

Application of FTIR Spectroscopy in the Determination of Antioxidant Efficiency in Sunflower Oil

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Abstract: Atmospheric oxygen can react spontaneously with lipids and other organic compounds causing structural degradation, which is ultimately responsible for the loss of quality in several chemical or natural products of economic or industrial importance. However, this oxidation process can be prevented or retarded by the addition of synthetic or natural antioxidants. A rapid and precise method for the evaluation of antioxidant activity in sunflower oil using Fourier transform infrared (FTIR) spectroscopy was developed. It was found that the intensity of the hydroperoxide absorption band in the infrared spectra was increased proportionally with the increase of hydroperoxide concentration, hence the activity of an antioxidant can be assessed by measuring its ability to inhibit hydroperoxide formation. Caffeic acid, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), Catechin, Gallic acid and TBHQ were examined for their antioxidant activities. The radical scavenging activity (RSA%) of the tested antioxidants using DPPH^e (1,1-diphenyl-2-picrylhydrazyl) assay was also measured. It was found that the FTIR spectroscopic technique could be applied well in the determination of the antioxidant activity of synthetic or natural antioxidants in sunflower oil.

Key words: Antioxidants, antioxidant activity, sunflower oil, oxidation, fourier transform infrared, DPPH, RSA%.

INTRODUCTION

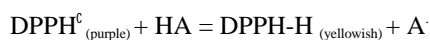
Oxidation of lipids in foods produces undesirable effects, including loss of fat-soluble vitamins, generation of off-flavors, palatability problems and even production of toxins that cause food poisoning^[1]. This oxidation leads to the formation of several products such as diene conjugates, malondialdehyde, 4-hydroxynonenal, volatile gases and hydroperoxides that can act as oxidation indicators. The peroxide value generally serves as a useful indicator of the extent of oxidation of lipids, fats and oils. An advantage of peroxide value determination is that it directly measures the lipid peroxides, which are the primary lipid oxidation products^[2].

The AOAC Official Method, 1990^[3] and the American Oil Chemists' Society method, 1989^[4] (both of which determine peroxide by using iodometric titration) lack sensitivity and require large amount of lipid. To improve the drawbacks of the official methods, a number of new methods were developed. The classical method for the determination of liberated iodine, titration with thiosulfate, has been replaced by iodine estimation as the triiodide anion^[5]. Another adaptation of the iodometric method is the determination of liberated iodine by coulometry^[6].

Spectrophotometric determination of ferric ions formed by the oxidation of ferrous ions by peroxides in the presence of xylenol orange (3,3'-bis [*N,N*-di {carboxymethyl}-amino methyl]-*o*-cresolsulphonaphthalein), also termed as the FOX (ferrous oxidation-xylenol orange method) has been used for determination of lipid hydroperoxides in liposomes and low-density lipoproteins^[7-9]. The International Dairy Federation (IDF) method^[10] for peroxide determination is a spectrophotometric method based on the ability of peroxides to oxidize ferrous ions to ferric ions. The method is limited to determination of peroxides in anhydrous milk fat.

Food manufacturers continue to add antioxidants during food processing to minimize lipid oxidation. An antioxidant may be defined as "a substance when present at low concentrations compared with those of an oxidizable substrate such as fats, proteins, carbohydrates or DNA, significantly delays or prevents oxidation of the substrate"^[11]. Antioxidants can act at different mechanisms in the oxidative sequence that involves lipids. For example, they may act by decreasing localized oxygen concentrations; preventing first-chain initiation by scavenging free radicals, such as hydroxyl radicals; binding metal ions in forms that will not generate new

radicals, and/or will not decompose lipid peroxides to peroxy or alkoxy radicals; decomposing peroxides by converting them to non-radical products, such as alcohols, and/or by chain-breaking whereby intermediate radicals, such as peroxy and alkoxy radicals, are scavenged to prevent continued hydrogen abstraction^[11]. The main mechanism of action of phenolic antioxidants (AH) is considered to be the scavenging of free radicals by hydrogen-donation, although other mechanisms may be involved^[12]. Much effort has been devoted to the development of procedures based on reaction of AH with different radical species of biological significance such as O₂^{·-}, OH[·], NO[·], or lipid peroxy radical LOO[·]^[13-15]. Tests using the reduction of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH[·]) in the presence of phenolic compounds are also quite popular among food scientists^[16-18]. These tests are based on the decrease of the absorbance of the radical solution according to the reaction:



This radical has been used for many decades to study the main mechanism of hydrogen-atom donation to free radicals from certain substrates or the antioxidant activity of compounds carrying -SH, -OH, and -NH groups^[19-23].

The infrared region of electromagnetic spectrum extends from 14000 to 50 cm⁻¹ and is divided into three areas: the far-infrared from 400 – 50 cm⁻¹; the mid-infrared region from 4000-400 cm⁻¹, which is a very interesting region of the spectrum for the study of organic compounds because the absorption bands are due to the vibration (vibrational spectroscopy) of a particular functional grouping; and the near infrared (NIR) from 14000 – 4000 cm⁻¹^[24]. The mid-infrared spectroscopy has been commonly used for the structural identification or qualitative determination of the 'fingerprint' of organic compounds because their functional groups display characteristic vibrational absorption frequencies in this infrared region of electromagnetic spectrum. The exact location of the corresponding bands depends on the influence of the rest of the molecule. Moreover, mid-infrared spectroscopy is useful in quantitative analysis applications. The intensities of the bands in the spectrum are proportional to the concentration of their respective functional groups as Lambert-Beer's law shows $A = \epsilon bc$, where A is the absorbance of the band, b is the path length, ϵ is a molar proportionality constant called molar absorptivity which is characteristic of each functional group and c is the concentration of the functional group.

Lipids study is a subject that may strongly profit from this spectroscopic technique, because they are composed of functional groups showing characteristic absorption bands in this region of electromagnetic spectrum^[25-27].

Evaluation of antioxidant activity of either synthetic or natural product usually goes through two main routes. First, measurement of its radical scavenging activity (e.g. with DPPH assay) and second, determining its efficiency in inhibiting primary or secondary oxidation products. As far as we know, there is a lack in the publication about the assessment of antioxidant activity using infrared spectroscopy. The aim of this study is to propose a new method using FTIR spectroscopy that may be used in evaluating antioxidant activity and which is more accurate and reproducible than other traditional methods.

MATERIALS AND METHODS

Standard reagents and solvents: Caffeic acid, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), Catechin, Gallic acid, DPPH[·] (1,1-diphenyl-2-picrylhydrazyl) and TBHQ were bought from Sigma-Aldrich GmbH, Steinheim, Germany.

All other used chemicals were either Analar or of analytical grade.

Estimation of radical scavenging activity (RSA %): The RSA% of the selected antioxidants was based on the method of Pekkarinen *et al*^[28] as described by Nenadis and Tsimidou^[22]. The decrease of the absorbency at 516 nm of DPPH[·] solution after addition of the antioxidant was measured in a glass cuvette (1cm width). An aliquot (2960µL) of 0.3 mM methanolic DPPH[·] solution was mixed with 40µL of 1mM methanolic antioxidant solution. The absorption was monitored at the start (t=0) and after 10 min (t=10). The results are expressed as RSA%:

$$\text{RSA\%} = \frac{\text{Abs}_b(t=0) - \text{Abs}_s(t=10)}{\text{Abs}_b(t=0)} \times 100$$

Where:

Abs_b = absorbance of blank (DPPH solution alone)

Abs_s = absorbance of sample (antioxidant + DPPH solution)

Sample preparation and oxidation experiment: Samples (25 g sunflower oil) in dark glass bottles were placed in dark in an air-circulating oven at 70 ± 1 °C over a 10-days period. Duplicate samples were withdrawn daily for iodometric determination of PV by AOCS method^[29] and duplicate samples were withdrawn for FTIR measurements.

FTIR instrumentation: All the FTIR measurements were carried out using a single beam Fourier Transform Infrared Spectrometer, FT/IR-430, Jascow, Japan. This instrument is equipped with a ceramic IR source, KBr beam splitter and DLATGS IR detector. The measurement wave range for this instrument is from 7800 to 400 cm^{-1} and the signal to noise ratio at resolution 4 cm^{-1} is $10^4 : 1$ at near 2200 cm^{-1} . The FTIR spectra of the samples were obtained in the spectral range 4000 to 400 cm^{-1} with a scanning speed of 2 mm/sec and resolution 4 cm^{-1} . The number of scans was set to "Auto".

Samples preparation for FTIR measurements: Duplicates of 5 μl of each oil sample were sandwiched between two transparent KBr disks. In this method, weights of 100 mg of KBr (IR grade) were gently ground in a special mortar for 30 sec. and then pressed into disks by a hydraulic pressing system (Riken Power, Riken Seiki Co LTD., Japan) using a 10 mm Pellet Die (Jascow, Japan). An oil rotary vacuum pump (Ulvac, Sinku Kiko Co., LTD., Japan) was used to evacuate air and moisture from the Die during the pressing of disks.

Processing of FTIR spectra: The FTIR spectra were processed using the "Spectra Analysis" computer program, which is included in the instrument software "Jascow Spectra Manager for Windows 95". By using this program, the positions and intensities of the IR bands were determined correctly. In addition, the peak height, peak area, half band width and absorbance ratios could be processed.

Fourier Transform Infrared vs. Iodometric PV: For validation of the infrared method to replace traditional (iodometric) PV method in evaluating antioxidant efficiency, a relation between FTIR hydroperoxide band intensities was plotted versus iodometric PV (meq. O_2/kg oil). A serial dilution of rancid sunflower oil of 200 meq. O_2/kg oil with fresh sunflower oil of peroxide zero was made and their corresponding FTIR spectra were recorded.

RESULTS AND DISCUSSIONS

The five antioxidants used, namely, Caffeic acid, Trolox, Catechin, Gallic acid, and TBHQ were examined for their radical scavenging activity (RSA%) using the DPPH assay as well as for their ability to inhibit hydroperoxide formation in the oil substrate (sunflower oil).

The decreasing order of RSA% was Gallic acid > Caffeic acid > Catechin > Trolox > TBHQ (Figure 1).

Table 1: The efficiency of different antioxidants as measured iodometrically in sunflower oil.

Samples	Antioxidant Efficiency (%)
Control	0.00
Trolox	96.58
TBHQ	96.17
Catechin	91.66
Gallic acid	87.13
Caffeic acid	75.06

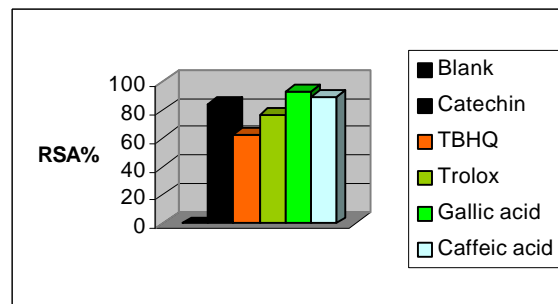


Fig. 1: Radical scavenging activity of selected antioxidants as measured by DPPH (1,1-diphenyl- β -picrylhydrazyl) assay.

The oil samples were submitted to oxidative conditions in dark in an air-circulating oven at 70 ± 1 $^{\circ}\text{C}$ over a period of 8-days. The peroxide value of all oil samples were determined periodically by the iodometric method as well as by recording their FTIR spectra. Fig. 2 shows the PV (meq. O_2/kg oil) over the period of oxidation.

Table 1 reveals that the decreasing order of the antioxidant efficiency was Trolox > TBHQ > Catechin > Gallic acid > Caffeic acid.

The antioxidant efficiency order was somewhat different from that of radical scavenging activity measured by DPPH assay mentioned above as the latter, although is a rapid screening test but it is influenced by the solvent used and the ability to form hydrogen bonding between the antioxidant and solvent molecules^[22].

Fig. 3 shows the infrared spectrum of non-oxidized sunflower oil with a very small band near 3470 cm^{-1} associated with the overtone of the glyceride ester carbonyl absorption. The band of hydroperoxide functional group near 3444 cm^{-1} in the non-oxidized oil was too small to be detected because hydroperoxides concentration was still low. The main characteristic absorption bands as displayed in the FTIR spectrum of sunflower oil is shown in Table 2.

As the oxidation progressed, the concentration of the hydroperoxide groups increases and its absorption band also increases. The hydroperoxide group gives a broad band which overlaps with that of the overtone of the

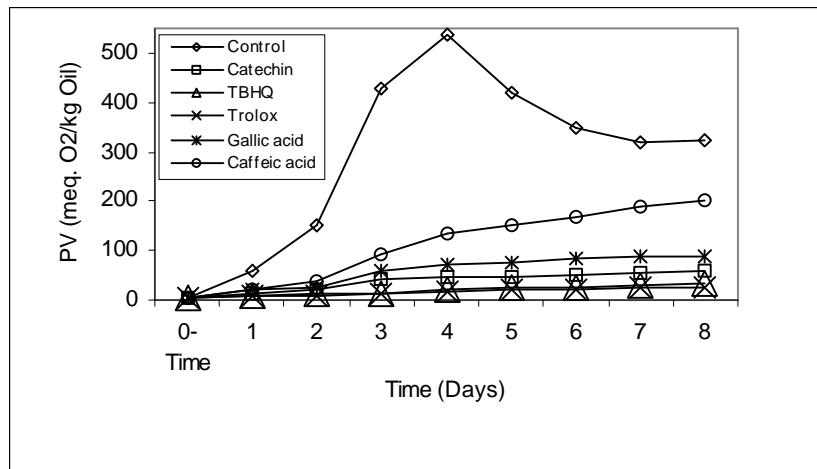


Fig. 2: PV (in meq. O₂/ kg oil) of the oil samples with and without antioxidant addition over the oxidation period (in days).

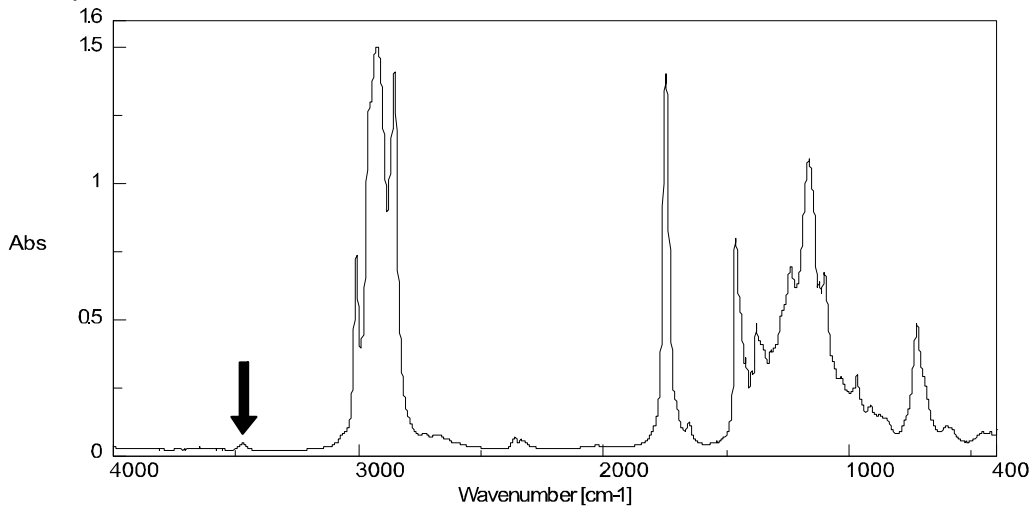


Fig. 3: FTIR spectrum of control (before oxidation) sunflower oil. (The arrow Points to the overtone of the glyceride carbonyl ester).

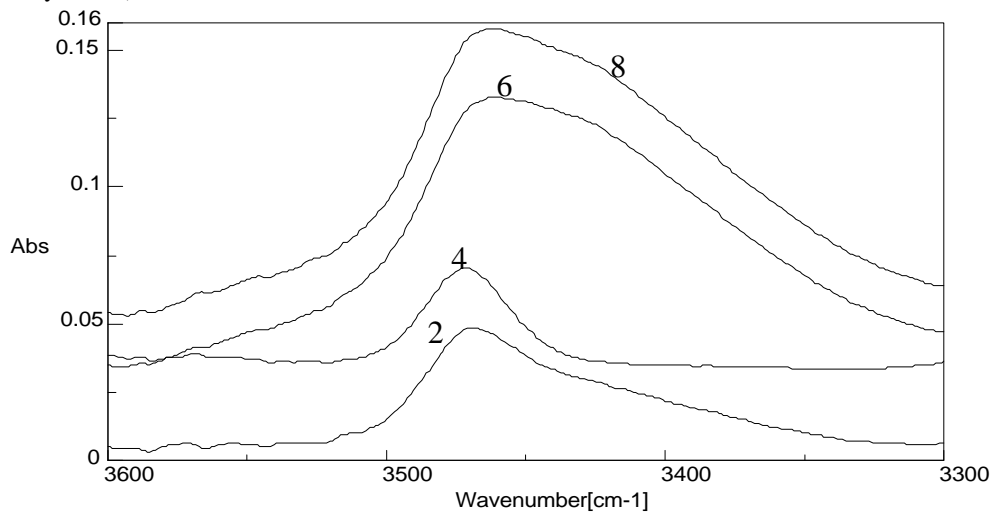


Fig. 4. FTIR spectra (3600-3300 cm⁻¹) of control sunflower oil (without antioxidants) during eight days of oxidation.

Table 2: shows the main characteristic absorption bands as displayed in the FTIR spectrum of sunflower oil which in agreement with that reported by Guillén, and Cabo^[30].

Frequency (cm ⁻¹)	Assignment & mode of vibration	Relative Intensity
3470	Overtone of -C-O ester	Weak
3006	Stretching of -C-H (cis)	Medium
2925	Asymmetric stretching of -C-H (CH ₂)	Very strong
2855	Symmetric stretching of -C-H (CH ₂)	Very strong
1746	Stretching of -C-O ester	Very strong
1650	Stretching of -C-C- (cis)	Very strong
1465	Bending (scissoring) of -C-H (CH ₂ , CH ₃)	Medium
1165	Stretching, bending of -C-O, -CH ₂	Strong
725	Bending (rocking) of - (CH ₂) _n -, -HC-CH-(cis)	Medium

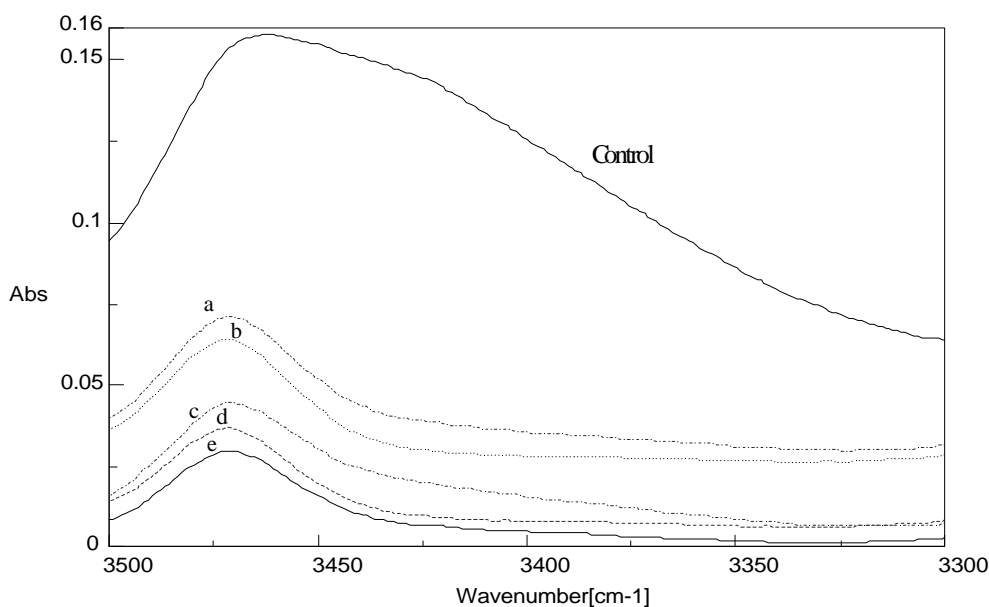


Fig. 5: FTIR spectra (3600-3300 cm⁻¹) of oxidized sunflower oil with and without antioxidants after 5 days oxidation; (control) oil without antioxidant, (a) with Caffeic acid, (b) with Gallic acid, (c) with Catechin, (d) with TBHQ and (e) with Trolox.

glyceride ester groups producing a decreasing in the frequency value of the glyceride original band together with an increasing in its absorbance. Frequency and absorbance of this band can give information about the hydroperoxide generation throughout the oxidation process^[24,30].

Fig. 4 shows a magnification of the spectral range 3600 – 3300 cm⁻¹ in the FTIR spectra of the oil samples exposed to oxidation (during days) without addition of antioxidants. The hydroperoxide band showed increased intensity and shifted towards shorter wave number (from ~3470 - 3460 cm⁻¹). So, we can make use of this phenomenon in evaluating the antioxidant efficiency of

any substance by measuring its ability to inhibit hydroperoxide formation, which can be detected in the IR spectrum of the oil. As we can see in fig. 5, the intensity of the hydroperoxide band of the control sample was much higher than those of samples containing antioxidants and the order of the band intensities was Caffeic acid > Gallic acid > Catechin > TBHQ > Trolox and hence the antioxidant efficiency order is the reverse.

Fourier Transform Infrared vs. Iodometric PV: Fig. 6 shows that there is a high correlation coefficient (0.96) between iodometric PV and FTIR hydroperoxide band intensities.

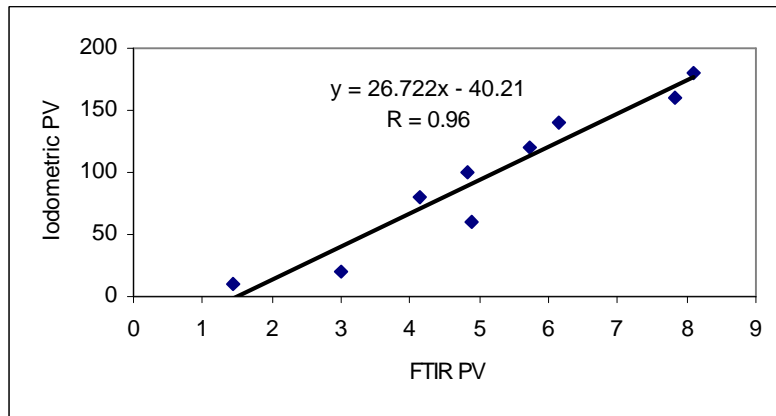


Fig. 6: Relation between FTIR hydroperoxide band intensities at 3470 cm⁻¹ and iodometric PV (meq.O₂ / kg oil).

This observation together with the observation of van de Voort *et al*^[31], revealed that determination of PV FTIR method is more accurate and precise than the iodometric PV because the latter method is very empirical and its results depends strongly on the standardization of all aspects of the procedure, make it possible to evaluate the antioxidant activity by measuring FTIR technique.

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