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Desenvolvimento e validação de um método analítico indicativo de estabilidade para o ácido nordihidroguaiarético

Development and validation of an indicative analytical method of stability for nordihydroguaiaretic acid

DOI:10.34117/bjdv6n1-168

Recebimento dos originais: 30/11/2019
Aceitação para publicação: 15/01/2020

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RESUMO

O ácido nordihidroguaiarético é um dos principais metabólitos gerados pela planta Larrea tridentata, a qual estudos clínicos comprovam sua segurança e eficácia em efeitos terapêuticos, como anti-inflamatório e antineoplásico. Alto potencial antioxidante é atribuído a esta molécula, devido a sua estrutura química ser composta por grupamentos catecóis. Atualmente, órgãos de regulamentação tais como ANVISA e FDA, delimitam diretrizes para os processos de registro e pós registro de medicamentos. Dentro desta esfera metodologias analíticas são exigidas para cumprimento de normas, como as resoluções da diretoria colegiada – RDC nº 166, 2017 e RDC nº 53, 2015. Este trabalho teve como objetivo desenvolver e validar um método analítico indicativo de estabilidade para a matéria prima do ácido nordihidroguaiarético por cromatografia líquida de alta eficiência. O desenvolvimento foi realizado através de testes iniciais, que envolveram otimizações da fase móvel, coluna,

concentração das soluções entre outros, até que a metodologia se demonstrou apta para ser validada. Em seguida, foi definido o perfil de degradação do ativo através do estudo de degradação forçada que tende a avaliar a aplicabilidade do método analítico na separação das impurezas geradas após condições de estresse. As condições foram definidas a partir de recomendações contidas em guias nacionais e internacionais que comtemplam estresses químicos e físicos. Os estresses químicos consistiram da exposição das amostras a condições de: hidrólise em ampla faixa de pH, além da oxidação por íons metálicos e reagente oxidante. Já as condições de degradação física consistiram da exposição das amostras a aquecimento, umidade e fotodegradação. Após concluir o desenvolvimento do método, seguiu-se com os parâmetros da validação: seletividade, linearidade, intervalo linear, precisão, exatidão e robustez. O sistema cromatográfico definido no desenvolvimento foi em modo gradiente utilizando como fase móvel água e metanol com fluxo de 1,0 mL/min, em um volume de injeção de 3 µL, coluna Agilent Eclipse XDB-C18 150 mm x 4,6 mm x 5 µm em temperatura ambiente e a detecção realizada em 283 nm. O método proposto demonstrou ser seletivo, pois os produtos de degradações gerados não interferiram na detecção e quantificação do NDGA, preciso, linear e robusto dentro da faixa de trabalho. Após realizar com êxito todas as análises pertinentes a validação da metodologia analítica, conclui-se que o método proposto é adequado para o dosear o ácido nordihidroguaiarético matéria-prima.

Palavras chaves: Larrea tridentata. Estudo de degradação forçada. Cromatografia líquida de Alta Eficiência.

ABSTRACT

The nordihydroguaiaretic acid is one of the main metabolites generated through the Larrea tridentata plant, to which clinical studies prove their safety and efficacy in therapeutic effects, such as anti-inflammatory and antineoplastic. High antioxidant potential is attributed to this molecule, because its chemical structure is composed of catechol groupings. Currently, regulatory agencies such as ANVISA and FDA, delimit guidelines for the registration processes and post registration of medicines. Within this sphere analytical methodologies are required to comply with norms, such as the resolutions of the collegial board of directors – RDC No 166, 2017 e RDC No 53, 2015. The objective of this work is to develop and validate an analytical method indicative of stability for the raw material for the nordihydroguaiaretic acid by high performance liquid chromatography. The development was carried out through initial tests, which involved mobile phase optimizations, column, concentration of solutions among others, until the methodology was shown to be validated. Next, the degradation profile of the active was defined through the study of forced degradation that tends to evaluate the applicability of the analytical method in the separation of the impurities generated after stress conditions. The conditions were defined based on recommendations contained in national and international guides that contemplate chemical and physical stresses. The chemical stresses consisted of the exposure of the samples to conditions of: hydrolysis in a wide pH range, besides the oxidation by metal ions and oxidizing reagent. The conditions of physical degradation consisted of exposure of the samples to heating, humidity and photodegradation. After completing the development of the method, we followed the validation parameters: specificity, forced degradation study, linearity, linear range, accuracy, precision (intra- and inter-day) and robustness. The chromatographic system defined in the development was in gradient mode using as mobile phase water and methanol with flow of 1.0 mL / min, in an injection volume of 3 µl, Agilent Eclipse column XDB-C18 150 mm x 4.6 mm x 5 µm at room

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temperature and the detection performed at 283 nm. The proposed method proved to be selective, since the degradation products generated did not interfere in the detection and quantification of NDGA, precise, exact and linear and robust within the working range. After successfully completing all relevant analyzes the validation of the analytical methodology, it is concluded that the proposed method is suitable for the determination of raw material nordihydroguaiarético acid.

Keywords: Larrea tridentata. Forced degradation study. High Performance Liquid Chromatography.

1. INTRODUCTION

The nordihydroguaiaretic acid (NDGA), which is considered a phenolic and anti-inflammatory antioxidant metabolite found in the resins on the leaf surface of the bush, was originally isolated from Larrea tridentata by Waller and Gisvold (1945), for the purpose of protection against herbivores, antimicrobial agent, UV radiation and water loss (LÜ *et al*., 2010; SNYPER, CASTRO, DESFORGES, 1989).

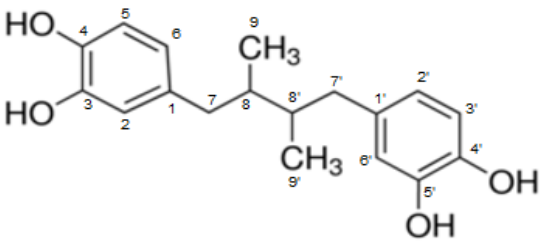
New drug development involves medicinal properties that explore and conduct clinical studies relevant to medical advances (SHEIKH, PHILEN, LOVE, 1997). Over time, NDGA has awoken researchers' interest in clinical studies after its antineoplastic, antiviral and anti-inflammatory effect was elucidated (LÜ *et al*., 2010).

Anti-inflammatory metabolism activities in the study are triggered after inhibiting arachidonic acid on 5-lipoxygenase activity reduces leukotriene of prostaglandin syntheses, subsequent decreases inflammation (WEST *et al*., 2004).

NDGA is a natural lignan that has the characteristic of 8 - 8 'position binding, chemically composed of polyphenols, presenting two halves of aliphatic carbon-bound catechols (PARACATU *et al*., 2015).

Figure 1 shows the chemical structure of NDGA formed by its four phenolic hydroxyl groups.

Figure 1 - NDGA Chemical Structure.



Source: **Adapted of LÜ et al., 2010.**

For a drug to reach the patient, it is important to point out that it is necessary to present data proving its raw material, that is, active pharmaceutical ingredient (API). Thus, analytical validations are performed to prove the safety and efficacy of such API.

In order to evaluate the selectivity of an analytical method, pharmaceutical companies performed forced degradation experiments, where IFA samples are subjected to hydrolysis conditions over a wide pH range, oxidative reactant oxidation and metal ions, dry heating, moisture and photodegradation, where the method's ability to separate and identify the degradation products formed in the most diverse scenarios to which the IFA may be exposed, such as: transport, storage and handling during the manufacture of the drug, is tested (ALSANTE *et al*., 2011; RAMAN *et al*., 2009). These requirements are established by guidelines determined by the National Health Surveillance Agency (ANVISA) as the Collegiate Board Resolution (RDC) No 166/ 2017 and RDC No 53/ 2015 (BRAZIL, 2017; BRAZIL, 2015).

Due to the lack of data in the literature regarding the forced degradation profile of the NDGA molecule, besides the inexistence of a selective analytical method for its quantification, this study aimed to develop and validate a raw material NDGA assay method, by high performance liquid chromatography (HPLC).

2. MATERIALS AND METHODS

2.2 MATERIALS

2.2.1 Chemicals and reagent

NDGA, was purchased from Sigma-Aldrich (USA). HPLC grade Acetonitrile (ACN) and Methanol (JT Baker), analytical grade hydrochloric acid (Neon, Brazil), copper sulfate (II), ethylenediamine tetraacetic acid (EDTA), hydrogen peroxide, iron chloride (III), sodium hydroxide were purchased Synth (Brazil) and high purity water (Millipore).

2.2.2 Instrumentation

The HPLC system consisted of the Alliance Waters 2695 (Milford, MA, USA) combined with a photodiode array wavelength detector (PDA) (Waters 2998). This system was equipped with a quaternary pump, an autosampler, an online degasser, and a column compartment with temperature control. Data acquisition, analysis, and reporting were performed using the Empower software 2 (Milford, MA, USA). The analysis was conducted

using a reverse phase C18 column (Eclipse XDB, Agilent) with a 5 µm particle size, 4.6 mm internal diameter and 150 mm length.

2.3 METHOD

2.3.1 Development of stability indicating HPLC method

For the separation of degradation products formed and the NDGA analyte, different chromatographic columns type, compositions, proportions and mobile phase flow, sample injection volume and adjustment of parameters related to system adequacy were evaluated (Table 1).

Table 1 - Tested conditions

Test No.	Composition of mobile phase	Column specification
1	Methanol: Water (75:25, V/V)	Waters Xbridge C18 250 x 4.6 mm x 5 µm
2	Methanol: Water (75:25, V/V)	Agilent Eclipse XDB C18 250 x 4.6 mm x 5 µm
3	ACN: Water (75:25, V/V)	Agilent Eclipse XDB C18 250 x 4.6 mm x 5 µm
4	ACN: Water (50:50, V/V)	Agilent Eclipse XDB C18 250 x 4.6 mm x 5 µm
5	ACN: Water (50:50, V/V)	Phenomenex Luna C18 250 x 4.6 mm x 5 µm
6	Methanol: Water (95:5, V/V / 5:95, V/V)	Agilent Eclipse XDB C18 250 x 4.6 mm x 5 µm
7	Methanol: Water (95:5, V/V / 5:95, V/V)	Agilent Eclipse XDB C18 150 x 4.6 mm x 5 µm

The development for the determination of forced degradation was carried out with acidic, alkaline, oxidative, metallic ion, photolytic, thermal and humidity stress (BRAZIL, 2015). Furthermore, peak purity of the NDGA were assessed from the spectral signals obtained by a photo diode array detector.

2.3.2 Validation of the analytical method

The parameters used for the validation followed the recommendations of the International Conference on Harmonization ICH (2005) guidelines, RDC 53 (2015) and RDC 166 (2017). Method validation evaluated the parameters of selectivity, forced degradation study, linearity, linear range, accuracy, precision (intra- and inter-day) and robustness.

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2.3.1.2 Solution Preparation

2.3.1.2.1 Diluent

A homogeneous mixture of methanol and water ratio (1: 1) V/V.

2.3.1.2.2 Sample Stock Solutions

Stock solutions of NDGA 60 µg L^{-1} was prepared by dissolving 3 mg NDGA sample in 50 mL of diluent.

2.3.1.2.3 Preparation of stressed/ degradation samples

A stock solution of NDGA was diluted up to 48 µg L^{-1} for sample control.

The acid degradation was performed using a stock solution of NDGA was diluted up to 48 µg L^{-1}. Further, 0.5 mL of 0.1 mol L^{-1} Hydrochloric acid (HCl) was added. The resulting solution was subjected for 90 min and subsequently neutralized with 0.1 mol L^{-1} NaOH. Similarly, alkaline hydrolysis of NDGA solution was carried out by adding 0.5 mL of 0.02 mol L^{-1} NaOH followed by neutralization with 0.02 mol L^{-1} HCl. The volume of both resulting solutions was adjusted to 5 mL using diluent.

The oxidative degradation was performed using H_2O_2 (0.01% V/V) and the volume was immediately adjusted to 5 mL using diluent. For metal ions degradation, the NDGA solution was performed using 0.5 mL of $CuSO_4$ 100 mmol L^{-1} for 24 hours. After this period of stress was added 0.5 mL of EDTA and the volume was adjusted to 5 mL using diluent.

The effect of heat, humidity and photolytic on NDGA was studied by exhibiting the solid-state substance to 60 ± 5 °C and 75 ± 5% relative humidity for 10 days, 200 W h/m^2 UV light and 1.2 million hours of visible light using photostability camera.

After the degradation period, the samples were prepared at the concentration of 48 µg L^{-1} respectively.

2.2.2.2 Selectivity

The selectivity evaluation was applied through results of forced degradation studies based on the degradation profile and spectral homogeneity analysis. In addition, the “white solution” of each stressor containing the diluent and the stressor was also analyzed.

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2.2.2.3 Linearity, linear range and accuracy

For linearity assessment, replicates (n=3) of five concentrations were analyzed ranging from 80 for 120% of the assay thus covering the range from 38.40 to 57.60 µg L^{-1} derived from the sample stock solutions with concentration of 60 µg L^{-1}. Further, the linearity curve was prepared by plotting the area of peak against the concentration of the respective solution injected.

Accuracy was verified along with linearity as it contains the same matrix. Results are determined by evaluating the nine determinations at low, medium and high concentrations (80, 100 and 120%) with three replicates of each.

2.2.2.4 Precision

Precision of the assay was determined by repeatability and intermediate precision for two consecutive days. To assess the repeatability of the method, six samples of NDGA (48 µg L^{-1}) were injected. These studies were repeated on two different days to determine the intermediate precision. The results were reported as relative standard deviation (RSD).

2.2.2.5 Robustness

Robustness study was performed by making small but deliberate changes related to the flow rate (0.9 and 1.1 mL/min), mobile phase ratio (Methanol 6% v/v and Methanol 4% v/v), temperature (30ºC and 25ºC) and diferente chromatographic columns (Phenomenex and Agilent) on NDGA solution (48 µg L^{-1}) using optimized chromatographic conditions.

To evaluate this parameter an experimental planning was performed by the Action Stat software, composed of four factors (Table 2).

Table 2 - Conditions employed in experimental design.

Parameters	Variation 1	Variation 2
	High level	Low level
Mobile phase methanol composition	6%	4%
Different column	Agilent	Phenomenex
Temperature	30°C	25°C
Mobile phase flow	1.1 mL/min	0.9 mL/min

The result of this test was determined by the percentage of recovery.

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Sample stability and different filters were also evaluated according to accuracy criterion.

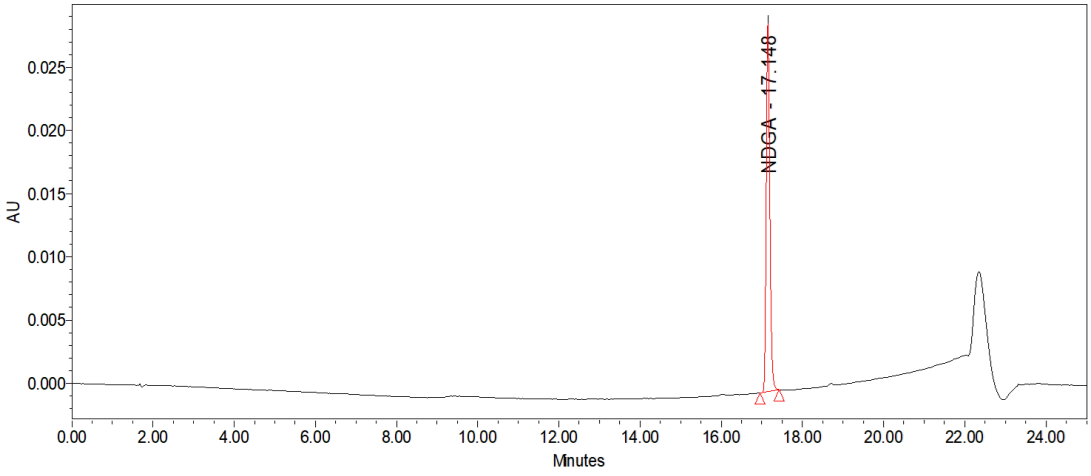
2.2.2.6 Results and discussion

2.2.2.6.1 Development of stability indicating HPLC method

From the preliminary results obtained during the development of the analytical method, the conditions of the methodology to be validated were determined.

The chromatographic system used during HPLC validation was conducted using methanol: water mobile phase (95:5, V / V / 5:95, V / V) and an Agilent Eclipse XDB- packing C18 150 mm x 4.6 mm x 5 µm chromatographic column. The injection volume was 10 µL and the flow rate were maintained at 1.0 mL/min. The wavelength was determined by evaluating the maximum absortivity, whose value was 283 nm. The retention time (Rt) of NDGA was found to be 17.0 min (Figure 2).

Figure 2 - HPLC chromatogram for NDGA solution (100 µg/mL).



All tests performed previous to this condition were not considered selective to be indicative of stability.

2.2.2.6.2 System Suitability

With the selected conditions, the repeatability of the injections was evaluated to prove the adequacy of the chromatographic system.

The results obtained showed that all performance parameters comply with requirements for system suitability. The value of RSD % of peak response was less than 2.0% and theoretical plates were more than 10.000.

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2.2.2.6.3 Forced degradation study

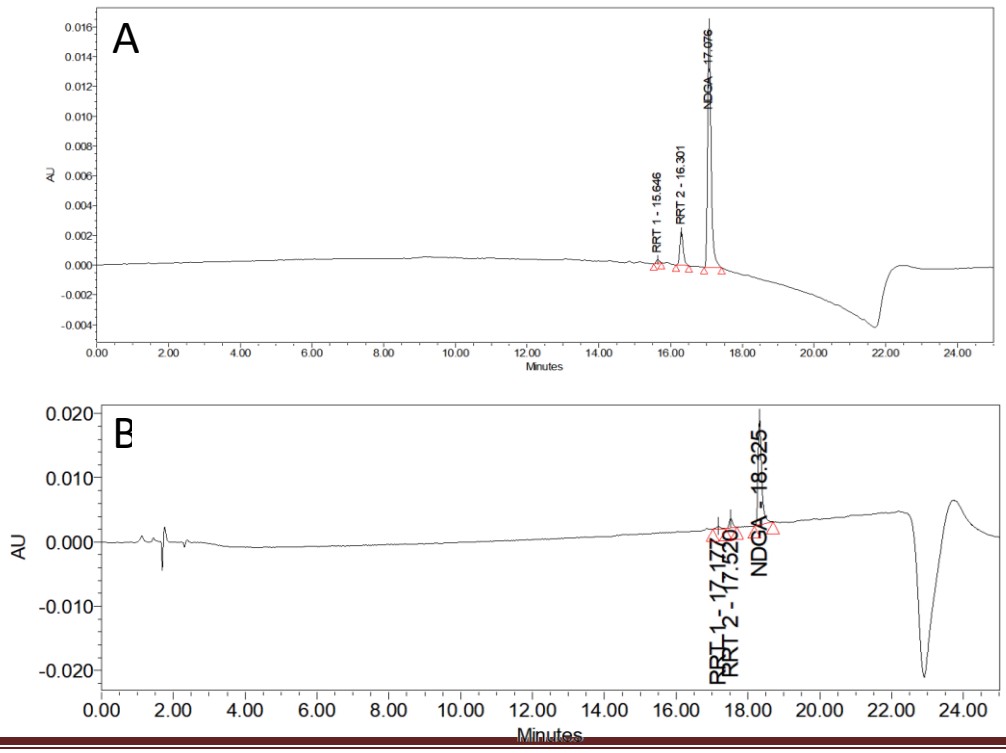
Stress tests were conducted in NDGA solution. Table 3 shows the results obtained during exposure of the sample to the degrading agent in chemical and physical degradation.

Table 3 - Percent degradation observed during forced degradation study.

Sr. No.	Degradation type	% Recovery	% degradation products	% Mass balance
1	Acidic degradation	86.61	9.69	96.30
2	Alkaline degradation	75.61	10.91	86.52
3	Oxidative degradation	89.87	6.72	96.59
4	Metal ions degradation	94.15	3.65	97.80
5	Heat degradation	97.88	3.37	101.25
6	Humidity degradation	97.72	0	97.72
7	Photolytic degradation	96.91	3.69	100.60

HPLC chromatograms obtained by acidic, alkaline, oxidative degradation, metal ions, heat, moisture and photolytic degradation are shown in figure 3.

Figure 3 - HPLC chromatogram for (A) Acidic degradation (B) Alkaline degradation (C) Oxidative degradation (D) Metal ions degradation (E) Heat degradation (F) Humidity degradation (G) Photolytic degradation.



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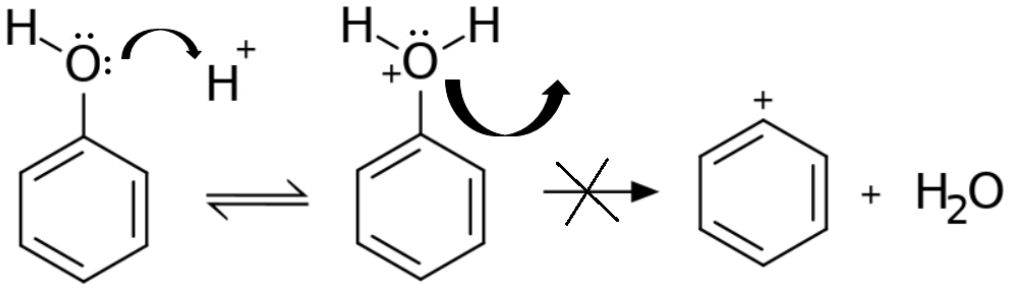
2.2.2.6.4 Acidic degradation

Phenol exposure to acidic stress (functional group with higher reactivity present in NDGA molecule) causes the protonation of phenyl alcohol. However, the formation of the phenyl carbocation by the water outlet is not observed, since the formation of such radical results in great loss of aromatic ring stability (Figure 4) (GROUTAS, 2002).

Figure 4 - Possible phenol reaction mechanism in acidic condition.



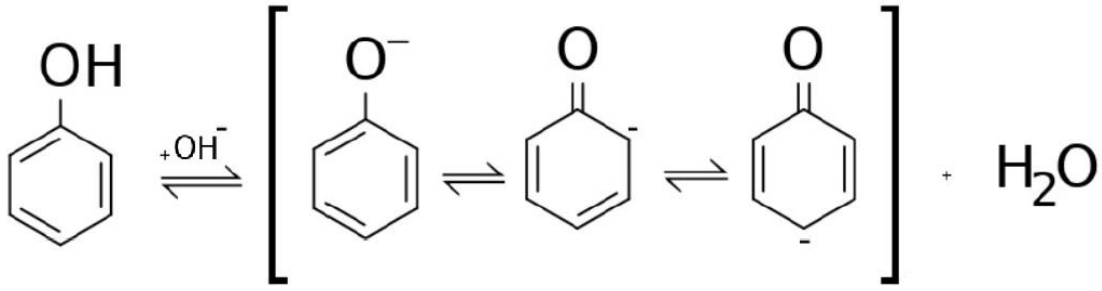
Source: GROUTAS, 2002.

The likely existence of chemical groups susceptible to protonation, such as alcohols, where when exposed to acidic pH conditions they protonate leaving the molecule more polar, this way you will have less interaction with the column. When the solution has been neutralized such functional groups return to their condition, presenting better interaction with the spine and being identified in the method.

2.2.2.6.5 Alkaline Degradation

In alkaline stress the deprotonation of the alcohol groups can occur, making the NDGA molecule too polar (Figure 5). However, when the solution is neutralized the molecule becomes nonpolar (MOUGEL *et al*., 2019).

Figure 5 - Phenol under alkaline conditions.



Source: MOUGEL *et al*., 2019.

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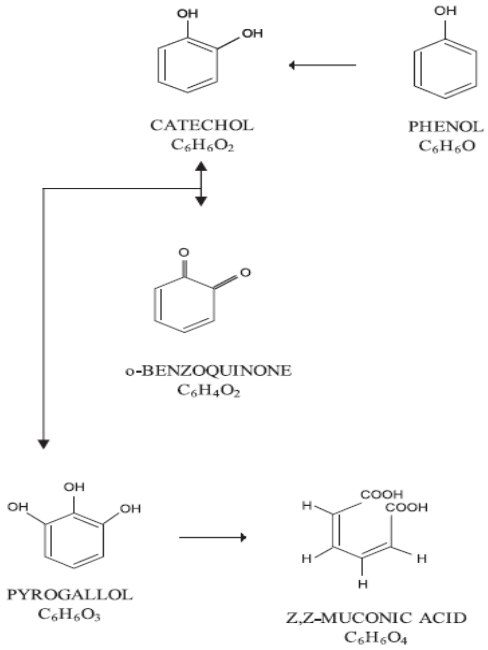
Therefore, it is concluded that neutralization is fundamental for the NDGA molecule under hydrolysis conditions, given the pH dependence of the NDGA molecule and its degradation products.

2.2.2.6.6 Oxidative degradation

The reaction with hydrogen peroxide is higher than estimated in theory, so these reactions can occur simultaneously or in single step (GE, LI, LISAK, 2019). Figure 6 shows that with the use of an oxidizing agent the catechol present in the NDGA molecule can lose the chromophore group, forming muconic acid (VILLOTA, LOMAS, CAMARERO, 2016).

Figure 6 - Possible reaction mechanism for intermediate phenol degradation.

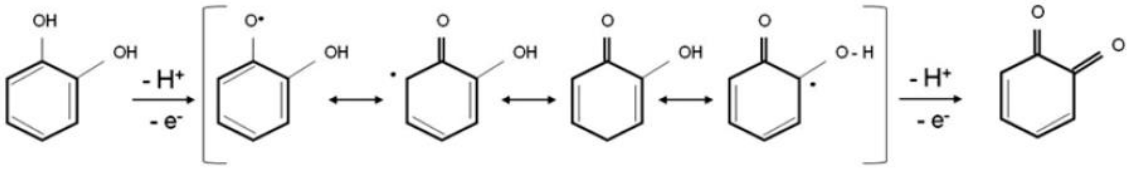


Source: VILLOTA, LOMAS, CAMARERO, 2016.

2.2.2.6.7 Metal ions degradation

Oxidation by metal ions may also occur hydroxyl deprotonation (Figure 7).

Figure 7 - Possible reaction mechanism for intermediate degradation of metal ions.



Source: VILLOTA, LOMAS, CAMARERO, 2016.

EDTA as a chelating agent prevents samples from analyzed in the solution by sequestering the metals present. When added EDTA in the solution, it will be complexed with the metal ions releasing NDGA molecules, which will be identified (ANTONIN *et al.*, 2011; OVIEDO, RODRÍGUEZ, 2003).

2.2.2.6.8 Physical degradation

According to the results, there was no significant decline in NDGA recovery under the conditions.

2.2.2.6.9 Selectivity

The results presented in the Figure 3 show the method analytical selectivity when compared to the NDGA retention time for all degrading solutions, the white solutions and impurities formed during the forced degradation study, as well as the peak purity tests that confirm the homogeneity spectral of NDGA peak in the stressed samples.

2.2.2.6.10 Linearity, linear range and accuracy

The equation of the calibration curve were y = 2.106x + 1777.6 with R2 = 0.9943 for NDGA.

The accuracy sample was analyzed under triplicate (Table 4).

Table 4 - Accuracy results for NDGA concentrations in sample solutions. (n = 3).

Standard solution (µg/mL)	Recovery (%)	RSD (%)
38.40	100.58	1.14
48.00	100.17	0.32
57.60	99.65	0.64

2.2.2.6.11 Precision

Developed HPLC method was found to be precise as indicated by % RSD (less than 1.3%) observed below than acceptance criteria (Table 5).

The DPR acceptance value will be adopted following the requirements of the Association of Official Analytical Chemists (AOAC).

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Table 5 - Precision 1st, 2nd day and Intermediate precision.

Level	Day	Response factor[1]	RSD (%)	Specification (%)	RSD (%) Intermediate Precision	Specification (%) Intermediate Precision
100%	1º	2383	1.2	RSD ≤ 1.3	1.8	RSD ≤ 2.0
		2449				
		2380				
		2363				
		2401				
		2390				
	2º	2334	1.1			
		2329				
		2323				
		2291				
		2363				
		2351				

[1]Signal relationship (area) / Signal concentration.

It is concluded that the method is precision due to the proximity of the results of the 1st and 2nd day precisions, meeting the established acceptance criteria.

2.2.2.6.12 Robustness

The method was evaluated by solution stability, filter, flow, mobile phase composition, temperature and different column manufacturers.

The stability of the sample was determined from the immediate injection to its preparation and injections after a few pre-established hours, as shown in Table 6.

Table 6 - Precision 1st, 2nd day and Intermediate precision.

Identification	Recovery (%)	Specification (%)
Sample 0 hour	100.00	98 – 102
Sample 3 hours	99.87	
Sample 10 hours	99.49	
Sample 20 hours	98.54	

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The NDGA sample was stable for a period of 20 hours after its preparation.

For filter robustness, the chromatographic profiles and the recovery against the control sample were evaluated (Table 7).

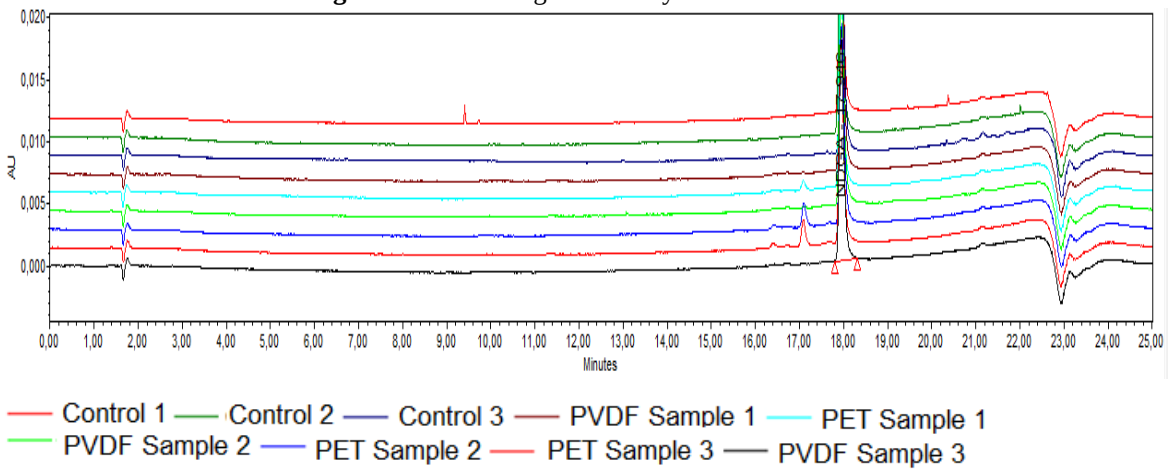
Table 7 - Filter robustness results.

Identification	Average recovery (%)	Specification (%)
Control sample	100.00	
PVDF[1] 0.45 µm	99.43	98 – 102
PET[2] 0.45 µm	96.73	

[1]Polyvinylidene fluoride
[2] Polyester

Based on the results presented, it is observed that the PVDF filter obtained less variation and when correlated the evaluation of the chromatographic profile expressed in Figure 8, it is possible to see that the PET filter shows peaks that were not detected in the control sample and the PVDF filter.

Figure 8 - Chromatogram overlay for filter evaluation.



The robustness of the method was proven after analysis of the experimental planning performed using Action Stat software, changing the analysis conditions as shown in Table 8.

Table 8 - Variations in chromatographic conditions in NDGA quantification.

Order	*A*[1]	*B*[2]	*C*[3]	*D*[4]	*Answer (%)*
1	-1	-1	1	1	99.97
2	1	1	-1	-1	102.33
3	1	1	1	1	98.33
4	-1	1	1	-1	98.79
5	1	-1	-1	1	99.22
6	-1	1	-1	1	98.71
7	1	-1	1	-1	99.92
8	-1	-1	-1	-1	99.11

[1] Mobile phase metanol composition;
[2] Different column;
[3] Temperature;
[4] Mobile phase flow.

3. CONCLUSIONS

The developed analytical method proved to be selective for the proposed purpose, because when compared the chromatographic profile of the NDGA solution against the white solutions, there were no elutions at the same retention time. The NDGA was susceptible to chemical degradation and not susceptible to physical degradation under stress conditions during FDS. Furthermore, the method presented linear response with accuracy and robustness within the range of 80 to 120% of the nominal sample concentration with a coefficient of determination of 0.9943 and precision (DPR <2.0). Finally, the parameters were conducted according to ICH, RDC No. 166/2017 and RDC No. 53/2015 it is concluded that the developed and validated method is suitable for NDGA assay.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

There was no funding received from any agency for any type ofwork included in this article or in the preparation of this article.

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