Photoacoustic Spectrometry: A Potential Tool For Future Antioxidant Test

Suparmo
Faculty of Agricultural Technology,
Gadjah Mada University, Yogyakarta, Indonesia

ABSTRACT

Peroxidation of membrane lipid is implicated with several health disorders and antioxidants are perceived capable of controlling the reaction. Quest for new natural antioxidant dominating research topics in the last decade. Test and validation of their usefulness becoming more important, especially in vivo on human subject. Current method for peroxidation tests, such as loss of polyunsaturated fatty acids, conjugated dienes, singlet oxygen, lipid hydroperoxides and malonaldehyde provide reliable data, however, the methods are lengthy and requiring blood or urine samples. New non-invasive and quicker methods are required.

Gas exhale by individual, that considered to be secondary product of lipid peroxidation are among markers candidates. Among the exhaled gases, ethylene, methane, ethane and pentane fall into the category. Ethylene and methane, however, are not specifically product of lipid oxidation. Ethylene is also produced during protein and carbohydrate oxidation, while methane is produced in a large amount by colon bacteria. Ethane and pentane are proved to be most good peroxidation marker and results are in a good correlation with the current methods. However, the two gases are produced in a very low concentration just slightly above that in ambient air. The detection of the two gases utilizing gas chromatography requiring special technique for air cleaning and concentration due to lack of sensitivity. The laser driven photoacoustic spectrometer is capable of detecting the two gases with more than 1000 times in sensitivity. Its uses in the detection of the two lipid peroxidation markers would be a potential for future peroxidation or antioxidant challenge test.

Key word: antioxidant, ethane, pentane, photoacoustic, peroxidation test

INTRODUCTION

Polyunsaturated fatty acids are found mainly as components of membrane lipids both cellular and sub cellular. They are the main determinant of microviscosity and fluidity properties of the membrane, therefore, directly influence enzyme and receptor function and signal transduction. They are prone to lipid peroxidation as a result of the extremely weak binding of the hydrogen atoms to the carbon chain between double bonds. These hydrogen atoms, therefore, are easily abstracted, and the resulting lipid radicals are in turn capable of subtracting new hydrogen atoms from adjacent poly-

unsaturated fatty acids. Thus, a chain reaction is induced, which, were demonstrated clearly in *in vitro* studies. Several reaction products are formed, which, many of them are able to propagate further lipid peroxidation process. Depending on the presence of peroxidicing products, dioxygen, and metal catalyst, a great variety of lipid radicals and more or less stable oxidation products may arise. It is among the overabundance of degradation products that the markers of *in vivo* oxidation have to be sought. The product must be solely formed by the peroxidation reaction or at least the extend of the reaction is the main determinant of product magnitude.

Peroxidation of membrane lipid is implicated with several health disorders and antioxidants are believed capable of controlling the reaction. Several antioxidants are prescribed as the disease prevention food supplements. Natural antioxidants are of the most popular food supplements that considered useful in maintaining good health, fighting against variety forms of stress, and avoiding several degenerative diseases. Many industries harvest top dollars from digging into traditional old medicine recipes and convert them into a new good looking tablet. Although the proof of the claimed health benefit was not substantiated clearly over the range of the application, such commodities are still sale very strongly. Many more ethnic medication perceived to have a certain health effect will certainly appear as new products in the market very soon.

Several industries do concern with producing hard proof in addition to the traditionally perceived health benefit from *in vitro* test, in animal and human assays. Several kind of tests for demonstrating antioxidation effect are available, however, each of them has its own limitation or even draw backs. Regulation mandates proof on human assay before health benefit is claimed, unfortunately, the test of antioxidation in animal or human assay is difficult to interpret. Simpler human test, even for presumptive test are yet still in quest.

Most test for effect of antioxidant resemble a challenge test for oxidation or peroxidation of samples exposed to unfavourable storage condition, such as high temperature and the presence of excessive oxygen gas. Difference in oxidation magnitudes or time required to reach certain oxidation level is considered effect of the antioxidant addition over control. This is easily done in *in vitro* test, however, in *in vivo* test several factors complicate the measurement. Test on human subject should not be done on a single procedure without confirmation by other procedure.

Photoacoustic (PA) Spectrometer operates based on conversion of light energy to acoustical energy. A gas absorbing a beam of light arise to its excited state and upon its decay to the ground state releases energy, which could be detected as sound signal by microphones in the PA cell. The intensity of the generated sound result from pressure fluctuation is proportional to the concentration of the absorbing trace molecule and to the light intensity. This very new technique has a very low detection limit, in a ppb level, and several other capabilities that, in many cases, far more favourable than the capability of current method utilizing gas chromatography.

End product of lipid oxidation emerged as gas exhale from the lung is more preferable over those requiring blood or urine samples for the ease of both the subject and the analytical procedure. This paper suggesting the use of photoacoustic spectrometry for a very sensitive, non-invasive and non-destructive method in the assessment of gas as marker of lipid peroxidation in human. *Performance of the PA spectrometer and choice of gas markers and their theoretical validation is discussed in this paper.*

Current Methods for Peroxidation Test

Loss of polyunsaturated fatty acids from a biological system is a simple and useful method for the detection of peroxidation process *in vitro*. However, for the *in vivo* process the interpretation is difficult because essential fatty acid deficiency usually indicates decreased intake of essential fatty acids, and/or energy or impaired absorption. However, the test has been applied to animal studies in which the relative abundance of fatty acid classes as a function of their number of

double bonds was expressed as the peroxidizability index, a low index indicating loss of polyunsaturated fatty acids (Halliwel *et al.*, 1992).

Conjugated dienes are rapidly formed once the chain reaction of lipid peroxidation has been initiated. Generally their formation is considered proof that the peroxidation has been taken place. In the presence of dioxygen, they form peroxyl radicals and lipid hydroperoxides. Their yield depends on the relative amounts of dienoic and polyenoic lipids. The determination of conjugated dienes in human body fluids is troubled with difficulties.(Banni *et.al.*, 1990, and Gutteridge and Halliwell, 1990)

The most abundant conjugated diene in human plasma is *cis-9-trans-11-octadeca dienoic acids*, a diene derivative of cis-9, cis-12 cis linoleic acid. It can be generated *in vitro* by exposing linoleic acid to free radicals in the presence of thiol-rich protein (Dormandy and Wickens, 1987). The *9,11-linoleic acid/9,12-linoleic acid ratio* has been advocated as a measure of lipid peroxidation in human plasma, bile, and exfoliated cells from cervix uteri. Lipid peroxidation is, however, not the only source of 9,11-linoleic acid in plasma, and its use as a lipid peroxidation marker has been questioned.

Lipid peroxidation is related with light emission due to the formation of excited-state carbonyls from peroxyl radicals and of singlet oxygen from triplet oxygen followed by decay back to the ground state (Halliwell and Chirico, 1993). The precise mechanism by which singlet oxygen is formed is not known. Chemiluminescence has been used extensively in studies on isolated cells and organs, especially blood specimens. The technique has been shown to be very sensitive and to correlate reasonably well with other measures of lipid peroxidation (Cadenas *et al.*, 1982.).

Lipid hydroperoxides represent the major initial reaction products of lipid peroxidation, which makes them attractive as a measure of peroxidation. They are unstable in the presence of metal ions, and their composition leads to the formation of conjugated dienes and numerous other secondary reaction products. Their measurement in the body fluids depends on the assessment of their decomposition products, notably malonaldehyde, with the thiobarbutyric reaction. The reaction is not specific and its time-dependent. Because it measures free aldehyde, the total amount of lipid peroxides is not adequately related. The assay involving thiobarbutiric acid-reactive material underestimates lipid peroxidation compared to spectrometric detection of lipid hydroperoxides (Zidenberg-Cherr *et al.*, 1991).

Malonaldehyde and 4-hydroxynonenal are the most important aldehydes formed as secondary lipid peroxidation products, although their reliability and specificity as markers of lipid peroxidation have been questioned. An aldehyde, such as 4-Hydroxynonenal and other aldehydes, including malonaldehyde, are immunogenic and cytotoxic due to their ability to react with several molecular species, such amino acids. The 4-hydroxynonenal has been shown to inhibit DNA as well as protein synthesis and to inhibit various enzyme systems. Furthermore, biological effects, such as the induction of chemotaxis and platelet aggregation, have been reported. Malonaldehyde is the single most abundant peroxidation product of polyunsaturated fatty acids, notably of those with more than two double bonds. Its assessment in body fluids through reaction with thiobarbutiric acid is relatively simple, that gain popularity in the study of lipid peroxidation. The interpretation of the result is often difficult, however, and only remotely quantitative. It should not be used in isolation without confirmation with other markers of lipid peroxidation. The thiobarbutiric assay for malonaldehyde or "thiobarbutiric acid reactive substances" (TBARS) should only regarded as a screening test suggestive of lipid peroxidation. Direct assessment of malonaldehyde by HPLC should remain the method of choice to permit the separation of malonaldehyde from several other molecules that react with thiobarbutiric acid (Bater and Black, 1987).

Hydrocarbons as Lipid Peroxidation Products

Hofvat et al. in 1964 were the first to experimentally relate hydrocarbon release to lipid peroxidation *in*

vitro. The evolution of hydrocarbon in the process of lipid oxidation in fact only a minor pathway. In most *in vitro* tests, it accounts for less than 1% of actual lipid peroxidation. Estimate was made revealing *in vitro* pentane production was in the magnitude of 0.01% of malonaldehyde production. The yield *in vivo* is probably much lower due to other factor, such as metabolism. Tappel and Dillard (1981) reported that pentane yield in the rat *in vivo* was about 0.2 mmol per mol of lipid peroxides. The odor of rancification of butter and other fat containing foods, a consequence of lipid peroxidation, is caused by volatile aldehyde, especially nonenal. Hexanal has been assessed in rat breath as a marker of lipid peroxidation, however, aldehyde have not been reported in human breath, aliphatic hydrocarbon are present instead.

In adult human, methane is the most abundant hydrocarbon exhaled. Its concentration in the breath averaging 0.122 ppm, a much higher than other hydrocarbon. The gas has not been found in infant and children breath, and its concentration in breath increase with age. Methane is produced almost exclusively by methanogenic bacteria in the gut as major end product of carbohydrate and protein fermentation. Therefore, the gas is not a candidate for gas marker of lipid peroxidation.

In previous study using gas chromatography on both ethane and pentane *in vitro* and in animal studies indicated that the sought of peroxidation marker was in favor of ethane over pentane. However, Pitkanen (1992) reported the study on erythrocyte *in vitro* shows pentane was more reliable over ethane and in human study it seems both products were generated from different pathways. Very little data shows utilization the two product as a marker of peroxidation process. Most probably because of different requirement of the sample handling system on GC preparation for both peroxidation products that make it difficult to combine them in one determination.

The predominant polyunsaturated fatty acids in the body are linoleic acid and arrachidonic, which are both of the n-6 family. The n-6 and n-3 ratio in the body is approximately 4:1. Lipid peroxidation would therefore primarily result in the production of pentane. In isolated hepatocytes treated with bromochloromethane, Gee and Tappel (1981) found ethane to be released at a rate approximately 1.5 times higher than pentane. A similar ratio was found by Dillard and Tappel (1979). As ethane is much easier in gas chromatography preparation, it is the preferred marker of lipid peroxidation.

Hydrocarbon Breath Test as a measure of Lipid Peroxidation

With the addition of certain techniques in concentration and purification of gas sample, the gas chromatography technique successfully records several hydrocarbon end products of lipid peroxidation in animal and human subjects. The technique should be conducted very tediously in a relatively long period. The use of photoacoustic spectrometer for the detection of these lipid peroxidation end products would be expected to be much simpler and more sensitive. Moreover, the method is capable in recording the time of changing when variables in treatment are applied.

Small molecule hydrocarbon ethane and pentane have been suggested as noninvasive markers of free-radical induced lipid peroxidation in humans. In *in vitro* studies, the evolution of ethane and pentane as end products of n-3 and n-6 polyunsaturated fatty acids, respectively, correlates very well with other markers of lipid peroxidation and even seems to be the most sensitive test available. In laboratory animals the use of both hydrocarbons as *in vivo* markers of lipid peroxidation has been validated extensively. Although there are other possible sources of hydrocarbons in the body, such as protein oxidation and colonic bacterial metabolism, these apparently are of limited importance and do not interfere with the interpretation of the hydrocarbon breath test.

Theoretically, the production of hydrocarbons relative to that of other end products of lipid peroxidation depends on variables, such as the local availability of iron(II) ions and dioxygen. These two variables are hard

to control. In addition, hydrocarbons are metabolized in the body, which especially influences the excretion of pentane (Pitkanen, 1992).

Relatively low of sensitivity of gas chromatography technique faced another additional problem in the detection of these markers. Because of the extremely low concentrations of ethane and pentane in human breath, which often are not significantly higher than those in ambient air, the hydrocarbon breath test requires a flawless technique regarding such factors as: (1) the preparation of the subject with hydrocarbon-free air to wash out ambient air hydrocarbons from the lungs, (2) the avoidance of ambient air contamination of the breath sample by using appropriate materials for sampling and storing, and (3) the procedures used to concentrate and filter the samples prior to gas chromatographic determination. For the gas chromatographic separation of hydrocarbons, open tubular capillary columns are preferred because of their high resolution capacity. Only in those settings where expired hydrocarbon levels are substantially higher than ambient air levels might washout prove to be unnecessary, at least in adults. Although many investigators have concentrated on one marker, it seems preferable to measure both ethane and pentane concurrently (Chandra and Spencer, 1963).

The results of the hydrocarbon breath test are not influenced by prior food consumption, but both vitamin E and b-carotene supplementation decrease hydrocarbon excretion (Tappel and Dillard, 1981). Nevertheless, the long-term use of a diet high in polyunsaturated fatty acids, such as in parenteral nutrition regimens, may results in increased hydrocarbon exhalation. Hydrocarbon excretion slightly increases with increasing age. Short-term increases follow physical and intellectual stress and exposure to hyperbaric dioxygen. Several other factors require further evaluation, including normal ranges in infants and children and the effects on the test of altered diffusion and local lipid peroxidation as a consequence of lung disease. The test seems to be unreliable in smokers, because smoking cigarettes results in impressive increase in ethane and penthane exhalation.

Hydrocarbon excretion is increased in a great variety of conditions in which lipid peroxidation was thought to be involved, which confirms both the reliability and the nonspecific nature of the test. Abnormal excretion has been documented in alcoholic and cholestatic liver disease, vitamin E deficiency, pulmonary disease, autoimmune disease, inflammatory bowel disease, ischemia-reperfusion injury, and neurologic disease. In many, if not most, conditions, increased lipid peroxidation is an epiphenomenon instead of playing a pathogenetic role. Therefore, the results of the hydrocarbon breath test should not be regarded in isolation but in the light of clinical and laboratory parameters, including other markers of lipid peroxidation (Cadenas *et al.*, 1982).

Kneepkens (1994) concluded that the hydrocarbon breath test, being noninvasive, has great potential for the assessment of the role of lipid peroxidation in clinical conditions, as well as for the detection and follow-up of lipid peroxidation-induced disease in clinical practice. Because the test is time-consuming and requires a flawless technique, it is yet unclear whether the test will ever proceed to become a clinical tool.

Laser based photoacoustic spectrometer

Effect of photoacoustic (PA) is generated upon conversion of light into acoustical energy. A gas absorbing a beam of light (i.e. infrared light as use in CO_2 laser) experiences a local temperature rise that is due to transfer of rotational and vibrational energy into translational energy. Modulating the light intensity generate temperature fluctuations and hence pressure fluctuations that can be recorded with a help of microphone. The intensity of the generated sound is proportional to the concentration of the absorbing trace molecule and to the light intensity and therefore high power light sources, such as lasers, are required for sensitive trace gas detection. As a detection cell a PA cell in the form of a resonator is operated reducing acoustical interference from surroundings and amplifying the acoustical signal.

When the instrument was used to measure gas emission from fruit, the fruit sample is covered in a closed cuvette which has an inlet and outlet lines. A gas carrier, usually compressed air, carries volatile compounds released by the sample to the outlet line. Gas in the outlet line heading to a cold trap removing interfering compounds (mainly water), subsequently, the gas flow enters the PA cell. When the sample is in a gas form, the gas could be injected directly to the line that goes to PA cell. By recording the PA signal on various laser lines multi-component gas mixtures can be analyzed. The spectrum of CO_2 laser line is presented in Figure 1. The spectrum of the CO_2 laser light (the infra red region) consists of 4 groups, namely group10 P (40 lines), 10R (28 lines), 9P (36 lines), and 9R (26 lines). The line or peak increase in height as additional energy enter as contribution of transition during the decay from excited state into ground state of the corresponding gas. For example, the presence of ethylene gas in the PA cell increases 10P 14 peak line as the energy released during the decay (transition of potential energy from excited state to its ground state) contributes to the spectrum of laser light (Harren. 1997 and Persijn. 2000).

In addition to the light source and the PA cell, the spectrometer is equipped with traps to clean the incoming gas carrier from gases that interfere with the intended signal or with validity of the data. CO_2 in the gas carrier interferes the PA signal, while hydrocarbon such as external ethylene interferes the data. The time response of the system depends on the number of compounds that needs to be analyzed, which approximately one minute per compound is required.

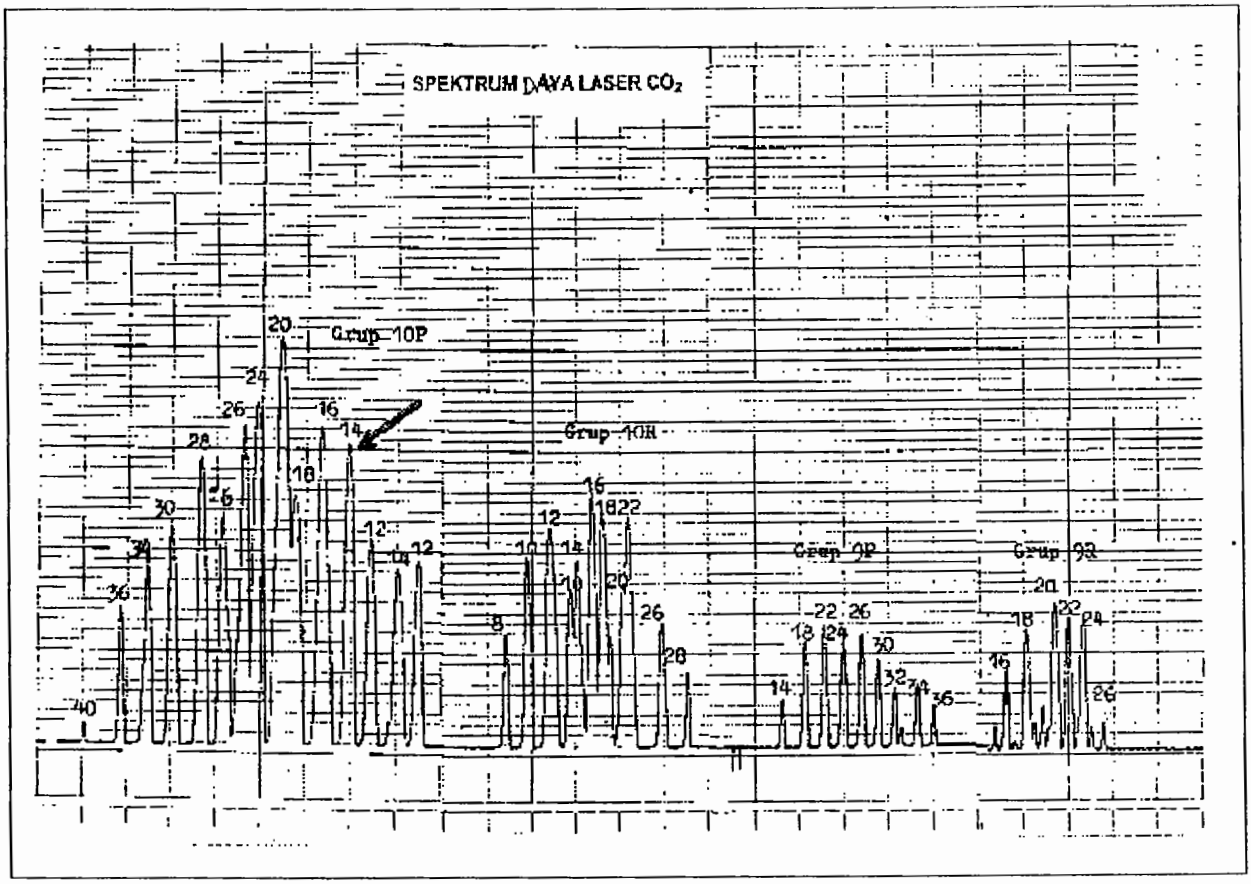

Figure 1. Spectrum of CO2 Laser Lines

The laser base PA spectrometer is capable of detecting several gases in a very low detection limit. This instrument offers *high sensitivity* and *fast time-response* for detection of several small molecule gases that absorbs light at infra red region. When the spectrometer is connected to several other instruments, such as several size of cuvettes, gas traps for gas clean-up system and computer, the instrument is very suitable for monitoring gas evolution over time from several samples. It offers a suitable tool for kinetic analysis of agricultural, medical and environmental research areas.

Several small gases that absorb light in infra red region showing peaks in the laser spectrum, and detectable by PA spectrometer. Table 1 representing several gases detectable by PA spectrometer with their corresponding detection limit as reported by Persijn *et.al.*, (2001). Ammonia, carbon disulfide, ethylene, and ozone are among gases that strongly absorb light in infra red region, and therefore have very low detection limit. For methane, ethane, ethanol and pentane, although the PA seems to have higher detection limit for these gases, it is still 1000 times more sensitive over gas chromatography.

Table 1. Detection limits of gases in laser based photoacoustic spectrometers *)

Name	Formula	Detection limit (ppb**)
Acetaldehyde	CH_3CHO	0.3
Ammonia	NH_3	0.02
Carbon disulfide	CS_2	0.01
Dimethyl sulfide	$S(CH_3)_2$	1
Ethane	C_2H_6	1
Ethanol	C_2H_5OH	3
Ethylene	C_2H_4	0.02
Methane	CH_4	1
Nitric oxide	NO	1
Ozone	O_3	0.05
Pentane	$CH_3(CH_2)_3CH_3$	3
Water vapour	H_2O	30

*) Adapted from Persijn, 2001. **) 1 ppb = $1:10^9$

Any of the above gases would be easily detected and monitored using FA spectrometer. *The instrument would be very useful in the peroxidation detection if any of the above gases is significant part of the lipid peroxidation product and no other mechanism contributes to the development of the gas.*

Snap-shot measurement of gases

Snapshot measurement of gases could be detected very easily in 2 minutes of time. Snap-shot measurements of ethylene emission rate of over 50 tropical fruits of Indonesia were performed using sample collected from local market in ready to consume stage. The measurement is done directly on a single fruit, which did not require accumulation time as that in gas chromatography technique. Emission rate of tropical fruit ranging from 0.1 nl/hr/fruit (citrus fruit) to 5000 nl/hr/fruit (soursop), while avocado emitted 4700 nl/hr/fruit or 33.3 nl/hr/g of fruit and banana emitted 150 nl/hr/fruit or 0.9 nl/hr/g of fruit (Suparmo,1998b and Persijn, 2001).

Recording dynamic biological changes

Emission of ethylene of fruit changes as affected by maturity process and environmental factors (Figure 2). Enzymatic synthesis of ethylene is an oxygen dependent is demonstrated in Figure 2A, while enzymatic synthesis process is affected by temperature is proved in Figure 2B. On Fig. 2A, ethylene emission runs normally as oxygen is used as gas carrier. Changing the gas carrier from oxygen to N_2 ceased the emission of ethylene showing proof that ethylene synthesis is oxygen dependent process. Changing the two gases on and off produces up and down in ethylene emission from the fruit sample. Ethylene emission stop when the carrier was changed into Nitrogen and the emission resumes when the carrier was changed back to oxygen again.

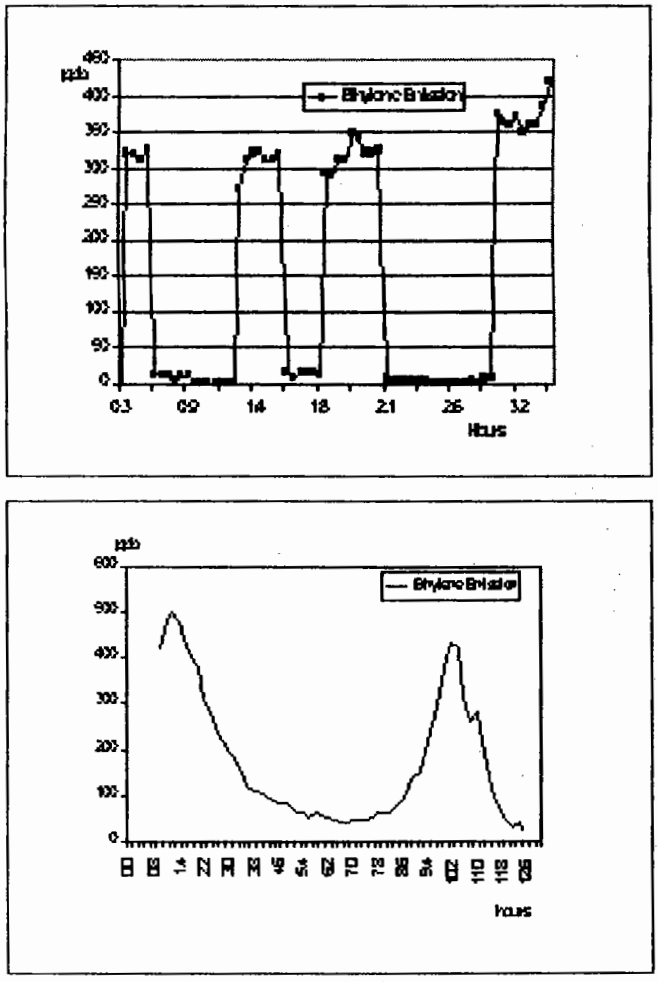

Figure 2. Dynamic changes of ethylene emission by fruit as affected by atmospheric composition (A) and temperature (B).

On Fig. 2B, (the temperature scale not shown in the graph) the first peak showing the start at 40°C cooling down to refrigeration temperature about 5°C and then up again reaching the second peak at 45°C and up to 60°C. Ethylene emission (shown on left scale) indicates that lowering temperature from ambient to refrigerator temperatures slowed down ethylene emission. The rate of ethylene emission was immediately restored as the temperature risen, however, when the temperature reached above the limit of it's tolerance level (45°C), the emission when down as temperature increases indicates capability to synthesize ethylene was impaired and never reached the normal level again. These findings demonstrated that gas detection of ethylene is much simpler and straight forward than the old enzymatic procedures which are very tedious and time consuming, while gas chromatography procedure is certainly fail to produce these findings. Although the measured compounds are released at relatively high rates the use of this spectrometer is justified since the fast time response of the spectrometer is needed to follow the quickly changing release rates that reflects changing in metabolism pattern.

UV-radiation damage on human subject

Research in Catholic University of Nijmegen,The Netherlands, successfully utilize the PA spectrometer in the detection of ethylene gas emission on human subject. The effect of UV-radiation on skin can be measured directly on the skin, or via analysis of the exhaled air. For measurements on breath, a commercial solar bench is used with a well defined spectrum of UV-A and UV-B. While the test person lies down and breathes clean air the exhale air is channeled into into the photoacoustic detector. Using a skin cuvette, which is equipped with inlet and outlet channels, a smaller area of the skin can be illuminated using a facial browner. The outlet air is directly channeled into the photoacoustic detector. Result indicates ethylene emission increase substantially upon UV radiation both in breath and skin analyses. The most interesting result in this finding is the fast and sharp responds of ethylene rise (from zero to 4 ppb) and cease upon exposure of UV radiation and the capability to produce result of such effect in a non-invasive way.

CONCLUSION

In future study such photoacoustic spectrometers will certainly be very useful to study trace gas emissions. The most important feature of the PA spectrometer is its extraordinary low detection limit that enable fast time response to follow the quickly changing gas-release rates in a non invasive way.

REFERENCES

Banni, S. Salgo, M.G.; Evans, R.W.; Coriongiu, F.P.; Lombardi, B. 1990. Conjugated diene and trans fatty acids in tissue lipids of rats fed an hepatocarcinogenic choline-devoid diet. *Carcinogenesis* **11**: 2053-2057

Bartoli, G.M.; Muller, A.; Cadenas, E.; Sies, H. 1983. Antioxidant effect of diethyldithiocarbamate on microsomal lipid peroxidation studies assessed by low-level chemiluminescence and alkane production. *FEBS Lett.* **164**:371-374,

Bater, C.W.; Black, M.S. 1987. Capillary chromatographic analysis of volatile organic compounds in the indoor environment. *J.Chromatogr. Sci.* **25**:60-64

Beaudry, R.M., 1999. The effect of O_2 and CO_2 partial pressure on selected phenomena affecting fruit and vegetable quality. Postharvest Biol. Technol. 15: 293-303

Beaulieu, J.C., Peiser, G, Salveit, M.E. 1997. Acetaldehyde is a causal agent responsible for ethanol induced ripening inhibition in tomato fruit, Plant Physiol. 133: 431-439

Burg, S.P. 1962. The physiology of ethylene formation, Annu. Rev. Plant Physi. 13: 265-302

Cadenas, E.; Wefers, H.; Muller, A.; Brigelius, R.; Sies, H. 1982. Active oxygen metabolites and their action in the hepatocyte: Studies on chemoluminescence responses and alkane production. *Agents Action* **11**(Suppl): 203-216

Chandra, G.R.; Spencer, M. 1963. A micro apparatus for absorption of ethylene and its use in determination of ethylene in exhales gases from human subjects. *Biochym. Biophys. Acta* **69**:423-425

Dillard, C.J.; Tappel, A.L. 1979. Volatile hydrocarbon and carbonyl products of lipid peroxidation: A comparison of pentane, ethane, hexanal and acetone as *in vivo* indices. *Lipids* **14**: 989-995

Dormandy, T.L.: Wickens, D.G. 1987. The experimental and clinical pathology of diene conjugation. *Chem. Phys. Lipids* **45**:353-364

Esterbauer, H. 1993. Cytotoxicity and genotoxicity of lipid-oxidation products. *Am. J. Clin. Nutr.* **57**:779S-786S

Fidler, J.C. 1951. A comparison of the aerobic and anaerobic respiration of apples. J. Exp. Bot. 2: 41-64.

Gee, D.L.; Tappel, A. I. 1981. Production of volatile hydrocarbons by isolated hepatocytes: An *in vitro* model for lipid peroxidation studies. *Toxicol. Appl. Pharmacol* **66**:112-120:

Gutteridge, J.M.C.; Halliwell, B. 1990. The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem.Sci.* **15**:129-135

Halliwel, B.; Gutteridge, J.M.C.; Cross, C.E. 1992. Free radicals, antioxidants, and human disease: Where are we now? *J.Lab.Clin.Med.* **119**:598-620

Halliwell, B.; Chirico, S. 1993. Lipid peroxidation: Its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* **57**:715S-125S

Harren, F.J.M, Reuss, J., 1997. Photoacoustic Spectroscopy. In: Encyclopedia of Applied Physics, G.L. Trigg (ed.), VCH Wennheim, Vol. 19, pp. 413-435

Kneepkens, C.M.F, Lepage G., and Roy C.C. 1994. The potential of the hydrocarbon breath test as a measure of lipid peroxidation. Free Radical Biology & Medicine,. Vol 17, No. 2 pp 127-160. Pergamon

Leshem YY, Wills RBH, Ku VVV. 1998. Evidence for the function of the free radical gas - nitric oxide (NO·) - as an endogenous maturation and senescence regulating factor in higher plants,

Plant Physiology and Biochemistry, 36 (11): 825-833

Mattheis, J.P., Buchanan, D.A., Fellman, J.K. 1991. Change in apple fruit volatiles afterstorage in atmospheres inducing anaerobic metabolism, J. Agric. Food. Chem. 39 (9): 1602-1605

Oomens, J, Zuckermann, H, Persijn, S.T., Parker, D.H., Harren, F.J.M., 1998. CO-laser-based photoacoustic trace-gas detection: applications in postharvest physiology, Appl. Phys. B 67 4: 459-466

Paldus, B.A. Spence, T.G., Zare, R.N., Oomens, J., Harren, F.J.M., Parker, D.H., Gmachl, C., Cappasso, F., Sivco, D.L., Baillargeon, J.N., Hutchinson, A.L., Cho, A.Y., 1999, Photoacoustic spectroscopy using quantum-cascade lasers Opt. Lett. 24 (3): 178-180

Peppelenbos, H.W. 1996. The use of gas exchange characteristics to optimize CA storage and MA packaging of fruits and vegetables, Ph.D. Thesis, Landbouw Universiteit Wageningen

Persijn, S.T., Veltman, R.H., Oomens, J., Harren, F.J.M., Parker, D.H. 2000. CO laser absorption coefficients for gases of biological relevance: water, CO_2, ethanol, acetaldehyde, and ethylene, Appl. Spectr. 1: 62-71

Persijn, S.T., Rofandi Hartanto, **Suparmo**, D.H. Parker, F.J.M. Harren . 2001. Application of sensitive trace gas spectrometers in crop storage. Postharvest Biology and Technology (19) 2: 129-137

Pesis, E., Fuchs, Y., Zaubermann., G. 1978. Cellulase activity and fruit softening in avocado, Plant Physiol. 61: 416-419

Pesis, E. 1995. Induction of fruit aroma and quality by post-harvest application of natural metaboltes or anaerobic conditions. In: Fruit analysis, modern methods of plant analysis. Linskens and Jackson (eds), Vol. 18., Springer-Verlag Berlin Heidelberg pp. 19-36

Pitkanen, O.M. 1992. Are ethane and pentane evolution and thiobarbituric acid reactivity specific for lipid peroxidation in erythrocyte membranes? *Scand J. Clin. Lab. Invest.* **52**:379-385

Riely, C.A.; Cohen, G.; Lieberman, M. 1974. Ethane evolution: A new index of lipid peroxidation. *Science* **183**:208-210

Slater, T.F. 1989. Overview of methods used for detecting lipid peroxidation. *Meth. Enzymol.* **105**: 69-81

Smith, M.T.; Thor, H.; Hartzell, P.; Orrelius, S. 1982. The measurement of lipid peroxidation in isolated hepatocytes. *Biochem. Pharmacol.* **31**: 19-26

Suparmo. 1998a. Perubahan emisi etilen beberapa buah tropis sebagai respons terhadap keadaan anoksid dan suhu yang diukur menggunakan spektrometer fotoakustik. Seminar Nasional Pangan. Bandung

Suparmo. 1998b. Laju emisi etilen buah-buahan tropis yang diukur menggunakan spektrofotometer fotoakustik. Prosiding Seminar Nasional Teknologi Pangan dan Gizi – Yogyakarta

Suparmo. 2002. Kadar Etilen Eksogen Pemicu Pematangan Buah Pisang Serta Perubahan Sifat Fisik Buah. Proc. Seminar Nasional Persatuan Ahli Teknologi Pangan Indonesia. Malang. A-111 – A117

Suparmo, 1998. Ethylene emission rate of tropical fruits measured using Photoacoustic Spectrometer. Proc. National Seminar of Food and Nasional Teknologi Pangan dan Gizi-Yogyakarta, hal : 398-404

Tappel, A.L.; Dillard, C.J. 1981. *In vivo* lipid peroxidation: Measurement via exhaled pentane and protection by vitamin E. *Fed. Proc.* **40:** 174-178

Veltman, R.H., Kho, R.M., van Schaik, A.C.R., Sanders, M.G., Oosterhaven, J. 2000. Ascorbic acid and tissue browning in pears (*Pyrus communis* L. cvs Rocha and Conference) under controlled

atmosphere conditions, Postharvest Biology and Technology (19) 2: 129-137

de Vries, H.S.M., Wasono, M.A.J., Harren, F.J.M., Woltering, E.J., van der Valk H.C.P.M., Reuss, J. 1996. Ethylene and CO_2 emission rates and pathways in harvested fruits investigated, in situ, by laser photodeflection and photoacoustic techniques, Postharvest Biology and Technology 8: 1-10

Wasono M.A.J., Muslim, Tranggono, **Suparmo**. 2000. Penerapan Spektrometer Fotoakustik Laser CO_2 Ekstrakavitas pada monitoring etilen buah pisang dalam kondisi atmosfir terkendali. Prosiding Simposium Fisika Nasional XVIII, April 2000: 433-439.

Wasono M.A.J., Muslim, Tranggono, **Suparmo**. A CO2 Laser Photoacoustic Spectroscopy in Trace Gas Monitoring of Tropical Fruits. Proc. Of the International Conf. on Opto-electronics and Laser Applications. ICOLA'02 , 2002, Jakarta.

Wasono M.A.J. 2000. Penerapan Teknik Spektroskopi Fotoakustik pada Penyelidikan Pengaruh Perlakuan Panas terhadap Buah Mangga Tropis (*Mangifera indica* var. Arum Manis). Journal Fisika Indonesia. 14- IV:51-63.

Wasono M.A.J., Muslim, Tranggono, **Suparmo**. Penerapan Spektrometer Fotoakustik Laser CO2 Semi *Sealed-off* pada Penentuan Koefisien Resistensi Buah Tertentu terhadap Difusi C_2H_4 menggunakan Gas SF_6 Sebagai Pelacak.. Prosiding Pertemuan dan Presentasi Ilmiah Penelitian Dasar Ilmu Pengetahuan dan Teknologi Nuklir P3TM-BATAN Yogyakarta, 2002

Woltering, E.J., Harren, F.J.M, Boerrigter, H.A.M. 1988. Use of a laser driven photoacoustic detection system for measurements of ethylene production in Cymbidium flowers, Plant Physiol. 88: 506-510

Zidenberg-Cherr, S. Olin, K.L.; Villanueva, J.; Tang, A.; Phinney, S.D.; Halsted, C.H.; Keen, C.L. 1991. Ethanol-induced changes in hepatic free radical defence mechanisms and fatty-acid composition in the miniature pig. *Hepatology* **13**:1185-1192