Smart manipulation of ratio spectra for resolving a pharmaceutical mixture of Methocarbamol and Paracetamol

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HIGHLIGHTS

• Application of smart and simple recently developed spectrophotometric methods manipulating the ratio spectra.
• The described methods are outstanding key for analysis of complex binary mixtures.
• Rapid methods without the need for sophisticated instruments or preliminary separation steps.
• Green, safe, economic, highly accurate and reproducible methods.
• The recently developed RDSM revealed higher selectivity and minimum data manipulation.

GRAPHICAL ABSTRACT

ABSTRACT

Two smart, specific, accurate and precise spectrophotometric methods manipulating ratio spectra are developed for simultaneous determination of Methocarbamol (METH) and Paracetamol (PAR) in their combined pharmaceutical formulation without preliminary separation. Method A, is an extended ratio subtraction one (EXRSM) coupled with ratio subtraction method (RSM), which depends on subtraction of the plateau values from the ratio spectrum. Method B is a ratio difference spectrophotometric one (RDM) which measures the difference in amplitudes of ratio spectra between 278 and 286 nm for METH and 247 and 260 nm for PAR. The calibration curves are linear over the concentration range of 10–100 µg mL⁻¹ and 2–20 µg mL⁻¹ for METH and PAR, respectively. The specificity of the developed methods was investigated by analyzing different laboratory prepared mixtures of the two drugs. Both methods were applied successfully for the determination of the selected drugs in their combined dosage form. Furthermore, validation was performed according to ICH guidelines; accuracy, precision and repeatability are found to be within the acceptable limits. Statistical studies showed that both methods can be competitively applied in quality control laboratories.

Introduction

Methocarbamol is 3-(2-methoxyphenoxy)-1,2-propanediol 1-carbamate (METH), with molecular formula of C₁₁H₁₅NO₅ [1,2]. Paracetamol is N-acetyl-p-aminophenol (PAR) Fig. 1b [1]. It is a centrally acting skeletal muscle relaxant, it relaxes skeletal muscles through depression of reflex impulse conduction within the spinal cord. Methocarbamol is prescribed to outpatients for acute muscle spasm as well as for the treatment of chronic spasitcity [3].
of headaches and other minor aches and pains and is a major ingredient in numerous cold and flu remedies [3].

Literature survey reveals that there are few analytical reports for the determination of the selected drugs in their pharmaceutical preparation by derivative and mathematical spectrophotometry [4–6], gas liquid chromatography [7] and high-performance liquid chromatography [8,9]. Even though the determination and validation of each drug either individually or in combination with other drugs is reported [10–13], the PAR–MET mixture is not yet official in any pharmacopoeia.

This study demonstrates the resolution power of newly introduced experimental spectrophotometric methods, namely; extended ratio subtraction method coupled with ratio subtraction method and ratio difference method for accurate determination of METH and PAR in bulk material and in combination tablet.

Theory of the proposed methods

Extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM)

Extended ratio subtraction method (EXRSM) starts with the ratio subtraction method (RSM) [14] which depends on that, if you have a mixture of two drugs X and Y having overlapped spectra and one of them is extended (Y), one can determine X by dividing the spectrum of the mixture by a known concentration of Y as a divisor Y. The division will give a new curve that represents X/Y + constant. If we measure this constant which is parallel to the wavelength axis in the region where Y is extended, then a new curve is obtained after subtraction of the constant. Then the zero order spectrum of component X could be obtained by multiplying the obtained ratio spectrum by the divisor Y. This can be summarized as the following:

\[
\frac{(X + Y)}{Y} = \frac{X}{Y} + \frac{Y}{Y} = \frac{X}{Y} + \text{constant}
\]

\[
\frac{X}{Y} \times Y = X
\]

Another extension of the already developed RSM method has been recently established namely extended ratio subtraction [15,16] to get the zero order spectrum of extended component (Y). It starts by dividing the obtained D0 spectrum of X by a known concentration of X as a divisor X to get the constant X/X for each concentration in the mixtures then follow the same procedure of the ratio subtraction through dividing each mixture by X, then subtracting the corresponding constant X/X and multiplying by (X):

\[
\frac{Y}{X} \times X = Y
\]

The concentration of X and Y were calculated from the corresponding regression equations (obtained by plotting the absorbance values of the zero order spectra of each drug at its \( \lambda_{\text{max}} \) against its corresponding concentrations).

Ratio difference spectrophotometric method (RDM)

Ratio difference spectrophotometric method [15–17] was recently developed for analyzing a mixture of two drugs X and Y having overlapped spectra. It depends on the amplitude difference between two wavelengths \( \lambda_1 \) and \( \lambda_2 \) in the ratio spectra of a mixture is directly proportional to the concentration of the component of interest; independence of the interfering component. if two drugs X and Y having overlapped spectra, you can determine X by dividing the spectrum of the mixture by a known concentration of Y as a divisor (Y). The division will give a new curve that represents \( \frac{X + Y}{Y} \) i.e. \( \frac{X}{Y} + \frac{Y}{Y} \), where \( \frac{Y}{Y} \) is a constant. By selecting 2 wavelengths \( \lambda_1 \) and \( \lambda_2 \) on the obtained ratio spectrum and subtracting the amplitudes at these two points, the constant \( \frac{Y}{Y} \) will be canceled along with any other instrumental error or any interference from the sample matrix. This can be summarized as the

\[
\frac{X + Y}{Y} = \frac{X}{Y} + \frac{Y}{Y} = \frac{X}{Y} + \text{constant}
\]

Suppose the amplitudes at the two selected wavelength are \( P_1 \) and \( P_2 \) at \( \lambda_1 \) and \( \lambda_2 \), respectively; by subtracting the two amplitudes the interfering substance Y shows no interference; then;

\[
P_1 - P_2 = \left( \frac{X}{Y} \right)_1 + \text{constant} - \left( \frac{X}{Y} \right)_2 + \text{constant}
\]

\[
P_1 - P_2 = \left( \frac{X}{Y} \right)_1 - \left( \frac{X}{Y} \right)_2
\]

where; \( P_1 \) is the peak amplitudes of the ratio spectrum at \( \lambda_1 \), \( P_2 \) is the peak amplitudes of the ratio spectrum at \( \lambda_2 \).

The concentration of X is calculated by using the regression equation representing the linear relationship between the differences of the ratio spectra amplitudes at the two selected wavelengths using Y as a divisor (Y) to the corresponding concentrations of drug (X). Similarly, Y could be determined by the same procedure using a known concentration of X as a divisor X.

Experimental

Instruments

Spectrophotometer: Shimadzu UV-1650 PC, dual-beam UV–visibile spectrophotometer (Japan), with matched 1-cm quartz cells, connected to an IBM-compatible PC and an HP-600 inkjet printer. Bundled, UV-PC personal spectroscopy software Version 3.7 was used to process the absorption and the derivative spectra. The spectral bandwidth was 2 nm with wavelength-scanning speed of 2800 nm min\(^{-1}\).

Materials and reagents

Pure samples were kindly supplied by Sigma Pharmaceutical Ind. Company, Cairo, Egypt. Their purity was found to be 100.63 ± 0.29 and 99.91 ± 0.56, for METH and PAR, respectively, according to the reported spectrophotometric method [5].

Pharmaceutical formulations, Methorelax\textsuperscript{®} tablets Batch No. 30888, were kindly supplied by Sigma Pharmaceutical Ind. company, Cairo, Egypt and were claimed to contain 400 mg of METH and 325 mg of PAR per tablet. All chemicals and reagents were of analytical grade and the solvents were of spectroscopic grade.

Standard solutions

METH and PAR standard solutions (0.2 and 0.1 mg mL\(^{-1}\), respectively), prepared by dissolving 20 mg of METH and 10 mg of PAR, separately, in a few milliliters of methanol into a 100-mL
Calibration curves were constructed from their standard solutions (0.2 and 0.1 mg mL\(^{-1}\), respectively) into two 10-mL volumetric flasks. Then the volumes were completed with methanol. The absorption spectra of the prepared solutions were recorded over the range 200–400 nm using methanol as a blank.

**Procedures**

**Spectral characteristics of METH and PAR**

Two aliquots equivalent to 120 μg of METH and 60 μg of PAR were transferred separately from their standard solutions (0.2 and 0.1 mg mL\(^{-1}\), respectively) into two 10-mL volumetric flasks. Then the volumes were completed with methanol. The absorption spectra of the prepared solutions were recorded over the range 200–400 nm using methanol as a blank.

**Linearity and construction of calibration curves**

Aliquots equivalent to (100–1000 μg) and (20–200 μg) of METH and PAR, respectively were separately transferred from their standard solutions (0.2 and 0.1 mg mL\(^{-1}\), respectively) into two series of 10-mL volumetric flasks. Then volumes were made-up with methanol. The spectra of the prepared standard solutions were scanned from 200 to 400 nm and stored in the computer.

**For extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM).** Calibration curves were constructed relating the absorbance of zero order spectra of METH at 274 nm and PAR at 248.3 nm versus the corresponding concentrations and regression equations were computed.

**For ratio difference spectrophotometric method (RDM).** The stored spectra of METH are divided by the spectrum of 20 μg mL\(^{-1}\) PAR while PAR spectra were divided by the spectrum of 200 μg mL\(^{-1}\) METH. Calibration curves of METH and PAR were constructed by plotting the difference between the amplitudes of ratio spectra at 278 & 286 nm for METH and 247 & 260 nm for PAR, versus the corresponding concentrations and the regression equations were computed.

**Application of extended ratio subtraction and ratio difference of ratio spectra for the determination of METH and PAR in laboratory prepared mixtures**

For preparation of laboratory mixtures, into a series of 10-mL volumetric flasks, aliquots equivalent to 100–300 μg mL\(^{-1}\) of METH and 100–200 μg mL\(^{-1}\) of PAR were accurately transferred from their standard solutions (0.2 and 0.1 mg mL\(^{-1}\), respectively) to obtain different ratios of the two drugs including the ratio of their commercial product. Subsequently, the volume was completed with methanol. The spectra of the prepared mixtures were scanned from 200 to 400 nm and stored in the computer.

**For extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM).** For determination of METH by RSM, the stored zero order absorption spectrum of each laboratory prepared mixture was divided by the absorption spectrum of standard PAR (20 μg mL\(^{-1}\)), then the amplitude in the plateau region at λ 290–305 nm (the constant) was recorded and subtracted from the obtained ratio spectra. Then by multiplying the obtained ratio spectra by PAR (20 μg mL\(^{-1}\)) yield the zero spectra of METH and accordingly, the concentration of METH is calculated using the corresponding regression equation at its \(\lambda_{\text{max}}\).

On the other hand, zero order absorption spectra of PAR could be obtained via EXRSM, where the absorption spectra of standard METH of the same concentrations of the mixtures were divided by standard METH (100 μg mL\(^{-1}\)) as a divisor to determine the constant values at plateau region (260–290 nm), then the same procedures as RSM were followed using METH (100 μg mL\(^{-1}\)) as a divisor. The concentration of PAR in each mixture is calculated using its corresponding regression equation at its \(\lambda_{\text{max}}\).

**Ratio difference spectrophotometric method (RDM).** The stored spectra of different laboratory prepared mixtures were divided separately by the absorption spectra of standard PAR (20 μg mL\(^{-1}\)) and standard METH (200 μg mL\(^{-1}\)) to obtain the ratio spectra for determination of METH and PAR, respectively. The difference between the amplitudes of ratio spectra at 278 & 286 nm for METH and 247 & 260 nm for PAR were calculated. The concentrations of the drugs were calculated from the corresponding computed regression equations.

**Application to pharmaceutical preparation**

To determine the content of METH and PAR in commercial tablets (each tablet labeled to contain 400 mg METH and 325 mg PAR), 20 tablets were weighed and finely powdered. An accurately weighed portion equivalent to 8 mg METH and 6.5 mg PAR was transferred to a 100-mL beaker. 50 mL of methanol was added, stirred using a magnetic stirrer for 30 min and filtered through 0.5 μm Whatman filter paper into a 100-mL volumetric flask. The residue was washed three times each with 10 mL of methanol and the solution was made up with the same solvent. From the above prepared solution, further dilutions were prepared in the obtained linearity ranges using the same solvent. The general procedure described above under each method was followed to determine the concentration of both drugs in their pharmaceutical formulation. The analysis was done in triplicates. Concentrations of METH and PAR in the prepared samples were calculated from the corresponding computed regression equations.

Standard addition technique was applied by mixing the powder content of the tablets with different increments of pure METH and PAR standards before proceeding in the above mentioned procedures.

**Results and discussion**

The classical analytical problem of spectrophotometric multicomponent analysis is that the analyte of interest is often accompanied by other co-formulated compounds absorbing in the same spectral region. In this case, spectral overlapping requires resolution by mathematical procedures.

This paper describes the application, validation and comparative study of two recently developed spectrophotometric methods manipulating ratio spectra for the determination of METH and PAR in their combined dosage form. The proposed methods are considered to be smart ones due to their practical simplicity and ability to cancel the interfering component along the whole spectrum then by the least mathematical steps either convert the ratio spectra to its original zero order one (RSM and EXRSM) or deal with ratio spectra directly without further steps (RDM). The zero order absorption spectra of pure drugs show overlapping which hinders their direct determination as shown in Fig. 2.

**Extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM)**

Extended ratio subtraction method (EXRSM) starts after the application of the ratio subtraction method (RSM) [14]. The RSM depends on that, if you have a mixture of METH and PAR, where the spectrum of PAR is more extended (Fig. 2), the determination of METH in the mixture could be done by scanning the zero order absorption spectra of the laboratory prepared mixtures (METH and PAR), dividing them by a carefully chosen concentration of standard PAR (20 μg mL\(^{-1}\)) as a divisor. This chosen concentration of the divisor gives the best regression over the proposed concentration range. This will produce new ratio spectra which represent METH/PAR + constant as shown in Fig. 3. Then, subtraction of the
values of these constants \((\text{PAR}/\text{PAR'})\) in the plateau region (290–305 nm) is done, as shown in Fig. 4. This is followed by multiplication of the obtained spectra by the divisor \(\text{PAR'} (20 \mu g \text{ mL}^{-1})\) to get the original zero order spectra of \(\text{METH}\), Fig. 5. Then the concentration of \(\text{METH}\) was calculated from the corresponding regression equation at 274 nm.

Furthermore, the concentration of \(\text{PAR}\) in the mixture could be obtained by the application of the new extended ratio subtraction method. In this method, the obtained spectra of \(\text{METH}\) were divided by a carefully chosen concentration of standard \(\text{METH}’ (100 \mu g \text{ mL}^{-1})\) producing ratio spectra that represent the constants \(\text{METH}/\text{METH}’\) in plateau (260–290 nm) as shown in Fig. 6. The previously scanned zero order absorption spectra of the laboratory prepared mixtures (\(\text{METH}\) and \(\text{PAR}\)) were divided by standard \(\text{METH}’ (100 \mu g \text{ mL}^{-1})\) as a divisor producing new ratio spectra which represent \(\text{PAR}/\text{METH}’ + \text{constant}\) as shown in Fig. 7. Then subtraction of these obtained constants \(\text{METH}/\text{METH}’\) as shown in Fig. 8, which is followed by multiplication of the obtained spectra by the divisor \(\text{METH}’ (100 \mu g \text{ mL}^{-1})\) produce the zero order spectra of \(\text{PAR}\), as shown in Fig. 9. Finally, the obtained spectra of \(\text{PAR}\) (Fig. 9) could be used for direct determination of \(\text{PAR}\) and the calculation of its concentration from the corresponding regression equation at 248.3 nm.

The extended ratio subtraction method has an advantage that the extended drug in the mixture could be determined at its \(\lambda_{\text{max}}\) which could not be achieved by the previously established ratio subtraction method [14]. Therefore, the two methods are considered to be complementary to each other since the two components of interest in the mixture could be determined [15]. The choice of the correct divisor as well as the determination of the plateau region for constant calculation are the most critical steps, so different divisor concentrations were tried and careful choice of the constant was considered for method optimization.

Linear relationships were obtained between the absorbance and the corresponding drug concentrations in the range of 2–20 \(\mu g \text{ mL}^{-1}\) and 10–100 \(\mu g \text{ mL}^{-1}\) for \(\text{PAR}\) at 248.3 nm and \(\text{METH}\) at 274 nm, respectively. The regression equations were computed and found to be:
$A_{\text{METH}} = 0.0105C + 0.0115 \quad r = 0.9999$

$A_{\text{PAR}} = 0.0839C + 0.0335 \quad r = 0.9999$

where $A$ is the absorbance, $C$ is concentration in $\mu$g mL$^{-1}$ and $r$ is the correlation coefficient. The mean percentage recoveries were 100.40 ± 0.67 and 99.77 ± 0.80, for METH and PAR, respectively, Table 1.

**Ratio difference spectrophotometric method (RDM)**

The most striking feature of the ratio difference method is its simplicity, rapidity, accuracy and minimal data manipulation aspects [15–17]. This is a newly developed method having the ability for solving severely overlapped spectra without prior separation; meanwhile it does not require any sophisticated apparatus or expensive computer programs.

The utilization of ratio difference method is to calculate the unknown concentration of a component of interest present in a sample matrix containing an interfering component. It uses the analytical data of the ratio spectrum at two accurately selected wavelengths $\lambda_1$ and $\lambda_2$ to nullify the interferent contribution. So the following requirements were applied: at the selected wavelength pair, the difference in analyte ratio spectrum have to be linear while the difference in interferent ratio spectrum is remaining zero with changing the concentration. Also, the difference in amplitude due to the analyte ratio spectrum at the two selected wavelengths should be as large as possible to reach good accuracy and sensitivity. Similarly, another two wavelengths are selected for the estimation of the second component (interferent). Thus, the overlapped spectra of the cited drugs suggested that a ratio difference method is a suitable method for the determination of METH and PAR in their combined dosage form.
For method optimization, some important decisions were carefully taken. Different divisor concentrations of METH and PAR were tried and different wavelength pairs were investigated to meet the method requirements. Ratio difference method starts by scanning the zero order absorption spectra of the laboratory-prepared mixtures (METH and PAR). For determination of METH, divide the previously scanned ratio spectra by a carefully chosen concentration of standard PAR \( (20 \mu g/mL \times C_0) \) as a divisor to produce new ratio spectra which represent \( \text{METH} / \text{PAR}_0 + \text{constant} \) as shown in Fig. 10. The amplitudes at 278 & 286 nm were selected and subtracted, so the constant \( \text{PAR} / \text{PAR}_0 \) was cancelled. Similarly, the difference of amplitudes at the two selected wavelengths (247 & 260 nm) using standard METH \( (200 \mu g/mL \times C_0) \) as a divisor were recorded for the estimation of PAR as shown in Fig. 11.

The concentration of METH and PAR were calculated using their corresponding regression equations. The regression equations for amplitude difference (278 & 286 nm for METH and 247 & 260 nm for PAR) were computed and found to be

\[
\text{RD}_{\text{METH}} = 0.0214C + 0.0082 \quad r = 0.9999
\]

\[
\text{RD}_{\text{PAR}} = 0.1847C + 0.0733 \quad r = 0.9999
\]

where \( \text{RD} \) is the amplitude difference, \( C \) is concentration (\( \mu g/mL \)) and \( r \) is the correlation coefficient. The mean percentage recoveries were 100.09 ± 0.95 and 99.78 ± 0.85, for METH and PAR, respectively, Table 1.

The validity of the suggested methods were checked in terms of accuracy by five determinations between 80% and 120% concentration levels and precision (repeatability and intermediate precision) at three concentration levels, Table 2.

In order to demonstrate the selectivity and applicability of the proposed methods, recovery studies were performed by analyzing laboratory prepared mixtures of the two drugs in different ratios including the commercial product ratio, Table 3. The proposed spectrophotometric ratio-spectra methods were successfully applied for the determination of METH and PAR in their combined
pharmaceutical formulation (Methorelax® tablets). Furthermore, the validity of the methods was assessed by applying the standard addition technique (Table 4). It shows that the developed methods are accurate and specific for determination of the cited drugs in co-formulated dosage form without interference of the pharmaceutical excipients.

From a comparative point of view to the previously reported 1DD and 2D methods [5,6], the suggested methods offer maximum accuracy and precision with minimum number of manipulation steps especially the RDM. Also, they offer higher correlation coefficient values that being an indication of the quality of the fitting of the data to the straight line, which is coincide with the fact that the
absorbance values did not cross the Beer’s–Lambert law limits which is not considered in 1DD method. Moreover, the derivatization step changes the minor features for each 4 nm ($\Delta \lambda$) along the spectrum, and the multiplication by scaling factor is a mandatory step which may increase the noise leading to the use of another smoothing step.

Results of the suggested methods for determination of METH and PAR were statistically compared with those obtained by applying the reported spectrophotometric method [5]. The calculated $t$- and $F$-values [18] were found to be less than the corresponding theoretical ones, confirming good accuracy and excellent precision (Table 5).

Conclusion

From the previous discussion, it could be concluded that the proposed RSM is very simple, accurate and does not require any sophisticated apparatus and is suitable for computer programs lacking access to derivatization step. Its main advantage is direct measurement of the drug at its characteristic $\lambda_{\text{max}}$, hence there is
a potential for greater sensitivity and accuracy. Its capability to determine only the less extended component is the major limitation. This is overcome by the coupling with the EXRSM for the determination of the component with extended spectrum. On the other hand, the developed RDM method has the advantages of being simpler and more selective than the conventional spectrophotometric ones as it does not need critical measurement at fixed wavelengths or any derivative calculation, hence signal to noise ratio is enhanced and by difference between two wavelengths, noise will be cancelled. The developed methods do not need sophisticated instruments or any prior separation steps and so they can be used as alternative methods to LC methods in laboratories lacking the required facilities for these techniques for the analysis of any binary mixture without any limitation. They could be used for routine analysis of METH and PAR in their available dosage form without any preliminary separation steps.

Acknowledgment

This manuscript is dedicated to the memory of deceased Professor M. Galal El-Bardicy, whose long interest in spectrophotometric methods inspired the rest of us in its pursuit.

| Table 3 | Determination of Methocarbamol and Paracetamol in their binary laboratory prepared mixtures by the proposed methods. |
| Ratios & Methocarbamol (recovery% ± SD) & Paracetamol (recovery% ± SD) |
| | RSM at 274 nm | RDM at 278–286 nm | ERSM at 248.3 nm | RDM at 247–260 nm |
| 1:2 | 101.30 ± 0.40 | 100.30 ± 0.23 | 99.02 ± 0.67 | 100.35 ± 0.20 |
| 1.5:1.25 | 100.97 ± 0.35 | 100.03 ± 0.42 | 101.69 ± 0.59 | 99.97 ± 0.48 |
| 1.5:1 | 99.84 ± 0.29 | 98.97 ± 0.33 | 100.03 ± 0.55 | 100.08 ± 0.41 |
| 2:1 | 100.71 ± 0.38 | 99.76 ± 0.31 | 98.51 ± 0.62 | 100.09 ± 0.55 |
| 1.6:1.3 | 100.62 ± 0.33 | 100.01 ± 0.42 | 100.90 ± 0.58 | 99.65 ± 0.53 |

* METH:PAR in μg mL⁻¹.

* The ratio in Methorelax® tablets.

| Table 4 | Determination of Methocarbamol and Paracetamol in pharmaceutical dosage form by the proposed methods and the application of standard addition technique. |
| Item & Methocarbamol (recovery% ± SD) & Paracetamol (recovery% ± SD) |
| | RSM at 274 nm | RDM at 278–286 nm | ERSM at 248.3 nm | RDM at 247–260 nm |
| Methorelax® tablets (B.N. 30888) | 101.49 ± 0.54 | 100.43 ± 0.38 | 101.08 ± 0.69 | 100.93 ± 0.67 |
| Standard addition | 100.62 ± 0.43 | 100.77 ± 0.54 | 100.53 ± 0.83 | 101.09 ± 0.73 |

| Table 5 | Statistical comparison between the results obtained by the proposed methods and the reported method [5] for the determination of Methocarbamol and Paracetamol in pure powder form. |
| Parameter & Methocarbamol & Reported method & Paracetamol & Reported method |
| & Mean & SD & Variance & RSM at 274 nm & RDM at 278–286 nm |
| & 100.57 | 0.32 | 6 | 0.10 | 1.25 | 0.635 |
| & 100.73 | 0.27 | 6 | 0.07 | 1.14 | 0.316 |
| & 100.68 | 0.29 | 6 | 0.08 | 2.45 | 0.497 |
| & 99.70 | 0.87 | 0.76 | 2.45 | 2.82 |
| & 99.92 | 0.33 | 0.11 | 2.82 | 0.33 |
| & 99.91 | 0.56 | 0.31 | 0.33 | 0.31 |

| Mean & SD & Variance | F ratio (5.05) & Student t-test (2,228) |
| & 0.10 | 1.25 | 0.635 | 0.316 | 0.497 | 0.038 |

Figures in parenthesis are the corresponding tabulated values at P = 0.05.

* Reported method is a ratio spectra derivative spectrophotometric method.

References