EDULAB FOR INSTRUCTORS: MAGNETTECH ESR5000

TEMPO-tations in the Kitchen: Discovering Antioxidants in Olive Oil

EPR of Antioxidants in Olive Oil

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Experiment Hashtag: #OliveOil, #Educate2Resonate

Keywords:

EPR, Olive Oil, Free Radicals, Antioxidants, Polyphenols, Food Analysis

Target Group:

Advanced Undergraduate or Graduate, General Chemistry, Analytical Chemistry, Food Chemistry, Food Safety and Control Laboratory, General Life Sciences

Objectives:

- Gain hands on experience with the Bruker Magnettech ESR5000 benchtop spectrometer while building upon the understanding of electron paramagnetic resonance (EPR) in a practical setting
- Apply EPR to a real-world sample
- Learn how to process and interpret EPR data and kinetics

Background of the Experiment:

The world we live in is a dangerous place for complex organisms. Radiation knocks the occasional molecule apart which produces reactive entities, called free radicals, that attach to other molecules in ways we might not want. Fortunately, over the last several billion years, our progenitors evolved mechanisms to shield themselves from these dangerous chemicals. This shield consists of a complex series of chemical reactions that metabolize these reactive molecules before they cause damage, repair damage if it happens, or outright capture and render harmless the reactive molecules directly. This last layer of protection uses molecules called antioxidants. Some organisms produce their own antioxidants while other get their antioxidants from what they eat.

It's useful to know if antioxidants are present in our food. We might prefer products that are high in these protective molecules if we have a choice. But food labels don't always give us that information. One antioxidant that is commonly listed is vitamin C, known to chemists and nutritionists as ascorbic acid. But much of the biochemistry that our bodies perform occurs in places where water, and hence vitamin C, is scarce. Our cells and their organelle are defined by cell or organelle membranes which are composed of lipids (a fancy term for fats). Membranes not only define our cellular systems but act as surfaces for a great deal of biochemical activity including the reactions that produce chemical energy by turning oxygen into water: a process that produces extremely reactive oxygen radicals (think of peroxide and superoxide). While we produce some antioxidants that protect our cells during these processes, we must get others from our food, and these antioxidants aren't part of the food labels.

The experiments described here that you will perform will give you a set of tools to measure antioxidant levels in food with high lipid content. We'll be studying olive oil but you can use this method on other foods as well.

We'll need some special equipment to do these experiments, but we will be able to get data we can't get any other way. The instrument we'll use, an EPR spectrometer, is purpose-built to measure molecules or atoms with unpaired elections, as free radicals are. Because electrons are magnetic, they can be made to absorb energy in a magnetic field. How they return that energy to the sample can give us detailed information about the environment the election is in. More importantly, for our purposes, because antioxidants attack free radicals, if we add free radicals to the sample and antioxidants are present, they will neutralize the free radical and make its signal go away.

Our experiment will therefore require a special kind of free radical; one that isn't particularly reactive but can be neutralized by antioxidants. All we need to do is keep track of the amount of this indicator molecule that is present in the sample that hasn't been neutralized.
The indicator molecule we will use has the official chemical name of 2,2,6,6-tetramethylpiperidine-N-oxyl. We'll use its nickname TEMPO.

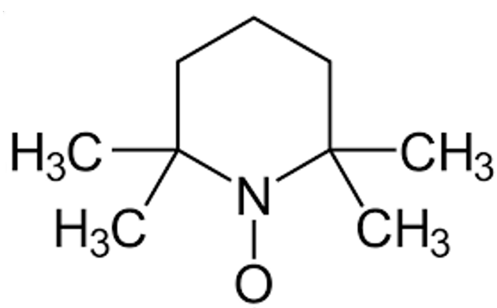

Figure 1 Chemical structure of 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO)

Glossary

EPR: Electron paramagnetic resonance or electron spin resonance spectroscopy is a method for studying materials that have unpaired electrons. The basic concepts of EPR are analogous to those of nuclear magnetic resonance, but the spins excited are those of the electrons instead of the atomic nuclei.

FREE RADICALS: An atom, molecule, or ion that has at least one unpaired valence electron

ANTIOXIDANTS: Antioxidants are compounds that inhibit oxidation, a chemical reaction that can produce free radicals. Their scavenging activity is different. Some of the most potent antioxidants are vitamin E, vitamin C, phenol alcohols, etc.

If we make a very dilute solution of TEMPO in olive oil and put it in our EPR spectrometer, we'll see a signal like this:

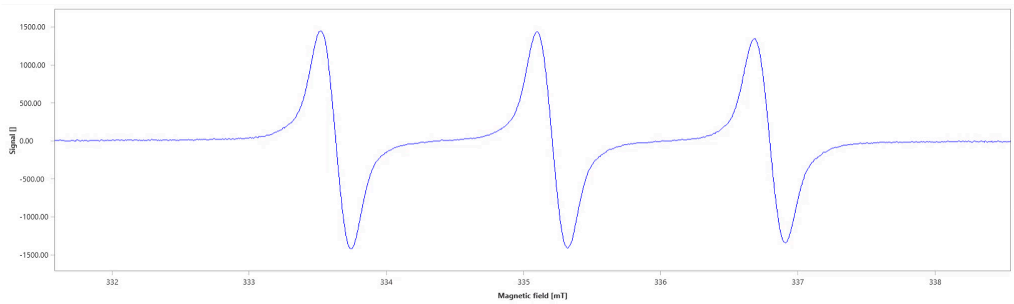

Figure 2 EPR spectrum of TEMPO

The three lines come from the magnetic field of the electron interacting with the magnetic field of the nitrogen nucleus. The magnetic field applied to the sample increases from left to right. For reasons we won't go into here, the electron only absorbs energy at a specific magnetic field. This absorption happens at three different magnetic fields because the magnetic field of the nitrogen nucleus adds to, subtracts from, or has no effect on, the applied magnetic field from the instrument.

If we collect EPR spectra over time, we can watch the signal to determine how fast it disappears and from that intuit how fast the free TEMPO radical is being neutralized. The more antioxidant in our sample, the faster the signal will disappear.

In this exercise, we will analyze olive and soybean oil to determine their antioxidant properties. We will monitor TEMPO free radical signal being reduced by antioxidants in the two types of oil and calculate the rate of reduction.

Preparation:

To ensure efficient completion of the experiments, it is recommended to form groups with a maximum of three students. The estimated time for sample preparation is approximately 30 min. The EPR experiments are expected to take approximately 2.5 hours in total including two samples of oil — olive and soybean (the second oil is optional). After completing the experiments, an additional 1.5 - 2 hours will be needed to write a report. It is assumed that students have already covered introductory concepts of EPR and have a basic understanding of instrumental parameters.

For comprehensive information on EPR basics and optimizing instrumental parameters, students can consult the Magnettech ESR5000 educational kit, which is also provided with the benchtop EPR spectrometer.

To perform these experiments, an installed Magnettech ESR5000 spectrometer is required.

Experimental Setup:

Materials:

- 95 % ethanol
- TEMPO
- 1 mm x 100 mm glass capillaries
- 1.5 ml Eppendorf tubes
- 1 ml glass vial
- Pipettes to measure variable amounts of liquid from 2 µl to 1 ml
- Balance accurate to at least 0.1 mg
- Capillary sealant
- Olive Oil (~ 5 ml)
- 2nd type of oil (optional)

Abbreviations

EPR: Electron Paramagnetic Resonance

Sample Preparation:

1. Make a stock solution of TEMPO in ethanol as follows:

- Weigh somewhere between 3-10 mg of TEMPO into a 1 ml glass vial. It's not important that you get a precise amount, but it is important that you know what that amount is. This is about 1-3 crystals of TEMPO.
- Calculate the amount of TEMPO necessary to make a 1 M solution. For example:

$$\frac{4.3\ \text{mg TEMPO}}{156.25\ \text{mg/mM}} = \frac{x\ \mu\text{l}}{1000\ \mu\text{l}}$$

$$4.3 \times 1000 / 156.25 = 27.5\ \mu\text{l}$$

This will make a 1 M solution. Gently invert the vial until the TEMPO dissolves (a couple of minutes).

2. Next make two dilutions:

- Take 10 µl of 1 M solution and dilute it in 990 µl EtOH to a final 10 mM.
- Take another 100 µl of 10 mM solution and dilute it in 990 µl EtOH to a final 1 mM.

3. Preparing the sample

- Add 1 ml of your sample of olive oil into a 1.5 ml plastic Eppendorf tube.
- Take 20 µl of the 1 mM TEMPO solution and pipette it into the Eppendorf tube with the oil. Make sure the tip of the pipette touches the side of the Eppendorf tube when you deliver the TEMPO into the olive oil. The final TEMPO concentration in the oil is 20 µM.
- Close the cap on the Eppendorf tube and carefully invert several times to mix the TEMPO into the olive oil. Ten times should do it.
- Open the Eppendorf tube with the olive oil and TEMPO and turn it on its side until the olive oil sample just barely comes up to the edge of the tube. Put the end of the capillary into the olive oil sample and allow the sample to fill the capillary. When the capillary is full, move it away from the sample (keep it horizontal or you will have your sample all over the floor) and bring the capillary sealant up to the end and push the capillary into the sealant with a slight twisting motion to trap the sample in the capillary (Figure 3). Wipe the capillary with a Kimwipe moistened with ethanol.

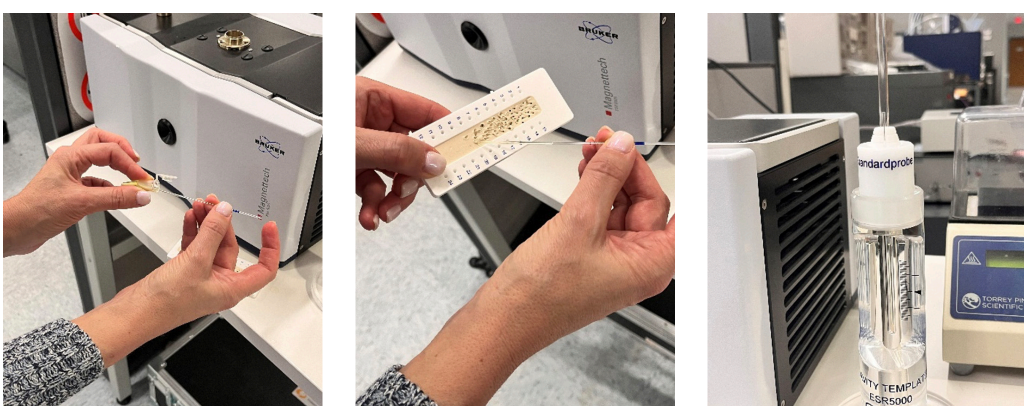

Figure 3 Sample preparation

- Place the capillary with the sample into a 4 mm quartz sample tube and use the cavity template for proper positioning as shown in Figure 3.

Notes

Experimental Procedure:

1. Start the EPR spectrometer up by turning on the power switch located on the back of the unit. Start the ESRStudio software. Connect to the spectrometer by clicking the Initialize button. Insert the oil sample carefully using the proper size sample holder.

2. Select the following parameters from Recipe editor tab:

Center Field B0	336 mT
Sweep	7 mT
Sweep Time	60 s
Modulation	0.2 mT
Frequency	100 kHz
Accumulations	1
Microwave power	10 mW

3. Switch the measurement type from 'Single measurement' to 'Kinetic series' and select 'Full Spectrum' option:

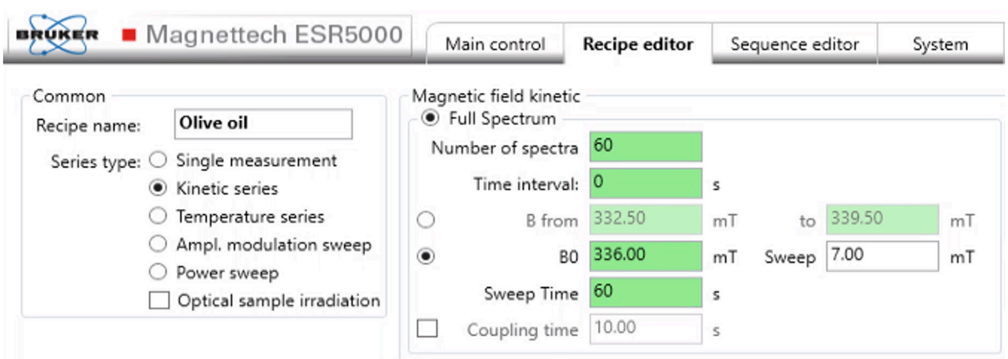

Figure 4 Choosing instrumental parameters

4. Select 60 for the number of spectra with a time interval of 0 sec. The total acquisition time for the experiment will be approximately 1 hour (60 spectra x 60 sec sweep time).

5. Save the parameters as a new recipe 'Olive oil' or another appropriate name if using a different type of oil.

6. Create a new container in ESRStudio and name it 'Oil antioxidants'.

7. Collect the spectra from the oil kinetics in this container.

Notes

Data Processing:

1. Apply digital filtering to all spectra. Right click on the highlighted folder, choose ‘Run postprocessing’, and select ‘0.02 mT’ for the digital filter. Select the option ‘Apply to container’:

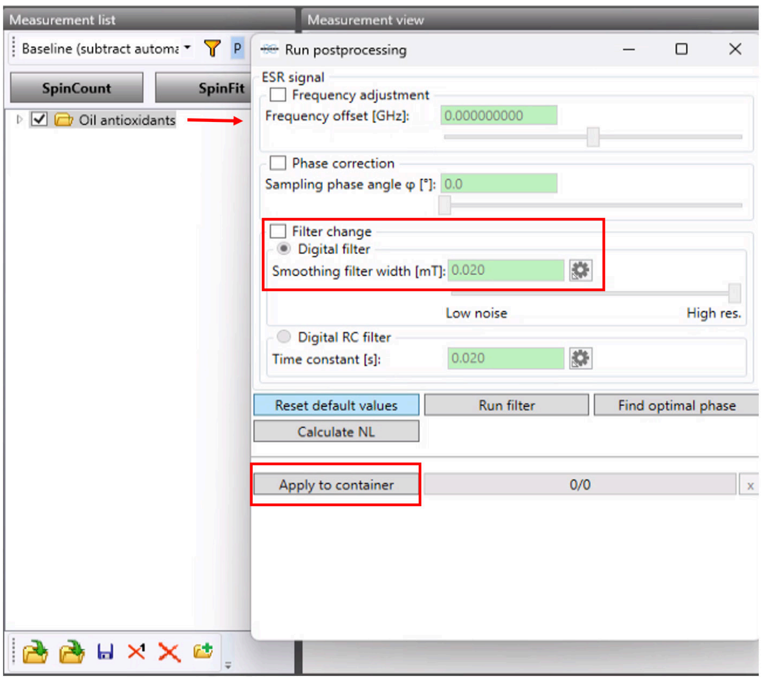

Figure 5 Choosing instrumental parameters

2. Enable the option ‘Show Evaluation View’ and create the function ‘Amplitude vs time’:

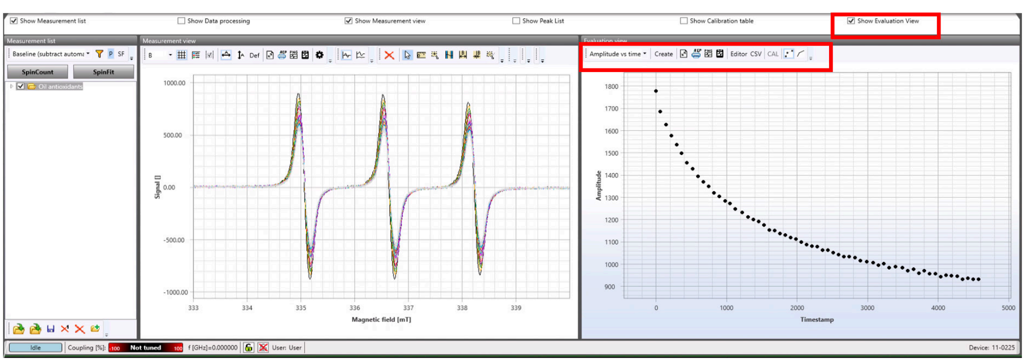

Figure 6 Creating Amplitude vs time in the Evaluation view

3. Export the kinetic curve as *.csv file.

Notes

Results & Discussion:

1. Plotting the EPR amplitude loss percentage.

   This is straight-forward. Assume that you have 100 % of your signal at the start of the experiment. Divide the rest of the data points by this value and multiply by 100 to get the percent change. Then plot the change in percent signal as a function of time (Figure 7). This is much easier if you use a spread sheet like Excel to do all the work for you:

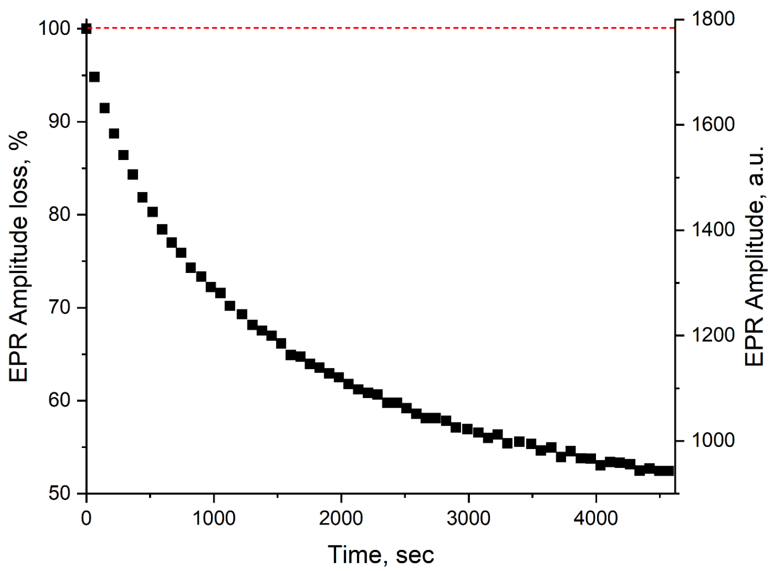

Figure 7 Calculating percentage loss

   This plot can be used to compare other types of olive oil if, and only if, the experimental parameters don't change. As long as you can reproduce your results by repeating this same experiment several times, you can use this method. However, there is a way to analyze the results that doesn't require this kind of experimental precision (but it does require that you know the concentration of your TEMPO in the sample).

2. Concentration calculation.

   Since we know the concentration of TEMPO in the sample, we can calculate the concentration in molecules per liter:

   20×10^{-6} moles/liter x 6.02×10^{23} molecules per mole = 1.204×10^{19} molecules/liter

   Assuming that our TEMPO sample at time 0 is 20 μM, its concentration is 1.204×10^{19} molecules/liter so the value of the signal at time 0 represents this concentration. Now we just need to calculate the ratio of spectrometer units per molecule.

   Let's say that our signal had an amplitude of 1778 arbitrary units at time zero (see Figure 7). This represents 1.204×10^{19} molecules/liter, so

   1.204×10^{19} molecules/liter / 1778 a.u. = 6.77×10^{15} molecules/liter/unit.

   If you want to go a step further, you can determine the change in concentration per unit time as a rate by using the formula:

$$\text{Rate} = -\,d[\text{Concentration}] / dt$$

Notes

Questions for Students:

1. **Why do you see a three-line EPR spectrum of TEMPO? Explain.**

   The structure of TEMPO (Figure 1) has one nitrogen nucleus and the unpaired electron interacts with it. In EPR the number of lines in the spectrum is predicted using the formula: Number of lines = 2 x n x I + 1, where n is the number of equivalent nuclei and I = 1 (nuclear spin for ^{14}N). This means that TEMPO will have a 3-line spectrum (Number of lines = 2 x 1 x 1 + 1 = 3).

   Bonus question: Can you predict how many you will see if TEMPO was isotope labeled with ^{15}N?

   Answer: For ^{15}N I = ½.

   This means that TEMPOL isotope labeled with ^{15}N will have a 2-line spectrum (number of lines = 2 × 1 × ½ + 1 = 2).

2. **What is the hyperfine splitting constant for TEMPO in oil? How did you measure it?**

   The distance between any two lines in the 3-line spectrum provides the value for the hyperfine splitting constant. Students can use the mouse ruler tool from ESRStudio software to read out the distance in the viewport as horizontal value.

3. **Why does the EPR peak-to-peak amplitude decrease over time? Explain.**

   Decreasing of TEMPO signal is related to the antioxidant properties of the edible oil of interest. The antioxidant molecules reduce TEMPO by donating one electron and converting it into hydroxylamine which is EPR silent.

4. **Assuming you have studied another oil and you got ~ 90 % reduction of TEMPO under identical experimental conditions. What conclusion can you make about the amount (or activity) of the antioxidant(s) in this second oil compared to the first?**

   More reduction of TEMPO simply means that the oil has more effective antioxidants. This is beneficial for the oil shelf-life and for your health.

Notes

Key Take Home Messages:

- Learn the effect of nuclear interactions with the unpaired electron and how to determine hyperfine splitting constants.
- Antioxidants are extremely important. They benefit your physical health.
- Antioxidant efficiency can be used to predict shelf-life in food products and to learn about antioxidant nutrients in olive oil and other edible oils.

References:

1. Ottaviani M. F. et al., *Electron paramagnetic resonance investigations of free radicals in extra virgin olive oils,* J. Agric. Food Chem. 49 (2001) 3691-3696

2. Lanza B. and Ninfali P., *Antioxidants in extra virgin olive oil and table olives: Connections between agriculture and processing for health choices,* Antioxidants 9 (2020) 41-58

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