

New ^1H NMR-Based Technique To Determine Epoxide Concentrations in Oxidized Oil

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S Supporting Information

ABSTRACT: A new method to determine epoxide concentrations in oxidized oils was developed and validated using ^1H NMR. Epoxides derived from lipid oxidation gave signals between 2.90 and 3.24 ppm, well separated from the signals of other lipid oxidation products. To calibrate, soybean oils with a range of epoxide concentrations were synthesized and analyzed using ^1H NMR by taking the *sn*-1,3 glycerol protons (4.18, 4.33 ppm) as internal references. The ^1H NMR signals were compared to the epoxide content determined by titration with hydrogen bromide (HBr)–acetic acid solution. As expected, the signal response increased with concentration linearly ($R^2 = 99.96\%$), and validation of the method gave results comparable to those of the HBr method. A study of the oxidative stability of soybean oil was performed by applying this method to monitor epoxides during thermal lipid oxidation. The epoxide content increased over time and showed a different trend compared to peroxide value (PV). A phenomenological model was suggested to model epoxides derived from lipid oxidation.

KEYWORDS: soybean oil, ^1H NMR, lipid oxidation, epoxides, validation

INTRODUCTION

Oils containing unsaturated fatty acids are susceptible to lipid oxidation, which brings about off-flavors and toxicity. In the study of lipid oxidation, much attention has been given to the measurement of primary oxidation products, such as hydroperoxides, because they are considered to be important intermediates in the oxidation process. Several methods have been developed to measure hydroperoxides,¹ including the commonly used method, peroxide value (PV), which is easy to perform, making it the most popular indication of lipid oxidation.² Generally, the formation of hydroperoxides is accompanied by the rearrangement of methylene-interrupted double bonds, which generates conjugated dienes.³ Conjugated dienes can be determined by measuring the absorption of ultraviolet–visible (UV–vis) light, and this technique has also been applied for the purpose of studying oxidative stability.⁴ The decomposition of hydroperoxides generates more active radicals and gives rise to the formation of secondary lipid oxidation products, including unsaturated aldehydes and short-chain volatiles. These products have also been successfully measured by UV–vis spectroscopy and headspace gas chromatography–mass spectrometry (GC-MS).¹ The generation of these products has been well studied; however, they are all associated with the decomposition of hydroperoxides, which fail to provide an overall description of the lipid oxidation process.

Epoxides have been reported to form from the rearrangement of the alkoxy radicals and thus were initially recognized as secondary oxidation products.⁵ In contrast, others have suggested that they form from intramolecular radical substitution with peroxides.⁶ More recently, epoxides have been considered as important intermediates, which may form from peroxy radicals directly, independently of hydroperoxides.⁷ However, they have been rarely monitored and

modeled during oxidative stability studies of oils. Accordingly, compared to the well-studied kinetic models of PV,^{8,9} much less information is available for kinetic modeling of epoxides in lipid oxidation. This lack of knowledge may lead to misunderstandings or underestimation of the extent of lipid oxidation.⁷ Monitoring epoxides should also provide a more comprehensive understanding of lipid oxidation mechanisms.

A number of methods have been developed to measure epoxides in oxidized oils, although all have associated challenges. The most widely used method to determine epoxide concentrations in epoxidized oil is the hydrogen bromide (HBr) method, involving direct titration of oil with HBr–acetic acid solution (AOCS Method Cd 9-57).² However, this method is not applicable to oxidized oils, because α - and β -unsaturated carbonyls and conjugated dienes formed during lipid oxidation can also react with HBr, resulting in overestimations of epoxide content. A number of other methods have been introduced recently to measure epoxides in oils, including utilization of 4-(*p*-nitrobenzyl)pyridine (NBP),¹⁰ *N,N*-diethylthiocarbamate (DTC),¹¹ and transmethylation.¹² However, these methods require derivatization of epoxides before analysis. Accordingly, they involve extra procedures for pretreatment and are therefore time-consuming. In addition, their applications to monitor epoxides formed during the oxidation process must be verified as a variety of other secondary oxidation products may interfere with the derivatization reactions.

^1H nuclear magnetic resonance (NMR) was recently applied to measure epoxides in oils by identifying the chemical shifts of

Received: April 6, 2015

Revised: May 25, 2015

Accepted: June 2, 2015

Published: June 2, 2015

epoxide groups, where a variety of internal standards were used.^{13,14} In one of these studies,¹³ the nondeuterated chloroform present in the deuterated chloroform was used as an internal reference for quantitative purposes. Generally, the main benefit of using the residual solvent signal as an internal standard is that it allows the sample to be recovered because the solvents are volatile and therefore easy to remove. However, the residual solvent content was found to vary from batch to batch, and its use for quantitative purposes requires external calibrants to determine its content in the deuterated solvent.¹⁵ Therefore, frequent calibrations are required when using the deuterated solvent as an internal standard. Moreover, because chloroform is easily evaporated, when it is used as an internal standard for quantification, special care must be taken in handling the samples and the solvents. In the other study,¹⁴ signals from methyl groups were used as the internal standard for quantification, but the applicability of this method to oxidized oils needs to be verified, because alkanes can be formed when oils undergo lipid oxidation,¹⁶ especially at high temperatures. Glycerol protons were found to be constant during oxidation in the absence of water;¹⁷ therefore, they can be used as alternative internal standards for ¹H NMR quantification, which will allow rapid sample preparation and easy handling.

In this study, a quantitative NMR method of measuring epoxides was developed and validated using soybean oil. This method represents significant improvement over other methods^{12–14} in that partially epoxidized oils are synthesized and used for quantification, instead of using epoxy fatty acid standards, so that signals are generated to mimic those that would be encountered in oxidized oils. Specifically, synthetic epoxidized soybean oils were made to establish a calibration curve, and used to validate the proposed method. The one-step sample preparation allowed rapid determination of epoxides at low levels without any addition of external references. As the objective of this study was to develop a method to monitor epoxides during lipid oxidation, the application of the proposed method to thermally oxidized soybean oils was also verified by conducting an accelerated study of oxidative stability.

MATERIALS AND METHODS

Materials. Commercial soybean oil was obtained from Clic International Inc. (Montreal, Canada). Hydrogen bromide–acetic acid solution (33 wt % in acetic acid) and deuterated chloroform were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Hydrogen peroxide (50%) was from Fisher Scientific (Ottawa, Canada). All chemicals were of analytical grade.

GC Analysis of Fresh Soybean Oil. The fatty acid profile of soybean oil was analyzed using gas chromatography with flame ionization detection (GC-FID). A modified version of the Global Organization for EPA and DHA Voluntary Monograph for Omega-3 was followed to convert triacylglycerols to fatty acid methyl esters (FAME).¹⁸ FAME were separated on a DB-23 column ((50%-cyanopropyl)methylpolysiloxane, 30 m, 0.25 mm i.d., 0.25 μm thickness) using helium as the carrier gas. The GC conditions were as follows: the initial temperature of the oven was held at 153 °C for 2 min and increased to 205 °C at 2.3 °C/min and held for 8.3 min; the FID was set to 270 °C, whereas the injector was operated in split mode (100:1) at 250 °C.¹⁹ The fatty acid profile was reported as percent of total mass.

Epoxidation. The fatty acid profile of the commercial soybean oil was obtained by GC-FID and the concentration of double bonds was therefore calculated using fatty acid proportional data. To prepare epoxidized soybean oil for characterization of epoxides, a molar ratio of 1:0.5:1.5 double bonds/formic acid/hydrogen peroxide was used for

epoxidation. The mixture of formic acid, hydrogen peroxide, and soybean oil was heated at 38 °C for 6 h.^{20,21}

To establish a calibration curve, epoxidized soybean oils with a range of different concentrations of epoxides were prepared by reducing the amount of the reagents (formic acid and hydrogen peroxide) used for epoxidation while maintaining the molar ratios of formic acid and hydrogen peroxide at 0.5:1.5 (1:3). As the amount of epoxides generated from lipid oxidation is usually small, much lower amounts (<10% of the original amount) of epoxidation reagents were used. For converting <2% double bonds in soybean oils to epoxy groups, 2 h instead of 6 h was used with heating at 38 °C. Ethyl acetate was used to recover the epoxidized oil, following the procedures in a previous study,²² and was then dried with anhydrous sodium sulfate. The solvent in the oil layer was evaporated under nitrogen, and the remaining oil was collected as the epoxidized soybean oil.

¹H NMR Analysis. The ¹H NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. All of the oil samples were prepared for ¹H NMR analysis by dissolving 0.1 g of sample in 2.0 mL of deuterated chloroform and placing 750 μL of the solution in a NMR tube (5 mm diameter, 8 in. in length, Wilmad-LabGlass, Vineland, NJ, USA). The acquisition parameters were modified from a previous study:²³ spectral width, 10080 Hz; relaxation delay, 3 s; number of scans, 64; acquisition time, 3.25 s; with a total acquisition time of 6.9 min for each sample. The signal of nondeuterated chloroform present in the deuterated chloroform was used as a reference for chemical shifts, and the signals associated with the *sn*-1,3 glycerol protons (4.18, 4.33 ppm) on the glycerol backbone were taken as an internal standard for quantification. All of the integrations for quantitative NMR were conducted using Topspin 2.1.

Epoxide Content Determined by the HBr Method. The concentration of epoxides in synthetic epoxidized soybean oil was determined by a modified AOCS Method Cd 9-57 using hydrogen bromide (HBr).² To accurately determine the low epoxide concentrations, diluted HBr–acetic acid solution and increased sample size were used. As HBr is sensitive to air and light, the HBr–acetic acid solution was standardized each time before use. Crystal violet solution (0.001 g/mL in acetic acid) was used as the indicator, and the samples were titrated to a blue-green end point. This determination was performed in triplicate for each sample.

To facilitate comparison with PV, the concentration of epoxides was reported as millimoles of epoxy groups per kilogram of oil (mmol/kg)

$$\text{epoxide content (mmol/kg)} = (V \times N \times 1000) / M$$

where *V* is the volume of HBr to titrate the sample in mL, *N* is the normality of the HBr solution in mol/L, and *M* is the mass of sample in g.

Characterization of Epoxides by ¹H NMR. The fresh and the epoxidized soybean oils were analyzed by ¹H NMR for characterization of epoxides. The signals were assigned by taking into account the descriptions of the chemical shifts from the previous work.¹³

Quantification of Epoxides by ¹H NMR. A standard curve is usually established by analyzing a series of standard solutions and plotting the signal responses versus the concentrations. However, when using NMR, this may contribute to large deviations from a linear relationship over a variety of concentration, as observed in previous work.²⁴ Therefore, rather than establishing a standard curve by making serial dilutions of a stock solution, a calibration curve method was used to keep the NMR samples at the same total lipid concentration of ~50 mM but with various epoxide contents.

A set of 20 synthetic epoxidized soybean oil samples were analyzed by ¹H NMR. The signals of the *sn*-1 and *sn*-3 glycerol protons (4.18, 4.33 ppm) were taken as an internal standard for quantification because they were well separated from other signals and remained constant during both epoxidation and oxidation. The area of the signal of epoxides was obtained by calibrating the area of the internal standard as 4.0000. The integrations were performed in triplicate. A calibration curve was established by plotting the epoxide area versus the epoxide concentration determined by using the modified AOCS Method Cd 9-57 described.²

Table 1. Test of Accuracy and Precision of the Proposed Method, Evaluated by the *p* Values of the Two-Sample *t* Test and the Acceptable Horwitz %RSDs, Respectively

sample	epoxide content (HBr method)		epoxide content (¹ H NMR)		two-sample <i>t</i> test <i>p</i> value	acceptable Horwitz %RSD
	mean ± SD (mmol/kg)	%RSD	mean ± SD (mmol/kg)	%RSD		
1	666.1 ± 4.7	0.71	660.7 ± 8.8	1.33	>0.10	2.65
2	325.9 ± 5.4	1.66	320.8 ± 5.8	1.81	>0.10	2.96
3	54.6 ± 0.8	1.49	55.6 ± 1.4	2.61	>0.10	3.87
4	11.7 ± 0.3	2.26	12.2 ± 0.5	3.73	>0.05	4.88

The validation of this quantitative NMR technique included the testing of linearity, parameters of accuracy, and repeatability.²⁵ Thus, the validation process was conducted following the guidelines previously addressed by the International Union of Pure and Applied Chemistry (IUPAC).²⁶ The statistical tests were conducted by Minitab 17. For the test of linearity, the regression and the residuals of the calibration curve were analyzed. For the estimation of accuracy and precision, four epoxidized samples were analyzed by ¹H NMR in five replicates. The average epoxide content of each sample was compared to the value determined according to AOCS Method Cd 9-57 for the test of trueness or bias, using a two-sample *t* test (Table 1). The precision was described by the relative standard deviation (%RSD) of the quintuplet results (Table 1) and characterized by the Horwitz equation.²⁷ According to the Horwitz equation, an acceptable Horwitz %RSD was calculated as

$$\%RSD = \frac{2}{3} \times 2^{(1-0.5 \log C)}$$

where *C* was the mass concentration of epoxides in decimal fraction. %RSD falling below this level indicated sufficient precision.²⁷

To assess precision using the Horwitz equation,²⁷ the epoxide content was calculated as mass fraction of the oxirane oxygen in the oil²

$$\text{epoxide content} = (V \times N \times 0.016) / M$$

where *V* is the volume of HBr to titrate the sample in mL, *N* is the normality of the HBr solution in mol/L, and *M* is the mass of sample in g.

The precision of the method was also evaluated using thermally oxidized soybean oils. Five soybean oil samples were incubated in open test tubes at 100 °C for 4, 8, 12, 16, and 20 days. Each sample was tested for its PV (AOCS Method Cd 8-53)² and epoxide content (the proposed method). The tests were conducted for each sample in triplicate (Table 2). The precision was described by the %RSD of the triplicate results.

Table 2. Peroxide Value and Epoxide Content Measured for Soybean Oil Heated at 100 °C for the Test of Repeatability, Expressed by %RSD

sample	peroxide value		epoxide content	
	mean ± SD (mmol/kg)	%RSD	mean ± SD (mmol/kg)	%RSD
1	47.2 ± 0.3	0.61	8.4 ± 0.4	4.15
2	48.3 ± 1.4	2.80	17.2 ± 0.6	3.58
3	89.1 ± 0.7	0.79	29.2 ± 1.1	3.85
4	87.6 ± 0.6	0.64	55.7 ± 1.5	2.69
5	60.0 ± 0.6	1.02	90.7 ± 2.0	2.20

Stability Studies of Soybean Oil. A stability study of soybean oil was conducted to monitor the changes in PV and epoxide content over the course of oxidation. Three open amber containers (125 mL) were used for incubations at 100 °C with each initially containing 30 g of soybean oil. Every 2 days, 2.5 g of oil was sampled from each container for analysis of its PV (AOCS Method Cd 8-53)² and epoxides (the proposed method). The tests of each oil sample were performed in triplicate. PV and epoxide content were plotted over time for comparison.

RESULTS AND DISCUSSION

Characterization of Epoxides Derived from Soybean Oil. The initial soybean oil contained 10.2 ± 0.1% palmitic acid (16:0), 21.9 ± 0.3% oleic acid (18:1n-9), 52.5 ± 0.8% linoleic acid (18:2n-6), and 7.7 ± 0.2% linolenic acid (18:3n-3). Therefore, the total unsaturation was estimated to be 5.46 mol of double bonds per kilogram of oil. The signals of epoxides derived from soybean oil were between 2.90 and 3.24 ppm; as the size of those signals increased, the size of the signals due to double bonds (~5.40 ppm) decreased correspondingly (Figure 1; Supplementary Table 1 in the Supporting Information). Thus, the epoxidation method used here was successful in epoxidizing the commercial soybean oil, generating obvious signals of epoxides in the ¹H NMR spectra.

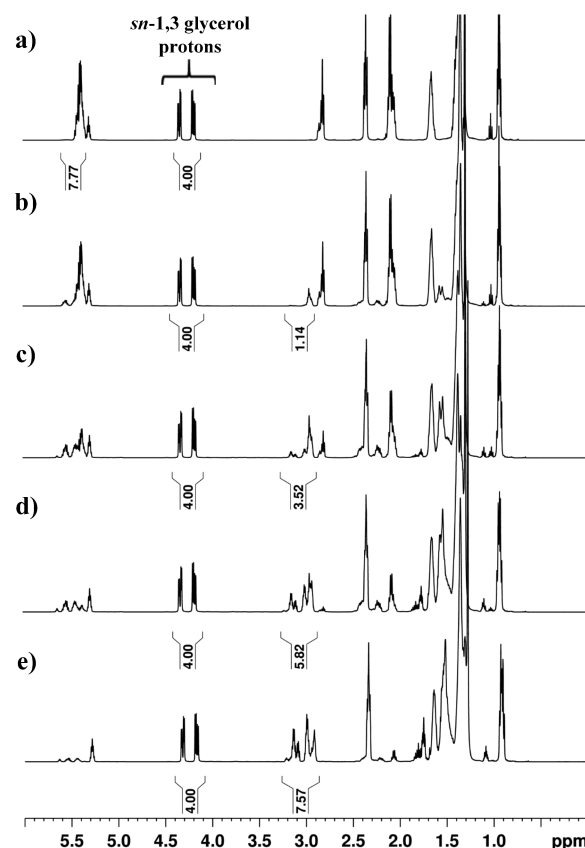


Figure 1. ¹H NMR spectrum of (a) fresh soybean oil and epoxidized soybean oils with epoxide contents of (b) 0.70 mol/kg, (c) 2.06 mol/kg, (d) 3.41 mol/kg, and (e) 4.16 mol/kg. The integrations of the double bonds (5.40–5.60 ppm in (a)) and the epoxides (2.90–3.24 ppm in (b–e)) were made by taking the signals of *sn*-1 and *sn*-3 glycerol protons (4.18 and 4.33 ppm) as internal standards. The area of the internal standard was calibrated as 4.00 as the number of *sn*-1 and *sn*-3 glycerol protons is 4.

On the basis of previous publications^{13,14} and our observation of the ¹H NMR spectra in Figure 1, we made the assignments as follows, where $-\text{CHOCH}-$ represents an epoxy group: δ 1.45–1.60 ($-\text{CH}_2-\text{CHOCH}-$), 1.70–1.85 ($-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$), 2.00–2.10 ($-\text{CH}_2-\text{CH}=\text{CH}-$), 2.80–2.85 ($-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$), 2.90–2.95 ($-\text{CHOCH}-$), 2.90–2.95 ($-\text{CHOCH}-\text{CH}_2-\text{CH}=\text{CH}-$), 3.00 ($-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$), 3.06–3.24 ($-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$), 5.35–5.45 ($-\text{CH}=\text{CH}-$), 5.40–5.60 ($-\text{CH}=\text{CH}-\text{CH}_2-\text{CHOCH}-$), 5.60–5.65 ($-\text{CHOCH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CHOCH}-$) (Supplementary Table 1). The range of chemical shifts of epoxides increased with the concentration of epoxides in the oil (Figure 1). For oils with a low epoxide content (Figure 1b), the signals of epoxides appeared around 2.90–2.95 ppm, which was likely related to the formation of the partially epoxidized linoleic acid and linolenic acid ($-\text{CHOCH}-\text{CH}_2-\text{CH}=\text{CH}-$), and overlapped with the less intense signal of epoxy oleic acid at 2.90–2.95 ppm ($-\text{CH}_2-\text{CHOCH}-\text{CH}_2-$). For oil samples with higher epoxide content (Figure 1c), the signals associated with diepoxides appeared at \sim 3.00 ppm ($-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$) and 3.09 and 3.16 ppm ($-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$), and their intensities increased with increasing concentration. The signal that indicated the presence of triepoxides appeared at 3.20 ppm, when \sim 62% of the original double bonds had been converted into epoxy groups (Figure 1d). In Figure 1e, the epoxidized soybean oil gave five signals between 2.90 and 3.24 ppm, with small signals due to double bonds remaining between 5.40 and 5.60 ppm, which indicated that \sim 76% of the double bonds were converted into epoxy groups.

Lipid oxidation is a relatively complex autoxidation process, so the structure of epoxy products may vary with the conditions of oxidation. Although the chromatography method employs common GC-FID instrumentation,²⁸ making it an accessible technique to many analysts, it does rely on the use of epoxy fatty acid methyl ester (FAME) standards for identification of the signals on the chromatograms. Any epoxide structures that vary from the standards may be overlooked during identification and cause underestimations of epoxide concentration. Moreover, the selectivity of quantitative NMR did not depend on the structure of epoxides, and therefore it gave an overview of all the epoxy groups formed in the oil. The chemical shifts of epoxides (2.90–3.24 ppm) were distinct from other oxidation products including alcohols (3.43–3.62 ppm), conjugated dienes (5.40–6.58 ppm), double bonds conjugated with carbonyls (6.08–6.82 ppm), hydroperoxides (8.30–8.90 ppm), and aldehydes (9.49–9.79 ppm).²⁹ In summary, ¹H NMR can be used to detect epoxides in oxidized soybean oils with superior selectivity, which is a major advantage over the chromatography method.

Quantification of Epoxides. Although fully epoxidized soybean oil gave signals between 2.90 and 3.24 ppm, oxidized oils, with relatively low epoxide content, gave signals between 2.90 and 3.10 ppm, mostly due to monoepoxides with or without adjacent double bonds (Supplementary Table 1). The epoxy protons were monitored for quantitative purposes because the size of the signals between 2.90 and 3.24 ppm was proportional to the amount of epoxy groups generated in the oil, regardless of the presence of saturated/unsaturated mono- and diepoxides. Although the signals at 1.45–1.60 ppm ($-\text{CH}_2-\text{CHOCH}-$) and 2.00–2.10 ppm ($-\text{CH}_2-\text{CH}=\text{CH}-$) were related to the generation of epoxides (Figure 1),

the change in size of the signals was not proportional to the amount of epoxy groups; therefore, the epoxy protons were the best indicators of formation of epoxides.

When signal response versus concentration was plotted, a calibration curve was established for epoxides in soybean oil with a good linear relationship. The regression equation was $y = 0.0151 + 0.0016x$ with an R^2 of 99.96%, where y represented the signal response and x was the concentration of epoxides (Figure 2). To our knowledge, this is the first demonstration of

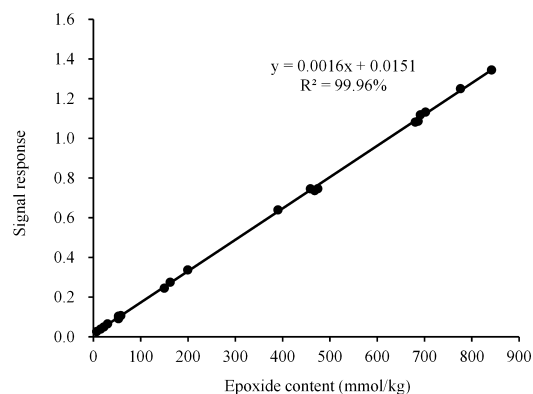


Figure 2. Fitted linear relationship by plotting signal response (y) versus the epoxide concentration (x) measured by the HBr method. Each point represents a single epoxidized soybean oil sample.

agreement between the ¹H NMR method and the HBr method for low epoxide concentrations. Residual analysis of all calibration points did not reveal any trends relating to the concentration of epoxides. The residuals passed the Anderson–Darling test and followed a normal distribution with a p value of 0.148. Thus, the linear relationship between the signal response and the epoxide content did not depend on the concentration of epoxides in the experimental range of concentrations (6.7–841.6 mmol/kg). A linear fit was found for the calibration curve ($F = 7.63$, $p < 0.001$). Therefore, the area ratio of the signals of epoxides/internal reference increased linearly with the epoxide content. Because epoxides were not found in the fresh soybean oil using the HBr method, the fresh soybean oil was considered a blank. The limit of quantification (LOQ) was therefore determined to be 6.3 mmol/kg by taking into account the standard deviation (σ) of the blank and the slope (s) of the calibration curve ($\text{LOQ} = 10\sigma/s$).³⁰

Accuracy testing was conducted on four epoxidized samples with different concentrations. As shown in Table 1, the proposed method gave results similar to those measured by AOCS Method Cd 9-57 (HBr method), with little bias and two-sample t tests showing no difference between the mean values determined by the HBr method and the proposed method for each sample (p value > 0.05). The precision of the proposed method (%RSD 1.33–3.73) was slightly poorer than that of the HBr method (%RSD 0.71–2.26), which was likely due to the errors introduced by manual integration of the ¹H NMR signals. The precision of both methods varied with concentration. Compared to the acceptable Horwitz %RSD values (2.65–4.88), the proposed method provided good precision (%RSD 1.33–3.73). Thus, quantitative NMR was shown to provide reliable results.

The objective of this work was to develop a quantitative method to determine epoxide content in oxidized oils for the study of lipid oxidation. Therefore, it was important to verify

the applicability of this method to thermally oxidized oils. The test of repeatability of the method was performed on five thermally oxidized soybean oil samples, which were heated at 100 °C for 4, 8, 12, 16, and 20 days (Table 2). There were contrasting patterns of PV and epoxide content with samples 1 and 2 (Table 2) having similar PVs (47.2 and 48.3 mmol/kg) but different epoxide contents (8.4 and 17.2 mmol/kg). Similarly, samples 3 and 4 (Table 2) had comparable PVs (89.1 and 87.6 mmol/kg) but very different epoxide contents (29.2 and 55.7 mmol/kg). The differing epoxide concentrations indicate that the two samples had undergone different oxidation processes, an observation that would be overlooked with just examination of PV. Thus, epoxide content provided a different evaluation of the extent of lipid oxidation compared to PV.

Compared to the HBr method,² the proposed method overcomes the problem of the interferences caused by other lipid oxidation products (α - or β -unsaturated carbonyls and conjugated dienes) when using the HBr method. Additionally, much less organic solvent is required for NMR analysis, because standardization of HBr solution is required before use of the reagent; the HBr method is also time-consuming. Compared to the previous methods of measuring epoxides, such as the NBP,¹⁰ DTC,¹¹ and transmethylation³¹ methods, the major advantage of quantitative NMR is its convenience. No derivatization is needed for analysis of epoxy compounds, and the preparation of an NMR sample is simple and rapid, making NMR a competitive approach to determine epoxides.

In this study, the *sn*-1,3 glycerol protons were taken as the internal standards instead of using the signals due to the residual chloroform¹³ or the methyl protons.¹⁴ As described earlier, methyl protons have limited applications as internal standards for oxidized oils, whereas the use of the residual solvent as standard may require external calibrants and special handling of the samples. Therefore, taking the signals related to the structure of triacylglycerol as internal standards can overcome these problems and still allow the sample to be recovered in its pure form, because no extra standard material is added. Unfortunately, information describing LOQ, accuracy, or precision was not provided in those previous studies,^{13,14} so it is impossible to compare the performance of the proposed method with the previous methods in those aspects.

The method described here is an original approach; rather than spiking fully epoxidized soybean oil or epoxy fatty acid standards into samples, synthetic partially epoxidized oils (<10% double bonds were converted into epoxy groups) were used to establish a calibration curve. The synthetic epoxidized oils with low epoxide levels gave signals (2.90–3.10 ppm) similar to those derived from the oxidation process, making it possible to establish a calibration curve mimicking the real situation. The use of synthetic epoxidized oils as calibration standards has a number of advantages. First, commercial unsaturated epoxy fatty acid standards and diepoxide standards are only available as a limited number of structures. The proposed method utilizes commonly used reagents, hydrogen peroxide and formic acid, to synthesize partially epoxidized oils with remaining double bonds, which avoids the limitation of the commercially available standards. Second, the extent of epoxidation can be controlled by the amount of reagents used; therefore, the range of the concentrations encompassed by the calibration curve is adjustable. When epoxides are expected at a relatively low level, epoxy groups are likely surrounded by double bonds, and only a small amount of reagents is needed. When epoxides are expected at a high level,

diepoxides can be synthesized by increasing the amount of epoxidation reagents. Third, the current epoxidation method can be easily applied to other kinds of oils,^{20,22} so this method will have a wide range of applications.

Stability Study of Soybean Oil. To further study the formation of epoxides during lipid oxidation, a stability study of soybean oil was performed at 100 °C for 12 days. The curves representing PV and epoxide content showed different patterns over time, although deviations were observed between the three samples determined at each test point (RSD between 1.62 and 10.63% for PV and between 0.61 and 8.69% for epoxide content; Figure 3). PV increased quickly in the first 8 days and

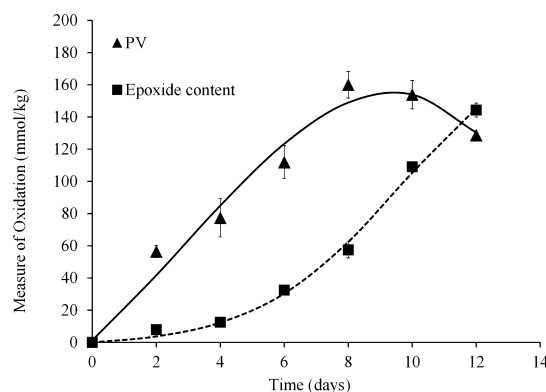


Figure 3. Peroxide value (PV) and epoxide content (mean \pm standard deviation, $n = 3$ for each point) in the stability study of soybean oil performed at 100 °C with model 2 shown for PV and with model 3 for the epoxide content. See Table 3 for model parameters.

then reached a peak, followed by a slow decline. However, no significant increase was found for the epoxide content in the first 4 days. Epoxide content increased drastically after the lag time (days 0–4) and was not observed to decrease in the experimental period. Compared to the stability study of sunflower oil at 100 °C in the previous study,¹³ lower amounts of epoxides were found in the soybean oil in this work. The two oils would have had different fatty acid compositions, which are well-known to influence the rate of formation and structure of lipid oxidation products. The surface area of oil during oxidation was also much greater in the previous work,¹³ likely leading to a faster oxidation process.³²

The PV and epoxide content were fitted by three selected models using the least-squares method (Table 3), including a second-order polynomial function, a third-order polynomial function, and a phenomenological model,⁹ all in the variable t , which represented the days of thermal oxidation. The polynomial functions were selected because PV showed a significant decrease after the peak value. The phenomenological model was previously introduced for modeling PV because hydroperoxides are known as intermediates, forming and decomposing at the same time. The parameters of the phenomenological model, $a(T)$, $k_f(T)$, and $t_{cf}(T)$, were temperature dependent, related to the formation and decomposition of hydroperoxides as intermediate products of lipid oxidation.⁹ In this work, this model was extensively fitted to the epoxide data because epoxides have also been considered as important intermediates.⁷ The mean square error (MSE) of each regression was used as a measure of the goodness of fit (Table 3). Specifically, PV was fitted by the second-order model $y(t) = -1.44t^2 + 28.83t + 1.32$ with an MSE of 163.94,

Table 3. Fit of the Selected Models to PV and Epoxide Content Observed in the Stability Study of the Soybean Oil

model 1:		$y(t) = at^2 + bt + c$			
	<i>a</i>	<i>b</i>	<i>c</i> ^a	MSE	
PV	-1.44	28.83	1.32	163.94	
epoxide content	1.10	-0.93	0.00	23.86	
model 2:		$y(t) = at^3 + bt^2 + ct + d$			
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i> ^a	MSE
PV	-0.16	1.35	18.12	1.32	88.26
epoxide content	0.00 ^b	1.08	-0.84	0.00	23.86
model 3: ^c		$y(t) = y_0 + [a(T) - y_0] \times \left\{ \frac{1}{1 + \exp[k_f(T)[t_{cf}(T) - t]]} - \frac{1}{1 + \exp[k_f(T)t_{cf}(T)]} \right\}$			
	<i>a</i> (<i>T</i>)	<i>k_f</i> (<i>T</i>)	<i>t_{cf}</i> (<i>T</i>)	MSE	
PV	204.15	0.47	2.17	220.47	
epoxide content	199.37	0.45	9.62	10.31	

^aThis constant was determined by the PV or epoxide content measured on the initial day (day 0). ^bThe value was 0.0014 in four

whereas the epoxide content was fitted by $y = 1.10t^2 - 0.93t$ with an MSE of 23.86. Increased order of polynomial function contributed to the lowest MSE for PV (MSE of 88.26 for the third-order model) but resulted in the same MSE for epoxide content (MSE of 23.86 for the third-order model) so a third-order model was not necessary for epoxides. In addition, PV and epoxide content were fitted by the phenomenological model (model 3 in Table 3), where the fit of PV gave a large MSE (MSE of 220.47), but the best fit was observed for the epoxide content (MSE of 10.31). Therefore, model 2, employing a third-order polynomial, was suggested for PV, whereas model 3, the phenomenological model, was best for epoxides (Figure 3). Further studies can be conducted to allow the model to be adapted to the formation of epoxides at different temperatures.

Although the physical meanings of these models are not discussed here, it is obvious that the curves of epoxide concentrations and PV show very different trends, indicating that the rearrangement of radicals is an important reaction during lipid oxidation and should be taken into consideration when monitoring the oxidation process. Epoxides are important lipid oxidation products, produced in large concentrations under the condition of this study, and their formation has a lag time in the initial period of lipid oxidation. As an additional indicator of oxidation, epoxide content provided more information about the extent of the process, which may enable a more comprehensive understanding of the mechanisms of lipid oxidation. With appropriate validation, this ¹H NMR technique should be easily applied to other oils.

■ ASSOCIATED CONTENT

Supporting Information

Integrals of the relevant signals in Figure 1 by taking the *sn*-1,3 glycerol protons as the internal standard (4.00), where overlapped peaks are deconvolved with Lorentzian line shapes. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01719.

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Funding

This work was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant to S.M.B.

Notes

The authors declare no competing financial interest.

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