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Laser photoacoustic spectroscopy: A powerful tool for measurement of trace gases of biological interest at sub-ppb level

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LASER PHOTOACOUSTIC SPECTROSCOPY: A POWERFUL TOOL FOR MEASUREMENT OF TRACE GASES OF BIOLOGICAL INTEREST AT SUB-PPB LEVEL

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A high sensitive photoacoustic system has been developed to monitor trace gases released by biological samples at concentrations of sub-ppb level. We have applied this technique to measure the release of the plant hormone ethylene during seed germination and ripening of climacteric fruits and of the marker ethylene as a result of doping cultures of human cells with heavy metals, of damaging living organisms by ionising radiation (X-ray) and of unleashing lipid peroxidation in lung epithelium following the inhalation of cigarette smoke.

Keywords: ethylene; lipid peroxidation; photoacoustic spectroscopy; plant physiology; spectrophone

1. INTRODUCTION

The photoacoustic (PA) spectroscopy is based on the effect discovered by Graham Bell in 1880 and consists of energy conversion of modulated light radiation to sound energy. A part of the energy absorbed by the sample under study is transformed into thermal energy due to the nonradiative transitions. Finally, the temperature variations determine the formation of acoustic waves that can be detected directly by appropriate sensors (sensitive microphones).

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The really stimulating impulse to the whole field of PA spectroscopy has been given by the invention of lasers. In this case, the spectral resolution and the sensitivity of the method have been increased by several orders of magnitude. Owing to its attractive characteristics, PA spectroscopy invaded the entire field of science, with applications in photochemistry (chemical processes monitoring such as reaction rates, equilibrium constants, enthalpies or identification of different compounds, even isotopes, isomers and radicals), agriculture (measurement of gaseous plant hormones), atmospheric studies (air pollutants, ozone depletion, explosives), medicine, biology and many others.

To increase the sensitivity of the method and to measure trace gases at concentrations of sub-ppb ($1\,\text{ppb} = 10^{-9}$ atm) level, we have designed, constructed and optimized a new spectrophone which was included in an experimental set-up based on a line-tunable, frequency-stabilised, CW CO_2 laser. The high sensitivity of the method allowed us to investigate some basic processes in plant physiology, such as the release of the plant hormone ethylene during seed germination or ripening of climacteric fruits and in lipid peroxidation as a result of doping cultures of human cells with heavy metals, damaging living organisms by ionising radiation (X-ray) and inhalation of cigarette smoke.

2. EXPERIMENTAL SET-UP

The experimental set-up used in our experiments is presented in Figure 1. As a radiation source we have used a home-built, line-tunable and frequency-stabilised CO_2 laser, since its emission spectrum overlaps with the absorption fingerprint of ethylene (C_2H_4) [1]. It emits CW radiation with an output power of 2–7 W and it is tunable between 9.2 and 10.8 μm on 57 different vibrational-rotational lines [2]. The laser beam is amplitude modulated by an optical chopper (C-980 type, DigiRad) and then focused by a ZnSe lens and introduced in the PA cell (spectrophone). For the experiments where the cleaning process of the PA cell lasts very long (e.g. cigarette smoke), a second PA cell can be used. In this case, a part of the laser radiation is diverted by a beam splitter. The laser power used to excite the sample gas inside the spectrophones is measured by a two channel powermeter (Rk-5720 model, Coherent Radiation).

The acoustic waves produced in the spectrophone are detected with four miniature electret microphones (EK 3033 type, Knowles), connected in series. The PA signal, proportional to the trace gas concentration is applied to a lock-in amplifier (SR 830 model, Stanford Research Systems), which detects and measures very small single frequency AC signals, usually buried in a larger random noise. The output signals of the lock-in amplifier

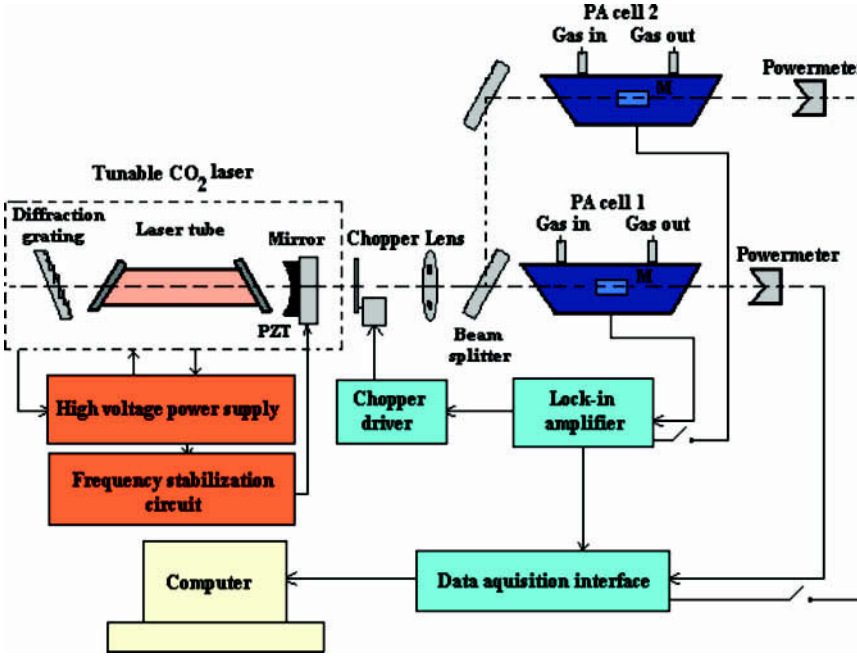

FIGURE 1 General scheme of the CO_2 laser based PA system.

and of the powermeter are then converted into digital signals by a 12-bit high speed A/D board (DAS 1601, Keithley) and processed by a computer [3].

The sensitive spectrophone (Fig. 2) consists of an open stainless steel pipe with small diameter (7 mm), excited in the first longitudinal mode, introduced inside a larger cylinder. Two buffer cavities, placed at the ends of the resonant tube, attenuate all resonance modes, except the longitudinal one and reduce noises produced by absorption of the cell windows [4]. The four sensitive microphones (sensitivity 10 mV/Pa each) are placed at the loops of the standing wave pattern for maximum operating signal. The battery fed microphones are mounted in a Teflon ring pulled out over the resonator tube. The two ends of the spectrophone are cut at Brewster angle and sealed with ZnSe windows.

Based on a two level system, one can obtain a formula for the voltage response of the PA system:

$$V = \alpha C P_L S_M c$$

where V[V] – photoacoustic signal (peak-to-peak value); α [cm^{-1} atm^{-1}] – gas absorption coefficient at a given wavelength; C [Pa cm W^{-1}] – cell constant; P_L [W] – CW laser power; S_M [V Pa^{-1}] – microphone sensitivity;

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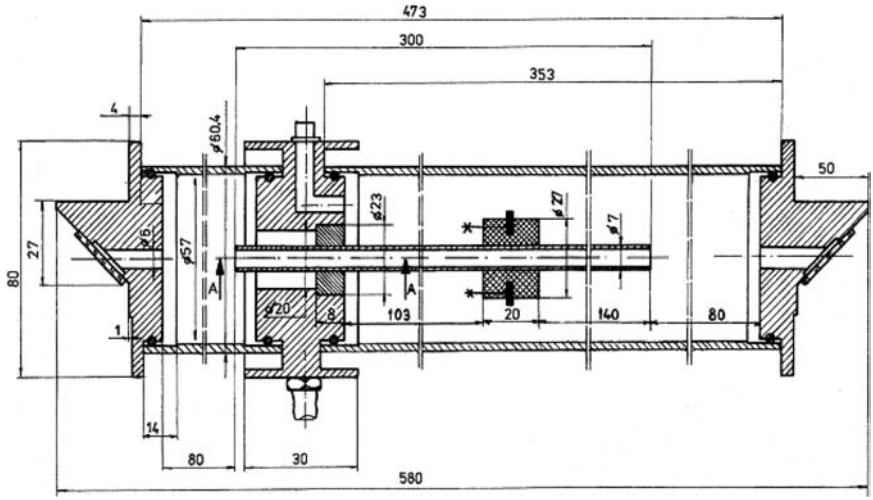

FIGURE 2 PA cell structure.

c [atm] – trace gas concentration ($1\,\text{ppb} = 10^{-9}\,\text{atm}$; $1\,\text{ppt} = 10^{-12}\,\text{atm}$). The product of cell constant and microphones sensitivity gives the responsivity (R [V cm W^{-1}]), which is a calibration constant (a merit factor of the cell). When the PA signal equals the noise voltage (at a signal-to-noise ratio SNR = 1), the minimum detectable concentration is obtained.

Our optimised spectrophone has the following characteristics: resonance frequency: 560 Hz; quality factor: 16.5; cell contant: $4600\,\text{Pa cm}\,W^{-1}$; and responsivity: $184\,\text{V cm}\,W^{-1}$ (one of the highest value reported in the literature for an extracavity PA cell).

To obtain reliable measurements, we have built a gas handling system (Fig. 3). Either certified nitrogen or synthetic air are used to transport the sample gas from the cuvette (or from a sample bag) to the PA cell. Both the mass flow (usually 1–1.5 l/h) and the total pressure inside the spectrophone are measured with digital instruments (MKS). To prevent the undesired supplementary absorption of the interfering gases, especially CO_2, a KOH – based scrubber was introduced before the PA cell.

In these conditions, our PA system is able to measure a minimum concentration of 0.2 ppb of C_2H_4 in synthetic air at atmospheric pressure.

3. RESULTS

We have investigated basic processes in plant physiology and in lipid peroxidation, where the release of trace gases (ethylene) is very low

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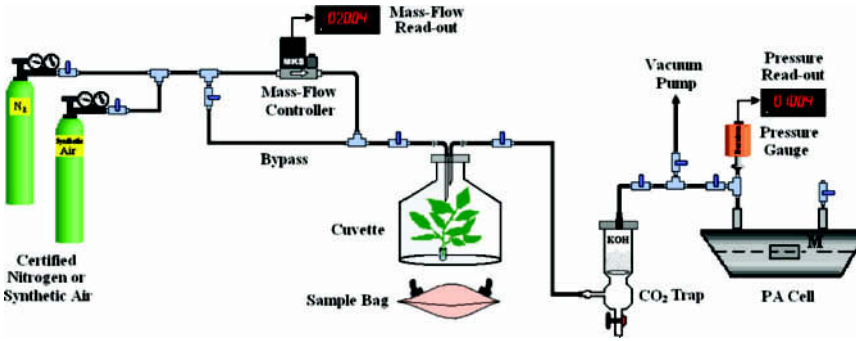

FIGURE 3 Experimental set-up of the gas handling system.

(0.2–2 ppb), impossible to be measured by others means (mass spectrometry or gas cromatography).

A. Plant physiology

Ethylene (C_2H_4) acts as a vegetal hormone produced by all plant tissues. It is transported by diffusion through plant tissues and increases the plasmatic membrane permeability. It has multiple effects on the cell metabolism: increases the oxidative processes, the transport inside the cells and the biodegradation of the organic acids and chlorophyll. Ethylene plays a major role in many metabolic processes: seed and bud dormancy, seed germination promotion, roots induction, development of plantlets (inhibitor of elongation and promotion of lateral shoots), grown promotion, leaf expansion, epinasty (downward curvature of leaves due to the growth of cells on the upper side of the petiole), flowering, wilting of flowers, fruit ripening (ethylene induces some biochemical modifications which produce polyalcohols, hydrocarbons and different oxygenated combinations responsible for the taste, aroma and texture of the fruit), aging and senescence of leaves and flowers and fianlly, the abscission of leaves and fruits.

The ethylene biosynthesis process in plants follows the MSAE pathway: L-methionine (amino acid) – SAM (S-adenosyl methionine) – ACC (aminocyclopropane-1-carboxylic acid) – C_2H_4.

Ethylene, or its precursor ACC, stimulate seed germination of many species at concentrations as low as 0.2 ppb. During germination, a complex cross-talking between several plant hormones exists.

Tomato is an useful model plant for studying ethylene action. Three tomato mutants altered in ripening process affects different steps in ethylene synthesis and perception, resulting in a delay of fruit maturation

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and pigmentation: *N*ever *r*ipe (*Nr*) is mutated in an ethylene receptor and exhibits delayed and incomplete fruit maturation; *r*ipening *in*hibitor (*rin*) is a delayed gene that causes the block of ripening before the respiratory burst; and *no*n *r*ipening (*nor*) shows pleiotropic effects analogue to *rin*. The aim of our study was to investigate the ethylene emission during seed germination of these 3 mutants, correlation with their germination ability and analysis of ethylene role on the loss of germinability during seed senescence [5].

The ethylene production per seed measured during seed germination and seedling elongation is presented in Figure 4. In these genes, ethylene influences not only fruit ripening, but also the seed germination. The germination index and the percentage of germination of the 5-years-old seeds of the mutants are higher in respect to the control (Ny – New Yorker), in spite of the lower ethylene production of germinating seeds. Conversely to other species, in 5-years-old tomato seeds an inverse correlation between ethylene production and percentage of germination exists. During seed senescence, ethylene accumulation occurs and some processes, triggered during germination, result altered. Further analysis are required to clarify the interaction between ethylene and other hormones like auxin, ABA and cytochinin.

Climacteric fruits show a respiratory rise during ripening (tomato, pear, fig, mango, banana), while others belong to nonclimacteric fruits (cherry, strawberry, lemon). Fruit ripening (yellowing, softening, respiration, autocatalytic ethylene production) and abscission are regulated by ethylene. During ripening, tomatoes show a strong increase in ethylene production

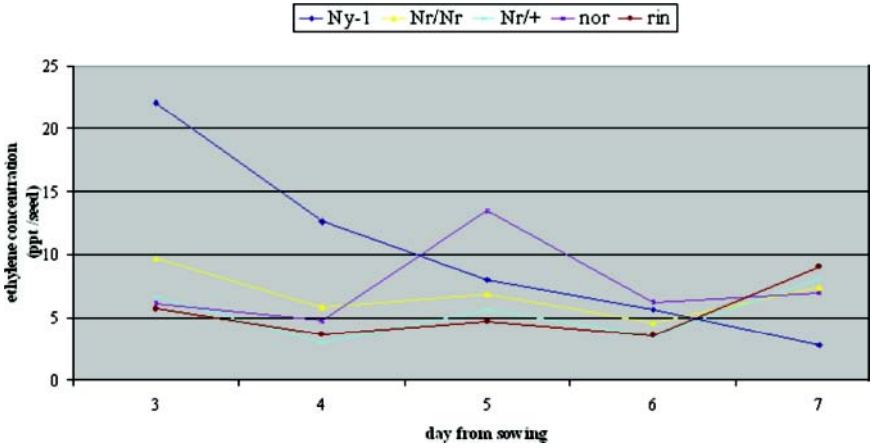

FIGURE 4 Ethylene production (ppt/seed) measured during seed germination and seedling elongation.

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coinciding with the climacteric rise in respiration (CO_2 production). Ethylene is also involved in the postmaturation processes, playing an important role in fruit ripening. Storage and shipping of fruits in terms of wounding effects, temperature, composition of atmospheric gases, postharvest pathogens or seal-packing conditions are important factors to establish the optimal environment necessary for their long term conservation. The aim of our experiments was to monitor the ethylene emission in plants and fruits at low temperature, together with the effect of the temperature at different ripening stages (important for optimization of different stages in agricultural procedures) and to study the effects of mechanical wounding of fruits.

Ethylene emission was monitored from plantlets at different temperatures and it saturates after about 20 min [6]. The temperature effect is evident in the emission intensity, which increases almost a factor four from 15° to 25°C. There is a remarkable decrease in the lag time for the gas emission at the optimum temperature for biosynthesis of ethylene (25°C). At temperatures lower than 22°C, this lag time is about 6 min, while drops to less than 2 min at 25°C. Temperature does not influence the ethylene emission of immature fruits (0.004 ppb/g and 0.005 ppb/g for 15°C and 25°C, respectively), while it becomes important when the ripening process is triggered in a maturated fruit (0.012 ppb/g and 1.56 ppb/g for 15°C and 25°C, respectively). The same result is obtained for plantlets. Temperature is important for ACC oxidase activity (decreased at low temperatures). Mechanical wounding exerts its effect at the step where SAM is converted to ACC, the direct precursor of ethylene; this step, regulated by the enzyme ACC synthase is rate limiting in the cascade of events leading to an increase of ethylene production.

B. Lipid peroxidation

The oxidative modification of biological molecules is an essential part of the normal biological activity in the human organism. An excess in some oxidant activities does cause injury to cells and tissues. Particular attention is devoted to the oxidant activity of free radicals. An increased free radical formation in the organism is involved in the pathophysiology of several diseases. One of the events generated by free radicals interaction with biomolecules is the oxidative degradation of fatty acids. Oxidative stress is the origin or cause of lipid peroxidation and, as a consequence, of a wide variety of pathophysiological processes. Lipid peroxidation is the free-radical-induced oxidative degradation of polyunsaturated fatty acids. Biomembranes and cells are thereby disrupted, causing cell damage and cell death. As a marker of free-radical-mediated damage in the human body, the measurement of the exhaled volatile hydrocarbons, such as ethylene (C_2H_4), is a good noninvasive method to monitor lipid peroxidation [7].

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We have studied lipid peroxidation as a consequence of ionising radiation and heavy metals in living cells. Most heavy metals have a toxic action on human cells and may induce lipid peroxidation. Cadmium is a toxic agent which is supposed to affect the transport of ion through the cell membrane. Cadmium and calcium ionic radii are similar, so Cd can be picked up through the Ca transport mechanism. On the other hand, the Cd permeability through the calcium channel is very poor, so Cd can be considered as a blocker of the calcium channel as well. We tried to determine the extent of the toxic action of Cd *in vitro* by monitoring the ethylene concentration in the breathing air of human cells cultured in a liquid medium to which cadmium chloride was added. Cells of the human leukemic T cell line (Jurkat) were kept in a culture in RPMI 1640 medium containing 10% FBS, 1% L-glutamine and 1% penicillin streptomycin at 37°C in a humidified incubator with 5% CO_2 and 95% air.

The measurement of ethylene before and after treatment of the culture of human cells with $CdCl_2$ has shown that the concentration has increased from 0.5 ppb for control (live cells) to 1.2–1.4 ppb for dead cells, both after 5 hours and 24 hours from the treatment. The increase of ethylene content clearly demonstrates that lipid peroxidation took place owing to the toxic effect of heavy metals (Fig. 5). The measurements were performed on

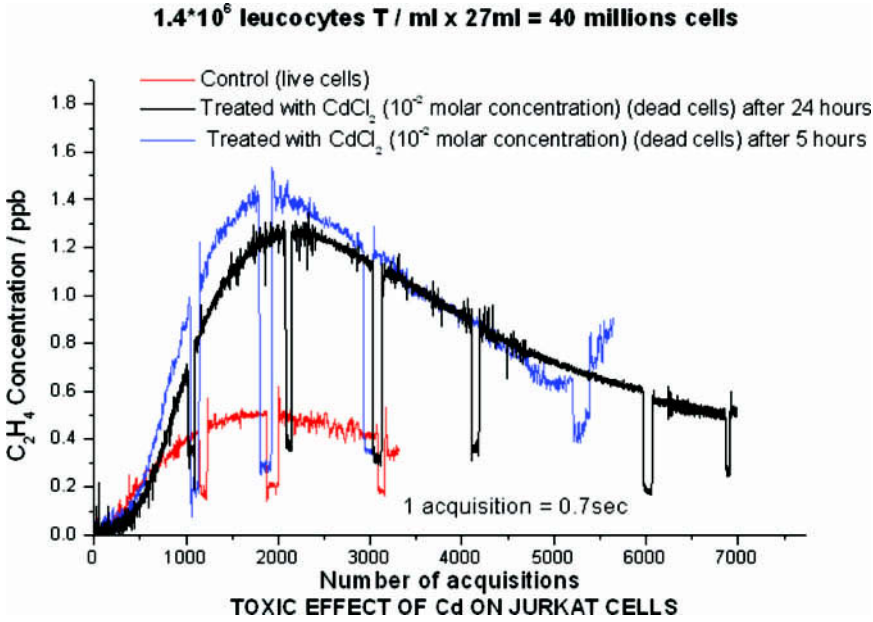

FIGURE 5 Toxic effect of Cd on Jurkat cells.

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10P(14) line of the CO_2 laser by means of a nitrogen flow-through system (25 ccm). The CO_2 laser, tuned off resonance on the 10P(20) line from time to time, causes a clear drop in the observed signal.

The radiation damage in living matter develops along complex chains of events that follow the absorption of energy: a) physical stage: the energy transfer from the radiation to the matter leads mainly to molecular excitations and ionization; b) chemical stage: the primary reactive species (free atoms and radicals, that are usually extremely unstable), undergo secondary single reactions or a succession of reactions among each other and with their environment, causing damage to molecules of biological importance; c) biological stage: molecular changes occurring in a living organism may cause alterations in the system organization, which become macroscopically observable as biological effects. A substantial part of the total ionising radiation effect concerns water radiolysis, water being a major component of living tissues present in all biological systems. Many water ions and radicals are generated inside tissues as primary reactive species. Aqueous free radicals are very reactive and induce oxidative degradation of phospholipids in cell membranes (lipid peroxidation). The aim of our investigation was to measure the X-ray induced ethylene emission in mice breath and to analyse breath exhaled from patients under external X-ray beam therapy for cancer treatment.

The laser-based analysis of exhaled breath is achieved according to the following pathway: production of the marker molecules inside cells and organs – diffusion through tissues – input into haematic flow – transport to the lungs – release in the breathing air – collection of a breath sample – assessment of the marker in the breath sample [8].

For the purpose of verifying the radioinduced effect, living mice (B6C3F1 and C57B1/6J male mice, between 3 and 6 months old) have been exposed to the total body action of a 250 kV X-ray apparatus GILARDONI model CHF-320-G. At 250 kV voltage and 15 mA current by using a 0.5 mm Cu filter, the measured dose rate was 90.1 cGy/min at 68.4 cm from the source. The value of the X-ray dose given to the treated mice (9 Gy total body) is comparable, as order of magnitude, to the therapeutic doses given to a human patient in the course of a radiotherapy cancer treatment. The mice were divided in treated and control groups. Each treated mouse received a substantial amount of X-rays in the whole body, while the control mouse received a zero dose. Samples of the breathing air has been collected before and after irradiation. The breathing air has been concentrated on active coal absorbing pellets for a time as long as 1.5 hours, successively expanded into 0.5 liters sample bags, and then transferred into the photoacoustic cell in order to perform the analysis of ethylene content. The PA analysis of ethylene content, by using the above described procedure, takes only few minutes and, after calibration, allows for immediate

data release. The radioinduced production of ethylene in the animal appears to be at clearly detectable levels, since the exhaled ethylene increases more than 4 times in the mouse breath after the irradiation (12.4 ppb for control mice before exposure, compared to 55.9 ppb for irradiated mice, after exposure) [9].

We followed up patients under external beam radiation therapy with X-ray, because they were affected by gut cancer. The irradiated area was the abdomen and they received a 1.8 Gy dose during each daily exposure in a field as large as 50 cm × 30 cm. No substantial radioinduced increase of ethylene content in the exhaled breath has been put into evidence. The results can be explained by assuming that the absorbed dose is too low to produce a detectable effect. Another possibility is that the ethylene production happens only at the time of irradiation and stops immediately after it. Therefore, a breath sampling during irradiation is necessary.

Many toxic components (heavy metals, free radicals, chemicals) in the inhaled cigarette smoke may induce ethylene formation by lipid peroxidation in the lung epithelium. The exhaled air from the subject being tested was collected inside aluminized bags and then the sample gas was transferred into the measurement PA cell. In all experiments, a high value of ethylene concentration was found immediately after smoking, followed by a slower decrease. A total concentration of 4040 ppb of ethylene was measured in cigarette smoke. In the exhaled breath of a smoker, we have found an ethylene concentration of 39 ppb immediately after smoking and even 1.4 ppb at half an hour from smoking a single cigarette, compared to 0.6 ppb as base (before smoking). Ethylene is dangerous for smokers because ethylene oxide is a chemical product that induces cancer in the lungs. For the moment, it is difficult to separate the exogenous and endogenous provenience of the ethylene in the smoker's breath.

4. CONCLUSIONS

The results obtained in all these applications have demonstrated that PA spectroscopy technique presents flexibility, possibility to detect various compounds with a single device, high stability, large dynamic range to measure low concentrations, good time response and high sensitivity in detection.

The measurement of trace gases released in atmosphere by some plants and fruits gives valuable information in selection of stress-resistant genotypes of agriculturally interesting plants. By using ethylene as a marker for lipid peroxidation, we were able to evaluate the effects of heavy metals on cultures of human cells, the ionising radiation (X-ray) damage in living organisms and the effects of inhalation of cigarette smoke.

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Based on these measurements we have determined the germination index and the percentage of germination of the mutants of tomato seeds, the effect of the temperature and of the mechanical wounding on fruits, the toxic action of heavy metals on human cells, the X-ray induced ethylene emission in animals and humans breath and the high level of ethylene in smokers breath following the inhalation of cigarette smoke.

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