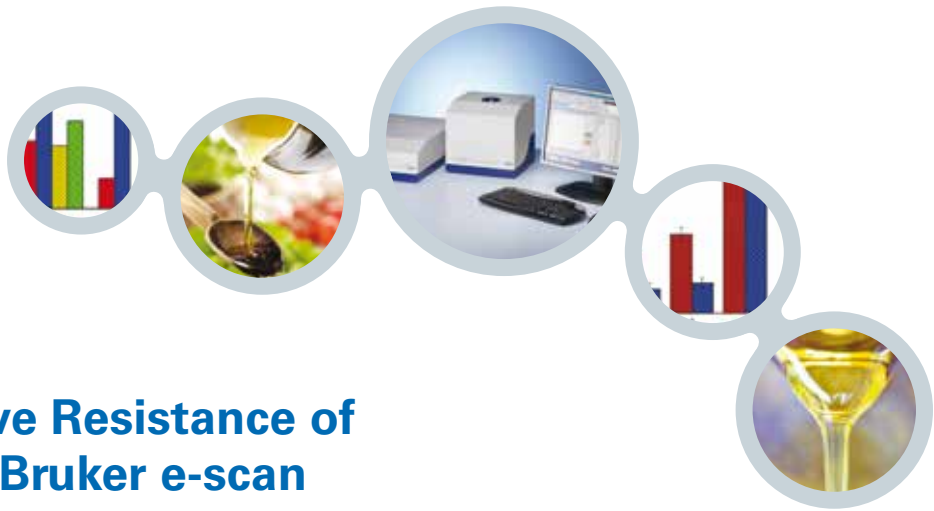
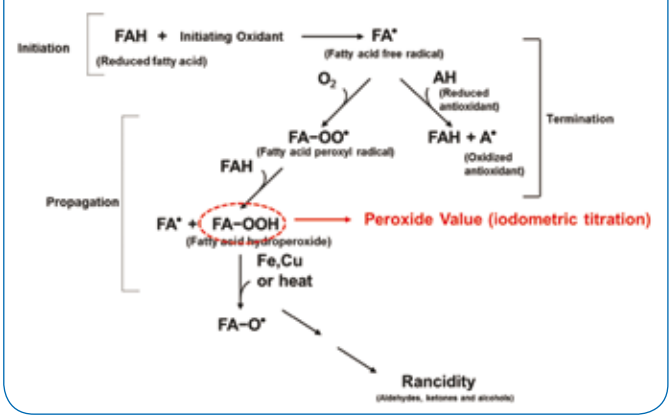


Measuring Oxidative Resistance of Olive Oil using the Bruker e-scan Bench-top EPR Spectrometer

The oxidative rancidity of vegetable oil is a major problem in food related industries. It occurs during storage and is due to the oxidation of unsaturated fatty acids and the subsequent formation of foul odor and taste. Extra virgin olive oil (EVOO) oxidation is of particular interest due to the complexity of its distribution channels around the world and the fact that it is an individually packaged product (its final quality reflects either positively or negatively on the producer). Oxidative stability is affected by a number of factors, such as oxygen, temperature, presence of metals and light. The resistance of EVOO to oxidation is related to the high levels of monounsaturated triacylglycerols and the presence of natural phenolic antioxidants. Electron paramagnetic resonance (EPR) is a useful tool to detect free radicals and to determine the level of free radical formation in olive oil during forced oxidation at different temperatures.

Figure 1. provides an example of a free radical chain reaction that results in rancidity. It starts with the oxidation of fatty acids to various fatty acid free radicals. These free radicals are usually carbon centered, and react rapidly with oxygen to form peroxyl radicals. Peroxyl radicals propagate further free radical formation by abstracting hydrogen atoms from other fatty acids. The peroxyl radicals are converted to hydroperoxides that either undergo thermolysis or react with metals (e.g., iron or copper) to form alkoxyl, peroxyl and carbon centered free radicals. This free radical chain reaction proceeds until it is terminated by an antioxidant.

Figure 1

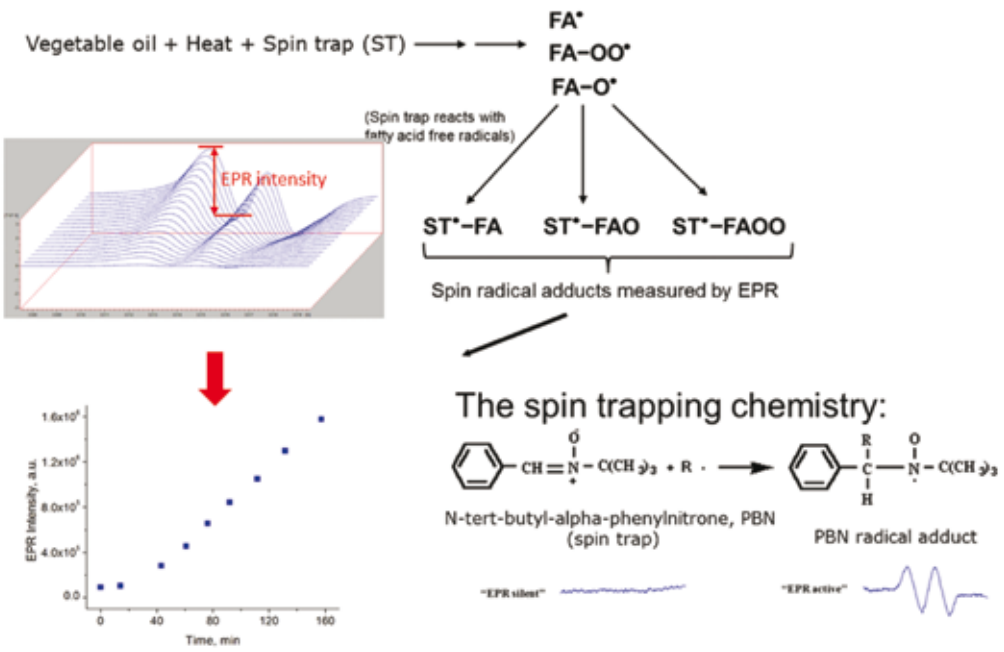


Simplified scheme for the free radical degradation of a fatty acid.

Antioxidants "intercept" a propagating free radical by donating an electron. A "good" antioxidant is oxidized to a stable non-reactive form. If the free radical process is not terminated in its early stages, bad flavor products (i.e., aldehydes, ketones, and alcohols) will form. See references 1–3 for further review of free radical reactions and antioxidants.

Various methods exist to measure the end products of rancidity. However, many of these methods require cumbersome HPLC separations and can be very non-specific. Fatty acid hydroperoxides (intermediates in the rancidity process) can be measured using iodometric titration. However, this method is time consuming, lacks sensitivity and because these hydroperoxides are unstable, can underestimate the true extent of rancidity.

Figure 2



Spin trapping in vegetable oil.

References 4–5 describe the various techniques commonly used for measuring rancidity. There has also been some previous work using EPR to measure oxidation in vegetable oil (references 6–7). Here, we describe a simple "forced oxidation" assay that uses EPR spin trapping to measure the fatty acid free radicals, themselves.

EPR (electron paramagnetic resonance, also called ESR, electron spin resonance) is a spectroscopic technique that measures samples with unpaired electrons and thus, is very useful for measuring free radicals. At the same time the technique is completely "blind" to molecules without unpaired electrons, which makes it very specific. Free radicals are "short-lived", so to increase our ability to detect them, we add a compound known as a spin trap. The spin trap reacts with the free radical to form a "spin adduct". Spin adducts are also free radicals, but they are more stable than the original radical which allows us to measure them using an EPR spectrometer. The general procedure for the EPR spin trapping assay is depicted below. In this note we describe how to use the EPR technique to measure rancidity in olive oil (Figure 2).

Experimental protocol

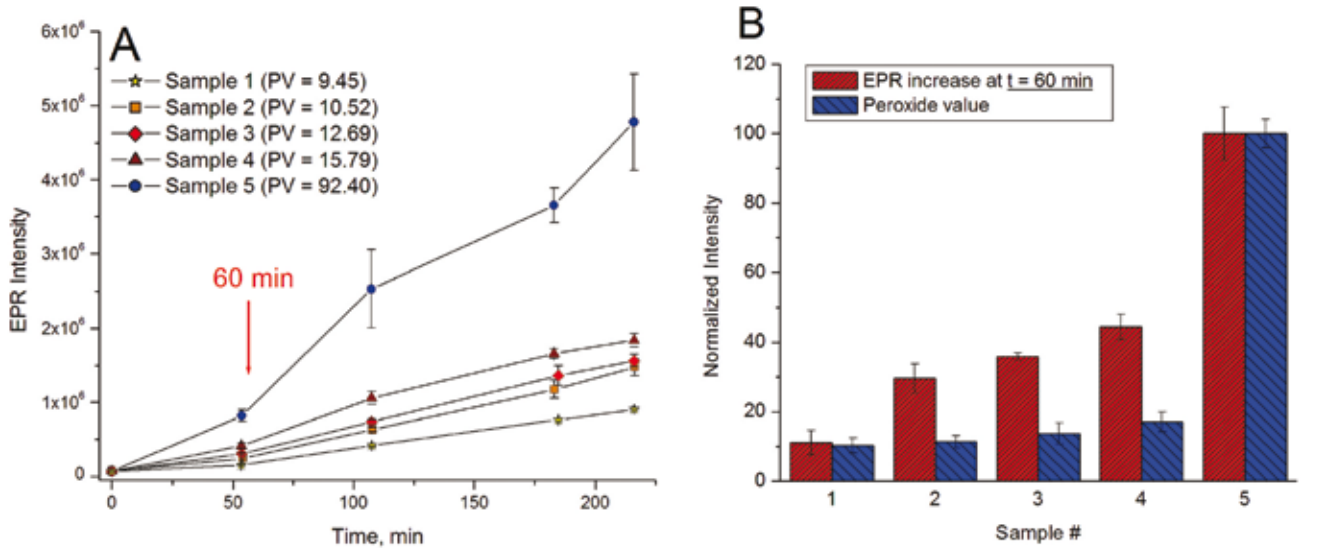
Materials needed:
1. Pipette pumps, pipette tips.
2. Test tubes.
3. Heater block
4. Olive oil sample.
5. Spin trap (N-tert--butylphenyl nitrone (PBN).
6. Bruker e-scan bench-top EPR spectrometer.
7. Sample cell and holder assembly.

Samples:
Olive oil samples were purchased from the American Oil Chemists' Society (AOCS) – Laboratory Proficiency Program. Peroxide value and EPR data were collected within 1 hour while the assay was performed at 70 °C. Peroxide values were measured using AOCS method CD8-53.

EPR Assay:

24 µL of a 2.5 M PBN (dissolved in ethanol) solution was added to 0.3 mL of the respective olive oil (final concentration 200 mM). The samples were mixed using a vortex and transferred immediately to a 100 µL capillary. Samples were then introduced to the EPR probehead which was pre-equilibrated at 70 °C using Bruker's ER4131VT variable temperature controller. After approximately 1 minute, the EPR measurements were started. Measurements were made approximately every 1 hour for almost 4 hour period. The relative quantity of spin adduct was measured from the peak-to-trough intensity of the middle EPR lines in Figure 2 Spin trapping in vegetable oil. These values were plotted versus the time of measurement to show the respective level of oxidation that occurred in each vegetable oil sample. The following spectrometer parameters were used: modulation frequency, 100 kHz; modulation amplitude, 1 gauss; microwave power, 20 mW; scan time, 5.24 sec.; sweep width, 12 gauss.

Figure 3



A: Peroxide values and EPR spin adduct formation data for the five AOCS samples.

B: Comparison of EPR data with PV data.

Results and Discussion

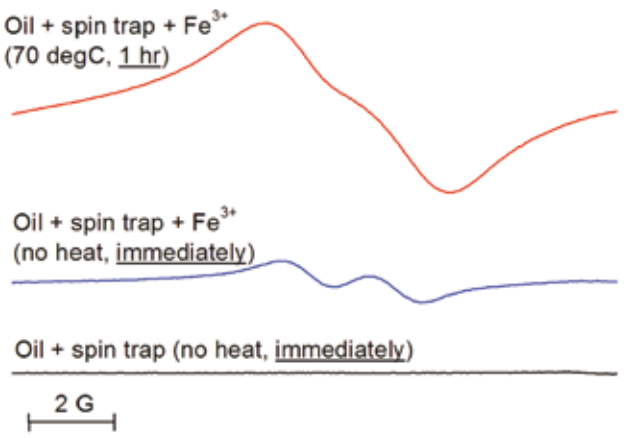
Performing the EPR assay was greatly simplified using the automated acquisition routine of Bruker's e-scan. spectrometer. Figure 2 also shows the resulting EPR triplet of doublets of the PBN radical adducts. The EPR intensity was measured as the peak-to-peak amplitude of the middle lines. The formation of the radical adducts and their time evolution was monitored by a 2D experiment (field sweep vs. time) (Figure 2). The assay was repeated for each of the samples described in the experimental section.

Figure 3 shows the time profiles of five olive oil samples containing 200 mM PBN with known peroxide values (PVs). The radical formation was accelerated at 70 °C. All the samples were heated continuously without PBN and the spin trap was added prior to each EPR measurement. Peroxide value determination was carried out by means of iodometric assay after 1 hour of punishment at 70 °C of olive oil samples (without the spin trap). As can be seen in Figure 3, the EPR signal increase correlated with the peroxide value data for the respective oil samples. To compare the EPR data with the PVs, the parameters were normalized to the strongest EPR signal at time point t = 60 min which also corresponded to the highest PV, i.e. sample 5 with PV = 92.40. After the normalization the results showed much higher EPR response compared to PVs' response suggesting better sensitivity for the EPR method.It is well known that peroxidized fatty acids produce free radicals when they react with pro-oxidants (e.g., metal ions) due to Fenton-type radical chemistry:

$$FA\text{-}OOH + Fe^{2+} \Rightarrow Fe^{3+} + RO^{\bullet} + OH^{-}$$
$$FA\text{-}OOH + Fe^{3+} \Rightarrow ROO^{\bullet} + H^{+} + Fe^{2+}$$

The addition of 100 µM Fe^{3+} greatly enhanced the EPR intensity and total extent of the spin radical formation immediately even at room temperature (Figure 4). More than five times increase was observed when the sample was heated at 70 °C for 1 hour in the presence of the metal ion. However, the PV data did not show significant increase immediately after the pro-oxidant treatment. Small changes in the PV values were detected after 1 hour of punishment but again, the percent change from the untreated control was much less that what was seen for the EPR result (Figure 5).

Figure 4

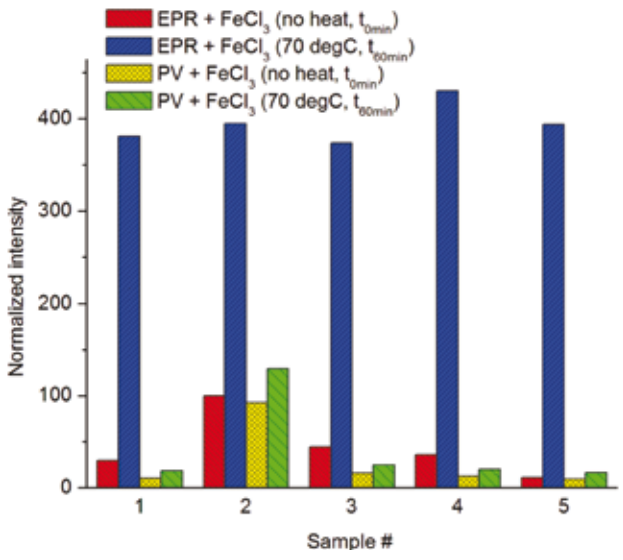


EPR data after pro-oxidant (100 µM Fe^{3+}) treatment.

Summary

EPR spin trapping provides a very simple and useful analytical tool for measuring rancidity (and the ability to resist rancidity) in vegetable oils. The sample handling is minimal and Bruker's fully automated EPR spectrometer made data acquisition and analysis both facile and reliable. These features give EPR an advantage over other techniques that are more labor intensive and prone to uncontrollable variation. There is a correlation between the EPR and PVs obtained from olive oil oxidation, thus the EPRt=60 min end point can be successfully used to detect early stage of oxidation process. In fact, our EPR studies have shown to be more sensitive than the peroxide value test which is because the EPR technique is able to trap free radicals both before and after hydroperoxide formation. These findings justify the further development of EPR methods for measuring and improving the resistance of edible oils to rancidity.

Figure 5



Normalized data (EPR vs. PV) after pro-oxidant (100 µM Fe^{3+}) treatment.

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