Global NEST Journal, Vol 14, No 1, pp 86-92, 2012

EVALUATION OF ARSENIC EFFECTS IN *Vicia faba* BY FTIR AND FTNIR SPECTROSCOPY

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Received: 20/12/11
Accepted: 31/01/12

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ABSTRACT

Arsenic is an ubiquitous and highly toxic metalloid widely distributed in the environment through both natural and anthropogenic pathways (Liu *et al.*, 2004) and its presence in food composites is a matter of concerns to the well being of both humans and animals. In fact, As has been recently found in drinking water (network and groundwater) in some Italian regions, including Lazio (Central Italy), at concentration ranging between 25 and 80 μg L^{-1}, higher than the limits set by law (10 μg L^{-1}) where Arsenic-contaminated groundwaters are often used in agriculture to irrigate crops for food and animal consumption; this determines that arsenic can enter human food chain. Inorganic or organic species of the metalloid arsenic occur in the environment. Either inorganic or organic As occurs in the environment and inorganic forms (iAs) are more toxic than the organic ones (oAs). Most of the human health effects of arsenic have been established based on epidemiologic studies, which have shown a significant association between the consumption of arsenic through drinking water and cancers of the skin, lung, bladder, liver, and kidney, neurologic disease, cardiovascular disease, as well as other non-malignant diseases. Arsenic is not an essential element for plants and its over-concentration in soils can generate toxicity phenomena. Its translocation from soil to plant constitutes one of the main human exposure ways. In this research we evaluated the effects of As exposure on *Vicia faba* seedlings by means of infrared (FTIR) and near infrared (FTNIR) spectroscopy to investigate molecular modifications caused by the interactions of plant with As. Both techniques showed relevant molecular modifications depending on As exposure. Molecular modifications evidenced by FTIR spectroscopy were mainly related to modified structures involving methyl groups of polysaccharides proteins and lipids, resulting better evidenced in meristem root samples. However, seconds derivative FTIR spectra did not show modification of the secondary structure of proteins.

The molecular modifications shown by FTNIR spectroscopy were mainly related to the bands of hydroxyl groups with carboxylic and methyl groups, involving hydrogen bonds between carbohydrates and nucleic acids and lipids. These molecular and structural modifications are determined by the direct introduction of As within the plant biomolecules.

KEYWORDS: inorganic Arsenic exposure effects, molecular modifications, *Vicia faba*, FTIR Spectroscopy, FTNIR Spectroscopy.

INTRODUCTION

Arsenic is an ubiquitous and highly toxic metalloid widely distributed in the environment through both natural and anthropogenic pathways (Liu *et al.*, 2006) and its presence in food composites is a matter of concerns to the well being of both humans and animals. In fact, As has been recently found in drinking water (network and groundwater) in some Italian regions, including Lazio (Central Italy), at concentrations ranging between 25 and 80 μg L^{-1}, higher than the limits set by law (10 μg L^{-1}) (Italian Legislative Decree 31/2001) where Arsenic-contaminated groundwaters are often used in agriculture to irrigate crops for food and animal consumption; this determines that arsenic can enter human food chain. Like other metals and metalloids, As resides in soils for long time, where it can either be taken up by plants or washed down into the groundwater, and present a risk to human health (Zhao *et al.*, 2010). A range of mitigation methods, from agronomic measures (e.g. raised beds, soil amendments), or plant breeding to genetic modification, may be employed to reduce As uptake by food crops (Brammer, 2009). Human exposure to food As contamination is a real concern just as exposure to drinking water (Meharg, 2004). Either inorganic or organic As occurs in the environment and inorganic forms (iAs) are more toxic than the organic ones (oAs). Most of the human health effects of arsenic have been established based on epidemiologic studies, which have shown a significant association between the consumption of arsenic through drinking water and cancers of the skin, lung, bladder, liver, and kidney, neurologic disease, cardiovascular disease, as well as other non-malignant diseases.

In a previous study (Sturchio *et al.*, 2011), we evaluated the effects of arsenic on food vegetables grown on different types of soils. We evaluated contamination effects on *Raphanus sativus* L. and *Lactuca sativa* L. cropping by using magnetic resonance imaging (MRI) and nuclear magnetic resonance (NMR). For both analytical approaches, we found indicators correlated to As contamination, chemical for NMR, such as modification of composition, and morphological for MRI, such as reorganisation of internal tissues. Furthermore, *Vicia faba* seedlings grown on polluted soils were used to evaluate the phytotoxic and genotoxic effects by comet assay, and the results of these tests were dependent on different soils. Due to the relevance of these results we tried to confirm them in this work where we studied the effects of arsenic contamination by means FTIR and FTNIR spectroscopy, and in addition As phytotoxic effects were also estimated, evaluating the effect of As alone and in combination with the Kipos biofertilizer. Kipos is used in agronomic practices to mitigate arsenic phytotoxic activity related to the presence in Kipos of humic, fulvic acids and several enzymes that complex arsenic, so reducing the phytoavailability (Maini, 2006).

METHODS AND MATERIALS

The experimental set up consisted of three steps; in the first step, fifteen aluminium basins, containing 25 seeds of *Vicia faba* placed in 250 g of quartz sand, irrigated with 40 ml of distilled water, were prepared; and incubated in climatic chamber (at 21° $\pm$ 1°C and 60 % w/w of humidity) for five days to allow the germination. After five days growth (i.e. the beginning of the second step) seedlings were sampled and placed in Petri dishes for the contamination by two different solutions, sodium arsenate dibasic heptahydrate (10 mg As L^{-1}) and sodium arsenate dibasic heptahydrate (10 mg As L^{-1}) mixed with commercial fertilizer (Kipos, containing a mixture of organic acids, humus, fulvic acids, and several enzymes). Distilled water was used as control test.

In the third step, seedlings were sampled after 18 hours exposure and washed repeatedly with distilled water, then placed in aluminum basins with quartz sand for the recovery step in climatic chamber at 21° $\pm$ 1 °C for 24 hours. At last, the primary roots were cut off, freeze-dried, and lyophilized in order to carry out FTIR and FTNIR spectroscopic analysis.

FTIR measurements were performed by a Jasco Fourier transform spectrophotometer mod. 410, equipped with a Pike Technology accessory for collecting spectra in diffuse reflectance mode, using a metal platform for placing samples. The instrument chamber was pre-heated to reduce atmospheric air interference depending on CO_2 mainly. Spectral acquisition was performed by means of 500 scans with 4 cm^{-1} of spectral resolution in the 650-4000 cm^{-1}range, using KBr as spectroscopic blank. The cosine function was applied as apodization method. Spectra were baseline correct and submitted to a 15 point smoothing filter for noise reduction. Then any spectrum was normalized by means of the Amide I band. All the spectra were saved as ASCII files.

FTNIR spectra were performed by a Jasco Fourier transform spectrophotometer mod. 420, equipped with a Pike Technology accessory for collecting spectra in diffuse reflectance mode. Spectra were

taken using a metal platform for placing samples, after 500 scans at 4 cm^{-1} of spectral resolution in the 3800-10000 cm^{-1} range, using KBr as spectroscopic blank. Spectra were baseline correct and submitted to the same 15 point smoothing filter for noise reduction, already reported for FTIR spectra. Then any spectrum was normalized by means of the band at 4030 cm^{-1}. All the spectra were saved as ASCII files.

RESULTS

FTIR spectroscopic investigation

FTIR spectra of primary roots (Figure 1) and root meristems (Figure 2) exposed to As and As with commercial fertilizer are reported and compared with the related control samples. Being normalized, intensity and peak shape changes represent quantitative and structural changes related to molecular modifications caused by As alone and in combination with Kipos biofertilizer exposure. In both typology of exposure and for both types of samples, significant molecular modifications are evidenced. In the case of primary root, polluted samples showed some change intensities (i.e., reduction) with respect to the control sample mainly related to polysaccharide content detected by the bands at 1160 and 3350 cm^{-1}. Lipid content changes related to vegetal and pigment compounds are also shown and detected by the changes of the large band between 2000 and 2400 cm^{-1} and by the changes of aliphatic chains between 2800 and 2950 cm^{-1} bands and 1350 and 1420 cm^{-1}. Other changes are shown by the P=O group of nucleic acids between 1210 and 1250 cm^{-1}. Analogous evidences are shown by the FTIR spectra of root meristems (Figure 2). Due to the several functional groups involved in molecular modifications we can suppose that As produces both quantitative and structural changes involving carbohydrates, proteins lipids an nucleic acids. A further study performed by second derivative FTIR spectra (Figure 3) let us suppose how direct structural effects of As on protein structure are negligible instead because in all the samples the same secondary structure bands at 1682 (β-sheet) and 1650 (α helix) cm^{-1} (Schweitzer-Stenner, 2006; Mecozzi *et al.*, 2011) show highly comparable shapes.

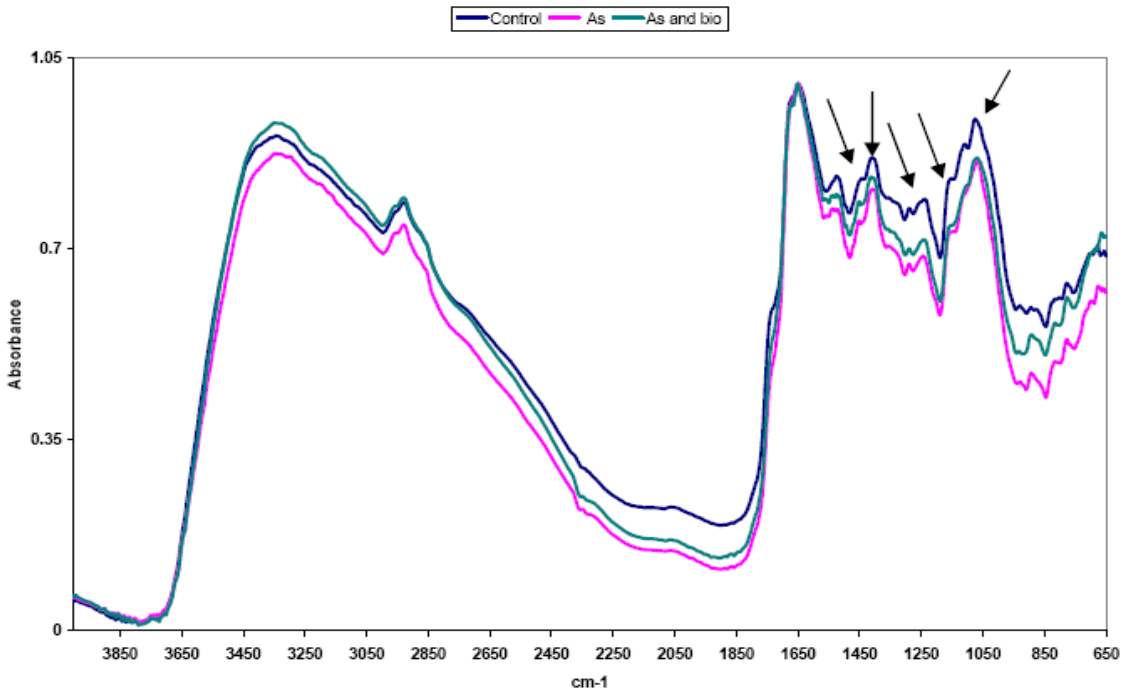

Figure 1. FTIR spectra of lyophilized primary root samples exposed As alone and in combination with Kipos biofertilizer. The arrows show the most significant molecular changes (see text)

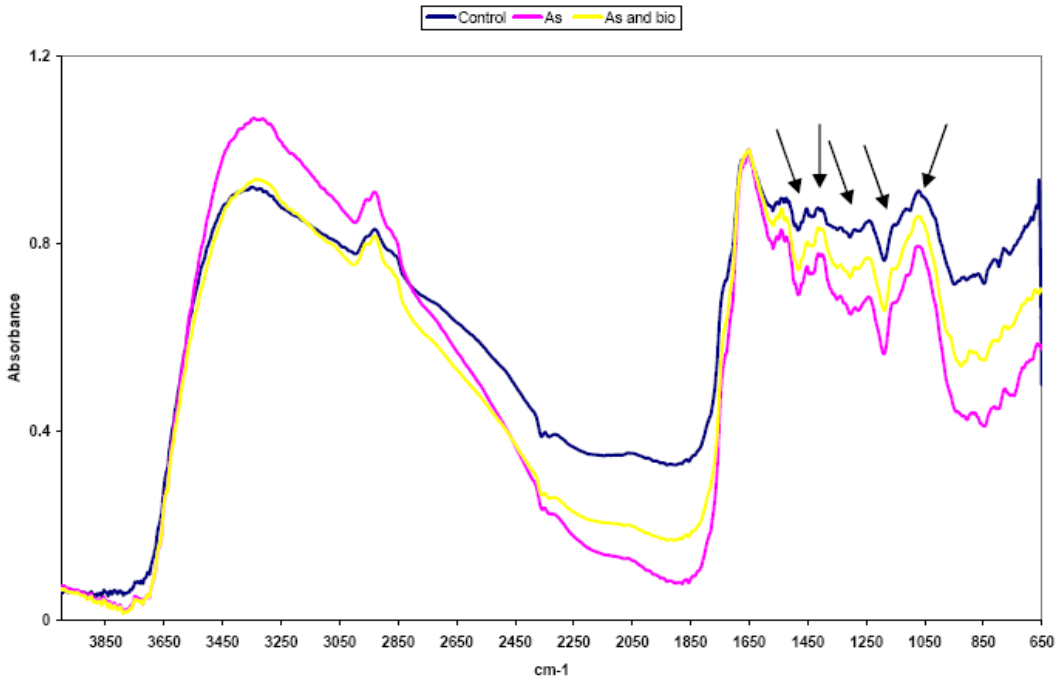

Figure 2. FTIR spectra of liophilyzed root meristem samples exposed to As alone and in combination with Kipos biofertilizer. The arrows show the most significant molecular changes (see text)

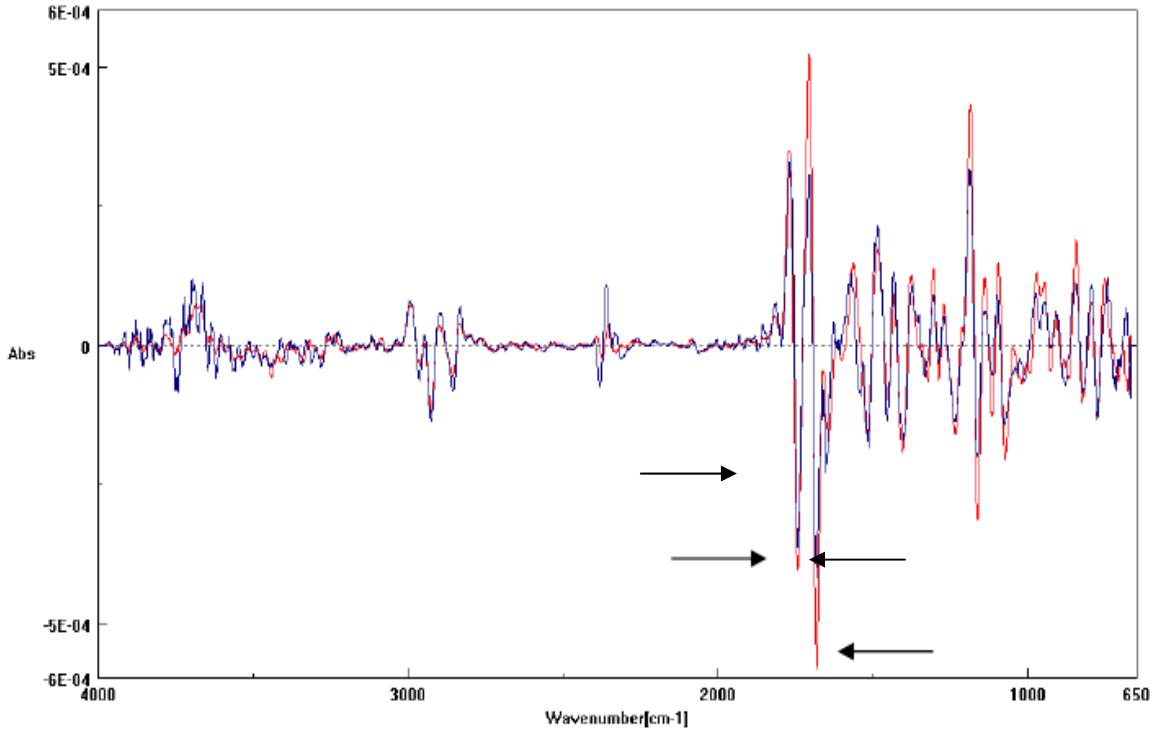

Figure 3. Example of primary root FTIR second derivative spectrum compared with the control one showing the presence of α-helix and β-sheet secondary protein structures in all the samples examined

A further evidence of this FTIR spectroscopic study using the second derivative shows that the presence of denaturated protein structures depending on As effects is not evident due to the absence of the band of the free coil (i.e. secondary structure) band of proteins, located close to the absorption at 1620 cm-1 (Schweitzer-Stenner, 2006; Mecozzi *et al.*, 2011). This is a specific finding,

pointing out that protein-As interactions are absent or negligible in the condition growth applied for all the experiments.

FTNIR spectroscopic investigation

FTNIR spectra reported in Figures 4 and 5 show additional information concerning structural modifications caused by As alone and in combination with Kipos biofertilizer exposure. In more details, in both samples, the most evident modifications resulting by FTNIR spectra concern the shape of the second overtone band of aliphatic –CH group between 8500 and 8000 cm^{-1} and the first overtone of –OH groups between 6400 and 6900 cm^{-1}. Other bands results unchanged and this is the case of two fundamental bands. The first band is that at 5100 – 5200 cm^{-1} one, related to the –OH and -C=O interaction between proteins and carbohydrates; the second band is that between 4500 and 4900 cm^{-1} related to the hydrogen bond between –C=O and –NH group of an α helix secondary structure (Mecozzi *et al.*, 2011).

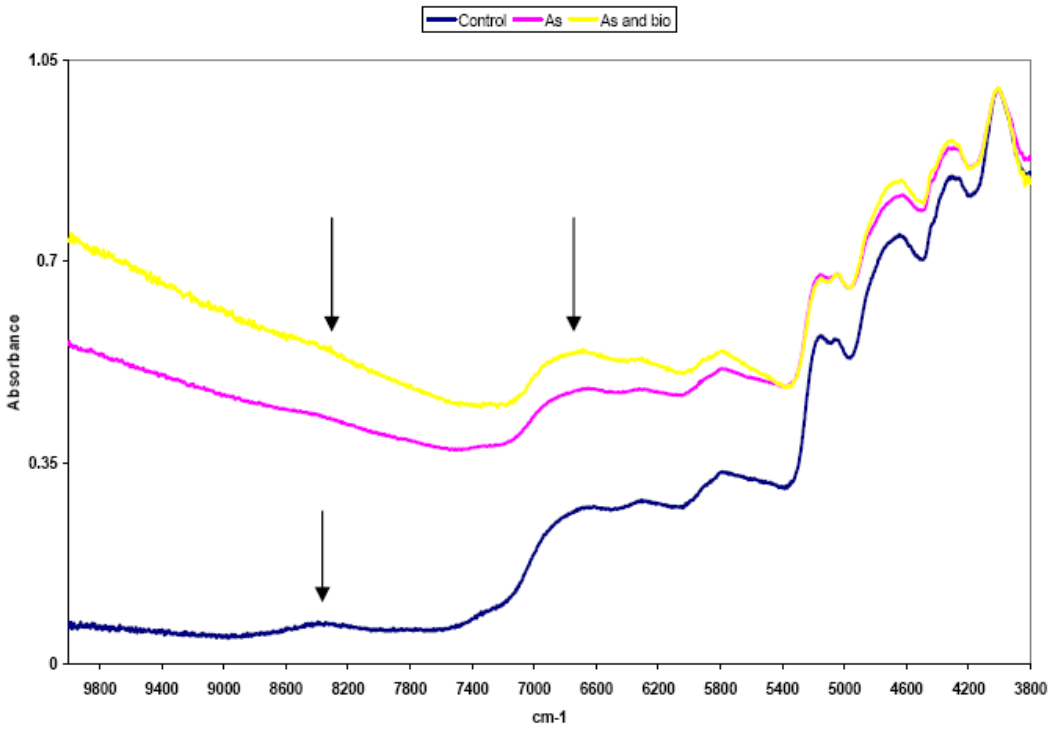

Figure 4. FTNIR spectra of primary root samples exposed to As alone and in combination with Kipos biofertilizer, showing the relevant shape modifications of the second overtone –CH and first overtone –OH bands

DISCUSSION

The FTIR spectra of primary roots and root meristems exposed to As and As with commercial fertilizer (Figures 1 and 2) are reported and compared with the related control samples. Being normalized, intensity and peak shape changes represent quantitative and structural changes related to molecular modifications caused by As exposure. Polluted primary root samples showed some change intensities (i.e., reduction) with respect to the control sample mainly related to polysaccharide content detected by the bands at 1160 and 3350 cm^{-1}. Lipid content changes related to vegetal and pigment compounds are also shown and detected by the changes of the large band between 2000 and 2400 cm^{-1} and by the changes of aliphatic chains between 2800 and 2950 cm^{-1} bands and 1350 and 1420 cm^{-1}. However, specific molecular changes can be directly related to As exposure.

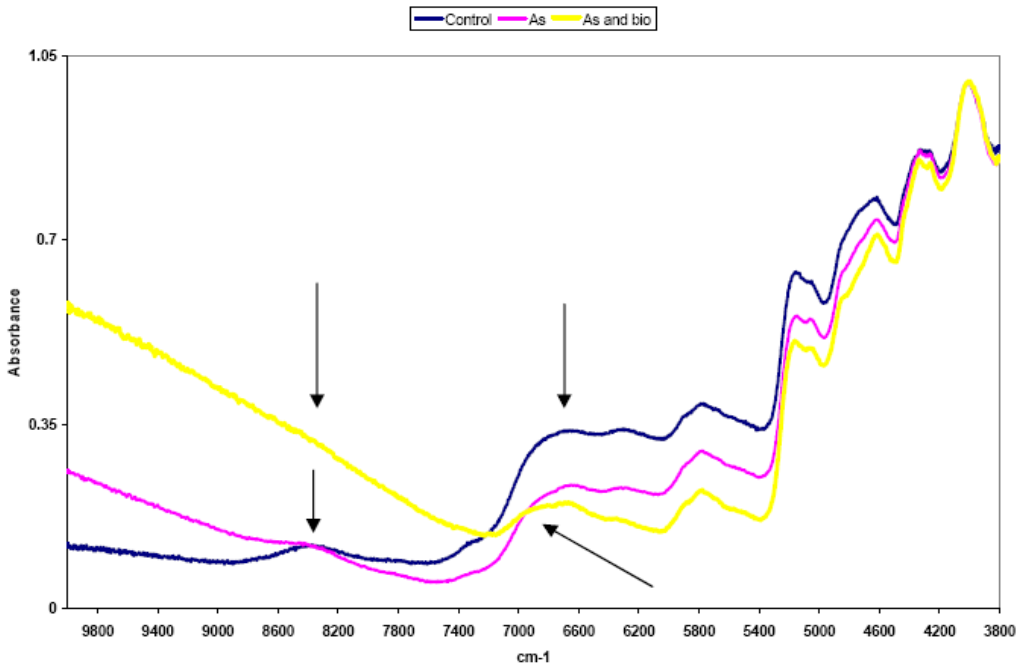

Figure 5. FTNIR spectra of root meristem samples exposed to As alone and in combination with Kipos biofertilizer, showing the relevant shape modification of the second overtone –CH and first overtone –OH bands

The chemical behaviour of arsenic is largely similar to that of phosphorus in soil where arsenate is taken up via the phosphate transport systems (Schultz and Joutti, 2007), and then, in the cell, arsenate, acting as a phosphate analogous can disrupt phosphate metabolism (Meharg and Whitaker, 2002). In particular, this mechanism can cause the replacing of P with As in the phosphate groups of DNA and the changes shown by the P=O group of nucleic acids between 1210 and 1250 cm^{-1} could be related to this partial substitution reaction.

A further study performed by second derivative FTIR spectra (Figure 3) let us suppose how direct structural effects of As on protein structure are negligible because all the samples showed the same secondary structure bands at 1682 (β-sheet) and 1650 (α helix) cm^{-1}.

Due to the several functional groups involved in molecular modifications, we can suppose that As produces major quantitative and structural changes involving carbohydrates, lipids and nucleic acids. FTNIR spectra, reported in Figures 4 and 5, showed additional information concerning structural modifications caused by As exposure. In more details, the most evident modifications resulting by FTNIR spectra concern the shape of the second overtone band of aliphatic –CH group, whereas other significant bands such as the 5200 cm^{-1} one are unchanged. This let us suppose that interaction between As and proteins is not significant as already supposed by the FTIR spectra of Figure 3.

Exposure to arsenate induces a large response in the synthesis and accumulation of glutathione and phytochelatin in vegetal cells playing an important mechanism of As detoxification (Zhao *et al.*, 2010) Moreover, the detoxification mechanism that is generally underinvestigated in plants involves As methylation according to the so called Challenger pathway, as previously established in fungi and bacteria; in fact methylated As species, such as MMA, DMA and trimethylarsine oxide (TMAO) have been found in plant samples (Meharg and Whitaker, 2002). So, we can reasonably suppose that the modified shape of second overtone –CH band is involved in the As methylation pathways. On the other hand, the presence of modified –CH band in FTNIR is also confirmed, though with a lesser intent, by the modified shape of -CH bending vibration bands shown by FTIR spectroscopy between 1350 and 1420 cm^{-1}.

So we can summarize that carbohydrates lipids and nucleic acids are more affected than proteins by the presence of As. Further studies are in progress to investigate the causes of the different spectral features observed between As and As with Kipos fertilizer exposed samples.

Acknowledgments
This study was supported by the joint project of the Italian Ministry of Health and the INAIL entitled “Development and application of innovative methodologies and techniques for risk assessment and health effects in occupational and environmental exposures (2008-2011)", Project 6, Scientific Coordinator: Dr. Elena Sturchio.

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