9,10-Phenanthrenequinone as a Mass-Tagging Reagent for Ultra-Sensitive Liquid Chromatography Tandem Mass Spectrometry Assay of Aliphatic Aldehydes in Human Serum

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Abstract

9,10-Phenanthrenequinone (PQ) was successfully used as a new mass-tagging reagent for sensitively labeling aliphatic aldehydes (C_3-C_{10}) prior liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). This reagent could overcome the drawbacks of previous amine or hydrazine-based reagents, such as lower sensitivity, formation of two stereoisomeric reaction products for each single analyte, need for longer derivatization time, and poor reactivity with aliphatic aldehydes. The PQ-aldehyde derivatives exhibited intense $[M+H]^+$ and a common product ion with ESI in the positive-ion mode. The derivatives were monitored at the transition of $[M+H]^+ \rightarrow m/z$ 231.9 with detection limits from 4.0 to 100 pM (signal to noise ratio = 3). 3-Phenylpropanal was used as an internal standard (IS) and the separation of the eight aldehydes and IS was achieved in less than 10 min employing gradient elution with methanol and ammonium formate buffer (20 mM, pH 4.0). The method employed salting out liquid-liquid extraction for aliphatic aldehydes form serum for the first time with excellent recoveries (92.6-110.8%). The developed method was validated and applied for quantification of the target aldehydes in serum of healthy volunteers (*n=14*).

Keywords: 9,10-Phenanthrenequinone; LC/ESI-MS/MS; aliphatic aldehydes; human serum.

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1. Introduction

Lipid peroxidation (LPO) has a prominent effects on many diseases like diabetes mellitus, atherosclerosis, and heart disease.[1–4] The *in vivo* LPO process is associated with degenerative free radical propagation reactions which are involved in the production of many carbonyl compounds. Among these compounds, small aliphatic aldehydes are considered main products of LPO.[5] Aldehydes are relatively more stable than free radicals and can spread inside or out of the cell attacking sites far from the original place of free radical-initiated actions.[6] Besides LPO, aldehydes can be produced endogenously from Maillard reaction, amine oxidases, or metabolic activations[4]. Damage caused by aldehydes in biological systems can disturb the function of proteins and enzymes, and lead to the formation of harmful DNA adducts. Thus, aldehydes are considered as key parameters for monitoring LPO, advanced glycation end products and activity of amine oxidases[4].

Elevated levels of hexanal, nonanal, and decanal in plasma of haemodialysis patients compared to control group suggested the contribution of these aldehydes to the intoxication caused by increased LPO in these patients. *n*-Alkanals have higher affinity for amino group than other types of aldehydes, which explains the highest negative correlation found only between the residual amino groups and total *n*-alkanals [7].

Additionally, propanal, butanal, pentanal, hexanal and nonanal have been proven to have cytoxic effect on rat and human hepatocytes, which was increased by increasing the length of carbon chain. However, these *n*-alkanals have intrinsic genotoxic activity on rat hepatocytes only, with no effect on human hepatocytes [8].

Aldehydes have been analyzed in different matrices by HPLC, mostly with UV or fluorescence (FL) detection, following derivatization. The most widely used derivatizing agents for aldehydes are those based on hydrazine nucleus including 2,4,6-trichlorophenylhydrazine(TCPH),[9] *N*-methyl-4-hydrazino-7-nitrobenzo-furazan(MNBDH),[10] 2,4-dinitrophenylhydrazine (DNPH)[11–16], dansylhydrazine (DNSH),[17] 4-(*N*,*N*-dimethylaminosulphony)-7-hydrazino-2,1,3 benzoxadiazole (DBD-H),[18] and 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-s-indacene (BODIPY-aminozide).[19] Some hydrazine-based reagents have also been used in combination with mass spectrometric (MS) detection such as DNPH[20,21], and DNSH.[22] Despite the large number of analytical methods depend on them; hydrazine-based reagents have some drawbacks such as probable interferences from other carbonyl compounds *e.g*. ketones and small carboxylic acids as well as the formation of stereoisomeric hydrazone derivatives as reaction products that may cause analytical errors. [23]

Some other reagents that are not based on hydrazine nucleus were utilized for aldehyde derivatization such as *O*-2,3,4,5,6-(penta-fluorobenzyl)hydroxylamine (PFBHA)[24,25], 2,3-diaminobenzene [26], 1,2-

diaminoanthraquinone[27], and 1,3-cyclohexanedione (CHD)[28,29]. Although these methods are suited for certain applications, inferior selectivity[24,25], need for relatively longer derivatization time[26,28], poor reactivity with aliphatic aldehydes[27], and poor sensitivity [28,29] may be weakness points. Interestingly, 1,2-di(2-furyl)-1,2-ethanedione (2,2`-furil) has been used in our laboratory as a safe, stable, and sensitive fluorogenic derivatizing agent for aliphatic aldehydes.[30,31]

Additionally, some reagents have been used for derivatization of aldehydes to improve their ionization characters prior MS detection *e.g.* 4-(2-(trimethylammonio)ethoxy)benzenaminium halide (4-APC),[32] 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benzenaminium dibromide,[33] CHD-based reagents,[34,35] and d_0/d_3-4-(1-methyl-1*H*-phenanthro[9,10-d]imidazol-2-yl) phenlamine.[36] Though, these methods have some limitations such as long reaction time of 3 hours [32–36], lower sensitivity [32–35], and poor selectivity [36].

As mentioned earlier, we recently reported a novel *α,β*-diketo derivatizing agent, 2,2`-furil, for quantification of aldehydes in biological fluids with high selectivity by HPLC-FL detection.[30,31] The inspiring chemical and analytical characteristics of the formed imidazole derivatives motivated us to utilize this reaction as a model to design a LC/ESI-MS/MS method with high sensitivity for the screening of aliphatic aldehydes (C_3-C_{10}) in human serum. In this study, several *α,β*-diketo reagents were evaluated to select the most proper one for

aldehyde derivatization. 9,10-Phenanthrenequinone (PQ, Fig. 1) was our reagent of choice that enables highly sensitive MS detection of the aldehydes in the sub-nanomolar levels. A study on the screening of C_3-C_{10} aldehydes in human serum was conducted demonstrating the applicability of the method in biological samples. The reaction of PQ with aldehydes has been used by Bai *et al.* [37]for determination of some aldehydes in beverage by non-aqueous capillary electrophoresis-diode array detection. Kishikawa *et al.* has been applied this reaction in a reverse way using benzaldehyde or 4-carbomethoxybenzaldehyde as derivatizing agents for determination of PQ by HPLC-FL [38,39]. Yet, the present study is the first report to use PQ as a mass-tagging reagent for the determination of aldehydes proving novelty of the developed method.

2. Experimental

2.1. Chemicals and reagents

Analytical grade reagents were used in this study and solvents were of LC/MS grade. PQ, 2,2'-pyridil, 4,4'-dimethoxybenzil (*p*-anisil), 1,10-phenanthroline-5,6-dione (PAD), ammonium acetate, and nonanal were obtained from Sigma Aldrich (St. Louis, MO, USA). Butanal, pentanal, hexanal, heptanal, octanal, decanal, glacial acetic acid, formic acid, and ultrapure water (LC/MS grade) were obtained from Wako Pure Chem. Ind. (Osaka, Japan). Propanal, benzil, and sodium chloride were from Nacalai Tesque INC. (Kyoto, Japan). Methanol and acetonitrile (LC/MS grade) were from Kanto Chem. Co. (Tokyo,

Japan). 3-Phenylpropanal (IS) and 2,2'-furil were purchased from Tokyo Chem. Ind. (Tokyo), while ammonium formate was obtained from Kishida Chem. Co. (Osaka). 4-Dimethylaminobenzil (DMAB) was synthesized in our laboratory in two steps; first we synthesized 4-dimethylaminobenzoin as reported by Levi and Hauser [40] then it was converted to DMAB using Fehling's solution. [41]

Stock solutions of the target aliphatic aldehydes (10.0 mM) were individually prepared in acetonitrile. Accurate volumes from the stock solutions of the eight aldehydes were mixed together and diluted with acetonitrile to obtain a mixed standard solution containing 4.0 µM of each aldehyde. Dilution of this solution was made as needed to obtain the required concentration levels. 3-Phenylpropanal (IS) solution (4.0 µM) was also prepared in acetonitrile. PQ solution (4.0 mM) was prepared in acetonitrile and ammonium acetate (2.0 M) was prepared in glacial acetic acid. The mixed standard solution was stored at -30 °C, while all other solutions were stored at 4 °C in the refrigerator.

2.2. Clinical samples

Serum samples from 14 healthy human volunteers (10 males and 4 females, mean age of 38.4 ± 13.1 years) were provided by Sasebo Chuo Hospital (Nagasaki, Japan). Serum samples were kept at -80 °C and gently thawed before analysis. All conducted studies were approved by the Ethics Committee of the School of Pharmaceutical Sciences, Nagasaki University, Japan.

2.3. Instrumentation and LC/ESI-MS/MS conditions

A Waters 2695 separation module (Waters Co., Milford, MA, USA) was employed for the separation of the studied aldehydes with a Cosmosil 3C_{18}-AR-II column (100 mm x 4.6 mm i.d., 3 µm particle size) from Nacalai Tesque INC. operated at 25 °C. Gradient elution was employed with a mobile-phase mixture of methanol (A) and ammonium formate buffer (20 mM, pH 4.0) (B). For the first 3 min of the run, the percentage of A was increased linearly from 70% to 100%. For the next 7 min the proportion of A was maintained at 100%. The initial composition of the mobile phase was re-established for 5 min before starting a new run. The mobile phase flow rate was set at 0.5 mL/min. MS analysis was accomplished by a Quattro micro™ triple-quadrupole mass spectrometer (Waters Co.) fitted with an electrospray ionization source. Acquisition of positive-ion ESI–MS data was done at a capillary voltage of 5 kV, source temperature of 120 °C, desolvation gas temperature of 350 °C and flow rate of 500 L/h, and cone gas flow rate of 40 L/h. Table 1 illustrates the cone voltage, collision voltage, precursor and product ions for the studied aldehydes (C_3-C_{10}). The PQ-aldehyde derivatives were detected using the multiple reaction monitoring (MRM) mode. This strategy was considered to quantify a specific product ion of *m/z* value of 231.9 by collision induced dissociation (CID). This product ion was assigned to 2-methyl-1*H*-phenanthro[9,10-*d*]imidazole moiety resulted from the cleavage of the PQ-aldehyde derivative (Fig. 1).

Extraction of serum samples was done with the assist of Himac CR 15 refrigerated centrifuge (Hitachi Koki Co., Ltd., Tokyo). Yamato HF-41 heating block (Tokyo) was used in the derivatization process. Horiba F22 pH-meter was used to adjust the pH of the buffer.

2.4. Derivatization procedure

Aliquots of 50 µL PQ (4.0 mM) and 100 µL of ammonium acetate (2.0 M) were mixed with 150 µL of aldehydes standard solution in a screw-capped vial, then heated for 30 min at 100 °C. After cooling, the solution was filtered with cellulose acetate membrane filter (0.45 µm) and 20 µL of the samples were injected to the LC/ESI-MS/MS for analysis *via* the auto injector. A reagent blank was prepared simultaneously.

2.5. Serum samples treatment by salting out liquid-liquid extraction

A volume of 200 µL of serum was mixed with 10 µL IS, 390 µL acetonitrile, and 0.2 g NaCl followed by centrifugation for 5 min at 6000 rpm. 150 µL of the upper phase was transferred to a screw-capped vial and the derivatization procedure was performed in the same way as for the standard solution.

2.6. Validation procedure

The Guidance for Industry on Bioanalytical Method Validation (Food and Drug Administration, FDA)[42] was followed for method validation. The validation procedure included determination of calibration curves, limit of quantification (LOQ), accuracy, precision, recovery, and stability study.

Calibration curves were generated for the eight studied aldehydes (C_3-C_{10}) by spiking serum samples at six concentration levels (including the LOQ) for each aldehyde and plotting the relative peak area (peak area of analyte/peak area of IS) *versus* concentration (nM). The LOQ was also determined for the eight aldehydes as the lowest concentration in the calibration curves which can be determined with satisfactory accuracy (80-120%) and precision (%relative standard deviation %RSD of ≤20%).[42] In addition, the limit of detection (LOD) was calculated as the concentration corresponding to signal to noise ratio (S/N) = 3.

The method was also evaluated regarding accuracy and precision by replicate analyses of serum samples spiked with the analytes at three concentrations within the working linearity ranges (0.5, 5.0, and 50 nM for each aldehyde). Each concentration was measured five times and the average was calculated. The difference of the average from the exact value expresses the accuracy. The intra-day precision was calculated as the %RSD for the peak areas of five injections *per* concentration within one day, and the inter-day precision was also measured as the %RSD for the peak areas of replicate determinations on five successive days at each concentration.[42]

Recovery studies were conducted to evaluate the efficiency of the extraction method. The recovery was calculated for the studied aldehydes at three concentration levels for each aldehyde (0.5, 5.0, and 50.0 nM) according to the following equation:

$$\%\text{Recovery} = \frac{\text{relative peak area of aldehyde extracted from serum samples}}{\text{relative peak area of aldehyde in un-extracted standard solution}} \text{x}100$$

Evaluation of the stability of the aldehydes and the IS solutions was conducted at room temperature for 6 h and at 4 °C for 1 week. Additionally, stability of the mass-tagging reagent PQ was tested at 4 °C up to 2 months. Also, the stability of the derivatized aldehydes and IS was determined over the anticipated resident time in the auto sampler, *i.e.* up to 48 h at 4 °C. All of the stability studies were performed by comparison of the results obtained for old solutions with those for newly prepared ones.

3. Results and Discussion

3.1. Preliminary screening for candidate derivatizing agents

Aldehydes are small hydrophilic molecules cannot be retained or separated on common LC columns resulting in weak or no signals at the MS detector. Chemical derivatization has been long applied to improve stability, MS detectability, and chromatographic behavior of aldehydes. As we previously reported,[30,31] the *α,β*-diketo compounds react with aldehydes in the presence of ammonium acetate co-reagent in a one-pot reaction at 90-100 °C forming a

stable imidazole derivative (Fig. 1). We initially screened several *α,β*-diketo reagents for selective and sensitive derivatization of the target aldehydes (C_3-C_{10}). The reagents investigated were PQ, PAD, 2,2′-pyridil, benzil, DMAB, *p*-anisil, and 2,2′-furil. We conducted the preliminary screening using positive-ion and negative-ion ESI-MS/MS modes and heptanal as a representative aldehyde (MS conditions for all reagents are summarized in Table S-1 (supplementary information)). The product ion spectra for the reagents-heptanal derivatives are shown in Fig. S-1 (supplementary information). For all of the reagents, the positive-ion mode showed better response than the negative-ion one (Fig. 2). Yet, the 2,2′-pyridil-heptanal derivative showed no distinct precursor ion in the negative-ion mode. The PQ-heptanal derivative has the simplest and clearest product ion spectrum among the tested reagents-heptanal derivatives (Fig. S-1), in addition it afforded the highest sensitivity in the positive-ion mode (Fig. 2). Hence, PQ was selected as the best derivatizing agent for further experiments. It is noteworthy that, all of the PQ derivatized aldehydes gave the corresponding $[M+H]^+$ as well as two common product ions with *m/z* values at 231.9 and 164.8 which were assigned to 2-methyl-1*H*-phenanthro[9,10-d]imidazole and fluorene moieties, respectively, produced by the cleavage of the PQ-aldehyde derivative during the CID (Fig. 1). Although the product ion of *m/z* 164.8 has a higher response (1.3 times) than that of *m/z* 231.9, the later has better S/N. So, the MRM acquisitions were performed at the product ion with *m/z* value of 231.9.

In order to monitor the selectivity of PQ for aldehydes in the presence of their isomers (ketones), we conducted the same reaction using acetone as a representative ketone. Then, we compared the obtained total ions spectrum of propanal (Fig. S-2 A, supplementary information) and that of its ketone isomer (acetone) (Fig. S-2 B, supplementary information). As illustrated in Fig. S-2 A, in addition to the reagent peak at about *m/z* 209, a distinct precursor ion of PQ-propanal derivative is shown at *m/z* 247, while in Fig. S-2 B, only the reagent peak appeared with no any precursor ion corresponding to acetone derivative of PQ. This finding conformed with the results obtained by our group which assured the selectivity of *α,β*-diketo compound to aldehydes in presence of acetone and 2-hydroxyacetophenone [31]. Also, carboxylic acid didn't interfere also. Acetic acid is used as a catalyst for this type of reaction and conducting the reaction in the presence of it without any aldehyde did not produce any products. In Fig. S-2 B where the reaction occur in presence of acetone (non-reactive substance toward our reaction as mentioned previously) and acetic acid (as a catalyst) we couldn't detect a precursor ion at 233 (if the carbonyl and hydroxyl group converted to diamine) or 249 (if only the carbonyl group reacted as in case of aldehydes). This confirmed the selectivity of PQ for aldehydes in presence of ketones and carboxylic acids, which is a very attractive advantage since most of the reported derivatizing reagents for aldehydes lack the selectivity [9–22,24,25,36].

3.2. Selection of the best derivatization conditions

Different derivatization conditions were investigated in order to obtain the highest derivatization efficiency. First, the effect of PQ concentration on the relative peak areas was studied using concentrations ranged from 1.0 to 12.0 mM. Constant response was obtained using 4.0 mM PQ, and further increase of the reagent concentration over this concentration did not affect the relative peak areas (Fig. 3A). Further, the influence of the solvent used for preparation of PQ was evaluated using methanol and acetonitrile. Higher signal was obtained using acetonitrile as a solvent, so 4.0 mM PQ solution in acetonitrile was used in further experiments.

The effect of the concentration of ammonium acetate was also investigated from 0.1 to 4.0 M. The highest relative peak areas were obtained using 1.5 M ammonium acetate then a plateau was reached up to 4.0 M where a decrease in the response was observed (Fig. 3B). Therefore, in this study 2.0 M of ammonium acetate was selected as the best concentration.

The reaction temperature was also studied from 40 to 110 °C, and the best response was obtained at 100 °C (Fig. 3C). Additionally, the derivatization time was investigated from 5 to 60 min. Increasing the reaction time produced a corresponding increase in the analytical response up to 30 min, after which no further increase was obtained indicating that the reaction has completed (Fig. 3D). Hence, the reaction was conducted at 100 °C for 30 min.

3.3. HPLC separation and MS/MS monitoring

The LC/ESI-MS/MS operational conditions were carefully investigated. 3-Phenylpropanal was selected as the IS since its chromatographic behavior (t_R = 7.3 min for IS and 6.3-9.2 min for the eight aldehydes) and extraction efficiency (102% for IS and 92.6-110.8% for the eight aldehydes) are similar to and perfectly matched with the studied aldehydes. Additionally, it is a non-physiological aldehyde with derivatization reactivity approximate to our target analytes. As well, 3-phenylpropanal has the same fragmentation pattern and product ion (*m/z* 231.9) like the studied aldehydes. Full scan product ion spectra of the IS derivative of PQ is shown in Fig. S-3.

The mass parameters including the cone and collision voltages were studied to obtain the maximum response for the eight analytes. Table 1 illustrates the parameters selected for each analyte and the IS yielded the highest sensetivity.

Besides, the mobile phase and the stationary phase were studied carefully to attain the best chromatographic behavior. In initial experiments, a mobile phase composed of 0.1 % formic acid in methanol: 0.1 % formic acid in water (8:2 v/v) was eluted isocratically on a C_{18} column (100 mm x 4.6 mm i.d., 3 µm particle size, from Nacalai Tesque INC.). Under these conditions, the separation of the eight analytes was not possible and there was a strong distortion in the peak shape for the eight aldehydes. Using the same mobile phase with a C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size, from Nacalai Tesque INC.) improved the peak shape of C_3-C_8 to some extent, while no separation could be achieved between C_3-C_7 aldehydes. Isocratic elution with a mobile phase consisted of methanol:

ammonium formate buffer (20 mM, pH 4.0) (9:1 v/v) and a C_{18} column (100 mm x 4.6 mm i.d, 3 µm particle size, from Nacalai Tesque INC.) produced a substantial improvement of the peak shape, yet, propanal and butanal were not efficiently separated. As well, the run time was long (22 min). Consequently, we decided to investigate gradient elution with methanol as solvent A and ammonium formate buffer (20 mM, pH 4.0) as solvent B, using the same column. The initial concentration of solvent A was studied from 60 to 90% and its final concentration was similarly studied from 90 to 100%. The gradient time was also studied from 1 to 5 min. Finally, based on resolution, sensitivity, and run time, we employed the gradient system described under section "2.3." for separation of the eight aldehydes. After these experimental investigations, the target aldehydes were separated within 10 min (propanal was eluted at t_R (mean±SD) = 6.34±0.02 min, butanal at t_R = 6.68±0.03 min, penatanl at t_R = 7.08±0.03 min, hexanal at t_R = 7.47±0.02 min, heptanal at t_R = 7.87±0.03 min, octanal at t_R = 8.28±0.03 min, nonanal at t_R = 8.74±0.03 min, decanal at t_R = 9.25±0.03 min, and the IS at 7.3±0.03 min).

3.4. Validation study

Validation of the developed LC-ESI/MS-MS method was established following The Guidance for Industry on Bioanalytical Method Validation.[42] The obtained results pertain to the efficiency, reproducibility, and precision of the

proposed method. Calibration functions were fitted by a linear model: $y = mx + b$ (y: relative peak area of aldehyde/IS, x: aldehyde concentration spiked to serum in nM, m: slope of the regression line, and b: intercept of the regression line) with excellent correlation coefficients > 0.999 (Table 2). The LOQ was also calculated for the eight aldehydes and found to be ranged from 0.05 to 0.25 nM with accuracy ranged from 94.03 to 107.3% and precision ≤17.32%. In addition, the LOD was determined for the studied aldehydes and found to be within the range of 0.004-0.1 nM (Table 2). Such results indicated the linearity, high sensitivity, and applicability of the method for quantification of trace levels of aldehydes in biological fluids.

Results of the study of accuracy, stated as the % deviation of the mean values from the true value, and precision (as %RSD) are illustrated in Table 3. As can be noted, the proposed method exhibited high accuracy (-13.8 to +9.0) and satisfactory precision (%RSD ranged from 0.1 to 11.7% in case of intra-day precision and from 1.4 to 13.4% for inter-day precision).

The recovery of the target aldehydes was estimated by comparing the response of extracted serum and un-extracted standard (denotes 100% recovery) at three concentrations (0.5, 5.0, and 50.0 nM). The recoveries were found to be ranged from 92.6 to 110.8% (Table 4).

The stability of the aldehydes and IS solutions was evidenced at room temperature up to 6 h, and at 4 °C up to 1 week. No significant variation was observed in both cases respect to the response of fresh solutions. Additionally, the

PQ solution showed high stability at 4 °C up to 2 months. The stability of derivatized aldehydes kept in sealed vials at 4 °C was demonstrated for 48 h which guarantees the stability of the processed samples during the resident time in the auto sampler.

The results of validation study agreed well with the requirements of The Guidance for Industry on Bioanalytical Method Validation [42] proving the suitability of the proposed method to screen the studied aldehydes in human serum.

3.5. Application of the proposed LC/ESI-MS/MS method to determination of aldehydes in human serum

For application of the developed LC/ESI-MS/MS method to the quantitation of aliphatic aldehydes (propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, and decanal) in human serum, a key factor to be considered is the choice of an efficient extraction procedure. In the course of this study, different extraction techniques were investigated including protein precipitation with methanol, sub-zero temperature liquid-liquid extraction with acetonitrile, and salting out liquid-liquid extraction using acetonitrile/NaCl. The best recoveries for the eight analytes (≥ 92.6 %) with accurate and reproducible results were attained using the salting out liquid-liquid extraction method (Fig. S-4).

Hence, the levels of the target aldehydes (C_3-C_{10}) were determined in the serum of 14 healthy volunteers using the developed method (Table 5). The eight target aldehydes were detectable in all serum samples. Fig. 4 shows a MRM chromatogram of the aldehydes detected in serum of a healthy volunteer. The concentrations of the eight aldehydes were calculated using the corresponding regression equations. The levels of the aldehydes found in serum using the proposed method are in accordance to those reported in the literature (Table 5). [19,21,31]

3.6. Comparison of the developed method and reported methods for aldehydes determination in biological fluids

For evaluation of the newly developed LC/ESI-MS/MS method, we compared it with the methods found in the literature for determination of aldehydes in biological fluids (Table 6). Our method has much lower LODs than the reported methods[14–16,19–22,25,31–35] yielding higher sensitivity of about 19 to 1000 folds. This high sensitivity made the proposed method well-suited for the trace quantification of aldehydes in biological fluids at sub-nanomolar concentration levels.

Additionally, the novel derivatizing agent PQ allows the MS/MS detection of the aldehydes without any interference from other carbonyl compounds. This advantage overcame the problem of selectivity associated with the use of hydrazine-derived reagents.[14–16,20–22] As well, the derivatization reaction is

a simple one-pot reaction giving a stable water-soluble reaction product does not require either extraction or evaporation/reconstitution steps prior analysis with LC/ESI-MS/MS. Additional advantages associated with the use of PQ reagent are its commercial availability and stability. Furthermore, the product ion spectra of PQ derivatives of aldehydes are in general clear and simple. Although our method has some drawbacks such as heating at 100 °C for 30 min, which is quite comparable to some of the reported methods [19,28–31,34]. However, the ultra-sensitivity and easiness of extraction from biological fluids with high efficiency still constitute an exceptional merit of the developed method.

In spite that our main target matrix is human serum, it is worth noting that the developed method exhibited superior sensitivity to most of the reported methods for determination of aldehydes in environmental samples like air and water.[10–13,17,24] Even though our method has a comparable sensitivity to the LC-MS/MS method of Sun *at al*. [36] our method is better than this one in terms of rapidness, selectivity and commercial availability of the derivatizing agent.

Although the LC-MS/MS instrument is expensive and needs special grade of solvents, it is considered the most powerful technique for metabolites determination, where it combines the separation efficiency of LC and the specificity and sensitivity of MS/MS.

Based on these results, we believe that our newly developed mass-tagging reagent PQ is very useful and superior to other available reagents for establishing

the levels of low and medium molecular weight aldehydes in biological fluids as LPO biomarkers.

4. Conclusions

A new, validated, ultra-sensitive, and precise LC/ESI-MS/MS method was developed for the monitoring of short and medium chain aliphatic aldehydes (C_3-C_{10}) as LPO biomarkers in human serum. It has been shown that aldehydes are readily derivatized with PQ forming imidazole derivatives that can be easily determined by MS with LOD values within the range of 4.0 to 100 pM. Aldehydes derivatization followed by LC/ESI-MS/MS yielded higher sensitivity than the existing HPLC-FL or UV detection methods. As well, our newly developed LC/ESI-MS/MS method is superior to other LC-MS/MS methods for aldehyde quantification with regard to sensitivity and rapidness. Thus, the developed method proved to be ideal for analyzing C_3-C_{10} aldehydes in human serum.

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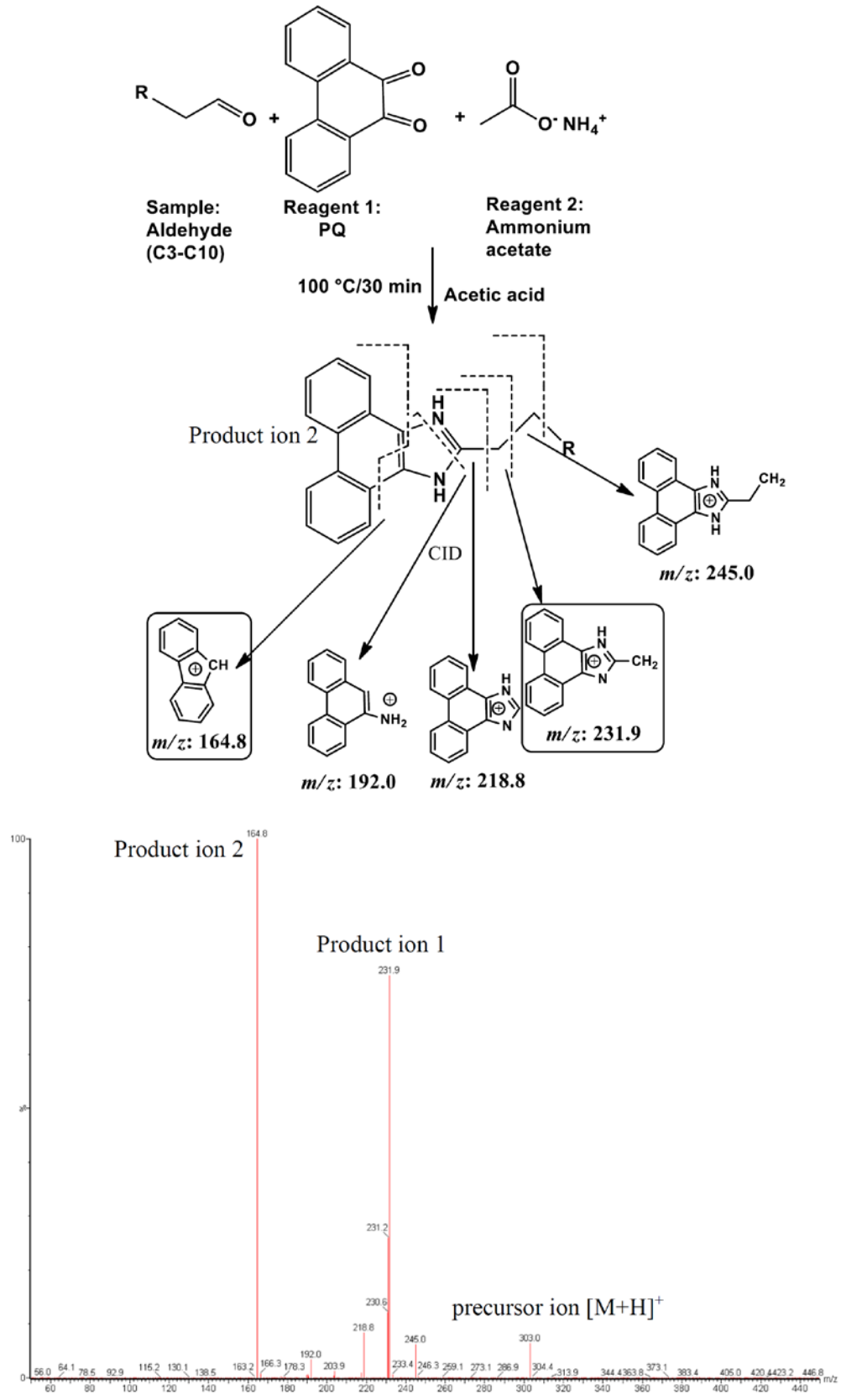

Fig.1.Reaction pathway of the target aliphatic aldehydes and PQ and the fragmentation pattern of the formed product (A), and MS/MS product ion spectrum of PQ-heptanal derivative (B)

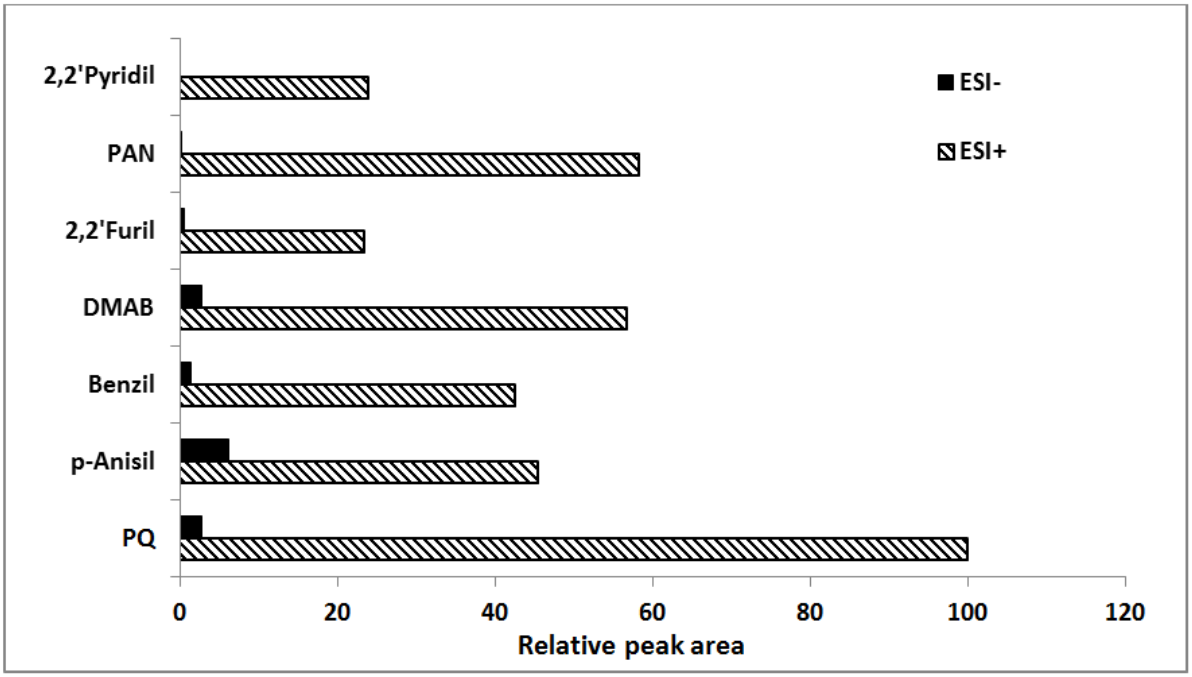

Fig.2. Relative peak areas of heptanal derivatives of the studied reagents using ESI^+ and ESI^- mode.

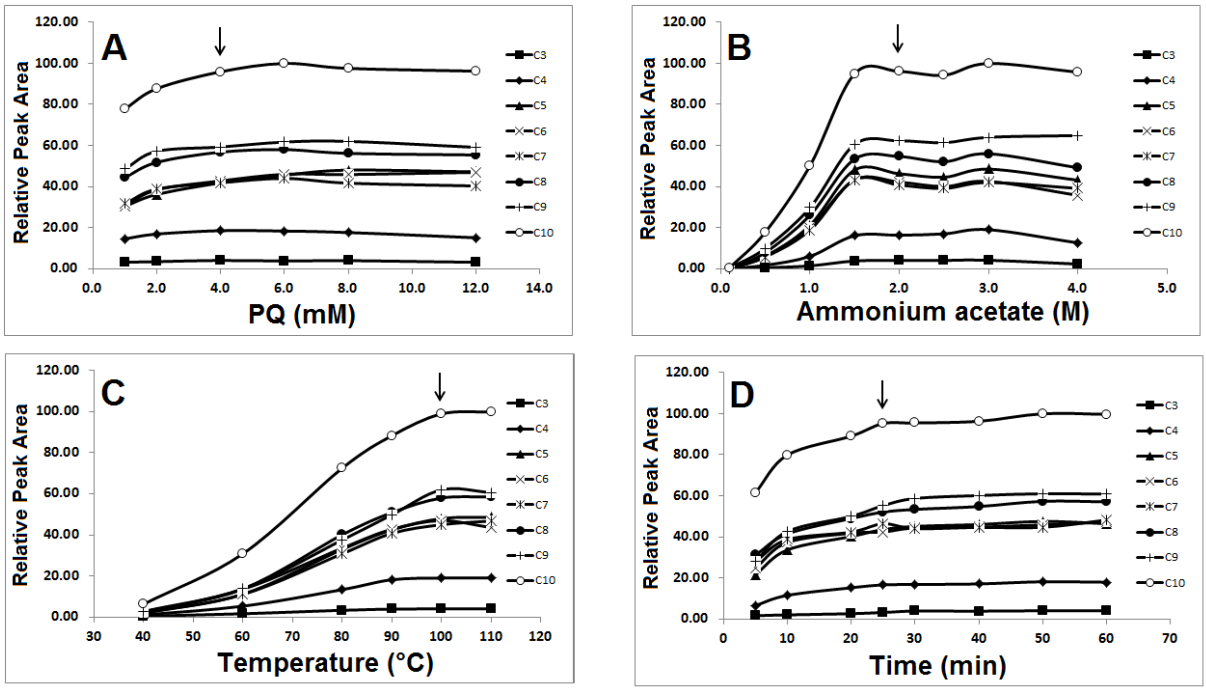

Fig.3. Effects of concentration of PQ (A) and ammonium acetate (B), and reaction temperature (C) and time (D) on the relative peak areas of the PQ- aldehydes derivatives (50.0 nM)

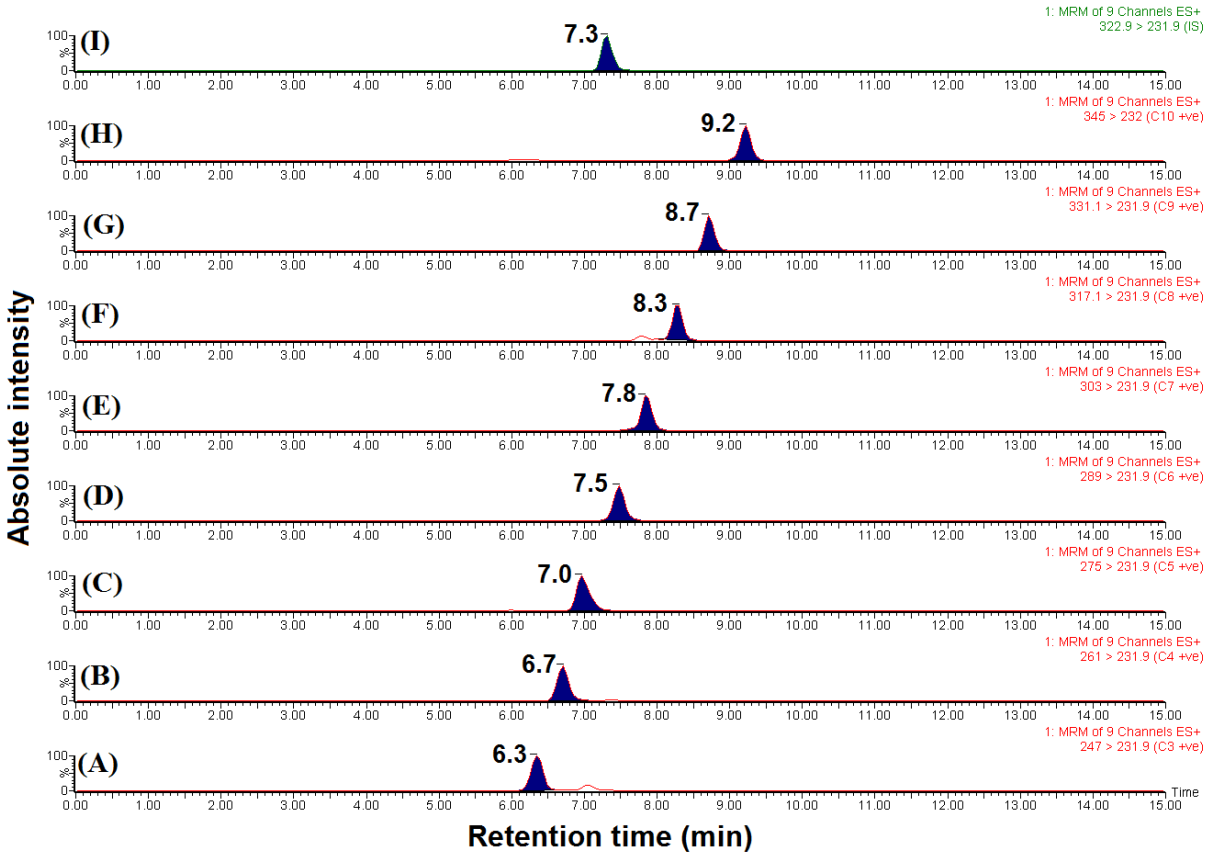

Fig.4. Representative chromatograms of the target aliphatic aldehydes and IS in serum after derivatization with PQ where (A) propanal, (B) butanal ,(C) pentanal, (D) hexanal, (E) heptanal, (F) octanal, (G) nonanal, (H) decanal, and (I) IS.

Table 1. Optimum MS/MS conditions for the analysis of PQ derivatives of the studied aliphatic aldehydes

Aldehyde	Cone voltage (V)	Collision voltage (V)	Parent ion	Daughter ion
Propanal	35	30	247.0	231.9
Butanal	40	30	261.0	231.9
Pentanal	45	30	275.0	231.9
Hexanal	50	30	289.0	231.9
Heptanal	50	30	303.0	231.9
Octanal	50	35	317.1	231.9
Nonanal	50	35	331.1	231.9
Decanal	50	35	345.0	232.0

Table 2: Collective regression data, limit of detections (LOD), limit of quantifications (LOQ), and accuracy and precision at LOQ for the studied aldehydes in serum

Compound	LOD[c], nM (fmol/injection)	LOQ[d], nM	Accuracy at LOQ (%)	Precision at LOQ (%RSD)	Range, nM	Regression equations [a], $n = 3$	r [b]
Propanal	0.100 (2.0)	0.25	106.15	12.53	0.25-200	Y= 0.0017 + 0.0010 X	0.9997
Butanal	0.050 (1.0)	0.10	109.4	17.32	0.1-200	Y= 0.0125 + 0.0044 X	0.9995
Pentanal	0.013 (0.26)	0.05	94.53	11.38	0.05-100	Y= 0.0590 + 0.0110 X	0.9991
Hexanal	0.027 (0.54)	0.05	107.30	8.64	0.05-100	Y= 0.0869 + 0.0100 X	0.9991
Heptanal	0.010(0.20)	0.05	97.75	9.88	0.05-100	Y= 0.0120 + 0.0112 X	0.9997
Octanal	0.012 (0.24)	0.05	95.09	6.86	0.05-100	Y= 0.0193 + 0.0143 X	0.9991
Nonanal	0.018 (0.36)	0.05	101.74	16.33	0.05-100	Y=0.0268 + 0.0151 X	0.9991
Decanal	0.004 (0.08)	0.01	94.03	12.70	0.01-50.0	Y= 0.0108 + 0.0254 X	0.9993

[a] Y= peak area, X= aldehyde concentration (nM).

[b] Correlation coefficient.

[c] S/N= 3.

[d] S/N≥ 5.

Table 3: Accuracy and precision of the proposed method for the determination of the studied aliphatic aldehydes in spiked serum samples.

Aldehydes	Spiked Conc. (nM)	Intra-day (*n* = 5)			Inter-day (*n* = 5)		
		Found conc.	Accuracy (% found)	Precision (% RSD)	Found conc.	Accuracy (%found)	Precision (%RSD)
Propanal	0.5	0.488	-2.4	7.2	0.521	4.2	10.5
	5	4.74	-5.2	6.0	5.01	0.2	3.2
	50	50.3	0.6	2.2	51.0	2.0	5.1
Butanal	0.5	0.488	-2.4	6.8	0.542	8.4	11.3
	5	4.94	-1.2	5.2	5.05	1.0	2.7
	50	50.2	0.4	1.9	50.7	1.4	4.2
Pentanal	0.5	0.431	-13.8	11.7	0.536	7.2	7.3
	5	5.05	1.0	7.1	5.00	0.0	1.9
	50	54.5	9.0	2.1	52.9	5.8	5.4
Hexanal	0.5	0.479	-4.2	7.7	0.532	6.4	6.9
	5	4.35	-13.0	10.6	4.94	-1.2	7.8
	50	50.5	1.0	0.5	50.1	0.2	1.7
Heptanal	0.5	0.505	1.0	5.2	0.507	1.4	10
	5	5.04	0.8	1.8	5.08	1.6	1.4
	50	49	-2.0	0.3	49.4	-1.2	1.9
Octanal	0.5	0.524	4.8	4.8	0.527	5.4	6.5
	5	5.07	1.4	3.4	5.22	4.4	1.7
	50	48.9	-2.2	0.6	52.4	4.8	8.2
Nonanal	0.5	0.496	-0.8	4.3	0.489	-2.2	13.4
	5	5.18	3.6	7.5	5.06	1.2	2.7
	50	49.7	-0.6	0.8	50.4	0.8	10.7
Decanal	0.5	0.544	8.8	9.4	0.497	-0.6	13.2
	5	4.99	-0.2	2.0	4.98	-0.4	4.1
	50	50.2	0.4	0.1	54.5	9.0	11.1

Table 4. Results of recovery study of the target of aliphatic aldehydes from spiked serum samples after salting out liquid-liquid extraction.

Aldehyde	Spiked amount (nM)	% Recovery	SD	Aldehyde	Spiked amount (nM)	%Recovery	SD
Propanal	0.5	97.2	14.5	**Heptanal**	0.5	96.6	5.8
	5	103.8	3.0		5	98.8	2.1
	50	110.8	2.0		50	96.6	2.7
Butanal	0.5	99.8	4.3	**Octanal**	0.5	98.6	8.8
	5	102.4	5.5		5	100.0	3.0
	50	103.8	2.9		50	99.2	1.6
Pentanal	0.5	106.2	4.5	**Nonanal**	0.5	96.8	3.3
	5	102.2	11.5		5	92.6	2.5
	50	98.2	3.0		50	95.8	0.7
Hexanal	0.5	96.8	6.2	**Decanal**	0.5	98.0	7.6
	5	104.6	6.9		5	99.2	3.2
	50	100.8	1.4		50	100.8	0.3

Table 5. Levels of the target aliphatic aldehydes in healthy human serum

Aldehydes	**Range, nM** [a]	**Mean ±SD , nM** [a]	**Previously reported level, nM** [17,19,29]	**Ref**
Propanal	7.2-68.8	26.2±17.1	34.0	[17]
Butanal	21.7-40.0	27.7±5.1	35.0	[17]
Pentanal	41.2-88.4	60.7±13.0	37.0	[17]
Hexanal	1.8-21.7	11.0±5.9	1.8 - 15.2	[19,29]
Heptanal	0.1-11.6	3.5±3.2	1.1 - 15.2	[19,29]
Octanal	0.1-11.9	2.6±3.4	1.7>	[17,29]
Nonanal	0.2-73.6	27.2±24.9	1.0≥ - 25.0	[17,29]
Decanal	0.1-2.1	1.1±0.6	0.8≥ - 1.5	[29]

[a] n=14, 10 Males + 4 Females, age = 38.4 ± 13.1 years

Table 6. Comparison of the proposed method and reported methods for determination of aldehydes in biological fluids

Analyte/matrix	Derivatization conditions		Analytical method	LOD, nM	%Recovery	Ref.
	Reagent	Temp °C/ time (min)				
C_6,C_7/human blood	DNPH	40/20	HPLC-UV	0.79-0.8	75.2-101.1	[14]
C_6,C_7/human blood	DNPH	40/10	HPLC-UV	7.9-2.34	67.84-70.28	[15]
C_6,C_7/human plasma	DNPH	N/A[a]	HPLC-UV	2.4-3.6	83-87	[16]
C_1-C_{12}/human serum	BODIPY-aminozide	60/30	HPLC-FL	0.43-0.69	95-105	[19]
C_6,C_7,C_9, malondialdehyde, α,β-unsaturated aldehydes/exhaled breath condensate	DNPH	RT/60	LC/APCI-MS/MS	1 (for C_6,C_7,C_9)	N/A[a]	[20]
C_6,C_7/human blood	DNPH	N/A[a]	LC/APCI-MS/MS	0.076-0.17	94-110	[21]
aldehydes and ketones/mice plasma	DNSH	RT/240	LC/ESI-MS/MS	0.2-4	70.3-111.8	[22]
C_6,C_7, acetone/human serum	PFBHA	40/8	GC/MS	0.24-0.32	88-92	[25]
C_6-C_{10}/human serum	2,2`-Furil	100/30	HPLC-FL	0.25-0.5	88-105	[31]
C_5-C_{10}, malondialdehyde, trans-2-pentanal/human urine	4-ACP	10/180	LC-MS/MS	3.0	N/A[a]	[32]
C_6-C_{10}, alkenals, hydroxyalkenals/human plasma	CDH	60/60	LC-MS/MS	(10-100 Pg)	N/A[a]	[34]
C_3-C_{10}/Serum	PQ	100/30	LC-MS/MS	0.004-0.1 (0.01-0.1 pg)	92.6-110.8	Proposed method

[a] N/A: Not available

Electronic Supplementary Material

9,10-Phenanthrenequinone as a New Derivatizing Reagent for Ultra-Sensitive and Selective LC/ESI-MS/MS Analysis of Aliphatic Aldehydes in Human Serum

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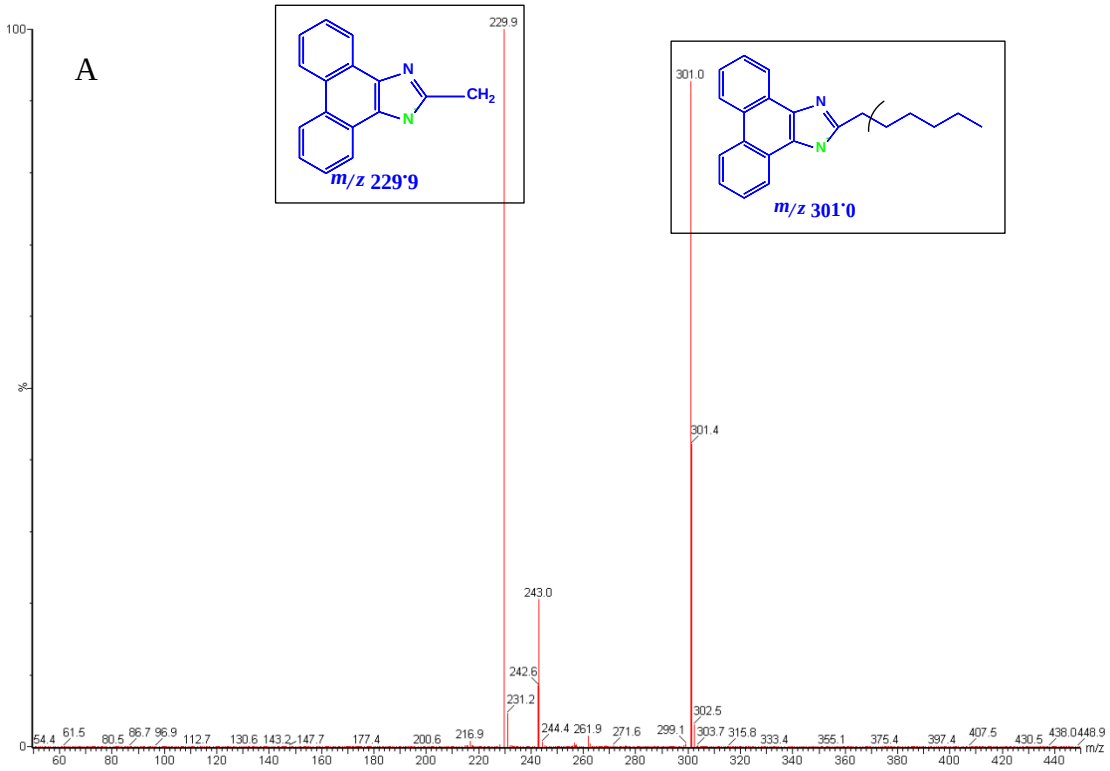
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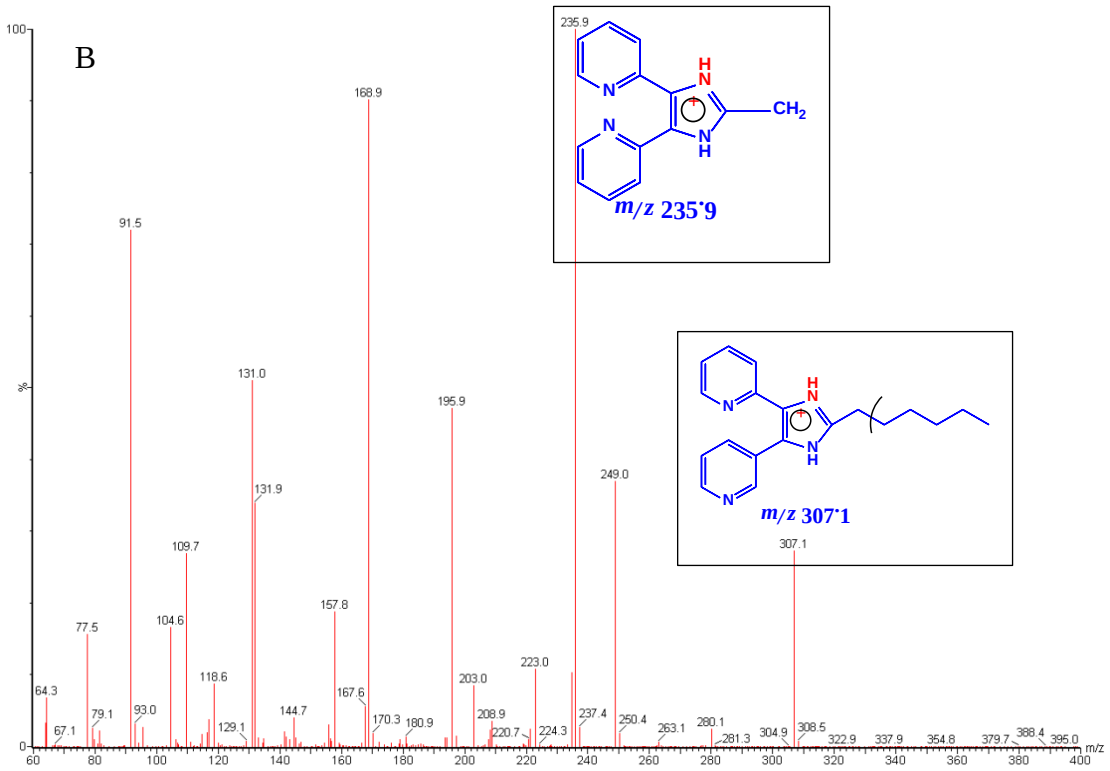
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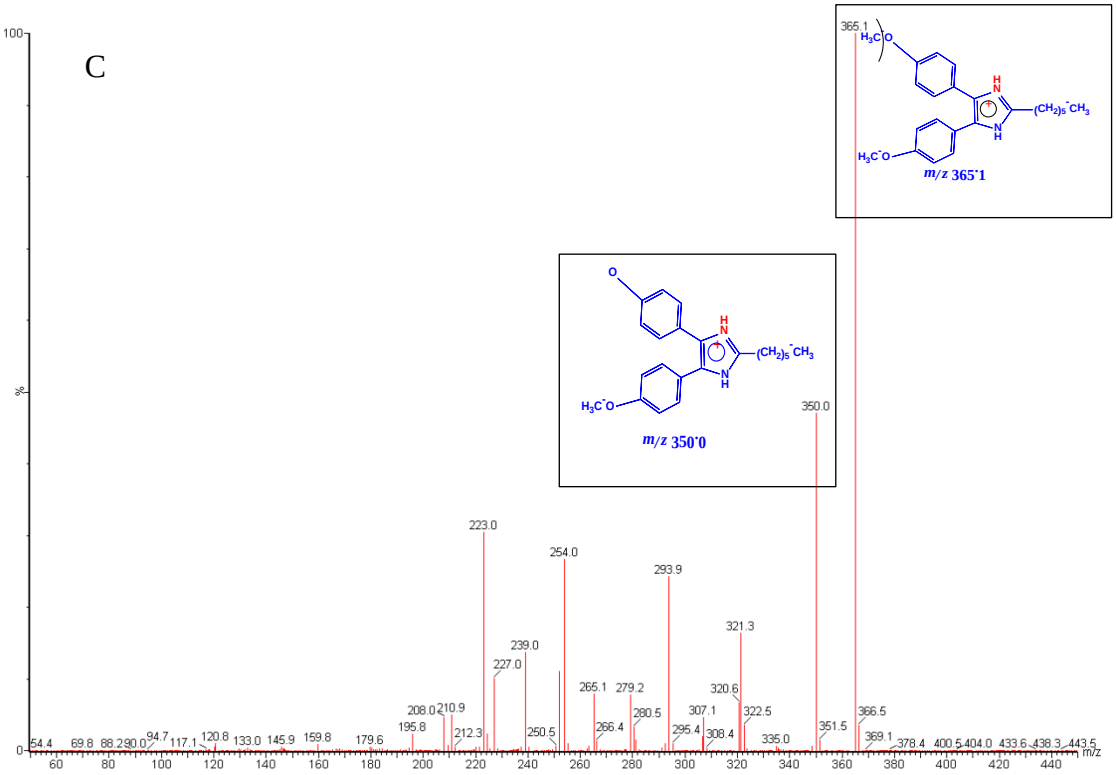
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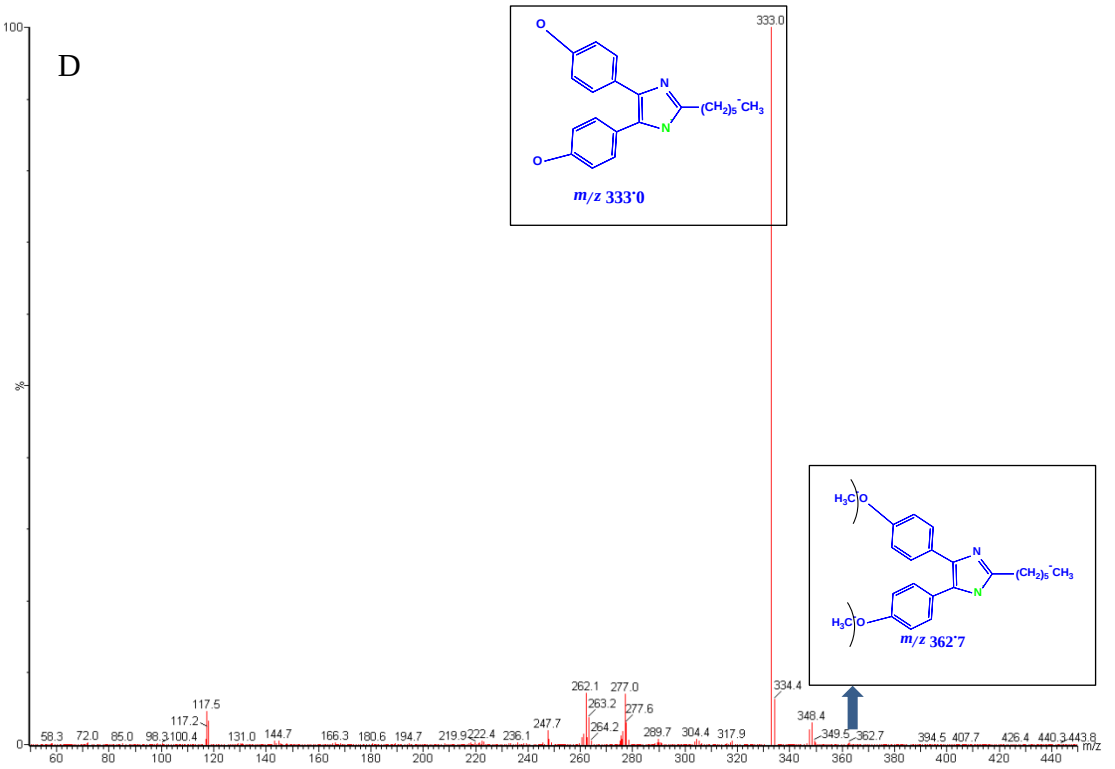
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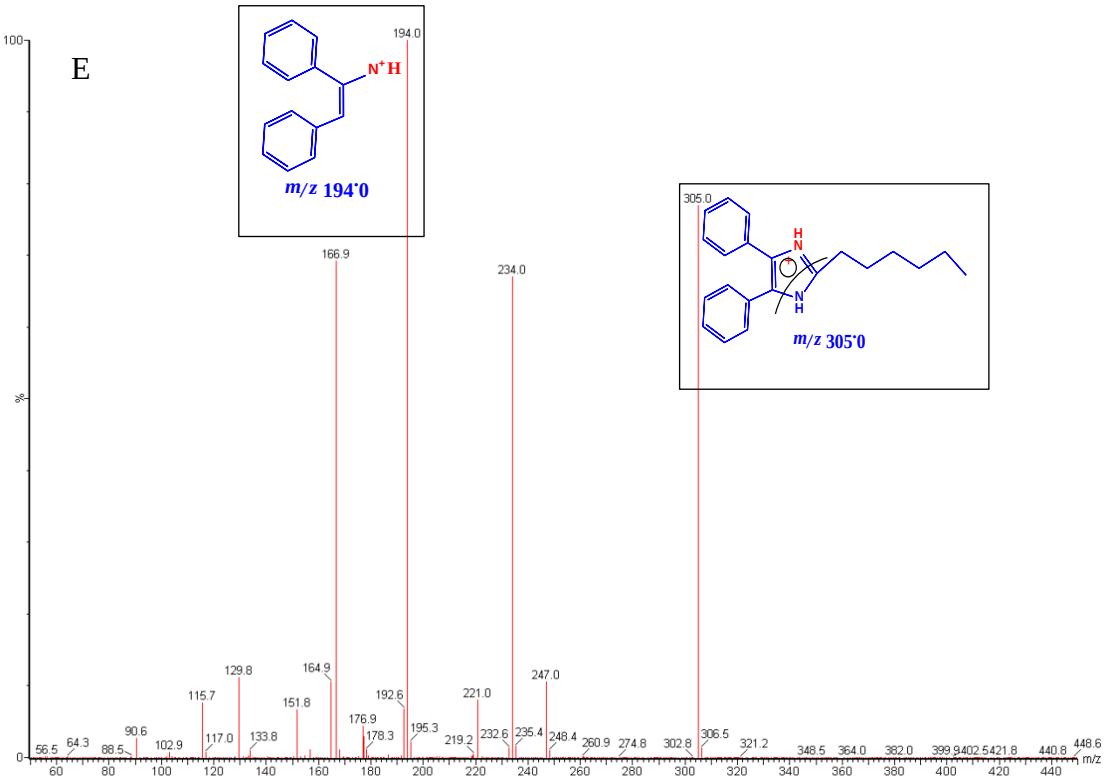
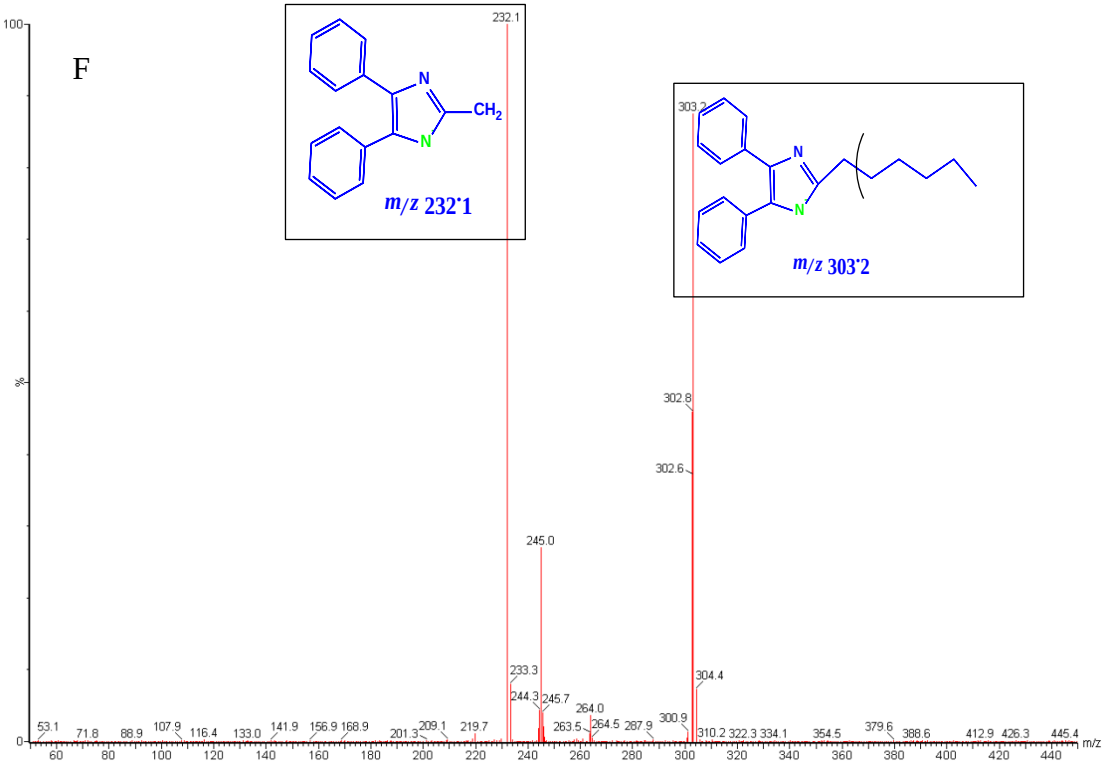
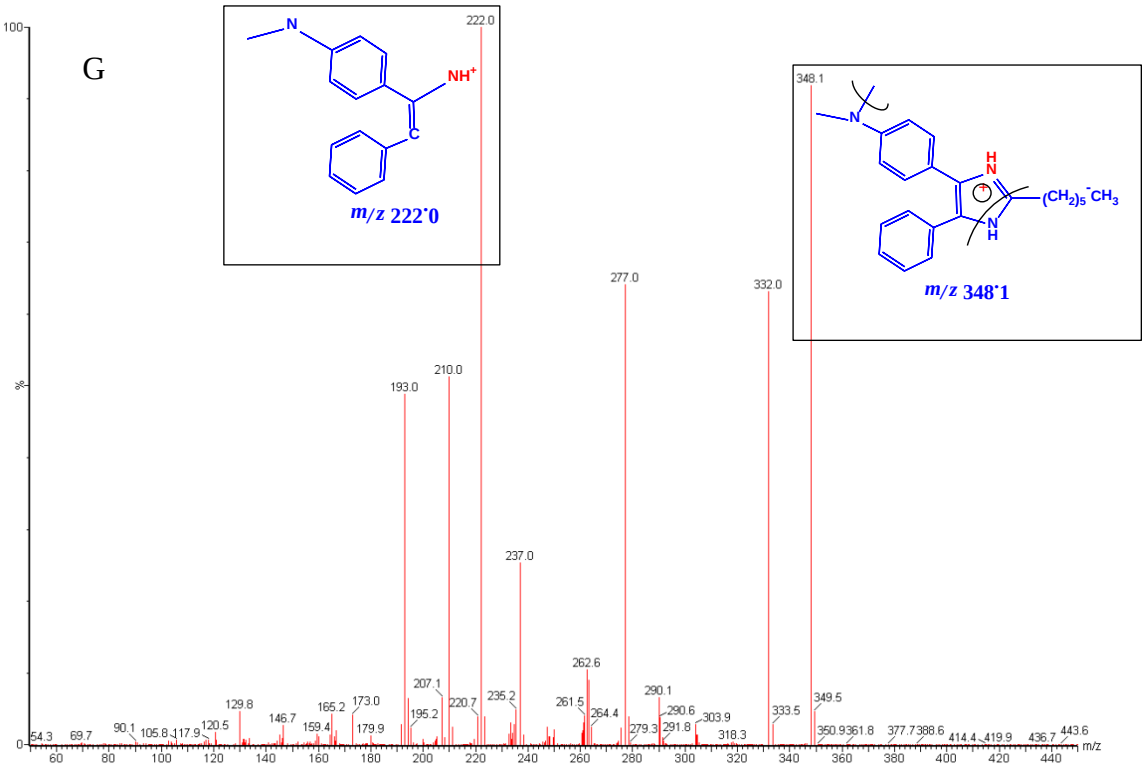

194.0
N+H
m/z 194.0
305.0
H
N
N
H
m/z 305.0
166.9
234.0
129.8
164.9
115.7
151.8
192.6
221.0
247.0
176.9
178.3
195.3
90.6
117.0
133.8
219.2
232.6
235.4
248.4
260.9
274.8
302.8
306.5
321.2
348.5
364.0
382.0
399.9
402.5
421.8
440.8
448.6
56.5
64.3
88.5
102.9
100
%
0
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
m/z

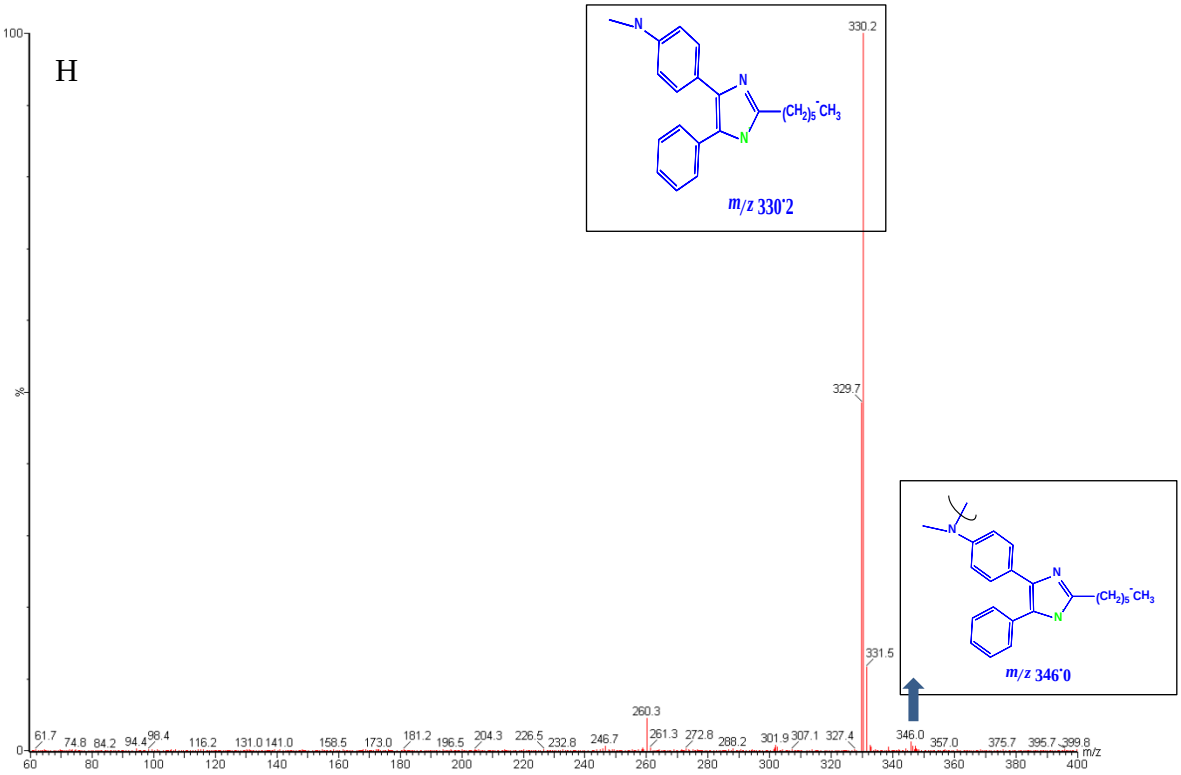
F


232.1
N
CH2
N
m/z 232.1
303.2
N
N
m/z 303.2
302.8
302.6
245.0
233.3
244.3
245.7
264.0
264.5
263.5
267.9
300.9
304.4
310.2
322.3
334.1
354.5
379.6
388.6
412.9
426.3
445.4
53.1
71.8
88.9
107.9
116.4
133.0
141.9
156.9
168.9
201.3
209.1
219.7
100
%
0
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
m/z

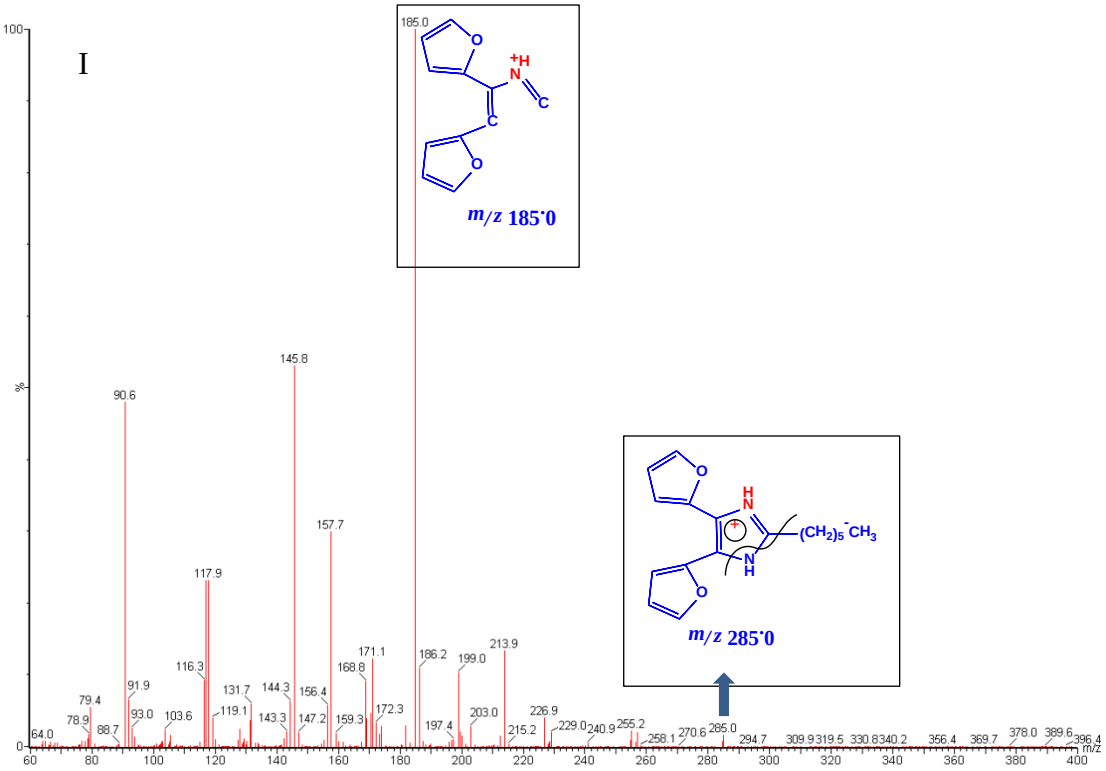
G


222.0
m/z 222.0
348.1
m/z 348.1
277.0
332.0
210.0
193.0
237.0
262.6
207.1
290.1
235.2
220.7
261.5
264.4
195.2
290.6
303.9
333.5
349.5
129.8
165.2
173.0
146.7
159.4
179.9
120.5
279.3
291.8
318.3
54.3
69.7
90.1
105.8
117.9
350.9
361.8
377.7
388.6
414.4
419.9
436.7
443.6
m/z

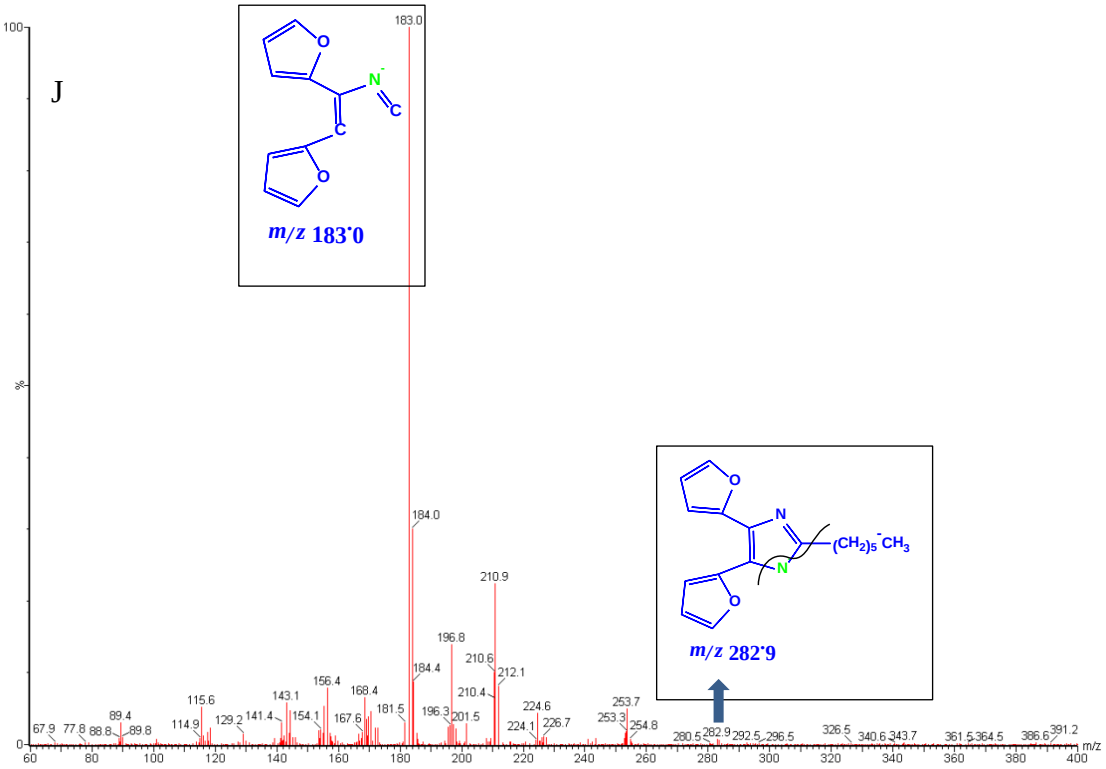
H


330.2
m/z 330.2
329.7
331.5
m/z 346.0
260.3
346.0
61.7
74.8
84.2
94.4
98.4
116.2
131.0
141.0
158.5
173.0
181.2
196.5
204.3
226.5
232.8
246.7
261.3
272.8
288.2
301.9
307.1
327.4
357.0
375.7
395.7
399.8
m/z

I


185.0
m/z 185·0
145.8
90.6
157.7
117.9
213.9
199.0
186.2
171.1
168.8
116.3
131.7
144.3
156.4
91.9
79.4
78.9
93.0
88.7
103.6
119.1
143.3
147.2
159.3
172.3
197.4
203.0
215.2
226.9
229.0
240.9
255.2
258.1
270.6
285.0
294.7
309.9
319.5
330.8
340.2
356.4
369.7
378.0
389.6
396.4
64.0
m/z
m/z 285·0

J


183.0
m/z 183·0
184.0
210.9
196.8
210.6
212.1
184.4
210.4
156.4
168.4
143.1
115.6
181.5
196.3
201.5
224.6
226.7
253.7
253.3
254.8
89.4
67.9
77.8
88.8
89.8
114.9
129.2
141.4
154.1
167.6
224.1
280.5
282.9
292.5
296.5
326.5
340.6
343.7
361.5
364.5
386.6
391.2
m/z
m/z 282·9

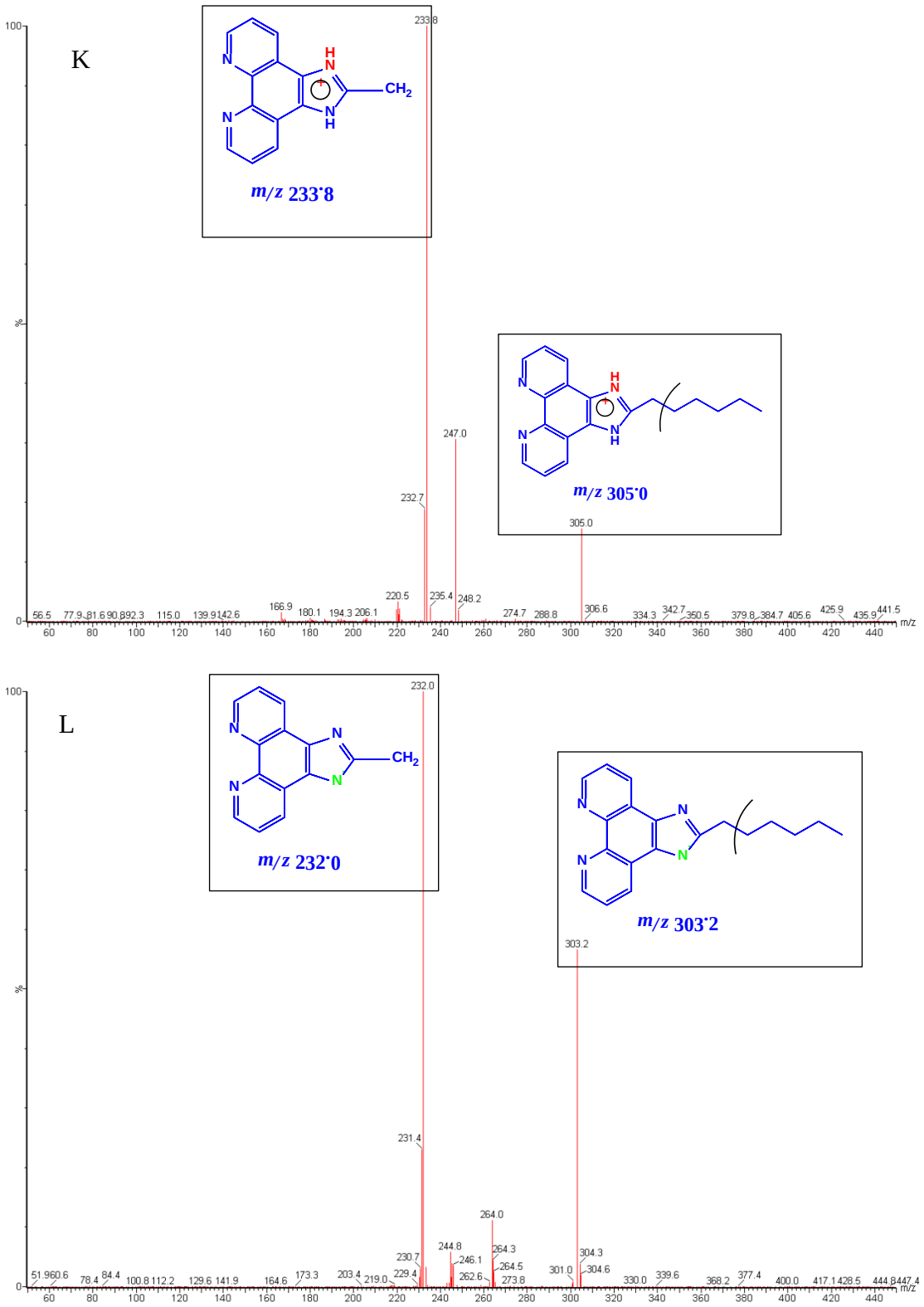


Fig. S-1: Daughter ion spectra of heptanal derivatives of (A) PQ using ESI^-, (B) Pyridl using ESI^+, (C,D) Anisil using ESI^+ and ESI^-, respectively, (E,F) Benzil using ESI^+ and ESI^-, respectively, (G,H) DMAB using ESI^+ and ESI^-, respectively, (I,J) Furil using ESI^+ and ESI^-, respectively, (K,L) PAD using ESI^+ and ESI^-, respectively.

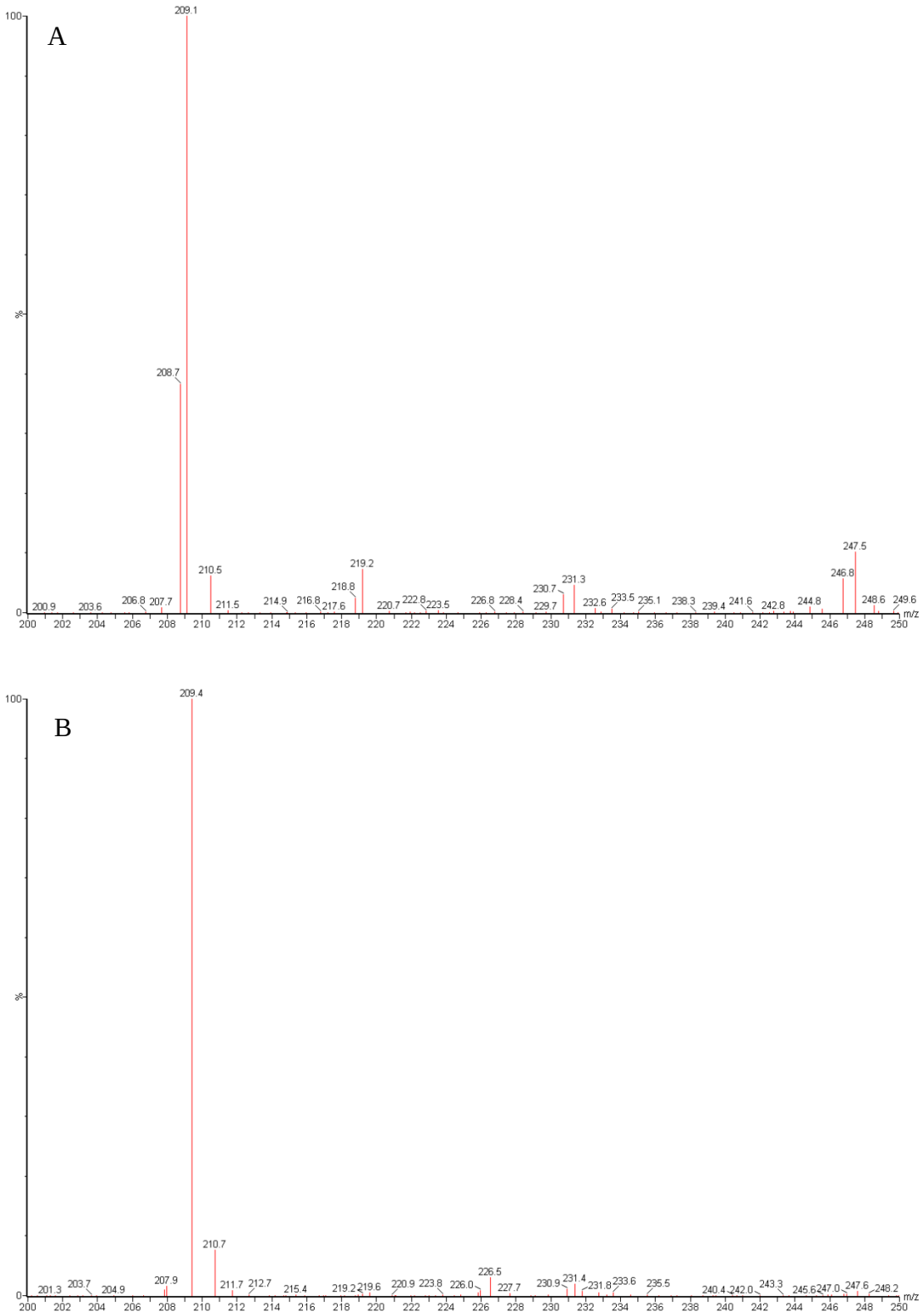


Fig. S-2: Total ion spectra of (A) propanal derivative resulted from the reaction of propanal with PQ and ammonium acetate in presence of acetic acid and (B) reaction mixture of acetone with PQ and ammonium acetate in presence of acetic acid using ESI^+.

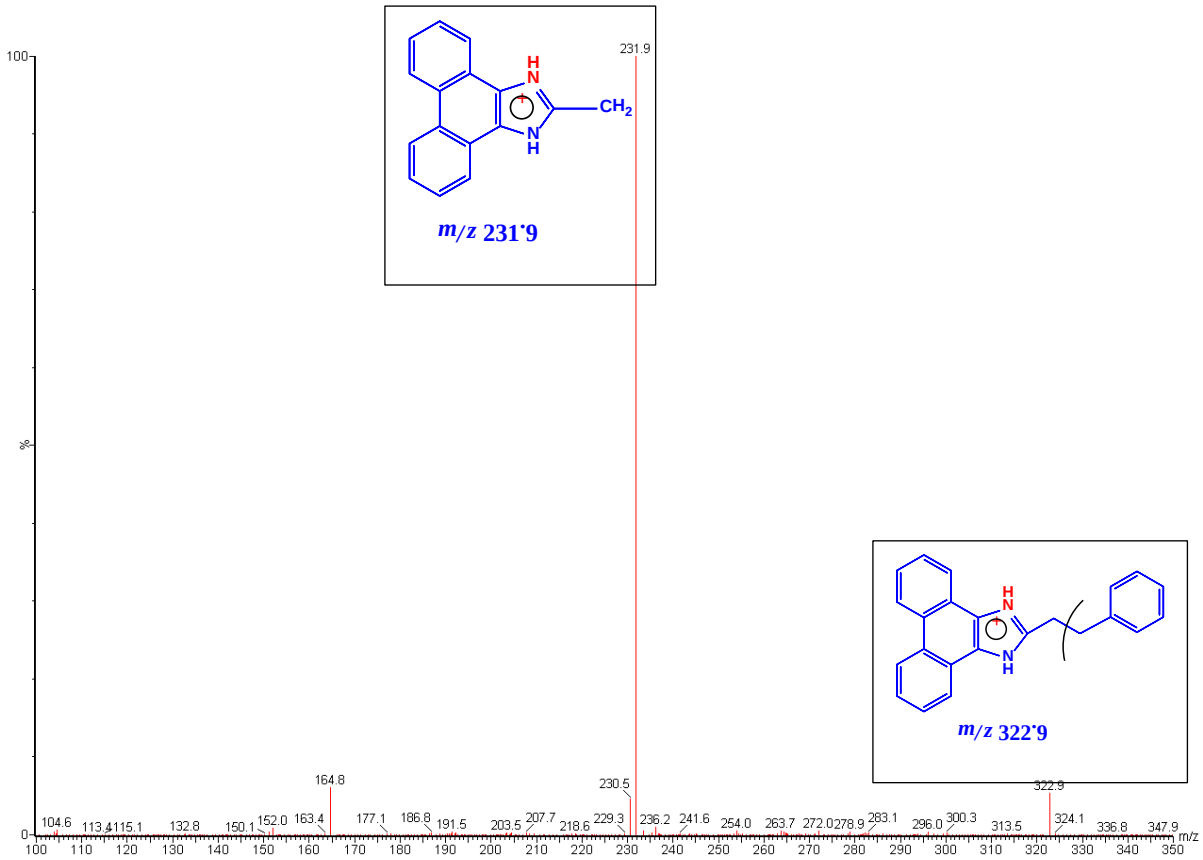


Fig. S-3. Daughter ion spectra of PQ-IS derivative

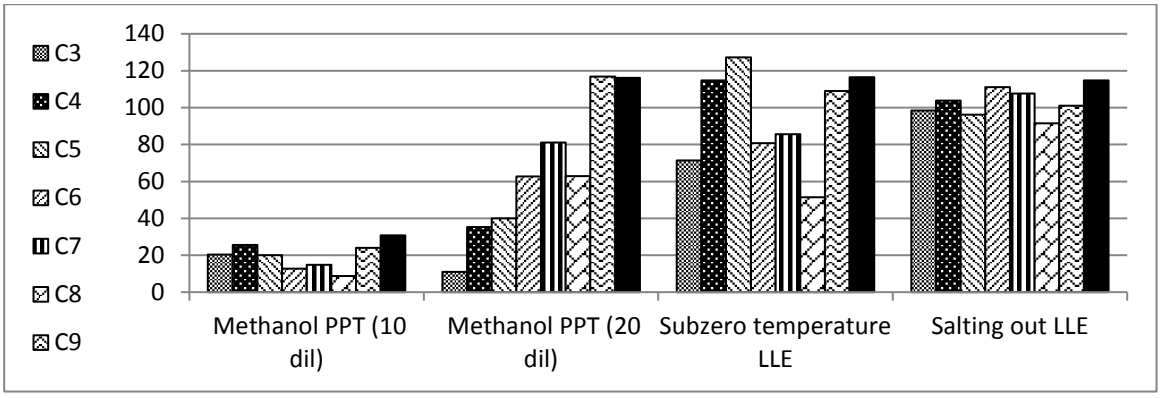


Fig. S-4. Recovery of the studied aldehydes from serum after protein precipitation (PPT) with methanol either with 10 or 20 time volume of that of serum, after subzero temperature liquid-liquid extraction (LLE), and after salting out LLE.

Table S1: The optimum MS/MS conditions for the studied α,β-diketo reagents

Reagent (ESI)	Capillary voltage (KV)	Cone voltage (V)	Collision voltage (V)	Precursor ion	Mean product ion (s)
PQ (+)	5.0	50	30	303.0	231.9, 164.9
PQ (-)	-3.0	-50	-30	301.0	230.0
Anisil (+)	5.0	55	30	365.1	350.1
Anisil (-)	-3.5	-40	-35	362.9	333.0
Benzil (+)	5.0	50	30	305.0	194.0
Benzil (-)	-3.5	-50	-30	303.2	232.1
DMAB (+)	5.0	50	50	348.0	222.0
DMAB (-)	-4.5	-35	-35	346.0	330.2
Furil (+)	5.0	40	35	285.0	185.0
Furil (-)	-3.5	-40	-35	282.9	183.0
Pyridil (+)	5.0	50	45	307.1	235.9
PAD (+)	3	50	35	305.0	233.8
PAD (-)	-3	-50	-30	303.0	232.0