Bulgarian Chemical Communications, Volume 48, Special Edition D, (pp. 18 – 26) 2016

A new study on the derivatization of malondialdehyde by anisidine reagent with use of UV-Vis, FT-IR, ^{1}HNMR, ^{13}CNMR techniques

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Received June 26, 2015, Revised September 10, 2015

Derivative methods of malondialdehyde (MDA) as a biomarker of oxidative stress, often desire high temperature and long heating time. Some of which activate a color reaction such as thiobarbituric acid reactive substances (TBA), diaminonaphtalene, pentafluorophenyl hydrazine, and phenylhydrazine after placing in that harsh condition. Our attempts were to introduce a new derivative reaction between malondialdehyde and Para methoxy amine (PMA) in order to identify MDA. The derivatization of malondialdehyde (MDA) with different concentration of para-methoxy aniline (anisidine) in an acidic medium and various time, temperature, and pH was investigated. The possible reaction of malondialdehyde and Anisidine was completed after 10 min at 75ºC in optimal condition. By spectrophotometric techniques, the complex formed in this process exhibits a highly specific UV spectrum with a sharp maximum at 400nm. ^{1}H NMR, ^{13}CNMR, and FT-IR were confirmed the formed adduct.

Keywords: malondialdehyde, anisidine, colorimetric reaction, derivatization

INTRODUCTION

Malondialdehyde (MDA), as one of the great significance individual aldehyde resulting from lipid peroxidation, is a reactive unsaturated dicarbonyl that can easily bind to macromolecules such as structural and functional proteins and nucleic acids [1-5]. As of now, the great number of analytical approaches, for determination of malondialdehyde (MDA), have been proposed various derivative methods. Most procedures based on the aldehyde reactivity of MDA employed hydrazine-based derivatization reagents [6,7] Still, the classical assay "2-thiobarbituric acid (TBA) is the most general applied technology in which two molecules of TBA change with one molecule of MDA to give a colored reaction product, which can be measured spectrophotometrically at 535 nm, or by fluorescence detection with excitation at 530 nm and emission at 550 nm. [8-12] In fact, the TBA assay is a non-specific method for MDA; therefore, further developed methods were strongly suggested by researchers with use of new color reagent for detection of this prominent biomarker in order to introduce specific method. [13-15] Derivative reagents, namely, thiobarbituricacid, diamino-naphtalene (DNPH), pentafluorophenylhydrazine (PFH), pPhenylhydrazine (PH), dansylhydrazine, methylhydrazine, 2,2,2-trifluoroethylhydrazine and FMOC-hydrazine after placing in high temperature, and long time have been proposed by researchers to measure MDA by expensive separation technology such as Reverse Phase-Liquid chromatography (**RP-HPLC**), Gas Chromatography–Mass Spectrometry (**GC-MS**), and Liquid chromatography–Mass Spectrometry (**LC-MS**, or alternatively **HPLC-MS**). [6] However, the resultof their investigation was far from satisfactory**.**

On the other hand, *p*-anisidine value would be a well-known method to measure the content of aldehydes (principally 2 alkenals and 2, 4-alkadienals) generated during the decomposition of hyroperoxides. *p*-methoxyaniline (anisidine or PMA) and the aldehyde compounds under acidic conditions provide yellowish products and absorb at 350 nm [16]. Besides, p-An Value is a valid indicator of oxidative rancidity in oils and fatty foods [17, 12]. However, no specific method has been developed for determination of malondialdehyde by anisidine reagent.

In this paper, based on colorimetric reaction, a new method for synthesis and identification of MDA was introduced. Investigation of complex formation (MDA-PMA) with use of variegated techniques was studied as well. In addition, it was expected that this procedure would assure a simple, selective, and sensitive measurement of MDA for further biological studies.

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EXPERIMENTAL

Material and methods

Malondialdehyde, tetra butyl ammonium salt (MDA, 96%) was purchased from SIGMA ALDRICH. In addition, para methoxyaniline, butylated hydroxytoluene, glacial acid acetic, HClO4 0.1 M diluted in glacial acetic acid, absolute ethanol, and sodium hydroxides were from Merck Company.

Solvent selection

To find an appropriate solvent, a possible reaction between malondialdehyde, tetra butyl ammonium salt (MDA, 96%) and para-methoxy aniline (anisidine, PMA) was evaluated. Therefore, different solvents such as HPLC-grade water, absolute ethanol, glacial acid acetic, and HCl were examined. Finally, absolute ethanol and glacial acetic acid were selected as two proper solvents for MDA and Para methoxy aniline, respectively.

Preparation of anisidine reagent

p-methoxy aniline (anisidine) as a reagent was prepared at 1000 $\mu M.L^{-1}$($\mu M = \mu M.L^{-1} = n$ mol/ ml) in glacial acetic acid, by dissolving 0.0123 g of Ansidine in 100 ml of glacial acetic acid. The malondialdehyde Tetra butyl ammonium salt in 10ml of absolute ethanol. **(**Figure.2**)** This stock solution remained stable for at least 6 months and stored at -20 ^{0}C in aliquots of 250 µl in dark place while the working standard solutions had to be prepared every day MDA solution was monitored by its absorbance at 267nm [6].

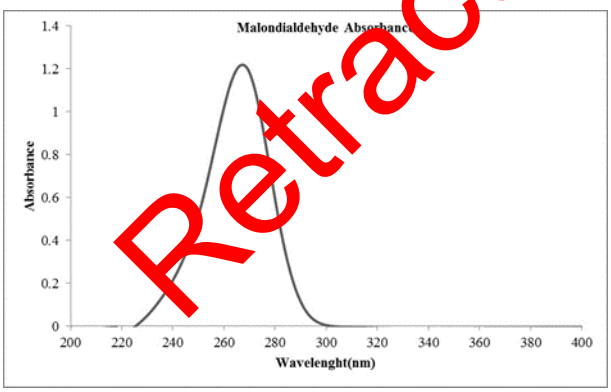

Fig. 2. Wavelength of malondialdhydetetrabutyl ammonium salt diluted in absolute ethanol

The process of derivatisation

First, seven clean glass test tubes were taken and labeled them (A-G). Second, different concentrations of MDA standard stock solutions were prepared (50, 25, 10.5, 5, 2.5, 1, and 0.5 µM) and diluted by absolute ethanol. Afterwards, two ml of prepared MDA solution was transferred to solution was protected from the light and was stored at room temperature for a daily basis. The maximum absorbance was at 273 nm (Figure.1).

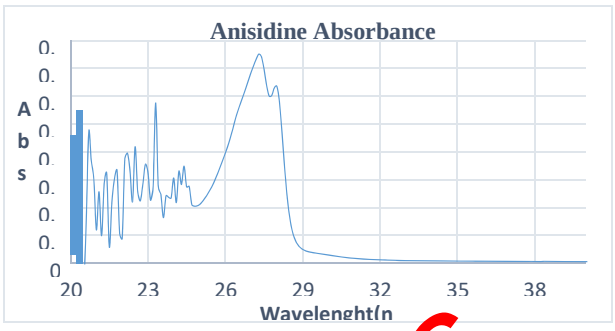

Fig. 1. Wavelength of anisidine diluted in glacial acetic acid

Anisidine was fully tested on room temperature in the dark place with two different concentrations (500 and 1000 µM) to investigate its stability in long period. Anisidine stability had a constant trend within 30 days.

2.4. Preparation of MDA stock solution

Standard stock solution of MDA (5000 $\mu M.L^{-1}$) was obtained by dissolving 0.0156 g

each labeled glass tubes .Then, two ml of anisidine (500 µM) as a color reagent was added to them and vortex the solution. Finally, the tubes were closed and placed them to any holder to keep the tubes upright during boiling at 90^{0}C. After 10 min, immediately, the tubes were removed and were placed in ice bath to stop the reaction. At least, 15 min was needed to be cooled down. Without any extraction steps, two ml of the clear yellowish complex of each glass tubes were loaded to absorption cell for reading the absorbance at 400-404 nm by UV-Vis.

Investigation of concentration, time, and temperature

Various concentrations of MDA standards (50, 12.5, 3.125 $\mu M.L^{-1}$) and anisidine reagent (30, 50, 100, 150, 250, 500 $\mu M.L^{-1}$) were prepared by further dilution of their stock solutions. After preparing anisidine reagent in glacial acetic acid, the solution should be protected from the light within the procedure. For activating the color, derivatization was performed at different reaction times (5, 10, 20, 30, and 40 min) and at different temperature (30, 70, and 90^{0}C) to assess the optimum conditions of MDA derivatization.

Process of complex (MDA-PMA) formation

An appropriate volumes and concentrations of anisidine, optimum temperature, and time were the significant parts of this study. In fact, in order to explore the colorimetric reaction between MDA and PMA, several tests with various concentrations of each have been done. Briefly, by our research work, various concentrations of MDA standards (50, 12/5, 3/125 µM) and anisidine reagent (150, 250, 500 µM) were prepared by further dilution of the stock solutions. Afterwards, 2 milliliter of prepared MDA solution (50) was transferred to a labeled glass tube (Complex MDA-PMA) .Then, two ml of anisidine (500 µM) as a color reagent was added to the labeled tube (Complex MDA-PMA) and vortex the solution. Finally, the tube was closed and placed them to any holder to keep the tubes upright during boiling at 90 ^{0}C, 70^{0}C, and 30^{0}C After 10 min, immediately, the tube were removed and placed in ice bath to stop reaction. At least, 10 min was needed to be cooled down. The optimum condition provided for stable yellowish color adduct (complex A) was considered as follows: (Time:20 min, Temperature75^{0}C, anisidine concentration 500 µM).

Synthesis of MDA-PMA adduct

The product (MDA-PMA) is not commercially available, but it can be prepared in laboratory condition. Formation of imine compound was necessary to evaluate the reaction and confirmation of MDA-PMA adduct. The derivatisation procedure is based on the principle of (0.5g) of MDA reacting with (0.4g) of anisidine prepared with glacial acetic acid at room temperature.

It was found that an imine compound is produced after a few seconds. Then, yellow-orange product was dried at 40 ^{0}C for 4 h in a vacuum drying oven. The structure of this compound was confirmed by different methods namely ^{1}HNMR, ^{13}CNMR, and FT-IR.

Effect of pH

The pH effect of the derivatisation reaction on the MDA value was investigated by previous scientists [3]. Although this new procedure was confirmed at pH= 7.4, the validity of the derivative method has been questioned in various pH (acidic or basic medium). In other words, the complete reaction should be evaluated in different pH for the future biological studies. For this reason, the pH ranges (3.2, 7.4, and 12.0) have been selected.

In normal pH (p H= 7.4), two ml of MDA (50 µM) was added to two ml of anisidine (500µM) in a 10 ml lab tube. Then, this reaction mixture was placed in the water bath for 10 min at 90^{0}C. The solution was cooled and ready for the analysis by UV/Vis spectrophotometer at 400-404nm.

In the basic medium, 200 µl of 6 M NaOH was added to 1000 µl of MDA in a 10ml lab tube. By placing this mixture at 60 ^{0} C in the water bath for 30 min, allow medium to be basic. After cooling, 200 µl of anisidine reagent (500µM) was added to this basic solution. The same as above, the reaction mixture was placed in water bath for 10 min at 90^{0}C. The solution was cooled and ready for the analysis by UV/Vis spectrophotometer at 400-404nm.

In the acidic medium, 250 µl of $HClO_4$ (0.1 M) diluted in glacial acetic acid was added to 1000 µl of MDA (50 µM) in a 10ml lab tube and vortex. Then, 200 µl of anisidine reagent (500µM) was added to the tube and finally this mixture was placed in water bath for 10 min at 90^{0}C. The solution was cooled and ready for the analysis by spectrophotometer at 400 nm.

Techniques

Possible structure of the formed adduct (complex MDA-PMA) under above condition was confirmed by UV-Vis spectrophotometry, FT-IR, ^{1}HNMR, and ^{13}CNMR.

Spectrophotometric analysis

After cooling in icy bath to stop the reaction, without any extraction steps, the solution was analyzed by spectrophotometer using a Lambda Bio 20 spectrometer (Perkin-Elmer, Rotkreuz, Switzerland) using 1-cm absorption cell. Spectrum of MDA-PMA solutions was recorded from 200 to 600 nm at a scanning speed of one nm/min against of the glacial acetic acid as a blank reaction mixture. Absorbance reading was done at 404nm. Data acquisition and processing were carried out with the Perkin-Elmer UV Winlab software.

FT-IR analysis

The infrared (IR) spectra of the samples were recorded in the range 400–4000 cm^{-1} on a Fourier transform infrared (FTIR) spectrometer (model Perkin-Elmer, Rotkreuz, Switzerland) using KBr pellet method.

Nuclear magnetic resonance spectroscopy

^{1}H, ^{13}C NMR spectra were performed on a 250 MHz Brüker (Germany) in deuterated chloroform for both experiments at room temperature to find whether the imine was formed or not.

RESULTS AND DISCUSSION

Influence of time in derivative reaction

The yellow complex under 30 ^{o}C cannot be produced, although time and concentration of anisidine equal to 500 µM have been raised. However, at 90 and 75^{o}C, reaction accelerates in short time (10min); this vital point can help us to consider the reaction of Imine formation or the same yellow complex in the real biological samples in order to determine MDA. As can be seen in figure 4, at 75^{o}C, formation of yellow complex indicates a complete reaction of MDA with PMA.

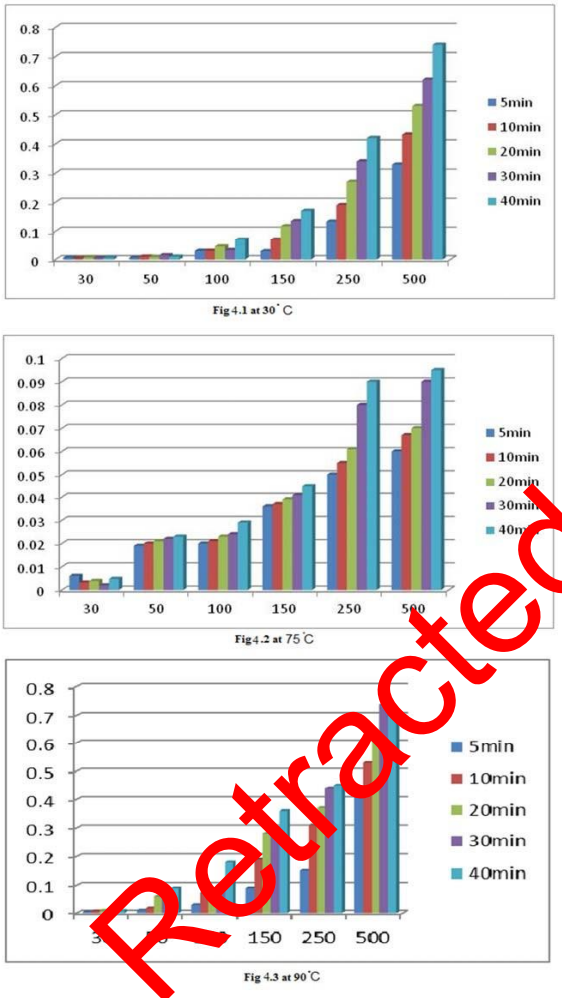

Fig.4. Formation of yellow complex at Different time (5, 10,20,30,40 min) and different temperature (30, 75,90^{0}C)

According to Figs. 4.1, 4.2, 4.3**,** the temperature of 75^{0}C indicates the complete derivatization and the formation of the colorimetric reaction. Moreover, at 90 ^{0}C, the reaction was carried out in a short time. However, at high level of temperature, the aldehyde levels may be raised in the real samples from the actual results which makes it difficult to evaluate the data [5].Therefore, to implement this method in biological samples, using 75 ^{0}C as an optimal temperature can be suited for the analysis. Furthermore, to obtain complete derivative reaction with anisidine (500µM) at 75 ^{0}C, 20 min would be a proper time. Actually, by using 250 µM or lower concentrations of anisidine, the complex (MDA-PMA) was remained at an intermediate state and the yellowish complex will not be formed and its result will not be suitable for the real samples. Therefore, the reaction is completed by anisidine (500 µM) with optimized temperature at 75^{0}C within 20 min. (Figure 5)

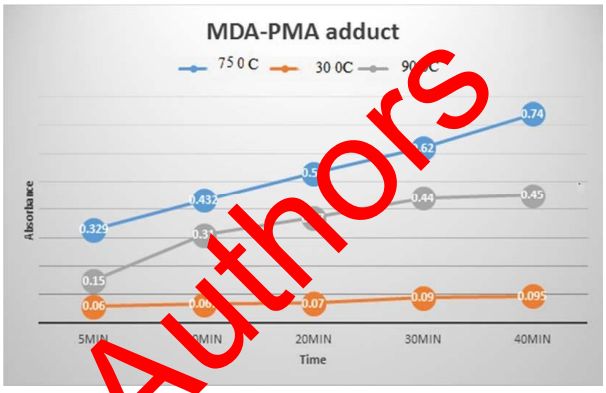

Fig. 5. The relationship between temperature and time in complex (MDA-PMA) formation (anisidine = 500 µM)

On the other hand, TFEH, and PFH as derivative reagents require harsh temperature to produce complex [6].Temperature effect on derivative procedure was considered as an important part in this research; therefore, the samples placed in a water bath at 30^{0}C, 75^{0}C and 90^{0}C and room temperature, then, cooled in icy bath.

To evaluate the optimum condition for formation of the complex [MDA-PMA], different concentrations of anisidine (30, 50, 100, 150, 250, 500 µM) was added to different concentrations of MDA (3.125, 12.5 and 50 µM) in different times (5, 10, 20, 30 and 40) and in separate runs.

The results of Fig. 5 indicate that yellow adduct was not formed at 30^{0}C and room temperature. Optimal temperature and time as the main part of derivation process were considered in this study. According to Fig.5, under low temperature (30^{0}C), the reaction was slow and proceeded to produce an intermediate compounds. Even, by increasing in the molar concentration of anisidine and time, it produces higher concentration of intermediate compounds; this indicates that derivative adduct could not be formed in this concentration and temperature. However, when the concentration of anisidine is 10 times higher than MDA concentration, the yellow color will reduce the form of intermediate compounds and enhance the form of complex.

It seems that the procedure was completed at 90^0C in 10 min which contribute to form the imine reaction or yellow adduct in this temperature while this should be considered that high temperature is not proper for this procedure, due to evaporation of malondialdehyde and solvent during derivative process, so this may be difficult to maintain the derivative concentrations.

On the other hand, at 30°C, there is no yellowish complex; therefore, it is not a proper temperature to assess the complex formation. Nevertheless, at 75°C, the reaction was completed in 40 min and the concentration of anisidine selected at 500 µm while Mao's method reaction time was 3 hours with FMOC reagent. [17]

As can be seen in Figure 6, at the wavelength of 300-380 nm, the wide peak is related to intermediate formation whereas in Figure 7, relatively high peak in the range of 400-404 nm indicates reduced concentration of intermediate and formation of adduct or the same considered complex.

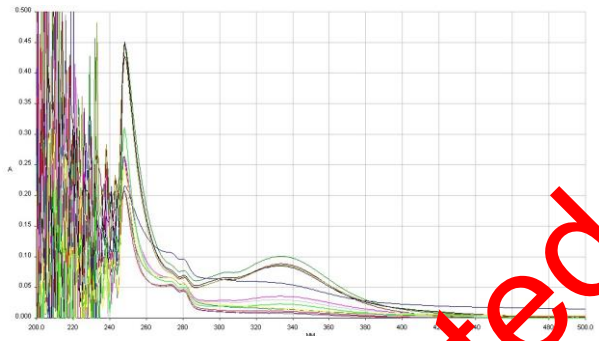

Fig. 6. Different concentration of anisidine with their absorbance spectra of derivative complex at 30 ^{0}C in complete reaction (Complete reaction)

As can be seen in Figure.7, this colorant has a strong absorbance peak in visible range in 400 nm related to electronic transference $\pi \rightarrow \pi^*$. The absorbance peaks in UV region is related to electron transference of benzene rings. [10] The obtained peak in visible range in the range of 300 to 395 is related to the formation of intermediates. The peak value decreases by high temperature. Likewise, by altering anisidine concentration, the adduct peak value increases.

Optimal condition for derivative procedure

The reaction of anisidine (para - methoxy aniline) with aldehyde groups can be considered as an addition reaction. The yellowish complex emerged from releasing H_2O molecules by which it presents a stable adduct. Investigating the reaction depended on MDA concentration indicated that the more molar concentration of anisidine, the more increase in the concentration of yellow complex (MDA-PMA). Derivatized adduct was evaluated with the molar ratios of 1/2:1 to 1:10 (MDA: Anisidine). Figure.4 and Figure.5 depict when the molar ratio of Anisidine concentration is (10[MDA]), the derivative solution attains to a higher level; thus, the derivatisation was completed at the molar ratio of 10:1.

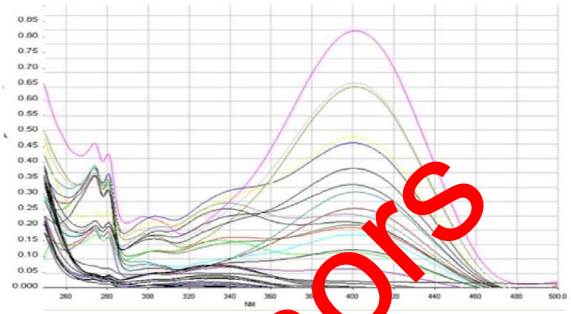

Fig.7. Different concentration of anisidine with their absorbance spectra of derivative complex at 90 ^{0}C in complete reaction (Complete reaction).

Stability

Current method is significantly different from Mao J, Zheng H, et al. [17]. Their method was time consuming procedure (4 hour) at 50 ^{0}C to reach near completion reaction [17]. In order to prevent false results, stability of malondialdehyde and anisidine as a crucial methodological aspect of this study was evaluated by UV-Vis spectrophotometry.

The absorbance reading of PMA (1000 µM and 500 µM) and MDA (stock solution (5000 µM) and its working standard solution (50 µM)) was done at 273 nm and 267 nm, respectively. It is clearly observed in Figure.8.1 that the solution of PMA was stable if it is protected from light.

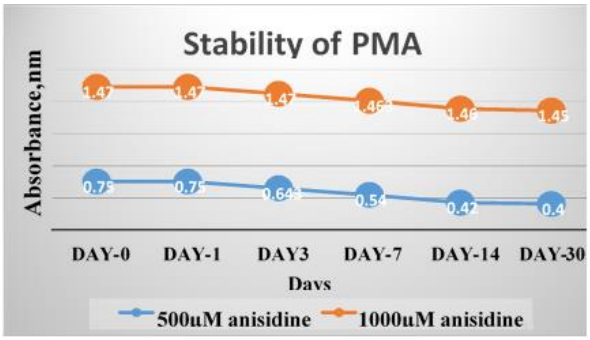

Fig. 8.1. Evaluation of PMA stability.

Nevertheless, it would be better to prepare 500 µM concentration of PMA at the time of the test. With regard to MDA stock solution stability, Figure.8.2depicts the satisfactory results of 5000 µM concentration of MDA. However, working standard solution did not prove to be stable over 30-days period. Thus, it is only possible to use stock solution to prepare working standard solution at the

time of test because MDA become degradation less than 24 hour.

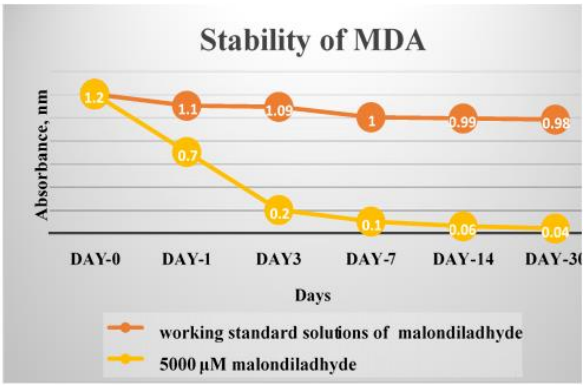

Fig. 8.2. Evaluation of MDA stability.

On the other hand, FMOC-hydrazine and DNPH (as derivative reagents) increased the derivative reaction under mild acidic conditions and lower temperatures, because of having identical hydrazine groups, which are strong nucleophiles and can readily react with aldehydes. These conditions prevent undesired artificial aldehyde formation generated during the sample pretreatment. The derivative reagents such as DNPH need several Liquid-Liquid extractions due to the excess of unreached DNPH and thus separation of the MDA-DNPH will be difficult. Moreover, because of DNPH instability, purification of DNPH should be performed on a daily basis. [17, 12]

Consequently, based on above aforementioned results, this method did not require multiple extraction steps for anisidine reagent and thus, no residue or cloudiness was appeared during and after derivative procedure, which made this procedure preferable over the other methods. Furthermore, the yellowish adduct has no hazardous material if keep it for a long time in a laboratory. A feature which is crucial in developing a precise and accurate quantitative method is that PMA was stable for at least 1 month at room temperature if it is protected from light whereas FMOC-hydrazone (as a reagent) is only stable for 72 h at room temperature [17,12].

The effect of pH

The influence of pH in this procedure was examined. As can be seen in Figures 9.1 and 9.2,derivative complex at different pH (3.0, 7.4, 12.2) were formed after 10 min at 90 ^{0}C. The maximum yield was achieved at 400 nm in each pH. The results may contribute to apply this procedure for determination of free and total MDA in biological samples at different pH for further studies.

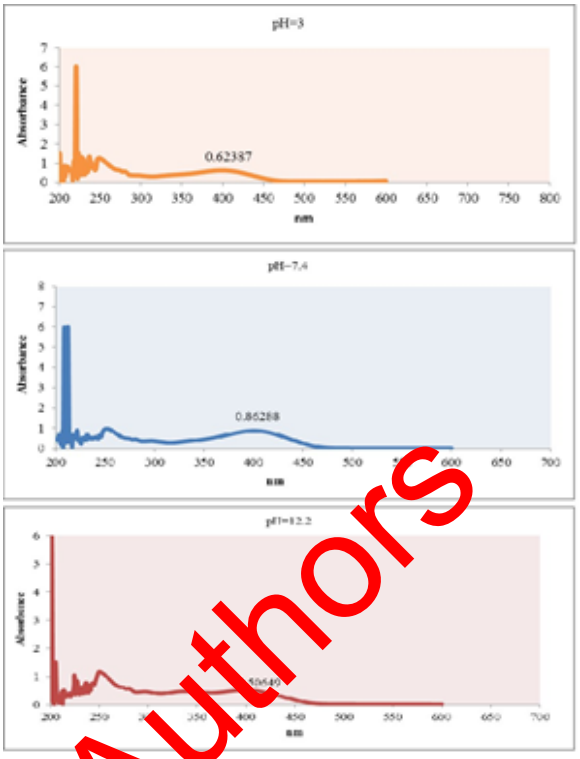

Fig.9.1. Effect of pH on complex formation

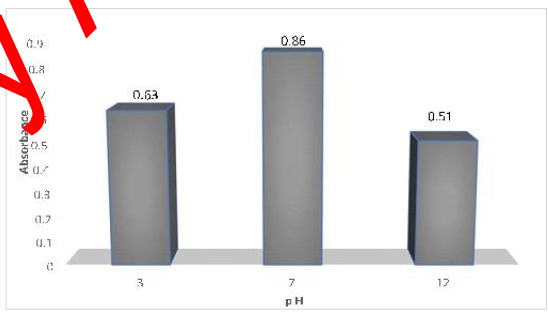

Fig. 9.2. Evaluation of different pH for complex formation.

Mechanism of imine formation

The formation of the imine is the initial step in this reaction. The imine was formed after reacting an amine (1^{0}) with aldehydes to form a dipolar intermediate. The reaction has two steps:

1-NH_2 preferentially approach to carbonyl groups of aldehydes

2- Elimination of water group.

In current study, imine formed during the synthesis procedure identified by ^{1}HNMR, ^{13}CNMR and FT-IR techniques were confirmed the adduct. The possible mechanism of MDA derivatization was depicted in Figure.10.

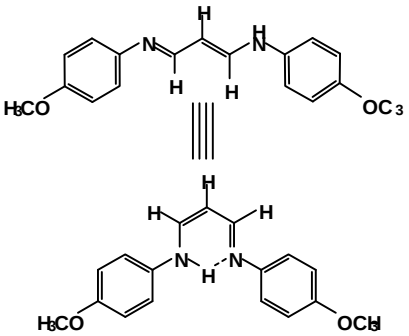

Fig. 10. The mechanism of MDA-PMA adduct.

The results of UV-Vis, FT-IR, and NMR

Spectrophotometric results

MDA and other carbonyl compounds naturally exist as byproducts of lipid peroxidation and prostaglandin biosynthesis. With regard to analytical approaches, it is better to derivatize MDA with reagents having high molar absorptivity at longer UV wavelengths (>254 nm). In fact, the high molar absorptivity and the close proximity of the absorbance maximum to ~ 300nm make a MDA derivative almost preferably suited for detection using a diode array system [9]. (Figure.11)

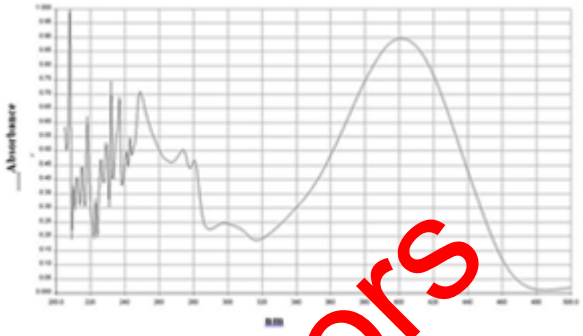

Fig. 11. A final spectrum of yellow adduct at 75^{0}C (50 µM MDA- 500 µM PMA)

FT-IR results

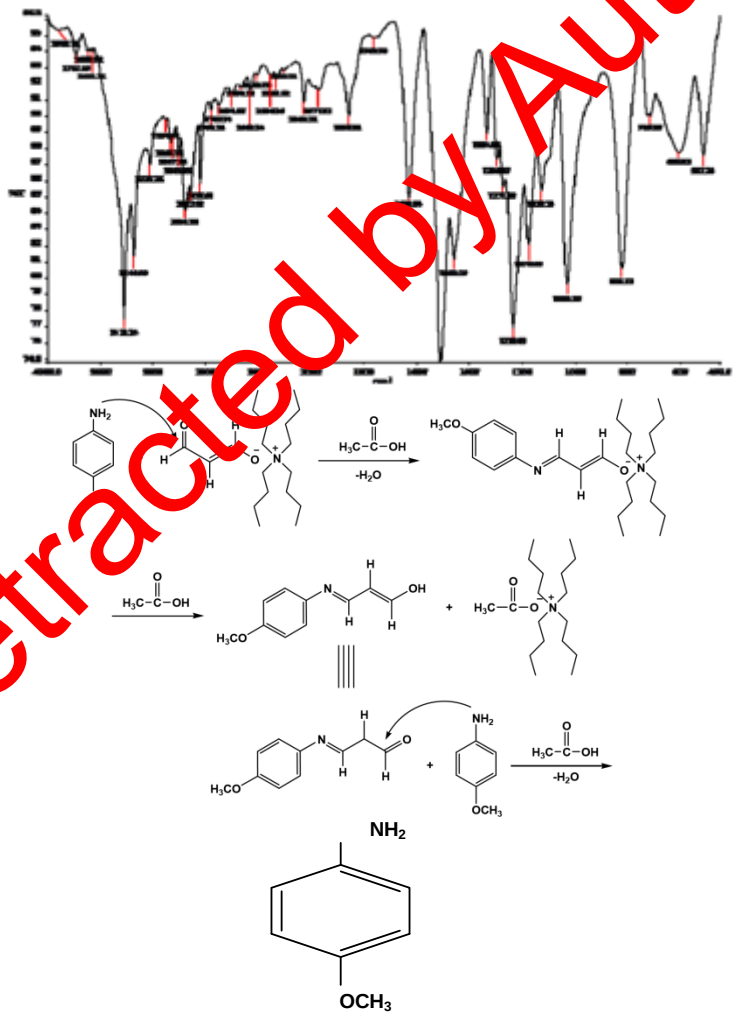

Fig. 12.1. IR-spectra of anisidine.

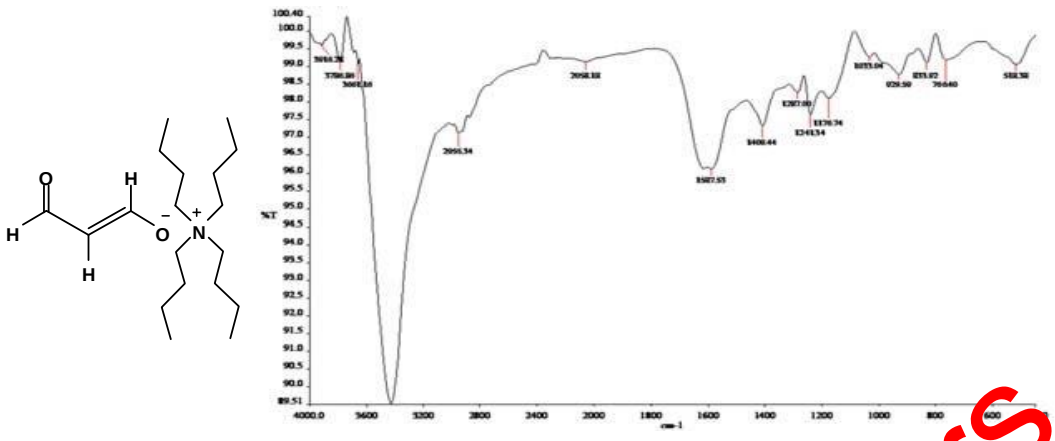

Fig. 12.2. IR spectrum of malondialdehydetetrabutyl ammonium salt molecule

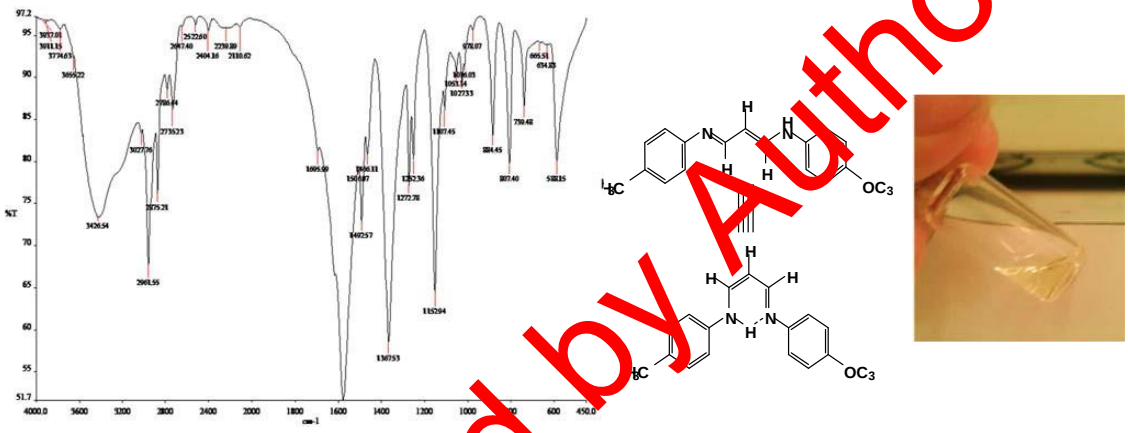

Fig. 12.3. IR spectrum of MDA-PMA and the proposed synthetic molecule

Table. 1. FT-IR results of MDA-PMA adduct

C-H = 2850 cm^{-1}	(NH group) = 3220 cm^{-1}
C-N (amine group) = 12[illegible] cm^{-1}	CH_3 (bending vibration) = 1287cm^{-1}
C-O (enole form) = [illegible]76 cm^{-1}	C=C (α, β unsaturated) = 1587 cm^{-1}
C=N (imide) = 16[illegible] cm^{-1}	N-H = 1409 cm^{-1}

The FT-IR spectrums of MDA-derivatisation, primary anisidine and malondialdehyde were confirmed in the reaction. IR spectra were different from spectra of MDA before and after derivative reaction. The important IR bands of the compounds along with their assignments are given in Figures 12.1, 12.2, and 12.3.

Analytical data. Important infrared spectral (cm^{-1}) bands of anisidine. Figure 12.1: IR-spectra of anisidine demonstrate the N-H tension bond appears close 3346 and 3232cm^{-1}.Another band at 3103 cm^{-1} was proved the aromatic CH tension. Furthermore, signals between 2838 cm^{-1} correspondent to the C-H aliphatic. Moreover, Band at 1510 shows the C=C and signals at 1334 and 1459 cm^{-1} depict the tension band of the CH_3 and N-H, respectively. The stretching vibration of the C-N and C-O band for the methoxy group were appeared at 1235 cm^{-1} and 1030, respectively.

Important infrared spectral (cm^{-1}) bands of malondialdhyde. Figure 12.2: IR-spectra of MDA show the C-N stretching band (calculated at 1272 cm^{-1}) related to the amine bands. The C-O stretching enol form band is clarified at 1152 cm^{-1} . The strong peak exhibits the aldehyde form of C=O at 1695 cm^{-1}. The enol form of C=C was verified by the strong band at 1580 cm^{-1} . Besides, the CH_3 and CH_2 bending at 1367 and 1492 $cm^{-1}$are roughly visible.

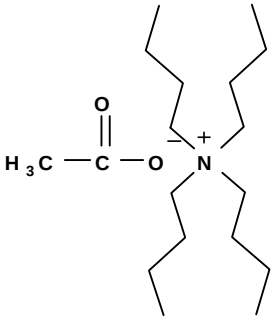

Fig. 13. The byproduct of MDA-PMA reaction.

IR spectrum of adduct. Figure 12.3: IR-spectra of adduct shows Tension signals were eliminated at 2786 and 2735 cm^{-1} (C-H) due to the reaction between anisidine and aldehyde group. Another band at 2955 cm^{-1} appeared as a result of *α,β*-unsaturated conjugated imides in <2955 cm^{-1} .

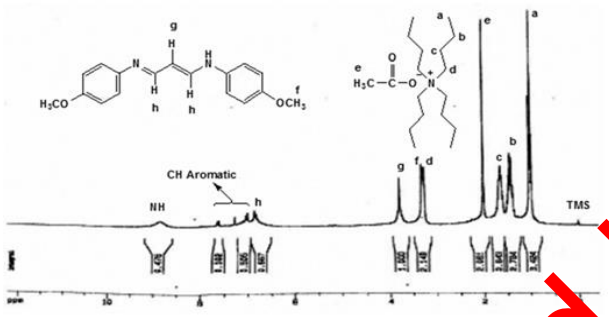

Fig.14.1:^{1}H NMR spectra of the adduct (MDA-PMA)

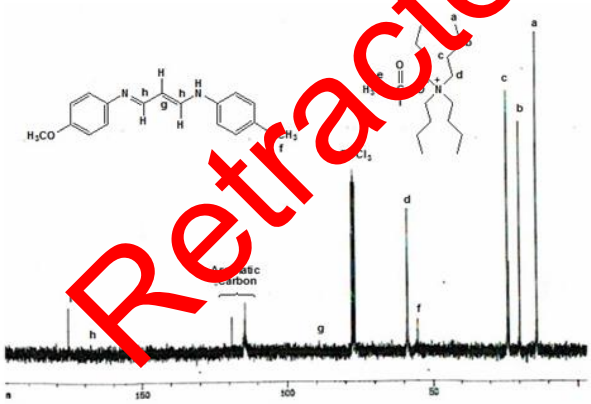

Fig.14-2.^{13}C NMR spectra of main production with glacial acid acetic and tetra butyl ammonium salt.

The new adduct being formed in acetic acid medium is conjugated imine aromatic. Not only is acetic acid as a catalyst for the reaction of amine and MDA but also make tetra-butyl amine separate from MDA and provide possible reaction for MDA and anisidine again.

The important infrared spectral (cm^{-1}) bands of MDA-PMA are considered as follows.

Ammonium ion peak as a byproduct was appeared in a strong state at 3450 cm^{-1} .This is the byproduct which has not been fully separated completely **(**Figure 13).

^{1}H NMR and ^{13}C NMR results

The structure of adduct (MDA-PMA) is confirmed by the ^{1}H-NMR and ^{13}C-NMR. The protons of NH group in $CDCL_3$ are shown in 9.8 ppm **(**Figure 14).

Acknowledgements: *The authors would like to express their sincere appreciation to Dr. Amir Ahmadi for her suggestions during this study.*

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