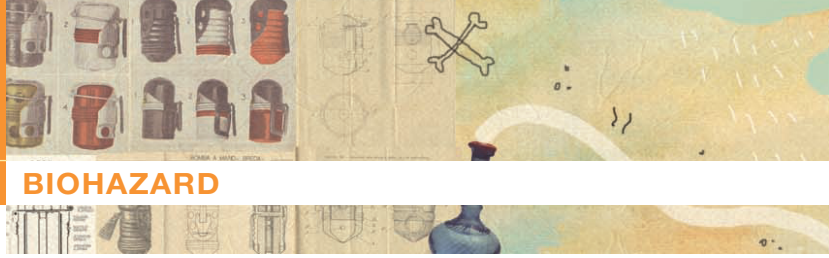

Dispositivi innovativi per agenti biologici e contaminanti alimentari

Il rilevamento di agenti biologici pericolosi necessita di strumenti che ne consentano un'identificazione tempestiva e accurata. In tale contesto, la spettroscopia Raman/SERS e la fotoacustica rappresentano strumenti rapidi e sensibili di monitoraggio e identificazione sia di agenti patogeni nell'ambiente e nel cibo, sia di sofisticazioni alimentari. Nel quadro della sicurezza ambientale e alimentare, si descrivono due nuovi sensori messi a punto per il rilevamento di agenti chimici e biologici nocivi per la salute.

Innovative devices for biohazards and food contaminants

The detection of dangerous biological agents requires tools that allow their early and accurate identification. In this context Raman/SERS and photoacoustics techniques represent fast and sensitive tools for monitoring and identifying both pathogens in the environment and food, and sophistication in food.
In the framework of environmental and food security, two new sensors are described, developed for the detection of biological and chemical agents dangerous for health.

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Introduction

Despite the long history of nations and people using bacteria agents as weapons [1, 2], and the use of Salmonella in 1984 for poisoning food in salad bars in Oregon, it is only in the last decade that biological weapons have received attention to a greater extent [3]. In fact in 2001 intentional release of endospores of *Bacillus anthracis* caused 19 infections, 5 deaths and 10 000 prescriptions of antibiosis (Center for Disease Control and Prevention 2002).
The endospore is a quiescent, rough, and non-reproductive structure (endurance forms) produced by bacteria in response of adverse environment conditions as well as lack of nutrients [4]. Thanks to their resistance to heat, radiation and desiccation, [5] the endospores can survive for many years, as long as the environmental conditions return good for germination and vegetative cells development [6, 7]. The genus Bacillus includes species endospore–forming as *B. anthracis*, that are important for health because of their capability to produce exo-toxins, the virulence factors, after germination. The characteristics of survival and resistance make endospores the ideal delivery vehicles for their distribution into the environment. The range of lethal dose of *B. anthracis* is from 500 to 55,000 inhaled spores [8], and the antibiosis treatments must begin within a day or two of inhalation [9].
The borderline between bioterroristic materials and contaminants, additives or better adulterants in goods and food is very narrow to rise the alarm also to everyday market consumers (e.g., botulinum toxin in canned foods). In fact, many examples are present in the market distribution chain as some specific adulterating substances like melamine, methanol, aspartame and ammonia, just to report the

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most famous. The adulterants to be detected by new sensors have been selected due to their presence in very common goods present daily on our tables, such as extra virgin oil, methanol, powder milk, fresh fish. In this respect, in EU countries regulations on production, manipulation, and distribution stages are very severe, especially in Italy [10].

In this context accurate, fast, and relatively simple methods of analysis to be implemented in early detection of suspicious materials (e.g., biohazards or contaminants) is essential to ward off disease outbreak and dispersion in the environment.

The Diagnostic and Metrology (DIM) Laboratory has contributed to the development of new monitoring tools with its background in spectroscopy field, in both EU and national projects.

Raman spectroscopy, and in particular the Surface Enhanced Raman Spectroscopy (SERS) technique, has recently attracted attention on homeland security for the capability in the identification and detection of microbes and bioagents (small particle detection). In this context, the DIM Laboratory is partner of the project "Rapid-Air Monitoring particle against Biological threats" (RAMBO) in the framework of the European Defense Agency (EDA) Joint Investment Program on Chemical, Biological, Radiological and Nuclear protection (CBRN protection). Main aim of this project is to develop an advanced combination of two sensors, a Raman technique devoted to the first alarm and the Polymerase Chain Reaction (PCR) to confirm the suspicious biological threats. It is expected to have high performances (towards the one spore limit), high selectivity, rapid response (<45 minutes), portability.

Conversely, the Laser PhotoAcoustic Spectroscopy (LPAS) has been implemented in the frame of the National Project SAL@CQO with the main aim of developing and applying innovative, easy-to-use product-instruments to be used by stakeholders or regulatory authorities in production chains, where food processing requires the continuous monitoring of quality and preservation of food included in the process.

Both Raman and LPAS offer the advantages to analyze samples without preparation, in real time, on line, and also in the remote mode.

Materials and methods

Biohazard measurement have been performed with

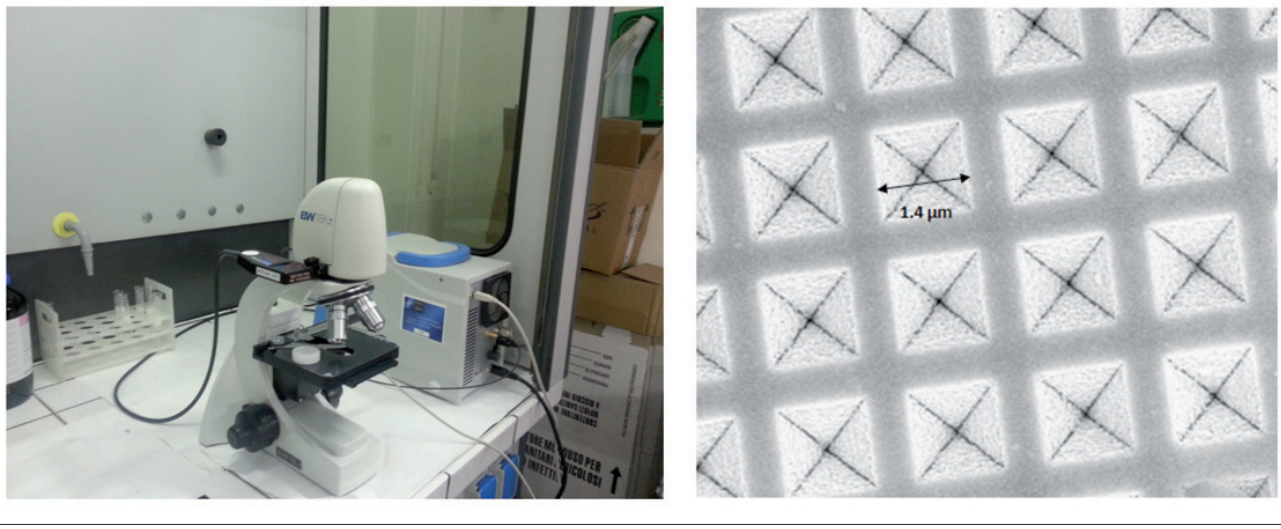


FIGURE 1 Micro Raman instrument (on the left) and SERS substrate (on the right) (image obtained with Scanning Electron Microscope (SEM) Leo1525, EHT=20 kW, WD=3 mm, aperture size=20 µm)

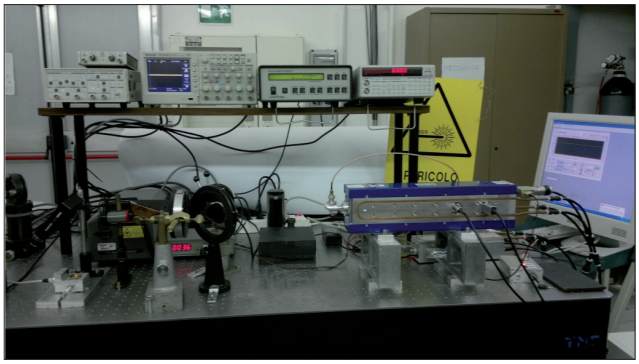


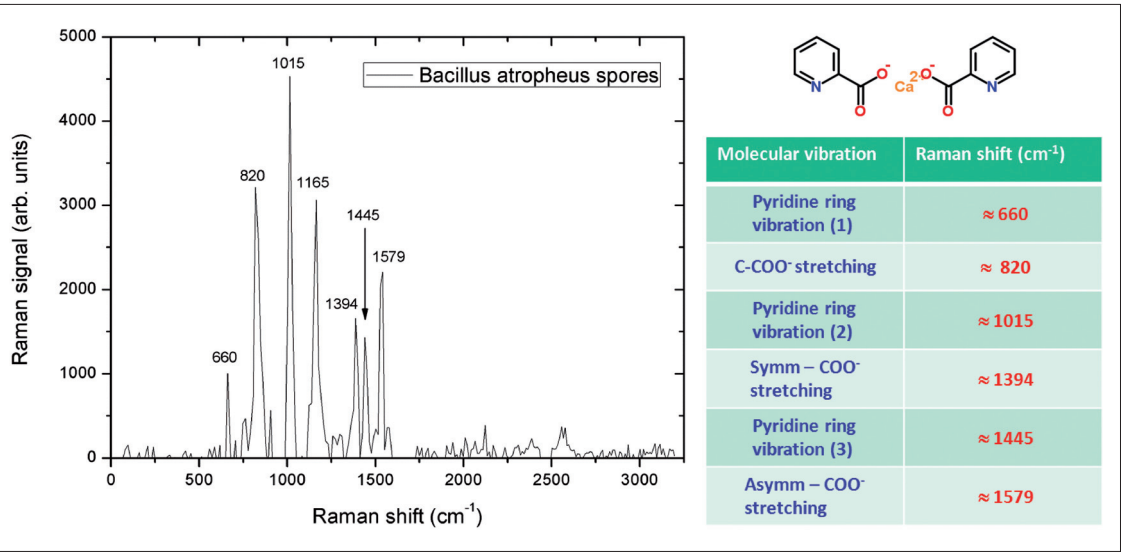
FIGURE 2 The LPAS apparatus with the control electronics in the background

the Surface Enhanced Raman Spectroscopy technique (SERS technique), and in particular with i-Raman BWTek system (http://bwtek.com/technology/raman-systems/), characterized by laser source @785 nm, spectral resolution of 3 cm^{-1}, range from 0 to 4000 cm^{-1} and CCD array of 2048 pixel (Fig. 1, left). The spectra were obtained with an acquisition time of 10 s. The lenses utilized are: 4x, 10x, 20x, 40x, 80x, 100x. SERS substrate (Klarite®, Renishaw Diagnostics inc.) are composed of regular arrays of inverted pyramidal pits, realized by depositing an Au layer (Fig. 1, right) with an active area of 4 mm x 4 mm.

In order to increase the selective capture of the bacteria vegetative cells, the appropriate bacteriophages were immobilized on an active SERS substrate (functionalization). The bacteriophages are a type of viruses that by means of their receptors, which are highly selective and reactive towards specific bacteria, infect them. The functionalization of commercially available SERS substrates has been successfully accomplished with a fairly good and reliable fill factor.

Given that *B. anthracis* is classified as *Risk Group 3 (very high dangerous microorganism)*, *Bacillus thuringiensis* (ATCC 10792) and *Bacillus atrophaeus* var. *globigii* (ATCC 9372) were used as a simulant. This bacteria are generally used as models due to their phylogenetical similarity with the most hazardous species [11]. Cell and spore concentrations were carefully adjusted in the range between 10^6 and 10^4 CFU/ml, respectively, while 10 µl of solution was dropped on the SERS sensor active area.

The LPAS set-up adopts a compact (40x11x7 cm) CW CO_2 laser source emitting in the mid IR 9 – 10 µm wavelength range, operated without water cooling and frequency stabilized by a dedicated software inserted in a feedback loop. The laser is grating



Molecular vibration	Raman shift (cm^{-1})
Pyridine ring vibration (1)	≈ 660
C-COO^- stretching	≈ 820
Pyridine ring vibration (2)	≈ 1015
Symm – COO^- stretching	≈ 1394
Pyridine ring vibration (3)	≈ 1445
Asymm – COO^- stretching	≈ 1579

FIGURE 3 SERS spectrum of *Bacillus atrophaeus* spores and band assignments

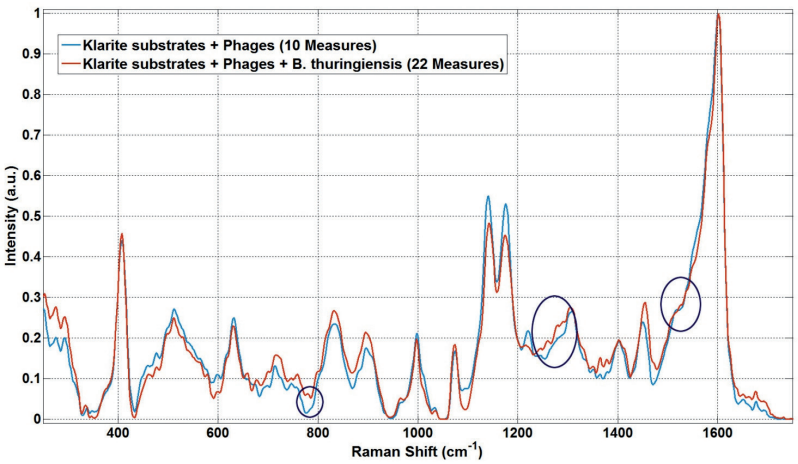


FIGURE 4 SERS spectra of vegetative cells of Bacillus thuringiensis (BT) on functionalized Klarite substrate

tunable through a single screw micrometer connected to a precise stepping motor, aimed at obtaining a computerized remote control (Fig. 2).

The LPAS apparatus has been implemented in discovering the possible methanol and melamine (2,4,6-triamino-1,3,5-triazine) contamination of food. The detection protocol is characterized by the absence of any requirement for chemical reagent purchase/disposal or sample pre-treatment and ease-of-use after initial method development. Spectra of methanol and ethanol alcohols were performed by the LPAS facility, arranged for the analysis of aqueous alcoholic mixtures directly in the photo-acoustic (PA) cell. Mixtures were analyzed at low alcohol concentration, down to 10 ppmv in water.

For monitoring milk adulteration with melamine, a home-made PA cell designed for analysis of solid mixtures was introduced in the LPAS apparatus.

Results

The detection of bacteria and spores, eventually dispersed in aerosol as biological weapons, can be obtained with a Raman spectrum of *B. atrophaeus* spores and *B. thuringiensis* vegetative cells (Fig. 3 and Fig. 4, respectively). The major constituent of the endospores is the dipicolinic acid (2,6-pyridinedicarboxylic acid; CaDPA) [5] is essential to the heat resistance, that for B anthracis spores represents 10 to 15% weight [12, 13]. A range of spectral features has been observed (660, 820, 1015, 1165, 1394, 1445 and 1549 cm^{-1}) mainly due to CaDPA, according to literature [13, 14] (Fig. 3, right).

The SERS spectra obtained to the Klarite® substrate functionalized with phages and inoculated with B. thuringiensis compared with Klarite® substrate functionalized without *B. thuringiensis* are showed in Figure 4.

Even if the spectra are very similar and, except for intensity, the phages signal is very strong and overlays that of the bacteria, slight differences are emerging around 1500 (very weak) 1300 and 800 cm^{-1} (Fig. 4, circle).

In order to highlight the spatial organization (e.g, a possible overlap between the attached molecules) of spores and cells, SEM investigation was performed on the same target material.

Figure 5 shows the SEM (LEO Gemini 1525 FEGSEM) images of spores of *B. atrophaeus* after deposition on the SERS sensor, while in Figure 6 the chain of

vegetative cells for B. thuringiensis dispersed in physiological solution is shown.
The left image in Figure 5 highlights the organization of spores in clusters of some tenths or more on the SERS sensor, even if some individual, scattered spores are also visible (Fig. 5, right). That condition is the best to carry out measurements.
The vegetative cells of *B. thuringiensis*, suspended in a physiological solution and organized in chain, are showed in Figure 6. The images a) and b) were acquired with Optical Microscope (NIKON Eclipse E400) at magnification 100x, while the images in c) and d) were obtained with LEO Gemini 1525 FEGSEM. The dimensions of the cells are compatible with B. thuringiensis size and shape. The arrow indicates a possible endospore formation.
In the case of contaminants, additives or adulterants in goods and food, LPAS spectra have been performed in the framework of the SAL@CQO project, for pure ethanol and methanol, and presented in Figure 7, respectively. Comparing the two spectral profiles, significant wavelength-dependent differences are observable in terms of relative absorption intensity, as expected.
Several spectral studies were performed for ethanol/methanol mixtures in water as well, by mixing different relative amounts of the two alcohols [15]. The differences existing among the recorded spectra are stressed by the Principal Component Analysis (PCA) method operated on the experimental records and shown in Figure 8, as a 2D view. From this analysis, it comes out that the aqueous solutions of pure or diluted alcohols cover non-overlapping areas on the first two Principal Components chart. This result is promising, and a second step experimentation is planned to take into account the role of interfering substances and to construct calibration curves. In particular, the calibration will be based on the LPAS analysis applied to a set of alcoholic mixtures, selected to cover a concentration range finalized to a practical use. In a further step, experiments will be performed to validate the method. In the last step, finalized to the exploitation of the results, this proof of concept will be applied to the real-time detection of methanol level in commercial beverage products.
Food adulterants, such as melamine powder, were monitored by LPAS, being a material commonly used as a fertilizer and in the production of laminates, plastics, and glues. In recent years, a fraudulent adulteration with melamine has been reported in milk products and pet food. In 2007, adulteration of pet food by melamine determined the illness and death of the animals that consumed the contaminated product [16, 17]. Two years later, 300,000 cases of

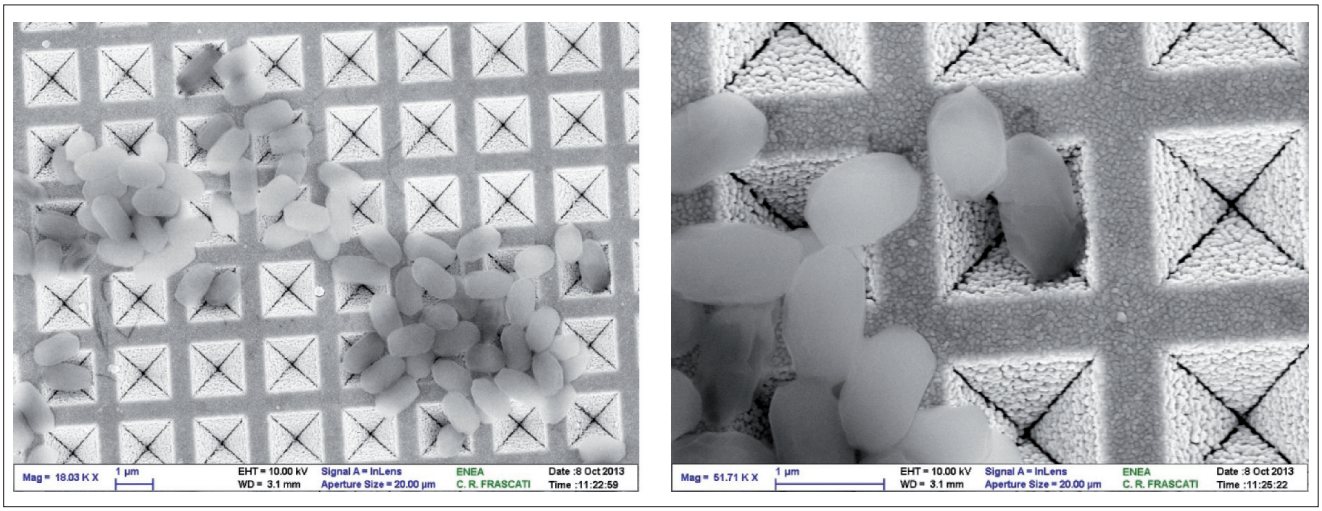


FIGURE 5 *Bacillus atrophaeus* spores SEM image (EHT=10 kW, WD=3.1 mm, aperture size=20 µm)

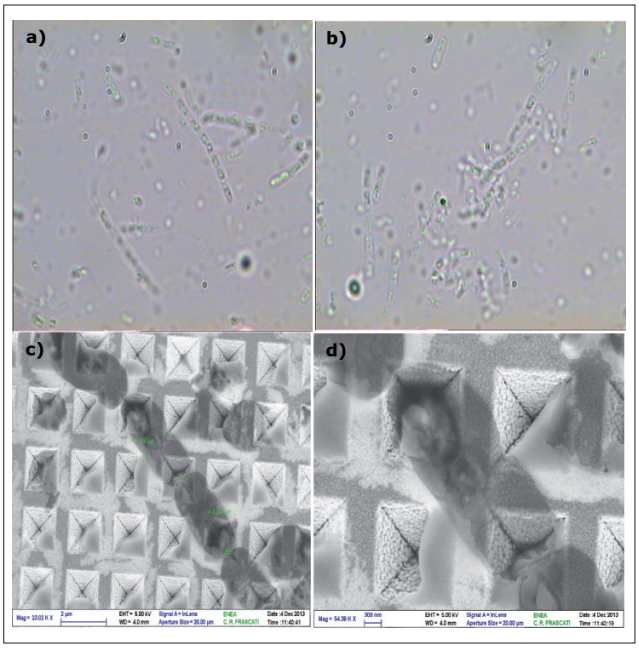


FIGURE 6 *Bacillus thuringiensis* cells organized in chain: a) and b): Optical Microscope images (NIKON Eclipse E400, magnification 100x); c) and d): SEM images LEO Gemini 1525 FEGSEM, EHT=5 kW, WD=4 mm, aperture size=20 µm

renal complications in children and at least 6 child deaths were ascertained in China, directly caused by milk adulterated with melamine. A reason for the adulteration of a food product with melamine is that its high nitrogen content increases the apparent protein content measured by standard analysis tests. This adulteration is difficult to reveal since standard chemical tests measure the total nitrogen content as an indication of the protein levels. The U.S. Food and Drug Administration (FDA) stated that a level of 1 ppm was a safe threshold for melamine in milk infant formula.

The LPAS spectra of pure milk and melamine mixtures are quite similar, but differences can be quantified in the statistical analysis presented in Figure 9. In this multivariate analysis it is evident that milk and melamine, as well as their mixtures, are clearly distinguishable from each other.

Conclusions

The easy-to-use sensors developed for early detection of biohazard in food and aerosols in the framework

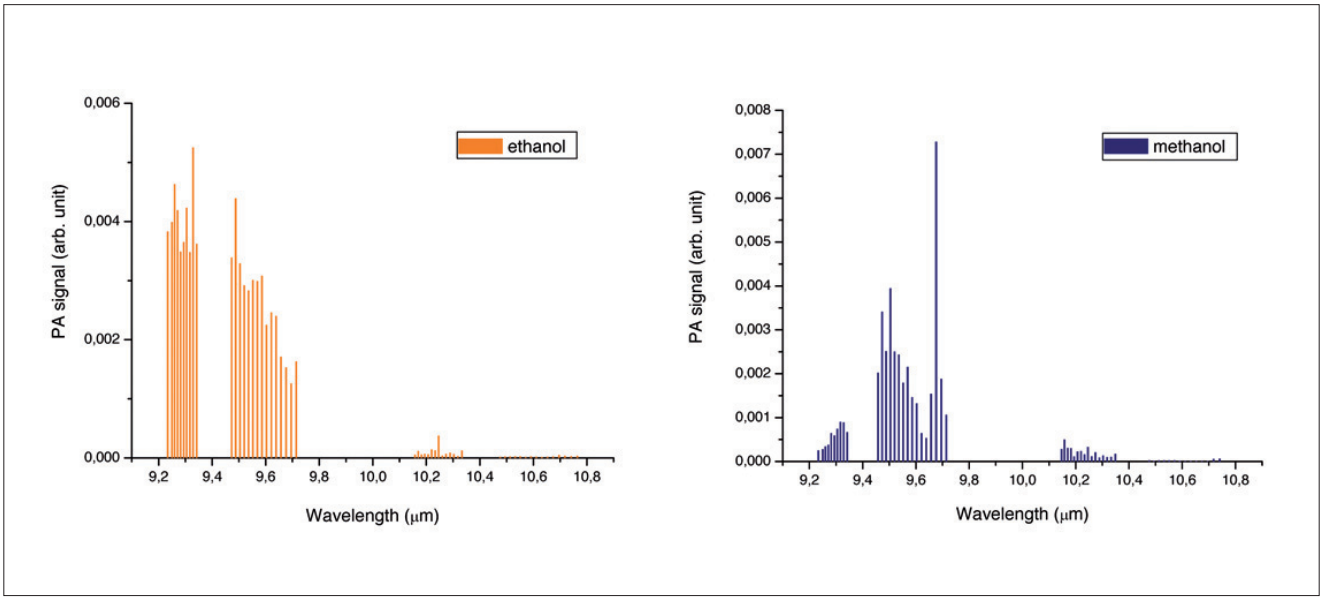


FIGURE 7 Photo-acoustic spectra measured with LPAS apparatus in the mid IR 9 – 10 µm wavelength range: pure ethanol (left); pure methanol (right)

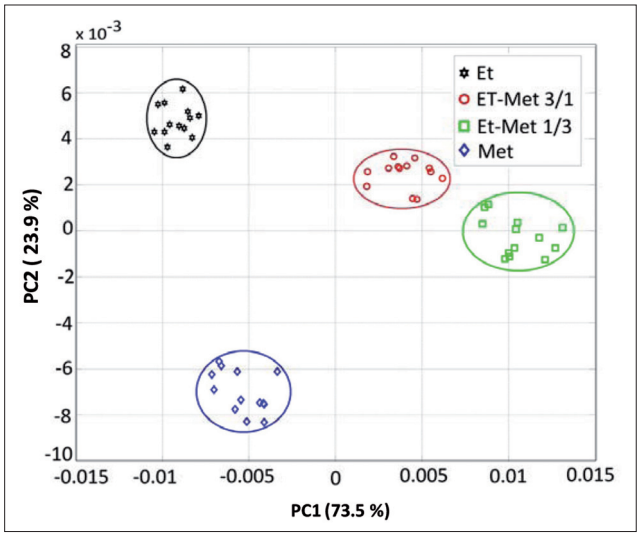


FIGURE 8 2D view of the PCA operated on ethanol/methanol mixtures in water, at different relative concentrations

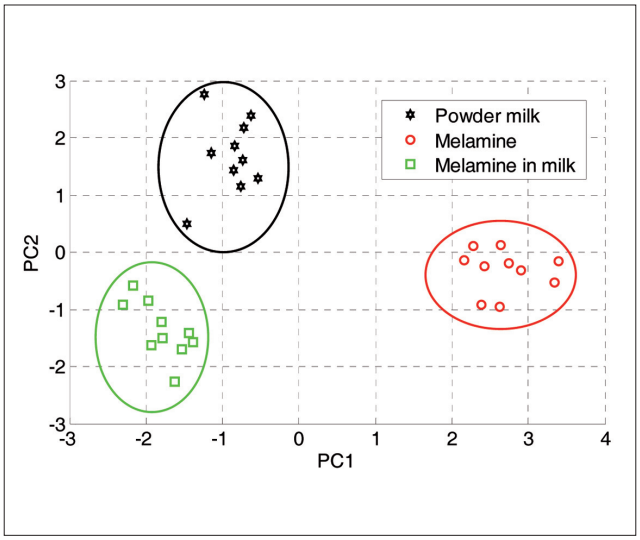


FIGURE 9 PCA result of the melamine experiment

of RAMBO and SAL@CQO projects have reached the capability for high-selectivity and sensitivity monitoring of both bacteria and contaminants.

In the case of bioterrorism attacks, SERS measurements were successfully performed even on few spores and cells, demonstrating its high sensitivity for early warning of biological threats. As for spores, the SERS spectra exhibited a satisfactory S/N ratio to identify the main spectral features that have been assigned to CaDPA. Also for cells originated from vegetation (like pollens), the spectra were assigned, even if the breakable physiology of the bacteria makes experiments more complicated. Work is in progress to define a routine data processing, that will allow to recognize and classify each spectrum on the basis of its peculiar SERS peaks.

The SAL@CQO project is aimed at developing and applying innovative, easy-to-use product-instruments to be used in production chains for performing a quality screening prior to food commercialization. The continuous monitoring of quality and preservation of food has been demonstrated by LPAS in distinguishing the presence of a specific additive in the relative food matrix by means of a multivariate analysis. The validation of the method is in progress, in cooperation with the Italian National Institute of Health (ISS) for a specific case study. The final demonstration will be based on the detection of a single food adulteration.

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