e-ISSN: 2687-6698 Research Article

Turkish Journal of Analytical Chemistry

TurkJAC

https://dergipark.org.tr/tr/pub/turkjac

Validation of stability-indicating high-performance liquid chromatography method for the determination of thymol in gelatin-based hydrogels

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Abstract

The aim of this study was to develop an analytical method to determine the amount of thymol loaded into the structure of a gelatin hydrogel developed to struggle varroosis infestation of honey bees and released into a model release system. Chromatographic separation was achieved using a high-performance liquid chromatograph, C18 column, and acetonitrile:water (75:25) mobile phase with an isocratic flow rate of 1 mL/min. To validate the method, specificity, precision, linearity, detection and measurement limit, accuracy, robustness, solution stability, and system suitability parameters were studied. Hydrolytic, thermal, oxidative, and photolytic degradation studies were performed for stress testing. The method is linear in the range of 0.25-15 µg/mL (R^2 = 0.999). LOD was calculated as 6.22 ng/mL and LOQ as 18.84 ng/mL. Intra-day and inter-day precision study %RSD values were ≤ 2%. The average recovery for the hydrogel was 100.3% (±2.12) and for the air sample was 99.9% (±3.14). The analytical method proved to be specific, linear, precise, and robust. This study presents a sensitive, convenient, and practical method for the detection of thymol in gelatin-based hydrogel structure and model release atmosphere.

Keywords: Controlled release, HPLC, hydrogel, thymol, validation, varroosis

1. Introduction

Thymol (known as 2-isopropyl-5-methylphenol) is a phenolic compound found in the extract and essential oil of *Thymus vulgaris* L. [1,2]. The utilization of thymol has been sanctioned for the management of pests encompassing bacteria, fungi, viruses, and parasites in both indoor and outdoor environments. [3]. Since the 1980s, thymol has been utilized in the management of varroosis, an infestation characterized by the proliferation of the mite *Varroa destructor* (*Anderson and Trueman*), which inflicts considerable damage on honey bee colonies [4,5]. When thymol is applied to the hive, it sublimates and diffuses into the hive atmosphere. In mites exposed to thymol vapour, GABA-gated chloride channels are blocked, resulting in excitation and convulsions in the central nervous system, leading to acaricidal effects [6]. However, when thymol is applied to colonies, acaricidal activity efficiency may vary because of environmental factors [7]. It is known that thymol has a highly volatile nature due to its structure and may decompose and cause loss in the environmental conditions in which it is applied [2,8–10]. Consequently, a hydrogel system was developed using gelatin polymer and thymol, hypothesizing that it could limit the volatility and degradation of thymol and enable its controlled release [11]. The development and validation of analytical methods to quantify active substances in such studies (e.g., loading efficiency and release tests) are crucial for the advancement of controlled release systems research and development [12,13] and for the quantification of thymol in air [14–17].

Qualitative and quantitative determination of thymol active substance can be done by chromatographic techniques such as high performance liquid chromatography (HPLC) systems equipped with UV (ultraviolet) or DAD (diode array detector).

Citation: O. Demir, M. Pulat, A. Bilgili, Validation of stability-indicating high-performance liquid chromatography method for the determination of thymol in gelatin-based hydrogels, Turk J Anal Chem, 7(2), 2025, 132–139.

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Received: December 20, 2024 **Accepted:** March 07, 2025
https://doi.org/10.51435/turkjac.1604250

Quantification of thymol in the animal skin matrix was performed by the HPLC-UV system at 278 nm in a method using an RP-C18 column and a mobile phase containing acetonitrile:water (35:65 v/v) [18].

In another study, determination of thymol in aromatic juice of Origanum onites L. was carried out using an HPLC-UV system at 254 nm and 273 nm using an XBridge C18 column and mobile phase containing acetonitrile:water (42:58 v/v) [19].

A method for the measurement of thymol in a tincture prepared with thymol, glycerol, ethyl alcohol, and purified water using a Microsob Varian C18 (4.6 × 250 mm, 5 µm) column and HPLC-UV system at 274 nm using a mobile phase containing acetonitrile:water (50:50 v/v) is reported [20].

It has been reported that the determination of thymol in a controlled release system in the form of a nanocapsule developed using Poly D, L-lactide-co-glycolide and Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) was performed by an HPLC-DAD system with an ODS Hypersil C18 (250 mm x 4.6 mm, 5 µm) column, acetonitrile:water (78:22 v/v) mobile phase, and a detection wavelength of 278 nm [21].

The migration amount of thymol loaded into polypropylene packaging films [12] and the release of thymol in films formed using silver nanoparticles and polylactic acid [13] were measured at 274 nm using an HPLC-UV system, acetonitrile:water (40:60 v/v) mobile phase, LiChrospher 100 RP18 column (250 mm × 5 mm × 5 µm, Agilent Technologies).

It was reported that the amount of thymol in swab samples taken from a cultural heritage mask exhibited in a museum was measured using a Jones C18 column (4 µm, 4.6 × 250 mm), acetonitrile:water (90:10 v/v) mobile phase, and HPLC-UV system at 225 nm [16].

In a study, the stability of thymol in Nigella sativa oil contained in gelatin capsules was investigated by HPLC-UV under different stress conditions. Inertsil ODS-3v C18 (250 mm × 4.6 mm 5-µm) column, 0.1 % aqueous formic acid:methanol (40:60 v/v) mobile phase, and measurements were made at 254 nm wavelength [22].

Although there are differences between studies in terms of analytical method components with placebo or matrix in which the amount of thymol is investigated, it is seen that methods are generally established with isocratic flow of acetonitrile, methanol and water or aqueous formic, C18 column and UV, DAD detectors in the wavelength range of 225-278 nm.

However, no published study was found for the determination of thymol in the developed CRS using gelatin polymer, glutaraldehyde crosslinker, sunflower oil, and thymol. For this reason, it was necessary to develop an analytical method to perform quantitative determination analyses for the gelatin based CRS.

The present study aims to develop and validate a stable HPLC-DAD method for the determination of thymol in a gelatin-based hydrogel and model release system atmosphere [11].

2. Material and methods

2.1. Reagents, solvents and materials

The hydrogel production materials included powdered bovine gelatin (Alfasol, s-Hertogenbosch, the Netherlands), glutaraldehyde (25%, Merck, Darmstadt, Germany), sunflower oil (Yudum, Ayvalık, Türkiye), thymol (99% purity, Sigma-Aldrich, St. Louis, MO, USA), and distilled water. For chromatographic analyses, a thymol reference standard (99.6% purity, Dr. Ehrenstorfer, Teddington, UK), a *p*-cumenol reference standard (99.6% purity, Dr. Ehrenstorfer, Augsburg, Germany), N-methyl-2-pyrrolidone (BASF Ludwigshafen, Germany), hydrochloric acid (%37, Scharlau, Sentmenat, Spain) sodium hydroxide (Sigma-Aldrich, St. Louis, MO, USA), Hydrogen peroxide (%30, (Sigma-Aldrich, St. Louis, MO, USA), acetonitrile (HPLC grade, Sigma-Aldrich, Darmstadt, Germany), and distilled water were used.

2.2. Instrumentation and analytical conditions

All chromatographic analyses were performed on an RP-HPLC-DAD (Dionex Ultimate 3000, Thermo Scientific, Waltham, Massachusetts, USA) using Chromeleon 7.2.9 chromatography software (Thermo Scientific, Waltham, Massachusetts, USA). Analytical separation was performed on an ACE 5 C18, 250x4.60 5 µm column (Advanced Chromatography Technologies Ltd, Aberdeen, Scotland). A PTFE 0.45 µm syringe tip filter (Isolab) was also used for sample preparation.

The mobile phase was adjusted isocratically at a flow rate of 1 mL/min in a ratio of 75 % acetonitrile and 25 % water from different lines. The detection wavelength was set to 277 nm, the column oven temperature to 25°C, the sample injection volume to 20 µL, and the analysis time to 7 min. Acetonitrile was used as the solvent, and acetonitrile:water (80:20) was used for dilutent.

2.2.1. Preparation of standard solutions

p-Cumenol and thymol standards were first prepared with acetonitrile at 1000 µg/mL. Stock solutions of *p*-cumenol 10 µg/mL, thymol 100 µg/mL, and thymol 10 µg/mL were then prepared by dilution with diluent. All solutions were placed in an ultrasonic bath for 20 min to ensure complete dissolution. All standards were filtered through a 0.45 µm PTFE (Isolab) syringe tip filter into glass vials prior to analysis by HPLC-DAD.

2.2.2. Preparation of blank, placebo and spike samples

For the hydrogel placebo sample, thymol-free sunflower oil was added to the centrifuge tubes. For the 80%, 100 %, and 120 % accuracy samples, a thymol/sunflower oil solution with a concentration of 500 mg/mL was added to the tubes. All tubes were then cross-linked by adding 10 % gelatin solution and 12.5 % glutaraldehyde solution prepared with water, respectively. After one night, the hydrogels were removed from the tubes, cut into 0.5 cm thick discs, and left to dry. The hydrogel discs containing 20, 25, 30 wt % thymol were cut into smaller pieces with the help of a scalpel and extracted with acetonitrile in a water bath at 70°C for 1 hour [11]. The resulting solutions passed through a 0.45 µm filter. After dilution with diluent, the solutions were analysed by HPLC-DAD.

For the preparation of air spike and blank samples, *p*-cumenol solution with a concentration of 10 µg/mL was added to the centrifuge tubes as an internal standard. Then, a thymol solution with a concentration of 100 µg/mL was added to the spike sample tubes with a final concentration of 0.25, 0.5, 1, 5, 10, and 15 µg/mL. All tubes were filled to volume with 10% N-methyl-2-pyrrolidone solution prepared with water. All solutions were kept in an ultrasonic bath for 20 min for complete dissolution. As a result, a blank sample with a *p*-cumenol concentration of 2 µg/mL and air spike samples with concentrations of 0.25, 0.5, 1, 5, 10, 15 µg/mL were prepared. The solutions were filtered through a 0.45 µm PTFE filter into glass vials and analysed by HPLC-DAD.

2.3. Validation of the HPLC method

The parameters of specificity, accuracy, precision, limit of detection, quantitative limit, linearity, robustness and system suitability [23,24], solution stability [23,25] were studied for the validation of the analytical method. The results were statistically analysed using Excel® (2010 version, Microsoft, Washington-USA).

2.3.1. Specificity:

The chemicals used in the analytical method, excipients used in the hydrogel, and structurally closely related substances such as *p*-cumenol were compared with the active substance thymol. The relevant chemical and excipient samples were injected into the HPLC-DAD system, and their interaction status was analysed in terms of retention times.

In addition, the thymol standard was subjected to hydrolytic, oxidative, thermal, and photolytic forced degradation conditions by stress test to investigate possible degradation product formation and interference [22]. For hydrolytic degradation, 2 mL of thymol solution with a concentration of 100 µg/mL was placed separately in 10 mL balloon jugs lined with aluminum foil. Then 2 mL each of 2 mol/L HCl or 1 mol/L NaOH solutions were added to the flasks. The flasks were sealed and kept at room temperature for one month. At the end of the period, the solutions were neutralised with 2 mol/L NaOH or 1 mol/L HCl. For oxidative degradation; 2 mL of thymol solution with a concentration of 100 µg/mL was placed separately in 10 mL flasks lined with aluminium foil. Then 0.5 mL of hydrogen peroxide solution (30%, v/v) was added to the flasks. The flasks were sealed and stored at room temperature for one month. For photodegradation, a 100 µg/mL concentration of thymol solution was placed in a 10 mL transparent flask and exposed to direct sunlight for 6 hours. For thermal degradation, thymol solution with a concentration of 100 µg/mL was placed in a 10 mL flask covered with aluminium foil and placed in a water bath set at 85 °C for 2 hours. All degradation studies were performed in triplicate. Degradation solutions were diluted with diluent to a thymol concentration of 10 µg/mL before analyses. All samples were filtered through a 0.45 µm filter and transferred to glass vials. They were then injected into the HPLC-DAD system.

2.3.2. Precision:

For intraday precision, 6 samples of thymol standard solution with a concentration of 10 µg/mL, hydrogel, and air spike samples were prepared, and 3 injections of each were made. At the end of this study, the %RSD value calculated according to the peak areas corresponding to each injection should be ≤ 3 [26]. For interday precision, samples of thymol standard solution with a concentration of 10 µg/mL, hydrogel and air spike samples prepared by different analysts were analyzed on 2 different days with 2 different HPLC devices. The %RSD value of the analysis results should be ≤ 2 [27].

2.3.3. Linearity, limit of detection (LOD) and limit of quantification (LOQ):

Solutions were prepared from thymol standard at concentrations of 0.25, 0.5, 1, 2.5, 5, 10, and 15 ug/mL. Samples were injected into the HPLC-DAD system 3 times [24]. The correlation coefficient calculated by plotting the concentration pilot curve against the peak areas should be at least 0.995 [26]. LOD is defined as the lowest amount of analyte that can be detected in a sample but does not need to be measured quantitatively under the specified experimental conditions, and LOQ is defined as the lowest amount of analyte in a sample that can be determined quantitatively with appropriate precision and accuracy [23]. These values can be calculated theoretically by regression of the calibration curve data [28]. For the verification of the theoretical limits, the standard prepared at the calculated LOQ level was injected into the HPLC-DAD system 6 times. The suitability was evaluated according to the criterion that

the RSD value between the measured field values was not more than 10% [20]. LOD was calculated with Equation 1, LOQ with Equation 2. (σ = standard deviation of the response, S = slope of the calibration curve)

$$LOD = 3.3\ \sigma/S \quad (1)$$

$$LOQ = 10\ \sigma/S \quad (2)$$

2.3.4. *Accuracy*

The recovery study was performed on hydrogel prepared with 80%, 100% and 120% concentration and air spike samples prepared with 0, 0.25, 0.5, 1, 2.5, 5, 10, and 15 µg/mL concentration for linearity study [29,30]. The recovery rate for hydrogel should be in the range of 95-105% [6] and the recovery for air should be in the range of 80-110% [5]. The percentage recovery rate was calculated separately for each concentration level with Equation 3.

$$\text{Recovery (\%)} = \frac{\text{Concentration of analysis results}}{\text{Theoretical concentration}} x\ 100 \quad (3)$$

2.3.5. *Robustness*

In order to test the robustness of the analytical method, it is recommended to evaluate the potential effects of changes to be made in at least 3 of the method components with factorial design on the analysis results [31]. For the robustness study, the test was carried out with changes made in the mobile phase flow rate, organic solvent ratio in the mobile phase and column temperature components presented in Table 3 [18]. The %RSD between the results obtained under normal conditions and the results obtained with the changes should be <2.0 [32].

2.3.6. *Solution stability:*

In order to evaluate the stability of analytical solutions, injections of *p*-cumenol and thymol standard solutions at a concentration of 10 µg/mL, which were kept at room and refrigerator conditions, were carried out into the HPLC DAD system at certain intervals. The difference between the peak areas obtained for the injections was calculated. The difference should be ≤ 2% for stability acceptance [23,25].

2.3.7. *System suitability:*

Six injections of 10 µg/mL concentration thymol solution were performed. After the analysis, the retention times and peak areas obtained were calculated for repeatability, and the theoretical plate number, resolution, peak symmetry parameters were calculated to evaluate the suitability in terms of pharmacopoeia limits [33,34].

3. Results and discussion

3.1. Results of HPLC method validation

3.1.1. *Specificity*

The chemicals used in the analytical method and the excipients used in the hydrogel not caused any interference in the retention time of thymol (Fig. 1 and Fig. 2). In the oxidative degradation experiment, thymol interacted with the free oxygen groups provided by hydrogen peroxide and almost completely degraded. This is expected in terms of the reported antioxidant properties of thymol [9,10]. In acidic degradation experiments, an average of 47% of thymol degraded, and in alkaline degradation experiments, 37%. Although degradation is also expected in acidic and basic conditions low levels [2], in present study, the degradation probably occurred at a high level due to long-term exposure. There was no significant change in the amount of thymol in photolytic and thermal degradation experiments. No interference was observed in the retention time of thymol with the formed degradation products (Fig. 3). The findings prove that the analytical method is stability-indicating.

3.1.2. *Precision:*

intraday and interday precision results are within the acceptance limits as presented in Table 1.

Table 1. Precision standard solution, hydrogel, and air spike samples (10 µg/mL)

	intra-day precision		inter-day precision	
	SD	% RSD	SD	% RSD
Thymol standard sol.	0.0175	0.17%	0.127	1.29%
Hydrogel	0.0333	0.33%	0.166	1.66%
Air Spike	0.0399	0.40%	0.174	1.74%

3.1.3. *Linearity, LOD and LOQ:*

It was calculated that the correlation coefficient (R^2) values calculated by plotting the concentration pilot curve against the peak areas were greater than or equal to 0.995 and that the method was linear in the concentration range studied (Fig. 4). Then, regression analysis was performed on the linearity graph data with Excel® package program (2010 version, Microsoft, Washington, USA) at a 95 % confidence level [28]. The remaining standard error of the y-axis data was calculated with the regression analysis. With the help of formulas, LOD was determined as 6.2261 ng/mL and LOQ as 18.866 ng/mL. The standard prepared at the LOQ level was injected into the HPLC-DAD system 6 times, and %RSD was calculated as 1.64. As a result of the evaluation, the detection limit was confirmed as 18.866 ng/mL [27].

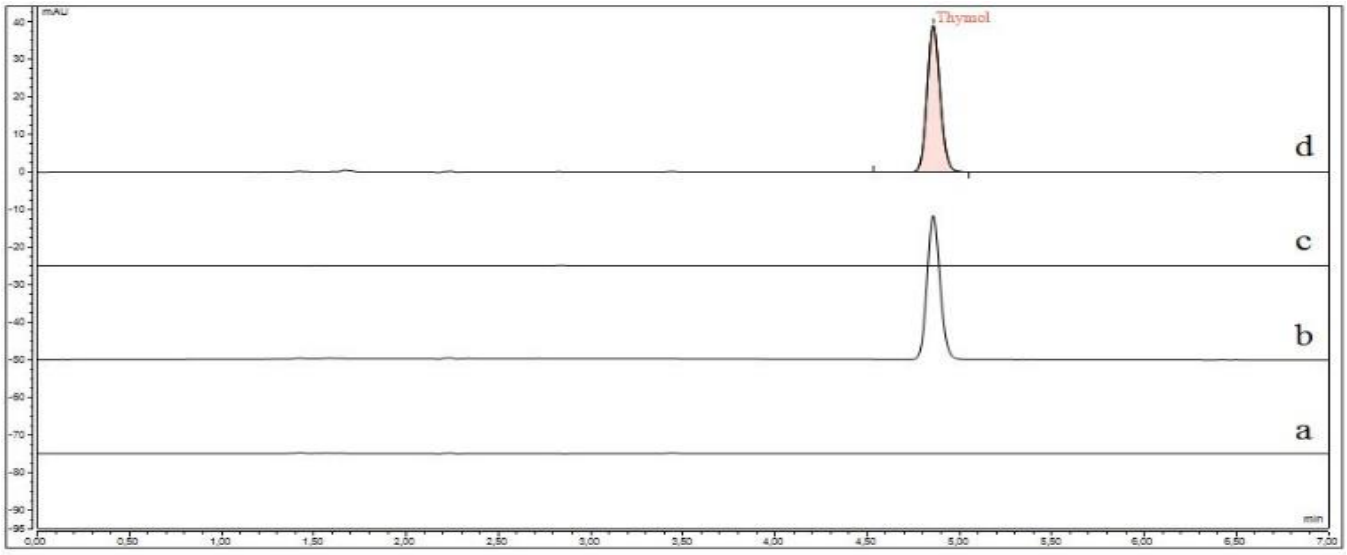

Figure 1. HPLC chromatograms of a) mobil phase, b) standard thymol solution (10 μg/mL), c) hydrogel plasebo, d) hydrogel sample (10 μg/mL thymol)

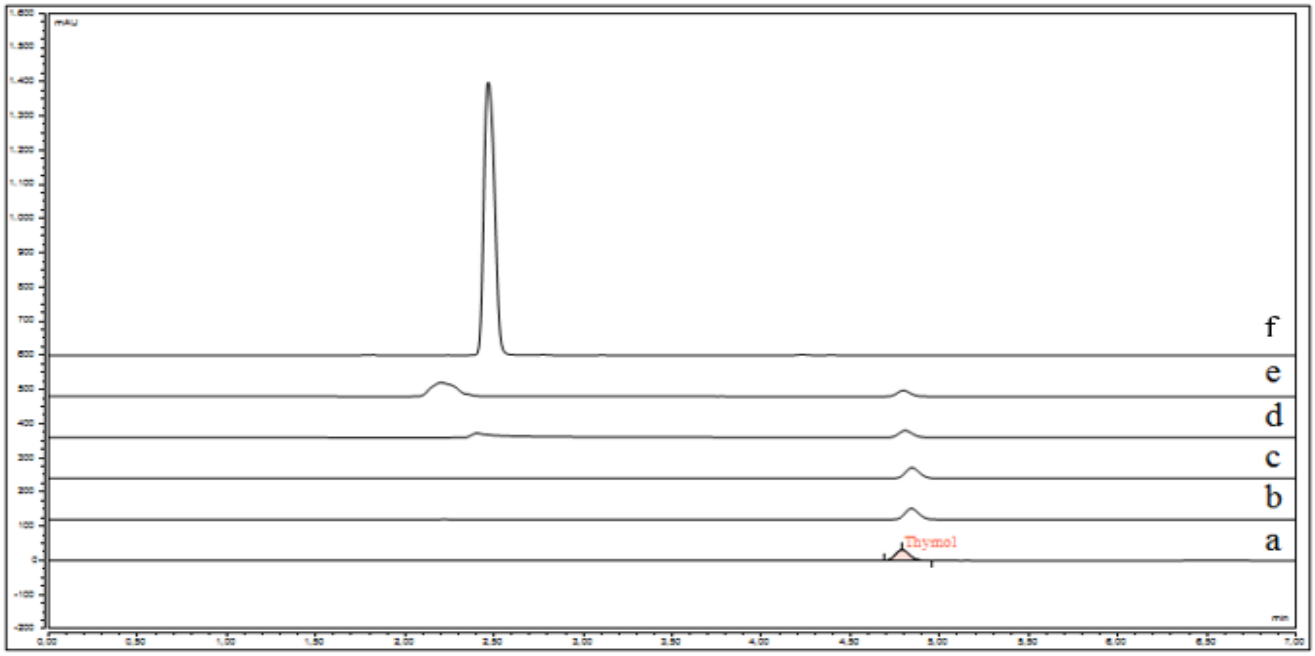

Figure 2. HPLC chromatograms of a) mobil phase, b) air blank sample (2 μg/mL *p*-cumenol) and c) air spike sample(2 μg/mL *p*-cumenol, 10 μg/mL thymol)

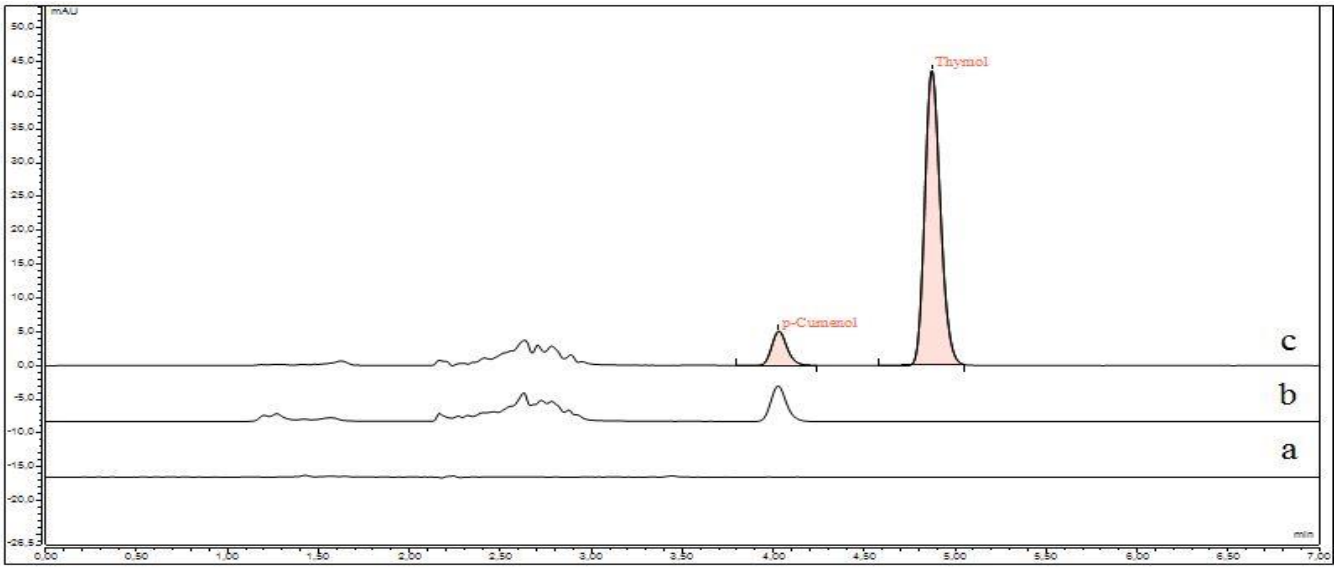

Figure 3. HPLC chromatograms of a) thymol standard solution, b) acid, c) base, d) oxidative, e) light and f) thermal induced forced degradation of thymol

3.1.4. Accuracy:

The mean recovery was calculated as 100.3% (±2.12) for hydrogel spike samples and 99.9% (±3.14) for air spike samples. The results are within the acceptance limits [26,30].

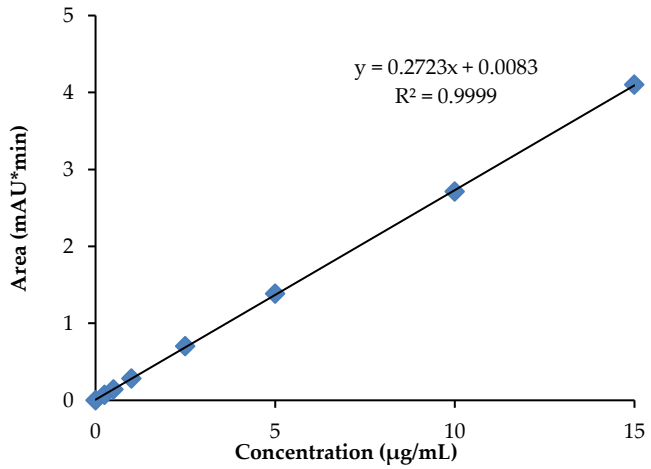

Figure 4. Linearity plot of thymol standard solutions (0-0.25-0.5-1-5-10-15 µg/mL)

Table 2. Robustness study results of thymol standard solution (10 µg/mL)

Mobil phase (acetonitril/water)	Column Temperature (°C)	Flow (mL/min)	Difference (%)
74-26 %	23	0.9	0.099
74-26 %	23	1.1	0.100
76-24 %	23	0.9	0.101
76-24 %	23	1.1	0.098
76-24 %	27	0.9	0.098
76-24 %	27	1.1	0.100
74-26 %	27	0.9	0.099
74-26 %	27	1.1	0.100

Table 3. System suitability test for thymol standard solution (10 µg/mL)

Parameter	Thymol	Acceptance limits
Hydrogel		
Repeatability – Retention time	0.08%	%RSD < 1%
Repeatability – Peak areas	0.18%	%RSD < 1%
Theoretical plates (N)	22597	N ≥ 2000
Resolution	7.06	≥ 2.0
Peak symmetry	1.1	0.8–1.8

3.1.5. Robustness:

As presented in Table 2, the difference in the comparison between the areas obtained with the changes made in the method and the areas obtained under normal conditions is less than 2%. The analytical method is successful in the robustness test against small changes [32].

3.1.6. Solution stability;

Thymol and *p*-cumenol standard solutions are stable for 42 hours at room temperature and 90 days under refrigerator conditions.

***3.1.7.** System suitability:*

As shown in Table 3, the results obtained for thymol standard solution 10 µg/mL meet the acceptance criteria [27,33,34].

In the present study, similar to previous studies, acetonitrile and water were preferred as HPLC system, DAD detector and mobile phase components [12,13,16,19–21]. The ideal ratio of mobile phase, acetonitrile, and water was determined as 75:25 (v/v), which provided ideal separation between the internal standard *p*-cumenol and thymol. In addition, no interference was observed between thymol peaks and chemicals used in the analytical method and excipients used in hydrogel production. Ideal resolution and peak symmetry values were obtained in the system suitability test using the ACE 5 C18 column, which has a similar structure to the columns used in previous studies. In the selection of reference wavelength, investigations were performed in the range of 225-278 nm, and optimum intensity and baseline appearance were obtained at the 277 nm wavelength.

Acetonitrile was successful in the dissolution of thymol and *p*-cumenol when used in standard solution and hydrogel extraction. However, it tended to volatilise when used in the model release system. For this reason, a 10 % N-methyl-2-pyrrolidone solution was tried in the model release medium. 10% N-methyl-2-pyrrolidone solution showed a successful performance in terms of recovery and stability of gaseous thymol.

Only one of the previous studies [22], which included methods for the determination of thymol, included a stress test for selectivity. In the present study, a stress test was performed under the specified conditions. As a result of thymol degradation in the oxidative degradation experiment, peaks of degradation products could be observed in the chromatogram. These peaks, which did not interfere with the thymol standard peak, could not be observed in the previous study [22]. This is thought to be due to the difference in the selectivity of the columns used in the methods.

In addition, an average of 47% of thymol was degraded in acidic degradation and 37% in alkaline degradation experiments. In a previous study, thymol was expected to show low levels of degradation under acidic and basic conditions [2], whereas in the present study, degradation was observed at high levels. This is probably due to the difference between the application times of acidic and basic conditions between the two methods.

In terms of peak retention time and analysis times, this method provides the advantage of faster thymol determination compared to previous studies [18–21].

When the LOD and LOQ values were compared with a reported study [18], it was determined that the LOD

and LOQ values of this study were at similar levels. For these reasons, it is thought that a rapid and sensitive method has been developed.

4. Conclusion

In this study, the analytical method required for the determination of thymol in gelatin-based controlled release system development stages was developed using HPLC-DAD. In order to ensure that the measurements made with the analytical method are consistently accurate and sensitive, the analytical method was tested and validated in terms of specificity, accuracy, precision, LOD, LOQ, linearity, robustness, system suitability and solution stability parameters determined for quantitative determination by HPLC. In addition, thymol degradation products were detected by stress test study. In conclusion, it is thought that this rapid and sensitive analytical method with stability indicator for thymol determination in gelatin-based hydrogel will contribute to the development of controlled release systems.

Acknowledgments

This study is derived from the first author's PhD thesis titled "Development of a Controlled Timol Release System for Varroosis Control and Investigation of its Effectiveness" (PhD Thesis, Ankara University, Ankara, Turkey, 2022). We would like to thank the T.C. Ministry of Agricultural and Forestry, General Directorate of Agricultural Research and Policies (TAGEM) (project no: TAGEM/HSGYAD/A/20/A5/P6/1943, Project Start-End date: 01.01.2020-01.01.2022)) a for their contribution to the financial support for this study.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author Contributions

The first, second, and third authors designed and planned the experiments. The first and second authors performed the experiments and contributed to the interpretation of the results. All the authors provided critical feedback and helped to analyze the research and shape the manuscript.

Availability of Data

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Statement

This study was carried out after animal experiments were approved by the Local Ethics Committee of Pendik Veterinary Control Institute (decision number: 202-17/2018).

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