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**DEPARTMENT OF CHEMISTRY
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Ph.D COURSE IN CHEMISTRY
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*" Innovative applications of stable carbon isotope ratio
in environmental and food chemistry"*

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*« Come un aquilone senza corda e
una farfalla senza ali, mia madre mi ha
insegnato a volare con i sogni »*

William H. McMurry III

Publications

- R. Cucciniello, A. Proto, M. Ricciardi, C. Pironti, O. Motta. Il tesoro dei sottoprodotti di reazione: dalla 2-monocloroidrina agli additivi per benzine. Libro degli Abstracts XV Congresso di Chimica Dell'ambiente e Dei Beni Culturali SCI ISBN:978-88-86208-95-6.
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Glossary

AOAC	Association of Official Analytical Chemists
ATP	Adenosine Triphosphate
CAM	Crassulacean acid metabolism
D₁	Pulse intervals of spectra
EA	Elemental Analyser
EC	European Commission
EEC	European Economic Community
ENV-CEN	European Committee for Standardisation
EU	European Union
FAO	Food and Agriculture Organisation
FT-IR	Fourier Transform Infrared
IOC	International Olive Council
IAEA	International Atomic Energy Association
IRMS	Isotope Ratio Mass Spectrometry
OIV	International Organization of Vine and Wine
	PDO Protected Designations of Origin
PGI	Protected Geographical Indication
¹³C/¹²C	The ratio of the isotope of carbon with atomic mass 13 to the isotope of carbon with atomic mass 12
¹³C NMR	Carbon nuclear magnetic resonance

$\delta^{13}\text{C}\text{‰}$	Isotope value refer to the international standard Vienna-Pee Dee Belemnite.
$^{15}\text{N}/^{14}\text{N}$	The ratio of the isotope of nitrogen with atomic mass 15 to the isotope of nitrogen with atomic mass 14
$\delta^{15}\text{N}\text{‰}$	"Delta nitrogen-15 $^{15}\text{N}/^{14}\text{N}$ per mil". The ratio expressed to the international standard N_2
NADP	Nicotinamide Adenosine Diphosphate
NIOSH	National Institute for Occupational Safety and Health, the United States federal agency for research and prevention of work-related injury and illness
NDIRS	Non-Dispersive Infrared Spectroscopy
NMAM	The analytical methods were reported in the manual of NIOSH
OAA	Oxaloacetic Acid
PEP	Phosphoenolpyruvate
PGA	Phosphoglycerate
PGAL	3-Phospho-glyceraldehyde
RuDP	Ribulose-1,5-diphosphate
R	Ratio between the heavier isotope and the lighter one
SNIF ^2H	Site Specific Natural Isotope Fractionation
NMR	Nuclear Magnetic Resonance spectroscopy
T_1	relaxation time
TSG	Traditional Speciality Guaranteed
V-PDB	Pee Dee Belemnite - Calcium carbonate used as an international standard for $\delta^{13}\text{C}\text{‰}$ scale

Abstract

In the last year, the stable isotope ratio analysis has become a useful tool with many applications in different scientific area. In particular the characteristic isotope signature of materials has permitted to identify authenticity and traceability of food sample and isotope composition has become a valuable marker in environmental studies. This work shows the applicability of analytical methods for isotopic carbon determination in food and environmental samples and the innovative use of $\delta^{13}\text{C}$ in cultural heritage as valuable tool to trace pollutant fate. The first part is dedicated to the improvement of spectroscopic methods as Fourier Transform Infrared (FT-IR) and Non-Dispersive Infrared spectroscopy (NDIRS) and their application to identify geographical origin in sample like pasta, cocoa, olive oil. The results conducted in order to assess the robustness of the two alternative methods respecting IRMS showed a strong correlation like a demonstration of the positive relationship between the tested analytical methods. A new method was developed ^{13}C NMR spectroscopy to determine the bulk $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio of inorganic carbonates and bicarbonates at natural abundance. In literature the use of ^{13}C NMR spectroscopy

was focused on ^{13}C position-specific isotope analysis of organic molecules; in this work it was reported the improvement of NMR methodology able to obtain stable carbon isotope ratio in bulk material using an internal standard.

The last part of project has regarded the application of isotope analysis to environmental studies and in the field of cultural heritage. The study was focused on identification of carbon dioxide sources thanks to stable carbon isotope composition and on correlation of carbon dioxide $\delta^{13}\text{C}$ value with presence of visitors and others pollutants such as NH_3 , NO_2 , BTEX, H_2S , SO_2 .

In fact the carbon isotope composition of carbon dioxide captured by passive sampler using a Ca-based solid absorbent ($\text{CaO}/\text{Ca}_{12}\text{Al}_{14}\text{O}_{33}$ 75:25 w/w) was analysed in three different archaeological places: Fruscione Palace and S.Pietro a Corte in Salerno (part of SNECS project), at The Last Supper of Da Vinci museum in Milan. The results of the isotope composition of CO_2 correlated with the variation of concentration in the air have proved to be a valid tool to follow the flux of people in the different area of museum and it could be used as a non-invasive marker for monitoring of filtering system.

Introduction to stable carbon isotope ratio

"...the masses of the elements are approximately expressed by whole numbers, where oxygen is taken as 16-with the exception of hydrogen itself. But the real interest, as we now see it, is not the whole number rule itself, but rather the departures from it. " Rutherford et al. 1929 talking about the experiments of F.W. Aston on masses measurements.

The first discovery of two isotopes of Cl was published by F.W. Aston, who has measured the atomic weight of chlorine by a different method and found substances with atomic weights of 35 and 37. Although other important scientists including J.J. Thomson, E. Rutherford, F. Soddy and J. Chadwick, G. Gamow were studying on masses of elements, this was the first evidence of two isotopes. The general assumption was that the isotopic compositions were homogeneously distributed throughout all substances; until 1925 was demonstrated that physical chemical processes could cause isotopic fractionation of light elements in natural substance.¹ During this period, Harold Urey (Nobel Prize in chemistry in 1934 for discovery of deuterium) sponsored his result on calculations about the isotopic fractionation using stable

isotope ratios of the light elements defined by mass spectrometer travelling to several important universities in Europe.² These studies had permitted to use stable isotope measurements in a wide range of applications, first of all for geochemistry studies and kinetic ratios of geochemical systems: crystal growth and mineral recrystallization; equilibrium and kinetic processes evaporation of water; different incorporation of CO₂ during photosynthesis.^{3,4,5} The principle was that there was a preferential fractionation of isotopes, with incorporating preferentially in one phase the heavy (or light) isotope relative to other coexisting phases. In last year the analytical instrumentation has been developed thanks to more sensibility and high precision of isotopic analysis about traditional elements such as H, C, N, O, S, which also had permitted to introduce the analysis of additional elements B, Cl, Si, Li, Mg, Ca, Cr, Fe, Cu, Zn, Se, and Mo. The most of natural materials contain one or more of these elements; therefore, stable isotopes ratio became a chemical parameter with important applications in various fields and types of scientific researches. For example, the carbon isotope composition was a useful tool in environmental sciences to monitor climate change^{7, 8} and the environmental impact on the

environment⁹. The carbon isotope composition has also found the extensive use in archaeological studies¹⁰⁻¹³ as well as in geochemical studies to monitor volcanic activity¹⁴ and also in noninvasive medicine tests to test the presence of *Helicobacter pylori* infection^{15, 16}.

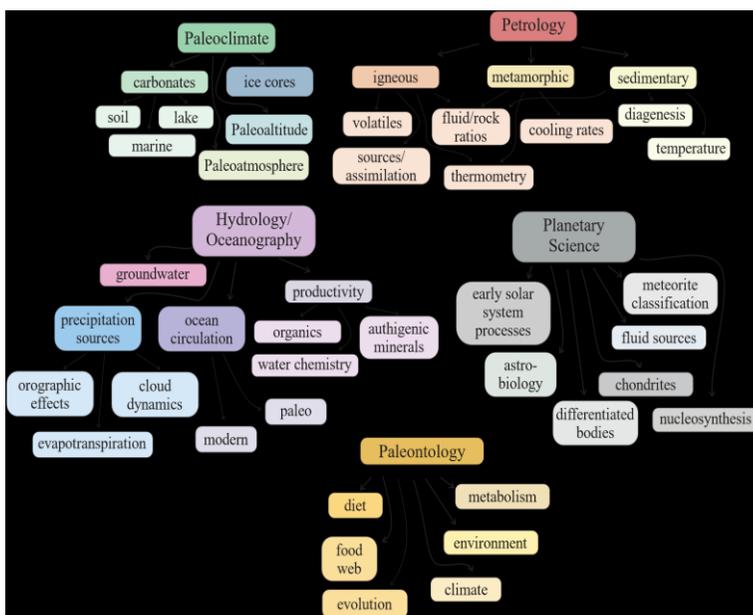


Figure 1. Examples of the types of fields using stable isotopes (from Z.D. Sharp. *Principles of Stable Isotope Geochemistry* 2017).

The study of the isotopic delta was considered of great importance also in the field of forensic science¹⁷ and food sciences¹⁸ to distinguish the presence of natural and synthetic substances of some ingredients or to trace the

origin product. This was possible because the value of the $^{13}\text{C} / ^{12}\text{C}$ and the isotopic composition of plants and their products were linked to the photosynthetic fixation of atmospheric carbon dioxide in organic compounds.

The carbon isotope ratio was expressed in $\delta\%$ relative to V-PDB (Vienna-Pee Dee Belemnite), according to the following IUPAC protocol:

$$\delta = (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}}$$

where R is the ratio between the heavier isotope and the lighter one.

1.1 Stable carbon isotopes fractionation in nature

Atmospheric carbon dioxide contains approximately 1.1% of isotope ^{13}C and 98.9% of ^{12}C . In the absence of human activity (industrial, agricultural, cities centers, etc), the $\delta^{13}\text{C}$ value of atmospheric CO_2 is -8‰, while was slowly becoming more negative in the cities due to the high number of vehicles and to combustion of fossil fuel ($\delta^{13}\text{C}$ for fossil fuel is approximately -30‰).¹⁹ In literature was reported the influence of environment and single processes on isotope carbon fractionation: for example, there were three classes of plants (C_3 , C_4 and CAM) with different isotopic pattern due to different photosynthetic

process and carbon dioxide storage; or stable carbon isotope ratio of carbonates influenced by the presence of carbon dioxide gas phase and some ion such as Magnesium, silicates, phosphates during growing process (dolomite, travertine, etc).

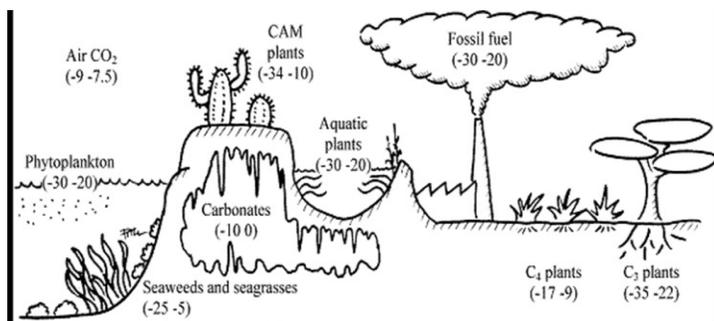


Figure 2. Carbon isotope composition of some natural substances.

During photosynthesis plants discriminated against ¹³C-¹²C due to small differences in chemical and physical properties influenced by the difference in mass. This discrimination could be used to identify various photosynthetic groups of plants. The most important reactions used by plants to fix CO₂ follow the C₃ and C₄ pathways.²⁰ Plants C₃ have used the Calvin cycle for CO₂ fixation and their carbon isotope compositions were in the range of -34 to -22 ‰.

In the cold temperate countries, with an atmospheric

concentration of carbon dioxide more than 50 ppm, the plants have used the Calvin cycle and CO₂ was fixed as molecules with three atoms of carbon (phosphoglyceraldehyde (PGAL) first sugar of photosynthesis), as showed in Figure 3. After the formation PGAL was used to produce others organic compounds: to synthesize ribulose biphosphate carboxylase (RuDP) thanks to a series of complex reactions driven by adenosine triphosphates (ATP); to synthesize the end product of photosynthesis, glucose.

The initial reaction of CO₂ with RuDP has produced an enrichment of ¹³C isotope associated with kinetic and equilibrium effects of process, even if the final δ¹³C‰ value of plant was influenced temperature, fertilization, salinity, CO₂ concentration, light intensity and photorespiration. In fact it was possible to distinguish the same plants from different countries of world: the δ¹³C‰ value of Italian wheat was different from the Australian one.

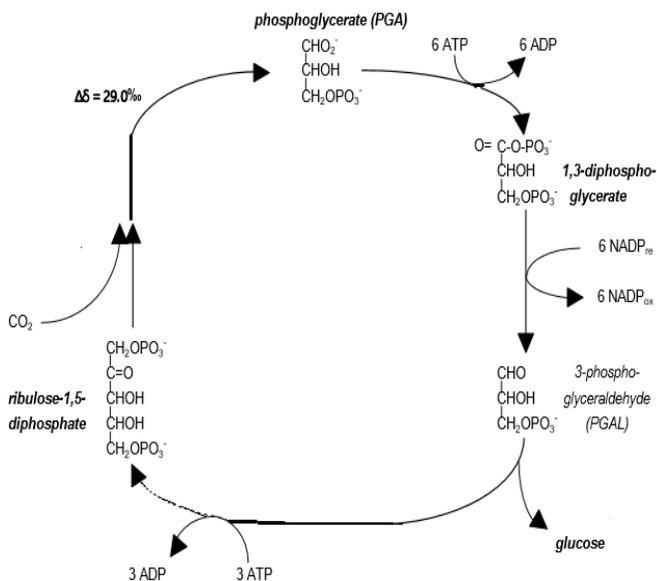


Figure 3. Calvin cycle to fix CO₂ for C₃ plants with expression of isotope fractionation.

C₄ plants have consisted of many tropical plants, including, for example, cane and cane sugar, using the Hatch-Stack cycle and had a higher isotopic delta value than C₃ plants. In this case, as reported in Figure 4., the plants could fix the carbon dioxide at low concentrations and in presence of high level of oxygen: carbon dioxide reacted with phosphoenolpyruvate to form oxaloacetic acid (OAA), a four carbon compound. The first step of reaction has produced lower ¹³C isotope enrichment, about 2.0 ‰. OAA was reduced to form malate or

aspartate; it could then oxidize to form a compound with three carbons and CO₂, which started the Calvin cycle to synthesize glucose.

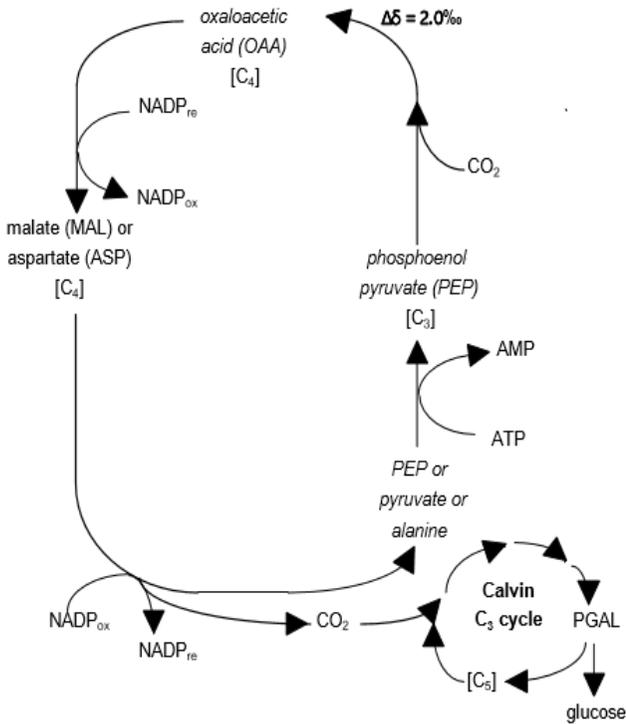


Figure 4. Hatch-Stack cycle to fix CO₂ for C₄ plants with expression of isotope fractionation.

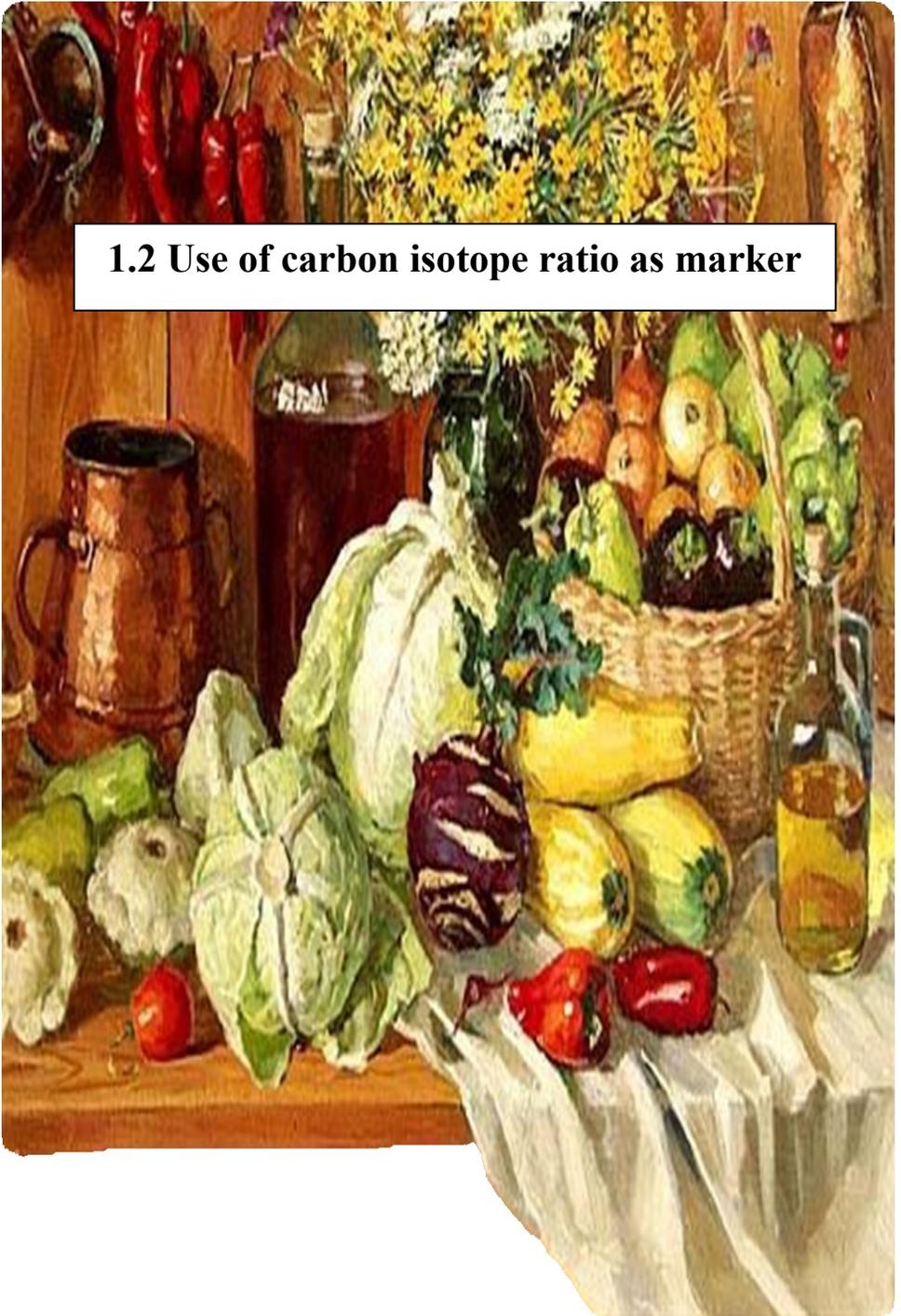
Another group of plants uses *Crassulacean* acid metabolism (CAM) as a photosynthetic pathway and their isotopic composition falls in the range of -33/ -11 ‰.²¹ The most famous plants were vanilla, pineapple, agave

and they used an improvement C₃ mechanism: during the night stomata were open and CO₂ was fixed as organic acids and stored in vacuoles; during the day when the stomata were closed to avoid the water loss, the plants used and the storage of high concentrations of organic acids and released the carbon to permit the photosynthesis. In summary, during the night the plants followed the Calvin cycle, while during the day used the same mechanism of C₄ plants; although the $\delta^{13}\text{C}$ value had a widely range influenced by local climatic conditions and if in some cases was predominant C₃ or C₄ pattern.

Thus the analysis of $\delta^{13}\text{C}$ composition of specific compounds had permitted to distinguish between C₃ and C₄ plants, to evaluate the presence of natural and non-natural additives used, and thus to certify the quality and purity of the analyzed samples. In the same way different carbon isotope ratio was considered in geochemistry samples, such as carbonates, to obtain information about groundwater and soil. Several studies have analyzed both aqueous and solid-phase components of terrestrial groundwater and results have indicated that the primary controls on precipitation of carbonates were a combination of water parameters such as pH, [HCO₃⁻], pCO₂, physical processes (e.g., temperature change,

degassing, steaming, evaporation, and dilution), hydrology and biotic activities.^{22, 23, 24}

1.2 Use of carbon isotope ratio as marker





1.2.1 Food chemistry

The safety and the authenticity of food was a critical point for consumers that come in contact with a wide variety of food from around the world. The General EU Food Regulation introduced criteria to identify safe or unsafe food and feed and only the safe food can be placed on the Union market. The public sensitivity was also influenced by some food scares and international scandals about products on market, such as the eggs contaminated by pesticide or presence of adulterants in olive oil, fruits juice and honey. For this reason European Union instituted a Traceability Regulation (178/2002/EC) which defined “food and feed traceability.” Moreover, one of the most important problems of industries was the requirement to protect against mislabeling the products manufactured according to rigorous criteria to guarantee the quality, safety and respect for the environment. These types of foods were certificated through Protected Geographical Indications (PGI), Protected Designations of Origin (PDO), and Traditional Specialties Guarantee (TSG), defined by European laws EC N. 510/2006 and 1151/2012. The authenticity of foods was identified

uncovering mislabeling of foods, substitution with cheaper ingredients or using adulterants, incorrect origin (such as geographical), species, or production method. In recent year the food industry needs international validated methods to screen food samples, to provide proof of authenticity and originality of food samples. Among the exploitable techniques stable isotope ratios analysis (SIRA), measured using isotope ratio mass spectrometry (IRMS), has taken on increasing importance in determining the authenticity of foods of animal origin for producers and control agencies. In the study of the geographic origin of food products²⁵⁻²⁸ giving the possibility for testing food authenticity^{29 - 31}, quality and typicality^{32 -34}, carbon isotope ratio has permitted to obtain specific information particularly when conventional analytical methods cannot provide unambiguous results.

For example, it was possible to identify the presence of natural or non-natural fatty acids in samples of chocolates and to discriminate about quality and origin of sample.

The different origin and types of fatty acids in cocoa butter have been studied and classified by stable carbon isotopes analysis. In previews studies the $\delta^{13}\text{C}$ value of cocoa butter was influenced by the presence of other

vegetable fats and by thermal or oxidative degradation processes during production (eg drying and roasting cocoa beans or the treatment of butter deodorization obtained from pre-tasting) or conservative process of cocoa butter and the other products (eg chocolate).³⁵

The evaluation of the carbon $^{13}\text{C} / ^{12}\text{C}$ isotopes ratio has also be used to characterize principal components of olive oil,³⁶ honey³⁷ and fruit juice³⁸.

Polyunsaturated fatty acids have become important natural additives with benefits for brain function and cardiovascular health. In literature was reported the addition of Omega-3s in conventional foods, including bread, milk, yogurt, pastry products and oil.³⁹ The carbon isotope analysis can also be applied to evaluate and quantify the omega-3s integrated into foods. At the same time, stable carbon isotope ratio was recognized a valid tool to evaluate the quality and the origin of durum wheat; this analysis has also been applied to monitor variations during the cooking process as well as to verify variations in composition of wheat.

The analytical methods used for stable isotope ratio were reported in Table 1. and there were only the isotopic methods that have been recognized according to EU Regulation for the detection of frauds. The laboratories,

which intend to verify the authenticity and originality in food samples, must use methods agreed in national legislation or in the absence of the rules other methods in accordance with scientific protocol.

Table 1. List of the current stable isotope ratio standard methods.

Method	Product	Stable isotope ratio
OIV-MA-AS311-05	Wine, must	(D/H) _I and (D/H) _{II} of ethanol
OIV-MA-AS312-07	Wine, must	¹³ C/ ¹² C of ethanol
OIV-MA-AS2-12	Wine, must	¹⁸ O/ ¹⁶ O of water
ENV 12140	fruit juice	¹³ C/ ¹² C of sugar
ENV 13070	fruit juice	¹³ C/ ¹² C of pulp
ENV 12141	fruit juice	¹⁸ O/ ¹⁶ O of water
AOAC 995.17	fruit juice	(D/H) _I and (D/H) _{II} of ethanol
AOAC 2004.01	fruit juice	¹³ C/ ¹² C of ethanol
	cheese	¹³ C/ ¹² C, ² H/ ¹ H, ¹⁵ N/ ¹⁴ N, ³⁴ S/ ³² S
	olive oil	of defatted cheese ¹³ C/ ¹² C, ² H/ ¹ H, ¹⁸ O/ ¹⁶ O of bulk olive oil
CEN, EN16466-1:2012; OIV-OENO 527-2015	Vinegar	(D/H) _I of acetic acid
CEN, EN16466-2:2012; OIV-OENO 510-2013	Vinegar	¹³ C/ ¹² C of acetic acid
CEN, EN16466-3:2012; OIV-OENO 511-2013	Vinegar	¹⁸ O/ ¹⁶ O of water

OIV: International Organization of Vine and Wine.

ENV-CEN: European Committee for Standardisation.

AOAC: Association of Official Analytical

Chemists. IOC: International Olive Council.

The Isotope Ratio Mass Spectrometry (IRMS) is the method of choice for the ¹³C measurements in terms of δ¹³C thanks to highly sensitive, up to 0.01%.⁴⁰ However

it was equally expensive system and new cheaper methods have been designed as alternatives to the IRMS for the ^{13}C analysis.⁴¹ In comparative studies was shown the reliability of alternative technique in measuring ^{13}C enrichment, due to be considered as a valid alternative to IRMS such as the use of NDIRS an accuracy of $\pm 0.4\%$.⁴²⁻⁴³ Moreover, spectroscopic methods proposed were based on the principle that substitution of ^{13}C and ^{12}C affected the distribution of vibrational and rotational energy of molecule and each distinct isotopomer of carbon dioxide has its own rotational - vibrational infrared spectrum. However, during experimental acquisition the peaks of isotopomers were overlapped and it was very difficult measure the contribution for each molecule. In the recent works, Kidness and Marr reported the measurement of $\delta^{13}\text{C}$ by using Fourier Transform Infrared Spectroscopy, utilizing high-resolution spectrometers (0.25 cm^{-1} , and 1 cm^{-1} at elevated sample pressures) obtained an accuracy of $\pm 12\%$ and $\pm 8\%$.^{44, 45} During PhD project was improved FTIR (Fourier Transform Infrared) analysis for food samples. The spectroscopic method used was developed by the group of Prof. Antonio Proto: CO_2 was absorbed into polystyrene films to provide sharp and well resolved IR absorption

bands for the ν^3 antisymmetric stretching mode of both $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ isotopomers.⁴⁶ This method has permitted to avoid overlapping of peaks and the good resolution was a result of the reduction in the rotational mode of CO_2 due to the interaction with the aromatic ring of polystyrene. By this way was obtained an accuracy of $\pm 2.5\%$.⁴⁶ In a previous work it was compared NDIRS and FTIR with IRMS for determination of ^{13}C -urea breath test in the non-invasive diagnosis of *Helicobacter pylori* infection¹⁵ showing that FTIR and NDIRS methods were as well as valid and accurate analytical tool to obtain a highly specific measurement of ^{13}C enrichment in breath samples and to discriminating between infected and non-infected subjects. Moreover, on the grounds of previews results, one of the aims of PhD project was the improvement of FT-IR and NDIRS methodology for identification of isotopic composition of food samples. The aim was focused not only the analytical methods but also on the preparation of sample to put out an easy and low cost protocol for isotopic analysis of foods. On the other hand, the research was based on development of new independent methodologies for $\delta^{13}\text{C}$ analysis able to validate the accuracy obtained by using the reference method such as IRMS. In this case, it was introduced ^{13}C

NMR spectroscopy to calculate bulk $\delta^{13}\text{C}$ in inorganic carbonates and bicarbonates.

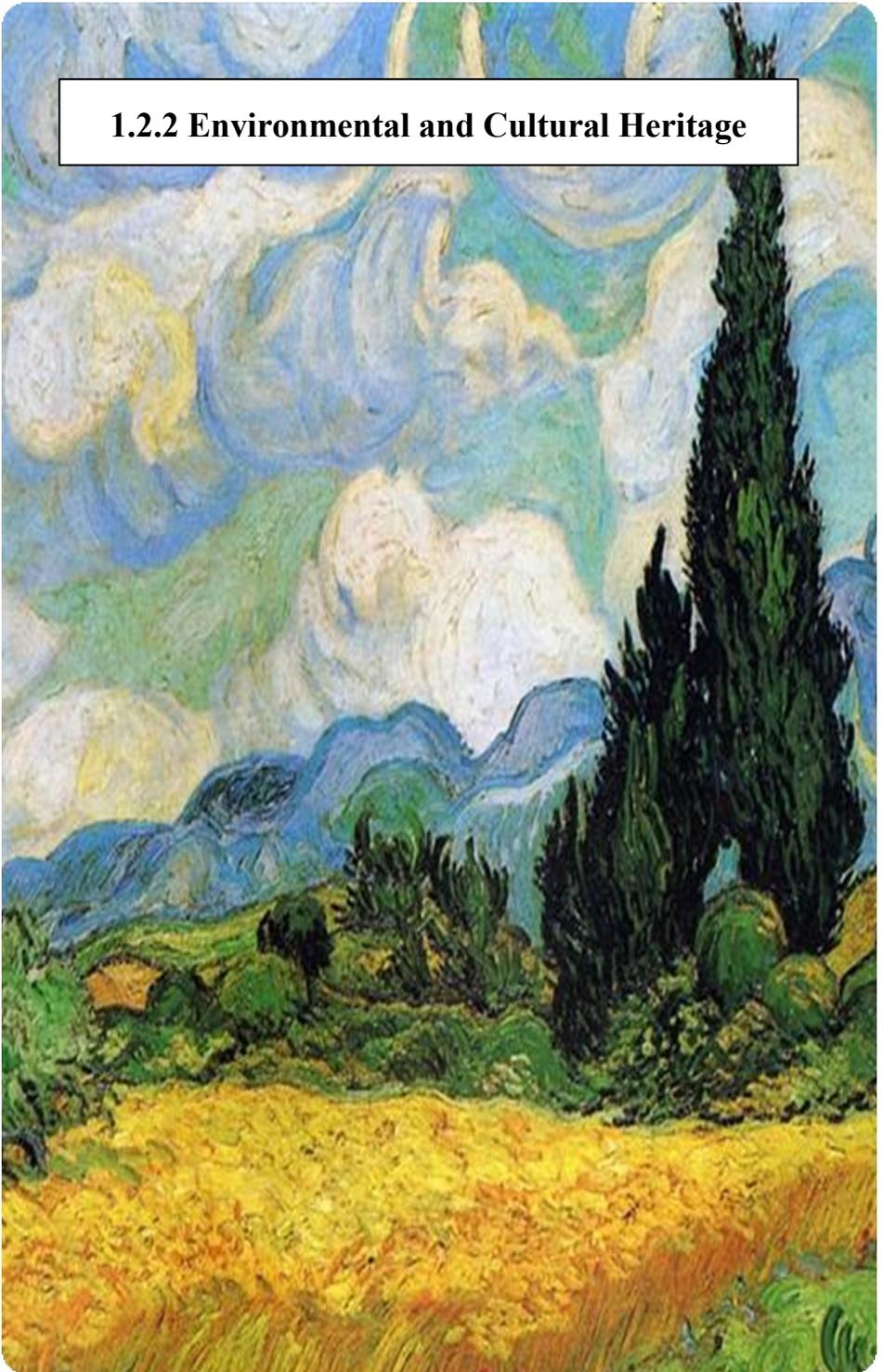
NMR spectroscopy was the official method applied by the OIV (International Organization of Vines and Wine) and by the European Commission to certify the presence of added sugar to wine.^{47, 48} In particular SNIF ^2H NMR (Site Specific Natural Isotope Fractionation Deuterium Nuclear Magnetic Resonance spectroscopy) has been used to measure the site-specific isotope ratios of D/H at natural abundance to control the origin of commercial products and to understand metabolic pathways. The advantage of SNIF ^2H NMR was the identification of isotope contribution for each atom of molecules to have a complete information about samples; however has required large amounts of products, long time of analysis and only molecules with low molecular weight could be analysed.

In the last years, irm- ^{13}C NMR spectroscopy was developed to conduct ^{13}C position-specific isotope ratio monitoring to analyse pharmaceutical, flavours and environmental samples with a precision better than 1%.⁴⁹ In literature Bayle and co-workers have reported the identification of geographic origin of vanillin and discrimination of its on the basis of the ^{13}C isotopic

profiles.⁵⁰ However, irm-¹³C NMR is not directly linked to international standards as required by EU Regulation and bulk $\delta^{13}\text{C}$ values from IRMS analysis was necessary to obtain the reduced molar fraction (f_i/F_i) of ¹³C used to the site-specific $\delta^{13}\text{C}$ values for each carbon in a target molecule.

So during the project ¹³C NMR spectroscopy was improved to determine the bulk ¹³C/¹²C carbon isotope ratio of inorganic carbonates and bicarbonates at natural abundance without IRMS analysis. Bayle and co-workers determined $\delta^{13}\text{C}$ using an dimethylsulfone as internal standard, while in this case was evaluated sodium acetate as internal standard and all parameters important to obtain quantitative and accurate analysis of carbon isotope ratio in bulk material.⁵⁰

1.2.2 Environmental and Cultural Heritage



1.2.2 Environmental and Cultural Heritage

In environmental sciences, the understanding of the origin and the evolution of contaminants was necessary for the decisions that must be taken by industrial companies and international agencies of healthy. The identification of the pollution sources in industrial site and the remediation activities, with possible temporary closure of industrial production, had a significant impact on the economy of the company. This problem carried out the requirement of appropriate tools, such as stable isotope ratio, to identify pollutants and their sources as well as to verify the performance of the proactive actions on the industrial sites.

The carbon isotope ratios of pollutants were analysed by an isotope ratio mass spectrometer coupled via a combustion interface to a gas chromatograph (GC-IRMS)⁵¹ or recently ²HNMR spectrometry has been applied for recognize degradation of gasoline additives in groundwater. The growth in level of carbon dioxide in the atmosphere has raised much interest in the chemical and physical implications of carbon sequestration. The mitigation of impacts of greenhouse gases on global warming was one of the most important aims of

International Organization, as showed during Climate Change Conference in Paris in 2015. In urban environments, carbon dioxide was emitted from different anthropogenic and biogenic sources, for this reason stable isotope analyses was a powerful tool for identification and for classification of the source of CO₂.^{52, 53} In some scientific works the isotopic composition of CO₂ was used to assess sources at local scale to discriminate emissions from vehicles from emissions generated by the biological activity in the urban areas.^{54, 55} On the other hand the disadvantage of this technique was that $\delta^{13}\text{CO}_2$ value was often influenced by spatially and temporally specific parameters. In the paper of A. Proto the isotopic ratio $\delta^{13}\text{CO}_2$ has been used as marker of carbon dioxide collected in three different area: a rural area where there were essentially plants and the contribution of carbon dioxide emission was attributed to sparing process of plants and $\delta^{13}\text{C}$ values ranging between -18.5 and -27% ; in urban environment $\delta^{13}\text{C}$ varying from -25.6 to -35% and the most important source was the combustion processes of fossil fuels; in civil inhabitation, near a kitchen, the burning of methane and other hydrocarbons was the source and stable carbon isotope value was from -40 to -45% .⁵³ This studies have showed the reliability of

$\delta^{13}\text{C}$ values as environmental tool to for identification of pollutants. Moreover, many studies were focused on sequestration of carbon dioxide and in particular geological storage was one of the most promising: CO_2 was captured, pressurized and pumped deep underground into geological formations. In this case was fundamental the studies on mineral carbonation reactions and the reactivity in aqueous solution, identification and characterization reaction products. NMR spectroscopy has been used as an ideal tool to study reaction of CO_2 in geologic formations, Diefenbacher and co-workers⁵⁶ have developed a methodology able to study the storage reaction in a water solution, at elevated pressure and temperature. Surface and others have also performed an *in-situ* measurement of MgCO_3 in the same condition of CO_2 sequestration in the presence of $\text{Mg}(\text{OH})_2$.⁵⁷ ^{13}C NMR spectroscopy was also attractive as quantitative, nondestructive, atom-specific spectroscopic method for the determination of $[\text{CO}_2]/[\text{HCO}_3^-]$ and $[\text{HCO}_3^-]/[\text{CO}_3^{2-}]$ ratios.⁵⁸ Solid-state ^{13}C NMR studies have also demonstrated that this methodology could be used as efficient tool to quantitatively distinguish and characterize magnesium carbonate phases, such as magnesite, hydromagnesite, dypingite and nesquehonite, with small

structural.⁵⁹

Thus, considering the high potential of NMR spectroscopy it was potentially applicable in material research studies of cultural heritage artefacts (carbonates, bicarbonates, marbles, salts etc.).⁶⁰

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2. Aims of work

Stable isotope analysis has gained increasing interest in the application of different scientific field; one of the most important regarded the food authenticity, quality and typicality, particularly in the areas where conventional analytical methods cannot provide unambiguous results. However, only IRMS was been recognized international technique for the investigation of food adulterations and other cheaper measuring equipment have been designed as alternatives in orders to enhance the choice of analyst.

In view of these requirements, this PhD project has been designed on three different aims:

- 1) The improvement of analytical methods for isotopic carbon determination in food samples and their application to identify geographical origin in sample like pasta, cocoa, olive oil. The spectroscopic methods such as FT-IR and NDIRS were cheapness and easy-to-operate respect to traditional methodology.
- 2) The development of ^{13}C NMR spectroscopy to determine the bulk $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio of inorganic carbonates and bicarbonates at natural abundance. In this case it were analysed all

parameters to obtained quantitative ^{13}C NMR analysis and thanks to the presence of internal standard could advance the quality of carbon isotope ratio determination of bulk material.

- 3) The application of isotopic carbon ratio analysis on samples which have considerable interest in several applications for environmental studies and in the field of cultural heritage. The intention is to correlate the isotopic composition with the variation of concentration in the air for the validation such as a tool to follow the flux of people in the different area of museum and to monitoring of filtering system.

3.1 Determination of isotopic composition

3.1.1 Isotopic Ratio Mass Spectrometer IRMS

A Delta Plus V Isotope Ratio Mass Spectrometer (ThermoFinnigan, Bremen, Germany) equipped with a Flash EA 1112 Elemental Analyzer (ThermoFinnigan) was used to measure $\delta^{13}\text{C}$. The $\delta^{13}\text{C}$ isotopic values were calculated using 2 homogenized in-house protein standards, which were themselves calibrated against international reference materials: L-glutamic acid USGS 40 (IAEA International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 for $^{13}\text{C}/^{12}\text{C}$. The measurement uncertainty, computed using the NORDTEST23-24 which combines the internal reproducibility with the performances results achieved in proficiency test FIT-PTS, was 0.3‰. The $\delta^{13}\text{C}$ values were reported relative to Vienna-Pee Dee Belemnite on a scale that was normalized by assigning a value of 46.6‰ to LSVEC lithium carbonate (IAEA).

3.1.2 FTIR infrared-Spectroscopy Fourier

Transform

For FTIR analysis of food samples, the sample was reduced to carbon dioxide gas in the same way as NDIRS analysis. Then, according to the procedure previously

described, 500 mg of BaCO₃ were introduced into a 10-ml glass flask with aPS (atactic polystyrene) film linked under the cap. Amorphous aPS film, with a thickness of 100-160 μm, was obtained by casting from 1.5% aPS chloroform solutions at room temperature. The flask was evacuated and adsorbed CO₂ was released by acidification with 2.5 mL of orthophosphoric acid. The polymeric film was left for 2 hours at room temperature into CO₂ gas and then introduced into the FTIR spectrometer.

Transmission infrared spectra in the midrange (4000-400 cm⁻¹) were acquired at a resolution of 2.0 cm⁻¹ and a scanning number of 32 with a Bruker Vector 22 FTIR spectrometer.

FTIR calibration was performed by using international standards purchased from the International Atomic Energy Agency (IAEA) (IAEA-CH-3, cellulose, δ¹³C= -24.5 ± 0.2‰; IAEA-C8, oxalic acid, δ¹³C= -18.3 ± 0.2‰; IAEA-CO-1, marble, δ¹³C= +2.5 ± 0.1‰; USGS41, L-glutamic acid, δ¹³C= +37.8 ± 0.2‰). The measurement uncertainty is 0.4‰, expressed as 2 SR (standard deviation of reproducibility).

3.1.3 NDIRS Non Dispersive Infrared Spectroscopy

For NDIRS (Non Dispersive Infrared Spectroscopy) analysis the food samples were preliminary reduced to carbon dioxide. The first step was combustion in atmosphere of pure oxygen at $T=1000^{\circ}\text{C}$; then carbon dioxide gas was collected in an impringer filled with 100 mL of barium hydroxide solution, $\text{Ba}(\text{OH})_2$, 0.3 M. This reaction started with a flash and carried on by itself. CO_2 reacted with $\text{Ba}(\text{OH})_2$ and it was quantitatively converted in barium carbonate, BaCO_3 .



Before analysis, BaCO_3 was washed with boiled water and dried under vacuum at 120°C for 1 hour.

All carbonates and bicarbonates were analyzed with the following procedure: 100 mg carbonate was introduced into a 10 mL glass flask, that was evacuated, and 2.5 mL of orthophosphoric acid was syringed to produce carbon dioxide. The CO_2 gas produced was collected in a specific aluminized bag. NDIRS spectroscopy was conducted by means of a Heli-FANplus analyzer (Medimars.r.l, Milan) equipped with a single beam non-dispersive infrared industrial photometer. The aluminized bags were directly

connected with the inlet ports of the NDIR spectrometer for sequential measurements. The NDIRS device was interfaced to a computer system that enables the software-guided measurement and calculation of results.

All the chemicals were purchased from Sigma Aldrich (Saint Louis, Missouri, USA).

NDIRS calibration was performed by using the international standard purchased from the International Atomic Energy Agency (IAEA) (marble, $\delta^{13}\text{C} = +2.5 \pm 0.1\%$). The measurement uncertainty was 0.6 %.

3.1.4 NMR Spectroscopy

The quantitative NMR spectra were recorded using a Bruker 600, with a probe accepting 5 mm o.d. tubes. The sample (0.1000 g), $\text{CH}_3^{13}\text{COONa}$ used as internal standard (0.0100 g) and the accurately weighed relaxation reagent $\text{Cr}(\text{acac})_3$ (0.0050 g), were added to 0.5 mL of deuterated solvent (D_2O) in order to lock the field to the frequency of the spectrometer. Gated decoupling techniques were applied in order to obtain quantitative results. The pulse angle was set at 90° and the pulse intervals, D , were selected ($D > 3T_1 \text{ max}$) on the basis of the longitudinal relaxation times, T_1 , first determined by the inversion recovery method.

Before analysis water insoluble carbonates were converted into soluble carbonates following the procedure: CaCO_3 was converted in water soluble Na_2CO_3 for ^{13}C NMR analysis according to the following procedure: a portion of 2 g of CaCO_3 was introduced into a 10 mL glass flask, that was evacuated, and 5 mL of orthophosphoric acid was syringed to produce carbon dioxide. The obtained CO_2 gas was collected in an impringer, filled with 20 mL of a saturated solution of sodium chloride and with 50 mL of NH_3 solution at 30-33% w/w; then it was quantitatively converted in NaHCO_3 and Na_2CO_3 . NaHCO_3 and Na_2CO_3 were removed by filtration, washed with distilled water and dried under vacuum at 300°C for 2 h to convert NaHCO_3 in Na_2CO_3 , following the well-known Solvay method. Na_2CO_3 was characterized by thermogravimetric analysis by using a Netzsch TG 209 apparatus. The analyses were carried out on samples with a mass of about 10 mg placed inside an alumina crucible. The sample temperature was then increased at a heating rate of $10^\circ\text{C min}^{-1}$ from room temperature up to 800°C under an inert atmosphere of nitrogen.

Typical values of the experimental parameters were as follows: spectrum width (SW) 3000 MHz, pulse width

(PW, for a pulse angle of 90°) $12\mu\text{s}$, memory size (SI) 32K, delay time 150 s, temperature (T) 300 K, zero filling (Z) 32K, number of transients (NS) 70, number of experiments per sample (NE) 4. Analyses time change in the range 1-2 h, based on the chemical difference of the compounds investigated.

3.1 Determination of FT-IR and NDIRS analysis to food samples



3.1 Determination of FT-IR and NDIRS analysis to food samples

The $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio was a chemical parameter with many important applications in the study of the geographic origin of food products giving the possibility for testing food authenticity, quality and typicality and the isotope ratio mass spectrometry was the technique currently used for the $\delta^{13}\text{C}$ determination. This latter is highly accurate (0.1‰) and sensitive (up to 0.01‰), but at the same time expensive and complex. In the first year the objective of project was to assess the reliability of spectroscopic methods such as FTIR and NDIRS techniques for determination of carbon stable isotope ratio of food sample. IRMS, NDIRS and FTIR were used to analyze samples of food, such as oil, durum, cocoa, pasta and sugar, in order to determine the natural abundance isotopic ratio of carbon in a parallel way. The results showed a close relationship among the results obtained by three different techniques. The main advantage in using FTIR and NDIRS is related to their cheapness and easy-to-operate in comparison to IRMS.

3.1.1 Material and Methods

This study was conducted on 19 selected samples typical components of the "Mediterranean Diet" such as pasta, oil, chocolate, cocoa, flour and sugar. These samples were collected within the framework of the Campus Project (Introduction and valorization of healthful foods and productive rationalization in the traditional industries of the Campania region) funded by the Italian Campania Region (POR CAMPANIA FESR 2007-2013). In Table 1 is reported a detailed description of the samples investigated.

Table 2. Detailed description of samples used for the $\delta^{13}\text{C}$ analysis.

Index	Sample	Composition
1	Oil	Commercial sunflower oil
2	Oil	Italian extravirgin olive oil 100%
3	Oil	Commercial extravirgin Olive Oil "100% Italian"
4	Oil	Extravirgin olive oil 100% Italian with 2% wt of fish oil (eith omega 3 and containing about 18% EPA ^a and 9% DHA ^b). Final oil should be about 0.4 g of EPA and 0.2 g DHA per 100g.
5	Oil	Extract of fish oil with about 18% EPA ^a and 9% DHA ^b
6	Wheatflour 0	Extract of fish oil with about 18% EPA ^a and 9% DHA ^b
7	Wheatflour 00	Italian wheat flour , a bit lessrefined about 70% of the grain, and a bit darker.
8	Cane Sugar	The softest, finest, Italian flour; very finely ground like a fine powder and very white.
9	Semolinaflour	Sucrose extracted from sugar cane
10	Semolinaflour	Semolina from Italian durum wheat
11	Semolinaflour	Semolina from Italian durum wheat
12	Pasta	Semolina from Italian durum wheat
13	Pasta	Italian durum wheat; lutein extracted from tomato skins local (20 mg/100 g)
14	Cornstarch	Italiandurumwheat
15	Chocolate	Derived from the corn grain
		Cocoa paste (70%) from Belgium; cocoabutter (20%); stevia + maltodextrin (produced by Nestevia); inulin (stardardprovided by Sigma Aldrich)

16	Cocoa	Cocoa 100%, the starting product obtained after extraction from plants
17	Cocoa	Cocoa 100%, the starting product obtained after extraction from plants
18	Chocolate	Cocoa paste (70%) from Belgium; cocoabutter (20%); stevia + maltodextrin (produced by Nestevia); inulin (standard provided by Sigma Aldrich)
19	Chocolate	Commercial dark chocolate, 50% cocoa. Ingredients: cocoa paste, sugar, butter, anhydrous vaccine, emulsifierlecithin, naturalvanillaflavor

^a EPA = Eicosapentaenoic acid; ^b DHA = docosahexaenoic acid.

3.1.2 Statistical analysis

Statistical analysis was performed using Sigma plot 12.0 software which was used for summary statistics (mean, standard deviation, coefficient of variation, linear regression and Bland Altman plot).

Linear regression analysis was used to determine the correlation between FTIR, NDIRS and IRMS. However, a good correlation does not certainly guarantee a good agreement between two methods. Bland and Altman did not state that in the analysis of measurement method comparison data, neither the correlation coefficient nor techniques such regression analysis are appropriate.^{5,7} They suggested to integrate these analyses by simply plotting the difference between the results of the methods against their mean. Bland Altman Bias plot was used to determine the 95% confidence limits of agreement and the bias between IRMS, FTIR and NDIRS results.

3.1.3 Results and discussions

The results of isotopic carbon analysis was reported in Table 3, food samples was analyzed by the three different methods (IRMS, NDIRS and FTIR) as mean value of three measures on each sample. To avoid some interferences and/or matrix effects on measurement, samples were selected with different composition, chemical nature and geographical origin.

Table 3. $\delta^{13}\text{C}$ values resulting from three different techniques.

Sample	Description	IRMS		NDIRS		FTIR	
		$\delta^{13}\text{C}$ (‰)	SEM*	$\delta^{13}\text{C}$ (‰)	SEM	$\delta^{13}\text{C}$ (‰)	SEM*
1	Oil ^a	-29.9	0.1	-28.5	0.2	-30.2	0.1
2	Oil ^b	-30.4	0.1	-30.2	0.1	-31.6	0.1
3	Oil ^c	-29.7	0.1	-29.7	0.2	-30.1	0.1
4	Oil ^d	-29.7	0.1	-29.8	0.1	-31.8	0.1
5	Oil ^e	-23.4	0.1	-22.9	0.2	-24.5	0.1
6	Wheat flour	-26.8	0.1	-26.5	0.2	-25.4	0.1
7	Wheat flour 00	-26.4	0.1	-24.1	0.2	-27.4	0.2
8	Cane sugar	-12.3	0.1	-15.6	0.2	-14.2	0.2
9	Semolina flour ^f	-25.5	0.1	-26.2	0.1	-25.7	0.1
10	Semolina flour ^g	-23.9	0.1	-23.5	0.3	-24.5	0.1
11	Semolina flour ^h	-26.9	0.1	-25.3	0.2	-25.4	0.2
12	Pasta ⁱ	-24.8	0.1	-25.2	0.2	-24.2	0.1
13	Pasta	-25.1	0.1	-25.0	0.1	-25.4	0.2
14	Corn Starch	-11.7	0.1	-13.8	0.2	-13.4	0.1
15	Chocolate ^l	-29.7	0.1	-28.5	0.1	-31.3	0.1

16	Cocoa ^m	-30.4	0.1	-29.1	0.3	-31.2	0.1
17	Cocoa ⁿ	-29.8	0.1	-28.4	0.1	-29.1	0.2
18	Chocolate ^o	-30.4	0.1	-28.1	0.1	-32.1	0.2
19	Chocolate ^p	-27.8	0.1	-26.4	0.1	-28.1	0.1

*SEM: Standard error of mean.

^a sunflower oil, ^b home made extravirgin olive oil, ^c extravirgin olive oil, ^d extravirgin olive oil with omega3, ^e fish oil extract, ^{f, g, h} different commercial semolina flour, ⁱ pasta with luteina, ^j dark chocolate 70% cocoa, ^m dark chocolate 50%, ^{m, n} different commercial cocoa, ^o dark chocolate with stevia, ^p dark chocolate with 50% cocoa.

The $\delta^{13}\text{C}$ values obtained show a very good agreement with values reported in the literature for similar food samples ^{1,2,3,8}. NDIRS and FTIR spectroscopies could be used to discriminate the isotopic domain characteristic of the particular kind of food.

The $\delta^{13}\text{C}$ values of olive oil, for example, respect the range of oil samples from -28.5 to -31.7 ‰ while the oil extracted from fish (see entry 5 in Table 3) has a quite different value (-22.9 to -24.5‰): olive plants have a C_3 photosynthetic pathway and their carbon composition was into the typical range -22 to -34‰ [5, 38] reflecting the different origin.

The same results were reported for flour samples (flour 0, flour 00, semolina and durum wheat flour) with an isotopic composition ranging from -27.4‰ to -24‰, the same range reported by Brescia et al. The $\delta^{13}\text{C}$ values of

chocolate and cocoa, from -26.4 to -32.0‰ show typical isotopic composition for C₃ plants (Calvin cycle). The C₄ plants, comprising most plants in the tropics including corn and sugar cane, use the Hatch-Stack cycle, and are isotopically heavier (-23 to -6‰) compared to C₃ plants.⁴ As reported in Table 3 cornstarch shows an isotopic composition ranging from -11.7 to -13.8‰ and cane sugar sample shows an isotopic composition in the range -12.3 to -15.6‰.

To better evidence the isotopic domains the delta values, obtained by the three methods, have been plotted vs the food samples in Figure 5.

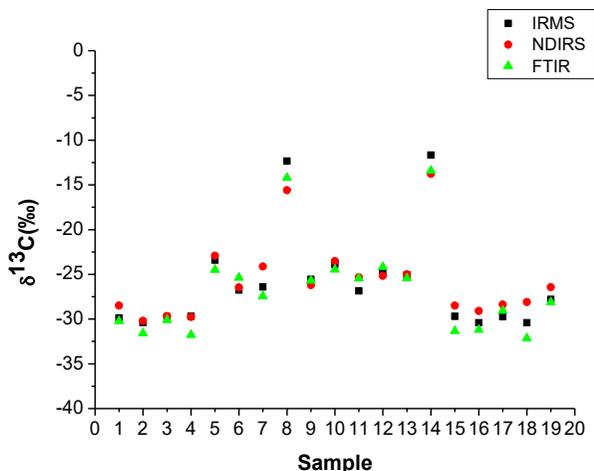


Figure 5. Summary of the delta values obtained by IRMS, NDIRS and FTIR.

The statistical analysis conducted in order to assess the robustness of the two alternative techniques, NDIRS and FTIR, with respect to the standard technique (IRMS) showed a strong correlation between the results like a demonstration of the positive relationship between the tested analytical methods.

Regression analysis performed reported a significant correlation ($r = 0.983$; regression line $y = 1.212x + 5.036$; $p\text{-value} < 0.001$; $n = 19$) for NDIRS and IRMS (Fig. 6a). A similar significant correlation ($r = 0.981$; regression line $y = 1.010x + 0.866$; $p\text{-value} < 0.001$; $n = 19$) between the methods was observed for IRMS and FTIR

(Fig. 6b) and for NDIRS and FTIR ($r = 0.953$; regression line $y = 0.807x - 4.130$; p -value < 0.001 ; $n = 19$ Fig. 2c).

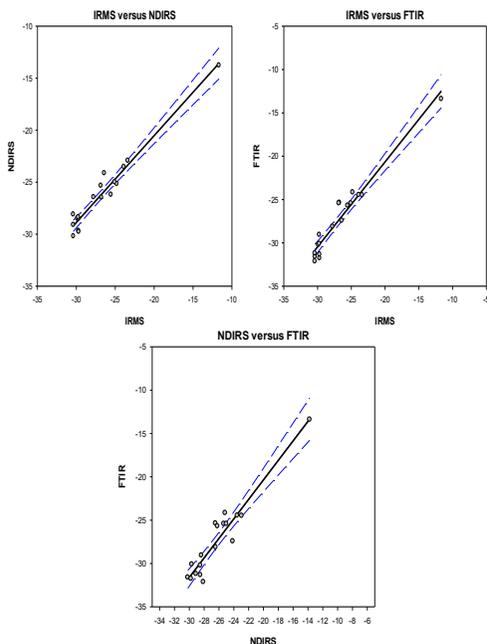


Figure 6. Linear regression line for the comparison between IRMS, NDIRS and FTIR.

Bland– Altman bias plot showed the agreement of the three methods making comparisons two by two. The relationship between the measurement error and true value (mean of the two methods) and the bias for the two methods were assessed by the use of limit of agreement (Fig. 6). Limit of agreement is defined as the limit where 95% of the differences would lie if the two methods are in

agreement. A plot of the difference between the methods against their mean was drawn and limits of agreement at 95% confidence level were -2.307 to 3.127 for the comparison between IRMS - NDIRS (Fig. 6a), -2.650 to 1.475 for the comparison between IRMS - FTIR (Fig. 6b) and -3.967 to 1.997 (Fig.6c) for FTIR - NDIRS. The differences were distributed within the limits of agreement, thus demonstrating that all methods were in agreement with acceptable bias of 0.407 (Fig. 7a), -0.587 (Fig. 7b) and 0.994 (Fig. 7c), respectively.

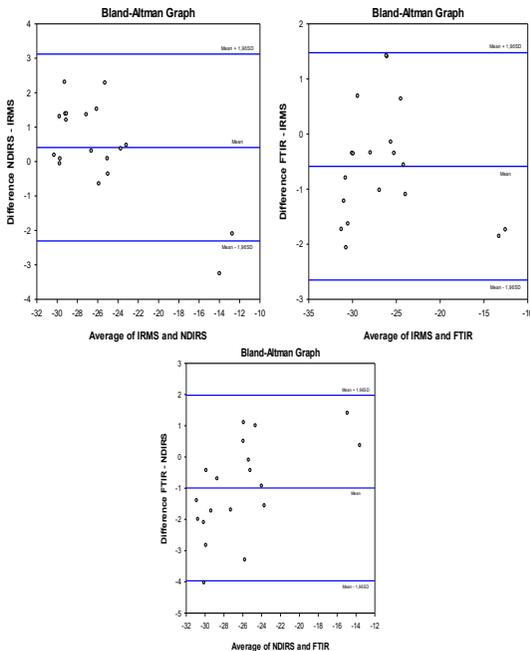


Figure 7. Altman-Bland plot. The difference in the delta values versus the average of the data pairs.

The $\delta^{13}\text{C}$ values of few samples showed some discrepancies with respect to IRMS (see samples 8,14 and 18 in Table 3), that could be due to different preliminary steps (combustion of sample (~1.0 g), recovery of the formed CO_2) required by spectroscopic analysis, while with IRMS the sample was a direct injected (0.8 mg) .

Anyway, generally values were good correlated in the 3 techniques and the differences between types of samples (e.g. C3 plants vs C4 plants). Therefore the spectroscopic methods can be used as a valuable alternative of IRMS for determining the authenticity of food, at least as first screening.

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3.2 Development of ^{13}C NMR method for bulk determination of carbon stable isotope composition in carbonates and bicarbonates samples



3.2 Development of ^{13}C NMR method for bulk determination of carbon stable isotope composition in carbonates and bicarbonates samples

The second part of project was focused on the first application of ^{13}C NMR (Nuclear Magnetic Resonance) spectroscopy to analyse the bulk $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio of inorganic carbonates and bicarbonates at natural abundance. In literature was reported the use of ^{13}C NMR spectroscopy (irm- ^{13}C NMR) to measure ^{13}C position-specific isotope of organic molecules with a precision of 1%.¹ Bayle and co-workers used this methodology to vanillin and identified its geographic origin thanks to the ^{13}C isotopic profiles.⁵

Moreover the technique has been extended to many applications, such as metabolic analyses², climate studies³, environmental studies⁴ and food chemistry. It is also the official method applied by the OIV (International Organization of Vines and Wine) as well as being adopted by the European Commission to control the addition of sugar to wine. The reliability of method was evaluated in comparison with the method chosen in the past for these measurements, i.e. Isotope Ratio Mass Spectrometry (IRMS), with very good results. The developed methodology analysis carried out the $^{13}\text{C}/^{12}\text{C}$ ratio of

carbonates and bicarbonates (≈ 50 - 100 mg) with a precision in the order of 1 ‰ in the presence of a relaxation agent, such as $\text{Cr}(\text{acac})_3$, and $\text{CH}_3^{13}\text{COONa}$ as internal standard. The method was applied not only to soluble inorganic carbonates and bicarbonates but also to insoluble carbonates by converting them to Na_2CO_3 , following a simple procedure and without observing isotopic fractionation.

3.2.1 Materials and Methods

All carbonate and bicarbonate, such as sodium carbonate (Na_2CO_3), potassium carbonate (K_2CO_3), cesium carbonate (Cs_2CO_3), calcium carbonate (CaCO_3), ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$), sodium hydrogen carbonate (NaHCO_3), potassium hydrogen carbonate (KHCO_3), ammonium hydrogen carbonate (NH_5CO_3), chromium acetylacetonate ($\text{Cr}(\text{acac})_3$) and sodium acetate ($\text{CH}_3^{13}\text{COONa}$) were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Oxalic acid (international standard) was purchased from the International Atomic Energy Agency (IAEA) as follows: IAEA-C8, oxalic acid, $\delta^{13}\text{C} = -18.3 \pm 0.2$.

To convert insoluble CaCO_3 in water soluble Na_2CO_3 for ^{13}C NMR analysis the procedure was the following: 2 g of

CaCO₃ was introduced into a 10 mL evacuated glass flask and 5 mL of orthophosphoric acid was syringed to produce carbon dioxide. The obtained CO₂ gas was collected in an impringer, filled with 20 mL of a saturated solution of sodium chloride and with 50 mL of NH₃ solution at 30-33% w/w; then it was quantitatively converted in NaHCO₃ and Na₂CO₃. NaHCO₃ and Na₂CO₃ were removed by filtration, washed with distilled water and dried under vacuum at 300°C for 2 h to convert NaHCO₃ in Na₂CO₃, following the well-known Solvay method. Na₂CO₃ was characterized by thermogravimetric analysis by using a Netzsch TG 209 apparatus. The analyses were carried out on samples with a mass of about 10 mg placed inside an alumina crucible. The sample temperature was then increased at a heating rate of 10°C min⁻¹ from room temperature up to 800°C under an inert atmosphere of nitrogen.

3.2.3 Linear calibration and validation

The development of a new method involved the optimization of the spectral parameters for ¹³C NMR analysis to obtain quantitative and precision analysis. The parameters analyzed were the relaxation time (T₁), the presence of the relaxation agent, the pulse intervals (D₁)

and the effect of an internal standard. In the first time was calculated the longitudinal relaxation times of carbonates and bicarbonates using the inversion-recovery sequence: the initial population was disturbed by inverting the spin populations through the application of a 180° pulse. In Table 4 were reported the relaxation times carried out from 16.050 s (in NaHCO₃) to 47.944 s (in K₂CO₃).

Table 4. Longitudinal relaxation times of carbonates and bicarbonates obtained by the inversion-recovery sequence at 600 MHz.

Sample	Chemical shift (ppm)	T ₁ ^{13C} (s)
Cs ₂ CO ₃	168.15	42.855
NaHCO ₃	161.20	16.050
K ₂ CO ₃	168.20	47.944
NH ₅ CO ₃	160.94	23.845
CH ₃ ¹³ CO ₂ Na	181.50	36.160

The carbonates have a long relaxation times and some hours were necessary to obtain quantitative spectra with NMR spectroscopy. In literature was reported the effect of the paramagnetic relaxing complex, such as Cr(acac)₃, that has permitted to reduce the pulse interval values from 10*T₁ to 3*T₁ and the analysis time to 1-2 h.⁵

Previously published reports adopted $D_1 = 10 * T_1$ for ^{13}C -NMR data acquisition^{1,5,6}; however, the best value obtained during experimental analysis was $D_1 = 3 * T_1$ for quantitative analysis of carbonates and bicarbonates. The integral value was optimized referring to $\delta^{13}\text{C}$ of Na_2CO_3 obtained by IRMS, and the integral value obtained from $D_1 > 3 * T_{1\text{max}}$ until was comparable.

The ^{13}C NMR wasn't directly linked to international standards and it was evaluated the use of an internal standard, such as sodium acetate, to avoid this disadvantage. One use of internal standard has been recently reported in the literature: the $\delta^{13}\text{C}$ determination of vanillin using dimethylsulfone as standard.³

Sodium acetate, $\text{CH}_3^{13}\text{CO}_2\text{Na}$ only enriched on carbonyl carbon, was chosen as suitable internal standard thanks to the similar spectroscopic parameters relaxation time (36.16 s) and chemical shifts (181.5 ppm) in the same range of the carbonates and bicarbonates investigated. The sample was prepared by dissolving $\text{CH}_3^{13}\text{CO}_2\text{Na}$, the sample and $\text{Cr}(\text{acac})_3$ in D_2O . Isotope carbon composition of the bulk sample was evaluated following the equations and linear calibration. The first step was the integration of spectra: normalization of the intensity of ^{13}C on carbonyl carbon of sodium acetate (internal standard) such as 1 and

comparison of the signal intensities of the sample were compared to the standard signal. $^{13}\text{C}_R$ is the molar carbon of the internal standard, $^{13}\text{C}_{\text{sample}}$ is the molar ^{13}C of the sample, obtained as the product of the molar carbon of the internal standard and the signal intensities of the sample (I_{sample}), as described in the following equations:

$$^{13}\text{C}_R = \frac{g_{\text{sodium acetate}}}{\text{molar mass}_{\text{sodium acetate}}}$$

$$^{13}\text{C}_{\text{sample}} = ^{13}\text{C}_R * I_{\text{sample}}$$

$$\frac{^{13}\text{C}}{^{12}\text{C}} = \frac{^{13}\text{C}_{\text{sample}}}{C_{\text{tot}} - ^{13}\text{C}_{\text{sample}}}$$

In Figure 8 was showed the calibration line using two carbonates, Cs_2CO_3 and K_2CO_3 , and two bicarbonates, NaHCO_3 and NH_5CO_3 . The calibration line was obtained by plotting the average absorption intensity ratios against the reference $\delta^{13}\text{C}$ values obtained by IRMS reported in Table 5, (correlation coefficient $r^2=0.9987$), leading to a standard deviation of 1.0 ‰ in terms of $\delta^{13}\text{C}$ units.

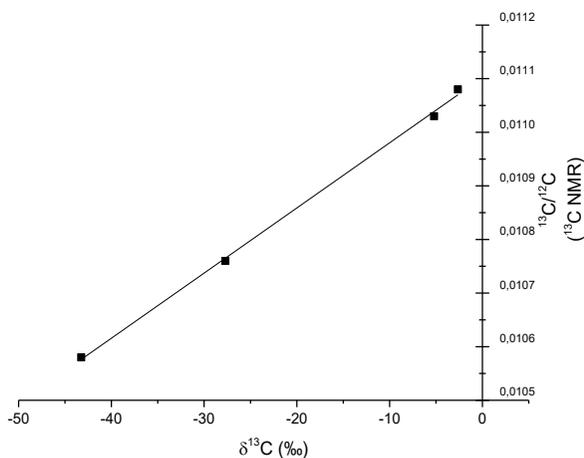


Figure 8. Linear calibration plot between the $^{13}\text{C}/^{12}\text{C}$ ratio obtained by ^{13}C -NMR and the $\delta^{13}\text{C}$ (‰) obtained by IRMS.

3.2.3 Results and discussions

The $\delta^{13}\text{C}$ of the sample was calculated on the basis of the linear calibration and the results were reported in Table 6. The $^{13}\text{C}/^{12}\text{C}$ ratio obtained by the ^{13}C -NMR experiments was determined independently for each carbonate and bicarbonate as the mean value out of four measurements for each sample. The values of $\delta^{13}\text{C}$ (‰) of standards ranged from -3.32 ‰ (in NaHCO_3) to -43.25 ‰ (in NH_5CO_3), in line with data reported in the literature for these samples.¹³

Table 5. ^{13}C NMR chemical shifts and isotope ratio obtained by $\delta^{13}\text{C}$ analysis*.

Sample	Chemical shift (ppm)	T_1 ^{13}C (s)	$\delta^{13}\text{C}$ (‰)	$^{13}\text{C}/^{12}\text{C}^*$ (NMR)
NaHCO ₃	161.20	16.050	-3.32	0.01113
Cs ₂ CO ₃	168.15	42.855	-5.20	0.01103
K ₂ CO ₃	168.20	47.944	-27.7	0.01076
NH ₅ CO ₃	160.94	23.845	-43.25	0.01058

* ^{13}C -NMR analyses are carried out in the presence of 0.005 g of Cr(acac)₃.

Table 6. Comparison of $\delta^{13}\text{C}$ values obtained.

Sample**	$\delta^{13}\text{C}$ (IRMS)	SEM *	$\delta^{13}\text{C}$ (NMR)	SEM *	$\delta^{13}\text{C}$ (NDIRS)	SEM *
Na ₂ CO ₃	-2.6	0.1	- 2.0	1.0	-2.4	0.2
KHCO ₃	-28.9	0.1	- 30.0	1.0	-29.7	0.2
CsHCO ₃	-33.0	0.1	- 32.0	1.0	-32.0	0.3
(NH ₄) ₂ CO ₃	-45.7	0.1	- 46.0	1.0	-45.5	0.1

*SEM: Standard error of mean.

* * ^{13}C -NMR analyses are carried out in the presence of the relaxation reagent (Cr(acac)₃).

The $\delta^{13}\text{C}$ values obtained by three different methodologies, IRMS, ^{13}C NMR and NDIRS, were very close to each other, carried out a good agreement among techniques. Precision value of 1‰ was obtained with ^{13}C NMR, in line with data obtained by ^{13}C position-specific isotope ratio monitoring¹. These results have demonstrated that the ^{13}C NMR methodology could be a valuable alternative to IRMS and NDIRS for the determination of carbon isotopic composition in bulk samples, at natural abundance.

However, the NMR analysis could be done in solution and some carbonates, for example calcium and barium carbonates, have a low solubility in water. It was necessary convert this compounds if want extend NMR methodology to the analysis of isotopic composition of insoluble calcium carbonates, which are of considerable interest due to their importance in several applications, such as environmental studies and in the field of cultural heritage.^{12, 13, 15}

Water insoluble carbonates were converted into sodium soluble carbonates following the procedure reported in the previews section. The synthesized carbonates were characterized by means of thermogravimetric analysis to ensure the quantitative conversion of NaHCO_3 in

Na_2CO_3 . In addition, $\delta^{13}\text{C}$ values of the initial insoluble carbonates as well as those of the obtained soluble carbonates were determined by IRMS and the results excluded any isotopic fractionation.

The obtained carbonates were then analyzed by IRMS, NDIRS and ^{13}C -NMR, three measurements for each sample and calcium carbonate showed a $\delta^{13}\text{C}$ of $-9.8 \pm 1.0\%$ obtained by NMR, which was very close value to the $\delta^{13}\text{C} = -9.0 \pm 0.1\%$ obtained by IRMS. The marble samples converted has the value of $\delta^{13}\text{C} = 1.0 \pm 1.0\%$ and $1.0 \pm 0.1\%$ by NMR and by IRMS, respectively.

Thanks to results NMR spectroscopy could be a valid tool to determine the carbon isotope composition of carbonate species in solution.

The carbon stable isotope composition was an interesting tool in determining the position-specific carbon isotope ratios, at natural abundance, by NMR spectroscopy to identify the origin, authenticity and traceability of several organic molecules in samples (some representative examples were glucose, glycerol, malic acid and vanillin)^{12,13-15}, the developed methodology was also applied for the first time to small organic molecules, such as oxalic acid. The sample was prepared by dissolving 0.0045 g $\text{CH}_3^{13}\text{CO}_2\text{Na}$, 0.120 g of oxalic acid and 5.0 mg

of $\text{Cr}(\text{acac})_3$ in 0.5 mL of D_2O . The ^{13}C spectrum of oxalic acid is reported in Figure 9.

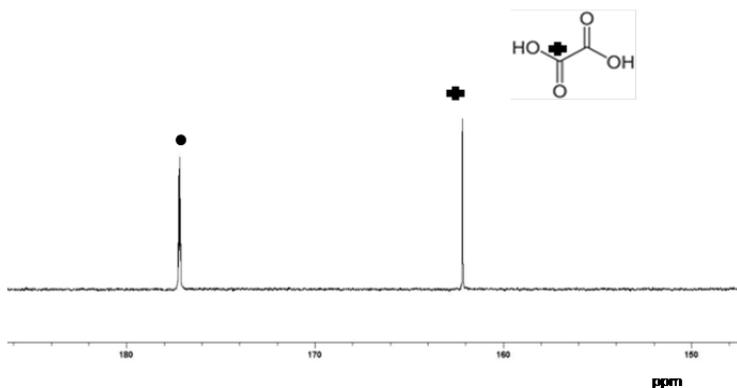


Figure 9. ^{13}C NMR spectrum of oxalic acid (✝ IAEA-C8 (International reference material for stable isotopes) in D_2O with $\text{CH}_3^{13}\text{CO}_2\text{Na}$ (●) and relaxation agent $\text{Cr}(\text{acac})_3$.

Taking into account that all molar carbon of sodium acetate is ^{13}C on carbonyl carbon with intensity such as 1, the signal intensities of oxalic acid correspond to 0.5282. Based on this assumption, $^{13}\text{C}_R$ represents the molar carbon of the internal standard, while $^{13}\text{C}_{\text{oxalic acid}}$ is the molar ^{13}C of oxalic acid obtained by multiplying the molar carbon of the internal standard with the signal intensities of the acid, as the following equations suggest:

$$^{13}\text{C}_R = \frac{0.0045 \text{ g}}{82.0343 \text{ g/mol}} = 5.485519329 \cdot 10^{-5} \text{ mol}$$

$$^{13}\text{C}_{\text{oxalic acid}} = ^{13}\text{C}_R * I_{\text{OA}} = 2.897446556 \cdot 10^{-5} \text{ mol}$$

$$\frac{^{13}\text{C}}{^{12}\text{C}}_{\text{oxalic acid}} = \frac{^{13}\text{C}_{\text{oxalic acid}}}{\text{C}_{\text{tot}} - ^{13}\text{C}_{\text{oxalic acid}}} = 0.01091$$

The $\delta^{13}\text{C}$ of oxalic acid was calculated on the basis of the linear calibration and the value obtained ($\delta^{13}\text{C} = -19.0 \pm 1.0\%$) was in line with that measured by IRMS analysis ($\delta^{13}\text{C} = -18.3 \pm 0.5\%$).

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3.4 Application of isotope carbon ratio for Cultural Heritage



3.4.1 S. Pietro a Corte and Palazzo Fruscione, a part of SNECS project

Fruscione Palace and S. Pietro a Corte are two important archaeological locations in Salerno and their preservation was the aim of national project (DATABENC-SNECS) growing with different research centers, universities and industries. The church of S. Pietro a Corte was situated in front of Fruscione Palace in a street near the city centre and together was a part of the same historical complex. The Church was composed of three levels: the first level was the *frigidarium* of ancient roman thermal baths dating from the 1st -2nd A.D.; the second level was a Christian place of worship from the 5th to the 7th century looking holy paintings on the walls; the third structural phase of the building, promoted by the king Arechi, was represented by autonomous structure of Cappella Palatina in the 8th century A.D. In the Fruscione Palace were preserved the remains of a Norman building, ancient mosaics and architectural elements of a roman thermal baths. The aim of the project SNECS (Social Networks delle Entità dei Centri Storici) was to collect and publish scientific, artistic, archaeological, literary, historical and philosophical knowledge of the local place supported by new technologies (mapping (GIS) modelling, aero-

photogrammetric, toponomastics), to obtained interactive atlas with high definition and flexibility.

Concern about atmospheric pollution and damage on monuments highlighted the relationship between the presence of pollutants at low concentration and degradation events.^{1, 2, 3} In "SNECS" project, during many social and cultural events as biennial exhibition of contemporary art, Luci d'artista organised at Fruscione Palace and S. Pietro a Corte, it was analysed the influence of tourists on environmental pollution correlated to isotope carbon composition of atmospheric carbon dioxide.

3.4.2 Material and Methods

The air monitoring of archaeological locations was developed using passive samplers. In particular, it was used the commercial RING from Aquaria Research Srl and the pollutants monitored were NH₃, NO₂, BTEX, H₂S, SO₂ with specific passive samplers. The methodologies used to prepare substrates and to analyse samples were described by National Institute for Occupational Safety and Health (NIOSH), the United States federal agency for research and prevention of work-related injury and illness. The analytical methods were reported in the manual of NIOSH (NMAM)⁴ that

contains recommendations for collection, sampling and analysis of contaminants in the workplace.

The methodologies used for different pollutants:

- n° 2018, Aliphatic Aldehydes;
- n° 1500, Hydrocarbons, BP 36°-216° C;
- n° 6013, Hydrogen Sulfide;
- n° 6014, Nitric Oxide and Nitrogen Dioxide;
- n°6004, Sulfur Dioxide;
- n° 6015, Ammonia.

The CO₂ was collected with passive sampler by using a Ca-based solid absorbent (CaO/Ca₁₂Al₁₄O₃₃ 75:25 w/w) and measurement of $\delta^{13}\text{C}$ was conducted by means of NDIRS. The monitoring campaigns were carried out during biennial exhibition of contemporary art and the following period at Fruscione Palace (October-November), while from April to January 2015 at S. Pietro a Corte. The samplers were located at 1,5 mt of height for three days, after it was extracted in solution and analysed according to international methodologies.

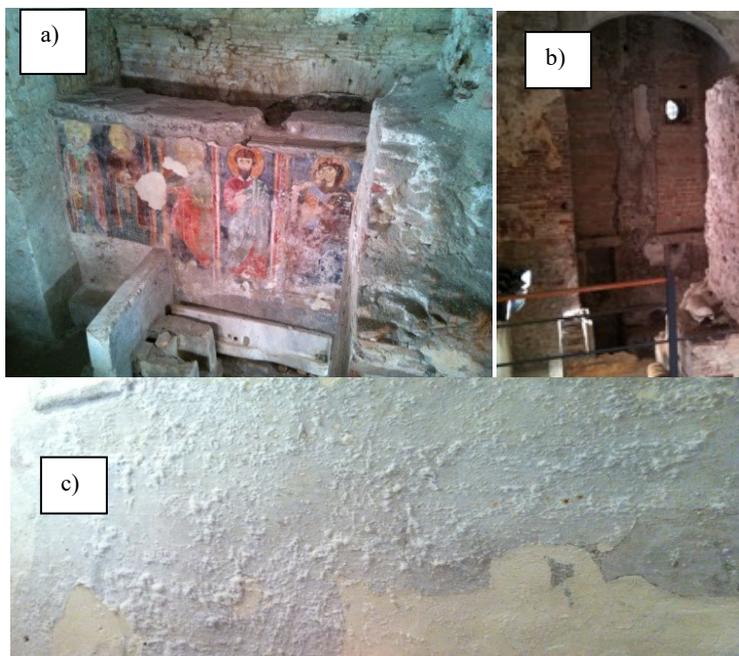


Figure 10. a) Paintings in S. Pietro a Corte with salt deposits; b) the first level of church with external sewer c) white powder deposits in S. Pietro a Corte.

Three samples of white powder deposits have been collected from the interior walls surfaces by means of a brush or a scalpel. The powder samples, about 10mg, was characterized by, XRD and thermogravimetric analysis by using a Netzsch TG with heating rate of $10^{\circ}\text{C min}^{-1}$ from room temperature up to 800°C under atmosphere of nitrogen. Ion chromatography (IC) has been employed for the quantification of the main anionic constituents of the deposits.: 10 mg of powder was treated with 10 mL of

MilliQ water and the solution have been sonicated before analyses with Dionex Dx-120 ion chromatograph (Dionex, Sunnyvale, CA, USA), equipped with a IonPac AS14 column (4mm x 250mm) at a flow rate of 1.0 ml min⁻¹ and a pressure of 1400 psi.

3.4.3 Results and Discussion

In the first part of project it was analysed the effect of visitors in Fruscione Palace on concentration of pollutants during contemporary arts exhibition and cultural events, the results were reported in Table 7.

Table 7. Concentrations of pollutants during and after cultural events.

	Before cultural events	During cultural events
NH ₃ (µg/m ³)	0,22	0,58
NO ₂ (µg/m ³)	0,67	1,74
Formaldeide (µg/m ³)	3,78	3,86
Benzene (µg/m ³)	6,95	10,34
Toluene (µg/m ³)	2,08	12,11
Etilbenzene (µg/m ³)	0,144	1,707
H ₂ S (µg/m ³)	0,068	0,042
δ ¹³ C (‰)	-25,4	-29,2

The values showed in Table 7 the strong correlation between greater presence of visitors during cultural events and increasing of concentration of pollutants, such as ammonia from 0,22 to 0,58 $\mu\text{g}/\text{m}^3$ and toluene from 2,08 to 12,11 $\mu\text{g}/\text{m}^3$. In the same way the values of $\delta^{13}\text{C}$ (‰) was influenced, passed from -25,4 ‰ to -29,2 ‰.

However, the concentrations of pollutants always respected the limit value of World Health Organization.

The monitoring of NO_2 , SO_2 and $\delta^{13}\text{C}$ was performed also in S. Pietro a Corte and in Table 8 were reported the results obtained from April 2015 to January 2016. During the period ranging from April to August values of pollutants and $\delta^{13}\text{C}$ (‰) had the same trend, value increased during autumn and winter season with increased of pollution, traffic and visitors. The strong correlation revealed that $\delta^{13}\text{C}$ value could be used such as a non-invasive marker for the air condition in the museum sites.

Table 8. Atmospheric concentration of pollutants in S. Pietro a Corte.

Month	$\delta^{13}\text{C}$ (‰)	NO_2 ($\mu\text{g}/\text{m}^3$)	SO_2 ($\mu\text{g}/\text{m}^3$)
April	-31,1	3,88	0,56
June	-26,5	2,5	0,2
July	-24,2	0,507	1,4
August	-20,5	0,320	0,14
October	-27,5	2,9	1,2
December	-31,5	3,9	0,75
January	-28,5	2,8	0,83

In the second part of project the white powder deposits collected were analysed to obtain a complete characterization of archaeological site statement. Powder deposits had adsorbed low quantity of water, less to 2%, and ion chromatography showed anionic constituents such as sulphate (64,32 ppm), nitrates (1127 ppm), chloride (0,88 ppm).

The concentration of nitrate and chloride ions could be a consequence of high humidity and the presence of salts on the top of stone derived from a puddle situated on the first level of S. Pietro a Corte. Nitrate salts were mainly

presented as potassium nitrate (in particular on *frigidarium*), as confirmed by XRD analysis.

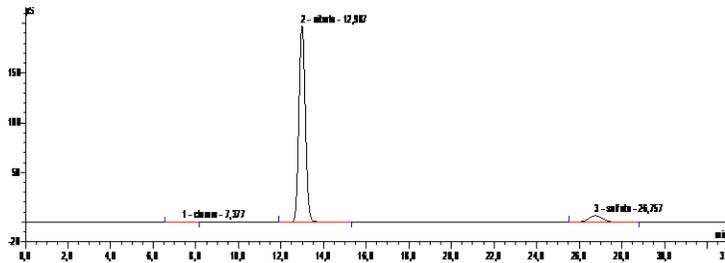


Figure 11. Ion chromatography of powder salt deposits.

During rainy season the level of puddle was growth and it was analysed by ion chromatography to determine correlation between different composition, chemical nature and origin of powder salts on the stone. Analysis reported the same concentration of the salts for sulphate (21,87 ppm), nitrates (29,37 ppm), fluoride (0,53 ppm) and phosphate (1,11 ppm).

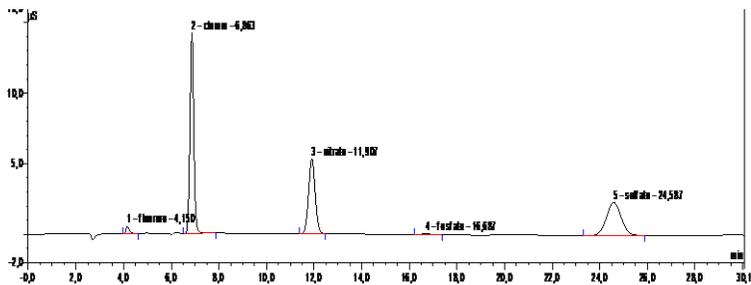


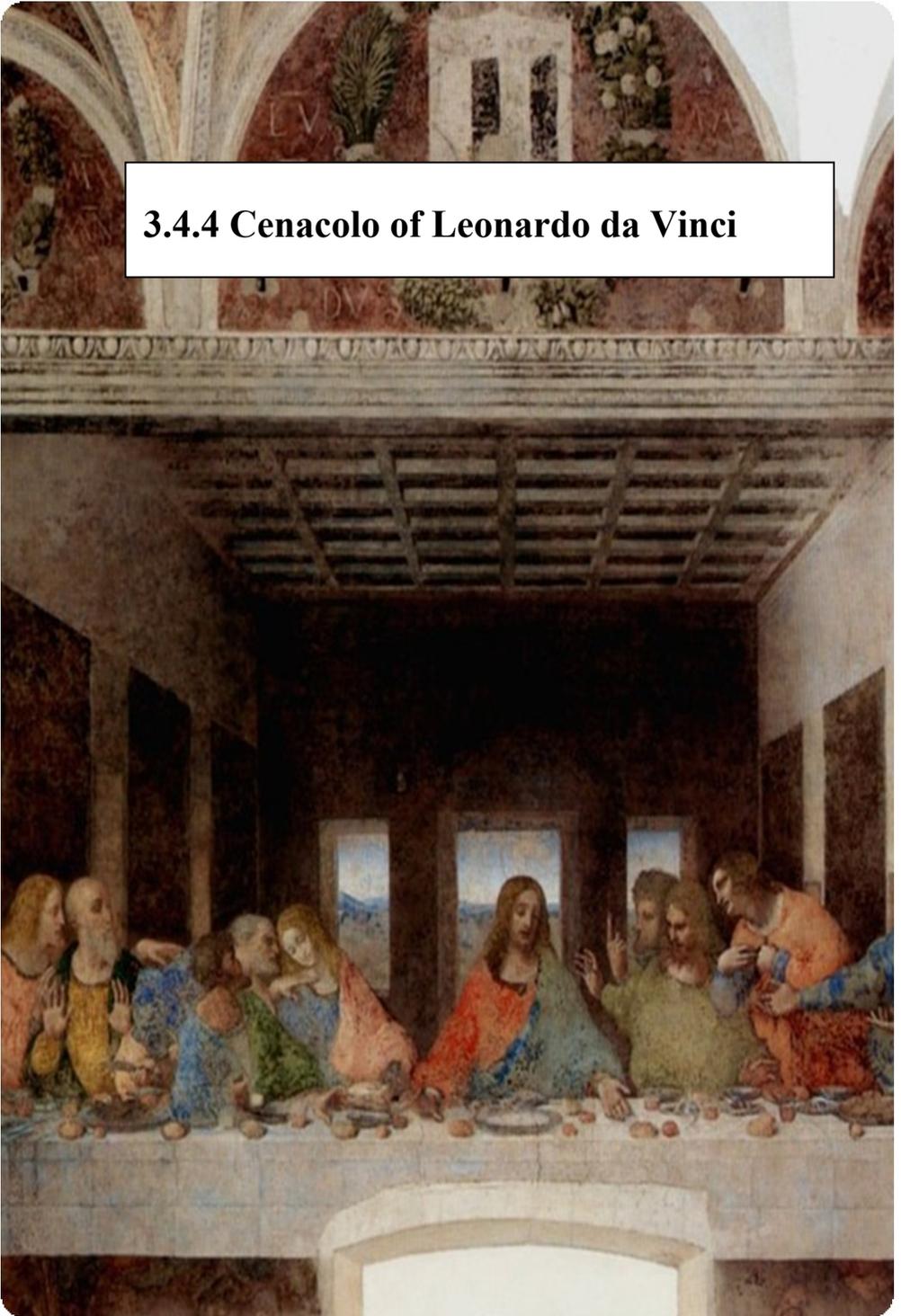
Figure 12. Ion chromatography of water in first level of S. Pietro a Corte.

In other investigations, it were analysed the chemical

composition of two rivers that delimited the centre of Salerno, Rafastia e Fusandola, to correlated the water in the church with underground drain. The river could be an important source of sulphates and chloride, via groundwater and capillary rise, and nitrate ions related to agricultural activities. It was possible the influence of others sources, such as the concentration of salt was a consequence of alluvial aquifer or sewers; moreover, the atmospheric pollution had an important rule for deposits on stone surface. However, ions composition wasn't a good parameter to distinguish the matrix correlation whit different sources. In literature was reported the possibilities to use isotopic nitrogen composition of compounds for identification of compounds.

In particular the samples of powder, extracted from waters were analysed by IRMS to obtain the $\delta^{15}\text{N}$ of salts. The results reported $\delta^{15}\text{N}$ value of +9.3 ‰ for powder salts and di +8,6‰ value for salts in waters, this was a clear correlation between salts and groundwater or sewers because the value of agricultural activities were more negative.

3.4.4 Cenacolo of Leonardo da Vinci



3.4.4 Cenacolo of Leonardo da Vinci

In the last year conservators and curators paid more attention to variability of microclimate conditions, contaminations, air pollution and influence of visitors on damage of museum. Atmospheric and gaseous pollutants could affect cultural heritage, especially the most fragile, such as the Last Supper of Leonardo da Vinci. The "Last Supper" was one of Leonardo da Vinci's most famous paintings, situated in the refectory of Santa Maria delle Grazie in Milan, Italy. This painting was majorly restored in the last century, however many projects analysed the risk of contamination from air pollution arising from its surrounding Milan area.⁵ For this reason the Leonardo's paint is protected by a filtering system and sophisticate system of ventilation, heating and humidity. In literature were reported the studies on the indoor/outdoor penetration of particulate matter in the Last Supper museum, in which were described that the sources of particulate matter (gasoline, vehicles, urban soil, and wood-smoke) obtained by source apportionment analysis contributed only for 11.2%. Although uncertainties on the mechanism of the particles dynamics and the gaseous contamination still persist. In this situation was analysed the isotope carbon composition of atmospheric carbon

dioxide in order to correlate the influence of visitors on preservation of archaeological sites. The $\delta^{13}\text{C}$ (‰) values have done a good information about the ventilation and filtration system inside museum, the active and passive sampling showed a variation of isotope composition in correlation with entrance of visitors.⁶

3.4.5 Material and Methods

The air monitoring of CO_2 was developed by passive sampler using a Ca-based solid absorbent ($\text{CaO}/\text{Ca}_{12}\text{Al}_{14}\text{O}_{33}$ 75:25 w/w) and active sampler based on ascarite sorbent, the collected carbon oxide was desorbed with acid treatment and measurement of $\delta^{13}\text{C}$ was conducted by means of NDIRS instrument. The monitoring campaigns were carried out during September, November, May and July: the passive samplers were located at 1,5 mt of height for six days, after it was analysed; while the active monitoring was conducted every 15 minutes in the same day from 7:00 am to 12:00am.

The map of samplers was reported in Figure 13: arrows suggested the way of visitors when they arrived in museum; numbers indicated passive samplers located in

different areas (entrance, cloister, and refectory where is the paintings, technical area, bookshop etc.). The external monitoring, in cloister, was done to have a reference of atmospheric value of $\delta^{13}\text{C}$ (‰).

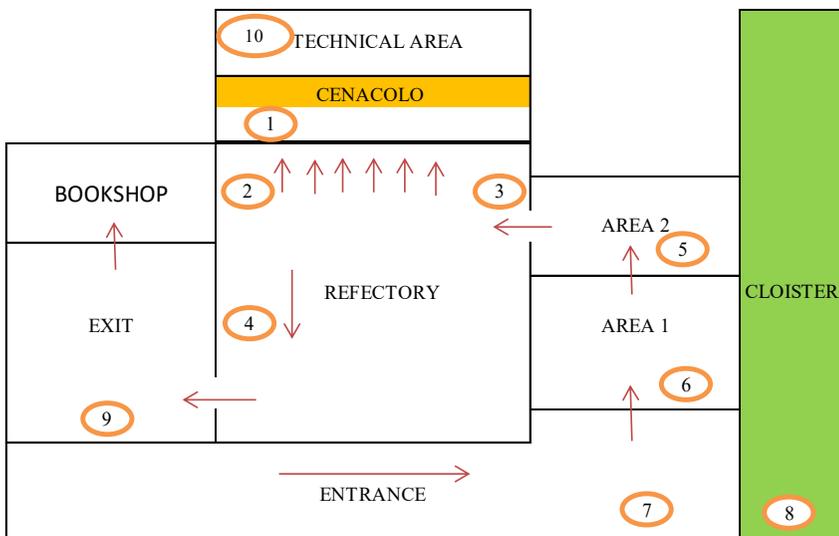


Figure 13. Map of the refectory of Santa Maria delle Grazie in Milan.

3.4.6 Results and Discussion

In the first part of project it was analysed the influence of visitors in museum of Cenacolo Vinciano on concentration of pollutants and the application of isotopic composition of carbon dioxide such as a marker of filtering system. The results of passively collecting were reported in Table 9.

Table 9. $\delta^{13}\text{C}$ (‰) value obtained from passively collecting.

Sampler	26-30 /09 $\delta^{13}\text{C}$ (‰)	09-14/11 $\delta^{13}\text{C}$ (‰)	23-29/05 $\delta^{13}\text{C}$ (‰)	17-24/07 $\delta^{13}\text{C}$ (‰)
1	-26,2	-29,7	-26,5	-23,3
2	-24,4	-32,1	-27,0	-22,0
3	-27,3	-31,2	-30,6	/
4	-25,1	-31,0	-34,7	-22,2
5	-19,7	-28,3	-33,8	-22,3
6	-22,4	-32,7	-28,8	-17,6
7	-25,5	-37,6	-30,1	-16,1
8	-26,6	-35,6	/	-22,3
9	-25,6	-21,0	/	-19,2
10	-21,9	-20,4	-20,7	-12,0

The values of $\delta^{13}\text{C}$ (‰) was influenced by numbers of visitors inside museum and by different area that it was analysed; the isotopic values of entrance (7) were more negative then area five and area six which had a filtering system of active carbon. The $\delta^{13}\text{C}$ (‰) of technical area (10) reflected the international value of human breath and did not change so much during monitoring , in fact passed from -21,9 ‰ to -20,7 ‰. The last values, concern to collecting of July, were a clear evidence of the variation

of condition in the museum and the reliability of isotopic composition: in this occasion the people of Church Santa Maria delle Grazie opened the windows of cloister, refectory, lighting candles and the isotopic values were increased looking alike values of external carbon dioxide. The same variation of isotopic carbon ratio was found out by active monitoring of carbon dioxide in the same day.

Table 10. $\delta^{13}\text{C}$ (‰) value obtained from active collecting.

$\delta^{13}\text{C}$ (‰)	$\delta^{13}\text{C}$ (‰)
-15,3	-11,3
-17,6	-18,2
-18,7	-17,7
-16,9	-18,2
-20,4	-16,3
-20,5	-11,1
-19,1	-21,0
-24,5	-11,0
-19,7	-23,5
-19,9	-16,1

At the early morning, when the museum was closed, the $\delta^{13}\text{C}$ (‰) values were -15,3 and -11,3, however if there was a group of visitors inside the isotopic value decreased

and was the same value of human breath. The active monitoring appeared a good tool to describe in real time the situation of carbon dioxide.

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Conclusions.

The important introduction of valuable alternative methods for the $\delta^{13}\text{C}$ was a consequence of new trend in analytical chemistry. The spectroscopic methods, like NDIRS, FTIR and NMR, were valuable alternatives to the traditional IRMS to determine stable carbon isotope ratio in food and environmental samples. The studies of food samples, pasta, cocoa, olive oil, have demonstrated a strong correlation between NDIRS, FTIR and IRMS methods. The results obtained by regression analysis carried out a significant correlation ($r = 0.983$) between NDIRS and IRMS techniques and a similar correlation ($r = 0.981$) between IRMS and FT- IR and for NDIRS and FT- IR ($r = 0.953$).

The advantage of spectroscopic methods was the lower cost respect to IRMS and the easy equipment required for analysis without well-trained operator. This could help the development of small centers able to analyze food authenticity, quality and typicality and to investigate falsifications and frauds.

Although the spectroscopic methods haven't provided the same sensibility of IRMS, they have given information about commodity, variety, composition and chemical nature different products, demonstrating the reliability of

techniques for isotope composition analysis of food.

In environmental sciences to understand the origin and the evolution of contaminants stable carbon isotope composition was an innovative marker; it was very important introduce methodologies able to describe all involved reactions. In this project the first application of ^{13}C NMR spectroscopy have the aim to analyse the bulk $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio, at natural abundance, in inorganic carbonates and bicarbonates with high accuracy (1.0 ‰). Measurement parameters, such as relaxation time (T_1), the presence of a relaxation agent, the pulse intervals (D_1) and the use of an internal standard were optimized to obtain quantitative analysis and to limit disadvantages such as time of analysis, absence of internal standard. The optimization of the experimental parameters has permit to reduce analysis time in the range of 1-2 h, based on the chemical difference of the compounds investigated.

The $\delta^{13}\text{C}$ values for carbonates/bicarbonates and oxalic acid obtained by ^{13}C NMR were in line with those obtained by other spectroscopic techniques (IRMS and NDIRS).

^{13}C NMR methodology can be used as a valuable alternative to IRMS and NDIRS for $\delta^{13}\text{C}$ analysis of

carbonate and bicarbonate matrices, extending the choice of techniques applicable for the determination of carbon isotopic composition.

The aim of NMR methodology improvement was correlated to the analysis of relevant sample for environmental studies and cultural heritage. In other studies was reported the monitoring of atmospheric pollutants as marker for analyse the influence of visitors on preservation of archaeological sites. The air monitoring of Fruscione Palace, San Pietro a Corte was developed using passive samplers and the pollutants monitored were NH_3 , NO_2 , BTEX, H_2S , SO_2 .

Effects of degradation were visible after several years, when restoration of monuments was already necessary and difficult; for this reason a good preservation of museum cannot leave out the control of external environment in which they are exposed. Historically, it was studied the influence of environmental components – humidity and temperature – on the preservation of Fine art works (monuments, paintings, manuscripts) and only in last year researches were focused on degradation linked to chemical interactions with gases and aerosols present in the urban environment. The importance of this study was to underline the presence of pollutants and not

only the concentration: because if there were pollutants, their effect was slower but long-lasting. In fact the monitoring reported a variation of ammonia concentration from 0,22 to 0,58 $\mu\text{g}/\text{m}^3$ and toluene concentration from 2,08 to 12,11 $\mu\text{g}/\text{m}^3$ linked to an increasing of traffic and visitors. At the same way the isotope carbon composition value increased during autumn and winter season with increased of pollution, traffic and visitors. The presence of salts in the upper part of the walls testifying high amount of water in the entire structure and it could be associated with the presence of drainage channels. The chemical identification of pollutants was essential to reduce the damage on architectural structures and archaeological heritage preserved in them, with a correct procedure of restoration such as limitation of entrance or use of filtration system.

The "Last Supper" was one of the most famous paintings, situated in the refectory of Santa Maria delle Grazie in Milan, Italy. This painting was restored in the last century and many studies have analysed the risk of contamination from atmospheric and gaseous pollutants in urban environment. In fact the Leonardo's paint is protected by a filtering system and system of ventilation, heating and humidity.

The values of $\delta^{13}\text{C}$ of carbon dioxide storage in refectory of museum showed a strong influence by numbers of visitors inside museum and by different location. The isotopic values of entrance were more negative than areas with filtering system based on active carbon, or the $\delta^{13}\text{C}$ (‰) of technical area reflected the international value of human breath $\delta^{13}\text{C}$ (‰) -23.0‰ and didn't change during monitoring. The air monitoring was developed using passive and active samplers and the same variation of isotopic carbon ratio was found out by active monitoring of carbon dioxide in the same day. The stable carbon isotope ratio have supported information about the ventilation and filtration system inside museum thanks to variation of isotope composition in correlation with entrance of visitors.

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