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Free Radical Generation Assays: New Methodology for Accelerated Oxidation Studies at Low Temperature in Complex Food Matrices

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A novel method for the rapid screening of antioxidant efficacy and oxidative stability in food and feed matrices has been developed. The analyses are described as free radical generation (FRG) assays. The new procedure combines the use of azo-initiators with analytical equipment that is widely used for antioxidant research such as the oxidative stability instrument and the oxygen bomb. The use of initiators instead of high temperatures as a driving force to increase the rate of oxidation improves the correlation between the accelerated screening of foodstuffs and real shelf life. The improved correlation can be mainly explained by the fact that food products are analyzed in their original status, maintaining all interfacial phenomena of the food matrix. Furthermore, the lower temperature of analysis reduces differences between the reaction kinetics of the assay and those of the oxidation during actual shelf life. Consequently, the correlation between the accelerated analysis and shelf life is improved, particularly when compared to accelerated oxidation at high temperatures. The FRG assays could be used successfully to evaluate the efficacy of natural antioxidants in heat-sensitive food products such as emulsions and meat products. A good correlation was observed between the accelerated tests and the oxidation parameters obtained from standard shelf-life evaluation. It was possible to successfully compare the efficacy of several antioxidants and to predict shelf life for these heat-sensitive food matrices.

KEYWORDS: Accelerated oxidation; free radical; antioxidant tests; food oxidation; FRG assay

INTRODUCTION

Research into antioxidants and oxidative stability is a time-consuming activity when protected samples are evaluated at normal storage conditions. Oxidation at room temperature or below is relatively slow, and signs of emerging rancidity appear only after several weeks or even months. Faster methods of analysis are needed for an efficient evaluation of antioxidant systems. Many conventional accelerated methods such as the oxidative stability instrument (OSI), oxygen bomb (OB), and Rancimat operate at high-temperature conditions to initiate the oxidation process (*1*, *2*). However, the reaction mechanism of the oxidation process typically changes at high temperatures (*3*). In most instances the corresponding activation energy of the new mechanism will be different, and a clear correlation between tests at higher temperatures and the actual shelf life is lost (*4*). Consequently, temperature-driven accelerated oxidation has always been controversial due to the difficulties that arise when one tries to promote oxidation without changing the original mechanism of oxidation or the status of the food matrix too strongly (*5*).

In attempts to circumvent these difficulties, a multitude of model systems have been developed that use chemically induced radical generation. Well-known applications of radical initiators in antioxidant research include the ORAC assay. This test can quantify the capability of antioxidants or serum to scavenge peroxyl radicals (*6*, *7*). However, it is difficult to link with lipid oxidation due to the use of aqueous buffer without the presence of a lipid. Previously, diazo-initiators were also used to oxidize lipids in other model systems. Some approaches evaluated the influence of antioxidants on the lipid oxidation of methyl linoleate dissolved in organic solvents or emulsified in buffer (*8*−*10*). Also, the inhibition of initiated peroxidation of micellar linoleic acid and rat plasma has been reported (*11*, *12*). Mostly these model systems are not relevant to complex food systems.

However, apolar azo-initiators theoretically have a good potential to mimic the natural oxidation process, combined with the ability to increase the rate of oxidation (*13*). Apolar initiators spend the majority of their lifetime in the apolar lipid phase, where they decompose into radicals, which will react immediately with the surrounding lipids to form lipid radicals. Such initiators are thought not to interfere with the other stages of the oxidation process such as decomposition of radicals, reactions of peroxides with transition metals, or the formation

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of aldehydes and short-chain free fatty acids. The mode of action of apolar initiators is more closely related to the natural oxidation process compared to the other principal techniques to accelerate oxidation. The prevalent method of increasing temperature will typically change the oxidation mechanism itself compared to nonaccelerated oxidation at storage temperature. The alternative utilization of high concentrations of transition metals also has important disadvantages because these metals will affect the initiation and decomposition of hydroperoxides. Simultaneously, the higher amount of metal ions makes it difficult to study the effect of chelators, an important class of preventive antioxidants.

Besides the importance of the specific technique used to accelerate oxidation, also the substrate is key to ensure that reliable results are obtained. The efficacy of antioxidants is greatly affected by many ingredients present in the food matrix such as salts, proteins, sugars, enzymes, pigments, and emulsifiers (*14*). Also, the interfacial phenomena in food products play a primordial role. The most accurate approach to study antioxidants in food is therefore to abandon model systems and to perform the analysis on the final food product (*3*). To achieve this goal, the initiation of lipid oxidation with diazo-initiators has been used in combination with OSI and OB to study the accelerated oxidation of food lipids and finished food products. The method has been further validated by comparison with oxidation under nonaccelerated conditions. Additionally, an attempt has been made to predict accurately the influence of antioxidants on the shelf life of some heat-sensitive food products.

MATERIALS AND METHODS

Chemicals. The reagents 2-thiobarbituric acid p.a. (TBA), trichloroacetic acid p.a. (TCA), and $Na_2S_2O_3$ were purchased from Merck-Eurolab. The compound 1,1,3,3-tetraethoxypropane was purchased from Sigma. Acetonitrile of HPLC grade, KH_2PO_4 p.a., NaOH p.a., acetic acid p.a., isooctane p.a., and potassium iodide were purchased from Acros.

A sample of 2,2′-azobis(2,4-dimethylpentanenitrile) [or 2,2′-azobis-(2,4-dimethylvaleronitrile), abbreviated AMVN] was obtained from DuPont Luxemburg. Both 2,2-azobis(2,4-dimethylpropanenitrile) [or 2,2′-azobis(isobutyronitrile), abbreviated AIBN] and 2,2′-azobis(2,4-dimethylbutanenitrile) were purchased from Aldrich. **DANGER:** AIBN and its decomposition products show high oral toxicity (approximate lethal doses in rats are 670 mg/kg for the azo-compound and 60 mg/kg for the decomposition product tetramethylsuccinonitrile). Suitable precautions need to be taken when this compound is used. Self-accelerating decomposition temperature (after storage for 7 days in large containers) is 35 °C for AMVN and 50 °C for AIBN and 2,2′-azobis-(2,4-dimethylbutanenitrile). During decomposition nitrogen is released and pressure can build up, but actual ignition is rare. For smaller samples stored in a freezer the risks are very low. Solutions of 1% or less present no unusual hazards. More concentrated solutions of 10% or more can present a hazard, but only when they are heated.

Dosing and Preparation of the Initiator. For the addition of the initiator sources to oils, fats, or emulsions, the initiator is gently ground in a mortar in the fume hood; mechanical grinding is unsuitable because of the liberation of excess heat. The ground material is added to the oil, fat, or emulsion, which is then heated to ~40 °C. The samples are magnetically stirred for a time that is typically ~15−30 min, but this may vary depending on the matrix. Because the oxidation process starts upon the first addition of the initiator, care should be taken that all samples are heated for the same amount of time. Alternatively, the initiator can be put on a suitable carrier: First, the initiator is dissolved in a suitable solvent such as acetone, and next the solution is mixed with a suitable carrier, such as silica gel. The gel is dried in air at ambient temperatures and then preferably stored at 0 °C or below. A powder is obtained that distributes very easily in a wide variety of samples. To add the initiator to meat, the initiator is also ground in a mortar and mixed directly with the meat. Once the initiator is diluted in the meat, it is safe to use additional mechanical blending.

Antioxidants. Two natural antioxidants were used: (1) A tocopherol-based formulation that contains 1% α-tocopherol, 11.40% δ-tocopherol, and 11.83% γ-tocopherol in a sunflower oil-based carrier. (2) A rosemary extract (RE) that contains 7.2% phenolic diterpenes and flavonoids.

Sample Matrices. Refined soybean oil was purchased from Vandemoortele (2.27 mmol of lipid peroxides/kg of oil). Refined rapeseed oil was purchased from Mosselman (1.08 mmol of lipid peroxides/kg of oil). Mayonnaise was prepared by mixing 8.4 g of sugar, 6.0 g of salt with a blend of 4 egg yolks, and 10 g of mustard brought to a pH of 4 by the addition of vinegar (8%). A total of 436 g of sunflower oil was added in portions (first 4 g, then 8 g, then 32 g) during mixing with a kitchen blender. After adding half of the oil, the antioxidants and/or radical initiators were added. The initiator was gently ground in a mortar before use. Minced pork meat was purchased from a local butcher. The pork meat was mixed with the antioxidants and/or initiators and thoroughly homogenized.

Quantification of TBA Reactive Substances (TBARS). In a 150 mL glass beaker, 10 g of a homogeneous food sample is mixed with 100 mL of a 5% TCA solution in deionized water. For a good extraction of TBARS out of solid fats, the solution is gently heated in the water bath to liquefy the fat prior to the extraction. The mixture is extracted for 15 min using a magnetic stirrer. Fats and oils should be extracted vigorously to ensure complete extraction of the malondialdehyde into the aqueous phase. The extracted mixture is centrifuged until a clear solution is obtained, and 5 mL of the supernatant is combined with 5 cm^3 of a 0.2% TBA solution in deionized water in a 20 mL test tube. The reaction mixture is homogenized and is then placed in a water bath at 95 °C for 30 min. Afterward, the reaction tubes are cooled in water. The solution is filtered using a 45 μm disposable filter. A 20 μL sample is examined by HPLC: autosampler, TSP AS1000; pump, TSP P2000; flow, 0.5 mL/min, isocratic; eluent, 200 mL of acetonirile/800 mL of 0.01 M KH_2PO_4 adjusted to a pH of 7 with 1 M NaOH; column, Nucleosil 100-5, C18, 250 × 4,6 mm + precolumn, Varian catalog no. CP 29370; detector, TSP Spectra Focus Scanning Detector, 532 nm; integrator, PC1000 System software. Quantification is performed by comparison with a standard curve based on 1,1,3,3-tetraethoxypropane (a diacetal of MDA). Note: 0.2 μg of 1,1,3,3-tetraethoxypropane corresponds to 0.065 μg of MDA.

Quantification of Peroxide Value. The mayonnaise emulsion was broken by overnight storage at −18 °C. An amount of 3 g of the lipid phase was transferred into a 50 mL beaker. Then 30 mL of 60% v/v acetic acid in isooctane is added. The mixture is stirred magnetically until the lipid phase is completely dissolved. Next, 500 μL of a saturated KI solution is added with stirring. After 1 min, 30 mL of deionized water is added. The mixture is titrated automatically with 0.01 N $Na_2S_2O_3$ using a Kyoto AT400/APB410 autotitrator with potentiometric detection of the equivalence point.

Measurement of the OSI. The OSI was measured on an Oxidative Stability Instrument (Omnion, Inc., Rockland, MA). The conductivity cell was filled with 60 mL of deionized water. The conductivity was closely monitored, and whenever a value >900 μS was obtained, the probe was cleaned and filled again. A glass round-bottom tube was filled with 10 g of fat or oil. Compressed air was used at 38 kPa. Analyses were performed at indicated temperatures. The software that runs the equipment reports the induction point automatically.

Measurement of Oxygen Consumption with the OB. A glass sample holder was filled with 50 g of sample and was placed in a stainless steel cylinder also referred to as a bomb (Seta 2652). The system was closed and purged with oxygen. A pressure transducer (Seta 2631) was installed on top of the cylinder. The oxygen bomb with an oxygen pressure of 345 kPa was then put in an oil bath at the selected temperature. The pressure was monitored as a function of time using a Seta Autoxidation Control Unit (Seta 2629).

RESULTS AND DISCUSSION

The use of free radical sources to accelerate lipid oxidation was investigated in combination with commercially available

Table 1. Relative Performance of Antioxidants in OSI as a Function of Analysis Temperature

	relative performance in OSI	
temperature (°C)	tocopherols	RE
100	100	57
80	100	93
60	100	122

Table 2. Acceleration of Oxidation with Different Diazo Radical Initiators Evaluated with OSI

radical initiator	OSI induction time (h)
control soybean oil	14.00
2,2′-azobis(2,4-dimethylvaleronitrile)	11.90
2,2′-azobis(isobutyronitrile)	12.64
2,2′-azobis(2,4-methylbutyronitrile)	10.70

equipment for antioxidant testing. Two widely used methods are the OSI and OB. Both techniques are temperature-accelerated methods, commonly performed at 98 °C. The mode of oxidation detection differs for the two methods. The OSI detects the formation of oxidation products using a conductivity probe, whereas in the OB method the oxygen pressure in the headspace is monitored. Food lipids are routinely used in both systems as the substrate instead of artificial model systems. Both the OSI and the OB methods were combined with the use of a free radical source to reduce the standard operating temperature of 98 °C.

Influence of Temperature-Driven Accelerated Oxidation on Antioxidant Performance in OSI. A clear example of problems associated with temperature-driven accelerated oxidation is shown in **Table 1**. Here the efficacy of mixed tocopherols and rosemary extract (RE) in lard was evaluated with OSI at different temperatures. When the OSI experiment was performed at 100 °C, the tocopherol blend was almost twice as efficient as the RE. At 80 °C both antioxidants had comparable efficacies. At the lowest temperature of 60 °C the RE outperformed the tocopherols. This example shows that it is very difficult to translate results obtained at high temperatures to shelf-life conditions due to the possible change of antioxidant performance.

Furthermore, in various food products high temperatures will also alter the structure and properties of the food product, which adds another level of complexity to the assessment of shelf life.

Evaluation of the Acceleration of the Oxidation Rate for a Series of Apolar Diazo-initiators. The radical initiating capacity of three different apolar initiators was evaluated in a vegetable oil matrix using OSI. An ethyl acetate stock solution containing 0.1 M of the initiator was prepared. An amount of 0.025 mL of the initiator solution was mixed with 10 g of soybean oil. The oil samples were analyzed with OSI at 98 °C (**Table 2**). The order of activity corresponds closely with the half-life times of these compounds reported by DuPont (*15*).

Evaluation of AMVN-Induced Lipid Oxidation in Bulk Oil with OSI at 50 °C. The conventional OSI method (AOCS Official Method Cd 12b-92) is an accelerated stress test, which evaluates the oxidation process continuously. Air is bubbled through a small amount of oil that is kept in a thermostatic heating block at a chosen temperature. The effluent gas, containing the volatile oxidation products, is trapped in deionized water. The conductivity of the water is recorded, and the induction point is defined as the time at which an inflection point occurs in the conductivity curve. The later in time the inflection occurs, the more stable is the oil against oxidation. Traditionally this method is performed at temperatures of 98 °C or higher to reduce the time of analysis. However, in combination with free radical generation the temperature can be reduced significantly to at least 40 °C. It is suggested to assign the term FRG−OSI to the method that combines the use of free radical generation with OSI. Combinations with other techniques can be assigned accordingly. A reduction of the analysis temperature to the range between 35 and 60 °C has several important advantages: phase changes are limited, there are minimal effects on the moisture content of the food and on the water activity, and also the partitioning of antioxidants or catalysts is similar to temperatures at the standard shelf life (*4*).

Table 3. Reproducibility of the Accelerated Oxidation of Soybean Oil at 50 °C

repetition	amount of AMVN (%)	OSI (h)	av (h)	SD (h)	relative error (%)
1	0.8	22.66			
2	0.8	22.46	22.84	±0.49	±2.14
3	0.8	23.39			
1	0.6	33.76			
2	0.6	33.63	33.82	±0.14	±0.41
3	0.6	33.48			
1	0.4	67.30			
2	0.4	66.00	66.42	±0.78	±1.17
3	0.4	65.90			

The AMVN-induced oxidation of soybean oil was evaluated in combination with OSI. A total of three different concentrations of AMVN (0.4, 0.6, and 0.8%) were used to accelerate the oxidation of soybean oil at 50 °C. All analyses were performed in triplicate. The results in **Table 3** show that the oxidation is remarkably accelerated. Normally the induction point in OSI for soybean oil at 50 °C will exceed 20 days. The time of analysis in this experiment could be reduced to <1 day.

The initiator AMVN was selected for further trials because it possesses strong radical-initiating properties and has low toxicity compared to other initiators. Furthermore, AMVN is lipophilic, which specifically promotes the formation of radicals in the lipid phase (*13*). The free radical generation by AMVN is much more closely related to the natural oxidation process compared to more polar initiators, which can distribute in the aqueous phase to produce nonlipid radicals. The dose−response relationship described in **Table 2** shows the good reproducibility of the experiment. The observed relative errors are comparable to conventional OSI analyses (*16*). Furthermore, the results show that the correlation between the AMVN concentration and induction time is not linear. In this case the curve starts to level out at ∼0.8% of the initiator. This concentration is still practical to use because the solubility of the initiator in the bulk oil is not an issue at a level of 0.8%. Higher concentrations will not yield an important decrease of the analysis time, and the preparation of the samples will be more demanding and time-consuming due to the solubility of the initiator.

Evaluation of AMVM-Induced Lipid Oxidation in Bulk Oil with the OB. The conventional OB test is an accurate tool to assess the oxidative stability of diverse materials ranging from pure fats to finished products. The OB continuously measures the change of pressure in the headspace over a food matrix under an atmosphere of pure oxygen in a sealed system at a pressure of 345 kPa. The pressure decrease in the headspace arises from the incorporation of oxygen into the lipid molecules due to oxidation. The induction point or the initial oxidation rate (correlated with the slopes of the curves) can be derived from

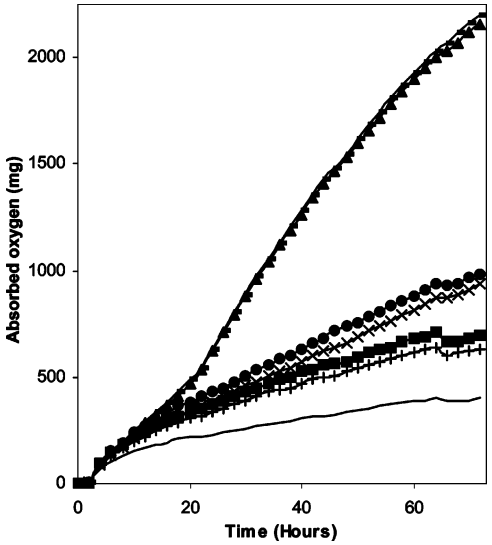

Figure 1. Accelerated oxidation of soybean oil in FRG–OB at 50 °C: (—) 0.5% AMVN (a); (▲) 0.5% AMVN (b); (●) 0.25% AMVN (a); (×) 0.25% AMVN (b); (■) 0.125% AMVN (a); (+) 0.125% AMNV (b); (—) negative control.

the resulting plots. These parameters can be used as a measure for oxidative stability of the food matrix. Stable products exhibit a slower rate of oxygen consumption with a delayed induction period.

In an FRG−OB assay a total of three different concentrations of AMVN (0.5, 0.25, and 0.125%) were used to accelerate the oxidation of soybean oil at 50 °C. All analyses were performed in duplicate (**Figure 1**). The results show that an induction point of ∼24 h is reached for the concentration of 0.5% in the OB, whereas for the FRG−OSI 0.8% of AMVN was needed to reach a similar induction time. This increase in oxidation rate can be attributed to the very high oxygen pressure in the bomb. This accelerates the formation of AMVN-derived peroxyl radicals, which in turn have a higher reactivity toward lipids than the corresponding carbon-centered radicals. Also, the FRG−OB assay proved to be very reproducible, and the variability is comparable with standard OB measurements

Validation of FRG Assays for the Prediction of Shelf Life and Antioxidant Performance: Shelf-Life Prediction of Mayonnaise. Mayonnaise is a typical example of a matrix that is destroyed during conventional accelerated oxidation studies. The emulsion tends to break at elevated temperatures, and the results therefore relate more to the bulk oil liberated from the emulsion during the analysis. Reduction of the temperature of analysis to ∼40 °C avoids destruction of the matrix and therefore gives a much more realistic view of the oxidative stability of the emulsion. However, when a mild temperature stress is used without additional initiator, then only minimal acceleration of oxidation was observed (*17*, *18*). The acceleration of oxidation with transition metals is undesirable for mayonnaises and dressings because chelators are commonly used in this type of product. The use of metal-initiated oxidation would prohibit research into this important class of preventive antioxidants.

The alternative FRG−OB assay was used to predict the possibility of replacing the synthetic antioxidant EDTA, which is commonly used in dressings, with a natural RE. For this purpose three groups of mayonnaise samples were evaluated with FRG−OB: (1) negative control without antioxidant; (2) positive control with 75 ppm of EDTA; and (3) treatment with 750 ppm of RE. A conventional validation assay was also conducted wherein identical samples were produced without initiator and stored at 22 °C for the measurement of peroxides over 58 days.

Table 4. Induction Times of Antioxidant-Treated Mayonnaise Measured with FRG–OB

	FRG–OSI induction time		
sample	induction time (h)	SD (h)	relative error (%)
control	57.3	±1.4	2.4
750 ppm of RE	73.8	±2.2	2.9
75 ppm of EDTA	74.3	±2.9	3.9

Table 5. Evolution of the Peroxide Values (Millimoles per Kilogram) of Antioxidant-Treated Mayonnaise as a Function of Time

	treatment	
day	750 ppm of RE	75 ppm of EDTA
13	6.49	7.23
33	9.40	9.99
58	21.74	19.43

Results for the FRG−OSI test (**Table 4**) and the evolution of peroxides (**Table 5**) indicated that 750 ppm of RE could replace 75 ppm of EDTA without changing the oxidative stability of the mayonnaise. Clearly a conventional long-term peroxide evolution assay gave results similar to these obtained by the accelerated FRG−OSI test.

The good predictability of shelf life in emulsions can be attributed to the similarity between the conditions of the accelerated oxidation and the natural oxidation process. The initiator produces radicals in only the lipid phase and has no direct influence on the aqueous phase. This situation relates to the natural oxidation process, wherein the majority of the radical reactions occur in the lipid phase. The initiator AMVN is known to generate radicals in the lipid phase only (*19*, *20*). Krainev et al. were able to prove with an EPR experiment that none of the AMVN-derived radical species can escape from the hydrophobic lipid environment (*13*). This avoids the formation of reactive oxygen species that can be considered artificial compared to the natural process, enhancing the correlation with the real shelf life of the emulsion.

Shelf-Life Prediction of Minced Pork. Accelerated evaluation of raw meat is impossible when high temperatures are used. The raw meat will start to cook, and the original food matrix will modify dramatically due to coagulation of the proteins, denaturation of the iron-containing hemoproteins (prooxidant), and destruction of enzyme activity (*21*−*23*). These transitions will not occur during FRG assays at 40 °C.

The correlation between FRG−OB data of antioxidant-treated minced pork (**Figure 2**) and the conventional nonaccelerated shelf life evaluated with TBARS (**Figure 3**) was investigated. For this purpose three groups of meat samples were evaluated: (1) negative control without antioxidant, (2) treatment with 1000 ppm of mixed tocopherols, and (3) treatment with 1000 ppm of RE. Similar samples were also produced without initiator and stored at 6 °C. The TBARS content was measured over a period of 8 days. The TBA values, which are commonly used to indicate shelf life, were compared with the predicted shelf life calculated from the results obtained with FRG−OB.

Again, the results from **Figures 2** and **3** yield the same conclusions, which is an important validation of the FRG assay. The control sample showed significant oxidation whether this was measured as oxygen absorption or as malondialdehyde production. Both antioxidants had a significant effect in reducing

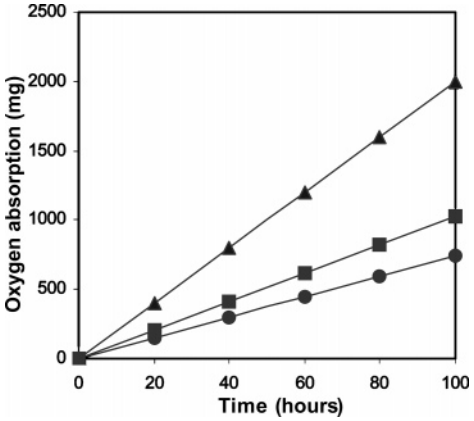

Figure 2. Regression curves of absorbed oxygen measured with FRG–OB: (▲) negative control; (■) 1000 ppm of tocopherols; (●) 1000 ppm of RE.

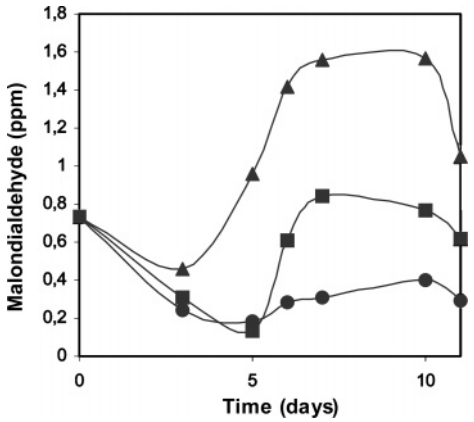

Figure 3. Malondialdehyde formation in antioxidant-treated minced pork: (▲) negative control; (■) 1000 ppm of tocopherols; (●) 1000 ppm of RE.

oxidation, and in both analytical systems RE was slightly superior to tocopherols (**Figures 2** and **3)**.

The average slopes obtained after linear regression of the FRG−OB curves had an average R^2 value of >0.98, and the standard deviations for the measurements were ∼5% (**Figure 2**). Because the slopes are proportional to the rate of the oxidation, they are useful to predict other parameters, which are also proportional to the rate of oxidation (e.g., malondialdehyde). The total surface beneath the TBA curves of **Figure 3** is also a measure of the total amount of malondialdehyde produced during the oxidation.

The slopes of the FRG−OB assays can be used to predict the expected TBA maximum, as this gives a good approximation of the surface beneath the TBA curve. The slopes for the different treatments indicate that the control sample oxidized about twice as fast as the sample treated with tocopherols. The treatment with RE was even more stable. When the TBA maximum of the control sample is used as a starting value, it is possible to use the FRG−OB results to estimate the TBA value of the two treatments. The slope of the control FRG−OB curve had a value of 0.334, which corresponds to a TBA value of 1.57. Therefore, the TBA maximum that would correspond to the slope of 0.172 for the tocopherol-treated sample and to 0.124 for the RE-treated sample can be calculated proportionally.

The results from **Table 6** show that it is possible to accurately predict the relative performance of the antioxidants based on the FRG−OB measurements. Also, quantitatively the TBARs give a good correlation with the experimental values, and this is an important validation of the method. The slightly lower correlation for the RE-treated sample may be due to the relatively low TBA values and the increased relative error for these smaller experimental values. Overall, the FRG−OB is seen to be a valuable method for identification and selection of antioxidant candidates. In combination with known oxidation parameters of the control sample, it is also possible to predict the effect of antioxidants on the final shelf life of the analyzed product.

Table 6. Prediction of TBA Values from FRG–OB Results

sample	slope in FRG–OB	exptl TBA max	predicted TBA max
control	0.334	1.57	
+ 1000 ppm of mixed tocopherols	0.172	0.84	0.81
+ 1000 ppm of RE	0.124	0.40	0.58

Conclusions. The combination of free radical generation with existing analytical equipment results in a straightforward assay that is easy to apply in practice. Both the OSI and OB could be combined successfully with free radical generation. The analysis of the oxidative stability of basic lipid materials proved to be highly reproducible for both methods. The oxidation process in the FRG assay proceeds much more quickly than in nonaccelerated studies. The time of analysis for soybean oil was reduced from >20 days in the traditional OSI assay at 50 °C to 24 h in the FRG−OSI. A comparable reduction of analysis time was observed for FRG−OB measurements.

The main advantage of FRG assays applied on complex food matrices is the improved ability to rapidly compare the qualitative performance of several antioxidants in an accelerated test. Several important weaknesses of traditional accelerated methods have been eliminated. First, the original status of the food matrix is easily retained because of the low temperatures used. Frankel et al. identified this as one of the most critical factors for antioxidant evaluation in food (*5*). The new assay also allows antioxidants with low thermal stability to be studied without the risk of degradation. Because the analyses can be carried out at temperatures of ≤40 °C, it is also possible to retain enzyme activity in food products. This may be an important feature in specific products where residual enzymes, for example, peroxidases or lipoxygenases, play a role in the oxidation process (*24*, *25*). Also, changes in antioxidant activity as a function of temperature are of no concern when the accelerated analyses are performed at temperatures close to the real storage temperature. This is important for many antioxidants that exhibit a change in antioxidant activity at higher temperatures. It was observed for rosemary extract and other antioxidants that the antioxidant efficacy declines at higher temperatures and that consequently the antioxidant performance can be underestimated with conventional accelerated techniques (*26*).

The quantification of real shelf life using FRG assays seems to be more accurate than for traditional methods that use increased temperatures. Whether FRG assays eventually will be able to replace nonaccelerated shelf-life studies remains to be investigated. To verify this possibility, many different types of food matrices need to be studied and methods need to be adjusted and validated in combination with correlation studies. Nevertheless, the FRG assays can be used as an alternative for traditional analytical methods. The technique is a valuable tool in antioxidant research for a rapid selection of promising antioxidant formulations or for initial screening of large libraries of antioxidant molecules from botanical sources for their antioxidant effect in specific food systems.

The accelerated oxidation at low temperatures opens new perspectives for the evaluation of the oxidative stability of heat-sensitive food products in a short period of time. It will be possible to analyze products without destruction of the original food matrix, which is expected to result in a better correlation with shelf life (*5*).

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