

Simultaneous determination of abamectin homologs H_2B_{1a} and H_2B_{1b} in gel formulation by high performance liquid chromatography

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Abamectin is a drug with antiparasitic properties used in several pharmaceutical formulations. The objective of this research was to develop and validate a high performance liquid chromatographic (HPLC) method for quantification of the two abamectin homologs (H_2B_{1a} and H_2B_{1b}) in gel formulation. This HPLC method was validated using a LichroCart® 100 RP-18 (125 x 4 mm, 5 μ m) column. The mobile phase contained of acetonitrile and water (95:5 v/v) with 1% acetic acid. The flow rate was 1.0 mL min⁻¹ and UV detection was performed at 245 nm. Mobile phase solutions were prepared containing a nominal concentration 185.2 μ g mL⁻¹ H_2B_{1a} and 9.6 μ g mL⁻¹ H_2B_{1b} . The method displayed good linearity in the concentration range of 148.1 – 222.3 μ g mL⁻¹ and 7.7 – 11.5 μ g mL⁻¹, for H_2B_{1a} and H_2B_{1b} , respectively, with a correlation coefficient of (r) > 0.99 for both compounds, calculated by the least mean squares method. Detection limits (DLs) were 2.8 μ g mL⁻¹ and 1.2 μ g mL⁻¹ and quantitation limits (QLs) were 8.6 μ g mL⁻¹ and 3.8 μ g mL⁻¹, for H_2B_{1a} and H_2B_{1b} , respectively. The method is simple, economical and efficient for the quantitative determination of abamectin H_2B_{1a} and H_2B_{1b} homologs in pharmaceutical preparations.

Uniterms: Abamectin homologs/validation method/gel formulation. Abamectin homologs. High Performance Liquid Chromatography (HPLC).

INTRODUCTION

Avermectins are a group of fermentation products obtained from a strain of *Streptomyces avermitilis* and are 16-membered macrocyclic lactones, and comprise several derivatives including abamectin (Xie *et al.*, 2011). Abamectin (Figure 1) is used in veterinary practice as an antiparasitic and in agriculture as an insecticide. Abamectin consists of a mixture of two homologous compounds: H_2B_{1a} (at least 80%) and avermectin H_2B_{1b} (no more than 20%) (Shoop, Mrozik, Fisher, 1995). Metabolic studies, have shown that abamectin H_2B_{1b} is generally metabolized more rapidly than the H_2B_{1a} homolog and therefore, H_2B_{1a} is the major residue found in tissues and biological fluids (Markus, Sherma, 1992). Abamectin was suspected causing adverse effects in the

aquatic environment, even in low concentrations, as it presents high toxicity to aquatic organisms, such as fish and microcrustaceans (Tisler, Erzen, 2006). Several different methods have been reported in scientific literature for the analyses of abamectin, such as, high performance liquid chromatography (HPLC) (Kulik *et al.*, 2011), liquid chromatography coupled to mass spectrometry (LC-MS) (Valenzuela *et al.*, 2000; Pozo *et al.*, 2003; Yoshii *et al.*, 2004; Xiaolin *et al.*, 2006; Thompson *et al.*, 2009; Rübensam *et al.*, 2011; Tao *et al.*, 2012; Campillo *et al.*, 2013; Ismail *et al.*, 2013), HPLC with fluorescence (Diserens, Henzelin, 1999; Souza *et al.*, 2003; Kolar *et al.*, 2004; Borges *et al.*, 2008; Cerkvenik-Flajsa *et al.*, 2010; Xie *et al.*, 2011; Rahman *et al.*, 2013), and UHPLC with fluorescence (Liu *et al.*, 2001; Romero-González *et al.*, 2011). The aim of this research was to validate a HPLC method for quantitative determination of abamectin homologs (H_2B_{1a} and H_2B_{1b}) in gel formulation, as this drug is widely used in veterinary practice. Abamectin consists of a mixture of two homologous compounds:

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H_2B_{1a} (at least 80%) and avermectin H_2B_{1b} (Shoop, Mrozik, Fisher, 1995). It is very important to separate and quantify both simultaneously since each homolog in abamectin has a specific concentration. The proposed method was developed and validated proposed to enable the separation and quantitative determination of the two abamectin homologs, in a short period of time; using a simple mobile phase in the chromatographic runs. The principal justification for this research was that there were very few methods described in literature for simultaneous quantitative determination of abamectin homologs, H_2B_{1a} and H_2B_{1b} . The proposed method presented a shorter analysis period compared to those described in literature, which is an advantage in quality control. It is a precise, and accurate method with can be used in routine analysis for quality control of gel formulation containing H_2B_{1a} and H_2B_{1b} .

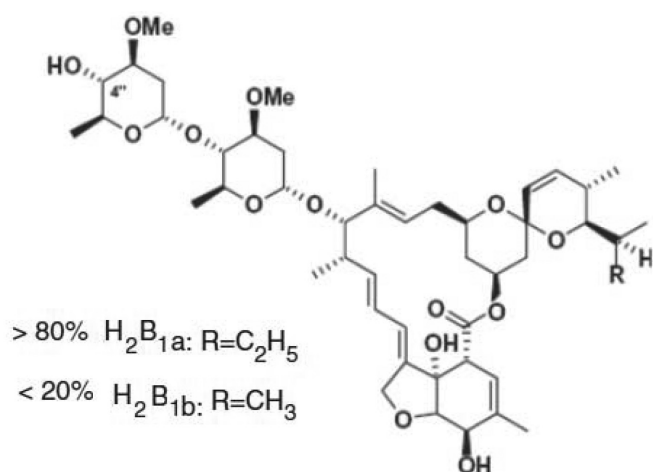


FIGURE 1 - Chemical structures the abamectin homologs (H_2B_{1a} and H_2B_{1b}) (Xie *et al.*, 2011).

MATERIAL AND METHODS

Chemicals and reagents

A mixture (98.7% purity) of abamectin H_2B_{1a} (93.8%) and H_2B_{1b} (4.9%) was donated by a pharmaceutical company. The gel formulation was a commercially available preparation containing 1% abamectin/ 10 g. Placebo was prepared in the laboratory containing the following excipients: hydroxyethylcellulose (1.00 g), EDTA (0.10 g), methyl paraben (0.20 g) and propyl paraben (0.10 g), imidazolidinyl urea solution 50% (0.60 g) and distilled water q.s.p 100.00 g.

The chromatographic grade methanol and acetonitrile were purchased from J. T. Baker® (Philipsburg, USA). Acetic acid was purchased from Vetec® (Rio de Janeiro, Brazil). Ultrapure water was obtained by using

a Milli-Q® water purification system (Millipore® Co., Milford, MA, USA).

The mobile phase consisted of acetonitrile and water (95:5 v/v) with 1% acetic acid. The flow rate was 1.0 mL min⁻¹ and UV detection was made at 245 nm. Solutions were prepared in the mobile phase containing 185.2 µg mL⁻¹ H_2B_{1a} and 9.6 µg mL⁻¹ of H_2B_{1b} .

Instrumentation

The proposed HPLC quantitative method was developed using the following equipment a solvent-delivery system, an auto-injector fitted with a 20 µL loop, an online degasification system, a column thermostat oven and an ultraviolet/visible (UV/VIS) with photodiode array detector. The output signal was monitored and integrated using Class-VP® 5.03 software (Shimadzu® Corporation, Kyoto, Japan). The mobile phase was filtered through a 0.45 µm PTFE Millipore® membrane (Millipore, Milford, USA). A Mettler AL204 analytical balance was used to weigh all compounds.

Procedures

Method validation

The method was validated according to the United States Pharmacopeia, 37th ed. (USP, 2014) and International Conference on Harmonization Guidelines (ICH, 2005).

Analytical curves

Analytical curves for abamectin were obtained using five different concentration levels, in each of the following ranges: 148.1 - 222.3 µg mL⁻¹ and 7.7 - 11.5 µg mL⁻¹, for H_2B_{1a} and H_2B_{1b} , respectively. Standard solutions were diluted with mobile phase and determinations were made in triplicate.

System suitability

Standard solutions containing 185.2 µg mL⁻¹ H_2B_{1a} and 9.6 µg mL⁻¹ H_2B_{1b} abamectin were prepared by dilution in the mobile phase. System suitability was determined from ten replicate injections of each standard solution.

Precision

Precision was obtained by determining the repeatability and intermediate precision. Repeatability was tested by analyzing six replicates of sample a concentration of 185.2 µg mL⁻¹ H_2B_{1a} and 9.6 µg mL⁻¹ H_2B_{1b} . Intermediate precision was obtained by performing the analysis, in triplicate on two different days using different analysts, using sample solutions in a concentration of 185.2 µg mL⁻¹ H_2B_{1a} and 9.6 µg mL⁻¹ H_2B_{1b} . Solutions were filtered

through a 0.45 µm Millipore® (PTFE) membrane before injection into the HPLC system.

Accuracy

Accuracy was assessed by determining the agreement between measured analyte concentrations of fortified and unfortified sample when a known amount of standard was added to the sample.

A quantity of 25.0 mg of abamectin standard (H₂B_{1a} and H₂B_{1b}) was weighed and transferred to a 25 mL volumetric flask. After addition of 20 mL mobile phase, the solution was sonicated for 5 min. The volume was completed with the same solvent (solution A). Final concentration was 1000.0 µg mL⁻¹. Aliquots of 0.8, 1.0 and 1.2 mL were transferred to 5 mL volumetric flasks. The volumes were completed with mobile phase.

An amount equivalent to 25.0 mg of the placebo gel formulation was weighed and transferred to a 25 mL volumetric flask. After addition of 20 mL of mobile phase, the solution was sonicated for 5 min. The volume was completed with the same solvent (solution B). Aliquots of 1.0 mL were transferred to 5 mL volumetric flasks. The volumes were completed with mobile phase.

As described in RE 899 placebo solutions were fortified by transferring 1.0 mL of sample solution (solution B) to 5 mL volumetric flasks, followed by addition of 0.8, 1.0 and 1.2 mL of standard solution (solution A). The volumes were completed with ultrapure water. Solutions were filtered through a 0.45 µm Millipore® (PTFE) membrane before injection into the HPLC system.

Stability test

Standard and sample solutions were prepared separately, as previously described, to obtain solutions containing 185.2 µg mL⁻¹ of H₂B_{1a} and 9.6 µg mL⁻¹ of H₂B_{1b}. These solutions were stored at 25°C and triplicate measurements were made during a period of 4 hours.

Robustness test

The UV detection wavelength and mobile phase composition were deliberately modified. Mobile phases consisted of acetonitrile and water (96:4 v/v) with 1% acetic acid and acetonitrile and water (94:6 v/v) with 1% acetic acid. UV detection was performed at 243 and 247 nm. The standard and sample solutions containing 185.2 µg mL⁻¹ H₂B_{1a} and 9.6 µg mL⁻¹ H₂B_{1b} were prepared by dilution in ultrapure water and injected in triplicate.

Stress testing

Stress testing was made as per Klick *et al.*, 2005. Evaluation was performed in neutral hydrolysis, acid

hydrolysis, alkaline hydrolysis and chemical oxidations.

Standard solution was prepared by transferring 25.0 mg of abamectin to a 25 mL volumetric flask. Sample solution was prepared by transferring 2500.0 mg of the gel formulation (equivalent to 25.0 mg of abamectin) to a 25 mL volumetric flask. To the standard and sample solutions, 20 mL of mobile phase were added and solutions sonicated for 5 min. Volume were completed with the same solvent. Aliquots of 1.0 mL of both solutions were transferred to 5 mL volumetric flasks. Neutral hydrolysis was performed by adding 1.0 mL of water, chemical oxidations were performed by adding 1.0 mL of 3% H₂O₂ solution, acid hydrolysis was performed by adding 1.0 mL of 1 mol L⁻¹ HCl solution and alkaline hydrolysis was performed by adding 1.0 mL of 1 mol L⁻¹ NaOH solution. After cooling the volumes were completed with mobile phase. The solutions were heated to 80 °C for 2 hours. The proposed method was used to analyze sample solutions in a concentration of 185.2 µg mL⁻¹ H₂B_{1a} and 9.6 µg mL⁻¹ H₂B_{1b}. Mobile phase was filtered through a 0.45 µm PTFE Millipore® membrane (Millipore, Milford, USA).

RESULTS AND DISCUSSION

Ideal conditions for obtaining a good separation of abamectin homologs (H₂B_{1a} and H₂B_{1b}) were achieved using a mobile phase consisting of acetonitrile and water (95:5 v/v) with 1% acetic acid. It was observed that pH adjustment was not required because the final pH of the mobile phase (4.47) was favorable for use considering abamectin pka (Figure 2). Mean retention times (RT) for H₂B_{1a} and H₂B_{1b} were, 2.6 and 2.3 min, respectively. The resulting chromatograms can be observed in Figure 4.

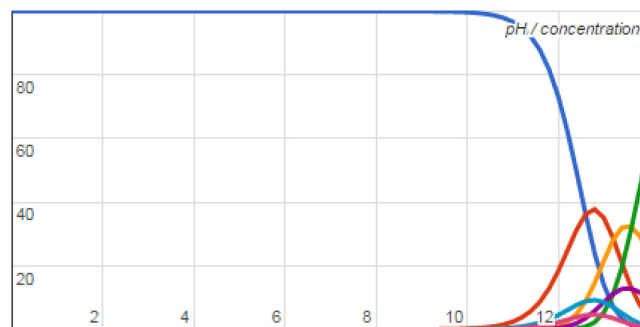


FIGURE 2 - Abamectin pkas observed in different pHs (Chemicalize, 2016).

Linearity

From analytical data, the responses obtained

TABLE I - Linear regression data obtained in the analyses of abamectin homologs H_2B_{1a} and H_2B_{1b} using the proposed HPLC method

Statistical parameters	H_2B_{1a}	H_2B_{1b}
Concentration range* ($\mu\text{g mL}^{-1}$)	148.1– 222.3	7.7 – 11.5
Regression equation	$y= 32305x + 1 \times 10^6$	$y= 15475x + 133889$
Correlation coefficient	0.9961	0.9960
Detection limit ($\mu\text{g mL}^{-1}$)	2.8	1.2
Quantitation limit ($\mu\text{g mL}^{-1}$)	8.6	3.8
F test **	379.50	371.01

* n= 5; ** value of 95%, 6.39.

for the two homologs were linear in the concentration ranges from: 148.1– 222.3 $\mu\text{g mL}^{-1}$ and 7.7 – 11.5 $\mu\text{g mL}^{-1}$, for H_2B_{1a} and H_2B_{1b} , respectively. These ranges were used based on ICH (ICH, 2005) which indicates a range from 80 to 120% of the test concentration for the assay of a drug substance or a finished drug product. Correlation coefficients were 0.9961 for H_2B_{1a} and 0.9960 for H_2B_{1b} . The F Test was also performed which confirm the proportionality of linear regression, resulting from analysis of variance. The experimental value must be greater than the default value for a confidence level of 95 % (Pimentel, Barros Neto, 1996). The value obtained was 10 times larger than the standard value (6.39) (Table I).

As important as the F test is residual analysis (Figure 3), which demonstrates an optimal distribution of the results (Pimentel, Barros Neto, 1995).

System suitability

Standard solutions were injected, and relative standard deviations (RSD) for each parameter were determined. In all cases, RSD values were less than 2%, which proves the reliability of the proposed analytical method (Table II).

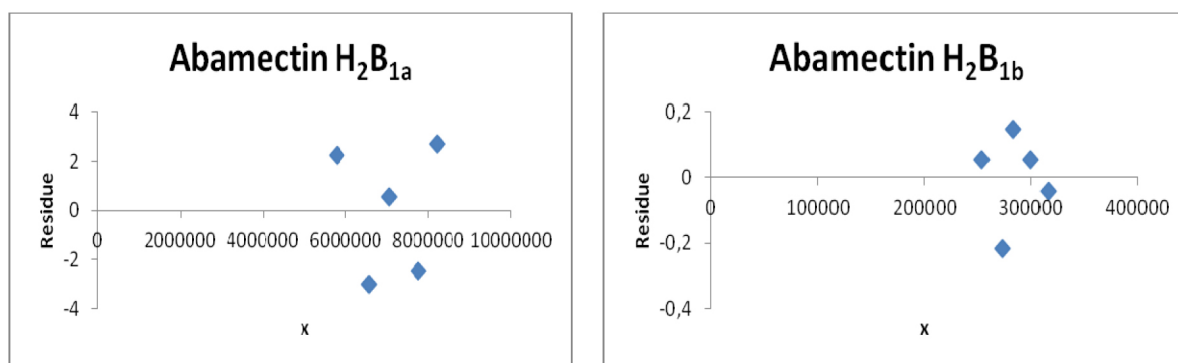
TABLE II - System suitability test results using final concentration of the sample solution: 185.2 $\mu\text{g mL}^{-1}$ H_2B_{1a} and 9.6 $\mu\text{g mL}^{-1}$ H_2B_{1b}

Parameters	H_2B_{1a} *	H_2B_{1b} *
Retention time	2.61± 0.38	2.28± 0.26
Area	6602238± 0.80	465191± 0.65
Peak height	854701± 0.84	24642± 0.68
Theoretical plates	4646± 0.48	4692± 0.51
Capacity factor	1.44± 0.45	1.13± 0.50
Assimetry	1.25± 0.00	1.33± 0.00

* Average of 10 determinations

Precision

Precision was obtained by determining repeatability and intermediate precision. The repeatability was conducted by analyzing six replicates of the sample at the concentration of 85.2 $\mu\text{g mL}^{-1}$ H_2B_{1a} and 9.6 $\mu\text{g mL}^{-1}$ H_2B_{1b} . Relative standard deviation (RSD) was determined. Intermediate precision was obtained by analyzing triplicates of the sample in the concentration of 185.2 $\mu\text{g mL}^{-1}$ H_2B_{1a} and 9.6 $\mu\text{g mL}^{-1}$ H_2B_{1b} . The RSD was determined after triplicate analysis of samples on two

**FIGURE 3** - Residual analysis of abamectin (H_2B_{1a} and H_2B_{1b}).

different days using two different analysts. The method presented good results for precision (Table III).

TABLE III - Precision analysis results obtained for the proposed HPLC method for determining abamectin homologs H₂B_{1a} and H₂B_{1b} in gel formulation

Precision	H ₂ B _{1a} (%)	H ₂ B _{1b} (%)
Repeatability ^a	92.29 ± 0.65	6.37 ± 0.06
RSD (%)	0.88	1.16
Intermediate precision ^b	92.07 ± 0.01	6.43 ± 0.03
	93.52 ± 0.01	6.57 ± 0.08
Mean value	92.80	6.50
RSD (%)	1.10	1.52

^a arithmetic mean value (n = 6); ^b arithmetic mean value (n = 2)

Accuracy

Recovery was evaluated by adding known amounts of abamectin H₂B_{1a} and H₂B_{1b} standard solutions to the abamectin gel sample. Recovery was evaluated at three different concentration levels. Triplicate determinations were performed at each concentration. Average recoveries of 98.62% ± 0.23 for H₂B_{1a} and 100.06% ± 0.95 for H₂B_{1b}, at three different concentration levels were obtained, indicating good accuracy for the proposed chromatographic method (Table IV).

TABLE IV - Recovery results for abamectin H₂B_{1a} and H₂B_{1b} homolog standard solutions added to sample and analyzed by the proposed HPLC method

Compound	Added amount (µg mL ⁻¹)	Found amount (µg mL ⁻¹)	Recovery (%)
H ₂ B _{1a}	150.62	148.15 ± 0.77	98.36
	187.53	185.26 ± 0.01	98.79
	225.13	222.27 ± 1.04	98.72
H ₂ B _{1b}	7.70	7.70 ± 0.07	100.00
	9.57	9.67 ± 0.01	101.04
	11.64	11.54 ± 0.01	99.14

Specificity

The placebo solution was prepared and analyzed using the proposed HPLC method. Results were compared with those obtained in the analyses of standard and sample. The excipients did not interfere in the method. The method was considered specific for the simultaneous determination of abamectin homologs H₂B_{1a} and H₂B_{1b} in gel formulation (Figure 4).

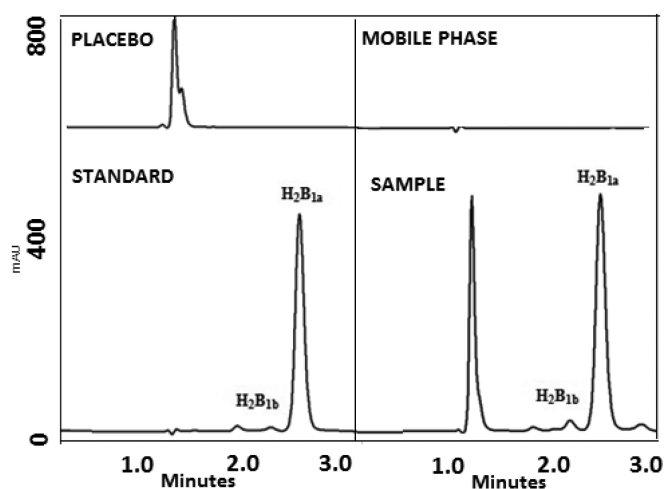


FIGURE 4 - Chromatograms of placebo, standard, sample and mobile phase solutions. Chromatographic conditions: concentrations: 185.2 µg mL⁻¹ for H₂B_{1a} and 9.6 µg mL⁻¹ for H₂B_{1b}, column: LichroCart® 100 RP-18 (125 x 4 mm, 5 µm), mobile phase: acetonitrile and water (95:5 v/v) with 1% acetic acid, flow rate: 1.0 mL min⁻¹, UV detection, at 245 nm and temperature, 25 ± 1 °C.

Detection limit (DL) and quantitation limit (QL)

The DL and QL were determined based on the standard deviation between response and slope of the curve. The theoretically obtained values for QL were cross-checked by performing analyses using the proposed HPLC method. DLs were 2.8 µg mL⁻¹ and 1.2 µg mL⁻¹, and QLs were 8.6 µg mL⁻¹ and 3.8 µg mL⁻¹, for H₂B_{1a} and H₂B_{1b}, respectively (Table I).

Stability of solutions

It is essential to evaluate the stability of standard and sample solutions to obtain reliable results. Standard and sample solutions were stored at 25 °C for 4 hours and after analyses the obtained results were compared with those obtained using freshly prepared solutions. No differences were observed in the instrumental responses under the described conditions. The RSDs obtained in the stability test were 1.19% and 0.44%, respectively for H₂B_{1a} and H₂B_{1b} (Table V), which were similar to those obtained using freshly prepared solutions.

Robustness

Deliberate changes in analytical parameters, did not lead to significant changes in the instrumental responses, and RSDs values were less than 2% in all cases. When the

TABLE V - Results from the stability test for abamectin H₂B_{1a} and H₂B_{1b}

Condition/RSD	H ₂ B _{1a} (%) ^a	H ₂ B _{1b} (%) ^a
Initial conditions	92.07± 0.01	6.43 ± 0.03
After stability test	93.63± 0.01	6.39 ± 0.01
RSD (%)	1.19	0.44

^a n= 3

wavelength was changed to 243 nm, the obtained RSDs were 0.86% and 0.99% and when changed to 247 nm, were 0.47% and 1.74% for H₂B_{1a} and H₂B_{1b}, respectively. When mobile phase composition was changed to 96:4 v/v acetonitrile and water with 1% acetic acid, RSDs were 0.10% and 0.44% for H₂B_{1a} and H₂B_{1b}, respectively. When changed to 94:6 v/v acetonitrile and water with 1% acetic acid, RSDs were 0.62% and 0.88% for H₂B_{1a} and H₂B_{1b}, respectively. All results obtained in the robustness test were similar to those under initial conditions. Thus, the proposed method can be considered reliable and robust.

Stress testing

Stress testing studies are used to assess drug substance and drug product stability to provide information

on possible degradation pathways, and to demonstrate the stability-indicating capability of the analysis methods used. These studies are performed under more drastic conditions than those used for accelerated stability tests (Klick *et al.*, 2005). Stress testing was conducted using neutral, acid and alkaline hydrolysis and chemical oxidation. After chemical oxidation and neutral hydrolysis, the results showed no significant changes in peak degradation, however, a different retention time was observed for the H₂B_{1a} standard substance. After both acid and alkaline hydrolysis, degradation was observed in both standard and sample substances (Figure 4).

The proposed method was appropriate for determining of abamectin homologs, H₂B_{1a} and H₂B_{1b}, in the presence of their degradation products, since all compounds could be separated, as seen in the chromatograms. Thus, the proposed HPLC method can be used in quality control as a stability- indicating method.

CONCLUSION

There are few methods described in literature for simultaneous quantitative determination of abamectin homologs H₂B_{1a} and H₂B_{1b}. Most methods described in literature just quantify total abamectin fraction.

The proposed HPLC method is simple, economic and

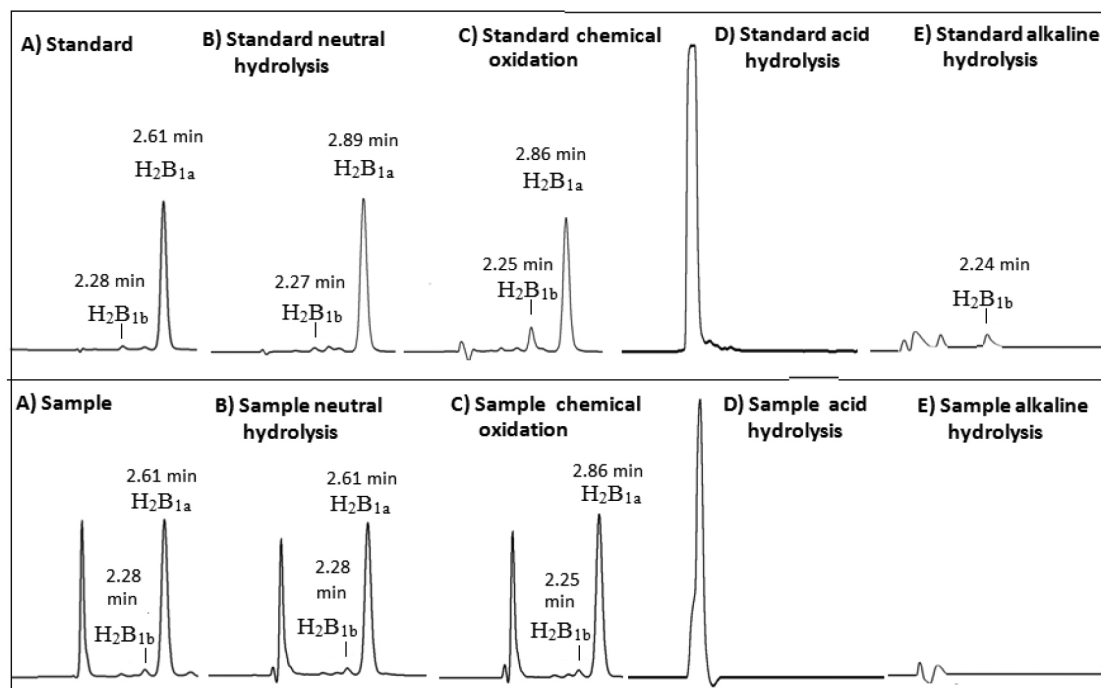


FIGURE 5 - Representative chromatograms standard reference homologs and sample solutions after stress testing. Concentration: 185.2 $\mu\text{g mL}^{-1}$ H₂B_{1a} and 9.6 μg H₂B_{1b}. Chromatograms: initial condition (A), neutral hydrolysis (B), chemical oxidation (C), acid hydrolysis (D), and alkaline hydrolysis (E). Chromatographic conditions: same as described in Figure 2

efficient for the separation and quantitative determination of the two homologs in gel formulation, and can thus be considered an important tool in quality control. The advantage of the proposed method over those described in scientific literature is the speed, leading to economy of solvents. It is very important to quantify these homologs as abamectin was identified as probable cause of adverse effects in the aquatic environment, even when present in low concentrations (Cerkvenik-Flajsa *et al.*, 2010). The excipients did not interfere in the analyses proving that the method presents selectivity. The proposed HPLC method proved to be useful and reliable and can be used to indicate the stability of routine analyses in quality control laboratories.

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