# Egyptian Journal of Chemistry

**http://ejchem.journals.ekb.eg/**

## Second and Third Derivative Spectrophotometric Determination of Liothyronine and Thyroxine in Human Serum

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**Abstract**

A simple and accurate spectrophotometric method for simultaneous determination was used in this research of liothyronine and thyroxine in human serum samples. The method created on the zero – crossing calculation for second and third derivative spectrophotometry. The calibration graphs are in the concentration spectrum, linear of $1.0 – 17.0$ µgmL$^{-1}$ and $1-18$ µgmL$^{-1}$ for liothyronine and thyroxine successively. The recoveries range from $93.78 – 102.68$ % for T3 and $93.47 – 103.25$ % for T4 with relative standard deviation less than 4.28% and 4.50% in all in stance for T3 and T4 respectively.

*Keyword:* liothyronine, thyroxine, derivative spectrophotometry

## 1. Introduction

Thyroid compounds are a group of hormones responsible for the regulation of a variety of biological functions, including basal metabolic rate, lipid, glucose and carbohydrate metabolism(1). Thyroxine (T4) and liothyronine or triidothyronine (T3 )are tyrosine-based hormones formed by the thyroid gland chemical structures shown in Fig. (1.0).(2, 3). The thyroid gland (Tg) is histologically characterizes. by bigfollicular tissue with a monolayer of cells, which producing T4 (90 percent) and T3 (10 percent) .T3 and T4 can be easily released into the systemic circulation because gland tissue is highly vascularized. Thyroid hormone secretion is well regulated by a negative feedback system that includes, in addition to the thyroid gland, the hypothalamus and pituitary gland.(4) .Normal thyroid hormone are 3.5–6.5 pmol/L and 9.5–21.5 pmol/L for T3 and T4, respectively. T3, which is found at much lower concentrations in the blood, has morebiological activities than T4. The low concentration of T3 usually present in serum, as well as the degradation of serum, are the two major challenges of serum T3 calculation (5, 6) The thyroglobulin was kept in the gland in the cavity that was activated by the thyrotropin releasing hormone and the proteolytic enzyme, the T3 and T4 were conjugated. As a result, the Tgb was isolated from the blood(7). Development

and growth, carbohydrate metabolism, oxygen intake, protein production, and fetal neurodevelopment are all regulated by the TH (T4) and (T3) (8, 9). On thyroglobulin molecules in the thyroid, both being circulated T4 and a minor percentage of being circulated T3 are synthesized. The majority of T3 in the blood is released enzymatically through T4 monodeiodination by particular within the cell deiodinases enzymes found in follicular cells and target tissue cells[8]. (T3) as well as (T4) are a TH activated by THS which is released and formed from the pituitary gland. The Tg creates both T3 and T4; however, T3 and T4 are not released in similar quantities. T4 is created entirely by the thyroid gland, while only (20.0%) of T3 is created directly by the thyroid gland. The extra thyroidal deiodination of T4 produces the remaining 80% of T3. The liver and/or kidneys are primarily responsible for extrathyroidal deiodination(10, 11).T3 determination is crucial in the early detection of thyroid disease. T3, and T4 concentrations in human blood rise, preventing the pituitary gland from producing TSH. TSH activity in the pituitary gland increases as the concentrations of T3, and T4 hormones diminution. T3 levels in women are raised during pregnancy as a result of estrogen treatment(11, 12).

Liothyronine (T3)

Thyroxine (T4)
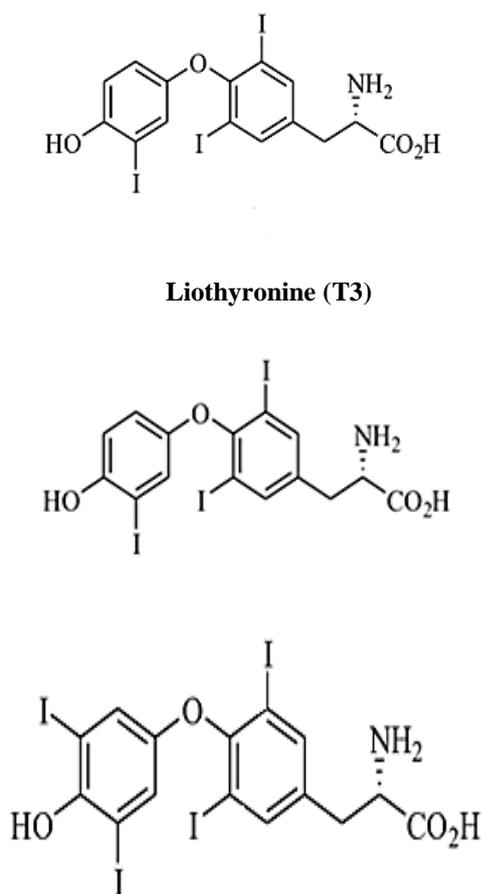
Fig. 1. Chemical structure of thyroid hormones

In recent years, analytical methods high performance liquid chromatography (HPLC),(7, 13, 14) liquid chromotography/ massspectrometry (LC/MS),(6), and liquid chromatography/tandem masss pectrometry (LC/MS/MS) (15, 16), isotope-dilution liquid chromatography tandem mass spectrometry (LC/MS/MS) (1          LC-MS/MS) (9)have been de          ermination of T4 and T3 in   human and animal   serum or plasma samples.
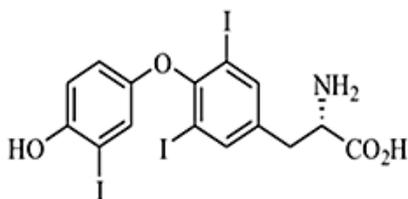
The aim of this work is to quantify (T3) and (T4) in human serum samples were utilizing derivative spectrophotometry. The method can directly determine the cited hormones simply. The developed calibration curves were utilized in deciding the concentration of these hormones in different human serum samples available with great accuracy and precision. This technique according to the quantities of the absolute value of the derivative spectrum of the combination of two hormones at a wavelength value where the absorbance of one of the mixture's components is reduced to zero. At this wavelength, the intensity is directly related to the other hormones.

The applications of derivation enable the separation of hidden signals and use them for quantitative purposes, because derivative leads to sharper zeroorder bands and gives higher signals in the resulting spectra. The spectra characteristics, for example peak height and noise level based on the parameters choice (19, 20).

## 2. Experimental
### 2.1. Apparatus

For spectrophotometric measurements, the spectrophotometer utilized was a Shimadzu UV-V, twin beam spectrophotometer (model UV 1800, Japan) with a fixed 1nm bandwidth and a 1 cm quartz cell., and a computer was coupled to the double beam spectrophotometer to record zero order spectra, and a computer loaded with software UV Probe program was used to record the different orders (1st, 2nd, 3rd, and 4th).

### 2.2. Reagents

All reagents used throughout this study are of analytical grad.

**2.2.1** Stock Solutions of T3 and T4 (Sigma Aldrich) (100 µg/mL): were made by dissolving 0.0100 g each of T3 and T4 in methanol, diluting to 100 mL in a volumetric flask, and storing at 4 °C. All of the stock solutions were kept for little less than two months. Daily working solutions were made by diluting stock solutions with methanol(2, 7) .

### 2.2.2 preparation of sample

**2.2.2.**1 Blood samples from humans were taken throughout the study, twenty-three samples were used. The samples ranged in age from 20.0 to 65.0 years for ten male and thirteen female for both normal and thyroide hormone disorder.  All of the samples were collected in Erbil city.

### 2.2.2.2 Extraction procedure

5.0 ml of blood was collected and put in to plastic tube then centrifuged at 2000 rounds per min for 10.0 min to separated serum from red blood cell. The extraction was carried out by vortexing 200 µL  of serum with 500 µL  of ethyl acetate for one minute. The mixture was shaken for 15.0

minutes at room temperature on a shaking table before being centrifuged for 3.0 minutes at 1800g. A second LLE process was carried out with 500 µL of ethyl acetate containing 10% formic acid. The samples were then centrifuged at 1800g for 3.0 minutes. Both supernatants were pooled and evaporated to dryness under nitrogen. The resulting pellet was dissolved in 100 µL of a methanol solution and spiked 5.0µg/ml of T3 and T4 standard solution add and used for determination (21).

## 2.3. Analysis by derivative UV spectrophotometry

Derivative spectrophotometry technique has been applied effectively for the simultaneous quantification of thyroid hormone in their combinations. In this investigation, various orders of derivative and various types of measurements were suggested; i.e. $1^{st}$, $2^{nd}$, $3^{rd}$ and $4^{rh}$ derivative for the same purpose.

### 2.3.1. Derivative spectrophotometric techniques (2D and 3D) for determination of T3 and T4

Aliquots of(1mL) from T3and T4 standard working solution, equivalent to 100 µg/mL for both hormones, was separately transferred into two 10.0 mL volumetric flasks, and then the volume was complete with methanol. The spectra of these two solutions. were scanned against a blank solution (methanol) and their absorptions were computed. The concentration of each hormones was quantified by constructing a calibration curve between the concentration of the hormone as abscissa and dA/dλ as ordinate at the determined zero crossing point of the other one. The 2D and 3D spectra were recorded under certain selected instrumental parameters as, Δλ, scaling factor and wavelength range, after that the working wavelengths of the two hormones, at the zero crossing points were recorded.

## 3. Results and discussion

### 3.1. Optimization of chemical parameters

Different solvents were studied to develop suitable methods of analysis methanol was the solvent of choice for all the suggested methods owing to the high solubility of the hormones in it. Further, in this solvent both hormones are stable for at least two months at 4 °C which is a good feature needed for any applicable method.

### 3.2. Selection of optimum apparatus conditions

The wavelength increment over which the derivatives are obtained Δλ is the most important instrumental factor influencing the form of the derivative spectra. In order to obtain a well-resolved broad peak with acceptable selectivity and sensitivity in the determination, this parameter must be tuned. Generally, noise decreases with an increase of Δλ, thus decrease the fluctuation in a derivative spectrum.

The scale factor must be investigated in order to determine if the device exhibits a spectral distortion effect. Furthermore, choosing this parameter allows for better reading of the analytical signal. The scaling factor was varied using the second derivative as 2, 4, 8, and 16, with 4 being chosen as the highest sensitivity without affecting the signal/noise ratio. By using the third derivative, the scaling factor was varied as; 2, 4, 8 and 16, a value of 8 was selected because it presents maximum sensitivity.

### 3.3 Normal Absorption Spectra of T3and T4

T3's normal UV absorption spectra closely overlap with T4's spectrum. The zero order absorption spectra of T3 and T4 and their mixture, using methanol as a solvent blank, were shown in Fig. (2.0).
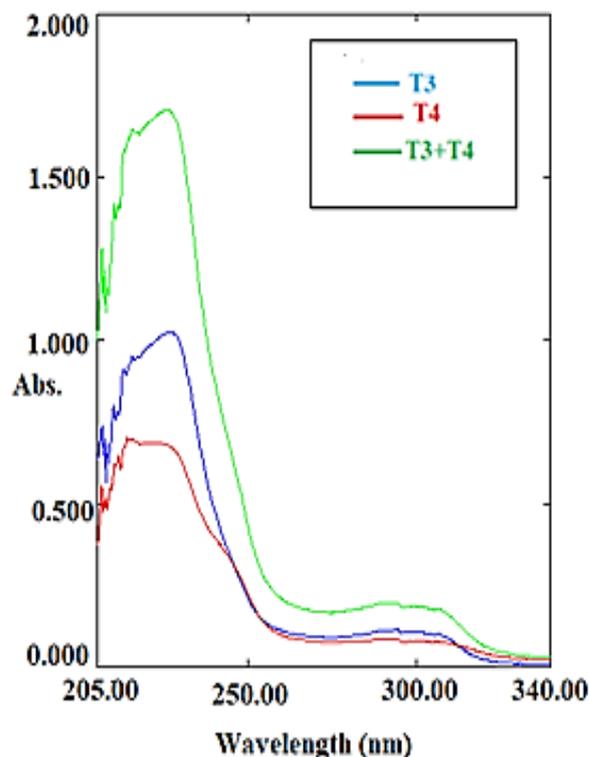


**Fig. 2 :Zero-order spectra of 15.0µg/ml of T3, 10.0 µg/ml of T4 and their mixture in methanol.**

### 3.4. Derivative spectrophotometric techniques for determination of T3 and T4

The 2D and 3D order DS gave the best outcomes as shown in Fig. (3.0) and (4.0) in compere with the other derivative orders. Graphically depending on different techniques of the 2nd and 3rd derivative spectra (peak-to-baseline, peak-to-peak and peak area). More than one relationship was established between the concentrations and signal or features of the derivative spectra cane obtained. Table (1.0) shows the statistical data of the calibration curves for determination of T3 and T4.
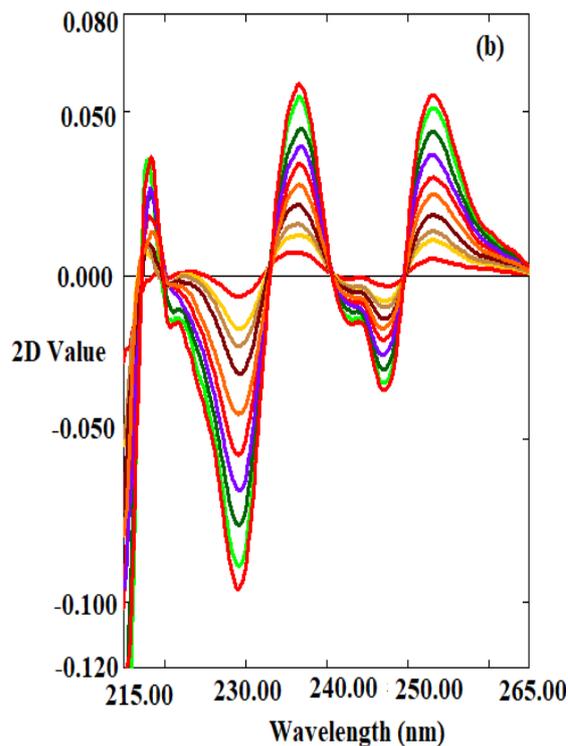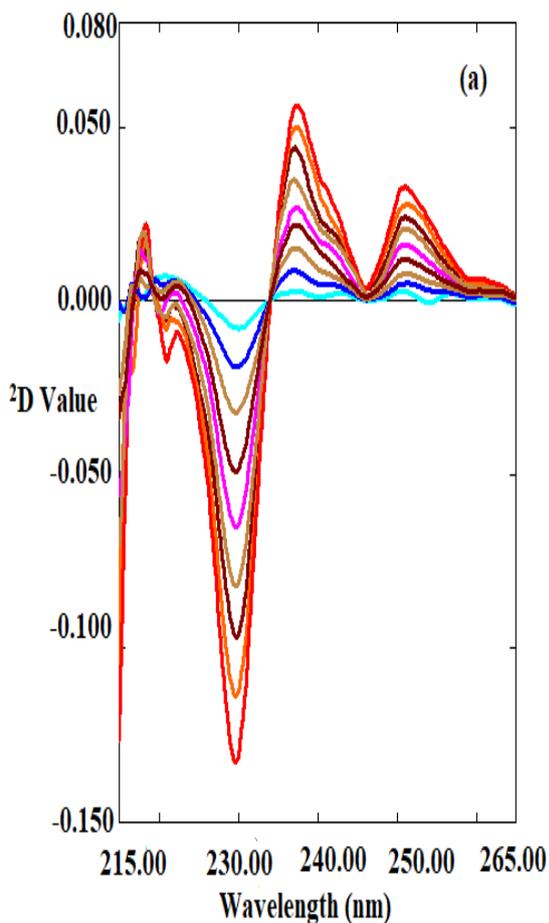


**Fig. 3.0: Second derivative spectra of different concentration of (a) T3, and (b) T4**
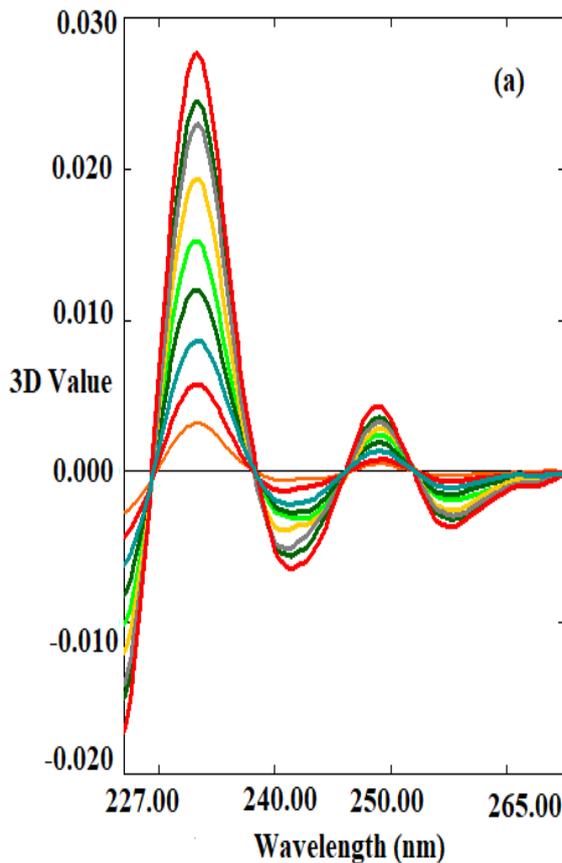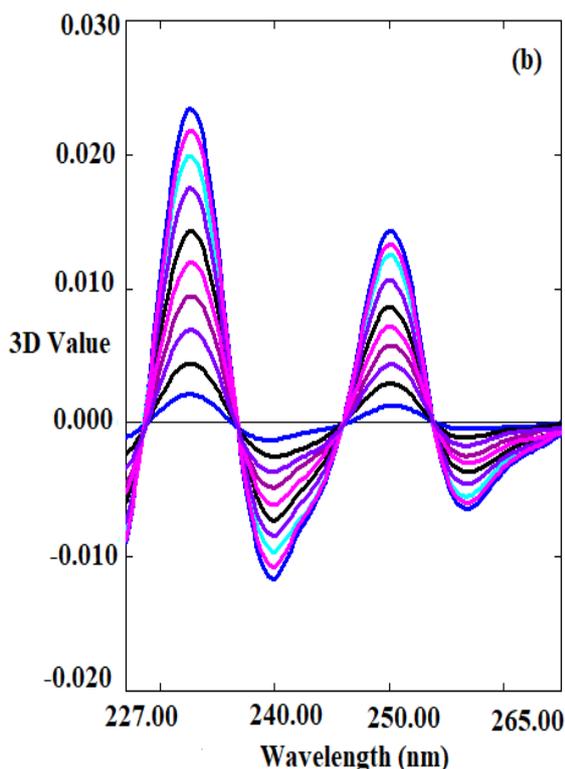
**Fig.4.0: Third derivative spectra of different concentration of (a) T3, and (b) T4**

### 3.4.1 Derivative spectrophotometric techniques for simultaneous determination of T3 and T4

The second order spectra of T3 and T4, also their zero crossing wavelengths were inferred in Fig. (5.0a). The selected wavelength, for the quantification of T3 was 232.75nm, 240.77nm and 249.93nm at this wavelength the amplitude is proportional to T3 concentration only (zero-crossing point of T4).On the other hand, the selected wavelength for the determination of T4 was 234.58 nm, because at this wavelength T4 peaks have amplitude values while the corresponding T3 peaks read zero (zero-crossing point for T3). So these wavelengths would be examined to be the optimal working wavelengths for simultaneous determination of $T_3$ and $T_4$ in their binary combinations. The 3D spectra of T3 and T4 are shown in Fig. (5.0 b), also their corresponding zero-crossing wavelengths were indicated in these figures.

Working wavelengths =236.55 nm for T3 quantification and =238.24 nm and 252.18 nm for T4 quantification were chosen because evaluations of the absolute value of the total derivative spectra performed at these wavelengths exhibited the best response to the analyte concentration (shown in the

**Table 1.0: The statistical parameters for determination of T3 and T4 using second and third derivative spectrophotometry method**

| Compounds | Technique of analysis | Wavelength (nm) | Linear range ( µg/ml) | Regression equation | r2 ( µg/ml) | LOD |
|---|---|---|---|---|---|---|
| [2]D T3 | Peak-to-baseline | 229.65 | 1.0 -17.0 | y= 0.0079x- 0.0054 | 0.9996 | 0.291 |
| | Peak-to-baseline | 237.54 | 1.0 – 17.0 | y= 0.0035x -0.0023 | 0.9993 | 0.776 |
| | Peak-to-baseline | 250.92 | 1.0 – 17.0 | y= 0.0021x- 0.0017 | 0.9981 | 0.109 |
| | Peak-to-peak | 229.65 – 237.54 | 1.0 – 17.0 | y= 0.011 5x-0.008 | 0.9998 | 0.776 |
| | Peak area | 219.60 – 234 | 2.0 – 17.0 | y= 0.049x - 0.0507 | 0.9992 | 1.165 |
| [3]D T3 | Peak-to-baseline | 233.21 | 1.0 – 17.0 | y= 0.0017x-0.0008 | 0.9997 | 0.137 |
| | Peak-to-baseline | 241.56 | 1.0 – 17.0 | y= 0.0003x+0.0002 | 0.9981 | 0.776 |
| | Peak-to-baseline | 248.84 | 1.0 – 17.0 | y= 0.0002x+0.0002 | 0.9982 | 0.825 |
| | Peak-to-peak | 241.56 – 248.84 | 2.0 – 17.0 | y= 0.0006x+0.0003 | 0.9993 | 0.366 |
| | Peak area | 229.60 – 238.60 | 2.0 – 17.0 | y= 0.0092x-0.0087 | 0.9994 | 0.136 |
| [2]DT4 | Peak-to-baseline | 229.28 | 1.0 – 18.0 | y= 0.0059x - 0.0096 | 0.9995 | 0.388 |
| | Peak-to-baseline | 236.69 | 1.0 – 18.0 | y= 0.0034x-0.002 | 0.9992 | 0.776 |
| | Peak-to-baseline | 247.11 | 1.0 – 18.0 | y= 0.0019x-0.0007 | 0.9992 | 0.915 |
| | Peak-to-baseline | 253.17 | 1.0 – 18.0 | y=0.0031x-0.0022 | 0.9992 | 0.776 |
| | Peak-to-peak | 229.28 – 236.69 | 1.0 – 18.0 | y=0.0093x-0.0118 | 0.9998 | 0.258 |
| | Peak-to-peak | 247.11 – 253.17 | 1.0 – 18.0 | y= 0.005x -0.0029 | 0.9995 | 0.466 |
| | Peak area | 219.60 – 233 | 2.0 – 18.0 | y= 0.0383x - 0.0587 | 0.9994 | 0.100 |
| | Peak area | 233 – 240.60 | 2.0 – 18.0 | y= 0.0149x -0.0069 | 0.9993 | 0.169 |
| | Peak area | 240.60 – 249.60 | 2.0 – 18.0 | y= 0.0099x - 0.0004 | 0.9995 | 0.211 |
| [3]D T4 | Peak-to-baseline | 232 | 1.0 – 18.0 | y= 0.0014x -0.0004 | 0.9993 | 0.425 |
| | Peak-to-baseline | 239.74 | 1.0 – 18.0 | y= 0.0006x +0.0002 | 0.9991 | 0.367 |
| | Peak-to-baseline | 250.12 | 1.0 – 18.0 | y= 0.0008x +0.0004 | 0.9997 | 0.367 |
| | Peak-to-peak | 239.74 -250.12 | 1.0 – 18.0 | y= 0.0014x +0.0005 | 0.9994 | 0.165 |
| | Peak area | 228.60 – 236.60 | 2.0 – 18.0 | y= 0.0059x -0.0004 | 0.9996 | 0.147 |
| | Peak area | 236.60 – 246 | 2.0 – 18.0 | y= 0.0039x -0.0031 | 0.9991 | 0.102 |
| | Peak area | 246 – 253.80 | 2.0 – 18.0 | y= 0.0037x -0.0015 | 0.9991 | 0.110 |

### 3.4.2 Calibration Curves and Statistical Data for Simultaneous Determination of T3 with T4 Using Second and Third Derivative Spectrophotometric Technique

Different mixture solutions of T3 and T4 were made in such a way that the T3 concentration remained constant 5.0 µg/mL with various concentrations of T4 for quantification of T4 in the presence of T3, normal, second and third derivative spectra of solutions were recorded. Likewise, different mixture solutions of T3 and T4 were prepared in a way that the concentration of T4 was kept constant 5.0 µg/mL with various concentrations of T3 for quantification of T3 in the presence of T4, normal, second and third derivative spectra of the solutions were taken. Table (2.0) demonstrates the results of statistical data of the calibration graphs using second and third derivative spectrophotometry for simultaneous quantification each of T3 and T4 in binary mixture as shown in Fig. (6.0 a), (6.0 b), (7.0a) and (7.0b).



**Fig. 5.0 (a) 2ⁿᵈ and (b) 3ʳᵈ spectra for ١.0 - 17.0 µg/mL T3 and ١.0-18.0 µg/mL T4 in methanol.**
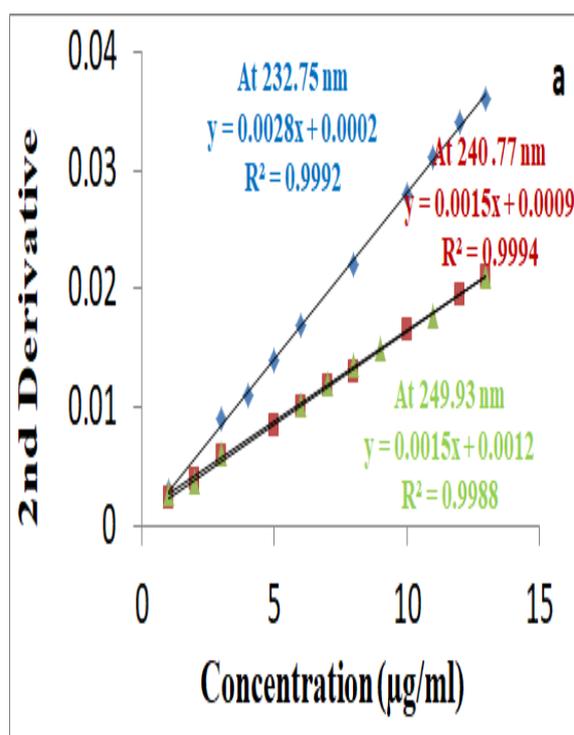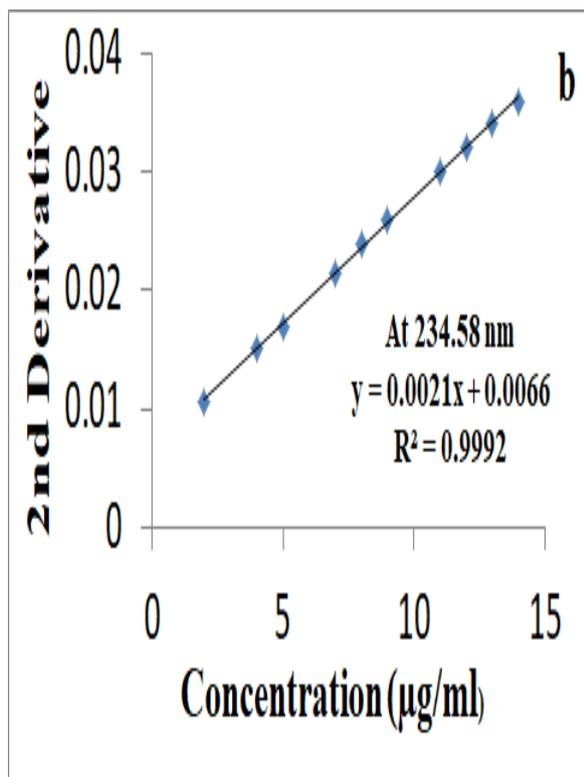
**Fig. 6.0 Calibration curves of second derivative spectrophotometric quantification of (a) T3 in the presence of 5.0 µg/mL T4, (b) T4 in the presence 5.0 µg/mL of T3, using zero-crossing technique.**
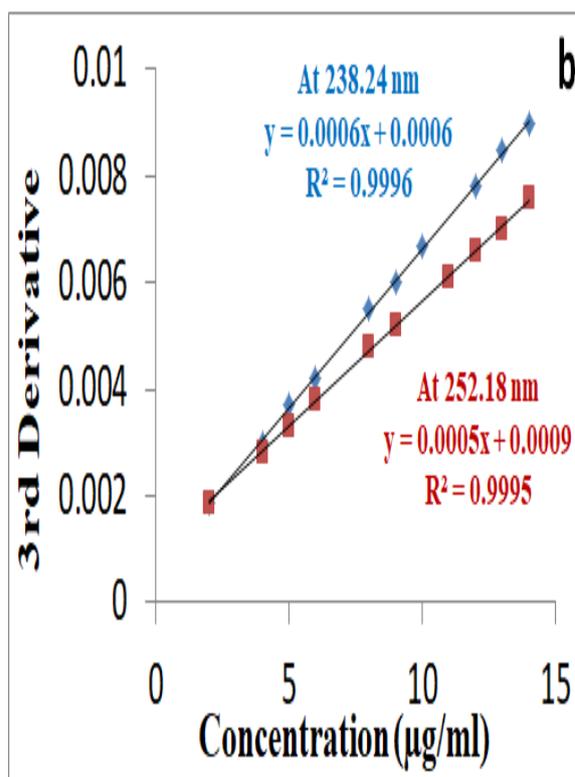


**Fig. 7.0 Calibration curves of third derivative spectrophotometric quantification of (a) T3 in the presence of 5.0 µg/mL T4, (b) T4 in the presence 5.0 µg/mL of T3, using zero-crossing technique.**
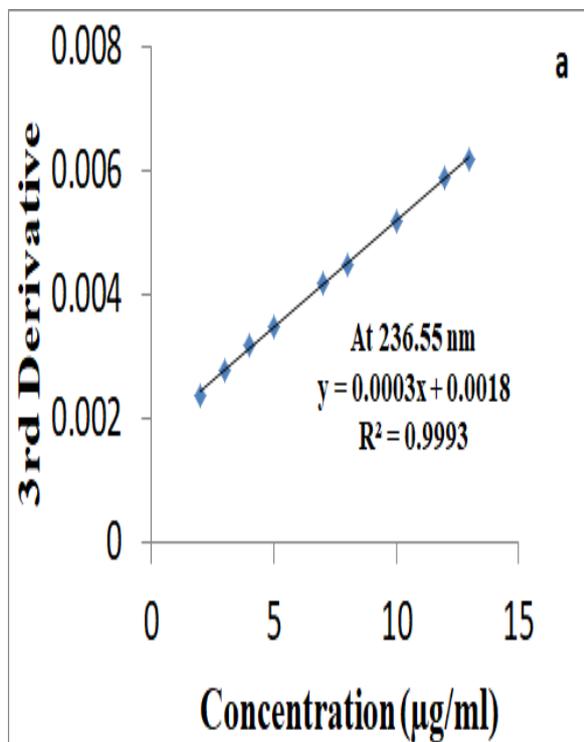
### 3.4.3 Accuracy and Precision

Under the optimum conditions, the accuracy and precision of the ($2^{nd}$ and $3^{rd}$ derivative spectrophotometric methods) for simultaneous determination of T3 with T4 in binary mixture under linearity were studied depending upon the values of the error percentage (Error %) and relative standard deviation percentage (RSD%) for five replicate measurements of three different standard concentrations. The results are illustrated in Table (3.0).

### 3.4.4 Study of Interferences

The effects of different compouns and ions on the simultaneous determination of T3 and T4 with the proposed 2D and 3D derivative spectrophotometric method have been studied. Results indicateed that the compounds under study did not interfere in the determination of analyates. As shown in Table (4.0).

**Table 2.0: The statistical parameters for determination of T3 and T4 using the proposed methods**

| Methods | Compoun | λmax (nm) | Linearity µg/mL | Regression equation | $r^2$ | LOD µg/mL |
|---|---|---|---|---|---|---|
| 2D and 3D derivative | T3 | 2D232.75 | 1.0 -13.0 | Y=0.0028x+0.0002 | 0.9992 | 0.100 |
| | | 2D240.77 | 1.0 -13.0 | y=0.0015x+0.0009 | 0.9994 | 0.155 |
| | | 2D249.93 | 1.0 - 13.0 | y =0.0015x+0.0012 | 0.9988 | 0.180 |
| | | 3D236.55 | 2.0 -13.0 | y=0.0003x+0.0018 | 0.9993 | 0.790 |
| 2D and 3D derivative | T4 | 2D234.58 | 2.0 – 14.0 | y=0.0021x+0.0066 | 0.9992 | 0.110 |
| | | ٣D238.٢٤ | 2.0 – 14.0 | y=0.0006x+0.0006 | 0.9996 | 0.550 |
| | | 3D252.18 | 2.0 -14.0 | y=0.0005x+0.0009 | 0.9995 | 0.660 |

### 3.4.5 Applications for quantification of T3 and T4 in human serum samples using the proposed method

The proposed 2D and 3D derivative zero-crossing technique for Simultaneous quantification of binary mixture of T3 with T4 was successfully applied with the aid of single standard addition technique for simultaneous determination of T3 and T4 and the cobas e 411 fully automated analyzer that uses Electro ChemiLuminescence technology for immunoassay analysis in human serum samples which collected in khanzad laboratory in Erbil city. Table (5.0) summarized the results of application and recovery study. s

### 3.4.6 Statistical analysis

Statistical study was achieved on the results found by the proposed method and cobas e411, for each T3 and T4 using Student's t- at P = 0.05, with the obtained data . No significant difference was initiated, as shown in Table( 6.0). Showing no significant difference between the T3 and T4 identification methods. Data analysis was performed by SPSS.

### 5. Conclusions

The main task of this study is to find fast, accurate, sensitive and cost effective spectrophotometric techniques for the spectrophotometry, second and third, derivative spectra of binary mixtures containing T3 and T4 were established. The results were in a good linearity, the high values of correlation coefficients indicate the good linearity of all calibration curves and the validity of Beer's law to derivative measurements. Satisfactory results were obtained for the recovery of each hormone in the mixtures. From recovery studies, 2D and 3D methods are the most suitable for quantification of T3 and T4 in binary combinations. The proposed techniques are selective and sensitive for simultaneous quantification of the human serum.

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**Table 3.0: Accuracy and Precision of the proposed methods for simultaneous determination of T3 and T4 in a binary mixture**

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| Compounds | Technique of analysis | Concentration (µg/mL) | Error% | RSD% |
|---|---|---|---|---|
| | Zero-crossing technique at $^2$D 232.75n | 1.0 | -3.57 | 4.28 |
| | | 8.0 | +2.67 | 2.63 |
| | | 13.0 | +1.65 | 2.46 |
| | Zero-crossing technique at $^2$D 240.77nm | 1.0 | +1.83 | 1.79 |
| | | 8.0 | +0.83 | 1.51 |
| | | 13.0 | -3.07 | 0.36 |
| T3 | Zero-crossing technique at $^2$D 249.93nm | 1.0 | -4.88 | 2.86 |
| | | 8.0 | -2.50 | 3.85 |
| | | 13.0 | -1.53 | 0.72 |
| | Zero-crossing technique at $^3$D 236.55nm | 2.0 | +1.36 | 1.67 |
| | | 8.0 | +1.39 | 1.09 |
| | | 13.0 | +1.11 | 0.71 |
| | Zero-crossing technique at $^2$D 234.58nm | 2.0 | +2.38 | 1.69 |
| | | 8.0 | +-3.57 | 1.23 |
| | | 14.0 | +0.34 | 0.35 |
| T4 | Zero-crossing technique at $^3$D 238.24nm | 2.0 | -4.16 | 2.50 |
| | | 8.0 | -2.80 | 2.44 |
| | | 14.0 | -1.21 | 3.57 |

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**Table 4.0: T. limit and error% of some interfering compounds on the Simultaneous quantification of binary mixtures of T$_3$ with T$_4$**

| Interfering Compound | T3 with T4 2D derivative | | | | T3 with T4 3D derivative | | | |
|---|---|---|---|---|---|---|---|---|
| | T3 | | T4 | | T3 | | T4 | |
| | T. limit µg/ml | Error% | T. limit µg/ml | Error% | T. limit µg/ml | Error% | T. limit µg/ml | Error% |
| Estradiol | 25 | +4.47 | 20 | +4.16 | 45 | +4.71 | 20 | -4.30 |
| Progesterone | 50 | -4.49 | 48 | -4.81 | 100 | -4.76 | 110 | -4.15 |
| Testosterone | 50 | +4.38 | 50 | +4.20 | 90 | +4.17 | 95 | +3.90 |
| Folic acid | 10 | -4.54 | 15 | -4.22 | 50 | +4.38 | 50 | +4.72 |
| Ascorbic acid | 40 | -4.48 | 50 | +4.38 | 35 | +4.27 | 50 | +4.33 |
| Uric acid | 35 | -4.60 | 45 | -4.33 | 65 | +4.11 | 85 | +4.10 |
| Creatinine | 25 | +4.93 | 40 | +4.49 | 60 | +4.31 | 75 | -4.07 |
| Cholesterole | 1500 | +4.62 | 1800 | +3.95 | 1800 | +4.01 | 2000 | +4.11 |
| Triglyceride | 1200 | +4.29 | 1500 | -4.40 | 2000 | +3.84 | 3000 | +4.16 |
| Urea | | | | | | | | |
| Glucose | Not efected | | | | | | | |
| Sodium ion | | | | | | | | |
| Potasium ion | | | | | | | | |
| Chloride ion | | | | | | | | |
| Calcium ion | | | | | | | | |
| Magnesium ion | | | | | | | | |
| Fe (III) | | | | | | | | |
| Fe(II) | | | | | | | | |

**Table 5.0: Simultaneous determination of T₃ and T₄ in human serum samples with their recoveries % using zero-crossing technique**

| No. of samples | Found (µg/ml) 2D proposed method | | Found (µg/ml)3D proposed method | | Recovery% 2D | | Recovery% 3D | | Found (µg/ml) by cobas e411 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T 3 | T4 | T3 | T4 | T3 | T4 | T3 | T4 | T3 | T4 |
| 1 | 0.372 | 0.254 | 0.375 | 0.256 | 94.35 | 96.45 | 95.66 | 98.04 | 0.381 | 0.263 |
| 2 | 0.261 | 0.105 | 0.264 | 0.111 | 98.46 | 97.14 | 101.51 | 96.39 | 0.270 | 0.116 |
| 3 | 0.312 | 0.188 | 0.31 | 0.187 | 94.15 | 97.87 | 95.61 | 98.38 | 0.325 | 0.194 |
| 4 | 0.151 | 0.222 | 0.154 | 0.226 | 102.64 | 96.84 | 97.40 | 101.32 | 0.162 | 0.233 |
| 5 | 0.397 | 0.095 | 0.399 | 0.096 | 96.17 | 95.78 | 98.74 | 103.25 | 0.416 | 0.101 |
| 6 | 0.108 | 0.111 | 0.111 | 0.113 | 96.29 | 96.39 | 97.29 | 97.34 | 0.117 | 0.119 |
| 7 | 0.402 | 0.112 | 0.408 | 0.115 | 98.50 | 98.21 | 96.98 | 98.26 | 0.416 | 0.120 |
| 8 | 0.114 | 0.161 | 0.117 | 0.164 | 94.87 | 98.13 | 95.72 | 97.56 | 0.123 | 0.172 |
| 9 | 0.306 | 0.304 | 0.310 | 0.309 | 98.30 | 99.34 | 99.35 | 98.70 | 0.322 | 0.315 |
| 10 | 0.201 | 0.270 | 0.204 | 0.271 | 95.18 | 100.74 | 98.03 | 94.13 | 0.213 | 0.279 |
| 11 | 0.262 | 0.237 | 0.269 | 0.235 | 102.68 | 102.12 | 98.51 | 94.92 | 0.279 | 0.245 |
| 12 | 0.368 | 0.118 | 0.371 | 0.118 | 98.29 | 93.88 | 94.78 | 96.61 | 0.377 | 0.125 |
| 13 | 0.235 | 0.116 | 0.237 | 0.115 | 98.60 | 101.72 | 95.68 | 96.52 | 0.246 | 0.123 |
| 14 | 0.355 | 0.133 | 0.359 | 0.135 | 94.23 | 103.05 | 98.88 | 95.45 | 0.365 | 0.144 |
| 15 | 0.071 | 0.171 | 0.078 | 0.176 | 98.67 | 98.24 | 96.15 | 98.86 | 0.083 | 0.183 |
| 16 | 0.302 | 0.162 | 0.307 | 0.160 | 99.39 | 95.67 | 98.69 | 93.47 | 0.317 | 0.174 |
| 17 | 0.656 | 0.15 | 0.659 | 0.156 | 95.12 | 94.49 | 93.78 | 98.51 | 0.663 | 0.161 |
| 18 | 0.528 | 0.137 | 0.526 | 0.135 | 98.15 | 97.08 | 94.33 | 101.73 | 0.535 | 0.142 |
| 19 | 0.163 | 0.116 | 0.167 | 0.115 | 95.04 | 94.33 | 97.6 | 98.30 | 0.172 | 0.126 |
| 20 | 0.121 | 0.115 | 0.123 | 0.118 | 96.50 | 95.67 | 96.25 | 99.45 | 0.129 | 0.124 |
| 21 | 0.600 | 0.181 | 0.603 | 0.185 | 97.37 | 96.68 | 99.04 | 98.72 | 0.614 | 0.192 |
| 22 | 0.303 | 0.236 | 0.381 | 0.236 | 97.97 | 97.37 | 95.78 | 95.90 | 0.312 | 0.243 |
| 23 | 0.241 | 0.241 | 0.243 | 0.242 | 95.48 | 98.75 | 98.23 | 99.17 | 0.250 | 0.248 |

**Table (6.0): Statistical comparison of the proposed methods with the cobas e411 for determination of T3 and T4 in human serum sample.**

| Methods | 2D | | 3D | | Cobas e411 | |
|---|---|---|---|---|---|---|
| | T3 | T4 | T3 | T4 | T3 | T4 |
| Mean | 0.297 | 0.171 | 0.303 | 0.173 | 0.308 | 0.180 |
| %RSD | 51.7163 | 35.9618 | 50.7672 | 35.6904 | 49.9015 | 34.3274 |
| n | 23 | 23 | 23 | 23 | 23 | 23 |
| Student t-test | 1.68 | 1.68 | 1.68 | 1.68 | 2.01 | 2.01 |
| p- value | 2.46 | 2.63 | 1.78 | 2.27 | 1.31 | 1.05 |

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