas like safeguarding biodiversity, refining production methods, and ensuring the authenticity of food.

In this evolving landscape marked by a surging appetite for top-tier quality foods, a preference for distinct culinary experiences, and a willingness to invest more in such products, inadvertently encourages the adulteration of premium foods. Moreover, the interplay of factors, including international trade, global market expansion, intricate supply chains, and the surge of e-commerce, provides fertile ground for instances of food fraud. Though certain adulterations may not carry immediate health risks—like mislabeling geographical origins—others hold the potential for health hazards stemming from toxic or allergenic substances. Despite the direct health implications, food fraud engenders skewed market competition and consumer deception.

Due to these compelling factors, the significance of food authentication has surged. It entails the analytical process of verifying label information related to a food's origin and production procedures (Danezis et al., 2016). This authentication process holds strategic importance for the food industry due to the escalating consumer interest in the quality and source of their food. This holds particularly true for consumers investing in pricier certified and high-value products, such as organic items or those with protected designations of origin (PDO) and protected geographical indications (PGI) (Caligiani et al., 2016).

1.5. Target and Non-Target Approaches

Methods that look for a predefined feature or adulteration are called "targeted methods", while methods that do not rely on analyzes of selected individual analytes because the molecules to be detected are not known in advance, but instead aim to examine the fingerprint as comprehensively as possible are called "non-targeted methods". (Ballin, N.Z, 2019; Cavanna et al,. 2018; Creydt et al, 2020)

The untargeted approach proves to be advantageous in situations where dealing with potential adulterers lacks prior information or when encountering non-traditional adulterations that are unlikely to be detected through conventional targeted methods. (Amaral, J. S., 2021)

1.6.Usage of GC-MS with Targeted and Untargeted Analyze

1.6.1.Targeted Analysis

GC-MS is commonly used for targeted analysis when the goal is to identify and quantify specific compounds of interest. The GC separates the mixture of compounds, and the mass spectrometer detects and measures

the mass-to-charge ratios of the analyte ions. The presence and concentration of the target analytes can be determined by comparing the mass spectra obtained from the sample to reference spectra or using calibration curves prepared with known standards. This approach is useful for quantifying known compounds, such as pesticides, drugs, or specific volatile organic compounds. (National Library of Medicine, 2019)

1.6.2.Untargeted Analysis

GC-MS can also be used for untargeted analysis when the goal is to obtain a comprehensive chemical profile of a sample without prior knowledge of the specific compounds present. In untargeted analysis, the GC-MS system is used to generate a complex fingerprint of the sample's chemical composition. The GC separates the mixture of compounds, and the mass spectrometer provides mass spectra for all the compounds eluting from the GC column. The obtained mass spectra can be compared to spectral libraries or databases, or analyzed using statistical methods to identify known compounds or discover new compounds. Untargeted GC-MS is useful for applications such as metabolomics, environmental analysis, and forensic analysis, where a broad analysis of the sample is desired. (National Library of Medicine, 2019)

It's important to note that the specific experimental setup, including the choice of column, mass spectrometer parameters, and data analysis methods, can be tailored to the targeted or untargeted approach. The selection of targeted or untargeted analysis using GC-MS depends on the research objectives and the information sought from the analysis.

Approach	Advantages	Disadvantages
Targeted metabolite analysis	• Low limit of detection • Usually quantitative • Data analysis and interpretation are easier • Metabolite data can be connected with pathways	• Limited number of compounds can be targeted • Non-targeted compounds are not considered • Purified standards of targeted compounds are required for quantification
Untargeted metabolite profiling	• Unbiased and comprehensive • High-throughput • Allows the discovery of new compounds not expected to be in the sample or not expected to be associated with the biological question	• Semi-quantitative • Larger number of false positives and false negatives • Many unknowns • Data interpretation can be challenging

Table 1 : Advantages & Disadvantages of Targeted Metabolite Analysis and Untargeted Metabolite Profiling (Jacob et al., 2018; Pinu et al., 2013; Patti et al., 2012; Villas-Bôas et al., 2005)

1.7. What is Fingerprint ?

Fingerprinting is a recently devised analytical technique that employs instrumental fingerprints to gather information concerning a material's characteristics, which are connected to its chemical makeup or rely on it. These characteristics may encompass the material's identity, specific physicochemical properties, natural attributes (biological, sensory, etc.), or the presence and quantity of certain chemical compounds within the material. This methodology is primarily used for monitoring food products, yet it has potential applications in various other domains, including botany, forensics, cultural heritage, petrochemistry, and pharmaceutics.

Instrumental fingerprints denote signals acquired from a particular material through an analytical instrument and subsequently stored in a suitable database. These signals inherently contain information about the chemical composition of the measured material, although this information remains concealed and is not overtly or explicitly displayed, nor does it pertain to any particular compounds. (Cuadros-Rodríguez et al., 2021)

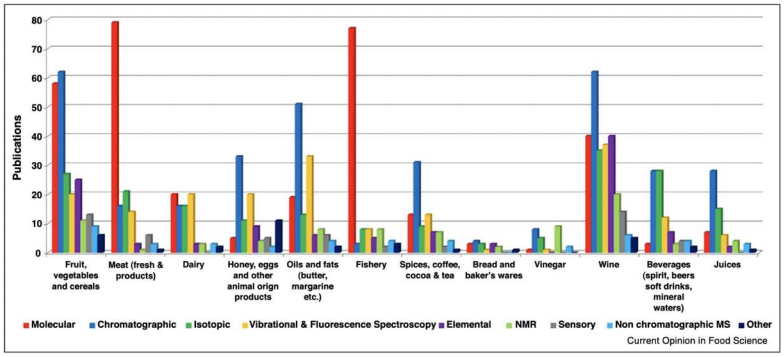

Figure 3. The usage amounts of the preferred analytical methods to find fingerprints of food varieties (Danezis et al., 2016)

In detection fingerprint, for dairy products chromatographic method is the second most used method with isotropic methods after molecular and Vibrational & Fluorescence Spectroscopy techniques. (Figure 3)

1.8.Fingerprint based methods

Analytical techniques known as fingerprinting-based methods rely on distinct characteristics or patterns, commonly referred to as "fingerprints," to distinguish and discern between various samples or substances. These methods leverage the exclusive features or profiles displayed by the samples, enabling their categorization, comparison, and identification. The following are examples of frequently employed fingerprinting-based methods.

1.8.1.DNA Fingerprinting

DNA fingerprinting, also known as DNA profiling or genetic fingerprinting, is a laboratory technique used to determine the probable identity of an individual based on the distinct nucleotide sequences found in specific regions of their DNA. This technique has various applications, such as in criminal investigations, forensic purposes, and paternity testing. The primary objective in these situations is to compare two DNA fingerprints - one from a known individual and the other from an unknown person - to establish a match. (National Human Genome Research Institute, 2023)

1.8.2.Chromatographic Fingerprinting

Chromatographic fingerprinting encompasses the utilization of chromatographic methods like gas chromatography (GC), high-performance liquid chromatography (HPLC), or thin-layer chromatography

(TLC) to separate and analyze intricate mixtures. Through this process, a distinct pattern or chromatogram is generated, enabling the comparison and identification of samples based on their chemical composition. Chromatographic methods yield various data, including retention time, peak intensity (area or height), and, when coupled with MS, peak m/z. In GC-MS, spectra consist of multiple m/z fragments from a single analyte, whereas in LC-MS, there is less fragmentation, and isotopic peaks can be detected. Furthermore, comprehensive two-dimensional chromatography introduces an additional chromatographic dimension by providing two retention times instead of one. This augmentation enhances the potential for profiling and identification.

A chromatographic profile expresses certain information about certain chemical components, while a chromatographic fingerprint contains non-specific and non-specific information that must be extracted by chemometric tools.

Chromatographic techniques:

GC-FID: Gas chromatography with flame ionization detection

GCeMS: Gas chromatography coupled to mass spectrometry

GC-(IT)MS: Gas chromatography coupled to mass spectrometry with a trap ionic ioniser

GC-(TOF)MS: Gas chromatography coupled to mass spectrometry with a time-of-flight analyser (Cuadros-Rodríguez et al., 2016)

In the realm of analytical techniques, fingerprinting involves various methods that offer non-selective insights into the composition of certain foods. The primary objective is to characterize or authenticate the food. The terminology of "fingerprinting" and "profiling" originates from the field of metabolomics.

Metabolomics has been applied over the past decades to gain a comprehensive understanding of diverse food materials, aiming to enhance and evaluate food quality. (Muroya et al., 2020)

Metabolomic methodologies encompass two distinct approaches. One is targeted, focusing on particular compounds or metabolic pathways. This approach necessitates forming initial hypotheses grounded in prior knowledge (Bohm et al., 2009; Tao et al., 2008). The other is untargeted and results in comprehensive chemical profiles, often referred to as fingerprints, offering a holistic view of the sample.

Metabolomics involves employing sophisticated analytical techniques to quantitatively analyze the metabolome or a portion of it. Apart from sampling and sample preparation, it encompasses chemical analysis and data analysis. While biochemistry traditionally focuses on measuring and quantifying individual or a few metabolites, metabolomics stands out by detecting a broader range of metabolite classes, utilizing diverse analytical methods, and requiring advanced signal processing and bioinformatics tools. (Cevallos-Cevallos et al., 2009)

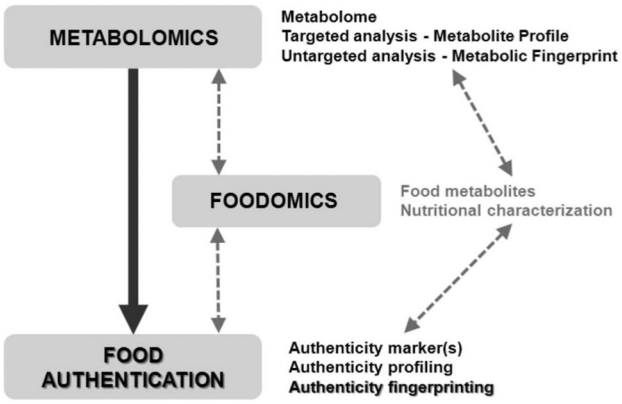

Figure 4: Food metabolomics, foodomics and food authentication
(Cuadros-Rodríguez et al., 2016)

Metabolomics provides a powerful tool to investigate the chemical composition of food, which can be used for both food authentication and foodomics studies. By identifying unique metabolite patterns, researchers can determine the authenticity of food products and gain insights into their potential health effects (Figure 4).

1.8.3. Spectroscopic Fingerprinting

Spectroscopic techniques utilize light to interact with matter, enabling the examination of specific aspects of a sample to gain insights into its composition or structure. As electromagnetic radiation, light exhibits various energies, and depending on these energies, diverse molecular characteristics of the sample can be investigated. (Hofmann, 2010)

Spectroscopy techniques are commonly categorized according to the wavelength region used, the nature of the interaction involved, or the type of material studied. Here there are types of spectroscopy:

- Infrared (IR) Spectroscopy
- Ultraviolet-Visible (UV/Vis) Spectroscopy
- Nuclear Magnetic Resonance (NMR) Spectroscopy
- Raman Spectroscopy
- X-Ray Spectroscopy

(PlatypusTechnologies. (n.d))

1.8.4.Mass Spectral Fingerprinting

Mass spectral fingerprinting involves the generation of unique mass spectra based on the fragmentation patterns of molecules. Mass spectrometry techniques, such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), are commonly used for this purpose. Mass spectral fingerprints are used for compound identification, classification, and comparison (Halket et al., 2005).

1.8.5.Metabolomic Fingerprinting

Metabolic fingerprinting, as a high-throughput analytical technique, primarily relies on spectroscopic methods to classify samples based on their origin or biological significance (Wang et al., 2012). It involves the use of techniques like nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS) to generate metabolomic profiles or fingerprints. These fingerprints are used to study metabolic changes, identify biomarkers, and classify samples based on their metabolic composition (Kosmides et al., 2013). Currently, cheese and yogurt component analyses are also performed through metabolomic approaches, such as GC-MS, NMR, and liquid chromatography-electrospray ionization-MS (LC-ESI/MS) (Selamat, 2021).

These are some examples of fingerprinting-based methods that are widely used in various scientific fields. Each method relies on specific characteristics or patterns to create unique profiles that can be used for identification, classification, or comparison of samples.

1.9.Relation between fingerprint and Gas Chromatography

Fingerprint modeling and analysis play a significant role in both food product discrimination and quality control.(Esteki et al., 2017) Due to the intricate nature of GC-MS fingerprints, it can be challenging to discern differences between similar samples, which may not be readily apparent. As a result, employing chemometrics modeling with machine learning methods becomes a promising approach to enhance the prediction of sensory data. (Bi et al., 2020)

The gas chromatography fingerprint technique offers several benefits, including rapid performance and cost-effectiveness. It enables uninterrupted analysis in both field and laboratory settings. Notably, this method has proven successful in scenarios where traditional production logging techniques lack efficacy. (Wen et al., 2005)

CHAPTER 2: GAS CHROMATOGRAPHY

2.1.Gas Chromatography

Gas chromatography refers to a set of analytical separation methods employed for examining volatile substances in the gas state. This technique involves dissolving and vaporizing the sample's constituents within a solvent, facilitating the separation of analytes by partitioning them between two phases: a stationary phase and a mobile phase. A chemically inert gas functions as the mobile phase, aiding in conveying the analyte molecules through a heated column. Unlike other chromatographic methods, gas chromatography uniquely doesn't rely on mobile phase-analyte interactions. Meanwhile, the stationary phase can be a solid

adsorbent, leading to gas-solid chromatography (GSC), or a liquid supported by an inert material, yielding gas-liquid chromatography (GLC). (Hopfer, 2022)

An injection port, a column, equipment for controlling carrier gas flow, ovens, and heaters to regulate temperatures of both the injection port and the column, an integrator chart recorder, and a detector constitute the key components of a standard gas chromatograph. (see Figure 5)
In gas-liquid chromatography, the process of compound separation involves introducing a solution sample containing desired organic compounds into the sample port, where it undergoes vaporization. Subsequently, the vaporized samples injected are conveyed by an inert gas, typically helium or nitrogen. This inert gas traverses a glass column filled with silica coated in a liquid phase. Substances less soluble in the liquid phase manifest quicker separation compared to those with higher solubility. This module aims to enhance comprehension of the separation and measurement methodologies, as well as the practical applications associated with gas chromatography. (Krugers, 1968.)

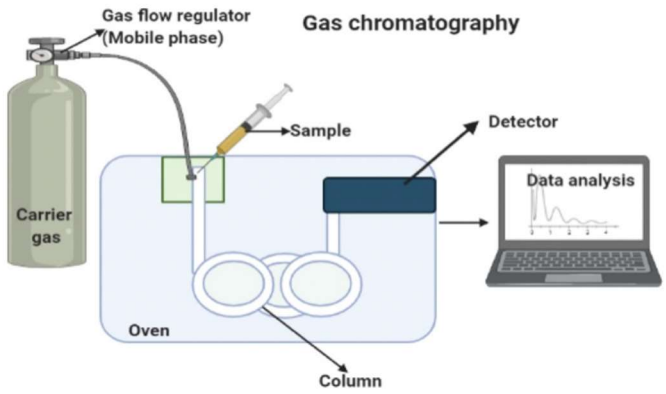

Figure 5. The parts of Gas Chromatography
(Kumari et al., 2022)

2.2.Mass Spectrometry

Built upon the ionization of sample molecules within the gaseous phase, mass spectrometry (MS) subsequently segregates and identifies the resultant ions based on their mass-to-charge ratio (m/z). The method of ionization and the desired purpose determine whether the ionized sample molecules are deliberately broken down to generate useful product ions. The findings are portrayed through a mass spectrum, graphically illustrating the relationship between ion abundance and m/z values. Having been initially documented in 1912 as a parabola spectrograph, the instrumental technique of mass spectrometry (MS) has evolved into widespread use. Over time, a multitude of advancements and refinements have transformed MS into a cornerstone technology, transitioning from its origins in physics laboratories to its present application in analytical chemistry and forensic science settings. (Smith, 2013)

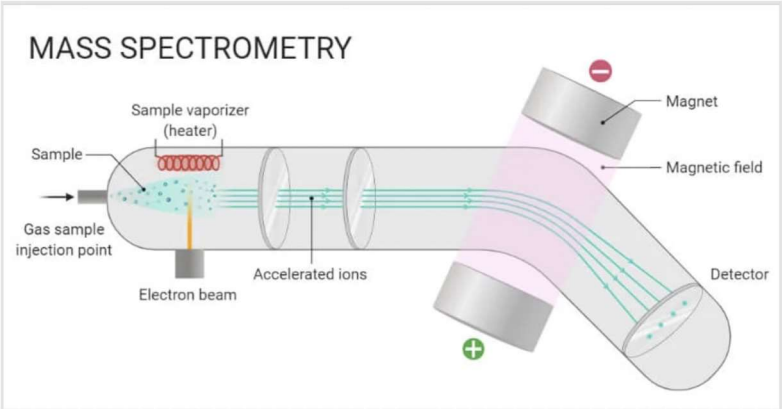

Figure 6: Schematic of mass spectrometer
(Khan Academy, n.d.)

2.3. GC-MS

The combination of Gas Chromatography (GC) and Mass Spectrometry (MS), known as GC/MS, is utilized for analyzing complex organic and biochemical mixtures. While GC excels in separating volatile and semi-volatile compounds with exceptional precision, it lacks the ability to identify them. On the other hand, MS can precisely identify and quantify most compounds by providing detailed structural information but struggles with their separation. Hence, it was not surprising that the idea of combining these two techniques was proposed soon after the development of GC in the mid-1950s. Gas chromatography and mass spectrometry are highly compatible methods in several aspects: both operate with vapor-phase samples, and both handle similar sample quantities and is a robust.(Hussain & Maqbool, 2014)

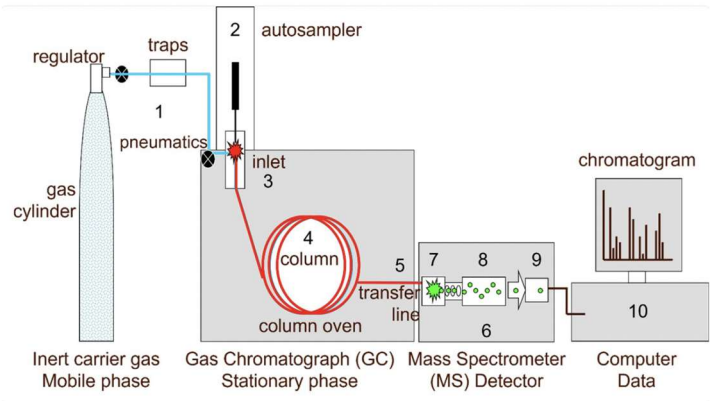

Figure 7: A simplified diagram of a gas chromatograph–mass spectrometer showing (1) carrier gas, (2) autosampler, (3) inlet, (4) analytical column, (5) interface, (6) vacuum, (7) ion source, (8) mass analyzer, (9) ion detector and (10) PC.

(Technologynetworks, 2022)

Figure 8: Appearance of GC System
Moore Analytical. (n.d.). GC-MS. Retrieved [23.07.2023], https://www.mooreanalytical.com/gc-ms/

2.4. Working Principle of GC-MS

GC-MS is a versatile analytical technique applicable to liquid, gaseous, or solid samples. The analysis commences within the gas chromatograph, where the sample undergoes efficient vaporization into the gas phase and is subsequently separated into its individual components through a capillary column coated with a stationary phase, either liquid or solid. An inert carrier gas like helium, hydrogen, or nitrogen propels the compounds through the system. Due to the separation, each compound elutes from the column at distinct times based on its boiling point and polarity, which is known as the compound's retention time. This capability enables GC to effectively resolve complex mixtures or sample extracts containing a multitude of compounds.

Following the exit from the GC column, the components are ionized and fragmented by the mass spectrometer, utilizing either electron or chemical ionization sources. Subsequently, the ionized molecules and fragments are accelerated through the mass analyzer, typically a quadrupole or ion trap, and separated based on their mass-to-charge (m/z) ratios. The data acquisition in GC-MS can be performed using full scan mode, covering a wide range of m/z ratios, or selected ion monitoring (SIM) mode, which focuses on specific masses of interest. In the final stages of the process, ion detection and analysis take place, with fragmented ions displaying their m/z ratios. The peak areas in the gas chromatogram are directly proportional to the quantity of the corresponding compound. For complex samples, multiple peaks will appear, and each peak generates a unique mass spectrum used for compound identification. By utilizing extensive commercially available libraries of mass spectra, it becomes possible to identify and quantify unknown compounds and target analytes. (Termofischer., (n.d)

2.5. Interpreting of GC results

Gas chromatography involves the passage of gases through a specially coated column designed to attract different components of the sample to varying degrees. This phase, known as the stationary phase, plays a crucial role in the separation of components, leading them to elute at distinct times and appear as peaks on the chromatogram. (InnovaTECH., 2018)

2.5.1.The X-Axis: Retention Time

Typically, in a gas chromatogram, the x-axis represents the temporal duration required for the analytes to traverse the column and reach the mass spectrometer detector. Evidently, the exhibited peaks correspond to the specific moments when individual components reached the detector.

The selection of the column type and the specific GC parameters (such as flow rate, injection temperature, oven temperature, etc.) significantly influence the retention time. Thus, for precise and reliable comparisons of retention times across distinct analyses or laboratories, it becomes imperative to employ identical parameters to ensure accuracy. (InnovaTECH, 2018)

2.5.2.The Y-Axis: Concentration or Intensity Counts

In a typical GC/MS chromatogram, the y-axis, represented by the peak area, serves as an indicator of the quantity of a specific analyte in the sample. This area measurement corresponds to the counts recorded by the mass spectrometer detector at the retention point. Nevertheless, it is essential to acknowledge that certain compounds exhibit a higher affinity for the detector, resulting in larger peak areas compared to their actual concentrations relative to other peaks on the chromatogram. This phenomenon is particularly observed in compounds that ionize readily. To surmount this challenge, our experts employ known concentration standards during the analysis to ensure precise quantification. Furthermore, when encountering unknown compounds, their identification is facilitated by comparing their retention times with those of known standards using alternative detectors. Subsequently, the mass spectrometer detector aids in confirming the identity of the compound through the analysis of its mass spectrum acquired during testing. (InnovaTECH, 2018)

In figure 5, we can see that “Fatty acid composition of Pecorino Romano cheese” as an example of gas chromatogram with x axis and y axis.

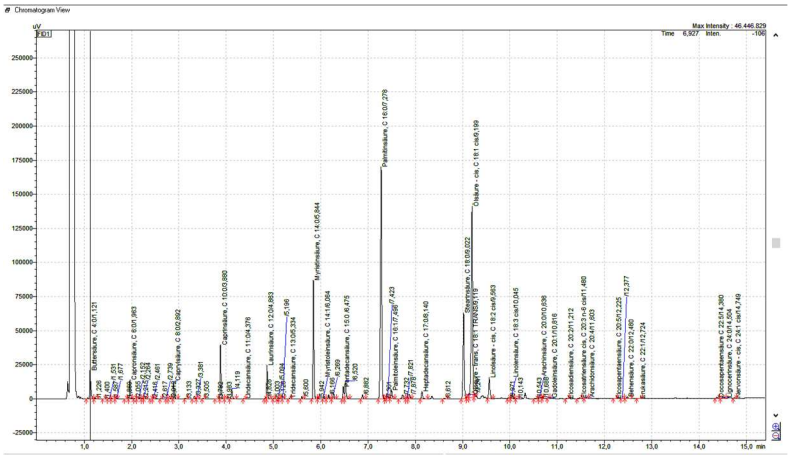

Figure 9: Fatty acid composition of a Pecorino Romano cheese on chromatogram
(The chromatogram data shared by Bilacon Laboratory GmbH)

In Figure 9, we can see those fatty acids form peaks of different sizes at different time intervals. In this chart, the fatty acid with the highest concentration in Pecorino Romano is "palmitic acid". From the point where the peak starts to the end on the Y axis, the "retention time" of that fatty acid, therefore, the rate of passing through the column can be seen. Small protrusions in the graph are not considered peaks, they are different non-fatty acids contained in Pecorino Romano cheese.

Pecorino Romano		
Fame	**Concentration [g/100g]**	**Corrected Concentration [g/100g]**
butyric acid, C 4:0	2,978	**3,34**
caproic acid, C 6:0	1,603	**1,80**
caprylic acid, C 8:0	1,316	**1,47**
capric acid, C 10:0	3,977	**4,46**
undecanoic acid, C 11:0	0,028	**0,03**
lauric acid, C 12:0	2,550	**2,86**
tridecanoic acid, C 13:0	0,072	**0,08**
myristic acid, C 14:0	9,739	**10,91**
myristoleic acid, C 14:1	0,203	**0,23**

pentadecanoic acid, C 15:0	1,111	**1,24**
pentadecenoic acid cis, C 15:1 cis		**0,00**
palmitic acid, C 16:0	26,905	**30,14**
palmitoleic acid, C 16:1	0,914	**1,02**
heptadecanoic acid, C 17:0	0,826	**0,93**
heptadecenoic acid, C 17:1		**0,00**
stearic acid, C 18:0	9,275	**10,39**
oleic acid - cis, C 18:1 cis	23,399	**26,21**
linoleic acid - cis, C 18:2 cis	2,441	**2,73**
linolenic acid, C 18:3 cis	0,465	**0,52**
Gamma Linolenic Acid - cis, C 18:3 cis		**0,00**
stearidonic acid C18:4		**0,00**
arachidic acid, C 20:0	0,368	**0,41**
gadoleic acid, C 20:1	0,055	**0,06**
eicosadienoic acid, C 20:2	0,049	**0,05**
eicosatrienoic acid cis, C 20:3 n-3 cis		**0,00**
eicosatrienoic acid cis, C 20:3 n-6 cis	0,134	**0,15**
arachidonic acid, C 20:4	0,180	**0,20**
eicosapentaenoic acid, C 20:5	0,057	**0,06**
uneicosanoic acid, C 21:0		**0,00**
behenic acid, C 22:0	0,239	**0,27**
erucic acid, C 22:1	0,060	**0,07**
docosadienoic acid - cis, C 22:2 cis		**0,00**
docosapentaenoic acid C 22:5 n-3	0,137	**0,15**
docosahexaenoic acid, C 22:6		**0,00**
tricosanoic acid, C 23:0		**0,00**
lignoceric acid, C 24:0	0,162	**0,18**
nervonic acid - cis, C 24:1 cis	0,024	**0,03**
oleic acid - trans, C 18:1 trans-6		**0,00**
oleic acid - trans, C 18:1 trans-9		**0,00**
oleic acid - trans, C 18:1 trans-11		**0,00**
linoleic acid C18:2 (cis/trans)		**0,00**
linoleic acid C18:2 (trans/cis)		**0,00**

linoleic acid C18:2 (trans/trans)		**0,00**
linolenic acid C18:3 (cis/cis/trans)		**0,00**
linolenic acid C18:3 (cis/trans/cis)		**0,00**
linolenic acid C18:3 (trans/cis/cis)		**0,00**
linolenic acid C18:3(cis/trans/trans)		**0,00**
linolenic acid C18:3(trans/cis/trans)		**0,00**
linolenic acid C18:3(trans/trans/cis)		**0,00**
linolenic acid C18:3(trans/trans/trans)		**0,00**
total fatty acids o. TFs	89,267	**100,0**
Factor o. TFs	1,120	
Saturated Fatty Acids	**61,149**	**68,50**
monounsaturated fatty acids	**24,655**	**27,62**
Polyunsaturated fatty acids	**3,463**	**3,88**
Omega - 3 - fatty acids	**0,659**	**0,74**
Omega - 5 - fatty acids	**0,000**	

Table 2: Fatty acid composition of Pecorino cheese.
(The data in the table shared by Bilacon Laboratory GmbH)

In Table 2, the numerical values of the gas chromatogram in Figure 1 are given. The barely visible peaks in Figure 1 are system disturbances, not fatty acid peaks. Therefore, if we assume the total concentration to be 100, when we subtract these system disturbances from the total concentration, we find the total fatty acid amount, which is given as 89,267 in Table 1. When we divide the concentration of each fatty acid by the total fatty acid concentration, we find the values in the yellow column titled “Corrected Concentration”.

2.6.AS AN ALTERNATIVE METHOD FAST GC

Gas chromatography, even though it is common analytical tool it has some disadvantages such as long analysis times. Speed of analysis can count one the most significant parameters in terms of sample output. The main objective of fast GC is to achieve a satisfactory level of resolving power in a shorter timeframe when compared to conventional GC. To accomplish this, fast GC utilizes advancements in column and instrument technology, along with optimized running conditions. This results in analysis times that are three to ten times faster while maintaining acceptable resolution levels.

Fast gas chromatography (GC) can be achieved by adjusting several analysis parameters. These parameters include the length and internal diameter (ID) of the column, the type of stationary phase, film thickness, carrier gas, linear velocity, oven temperature, and ramp rate. (Schrenk & Cartus, 2017)

CHAPTER 3: GC APPLICATION on ITALIAN CHEESE PRODUCTS

In this section, it is shown an overview of the published 3 main articles and supporter articles about GC applications on some Italian cheese products such as Parmigiano Reggiano, Pecorino Romano, Provola dei Nebrodi and also their history, method of production. These studies are based on the analysis of cheese contents by gas chromatography method in order to understand whether various Italian cheeses comply with quality standards.

These cheeses are subject to strict control due to their high added value and recognition, and this section will also contribute to an understanding of the role of Gas Chromatography in their inspection.

3.1.Parmigiano Reggiano

Parmigiano Reggiano is quate important cheese in terms of traditional production, high quality, long aging duration, versatility and taste and nutritional value. The importance of detecting specific fatty acids in Parmigiano Reggiano lies in its impact on the cheese's flavor, quality, and authenticity. Fatty acids play a crucial role in determining the taste and aroma of the cheese. They also contribute to the texture and melting properties of the cheese during cooking. By analyzing the fatty acid profile, cheese producers can ensure the consistency and genuineness of their product, adhering to the traditional standards of Parmigiano Reggiano.

According to the European Commission's definition, 'Parmigiano Reggiano' is described as a hard cheese made from raw cow's milk, which is partially skimmed by allowing the cream to rise to the surface. The cheese is scalded and slowly matured. The milk must not undergo any heat treatment and has to come from cows fed primarily on fodder obtained in the area of origin. The cheese must be matured for at least 12 months. 'Parmigiano Reggiano 'can be sold whole, in portions or grated.

THE SHAPE & APPEARANCE	THE CONTENT	THE TEXTURE
Cylindrical with a slightly convex or virtually straight heel and flat faces with a slightly raised edge	The cheese contains no additives	The body of cheese is fine-grained and flaky in texture
Flat faces have a diameter of 35 cm to 45 cm and the heel height is 20 cm to 26 cm.	The minimum fat content in dry matter is 32%	Characteristic aroma and taste are fragrant, delicate and flavour some without being pungent
Minimum weight of each cheese is 30 kg	It has a cyclopropane fatty acid ratio of less than 22 mg per 100 g of fat	

Outer rind is a natural straw colour		
Rind is approximately 6 mm thick		

Table 3: Characteristics of Parmigiano Reggiano
(EC, 2016)

Figure 10: 12 months aged Parmigiano Reggiano cheese

According to European Regulations 510/2006 and 1151/12, the name PDO Parmigiano Reggiano is only given to cheese manufactured from milk produced in a small region of Italy that includes Parma, Reggio Emilia, Modena, Mantova, and Bologna with strict traditional methods. The use of silaged feed is specifically forbidden by the Parmigiano Reggiano production criteria, which also include particular rules for cow diets.
Insights regarding the make-up of their feed and the rumen's fermentation patterns can be gained from the complex profile of fatty acids present in milk fat. Furthermore, elements like the milk's geographic region of origin and the diet that cows are fed have an impact on the fatty acid content of the liquid. It has been proposed that the fatty acid composition can be used to predict the nutritional composition of the cow and the altitude at which the bulk milk is generated.

Herein lies a study in which Cyclopropane, a marker that should remain undetectable as per the fatty acid profile of Parmigiano Reggiano, was identified through employment of the GCMS instrument.

3.1.2.Quantitative GC-MS Method for Cyclopropane Fatty Acid Detection in Parmigiano Reggiano

The nutritional value of dairy foods is significantly impacted by their fatty acid (FA) composition, a parameter that has been extensively acknowledged. The modulation of this composition is well-documented to be influenced by the animal's diet (Nudda et al., 2008; Shingfield et al., 2013). The FA profile exhibited by forages, which are crucial in this context, can be subject to alteration due to a range of factors including systems, seasons, and processing. This phenomenon is substantiated by previous research findings (Lolli et al., 2021). Cyclopropanes, distinct fatty acids exclusive to certain bacterial species, play a vital role. The presence of cyclopropanes within Parmigiano Reggiano cheese serves as an authenticity indicator, signifying adherence to traditional production methods within designated regions. Incorporation of low-quality cheeses with grated Parmigiano Reggiano has become a concern, thus prompting exploration of spectroscopic methods. These methods aim to determine both the geographical origins and the degree of ripeness in Parmigiano Reggiano cheese. Additional techniques utilized to assess ripeness encompass measurements of free amino acids, oligopeptides, and D-amino acids (Caligiani et al., 2016). As a consequence, cyclopropane fatty acids (CPFAs) have emerged as impactful lipid biomarkers for monitoring ruminant feeding systems. They are particularly valuable in ensuring compliance with quality standards like protected geographical indications (PGI) and protected designation of origin (PDO). This significance becomes particularly pronounced when verifying claims of excluding silages from cattle rations, as proclaimed by producers (Lolli et al., 2020; Caligiani et al., 2016). Caligiani et al. formulated a specialized quantitative method using gas chromatography-mass spectrometry (GC-MS) to analyze cyclopropane fatty acids (CPFA), such as lactobacillus and dihydrosterculic acid. These acids, inherent in bacterial membranes, have been recently detected in dairy products and milk sourced from corn silage-fed cows. The limit of detection and quantitation for CPFA was respectively established at 60 and 200 mg/kg of cheese fat. Moreover, the intralaboratory precision, assessed across three concentration levels, met the standards outlined by the Horwitz equation. The method was applied to 304 samples of PDO cheeses with certified origins, including Parmigiano Reggiano (Italy), Grana Padano (Italy), Fontina (Italy), Comté (France), and Gruyere (Switzerland). The results revealed the absence of CPFA in all cheeses whose Production Specification Rules explicitly prohibited silage usage (Parmigiano Reggiano, Fontina, Comte, and Gruyere). Conversely, CPFA were detected in varying concentrations (300−830 mg/kg of fat) within all Grana Padano cheese samples (where silages were permitted). An experiment involving a mixture of grated Parmigiano Reggiano and Grana Padano demonstrated the method's ability to identify counterfeiting with up to 10−20% Grana Padano content.

sample	use of silages	no. of samples	no. of samples positive to CPFA[a]	mean (mg/kg fat) ± SD	range (min−max)
Parmigiano Reggiano	forbidden	200	0	nd	nd
Fontina	forbidden	16	0	nd	nd
Comté	forbidden	10	0	nd	nd
Gruyère	forbidden	10	0	nd	nd
Grana Padano	permitted	68	68	540 ± 110	300−830

[a]Positivity for CPFA > 60 mg/kg fat (LOD).

Table 4: Presence of CPFA in cheeses of known origin
(Caligiani et al., 2016)

The hypothesis suggesting CPFA's potential as an indicator for cheese made from silage-fed cattle is reinforced by the findings presented in Table 4. The data provided herein can be viewed as an initial endeavor to establish a CPFA presence database within PDO cheeses.

3.1.3. Analyzing Cyclopropane Fatty Acids: A Comparative Study of GC-MS and NMR Methods for Profiling and Quantification

While gas chromatography methods take the lead in the qualitative and quantitative determination of CPFAs in milk and dairy products, there are also alternative methods in use. Gas chromatography analysis demands the time-consuming derivatization of samples, which carries the risk of interference from by-products and the use of substantial solvent quantities. For instance, milk contains over 400 different fatty acids. In the case of cyclopropane fatty acids, Lolli et al. (2018) achieved their separation in cheese fat using an apolar capillary column. However, this column is not well-suited for optimal fatty acid separation, potentially leading to interferences due to changes in the food matrix. Moreover, other cyclopropane fatty acids might be present but remain undetected due to signal overlap with the most abundant fatty acids. Therefore, the availability of an alternative method to confirm the presence of the cyclopropane ring and potentially enable accurate CPFA quantification is of importance. Additionally, the development of a rapid method that provides the required analytical information with minimal sample preparation would be beneficial. NMR spectroscopy stands out as such an analytical tool, sidestepping the need for sample derivatization and offering the advantage of quick data acquisition, as stated by Lolli (2018).

3.2.Volatile Fraction Analysis by using HS-SPME/GC-MS

Utilized across a wide range of cheeses, headspace solid-phase micro-extraction (HS-SPME) stands as a technique with numerous benefits. It offers ease of use, a relatively swift approach, and ample sensitivity. Moreover, it eliminates the need for organic solvents. (Bezerra, 2016)

Di Donato et al.,2021; M. Ziino et al., 2005 evaluated the potential of the volatile profile in the characterization and differentiation of high value-added Italian cheeses such as Pecerino and Provola dei Nebrodi, respectively, using HS-SPME/GC–MS.

The food identity verification application of these cheeses was described by analyzing their characteristics and volatile compounds using HS-SPME/GC-MS application as described below.

3.2.1.Pecorino Romano PDO

Pecorino, the collective name for Italian cheeses made from sheep's milk, includes various kinds that differ based on the place of production, the maturing procedure, the salting methods, and the degree of aging.

These factors contribute to local diversity, influencing distinct flavors that highlight the unique terroir and regional palate across Italy. (Schirone et al., 2012)

Several Pecorino cheese varieties, including Pecorino Sardo, Pecorino Romano, Pecorino Toscano, Pecorino Siciliano, Fiore Sardo, and Canestrato Pugliese, have recently been given the protected designation of origin (PDO) status under EU legislation. This seal ensures that particular cheeses are produced in specified areas using traditional methods and in compliance with accepted criteria. However, the "Protected Designation of Origin" is absent from a large number of Pecorino cheeses made in Italy. (Cevoli et al., 2011)
'Pecorino Romano 'PDO is a hard, cooked cheese produced exclusively from fresh full-fat sheep's milk obtained from sheep bred. When it is marketed for human consumption, it is cylindrical in shape with flat sides and the diameter of its flat sides is between 25 and 35 cm. Its weight varies from 20 to 35 kg, according to the size of the cheese wheel. The crust is fine, and ivory or natural straw in colour, and is sometimes covered with special protective covers. The rind has a compact or slightly open structure; when cut it is of a colour varying between white and a more or less intense straw colour. The taste is aromatic and slightly spicy for the table cheese, and spicy, intense and pleasant at the advanced stage of maturing for the grating cheese. 'Pecorino Romano 'has a characteristic aroma. Fat content of the dry matter: not less than 36 %. (EC, 2009)

3.2.2.History of Pecorino Romano PDO

Pecorino Romano PDO, the oldest and most renowned Italian pecorino cheese, has a rich history dating back to the Roman Empire. Its manufacturing method, similar to the present one, was described by Verrone and Pliny the Elder. Historical records indicate its presence in the daily lives of the Sabine populations in Lazio for at least 2,000 years, and its name reflects this historical heritage.
During the Roman Empire, Pecorino Romano held great significance. It was considered the perfect condiment for banquets in imperial palaces, and its long shelf life made it a staple food for Roman legions during their travels. The cheese provided essential nutrients such as calcium, protein, and salt, which were crucial for restoring strength and vitality to weary soldiers. Moreover, its high salt content made it an ideal product for long-term storage.
In 227 BC, Pecorino Romano production expanded to Sardinia, where similar environmental conditions and sheep breeding practices existed. In the early 1900s, the cheese played a vital role in the economic development of the island, highlighting its valuable resources: pastures and sheep. Pecorino Romano's exceptional durability allowed Italian immigrants to enjoy this cheese even in the United States, as it could withstand the lengthy journey by ship when other types of cheese would not.
Today, Pecorino Romano continues to be produced in the same exclusive areas of origin, using the natural processes passed down through centuries. The only difference from the past is that curdling, salting, and

aging now take place in modern dairies equipped with advanced technology for hygiene and health purposes.

Developing analytical methods for the verification and tracking of certified dairy products such as Pecorino Romano PDO is important to protect their geographical indications and designations of origin from possible fraud, given their higher market value compared to other similar dairy products. Cheeses of the same variety but from different origins often exhibit variations due to the influence of local traditions on the technologies utilized in raw material transformation. These technologies encompass factors such as starters, coagulants, curd heating temperatures, and ripening conditions. (Karoui, R., & De Baerdemaeker, J. (2007))
Gas chromatography (GC) combined with static or dynamic headspace solid-phase microextraction (SPME) emerges as the optimal approach to assess the quality and authenticity of dairy products. This technique allows for the analysis of flavor compounds extracted either directly from milk or from dairy products sourced from various countries.

Accordingly, there is a study in which the volatile fraction determination of HS-SPME/GC-MS was used to determine the differentiation of Typical Italian Pecorino cheeses, and it was as follows:

3.2.3.HS-SPME/GC–MS volatile fraction determination and chemometrics for the discrimination of typical Italian Pecorino cheeses

Di Donato et al. (2021), evaluated the potentiality of the volatile profile in the characterization and discrimination of three typical high- added value Italian Pecorino cheeses: PDO Pecorino Romano (PR), PDO Pecorino Sardo (PS) and Pecorino di Farindola (PF), included by Slow Food Foundation for Biodiversity in the list of traditional food to safeguard.
The study utilized GC-MS in conjunction with HS-SPME to differentiate between the three distinct types of Pecorino cheeses. SPME, a solvent-free and straightforward sample pretreatment technique, was employed for the qualitative and semi-quantitative analysis of volatile compounds. This combination of techniques is highly suitable for extracting flavor compounds from milk or dairy products of various origins, making it an optimal choice for this analysis.

47 Pecorino samples were analyzed by HS-SPME/GC-MS and two discriminative approaches, LDA and PLS-DA, were used for classification. Samples were collected from multiple producers covering at least two production cycles and varying maturation times to test the strength of the classification models.

The limited availability of samples with certain labels (PDO for PS and PR, Slow Food Presidium for PF) resulted in a small number of objects for the calibration and validation sets, with each misclassification significantly affecting the performance of the model. However, despite natural flavor changes due to maturation time and seasonal effects, analysis of volatile compounds with discriminative classifiers yielded satisfactory results that correctly classified most samples. The approaches achieved an overall classification

rate of 87.5% on external validation and only two test objects were misclassified. In particular, a misclassification (from the PF class) occurred in both classifiers. As a result, the Pecorino effectively demonstrated its origin and cheesemaking process.

3.2.4.Provola dei Nebrodi

Provola dei Nebrodi, a Sicilian semi-hard pasta filata cheese, is renowned and enjoyed not only in Sicily but throughout Italy. It is made from full-fat, raw milk, predominantly sourced from cows, although occasionally up to 40% of ewes' and goats' milk might be added. The cheese is produced in various locations in the Nebrodi Mountains near Messina. (Ziino et al., 2005)

This cheese is distinctively shaped like a pear with a short neck and a small round top. Its rind is smooth, shiny, thin, and yellowish gold, while the interior appears straw-colored and compact. Provola dei Nebrodi can be consumed either fresh after a one-month ripening period or aged for three to four months.

Recognized as a 'historical dairy product' by Regione Sicilia, Provola dei Nebrodi is crafted using a traditional method involving coagulation with lamb or kid rennet paste without adding any starter cultures. This artisanal approach leads to some variability in the cheeses produced by different farms. As a result, the Sicilian Department of Agriculture has initiated projects to characterize, protect, promote, and authenticate Provola dei Nebrodi cheese.

The flavor of Provola dei Nebrodi plays a crucial role in its acceptability to consumers. The development of flavor depends on various processes such as proteolysis, lipolysis, and the metabolism of residual lactose, lactate, and citrate. These changes are mainly influenced by enzymes from milk, rennet, and microorganisms, as well as non-enzymatic reactions.

The production of Nebrodi Provola, also known as "Provola a sfoglia" or "Provola del Casale," is confined to a specific geographical region. This area includes various municipalities encompassing the hills and mountains, such as Alcara Li Fusi, Basicò, Capizzi, Caronia, Castel di Lucio, Castell'Umberto, Cerami, Cesarò, Floresta, Galati Mamertino, Librizzi, Longi, Maniace, Militello Rosmarino, Mirto, Mistretta, Montalbano Elicona, Motta d'Affermo, Naso, Patti, Pettineo, Raccuja, Randazzo, Roccella Valdemone, San Fratello, San Marco d'Alunzio, San Piero Patti, San Salvatore di Fitalia, San Teodoro, Sant'Agata Militello, Sant'Angelo di Brolo, Santa Domenica Vittoria, Santo Stefano di Camastra, Tortorici, Tripi, Troina, Tusa, Ucrìa, and other Nebrodi municipalities within the region. (NonsoloCibus , n.d)

Figure 11: PDO Provola dei Nebrodi cheese

3.2.5.History of Provola dei Nebrodi

Provola dei Nebrodi originated in the Floresta region and later spread to surrounding areas. Historian Antonino Uccello and priest Gaetano Salamone collected personal testimonies from cheese producers experienced in preserving traditional cheese production methods in their 1872 books "Bovari, pastori e curatuli" and "Technical-Practical Manual of Agriculture and Pastoralism".

From a historical perspective, it is known that provola has historically been recorded in accounting records as a payment in kind for pasture leases over the years. The Nebrodi area has a long-standing custom of making various provolas utilizing tuma to make figured cheeses. These cheeses were first formally displayed at the "Sicilian Ethnographic Exhibition" in Palermo in 1892. On particular feasts and occasions, these cheeses, which have a significant folklore significance, are offered as presents. (The Nonsolocibus Association. (n.d.))

3.2.6. Characterization of "Provola dei Nebrodi", a typical Sicilian cheese, by volatiles analysis using SPME-GC/MS

M. Ziino , 2005 studied the identification of volatile compounds of Provola dei Nebrodi cheese and the evaluation of changes in their concentrations during ripening. In doing so, the analysis of the volatile fraction of Provola dei Nebrodi was carried out using Solid Phase Microextraction High Resolution Gas Chromatography/Mass Spectrometer. This approach is considered the most suitable analytical technique for extracting flavor compounds. Analysis of volatile compounds in cheese often requires their isolation and concentration before gas chromatography is performed. It involves using a silica fiber coated with a suitable stationary phase to isolate and concentrate the desired compounds and offers a simple and solvent-free method of sample preparation without the need for sample manipulation. Cheeses were sampled and

analyzed at four different maturation stages (0, 7, 30 and 90 days). A total of 60 compounds were identified: fatty acids (11), esters (15), lactones (2), methyl ketones (8), aldehydes (9), alcohols (4), hydrocarbons (3), terpenes (1), chlorinated compounds (1) and aromatic compounds (6). The main components were found to be hexanoic and octanoic acids and ethylhexanoate. 60 components were present in all samples regardless of maturation stage, but their ratios showed statistically significant differences (Po0:01 or Po0:05) with aging. Fatty acids and esters increased, while aldehyde content greatly decreased after 30 days. The profile of the identified volatile compounds looked different from other macaroni filata cheeses.

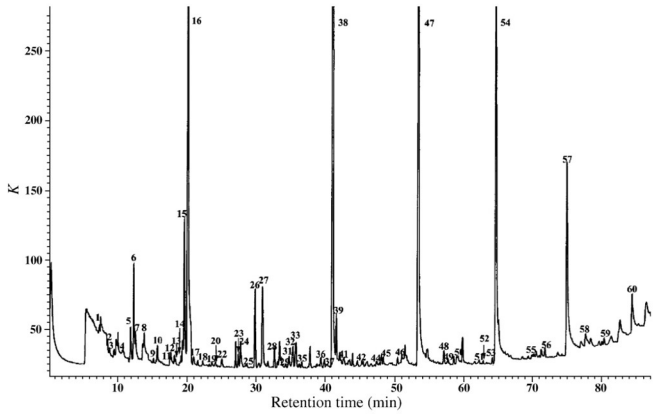

Figure 12: Typical TIC chromatogram of volatile compounds of Provola dei Nebrodi cheese (Ziino,2004)

Figure 12 shows a typical HRGC/MS chromatogram of the volatile fraction extracted by SPME of Provola dei Nebrodi cheese. Sixty components were identified: fatty acids from C2 to C12 and methyl, ethyl, propyl and butyl esters from C4 to C14; d-lactones; aldehydes from C6 to C11; primary and secondary alcohols from C4 to C9; methyl ketones; aromatic compounds; hydrocarbons and terpenes.

Cheese flavor finds its distinctiveness in the presence of free fatty acids (FFAs), holding significant roles that vary according to the cheese type. In aged Italian cheese, they noteworthily contribute to the primary flavor profile (Woo & Lindsay, 1984). Mean values of FFA peak areas in chromatograms at different ripening stages are presented in Table 3. Provola dei Nebrodi, notably, contains all short-chain and medium-chain linear saturated fatty acids within its volatile fraction. Notably, hexanoic acid consistently registers the highest area value across samples and ripening stages, followed by octanoic, butanoic, and decanoic acids, descending in significance. Additionally, acetic, propanoic, and butanoic acids display accelerated increases, attaining higher concentrations after 7 days, whereas hexanoic, heptanoic, octanoic, nonanoic, decanoic, decenoic, and dodecanoic acids reach their zenith after 30 days. In Provola dei Nebrodi, the principal driver of lipolysis originates from pregastric esterase activity in the rennet and, to some extent, lipo-protein lipase of raw milk (McSweeney & Sousa, 2000). Acetic acid, on the other hand, stems from various processes, including lactose oxidation by lactic acid bacteria under anaerobic conditions, and the catabolism (oxidative deamination and decarboxylation) of alanine and serine by lactic acid bacteria (Ur-Rehman et al., 2000). Propanoic acid, primarily, results from lactate metabolism by Propionibacterium sp. (Steffen et al., 1987).

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	*X*	SD	*X*	SD	*X*	SD
35	1594	Methyl decanoate	24b	28	34a	21	55a	34	47a	22
2	900	Ethyl acetate	51c	23	97b	47	176a	72	82c	62
6	1037	Ethyl butanoate	782	479	1122	1088	1344	1660	825	489
16	1230	Ethyl hexanoate	1965	1819	3086	3345	3848	5387	2496	1738
21	1331	Ethyl propanoate	26b	29	47a	37	67a	55	40a	23
26	1430	Ethyl octanoate	654B	345	883B	554	1484AB	1712	1785A	1511
39	1639	Ethyl decanoate	665cb	356	520c	223	1101ab	653	1334a	712
53	2048	Ethyl tetradecanoate	26c	17	43b	23	52ab	37	70a	27
9	1116	Propyl butanoate	76c	89	148cb	210	192ba	216	204a	88
19	1316	Propyl hexanoate	48c	63	106b	127	197b	238	646a	379
31	1523	Propyl octanoate	17c	7	23c	14	52b	18	378a	165
43	1722	Propyl decanoate	26b	16	30b	19	107a	37	143a	66
15	1214	Butyl butanoate	47b	69	71b	107	95b	140	146a	86
25	1411	Butyl hexanoate	13c	10	35b	12	124b	168	243a	107
37	1612	Butyl octanoate	18c	9	34b	16	69a	26	101a	43

Table 5: Esters in the volatile fraction of Provola dei Nebrodi at four different ripening times

Esters are common constituents of the volatile fraction of cheese (Ur-Rehman et al., 2000). Esters are mainly produced by enzymatic or chemical reaction of fatty acids with primary alcohols (Engels et al., 1997) and the alcohol concentration is usually the limiting factor in ester production; moreover, they can also be formed by trans esterification of partial glycerides (Holland, 2004).

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	*X*	SD	*X*	SD	*X*	SD
56	2200	δ-Decalactone	67	24	207	221	150	99	98	54
59	2386	δ-Dodecalactone	47	34	56	19	76	30	53	24

Table 6 : Lactones in the volatile fraction of Provola dei Nebrodi at four different ripening times (Ziino,2004)

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	*X*	SD	*X*	SD	*X*	SD
1	830	Acetone	1c	0	155ab	175	201a	140	61b	61
3	903	2-Butanone	35b	21	94a	67	151a	75	44b	45
4	983	2-Pentanone	6b	6	120a	135	175a	176	12b	17
11	1183	2-Heptanone	215	273	254	322	479	794	129	79
23	1390	2-Nonanone	311	376	394	505	1171	1896	485	574
30	1518	3,5 Octadien-2-one	109a	36	150a	107	148a	62	54b	19
36	1599	2-Undecanone	86B	24	121B	109	270A	301	241A	188
52	2023	2-Pentadecanone	12c	7	19b	6	42a	23	30a	5

Table 7 : Methyl ketones in the volatile fraction of Provola dei Nebrodi at four different ripening times (Ziino,2004)

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	*X*	SD	*X*	SD	*X*	SD
8	1084	Hexanal	1373a	1741	1735a	2139	844a	824	75b	87
12	1192	Heptanal	729a	825	802a	1096	518a	673	1b	0
18	1301	Octanal	363a	455	404a	528	148a	187	47b	73
24	1391	Nonanal	482	609	687	1016	562	781	187	245
14	1224	2-Hexenal (E)	360a	290	245a	187	149a	104	46b	42
20	1330	2-Heptenal (E)	251a	125	250a	131	128b	89	4c	4
33	1540	2-Nonenal	309a	172	441a	243	258a	175	77b	66
40	1647	2-Decenal	222a	46	209a	58	141b	52	34c	13
45	1755	2-Undecenal	65a	25	48a	11	66a	40	33b	35

Table 8 : Aldehydes in the volatile fraction of Provola dei Nebrodi at four different ripening times (Ziino,2004)

The mean values of aldehyde peak areas in the chromatograms are reported in Table 9. Only saturated and 2-unsaturated aldehydes were found in the volatile fraction of Provola dei Nebrodi.

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	X	SD	X	SD	X	SD
10	1152	1-Butanol	18d	5	60c	66	84b	40	238a	219
22	1351	1-Hexanol	62B	90	101B	136	132AB	103	175A	129
28	1488	2-Ethyl-1-hexanol	158	121	101	86	198	237	94	47
29	1515	2-Nonanol	8b	13	39a	46	76a	100	83a	82

Table 9 : Alcohols in the volatile fraction of Provola dei Nebrodi at four different ripening times
(Ziino,2004)

Alcohols were found at low levels in Provola dei Nebrodi cheese as seen in Table 9.

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	X	SD	X	SD	X	SD
42	1701	Heptadecane	36b	26	26b	9	59a	24	56b	21
46	1801	Octadecane	37	27	37	26	57	47	55	36
49	1940	Nonadecane	18b	9	43a	11	23b	8	6c	3
13	1204	Limonene	180	212	264	313	351	564	71	70
5	1022	Chloroform	40	12	53	22	122	76	628	1065

Table 10: Hydrocarbons, terpenes, and chloroform in the volatile fraction of Provola dei Nebrodi at four different ripening times
(Ziino,2004)

Three linear hydrocarbons (heptadecane, octadecane and nonadecane) were detected in the volatile fraction of Provola dei Nebrodi cheese, together with limonene and chloroform, as seen in Table 10.

Peak no.	LRI	Compounds	0 days		7 days		30 days		90 days	
			X	SD	X	SD	X	SD	X	SD
7	1047	Toluene	277	253	529	587	542	523	297	210
17	1264	Styrene	90	78	83	79	48	12	100	105
41	1660	Acetophenone	75	61	131	122	178	231	94	95
32	1533	Benzaldehyde	178	76	233	125	178	93	169	87
48	1918	Phenylethanol	105	101	129	148	156	170	113	94
51	2015	Phenol	18b	7	28a	8	36a	13	13c	12

Table 11:Aromatic compounds in the volatile fraction of Provola dei Nebrodi at four different ripening times
(Ziino,2004)

Six aromatic compounds (two hydrocarbons, one ketone, one aldehyde and two alcohols were found in the samples analysed (Table11). The amount of toluene, styrene, acetophenone, benzaldehyde and 2-phenyl-1-ethanol did not show any significant variation during ripening ($P > 0.05$); phenol, on the other hand, showed an increase over the first 7 days, and a decrease at the end of ripening.

CHAPTER 4: CONCLUSION

In conclusion, the significance of food product authentication and fraud detection has gained widespread recognition in contemporary society. The ongoing pursuit of innovative techniques and analytical methods underscores the commitment to achieving this crucial goal. While traditional approaches persist, the escalating demand for newer authentication methodologies driven by cost-effectiveness and rapid protocols is evident, fueled by the active involvement of consumers, regulatory bodies, and the food industry.

Contemporary authentication analysis, centered around the detection and measurement of diverse chemical parameters, holds the potential to distinguish between various food samples. Beyond ensuring economic

integrity, this approach introduces critical health and safety dimensions to the food industry. Consequently, global policies emphasize stringent monitoring and quality control of food products to safeguard public health and trust.

Gas chromatography has emerged as an indispensable verification method, employed by control laboratories to satisfy the rising consumer appetite for authentic and original food items. Its pivotal role in maintaining the quality of diverse food products, notably esteemed Italian cheeses like PDOs, protects the industry's reputation while fulfilling consumers' preferences for genuine and premium goods. Given the high economic value and reputation of Italian cheeses, the specific importance of traceability and authentication becomes even more evident. Inspecting and ensuring the authenticity of these cheeses is crucial to preserve their integrity, protect against counterfeit products, and maintain consumer confidence.

As gas chromatography seamlessly integrates with a versatile range of analytical instruments, it is poised to maintain its central position in food authentication for the foreseeable future. Moreover, ongoing advancements aimed at addressing its limitations are expected to perpetuate its role in upholding the authenticity and quality of food products, contributing to consumer confidence and the integrity of the food supply chain.

REFERENCES

Amaral, J. S. (2021). Target and non-target approaches for food authenticity and traceability. *Foods*, *10*(1), 172.

Ballin, N. Z., & Laursen, K. H. (2019). To target or not to target? Definitions and nomenclature for targeted versus non-targeted analytical food authentication. *Trends in Food Science & Technology*, *86*, 537-543.

Bezerra, T. K. A., Araujo, A. R. R., Arcanjo, N. M. D. O., Silva, F. L. H. D., Queiroga, R. D. C. R. D. E., & Madruga, M. S. (2016). Optimization of the HS-SPME-GC/MS technique for the analysis of volatile compounds in caprine Coalho cheese using response surface methodology. Food Science and Technology, 36, 103-110.

Bi, K., Zhang, D., Qiu, T., & Huang, Y. (2019). GC-MS fingerprints profiling using machine learning models for food flavor prediction. *Processes*, *8*(1), 23.

Bohm, D. A., Stachel, C. S., & Gowik, P. (2009). Multi-method for the determination of antibiotics of different substance groups in milk and validation in accordance with Commission Decision 2002/657/EC. *Journal of Chromatography A*, *1216*(46), 8217-8223.

Caligiani, A., Nocetti, M., Lolli, V., Marseglia, A., & Palla, G. (2016). Development of a quantitative GC–MS method for the detection of cyclopropane fatty acids in cheese as new molecular markers for Parmigiano Reggiano authentication. *Journal of agricultural and food chemistry*, *64*(20), 4158-4164.

Cavanna, D., Righetti, L., Elliott, C., & Suman, M. (2018). The scientific challenges in moving from targeted to non-targeted mass spectrometric methods for food fraud analysis: A proposed validation workflow to bring about a harmonized approach. *Trends in Food Science & Technology*, *80*, 223-241.

Cevallos-Cevallos, J. M., Reyes-De-Corcuera, J. I., Etxeberria, E., Danyluk, M. D., & Rodrick, G. E. (2009). Metabolomic analysis in food science: a review. *Trends in Food Science & Technology*, *20*(11-12), 557-566.

Cevoli, C., Cerretani, L., Gori, A., Caboni, M. F., Toschi, T. G., & Fabbri, A. (2011). Classification of Pecorino cheeses using electronic nose combined with artificial neural network and comparison with GC–MS analysis of volatile compounds. *Food Chemistry*, *129*(3), 1315-1319.

Creydt, M., & Fischer, M. (2020). Food authentication in real life: How to link nontargeted approaches with routine analytics? *Electrophoresis*, *41*(20), 1665-1679.

Danezis, G. P., Tsagkaris, A. S., Brusic, V., & Georgiou, C. A. (2016). Food authentication: state of the art and prospects. *Current Opinion in Food Science*, *10*, 22-31.

Di Donato, F., Biancolillo, A., Mazzulli, D., Rossi, L., & D'Archivio, A. A. (2021). HS-SPME/GC–MS volatile fraction determination and chemometrics for the discrimination of typical Italian Pecorino cheeses. *Microchemical Journal*, 165, 106133.

EC.(2009). COMMISSION REGULATION (EC) No 1030/2009 of 29 October 2009 approving minor amendments to the specification of a name registered in the register of protected designations of origin and protected geographical indications (Pecorino Romano (PDO)). *Official Journal of the European Union*, number

EC. (2020). *Knowledge Centre for Food Fraud and Quality*. *Food Fraud and Quality*. Retrieved from https://knowledge4policy.ec.europa.eu/food-fraud-quality/topic/food-fraud_en (Last updated: January 14, 2020).

Engels, W. J. M., Dekker, R., De Jong, C., Neeter, R., & Visser, S. (1997). A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. *International Dairy Journal*, *7*(4), 255-263.

Esteki, M., Farajmand, B., Amanifar, S., Barkhordari, R., Ahadiyan, Z., Dashtaki, E., ... & Vander Heyden, Y. (2017). Classification and authentication of Iranian walnuts according to their geographical origin based on gas chromatographic fatty acid fingerprint analysis using pattern recognition methods. *Chemometrics and Intelligent Laboratory Systems*, *171*, 251-258.

FAO. 2021. *Food fraud – Intention, detection and management.* Food safety technical toolkit for Asia and the Pacific No. 5. Bangkok.

Halket, J. M., Waterman, D., Przyborowska, A. M., Patel, R. K., Fraser, P. D., & Bramley, P. M. (2005). Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. *Journal of experimental botany*, *56*(410), 219-243.

Hassoun, A., Måge, I., Schmidt, W. F., Temiz, H. T., Li, L., Kim, H. Y., ... & Cozzolino, D. (2020). Fraud in animal origin food products: Advances in emerging spectroscopic detection methods over the past five years. *Foods*, *9*(8), 1069.

Holland, R. (2004). Esterases of lactic acid bacteria and cheese flavour. *IDF symposium on cheese: ripening, characterization & technology*, Prague, Czech Republic, March 21–25, 2004. p. 24.

Abdumajitovich, B. S. (2022). ANALYZING THE CHEMICAL COMPOSITION OF THE STRECH USED FOR FOOD PACKAGING WITH A "GASCHROMATOGRAPHY" METHOD. *Editorial board*, 15.

Hussain, S. Z., & Maqbool, K. (2014). GC-MS: Principle, Technique and its application in Food Science. *International Journal of Current Science*, *13*, 116-126.

InnovaTECH. (April 23, 2018). A Beginner's Guide: How to Interpret Gas Chromatography Mass Spectrometry Results. Retrieved May 18, 2023, from https://www.innovatechlabs.com/newsroom/1841/how-to-interpret-gas-chromatography-mass-spectrometry-results/

Jacob, M., Malkawi, A., Albast, N., Al Bougha, S., Lopata, A., Dasouki, M., & Rahman, A. M. A. (2018). A targeted metabolomics approach for clinical diagnosis of inborn errors of metabolism. *Analytica chimica acta*, *1025*, 141-153.

Karoui, R., & De Baerdemaeker, J. (2007). A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products. *Food chemistry*, *102*(3), 621-640.

Khan Academy. (n.d.). *Mass spectrometry*. Retrieved July 23, 2023, from https://www.khanacademy.org/science/ap-chemistry-beta/x2eef969c74e0d802:atomic-structure-and-properties/x2eef969c74e0d802:mass-spectrometry-of-elements/v/mass-spectrometry

Kumari, V. C., Patil, S. M., Ramu, R., Shirahatti, P. S., Kumar, N., Sowmya, B. P., ... & Patrick-Iwuanyanwu, K. C. (2022). Chromatographic techniques: types, principles, and applications. In *Analytical techniques in biosciences* (pp. 73-101). Academic Press.

Krugers, J., Instrumentation in Gas Chromatography. Centrex Publishing Company-Eindhoven, Netherlands, 1968.

Lolli, V., Marseglia, A., Palla, G., Zanardi, E., & Caligiani, A. (2018). Determination of cyclopropane fatty acids in food of animal origin by 1H NMR. *Journal of Analytical Methods in Chemistry*, *2018*.

Lolli, V., Zanardi, E., Moloney, A. P., & Caligiani, A. (2020). An overview on cyclic fatty acids as biomarkers of quality and authenticity in the meat sector. *Foods*, *9*(12), 1756.

Lolli, V., Renes, E., Caligiani, A., de la Fuente, M. A., & Gómez-Cortés, P. (2021). Cyclopropane Fatty Acids as Quality Biomarkers of Cheeses from Ewes Fed Hay-and Silage-Based Diets. *Journal of Agricultural and Food Chemistry*, *69*(33), 9654-9660.

Montgomery H, Haughey SA, Elliott CT. Recent food safety and fraud issues within the dairy supply chain (2015-2019). Glob Food Sec. 2020 Sep;26:100447. doi: 10.1016/j.gfs.2020.100447. Epub 2020 Oct 16. PMID: 33083214; PMCID: PMC7561604.

Moore Analytical. (n.d.). GC-MS. Retrieved [23.07.2023], https://www.mooreanalytical.com/gc-ms/

Muroya, S., Ueda, S., Komatsu, T., Miyakawa, T., & Ertbjerg, P. (2020). MEATabolomics: Muscle and meat metabolomics in domestic animals. *Metabolites*, *10*(5), 188.

National Library of Medicine. (2019, October 18).Untargeted/Targeted 2D Gas Chromatography/Mass Spectrometry Detection of the Total Volatile Tea Metabolome, Retrieved May 11, 2023, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6832143/

NonsoloCibus - Association for the Promotion and Improvement of Typical Regional Productions.(n.d).Provola dei Nebrodi. https://www.nonsolocibus.it/provola-dei-nebrodi/

Nudda, A., Palmquist, D. L., Battacone, G., Fancellu, S., Rassu, S. P. G., & Pulina, G. (2008). Relationships between the contents of vaccenic acid, CLA and n− 3 fatty acids of goat milk and the muscle of their suckling kids. *Livestock Science*, *118*(3), 195-203.

Ur-Rehman, S., Banks, J. M., Brechany, E. Y., Muir, D. D., McSweeney, P. L. H., & Fox, P. F. (2000). Influence of ripening temperature on the volatiles profile and flavour of Cheddar cheese made from raw or pasteurised milk. International Dairy Journal, 10, 55–65.

Patti, G. J., Yanes, O., & Siuzdak, G. (2012). Metabolomics: the apogee of the omics trilogy. *Nature reviews Molecular cell biology*, *13*(4), 263-269.

Pinu, F. (2013). Sauvignon blanc metabolomics: Metabolite profile analysis before and after fermentation (Doctoral dissertation, ResearchSpace@ Auckland).

Schirone, M., Tofalo, R., Visciano, P., Corsetti, A., & Suzzi, G. (2012). Biogenic amines in Italian Pecorino cheese. *Frontiers in microbiology*, *3*, 171.)

Schrenk, D., & Cartus, A. (Eds.). (2017). *Chemical contaminants and residues in food*. Woodhead Publishing.

Shingfield, K. J., Bonnet, M., & Scollan, N. D. (2013). Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal*, *7*(s1), 132-162.

Smith RW. Mass spectrometry. In: JA Siegel, PJ Saukko, MM Houck, eds. *Encyclopedia of Forensic Sciences*. 2nd ed. Academic Press; 2013: 603-608.

Steffen, C., Flueckiger, E., Bosset, J. O., & Ruegg, M. (1987). Swiss-type Varietes. Cheese: Chemistry, Physics and Microbiology

Tao, N., DePeters, E. J., Freeman, S., German, J. B., Grimm, R., & Lebrilla, C. B. (2008). Bovine milk glycome. *Journal of dairy science*, *91*(10), 3768-3778.

Technologynetworks. (2022, June 10). GC-MS: Principle, Instrument, and Analyses, and GC-MS/MS. Retrieved June 23, 2023, from https://www.technologynetworks.com/analysis/articles/gc-ms-principle-instrument-and-analyses-and-gc-msms-362513

Termofischer. (n.d). Gas Chromatography-Mass Spectrometry (GC-MS) Information. Retrieved May 18, 2023, from https://www.thermofisher.com/de/de/home/industrial/mass-spectrometry/mass-spectrometry-learning-center/gas-chromatography-mass-spectrometry-gc-ms-information.html

van Asselt E.D., van der Fels-Klerx H.J., Marvin H.J.P., van Bokhorst-van de Veen H., Groot M.N. Overview of food safety hazards in the European dairy supply chain. *Compr. Rev. Food Sci. Food Saf.* 2017;16(1):59–75. doi: 10.1111/1541-4337.12245.

Villas-Bôas, S.G.; Rasmussen, S.; Lane, G.A. Metabolomics, or metabolite profiles? Trends Biotechnol. 2005, 23, 385–386.

Wen, Z., Zhu, D., Tang, Y., Li, Y., & Zhang, G. (2005). The application of gas chromatography fingerprint technique to calculating oil production allocation of single layer in the commingled well. *Chinese Journal of Geochemistry*, *24*, 257-261.

Ziino, M., Condurso, C., Romeo, V., Giuffrida, D., & Verzera, A. (2005). Characterization of “Provola dei Nebrodi”, a typical Sicilian cheese, by volatiles analysis using SPME-GC/MS. *International Dairy Journal*, *15*(6-9), 585-593.