Determination of the Full Synthetic Antioxidant Present in Biodiesel from Raw Materials Used Worldwide During its Storage and Transportation by Gas Chromatography: A Metrological Approach

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Abstract: *Biodiesel is a renewable fuel composed by alkyl esters extracted from vegetable oils and/or animal fats. Due to its composition, it is susceptible to oxidation reactions, which affect quality and suitability use of the fuel. In order to prevent some of these oxidation problems and ensure the product suitability covering quality requirements, biodiesel producers have used synthetic antioxidants in their production processes. Here we used gas chromatography with flame ionization detector to develop an analytical method to determine synthetic antioxidants in final biodiesel. The development stages covered method optimization, limitations evaluation and the method validation according to international protocols and suitable statistical parameters. The recovery values were between 92 and 106%, and uncertainty of measurement has presented a shift from 1 up to 40% in the application range. Considering that in Brazil soybean oil is responsible for about 80 % of all raw materials used in biodiesel production, the method is very useful. In worldwide level, as canola and corn have a significant unsaturated ester composition, as soybean, the method is also applicable.*

Keywords: *biodiesel, method validation, flame ionization detector, uncertainty of measurement.*

1. INTRODUCTION

There is a large set of raw materials which can be used to produce biodiesel, including soybean, cotton, palm, peanut, canola, sunflower, coconut oils, and animal fats (beef tallow in Brazil) [1]. Biodiesel is a fatty acid alkyl ester, which is susceptible to oxidation when exposed to the air, reacting and forming oxidation products that can affect the fuel quality. For this reason, the oxidation stability of the biodiesel has been a main objective of many researches [2-11]. A quality parameter for this property was added to many quality requirements [12-15], using the Rancimat method [16]. To ensure the quality of the produced biodiesel, synthetic antioxidants have been added to production processes [11]. Thus, after biodiesel certification and commercialization, the product can be kept under storage conditions up to six months before being mixed with fossil diesel fuel, at a certain proportion as determined by Brazilian Government [17-18]. During storage, depending on the air exposure and temperature conditions, loss of fuel quality related to deterioration is possible. Considering that in Brazil about 80% of all biodiesel is produced from soybean oil [1], a raw material that generates an alkyl ester suitable to oxidation, synthetic antioxidants are necessary [19], thus the availability of reliable analytical methods to determine synthetic antioxidants contents after biodiesel production is useful. By using these methods, a synthetic antioxidant concentration may be easily evaluated, in function of the storage time, for example.

Biodiesel certification laboratories in Brazil must fulfill many requirements concerning their assays results quality [20]. Part of these requirements is based on international guidelines: the quality standards for assays or calibration laboratories [21]. In these guidelines, validation of the method is mandatory. When a method is proposed, it is necessary to fulfill some stages in order to be validated. Therefore, the proposal of an analytical method to determine synthetic antioxidants in biodiesel, tert-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) – the most used for biodiesel in Brazil – must firstly be validated to be used in certification laboratories. In this scene of the quality assurance of the result, the uncertainty of measurement is one of the parameters related to the measurement result. Nowadays it is accepted that the measurement result is not completed in the

absence of its uncertainty. To guarantee the comparison between two results, the uncertainty of each one is mandatory, for example [22]. The ISO/IEC 17025 [21] treats the uncertainty of measurement evaluated according to theoretical and practical knowledge about the method as one of the used techniques to evaluate the analytical performance. Then the uncertainty of measurement evaluation is directly related to the method validation.

Among the methods to determine synthetic antioxidants in biodiesel available in the literature, the electrochemical techniques use has been highlighted [23-26], mainly at the TBHQ determination, which can be oxidized to quinone generating a current signal when a potential is applied. Other important available information is about the performance of antioxidants. Studies have pointed TBHQ as the most effective for biodiesel [27-29]. However, electrochemical techniques are difficult to be applied in certification laboratories due to unusual instrumentation and materials in common certification analysis routine. Here we propose a method to determine commercial TBHQ and BHT by using a single instrumentation, which is common among the biodiesel industry laboratories. This instrumentation is already in use in routine methods cited in biodiesel specifications [30-31]. In addition to the development of this method, this work evaluated the suitability of this method in commercial biodiesel samples, and its validation as a method in accordance to international protocols, including the uncertainty of measurement.

2. MATERIALS AND METHODS

2.1. Instrumentation

Agilent 6890 gas chromatography instrument with flame ionization detector (GC-FID) and cool on-column injection was used to develop the method. The selected column was high temperature non-polar phase, compound by 95% of dimethylpolisiloxane and 5% phenylmethylpolisiloxane, with dimensions 30 m x 0.25 mm x 0.10 µm. This instrumentation is suitable to ASTM D6584 [31] and EN14105 [30] methods, whose determine free glycerol and total glycerides in biodiesel. These methods are prescribed in specifications requirements and have been used for the certification laboratories.

2.2. Reagents, Standards and Samples

Commercial synthetic antioxidants TBHQ and BHT (Biocapital Participações S/A, Charqueada, São Paulo) were used. These compounds were chosen as synthetic antioxidants applied in biodiesel production have different purity grades from an analytical grade. This work has the goal to determine the full synthetic antioxidant present in biodiesel samples, and therefore these materials have a better suitability for the proposed method. For the manufacturers related to biodiesel commercial chain, the most relevant information is the full antioxidant amount in the product, not the pure TBHQ amount. It occurs because the full antioxidant amount is minutely measured in the production process to be added. In addition, according Brazilian National Agency of Petroleum (ANP) data, no biodiesel producer had used different synthetic antioxidants from TBHQ and BHT, in 2012 [32]. Chemicals used in the method development and determinations were analytical grade. N-N-Trymethylsylyltryfluoroacetamide (MSTFA) (Merck) was used, besides pyridine as sylanization solvent and n-Heptane was used as final solvent (Vetec, Rio de Janeiro, Brazil). Commercial samples without synthetic antioxidants (from beef tallow, which is a raw material that does not need antioxidant addition to fulfill the specification requirements) were used as blank and recovery tests. Commercial samples from soybean oil were used to verify the method performance and suitability.

2.3. Analytical Procedures and Sample Preparation

The gas chromatography system conditions were similar to the ASTM D6584 and EN14105 methods (Table 1), including the sylanization with MSTFA step. The sylanization is mandatory because synthetic antioxidants have hydroxyl group (-OH), which hinders the GC elution without sylanization [33-34]. These conditions were chosen as "start point" to method development because these experimental parameters are known and applied in biodiesel assurance quality laboratories. An analytical curve was carried out, with known synthetic antioxidants amounts, from stock solutions, prepared according to Table 2. From these solutions, calibrations solutions were then prepared. The chosen stock solution solvent was pyridine, because it is an excellent solvent for both interest compounds and biodiesel. Besides, it allows sylanization reactions with high yield and is a solvent available in all certification laboratories. We could use other solvent with less toxicity (acetonitrile for

example), but in this case an additional extraction step would be needed, increasing the time for analysis. In addition, new sylanization conditions (time and temperature of reaction) should be tested [35], while the pyridine use with MSTFA at room temperature is quantitative [36].

Table1. *GC conditions for the proposed method.*

Injector		
Cool on-column		
Injection size	1 μL	
Temperature	Follows oven	
Oven program		
Stage	**Temperature**	**Holding time**
Initial	50 °C	1 min
Stage 1	15 °C min^{-1} until 180 °C	0 min
Stage 2	7 °C min^{-1} until 230 °C	0 min
Stage 3	30 °C min^{-1} until 380 °C	10 min
Detector		
Type	Hydrogen flame ionization	
Temperature	380 °C	
Hydrogen flow	35 mL min^{-1}	
Synthetic air flow	450 mL min^{-1}	
Nitrogen flow (make up)	30 mL min^{-1}	
Full time	About 35 min	
Carrier Gas		
Type	Helium	
Flow	3 mL min^{-1}	

Table2. *Stock Solutions and Calibration Solutions*

Stock Solution			
Antioxidant	Antioxidant amount /g	Final volume /mL	Final Concentration / mg mL^{-1}
Commercial TBHQ	0.2062	25	8.248
Commercial BHT	0.2039	25	8.156

Calibration Solutions					
Amounts	Mass Levels				
	1	2	3	4	5
Stock solution / μL	20	40	60	80	100
Pyridine / μL	80	60	40	20	0
MSTFA / μL	100	100	100	100	100
n-Heptane / mL	10	10	10	10	10
Obtained antioxidant mass in solutions / mg	0.16496	0.33712	0.50568	0.65984	0.82480

After stock solutions segregation, the sylanization reaction was carried out by adding 100 μL of MSTFA, shaking carefully and leaving the flasks in rest for 20 minutes to react at room temperature. The final dilution was performed with 10 mL of n-Heptane. The solutions were, then, ready to be injected in the gas chromatography system. For analysis, a sample weighting 100 mg was diluted in 200 μL of pyridine, after that the MSTFA sylanization was carried out, to the final dilution with n-Heptane, in same way of calibration solutions. The obtained mass from analytical curve result was converted according the exact mass sample, to obtain the final analytical result, in mg kg^{-1}. The maximum water content in the samples was fixed as 500 mg kg^{-1}, because samples with higher water contents may show peak irregularities in chromatograms, which probably occurs due to humidity affecting sylanization reaction in a quantitative manner [36].

2.4. Validation Procedures and Uncertainty of Measurement

Table 3 shows the selected validation protocol, which was based in international protocols [37-39]. The evaluated parameters were: accuracy, precision, linearity, limits of detection and quantification (LOD/LOQ), sensitivity, stability and uncertainty of measurement. The most innovative methodologies used at the parameters evaluation for this work are detailed as follows.

Table3. *Applied Method Validation Protocol.*

Parameter	Methodology	Acceptance criteria	Result for TBHQ	Result for BHT
Accuracy	Recovery test [38]	70 – 120 % in at least three concentration levels	93-106 %	92-98 %
Precision	Relative standard deviation (RSD) for 10 samples [38]	RSD ≤ 1%	0.33 %	0.14 %
Area repeatability	Peak area RSD from a calibration solution [39]	RSD ≤ 1%	1.0 %	0.8 %
Retention time repeatability	Retention time RSD from a calibration solution [39]	RSD ≤ 1%	0.06 %	0.06 %
Linearity	Correlation coefficient (*r*) evaluation [39]	$r \geq 0.999$	0.999	0.999
LOD	Crossing between prevision limit and analytical curves [40]	LOD ≤ 70% of first level in analytical curve	54 mg kg^{-1}	268 mg kg^{-1}
LOQ		LOQ ≤ first level in analytical curve	108 mg kg^{-1}	536 mg kg^{-1}
Selectivity	Chromatograms evaluation [39]	None co-elution	No co-elution	No co-elution
Stability	Injections of the same sample along the time	-	18 h	18 h
Application range	Definition LOQ – last concentration level in analytical curve [39]	-	108-8248 mg kg^{-1}	536-8124 mg kg^{-1}
Uncertainty of Measurement (U)	Estimated according guides [40-42]	U ≤ 20% of the result	-	-

For linearity, in addition to the correlation coefficient evaluation, the linear regression type has been evaluated for each synthetic antioxidant. To do that, Jacknife outliers test, Ryan-Joiner residue normality test, Durbin-Watson residue independence and Brown-Forsythe homocedasticity test were performed in sequence [40]. In case of disapproval at one of these tests, the weigthed least square method (WLSM) was used for linear regression, instead of ordinary least square method (OLSM).

For LOD/LOQ, a crossing between the prevision limit and analytical curves (with 95% of confidence level) methodology was used. It is not common, but is more conservative than the crossing between confidence limit and analytical curves [41]. Appendix A brings details about this.

For Uncertainy of Measurement, a methodology of combined concepts of *The Guide to the Expression of Uncertainty in Measurement* [42], *Eurachem / CITAC Guide* [43] and *Eurachem / Relacre* 2 [44] was applied. After the measurement equation establishment, the error sources mapping was carried out by Ishikawa Diagram. The uncertainty sources were calculated in group way, then the sensitivity coefficients were obtained before the final uncertainty.

3. RESULTS AND DISCUSSION

3.1. Synthetic Antioxidants Identification and Quantification

In method development, the TBHQ and BHT retention times were not coincident with free glycerol and 1,2,4-butanetriol retention times. 1,2,4-butanetriol is the internal standard used in glycerol determination [30]. Consequentely, the proposed method could be used with glycerides analysis simultaneously. Figure 1 shows the chromatograms whose prove this information, besides two analytical curves (for TBHQ and BHT).
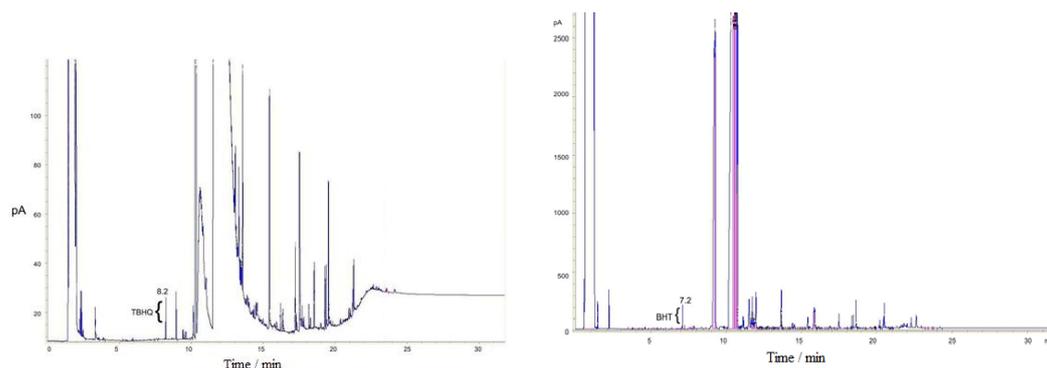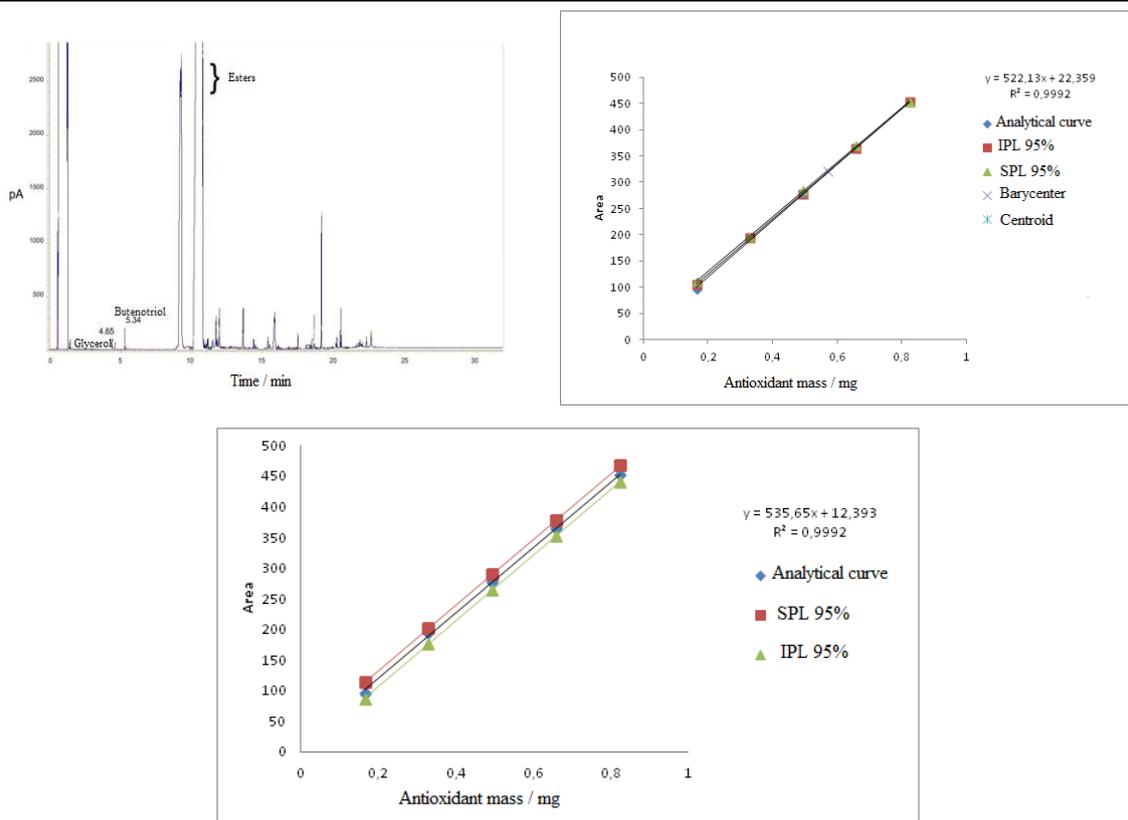
Fig1. *Full Chromatograms for the TBHQ (a), BHT (b) and biodiesel sample with high free glycerol content (c). In addition, analytical curves for TBHQ (d) and BHT (e) antioxidants.*

3.2. Method Validation

Table 3 summarizes the validation parameters evaluated, methodologies, acceptance criteria and validation results. According to the presented data, the recovery stayed between 92-106%. We can also highlight the absence of certified reference material (CRM) for a better accuracy test. In this case, these recovery results were satisfactory. Actually, a range between 70-120% has been accepted in chromatography [39]. For precision, the obtained values were also satisfactory, fulfilling the acceptance criteria (maximum RSD of 1.0%), at same way for areas and retention times repeatability.

In linearity evaluation, the residues graphic (Figure 2) for BHT have indicated a more random distribution than TBHQ. In addition, after the test results (see section 2.4), WLSM was used for TBHQ determination and OLSM was used for BHT determination.
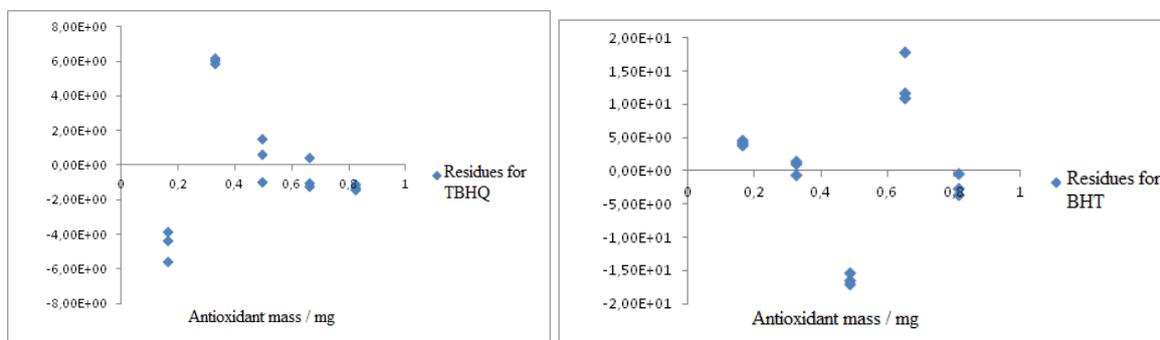

Fig2. *Graphical of residues for TBHQ (a) and BHT (b)*

In LOD/LOQ evaluation, the TBHQ results were significantly lowers than BHT data, because the WLSM use. Samples without santioxidants were also spiked with amounts near to LOD/LOQ levels, to evaluate the method performance in low concentrations. Table 4 presents the obtained data in these determinations, whose are compared with theoretical data from validation. For LODs, the recovered and theoretical values were almost the same; for LOQs, the recovery results were in 98-111% interval, therefore, in the established recovery range.

Table4. *LOD/LOQ evaluation.*

Concentrations / mg kg^{-1}	TBHQ	BHT
Obtained LOD	54	268
Spiked concentration in beef tallow biodiesel sample for LOD test	60	289
Concentration read for LOD test	69	324
Obtained LOQ	108	536
Spiked concentration in beef tallow biodiesel sample for LOQ test	98	554
Concentration read for LOQ test	109	561

For selectivity evaluation, qualitative criterion was applied – no obtained samples chromatograms have indicated any co-elution. For stability, sample injections were carried out along the time to verify at least 2% variation on the antioxidant concentration. The proposed method does not use internal standard, so a short stability time was expected. Figure 3 shows graphic which allow the estimative for stability time of 24 hours. Due laboratory limitations, a stability time for the method without new calibration was established as 18 hours, at a conservative way.
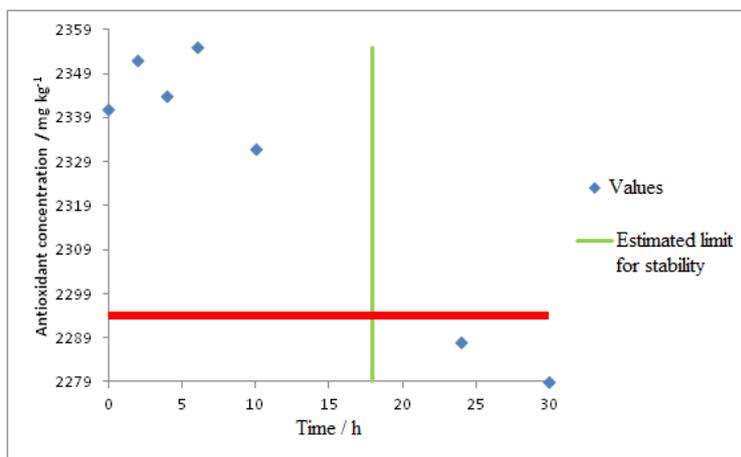

Fig3. *Study of stability in method validation procedure. (The selected limit of 18 h has been considered as conservative).*

3.3. Uncertainty of Measurement

Tables 5 and 6 show the final uncertainty calculation for the proposed method. The errors sources mapping (Ishikawa Diagram) are illustrated in Figure 4. The error sources mapping is in accordance to the following measurement equation:

$$C = \frac{m_{ant}}{m_{biod}} \times 10^6$$

Where:

C is the antioxidant concentration, in mg kg^{-1};

m_{ant} is the obtained antioxidant mass from analytical curve, in mg;

m_{biod} is the sample mass.

Table5. *Final Uncertainty calculation for a result as 1700 mg kg^{-1} of TBHQ. (k = coverage factor) (*) See details about sensitivity coefficient calculation in appendix B.*

| Uncertainty source	Value	Probability distribution	Sensitivity coefficient*	Contribution %
Linear regression	0.00223 mg	Normal	10000 mg^{-1}	21.25
Sample mass	0.67823 mg	Normal	-17 mg^{-1}	1.41
Combined calibration solutions	0.0078 mg	Normal	10000 mg^{-1}	64.71
Repeatability	17.22 mg kg^{-1}	Normal	1.00	12.62
Combined uncertainty	**Effective degrees of freedom**	**k**	**Expanded Uncertainty** (confidence level= 95 %)	
48.4649	556	2.00	96.9297 mg kg^{-1}	
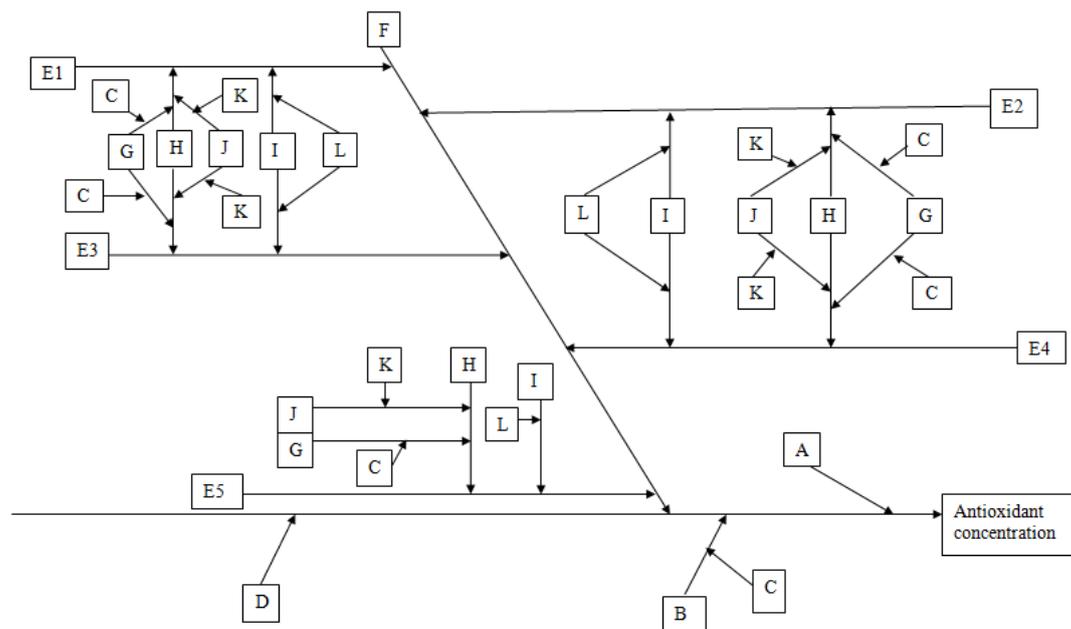
Fig4. *Ishikawa Diagram for errors sources mapping. A: uncertainty of linear regression; B: uncertainty of sample mass; C: uncertainty of mass measurement, covers resolution, calibration, random behavior and eccentricity; D: uncertainty of measurement repeatability; E1 to E5: uncertainty of calibration solutions 1 to 5, respectively; F: uncertainty of combined calibration solutions; G: uncertainty of antioxidant mass, to prepare stock solution; H: uncertainty of stock solution concentration; I: uncertainty of a measured volume by a pipette; J: uncertainty of a measured volume by a volumetric flask; K: uncertainty of volume measurement, covers calibration, random behavior and repeatability of the volumetric flask; L: the same of K, covers resolution, calibration, random behavior and repeatability of a pipette.*

The main axis in Ishikawa Diagram has all the measurement equation variables, besides the measurement repeatability and combined calibration solutions errors. The sum of the standard deviations for each error source was done in agreement with *ISO GUM guide* [42]. To evaluate uncertainty of linear regression and combined calibration solutions, the *Eurachem / Relacre* 2 [44] formalism was used.

According to data contained in tables 5 and 6, the final uncertainty for TBHQ was lower than BHT. It can be explained in function of WLSM use in TBHQ case. For TBHQ the linear regression (with WLSM use) uncertainty contribution was about 21% of the final uncertainty, while for BHT it was about 76% (with OLSM use). However, for BHT only a defined math function (with acceptable confidence level) could be drawn between uncertainty and antioxidant concentration (Figure 5). This math function allows providing the uncertainty of an unknown sample determination without a re-calculation of uncertainty. However, for TBHQ it was not possible, possibly due to WLSM use, causing a random uncertainty increasing in application range.

Table6. *Final Uncertainty calculation for a result as 1700 mg kg^{-1} of BHT. (k = coverage factor) (*) See details about sensitivity coefficient calculation in appendix B.*

| Uncertainty source	Value	Probability distribution	Sensitivity coefficient*	Contribution %
Linear regression	0.00773 mg	Normal	10000 mg^{-1}	76.34
Sample mass	0.67823 mg	Normal	-17 mg^{-1}	0.43
Combined calibration solutions	0.0078 mg	Normal	10000 mg^{-1}	19.44
Repeatability	17.22 mg kg^{-1}	Normal	1.00 mg^{-1}	3.79
Combined uncertainty	**Effective degrees of freedom**	**k**	**Expanded Uncertainty** (confidence level= 95 %)	
88.4172	6166	2.00	176.8343 mg kg^{-1}	
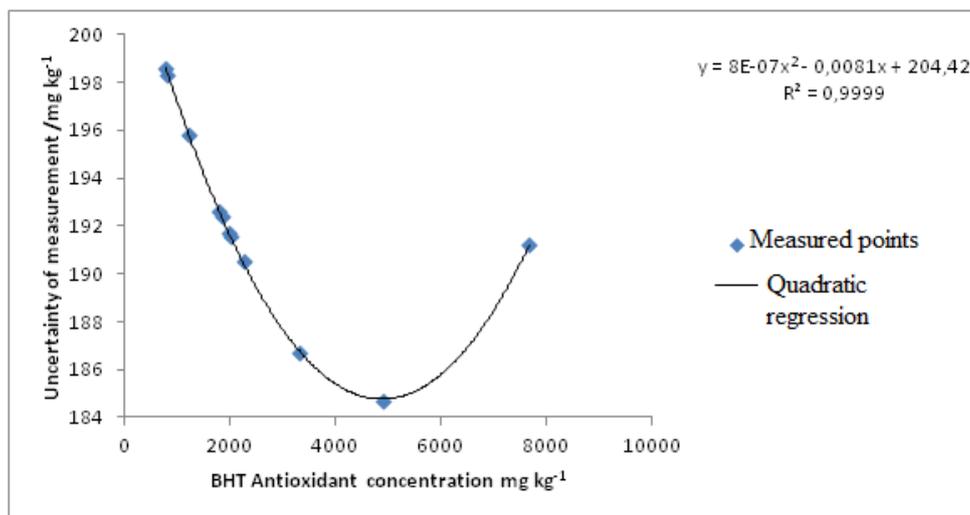
Fig5. *Math function illustration between BHT antioxidant concentration and uncertainty of measurement*

3.4. Application to Biodiesel Samples

Table 7 shows the synthetic antioxidants contents for commercial samples, from Brazilian market. The application range of the proposed method has been suitable for the tested samples even the most of samples have presented contents of approximately 1000 mg kg^{-1}.

Table7. *Concentration values (n=3) for antioxidants in biodiesel samples, with confidence level of 95% by GC-FID.*

Sample / raw material	TBHQ content / mg kg^{-1}	BHT content / mg kg^{-1}
Soybean oil	845 ± 171	-
Soybean oil	-	1244 ± 179
Beef Tallow	-	-
Blend Soybean oil / Beef Tallow	-	-
Soybean oil	1955 ± 87	-
Soybean oil	1332 ± 108	-
Soybean oil	-	998 ± 180
Soybean oil	-	852 ± 181
Soybean oil	-	1008 ± 180
Soybean oil	952 ± 152	-
Blend Soybean oil / Beef Tallow	-	-

4. CONCLUSIONS

A method for determination of synthetic antioxidants concentration in biodiesel samples is proposed in this work. The analytical technique already is used in assurance of quality control by certification laboratories at their routines. Therefore, the proposed method is easily applicable for the biodiesel producers who want to monitor the antioxidants contents during storage or transportation.

The proposed method has been validated in individual laboratory, according international protocols criteria, for TBHQ and BHT antioxidants. The obtained LODs/LOQs were satisfactory for the verified contents in the biodiesel samples. In addition, two types of linear regression (WLSM and OLSM) have been compared. The WLSM use has contributed for lowers LODs/LOQs and uncertainty of measurement. At least for BHT, a reliable math function between uncertainty and antioxidant concentration was also verified. In method application, the suitability was higher for biodiesel samples of soybean oil than beef tallow, as expected due to unsaturated ester composition in soy. As in Brazil, soybean oil is responsible for about 80% of all raw materials used in biodiesel production, the method is very useful. In worldwide level, as canola and corn have a significant unsaturated ester composition, as soybean, the method is also applicable.

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APPENDIX A

LOD and LOQ graphic calculations (see section 2.4). This calculation is based on crossing between prevision / analytical curves. Figure A.1 shows the pertinent curves. For LOD, the superior prevision limit (SPL) curve intercept is interpolated on analytical curve. For LOQ, the same point is interpolated on inferior prevision limit (IPL) curve.
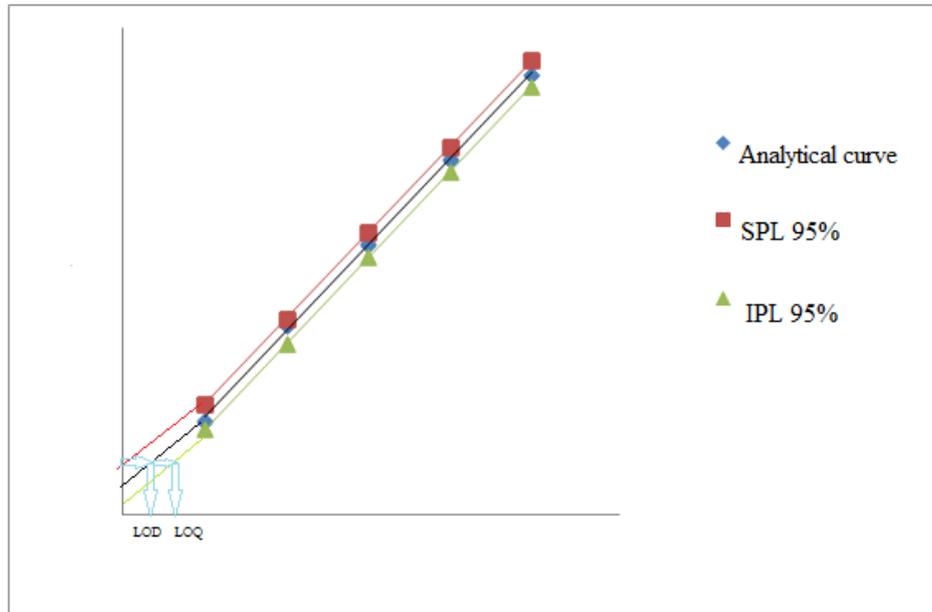
FigA.1. *Crossing between prevision / analytical curves.*

APPENDIX B

Sensitivity Coefficients calculation for final uncertainty. Based on measurement equation (see section 3.3).

For m_{ant}:

$$c_{mant} = \frac{\partial C}{\partial m_{ant}} = \frac{1}{m_{biod}} * 10^6 = \frac{1}{100} * 10^6 = 10\ 000\ mg^{-1}\ (B.1)$$

For m_{biod}:

$$c_{mbiod} = \frac{\partial C}{\partial m_{biod}} = -\frac{m_{ant}}{m_{biod}^2} * 10^6 = -\frac{0.17}{100^2} * 10^6 = -17\ mg^{-1}\ (B.2)$$

Authors' Biography

Waldemar Pacheco De Oliveira Filho, He has a degree in Chemistry at the Institute of Chemistry at the University of Brasilia (2003) and a PhD in Analytical Chemistry from the Institute of Chemistry of São Carlos, University of Sao Paulo (2013). He is currently Regulation Specialist from National Agency of Petroleum (ANP, Brazil), which is the agency from Brazilian government to regulate the petroleum, fuels and biofuels market. He has evaluated the suitability of the international test methods to certificate biodiesel for Brazilian scene since 2006. At the same time he has applied good laboratory pratices and metrological tools to optimize new test methods for biodiesel certification. Gas chromatography, mass spectrometry, atomic and molecular spectroscopy are among his most studied analytical techniques in this context.

Maria Olímpia Oliveira Rezende, She has a degree in Chemistry at the Institute of Chemistry at the University of São Paulo (1980) and a PhD in Chemistry (Analytical Chemistry) from the same university (1987), developing theme linked to Environmental Chemistry. She was a visiting professor at Oklahoma State University, from 1992 to 1994. She is currently an associate professor of the Institute of Chemistry of São Carlos, University of Sao Paulo. She has more than 110 published articles as well as books and book chapters. She led a project at the Laboratory of Chemical Residues at the São Carlos campus of the University of Sao Paulo, which deals, recovers and recycles chemical residues from all the campus labs. She has experience in Chemistry with emphasis on Trace Analysis and Environmental Chemistry, acting on the following topics: soil organic matter, vermicomposting, treatment of effluents and development of bioherbicides.