Workshop proceedings

Electron microscopy and imaging techniques in food science

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THE SCANNING ELECTRON MICROSCOPE IN FOOD INDUSTRY: APPLICATIONS AND EXAMPLES

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Scanning Electron Microscope (SEM) usage has strongly increased over the past decades, dealing with a number of interest fields that has constantly grown, from semiconductors, to Metallurgy, to Chemistry, to Life Science, just to cite a few.

The technological improvement, together with the development of user friendly interface with the user, made the SEM much more appealing even to scientist who were not familiar with this technique, extending their research capability to boundaries that were not in their focus just a few years ago.

Here, the great step ahead with respect to the classical microscopy, lies on being able to go much further than the optical microscope resolution limit (i.e.: a few hundreds of nm) down to even less than one nm.

In Life Science the complication arises from the nature of the sample, that usually contains water, it is not stable under vacuum, is very often non electrically-conductive and, therefore, not easily suitable for a SEM analysis where vacuum is needed as well as a conductive specimen would be the desirable condition.

Instead of making use of a light beam as a probe medium as it happens in the optical microscope, the SEM uses an electron beam as a probe, that is regularly scanned by a computer onto the specimen surface to be analysed, the beam being coupled to a detector and the computer itself that reproduces the specimen topography.

In order to have enough mean free path for the electrons, from the electron source down to the specimen vacuum must be produced, to prevent air from stopping or just scattering the electron beam off the electron path axis. This is why vacuum is needed in this microscope setup.

There are different ways to manage the food samples for SEM analysis: from freezing the sample down to low temperatures, to prevent itself from degaussing (i.e.: changing its morphology and structure) when in vacuum, to coating it by conductive and impermeable films that prevent water from being dispersed into vacuum, to dehydrate the sample if loss of water does not imply a change in the imaging expectations, and so on.

This presentation deals with an overview about sample preparation for Food Science related samples that are then supposed to be measured by SEM, a discussion on the challenges and opportunity that an SEM offers in the field of analysis of the Food Science, and a session dedicated to the application and examples of what can be done by using an SEM in food applications.

It is discussed the sample issues for a good SEM analysis, how to properly manage the sample itself, the usage of the instrument and the results, with a special focus toward the ones who are not expert in SEM practise, though they are used to analyse Food related samples for their Research areas.

OPTICAL IMAGING IN FOOD ASSESSMENT: TECHNIQUES AND INSTRUMENTS

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The visual perception is one of the most ancient and useful approach for food assessment. Nowadays the extension of the visual perception is represented by the optical imaging which is based on the detection of ultraviolet and near infrared photons beyond visible photons. This extension is possible thanks to the development of new instruments, light sources (lasers and LEDs) and very sensitive detectors (photomultipliers and charge coupled devices – CCDs). Here the attention is focused on the detection of light with optical imagers and confocal microscopes.

Generally, the light processes detected with these instruments are: fluorescence, phosphorescence and bioluminescence. When a beam of light passes through the matter the photons can excite a molecule to a higher vibrational energy level. The excited molecule rapidly losses part of the energy as heat and relaxes to the previous state with the emission of a photon with lower energy. The process, called fluorescence, is almost immediate (10^{-8} s). Sometimes the excited molecule can stay in an intermediate energy level and returns at the ground level at significantly slower time scales. The emission, called phosphorescence, is thus retarded with respect the excitation. The third process is the bioluminescence which is a form of chemiluminescence which happens in biological systems.

The optical imagers are composed by a dark chamber to prevent the contamination of light from outside and a very sensitive CCD camera with very low noise level. They are also equipped with a light lamp and many excitation and emission filters which are used in fluorescence modality. The autofluorescence of cells and tissues can be used as endogenous fluorescent markers, instead commercially available fluorescent molecules as exogenous markers. They can be selected among a great variety and with the required optical properties. The contemporary use of two or three markers are also very interesting. In case of bioluminescence modality, the lamp is off and no filters are used to detect the light. The optical imaging technique is cheap, repeatable, allowing very simple samples preparation and it offers the advantages of fast acquisitions (1 sec to 5 min, depending of the modalities).

The confocal microscopes are an improvement of the classic optical microscopes designed for increasing optical resolution and contrast by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. They permit high resolution in thin section using autofluorescence, exogenous fluorescence and allowing the 3D reconstruction of the foods' properties and structures.[1] The structure of a food affects how it breaks down in our mouths and our perception of its flavor but also the release and bioavailability of minerals, vitamins and polyphenols in our digestive system. Thus food structures are increasingly being recognized as important in technology innovation for the development of healthier foods.[2]

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OPTICAL PROPERTIES OF FOODS: POTENTIALITIES AND PROSPECTS

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Food analysis is the discipline dealing with the development, application and study of analytical procedures for characterizing the properties of foods and their constituents. Many investigative procedures are used to provide information about a wide variety of different characteristics of foods, including their composition, structure, physicochemical properties and sensory attributes.

Optical properties of food are a good instrument to evaluate many parameters as presence of bacteria, product quality, freshness or state of preservation or conservation type.

Normally in the food and raw materials industry methods of quality and sterility control are used (which detect the presence of bacteria, fungi or yeasts) through luminometric quantitative methods that relate the presence of living organisms with the emission of photons. But in this case optical properties of luciferase - luciferine system are used to measure presence and quantity of ATP.

Intrinsic optical properties of food could be assessed with no difficulty in case of vegetable foods. The fluorescence emission of chlorophyll (680 and 700 nm) is measurable in a non invasive and relative easy way and it is indicative of the changes in the morphology, anatomy and physiology of the plant itself. Chlorophyll fluorescence could be used as an early stress indicator. In particular, the fluorescence could provide details on the ability of a plant to tolerate environmental stresses and their capacity to damage the structure of the plant. So it is possible to detect cultivation mode, mode and timing of harvest and post-harvest preservation methods.

Also, food of animal origin contains molecules, as peptides or proteins or others components, that have intrinsic optical properties of photon emission. All these components could be affected by how they are to be processed, handled, stored, and conserved. In this case the cause of photon emission is unknown so only with the use of a multimodal approach could it be possible to characterize the structure and identity of the source of this emission.

The measurement of the photon emission could be also useful to highlight the presence of food additives or ingredients if they have, as it is often the case, intrinsic optical characteristics of photons emission with fluorescence or phosphorescence modality. Besides thanks to basic research studies it could be also monitored the presence of these components in living organism, their biodistribution and tissue accumulation characteristics through experimental model where they are viewable.

Food safety and quality control could be investigate through optical properties of the food but also the study of optical properties of food is a non invasive way to monitor the impact on human health of the use of additive, preservative and food processing mode.

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POTENTIAL OF MAGNETIC RESONANCE IMAGING IN FOOD SCIENCE

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Magnetic Resonance Imaging (MRI) is a powerful diagnostic modality providing high resolution, non-invasive and multi-parametric images of the human body. Dedicated equipment, mainly developed for imaging of small laboratory animals, can reach the limit of MRI microscopy that generally is assumed to be 100 microns.[1] NMR, and particularly its microscopy version, also known as MRI, has been applied to investigate different kind of foods: fruit, vegetables, meat, dairy products, cereals.[2] The non-invasiveness of MRI acquisitions, which is one of the most relevant features of diagnostic MRI, is advantageous also in food science since it allows dynamic studies to be performed during the preparation process of food or during fruit ripening. For example, MRI has been applied in dynamic studies to investigate de-hydration, cheese brining, fish salting, thermal processing of cereals.[2] Moreover, it is worthwhile to mention the studies performed to unveil the geographical origin of food growth,[3] or to measure fat content in ham.[2] Interestingly, most of MRI modalities used in the clinics can be translated in food science. For example T2-weighted images can provide high resolution images of vegetables with superb morphological detail and clear discrimination of regions containing free water. Localized spectroscopy is used in humans to detect and quantify lipids or metabolites and can be applied in food science as well to quantify lipids or sugar concentration. Diffusion tensor imaging (DTI) allows to detect axonal fiber in the brain, but can also be used in several fiber-rich vegetables as celery[4] or fennel to detect the organization of vegetal fiber. Finally dynamic contrast enhanced MRI (DCE-MRI), used in humans to study in vivo tissue perfusion, has been recently used to study fluid transportation in tomatoes.[5]

In conclusion, MRI at microscopic resolution has a great potential in food science that has not been fully exploited at the present, mainly due to the high costs of the equipment and the requirement of specialized personnel.

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MEAT COMPOSITION, FROM MICROSCOPY TO CHEMISTRY BY WAY OF DIAGNOSTIC IMAGING

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The increasing demand of a real-time monitoring of food products has encouraged the application of non-invasive techniques. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) proved to be very accurate and valuable tools in estimating body and carcass composition in farm animals.[1] CT has been successfully used for the characterization of food Italian products such as salami, providing a precise evaluation of fat percentage, also assessing its spatial distribution.[2] Manzocco and colleagues demonstrated that MRI has great potential in monitoring the evolution of dry curing in S. Daniele hams.[3]

In our experience, helical CT proved to be a fast tool in the classification of different meat cuts, deriving from adult cow and destined for the preparation of aircured products as "lean meat" or "fat meat", both in fresh and frozen samples.

Histological studies confirmed that CT clearly distinguishes adipose and connective tissue infiltration within muscles and that semi-quantitative analysis of infiltration degree can be achieved. These data were further supported by the chemical analysis of meat samples corresponding to the same region of interest observed in both CT and histologic investigations; dry matter, crude proteins, crude fat and ash contents, calculated following standard international methods,[4] varied in fact depending on the fat infiltrated extent, according with CT images. We finally observed that CT could be used in the evaluation of the same products at the end of ripening, without removing the outer envelope.

These results are important for beef and meat industrial processing sector, suggesting that CT could be employed as an on-line instrument in abattoir and dry-cured meat industry in classifying the products at the beginning of the manufacturing even they are frozen. Moreover, it might represent a rapid and non-invasive technique for quality check at the end of the production line and for the assignment of the most appropriate nutritional and commercial value to different products.

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MRI (AND NOT ONLY) IN FOOD SCIENCE

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Food is any substance consumed to provide nutritional support for the body. It is usually of plant or animal origin, and contains essential nutrients, such as carbohydrates, fats, proteins, vitamins, or minerals. The whole food industry covers several areas from farming and food production, packaging and distribution, to retail and catering including subareas like regulation, education, R&D, manufacturing, agriculture, processing, marketing, wholesale and distribution, and for Europe it represents the second largest industry (after metal industry) with Euro 917 billions 310.000 companies and 4.8 millions employees, equal to 14% of the total manufacturing sector (EU-27 - 2013 http://ec.europa.eu/enterprise/sectors/food/eu-market/index_en.htm).

With a wide range of products, Bruker (www.bruker.com) offers solutions in most of these fields. In particular Bruker offers a wide portfolio of imaging techniques like magnetic resonance imaging (MRI), high resolution microtomography (microCT), PET, SPECT, CT, Optical fluorescence and bioluminescence, Magnetic Particle Imaging (MPI).

These techniques are routinely used in the fields of food quality and safety, food technology, raising, seafood, preparation of fresh products, manufacture of prepared food products and warehousing.

MRI has the advantage of being a non-destructive, non-invasive, molecule specific (fat, water, alcohol, ...), nucleus specific (^{1}H, ^{31}P, ^{13}C, ^{23}Na, ^{19}F, etc.) technique mainly based on the detection water (or generally speaking 1H) content in tissues/materials, under several contrast parameters as density, mobility, relaxation, diffusion and flow with a typical image resolution down to tens of microns.

It allows 2D/3D spatial localization, resolution of the information in the temporal dimension even with dynamic and real time capabilities. As a non-destructive technique it's very useful in longitudinal studies. A non-exhaustive list of examples of MRI applications in the food industry are the cooking process of pasta, single onion cell imaging, leaves structure, melon infection studies, plants roots growing, water uptake followed by drying of a barley corn, bread dough leavening and yoghurt homogeneity determination.

A good alternative to MRI is represented by micro-tomography (microCT). It allows a complete quantitative 3D volume insight of any food during its preparation as well as in the packaging with a resolution down to a nanometer. Examples of uCT application in the food industry are the distribution of coffee powder in coffee chowders, potato chips distribution of air/oil bubbles, maple seeds fine structure, determination of fat content in fish food, degradation of apples, studies on ice cream homogeneity.

In conclusion imaging techniques represent an innovative tool for the food industry and bridge the gap between macroscopic observation and microscopy techniques.

TECHNIQUES FOR THE ULTRASTRUCTURAL ANALYSIS OF FOOD

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Ultrastructural techniques are powerful tools by which a wide variety of data can be obtained on the structure and composition of food. In particular, transmission electron microscopy allows to gain evidence on the preservation state of food, to investigate the processing-induced changes, and to distinguish food typicalities, while scanning electron microscopy provides detailed information on the outer and cutting surfaces of food products. X-ray microanalysis at electron microscopy can be used to detect the occurrence of specific chemical elements which can be either naturally present or may contaminate food during processing or packaging. These techniques are often used to examine meat or fruit, but they could also be applied to more specialized investigations such as the analysis of microbial components with probiotic activity or of nanoparticulates. Ultrastructural techniques should therefore integrate the conventional histological analyses and be extensively applied, in future, for food characterization and safety control, thus being a helpful support for the protection of public health.

IMAGING TECHNIQUES APPLIED TO THE STUDY OF GRAPEVINE BERRY RIPENING AND POSTHARVEST DEHYDRATION

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Grapevine berry development is characterized by many metabolic and structural changes that determine the final quality of the fruit at harvest. These include the pericarp tissue softening, the flesh cell volume increase due to water and sugar import, and the accumulation of anthocyanis and other flavonoids in the epidermal and hypodermal cells of the skin. For particular enological purposes (i.e. the production of wines like Recioto and Amarone), in the Verona area grapes are placed in ventilated rooms, for a period of about three months after harvest. During this time berries undergo several physical and biochemical changes in part due to a slow but constant water loss. We performed analyses using several different imaging and microscopy techniques to explore their potential application in revealing structural and composition features of grape berries through development and during the postharvest dehydration phases. The preliminary results showed that NMR is a powerful non-destructive technique to study the structural changes occurring in berry tissues along development. On the other hand, SEM microscopy was particularly helpful in revealing differences in the outermost cell layers of berry skin in different grapevine varieties. Finally, using optical imaging spectroscopy we were able to select combination of excitation/emission wavelengths able to discriminate whole grape berries (i) at different dehydration levels, (ii) subjected to different dehydration condition and (iii) of different varieties. Overall these preliminary data indicate that the microscopy and imaging approaches used in this study represent promising tools for the detailed description of the changes that characterize grape berries during the on-vine growth and ripening stages and the off-vine dehydration process.

DISCOVERY THE MICROORGANISMS OF FOODS AND BEVERAGES BY ELECTRON MICROSCOPY

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The study of microorganisms has gone hand in hand with microscopy since Antoni van Leeuwenhoek observed and described single-celled organisms in 1684 using handcrafted simple microscopes. However, the ability to resolve structures using light microscopes is though limited to around 200 nm. With the development of electron microscopy (EM) techniques, the resolution limits of light optical microscopy was breached, and a dramatic enhancement of the level of detail in cell morphology has been achieved, as well as the possibility to reveal the cellular ultrastructure.

Since the 1950s, EM technologies have been used in microbiological research and remain essential tools for the users to explore the microbial world and provide novel insights into a wide variety of aspects of modern microbiology.

This presentation demonstrated some application of EM related to the microorganisms associated with foods and beverages, with special attention to the pro-technological and the probiotic organisms.

Selected scanning EM (SEM) micrographs showing pure cultures of single bacteria, yeasts and moulds were presented to visualize the different shapes, sizes and arrangements of cells. This basic information on morphological features is fundamental in the taxonomic characterization of new culturable food-borne isolates. In addition, the description of novel genera and species should include morphology to ensure a rich polyphasic characterization. As an example, in the proposal of *Zygosaccharomyces gambellarensis*, a novel yeast isolated from an Italian “passito” style wine, we have reported a detailed description of the cell shape and morphology as observed in photomicrographs.

SEM also plays a paramount role for assessing the three-dimensional structure of microbial populations present in complex food samples, and can unveil food-microbe and microbe-microbe interactions thought to be crucial for obtaining the desired product quality. Examples of such observations were reported: SEM images of yogurt and cheese revealed the presence of micro-colonies of bacilli and cocci in micro-holes of the matrices. A carpet of yeasts and bacteria colonized the surface of Kefir grains, and fibrillar material was also observed among cells. During the first months of ripening, the San Daniele ham was covered by an agglomerate of moulds, yeasts and bacteria, that are important for a proper drying. Development of *Botrytis cinerea* in its noble form in grapevine berries during withering can be followed by EM examination and linked to peculiar sensory traits of the obtained wine.

Finally, in this post-genomic era, the identification of novel genome features coding for specific morphological characters, such as presence of pilus gene clusters in a probiotic bacterium, needs validation through appropriate EM observations. Thus the close link between microbiology and microscopy is continuing to increase our understanding of the beneficial effects of microbes in food.