

## AUTHOR

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## HPLC Analysis of Phenolic Antioxidants in Edible Fats and Oils supporting Food Quality Control and Label Transparency

### Introduction

Fats and oils are used in cooking as a frying medium or as an ingredient in baking. Autoxidation of lipids in the fats and oils

proceeds as a free-radical chain reaction that leads to rancidity causing foul flavors and smells.<sup>1</sup> Phenolic antioxidants may be added to fats and oils to prevent oxidation and rancidity by functioning as free-radical terminators.<sup>1</sup> Food additives, such as these, are regulated globally under various regulatory organizations as long-term elevated exposures leads to increased incidence of certain cancers.<sup>2-4</sup> Depending on the antioxidant, application, and the jurisdiction, concentrations shall not exceed 2 µg/g (2 ppm).<sup>3,4</sup>

It is important for manufacturers producing food stuffs containing phenolic antioxidants to employ robust analytical methods to ensure product consistency and maintain regulatory compliance. Also, for laboratories and regulatory agencies analyzing food stuffs containing phenolic antioxidants, this method allows labeling transparency for fair market competition and consumers' trust.

This application note demonstrates a fast, robust, and sensitive liquid chromatographic method for the analysis of ten commonly used phenolic antioxidants (Figure 1) and builds upon prior work.<sup>5,6</sup>

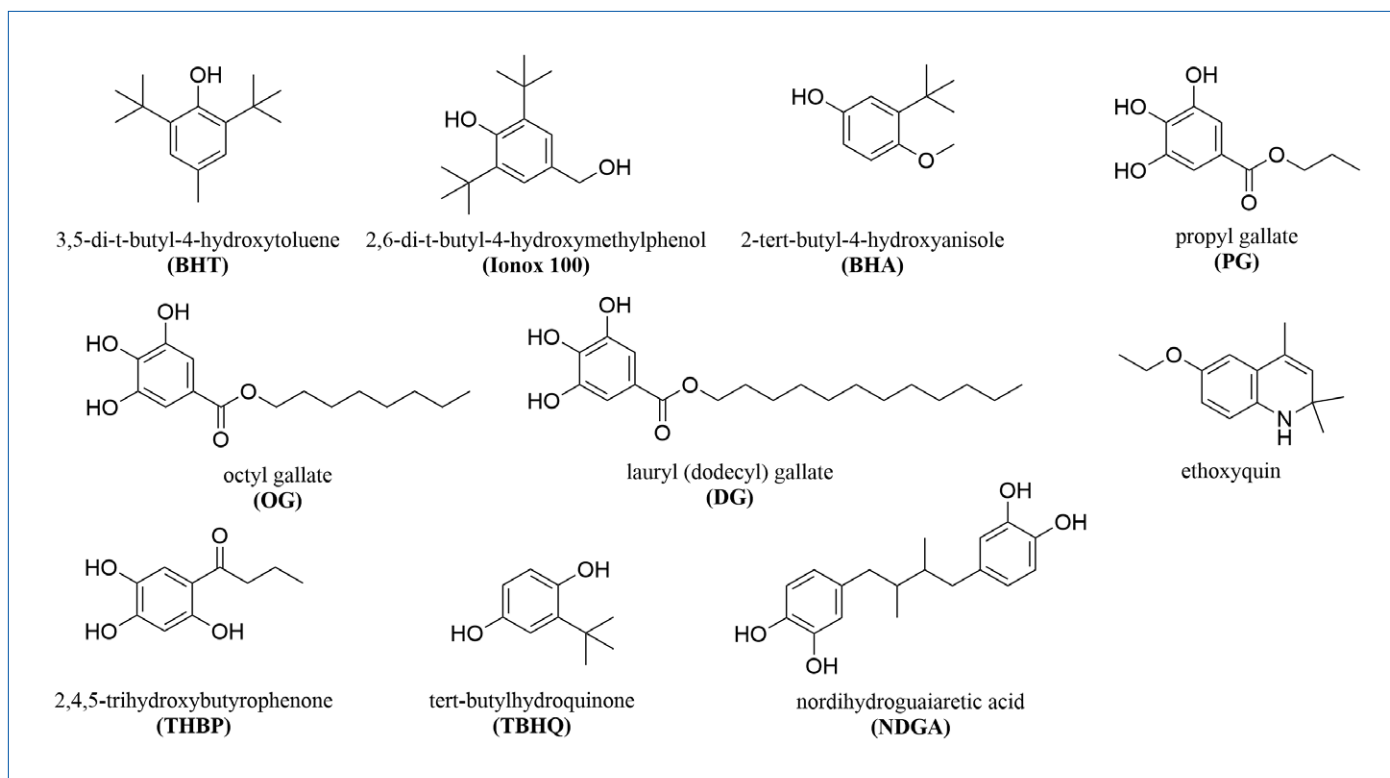


Figure 1: Chemical structures of the ten common phenolic antioxidants analyzed in this study with their common abbreviations in bold.

## Experimental

### Hardware/Software

Chromatographic separation was achieved using a PerkinElmer LC 300™ HPLC System, consisting of an LC 300 HPLC Pump and an LC 300 HPLC Autosampler equipped with an integrated column oven. Detection was achieved using an LC 300 Photodiode Array Detector (PDA). Instrument control, analysis and data processing were performed using the PerkinElmer SimplicityChrom™ CDS Software.

### Method Parameters

The LC parameters are shown in Table 1.

### Solvents, Standards and Samples

All solvents and diluents used were HPLC grade. Unless otherwise specified, standard and sample extract dilutions were prepared using a 1:1 (v/v) acetonitrile/methanol mixture.

The phenolic antioxidant standard kit #2 (catalog# 40048-U) was obtained from Supelco® (Irvine, CA). This included nordihydroguaiaretic acid (NDGA), propyl gallate (PG), octyl gallate (OG), lauryl gallate (dodecyl gallate; DG), 2-tert-butyl-4-hydroxyanisole (BHA), 2,6-di-t-butyl-4-hydroxymethylphenol (Ionox 100), tert-butylhydroquinone (TBHQ), 3,5-di-t-butyl-4-hydroxytoluene (BHT) and ethoxyquin. In addition, a

Table 1: LC Parameters.

LC Parameters					
Column	PerkinElmer Epic Biphenyl, 3 μm, 3.0 x 100 mm (Part# 123191-EBPH)				
Mobile Phase	Solvent A: Water with 0.05% phosphoric acid				
	Solvent B: 75/25 (v/v) acetonitrile/methanol				
	Solvent program: Linear Gradient				
	Step	Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
	1	0.0	1.00	75	25
	2	3.5	1.00	52.7	47.3
	3	4.7	1.00	52.7	47.3
	4	8.0	1.00	0	100
Analysis Time	8 min.; Equilibration Time: 2 min.				
Pressure	5400 psi/370 bar maximum				
Oven Temperature	35 °C				
Sample Temperature	4 °C				
Injection Volume	5 μL (Partial loop)				
PDA Wavelength	Analytical: 280 nm		Reference: 480 nm		Bandwidth: 10 nm
Data Collection Rate	5 pts/sec (Hz)				
PDA Flow cell	10 mm (standard)				

2,4,5-trihydroxybutyrophenone standard (THBP; catalog# 2620-1-X9) was obtained from SynQuest® (Alachua, FL).

Individual 1000- $\mu\text{g}/\text{mL}$  stock standard solutions were prepared by dissolving 50 mg of each standard into 25 mL of methanol in a 50-mL volumetric flask and brought to volume with acetonitrile. A 100- $\mu\text{g}/\text{mL}$  working solution was then prepared by combining 2.5 mL of each stock standard solution in a 25-mL volumetric flask. This also served as the Level 6 calibration standard.

Additional calibrants were prepared by diluting the working solution to concentration levels of 75, 50, 25, 10, and 1  $\mu\text{g}/\text{mL}$  using a 1:1 (v/v) acetonitrile/methanol mixture to give a 6-level calibration set. All calibrants were stored in amber vials at 4 °C for no more than a week to prevent thermal and photodecomposition.

Six different commercial samples were purchased from a few local supermarkets including flavored oil, organic oil, and a vegetable shortening sample, which had TBHQ listed in its ingredients.

Approximately 5.5 g of liquid oil sample was diluted with 10 mL of hexane in a 125-mL separatory funnel and extracted with 10 mL portions of acetonitrile.<sup>7,8</sup> For solid samples, 3 g were liquefied in a water bath at 60 °C before combining with hexane and extraction with acetonitrile. The resulting portions were combined in a 50-mL round-bottom flask and evaporated with nitrogen to about 1-2 mL and reconstituted to 6 mL with a 1:1 (v/v) acetonitrile/methanol mixture. Samples were filtered using 0.22- $\mu\text{m}$  nylon syringe filters into 2-mL amber HPLC vials prior to injecting 5  $\mu\text{L}$  for analysis. Extracted samples were stored at in amber vials 4 °C for no more than 24 hours.

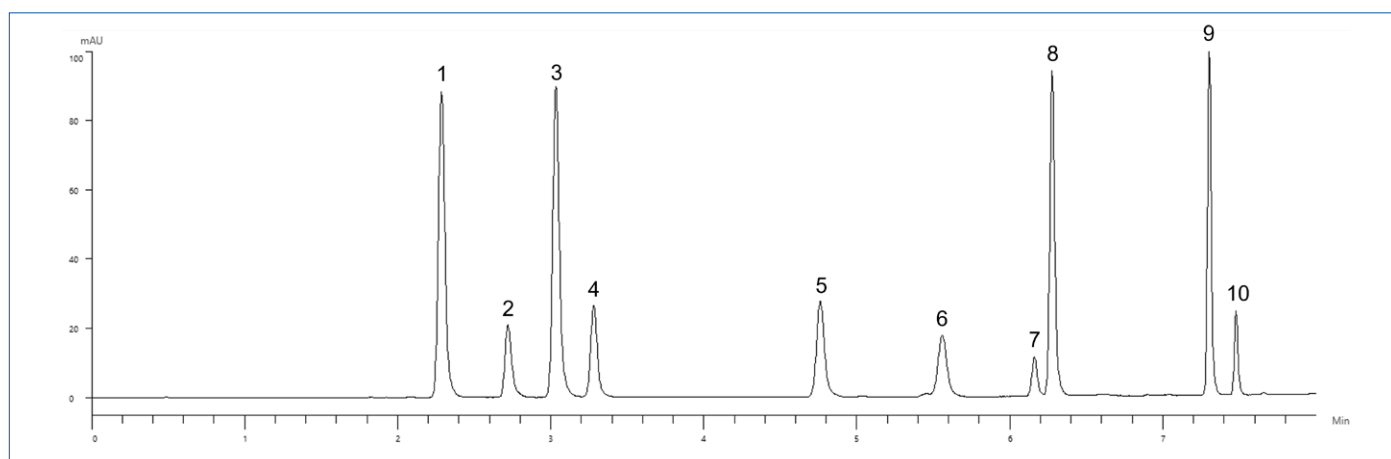


Figure 2: Chromatogram of the 25- $\mu\text{g}/\text{mL}$  phenolic antioxidant standard. 1. PG, 2. Ethoxyquin, 3. THBP, 4. TBHQ, 5. NDGA, 6. BHA, 7. Ionox 100, 8. OG, 9. DG, 10. BHT.

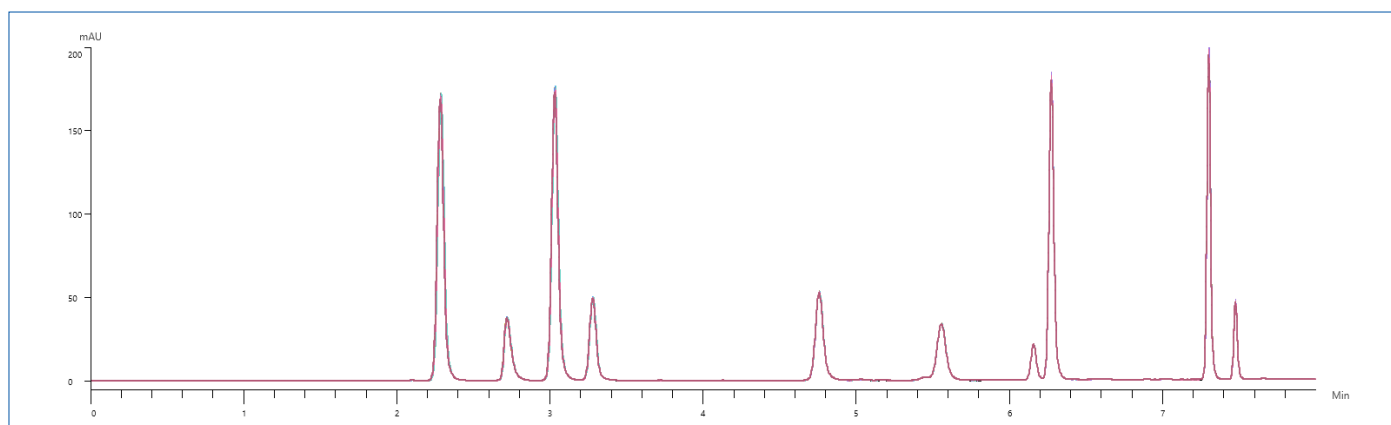


Figure 3: Chromatographic overlay of 16 replicate injections of the 50- $\mu\text{g}/\text{mL}$  standard.

## Results and Discussion

The chromatogram of the 25- $\mu\text{g}/\text{mL}$  standard is shown in Figure 2, with all eight analytes eluting in just under eight minutes. This method provides excellent chromatographic

resolution between all closely eluting peaks. A resolution of 3.0 or greater was achieved between most peaks with Ionox 100 and OG having a resolution of 1.8.

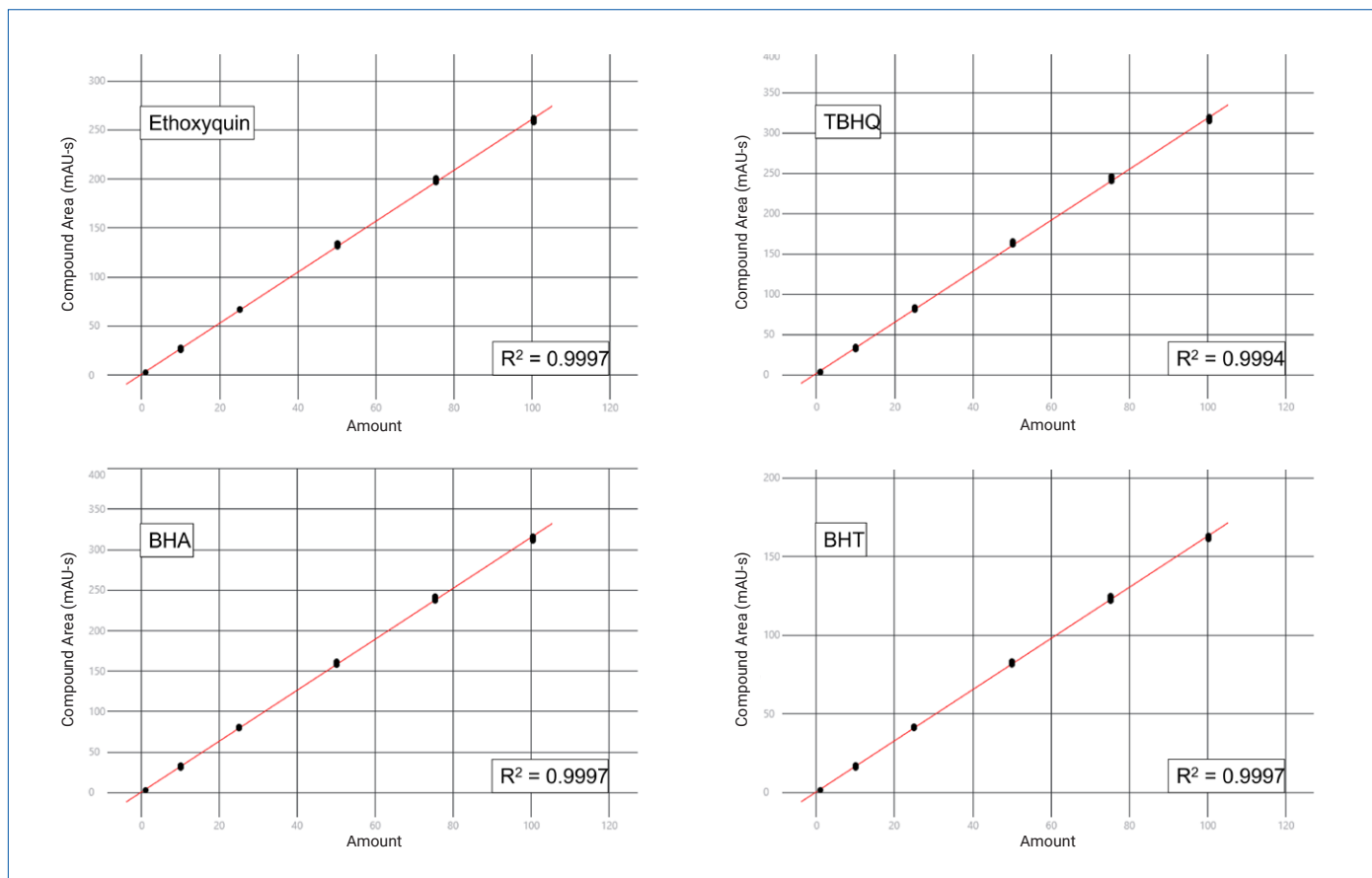


Figure 4: Results of the 6-level calibration set for ethoxyquin, TBHQ, BHA, and BHT.

Figure 3 shows an overlay of 16 replicate injections of the 25- $\mu\text{g}/\text{mL}$  standard, demonstrating exceptional reproducibility. Retention time %RSDs were below 0.06% ( $n=16$ ), with most analytes below 0.03%. Peak area %RSDs were below 0.78% for all analytes ( $n=16$ ).

Figure 4 shows the calibration results of four selected analytes over a concentration range of 1 to 100  $\mu\text{g}/\text{mL}$ . All analytes followed a linear (1<sup>st</sup> order) fit and had  $R^2$  coefficients above 0.999 ( $n=7$  at each level).

As listed in Table 2, the limits of detection (LOD,  $S/N \geq 3:1$ ) and limits of quantitation (LOQ,  $S/N \geq 10:1$ ) were obtained for each analyte and were calculated from the standard deviation of the signal response of the curve and the slope of the calibration curve. All analytes have LOQ values well below both US and EU regulations for food additives.<sup>3,4</sup>

Table 2: Figures of merit including  $R^2$ , LOD, and LOQ for the ten analytes of interest, in order of elution, along with the most stringent regulation limit from either the US or EU.

Analyte	$R^2$	Calculated LOD ( $\mu\text{g}/\text{mL}$ )	Calculated LOQ ( $\mu\text{g}/\text{mL}$ )	Regulation Limit ( $\mu\text{g}/\text{mL}$ ) <sup>3,4</sup>
PG	0.9998	0.356	1.079	100
Ethoxyquin	0.9997	0.502	1.520	3
THBP	0.9998	0.387	1.173	200
TBHQ	0.9994	0.659	1.998	200
NDGA	0.9998	0.365	1.106	N/A
BHA	0.9997	0.441	1.336	20
Ionox 100	0.9996	0.535	1.622	N/A
OG	0.9998	0.436	1.322	100
DG	0.9998	0.398	1.206	100
BHT	0.9997	0.448	1.357	20

Using the same chromatographic conditions, the six different oil and fat samples were analyzed. Chromatographic results for three of the samples with the 25- $\mu\text{g}/\text{mL}$  standard overlaid are shown in Figures 5, 6, and 7.

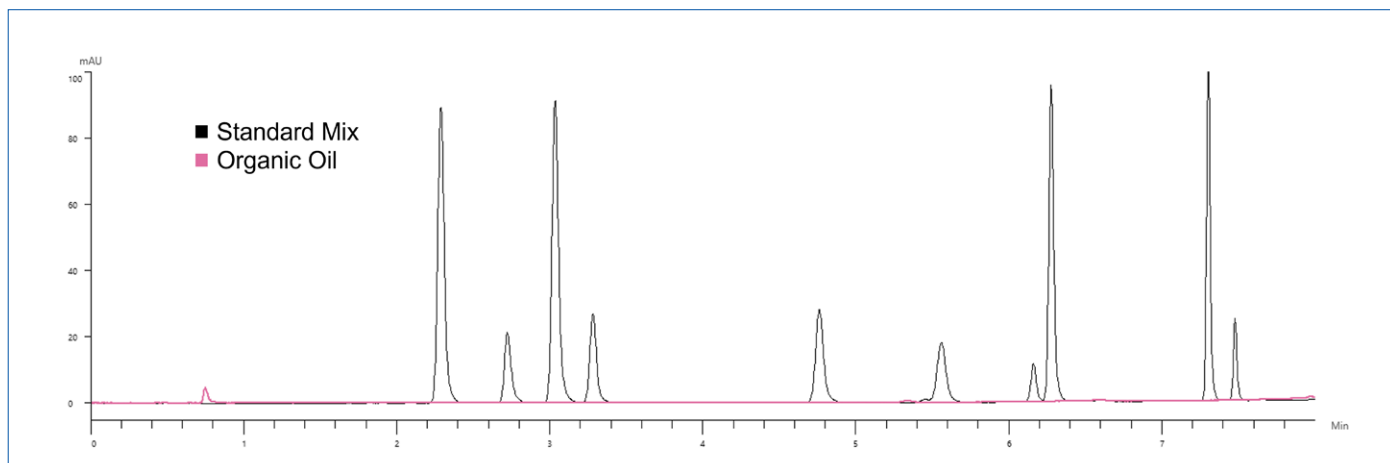


Figure 5: Chromatogram of organic oil sample.

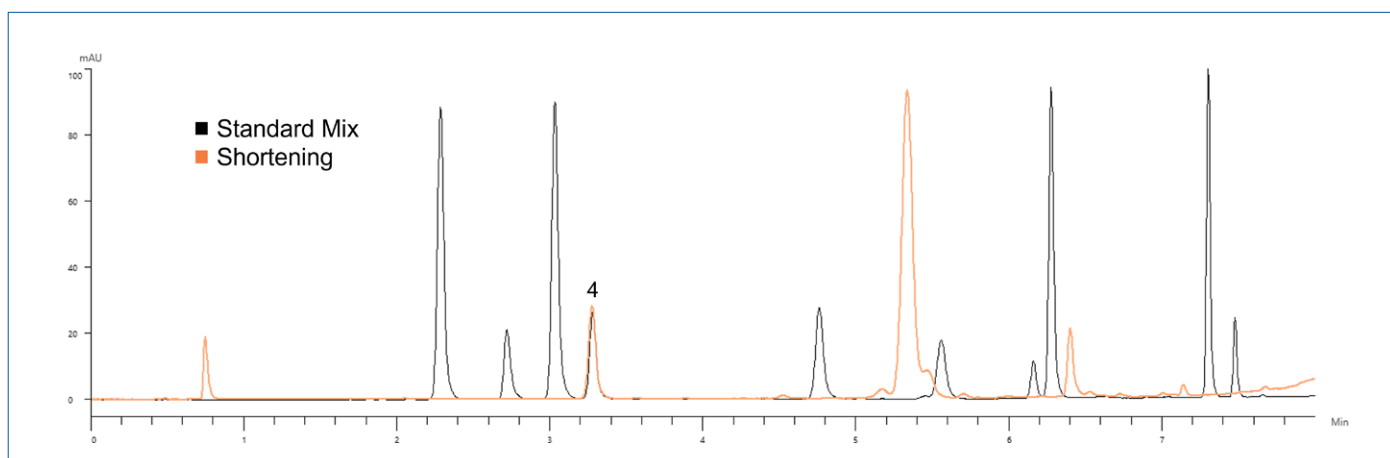


Figure 6: Chromatogram of shortening sample.

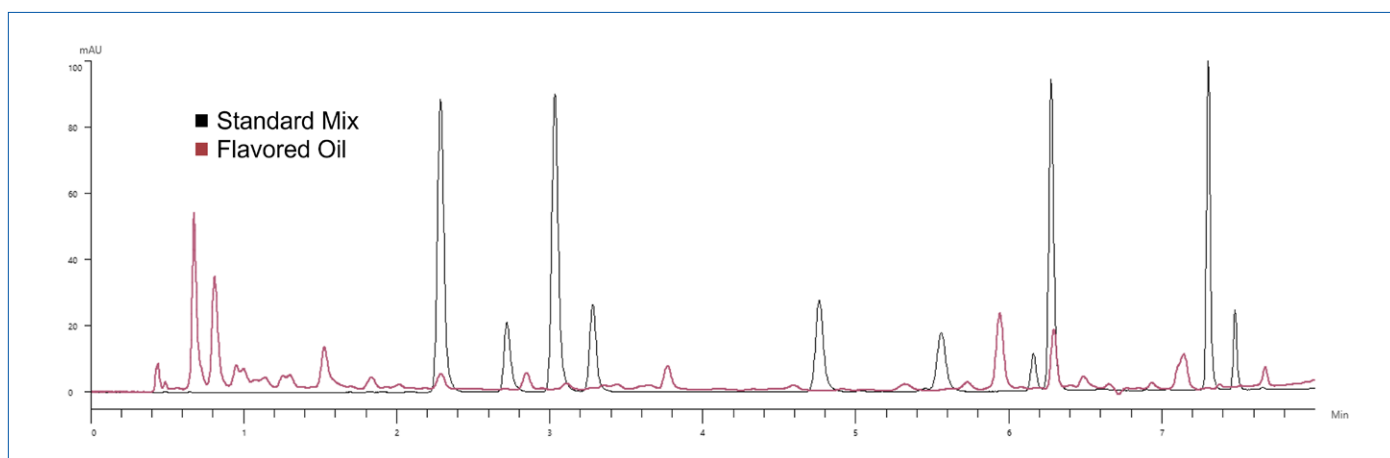


Figure 7: Chromatogram of flavored oil sample.

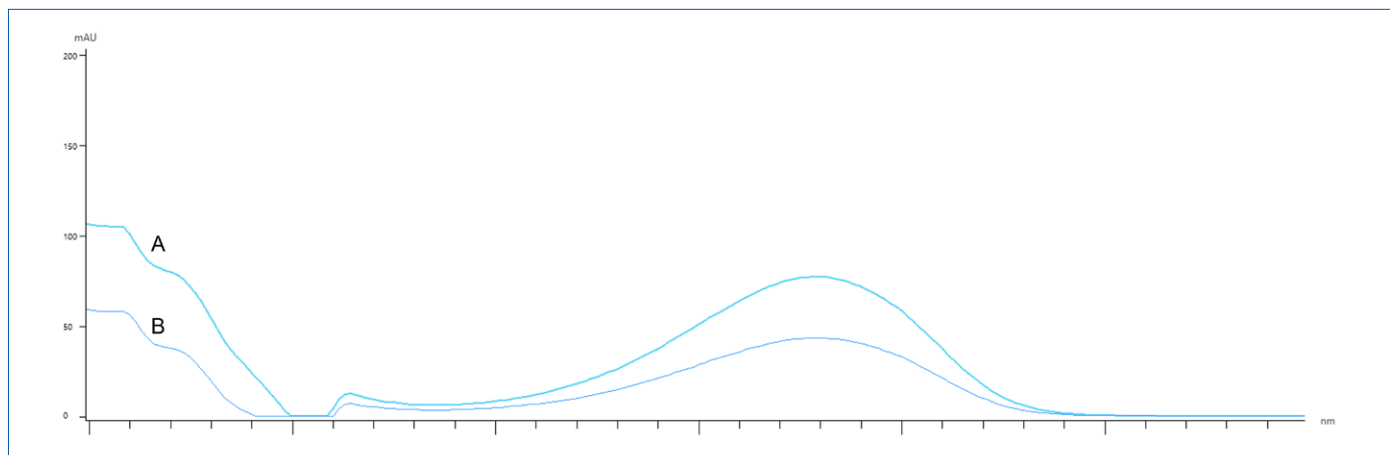


Figure 8: Comparison of PDA spectra for TBHQ in the library (A) and the shortening sample (B).

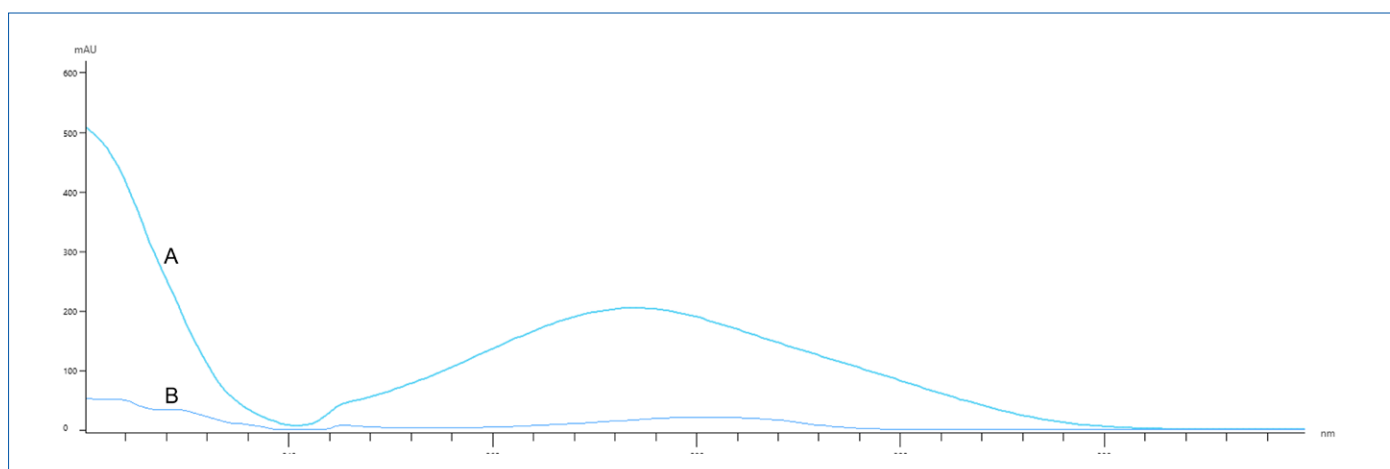


Figure 9: Comparison of PDA spectra for OG in the library (A) and the flavored oil sample (B).

The organic oil sample was determined not to contain any of the analytes of interest. Only two commercial samples, the shortening and flavored oil, had peaks at similar retention times to the phenolics analyzed. To aid in confirmation of the identity of the eluent, a spectral library for each of the phenolics was created using the 25- $\mu\text{g}/\text{mL}$  standard. Figure 8 and Figure 9 show the comparison of the absorption spectra for the unknown peak and that of the library match. This method allowed for confirmation of TBHQ in the shortening sample. By back-calculating the concentration in the original sample, it was determined that the shortening sample contained approximately 56 ppm of TBHQ, which is lower than both limits set by the US and EU. The actual concentration could not be verified as it was not provided in the product's label claim. There was no spectral match for OG in the flavored oil sample. Other oil samples also had peaks at other retention times than the analytes of interest. Further identification of other peaks present in the chromatograms was not pursued.

## Conclusion

For product consistency and labeling transparency for fair market competition and consumers' trust a robust HPLC-method for analyzing food stuffs containing phenolic antioxidants is needed to ensure compliance and safety. This work has demonstrated the fast and robust chromatographic separation and quantitation of ten commonly used phenolic antioxidants using the LC 300 HPLC System with PDA detection. The results exhibited excellent retention time repeatability, as well as excellent linearity over the tested concentration ranges. The method also affords LOQs of < 2.0  $\mu\text{g}/\text{mL}$  for all analytes.

This work specifically focused on the analysis of six different commercially available oil and shortening samples, one of which claimed to use an antioxidant. Analytical results were consistent with the ingredients label for each sample and all antioxidants were found to be below the maximum concentration limits set by the EU Commission Regulation and the FDA.

## References

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4. European Parliament and Council, Directive No. 1130/2011.
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## Consumables Used

Component	Part Number
0.22 $\mu$ m Nylon Syringe Filter 17 mm dia.	02542881
9 mm Screw Amber Vials (2-mL)	N9307802
9 mm PTFE/silicone (pre-slit) Screw Caps	N9306203
Pristine Acetonitrile, HPLC Grade	N9304928
Pristine Methanol, HPLC Grade	N9304931
Epic Biphenyl, 3 $\mu$ m, 3.0 x 100 mm Column	123191-EBPH