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Spectrophotometric Assay of Thymol in Various Samples by Coupling with Diazotized *p*-Nitroaniline

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ABSTRACT

A simple and rapid spectrophotometric method for the trace determination of trace amount of thymol in various samples has been established. The method is based on the coupling reaction of thymol with diazotized *p*-nitroaniline in basic medium to form an intense pinkish-red, water-soluble and stable mono azo dye which shows maximum absorption at 513nm. A plot of absorbance with thymol concentration gives a straight line indicating that Beer's law has been obeyed over the range 1-300 μg/25ml, i.e., 0.04-12 ppm with a molar absorptivity of 2.48×10^4 l mol^{-1} cm^{-1}, and Sandell sensitivity index of 0.006 μg/cm^2. The method does not resort to neither temperature control nor to solvent extraction. The effect of organic solvents on the spectrophotometric properties of the azo dye, the composition and stability constant and interference due to foreign compounds have been worked out. The method has been applied to the determination of thymol in pharmaceutical, oil and waters.

التقدير الطيفي للثايمول في نماذج مختلفة بازدواجه مع الكاشف بارا-نايترو أنيلين المؤزوت

الملخص

تم تثبيت طريقة بسيطة وسهلة وسريعة لتقدير كميات متناهية في الصغر من الثايمول في نماذج مختلفة، تعتمد الطريقة على تفاعل ازدواج الثايمول مع كاشف بارا-نايترو أنيلين المؤزوت في محيط قاعدي لتكوين صبغة أحادية وردية-حمراء ذائبة في الماء والتي تعطي أعلى امتصاصية عند 513 نانوميتر. أظهر منحني الامتصاصية خطاً مستقيماً والذي يؤكد على أنه يتبع قانون بير عند مدى تراكيز الثايمول 1-30 مايكروغرام/25مل أي (0.04-12) جزء/مليون جزء، وأن الامتصاصية المولارية 2.48×10^4 لتر.مول$^{-1}$.سم$^{-1}$ ودلالة ساندل للحساسية 0.006 مايكروغرام/سم^2. بالإضافة إلى أن الطريقة لا تحتاج إلى السيطرة على درجات الحرارة أو الاستخلاص بالمذيب، لقد تم دراسة تأثير عدد من المذيبات العضوية وتركيب الصبغة وثابت الاستقرارية والمتداخلات. ولقد طبقت الطريقة لتقدير الثايمول في المستحضرات الصيدلانية والزيت والمياه.

INTRODUCTION

Thymol is a constituent of oil of thyme, a naturally occurring mixture of compounds in the plant Thymus vulgaris L., or thyme. Thymol is an active ingredient in pesticide products registered for use as animal repellents, fungicides/fungistats, medical disinfectants, tuberculocides and virucides. Thymol has also many non-pesticidal uses, including use in perfumes, food flavoring, mouth washes, pharmaceutical preparations, cosmetics and also as a stabilizer to several therapeutic agents, including halothane (EPA, 1993; Szentandrassy et al., 2003 and Dewick, 2002). Therefore, its spectrophotometric assay seems desirable (Younis and Bashir, 1995).

Thymol and some other phenolic compounds in volatile oils and plants have been determined by two spectrophotometric methods. The first one is based on the reaction with 5% sodium nitrite solution and 10% hydrochloric acid solution followed by the addition of 10% sodium hydroxide solution to give the yellow nitroso colored compound which is measured at 396nm (Enaam, 1996). The second method depends on the reaction of thymol with a known excess of potassium permanganate solution in a neutral medium and measuring the excess unreacted amount of reagent after 5 min at 520 nm (25±5°)C. The decrease in the absorbance is related to thymol concentration. Menthol, anisole and methyl salicylate have been found not to interfere (Enaam, 1996).

Another method is based on coupling with diazotized 2,4,6-trimethylaniline reagent in borate buffer solution calls for the use of SDS micellar medium (to increase water solubility of reagent and to catalyze the coupling reaction), heating at 90°C for at least 2 min in a water-bath and the need to prepare the reagent in situ (Romer et al., 1994). Ahmad and Aesa (2004) developed a simple method for the determination of thymol in smicks ointment using *p*-aminophenol via oxidative coupling reaction in aqueous alkaline solution at 594nm as the maximum absorption, the linear calibration range was from 0.4-20ppm with a molar absorptivity of 1.65×10^{4} l.mol^{-1}.cm^{-1}, relative *standard* deviation of the method was less than 1.1%, and the accuracy was 100.07%. While Mohammad (2005) described a spectrophotometric method for the determination of thymol in mouth-washes and creams of pharmaceutical preparation, based on the oxidative coupling reaction of thymol with *p*-phenylenediamine reagent in the presence of sodium metaperiodate in alkaline medium. Under these conditions, the dye formed absorbs maximum at 550nm with a molar absorptivity of 7.45×10^{3} l.mol^{-1}.cm^{-1} and Beer's law obeyed over the concentration range of 0.4-24ppm. Other methods for thymol determination including chromatographic (Nazal et al., 2002 and Kohert et al., 2002), flow injection(Rodriguez et al., 1999) and steam distillation-extraction (Bartak et al., 2000) are also available.

In the present work, the stable diazotized *p*-nitroaniline reagent has been proposed to determine thymol in various samples by the azo-coupling reaction.

EXPERIMENTAL

Reagents

All Chemicals used are of the highest purity available.

Thymol (100μg/ml) solution

This solution is prepared by dissolving 0.0100g of thymol (BDH) in 5ml of 96% ethanol (Al-Tharthar, Iraq) then the volume is completed to 100ml with distilled water in a volumetric flask. This solution is then transferred to a dark bottle where it is stable for at least 1 month.

Diazotized p-nitroaniline (25mM) solution

This reagent solution is prepared by dissolving 0.3453g of *p*-nitroaniline (Fluka) in ~80ml distilled water containing 8ml of 5M HCl (Fluka) (heating is required to hasten dissolution) then cooled to about 5°C. A 0.1760g-amount of sodium nitrite (BDH) is added and the mixture is stirred occasionally for ~15min then the volume is diluted to 100ml with additional cooled (5°C) distilled water. This solution is transferred to a dark bottle and kept in a refrigerator where it is stable for 3 months.

Diazotized p-nitroaniline (5mM) solution

This solution has been prepared by diluting 20ml of the 25mM solution of diazotized
p-nitroaniline solution to 100ml with cooled distilled water in a volumetric flask and then kept in a dark bottle where it is stable for 1 week.

Sodium hydroxide (1M) solution

Instruments

Spectral measurements, are performed, using Shimadzu, UV-160, Cintra 5GBC, and CECIL-CE 1021 digital single beam, with 1-cm plastic cells.
pH readings are measured using Philips PW 9420 pH meter.

Procedure

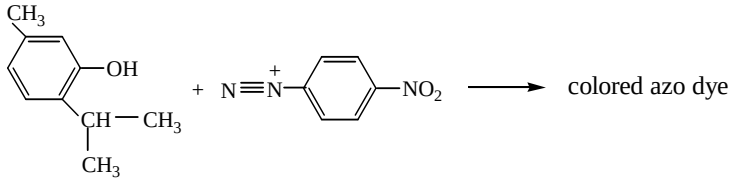
To a series of 25ml volumetric flasks are added 5-300 μg of thymol. 1 ml of diazotized *p*-nitroaniline reagent (5mM) solution and 3ml of sodium hydroxide (1M) solution are then added and the mixture is diluted to the mark with distilled water. The absorbances are measured against the corresponding reagent blank at 513nm using 1-cm plastic cells.

For the subsequent experiments, 50μg of thymol is taken and the final volumes are 25ml.

RESULTS AND DISCUSSION

Principle of the color reaction

Thymol, as other phenols can couple with diazotized reagents in alkaline medium as the preliminary experiments have confirmed (Romero et al., 1994). The present method is based on the coupling of thymol with diazotized *p*-nitroaniline in basic medium to form an intensity-coloured azo dye:



The intensity of the dye formed has been found to be proportional to the amount of thymol originally present in solution.

Study of the optimum reaction conditions

The effects of various parameters on the optical properties of the azo dye have been studied and the reaction conditions are optimized.

Choice of diazotized reagent and amount

Diazotized *p*-nitroaniline reagent has been selected in this study for the following reasons: (i) the strongest diazonium electrophile ever used (due to the presence of the strong electron–withdrawing nitro group). (ii) the strongest color contrast observed in its azo dye production (due to the presence of the *p*-nitro resonating group), (iii) the most sensitive diazo-coupling reaction diazotized *p*-nitroaniline reagent can give with aromatic components Rahim et al., 1986 and Ahmed et al., 1987), (iv) the solution of the diazotized *p*-nitro-aniline reagent is stable for long times (>1 month) if kept in cold and dark conditions, and (v) the color of the diazotized reagent solution is faint yellow thus giving lower blank values.

The effect of the amount of diazotised *p*-nitroaniline reagent on the absorbance of the resuling azo dye has next been investigated. The experimental results have shown that 1ml of 5mM of diazotized *p*-nitroaniline solution is optimum because: (i) the absorbance of the resulting dye becomes independent when higher amounts of diazotized reagent are used, (ii) the blank value is low, (iii) the $r_{10\text{-}100\mu g\ thymol}$ = 0.9999 and the sensitivity of the colored reaction is high.

Effect of base

To produce the colored azo dye with thymol upon coupling with diazotized *p*-nitroaniline reagnet, only basic medium can achieve forming the azo dye with useful analytical properties. Therefore, different amounts 1-5ml of 1M solutions) of various bases have been tried for the purpose of producing intense colored dye, strong color contrast and lower blank value. Sodium hydroxide, potassium hydroxide and sodium carbonate have been tried for the purpose. The experimental data have shown that sodium hydroxide and potassium hydroxide produce the pinkish-red azo dye with approximately equal intensity, while sodium carbonate gives rise to a yellow

dye (λ_{max}=409 nm) with half an intensity as sodium and potassium hydroxides, which is apparantly due to pH variation. The pinkish-red dye develops only in basic medium of pH>10. The maximum pH reached by sodium carbonate (5ml of 0.25M) solution is 9.5. Consequently, 3ml of 1M NaOH solution have been recommended for the subsequent experiments.

Effect of surfactants

The presence of surfactants in a colored mixture solution frequently leads to an increase in the absorbance and a shift in the wavelength to higher values (Escrig-Tena et al., 1998). This led to a test of the effect of surfactant addition to the reaction mixture. In this respect, sodium dodecyl sulfate (anionic surfactant), cetyltrimethylammonium bromide (cationic surfactant) and Triton x-100 (non-ionic surfactant) have been introduced. The experimental data reveal that non of the surfactants give very useful results from the analytical point of view. Therefore, surfactants have been omitted in the subsequent experiments.

Development time and stability period

To test the effect of time on the absorbance of the colored dye at the wavelength of maximum absorption at 513nm, the colored dye has been prepared from different amounts of thymol, under the optimal experimental conditions, and the absorbances are measured at different intervals of time upto 60min. The experimental results have shown that the colored dye develops immediately and the absorbance remains maximum and constant for at least 60min. The stability period of at least 60min is sufficient for many measurements to be made.

Absorption spectra

When a dilute solution of thymol, under the above-established conditions, is mixed with diazotized *p*-nitroaniline in the presence of sodium hydroxide, a pinkish-red colored dye immediately formed. This shows maximum absorption at 513nm in contrast to the colored reagent blank which shows no absorption. Fig. 1 Shows the absorption spectra. The wavelength of maximum absorption at 513nm is still used for the subsequent investigations.

Analytical properties of the method

The calibration graph is linear up to at least (<2.5 absorbance unit) 300μg thymol per 25ml final volume with a linear regression equation of: A=0.1897C +0.0303 (where A is the absorbance of the final colored solution and C is the final thymol concentration in ppm) with a correlation coefficient (r)=0.9996 (n=16). The apparent molar absorptivity of the colored dye, with respect to thymol, at the wavelength of maximum absorption at 513nm, is 3.17×10^4 $l.mol^{-1}.cm^{-1}$, which indicates a sensitive colour reaction. The relative error of the calibration graph is ±1.3 and the relative standard deviation is ±1.2% to ±2.8%, depending on the concentration level of thymol in solution.

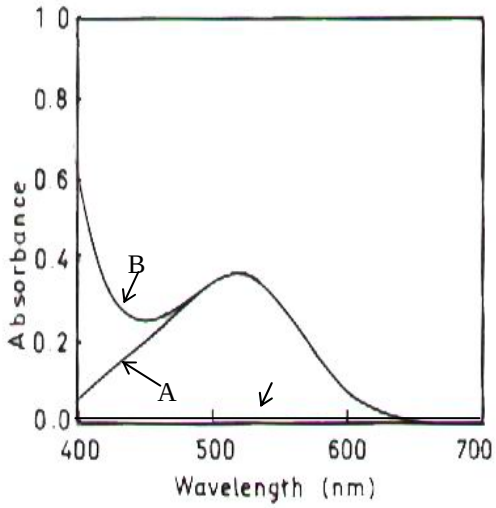
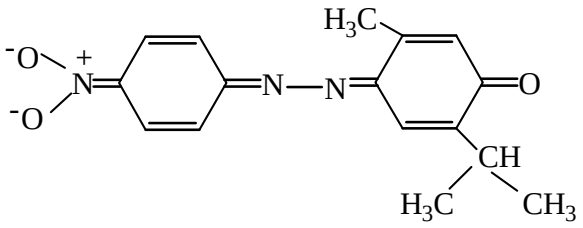


Fig. 1: Absorption spectra of 50µg of thymol/25ml treated according to the procedure and measured against: (A) blank; (B) distilled water and (C) blank against distilled water.

Nature of the dye

To establish the composition (ratio of thymol to diazotized *p*-nitroaniline reagent) of the pinkish-red azo dye formed, Job's method of continous variations and mole-ratio method have been used. The resulting data reveal that the dye has been formed by the reaction of thymol with diazotized *p*-nitroaniline reagent in a ratio of 1:1, indicating a mono azo dye with probably of the following structure:



The apparent stability constant of the azo dye in aqueous solution, under the conditions of experimental procedure, has been calculated, and found to be 1.6×10^{8} M^{-1}

Effect of organic solvents

To test the effect of organic solvents on the optical properties of the colored mono azo dye, the reaction mixture has been diluted using different organic solvents and the data are shown in Table 1.

Table 1: Effect of water and organic solvents on the optical properties of the azo dye.

Solvent	Color		λ_{max} (nm)	ε, $l.mol^{-1}cm^{-1}$
	Sample	Blank		
Acetic acid	Yellow	Colorless	398	2.2×10^4
Acetone	Violet	Yellow	575	4.4×10^4
Dioxane	Violet	Yellow	554	3.9×10^4
Ethanol	Violet	Yellow	553	3.4×10^4
Formic acid	Orange	Colorless	474	3.2×10^4
iso-Butanol	-	-	Two layers	-
Methanol	Violet	Pale yellow	534	3.5×10^4
n-Propanol	Violet	Yellow	548, 553	3.6×10^4
Pyridine	Blue	Yellow	Turbid	-
Water	Pinkish-red	Colorless	513	3.2×10^4

From the above table, it can be shown that alcohols (ethanol, methanol and propanol) exert a bathochromic shift on the dye with a comparable molar absorptivity. This might be due to an organic hydrogen-bond interaction with the azo group. While acids (acetic and formic acids) exert a blue shift due to their protonation effect which might change the quinone form of the dye (a high conjugated system) to the phenolic form (a system of lower conjugation). Ketones (acetone) and ethers (dioxane) exert similar effects, in the direction of increasing both λ_{max} and ε compared to water, is due to hydropholic interaction with the dye in the excited state.

Effect of interferences

In order to assess the possible analytical applications of the present proposed method, the interfering effects of excipients at various levels on the determination of 50μg of thymol by the proposed method have been examined and the results are given in Table 2.

Table 2: Effect of excipients on the determination of 50μg of thymol

Exceipent	Error% μg of excipient added				
	500	1000	2000	3000	5000
Glucose	0.0	+1.5	+1.9	+2.2	+3.6
Acacia	+1.5	-	+2.9	+3.1	+2.9
Vanillin	0.0	+0.7	+1.9	+3.6	+3.9
Glycerol	+1.0	+1.7	+3.9	+3.6	+3.4
Starch	+1.1	-1.3	+1.6	+1.5	+0.8
Arabic Gum	-1.3	-0.3	+1.2	+1.1	+1.1

From the above table, it can be observed that non of the excipients can introduce significant interference.

Application of the method

Determination in a drug

Thymol as one component of a Syrian pharmaceutical preparation (Thymopectol, Surp, Medico Labs., Homs, Syria. This drug is certified to contain 1600mg thymol/100ml) has been certified by the present method and the recovery is found to be ~94%. While by the standard 4-aminoantipyrine method (Farino et al., 1981) the recovery is 101.6%. The experimental t-test found to be $[t_4^{95\%}(\text{exp.}) = 0.18]$, while the tabulated value is 2.776 at confidence level 95% and for four degrees of freedom, which indicate that this method is reliable for application.

Determination in oil

The content of an oil for thymol(*) has been found by the present method to be 3.75%. While by the standard 4-aminoantipyrine method (Farino et al., 1981) the content is found to be 2.5%. The experimental t-test for four degrees of freedom and at confidence level 95% is $[t_4^{95\%}(\text{exp.}) = 2.45]$ which is less than the tabulated value; therefore this method is reliable and can be used it for this application.

Determination of thymol in sea water

Thymol may find its way into sea water by ship accidient (Younis and Bashir, 1995). Synthetic sea water (Henriksen, 1965) has been prepared and thymol has been assayed in sea water by the present method, and the results show that the average recovery of 10-200μg thymol/1ml of sea water is 99.6%.

Determination of thymol in river water

The compound may find its way into river water through the discharge of some factories (Younis and Bashir, 1995). Thymol has been assayed in Tigris river by the present method and the results show that the average recovery of 10-200μg thymol/1-5ml of Tigris river is 100.7%.

Determination of thymol in well water

Thymol in well water has been assayed in well water by the present method and the average recovery of 10-100μg thymol/1/5ml of well water is 103.5%.

Comparison of methods

The table below shows the comparison between the present method and another spectrophotometric method.

Table 3: Comparison of Methods

Analytical parameters	Present method	Literature method (Mohammad, 2005)
Temperature (°C)	Room temperature	Room temperature
λ_{max} (nm)	513	550
Reaction medium	Aqueous	Aqueous
Type of reaction	Diazo-coupling	Oxidative coupling
Reagent	Diazotized *p*-nitroaniline	*p*-phenylenediamine + sodium metaperiodate
Beer's law range (ppm)	0.04-12	0.4-24
Molar absorptivity ($l.mol^{-1}.cm^{-1}$)	2.48×10^4	7.45×10^3
Sandell sensitivity index ($\mu g.cm^{-2}$)	0.006	0.02
Colour of the dye	Pinkish - red	violet
Composition of the dye	1:1	1:1
Application of the method	Pharmaceutical, oil and waters	Pharmaceutical preparations

The present method is more sensitive than the most recently-published method on thymol (Mohammad, 2005).

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