Photoacoustic Spectrometry: A Potential Tool For Future Antioxidant Test

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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 color 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 specific 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 antioxidant challenges test.

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

INTRODUCTION

Polyunsaturated fatty acids are found mainly as components of membrane lipids both cellular and subcellular. 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 was demonstrated clearly in in vitro studies. Several reaction products are formed, which, among others, are able to propagate further lipid peroxidation process. Depending on the presence of peroxidizing products, dioxygen, and metal catalyst, a great variety of lipid radicals and more or less stable oxidation products may arise. It is among the overwhelming abundance 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 extent 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 some dollars from digging into traditional old medicine recipes and convert them into a new good locking tablet. Although the proof of the claimed health benefit was not substantiated clearly over the range of the application, such commodities are still salable very strongly. Many more ethnic medicine 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 antioxidant effect are available, however, each of them has its own limitation or even draw backs. Regulation mandates, in particular, the test of antioxidant in animal or human assay is difficult to interpret. Simpler human eat, 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 conditions, 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 an in vitro test, however, in an 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.

Photocoustic (PA) Spectrometer operates based on conversion of light energy to acoustical energy. A gas absorbing a beam of light arises 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 exhaled from the lungs is more preferable over these requiring blood or urine samples for the case of both the subject and the analytical procedure. This paper suggesting the use of photocoustic 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 Method for Peroxidation Test
Lost of polymer saturated fatty acids from a biological system is a simple and useful method for the detection of peroxidation process in vivo. 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 new index indicating loss of polyunsaturated fatty acids (Halliwell 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 (Barni et al., 1990, and Gutteridge and Halliwell, 1990).

The most abundant conjugated diene in human plasma is cis-9,trans-11-octadecadienoic acid, a diene derivative of cis-9, cis-12 cis-linoelitic 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 thiobarbituric 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 involves thiobarbituric acid-reactive material which overestimates lipid peroxidation compared to spectrometric detection of lipid hydroperoxides (Zdenek-Cier et al., 1991).

Malonaldehyde and 4-hydroxyxynonenal 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-Hydroxyxynonenal and other aldehydes, including malonaldehyde, are immunogenic and cytotoxic due to their ability to react with several molecular species, such as amino acids. The 4-hydroxyxynoanal has been shown to inhibit DNA as well as protein synthesis and to inhibit various enzyme systems. Furthermore, biological effects, such as the inductive 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 thiobarbituric 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 conformation with other markers of lipid peroxidation. The thiobarbituric assay for malonaldehyde or "thiobarbituric 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 thiobarbituric acid (Bate 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 is 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 fomaldehyde production. The yield in *in vitro* 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 ranntication of butter and other fat containing foods, a consequence of lipid peroxidation, is caused by volatile aldehyde, especially acrolein. Hexanal has been assessed to 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 averaged 0.122 ppm, 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 studies using gas chromatography on both ethane and pentane in *in vivo* 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 excretoxy in *in vivo* showed pentane was more reliable than ethane and in human study it seems both products were generated from different pathways. Very little data shows utilization of the two products 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 order 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, Go 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 marker; of free radical induced lipid peroxidation in humans. In *in vivo* studies, the evolution of ethane and pentane as end products of n3 and n6 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
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 result 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 pentane 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 pathogenic 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).

Koopmans (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 CO2 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 line. A gas carrier, usually compressed air, carries volatile compounds released by the sample to the outlet line. Gas in, the outlet line leading 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 CO₂ laser line is presented in Figure 1.

The spectrum of the CO₂ laser light (the infra red region) consists of 4 groups, namely group 10 F (40 lines), 10 R (25 lines), 9 F (36 lines), and 9 R (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 10 F 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 (Hurren, 1997 and Pevijn, 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. CO₂ is 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](image-url)
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 in 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 100 times more sensitive over gas chromatography.

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Detection limit (ppb**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaldhey</td>
<td>CH₃CHO</td>
<td>0.3</td>
</tr>
<tr>
<td>Ammonia</td>
<td>NH₃</td>
<td>0.02</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>CS₂</td>
<td>0.01</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>S(CH₃)₂</td>
<td>1</td>
</tr>
<tr>
<td>Ethane</td>
<td>C₂H₆</td>
<td>1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>C₂H₅OH</td>
<td>3</td>
</tr>
<tr>
<td>Ethylene</td>
<td>C₂H₄</td>
<td>0.02</td>
</tr>
<tr>
<td>Methane</td>
<td>CH₄</td>
<td>1</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO</td>
<td>0.05</td>
</tr>
<tr>
<td>Ozone</td>
<td>O₃</td>
<td>1</td>
</tr>
<tr>
<td>Pentane</td>
<td>CH₃(CH₂)₂CH₃</td>
<td>3</td>
</tr>
<tr>
<td>Water vapour</td>
<td>H₂O</td>
<td>30</td>
</tr>
</tbody>
</table>

(*) Adapted from Persijn, 2001. (**) 1 ppb = 1 x 10⁻⁹

Any of the above gases would be easily detected and monitored using PA 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.

Snapshot measurement of gases

Snapshot measurement of gases could be detected very easily in 2 minutes of time. Snap-shot measuremenets of ethylene emission rate of over 59 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 is in gas chromatography technique. Emission rate of tropical fruit ranging from 0.1 nl/h/fruit (citrus fruit) to 5000 nl/h/fruit (couscop), while avocado emitted 4700 nl/h/fruit or 33.3 nl/h/g of fruit and banana emitted 150 nl/h/fruit or 0.8 nl/h/g of fruit (Suparno, 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 rate normally as oxygen is used as gas carrier. Changing the gas carrier from oxygen to N₂ 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.
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 refrigeration 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 its tolerance level (45°C), the emission when down as temperature increased 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 straightforward than the old enzymatic procedures which are very tedious and time consuming, while gas chromatography procedure is certainly fast 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 is Catholic University of Nijmegen, The Netherlands, successfully utilize the PA spectrometer in the detection of ethylene gas emissions on human subjects. 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 exhaled air is channelled into the photacoustic detector. Using a skin cuvette, which is equipped with inlet and outlet channels, a smaller area of the skin can be illuminated rising a facial browser. The outlet air is directly channelled into the photacoustic detector. Results indicate 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 response 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 photacoustic 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.

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